METHODS FOR COLLECTION AND ANALYSIS OF AQUATIC BIOLOGICAL AND MICROBIOLOGICAL SAMPLES

Techniques of Water-Resources Investigations
of the United States Geological Survey

Book 5, Chapter A4

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METHODS FOR COLLECTION AND ANALYSIS OF AQUATIC BIOLOGICAL AND MICROBIOLOGICAL SAMPLES

By L. J. Britton and P. E. Greeson, Editors

Techniques of Water-Resources Investigations
of the United States Geological Survey
Book 5, Chapter A4

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DEPARTMENT OF THE INTERIOR

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PREFACE

The series of chapters on techniques describes methods used by the U.S. Geological Survey for planning and conducting water-resources investigations. The material is arranged under major subject headings called books and is further subdivided into sections and chapters. Book 5 is on laboratory analysis. Section A is on water. The unit of publication, the chapter, is limited to a narrow field of subject matter. "Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples" is the fourth chapter to be published under Section A of Book 5. The chapter number includes the letter of the section.

This chapter was prepared by several aquatic biologists and microbiologists of the U.S. Geological Survey to provide accurate and precise methods for the collection and analysis of aquatic biological and microbiological samples.

Use of brand, firm, and trade names in this chapter is for identification purposes only and does not constitute endorsement by the U.S. Geological Survey.

This chapter supersedes "Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples" edited by P.E. Greeson, T.A. Ehlke, G.A. Irwin, B.W. Lium, and K.V. Slack (U.S. Geological Survey Techniques of Water-Resources Investigations, Book 5, Chapter A4, 1977) and also supersedes "A Supplement to--Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples" by P.E. Greeson (U.S. Geological Survey Techniques of Water-Resources Investigations, Book 5, Chapter A4), Open-File Report 79-1279, 1979.

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CONVERSIONS FACTORS

Metric units (International System) in this report may be converted to inch-pound units by using the following conversion factors:

Multiply metric unit	By	To obtain inch-pound unit
centimeter (cm)	0.3937	inch
cubic meter (m^3)	35.31	cubic foot
gram (g)	0.03527	ounce, avoirdupois
gram per cubic meter (g/m^3)	62.45×10^{-6}	pound per cubic foot
gram per cubic meter per hour [$(g/m^3)/h$]	62.45×10^{-6}	pound per cubic foot per hour
kilogram (kg)	2.205	pound, avoirdupois
kilogram per square centimeter (kg/cm^2)	14.22	pound per square inch
liter (L)	0.2642	gallon
meter (m)	3.281	foot
meter per second (m/s)	3.281	foot per second
microgram (µg)	35.27×10^{-8}	ounce, avoirdupois
microliter (µL)	26.42×10^{-8}	gallon
micrometer (µm)	39.37×10^{-6}	inch
milligram (mg)	35.27×10^{-5}	ounce, avoirdupois
milliliter (mL)	26.42×10^{-5}	gallon
millimeter (mm)	0.3937	inch
square centimeter (cm^2)	0.155	square inch
square kilometer (km^2)	0.3861	square mile
square meter (m^2)	10.76	square foot
square millimeter (mm^2)	1.550×10^{-3}	square inch

Inch-pound units in this report may be converted to metric units (International System) by using the following conversion factors:

Multiply inch-pound unit	By	To obtain metric unit
acre-foot (acre-ft)	1,233	cubic meter
cubic foot per second (ft^3/s)	0.02832	cubic meter per second
foot (ft)	0.3048	meter
inch (in.)	25.4	millimeter
mile (mi)	1.609	kilometer
ounce, fluid	0.02957	liter
pound, avoirdupois (lb)	453.6	gram
pound per square inch (psi)	703.1	kilogram per square meter
square inch (in^2)	6.452	square centimeter
square mile (mi^2)	2.59	square kilometer

Degree Celsius (°C) may be converted to degree Farhenheit (°F) by using the following equation:

$$°F = 9/5\ (°C+32).$$

Degree Fahrenheit (°F) may be converted to degree Celsius (°C) by using the following equation:

$$°C = 5/9\ (°F-32).$$

The following terms and abbreviations also are used in this report:

disintegrations per minute (dpm)
gram per liter (g/L)
gram per milliter (g/mL)
liter per milligram multiplied by centimeter (L/mg)×cm
lumens per square meter (lumens/m^2)
microcurie (μCi)
microcurie per microgram (μCi/μg)
microcurie per milliliter (μCi/mL)
microgram-atoms per liter (μg-atoms/L)
microgram per liter (μg/L)
microgram per milliliter (μg/mL)
millicurie (mCi)
milligram carbon per cubic meter per day [mg(C/m^3)/d]
milligram carbon per cubic meter per hour [mg(C/m^3)/h]
milligram carbon per square meter per day [mg(C/m^2)/d]
milligram oxygen per cubic meter per day [mg(O_2/m^3)/d]
milligram oxygen per cubic meter per hour [mg(O_2/m^3)/h]
milligram oxygen per square meter per day [mg(O_2/m^2)/d]
milligram per cubic meter (mg/m^3)
milligram per liter (mg/L)
milligram per liter per acre-foot [(mg/L)/acre-ft]
milligram per square meter (mg/m^2)
milliliter per minute (mL/min)
millivolt (mV)
nanometer (nm)
revolutions per minute (r/min)
volt (V)
Watt (W)

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L. J. Britton and P. E. Greeson, Editors

ABSTRACT

Chapter A4 contains methods used by the U.S. Geological Survey to collect, preserve, and analyze water to determine its biological and microbiological properties. Part 1 consists of detailed descriptions of more than 45 individual methods, including those for bacteria, phytoplankton, zooplankton, seston, periphyton, macrophytes, benthic invertebrates, fish and other vertebrates, cellular contents, productivity, and bioassays. Each method is summarized, and the applications, interferences, apparatus, reagents, analyses, calculations, reporting of results, precisions, and references are given. Part 2 consists of a glossary. Part 3 is a list of taxonomic references.

INTRODUCTION

The U.S. Department of the Interior has the basic responsibility for the appraisal, conservation, and efficient use of the Nation's natural resources, including water as a resource, as well as water involved in the use and development of other resources. As one of the several agencies of the U.S. Department of the Interior, the U.S. Geological Survey's primary responsibility in relation to water is to assess its availability and use as a natural resource. The U.S. Geological Survey's responsibility for water appraisal includes not only assessments of the location, quantity, and availability of water but also determinations of water quality. Inherent in this responsibility is the need for extensive water-quality studies related to the physical, chemical, and biological adequacy of natural and developed surface- and ground-water resources. Included, also, is the need for supporting research to increase the effectiveness of these studies.

As part of its mission, the U.S. Geological Survey is responsible for providing a large part of the water-quality data for rivers, lakes, and ground water that is used by planners, developers, water-quality managers, and pollution-control agencies. A high degree of reliability and standardization of these data is paramount.

This chapter was prepared to provide accurate and precise methods for the collection and analysis of aquatic biological and microbiological samples, primarily from freshwater. Although excellent and authoritative manuals on

aquatic biological analyses are available, their methods and procedures often are diverse. The purpose of this chapter is to provide, in a single publication, the methods used by the U.S. Geological Survey in conducting biological investigations.

The work of the U.S. Geological Survey in aquatic biology and microbiology ranges from research to the collection of biological information from onsite investigations and from a nationwide network of water-quality stations. The objectives vary so widely that it is impractical to tailor methods to fit all possible requirements. In general, the methods herein apply to the collection of biological information.

It is clear from the accelerating rate of publication of reports on the subject of aquatic biology that new and improved methods are being developed in response to man's increasing awareness of his environment. A technique that represents the state-of-the-art today may be outdated tomorrow. The author of a manual of techniques may have the impression of taking a "grab sample" from a changing stream of new developments, although it is possible t a degree to integrate the experience of the past and to select the most appropriate methods from an ever-growing number of methods.

A methods manual is only one of several tools available to the investigator. At best, it can indicate to him "how to"; it can never indicate to him "what to"; nor can it indicate to him what a specific numerical value means. Entire volumes have been written on subjects, for example, primary productivity, to which this chapter can devote only a few pages. It is emphasized that the successful investigator must keep abreast of the new developments, both in methodology and in the understanding of aquatic ecosystems.

Safety procedures, especially with use of hazardous chemicals or equipment, micro-organisms that may produce human disease, water that may contain bacteria, and radioactive substances, should be recognized and manufacturers' instructions followed when using the methods in this chapter. Special attention is called to a number of hazardous materials within the individual methods and serves to emphasize safety concerns.

PART 1: DESCRIPTION OF METHODS

BACTERIA

Introduction

Bacteria can be collected, observed, and counted directly using the highest resolution of the light microscope. A method for counting total bacteria by epifluorescence is included in this chapter; however, the method is somewhat difficult and may not be appropriate for general use. Of far greater applicability are methods whereby the bacteria in a measured volume of water are placed in contact with material on which they can grow. After a suitable time, each bacterium in the sample will multiply into an easily visible colony. The number of colonies is extrapolated from the number of bacteria in the original sample. The first method in the following section provides an approximation of the total bacterial population. Because all culture methods are selective, a total count of the bacteria in a habitat is impossible using this technique. However, uniform methods permit comparison of results by different investigators. The remaining methods given are designed to be selective for specific groups of bacteria. These methods will provide an estimate of the number of bacteria in an environment, but no information is obtained about the activity of the organisms in the ecosystem being studied.

Most-probable-number (MPN) methods, using multiple-dilution tubes, can be used to estimate the size of a bacterial population without counting either single cells or colonies (Meynell and Meynell, 1970). Several dilutions of a sample are made and aliquots are inoculated into suitable media. The method requires either that the media be selective for a specific group of bacteria and allow only those organisms to grow or that some readily identifiable product be produced. The dilutions, including the most dilute samples used, need to contain no bacterial cells of the type under study (dilution to extinction). Based on the distribution of positive and negative cultures, the MPN of bacteria in the original sample is calculated.

MPN tables are included with each applicable method. These tables are based on those published in "Standard Methods" by the American Public Health Association and others (1985); however, the tables have been modified to include the procedures specified in "Techniques of Water-Resources Investigations" methods. All MPN tables use 1-, 0.1-, and 0.01-mL sample volumes and express MPN per 1 or 100 mL depending on how the count is to be reported. Examples included with each method illustrate the calculation of MPN if sample volumes other than 1, 0.1, and 0.01 mL are used.

The membrane-filter (MF) method has attained widespread application in microbiology principally because it is simple and quick to perform (Bordner and others, 1977). Also, it is statistically more reliable than the MPN method. A brief discussion of the merits and limitations of the MF method are appropriate at this time because precision and accuracy are dependent to a great extent on careful attention to procedural details.

Membrane filters used in microbiology are inert plastic films about 125 μm thick. The membranes are available in a variety of chemical types, each designed for a particular application. It is imperative that the analyst select a type intended for bacterial application. Whatever the type, the membrane is about 80 percent void with pores of uniform size. Pore sizes of 0.45 or 0.7 μm (Green and others, 1975; Sladek and others, 1975; American Public Health Association and others, 1985) are the most common sizes used in microbiology because the type of bacteria most often counted is larger than 0.5 μm. Membranes with pore size less than 0.45 μm are available but are used less commonly in microbiology because of their susceptibility to clogging. Filters are manufactured in many sizes from about 13 to 293 mm in diameter, but only the 47-mm diameter size is used commonly in microbiology. The useful shelf life of membrane filters is 1 year (American Public Health Association and others, 1985).

Bacterial analysis begins with sample collection, which is described in a general way in this introduction. Media and equipment preparation are described with each specific method. At some point in each method, a sample aliquot is passed through a filter. Membrane filters have a rapid flow rate initially due to the large void volume, but the filter will clog quickly if the sample is turbid. For this and other reasons, the MF method generally is not suitable for turbid waters. Even with relatively clear waters, sample filtration generally is limited to about 100 to 250 mL per filter. If it is necessary to filter a larger volume of sample, as with the isolation of Salmonella, it is permissible to divide a sample volume between several filters.

After filtration, the bacteria may be arrayed singly, paired, or in chains on the surface of the membrane. They cannot be seen without magnification; therefore, the filters must be incubated for a time sufficient for the individual cells to grow into visible colonies. After filtration, the filter is aseptically placed in a petri dish containing solid (agar) medium. Liquid-broth medium is not recommended for use in the Water Resources Division. Incubation is allowed to proceed at 35 °C for 24 to 48 hours for total coliform and fecal streptococcal bacteria or at 44.5 °C for 24 hours for fecal coliform bacteria. It is very important that the temperature be held within the limits established for each method. Recent work (Green and others, 1975) indicated that many more cells are retained on the surface of the membrane than actually grow. Use of broth media is not recommended because optimum cell growth depends on an adequate nutrient supply, and solid (agar) media have been found to yield larger colony counts than broth-grown media cultures. This is due to the larger volume (6.5 mL compared to 1.8 mL) of medium used in the agar technique. During incubation, the petri dishes generally will lose moisture and dry. This is particularly true of dry (air) incubators at 44.5±0.2 °C. The result of drying serves to inhibit bacterial growth, thus underestimating the true population. To prevent this from occurring, the petri dishes should be checked for proper sealing before incubation. Cracked dishes should be discarded.

When the individual cells have multiplied to visible colonial size (usually 24- to 48-hour incubation), the colonies must be counted. The counting procedure is based on enumerating all colonies of a specific color, regardless of size or shape. Each bacterial method has different colony

identification criteria. After a count has been made, the result is calculated and reported in terms of number of colonies per milliliter or 100 mL of sample.

Media used in many of the methods described in this manual are commercially available in a pre-mixed, dehydrated form. Unopened containers of nutrient media should not be stored for more than 1 year. The shelf life of opened containers of media is highly variable; to extend the shelf life of opened containers, the media should be stored in a dessicator.

Collection

If valid information about the number and type of bacteria present in an environment is to be obtained, care must be taken before, during, and after sampling. A valid sample will be representative of the organisms present at the site under study and will be uncontaminated by extraneous organisms. After such a soil or water sample has been obtained, it must be processed as quickly as possible and carefully maintained so the bacterial populations do not change extensively.

The study objective is of overriding importance, and the final determination of the best sampling method, frequency of sample collection, and number and distribution of sampling sites is left to the judgment of the investigator. The sites and methods used for sampling of bacteria need to correspond as closely as possible to those selected for chemical and other biological sampling.

Some of the general guidelines for collecting soil and water samples given by Hem (1985), Guy and Norman (1970), and Wood (1976, p. 1-7) can be applied to microbiological work. However, collecting valid samples for bacterial analysis is more difficult because extra care is required to avoid contamination and because micro-organisms rarely are distributed randomly. Bacteria within any habitat or microhabitat probably will have a clumped or patchy distribution. Localized differences in chemical and physical characteristics, such as Eh, pH, temperature, nutrient availability, and dissolved-oxygen concentration, will affect the size and distribution of the bacterial population.

Although guidelines for sample collection are provided in this section, it is impossible to provide detailed instructions on sample collection for all possible circumstances. More extensive discussions of microbiological sampling are given in the following:

1. Surface water--Rodina (1972), Collins and others (1973), and Skinner and Shewan (1977).

2. Ground water--Dunlap and McNabb (1973), Dunlap and others (1977), and Scalf and others (1981).

3. Soil--Black (1965), Parkinson and others (1971), and Williams and Gray (1973).

Surface Water

The location of sampling sites and the frequency of sampling are critical factors in obtaining meaningful data about bacterial density in any water body. In lakes, reservoirs, deep rivers, and estuaries, bacterial abundance may vary laterally, with depth, and with time of day. Generally, multiple samples collected at different depths and sites within a study area yield more reliable data than do single samples. Water in small, fast-flowing streams is likely to be well mixed. A point sample, collected at a single transverse position located at the centroid of flow, may be adequate (Goerlitz and Brown, 1972).

To collect a sample of water at the surface, open a sterile milk dilution bottle or equivalent sample container, grasp it near its base, and plunge it, neck downward, below the water surface. Allow the bottle to fill by slowly rotating the bottle until the neck points slightly upward. The mouth of the bottle must be directed into the current. If there is no current, as in a lake, a current should be created artificially by pushing the bottle horizontally forward in a direction away from the hand (American Public Health Association and others, 1985).

Several types of microbiological sampling apparatus are available that collect a water sample at depth. Samplers of the Kemmerer or Van Dorn type have been used, but their use is discouraged; most of these devices are not autoclavable, and the metallic parts, if present, can have bacteriocidal effects if they remain in contact with the sample for a prolonged period of time. Niskin and ZoBell samplers (Rodina, 1972) collect a sample in either a sterile plastic bag or a sterile bottle. All of these sampling devices are triggered by a messenger and collect samples at one point in the water column.

Samplers, such as the D-77 and DH-80, available from the Federal Interagency Sedimentation Project, St. Anthony Falls, Hydraulic Laboratory, Minneapolis, Minn., can be used for collecting depth-integrated samples from flowing water. The sampler's nozzle and chamber are autoclavable.

Ground Water

Obtaining a valid sample of ground water for microbiological examination requires care in well construction and sampling technique. During well construction, the potential for contamination by the extraneous introduction of nutrients and bacteria needs to be minimized.

Generally, the water in the casing and in proximity to the well is not representative of the ground water at a distance from the well. Oxidation-reduction and nutrient conditions generally are different near the well where bacteria may be present in greater numbers than in the aquifer some distance from the well. There is no general rule for the number of times that water in the well casing must be cleared before collecting water samples for bacterial analysis. The volume of pumping necessary will depend on site-specific conditions and the purpose of the investigation. Public-supply, industrial, or irrigation wells, which are pumped continuously, may give the most representative sample of aquifer water.

The possibility of external contamination during sampling can be lessened by sterilizing all materials that will come in contact with the water sample; however, this may be difficult during some onsite conditions. Water within 25 ft of land surface can be collected by peristaltic and other low-volume suction pumps fitted with sterile hoses. For studies that require water samples collected deeper than 25 ft, other types of pumps must be used. Gas-powered, all-glass pumps that can be heat sterilized have been developed, but these are fragile and require special care (Tomson and others, 1980). Gas-powered squeeze pumps that fit into small-diameter wells and that may be autoclaved also have been developed (Koopman, 1979). Portable submersible pumps commonly are the most convenient sampling devices. Although they may be difficult or impossible to sterilize, these pumps can be disinfected by recirculating a chlorine solution.

Soil and Sediment

Collect soil samples using sterile procedures and place in sterile glass, polypropylene or teflon bottles, or Whirl-Pak bags. Avoid exposing soil samples to heat or drying. If the sample is not processed on the day of collection, it may be stored at 4 °C for 1 to 2 weeks in the closed container, provided that the container is pinholed for aeration. Just prior to processing, pass the entire sample through a 10-mesh sieve (2,000 μm) and mix thoroughly before taking an aliquot for analysis. If desired, a separate subsample may be taken for determination of dry weight (Clark, 1965).

Bottom-material sampling devices suitable for use in anaerobic environments are available. The simplest device, useful in soft muds and mucks, consists of a length of thin-wall plastic or metal tubing. The tube is pushed into the soil to the desired depth, and the open end is sealed with a rubber stopper. The entire assembly then is withdrawn. The core should remain in place because of the suction effect exerted by the closed air chamber above the core. In deep water, a remote-operating core sampler, such as the K-B type (Wildlife Supply Co., or equivalent), may be required. Fine-grained material may be sampled by inserting a large bore hypodermic syringe or cannula through holes drilled through the side of the coring tube. If a core is to be subdivided, contaminants from the coring device should be removed by trimming the perimeter of the core with sterile instruments.

Sample Containers

Samples for microbiological examination must be collected and held in containers that have been carefully cleaned and sterilized by autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for at least 15 minutes. Narrow-mouth bottles (milk dilution) are the preferred sample containers. Caps or stoppers must be loosened during autoclaving to allow the steam to contact all surfaces. Alternatively, dry glassware may be sterilized in a hot air oven at 170 °C for a minimum of 2 hours. Presterilized plastic bags (Whirl-Pak, or equivalent) are commercially available and may be suitable for soil or bottom-material samples but are not recommended for collection of water samples for bacterial analysis.

Sample containers must be constructed of a material that can be sterilized and that is resistant to the solvent action of water. Borosilicate glass or plastic that can be autoclaved without distortion or the production of toxic compounds are acceptable materials. Containers made of polypropylene and teflon are autoclavable.

Containers may be of any suitable size and shape; they must allow a sufficient volume of sample to be collected and maintain the sample uncontaminated until analyses are complete. When the sample is collected, ample air space must be left in the container to facilitate mixing of the sample by shaking.

Bottle closures must be water tight. Ground-glass-stoppered bottles are acceptable, as are bottles with plastic screwcap closures, provided that, during sterilization, no bacteriostatic or nutritive compounds are produced.

Dechlorination

A dechlorinating agent should be added to sample bottles used to collect water containing residual chlorine. Sodium thiosulfate is a satisfactory dechlorinating agent that will neutralize any residual chlorine and prevent continuing bacteriocidal action prior to sample processing. Add 0.1 mL of a 10-percent solution of sodium thiosulfate to each 120-mL sample container prior to sterilization (American Public Health Association and others, 1985). This concentration of sodium thiosulfate will neutralize a sample containing about 15 mg/L of residual chlorine.

Chelating Agent

A chelating agent should be added to water samples suspected of containing greater than 0.01 mg/L of heavy metals, such as copper, nickel, or zinc. Add 0.3 mL of a 15-percent solution of ethylenediaminetetraacetic acid (EDTA) tetrasodium salt to each 120-mL sample bottle prior to sterilization (Bordner and others, 1978).

Preservation and Storage

A general rule in working with micro-organisms is that the more rapidly the samples are processed, the more accurate the results will be. The chemical and biological characteristics of the sample will change during storage and no longer will be representative of conditions at the sampling site. Therefore, microbiological analysis should begin as soon as possible after collection, preferably within 1 hour and not more than 6 hours. Samples should be iced or refrigerated, but never frozen, and kept in the dark during the holding period. Sample containers should not be totally immersed in water during storage. Under no circumstances should samples be exposed to direct sunlight. If it is impossible to transport the sample to the laboratory within the required period of time, onsite analytical procedures should be considered.

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Standard Plate Count (Membrane-Filter Method)
(B-0001-85)

Parameter and Code:
Total plate count, TPC medium, 35 °C, 24 hours
(colonies/mL): 31751

The standard plate count is an empirical method for estimating the aerobic, heterotrophic bacterial population in a water sample. Because the nutrient and environmental requirements of certain bacteria are unique, the colony counts derived by this method generally underestimate the natural population. Anaerobic bacteria and many species of autotrophic bacteria will not grow on the specified medium, and for these, other methods must be used.

1. Applications

The method is applicable for all water with a dissolved-solids concentration of less than 20,000 mg/L. The test is performed using the agar-plate method (Bordner and others, 1978; American Public Health Association and others, 1985).

2. Summary of method

The sample is filtered onsite immediately after collection, and the filter is placed on tryptone glucose extract (TPC) agar. After incubation at 35±0.5 °C for 24±2 hours, the colonies are counted. Staining is used to enhance the contrast between the bacterial colonies and the filter.

3. Interferences

3.1 Suspended materials may not permit the filtration of sample volumes sufficient to produce significant results. Water samples with a large suspended-solids concentration may be divided between two or more membrane filters.

3.2 Some species of bacteria and fungi exhibit a spreading type of growth, and a single colony may cover the entire surface of the filter, obscuring other colonies.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

Figure 1.--Portable water laboratory. (Photograph courtesy of Millipore Corp., Bedford, Mass.)

4.1 Alcohol burner, glass or metal, containing ethyl alcohol for flame sterilizing of forceps.

4.2 Aluminum seals, one piece, 20 mm.

4.3 Bottles, milk dilution, screwcap.

4.4 Bottles, serum.

4.5 Crimper, for attaching aluminum seals.

4.6 Decapper, for removing aluminum seals from spent tubes.

4.7 Filter-holder assembly* and syringe that has a two-way valve* or vacuum hand pump.

4.8 Forceps*, stainless steel, smooth tips.

4.9 Graduated cylinders, 100-mL capacity.

4.10 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.11 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.12 Incubator*, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.13 Membrane filters, white, grid, sterile, 0.45-µm pore size, 47-mm diameter, and absorbent pads.

4.14 Microscope, binocular wide-field dissecting-type, and fluorescent lamp.

4.15 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.16 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.17 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.18 Plastic petri dishes with covers, disposable, sterile, 50×12 mm.

4.19 Rubber stoppers, 13×20 mm.

4.20 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

Figure 2.--Portable heaterblock incubator. (Photograph courtesy of Millipore Corp., Bedford, Mass.)

4.21 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.22 Thermometer, having a temperature range of at least 40 to 100 °C.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 Distilled or deionized water.

5.3 Ethyl alcohol, 95-percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methyl alcohol also may be used for sterilization.

5.4 Methyl alcohol, absolute, for sterilizing filter-holder assembly.

5.5 Methylene blue staining solution. Add 3 g methylene blue dye to 300 mL of 95-percent ethyl alcohol. Dissolve 0.1 g of potassium hydroxide (KOH) in 1 L of distilled water. Add to the alcoholic methylene blue solution and mix well.

5.6 Tryptone glucose extract agar. Prepare medium according to manufacturer's instructions, using agar. Heat while stirring vigorously until the solution becomes clear. Remove from heat immediately when clear. (Prevent scorching or boiling over of the medium.) The agar must be dispensed into suitably capped containers and sterilized in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes before the medium is added to presterilized petri dishes (see 6.1).

6. Analysis

The volume of the sample to be filtered depends on the expected bacterial density of the water being tested, but the volume should be enough that, after incubation, at least one of the membrane filters will contain from 20 to 150 colonies. When there are no existing data on the bacterial density of a given sample, the quantities must be determined by trial. The following guidelines may be helpful for unknown water: unpolluted ground water, 10- and 50-mL samples; unpolluted surface water, 0.001-, 0.01-, 0.1-, and 1-mL samples.

6.1 Pour the agar medium at 45 to 50 °C into a petri dish bottom to a depth of about 4 mm (6-7 mL). Replace petri dish tops (not tightly, to prevent excessive condensation) and allow agar to solidify.

6.2 Sterilize filter-holder assembly (Note 1). In the laboratory, wrap the funnel and filter base parts of the assembly separately in kraft paper or polypropylene bags and sterilize in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Steam must contact all surfaces to ensure complete sterilization. Cool to room temperature before use.

Note 1: Onsite sterilization of filter-holder assembly needs to be in accordance with the manufacturer's instructions but usually involves application and ignition of methyl alcohol to produce formaldehyde. Autoclave sterilization in the laboratory prior to the trip to the sampling site is preferred. Sterilization must be performed at all sites.

6.3 Assemble the filter holder and, using flame-sterilized forceps (Note 2), place a sterile membrane filter over the porous plate of the assembly, grid side up. Carefully place funnel on filter to avoid tearing or creasing the membrane.

Note 2: Flame-sterilized forceps--Dip forceps in ethyl or methyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

6.4 Shake the sample vigorously about 25 times to obtain an equal distribution of bacteria throughout the sample before transferring a measured portion of the sample to the filter-holder assembly.

6.4.1 If the volume of sample to be filtered is 10 mL or more, transfer the measured sample directly onto the dry membrane.

6.4.2 If the volume of the sample is between 1 and 10 mL, pour about 20 mL sterilized buffered dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of bacteria.

6.4.3 If the volume of original water sample is less than 1 mL, proceed as in 6.4.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle (Note 3) in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Filter this volume
1:10	11 milliliters of original sample	1 milliliter of 1:10 dilution
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	1 milliliter of 1:10 dilution	1 milliliter of 1:1,000 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution

Note 3: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be filtered within 20 minutes after preparation.

6.5 Apply vacuum and filter the sample. When vacuum is applied using a syringe fitted with a two-way valve, proceed as follows: Attach the filter-holder assembly to the inlet of the two-way valve with plastic tubing. Draw the syringe plunger very slowly on the initial stroke to avoid the danger of air lock before the assembly fills with water. Push the plunger forward to expel air from the syringe. Continue until the entire sample has been filtered. If the filter balloons or develops bubbles during sample filtration, disassemble the two-way valve and lubricate the rubber valve plugs lightly with stopcock grease. If a vacuum hand pump is used, do not exceed a pressure of 25 cm to avoid damage to bacteria.

6.6 Rinse sides of funnel twice with 20 to 30 mL of sterile buffered dilution water while applying vacuum.

6.7 Maintaining the vacuum, remove the funnel from the base of the filter-holder assembly and, using flame-sterilized forceps, remove the membrane filter from the base and place it on the agar in the plastic petri dish, grid side up, using a rolling action at one edge. Use care to avoid trapping air bubbles under the membrane (Note 4).

Note 4: Hold the funnel while removing the membrane filter and place it back on the base of the assembly when the membrane filter has been removed. Placement of the funnel on anything but the base of the assembly may result in contamination of the funnel.

6.8 Place top on petri dish and proceed with filtration of the next volume of water. Filter in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations.

6.9 Clearly mark the lid of each plastic petri dish indicating location, time of collection, time of incubation, sample number, and sample volume. Use a waterproof felt-tip marker or grease pencil.

6.10 Inspect the membrane in each petri dish for uniform contact with the agar. If air bubbles are present under the filter (indicated by bulges), remove the filter using sterile forceps and roll onto the agar again.

6.11 Close the plastic petri dish by firmly pressing down on the top.

6.12 Incubate the filters in the tightly closed petri dishes in an inverted position (agar and filter at the top) at 35±0.5 °C for 24±2 hours. Filters need to be incubated within 20 minutes after placement on medium.

6.13 After incubation, saturate an absorbent pad with 1.8 mL of methylene blue staining solution.

6.14 Transfer incubated filter with developed colonies to the newly saturated pad and wait 15 minutes.

6.15 Count the colonies, which will be dark blue against a lighter color background. The counts are best made using 10X to 15X magnification. Illumination is not critical.

6.16 Autoclave all cultures at 121 °C at 1.05 kg/cm2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 If only one filter has a colony count between the ideal of 20 and 150, use the equation:

$$\text{Colonies/mL} = \frac{\text{Number of colonies counted}}{\text{Volume of original sample filtered (milliliters)}} .$$

7.2 If all filters have colony counts less than the ideal of 20 colonies or greater than 150 colonies, calculate using the equations in 7.5 for only those filters having at least one colony and not having colonies too numerous to count. Report results as number per milliliter, followed by the statement, "Estimated count based on nonideal colony count."

7.3 If no filters contain colonies, report a maximum estimated value. Assume a count of one colony for the largest sample volume filtered, then calculate using the equation in 7.1. Report the results as less than (<) the calculated value.

7.4 If all filters have colonies too numerous to count, report a minimum estimated value. Assume a count of 150 for the smallest sample volume filtered, then calculate using the equation in 7.1. Report the results as greater than (>) the calculated value.

7.5 Sometimes two or more filters of a series will produce colony counts within the ideal counting range. Make colony counts for all such filters. The method for calculating and averaging is as follows (Note 5):

$$\begin{array}{r} \text{Volume filter 1} \\ +\ \text{Volume filter 2} \\ \hline \text{Volume sum} \end{array} \qquad \begin{array}{r} \text{Colony count filter 1} \\ +\ \text{Colony count filter 2} \\ \hline \text{Colony count sum} \end{array}$$

$$\text{Colonies/mL} = \frac{\text{Colony count sum}}{\text{Volume sum (milliliters)}} .$$

Note 5: Do not calculate the total colonies per milliliter for each volume filtered and then average the results.

8. Reporting of results

Report number of colonies per milliliter to two significant figures and designate as "standard plate count at 35 °C." Never report a count as less than one.

9. Precision

No numerical precision data are available.

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R. H., Winter, J. A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Total Coliform Bacteria (Membrane-Filter Method)

Immediate Incubation Test
(B-0025-85)

Parameter and Code:
Coliform, membrane filter, immediate M-Endo medium
(colonies/100 mL): 31501

The standard test for presence of members of the coliform group may be made by using the following membrane-filter method or by using the multiple-tube test described in the "Presumptive Test," "Presumptive Onsite Test," and "Confirmation Test" subsections in the "Total Coliform Bacteria (Most-Probable-Number, MPN, Method)" section, or in Bordner and others (1978) and American Public Health Association and others (1985).

The coliform group is defined as the aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose with gas formation at 35 °C within 48 hours. For the purposes of the methods described in the following paragraphs, the coliform group is defined as all the organisms that produce colonies with a golden-green metallic sheen when incubated at 35 °C on M-Endo medium within 24 hours.

1. Applications

The membrane-filter method is applicable to fresh and saline water. The test is performed using the agar-plate method.

2. Summary of method

The sample is filtered onsite immediately after collection, and the filter is placed on a nutrient medium designed to stimulate the growth of members of the coliform group and to suppress the growth of most noncoliform organisms. After incubation at 35±0.5 °C for 22 to 24 hours, the colonies are counted.

3. Interferences

3.1 Suspended materials may inhibit the filtration of sample volumes sufficient to produce significant results. Coliform colony formation on the filter may be inhibited by large numbers of noncoliform colonies, by the presence of algal filaments and detritus, or by toxic substances.

3.2 Water samples having a large suspended-solids concentration may be divided between two or more membrane filters. The multiple-tube test, which is described in this chapter, will give the most reliable results when suspended-solids concentration are large and coliform counts are small.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 Alcohol burner, glass or metal, containing ethyl alcohol for flame sterilizing of forceps.

4.2 Aluminum seals, one piece, 20 mm.

4.3 Bottles, milk dilution, screwcap.

4.4 Bottles, serum.

4.5 Crimper, for attaching aluminum seals.

4.6 Decapper, for removing aluminum seals from spent tubes.

4.7 Filter-holder assembly* and syringe that has a two-way valve* or vacuum hand pump.

4.8 Forceps*, stainless steel, smooth tips.

4.9 Graduated cylinders, 100-mL capacity.

4.10 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.11 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.12 Incubator*, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.13 Membrane filters, white, grid, sterile, 0.45- or 0.7-µm mean pore size, 47-mm diameter, and absorbent pads.

4.14 Microscope, binocular wide-field dissecting-type, and fluorescent lamp.

4.15 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.16 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.17 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.18 Plastic petri dishes with covers, disposable, sterile, 50×12 mm.

4.19 Rubber stoppers, 13×20 mm.

4.20 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.21 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.22 Thermometer, having a temperature range of at least 40 to 100 °C.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 Distilled or deonized water.

5.3 Ethyl alcohol, 95-percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methyl alcohol also may be used for sterilization.

5.4 M-Endo agar. Add 4.8 g of M-Endo broth MF to 100 mL 2 percent nondenatured ethyl alcohol, then add 1.5 g agar. Stir well and place the beaker containing the medium in a boiling water bath and heat the medium to 96 °C, stirring constantly. Do not autoclave the medium. When the medium begins to boil, promptly remove from heat and cool to 45 to 50 °C. Pour

to a depth of 4 mm (6-7 mL) in 50-mm petri dish bottoms. When the medium solidifies, store the prepared petri dishes at 2 to 10 °C for a maximum period of 4 to 5 days.

5.5 Methyl alcohol, absolute, for sterilizing filter-holder assembly.

6. Analysis

The volumes of the sample to be filtered depend on the expected bacterial density of the water being tested, but the volumes should be enough that, after incubation, at least one of the membrane filters will contain from 20 to 80 total coliform colonies and not more than 200 of all types (total coliform plus noncoliform colonies). It is extremely important that the limitation on total coliform colonies be observed, otherwise the medium used in the method may not support development of the characteristic metallic sheen. If the upper limit of 80 total coliform colonies per membrane filter is exceeded, interferences from crowding, deposits of extraneous material, and other factors will give questionable results.

The lower limit of 20 total coliform colonies per membrane filter is arbitrarily set as a number below which statistical validity becomes questionable. However, even with a bacterial population of 200 or fewer colonies (coliform plus noncoliform) per 100 mL of sample, fewer than 20 total coliform colonies will be present on the membrane filter of some samples.

The following sample volumes are suggested for filtration:

1. Unpolluted raw surface water: 0.1-, 0.4-, 1.5-, 6-, 25-, and 100-mL samples will include a range of 20 to 80,000 total coliform colonies per 100 mL using the criterion of 20 to 80 total coliform colonies on a filter as an ideal determination.

2. Polluted raw surface water: 0.002-, 0.006-, 0.025-, 0.1-, 0.4-, and 1.6-mL samples will include a range of 1,200 to 4,000,000 total coliform colonies per 100 mL.

6.1 Sterilize filter-holder assembly (Note 1). In the laboratory, wrap the funnel and filter base parts of the assembly separately in kraft paper or polypropylene bags and sterilize in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Steam must contact all surfaces to ensure complete sterilization. Cool to room temperature before use.

Note 1: Onsite sterilization of filter-holder assembly needs to be in accordance with the manufacturer's instructions but usually involves application and ignition of methyl alcohol to produce formaldehyde. Autoclave sterilization in the laboratory prior to the trip to the sampling site is preferred. Sterilization must be performed at all sites.

6.2 Assemble the filter holder and, using flame-sterilized forceps (Note 2), place a sterile membrane filter over the porous plate of the assembly, grid side up. Carefully place funnel on filter to avoid tearing or creasing the membrane.

Note 2: Flame-sterilized forceps--Dip forceps in ethyl or methyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

6.3 Shake the sample vigorously about 25 times to obtain an equal distribution of bacteria throughout the sample before transferring a measured portion of the sample to the filter-holder assembly.

6.3.1 If the volume of sample to be filtered is 10 mL or more, transfer the measured sample directly onto the dry membrane.

6.3.2 If the volume of the sample is between 1 and 10 mL, pour about 20 mL sterilized buffered dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of bacteria.

6.3.3 If the volume of original water sample is less than 1 mL, proceed as in 6.3.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle (Note 3) in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Filter this volume
1:10	11 milliliters of original sample	1 milliliter of 1:10 dilution
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	1 milliliter of 1:10 dilution	1 milliliter of 1:1,000 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution

Note 3: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be filtered within 20 minutes after preparation.

6.4 Apply vacuum and filter the sample. When vacuum is applied using a syringe fitted with a two-way valve, proceed as follows: Attach the filter-holder assembly to the inlet of the two-way valve with plastic tubing. Draw the syringe plunger very slowly on the initial stroke to avoid the danger of air lock before the assembly fills with water. Push the plunger forward to expel air from the syringe. Continue until the entire sample has been filtered. If the filter balloons or develops bubbles during sample filtration, disassemble the two-way valve and lubricate the rubber valve plugs lightly with stopcock grease. If a vacuum hand pump is used, do not exceed a pressure of 25 cm to avoid damage to bacteria.

6.5 Rinse sides of funnel twice with 20 to 30 mL of sterile buffered dilution water while applying vacuum.

6.6 Maintaining the vacuum, remove the funnel from the base of the filter-holder assembly and, using flame-sterilized forceps, remove the membrane filter from the base and place it on the agar in the plastic petri dish, grid side up, using a rolling action at one edge. Use care to avoid trapping air bubbles under the membrane (Note 4).

Note 4: Hold the funnel while removing the membrane filter and place it back on the base of the assembly when the membrane filter has been removed. Placement of the funnel on anything but the base of the assembly may result in contamination of the funnel.

6.7 Place top on petri dish and proceed with filtration of the next volume of water. Filter in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations.

6.8 Clearly mark the lid of each plastic petri dish indicating location, time of collection, time of incubation, sample number, and sample volume. Use a waterproof felt-tip marker or grease pencil.

6.9 Inspect the membrane in each petri dish for uniform contact with the agar. If air bubbles are present under the filter (indicated by bulges), remove the filter using sterile forceps and roll onto the agar again.

6.10 Close the plastic petri dish by firmly pressing down on the top.

6.11 Incubate the filters in the tightly closed petri dishes in an inverted position (agar and filter at the top) at 35±0.5 °C for 22 to 24 hours. Filters need to be incubated within 20 minutes after placement on medium.

6.12 Using forceps, remove the filters and allow to dry for at least 1 minute on an absorbent surface. Membranes that have colonies having poor sheen production can be allowed to dry completely. This will enhance sheen production.

6.13 Count the number of coliform sheen colonies, that is, dark colonies having a golden-green metallic sheen. The sheen may cover the entire colony or appear only in a central area or on the periphery. The color plate in Millipore Corp. (1973, p. 42) may be helpful in identifying total coliform colonies. The counts are best made using 10X to 15X magnification. Place the illuminator (fluorescent) as directly above the filter as possible.

6.14 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 If only one filter has a colony count between the ideal of 20 and 80, use the equation:

$$\text{Total coliform colonies/100 mL} = \frac{\text{Number of colonies counted} \times 100}{\text{Volume of original sample filtered (milliliters)}} .$$

7.2 If all filters have counts less than the ideal of 20 colonies or greater than 80 colonies, calculate using the equations in 7.5 for only those filters having at least one colony and not having colonies too numerous to count. Report results as number per 100 mL, followed by the statement, "Estimated count based on nonideal colony count."

7.3 If no filters develop characteristic total coliform colonies, report a maximum estimated value. Assume a count of one colony for the largest sample volume filtered, then calculate using the equation in 7.1. Report the results as less than (<) the calculated value per 100 mL.

7.4 If all filters have colonies too numerous to count, report a minimum estimated value. Assume a count of 80 total coliform colonies for the smallest sample volume filtered, then calculate using the equation in 7.1. Report the results as greater than (>) the calculated value per 100 mL.

7.5 Sometimes two or more filters of a series will produce colony counts within the ideal counting range. Make colony counts for all such filters. The method for calculating and averaging is as follows (Note 5):

$$\frac{\begin{array}{r}\text{Volume filter 1} \\ + \text{Volume filter 2}\end{array}}{\text{Volume sum}} \qquad \frac{\begin{array}{r}\text{Colony count filter 1} \\ + \text{Colony count filter 2}\end{array}}{\text{Colony count sum}}$$

$$\text{Total coliform colonies/100 mL} = \frac{\text{Colony count sum} \times 100}{\text{Volume sum (milliliters)}} .$$

Note 5: Do not calculate the total coliform colonies per 100 mL for each volume filtered and then average the results.

8. Reporting of results

Report total coliform concentration as total coliform colonies per 100 mL, M-Endo immediate incubation at 35 °C as follows: less than 10 colonies, whole numbers; 10 or more colonies, two significant figures.

9. Precision

No numerical precision data are available.

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R. H., Winter, J. A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Millipore Corp., 1973, Biological analysis of water and wastewater: Bedford, Mass., Application Manual AM302, 84 p.

Total Coliform Bacteria (Membrane-Filter Method)

Delayed Incubation Test
(B-0030-85)

Parameter and Code:
Coliform, membrane filter, delayed M-Endo medium
(colonies/100 mL): 31503

The delayed incubation test is not a substitute for the immediate incubation test. Results obtained from these two tests are not comparable.

1. Applications

The method is applicable to fresh and saline water. It is used when it is not possible to begin incubation of samples at the specified temperature within 6 hours of collection. Within 72 hours, the membranes must be transferred to a nutrient medium and normal incubation started. The applicability of the delayed incubation test for a specific water source can be determined by comparative test procedures with conventional methods.

2. Summary of method

The sample is filtered onsite immediately after collection, and the filter is placed on a holding medium and shipped to the laboratory. The holding medium maintains the viability of the coliform organisms and generally does not permit visible growth during the time of transit. The coliform determination is completed in the laboratory by transferring the membrane to a growth medium, incubating at 35±0.5 °C for 20 to 22 hours, and counting the typical coliform colonies.

3. Interferences

3.1 Suspended materials may inhibit the filtration of sample volumes sufficient to produce significant results. Coliform colony formation on the filter may be inhibited by large numbers of noncoliform colonies, by the presence of algal filaments and detritus, or by toxic substances.

3.2 Water samples having a large suspended-solids concentration may be divided between two or more membrane filters.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 Alcohol burner, glass or metal, containing ethyl alcohol for flame sterilizing of forceps.

4.2 Aluminum seals, one piece, 20 mm.

4.3 Bottles, milk dilution, screwcap.

4.4 Bottles, serum.

4.5 Crimper, for attaching aluminum seals.

4.6 Decapper, for removing aluminum seals from spent tubes.

4.7 Filter-holder assembly* and syringe that has a two-way valve* or vacuum hand pump.

4.8 Forceps*, stainless steel, smooth tips.

4.9 Graduated cylinders, 100-mL capacity.

4.10 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.11 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.12 Incubator*, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.13 Membrane filters, white, grid, sterile, 0.45- or 0.7-µm mean pore size, 47-mm diameter, and absorbent pads.

4.14 Microscope, binocular wide-field dissecting-type, and fluorescent lamp.

4.15 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.16 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.17 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.18 Plastic petri dishes with covers, disposable, sterile, 50×12 mm.

4.19 Rubber stoppers, 13×20 mm.

4.20 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.21 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.22 Thermometer, having a temperature range of at least 40 to 100 °C.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 Cyclohexamide. Dissolve 500 mg of cyclohexamide in 10 mL distilled water. The cyclohexamide solution needs to be refrigerated; storage should not exceed 6 months.

CAUTION.--Cyclohexamide is a powerful skin irritant and needs to be handled according to the manufacturer's directions. Add 1 mL of cyclohexamide solution to 100 mL of M-Endo preservative medium described in 5.6.

5.3 Distilled or deonized water.

5.4 Ethyl alcohol, 95-percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methyl alcohol also may be used for sterilization.

5.5 M-Endo agar. Add 4.8 g of M-Endo broth MF to 100 mL 2 percent nondenatured ethyl alcohol, then add 1.5 g agar. Stir well and place the beaker containing the medium in a boiling water bath and heat the medium to 96 °C, stirring constantly. Do not autoclave the medium. When the medium begins to boil, promptly remove from heat and cool to 45 to 50 °C. Pour

to a depth of 4 mm (6-7 mL) in 50-mm petri dish bottoms. When the medium solidifies, store the prepared petri dishes at 2 to 10 °C for a maximum period of 4 to 5 days.

5.6 M-Endo preservative medium. Add 4.8 g M-Endo broth MF to 100 mL 2 percent nondenatured ethyl alcohol in a beaker and stir for 3 minutes. Place the beaker on a hot plate and heat to boiling, stirring constantly. (Prevent scorching or boiling over of the medium.) When the medium reaches the boiling point, promptly remove from heat and cool to less than 45 °C. Do not sterilize by autoclaving. To 100 mL of M-Endo broth, add 3.2 mL 12 percent sodium benzoate solution. Store the finished medium in the dark at 2 to 10 °C for a maximum period of 4 to 5 days.

5.7 Methyl alcohol, absolute, for sterilizing filter-holder assembly.

5.8 Sodium benzoate solution, 12 percent. Dissolve 12 g sodium benzoate ($C_7H_5NaO_2$) in sufficient distilled water to make 100 mL. Sterilize by filtration through a 0.45-μm pore-size membrane filter or autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Discard unused solution after 6 months.

6. Analysis

The volumes of sample to be filtered depend on the expected bacterial density of the water being tested, but the volumes should be enough that, after incubation, at least one of the membrane filters will contain from 20 to 80 total coliform colonies and not more than 200 of all types (total coliform plus noncoliform colonies). It is extremely important that the limitation on total coliform colonies be observed, otherwise the medium used in the method may not support development of the characteristic metallic sheen. If the upper limit of 80 total coliform colonies per membrane filter is exceeded, interferences from crowding, deposits of extraneous material, and other factors will give questionable results.

The lower limit of 20 total coliform colonies per membrane filter is arbitrarily set as a number below which statistical validity becomes questionable. However, even with a bacterial population of 200 or fewer colonies (coliform plus noncoliform) per 100 mL of sample, fewer than 20 total coliform colonies will be present on the membrane filter of some samples.

The following sample volumes are suggested for filtration:

1. Unpolluted raw surface water: 0.1-, 0.4-, 1.5-, 6-, 25-, and 100-mL samples will include a range of 20 to 80,000 total coliform colonies per 100 mL using the criterion of 20 to 80 total coliform colonies on a filter as an ideal determination.

2. Polluted raw surface water: 0.002-, 0.006-, 0.025-, 0.1-, 0.4-, and 1.6-mL samples will include a range of 1,200 to 4,000,000 total coliform colonies per 100 mL.

6.1 Place a sterile absorbent pad in the bottom (larger half) of each sterile plastic petri dish using flame-sterilized forceps (Note 1).

Note 1: Flame-sterilized forceps--Dip forceps in ethyl or methyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

6.2 Saturate each pad with about 2 mL M-Endo preservative medium and tilt the petri dish to expel excess liquid. Replace petri dish tops (not tightly to prevent excessive condensation).

6.3 Sterilize filter-holder assembly (Note 2). In the laboratory, wrap the funnel and filter base parts of the assembly separately in kraft paper or polypropylene bags and sterilize in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Cool to room temperature before use.

Note 2: Onsite sterilization of filter-holder assembly needs to be in accordance with the manufacturer's instructions but usually involves application and ignition of methyl alcohol to produce formaldehyde. Autoclave sterilization in the laboratory prior to the trip to the sampling site is preferred. Sterilization must be performed at all sites.

6.4 Assemble the filter holder and, using flame-sterilized forceps, place a sterile membrane filter over the porous plate of the assembly, grid side up. Carefully place funnel on filter to avoid tearing or creasing the membrane.

6.5 Shake the sample vigorously about 25 times to obtain an equal distribution of bacteria throughout the sample before transferring a measured portion of the sample to the filter-holder assembly.

6.5.1 If the volume of sample to be filtered is 10 mL or more, transfer the measured sample directly onto the dry membrane.

6.5.2 If the volume of sample is between 1 and 10 mL, pour about 20 mL sterilized buffered dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of bacteria.

6.5.3 If the volume of original water sample is less than 1 mL, proceed as in 6.5.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle (Note 3) in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Filter this volume
1:10	11 milliliters of original sample	1 milliliter of 1:10 dilution
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	1 milliliter of 1:10 dilution	1 milliliter of 1:1,000 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution

Note 3: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be filtered within 20 minutes after preparation.

6.6 Apply vacuum and filter the sample. When vacuum is applied using a syringe fitted with a two-way valve, proceed as follows: Attach the filter-holder assembly to the inlet of the two-way valve with plastic tubing. Draw the syringe plunger very slowly on the initial stroke to avoid the danger of air lock before the assembly fills with water. Push the plunger forward to expel air from the syringe. Continue until the entire sample has been filtered. If the filter balloons or develops bubbles during sample filtration, disassemble the two-way valve and lubricate the rubber valve plugs lightly with stopcock grease. If a vacuum hand pump is used, do not exceed a pressure of 25 cm to avoid damage to bacteria.

6.7 Rinse sides of funnel twice with 20 to 30 mL of sterile buffered dilution water while applying vacuum.

6.8 Maintaining the vacuum, remove the funnel from the base of the filter-holder assembly and, using flame-sterilized forceps, remove the membrane filter from the base and place it on the broth-soaked absorbent pad in the plastic petri dish, grid side up, using a rolling action at one edge. Use care to avoid trapping air bubbles under the membrane (Note 4).

Note 4: Hold the funnel while removing the membrane filter and place it back on the base of the assembly when the membrane filter has been removed. Placement of the funnel on anything but the base of the assembly may result in contamination of the funnel.

6.9 Place top on petri dish and proceed with filtration of the next volume of water. Filter in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations.

6.10 Clearly mark the lid of each plastic petri dish indicating location, time of collection, time of incubation, sample number, and sample volume. Use a waterproof felt-tip marker or grease pencil.

6.11 Inspect the membrane in each petri dish for uniform contact with the saturated pad. If air bubbles are present under the filter (indicated by bulges), remove the filter using sterile forceps and roll onto the absorbent pad again.

6.12 Close the plastic petri dish by firmly pressing down on the top.

6.13 Place the petri dish containing the membrane filter in an insulated shipping container and mail. The container needs to arrive in the laboratory within 72 hours. Limited bacterial growth sometimes occurs on the preservative medium when high temperatures are encountered.

6.14 In the laboratory, transfer the membrane from the petri dish in which it was shipped to a fresh sterile petri dish containing M-Endo agar. Use sterile forceps and ensure a good contact between the filter and medium.

6.15 Incubate the filters in the tightly closed petri dishes in an inverted position (agar and filter at the top) at 35±0.5 °C for 20 to 22 hours. Filters need to be incubated within 20 minutes after placement on medium.

6.16 Using forceps, remove the filters and allow to dry for at least 1 minute on an absorbent surface. Membranes that have colonies having poor sheen production can be allowed to dry completely. This will enhance sheen production.

6.17 Count the number of coliform sheen colonies, that is, dark colonies having a golden-green metallic sheen. The sheen may cover the entire colony or appear only in a central area or on the periphery. The color plate in Millipore Corp. (1973, p. 42) may be helpful in identifying total coliform colonies. The counts are best made using 10X to 15X magnification. Place the illuminator (fluorescent) as directly above the filter as possible.

6.18 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 If only one filter has a colony count between the ideal of 20 and 80, use the equation:

$$\text{Total coliform colonies/100 mL} = \frac{\text{Number of colonies counted} \times 100}{\text{Volume of original sample filtered (milliliters)}} .$$

7.2 If all filters have counts less than the ideal of 20 colonies or greater than 80 colonies, calculate using the equations in 7.5 for only those filters having at least one colony and not having colonies too numerous to count. Report results as number per 100 mL, followed by the statement, "Estimated count based on nonideal colony count."

7.3 If no filters develop characteristic total coliform colonies, report a maximum estimated value. Assume a count of one colony for the largest sample volume filtered, then calculate using the equation in 7.1. Report the results as less than (<) calculated value per 100 mL.

7.4 If all filters have colonies too numerous to count, report a minimum estimated value. Assume a count of 80 total coliform colonies for the smallest sample volume filtered, then calculate using the equation in 7.1. Report the results as greater than (>) the calculated value per 100 mL.

7.5 Sometimes two or more filters of a series will produce colony counts within the ideal counting range. Make colony counts for all such filters. The method for calculating and averaging is as follows (Note 5):

$$\begin{array}{rr} \text{Volume filter 1} & \text{Colony count filter 1} \\ + \text{Volume filter 2} & + \text{Colony count filter 2} \\ \hline \text{Volume sum} & \text{Colony count sum} \end{array}$$

$$\text{Total coliform colonies/100 mL} = \frac{\text{Colony count sum} \times 100}{\text{Volume sum (milliliters)}} .$$

Note 5: Do not calculate the total coliform colonies per 100 mL for each volume filtered and then average the results.

8. Reporting of results

Report the total coliform concentration as total coliform colonies per 100 mL, M-Endo delayed incubation at 35 °C as follows: less than 10 colonies, whole numbers; 10 or more colonies, two significant figures.

9. Precision

No numerical precision data are available.

10. References cited

Millipore Corp., 1973, Biological analysis of water and wastewater: Bedford, Mass., Application Manual AM302, 84 p.

Total Coliform Bacteria (Most-Probable-Number, MPN, Method)

Presumptive Test
(B-0035-85)

Parameter and Code:
Coliform, presumptive (MPN): 31507

1. Applications

This method is applicable to fresh and saline water, water having large suspended-solids concentration, and water having large populations of non-coliform bacteria.

2. Summary of method

Decimal dilutions of multiple sample aliquots are inoculated into lauryl tryptose broth. The cultures are incubated at 35±0.5 °C and examined after 24 and 48 hours for evidence of growth and gas production. The most probable number (MPN) of coliform organisms in the sample is determined from the distribution of gas-positive cultures among the inoculated tubes or serum bottles. Do not use the presumptive test unless the confirmation test (B-0045-85) also is done.

3. Interferences

Large concentrations of heavy metals or toxic chemicals may interfere when large volumes of sample are added to small volumes of concentrated lauryl tryptose broth. Certain noncoliform organisms can ferment lactose with gas formation.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 Aluminum seals, one piece, 20 mm.

4.2 Bottles, milk dilution, screwcap.

4.3 Bottles, serum.

4.4 Crimper, for attaching aluminum seals.

4.5 Culture tubes and durham (fermentation) tubes. Two combinations of culture tubes and durham (fermentation) tubes may be used. The choice will depend on the volume of water to be tested. The durham tube, used to detect gas production, must be completely filled with medium and at least partly submerged in the culture tube. The following combinations have been satisfactory:

4.5.1 For testing 10-mL aliquots, use borosilicate glass culture tubes, 20×150 mm; tube caps, 20 mm; and use borosilicate glass culture tubes, 10×75 mm, as durham tubes.

4.5.2 For testing 1-mL or small aliquots, use borosilicate glass culture tubes, 16×125 mm; tube caps, 16 mm; and use flint glass culture tubes, 6×50 mm, as durham tubes.

4.6 Culture-tube rack, galvanized, for 16- and 20-mm culture tubes.

4.7 Decapper, for removing aluminum seals from spent tubes.

4.8 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.9 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.10 Incubator*, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.11 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.12 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.13 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.14 Rubber stoppers, 13×20 mm.

4.15 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.16 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal

eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 Distilled or deionized water.

5.3 Lauryl tryptose broth. Use premixed lauryl tryptose broth or lauryl sulfate broth, and prepare according to directions on bottle label. The medium also may be prepared according to American Public Health Association and others (1985).

5.3.1 Place 10 mL of medium containing 71.2 g/L lauryl tryptose broth or lauryl sulfate broth in a 20×150-mm culture tube for each 10-mL aliquot of sample to be tested.

5.3.2 Place 10 mL of medium containing 35.6 g/L lauryl tryptose broth or lauryl sulfate broth in a 16×125-mm culture tube for each 1-mL or smaller aliquot of sample to be tested.

5.3.3 In each culture tube, place an inverted (mouth downward) durham tube (fig. 3). Sterilize culture tubes in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes as soon as possible after dispensing medium. Air will be expelled from the inverted durham tubes during heating; each will fill completely with medium during cooling. Discard any culture tubes in which air bubbles are visible in the durham tubes.

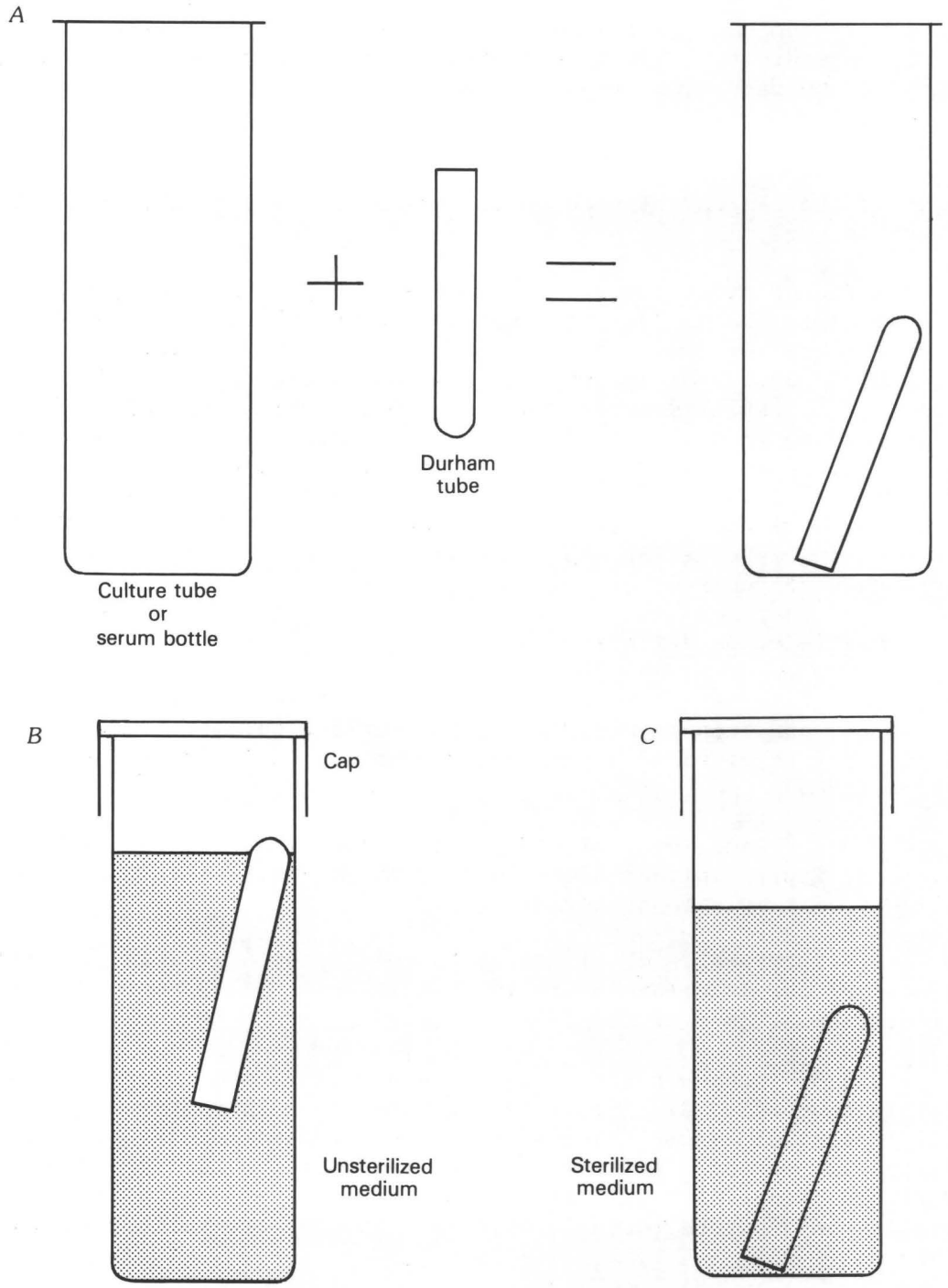


Figure 3.--Preparation of culture tube or serum bottle: (A) Invert durham tube inside culture tube or serum bottle; (B) add unsterilized medium and cap; (C) durham tube fills with medium following sterilization.

6. Analysis

Two questions must be answered when planning a multiple-tube test:

1. What volumes of water need to be tested?

2. How many culture tubes of each volume need to be tested?

Choose a range of volumes so positive and negative results are obtained throughout the range tested. The method fails if only positive or only negative results are obtained when all volumes are tested. The number of culture tubes used per sample volume depends on the precision required. The greater the number of tubes inoculated with each volume, the greater the precision, but the effort involved and expense also are increased. A five-tube series is described below. The following sample volumes are suggested:

1. Unpolluted raw surface water: 0.1-, 1-, and 10-mL samples will include an MPN range of <2 to ≥2,400 coliforms per 100 mL.

2. Polluted raw surface water: 0.001-, 0.01-, 0.1-, and 1-mL samples will include an MPN range of 20 to 240,000 coliforms per 100 mL.

6.1 Set up five culture tubes of lauryl tryptose broth for each sample volume to be tested.

6.1.1 If the volume to be tested is 0.1 mL or more, transfer the measured samples directly to the culture tubes using sterile pipets (Note 1).

6.1.2 If the volume of original water sample is less than 0.1 mL, proceed as in 6.1.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Size of inoculum
1:10	----------	0.1 milliliter of original sample
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	----------	0.1 milliliter of 1:100 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution
1:100,000	----------	0.1 milliliter of 1:10,000 dilution

Note 1: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be inoculated within 20 minutes after preparation.

6.2 Clearly mark each set of culture tubes indicating location, time of collection, sample number, and sample volume. Code each tube for easy identification.

6.3 Place the inoculated culture tubes in the culture-tube rack and incubate at 35±0.5 °C for 24±2 hours. Tubes must be maintained in an upright position.

6.4 Remove culture tubes from incubator and examine. Gas in any quantity in the durham tube, even a pinhead-sized bubble, constitutes a positive test (fig. 4). The appearance of an air bubble must not be confused with actual gas production. If the gas is formed as a result of fermentation, the broth medium will become cloudy. Active fermentation may be indicated by the continued appearance of small bubbles of gas in the medium outside the durham tube when the culture tube is shaken gently (Bordner and others, 1978; American Public Health Association and others, 1985).

6.5 After submitting all gas-positive culture tubes to the confirmation test (B-0045-85), autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

6.6 Return all gas-negative culture tubes to incubator and incubate at 35±0.5 °C for an additional 24±2 hours.

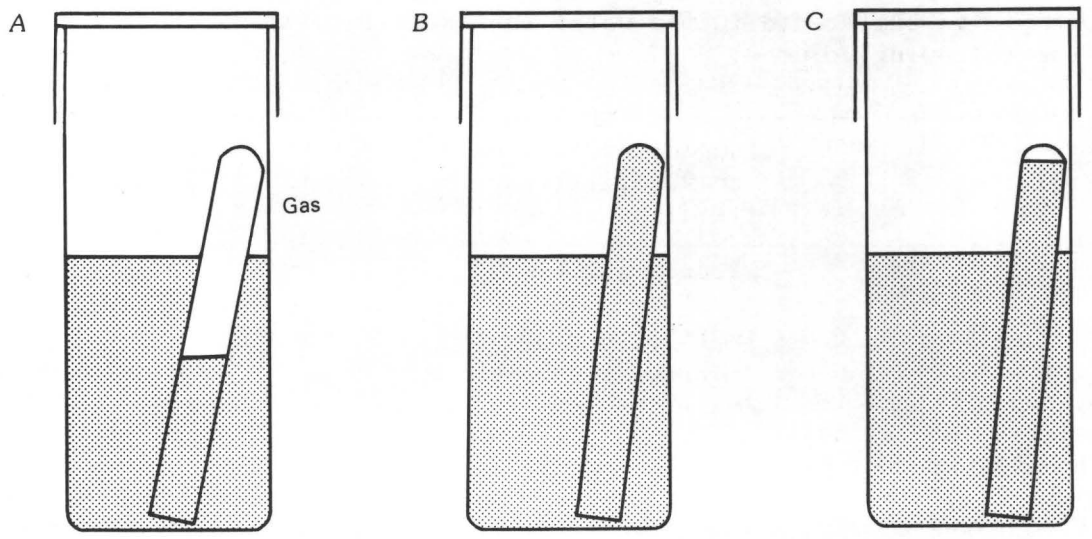


Figure 4.--Examination for gas formation: (A) Positive; (B) negative; (C) positive.

6.7 Remove culture tubes from incubator and examine for gas formation. Autoclave all remaining tubes of lauryl tryptose broth as in 6.5 before discarding.

7. Calculations

7.1 Record the number of gas-positive culture tubes at 24 and 48 hours occurring for all sample volumes tested.

7.2 When more than three volumes are tested, use results from only three of them when computing the MPN. To select the three dilutions for the MPN index, use as the first, the smallest sample volume in which all tests are positive (no larger sample volume having any negative results) and the next two succeeding smaller sample volumes (Bordner and others, 1978; American Public Health Association and others, 1985).

7.3 In the examples listed below, the number in the numerator represents positive culture tubes; the denominator represents the total number of tubes inoculated.

Example	Decimal dilutions				Combination of positives
	1 milliliter	0.1 milliliter	0.01 milliliter	0.001 milliliter	
a	5/5	5/5	2/5	0/5	5-2-0
b	5/5	4/5	2/5	0/5	5-4-2
c	0/5	1/5	0/5	0/5	0-1-0
d	5/5	3/5	1/5	1/5	5-3-2
e	5/5	3/5	2/5	0/5	5-3-2

In example c, the first three dilutions need to be taken to place the positive results in the middle dilution. When a positive result occurs in a dilution larger than the three chosen according to the guideline, as in d, it needs to be placed in the result for the largest chosen dilution as in e (Note 1).

(Note 1: The largest dilution has the smallest concentration of the sample; the largest dilution in the preceding table is 0.001.

7.4 The MPN for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-mL dilutions are used are listed in table 1. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value in table 1 needs to be corrected for the dilutions actually used. To do this, divide the value in table 1 by the dilution factor of the first number in the three-number sequence (the culture tubes having the largest concentration of the sample). For example, if dilutions of 0.1, 0.01, and 0.001 mL are used, divide the value in table 1 by 0.1 mL. MPN tables for other combinations of sample volumes and numbers of tubes at each level of inoculation are in American Public Health Association and others (1985).

Table 1.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used

[mL, milliliters; MPN, most probable number; ---, not applicable; modified from American Public Health Association and others, 1985]

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
0	0	0	<20	---	---
0	0	1	20	<5	70
0	1	0	20	<5	70
0	2	0	40	<5	11
1	0	0	20	<5	70
1	0	1	40	<5	110
1	1	0	40	<5	110
1	1	1	60	<5	150
1	2	0	60	<5	150
2	0	0	50	<5	130
2	0	1	70	10	170
2	1	0	70	10	170
2	1	1	90	20	210
2	2	0	90	20	210
2	3	0	120	30	280
3	0	0	80	10	190
3	0	1	110	20	250
3	1	0	110	20	250
3	1	1	140	40	340
3	2	0	140	40	340
3	2	1	170	50	460
4	0	0	130	30	310
4	0	1	170	50	460
4	1	0	170	50	460
4	1	1	210	70	630
4	1	2	260	90	780
4	2	0	220	70	670
4	2	1	260	90	780
4	3	0	270	90	800
4	3	1	330	110	930
4	4	0	340	120	930

Table 1.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used--Continued

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
5	0	0	230	70	700
5	0	1	310	110	890
5	0	2	430	150	1,100
5	1	0	330	110	930
5	1	1	460	160	1,200
5	1	2	630	210	1,500
5	2	0	490	170	1,300
5	2	1	700	230	1,700
5	2	2	940	280	2,200
5	3	0	790	250	1,900
5	3	1	1,100	310	2,500
5	3	2	1,400	370	3,400
5	3	3	1,800	440	5,000
5	4	0	1,300	350	3,000
5	4	1	1,700	430	4,900
5	4	2	2,200	570	7,000
5	4	3	2,800	900	8,500
5	4	4	3,500	1,200	10,000
5	5	0	2,400	680	7,500
5	5	1	3,500	1,200	10,000
5	5	2	5,400	1,800	14,000
5	5	3	9,200	3,000	32,000
5	5	4	16,000	6,400	58,000
5	5	5	≥24,000	---	---

7.5 Example: The following results were obtained with a five-tube series:

Volume (milliliters)	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Results	5/5	5/5	3/5	1/5	0/5.

Using 10^{-6}, 10^{-7}, and 10^{-8} mL sample volumes, the test results indicate a sequence of 5-3-1 for which the MPN (table 1) is 1,100. Dividing by 10^{-6}, the MPN is computed to be 11×10^8 total coliform bacteria per 100 mL and 95-percent confidence limits of 3.1×10^8 and 25×10^8 total coliform bacteria per 100 mL.

8. Reporting of results

Report total coliform concentrations as MPN total coliforms per 100 mL as follows: less than 10, whole numbers; 10 or more, two significant figures.

9. Precision

9.1 Precision of the MPN method increases as the number of culture tubes is increased. It increases rapidly as the number of tubes increases from 1 to 5, but then it increases at a slower rate making the gain, when using 10 tubes instead of 5, much less than is achieved by increasing the number of tubes from 1 to 5. Variance as a function of the number of tubes inoculated from a tenfold dilution series is given below:

Number of culture tubes at each dilution	Variance for tenfold dilution series
1	0.580
3	.335
5	.259
10	.183

9.2 The 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-mL dilutions are used are listed in table 1.

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R. H., Winter, J. A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Total Coliform Bacteria (Most-Probable-Number, MPN, Method)

Presumptive Onsite Test
(B-0040-85)

Parameter and Code:
Coliform, presumptive (MPN): 31507

1. Applications

This method is applicable to fresh and saline water, water having large suspended-solids concentration, and water having large populations of non-coliform bacteria. It is suitable for application at the sampling site to eliminate sample transport and storage.

2. Summary of method

Decimal dilutions of multiple sample aliquots are inoculated into lauryl tryptose broth. The cultures are incubated at 35±0.5 °C and examined after 24 and 48 hours for evidence of growth and gas production. The most probable number (MPN) of coliform organisms in the sample is determined from the distribution of gas-positive cultures among the inoculated serum bottles. The method described in this section is similar to the total coliform MPN method (presumptive test, B-0035-85) except provision is made for the incubation of samples onsite. Do not use the presumptive onsite test unless the confirmed test (B-0045-85) also is done.

3. Interferences

Large concentrations of heavy metals or toxic chemicals may interfere when large volumes of sample are added to small volumes of concentrated lauryl tryptose broth. Certain noncoliform organisms can ferment lactose during gas formation.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 Aluminum seals, one piece, 20 mm.

4.2 Bottles, milk dilution, screwcap.

4.3 Bottles, serum.

4.4 Crimper, for attaching aluminum seals.

4.5 Decapper, for removing aluminum seals from spent tubes.

4.6 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.7 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.8 Incubator* for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.9 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.10 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.11 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.12 Rubber stoppers, 13×20 mm.

4.13 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.14 Serum bottles and durham (fermentation) tubes. Two combinations of serum bottles and durham (fermentation) tubes may be used. The choice will depend on the volume of water to be tested. The durham tube, 6×25 mm test tubes, used to detect gas production, must be completely filled with medium and at least partly submerged in the serum bottle. The following combinations have been satisfactory:

4.14.1 For testing 10-mL aliquots, use borosilicate glass serum bottles, 20-mL capacity.

4.14.2 For testing 1-mL or smaller aliquots, use borosilicate glass serum bottles, 10-mL capacity.

4.15 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months). Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 Distilled or deionized water.

5.3 Ethyl alcohol, 70 percent. Dilute 74 mL 95-percent ethyl alcohol to 100 mL using distilled water. Undiluted isopropanol (ordinary rubbing alcohol) may be used instead of 70-percent ethyl alcohol.

5.4 Lauryl tryptose broth. Use premixed lauryl tryptose broth or lauryl sulfate broth, and prepare according to directions on bottle label. The medium also may be prepared according to American Public Health Association and others (1985).

5.4.1 Place 10 mL of medium containing 71.2 g/L lauryl tryptose broth or lauryl sulfate broth in a 20-mL serum bottle for each 10-mL aliquot of sample to be tested.

5.4.2 Place 10 mL of medium containing 35.6 g/L lauryl tryptose broth or lauryl sulfate broth in each 10-mL serum bottle for each 1-mL or smaller aliquot of sample to be tested.

5.4.3 In each serum bottle, place an inverted (mouth downward) durham tube (fig. 3). Place rubber stopper in mouth of bottle and attach aluminum seal using crimper. Sterilize bottles in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes as soon as possible after dispensing medium. Air will be expelled from the inverted durham tubes during heating; each will fill completely with medium during cooling. Discard any bottle in which air bubbles are visible in the durham tube.

6. Analysis

Two questions must be answered when planning a multiple serum-bottle test:

1. What volumes of water need to be tested?

2. How many serum bottles of each volume need to be tested?

Choose a range of volumes so positive and negative results are obtained throughout the range tested. The method fails if only positive or only negative results are obtained when all volumes are tested. The number of serum bottles used per sample volume depends on the precision required. The greater the number of bottles inoculated with each volume, the greater the precision, but the effort involved and expense also are increased. A five serum-bottle series is described below. The following sample volumes are suggested:

1. Unpolluted raw surface water: 0.1-, 1-, and 10-mL samples will include an MPN range of <2 to ≥2,400 coliforms per 100 mL.

2. Polluted raw surface water: 0.001-, 0.01-, 0.1-, and 1-mL samples will include an MPN range of 20 to 240,000 coliforms per 100 mL.

6.1 Set up five serum bottles of lauryl tryptose broth or lauryl sulfate broth for each sample volume to be tested.

6.1.1 If the volume to be tested is 0.1 mL or more, transfer the measured samples directly to the serum bottles using presterilized disposable hypodermic syringes (Note 1).

6.1.2 If the volume of original water sample is less than 0.1 mL, proceed as in 6.1.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Size of inoculum
1:10	----------	0.1 milliliter of original sample
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	----------	0.1 milliliter of 1:100 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution
1:100,000	----------	0.1 milliliter of 1:10,000 dilution

Note 1: Use a sterile hypodermic syringe for each serum bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be inoculated within 20 minutes after preparation.

6.2 When using serum bottles with rubber septums, proceed as follows:

6.2.1 Remove the inserts from the metal caps and swab the exposed area of the rubber septum using a bit of cotton saturated with 70-percent ethyl alcohol, undiluted isopropanol, or disinfectant.

6.2.2 Carefully invert a serum bottle so that the rubber septum is at the bottom. Inoculate the medium by carefully puncturing the septum with the sterile hypodermic syringe and insert the needle until only the beveled tip is inside the bottle. Discharge the contents of the syringe into the bottle and withdraw the needle. Agitate the bottle gently to mix the contents.

6.2.3 Carefully return serum bottle to the normal, upright position with septum at top. Make sure that the inverted durham tube is completely filled with medium and no residual bubbles remain in the durham tube.

6.3 Clearly mark each set of serum bottles indicating location, time of collection, sample number, and sample volume. Code each bottle for easy identification.

6.4 Place the inoculated serum bottles in the incubator and incubate at 35±0.5 °C for 24±2 hours. Bottles must be maintained in an upright position.

6.5 Remove serum bottles from incubator and examine. Gas in any quantity in the durham tube, even a pinhead-sized bubble, constitutes a positive test (fig. 4). The appearance of an air bubble must not be confused with actual gas production. If the gas is formed as a result of fermentation, the broth medium will become cloudy. Active fermentation may be indicated by the continued appearance of small bubbles of gas in the medium outside the durham tube when the bottle is shaken gently (Bordner and others, 1978; American Public Health Association and others, 1985).

6.6 After submitting all gas-positive serum bottles to the confirmation test (B-0045-85), autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

6.7 Return all gas-negative serum bottles to incubator and incubate at 35±0.5 °C for an additional 24±2 hours.

6.8 Remove serum bottles from incubator and examine for gas formation. Autoclave all remaining bottles of lauryl tryptose broth as in 6.6 before discarding.

7. Calculations

7.1 Record the number of gas-positive serum bottles occurring for all sample volumes tested.

7.2 When more than three volumes are tested, use results from only three of them when computing the MPN. To select the three dilutions for the MPN index, use as the first, the smallest sample volume in which all tests are positive (no larger sample volume having any negative results) and the next two succeeding smaller sample volumes (Bordner and others, 1978; American Public Health Association and others, 1985).

7.3 In the examples listed below, the number in the numerator represents positive serum bottles; the denominator represents the total number of bottles inoculated.

Example	Decimal dilutions				Combination of positives
	1 milliliter	0.1 milliliter	0.01 milliliter	0.001 milliliter	
a	5/5	5/5	2/5	0/5	5-2-0
b	5/5	4/5	2/5	0/5	5-4-2
c	0/5	1/5	0/5	0/5	0-1-0
d	5/5	3/5	1/5	1/5	5-3-2
e	5/5	3/5	2/5	0/5	5-3-2

In example c, the first three dilutions need to be taken to place the positive results in the middle dilution. When a positive result occurs in a dilution larger than the three chosen according to the guideline, as in d, it needs to be placed in the result for the largest chosen dilution as in e (Note 1).

Note 1: The largest dilution has the smallest concentration of the sample; the largest dilution in the preceding table is 0.001.

7.4 The MPN for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-mL dilutions are used are listed in table 2. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value from table 2 needs to be corrected for the dilutions actually used. To do this, divide the value from table 2 by the dilution factor of the first number in the three-number sequence (the serum bottles having the largest concentration of the sample). For example, if dilutions of 0.1, 0.01, and 0.001 mL are used, divide the value in table 2 by 0.1 mL. MPN tables for other combinations of sample volumes and numbers of bottles at each level of inoculation are in the American Public Health Association and others (1985).

Table 2.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used

[mL, milliliters; MPN, most probable number; ---, not applicable; modified from American Public Health Association and others, 1985]

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
0	0	0	<20	---	---
0	0	1	20	<5	70
0	1	0	20	<5	70
0	2	0	40	<5	11
1	0	0	20	<5	70
1	0	1	40	<5	110
1	1	0	40	<5	110
1	1	1	60	<5	150
1	2	0	60	<5	150
2	0	0	50	<5	130
2	0	1	70	10	170
2	1	0	70	10	170
2	1	1	90	20	210
2	2	0	90	20	210
2	3	0	120	30	280
3	0	0	80	10	190
3	0	1	110	20	250
3	1	0	110	20	250
3	1	1	140	40	340
3	2	0	140	40	340
3	2	1	170	50	460
4	0	0	130	30	310
4	0	1	170	50	460
4	1	0	170	50	460
4	1	1	210	70	630
4	1	2	260	90	780
4	2	0	220	70	670
4	2	1	260	90	780
4	3	0	270	90	800
4	3	1	330	110	930
4	4	0	340	120	930

Table 2.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used--Continued

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
5	0	0	230	70	700
5	0	1	310	110	890
5	0	2	430	150	1,100
5	1	0	330	110	930
5	1	1	460	160	1,200
5	1	2	630	210	1,500
5	2	0	490	170	1,300
5	2	1	700	230	1,700
5	2	2	940	280	2,200
5	3	0	790	250	1,900
5	3	1	1,100	310	2,500
5	3	2	1,400	370	3,400
5	3	3	1,800	440	5,000
5	4	0	1,300	350	3,000
5	4	1	1,700	430	4,900
5	4	2	2,200	570	7,000
5	4	3	2,800	900	8,500
5	4	4	3,500	1,200	10,000
5	5	0	2,400	680	7,500
5	5	1	3,500	1,200	10,000
5	5	2	5,400	1,800	14,000
5	5	3	9,200	3,000	32,000
5	5	4	16,000	6,400	58,000
5	5	5	≥24,000	---	---

7.5 Example: The following results were obtained with a five serum-bottle series:

Volume (milliliters)	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Results	5/5	5/5	3/5	1/5	0/5.

Using 10^{-6}, 10^{-7}, and 10^{-8} mL sample volumes, the test results indicate a sequence of 5-3-1 for which the MPN (table 2) is 1,100. Dividing by 10^{-6}, the MPN is computed to be 11×10^8 total coliform bacteria per 100 mL and 95-percent confidence limits of 3.1×10^8 and 25×10^8 total coliform bacteria per 100 mL.

8. Reporting of results

Report total coliform concentrations as MPN total coliforms per 100 mL as follows: less than 10, whole numbers; 10 or more, two significant figures.

9. Precision

9.1 Precision of the MPN method increases as the number of serum bottles is increased. It increases rapidly as the number of bottles increases from 1 to 5, but then it increases at a slower rate making the gain, when using 10 bottles instead of 5, much less than is achieved by increasing the number of bottles from 1 to 5. Variance as a function of number of bottles inoculated from tenfold dilution series is listed below:

Number of serum bottles at each dilution	Variance for tenfold dilution series
1	0.580
3	.335
5	.259
10	.183

9.2 The 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-mL dilutions are used are listed in table 2.

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R. H., Winter, J. A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Total Coliform Bacteria (Most-Probable-Number, MPN, Method)

Confirmation Test
(B-0045-85)

Parameter and Code:
Coliform, confirmed (MPN): 31505

All gas-positive cultures from the presumptive test (B-0035-85 or B-0040-85) need to be verified by the confirmation test. When the membrane-filter method is used, some members of the coliform group may react atypically and not produce the characteristic colonies on M-Endo medium. Thus, the identity of suspected coliform colonies need to be verified. Geldreich and others (1967) discussed verification and other aspects of the membrane-filter method.

Because coliform organisms are defined on the basis of their ability to ferment lactose with gas formation at 35±0.5 °C within 48 hours, verification is readily accomplished by using the lactose fermentation-tube method described in this section. Only a minimum of special equipment is needed. Ready-to-use sterile media are commercially available.

1. Applications

The confirmation test is applicable to all gas-positive cultures from the presumptive test and to coliform colonies produced by the membrane-filter method. Initiation of the confirmation test needs to be made immediately for gas-positive cultures from the presumptive test and as soon as possible after completion of the membrane-filter method, but not later than 24 hours.

2. Summary of method

2.1 Material from selected colonies on the membrane filters is placed in tubes of sterile lactose broth and incubated at 35±0.5 °C for 48 hours. Material from these tubes indicating gas formation within 48 hours or gas-positive cultures from the presumptive test are placed in tubes of sterile, brilliant green lactose bile broth. Gas production in the brilliant green lactose bile broth at 35±0.5 °C within 48 hours confirms the presence of coliform bacteria.

2.2 The confirmation test is compatible with the procedure described by Bordner and others (1978) and the American Public Health Association and others (1985).

3. Interferences

Certain noncoliform organisms can ferment lactose with gas formation, but their presence in this double enrichment method is unlikely.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 Aluminum seals, one piece, 20 mm.

4.2 Bunsen burner, for sterilizing inoculating loop.

4.3 Crimper, for attaching aluminum seals.

4.4 Culture tubes and durham (fermentation) tubes. Two combinations of culture tubes and durham (fermentation) tubes may be used. The choice will depend on the volume of water to be tested. The durham tube, used to detect gas production, must be completely filled with medium and at least partly submerged in the culture tube. The following combinations have been satisfactory:

4.4.1 For testing 10-mL aliquots, use borosilicate glass culture tubes, 20×150 mm; tube caps, 20 mm; and use borosilicate glass culture tubes, 10×75 mm, as durham tubes.

4.4.2 For testing 1-mL or smaller aliquots, use borosilicate glass culture tubes, 16×125 mm; tube caps, 16 mm; and use flint glass culture tubes, 6×50 mm, as durham tubes.

4.5 Culture-tube rack, galvanized, for 16- and 20-mm culture tubes.

4.6 Decapper, for removing aluminum seals from spent tubes.

4.7 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.8 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.9 Incubator*, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having a more precise temperature regulation, is satisfactory for laboratory use.

4.10 Inoculating loop, platinum-iridium wire, 3 mm, Brown and Sharpe gauge 26.

4.11 Needle holder.

4.12 Rubber stoppers, 13×20 mm.

4.13 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Brilliant green lactose broth, prepackaged brilliant green lactose broth in 16×125-mm tubes and fermentation shell. The medium also may be prepared according to American Public Health Association and others (1985). Use brilliant green bile, 2 percent, or brilliant green bile broth, 2 percent, and prepare according to directions on bottle label. Place 10 mL of medium in a culture tube for each colony to be tested. In each culture tube, place an inverted (mouth downward) durham tube (fig. 3). Sterilize culture tubes in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes as soon as possible after dispensing medium. Air will be expelled from the inverted durham tube during heating; each will fill completely with medium during cooling. Discard any culture tube in which air bubbles are visible in the durham tube.

5.2 Lauryl tryptose broth, prepackaged lauryl tryptose broth in 16×125-mm tubes and fermentation shell, or use premixed lauryl tryptose broth or lauryl sulfate broth, and prepare according to directions on bottle label. The medium also may be prepared according to American Public Health Association and others (1985).

5.2.1 Place 10 mL of medium in a culture tube for each colony to be tested.

5.2.2 In each culture tube, place an inverted (mouth downward) durham tube (fig. 3). Sterilize tubes in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes as soon as possible after dispensing medium. Air will be expelled from the inverted durham tube during heating; each will fill completely with medium during cooling. Discard any tube in which air bubbles are visible in the durham tube.

6. Analysis

6.1 Complete the membrane-filter method for total coliform bacteria according to procedures described in this chapter.

6.2 Select a colony or colonies to be confirmed for total coliform bacteria from the incubated membrane filters.

6.3 Sterilize the inoculating loop by flaming in the burner. The long axis of the wire needs to be held parallel to the cone of the flame so the entire end of the wire and loop is heated to redness.

6.4 Remove from flame and allow the wire to cool for about 10 seconds. Do not allow the inoculating loop to contact any foreign surface during the cooling period. When cool, touch the loop lightly to the colony. Part of the colony material will adhere to the wire.

6.5 Uncap a culture tube containing lauryl tryptose broth and hold it at an angle of about 45°. Insert the inoculating loop and colony material into the tube. Rub the wire loop and attached bacteria against the side of tube at the liquid meniscus to disperse the bacteria in the liquid.

6.6 Recap the culture tube. Flame the inoculating loop and inoculate additional tubes as in 6.5 until all colonies to be tested have been placed into broth in separate tubes. Place the inoculated tubes in the culture-tube rack and incubate at 35±0.5 °C for 24±2 hours.

6.7 Remove culture tubes from incubator and examine. Gas in any quantity in the durham tube constitutes a positive test (fig. 4). Return all gas-negative tubes to incubator and incubate at 35±0.5 °C for an additional 24±2 hours.

6.8 Using a sterile inoculating loop, transfer one loopful of broth from each culture tube indicating gas to a culture tube of sterile brilliant green lactose broth. Sterilize the loop after each transfer.

6.9 Autoclave all gas-positive culture tubes of lauryl tryptose broth at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

6.10 Incubate the culture tubes of brilliant green lactose broth at 35±0.5 °C for 48±3 hours.

6.11 Examine the remaining culture tubes of lauryl tryptose broth. Transfer one loopful of material from each tube producing gas to a culture tube of brilliant green lactose broth as in 6.8 and continue as in 6.10. If no gas appears in the tube of lauryl tryptose broth within 48±3 hours, the original colony was not of the coliform group. Autoclave all tubes of lauryl tryptose broth as in 6.9 before discarding.

6.12 Examine culture tubes of brilliant green lactose broth after 24±2 and 48±3 hours. The formation of gas in any quantity in the durham tube constitutes a positive confirmation for the presence of total coliform bacteria. If no gas appears in the tube of brilliant green lactose broth within 48±3 hours, the original colony was not of the coliform group, even though gas was produced in the tube of lauryl tryptose broth.

6.13 Culture tubes of brilliant green lactose broth need to be autoclaved as in 6.9 before discarding.

7. Calculations

No calculations are necessary.

8. Reporting of results

Results of the total coliform confirmation test are included in the results of the membrane-filter and presumptive tests for total coliform bacteria.

9. Precision

No precision data are available.

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R. H., Winter, J. A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Geldreich, E. E., Jeter, H. L., and Winter, J. A., 1967, Technical considerations in applying the membrane filter procedure: Health Lab Science, v. 4, p. 113-125.

Fecal Coliform Bacteria (Membrane-Filter Method)

Immediate Incubation Test
(B-0050-85)

Parameter and Code:
Coliform, fecal, 0.7-µm, M-FC media at 44.5 °C
(colonies/100 mL): 31625

Fecal coliforms are those organisms of the coliform group that are present in the intestines and feces of warm-blooded animals. They are capable of producing gas from lactose in a suitable culture medium at 44.5 °C. Bacterial organisms from other sources generally cannot produce gas when subjected to the same conditions (Bordner and others, 1978; American Public Health Association and others, 1985).

For the purpose of the method described in this section, the fecal coliform group is defined as all organisms that produce blue colonies when incubated at 44.5±0.2 °C within 24 hours on M-FC medium. The nonfecal coliform colonies are gray to cream colored.

1. Applications

The method is applicable to fresh and saline waters.

2. Summary of method

The sample is filtered onsite immediately after collection, and the filter is placed on a nutrient medium containing a pH-sensitive color indicator. Filters are incubated at a temperature of 44.5±0.2 °C for 24 hours in an incubator to suppress growth of nonfecal coliform bacteria, thereby selectively favoring growth of fecal coliforms.

3. Interferences

3.1 Suspended materials may inhibit the filtration of sample volumes sufficient to produce significant results. Fecal coliform colony formation on the filter may be inhibited by large numbers of noncoliform colonies, by the presence of algal filaments and detritus, or by toxic substances.

3.2 Water samples having a large suspended-solids concentration may be divided between two or more membrane filters. The multiple-tube method, which is described in this chapter, will give the most reliable results when suspended-solids concentrations are large and fecal coliform counts are small.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 Alcohol burner, glass or metal, containing ethyl alcohol for flame sterilizing of forceps.

4.2 Aluminum seals, one piece, 20 mm.

4.3 Bottles, milk dilution, screwcap.

4.4 Bottles, serum.

4.5 Crimper, for attaching aluminum seals.

4.6 Decapper, for removing aluminum seals from spent tubes.

4.7 Filter-holder assembly* and syringe that has a two-way valve* or vacuum hand pump.

4.8 Forceps*, stainless steel, smooth tips.

4.9 Graduated cylinders, 100-mL capacity.

4.10 Hydrodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.11 Hydrodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.12 Incubator*, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.13 Membrane filters, white, grid, sterile, 0.7-μm pore size, 47-mm diameter.

4.14 Microscope, binocular wide-field dissecting-type, and fluorescent lamp.

4.15 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.16 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.17 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.18 Plastic petri dishes with covers, disposable, sterile, 50×12 mm.

4.19 Rubber stoppers, 13×20 mm.

4.20 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.21 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.22 Thermometer, having a temperature range of at least 40 to 100 °C.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 Distilled or deionized water.

5.3 Ethyl alcohol, 95-percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methyl alcohol also may be used for sterilization.

5.4 Methyl alcohol, absolute, for sterilizing filter-holder assembly.

5.5 M-FC agar. Add 5.2 g M-FC agar to 100 mL distilled water. Do not autoclave. Heat to 90 °C in a water bath stirring occasionally, then add 1 mL rosolic acid solution. Continue heating for a maximum of 1 minute, then remove from heat and allow to cool to 50 °C. Pour to a depth of 4 mm (6-7 mL) in 50-mm petri dish bottoms. Replace petri dish tops loosely until medium solidifies, then close tightly and store the prepared petri dishes at 2 to 10 °C for a maximum of 72 hours; preferably the medium should not be stored for more than 24 hours.

5.6 Rosolic acid solution. Add 10 mL 0.2 N NaOH to 0.10 g rosolic acid crystals. Stir vigorously to dissolve. Do not heat. Store in the dark at room temperature for a maximum of 2 to 3 weeks. Discard if color changes from deep red to orange.

6. Analysis

The volumes of the sample to be filtered depend on the expected bacterial density of the water being tested, but the volumes should be enough that after incubation, at least one of the membrane filters will contain from 20 to 60 fecal coliform colonies.

The following sample volumes are suggested for filtration:

1. Unpolluted raw surface water: 0.1-, 0.3-, 1-, 3-, 10-, 30-, and 100-mL samples will include a range of 20 to 60,000 fecal coliforms per 100 mL using the criterion of 20 to 60 coliform colonies on a filter as an ideal determination.

2. Polluted raw surface water: 0.01-, 0.03-, 0.1-, 0.3-, 1-, and 3-mL samples will include a range of 670 to 600,000 fecal coliforms per 100 mL.

6.1 Sterilize filter-holder assembly (Note 1). In the laboratory, wrap the funnel and filter base parts of the assembly separately in kraft paper or polypropylene bags and sterilize in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Steam must contact all surfaces to ensure complete sterilization. Cool to room temperature before use.

Note 1: Onsite sterilization of filter-holder assembly needs to be in accordance with the manufacturer's instructions but usually involves application and ignition of methyl alcohol to produce formaldehyde. Autoclave sterilization in the laboratory prior to the trip to the sampling site is preferred. Sterilization must be performed at all sites.

6.2 Assemble the filter holder and, using flame-sterilized forceps (Note 2), place a sterile membrane filter over the porous plate of the assembly, grid side up. Carefully place funnel on filter to avoid tearing or creasing the membrane.

Note 2: Flame-sterilized forceps--Dip forceps in ethyl or methyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

6.3 Shake the sample vigorously about 25 times to obtain an equal distribution of bacteria throughout the sample before transferring a measured portion of the sample to the filter-holder assembly.

6.3.1 If the volume of sample to be filtered is 10 mL or more, transfer the measured sample directly onto the dry membrane.

6.3.2 If the volume of sample is between 1 and 10 mL, pour about 20 mL sterilized buffered dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of bacteria.

6.3.3 If the volume of original water sample is less than 1 mL, proceed as in 6.3.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle (Note 3) in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Filter this volume
1:10	11 milliliters of original sample	1 milliliter of 1:10 dilution
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	1 milliliter of 1:10 dilution	1 milliliter of 1:1,000 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution

Note 3: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be filtered within 20 minutes after preparation.

6.4 Apply vacuum and filter the sample. When vacuum is applied using a syringe fitted with a two-way valve, proceed as follows: Attach the filter-holder assembly to the inlet of the two-way valve with plastic tubing. Draw the syringe plunger very slowly on the initial stroke to avoid the danger of air lock before the assembly fills with water. Push the plunger forward to expel air from the syringe. Continue until the entire sample has been filtered. If the filter balloons or develops bubbles during sample filtration, disassemble the two-way valve and lubricate the rubber valve plugs lightly with stopcock grease. If a vacuum hand pump is used, do not exceed a pressure of 25 cm to avoid damage to bacteria.

6.5 Rinse sides of funnel twice with 20 to 30 mL of sterile buffered dilution water while applying vacuum.

6.6 Maintaining the vacuum, remove the funnel from the base of the filter-holder assembly and, using flame-sterilized forceps, remove the membrane filter from the base and place it on the agar surface in the plastic petri dish, grid side up, using a rolling action at one edge. Use care to avoid trapping air bubbles under the membrane (Note 4).

Note 4: Hold the funnel while removing the membrane filter and place it back on the base of the assembly when the membrane filter has been removed. Placement of the funnel on anything but the base of the assembly may result in contamination of the funnel.

6.7 Place top on petri dish and proceed with filtration of the next volume of water. Filter in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations.

6.8 Clearly mark the lid of each plastic petri dish indicating location, time of collection, time of incubation, sample number, and sample volume. Use a waterproof felt-tip marker or grease pencil.

6.9 Inspect the membrane in each petri dish for uniform contact with the agar. If air bubbles are present under the filter (indicated by bulges), remove the filter using sterile forceps and roll onto the agar again.

6.10 Close the plastic petri dish by firmly pressing down on the top.

6.11 If using a water-bath incubator, place each petri dish in a waterproof plastic bag or seal the dish with waterproof plastic tape.

6.12 Incubate the filters in the tightly closed petri dishes in an inverted position (agar and filter at the top) at 44.5±0.2 °C for 24±2 hours. Filters need to be incubated within 20 minutes after placement on medium.

6.13 Count the fecal coliform colonies (blue color) within 20 minutes after the dishes have been removed from the incubator. M-FC medium is very selective, and growth of colonies other than fecal coliform is inhibited. Colonies that are not fecal coliform will be gray to cream colored. The color plate in Millipore Corp. (1973, p. 42) may be helpful in identifying fecal coliform colonies. The counts are best made using 10X to 15X magnification.

6.14 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 If only one filter has a colony count between the ideal of 20 and 60, use the equation:

$$\text{Fecal coliform colonies/100 mL} = \frac{\text{Number of colonies counted} \times 100}{\text{Volume of original sample filtered (milliliters)}} .$$

7.2 If all filters have counts less than the ideal of 20 colonies or greater than 60 colonies, calculate using the equations in 7.5 for only those filters having at least one colony and not having colonies too numerous to count. Report results as number per 100 mL, followed by the statement, "Estimated count based on nonideal colony count."

7.3 If no filters develop characteristic fecal coliform colonies, report a maximum estimated value. Assume a count of one colony for the largest sample volume filtered, then calculate using the equation in 7.1. Report the results as less than (<) the calculated value per 100 mL.

7.4 If all filters have colonies too numerous to count, report a minimum estimated value. Assume a count of 60 coliform colonies for the smallest sample volume filtered, then calculate using the equation in 7.1. Report the results as greater than (>) the calculated value per 100 mL.

7.5 Sometimes two or more filters of a series will produce colony counts within the ideal counting range. Make colony counts for all such filters. The method for calculating and averaging is as follows (Note 5):

$$\begin{array}{rr} \text{Volume filter 1} & \text{Colony count filter 1} \\ + \text{Volume filter 2} & + \text{Colony count filter 2} \\ \hline \text{Volume sum} & \text{Colony count sum} \end{array}$$

$$\text{Fecal coliform colonies/100 mL} = \frac{\text{Colony count sum} \times 100}{\text{Volume sum (milliliters)}} .$$

Note 5: Do not calculate the fecal coliform colonies per 100 mL for each volume filtered and then average the results.

8. Reporting of results

Report fecal coliform concentration as fecal coliform colonies per 100 mL as follows: less than 10 colonies, report whole numbers; 10 or more colonies, two significant figures.

9. Precision

No numerical precision data are available. However, the method gives 93-percent accuracy for differentiating between fecal coliforms and coliforms from other sources (American Public Health Association and others, 1985).

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R. H., Winter, J. A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Millipore Corp., 1973, Biological analysis of water and wastewater: Bedford, Mass., Application Manual AM302, 84 p.

Fecal Coliform Bacteria (Most-Probable-Number, MPN, Method)

Presumptive Test
(B-0051-85)

Parameter and Code:
Coliform, fecal, EC broth at 44.5 °C (MPN): 31615

1. Applications

This method is applicable to fresh and saline water, water having large suspended-solids concentration, and water having large populations of non-coliform bacteria.

2. Summary of method

Decimal dilutions of multiple sample aliquots are inoculated into lauryl tryptose broth. The cultures are incubated at 35±0.5 °C and examined after 24 and 48 hours for evidence of growth and gas production. Positive cultures at 24 and 48 hours are transferred to EC broth, incubated at 44.5±0.2 °C for 24 hours, and examined for growth and gas production. The MPN of fecal coliform bacteria in the sample is determined from the distribution of gas-positive cultures among the inoculated EC broth culture tubes.

3. Interferences

Large concentrations of heavy metals or toxic chemicals may interfere when large volumes of sample are added to small volumes of concentrated lauryl tryptose broth.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Aluminum seals, one piece, 20 mm.

4.2 Bottles, milk dilution, screwcap.

4.3 Bottles, serum.

4.4 Bunsen burner, for sterilizing inoculating loop.

4.5 Crimper, for attaching aluminum seals.

4.6 Culture tubes and durham (fermentation) tubes. The sizes of the culture tube and durham tube, used for the detection of gas production, should enable the durham tube to completely fill with medium and at least partly submerge in the culture tube. The specific choice of culture tubes and durham tubes depends on the volume of water to be tested and whether the test is to be done in the laboratory or onsite. The procedure described below specifies the use of culture tubes as culture vessels. Serum bottles may be more

appropriate as culture vessels if samples are to be inoculated and incubated onsite. Apparatus needed for an onsite procedure is described in "Presumptive Onsite Test" (B-0040-85) subsection of the "Total Coliform Bacteria" section. The following combinations have been satisfactory.

4.6.1 For testing 10-mL aliquots, use borosilicate glass culture tubes, 20×150 mm; tube caps, 20 mm; and use borosilicate glass culture tubes, 10×75 mm, as durham tubes.

4.6.2 For testing 1-mL or smaller aliquots, use borosilicate glass culture tubes, 16×125 mm; tube caps, 16 mm; and use flint glass culture tubes, 6×50 mm, as durham tubes.

4.7 Culture-tube rack, galvanized, for 16- and 20-mm culture tubes.

4.8 Decapper, for removing aluminum seals from spent tubes.

4.9 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.10 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.11 Incubator*, for operation at a temperature of 35±0.5 °C, or water bath, capable of maintaining a temperature of 35±0.5 °C.

4.12 Incubator, water bath, for operation at 44.5±0.2 °C. Precise, uniform temperature control is essential.

4.13 Inoculating loop, platinum-iridium wire, 3 mm, Brown and Sharpe gauge 26.

4.14 Needle holder.

4.15 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.16 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.17 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.18 Rubber stoppers, 13×20 mm.

4.19 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.20 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months). Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 Distilled or deionized water.

5.3 EC medium or broth. Use premixed EC medium or broth, and prepare according to directions on bottle label. The medium also may be prepared according to American Public Health Association and others (1985) (Note 1).

Note 1: Because the number of positive lauryl tryptose cultures is unknown at the time of medium preparation, it is advisable to prepare a sufficient number of culture tubes of medium to enable inoculation of the maximum number of positives.

5.3.1 Place 10 mL of medium containing 37 g/L of EC medium or broth in a 20×150-mm culture tube for each culture tube or serum bottle of lauryl tryptose broth prepared in 5.4.

5.3.2 In each culture tube, place an inverted (mouth downward) 10×75-mm durham tube (fig. 3). Sterilize tubes at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes as soon as possible after dispensing medium. Air will be expelled from the inverted durham tubes during heating; each will fill completely with medium during cooling. Discard any culture tube in which air bubbles are visible in the durham tube. Prepared medium may be retained at 4 °C for no longer than 96 hours.

5.4 Lauryl tryptose broth. Use premixed lauryl tryptose broth or lauryl sulfate broth, and prepare according to directions on bottle label. The medium also may be prepared according to American Public Health Association and others (1985).

5.4.1 Place 10 mL of medium containing 71.2 g/L lauryl tryptose broth or lauryl sulfate broth in a 20×150-mm culture tube for each 10-mL aliquot of sample to be tested.

5.4.2 Place 10 mL of medium containing 35.6 g/L of lauryl tryptose broth or lauryl sulfate broth in a 16×125-mm culture tube for each 1-mL or smaller aliquot of sample to be tested.

5.4.3 In each culture tube, place an inverted (mouth downward) durham tube (fig. 3). Sterilize tubes in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes as soon as possible after dispensing medium. Air will be expelled from the inverted durham tube during heating; each will fill completely with medium during cooling. Discard any tube in which air bubbles are visible in the durham tube.

6. Analysis

Two questions must be answered when planning a multiple-tube test:

1. What volumes of water need to be tested?

2. How many culture tubes of each volume need to be tested?

Choose a range of volumes so positive and negative results are obtained throughout the range tested. The method fails if only positive or only negative results are obtained when all volumes are tested. The number of culture tubes used per sample volume depends on the precision required. The greater the number of tubes inoculated with each volume, the greater the precision, but the effort involved and expense also are increased. A five-tube series is described below. The following sample volumes are suggested:

1. Unpolluted raw surface water: 0.1-, 1-, and 10-mL samples will include an MPN range of <2 to ≥2,400 coliforms per 100 mL.

2. Polluted raw surface water: 0.001-, 0.01-, 0.1-, and 1-mL samples will include an MPN range of 20 to 240,000 coliform organisms per 100 mL.

6.1 Set up five culture tubes of lauryl tryptose broth for each sample volume to be tested.

6.1.1 If the volume to be tested is 0.1 mL or more, transfer the measured samples directly to the culture tubes using sterile pipets (Note 2).

6.1.2 If the volume of original water sample is less than 0.1 mL, proceed as in 6.1.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Size of inoculum
1:10	----------	0.1 milliliter of original sample
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	----------	0.1 milliliter of 1:100 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution
1:100,000	----------	0.1 milliliter of 1:10,000 dilution

Note 2: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be inoculated within 20 minutes after preparation.

6.2 Clearly mark each set of culture tubes indicating location, time of collection, sample number, and sample volume. Code each tube for easy identification.

6.3 Place the inoculated culture tubes in the culture-tube rack and incubate at 35±0.5 °C for 24±2 hours. Tubes must be maintained in an upright position.

6.4 Remove culture tubes from incubator and examine. Gas in any quantity in the durham tube, even a pinhead-sized bubble, constitutes a positive test (fig. 4). The appearance of an air bubble must not be confused with actual gas production. If the gas is formed as a result of fermentation, the broth medium will become cloudy. Active fermentation may be indicated by the continued appearance of small bubbles of gas in the medium outside the durham tube when the culture tube is shaken gently (Bordner and others, 1978; American Public Health Association and others, 1985).

6.5 Sterilize the inoculating loop by flaming in the burner. The long axis of the wire must be held parallel to the cone of the flame so the entire end of the wire and loop is heated to redness. Remove from flame and allow wire to cool for about 10 seconds. Do not allow the loop to contact any foreign surface during the cooling period.

6.6 Gently shake and uncap a positive culture tube of lauryl tryptose broth. Insert the inoculating loop beneath the liquid surface and carefully withdraw a loopful of culture. Uncap a tube of EC broth and insert the loop beneath the medium surface. Gently swirl the loop to disperse bacteria in the medium.

6.7 Recap both culture tubes. Flame the inoculating loop and inoculate additional tubes until all positive cultures have been transferred to EC broth. Sterilize the loop after each transfer. Place the culture-tube racks of inoculated EC tubes into a water-bath incubator and incubate at 44.5±0.2 °C for 24±2 hours. Place all inoculated EC tubes in the water bath as soon as possible and always within 30 minutes.

6.8 Return remaining gas-negative culture tubes of lauryl tryptose broth to the incubator and incubate at 35±0.5 °C for an additional 24±2 hours.

6.9 Autoclave all gas-positive culture tubes of lauryl tryptose broth at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

6.10 Remove culture tubes of EC broth and examine for gas production. Gas in any quantity indicates a positive test for fecal coliforms.

6.11 Remove remaining culture tubes of lauryl tryptose broth and examine for gas production. Transfer any positive cultures to EC broth and incubate as in 6.7 and 6.8.

6.12 Autoclave all gas-positive culture tubes of lauryl tryptose broth and EC broth before discarding as in 6.9.

6.13 Remove remaining culture tubes of EC broth incubated in 6.11 and examine for gas production.

6.14 Autoclave all culture tubes of EC broth before discarding as in 6.9.

7. Calculations

7.1 Record the number of gas-positive culture tubes of lauryl tryptose broth and EC broth at 24 and 48 hours for all sample volumes tested. Determine presumptive MPN of total coliform bacteria from the number of positive tubes of lauryl tryptose broth. Determine MPN of fecal coliform bacteria from the number of positive tubes of EC broth.

7.2 When more than three volumes are tested, use results from only three of them when computing the MPN. To select the three dilutions for the MPN index, use as the first, the smallest sample volume in which all tests are positive (no larger sample volume having any negative results) and the next two succeeding smaller sample volumes (Bordner and others, 1978; American Public Health Association and others, 1985).

7.3 In the examples listed below, the number in the numerator represents positive culture tubes; the denominator represents the total number of tubes inoculated.

Example	Decimal dilutions				Combination of positives
	1 milliliter	0.1 milliliter	0.01 milliliter	0.001 milliliter	
a	5/5	5/5	2/5	0/5	5-2-0
b	5/5	4/5	2/5	0/5	5-4-2
c	0/5	1/5	0/5	0/5	0-1-0
d	5/5	3/5	1/5	1/5	5-3-2
e	5/5	3/5	2/5	0/5	5-3-2

In example c, the first three dilutions need to be taken to place the positive results in the middle dilution. When a positive result occurs in a dilution larger than the three chosen according to the guideline, as in d, it needs to be placed in the result for the largest chosen dilution as in e (Note 3).

Note 3: The largest dilution has the smallest concentration of the sample; the largest dilution in the preceding table is 0.001.

7.4 The MPN for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-mL dilutions are used are listed in table 3. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value in table 3 needs to be corrected for the dilutions actually used. To do this, divide the value in table 3 by the dilution factor of the first number in the three-number sequence (the culture tubes having the largest concentration of the sample). For example, if dilutions of 0.1, 0.01, and 0.001 mL are used, divide the value in table 3 by 0.1 mL. MPN tables for other combinations of sample volumes and numbers of tubes at each level of inoculation are in American Public Health Association and others (1985).

7.5 Example: The following results were obtained with a five-tube series:

Volume (milliliters)-----	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Results-----------------	5/5	5/5	3/5	1/5	0/5.

Using 10^{-6}, 10^{-7}, and 10^{-8} mL sample volumes, the test results indicate a sequence of 5-3-1 for which the MPN (table 3) is 1,100. Dividing by 10^{-6}, the MPN is computed to be 11×10^{8} fecal coliform bacteria per 100 mL and 95-percent confidence limits of 3.1×10^{8} and 25×10^{8} fecal coliform bacteria per 100 mL.

8. Reporting of results

Report presumptive fecal coliform concentrations as MPN fecal coliforms per 100 mL as follows: less than 10, whole numbers; 10 or more, two significant figures.

Table 3.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used

[mL, milliliters; MPN, most probable number; ---, not applicable; modified from American Public Health Association and others, 1985]

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
0	0	0	<20	---	---
0	0	1	20	<5	70
0	1	0	20	<5	70
0	2	0	40	<5	11
1	0	0	20	<5	70
1	0	1	40	<5	110
1	1	0	40	<5	110
1	1	1	60	<5	150
1	2	0	60	<5	150
2	0	0	50	<5	130
2	0	1	70	10	170
2	1	0	70	10	170
2	1	1	90	20	210
2	2	0	90	20	210
2	3	0	120	30	280
3	0	0	80	10	190
3	0	1	110	20	250
3	1	0	110	20	250
3	1	1	140	40	340
3	2	0	140	40	340
3	2	1	170	50	460
4	0	0	130	30	310
4	0	1	170	50	460
4	1	0	170	50	460
4	1	1	210	70	630
4	1	2	260	90	780
4	2	0	220	70	670
4	2	1	260	90	780
4	3	0	270	90	800
4	3	1	330	110	930
4	4	0	340	120	930

Table 3.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used--Continued

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
5	0	0	230	70	700
5	0	1	310	110	890
5	0	2	430	150	1,100
5	1	0	330	110	930
5	1	1	460	160	1,200
5	1	2	630	210	1,500
5	2	0	490	170	1,300
5	2	1	700	230	1,700
5	2	2	940	280	2,200
5	3	0	790	250	1,900
5	3	1	1,100	310	2,500
5	3	2	1,400	370	3,400
5	3	3	1,800	440	5,000
5	4	0	1,300	350	3,000
5	4	1	1,700	430	4,900
5	4	2	2,200	570	7,000
5	4	3	2,800	900	8,500
5	4	4	3,500	1,200	10,000
5	5	0	2,400	680	7,500
5	5	1	3,500	1,200	10,000
5	5	2	5,400	1,800	14,000
5	5	3	9,200	3,000	32,000
5	5	4	16,000	6,400	58,000
5	5	5	≥24,000	---	---

9. Precision

9.1 Precision of the MPN method increases as the number of culture tubes is increased. It increases rapidly as the number of tubes increases from 1 to 5, but then it increases at a slower rate making the gain, when using 10 tubes instead of 5, much less than is achieved by increasing the number of tubes from 1 to 5. Variance as a function of number of tubes inoculated from a tenfold dilution series is listed below:

Number of culture tubes at each dilution	Variance for tenfold dilution series
1	0.580
3	.335
5	.259
10	.183

9.2 The 95-percent confidence limits for various combinations of positive and negative results, when five 1-, five 0.1-, and five 0.01-mL dilutions are used are listed in table 3.

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R. H., Winter, J. A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Fecal Streptococcal Bacteria (Membrane-Filter Method)

Immediate Incubation Test
(B-0055-85)

Parameter and Code:
Streptococci, fecal, MF, KF agar
(colonies/100 mL): 31673

Fecal streptococci are increasingly used as indicators of substantial contamination of water because the normal habitat of these organisms is the intestines of man and animals. Fecal streptococcal data verify fecal pollution and may provide additional information concerning the recency and probable origin of pollution.

1. Applications

The method is applicable to most types of water.

2. Summary of method

The sample is filtered onsite immediately after collection, and the filter is placed on a nutrient medium designed to stimulate the growth of fecal streptococci and to suppress the growth of other organisms. After incubation at 35±0.5 °C for 48 hours, the red or pink colonies are counted.

3. Interferences

3.1 Suspended materials may inhibit the filtration of sample volumes sufficient to produce significant results. Streptococcal colony formation on the filter may be inhibited by large numbers of nonstreptococcal colonies, by the presence of algal filaments and detritus, or by toxic substances.

3.2 Water samples having a large suspended-solids concentration may be divided between two or more membrane filters. The multiple-tube method, which is described in this chapter, will give the most reliable results when suspended-solids concentrations are large and streptococcal counts are small.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 Alcohol burner, glass or metal, containing ethyl alcohol for flame sterilizing of forceps.

4.2 Aluminum seals, one piece, 20 mm.

4.3 Bottles, milk dilution, screwcap.

4.4 Bottles, serum.

4.5 Crimper, for attaching aluminum seals.

4.6 Decapper, for removing aluminum seals from spent tubes.

4.7 Filter apparatus, sterile, complete with membrane filter, 0.22-μm mean pore size, 25-mm diameter.

4.8 Filter-holder assembly* and syringe that has a two-way valve* or vacuum hand pump.

4.9 Forceps*, stainless steel, smooth tips.

4.10 Graduated cylinders, 100-mL capacity.

4.11 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.12 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.13 Incubator*, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.14 Membrane filters, white, grid, sterile, 0.45-μm mean pore size, 47-mm diameter.

4.15 Microscope, binocular wide-field dissecting-type, and fluorescent lamp.

4.16 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.17 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.18 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.19 Plastic petri dishes with covers, disposable, sterile, 50×12 mm.

4.20 Plastic syringe, disposable, 20-mL capacity.

4.21 Rubber stoppers, 13×20 mm.

4.22 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.23 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.24 Thermometer, having a temperature range of at least 40 to 100 °C.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 Distilled or deionized water.

5.3 Ethyl alcohol, 95-percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methyl alcohol also may be used for sterilization.

5.4 KF streptococcus agar. Suspend 7.64 g KF streptococcus agar in 100 mL distilled water. Do not autoclave. Stir and heat to boiling in a water bath. Once boiling starts, heat an additional 5 minutes. Remove from heat and cool to 50 to 60 °C. Add 1 mL 1-percent TTC solution after the medium has cooled to less than 60 °C. If commercially prepared 1-percent sterile TTC solution is used, swab the rubber septum on the vial with 95-percent ethyl alcohol before use. Remove 1 mL using a sterile, disposable

hypodermic syringe. When medium has cooled to approximately 50 °C, pour medium into 50×12-mm petri dishes to a depth of 4 mm (6-7 mL). When medium solidifies, store the prepared plates in a refrigerator. Discard after 2 weeks if sterile TTC was used and after 24 hours if unsterilized TTC was used.

5.5 Methyl alcohol, absolute, for sterilizing filter-holder assembly.

5.6 TTC solution. Sterile 1-percent TTC solution is available from commercial sources. Alternatively, prepare a 1-percent sterile solution by dissolving 0.1 g triphenyltetrazolium chloride in 10 mL distilled water. Filter the solution aseptically through a 0.22-μm-membrane filter into a sterile, capped test tube. Store sterilized TTC solution at 2 to 8 °C in darkness and discard after container has been opened for 1 month or if contamination occurs, as indicated by color change or turbidity. As an expedient, substitute freshly prepared unsterilized TTC solution if the KF medium will be used promptly. TTC solution cannot be sterilized by heat.

6. Analysis

The volumes of the sample to be filtered depend on the expected bacterial density of the water being tested, but the volumes should be enough that, after incubation, at least one of the membrane filters will contain from 20 to 100 fecal streptococcal colonies.

Fecal streptococci generally are present in fewer numbers than coliform bacteria; therefore, the filtered volume of sample needs to be larger than that used for other indicator bacterial determinations. When filtering water of unknown quality, the following sample volumes are suggested: 0.05, 0.25, 1, 5, 25, and 100 mL. This will include a range of 20 to 200,000 fecal streptococci per 100 mL using the criterion of 20 to 100 colonies on a filter as an ideal determination.

6.1 Pour agar medium at 45 to 50 °C into bottom (larger half) of each sterile plastic petri dish to a depth of about 4 mm (6-7 mL). Pads are not used. Replace petri dish tops.

6.2 Sterilize filter-holder assembly (Note 1). In the laboratory, wrap the funnel and filter base parts of the assembly separately in kraft paper or polypropylene bags and sterilize in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Steam must contact all surfaces to ensure complete sterilization. Cool to room temperature before use.

Note 1: Onsite sterilization of the filter-holder assembly needs to be in accordance with the manufacturer's instructions but usually involves application and ignition of methyl alcohol to produce formaldehyde. Autoclave sterilization in the laboratory prior to the trip to the sampling site is preferred. Sterilization must be performed at all sites.

6.3 Assemble the filter holder and, using flame-sterilized forceps (Note 2), place a sterile membrane filter over the porous plate of the assembly, grid side up. Carefully place funnel on filter to avoid tearing or creasing the membrane.

Note 2: Flame-sterilized forceps--Dip forceps in ethyl or methyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

6.4 Shake the sample vigorously about 25 times to obtain an equal distribution of bacteria throughout the sample before transferring a measured portion of the sample to the filter-holder assembly.

6.4.1 If the volume of the sample to be filtered is 10 mL or more, transfer the measured sample directly onto the dry membrane.

6.4.2 If the volume of sample is between 1 and 10 mL, pour about 20 mL of sterilized buffered dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of bacteria.

6.4.3 If the volume of original water sample is less than 1 mL, proceed as in 6.4.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle (Note 3) in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Filter this volume
1:10	11 milliliters of original sample	1 milliliter of 1:10 dilution
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	1 milliliter of 1:10 dilution	1 milliliter of 1:1,000 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution

Note 3: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be filtered within 20 minutes after preparation.

6.5 Apply vacuum and filter the sample. When vacuum is applied using a syringe fitted with a two-way valve, proceed as follows: Attach the filter-holder assembly to the inlet of the two-way valve with plastic tubing. Draw the syringe plunger very slowly on the initial stroke to avoid the danger of air lock before the assembly fills with water. Push the plunger forward to expel air from the syringe. Continue until the entire sample has been filtered. If the filter balloons or develops bubbles during sample filtration, disassemble the two-way valve and lubricate the rubber valve plugs lightly with stopcock grease. If a vacuum hand pump is used, do not exceed a pressure of 25 cm to avoid damage to bacteria.

6.6 Rinse sides of funnel twice with 20 to 30 mL of sterile buffered dilution water while applying vacuum.

6.7 Maintaining the vacuum, remove the funnel from the base of the filter-holder assembly and, using flame-sterilized forceps, remove the membrane filter from the base and place it on the agar surface in the plastic petri dish, grid side up, using a rolling action at one edge. Use care to avoid trapping air bubbles under the membrane (Note 4).

Note 4: Hold the funnel while removing the membrane filter and place it back on the base of the assembly when the membrane filter has been removed. Placement of the funnel on anything but the base of the assembly may result in contamination of the funnel.

6.8 Place top on petri dish and proceed with filtration of the next volume of water. Filter in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations.

6.9 Clearly mark the lid of each plastic petri dish indicating location, time of collection, time of incubation, sample number, and sample volume. Use a waterproof felt-tip marker or grease pencil.

6.10 Inspect the membrane in each petri dish for uniform contact with the agar. If air bubbles are present under the filter (indicated by bulges), remove the filter using sterile forceps and roll onto the agar again.

6.11 Close the plastic petri dish by firmly pressing down on the top.

6.12 Incubate the filters in the tightly closed petri dishes in an inverted position (agar and filter at the top) at 35±0.5 °C for 48±2 hours. Filters need to be incubated within 20 minutes after placement on medium.

6.13 Count all red or pink colonies as fecal streptococci. The color plate in Millipore Corp. (1973, p. 42) may be helpful in identifying fecal streptococcal colonies. The counts are best made using 10X to 15X magnification. Illumination is not critical.

6.14 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 If only one filter has a colony count between the ideal of 20 and 100, use the equation:

$$\text{Fecal streptococcal colonies/100 mL} = \frac{\text{Number of colonies counted} \times 100}{\text{Volume of original sample filtered (milliliters)}} .$$

7.2 If all filters have counts less than the ideal of 20 colonies or greater than 100 colonies, calculate using the equations in 7.5 for only those filters having at least one colony and not having colonies too numerous to count. Report results as number per 100 mL, followed by the statement, "Estimated count based on nonideal colony count."

7.3 If no filters develop characteristic fecal streptococcal colonies, report a maximum estimated value. Assume a count of one colony for the largest sample volume filtered, then calculate using the equation in 7.1. Report the results as less than (<) the calculated value per 100 mL.

7.4 If all filters have colonies too numerous to count, report a minimum estimated value. Assume a count of 100 fecal streptococcal colonies for the smallest sample volume filtered, then calculate using the equation in 7.1. Report the results as greater than (>) the calculated value per 100 mL.

7.5 Sometimes two or more filters of a series will produce colony counts within the ideal counting range. Make colony counts for all such filters. The method for calculating and averaging is as follows (Note 5):

$$\frac{\begin{array}{r}\text{Volume filter 1}\\ +\ \text{Volume filter 2}\end{array}}{\text{Volume sum}} \qquad \frac{\begin{array}{r}\text{Colony count filter 1}\\ +\ \text{Colony count filter 2}\end{array}}{\text{Colony count sum}}$$

$$\text{Fecal streptococcal colonies/100 mL} = \frac{\text{Colony count sum} \times 100}{\text{Volume sum (milliliters)}} .$$

Note 5: Do not calculate the fecal streptococcal colonies per 100 mL for each volume filtered and then average the results.

8. Reporting of results

Report the fecal streptococcal concentration as fecal streptococcal colonies per 100 mL as follows: less than 10 colonies, whole numbers; 10 or more colonies, two significant figures.

9. Precision

No numerical precision data are available.

10. References cited

Millipore Corp., 1973, Biological analysis of water and wastewater: Bedford, Mass., Application Manual AM302, 84 p.

Fecal Streptococcal Bacteria (Membrane-Filter Method)

Confirmation Test
(B-0060-85)

Parameter and Code: Not applicable

KF agar medium stimulates the growth of fecal streptococci. A few other types of bacteria, chiefly nonfecal streptococci, may appear occasionally on this medium. Colonies of nonfecal streptococci typically are very small but exhibit the characteristic red or pink coloration and could be counted as fecal streptococci in the membrane-filter method. Suspected colonies may be confirmed according to this test.

The fecal streptococcal bacteria are distinguished from other bacteria by the following three characteristics: (1) They lack the enzyme catalase; (2) they can grow at 45±0.5 °C; and (3) they grow in 40-percent bile. The confirmation test uses these three characteristics as criteria for identification. The procedure is similar to that in Bordner and others (1978) and the American Public Health Association and others (1985).

1. Applications

The confirmation test is applicable to fecal streptococcal colonies produced by the membrane-filter method. Confirmation must be made as soon as possible after completion of the membrane-filter method, but not later than 24 hours.

2. Summary of method

Cells from colonies to be tested are streaked on brain-heart infusion agar slants. Cells from the slants are tested for the presence of catalase and for the ability to grow at 45±0.5 °C and in the presence of 40-percent bile. Absence of catalase and growth at 45±0.5 °C and in 40-percent bile constitute a positive test for fecal streptococci. Presence of catalase or failure to grow at 45±0.5 °C or in 40-percent bile indicate that the original colony was not of the fecal streptococcal group.

3. Interferences

As far as is known, only fecal streptococci show the pattern of results described in this method.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the

manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 Bunsen burner, for sterilizing inoculating loop.

4.2 Culture tubes, borosilicate glass, 16×150 mm, and culture-tube caps, 16 mm.

4.3 Culture-tube rack, galvanized, for 16-mm tubes.

4.4 Incubator*, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.5 Inoculating loop, platinum-iridium wire, 3 mm, Brown and Sharpe gauge 26.

4.6 Microscope slides, glass, 76×25 mm.

4.7 Needle holder.

4.8 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Brain-heart infusion agar. Add 52 g brain-heart infusion agar to 1 L distilled water. Heat in a water bath and vigorously stir until solution becomes clear. Remove from heat immediately on clearing. Place 5 mL of hot solution in each of twelve 16×150-mm culture tubes. CAUTION:--Do not allow solution to cool below 45 °C or it will solidify.

Cap each tube. Sterilize at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Remove from sterilizer and set tubes of molten agar at an angle of about 20° from the horizontal (fig. 5). Allow to cool until the solution solidifies.

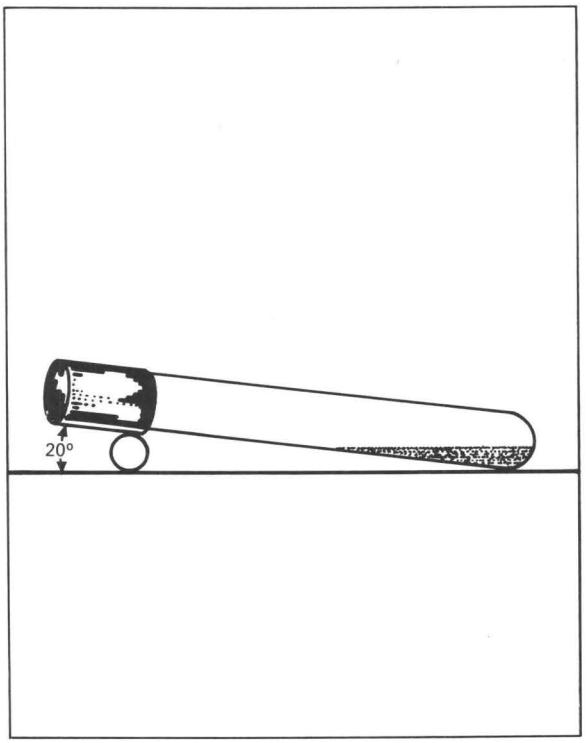


Figure 5.--Preparation of agar slant.

5.2 Brain-heart infusion broth. Add 37 g brain-heart infusion broth to 1 L distilled water. Stir until dissolved. Place 6 mL of broth in each of twelve 16×150-mm culture tubes. Cap each tube. Sterilize at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

5.3 Brain-heart infusion-40-percent bile broth. Add 37 g brain-heart infusion broth to 1 L distilled water. Stir until dissolved. Place 6 mL of brain-heart infusion broth in each of twelve 16×150-mm culture tubes. Cap each tube. Sterilize at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

Add 100 g oxgall to 1 L distilled water. Stir until dissolved. Place 4 mL of 10-percent oxgall solution in each of twelve 16×150-mm culture tubes. Cap each tube. Sterilize at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

Remove caps from a tube of sterile 10-percent oxgall solution and a tube of sterile brain-heart infusion broth. Quickly pour the oxgall solution into the brain-heart infusion-broth tube and recap.

5.4 Distilled or deionized water.

5.5 Hydrogen peroxide solution, 3 percent.

5.6 Potassium iodide, crystals.

6. Analysis

6.1 Complete the membrane-filter method for fecal streptococcal bacteria according to procedures described in this chapter.

6.2 Select a colony or colonies to be confirmed for fecal streptococcal bacteria from the incubated membrane filter.

6.3 Sterilize the inoculating loop by flaming in the burner. The long axis of the wire needs to be held parallel to the cone of the flame so the entire end of the wire and loop is heated to redness.

6.4 Remove from flame and allow the wire to cool for about 10 seconds. Do not allow the inoculating loop to contact any foreign surface during the cooling period. When cool, touch the loop lightly to a single, well-isolated colony. Part of the colony material will adhere to the wire.

6.5 Uncap a culture tube of a brain-heart infusion-agar slant and hold it at an angle of about 45° with the flat surface of the slant upward (fig. 6). Insert the inoculating loop and colony material into the tube. Starting at the base of the slant, lightly rub the loop against the agar, moving toward the top, in a zig-zag pattern (fig. 6).

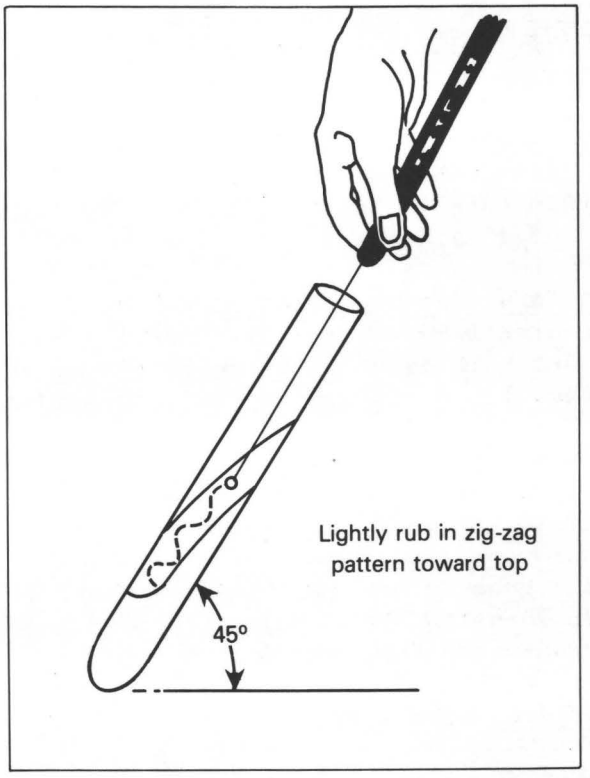


Figure 6.--Method of streaking on an agar slant.

6.6 Recap the culture tube. Flame the inoculating loop and inoculate additional tubes as in 6.4 and 6.5 until all colonies to be tested have been placed on agar in separate tubes. Place the inoculated tubes in the culture-tube rack and incubate at 35±0.5 °C for 24 to 48 hours.

6.7 Remove the culture tubes from the incubator and examine. Growth will be evident as a translucent, glistening film on the surface of the agar.

6.8 Test the potency of the hydrogen peroxide solution by placing a few milliliters in a test tube and adding a few potassium iodide crystals. A brown coloration and the appearance of bubbles in the solution indicate that the hydrogen peroxide solution is acceptable for use. If these reactions do not occur, discard and obtain a fresh hydrogen peroxide solution.

6.9 Flame the inoculating loop and allow to cool. Immediately uncap a culture tube of brain-heart infusion agar having growth. Remove a loopful of growth from the tube and smear on a clean glass slide. Add a few drops of freshly tested 3-percent hydrogen peroxide solution to the material on the slide. Immediately watch the slide for bubble formation. Observation of bubble formation may be facilitated by use of a low-power microscope. The absence of bubbles constitutes a negative catalase test indicating a probable fecal streptococcal culture, and the confirmation test should be continued. The presence of bubbles constitutes a positive catalase test indicating the presence of a nonstreptococcal bacteria, and the test may be terminated at this point.

6.10 Proceed as follows for all catalase-negative cultures. Uncap one culture tube each of brain-heart infusion broth and brain-heart infusion-40-percent bile broth. Using a flamed inoculating loop, transfer one loopful of material from the brain-heart infusion-agar slant to one of the tubes. Reflame the loop and transfer a loopful of material from the agar slant to the other tube. Recap the tubes.

6.11 Flame the inoculating loop and inoculate additional culture tubes as in 6.9 until all catalase-negative cultures have been placed in separate tubes of brain-heart infusion broth and brain-heart infusion-40-percent bile broth.

6.12 Place the inoculated culture tubes of brain-heart infusion broth in a culture-tube rack and incubate at 45±0.5 °C for 48±3 hours. Include tubes of uninoculated medium as controls.

6.13 Place the inoculated culture tubes of brain-heart infusion-40-percent bile broth in a culture-tube rack and incubate at 35±0.5 °C for 72±4 hours. Include tubes of uninoculated medium as controls.

6.14 Remove culture tubes from incubator and examine. Appearance of turbidity in the inoculated tubes, when compared to the controls, constitutes a positive test for growth.

Appearance of growth in the brain-heart infusion broth and the brain-heart infusion-40-percent bile broth constitutes a positive confirmation for the presence of fecal streptococci in the original colony. Absence of growth in either or both culture tubes indicates that the original colony was not of the fecal streptococcal group.

6.15 Autoclave all inoculated culture tubes and smeared slides at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

No calculations are necessary.

8. Reporting of results

Results of the fecal streptococcal confirmation test are included in the colony counts for fecal streptococcal bacteria.

9. Precision

No precision data are available.

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R. H., Winter, J. A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Fecal Streptococcal Bacteria (Most-Probable-Number, MPN, Method)

Presumptive and Confirmation Test
(B-0065-85)

Parameter and Code:
Streptococci, fecal (MPN): 31677

1. Applications

This method is not applicable to saline water. It is applicable to fresh water having large suspended-solids concentration and large populations of nonstreptococcal bacteria.

2. Summary of method

2.1 Decimal dilutions of multiple sample aliquots are inoculated into azide dextrose broth. The cultures are incubated at 35±0.5 °C and examined after 24 and 48 hours for evidence of growth. Positive cultures at 24 or 48 hours constitute a positive presumptive test for fecal streptococci.

2.2 Positive cultures at 24 and 48 hours are inoculated into ethyl violet azide broth and incubated at 35±0.5 °C and examined after 24 hours. Negative ethyl violet azide cultures after 24-hour incubation are reinoculated with original positive presumptive cultures of azide dextrose broth, incubated, and examined again after an additional 24 hours. Growth in ethyl violet azide after 24 or 48 hours constitutes a positive confirmation test for fecal streptococci.

3. Interferences

Certain members of the streptococcal group from soil, vegetative, and insect sources will test positive in this procedure; therefore, the test should be used concurrently with tests for other fecal indicators to substantiate the sanitary significance of the results (American Public Health Association and others, 1985). Differentiation of the streptococcal group requires additional taxonomic tests (Bordner and others, 1978, p. 144-153).

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 Aluminum seals, one piece, 20 mm.

4.2 Bottles, milk dilution, screwcap.

4.3 Bottles, serum.

4.4 Bunsen burner, for sterilizing inoculating loop.

4.5 Crimper, for attaching aluminum seals.

4.6 Culture tubes. The size and the type of culture tube used depend on the volume of water to be tested and whether the test is to be done in the laboratory or onsite. The procedure described below specifies the use of test tubes as culture vessels. Serum bottles may be more appropriate as culture vessels if samples are to be inoculated and incubated onsite. Apparatus needed for an onsite procedure is described in the "Presumptive Onsite Test" (B-0040-85) subsection of the "Total Coliform Bacteria" section.

4.6.1 For testing 10-mL aliquots, use borosilicate glass culture tubes, 20×150 mm; tube caps, 20 mm.

4.6.2 For testing 1-mL or smaller aliquots, use borosilicate glass culture tubes, 16×125 mm; tube caps, 16 mm.

4.7 Culture-tube rack, galvanized, for 16- and 20-mm culture tubes.

4.8 Decapper, for removing aluminum seals from spent tubes.

4.9 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.10 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.11 Incubator*, for operation at a temperature of 35±0.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.12 Inoculating loop, platinum-iridium wire, 3 mm, Brown and Sharpe gauge 26.

4.13 Needle holder.

4.14 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.15 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.16 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.17 Rubber stoppers, 13×20 mm.

4.18 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.19 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Azide dextrose broth. Use premixed azide dextrose broth, and prepare according to directions on bottle label. The medium also may be prepared according to American Public Health Association and others (1985).

5.1.1 Place 10 mL of medium containing 69.4 g/L azide dextrose broth in a 20×150-mm culture tube or a serum bottle for each 10-mL aliquot of sample to be tested.

5.1.2 Place 10 mL of medium containing 34.7 g/L azide dextrose broth in a 16×125-mm culture tube or a serum bottle for each 1-mL or smaller aliquot to be tested.

5.1.3 Sterilize capped culture tubes or serum bottles of media in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

5.2 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months). Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at

121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.3 Distilled or deionized water.

5.4 Ethyl violet azide (EVA) broth. Use premixed EVA broth, and prepare according to directions on bottle label (Note 1).

Note 1: Because the number of positive azide dextrose broth cultures is unknown at the time of medium preparation, prepare a sufficient number of culture tubes containing ethyl violet azide broth to enable inoculation of the maximum number of positives.

5.4.1 Place 10 mL of medium containing 35.8 g/L EVA broth in a 16×125-mm culture tube for each culture tube or serum bottle of azide dextrose broth prepared in 5.1.

5.4.2 Sterilize capped culture tubes or serum bottles of media in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

6. Analysis

Two questions must be answered when planning a multiple-tube test:

1. What volumes of water need to be tested?

2. How many culture tubes of each volume need to be tested?

Choose a range of volumes so positive and negative results are obtained throughout the range tested. The method fails if only positive or only negative results are obtained when all volumes are tested. The number of culture tubes used per sample volume depends on the precision required. The greater the number of tubes inoculated with each volume, the greater the precision, but the effort involved and expense also are increased. A five-tube series is described below. Order-of-magnitude estimates can be made using a one-tube series.

6.1 Set up five culture tubes of azide dextrose broth for each sample volume to be tested.

6.1.1 If the volume to be tested is 0.1 mL or more, transfer the measured samples directly to the culture tubes using sterile pipets (Note 1).

6.1.2 If the volume of original water sample is less than 0.1 mL, proceed as in 6.1.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Size of inoculum
1:10	----------	0.1 milliliter of original sample
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	----------	0.1 milliliter of 1:100 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution
1:100,000	----------	0.1 milliliter of 1:10,000 dilution

Note 1: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be inoculated within 20 minutes after preparation.

6.2 Clearly mark each set of culture tubes indicating location, time of collection, sample number, and sample volume. Code each tube for easy identification.

6.3 Place the inoculated culture tubes in the culture-tube rack and incubate at 35±0.5 °C for 24±2 hours. Tubes must be maintained in an upright position. Include a tube of uninoculated medium as a control.

6.4 Remove the inoculated culture tubes from the incubator and examine each tube for the presence of turbidity. Any quantity of turbidity in the inoculated tubes, when compared to the control, constitutes a positive presumptive test for fecal streptococci.

6.5 Sterilize the inoculating loop by flaming in the burner. The long axis of the wire needs to be held parallel to the cone of the flame so the entire end of the wire and loop is heated to redness.

6.6 Remove from flame and allow wire to cool for about 10 seconds. Do not allow the inoculating loop to contact any foreign surface during the cooling period.

6.7 Gently shake and uncap a positive culture tube of azide dextrose broth. Insert the inoculating loop beneath the liquid surface and carefully withdraw a loopful of culture. Uncap a tube of EVA broth and insert the loop of culture beneath the liquid surface. Gently swirl the loop to disperse the

bacteria. Repeat this procedure twice more, flaming the loop between inoculations, until three loopfuls of culture have been transferred to the tube containing the EVA broth.

6.8 Recap both culture tubes. Flame the inoculating loop and inoculate additional tubes as in 6.7, transferring three loopfuls of culture to each tube, until all positive cultures have been transferred to EVA broth.

6.9 Return all positive and negative culture tubes of azide dextrose broth and inoculated tubes of EVA broth to the incubator and incubate at 35±0.5 °C for 24±2 hours.

6.10 Remove all culture tubes from the incubator and examine. A positive culture on EVA broth is indicated by a purple button of growth at the bottom of the tube or occasionally by dense turbidity. Sterilize positive EVA broth tubes in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

6.10.1 Reinoculate any negative EVA broth culture tubes using an additional three loopfuls of the original positive azide dextrose broth as in 6.7. Discard the original positive presumptive tubes after autoclaving.

6.10.2 Inoculate into EVA broth material from any additional culture tubes of azide dextrose broth that have become positive during the preceding 24±2-hour incubation.

6.10.3 Return remaining positive azide dextrose broth culture tubes and remaining EVA broth tubes to the incubator and incubate as in 6.3.

6.11 Remove all culture tubes from the incubator and examine.

6.11.1 Discard after autoclaving any EVA broth culture tubes that remain negative after reinoculation in 6.10.1.

6.11.2 Reinoculate any negative EVA broth culture tubes from 6.10.2 with three loopfuls of original positive azide dextrose broth cultures.

6.11.3 Sterilize and discard all remaining culture tubes of azide dextrose broth cultures and all positive tubes of EVA broth.

6.11.4 Return remaining culture tubes of EVA broth to the incubator and incubate as in 6.3.

6.12 Remove the last EVA broth culture tubes and examine. Discard all remaining tubes after autoclaving.

7. Calculations

7.1 Record the number of positive culture tubes occurring for all sample volumes tested. Calculate presumptive fecal streptococci from the total number of positive tubes of azide dextrose broth. Use the number of positive tubes of EVA broth to determine the most probable number of confirmed fecal streptococci.

7.2 When more than three volumes are tested, use the results from only three of them when computing the MPN. To select the three dilutions for the MPN index, use as the first, the smallest sample volume in which all tests are positive (no larger sample volume having any negative results) and the next two succeeding smaller sample volumes (American Public Health Association and others, 1985).

7.3 In the examples listed below, the number in the numerator represents positive culture tubes; the denominator represents the total number of tubes inoculated.

Example	Decimal dilutions				Combination of positives
	1 milliliter	0.1 milliliter	0.01 milliliter	0.001 milliliter	
a	5/5	5/5	2/5	0/5	5-2-0
b	5/5	4/5	2/5	0/5	5-4-2
c	0/5	1/5	0/5	0/5	0-1-0
d	5/5	3/5	1/5	1/5	5-3-2
e	5/5	3/5	2/5	0/5	5-3-2

In example c, the first three dilutions need to be taken to place the positive results in the middle dilution. When a positive result occurs in a dilution larger than the three chosen according to the guideline, as in d, it needs to be placed in the result for the largest chosen dilution as in e (Note 2).

Note 2: The largest dilution has the smallest concentration of the sample; the largest dilution in the preceding table is 0.001.

7.4 The MPN for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-mL dilutions are used are listed in table 4. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value in table 4 needs to be corrected for the dilutions actually used. To do this, divide the value in table 4 by the dilution factor of the first number in the three-number sequence (the culture tubes having the largest concentration of the sample). For example, if dilutions of 0.1, 0.01, and 0.001 mL are used, divide the value in table 4 by 0.1 mL. MPN tables for other combinations of sample volumes and number of tubes at each level of inoculation are in American Public Health Association and others (1985).

Table 4.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used

[mL, milliliters; MPN, most probable number; ---, not applicable; modified from American Public Health Association and others, 1985]

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
0	0	0	<20	---	---
0	0	1	20	<5	70
0	1	0	20	<5	70
0	2	0	40	<5	11
1	0	0	20	<5	70
1	0	1	40	<5	110
1	1	0	40	<5	110
1	1	1	60	<5	150
1	2	0	60	<5	150
2	0	0	50	<5	130
2	0	1	70	10	170
2	1	0	70	10	170
2	1	1	90	20	210
2	2	0	90	20	210
2	3	0	120	30	280
3	0	0	80	10	190
3	0	1	110	20	250
3	1	0	110	20	250
3	1	1	140	40	340
3	2	0	140	40	340
3	2	1	170	50	460
4	0	0	130	30	310
4	0	1	170	50	460
4	1	0	170	50	460
4	1	1	210	70	630
4	1	2	260	90	780
4	2	0	220	70	670
4	2	1	260	90	780
4	3	0	270	90	800
4	3	1	330	110	930
4	4	0	340	120	930

Table 4.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used--Continued

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
5	0	0	230	70	700
5	0	1	310	110	890
5	0	2	430	150	1,100
5	1	0	330	110	930
5	1	1	460	160	1,200
5	1	2	630	210	1,500
5	2	0	490	170	1,300
5	2	1	700	230	1,700
5	2	2	940	280	2,200
5	3	0	790	250	1,900
5	3	1	1,100	310	2,500
5	3	2	1,400	370	3,400
5	3	3	1,800	440	5,000
5	4	0	1,300	350	3,000
5	4	1	1,700	430	4,900
5	4	2	2,200	570	7,000
5	4	3	2,800	900	8,500
5	4	4	3,500	1,200	10,000
5	5	0	2,400	680	7,500
5	5	1	3,500	1,200	10,000
5	5	2	5,400	1,800	14,000
5	5	3	9,200	3,000	32,000
5	5	4	16,000	6,400	58,000
5	5	5	≥24,000	---	---

7.5 Example: The following results were obtained with a five-tube series:

Volume (milliliters)	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Results	5/5	5/5	3/5	1/5	0/5.

Using 10^{-6}, 10^{-7}, and 10^{-8} mL sample volumes, the test results indicate a sequence of 5-3-1 for which the MPN (table 4) is 1,100. Dividing by 10^{-6}, the MPN is computed to be 11×10^{8} streptococcal bacteria per 100 mL and 95-percent confidence limits of 3.1×10^{8} and 25×10^{8} streptococcal bacteria per 100 mL.

8. Reporting of results

Report fecal streptococcal concentration as MPN fecal streptococci per 100 mL as follows: less than 10, whole numbers; 10 or more, two significant figures.

9. Precision

9.1 Precision of the MPN method increases as the number of culture tubes is increased. It increases rapidly as the number of tubes increases from 1 to 5, but then it increases at a slower rate making the gain, when using 10 tubes instead of 5, much less than is achieved by increasing the number of tubes from 1 to 5. Variance as a function of the number of tubes inoculated from a tenfold dilution series is listed below:

Number of culture tubes at each dilution	Variance for tenfold dilution series
1	0.580
3	.335
5	.259
10	.183

9.2 The 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-mL dilutions are used are listed in table 4.

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Bordner, R. H., Winter, J. A., and Scarpino, Pasquale, eds., 1978, Microbiological methods for monitoring the environment, water and wastes: Cincinnati, Ohio, U.S. Environmental Protection Agency, EPA-600/8-78-017, 338 p.

Nitrifying Bacteria (Most-Probable-Number, MPN, Method) (B-0420-85)

Parameter and Code:
Nitrifying bacteria (MPN): 31854

Nitrification is the biological oxidation of reduced nitrogen compounds to nitrite and nitrate. Most commonly, the initial substance is ammonium, and the final product is nitrate. The process has two distinct steps, each mediated by a specific group of bacteria. The Nitrosomonas group, which includes several genera of bacteria, oxidizes ammonium (NH_4^+) only to nitrite (NO_2^-) as shown:

$$NH_4^+ + 3/2\ O_2 \longrightarrow NO_2^- + 2H^+ + H_2O\ .$$

The Nitrobacter group of bacteria oxidizes NO_2^-, but not NH_4^+ or any other reduced nitrogen compound, to nitrate (NO_3^-) as shown:

$$NO_2^- + 1/2\ O_2 \longrightarrow NO_3^-\ .$$

Hydrogen ions produced by the oxidation of NH_4^+ to NO_3^- may be of some geochemical significance because the excess acid can dissolve minerals and can serve as the catalyst in exchange reactions on clays. Nitrification is important in soils because the process controls the supply of NO_3^- used by higher plants. In surface waters, nitrification contributes to oxygen demand.

The organisms, Nitrosomonas and Nitrobacter, are autotrophic bacteria; they obtain their energy from the inorganic oxidations indicated in the preceding paragraph and use carbon dioxide as a source of cellular carbon. Media used to isolate these bacteria are assumed to be free of organic carbon. This assumption is valid initially, and only nitrifiers will grow on the media; however, as these autotrophs grow, they release cell substances to the media, and heterotrophs may develop.

The medium for isolating Nitrosomonas contains NH_4^+. Appearance of NO_2^- in the inoculated cultures, but not in the control cultures, presumptively indicates the presence of Nitrosomonas in the sample. A negative test is not sufficient evidence to prove that Nitrosomonas is absent because NO_2^- produced by Nitrosomonas can be oxidized to NO_3^- by Nitrobacter. Therefore, a positive test for either NO_2^- or NO_3^- in the inoculated cultures indicates the presence of Nitrosomonas. The medium for isolating Nitrobacter contains NO_2^-; disappearance of NO_2^- from the inoculated cultures, but not from the control cultures, presumptively indicates the presence of Nitrobacter.

1. Applications

The method described is similar to that described by Alexander and Clark (1965) and is applicable to all types of soil and fresh and saline water.

2. Summary of method

Decimal dilutions of multiple sample aliquots are inoculated into organic-carbon-free media containing NH_4^+ ions for Nitrosomonas isolation or NO_2^- ions for Nitrobacter isolation. After incubation at 28±1 °C for 21 days, the inoculated cultures and control cultures are tested for the presence of NO_2^-. The most-probable-number (MPN) of each group of nitrifying bacteria is determined from the distribution of positive and negative tests among the inoculated tubes.

3. Interferences

No interferences are known for the procedure.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Aluminum seals, one piece, 20 mm.

4.2 Bottles, milk dilution, screwcap.

4.3 Bottles, serum.

4.4 Crimper, for attaching aluminum seals.

4.5 Culture tubes and caps, borosilicate glass culture tubes, 16×125 mm; tube caps, 16 mm.

4.6 Culture-tube rack, galvanized, for 16-mm culture tubes.

4.7 Decapper, for removing aluminum seals from spent tubes.

4.8 Glass beads, solid, 3 mm may be necessary for soil samples.

4.9 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.10 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.11 Incubator, for operation at a temperature of 28±1 °C, or water bath capable of maintaining a temperature of 28±1 °C.

4.12 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.13 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.14 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.15 Rubber stoppers, 13×20 mm.

4.16 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.17 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Ammonium calcium carbonate medium for MPN of Nitrosomonas. To 1 L distilled water, add 0.5 g ammonium sulfate [$(NH_4)_2SO_4$], 1 g potassium phosphate dibasic (K_2HPO_4), 0.03 g ferrous sulfate ($FeSO_4 \cdot 7H_2O$), 0.3 g sodium chloride (NaCl), 0.3 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$), and 7.5 g calcium carbonate ($CaCO_3$). Place 3 mL of medium in each culture tube; cap and autoclave at 121° at 1.05 kg/cm^2 (15 psi) for 15 minutes.

5.2 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months). Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.3 Dilution water for soils. For dilution blanks, place 95 mL distilled water and approximately three dozen, 3-mm diameter, glass beads in a milk dilution bottle. For each 95-mL dilution blank, also prepare 5 dilution blanks of 90 mL distilled water in milk dilution bottles. Omit the glass beads from the 90-mL dilution blanks. Autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.4 Distilled or deionized water.

5.5 Ethyl alcohol, 70 percent. Dilute 74 mL of 95-percent ethyl alcohol to 100 mL using distilled water. Undiluted isopropanol (ordinary rubbing alcohol) may be used instead of 70-percent ethyl alcohol.

5.6 Nitrite calcium carbonate medium for MPN of Nitrobacter. To 1 L distilled water, add 0.006 g potassium nitrite (KNO_2), 1 g potassium phosphate dibasic (K_2HPO_4), 0.3 g sodium chloride (NaCl), 0.1 g magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 1 g calcium carbonate ($CaCO_3$), and 0.3 g calcium chloride ($CaCl_2$). Place 3 mL of medium in each culture tube; cap and autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes.

5.7 Nitrite-test reagent. Add 200 mL concentrated phosphoric acid (specific gravity 1.69) and 20 g sulfanilamide to approximately 1.5 L demineralized water. Dissolve completely (warm if necessary). Add 1 g N-1 naphthylethylenediamine dihydrochloride and dissolve completely. Dilute to 2 L using demineralized water. Store in an amber bottle and refrigerate. The reagent must be at room temperature when it is used. The reagent is stable for approximately 1 month.

5.8 Zinc copper manganese dioxide mixture. Mix together 1 g powdered zinc metal (Zn), 0.1 g powdered copper (Cu), and 1 g powdered manganese dioxide (MnO_2).

6. Analysis

Two questions must be answered when planning a multiple-tube test:

1. What volumes of water need to be tested?

2. How many culture tubes of each volume need to be tested?

Choose a range of volumes so positive and negative results are obtained throughout the range tested. The method fails if only positive or only negative results are obtained when all volumes are tested. The number of culture tubes used per sample volume depends on the precision required. The greater the number of tubes inoculated with each volume, the greater the precision, but the effort involved and expense also are increased. For general use, the three-tube series is recommended and is described in this section. Order-of-magnitude estimates can be made using a one-tube series. The following test volumes are suggested:

1. For water samples, use volumes of 1, 0.1, 0.01, 0.001, and 0.0001 mL.

2. For soil samples, use dilutions of 10^{-2} to 10^{-6} mL.

It may be advisable to do an order-of-magnitude estimate prior to undertaking an extensive sampling program.

6.1 Before starting the analysis, clear an area of the laboratory bench and swab it using a bit of cotton moistened with 70-percent ethyl alcohol, undiluted isopropanol, or disinfectant.

6.2 Set out three culture tubes of ammonium calcium carbonate medium and three tubes of nitrite calcium carbonate medium for each volume to be tested. For each dilution series, set aside one extra tube of each medium as an uninoculated control tube.

6.2.1 If the volume to be tested is 0.1 mL or more, transfer the measured samples directly to the culture tubes using sterile pipets (Note 1). Carefully remove caps from sterile tubes to avoid contamination.

6.2.2 If the volume of the desired sample aliquot is less than 0.1 mL, proceed as in 6.2.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Size of inoculum
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	----------	0.1 milliliter of 1:100 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution
1:100,000	----------	0.1 milliliter of 1:10,000 dilution
$1:10^6$	1 milliliter of 1:10,000 dilution	1 milliliter of $1:10^6$ dilution
$1:10^7$	----------	0.1 milliliter of $1:10^6$ dilution

Note 1: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be inoculated within 20 minutes after preparation.

6.2.3 Dilution series of soil samples are prepared as follows: Transfer 10 g of soil to a dilution blank containing 95 mL water and glass beads. Cap the bottle and shake vigorously for 1 minute. Immediately transfer 10 mL from the center of the suspension to a 90-mL dilution blank and shake. Continue transferring 10-mL portions to 90-mL dilution blanks until the desired dilution is reached.

6.3 Clearly mark each set of inoculated culture tubes indicating location, time of collection, sample number, and sample volume. Code each tube for easy identification.

6.4 Place the inoculated culture tubes and control tubes in a culture-tube rack and incubate at 28±1 °C for 21 days. Clearly defined results will occur only if the bacteria consume all the NO_2^-(or convert all NH_4^+ to NO_2^-). For this reason, incubation should always be for 21 days.

6.5 Test for the production of NO_2^-. After incubation, add 0.5 mL of the nitrite-test reagent to each inoculated culture tube and control tube. Observe the contents of each tube for the development within 5 minutes of a reddish color.

CAUTION.--Nitrite-test reagent contains acid and must be handled carefully.

6.6 Growth of Nitrosomonas usually is evidenced by a brick-red color at the bottom of a culture tube and a purplish-red coloration in the overlying liquid. Control tubes and inoculated tubes having no NO_2^- may turn faintly pink; thus, it is imperative that uninoculated control tubes be used in color comparison.

6.7 To all culture tubes of ammonium calcium carbonate medium (Nitrosomonas) that do not develop a purplish-red color within 5 minutes, add a small pinch of the zinc copper manganese dioxide mixture. If a reddish color develops, record the culture tube as positive for Nitrosomonas on the basis that the initial negative reading for NO_2^- indicated that the NO_2^- produced by Nitrosomonas was oxidized to NO_3^- by Nitrobacter.

6.8 Record as positive for Nitrobacter all culture tubes of nitrite calcium carbonate medium that do not develop the characteristic purplish-red color formed by the reaction of NO_2^- with the nitrite-test reagent.

6.9 A positive result in a control culture tube indicates a contamination of the medium and results of the test, therefore, are invalid.

6.10 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

Record the number of positive inoculated culture tubes occurring for all sample volumes tested. When more than three volumes are tested, use results from only three of them when computing the MPN. To select the three dilutions for the MPN index, use as the first, the smallest sample volume in which all tests are positive (no larger sample volume having any negative results) and the next two succeeding smaller sample volumes (American Public Health Association and others, 1985).

In the examples listed below, the number in the numerator represents positive culture tubes; the denominator represents the total number of tubes inoculated.

Example	Decimal dilutions				Combination of positives
	1 milliliter	0.1 milliliter	0.01 milliliter	0.001 milliliter	
a	3/3	3/3	2/3	0/3	3-2-0
b	0/3	1/3	0/3	0/3	0-1-0
c	3/3	2/3	1/3	1/3	3-2-2
d	3/3	2/3	2/3	0/3	3-2-2

In example b, the three dilutions need to be taken to place the positive results in the middle dilution. When a positive result occurs in a dilution larger than the three chosen according to the guideline, as in c, it needs to be placed in the result for the largest chosen dilution as in d (Note 2).

Note 2: The largest dilution has the smallest concentration of the sample; the largest dilution in the preceding table is 0.001.

7.3 The MPN for various combinations of positive and negative results when three 1-, three 0.1-, and three 0.01-mL dilutions are used are listed in table 5. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value in table 5 needs to be corrected for the dilutions actually used. To do this, divide the value in table 5 by the dilution factor of the first number in the three-number sequence (the culture tubes having the largest concentration of the sample). For example, if dilutions of 0.1, 0.01, and 0.001 mL are used, divide the value in table 5 by 0.1 mL. MPN tables for other combinations of sample volumes and numbers of tubes at each level of inoculation are in American Public Health Association and others (1985).

7.4 If only one culture tube is inoculated at each decimal dilution level, record the smallest dilution showing a positive response compared to the largest dilution showing a negative response. Record the results as a range of numbers, for example 100 to 1,000 nitrifying bacteria per milliliter. If all tubes are positive, record the result as a number greater than that indicated by the value of the largest dilution of the series. For example, 1-, 0.1-, and 0.01-mL samples are tested, and all tubes are positive at the end of the test. Record the result as greater than 100 nitrifying bacteria per milliliter (greater than 10^4 nitrifying bacteria per 100 mL).

Table 5.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when three 1-, three 0.1-, and three 0.01-milliliter dilutions are used

[mL, milliliters; MPN, most probable number; ---, not applicable; modified from American Public Health Association and others, 1985]

Number of culture tubes indicating positive reaction out of:			MPN index per 100 mL	95-percent confidence limits	
Three of 1 mL each	Three of 0.1 mL each	Three of 0.01 mL each		Lower	Upper
0	0	0	<30	---	---
0	0	1	30	<5	90
0	1	0	30	<5	130
1	0	0	40	<5	200
1	0	1	70	10	210
1	1	0	70	10	230
1	1	1	110	30	360
1	2	0	110	30	360
2	0	0	90	10	360
2	0	1	140	30	370
2	1	0	150	30	440
2	1	1	200	70	890
2	2	0	210	40	470
2	2	1	280	100	1,500
3	0	0	230	40	1,200
3	0	1	390	70	1,300
3	0	2	640	150	3,800
3	1	0	430	70	2,100
3	1	1	750	140	2,300
3	1	2	1,200	300	3,800
3	2	0	930	150	3,800
3	2	1	1,500	300	4,400
3	2	2	2,100	350	4,700
3	3	0	2,400	360	13,000
3	3	1	4,600	710	24,000
3	3	2	11,000	1,500	48,000
3	3	3	≥24,000	---	---

7.5 Examples of test results and calculations are listed below.

7.5.1 The following results were obtained with a three-tube series:

[-, negative; +, positive]

Volume (milliliters)	Culture tube number 1	2	3	Result
0.1	+	+	+	3/3
0.01	+	+	+	3/3
0.001	+	+	-	2/3
0.0001	-	-	-	0/3

Following the guideline given above and using 0.01-, 0.001-, and 0.0001-mL sample volumes, the test results indicate a sequence of 3-2-0. From this, an MPN of 930 is indicated (table 5). Dividing by 0.01 mL to correct for the effect of dilution, the MPN of the sample is 9.3×10^4 nitrifying bacteria per 100 mL. The 95-percent confidence limits are 1.5×10^4 and 38×10^4 nitrifying bacteria per 100 mL.

7.5.2 The following results were obtained with a three-tube series:

Volume (milliliters)	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Results	3/3	3/3	2/3	1/3	0/3.

Using 10^{-6}, 10^{-7}, and 10^{-8} mL sample volumes, the test results indicate a sequence of 3-2-1 for which the MPN (table 5) is 1,500. Dividing by 10^{-6}, the MPN is computed to be 15×10^8 nitrifying bacteria per 100 mL and 95-percent confidence limits of 3.0×10^8 and 44×10^8 nitrifying bacteria per 100 mL.

7.5.3 The following results were obtained with a three-tube series:

Volume (milliliters)	1	0.1	0.01	0.001
Results	0/3	1/3	0/3	0/3.

Use the sequence of 0-1-0 for which the MPN is 30 and 95-percent confidence limits of <5 and 130 (table 5).

7.6 The various combinations listed in table 5 represent those most likely to be obtained. Other combinations are statistically unlikely. If unlikely combinations are obtained, it is probable either that the multiple-tube technique is inapplicable or that errors of manipulation have occurred.

8. Reporting of results

Report concentration of nitrifying bacteria as MPN Nitrosomonas and MPN Nitrobacter per 100 mL for water samples or as MPN per 100 g for soil samples as follows: less than 10, whole numbers; 10 or more, two significant figures. Indicate the method of expressing unit weight (wet or dry) of soil samples.

9. Precision

9.1 Precision of the MPN method increases as the number of culture tubes is increased. It increases rapidly as the number of tubes increases from 1 to 5, but then it increases at a slower rate making the gain, when using 10 tubes instead of 5, much less than is achieved by increasing the number of tubes from 1 to 5. Variance as a function of the number of tubes inoculated from a tenfold dilution series is listed below:

Number of culture tubes at each dilution	Variance for tenfold dilution series
1	0.580
3	.335
5	.259
10	.183

9.2 The 95-percent confidence limits for various combinations of positive and negative results when three 1-, three 0.1-, and three 0.01-mL dilutions are used are listed in table 5.

10. References cited

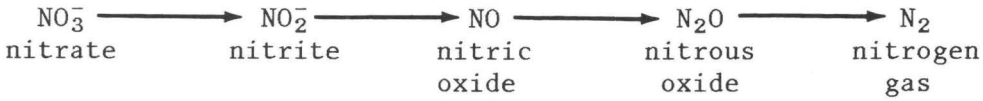
Alexander, Martin, and Clark, F. E., 1965, Nitrifying bacteria, in Black, C. A., ed., Methods of soil analysis: Madison, Wis., American Society of Agronomy, Part 2, p. 1477-1483.

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Denitrifying and Nitrate-Reducing Bacteria (Most-Probable-Number, MPN, Method) (B-0430-85)

Parameter and Code:
Denitrifying bacteria (MPN): 31856

Some bacteria reduce the nitrogen (N) atom of nitrate (NO_3^-). This occurs by a sequence of reactions that may stop at the level of nitrite (NO_2^-) or proceed to completion with the production of gaseous N compounds. The following pathway indicates the steps involved:



The bacteria that cause these reactions can be referred to collectively as nitrate-reducers or nitrate-respirers. Organisms that do only the first step produce NO_2^- and sometimes are called nitrite-accumulators. They also are commonly referred to by the more general terms nitrate-reducers or nitrate-respirers. The term denitrifiers is more specific and is used for those bacteria that remove N from the system by producing gaseous end products.

Regardless of the final product, the bacteria involved are using the N atom as a sink for the electrons generated during the oxidation of their energy source. Because these denitrifying bacteria also use oxygen as a terminal electron acceptor (aerobic respiration) and will do so as long as oxygen is available, NO_3^- and other oxidized N forms will not be reduced until oxygen has been depleted. Essentially, the bacteria continue respiration even though NO_3^- or NO_2^- has replaced oxygen in their metabolism.

A large and diverse group of bacteria causes NO_3^- reduction and denitrification. Typically, the number of nitrite-accumulators in an environment is greater than the number of denitrifiers. Species in the following genera are believed to be most significant in denitrification in soil: Pseudomonas, Alcaligenes, and Flavobacterium (Gamble and others, 1977). Bacillus and Paracoccus species may be significant in some environments.

Because of the diversity of the group of organisms responsible for NO_3^- reduction and denitrification, the environmental conditions necessary for the processes to occur are not too restrictive. Ranges reported for pH (5-9) and temperature (15-65 °C) are quite broad (Focht and Verstraete, 1977). Various types of soil, sediment, fresh and saline water, and sewage-treatment systems support NO_3^- reduction and denitrification. There are two environmental factors that have an important effect on NO_3^- reduction: A suitable energy source (usually a carbon-containing compound) must be available, and oxygen must be absent because it will be used in preference to NO_3^- by denitrifying and nitrate-respiring bacteria. However, denitrification can take place in apparently well-aerated systems due to the existence of anaerobic microsites.

1. Applications

The method is for the determination of the most probable number (MPN) of nitrate-reducing and denitrifying bacteria. The method is applicable to all types of soil and fresh water.

2. Summary of method

2.1 Samples of soil or water and decimal dilutions thereof are inoculated into nutrient broth containing 0.1 percent potassium nitrate (KNO_3). The cultures are incubated at 28±1 °C for 14 days and scored for gas production, production of NO_2^-, and loss of NO_3^-. The MPN of denitrifiers in the sample is determined by the distribution of culture tubes indicating gas production and loss of NO_3^-. Nitrate-reducers (nitrite-accumulators) in the sample may be isolated by the distribution of tubes containing NO_2^-.

2.2 The method is similar to that of Focht and Joseph (1973) and depends on trapping the gas produced and detecting any NO_2^- or NO_3^- remaining in the culture tube.

3. Interferences

Large concentrations of heavy metals or toxic chemicals in the soil or water sample to be tested may interfere.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Aluminum seals, one piece, 20 mm.

4.2 Bottles, milk dilution, screwcap.

4.3 Bottles, serum.

4.4 Crimper, for attaching aluminum seals.

4.5 Culture tubes and caps, borosilicate glass, screwcap culture tubes, 16×125 mm. Larger screwcap tubes may be used if larger volumes of water are analyzed. Screwcap tubes will slow diffusion of oxygen from the atmosphere and promote anaerobic conditions.

4.6 Culture-tube rack, use any rack appropriate for culture tubes being used.

4.7 Decapper, for removing aluminum seals from spent tubes.

4.8 Durham (fermentation) tubes. The durham tube, used to detect gas production, must be completely filled with medium and at least partly submerged in the culture tube. For 16×125-mm culture tubes, use 6×50-mm durham tubes.

4.9 Glass beads, solid, 3 mm, may be necessary for soil samples.

4.10 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.11 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.12 Incubator, for operation at a temperature of 28±1 °C or water bath capable of maintaining a temperature of 28±1 °C.

4.13 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.14 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.15 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.16 Rubber stoppers, 13×20 mm.

4.17 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.18 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months). Dispense in milk dilution or serum bottles (capped with rubber stoppers and crimped with aluminum seals) in

quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 Dilution water for soil. For dilution blanks, place 95 mL distilled water and approximately three dozen, 3-mm diameter, glass beads in a milk dilution bottle. For each 95-mL dilution blank, also prepare 5 dilution blanks of 90 mL distilled water in milk dilution bottles. Omit the glass beads from the 90-mL dilution blanks. Autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.3 Distilled or deionized water.

5.4 Ethyl alcohol, 70 percent. Dilute 74 mL 95-percent ethyl alcohol to 100 mL using distilled water. Undiluted isopropanol (ordinary rubbing alcohol) may be used instead of 70-percent ethyl alcohol.

5.5 Nitrate broth. Use nitrate broth or nutrient broth, plus 0.1 percent KNO_3. Prepare according to directions on bottle label. Place 9 mL medium in a 16×125-mm culture tube for each 1-mL or smaller aliquot of sample to be tested. In each culture tube, place an inverted (mouth downward) durham tube (fig. 3). Place caps on culture tubes. Sterilize tubes in upright position at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes as soon as possible after dispensing medium. Loosen screwcaps prior to sterilizing and tighten when tubes have cooled. Air will be expelled from the inverted durham tubes during heating; each will fill completely with medium during cooling. Discard any culture tube in which air bubbles are visible in the durham tube.

5.6 Nitrite-test reagent. Add 200 mL concentrated phosphoric acid (specific gravity 1.69) and 20 g sulfanilamide to approximately 1.5 L demineralized water. Dissolve completely (warm if necessary). Add 1 g N-1 naphthylethylenediamine dihydrochloride and dissolve completely. Dilute to 2 L using demineralized water. Store in an amber bottle and refrigerate. The reagent must be at room temperature when it is used. The reagent is stable for approximately 1 month.

5.7 Zinc copper manganese dioxide mixture. Mix together 1 g powdered zinc metal (Zn), 1 g powdered manganese dioxide (MnO_2), and 0.1 g powdered copper (Cu).

6. Analysis

Two questions must be answered when planning a multiple-tube test:

1. What volumes of water need to be tested?

2. How many culture tubes of each volume need to be tested?

Choose a range of volumes so positive and negative results are obtained throughout the range tested. The method fails if only positive or only negative results are obtained when all volumes are tested. The number of culture tubes used per sample volume depends on the precision required. The greater the number of tubes inoculated with each volume, the greater the precision, but the effort involved and expense also are increased. For general use, the three-tube series is recommended and is described in this section. Order-of-magnitude estimates can be made using a one-tube series. Increased precision can be obtained using a five-tube series. The following test volumes are suggested:

1. For water samples, use volumes of 1, 0.1, 0.01, 0.001, and 0.0001 mL.

2. For sewage or heavily polluted water samples, use volumes of 10^{-2} to 10^{-6} mL.

3. For soil samples, use dilutions of 10^{-2} to 10^{-6} mL.

It may be advisable to do an order-of-magnitude estimate prior to undertaking an extensive sampling program.

6.1 Before starting the analysis, clear an area of the laboratory bench and swab it using a bit of cotton moistened with 70-percent ethyl alcohol, undiluted isopropanol, or disinfectant.

6.2 Set out three culture tubes of nitrate broth for each volume to be tested. For each dilution series, set aside one extra tube of medium as an uninoculated control tube.

6.2.1 If the volume to be tested is 0.1 mL or more, transfer the measured samples directly to the culture tubes using sterile pipets (Note 1). Carefully remove caps from sterile tubes to avoid contamination.

6.2.2 If the volume of the desired sample aliquot is less than 0.1 mL, proceed as in 6.2.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Size of inoculum
1:10	----------	0.1 milliliter of original sample
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	----------	0.1 milliliter of 1:100 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution
1:100,000	----------	0.1 milliliter of 1:10,000 dilution

Note 1: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the organisms in the sample. Diluted samples need to be inoculated within 20 minutes after preparation.

6.2.3 Dilution series of soil samples are prepared as follows: Transfer 10 g of soil to a dilution blank containing 95 mL water and glass beads. This is a 1:10 dilution. Cap the bottle and shake vigorously for 1 minute. Immediately transfer 10 mL from the center of the suspension to a 90-mL dilution blank and shake. This is a 1:100 dilution. Continue transferring 10-mL portions to 90-mL dilution blanks until the desired dilution is reached.

6.3 Clearly mark each set of inoculated culture tubes indicating location, time of collection, sample number, and sample volume. Code each tube for easy identification.

6.4 Place the inoculated culture tubes and control tubes in a culture-tube rack and incubate tubes at 28±1 °C for 14 days.

6.5 Examine the culture tubes after 14 days. Each tube will be examined for three characteristics in the following order: gas formation, production of NO_2^-, and removal of NO_3^-. A flow diagram of the test procedure for each culture is shown in figure 7.

6.5.1 Gas production is determined by examining the durham tube for gas bubbles (fig. 4). Any bubble is presumptive evidence for denitrification; however, a check for removal of NO_3^- is advised.

6.5.2 Test for the production of NO_2^-. Add 0.5 mL nitrite-test reagent to each inoculated culture tube and control tube. Tubes that show a red color are positive for NO_2^-.

CAUTION.--Nitrite-test reagent contains acid and must be handled carefully.

6.5.3 Test for the presence of NO_3^-. To all culture tubes that remain colorless or have only a light pink color, add about 50 mg zinc copper manganese dioxide mixture. This mixture of metals reduces any NO_3^- remaining in the tube to NO_2^-. The NO_2^- reacts with the nitrite-test reagent already in the tube to give a deep red color. If the red color develops within 5 minutes, record the tube as positive for NO_3^-.

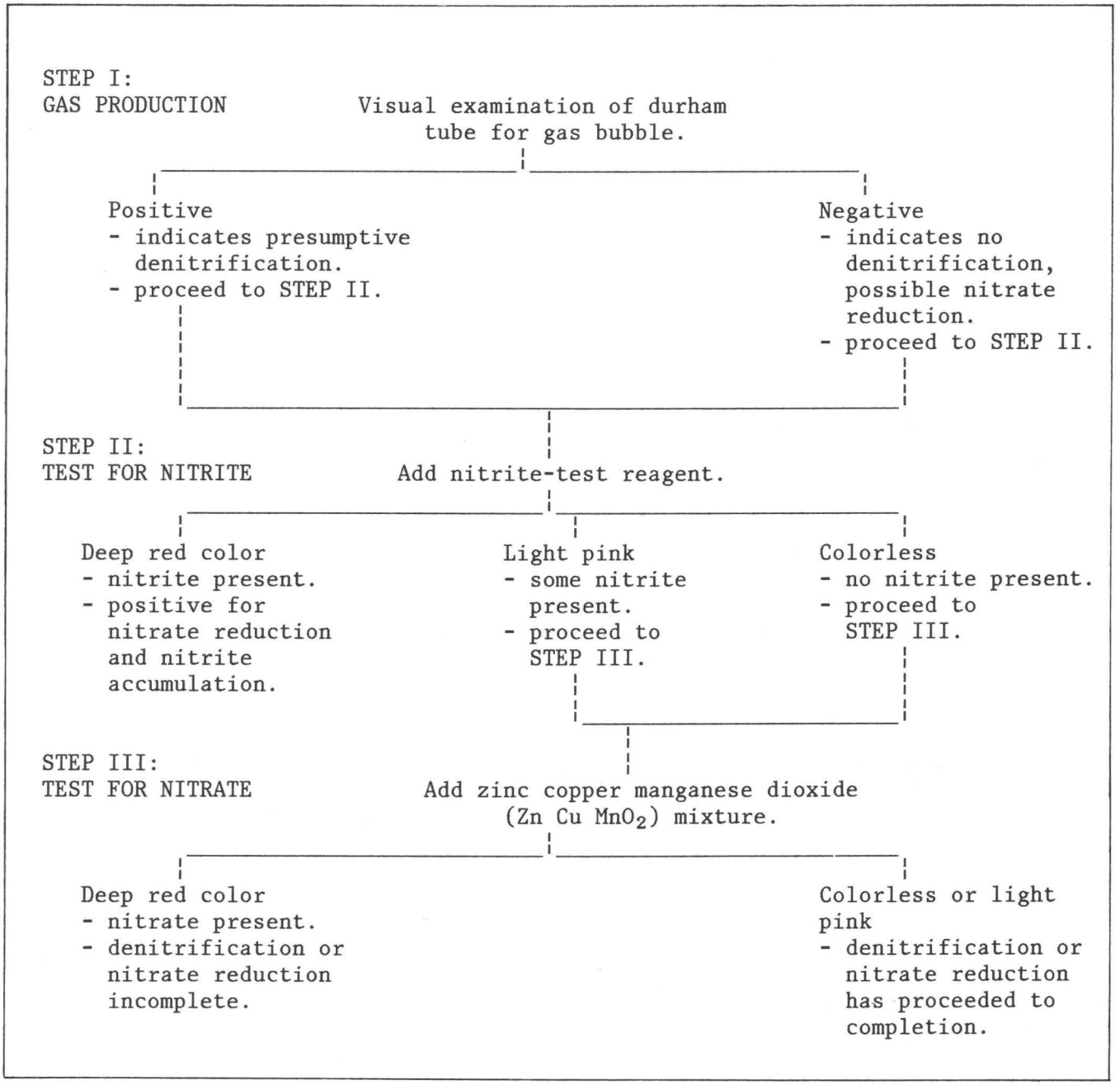


Figure 7.--Flow diagram showing the test procedure for each culture of denitrifying or nitrate-reducing bacteria.

6.5.4 Examples of possible results for any given culture tube and interpretation:

[-, negative; +, positive]

Sample	Gas	Nitrite	Nitrate
A	-	-	+
B	+/	-	-
C	-	+	not tested
D	-	+	+
E	+	+	-
F	+	-	+
G	-	-	-

Sample A: Negative for denitrification.
Negative for nitrate reduction.

Sample B: Positive for denitrification.

Sample C: Negative for denitrification.
Positive for nitrate reduction.

Sample D: Negative for denitrification.
Positive for nitrate reduction.

Sample E: Positive for denitrification.
Positive for nitrate reduction.

Sample F: Inconclusive.

Sample G: NO_3^- has been removed, although there is no accumulation of NO_2^- and no apparent gas production. It is possible that nitrous oxide (N_2O), which is soluble in water, has been produced. It also is possible that the NO_3^- has been reduced to some other unknown compound.

6.6 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculation

7.1 Record the number of positive inoculated culture tubes occurring for all sample volumes tested. When more than three volumes are tested, use results from only three of them when computing the MPN. To select the three dilutions for the MPN index, use as the first, the smallest sample volume in which all tests are positive (no larger sample volume having any negative results) and the next two succeeding smaller sample volumes (American Public Health Association and others, 1985).

7.2 In the examples listed below, the number in the numerator represents positive culture tubes; the denominator represents the total number of tubes inoculated.

Example	Decimal dilutions				Combination of positives
	1 milliliter	0.1 milliliter	0.01 milliliter	0.001 milliliter	
a	3/3	3/3	2/3	0/3	3-2-0
b	0/3	1/3	0/3	0/3	0-1-0
c	3/3	2/3	1/3	1/3	3-2-2
d	3/3	2/3	2/3	0/3	3-2-2

In example b, the three dilutions need to be taken to place the positive results in the middle dilution. When a positive result occurs in a dilution larger than the three chosen according to the guideline, as in c, it needs to be placed in the result for the largest chosen dilution as in d (Note 2).

Note 2: The largest dilution has the smallest concentration of the sample; the largest dilution in the preceding table is 0.001.

7.3 The MPN for various combinations of positive and negative results when three 1-, three 0.1-, and three 0.01-mL dilutions are used are listed in table 6. If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN value in table 6 needs to be corrected for the dilutions actually used. To do this, divide the value in table 6 by the dilution factor of the first number in the three-number sequence (the culture tubes having the largest concentration of the sample). For example, if dilutions of 0.1, 0.01, and 0.001 mL are used, divide the value in table 6 by 0.1 mL. MPN tables for other combinations of sample volumes and numbers of tubes at each level of inoculation are in American Public Health Association and others (1985).

7.4 If only one culture tube is inoculated at each decimal dilution level, record the smallest dilution indicating a positive response compared to the largest dilution indicating a negative response. Record the results as a range of numbers, for example 100 to 1,000 denitrifying bacteria per milliliter. If all tubes are positive, record the result as a number greater than that indicated by the value of the largest dilution of the series. For example, 1-, 0.1-, and 0.01-mL samples are tested, and all tubes are positive at the end of the test. Record the result as greater than 100 denitrifying bacteria per milliliter.

Table 6.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when three 1-, three 0.1-, and three 0.01-milliliter dilutions are used

[mL, milliliters; MPN, most probable number; ---, not applicable; modified from American Public Health Association and others, 1985]

Number of culture tubes indicating positive reaction out of:			MPN index per 1 mL	95-percent confidence limits	
Three of 1 mL each	Three of 0.1 mL each	Three of 0.01 mL each		Lower	Upper
0	0	0	<0.3	---	----
0	0	1	.3	<0.05	0.9
0	1	0	.3	<.05	1.3
1	0	0	.4	<.05	2.0
1	0	1	.7	.1	2.1
1	1	0	.7	.1	2.3
1	1	1	1.1	.3	3.6
1	2	0	1.1	.3	3.6
2	0	0	.9	.1	3.6
2	0	1	1.4	.3	3.7
2	1	0	1.5	.3	4.4
2	1	1	2.0	.7	8.9
2	2	0	2.1	.4	4.7
2	2	1	2.8	1.0	15.0
3	0	0	2.3	.4	12.0
3	0	1	3.9	.7	13.0
3	0	2	6.4	1.5	38.0
3	1	0	4.3	.7	21.0
3	1	1	7.5	1.4	23.0
3	1	2	12.0	3.0	38.0
3	2	0	9.3	1.5	38.0
3	2	1	15.0	3.0	44.0
3	2	2	21.0	3.5	47.0
3	3	0	24.0	3.6	130.0
3	3	1	46.0	7.1	240.0
3	3	2	110.0	15.0	480.0
3	3	3	≥240.0	---	---

7.5 Examples of test results and calculations are listed below.

7.5.1 The following results were obtained with a three-tube series

[-, negative; +, positive]

Volume (milliliters)	Culture tube number 1	2	3	Result
0.1	+	+	+	3/3
0.01	+	+	+	3/3
0.001	+	+	-	2/3
0.0001	-	-	-	0/3

Following the guideline in 7.3 and using 0.01-, 0.001-, and 0.0001-ml sample volumes, a sequence of 3-2-0 is indicated. From this, an MPN of 9.3 is indicated (table 6). Dividing by 0.01 mL to correct for the effect of dilution, the MPN of the sample is 930 denitrifying bacteria per milliliter. The 95-percent confidence limits are 150 and 3,800.

7.5.2 The following results were obtained with a three-tube series:

Volume (milliliters)-----	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Results-----------------	3/3	3/3	2/3	1/3	0/3.

Using 10^{-6}, 10^{-7}, and 10^{-8} mL sample volumes, the test results indicate a sequence of 3-2-1 for which the MPN (table 6) is 15.0. Dividing by 10^{-6}, the MPN is computed to be 15×10^{6} denitrifying bacteria per milliliter and 95-percent confidence limits of 3.0×10^{6} and 44×10^{6} denitrifying bacteria per milliliter.

7.5.3 The following results were obtained with a three-tube series:

Volume (milliliters)-------	1	0.1	0.01	0.001
Results-------------------	0/3	1/3	0/3	0/3.

Use the sequence of 0-1-0 for which the MPN is 0.3 and 95-percent confidence limits of <0.05 and 1.3.

7.6 The various combinations listed in table 6 represent those most likely to be obtained. Other combinations are statistically unlikely. If unlikely combinations are obtained, it is probable either that the multiple-tube technique is inapplicable or that errors of manipulation have occurred.

8. Reporting of results

Report the concentration of denitrifying or nitrate-reducing bacteria, or both, as MPN per milliliter for water samples or as MPN per gram for soil samples as follows: less than 10, whole numbers; 10 or more, two significant figures. Indicate the method of expressing unit weight (wet or dry) of soil samples.

9. Precision

9.1 Precision of the MPN method increases as the number of culture tubes is increased. It increases rapidly as the number of tubes increases from 1 to 5, but then it increases at a slower rate making the gain, when using 10 tubes instead of 5, much less than is achieved by increasing the number of tubes from 1 to 5. Variance as a function of the number of tubes inoculated from a tenfold dilution series is listed below:

Number of culture tubes at each dilution	Variance for tenfold dilution series
1	0.580
3	.335
5	.259
10	.183

9.2 The 95-percent confidence limits for various combinations of positive and negative results when three 1-, three 0.1-, and three 0.01-mL dilutions are used are listed in table 6.

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

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Sulfate-Reducing Bacteria (Most-Probable-Number, MPN, Method) (B-0400-85)

Parameter and Code:
Sulfate-reducing bacteria (MPN): 31855

Sulfate-reducing bacteria commonly are found in environments where reducing conditions prevail, such as ground water, the hypolimnion of stratified lakes, saturated soil, and mud from lake bottoms and stream bottoms. The geochemical implications of sulfate-reducing bacteria have been discussed by Kuznetsov and others (1963). Although many species of bacteria reduce sulfate during the synthesis of sulfur-containing amino acids, four genera of obligate anaerobic bacteria use sulfate reduction as a major energy-yielding reaction and produce large quantities of hydrogen sulfide. These are Desulfovibrio, Desulfotomaculum, Desulfomonas, and Desulfobulbus.

1. Applications

The method described in this section is similar to the sulfate-reducing bacteria test given in the American Petroleum Institute (1965). The method is applicable for all water, including brine with large salt concentrations.

2. Summary of method

2.1 Samples are collected and handled using techniques that minimize exposure to oxygen. The samples are incubated at 18 to 25 °C for 28 days, and results are recorded. The most probable number (MPN) of organisms in the sample is determined from the positive and negative responses among a number of inoculated serum bottles of suitable culture medium.

2.2 The sulfate-reducing bacteria are cultivated on a medium containing lactate as a carbon and energy source. Growth is enhanced in the presence of yeast extract. Ascorbic acid is present as a reducing agent. Hydrogen sulfide produced by the bacteria reacts with ferrous iron to produce an inky blackening of the culture medium. Blackening of the culture medium is a positive response and indicates the presence of sulfate-reducing bacteria.

3. Interferences

3.1 Other species of facultative and obligate anaerobic bacteria can grow in the lactate-yeast extract broth and produce a turbidity in the medium, but only sulfate reducers will produce the characteristic inky blackening.

3.2 According to Postgate (1959), the Eh of the culture medium must be less than -200 mV for initiation of growth of sulfate-reducing bacteria. The presence of traces of oxygen will render the medium unsuitable.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Cotton balls.

4.2 Decapper, for removing aluminum seals from spent serum bottles.

4.3 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.4 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.5 Rubber stoppers, 13×20 mm.

4.6 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines given in the "Collection" subsection of the "Bacteria" section.

4.7 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Distilled or deionized water.

5.2 Ethyl alcohol, 70 percent. Dilute 74 mL 95-percent ethyl alcohol to 100 mL using distilled water. Undiluted isopropanol (ordinary rubbing alcohol) may be used instead of 70-percent ethyl alcohol.

5.3 Sulfate API broth. Ready-to-use presterilized medium packed in 10-mL serum bottles.

6. Analysis

Two questions must be answered when planning a multiple serum-bottle test:

1. What volumes of water need to be tested?

2. How many serum bottles of each volume need to be tested?

Choose a range of volumes so positive and negative results are obtained throughout the range tested. The method fails if only positive or only negative results are obtained when all volumes are tested. The number of serum bottles used per sample volume depends on the precision required. The greater the number of bottles inoculated with each volume, the greater the precision, but the effort involved and expense also are increased. For general use, the three serum-bottle series is recommended and is described in this section. Order-of-magnitude estimates can be made using a one serum-bottle series. Increased precision can be obtained using a five serum-bottle series. The following test volumes are suggested: For water samples, use volumes of 1, 0.1, 0.01, 0.001, and 0.0001 mL. It may be advisable to do an order-of-magnitude estimate prior to undertaking an extensive sampling program.

6.1 Remove the inserts from the metal caps of the serum bottles and swab the exposed area of the rubber septa using a bit of cotton saturated with 70-percent ethyl alcohol, undiluted isopropanol, or disinfectant.

6.2 Using a sterile syringe, withdraw 1 mL of sample.

6.3 Invert a serum bottle so the rubber septum is at the bottom. Inoculate the medium by carefully puncturing the septum with the sterile hypodermic syringe and insert the needle until only the beveled tip is inside the bottle. Discharge the contents of the syringe into the bottle and withdraw the needle. Agitate the bottle vigorously.

6.4 Using a new sterile syringe, withdraw 1 mL from the previously inoculated serum bottle and then inoculate a fresh bottle as in 6.3.

6.5 To conserve time and reagents, a scheme such as given in the following example is recommended. Suppose it is desired to test 0.1, 0.01, and 0.001 mL of a given water sample:

6.5.1 Set out 10 serum bottles of culture medium.

6.5.2 Prepare them as in 6.1.

6.5.3 Withdraw 1 mL of sample as in 6.2 and inoculate one serum bottle of medium as in 6.3.

6.5.4 Using the dilution prepared in 6.5.3, inoculate three fresh serum bottles of culture medium as in 6.4 to prepare the 0.1-mL dilutions.

6.5.5 Using one of the dilutions prepared in 6.5.4, inoculate three fresh serum bottles of culture medium as in 6.4 to prepare the 0.01-mL dilutions.

6.5.6 Using one of the dilutions prepared in 6.5.5, inoculate three fresh serum bottles of culture medium as in 6.4 to prepare the 0.001-mL dilutions.

Similar schemes can be established for other combinations using any number of bottles per dilution level.

6.6 Clearly mark each set of inoculated serum bottles indicating location, time of collection, sample number, and sample volume. Code each bottle for easy identification.

6.7 Incubate serum bottles at room temperature (18 to 25 °C) for 28 days. Do not consider serum bottles that turn black within 2 hours as positive because this probably is due to the presence of sulfide ion in the sample. Subcultures of these false positives may be made after 1 week following the guidelines in 6.1 through 6.3.

6.8 Examine the serum bottles after 28 days. Record as positive all bottles that have substantial quantities of black precipitate. When shaken, the bottles should assume an inky black appearance. Record as negative all bottles in which the medium is turbid but only slightly grayish.

6.9 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 Record the number of positive inoculated serum bottles occurring for all sample volumes tested. When more than three volumes are tested, use results from only three of them when computing the MPN. To select the three dilutions for the MPN index, use as the first, the smallest sample volume in which all tests are positive (no larger sample volume having any negative results) and the next two succeeding smaller sample volumes (American Public Health Association and others, 1985).

7.2 In the examples listed below, the number in the numerator represents positive serum bottles; the denominator represents the total number of bottles inoculated.

Example	Decimal dilutions				Combination of positives
	1 milliliter	0.1 milliliter	0.01 milliliter	0.001 milliliter	
a	3/3	3/3	2/3	0/3	3-2-0
b	0/3	1/3	0/3	0/3	0-1-0
c	3/3	2/3	1/3	1/3	3-2-2
d	3/3	2/3	2/3	0/3	3-2-2

In example b, the three dilutions need to be taken to place the positive results in the middle dilution. When a positive result occurs in a dilution larger than the three chosen according to the guideline, as in c, it needs to be placed in the result for the largest chosen dilution as in d (Note 1).

Note 1: The largest dilution has the smallest concentration of the sample; the largest dilution in the preceding table is 0.001.

The MPN for various combinations of positive and negative results when three and five 1-, 0.1-, and 0.01-mL dilutions are used are listed in tables 7 and 8.

Table 7.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when three 1-, three 0.1-, and three 0.01-milliliter dilutions are used

[mL, milliliters; MPN, most probable number; ---, not applicable; modified from American Public Health Association and others, 1985]

Number of serum bottles indicating positive reaction out of:			MPN index per 1 mL	95-percent confidence limits	
Three of 1 mL each	Three of 0.1 mL each	Three of 0.01 mL each		Lower	Upper
0	0	0	<0.3	---	---
0	0	1	.3	<0.05	0.9
0	1	0	.3	<.05	1.3
1	0	0	.4	<.05	2.0
1	0	1	.7	.1	2.1
1	1	0	.7	.1	2.3
1	1	1	1.1	.3	3.6
1	2	0	1.1	.3	3.6
2	0	0	.9	.1	3.6
2	0	1	1.4	.3	3.7
2	1	0	1.5	.3	4.4
2	1	1	2.0	.7	8.9
2	2	0	2.1	.4	4.7
2	2	1	2.8	1.0	15.0
3	0	0	2.3	.4	12.0
3	0	1	3.9	.7	13.0
3	0	2	6.4	1.5	38.0
3	1	0	4.3	.7	21.0
3	1	1	7.5	1.4	23.0
3	1	2	12.0	3.0	38.0
3	2	0	9.3	1.5	38.0
3	2	1	15.0	3.0	44.0
3	2	2	21.0	3.5	47.0
3	3	0	24.0	3.6	130.0
3	3	1	46.0	7.1	240.0
3	3	2	110.0	15.0	480.0
3	3	3	≥240.0	---	---

Table 8.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used

[mL, milliliters; MPN, most probable number; ---, not applicable; modified from American Public Health Association and others, 1985]

Number of serum bottles indicating positive reaction out of:			MPN index per 1 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
0	0	0	<0.2	---	---
0	0	1	.2	<0.05	0.7
0	1	0	.2	<.05	.7
0	2	0	.4	<.05	1.1
1	0	0	.2	<.05	.7
1	0	1	.4	<.05	1.1
1	1	0	.4	<.05	1.1
1	1	1	.6	<.05	1.5
1	2	0	.6	<.05	1.5
2	0	0	.5	<.05	1.3
2	0	1	.7	.1	1.7
2	1	0	.7	.1	1.7
2	1	1	.9	.2	2.1
2	2	0	.9	.2	2.1
2	3	0	1.2	.3	2.8
3	0	0	.8	.1	1.9
3	0	1	1.1	.2	2.5
3	1	0	1.1	.2	2.5
3	1	1	1.4	.4	3.4
3	2	0	1.4	.4	3.4
3	2	1	1.7	.5	4.6
4	0	0	1.3	.3	3.1
4	0	1	1.7	.5	4.6
4	1	0	1.7	.5	4.6
4	1	1	2.1	.7	6.3
4	1	2	2.6	.9	7.8

Table 8.--Most-probable-number (MPN) index and 95-percent confidence limits for various combinations of positive and negative results when five 1-, five 0.1-, and five 0.01-milliliter dilutions are used--Continued

Number of serum bottles indicating positive reaction out of:			MPN index per 1 mL	95-percent confidence limits	
Five of 1 mL each	Five of 0.1 mL each	Five of 0.01 mL each		Lower	Upper
4	2	0	2.2	0.7	6.7
4	2	1	2.6	.9	7.8
4	3	0	2.7	.9	8.0
4	3	1	3.3	1.1	9.3
4	4	0	3.4	1.2	9.3
5	0	0	2.3	.7	7.0
5	0	1	3.1	1.1	8.9
5	0	2	4.3	1.5	11.0
5	1	0	3.3	1.1	9.3
5	1	1	4.6	1.6	12.0
5	1	2	6.3	2.1	15.0
5	2	0	4.9	1.7	13.0
5	2	1	7.0	2.3	17.0
5	2	2	9.4	2.8	22.0
5	3	0	7.9	2.5	19.0
5	3	1	11.0	3.1	25.0
5	3	2	14.0	3.7	34.0
5	3	3	18.0	4.4	50.0
5	4	0	13.0	3.5	30.0
5	4	1	17.0	4.3	49.0
5	4	2	22.0	5.7	70.0
5	4	3	28.0	9.0	85.0
5	4	4	35.0	12.0	100.0
5	5	0	24.0	6.8	75.0
5	5	1	35.0	12.0	100.0
5	5	2	54.0	18.0	140.0
5	5	3	92.0	30.0	320.0
5	5	4	160.0	64.0	580.0
5	5	5	≥240.0	---	---

If a series of decimal dilutions other than 1, 0.1, and 0.01 mL is used, the MPN values in tables 7 and 8 need to be corrected for the dilutions actually used. To do this, divide the values in tables 7 and 8 by the dilution factor of the first number in the three-number sequence (the serum bottles having the largest concentration of the sample). For example, if dilutions of 0.1, 0.01, and 0.001 mL are used, divide the values in tables 7 and 8 by 0.1 mL. MPN tables for other combinations of sample volumes and number of serum bottles or culture tubes at each level of inoculation are in American Public Health Association and others (1985).

7.5 If only one serum bottle is inoculated at each decimal dilution level, record the smallest dilution showing a positive response compared to the largest dilution showing a negative response. Record the results as a range of numbers, for example 100 to 1,000 sulfate-reducing bacteria per milliliter. If all bottles are positive, record the result as a number greater than that indicated by the value of the largest dilution of the series. For example, 1-, 0.1-, and 0.01-mL samples are tested, and all tubes are positive at the end of the test. Record the result as greater than 100 sulfate-reducing bacteria per milliliter.

7.6 Examples of test results and calculations are listed below.

7.6.1 The following results were obtained with a three serum-bottle series:

[-, negative; +, positive]

Volume (milliliters)	Serum bottle number 1	2	3	Result
0.1	+	+	+	3/3
0.01	+	+	+	3/3
0.001	+	+	-	2/3
0.0001	-	-	-	0/3

Following the guideline in 7.3 and using 0.01-, 0.001-, and 0.0001-mL sample volumes, a sequence of 3-2-0 is indicated. From this, an MPN of 9.3 is indicated (table 7). Dividing by 0.01 mL to correct for the effect of dilution, the MPN of the sample is 930 sulfate-reducing bacteria per milliliter. The 95-percent confidence limits are 150 and 3,800.

7.6.2 The following results were obtained with a five serum-bottle series:

Volume (milliliters)	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Results	5/5	5/5	3/5	1/5	0/5.

Using 10^{-6}, 10^{-7}, and 10^{-8} mL sample volumes, the test results indicate a sequence of 5-3-1 for which the MPN (table 8) is 11.0. Dividing by 10^{-6}, the MPN is computed to be 11×10^{6} sulfate-reducing bacteria per milliliter and 95-percent confidence limits of 3.1×10^{6} and 25×10^{6} sulfate-reducing bacteria per milliliter.

7.6.3 The following results were obtained with a three serum-bottle series:

Volume (milliliters)	1	0.1	0.01	0.001
Results	0/3	1/3	0/3	0/3.

Use the sequence of 0-1-0 for which the MPN is 0.3 and 95-percent confidence limits of <0.05 and 1.3 (table 7).

8. Reporting of results

8.1 For one serum-bottle series, report the data as a range of numbers.

8.2 For a multiple serum-bottle series, report results as MPN of sulfate-reducing bacteria per milliliter as follows: less than 10, whole numbers; 10 or more, two significant figures.

9. Precision

9.1 Precision of the MPN method increases as the number of serum bottles is increased. It increases rapidly as the number of bottles increases from 1 to 5, but then it increases at a slower rate making the gain, when using 10 bottles instead of 5, much less than is achieved by increasing the number of bottles from 1 to 5. Variance as a function of the number of bottles inoculated from a tenfold dilution series is listed below:

Number of serum bottles at each dilution	Variance for tenfold dilution series
1	0.580
3	.335
5	.259
10	.183

9.2 The 95-percent confidence limits for various combinations of positive and negative results when three and five 1-, 0.1-, and 0.01-mL dilutions are used are listed in tables 7 and 8.

10. References cited

American Petroleum Institute, 1965, Recommended practice for biological analysis of subsurface injection waters (2d ed.): Dallas, Tex., American Petroleum Institute Division Production API RP 38, 6 p.

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Postgate, J. R., 1959, Sulfate reduction by bacteria, in Clifton, C. E., Raffel, Sidney, and Starr, M. P., eds., Annual review of microbiology: Palo Alto, Calif., Annual Reviews, Inc., p. 505-520.

Total Bacteria (Epifluorescence Method)
(B-0005-85)

Parameter and Code:
Bacteria, total count, epifluorescence (number/mL): 81803

Epifluorescent microscopy is one method for determining the bacterial density in water. It has the advantage of being more rapid than viable count methods (standard plate count, membrane filter, and most probable number). However, bacterial densities determined by epifluorescent microscopy are not directly comparable to viable cell counts or to other biomass measurements, such as adenosine triphosphate (ATP). Direct microscopic counts usually are greater than viable counts for two principal reasons. First, cells that are living as well as dead at the time of collection will be counted by direct microscopy. Second, only a fraction of the total bacteria is enumerated in a viable count.

1. Applications

The epifluorescence method is suitable for all water, except that having a large suspended-sediment concentration. It is similar to other published methods (Hobbie and others, 1977; Dutka, 1978).

2. Summary of method

A water sample is collected and preserved onsite using formaldehyde. In the laboratory, an aliquot of the sample is mixed with a fluorescent dye and filtered through a black membrane filter. The membrane filter is mounted on a microscope slide and viewed at 1,000X using epifluorescent microscopy. Bacteria and other life forms appear green, orange, or red against a black background. The number of bacteria per milliliter in the sample is calculated from the average bacterial density per microscopic field.

3. Interferences

Bacteria absorbed on particulate matter are difficult to isolate and the number may be underestimated. Fluorescence of nonbacterial matter, such as algae, protozoa, and fungi, also may cause enumeration errors. Some surfactants prevent the fluorescent dye from attaching to the bacteria or may remove dye from the membrane filter making analysis impossible. Excessive sediment on the filter makes it difficult to view underlying cells.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Bottles, milk dilution, screwcap.

4.2 Cover slips, 25-mm circles.

4.3 Filter-holder assembly, 25 mm.

4.4 Filter-holder assembly, 47 mm.

4.5 Flasks, 1 L, erlenmeyer (borosilicate glass).

4.6 Laboratory film, parafilm.

4.7 Membrane filters, cellulose, 0.45-μm pore size, 25-mm diameter.

4.8 Membrane filters, polycarbonate, 0.2-μm pore size, 25-mm diameter.

4.9 Membrane filters, white, grid, sterile, 0.45-μm pore size, 47-mm diameter.

4.10 Membrane forceps.

4.11 Microscope, with lamp, heat filter, red attenuation filter, beam splitter, barrier filter, exciter filter, or equivalent apparatus.

4.12 Microscope slides, 25×75 mm.

4.13 Pipets, 1-mL capacity, sterile.

4.14 Pipets, 10-mL capacity, sterile.

4.15 Plastic petri dishes with covers, disposable, sterile, 50×12 mm.

4.16 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.17 Stage micrometer.

4.18 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.19 Test tubes, 16x100 mm, glass, disposable.

4.20 Vacuum filtering flask.

4.21 Vacuum pump.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acridine orange, 0.1 percent. Dissolve 0.1 g acridine orange in 97 mL distilled water, then add 3 mL 37-percent formaldehyde solution. Filter solution through a 0.45-µm membrane filter to remove insoluble dye and store in an amber or black bottle in darkness. The acridine orange solution is stable for approximately 1 month at room temperature.

CAUTION.--Acridine orange resulted in mutagenic activity in the Ames test and needs to be treated with care.

5.2 Distilled or deionized water.

5.3 Formaldehyde preservative, 37-percent formaldehyde solution.

5.4 Immersion oil, low fluorescence.

5.5 Irgalan black solution, 0.2 percent. Dissolve 2 g irgalan black in 1 L distilled water containing 2 percent acetic acid.

5.6 Particle-free sterile distilled or deionized water. Filter distilled water through a 0.45-µm membrane filter and transfer into a 1-L screwcap erlenmeyer flask. Sterilize by autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes.

6. Analysis

6.1 Preserve the sample, immediately after collection, by the addition of formaldehyde solution (37 percent) at the rate of 5 mL of formaldehyde to 95 mL of sample. Record the volume of preservative added. Maintain the sample in a cool, dark location prior to analysis but prevent from freezing. Refrigeration is ideal but is not required. Sample analysis needs to be completed within 1 month of collection.

6.2 Soak the polycarbonate membrane filters in irgalan black solution for 8 to 24 hours. Rinse the filters in two successive sterile particle-free distilled water rinses and place in a sterile petri dish prior to use.

6.3 Shake the water sample vigorously for 10 seconds to distribute the contents evenly.

6.4 Using a sterile pipet, place 0.5 mL acridine orange solution into a 16×100-mm test tube. Place a 4.5 mL sample into the test tube or a 4.5-mL combination of sample plus particle-free distilled water. Cover the test tube with a small piece of parafilm and invert several times to mix. Let stand for 2 (or as much as 30) minutes.

6.5 Assemble the 25-mm filter-holder assembly with a cellulose membrane filter (0.45 µm, 25-mm diameter) on the bottom and a polycarbonate filter (0.2 µm, 25-diameter) on top. Attach vacuum pump to vacuum filtering flask.

6.6 Filter the acridine orange containing sample at 0.5 bar (15 in. of vacuum) until the filter just becomes dry. Rinse the test tube using about 5 mL of particle-free sterile distilled water and filter as before to rinse particulate matter from the inner surface of the filter-holder assembly.

6.7 When the polycarbonate filter just becomes dry, place it on a microscope slide. Allow to dry for an additional minute, place a drop of immersion oil on the filter, and add a cover slip.

6.8 Examine the preparation under epifluorescent microscopy at 100X following the manufacturer's instructions for the unit. When the filter surface is in focus, change to high dry (450X) and scan the filter looking for problems such as poor dispersion or excessive fluorescence. If the filter has no apparent problems, add a drop of immersion oil to the cover slip and change to 1,000X magnification. Count the bacteria either within the entire field or within the area enclosed by an ocular grid. Bacterial enumeration is easiest using a Whipple or similar ocular grid. Ideally, each microscopic field should have 5 to 50 bacteria. Generally, most bacteria fluoresce green, but a few also may fluoresce orange or red. Only objects having clearly discernible bacterial morphology should be counted. Count each field separately. Count at least 10 random fields until a total of 300 or more bacteria are counted. If the preparation is too concentrated or dilute, prepare another mount with a different sample volume.

7. Calculations

7.1 Calculate the number of bacteria per milliliter as follows:

$$\text{Bacteria/mL} = \frac{\dfrac{\text{Average count per field} \times \text{effective filter area (square millimeters)}}{\text{field area (square millimeters)}}}{\text{Sample volume filtered (milliliters)} \times \text{dilution factor}} .$$

The effective filter area is the area of filter exposed to the water sample. The 25-mm filter-holder assembly described in the "Apparatus" subsection has an effective filter diameter of 16 mm or an effective filter area of 201 mm^2. Other types of filter-holder assemblies may have different effective filter areas. The field area must be determined for each microscope using a stage micrometer and following the procedure described by the American Public Health Association and others (1985). The dilution factor corrects for the addition of preservative as follows:

$$\text{Dilution factor (Note 1)} = \frac{\text{Sample volume (milliliters)}}{\text{Sample volume (milliliters)} + \text{preservative (milliliters)}} .$$

Note 1: Addition of 5 mL of formaldehyde to 95 mL of sample will give a dilution factor of 0.95.

7.2 Example calculation:

95 mL of sample + 5 mL preservative = dilution factor 0.95

Sample volume = 2 mL

Effective filter area = 201 mm^2

Field area of microscope = 2.303×10^{-3} mm^2

Total bacteria per field = 60

$$\text{Bacteria/mL} = \frac{(60)\ \frac{201}{2.303 \times 10^{-3}}}{(2)\ (0.95)}$$

$$= \frac{(60)\ (0.8727 \times 10^{5})}{(2)\ (0.95)}$$

$$= 2{,}756{,}130$$

$$= 2{,}760{,}000\ .$$

8. Reporting of results

Report the bacterial density as bacteria per milliliter as follows: three significant figures.

9. Precision

The precision is dependent on the density of bacteria in the sample and the quantity of nonbacterial debris. For typical samples, the precision is approximately ±10 percent.

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C., American Public Health Association, 1,268 p.

Dutka, B. J., ed., 1978, Methods for microbiological analysis of waters, wastewaters, and sediments: Burlington, Canada Centre for Inland Waters, 288 p.

Hobbie, J. E., Daley, R. J., and Jasper, S., 1977, Use of nuclepore filters for counting bacteria by fluorescence microscopy: Applied and Environmental Microbiology, v. 33, p. 1225-1228.

Salmonella and Shigella (Diatomaceous-Earth and Membrane-Filter Method) (B-0100-85)

Parameter and Code: Not applicable

Pathogenic bacteria of the genera Salmonella and Shigella may be isolated from water by similar methods. The genus Salmonella comprises more than 1,000 varieties, all of which are potentially pathogenic to humans. The more common diseases caused by Salmonella include typhoid and paratyphoid fever and salmonellosis. Because morphologically and physiologically similar Salmonella varieties can cause different diseases, Salmonella identification involves serology, which is specific for a particular type of Salmonella. The members of the genus Shigella are all potentially pathogenic and are similar to Salmonella in many aspects. Shigella causes acute bacillar dysentery, also known as shigellosis.

Salmonella and Shigella can inhabit the gastrointestinal tract of humans. The bacteria pass with the feces. These organisms share the same native environment and travel the water route along with fecal coliforms. The pathogens in water form an extremely small part of the total bacterial population because of excessive numbers of coliforms. Geldreich (1970) reported isolation of Salmonella in less than 27.6 percent of freshwater samples when the fecal coliform concentration was less than 200 colonies per 100 mL. Salmonella was isolated in 85.2 percent of water samples having fecal coliform concentrations between 200 and 2,000 colonies per 100 mL and was isolated in 98.1 percent of samples having fecal coliform concentrations exceeding 20,000 colonies per 100 mL.

Because of the small occurrence of pathogenic bacteria in most water, large volumes of sample must be filtered. In addition, selective enrichment culture is necessary to increase the population density of the pathogens so that detection is possible. Thus, the procedure is qualitative only. Quantification of pathogens in an original sample cannot be determined readily by this method.

This method is approved for use in the Water Resources Division by those individuals who have special training and knowledge in the handling of pathogenic organisms. Extreme care must be taken because the method provides for the reproduction and enhancement of growth of pathogenic bacteria. Following completion of tests, all cultures must be destroyed and all equipment sterilized by autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 30 minutes.

1. Applications

The method is applicable for all fresh and estuarine water. Very few reports of the occurrence of Salmonella and Shigella in marine environments are available except to indicate that sediment may be an important source.

2. Summary of method

2.1 Samples are collected using sterile procedures to avoid contamination, while minimizing exposure of onsite personnel to possible pathogens. Several liters of water are filtered through either diatomaceous earth or a

membrane filter. The bacteria-laden diatomaceous earth or membrane filter is divided into parts for inoculation into suitable enrichment media. Selenite and tetrathionate broth media are recommended for all Salmonella and most Shigella determinations.

2.2 Selective solid media plates are streaked at 24-hour intervals for as much as 5 days after incubation at 41.5 °C. Colonies that appear on the selective media having typical Salmonella or Shigella characteristics are purified and further classified by biochemical reactions. Several non-pathogenic organisms share some important biochemical characteristics with the Salmonella and Shigella groups. For this reason, many differential biochemical tests are necessary for presumptive identification of the pathogenic Enterobacteriaceae, of which Salmonella and Shigella are members. Identification cannot be done until the bacteria is verified serologically. A diagrammatic identification scheme is shown in figure 8.

3. Interferences

The membrane-filter method may not work with water having a large suspended-solids concentration. Additionally, many bacteria, other than Salmonella and Shigella, growing in the enrichment media make isolation and identification of the pathogenic Enterobacteriaceae difficult, even for experienced investigators. Cultures used for inoculation of media in biochemical tests must be pure; if not, false results will be obtained.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 Bacteriological transfer loops and needles.

4.2 Bottles, milk dilution, screwcap.

4.3 Diatomaceous earth.

4.4 Durham tubes, flint glass, 6×50 mm.

4.5 Filter-holder assembly* and syringe that has a two-way valve* or vacuum hand pump.

4.6 Flasks, 125-mL, screwcap, erlenmeyer.

4.7 Forceps*, stainless steel, smooth tips.

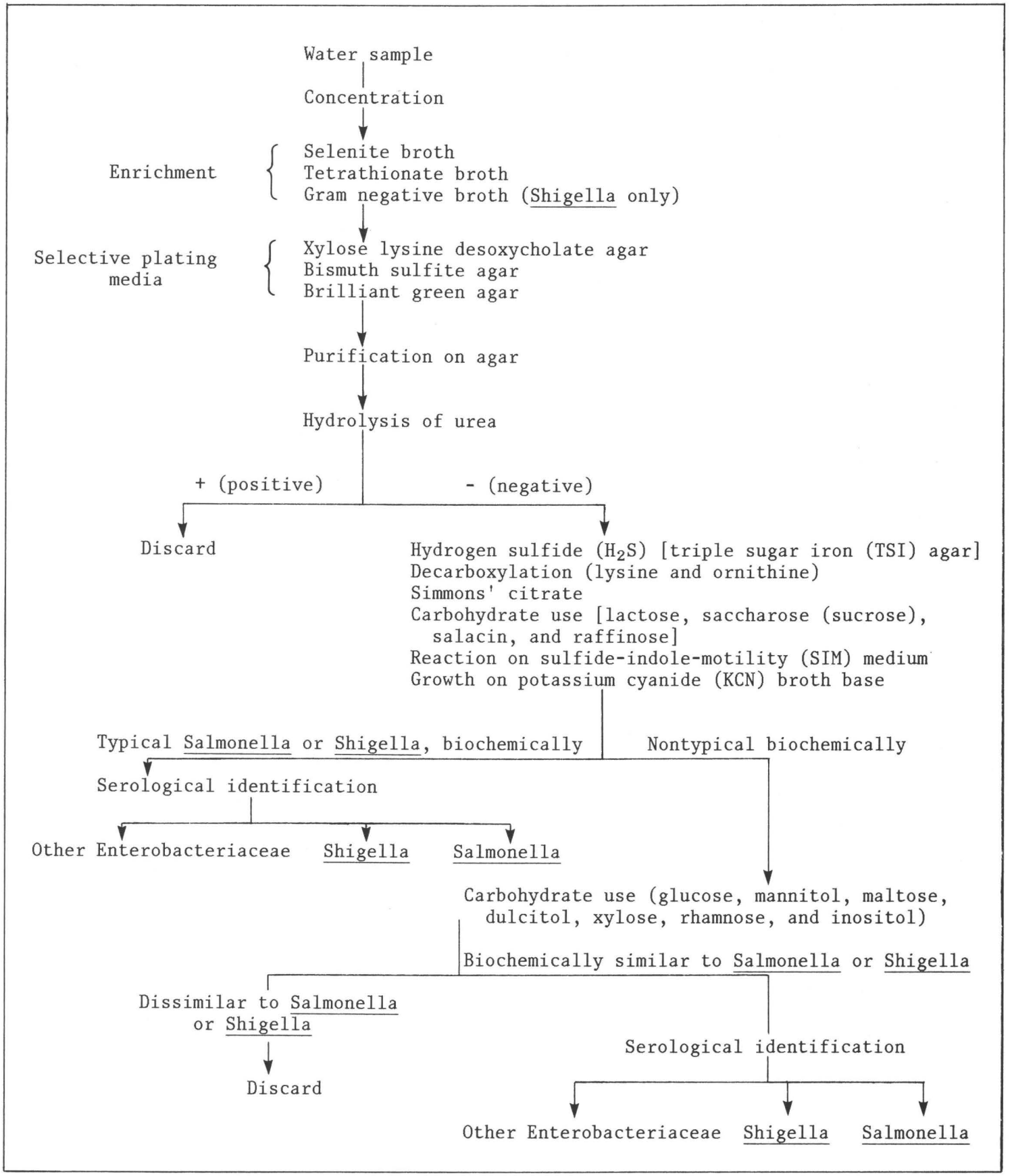


Figure 8.--Identification scheme for Salmonella and Shigella.

4.8 Hot plate, or kitchen stove.

4.9 Incubator*, for operation at a temperature of 35±0.5 °C and 41.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use.

4.10 Laboratory balance, with sensitivity to 0.01 g.

4.11 Membrane filters, white, grid, sterile, 0.45-µm mean pore size, 47-mm diameter, and absorbent pads.

4.12 Microscope slides, 25×75 mm.

4.13 Plastic petri dishes with covers, disposable, sterile, 100×15 mm.

4.14 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section. Care when collecting the sample is advised to preclude the possibility of contamination of the sample or the collector. Sterile, disposable gloves are recommended. A minimum of 2 L of sample is necessary for filtration. Because this procedure will be used for qualitative determinations, samples representative of mean flow of a stream generally are not required.

4.15 Scissors, autoclavable.

4.16 Spatula, laboratory, 120×20 mm.

4.16 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.18 Test tubes, borosilicate glass, 16×150 mm, and tube caps, 16 mm.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Agar.

5.2 Bismuth sulfite agar.

5.3 Brilliant green agar.

5.4 Decarboxylase base Moeller.

5.5 Distilled or deionized water.

5.6 Ethyl alcohol, 95-percent denatured or absolute ethyl alcohol for slevilizing equipment. Absolute methyl alcohol also may be used for sterilization.

5.7 GN (gram negative) broth.

5.8 KCN (potassium cyanide) broth base.

5.9 Kliger iron agar.

5.10 Lactose.

5.11 L-lysine HCL.

5.12 L-ornithine HCL.

5.13 Potassium cyanide (KCN), powdered, reagent grade.

5.14 Purple broth base.

5.15 Raffinose.

5.16 Saccharose.

5.17 Salicin.

5.18 Salmonella H Antiserum Kit.

5.19 Salmonella O Antiserum Kit.

5.20 Selenite broth.

5.21 SIM (sulfide-indole-motility) medium.

5.22 Simmons' citrate agar.

5.23 Sucrose.

5.24 Tetrathionate broth.

5.25 TSI (triple sugar iron) agar.

5.26 Urea agar base.

5.27 Veal infusion broth.

5.28 XLD (xylose lysine desoxycholate) agar.

Note 1: It is important that manufacturer's instructions be followed closely in the preparation and storage of all media. If onsite inoculation is intended, discretion is advised in the final dispensing of selenite and tetrathionate broth. The container must allow room for diatomaceous earth and membrane filters and must fit in an onsite incubator.

6. Analysis

6.1 Sterilize filter-holder assembly (Note 2). In the laboratory, wrap the funnel and filter base parts of the assembly separately in kraft paper or polypropylene bags and sterilize in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Steam must contact all surfaces to ensure complete sterilization. Cool to room temperature before use.

Note 2: Onsite sterilization of filter-holder assembly needs to be in accordance with the manufacturer's instructions but usually involves application and ignition of methyl alcohol to produce formaldehyde. Autoclave sterilization in the laboratory prior to the trip to the sampling site is preferred. Sterilization must be performed at all sites.

6.2 Assemble the filter-holder assembly and, using flame-sterilized forceps (Note 3), place a sterile membrane filter over the porous plate of the assembly, grid side up, or a sterile absorbent pad in the funnel part of the filter-holder assembly (6.4). Carefully place funnel on filter to avoid tearing or creasing the membrane.

Note 3: Flame-sterilized forceps--Dip forceps in ethyl or methyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

6.3 Shake the sample vigorously about 25 times to obtain an equal distribution of bacteria throughout the sample before transferring a measured portion of the sample to the filter-holder assembly.

6.4 Concentration: the sample must be concentrated before inoculation into selective media. Two procedures are available for concentration--diatomaceous-earth filtration and membrane filtration.

6.4.1 Diatomaceous-earth filtration: Place a sterile 47-mm diameter absorbent pad in the funnel part of the filter-holder assembly and fill the neck halfway with diatomaceous earth. Pour 2 L of sample slowly into the funnel and apply vacuum. When the sample has been completely filtered, transfer equal parts of the diatomaceous earth to the selective growth media (Note 4).

Note 4: Not all bacteria are retained; the filtrate will contain some bacteria and possibly pathogens.

6.4.2 Membrane filtration: Filter 2 L (minimum) of sample through a 0.45-μm mean pore size membrane filter. Because of the small pore diameter, a 47-mm diameter membrane filter will clog quickly unless the water is relatively free of suspended material. Larger diameter filters, such as 100 or 150 mm, may be used if suitable filter-holder assemblies are available. When filtration is complete, remove the filter from the assembly, cut with sterile scissors, and transfer equal-sized pieces of the filter to selective growth media. Record volume of sample that was filtered.

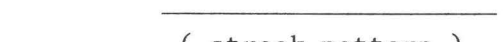
6.5 If isolation of Salmonella is desired, transfer one-half of the diatomaceous earth or membrane filter(s) to previously prepared and prewarmed (41.5 °C) flasks of selenite and tetrathionate broth. Prepare flasks by placing 50-mL aliquots of appropriate broth medium in sterilized 125-mL screwcap erlenmeyer flasks. If only Shigella is desired, transfer one-half of the diatomaceous earth or membrane filter(s) to GN broth. GN broth cannot be used to isolate Salmonella.

6.6 Immediately place inoculated flasks into an incubator preset at 41.5 °C. No more than 24 hours may elapse between incubation and subsequent culture transfers (6.5).

6.7 After arrival at the laboratory, transfer primary culture flasks to a laboratory incubator prewarmed to 41.5 °C and prepare selective media. For Salmonella, use brilliant green agar, bismuth sulfite agar, and XLD agar. XLD agar also may be used for Shigella. One to four petri dishes of each medium will be needed for every primary (broth) culture.

6.8 After incubation periods indicated in this paragraph and using bacteriological needles, streak broth cultures having evidence of bacterial growth onto media prepared in 6.4. Selenite broth cultures displaying growth become turbid and develop orange-red coloration. Optimum recovery of Salmonella from selenite broth is obtained after incubation at 41.5 °C for 24 hours, but additional streaking after 48 and 72 hours may be needed to recover some slower growing strains. Incubate tetrathionate cultures for 48 hours before streaking. Repeated streaking from tetrathionate cultures may be necessary for as much as 5 days to recover all Salmonella. Streak the GN broth after 24-hour incubation only. Streak using care and precision so isolated colonies will grow in a discrete pattern (Note 5).

Note 5: The following streak pattern will give good results if care is taken to flame the needle after streaking each section:



(streak pattern)

6.9 Incubate inoculated petri dishes in an inverted (upside down) position at 41.5 °C. Incubate XLD agar petri dishes for 24 hours. Incubate all other petri dishes for 48 hours.

6.10 After incubation, inspect the petri dishes for Salmonella or Shigella colonies. The petri dishes usually have luxuriant bacterial growth, so care and discretion are necessary in the selection of possible colonies of pathogens. On brilliant green agar, Salmonella typically forms pinkish-white colonies having a red background (if well isolated). If the petri dish is overgrown with colonies, Salmonella may be indistinguishable from the usually more numerous nonpathogens. On bismuth sulfite agar, Salmonella develops black colonies that may or may not have a metallic sheen; sometimes a halo is produced around the colony. A few Salmonella strains develop a green, rather than black, coloration on bismuth sulfite agar. Therefore, isolate some green colonies. On XLD agar, Shigella forms red colonies, and Salmonella produces black-centered red colonies.

6.11 Carefully transfer all suspected Salmonella or Shigella colonies, using a sterile bacteriological loop, to fresh agar petri dishes. Incubate at 41.5 °C for 48 hours. Continue repeated examination, streaking, and incubation of suspected Salmonella and Shigella until pure cultures are obtained.

6.12 After the suspected Salmonella or Shigella colonies have been developed in pure culture, subject them to a series of biochemical tests. If cultures are still positive for Salmonella or Shigella following the biochemical testing, serological confirmation must be done. In some areas, State or local health departments may be able to perform the biochemical and serological testings. If not, use the scheme in figure 8.

Biochemical identification of large numbers of cultures is expensive and time consuming. It should not be attempted independently without previous training and experience in reading reactions and interpreting results. Additionally, care must be used in working with cultures if laboratory-acquired infections are to be avoided.

There are many published identification schemes for Salmonella and Shigella. Publications by Brezenski and Russomanno (1969), Claudon and others (1971), Presnell and Miescier (1971), and Edwards and Ewing (1972) describe various methods for the identification procedure. The manufacturers of bacteriological media also provide useful leaflets about certain testing procedures. Difco Laboratories publications (1968, 1969a, 1969b, 1971a, 1971b) are available on request to Difco Laboratories.

If local identification of a suspect culture is desired, first check for the production of urease. Salmonella and Shigella always are negative for urease production using the Christensen method (Difco Laboratories, 1969b). Screen urease negative cultures for biochemical action as follows: Lysine and ornithine decarboxylation using the Moeller method (Difco Laboratories, 1969a); citrate using the Simmons method (Difco Laboratories, 1953); hydrogen sulfide production on TSI; fermentation of lactose, saccharose (sucrose), salicin, and raffinose; growth in KCN broth; and action on SIM medium. Procedural details are listed in table 9.

If biochemical tests (table 10) indicate the isolated culture may be Salmonella or Shigella, identify serologically.

6.13 Serological identification. Serological identification of Salmonella or Shigella should be carried out as described by Edwards and Ewing (1972) and American Public Health Association and others (1985). Difco Laboratories (1971b) developed one procedure for the serological identification of Salmonella.

A brief description of the serological process may improve the nonserologist's understanding. If an organism is exposed to a foreign body, such as a bacterial cell, part of the organism's defense is the production of a specific protein, called an antibody, that renders the bacterium harmless or nonvirulent. Antibodies are found in the plasma fraction of the blood; hence, blood serum that contains antibodies against, for example, Salmonella, is called antiserum. Antiserum, if specific for a certain bacterium, will cause clumping of the bacteria. The clumping can be observed under 100X magnification. The serological process is so specific that more than 1,000 different Salmonella types (serotypes) have been identified.

A foreign body that stimulates the production of an antibody is called an antigen. Salmonella has two main types of antigens, the O (somatic or intracellular) antigens and H (flagellar) antigens. The O antigens are heat stable and provide basic differentiation into groups of bacteria. The H antigens are heat labile and are used for differentiation within a bacterial group. Occasionally another somatic antigen, termed Vi, is observed. The Vi antigen can block activity of an O antigen and must be inactivated by heat during the serological grouping tests.

The serological procedure for the identification of Shigella is similar to that of Salmonella; therefore, only the Salmonella serology is further detailed. A simplified scheme devised by Spicer and Edwards (Difco Laboratories, 1971b) can be used for tentative serological identification of Salmonella using minimal effort. The O antigen is identified first using Salmonella O antiserum. If the results are positive (clumping occurs), the culture is one of the genus Salmonella. If only verification that the culture is a Salmonella is needed, the O antigen analysis is sufficient.

If further identification is desired, the H antigen needs to be determined using Salmonella H antiserum. In this step, most Salmonella can be classified into a specific serotype. A diagrammatic serological scheme for Salmonella is shown in figure 9.

All cultures not retained for serological testing should be autoclaved at 121 °C at 1.05 kg per/cm^2 (15 psi) for 15 to 30 minutes before discarding.

Table 9.--Biochemical test procedures for Salmonella and Shigella

[°C, degrees Celsius; mL, milliliters; H_2S, hydrogen sulfide; TSI, triple sugar iron; SIM, sulfide-indole-motility; KCN, potassium cyanide]

Test	Media requirements	Media preparation	Inoculation and incubation	Typical result
Urease.	Urea agar base or agar.	Prepare medium in slants with generous butts.	Make one streak along entire length. Do not inoculate butt. Incubate at 37 °C for 24 hours.	Salmonella and Shigella are negative (no color change). Others turn medium pink within 24 hours.
Decarboxylation of lysine and ornithine.	Decarboxylase base, L-lysine, and L-ornithine.	Use amino acids at 0.5 percent, added to base medium. Ornithine must be adjusted to pH 6.5 with I N sodium hydroxide. Dispense in 5-mL quantities in screwcap tubes.	Inoculate with a 24-hour agar slant culture. Screw caps on tightly and incubate at 37 °C for 24 hours.	Reddish violet, if positive; yellow, if negative. Salmonella, usually positive and Shigella, negative, on lysine; variable on ornithine (see table 10).
Citrate.	Simmons' citrate agar.	Prepare medium in slants with generous butts.	Make one streak along length and stab the butt using a needle. Incubate at 37 °C for 24 to 48 hours.	Shigella is negative (green color). Most Salmonella are positive (deep blue).
H_2S production.	TSI agar.	Prepare medium in slants with generous butts.	Streak slant heavily along entire length and stab the butt. Incubate at 37 °C for 24 hours.	Salmonella has red slant, yellow butt, positive for H_2S production (blackening), gas variable. Shigella has red slant, yellow butt, negative for H_2S production (no blackening).

Table 9.--Biochemical test procedures for Salmonella and Shigella--Continued

Test	Media requirements	Media preparation	Inoculation and incubation	Typical result
Carbohydrate use.	Purple broth base, lactose, saccarose sucrose), salicin, and raffinose.	Sterilize base and sugar separately, the latter by filter. Use 0.5 to 1 percent sugar. Add after sterilizing base in test tubes with durham tubes.	Inoculate from 24-hour agar slant culture. Incubate at 37 °C. Examine daily for 7 days.	A positive reaction is production of acid (yellow color) with or without gas (bubbles in durham tube). Salmonella is negative.
SIM.	SIM medium and indole test strips.	Dispense in test tubes half full. Sterilize, allow to harden upright. Put a test strip in each tube.	Inoculate with needle from 24-hour agar slant culture. Stab in center to 1/2 depth. Incubate at 37 °C for 24 to 48 hours.	If indole is produced, paper turns pink. Medium blackens if H_2S is produced. Salmonella is negative for indole; may produce H_2S.
KCN.	KCN broth base KCN reagent, powder.	Sterilize base separately. Prepare 0.5 percent KCN solution and add 1.5 mL to 100 mL sterile base. Dispense 2 mL in test tubes and close with sterile paraffined stoppers.	Inoculate from 24-hour KCN broth culture without KCN. Incubate at 37 °C for 48 hours.	Salmonella and Shigella will not grow. Other Enterobacteriaceae may grow (see table 10).

Table 10.--Differentiation of Enterobacteriaceae by biochemical tests

[+, 90 percent or more cultures positive within 1- or 2-day incubation; - or +, majority of cultures negative; -, 90 percent or more cultures negative; d, different biochemical reactions (+, (+), -); + or -, majority of cultures positive; d^w, different biochemical reactions (+, (+), -), weak reaction; (+), delayed positive reaction; - or (+), majority of cultures negative (delayed reaction of 3 days or more); (+) or +, majority of reactions delayed, some occur within 1 or 2 days; + or (+), 90 percent or more positive (delayed reaction of 3 days or more); °C, degrees Celsius; from Edwards and Ewing, 1972]

Test or substrate	Escherichieae		Edward-sielleae	Salmonelleae		
	Escherichia	Shigella	Edwardsiella	Salmonella	Arizona	Citrobacter
Indol	+	- or +	+	-	-	-
Methyl red	+	+	+	+	+	+
Voges-proskauer	-	-	-	-	-	-
Simmons' citrate	-	-	-	d	+	+
Hydrogen sulfide (TSI)	-	-	+	+	+	+ or -
Urease	-	-	-	-	-	d^w
KCN	-	-	-	-	-	+
Motility	+ or -	-	+	+	+	+
Gelatin (22 °C)	-	-	-	-	(+)	-
Lysine decarboxylase	d	-	+	+	+	-
Arginine dihydrolase	d	- or (+)	-	(+) or +	+ or (+)	d
Ornithine decarboxylase	d	d(1)	+	+	+	d
Phenylalanine deaminase	-	-	-	-	-	-
Malonate	-	-	-	-	+	d
Gas from glucose	+	-(1)	+	+	+	+
Lactose	+	-(1)	-	-	d	d
Saccharose (Sucrose)	d	-(1)	-	-	-	d
Mannitol	+	+ or -	-	+	+	+
Dulcitol	d	d	-	d(2)	-	d
Salicin	d	-	-	-	-	d
Adonitol	-	-	-	-	-	-
Inositol	-	-	-	d	-	-
Sorbitol	+	d	-	+	+	+
Arabinose	+	d	-	+(2)	+	+
Raffinose	d	d	-	-	-	d
Rhamnose	d	d	-	+	+	+

Table 10.--Differentiation of Enterobacteriaceae by biochemical tests--Continued

Test or substrate	Klebsiella	Klebsielleae: Enterobacter: Cloacae	Enterobacter: Aerogenes	Enterobacter: Hafniae 37 °C	Enterobacter: Hafniae 22 °C	Enterobacter: Liquefaciens 37 °C	Enterobacter: Liquefaciens 22 °C	Serratia	Pectobacterium 25 °C
Indol	- or +	-	-	-	-	-	-	-	- or +
Methyl red	-	-	-	+ or -	-	+ or -	- or +	- or +	+ or -
Voges-proskauer	+	+	+	+ or -	+	- or +	+ or -	+	- or +
Simmons' citrate	+	+	+	(+) or -	d	+	+	+	d
Hydrogen sulfide (TSI)	-	-	-	-	-	-	-	-	-
Urease	+	+ or -	-	-	-	d	-	d^{W}	d^{W}
KCN	+	+	+	+	+	+	+	+	+ or -
Motility	-	+	+	+	+	d	+	+	+ or -
Gelatin (22 °C)	-	(+) or -	- or (+)		-		+	+	+ or (+)
Lysine decarboxylase	+	-	+	+	+	+ or -	+	+	-
Arginine dihydrolase	-	+	-	-	-	-	-	-	- or +
Ornithine decarboxylase	-	+	+	+	+	+	+	+	-
Phenylalanine deaminase	-	-	-	-	-	-	-	-	-
Malonate	+	+ or -	+ or -	+ or -	+ or -	-	-	-	- or +
Gas from glucose	+	+	+	+	+	+	+	+ or -(3)	- or +
Lactose	+	+	+	- or (+)	- or (+)	d	(+)	- or (+)	d
Saccharose (Sucrose)	+	+	+	d	d	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+
Dulcitol	- or +	- or +	-	-	-	-	-	-	-
Salicin	+	+ or (+)	+	d	d	+	+	+	+
Adonitol	+ or -	- or +	+	-	-	d	d	d	-
Inositol	+	d	+	-	-	+	+	d	-
Sorbitol	+	+	+	-	-	+	+	+	-
Arabinose	+	+	+	+	+	+	+	-	+
Raffinose	+	+	+	-	-	+	+	-	+ or (+)
Rhamnose	+	+	+	+	+	-	-	-	d

Table 10.--Differentiation of Enterobacteriaceae by biochemical tests--Continued

Test or substrate	Proteeae					
	Proteus				Providencia	
	Vulgaris	Mirabilis	Morganii	Rettgeri	Alcali-faciens	Stuartii
Indol	+	-	+	+	+	+
Methyl red	+	+	+	+	+	+
Voges-proskauer	-	- or +	-	-	-	-
Simmons' citrate	d	+ or (+)	-	+	+	+
Hydrogen sulfide (TSI)	+	+	-	-	-	-
Urease	+	+	+	+	-	-
KCN	+	+	+	+	+	+
Motility	+	+	+	+	+	+
Gelatin (22 °C)	+ or (+)	+	-	-	-	-
Lysine decarboxylase	-	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-	-
Ornithine decarboxylase	-	+	+	-	-	-
Phenylalanine deaminase	+	+	+	+	+	+
Malonate	-	-	-	-	-	-
Gas from glucose	+ or -	+	d	- or +	+ or -	-
Lactose	-	-	-	-	-	-
Saccharose (Sucrose)	+	d	-	d	d	d
Mannitol	-	-	-	+ or -	-	d
Dulcitol	-	-	-	-	-	-
Salicin	d	d	-	d	-	-
Adonitol	-	-	-	d	+	-
Inositol	-	-	-	+	-	+
Sorbitol	-	-	-	d	-	d
Arabinose	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-
Rhamnose	-	-	-	+ or -	-	-

(1) Certain biotypes of S. flexneri produce gas; S. sonnei cultures ferment lactose and sucrose slowly and decarboxylate ornithine.

(2) S. typhi, S. cholerae-suis, S. enteritidis bioserv. Paratyphi A and Pollorum, and a few others ordinarily do not ferment dulcitol promptly. S. cholerae-suis does not ferment arabinose.

(3) Gas volumes produced by cultures of Serratia, Proteus, and Providencia are small.

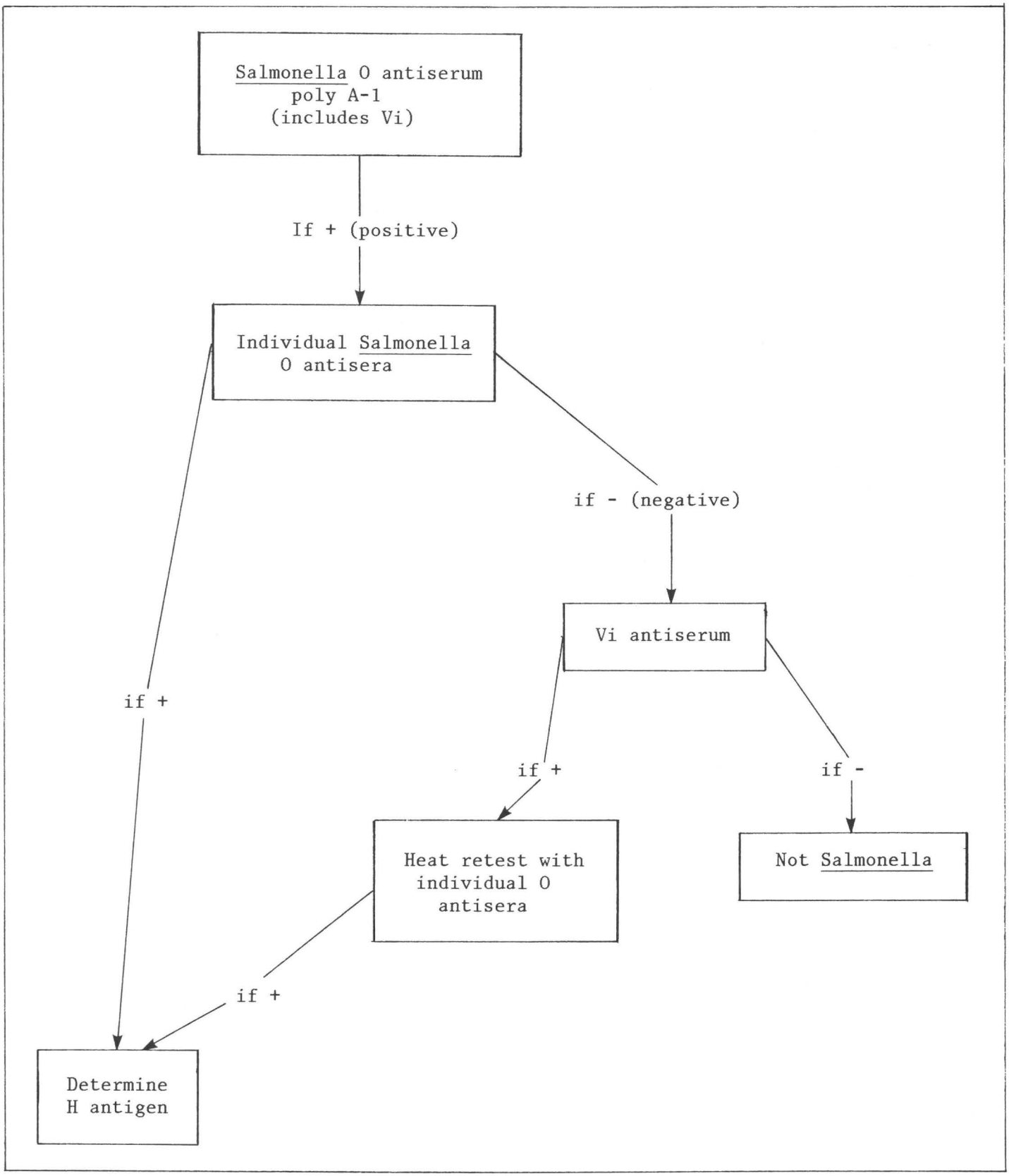


Figure 9.--Salmonella serology (from Difco Laboratories, 1971b).

If Difco reagents are used for serological identification, the procedure is as follows:

6.13.1 Somatic O Antigen Analysis (Difco Laboratories, 1971b).

1. Only micro-organisms that give typical Salmonella reactions culturally and biochemically should be tested.

2. Colonies growing on TSI agar or Kligler iron agar are satisfactory.

3. Prepare a dense suspension of the organisms to be tested by suspending the growth from an 18-hour TSI agar slant in 0.5 mL of 0.85 percent sodium chloride solution. This should produce a dense homogeneous suspension approximating 50 times that of a McFarland barium sulfate standard. Care must be taken to ensure an even suspension.

4. Using a wax pencil, mark a microscope slide or glass plate into sections about 1 cm square.

5. Place a drop (0.05 mL) of the appropriate Salmonella O antiserum poly on the ruled section of slide or plate as shown.

Antiserum	NaCl
Bacteria	Bacteria

6. Place one drop of 0.85 percent sodium chloride solution in the square adjacent to the one containing the antiserum. This will serve as a negative control of the bacterial suspension.

7. Using a clean bacteriological loop, transfer a loopful (0.05 mL) of the bacterial suspension (step 3) to the square containing sodium chloride solution. Mix bacterial and sodium chloride solutions thoroughly to obtain an even mixture.

8. Transfer a second loopful of bacterial suspension (step 3) to the square containing the antiserum. Mix bacterial solutions and antiserum thoroughly to obtain an even mixture.

9. Positive agglutination will be completed within 1 to 2 minutes. A delayed or partial agglutination should be considered negative.

10. If positive agglutination occurs, identify the group to which the bacterium belongs by using the desired individual Salmonella O antisera groups in the same manner as described in steps 5 through 9 for the Salmonella O antiserum poly.

11. If the culture reacts with Salmonella O antiserum poly A-1, step 10, but does not react with the specific Salmonella O antisera groups, it should be checked using Salmonella Vi antiserum by the method described in steps 5 through 9. If there is no agglutination caused by Salmonella Vi antiserum at this point, the culture may be regarded as not of the Salmonella genus. If the culture reacts

with the Salmonella Vi antiserum, the culture suspension should be heated in a boiling water bath for 10 minutes and cooled. After cooling, the heated culture should be retested using the desired individual Salmonella O antisera groups and the Salmonella Vi antiserum. If the culture does not react with the Vi antiserum after heating but reacts with the Salmonella O antiserum group D, factor 9, it is most likely Salmonella typhi and should be confirmed using Salmonella H antiserum d and an unheated culture.

12. If the heated culture in step 11 continues to react with the Vi antiserum and does not react with any of the Salmonella O antisera, the culture may be classified as a member of the Citrobacter (Citrobacter freundii) group. Edwards and Ewing (1972) recommended resubmitting for further biochemical tests all cultures having a typical reaction with Salmonella Vi antiserum and Salmonella O antiserum (poly or individual groups). They recommend using lysine decarboxylase broth and KCN broth. This step will aid in the elimination of the Citrobacter group (Bethesda-Ballerup) of bacteria.

13. Cultures having positive agglutination with Salmonella O antiserum groups may be analyzed further for their H antigens using the appropriate Salmonella H antisera, if necessary.

6.13.2 Flagellar H Antigen Analysis (Difco Laboratories, 1971b). For final identification of the Salmonella serotypes within a group, as determined by the Salmonella O antisera, it is necessary to determine the H antigens and the phase of the bacterium. Use tube-test procedure developed by Edwards and Bruner (1947). It is necessary to have a motile bacterium when testing for H antigens. Usually TSI broth cultures of fresh isolates are satisfactory for use as antigens. Occasionally, it is necessary to increase the motility of the test bacteria by making several consecutive transfers in SIM medium. This is a semi-solid medium that permits visual determination of bacterial movement. If the bacterium grows well on SIM medium, the biochemical test procedure is described in table 9. Inoculate the test tubes slightly below the surface of the medium by the stab method. Incubate the tubes at 41.5 °C for 18 to 20 hours. Transfer only those bacteria that have migrated to the bottom of the tube when making successive cultures. After several transfers, if the bacteria in the culture travel 50 to 60 mm through the medium in 18 to 20 hours, it is ready for use.

1. Inoculate a veal infusion broth using the motile bacteria from the last transfer (in motility medium) and incubate at 41.5 °C overnight.

2. Inactivate the culture using equal volumes of culture and 0.6 percent physiological saline solution (6 mL of 40 percent formaldehyde solution plus 8.5 g sodium chloride in 1 L distilled water).

3. Dilutions containing Salmonella H antisera depend on which sera are to be used. In general, use a 1:1,000 dilution with the majority of the H sera. This is done by diluting the rehydrated antiserum in a ratio of 0.1 mL antiserum to 33 mL 0.85-percent sodium chloride solution. A few of the specific single-factor sera must be used at a 1:500 dilution because extensive absorption is necessary to render them specific. The 1:500 dilution is recommended when Salmonella H antisera s, z13, z15, and z28 are used. To prepare a 1:500 dilution, add 0.1 mL rehydrated antiserum to 16 mL 0.85-percent sodium chloride solution. When using Salmonella H antiserum poly a-z, use a dilution of 1:100. To obtain this dilution, add 1 mL rehydrated polyvalent antiserum to 33 mL 0.85-percent sodium chloride solution. Salmonella H antisera poly A, B, C, D, E, and F, however, are used at a 1:1,000 dilution as prepared above. Prepare only the quantity of diluted Salmonella H sera that can be used in any given day. Discard all excess.

4. Add 0.5 mL of the appropriate serum dilution to Kahn-type serological tubes.

5. Add 0.5 mL of the antigen and incubate in a water bath at 50 °C for 1 hour.

6. Observe the agglutination and record. Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

Not applicable.

8. Reporting of results

Report results only as positive or negative for Salmonella or Shigella in the sample. Record the sample volume if it is known.

9. Precision

No precision data are available.

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Pseudomonas Aeruginosa (Membrane-Filter Method) (B-0105-85)

Parameter and Code:
Pseudomonas aeruginosa, MF (colonies/100 mL): 71220

The occurrence of Pseudomonas aeruginosa is of increasing concern because it is frequently the causative agent of skin, ear, eye, nose, and throat infections among those engaged in water-contact sports. P. aeruginosa also has often been implicated as the cause of some hospital-acquired infections. P. aeruginosa is a natural inhabitant of soil, surface water, and vegetation. The majority of the strains identified as P. aeruginosa are nonpathogenic to humans. However, the appearance and biochemical characteristics of pathogenic strains are indistinguishable from nonpathogenic P. aeruginosa (in the method reported here) so that caution should be observed while handling all pseudomonas cultures. P. aeruginosa is a gram-negative, rod-shaped bacterium, motile by monotrichous polar flagella. Most strains produce a variety of pigments, some of which are used for identification in this method. A fluorescent greenish-blue pigment and pyocyanin, a blue pigment, are the most common, but some strains also produce pyorubin, a brownish-red pigment. An incubation temperature of 41.5±0.5 °C is used because other fluorescent pseudomonads, such as P. fluorescens, will not grow at, or above, 41 °C.

The presence of P. aeruginosa in water used for swimming has caused health concern. Presently, insufficient work has been done to indicate safe limits of P. aeruginosa in bathing waters. Brodsky and Nixon (1974) reported that 43 percent of the swimming pools studied had greater than 18 P. aeruginosa per 100 mL and 77 percent had a count of greater than 160 P. aeruginosa per 100 mL. The occurrence and pathogenicity of P. aeruginosa in surface water is not well known except that P. aeruginosa is widely distributed in all water.

1. Applications

The method is applicable to all water that does not have large suspended-solids concentration.

2. Summary of method

A water sample is filtered through a 0.45-µm pore size membrane filter (0.7-µm filters would allow passage of the pseudomonads). The membrane filter is placed on m-PA agar and incubated at 41.5±0.5 °C for 48±2 hours. After incubation, colonies having typical diffuse brown pigment are counted. Typical colonies may be verified by reaction on skim milk agar.

3. Interferences

Suspended materials may inhibit the filtration of sufficiently large sample volumes to produce statistically valid results. In addition, some suspended material is toxic to bacteria and inhibits their growth. If suspended material is a problem, the multiple-tube method described by the American Public Health Association and others (1985) may be used to estimate P. aeruginosa numbers.

4. Apparatus

All materials used in microbiological testing need to be free of agents that inhibit bacterial growth. Most of the materials and apparatus listed in this section are available from scientific supply companies.

The following apparatus list assumes the use of an onsite kit for microbiological water tests, such as the portable water laboratory (Millipore, or equivalent). If other means of sample filtration are used, refer to the manufacturer's instructions for proper operation of the equipment. Items marked with an asterisk (*) in the list are included in the portable water laboratory (fig. 1).

4.1 Alcohol burner, glass or metal, containing ethyl alcohol for flame sterilizing of forceps.

4.2 Aluminum seals, one piece, 20 mm.

4.3 Analytical balance, with sensitivity of 0.1 mg.

4.4 Bacteriological transfer needle.

4.5 Bottles, milk dilution, screwcap.

4.6 Bottles, serum.

4.7 Crimper, for attaching aluminum seals.

4.8 Decapper, for removing aluminum seals from spent tubes.

4.9 Filter-holder assembly* and syringe that has a two-way valve* or vacuum hand pump.

4.10 Forceps*, stainless steel, smooth tips.

4.11 Graduated cylinders, 100-mL capacity.

4.12 Hypodermic syringes, sterile, 1-mL capacity, equipped with 26-gauge, 3/8-in. needles.

4.13 Hypodermic syringes, sterile, 10-mL capacity, equipped with 22-gauge, 1- to 1½-in. needles.

4.14 Incubator*, for operation at a temperature of 41.5±0.5 °C. A portable incubator as provided in the portable water laboratory, or heaterblock (fig. 2), which operates on either 115 V ac or 12 V dc, is convenient for onsite use. A larger incubator, having more precise temperature regulation, is satisfactory for laboratory use. A water bath capable of maintaining a temperature of 41.5±0.5 °C also is satisfactory.

4.15 Membrane filters, white, grid, sterile, 0.45-µm pore size, 47-mm diameter.

4.16 Microscope, binocular wide-field dissecting-type, and fluorescent lamp.

4.17 pH meter.

4.18 Pipets, 1-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.19 Pipets, 10-mL capacity, sterile, disposable, glass or plastic, having cotton plugs.

4.20 Pipettor, or pi-pump, for use with 1- and 10-mL pipets.

4.21 Plastic petri dishes with covers, disposable, sterile, 50×12 mm.

4.22 Plastic petri dishes with covers, disposable, sterile, 100×15 mm.

4.23 Rubber stoppers, 13×20 mm.

4.24 Sample-collection apparatus. Use an appropriate device for collecting a representative sample from the environment to be tested, following guidelines in the "Collection" subsection of the "Bacteria" section.

4.25 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.26 Thermometer, having a temperature range of at least 40 to 100 °C.

4.27 Whirl-Pak, 18 oz.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Buffered dilution water. Dissolve 34 g potassium dihydrogen phosphate (KH_2PO_4) in 500 mL distilled water. Adjust to pH 7.2 using 1 N sodium hydroxide (NaOH). Dilute to 1 L using distilled water. Sterilize in dilution bottles at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Add 1.25 mL KH_2PO_4 solution to 1 L distilled water containing 0.1 percent peptone. (Do not store KH_2PO_4 solutions for more than 3 months.) Dispense in milk dilution or serum

bottles (capped with rubber stoppers and crimped with aluminum seals) in quantities that will provide 99±2 mL after autoclaving at 121 °C at 1.05 kg/cm^2 (15 psi) for 20 minutes. Allow enough space between bottles for steam to circulate during autoclaving. Loosen caps prior to sterilizing and tighten when bottles have cooled.

5.2 Distilled or deionized water.

5.3 Ethyl alcohol, 95-percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methyl alcohol also may be used for sterilization.

5.4 m-PA agar. This agar medium is not available in dehydrated form and requires preparation from the basic ingredients. The composition of m-PA agar is listed in table 11. To prepare m-PA agar, combine all ingredients, except antibiotics, and adjust to pH 6.5 using 1 N NaOH. Sterilize at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Cool to 55 to 60 °C and aseptically readjust to pH 7.1±0.1. This is done by removing small aliquots of medium to check the pH after adding 1 N NaOH. If the quantities in table 11 are followed, approximately 1.1 mL of 1 N NaOH is needed at this point to attain pH 7.1. After the pH of 7.1 has been achieved, add the antibiotics listed in table 11, using a gentle swirling motion. Pour the medium into 50-mm diameter petri dishes to a depth of 4 mm (6-8 mL) when the melted medium has cooled to 50 °C or less.

5.5 Skim milk agar

Solution A:

Skim milk	100 g
Distilled water	500 mL

Solution B:

Nutrient broth	12.5 g
Sodium chloride (NaCl)	2.5 g
Agar	15 g
Distilled water	500 mL

Heat solutions separately to boiling and dispense in convenient volumes (such as 75 mL in 160-mL milk dilution bottles). Sterilize at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Cool to approximately 60 °C, then combine equal volumes of solution A and B, and pour into 100-mm petri dishes to a depth of 4 mm (15 mL). After solidification occurs, store the petri dishes in a plastic bag at 2 to 5 °C (refrigerated) for not more than 2 weeks. Sterile skim milk agar (solutions uncombined) also may be refrigerated for 2 weeks and can be melted and combined as needed.

Table 11.--Composition of m-PA agar

Ingredients	Quantity
L-lysine HCl	2.5 grams
Sodium chloride	2.5 grams
Yeast extract	1.0 gram
Xylose	1.25 grams
Sucrose	.62 gram
Lactose	.62 gram
Phenol red	.04 gram
Ferric ammonium citrate	.40 gram
Sodium thiophosphate	3.40 grams
Agar	7.5 grams
Distilled water	500 milliliters
Antibiotics:	
Sulfapyridine	88 milligrams
Kanamycin	4.25 milligrams
Naladixic acid	18.5 milligrams
Actidione	75 milligrams

6. Analysis

6.1 Sterilize filter-holder assembly (Note 1). In the laboratory, wrap the funnel and filter base parts of the assembly separately in kraft paper or polypropylene bags and sterilize in the autoclave at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 minutes. Steam must contact all surfaces to ensure complete sterilization. Cool to room temperature before use.

Note 1: Onsite sterilization of the filter-holder assembly needs to be in accordance with the manufacturer's instructions but usually involves application and ignition of methyl alcohol to produce formaldehyde. Autoclave sterilization in the laboratory prior to the trip to the sampling site is preferred. Sterilization must be performed for all sites.

6.2 Assemble the filter holder and, using flame-sterilized forceps (Note 2), place a sterile membrane filter over the porous plate of the assembly, grid side up. Carefully place funnel on filter to avoid tearing or creasing the membrane.

Note 2: Flame-sterilized forceps--Dip forceps in ethyl or methyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

6.3 Shake the sample vigorously about 25 times to obtain an equal distribution of bacteria throughout the sample before transferring a measured portion of the sample to the filter-holder assembly.

6.3.1 If the volume of sample to be filtered is 10 mL or more, transfer the measured sample directly onto the dry membrane. For most surface water, sample volumes of 10, 40, 100, and 200 mL are suggested. Filtration volumes more than 100 mL need to be split between two or more filters.

6.3.2 If the volume of sample is between 1 and 10 mL, pour about 20 mL sterilized buffered dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of bacteria.

6.3.3 If the volume of original water sample is less than 1 mL, proceed as in 6.3.1 after preparing appropriate dilutions by adding the sample to buffered dilution water in a sterile milk dilution bottle (Note 3) in the following volumes:

Dilution	Volume of sample added to 99-milliliter milk dilution bottle	Filter this volume
1:10	11 milliliters of original sample	1 milliliter of 1:10 dilution
1:100	1 milliliter of original sample	1 milliliter of 1:100 dilution
1:1,000	1 milliliter of 1:10 dilution	1 milliliter of 1:1,000 dilution
1:10,000	1 milliliter of 1:100 dilution	1 milliliter of 1:10,000 dilution

Note 3: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer, close and shake the bottle vigorously at least 25 times to maintain distribution of the bacteria in the sample. Diluted samples need to be filtered within 20 minutes after preparation.

6.4 Apply vacuum and filter the sample. When vacuum is applied using a syringe fitted with a two-way valve, proceed as follows: Attach the filter-holder assembly to the inlet of the two-way valve with plastic tubing. Draw the syringe plunger very slowly on the initial stroke to avoid the danger of air lock before the assembly fills with water. Push the plunger forward to expel air from the syringe. Continue until the entire sample has been filtered. If the filter balloons or develops bubbles during sample filtration, disassemble the two-way valve and lubricate the rubber valve plugs lightly with stopcock grease. If a vacuum hand pump is used, do not exceed a pressure of 25 cm to avoid damage to bacteria.

6.5 Rinse sides of funnel twice with 20 to 30 mL of sterile buffered dilution water while applying vacuum.

6.6 Maintaining the vacuum, remove the funnel from the base of the filter-holder assembly and, using flame-sterilized forceps, remove the membrane filter from the base and place it on the agar surface in the plastic petri dish, grid side up, using a rolling action at one edge. Use care to avoid trapping air bubbles under the membrane (Note 4).

Note 4: Hold the funnel while removing the membrane filter and place it back on the base of the assembly when the membrane filter has been removed. Placement of the funnel on anything but the base of the assembly may result in contamination of the funnel.

6.7 Place top on petri dish and proceed with filtration of the next volume of water. Filter in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations.

6.8 Clearly mark the lid of each plastic petri dish indicating location, time of collection, time of incubation, sample number, and sample volume. Use a waterproof felt-tip marker or grease pencil.

6.9 Inspect the membrane in each petri dish for uniform contact with the agar. If air bubbles are present under the filter (indicated by bulges), remove the filter using sterile forceps and roll onto the agar again.

6.10 Close the plastic petri dish by firmly pressing down on the top.

6.11 Incubate the petri dishes with filters in an inverted position (agar and filter at the top) at 41.5±0.5 °C for 48±2 hours. Filters need to be incubated within 20 minutes after placement on medium. If a water-bath incubator is used, the petri dishes should either be taped to prevent water entry or the dishes put into Whirl-Pak, or equivalent plastic bags. The dishes must be incubated below the water surface.

6.12 After incubation, remove petri dish lids and count typical colonies at 15X magnification. Angle of illumination is not critical. P. aeruginosa colonies are dark brown, have an irregular margin, and are almost flat. A light-brown pigment diffusing radially away from the colony is usually visible. Petri dishes having between 20 and 80 P. aeruginosa colonies are considered to be ideal for counting purposes and should be used for calculation, if possible.

6.13 Some of the colonies counted as P. aeruginosa should be confirmed by determining growth on skim milk agar. Sterilize the bacteriological transfer needle (sterile round toothpicks also are suitable) by flaming in the burner. The long axis of the needle needs to be held parallel to the cone of the flame so that the entire length of the needle is heated to redness. Remove from flame and allow the needle to cool for about 10 seconds. Do not allow the needle to contact any foreign surface during the cooling period. When cool, remove a small portion of a colony using the sterilized needle and lightly streak the skim milk agar surface. Several such transfers may be made to each petri dish [multiple (24) well petri dishes are useful], sterilizing the needle between each inoculation. Every inoculation should have appropriate notation to identify the source.

6.14 Invert and incubate each inoculated petri dish at 20 to 35 °C for 24 to 48 hours. P. aeruginosa causes casein hydrolysis (clearing of the agar) where growth occurs. A yellow-green diffusible pigment should be visible when the petri dish is viewed from the side.

6.15 Autoclave all cultures at 121 °C at 1.05 kg/cm^2 (15 psi) for 15 to 30 minutes before discarding.

7. Calculations

7.1 If only one filter has a colony count between the ideal of 20 and 80, use the equation:

$$\text{P. aeruginosa (colonies/100 mL)} = \frac{\text{Number of colonies counted} \times 100}{\text{Volume of original sample filtered (milliliters)}}.$$

7.2 If all filters have counts less than the ideal of 20 colonies or greater than 80 colonies, calculate using the equation in 7.5 for only those filters having at least one colony and not having colonies too numerous to count. Report results as the number per 100 mL, followed by the statement, "Estimated count based on nonideal colony count."

7.3 If no filters develop characteristic P. aeruginosa colonies, report a maximum estimated value. Assume a count of one colony for the largest sample volume filtered, then calculate using the equation in 7.1. Report the results as less than (<) the calculated value per 100 mL.

7.4 If all filters have colonies too numerous to count, report a minimum estimated value. Assume a count of 80 P. aeruginosa colonies for the smallest sample volume filtered, then calculate using the equation in 7.1. Report the results as greater than (>) the calculated value per 100 mL.

7.5 Sometimes two or more filters of a series will produce colony counts within the ideal counting range. Make colony counts on all such filters. The method for calculating and averaging is as follows (Note 5):

$$\frac{\text{Volume filter 1} + \text{Volume filter 2}}{\text{Volume sum}} \qquad \frac{\text{Colony count filter 1} + \text{Colony count filter 2}}{\text{Colony count sum}}$$

$$\text{P. aeruginosa colonies/100 mL} = \frac{\text{Colony count sum} \times 100}{\text{Volume sum (milliliters)}}$$

Note 5: Do not calculate the P. aeruginosa colonies per 100 mL for each volume filtered and then average the results. If a large filtered volume was divided between several filters, make the count using the equations as in 7.5. Such counts are considered to be in the ideal range if the sum of the colonies is between 20 and 80 colonies.

8. Reporting of results

Report P. aeruginosa concentration as P. aeruginosa colonies per 100 mL as follows: less than 10 colonies, whole numbers; 10 or more colonies, two significant figures.

9. Precision

Carson and others (1975) reported a mean recovery of 95 percent of naturally occurring P. aeruginosa using m-PA agar.

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PHYTOPLANKTON

Introduction

Phytoplankton are unicellular algae existing as single cells, colonies, chains, or filaments that generally are transported passively (some forms are active swimmers) by currents and turbulent mixing. Morris (1967) divides the planktonic algae into nine taxonomic divisions, including the blue greens (Cyanophyta), greens (Chlorophyta), diatoms (Bacillariophyta), dinoflagellates (Pyrrophyta), and five other divisions of flagellates. The range of sizes among phytoplankton cells or colonies is diverse (ranging from about 1 to about 1,000 μm) and has been partitioned into four size classes by Wetzel (1975): macroplankton (more than 500 μm), netplankton (50-500 μm), nannoplankton (10-50 μm), and ultraplankton (less than 10 μm). Physiological processes of planktonic algae can profoundly affect (and indicate) the productivity and quality of natural water. Their photosynthetic assimilation of carbon dioxide and production of organic matter provide a (the) primary food source for other trophic levels, including harvestable species; they also affect the concentration of dissolved gases (carbon dioxide, oxygen), inorganic nutrients (nitrogen, phosphorus, silica, and trace elements), and dissolved organic substances. Phytoplankton blooms can severely affect water quality, either through the production of toxins that lead to fish kills or threats to human health or through the decomposition of organic matter that can deplete oxygen.

Integrated studies of aquatic ecosystems need to include measurements of phytoplankton biomass and composition. Measurement of bulk constituents [chlorophyll *a*, adenosine triphosphate (ATP), and particulate organic carbon or nitrogen] can be used as indices of biomass, while particle counters can provide information about size distribution. However, these methods have interferences from nonphytoplankton particulate matter (detritus, bacteria, microzooplankton, and sediment). The only method of determining the species composition of phytoplankton communities is by microscopic enumeration and identification. Although time consuming and laborious, this method can offer valuable information. Knowledge of species composition can indicate the causes of seasonal changes in biomass, can be useful as tracers for different water masses, and can indicate stresses imposed by pollutants that may not be evident from measurements of biomass alone. Estimates of cell size and measurements of cell-size distribution also can provide an accurate measurement of phytoplankton biomass [as biovolume, which can be converted to carbon (Strathman, 1967)].

Collection

There is no single best method for collecting and enumerating phytoplankton samples because phytoplankton types and abundance differ spatially and temporally. Therefore, it is necessary to choose a sampling strategy and method most consistent with the goals of a given water-quality study. For example, frequent collection of a depth-integrated sample at one representative site may be appropriate for a monitoring study; whereas a detailed spatial grid may be more appropriate for assessing the effects of a point

source of a pollutant. Sampling in those areas having the greatest environmental variability or having rapid temporal change needs to be intensified. Sournia (1978) has compiled a detailed manual of phytoplankton methods that includes a discussion of sampling strategy and statistical analyses.

A phytoplankton sample consists of a volume of water (usually 100 mL to 1 L) that is stored in a graduated polyethylene or glass bottle. Dissolution of weakly silicified diatoms is minimized in bottles made of soft glass (Banse, 1974). To ensure maximum correlation of results, the sample site and method used need to correspond as closely as possible to those selected for chemical and microbiological sampling. If a living sample is to be examined, it can be maintained at 3 to 4 °C for 24 hours or it can be kept cool and darkened for 3 to 4 hours. Extended storage requires use of a preservative. Two preservatives commonly are used:

1. To each 100 mL of sample, add 3 mL 34 to 70 percent aqueous formaldehyde solution (100 percent formalin), 0.5 mL 20 percent detergent solution, and 0.1 mL cupric sulfate solution. This preservative maintains cell coloration and is effective indefinitely but may distort the cell shape of species and cause loss of flagella.

2. Lugol's solution using acetic acid (Rodhe and others, 1958) will stain cells (and other organic particles) brownish yellow and will maintain cell morphology of flagellates. To each 100 mL of sample, add 1 mL Lugol's solution having 10 percent acetic acid.

Phytoplankton samples can be collected using a water-sampling bottle, depth-integrating sampler, net, or pump. Most water-sampling bottles consist of a cylindrical tube that has stoppers at each end and a closing device that is activated by a messenger. The bottle is lowered into the water in the open position to a desired depth, tripped, and retrieved. Most common examples of bottles are the Kemmerer (fig. 10), Van Dorn-type (fig. 11), the Nansen, the Fjarlie, and the Niskin. These bottles are available in a variety of sizes, having capacities from 0.2 to more than 30 L, and are constructed of brass, clear acrylic, or polyvinyl chloride. Advantages of water-sampling bottles include: (1) Quantitative samples can be collected that include nannoplankton and ultraplankton; (2) samples of a known volume can be obtained from a precise depth; (3) bottles can be hung in arrays to collect simultaneous samples at a variety of depths; and (4) bottles are light and do not require auxiliary equipment. However, they are difficult to handle in strong currents.

Depth-integrating samplers are used to collect quantitative samples representative of a cross section of a stream or the water column of a lake, reservoir, stream, or estuary (Schröder, 1969; Lewis and Saunders, 1979; Wetzel and Likens, 1979). The simplest depth-integrating sampler is a length of garden hose or flexible tubing that is weighted on one end (Lund, 1949). The weighted end is lowered through the desired sampling depth of the water column, and the open end then is pinched off to secure the sample within the hose as it is raised to the surface.

Figure 10.--Kemmerer water-sampling bottle. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

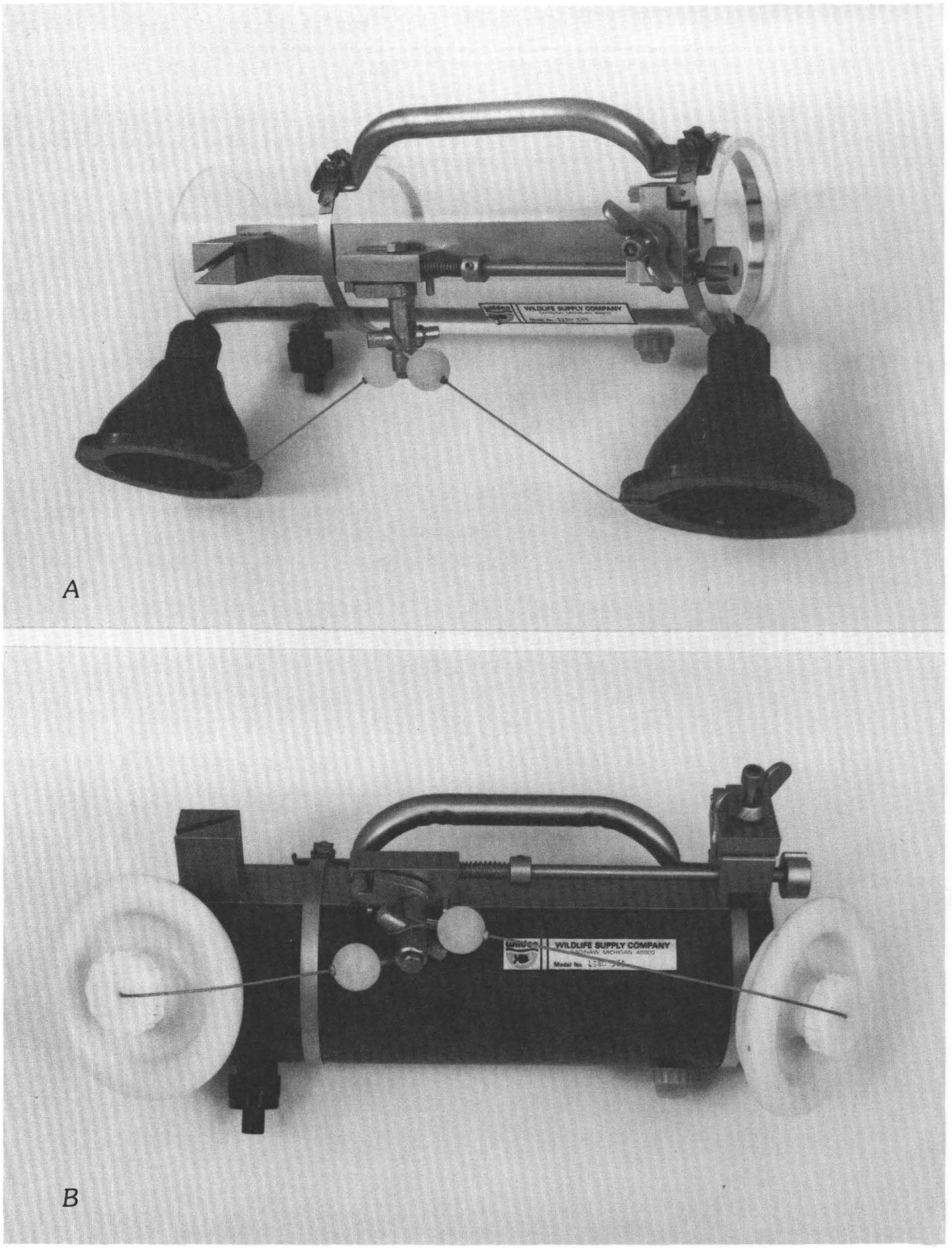

Figure 11.--Van Dorn-type water-sampling bottle: (A) Alpa bottle; (B) Beta bottle. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

A sampler, such as the D-77 sampler (fig. 12), can be used for depth-integrating sample collection. This sampler is made of aluminum or bronze and has a built-in cap and nozzle that can be sterilized and will collect a 3-L sample. A depth-integrating sampler designed specifically for collecting phytoplankton is described in Fee (1976). This sampler is a modification of the Van Dorn-type water-sampling bottle and has release mechanisms to clamp the sample-inflow and air-escape hoses. The sample-inflow hose goes to the bottom of the sampler, and the air-escape hose to the bottom of the cap. The sampler is lowered to the desired depth, a messenger is released, and the release of the two hoses starts the sampler. For stream sampling, the equal-transit method developed by Guy and Norman (1970) is useful. In this method, the standard suspended-sediment sampler is used to collect samples at a number of equally spaced verticals in the cross section. Samples collected in each vertical are composited into a single sample that has been discharge-weighted and is representative of the entire cross section.

Advantages of depth-integrating samplers include: (1) Quantitative samples can be collected that include nannoplankton and ultraplankton; (2) samples of a known volume can be obtained; (3) these samplers provide the only means of collecting a truly representative sample of phytoplankton within a water column or in a stream cross section; and (4) many are light and can be used without auxiliary equipment. However, sample collection may be time consuming using some of these samplers, and some are heavy and do require auxiliary equipment. In addition, these samplers may not be adequate for use during high flow.

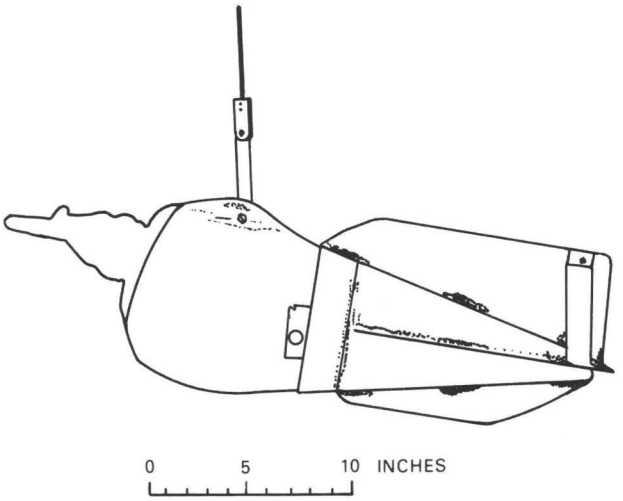

Figure 12.--D-77 depth-integrating sampler. (Sketch based on photograph courtesy of St. Anthony Falls Hydraulic Laboratory, Minneapolis, Minn.)

Plankton nets have been used widely as sampling devices in phytoplankton investigations because they enable filtration of a large volume of water; however, nets selectively retain only the largest phytoplankton cells. Margalef (1969) assumed that only 10 percent of all algal cells are retained by nets having a mesh size of 40 μm. However, phytoplankton collection using nets may be appropriate for qualitative studies of large planktonic algae. Most qualitative samplers are cone-shaped nets that are towed slowly from a bridle and that funnel trapped material into a bucket (fig. 13). Nets come in a variety of mesh size, have openings ranging from 0.5 μm to 5 mm, and usually are constructed of woven synthetic filaments (monofilament nylon or polyester) that resist chemicals and have stable mesh geometry. Nets can be towed vertically, horizontally, or obliquely to collect integrated samples. Closing nets, such as the Birge sampler (Welch, 1948), can be lowered to a selected position, activated, and then closed by messenger to sample only at a specific depth. Advantages of nets include: (1) They provide a simple means of collecting qualitative samples of macroplankton, netplankton, and some nannoplankton; (2) they can be adapted with a flowmeter for collecting semiquantitative samples; (3) the mesh size can be chosen, within limits, to collect planktonic algae of selected sizes; (4) large species are collected; and (5) nets are relatively inexpensive and easy to operate from a small boat. Disadvantages include: (1) They do not collect quantitative samples; (2) they exclude ultraplankton and some nannoplankton (these forms often constitute a majority of phytoplankton biomass); (3) they are not suitable for collection in very shallow water or water having large algal populations; and (4) clogging by vascular plants, detritus, and dense populations of algae can be a problem, particularly with fine-mesh nets.

Pumps can be used to collect qualitative or quantitative samples of phytoplankton (Aron, 1958; Fee, 1976; Schemel and Dedini, 1979). The basic design consists of a centrifugal (impeller) or reciprocating (piston or diaphragm) pump connected to a hose that is lowered to the sampling depth, a base, and a collecting net and bucket. The centrifugal pumps probably are least damaging to algae. Quantitative samples can be collected by measuring the flow rate of the pumped stream using either a volume register or a calibrated container. Advantages of pumps include: (1) Quantitative samples of macroplankton, netplankton, and some nannoplankton can be collected quickly; (2) discrete samples from known depths can be collected; (3) the sampling hose can be moved during sampling to collect a depth-integrated sample; and (4) the pumps can be used in shallow water. In addition, pumps are good for point samples but may induce erroneous respiration and productivity values. Disadvantages include: (1) Pumps usually are bulky, expensive, and require an electrical source; and (2) they may break algal chains and colonies, or physiologically stress planktonic algae.

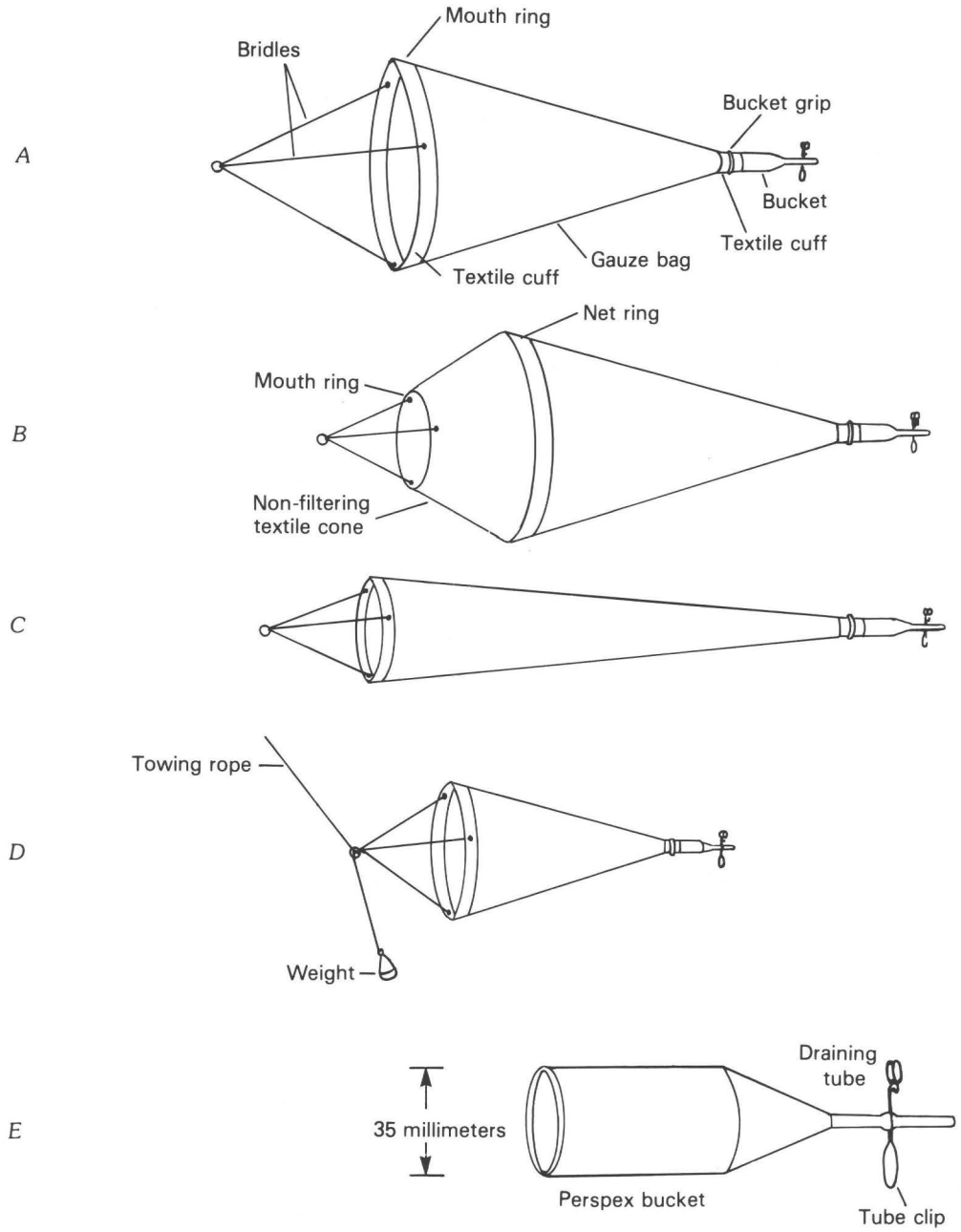

Figure 13.--Phytoplankton sampling nets and accessories: (A) Standard net. The length of standard nets normally is 2 to 3 times the mouth diameter. (B) Fine-mesh net that has decreased mouth diameter. A tapering nonfiltering textile sleeve is inserted between the large net ring and the smaller mouth ring. (C) Extra long, fine-mesh standard net. (D) Standard net attached to the towing rope, and a weight in front of the mouth. (E) Plankton-collecting bucket made of clear perspex material. Diameter of the bucket is 30 to 100 millimeters (here 35 millimeters); length of the cylindrical part is 50 to 200 millimeters (here 65 millimeters). The bucket is attached to the net tail by textile tape or a specially made metal grip (from Sournia, 1978; reproduced by permission of UNESCO).

Precision

The precision of estimated phytoplankton cell densities is essential for comparing estimated population densities in different samples; however, calculation of the exact precision of population estimates is difficult for two reasons. First, accurate statistical analysis requires knowledge of the frequency distribution of algal cells in nature, in aliquots of samples, and in counting chambers. Second, most sampling programs involve multiple stages of subsampling (for example, onsite population ⟶ sample ⟶ aliquot ⟶ microscopic field). Each stage of subsampling adds a new component of variability to the data (Venrick, 1978). If the distribution of phytoplankton cells is random (that is, conforms to a Poisson distribution), then the precision of cell counts can be estimated from the formulas in the following paragraphs. Departures from a random distribution are common, usually because of clumping or aggregation, and can be determined using the chi-squared test (Lund and others, 1958). Assuming that phytoplankton cells are not densely aggregated in counting chambers, the following procedures can provide reasonable estimates of counting precision (Venrick, 1978).

If phytoplankton are counted in $\underline{n}$ random microscopic fields of only one aliquot from one sample, then the precision of only the mean number of cells in that one aliquot can be estimated. This may not represent the overall precision of a multilevel sampling program, and it certainly overestimates the precision of population estimates when phytoplankton are spatially heterogeneous. When the number of cells enumerated per chamber is small (less than 50), the confidence limits for a count can be estimated using figure 14. If more than 50 cells are enumerated per chamber, Venrick (1978) suggests using the normal approximation, where confidence limits around the total count (at the 1-α level of significance) are indicated by

$$\Sigma\underline{x} \pm \underline{z}\alpha \sqrt{\Sigma\underline{x}} \quad ,$$

where $\Sigma\underline{x}$ is the total count of cells; and
$\underline{z}\alpha$ is the normal variate (tabulated in most statistics texts).

Precision increases in proportion to the square root of the total number of cells counted, as listed in table 12. This table can be used to determine the number of cells that should be enumerated for a desired level of precision. For example, if 100 cells are enumerated, we can say with 95-percent certainty that the true count does not vary from the mean estimated count by more than 20 percent. Enumeration of 400 cells ensures a precision that is within 10 percent of the mean count.

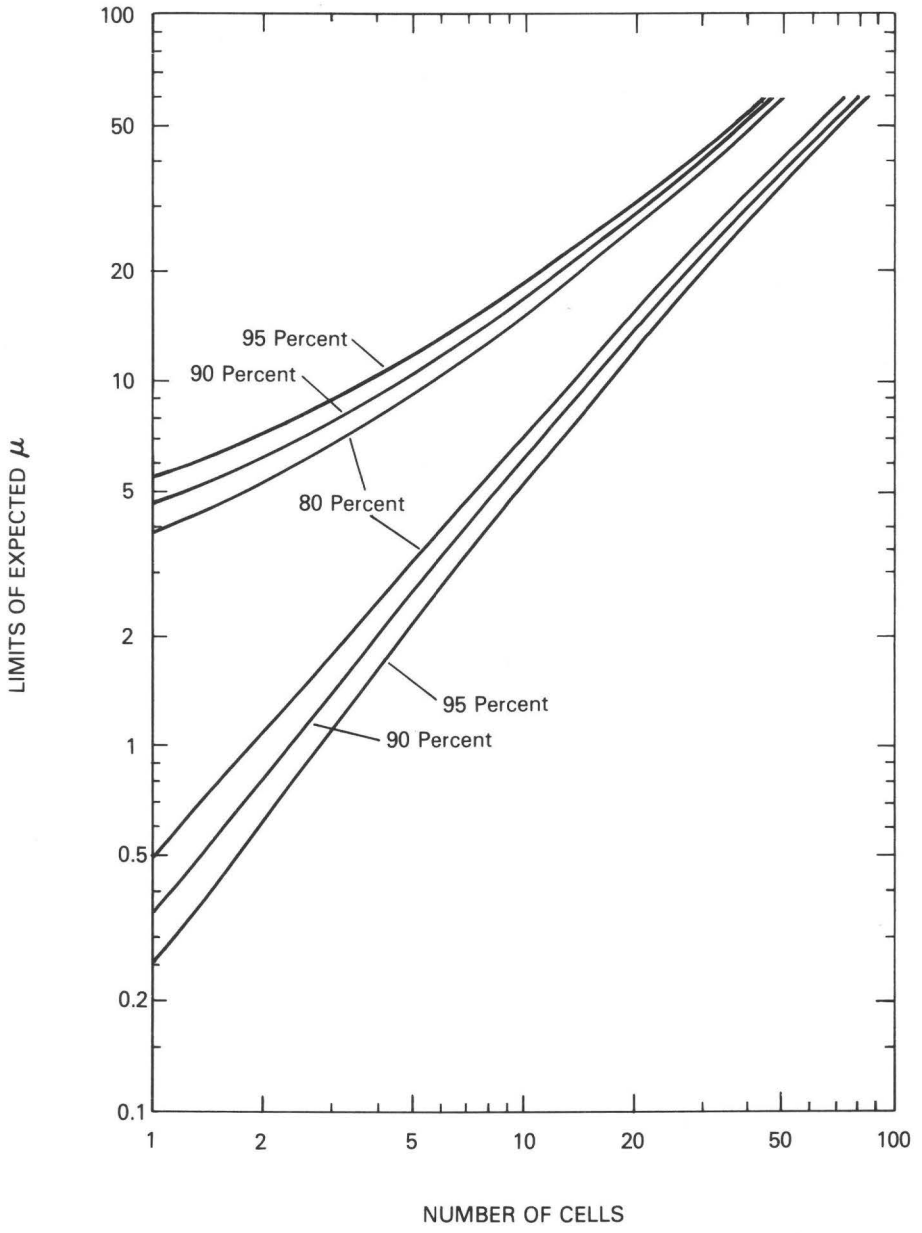

Figure 14.--Limits of expectation of phytoplankton population means, based on single estimates of abundance from a Poisson distribution, at three levels of significance: 95, 90, and 80 percent (from Sournia, 1978; reproduced by permission of UNESCO).

In the instance where replicate chambers are enumerated from one or more aliquots from one or more samples, total variance of counts from all subsampling stages can be estimated. Venrick (1978) recommends use of the studentized normal variate (t) when the mean number of counts per chamber $\bar{x}$ ($\bar{x} = \Sigma x/N$) is greater than 50. Confidence limits around the mean thus are

$$\bar{x} = t\alpha,\ N\text{-}1\ \sqrt{\bar{x}/N}\ ,$$

where N is the number of chambers enumerated.

Table 12.--Approximate 95-percent confidence limits for the number of cells counted, assuming a random distribution (from Lund and others, 1958)

[Precision is the maximum expected departure from the count, expressed as a percentage of the count]

Number of cells counted	95-percent confidence limit[1]	Precision (percent of the count)
4	0-8	±100
16	8-24	±50
25	15-35	±40
100	80-120	±20
400	360-440	±10
1,600	1,520-1,680	±5

[1]For some colonies, the confidence limits in terms of number of cells can be calculated by finding the confidence limits for the complete count of phytoplankton, and then multiplying these by the mean number of cells per colony in these same phytoplankton (Lund and others, 1958).

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Counting-Cell Method
(B-1505-85)

Parameter and Code:
Phytoplankton, total (cells/mL): 60050

Aliquots from phytoplankton samples that previously may have been concentrated or diluted are placed in one of four different counting cells and then examined under a conventional light microscope. Each counting cell is appropriate for a specific range of cell sizes. The Sedgwick-Rafter cell is most appropriate for enumerating macroplankton and netplankton; the Palmer-Maloney cell is appropriate for nannoplankton; and the Hemacytometer and Petroff-Hausser cells are most efficient for enumerating ultraplankton. Efficient counting schemes may require use of two different counting-cell types to ensure inclusion of both large and small phytoplankton.

The counting-cell method is one of several procedures for determining the concentration of phytoplankton. The method is performed easily and provides reasonably reproducible data when used with a calibrated microscope equipped with an eyepiece measuring device, such as the Whipple ocular micrometer (American Public Health Association and others, 1985).

The counting-cell method is much less time consuming than the membrane-filter method. The disadvantage of the method is that the Sedgwick-Rafter counting cell, for example, does not provide for use of a high-power microscope objective. However, the kinds of phytoplankton present in a sample may be determined by high-power magnification prior to using this counting cell.

The Sedgwick-Rafter cell is too thick to use with high-power microscope objectives. Observation of fine structure necessary for identification of some phytoplankton thus is not possible. Furthermore, counting of individual cells, especially filamentous species, is limited. Thinner walled counting cells, which can be used with high-power objectives, are available commercially. Most common is the biomedical hemacytometer, a single piece of thermal- and shock-resistant glass that has an H-shaped trough forming two counting areas. Raised supports hold a cover glass the proper distance above the counting areas. Most hemacytometers have a slight recession on the underside of the chamber to decrease the possibility of accidentally scratching the viewing area and have a thin, metallized deposit on the ruled area to enhance contrast. The primary disadvantage of the hemacytometer, in contrast to the Sedgwick-Rafter cell, for phytoplankton enumeration is that counts are more time consuming, and large cells are not distributed evenly.

1. Applications

The method is suitable for all water.

2. Summary of method

An aliquot of a thoroughly mixed phytoplankton sample is placed in a counting cell (chamber) and examined microscopically. The number of algal cells present in random fields is counted. The density of phytoplankton in the sample, as cells per milliliter, is calculated.

3. Interferences

The enumeration and identification of phytoplankton is impaired by large concentrations of suspended sediment or detritus that obscure micro-organisms. Previously used sample bottles and counting cells must be scrubbed thoroughly to remove adherent diatoms and other materials.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Balance, that has an automatic tare.

4.2 Centrifuge, either swing-out or fixed-head cup-type, 3,000 to 4,000 r/min, 15- to 50-mL conical or 100-mL pear-shaped centrifuge tubes and simple siphoning or suction device to remove excess fluid after centrifugation.

4.3 Counting cells for conventional microscope.

4.3.1 Sedgwick-Rafter counting cell (fig. 15A) and cover glass, 50×20×1 mm.

4.3.2 Palmer-Maloney cell (fig. 15B), and 22-mm no. 1½ cover glass.

4.3.3 Hemacytometer (fig. 15C), 0.1 mm deep, having Improved Neubauer ruling, and cover glasses.

4.3.4 Petroff-Hausser cell (fig. 15D), 0.02 mm deep, having Improved Neubauer ruling.

4.4 Microscope, either conventional light microscope or equivalent. Bright field condensor and objectives are required, and phase contrast is desirable, particularly for taxonomic examination. A series of objectives needs to be available (10X, 20X, and 40X), and 100X phase-contrast oil-immersion objectives need to be available for examination of ultraplankton. The microscope needs to be equipped with a movable mechanical stage that has vernier scales.

4.5 Pipet, Pasteur, 1 mL, disposable.

4.6 Sample containers, glass or graduated polyethylene bottles and screwcaps, 100 mL to 1 L.

4.7 Stage micrometer, 2-mm scale divided into 200×0.01-mm units mounted on 25×75-mm slide.

4.8 Water-sampling bottle, or nets. Depth-integrated samplers are discussed in Guy and Norman (1970) and in Wetzel and Likens, (1979).

4.9 Whipple disc placed in one ocular of the microscope.

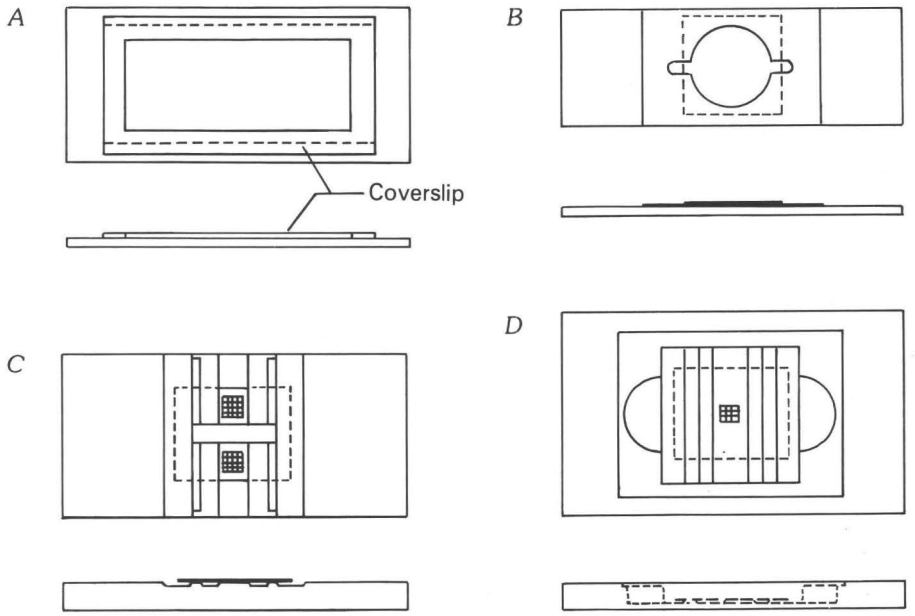

Figure 15.--Phytoplankton counting cells: (A) Sedgwick-Rafter; (B) Palmer-Maloney; (C) Hemacytometer; and (D) Petroff-Hausser (from Sournia, 1978; reproduced by permission of UNESCO).

5. Reagents

5.1 Cupric sulfate solution, saturated. Dissolve 21 g cupric sulfate ($CuSO_4$) in 100 mL distilled water.

5.2 Detergent solution, 20 percent. Dilute 20 mL liquid detergent, phosphate free, to 100 mL using distilled water.

5.3 Distilled or deionized water.

5.4 Ethyl alcohol, 90 percent, for cleaning counting slides.

5.5 Formaldehyde cupric sulfate solution. Mix 1 L 40 percent aqueous formaldehyde containing 10 to 15 percent methyl alcohol with 1 mL cupric sulfate solution.

5.6 Lugol's solution plus acetic acid. Dissolve 10 g iodine (I_2) crystals and 20 g potassium iodide (KI) in 200 mL distilled water. Add 20 mL glacial acetic acid a few days prior to use, and store in an amber glass bottle (Vollenweider, 1974).

6. Analysis

Phytoplankton samples need to be examined under two different magnifications: low power (80X to 200X) to ensure inclusion of large, usually rare, species; and high power (200X to 1,000X, using oil immersion, if possible) to

facilitate identification and to ensure inclusion of ultraplankton. Phytoplankton in the entire slide mount often can be counted using low magnification, but random fields need to be selected at high magnification until a sufficient number of units (cells, filaments, chains, or colonies) have been enumerated for the desired level of precision. Use of a Whipple disc in one ocular will demarcate the microscopic field into a defined, easily viewed grid of 100 squares. When making the counts, enumerate only forms that lie completely inside the grid and those that intersect two of the outer grid borders. If a large number of colonies or filaments appear within the field, determine the average number of cells in an average-size colony or filament and multiply by the number of colonies or filaments present. Count only viable cells, those having protoplasm or pigments. Identify all forms to some predetermined taxonomic level (species level is preferred); count and describe unidentifiable cells.

The volume of original, unconcentrated sample to be examined will vary, depending on sediment content and density of phytoplankton; the volume commonly will range between 25 mL (for eutrophic water or water that has large suspended-sediment concentrations) and 100 mL (for oligotrophic water). Net samples may not require further concentration.

6.1 A variety of counting cells, as well as a conventional light microscope, have been used to enumerate phytoplankton samples. The four types described here (fig. 15) vary in the volume of sample they hold and in the depth of the sample chamber. Therefore, each is suited to a particular size and abundance of planktonic algae. The smaller cells are ruled to enable easy calculation of cell density from tallies within the chamber grid. The Sedgwick-Rafter cell (McAlice, 1971) has a rectangular chamber 1 mm deep that holds 1 mL. The Palmer-Maloney cell (Palmer and Maloney, 1954) has a circular chamber 0.4 mm deep that holds 0.1 mL. Hemacytometers, having Improved Neubauer ruling (Guillard, 1973), are 0.1 mm deep and have two counting grids composed of nine 1-mm squares (sample volume thus is 0.0018 mL). The Petroff-Hausser cell is 0.02 mm deep, has one chamber that has Improved Neubauer ruling, and holds 0.00018 mL; it is designed for cells of bacterial dimension.

6.2 If phytoplankton abundance is sufficiently great to impede enumeration, dilute samples (serially, if necessary) using distilled water. More often, samples collected using a water-sampling bottle must be concentrated to ensure a sufficient density of phytoplankton on counting cells to enable statistically reliable estimation of population abundance. Concentrate samples by settling or centrifuging.

6.3 Allow the sample to settle in the sample container for 4 hours per centimeter of depth to be settled. After settling, weigh the sample container on an automatic tare balance.

6.4 Carefully siphon the supernatant to avoid disturbance of the settled material. Place sample container and remaining sample on balance and weigh. The decrease in weight (in grams) is equivalent to the number of milliliters of supernatant removed. Use the same method to obtain the volume of concentrate. Use centrifugation to concentrate either live or preserved samples.

Using a swing-out or fixed-angle cup-type centrifuge, spin balance samples in 15- to 50-mL conical tubes at about 1,500 r/min (200 × gravity) for 20 to 30 minutes. Siphon a measured volume of supernatant and then disperse the phytoplankton concentrate in the remaining volume of water.

6.5 Use of the Sedgwick-Rafter and Palmer-Maloney cells is similar. With the counting cell on a flat surface, place a no. 1½ cover glass across the cell. Thoroughly mix the sample, remove a 1-mL (0.1 mL for Palmer-Maloney) aliquot using a large-bore Pasteur pipet and transfer the aliquot to the counting cell. Place the cover glass over the counting cell and allow the phytoplankton to settle. Carefully place the cell on the mechanical stage of a calibrated microscope, and enumerate phytoplankton as described in 6. Because neither of these counting cells is ruled, enumeration is facilitated by use of a Whipple disc.

6.6 To fill a hemacytometer, place a clean cover glass onto the counting-chamber supporting ribs. Using a smooth-tipped pipet, place a drop of homogenized sample in the V groove of the metal surface at the edge of the cover glass. The sample will be drawn rapidly into the space between the cover glass and the ruled area of the cell. Any overflow will draw phytoplankton into the moat, and the chamber will have to be cleaned and refilled. Allow phytoplankton to settle and examine the ruled counting area using low power (80X to 200X) to ensure an even distribution of phytoplankton over the grid. Count using high power (200X to 1,000X) and tally cells in a sufficient number of grid squares to ensure the desired level of precision.

6.7 Wash all counting cells using 90-percent ethyl alcohol or phosphate-free detergent and then distilled water.

7. Calculations

The following procedure will provide estimates of phytoplankton population density from tallied counts of algal cells from subsamples enumerated on microscopic slides or counting cells.

7.1 If the sample has been collected by net or if a bottle sample has been either diluted or concentrated by centrifugation-siphoning, calculate the concentration factor, $\underline{c}$ (volume of water represented by a volume of processed sample). The factor $\underline{f}$ corrects for the volume of preservative added:

$$\underline{f} = \frac{\text{Volume of water collected + volume of preservative added}}{\text{Volume of water collected}} ;$$

$$\text{Net sample } \underline{c} = \frac{\text{Volume of water passed through the net}}{\text{Volume of preserved sample}} \times \underline{f} \text{ ; and}$$

$$\text{Bottle sample } \underline{c} = \frac{\text{Volume of water collected}}{\text{Final volume of concentrated or diluted sample}} \times \underline{f} .$$

7.2 For ruled counting cells, calculate the area, $\underline{a}$ (square millimeters), represented by one microscopic field (or Whipple disc grid) using a stage micrometer. This needs to be done for each magnification used for enumeration. For example, if enumeration is done using a Whipple disc at 125X, $\underline{a}$ = 0.49 mm^2.

7.3 For unruled counting cells, calculate the area, $\underline{A}$ (square millimeters), that the sample covers on the counting cell or membrane filter. For the Sedgwick-Rafter cell, $\underline{A}$ = 1,000 mm^2; for the Palmer-Maloney cell, $\underline{A}$ = 250 mm^2.

7.4 Sum the total number of units, $\underline{T}$ (cells, colonies, or filaments × number of cells per colony or filament), tallied within $\underline{n}$ microscopic fields:

$$\underline{T} = \sum_{\underline{i}=1}^{\underline{n}} \underline{x}_{\underline{i}} ,$$

where $\underline{x}_{\underline{i}}$ is total number of units counted in field $\underline{i}$.

7.5 For unruled counting cells, calculate the total volume, v (milliliters), of the original sample represented by $\underline{n}$ microscopic fields:

$$\underline{v} = \underline{c} \times \underline{n} \times \underline{a}/\underline{A} \times \underline{V} ,$$

where $\underline{V}$ is the volume (milliliters), of preserved sample that was settled, filtered, or placed directly into a counting cell.

7.6 For ruled counting cells (hemacytometer), calculate the total volume, $\underline{v}$ (milliliters), of original sample represented by $\underline{n}$ 1-mm squares of the hemacytometer:

$$\underline{v} = \underline{c} \times \underline{n} \times 0.0001,$$

where the volume of sample represented by one square is 0.0001 mL.

7.7 Calculate the population density, $\underline{D}$ (cells per milliliter), of phytoplankton in the original sample:

$$\underline{D} = \underline{T}/\underline{v} .$$

8. Reporting of results

Report phytoplankton density to two significant figures.

9. Precision

See "Precision" subsection in the "Phytoplankton" section.

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Inverted-Microscope Method
(B-1520-85)

Parameter and Code:
Phytoplankton, total (cells/mL): 60050

1. Applications

The method is suitable for all water.

2. Summary of method

The inverted-microscope method enables the observation of the phytoplankton in an aliquot of water at high-power magnification without disrupting or crushing delicate phytoplankton. Phytoplankton are concentrated by settling to the bottom of a vertical-tube sedimentation apparatus (Utermohl, 1958; Lovegrove, 1960; Hasle, 1978). Lund and others (1958) reported that all known phytoplankton can be settled. After settling, an aliquot of phytoplankton sample is poured into a phytoplankton counting cell or sedimentation apparatus (fig. 16). The phytoplankton settle onto a microscope cover glass that forms the bottom of the sedimentation apparatus, and the settled phytoplankton are observed from beneath, using an inverted microscope. Because this method enables use of the high-powered dry and oil-immersion objectives on the microscope, ultraplankton can be identified and enumerated.

3. Interferences

The enumeration and identification of phytoplankton is impaired by large concentrations of suspended sediment or detritus that obscure micro-organisms. Previously used sample bottles and counting cells must be scrubbed thoroughly to remove adherent diatoms and other material. Convection currents and air bubbles in the sedimentation tube can interfere with settling of phytoplankton.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Balance, that has an automatic tare.

4.2 Cover glass, 22-mm diameter, no. 1 and no. 1½.

4.3 Inverted microscope.

4.4 Pipet, serological, 1 mL.

4.5 Plankton counting cell, 26×76-mm glass slide that has a 12-mm circular hole, covered by cementing no. 1½ cover glass to slide, and a no. 1½ cover glass for top of cell.

4.6 Rubber cement, for attaching cover glass to the counting cell.

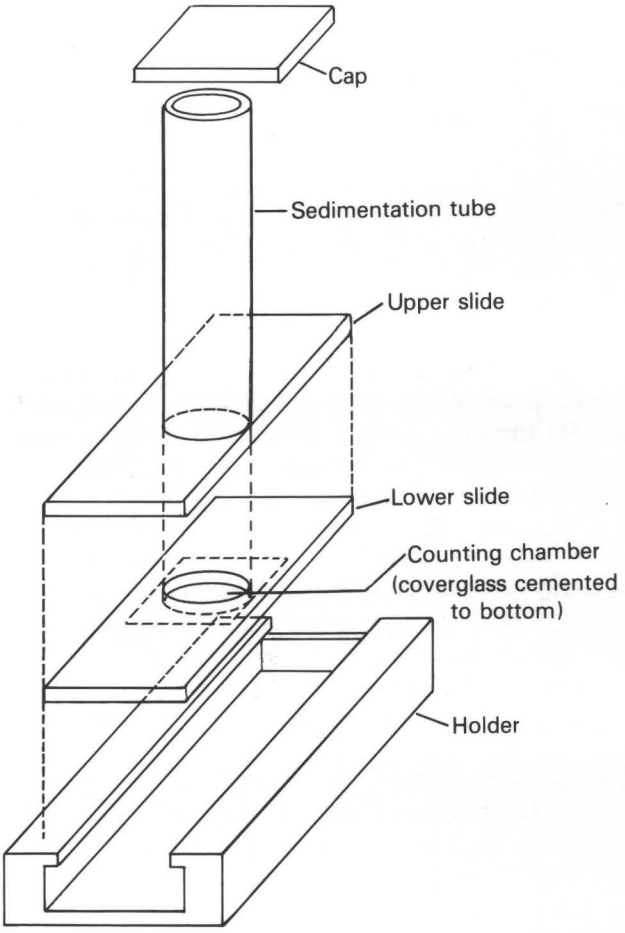

Figure 16.--Phytoplankton counting cell and sedimentation apparatus (modified from Lovegrove, 1960).

4.7 Sample containers, glass or graduated polyethylene bottles and screwcaps, 100 mL to 1 L.

4.8 Sedimentation apparatus, of the type described by Lovegrove (1960) and Hasle (1978), consisting of a sedimentation tube that connects to a counting cell and a bottom cover glass (fig. 16).

4.9 Stage micrometer, 2-mm scale divided into 200×0.01-mm units, mounted on 25×75-mm slide.

4.10 Water-sampling bottle, or nets. Depth-integrated samplers are discussed in Guy and Norman (1970) and in Wetzel and Likens (1979).

4.11 Whipple disc, placed in one ocular of the microscope.

5. Reagents

5.1 Cupric sulfate solution, saturated. Dissolve 21 g cupric sulfate ($CuSO_4$) in 100 mL distilled water.

5.2 Detergent solution, 20 percent. Dilute 20 mL liquid detergent, phosphate free, to 100 mL using distilled water.

5.3 Distilled or deionized water.

5.4 Formaldehyde cupric sulfate solution. Mix 1 L 40 percent aqueous formaldehyde containing 10 to 15 percent methyl alcohol with 1 mL cupric sulfate solution.

5.5 Lugol's solution plus acetic acid. Dissolve 10 g iodine (I_2) crystals and 20 g potassium iodide (KI) in 200 mL distilled water. Add 20 mL glacial acetic acid a few days prior to use; store in an amber glass bottle (Vollenweider, 1974).

6. Analysis

Phytoplankton samples need to be examined using two different magnifications: low power (80X to 200X) to ensure inclusion of large, usually rare, species; and high power (200X to 1,000X, using oil immersion, if possible) to facilitate identification and to ensure inclusion of ultraplankton. Phytoplankton in the entire slide mount often can be counted using low magnification, but random fields need to be selected at high magnification until a sufficient number of units (cells, filaments, chains, or colonies) have been enumerated for the desired level of precision. Use of a Whipple disc in one ocular will demarcate the microscopic field into a defined, easily viewed grid of 100 squares. When making the counts, enumerate only forms that lie completely inside the grid and those that intersect two of the outer grid borders. If a large number of colonies or filaments appear within the field, determine the average number of cells in an average-size colony or filament and multiply by the number of colonies or filaments present. Count only viable cells, those having protoplasm or pigments. Identify all forms to some predetermined taxonomic level (species is preferrable); count and describe unidentifiable cells.

The volume of original, unconcentrated sample to be examined will vary, depending on sediment content and density of phytoplankton; the volume commonly will range between 25 mL (for eutrophic water or water that has large suspended-sediment concentration) and 100 mL or more (for oligotrophic water). Net samples may not require further concentration.

6.1 If using the sedimentation apparatus (fig. 16), proceed to 6.5. If using the plankton counting cell, proceed as follows. If concentration is necessary, allow the sample to settle undisturbed in the sample container for 4 hours per centimeter of depth to be settled. After settling, weigh the sample container on an automatic balance.

6.2 Carefully siphon the supernatant to avoid disturbance of the settled material. Place sample container and remaining sample on the balance and weigh. The decrease in weight (in grams) is equivalent to the number of milliliters of supernatant removed. Use the same method to obtain the volume of concentrate.

6.3 Mix the concentrated sample well (but not vigorously) and pipet an appropriate volume into each of two plankton counting cells. Slide cover glass into place.

6.4 Place the plankton counting cell on the mechanical stage of a calibrated microscope. Proceed to 6.10.

6.5 To prepare the sedimentation apparatus, cement a no. 1 cover glass to the bottom of the lower slide to form the bottom of the counting cell (fig. 16). When dry, remove the excess rubber cement from the inside of the counting cell using a knife.

6.6 Test for leaks. Coat the underside of the upper slide (fig. 16) with vacuum grease and press onto the lower slide to form a watertight seal. Assemble the sedimentation apparatus and fill with distilled water so the meniscus bulges slightly above the top of the sedimentation tube. Slide the cap over the top to seal the tube. Let stand overnight and check for water loss in the morning.

6.7 If no leaks are detected, thoroughly mix a sample by inverting it at least 40 times, and then fill the sedimentation apparatus and apply the cap as described in 6.6 (Note 1). Allow 4 hours settling time per 1 cm of sedimentation-tube length. The volume of sample is dependent on the density of phytoplankton. In oligotrophic water, 100 mL or more of sample may be required; in eutrophic water, 25 mL or less of sample may be sufficient. The 25-mL volume is most commonly used. Dilute the samples if necessary.

Note 1: Air bubbles on the sides of the sedimentation tube can be prevented if the water sample and the sedimentation apparatus are at the same temperature when the sample is added. The apparatus needs to be maintained at a constant temperature to avoid convection currents, which can interfere with settling.

6.8 After settling, isolate the phytoplankton in the counting cell from the remainder of the sedimentation apparatus. To separate the sedimentation tube and upper slide from the lower slide and counting cell (fig. 16), move the sedimentation tube to one side, dividing the water column. Remove the tube cap and siphon or pipet off the supernatant. Remove the empty sedimentation tube.

6.9 Remove the lower slide that has the counting cell from the holder (fig. 16). Place the cap over the top of the counting cell to form a closed cell. If an air bubble remains under the cap, move it to one side of the cell and carefully add distilled water to fill the void. Replace the tube cap and put the slide on the inverted microscope.

6.10 Three basic procedures exist for microscopically enumerating and identifying concentrated phytoplankton samples. Although specific materials and methods vary between these procedures, the general counting procedure is identical.

7. Calculations

The following procedure will provide estimates of phytoplankton population density from tallied counts of phytoplankton cells from subsamples enumerated on microscopic slides or counting cells.

7.1 If the sample has been collected by net or if a bottle sample has been either diluted or concentrated by centrifugation-siphoning, calculate the concentration factor, $\underline{c}$ (volume of water represented by a volume of processed sample). The factor $\underline{f}$ corrects for the volume of preservative added:

$$\underline{f} = \frac{\text{Volume of water collected + volume of preservative added}}{\text{Volume of water collected}} ;$$

$$\text{Net sample } \underline{c} = \frac{\text{Volume of water passed through the net}}{\text{Volume of preserved sample}} \times \underline{f} \text{ ; and}$$

$$\text{Bottle sample } \underline{c} = \frac{\text{Volume of water collected}}{\text{Final volume of concentrated or diluted sample}} \times \underline{f} .$$

7.2 For ruled counting cells, calculate the area, $\underline{a}$ (square millimeters), represented by one microscopic field (or Whipple disc grid) using a stage micrometer. This needs to be done for each magnification used for enumeration. For example, if enumeration is done using a Whipple disc at 125X, $\underline{a} = 0.49$ mm^2.

7.3 For inverted-microscope counting cells that have a bottom plate that has a diameter of 25 mm, the area is $\underline{A} = 491$ mm^2.

7.4 Sum the total number of units, $\underline{T}$ (cells, colonies, or filaments × number of cells per colony or filament), tallied within $\underline{n}$ microscopic fields:

$$\underline{T} = \sum_{\underline{i}=1}^{\underline{n}} \underline{x}_{\underline{i}} ,$$

where $\underline{x}_{\underline{i}}$ is total number of units counted in field $\underline{i}$.

7.5 For unruled counting cells, calculate the total volume, $\underline{v}$ (milliliters), of the original sample represented by $\underline{n}$ microscopic fields:

$$\underline{v} = \underline{c} \times \underline{n} \times \underline{a}/\underline{A} \times \underline{V} ,$$

where $\underline{V}$ is the volume (milliliters), of preserved sample that was settled, filtered, or placed directly into a counting cell.

7.6 Calculate the population density, $\underline{D}$ (cells per milliliter), of phytoplankton in the original sample:

$$\underline{D} = \underline{T}/\underline{v}$$

8. Reporting of results

Report phytoplankton density to two significant figures.

9. Precision

See "Precision" subsection in the "Phytoplankton" section.

10. References cited

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Wetzel, R. G., and Likens, G. E., 1979, Limnological analyses: Philadelphia, W. B. Saunders, 357 p.

Permanent-Slide Method for Planktonic Diatoms
(B-1580-85)

Parameter and Code: Not applicable

This method enables preparation of permanent mounts using a minimum of time and equipment. Numerous alternative methods for clearing diatom frustules (cell walls) and mounting exist in the literature. Alternative methods for clearing include nitric acid digestion of tissue on the slide (Knudsen, 1966), sulfuric acid and potassium permanganate (Hasle and Fryxell, 1970), hydrochloric acid (HCl) (Cupp, 1943), and potassium permanganate and HCl (Hasle, 1978). Hydrogen peroxide and potassium permanganate (Von der Webb, 1953), hydrogen peroxide and ultraviolet light (Swift, 1967), and hydrogen peroxide after mild heating (Wong, 1975) also have been used for tissue digestion. The reader is referred to the original papers for the details of these procedures.

1. Applications

This qualitative method is suitable for all water. Advantages of the method are that a permanent mount is prepared, and clearing of the cells enhances observation of frustule detail. The method, therefore, is important in the taxonomic study of diatoms.

2. Summary of method

The diatoms in a sample are concentrated, the cells are cleared, and a permanent mount is prepared. The mount is examined microscopically, and the number of diatom taxa is calculated from strip counts.

3. Interferences

3.1 Inorganic particulate matter, including salt crystals, interferes with mount preparation but can be decreased by sample washing.

3.2 The method does not distinguish living from dead diatoms.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Balance, that has an automatic tare.

4.2 Centrifuge, either swing-out or fixed-head cup-type, 3,000 to 4,000 r/min, 15- to 50-mL conical or 100-mL pear-shaped centrifuge tubes, and simple siphoning or suction device to remove excess fluid after centrifugation.

4.3 Cover glasses 18×18 or 22×22 mm, no. 1½, and microscope slides, glass, 76×22 mm.

4.4 Forceps, curved tip.

4.5 Graduated cylinders, plastic, of sufficient capacity (100 and 500 mL, and 1 L are convenient sizes) for measuring known volumes of water samples.

4.6 Hotplate, thermostatically controlled to 538 °C. It is convenient to have a second hotplate for operation at about 93 to 121 °C as described in 6.8.

4.7 Microscope, conventional light microscope, or equivalent. Bright field condensor and objectives are required, and phase contrast is desirable, particularly for taxonomic examination. A series of objectives needs to be available (10X, 20X, and 40X), and 100X phase-contrast oil-immersion objectives need to be available for examination of smaller sized diatoms. The microscope needs to be equipped with a movable mechanical stage that has vernier scales.

4.8 Pipets, 1-mL or 10-mL capacity, sterile.

4.9 Sample containers, glass or graduated polyethylene bottles and screwcaps, 100 mL to 1 L.

4.10 Water-sampling bottle, or nets. Depth-integrated samplers are discussed in Guy and Norman (1970) and in Wetzel and Likens (1979).

4.11 Whipple disc, placed in one ocular of the microscope.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Cupric sulfate solution, saturated. Dissolve 21 g cupric sulfate ($CuSO_4$) in 100 mL distilled water.

5.2 Detergent solution, 20 percent. Dilute 20 mL liquid detergent, phosphate free, to 100 mL using distilled water.

5.3 Distilled or deionized water.

5.4 Formaldehyde cupric sulfate solution. Mix 1 L 40 percent aqueous formaldehyde containing 10 to 15 percent methyl alcohol with 1 mL cupric sulfate solution.

5.5 Immersion oil. Cargille's nondrying type A.

5.6 Lugol's solution plus acetic acid. Dissolve 10 g iodine (I_2) crystals and 20 g potassium iodide (KI) in 200 mL distilled water. Add 20 mL glacial acetic acid a few days prior to use; store in an amber glass bottle (Vollenweider, 1974).

5.7 Mounting medium (table 13). Generally, mounting media should have a refractive index different than that of diatom frustules. Diatom frustules have a refractive index of approximately 1.15 (Reid, 1978).

Table 13.--Synthetic mounting media in general use for permanent mount of planktonic diatoms

[Adapted from Reid, 1978; reproduced by permission of UNESCO; --, not available]

Media	Refractive index, n	Solvent	Other information
Aroclor	1.63	Xylene.	Good for diatoms.
Clearax	1.67	Xylene, acetone.	Good for diatoms.
Clearmount	1.51	Xylene, benzene, toluene, alcohol, dioxan, and other solvents.	Conserves stains.
Euparal	1.48	Xylene, alcohol.	Mixture of natural and synthetic resins; can be used immediately after 95-percent alcohol application; intensifies hematoxylin stains.
Hyrax	1.63	Xylene, benzene, toluene.	Expensive; good for diatoms (Hanna, 1930).
Naphrax	1.72	Xylene, toluene, acetone.	Good for diatoms (Fleming, 1943, 1954).
Permount	--	Toluene.	Conserves stains: does not yellow.
Pleurax	1.75	Alcohol.	Good for delicate diatoms. Procedure for mixing in Hanna (1949).

6. Analysis

6.1 If the sample contains great numbers of phytoplankton, as typically occurs in eutrophic water, dilute the sample. To dilute, thoroughly mix 50 mL sample with 50 mL distilled water (1:1 dilution) and proceed to 6.2. If microscopic examination reveals a concentration of phytoplankton still too numerous to count, thoroughly mix 50 mL 1:1 dilution with 50 mL distilled water (1:4 dilution). Make additional dilutions as appropriate.

6.2 If concentration is necessary, allow the sample to settle undisturbed in the sample container for 4 hours per centimeter of depth to be settled. After settling, weigh the sample container on an automatic tare balance.

6.3 Carefully siphon the supernatant to avoid disturbance of the settled material. Place sample container and remaining sample on balance and weigh. The decrease in weight (in grams) is equivalent to the number of milliliters of supernatant removed. Use the same method to obtain the volume of concentrate.

6.4 If the sample was collected from seawater or saline lakes, wash the sample, using distilled water, at least three times to ensure that the permanent mounts are not obscured by salt crystals. Add about 10 mL distilled water to the concentrate in the centrifuge tube, gently shake the tube to suspend the residue, fill the tube with distilled water, and centrifuge for 20 minutes. Decant the supernatant fluid and repeat the washing process two more times.

6.5 Place two or three drops of the concentrate on each of three or four cover glasses.

6.6 With the concentrate side up, place the cover glass on a hotplate and heat, slowly at first to prevent splattering, to about 538 °C (a higher temperature will melt diatom valves) for 30 minutes.

6.7 Remove cover glass from the hotplate and cool.

6.8 Place a drop of mounting medium (table 13) on a microscope slide and heat at about 93 to 121 °C for 3 to 4 minutes.

6.9 Invert the cover glass, concentrate side down, on the heated medium. Apply slight pressure to the cover glass (for example, with a pencil eraser) until visible air bubbles dissappear. Remove slide from hotplate and allow to cool. If bubbles still are present under the cover glass, heat the slide and gently apply additional pressure to the cover glass. Label slide to identify sample.

6.10 Examine the slide using the 1,000X objective (oil immersion). Count and identify diatom taxa found in several lateral strips the width of the Whipple disc. Identify and tabulate 200 to 300 diatom cells, if possible. Generally, at least 100 individuals of the most common species should be enumerated. Ignore frustule fragments. Thin-walled forms, such as

Rhizosolenia eriensis and Melosira crenulata, may be difficult to observe when using this method (Weber, 1966, p. 3). If a microscope that has a mechanical stage is used, recording of the x and y coordinates of lateral strips or individual cells enables investigators to later recheck and verify identification (Wong, 1975).

7. Calculations

$$\text{Percent occurrence of each species} = \frac{\text{Number of diatoms of a given species}}{\text{Total number of diatoms tabulated}} \times 100 .$$

8. Reporting of results

Report percentage composition of diatoms to the nearest whole number. Report taxa and number of diatoms per taxa.

9. Precision

No numerical precision data are available.

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ZOOPLANKTON

Introduction

The zooplankton are the animal part of the plankton. In general, they predominantly are composed of free-living, nonphotosynthetic protozoa, rotatoria, and crustacea. They are found in a variety of aquatic habitats, although usually they are absent or occur in small numbers in rapid streams. Zooplankton are important contributors to aquatic ecosystem metabolism because they are grazers of phytoplankton and bacteria and are important predators. Fish and certain invertebrate groups also use zooplankton as a food source. Zooplankton, therefore, can have a substantial effect on the structure and functioning of aquatic ecosystems.

Zooplankton characteristically have patchy distributions in aquatic ecosystems. They are rarely distributed randomly or uniformly. Additionally, vertical differences in zooplankton abundance on a daily and seasonal basis commonly are observed and are caused by the diurnal vertical migration of certain groups of zooplankton in response to changes in illumination. The fact that zooplankton are heterogeneous in their areal and vertical distribution must be considered in any investigation of the zooplankton. No single method of sampling can sample conclusively and accurately the entire zooplankton community.

Collection

There are several methods available for the collection of zooplankton. These methods are grouped into two categories based, in part, on the size of the zooplankton being collected. Zooplankton smaller than 200 µm are considered microzooplankton; this includes protozoa and small rotifers (Tranter and Fraser, 1968; Tonolli, 1971). They are readily collected by water-sampling bottles, water cores or tubes, and water pumps, followed by concentration of the sample onsite or in the laboratory. Collection also is facilitated by the use of plankton traps. Larger zooplankton, including the crustacea and larger rotifers, can be collected using various equipment that filter the zooplankton from the water through a net (Tonolli, 1971). These devices include unmetered tow nets (Wisconsin- or Birge-type), metered tow nets (Clarke-Bumpus sampler), and plankton traps (Schindler-Patalas trap).

There are several types of net mesh and sizes available for use in net sampling devices. The choice of mesh size and net design depends on the abundance of the zooplankton and the towing speed of the net. Nets of 202-µm mesh generally are used during U.S. Geological Survey studies. Smaller net sizes can be used for the purpose of collecting microzooplankton; however, clogging becomes an important factor using mesh sizes less than 65 µm (Steedman, 1976). Although the collector need not be restricted to the 202-µm mesh size, the mesh size used needs to be reported when presenting zooplankton results.

Detailed collection methods are discussed in Tranter and Fraser (1968), Schwoerbel (1970, p. 37-52), Edmondson and Winberg (1971, p. 1-20), Lind (1979, p. 100-115), and Wetzel and Likens (1979, p. 161-166). The study objectives need to be considered when selecting appropriate methods of collection. However, to ensure maximum correlation of results, the sample sites and methods used for zooplankton need to correspond as closely as possible to those selected for other biological, microbiological, and chemical sampling.

Water-sampling bottles can be used to collect a sample representative of the zooplankton density at a particular depth in ponds, lakes, reservoirs, estuaries, and deep rivers. This method is appropriate for collection when information on the vertical distribution of all zooplankton (including microzooplankton) is required. Water-sampling bottles that enable collection, cause minimal disturbance of water passage into the bottle, and minimize avoidance reactions by the zooplankton are desirable (Tonolli, 1971). Van Dorn-type water-sampling bottles, or equivalent, are an adequate collection device for zooplankton.

Depth-integrating samplers are used to collect a sample representative of the entire flow of a stream (Guy and Norman, 1970; Goerlitz and Brown, 1972). For small streams, a depth-integrated sample, or a point sample, at a single transverse position located at the centroid of flow may be adequate. Depth-integrating samplers are discussed in Guy and Norman (1970).

Following collection, the contents from the water-sampling bottle or depth-integrating samplers are poured through an appropriate monofilament screen cloth (202 μm could be used, but it will enable microzooplankton to pass through), which retains the zooplankton for identification and enumeration or for biomass determinations. The advantage of water-sampling-bottle collection is negated by filtering the zooplankton through an inappropriate screen cloth that damages them or through a mesh size that lets microzooplankton pass through (Tonolli, 1971).

A sampling tube or water core can be used when information about the vertical distribution of all zooplankton (including microzooplankton) is not required. One limitation of this method is that good swimmers can avoid capture. This collection device consists of a weighted thin-walled rubber or plastic tube, having a closing device for collection of a relatively large vertical column of water and its associated zooplankton.

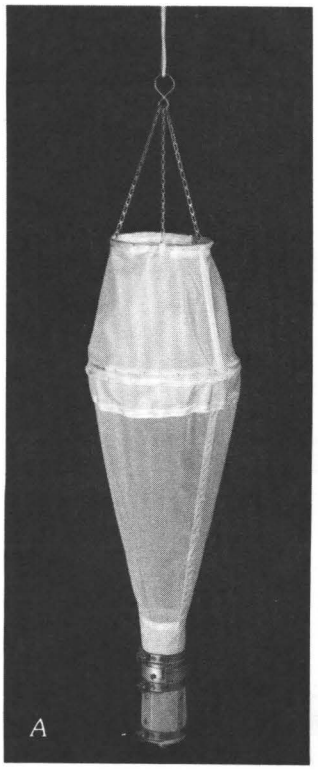
To collect a sample, the flexible tube is lowered to the desired depth. The sampling core is retrieved by pulling on a rope that is connected between two rings about 10 cm apart at the base of the tube. Pulling on the rope closes the tube. The advantage of this method is that the entire water column can be sampled using a relatively simple device (Tonolli, 1971, p. 4). Following collection, the contents are filtered through an appropriate mesh-size monofilament screen cloth (less than or equal to 202 μm), which retains the zooplankton for identification and enumeration or for biomass determination.

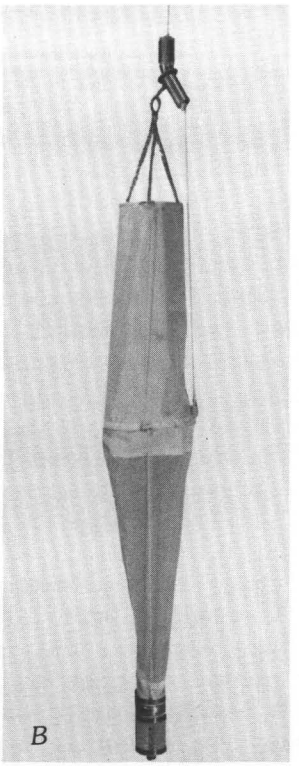
The water-pump method has the advantage of easily collecting large volumes of water from various depths. However, the problem of avoidance by larger zooplankton may be encountered (Tonolli, 1971). A hand pump or electric pump is attached to a relatively large diameter tube, which in turn is weighted at the bottom. The tube is lowered to a preselected depth and flushed with a volume of water three times the tube's volume to eliminate water that entered the tube during lowering. A known quantity of water then is pumped and filtered through an appropriate mesh-size monofilament screen cloth (less than or equal to 202 μm), which retains the zooplankton for identification and enumeration or for biomass determination.

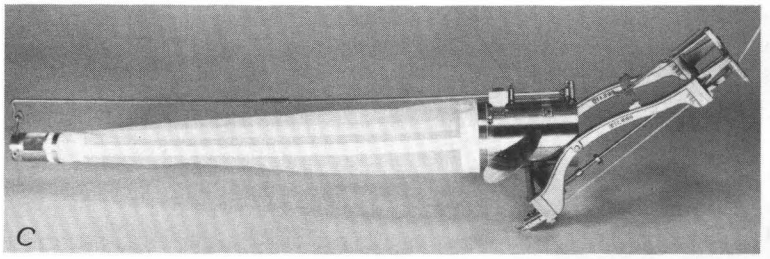
Unmetered plankton nets are useful in qualitative investigations of the zooplankton when complete quantitative data are not required. It is a fairly simple technique that permits relative comparisons of zooplankton communities (Tonolli, 1971). The entire water column is sampled easily by using plankton nets in vertical hauls. Wisconsin-type (open) (fig. 17A) and Birge-type (closed) (fig. 17B) plankton nets are examples of the nets suitable for this method. The zooplankton are collected by lowering the net to a known depth and raising it at a constant speed to the surface. Wisconsin-type plankton nets may become clogged and lose sampling efficiency during long retrieval. Birge-type plankton nets that can be closed at a preselected depth by dropping a messenger are advantageous for these conditions. In general, a large ratio of filtering surface to mouth-opening area decreases clogging. Therefore, long nets are more efficient than short nets. After retrieval, the filtering cone then is cleared of zooplankton by rapidly lowering and raising the net in the water, without submerging the net opening, and then bringing the net completely out of the water. Alternatively, the filtering cone of the plankton net can be cleared by repeated washing using water. These procedures concentrate the zooplankton in the removable plankton bucket, located at the bottom of the net. The zooplankton are washed from the plankton bucket into a sample container for identification and enumeration or filtered through an appropriate mesh-size monofilament screen cloth for biomass determination.

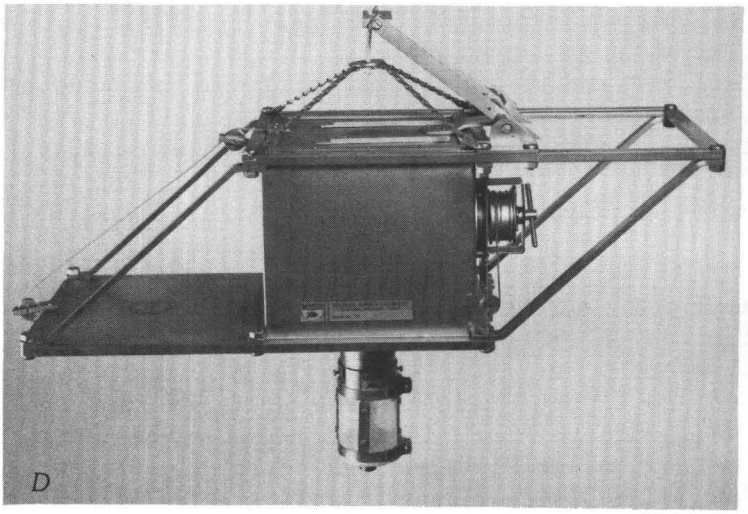
The volume of water (V) filtered through the Wisconsin- and Birge-type nets is calculated as $V = \pi r^2 d$, where r = radius of the mouth of the net and d = tow length through the water column (entire length of tow for the Wisconsin-type net and length of tow before closing for the Birge-type net). This assumes that the filtering efficiency of the net is 100 percent. The actual efficiency of the net generally will be less than 100 percent (Tonolli, 1971).

The Clarke-Bumpus plankton sampler is a metered tow net that enables quantitative sampling of the zooplankton in either horizontal or vertical tows (fig. 17C). This device consists of a net and flowmeter mounted on a horizontal frame. The net is opened and closed using a messenger. By knowing the initial and final reading on the counter of the flowmeter, the volume of water that has passed through the net can be determined (Schwoerbel, 1970, p. 45; Tonolli, 1971, p. 6-12). Thus, the Clarke-Bumpus plankton sampler has an advantage over the Wisconsin-type net or Birge-type net, because the exact volume of water passing through the net is known. However, clogging can become important when samples are collected from water that has dense zooplankton populations, because of the large volumes filtered by the Clarke-Bumpus plankton sampler (Tonolli, 1971; Wetzel and Likens, 1979).









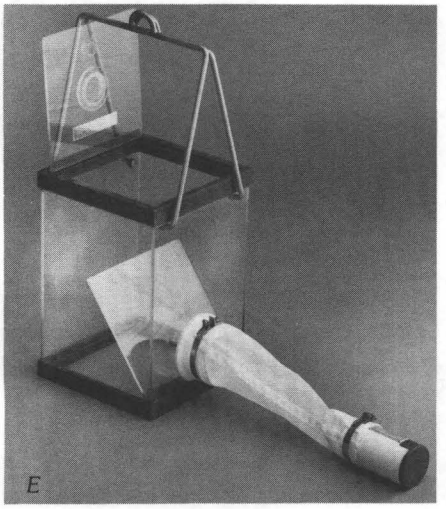


Figure 17.--Zooplankton collecting devices: (A) Wisconsin-type (open) plankton net; (B) Birge-type (closed) plankton net; (C) Clarke-Bumpus plankton sampler; (D) Juday plankton trap; (E) Schindler-Patalas plankton trap. (Photographs courtesy of Wildlife Supply Co., Saginaw, Mich.)

When collecting a sample, the initial reading of the flowmeter is recorded. The sampler is lowered to the selected depth, and the net is opened by dropping a messenger. After towing the sampler for a known interval of time or distance, the net is closed using another messenger, and the net is retrieved. The final reading on the flowmeter then is recorded. The net is washed, and the zooplankton are concentrated into the removable bucket. The zooplankton then are washed from the plankton bucket into a sample container for identification and enumeration or filtered through an appropriate mesh-size monofilament screen cloth for biomass determination.

For horizontal hauls, a moving boat is required. Also, a clinometer and cable depressor are necessary to ensure the haul is collected at a known depth. Further detailed discussion of the use of this device is presented by Tonolli (1971).

Plankton traps are used for point sample collection of the water column when information about the vertical distribution of the zooplankton is required. This method is suitable for capture of microzooplankton and larger zooplankton. There are two basic types of plankton traps, those requiring a messenger for closing [Juday trap, (fig. 17D)](Juday, 1916) and one that does not [Schindler-Patalas trap (fig. 17E)] (Schindler, 1969). The Juday trap is lowered to a predetermined depth and closed by a messenger. The trap then is retrieved, and the water drains through an attached plankton bucket, concentrating the zooplankton. The Schindler-Patalas trap, constructed using transparent Plexiglas, has two swinging lids that facilitate collection by lowering to a predetermined depth and then raising the trap to the water surface. A mesh-covered hole in the top lid enables the contents of the trap to be filtered through the attached net. The contents of the net are washed readily into the detachable plankton bucket (Schindler, 1969). Once the zooplankton have been concentrated in the plankton bucket of either the Juday trap or the Schindler-Patalas trap, the zooplankton are washed into a sample container for identification and enumeration or filtered through a 202-μm (or less, to include the microzooplankton) mesh-size monofilament screen for biomass determination. The advantages of the Schindler-Patalas trap are that it does not have a messenger activated tripping system, filtering occurs during raising, and it is less subject to the avoidance reactions by zooplankton encountered using water-bottle samplers, tow nets, and metal traps because it is transparent.

Samples collected for biomass determination on mesh-size monofilament screen cloth are handled as follows. Wash the screen cloth containing the zooplankton by dipping in distilled water several times, place in a plastic bag or other suitable sample container, and preserve onsite by freezing using dry ice. Keep frozen until gravimetric determinations can be made (Committee on Oceanography, Biological Methods Panel, 1969, p. 57). Additional information about sample preparation onsite prior to biomass determination is presented in Beers (1976, p. 74-76).

Samples collected for identification and enumeration are narcotized using an appropriate agent. A simple method is the addition of a commercial soda water (10-15 percent of total sample volume) to the sample, resulting in carbon dioxide excess. Narcotization prevents contraction and distortion of the

zooplankton when fixed using preservative that enables ready identification in the preserved state (Steedman, 1976). Following narcotization, preserve the samples using neutralized formaldehyde (approximately 2-4 percent of total sample volume) solution (5 percent formalin). Add several drops of glycerin (approximately 5 percent of total sample volume) to the sample to prevent drying during storage. If samples collected for biomass determination cannot be kept frozen, preserve using 2 percent neutralized formaldehyde solution, but use the selected sample-preservation method consistently throughout the study.

For identification and enumeration and for biomass determinations, label the sample to indicate the volume of water filtered or to indicate the information needed to determine the volume. For example, record the length of a vertical net haul and the diameter of the net opening. Also, the date and site location should be included, the order of collection when replicate sampling is used, and collection device and mesh size of any screen cloth used.

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Counting-Cell Method
(B-2501-85)

Parameter and Code:
Zooplankton, total (organisms/m^3): 70946

1. Applications

The method is suitable for all water.

2. Summary of method

Samples of the zooplankton community are collected, preserved, and examined microscopically for numbers and types of zooplankton per unit volume of water sampled.

3. Interferences

Suspended materials in the water and abundant algae may interfere with the collection and microscopic examination of zooplankton.

4. Apparatus

Methods and equipment for the collection of zooplankton and their examination for identification and enumeration are described briefly in this section and are described in more detail in Welch (1948), Tranter and Fraser (1968), Schwoerbel (1970), Edmondson and Winberg (1971), Steedman (1976), Lind (1979), Wetzel and Likens (1979), and American Public Health Association and others (1985). Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Beaker, 250-mL capacity, for use as a mixing vessel for zooplankton samples.

4.2 Clarke-Bumpus plankton sampler that has 202-μm mesh netting. An impeller at the net opening registers the volume of water filtered through the net. The Clarke-Bumpus plankton sampler is used most often for horizontal tows, but it also may be used for vertical tows (fig. 17B).

4.3 Counting cells. A petri dish, half, that has etched grid on the bottom, is a convenient open counting cell. The construction of large volume counting cells is discussed in Edmondson (1971, p. 131). Open counting cells are used for counting subsample aliquots larger than 1 mL. Closed counting cells are used for smaller subsamples. Sedgwick-Rafter counting cells, 50×20×1 mm and cover glass are used in counting small samples. Small organisms (less than 10 μm) are identified more easily and counted using thinner counting cells, such as the Palmer-Maloney cell or standard medical hemacytometer (Edmondson, 1971).

4.4 Graduated cylinders, plastic, of sufficient capacity (100 and 500 mL and 1 L are convenient sizes) for measuring known volumes of water samples.

4.5 Microscope, binocular, flat-field, zoom lens, and illuminator for the smaller zooplankton. For the larger zooplankton, a binocular wide-field dissecting microscope is adequate.

4.6 Nylon monofilament screen cloth, 202-μm mesh opening.

4.7 Piston or Hensen-Stempel pipet, 4-mm diameter or 5-mL capacity, for obtaining subsamples from zooplankton samples. A 1-mL Hensen-Stempel pipet is convenient for use with Sedgwick-Rafter counting cells.

4.8 Plankton nets, Wisconsin-type, open, or Birge-type, closing. The closing plankton nets have greater sampling flexibility in deep-water bodies because they can be closed at any selected depth (fig. 17A).

4.9 Plankton trap (Juday type), a 10-L closing box, attached plankton bucket that has 202-μm mesh openings and that has messenger closing (fig. 17C), or transparent Plexiglas type that does not require messenger closing [Schindler-Patalas type (fig. 17D)].

4.10 Sample containers, glass or plastic bottles, vials, or sealable plastic bags. However, bags are subject to leakage during prolonged storage.

4.11 Sampling tube or water core, a weighted thin-walled rubber or plastic tube that has a closing device for collecting a relatively large vertical column of water and its associated zooplankton (Edmondson and Winberg, 1971, p. 4).

4.12 Spatula, for stirring samples.

4.13 Water pump, and attached rubber or plastic hose. Water is pumped through a net having a mesh size of 202 μm to retain the zooplankton (Committee on Oceanography, Biological Methods Panel, 1969, p. 48).

4.14 Water-sampling bottle, Van-Dorn type. Depth-integrating samplers are described in Guy and Norman (1970).

4.15 Whipple disc, placed in one ocular of the microscope.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Detergent solution, 20 percent. Dilute 20 mL liquid detergent, phosphate free, to 100 mL using distilled water.

5.2 Distilled or deionized water.

5.3 Formaldehyde solution, 2 percent. Dilute 5 mL 37 to 40 percent aqueous formaldehyde solution (formalin) to 100 mL using distilled water (Note 1).

Note 1: Commercial formaldehyde solution is slightly acid and may be neutralized by maintaining a small deposit of sodium or calcium carbonate in the stock bottle.

5.4 Glycerin, used to prevent drying of stored zooplankton samples.

5.5 Narcotizing agent (soda water, Schweppes, Canada Dry, or equivalent).

6. Analysis

6.1 Empty the contents of the entire sample into a graduated cylinder and adjust the volume to some convenient value, such as 50, 100, or 200±5 mL, by adding preservative solution. Because of the difficulty in examining the zooplankton in formalin preservative, tap water also can be used.

6.2 Pour the suspension in the graduated cylinder into an appropriate size beaker. Stir the contents of the beaker irregularly using a spatula to produce a random distribution of the zooplankton in the beaker. Take a subsample from the beaker for counting.

6.3 Count the zooplankton as in 6.4 or 6.5. Use the taxonomic keys in Edmondson (1959), Needham and Needham (1962), and Pennak (1978) to identify the different taxa of zooplankton for qualitative analysis and for the calculations of percent species composition.

6.4 Closed counting-cell method--Sedgwick-Rafter method.

6.4.1 With the counting cell on a flat surface, place the cover glass across the cell. Take a subsample as described in 6.2 by removing a 1-mL aliquot using a Hensen-Stempel pipet and transfer the aliquot to the cell. As the cell fills, the cover glass often will rotate slowly and cover the inner part of the cell, but the cover glass must not float above the rim of the cell. Allow the cell to stand for 15 to 20 minutes so the contents will settle.

6.4.2 Carefully place the counting cell on the mechanical stage of a microscope calibrated using a Whipple disc. Count the entire contents of the cell at 100X magnification. Alternatively, count several horizontal transects where the percent of the total contents of the cell is determined by the use of the Whipple disc. Count at least two subsamples from the beaker using the cell. The Sedgwick-Rafter method is not suitable for some large zooplankton because they do not fit in the cell under a cover glass.

6.5 Open counting-cell method. In this method, the entire contents from the beaker are counted. Using the etched or painted guidelines on the bottom of the Sedgwick-Rafter counting cell, count the zooplankton in random sections to determine an average density. A binocular wide-field dissecting microscope is adequate to count the zooplankton. Take care not to disturb the placement of the zooplankton in the open cell when counting, or the counting process will have to be started again. Several drops of liquid detergent can be added

to the open-cell subsample to decrease surface tension and prevent floating of the zooplankton on the surface. The open counting-cell method enables easy access to the subsample contents to enable manipulation of individual zooplankton for easier identification or removal for closer examination using a binocular flatfield microscope.

6.6 If the sample is to be retained, proceed as follows: After counting of the sample has been completed, return all the sample to the beaker and allow to settle overnight. Remove enough of the supernatant liquid to enable the return of the sample contents to the original sample container. Add preservative to ensure the integrity of the sample.

7. Calculations

7.1 Sedgwick-Rafter method:

Total zooplankton per cubic meter

$$= \frac{(\text{Zooplankton per cell}) \times (\text{volume of sample, milliliters})}{\text{Volume of water sampled (liters)}}$$

$$\times \frac{1{,}000\ \text{L}}{\text{Cubic meters}} .$$

7.2 Open counting-cell method, section counts:

Total zooplankton per cubic meter

$$= \frac{\begin{array}{c}(\text{Average count per section}) \\ \times (\text{number of sections}) \\ \times [\text{total volume of concentrated sample (milliliters)}]\end{array}}{\begin{array}{c}[\text{Volume of counting cell (milliliters)} \\ \times (\text{volume of water sampled (liters)}]\end{array}}$$

$$\times \frac{1{,}000\ \text{L}}{\text{Cubic meters}} .$$

7.3 Percent taxon composition in sample

$$= \frac{\text{Number of zooplankton of a particular taxon}}{\text{Total number of zooplankton of all taxa}} \times 100 .$$

8. Reporting of results

Report zooplankton densities as total number of organisms per cubic meter to two significant figures.

9. Precision

No numerical precision data are available.

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Gravimetric Method for Biomass
(B-2520-85)

Parameters and Codes:
Zooplankton, dry weight (g/m^3): 70947
Zooplankton, ash weight (g/m^3): 70948

1. Applications

The method is suitable for all water.

2. Summary of method

Samples of the zooplankton community are collected from known volumes of water. The dry weight and ash weight are determined, and the weight of ash-free matter, an estimate of organic weight per unit volume of the water sampled, is calculated.

3. Interferences

Suspended materials in the water may interfere with sample collection. Inorganic matter in the sample will cause erroneously large dry and ash weights. Nonliving organic matter, as well as living plant and bacteria material, in the sample will cause erroneously large dry and ash-free weights.

4. Apparatus

Methods and equipment for the collection of zooplankton for biomass determination have been described in the "Collection" subsection of the "Zooplankton" section and are presented in more detail in Tranter and Fraser (1968), Schwoerbel (1970), Steedman (1976), Wetzel and Likens (1979), and American Public Health Association and others (1985). Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Balance, capable of weighing to at least 0.1 mg.

4.2 Beaker, 250-mL capacity, for use as a mixing vessel for zooplankton samples.

4.3 Clarke-Bumpus plankton sampler that has 202-µm mesh netting. An impeller at the net opening registers the volume of water filtered through the net. The Clarke-Bumpus plankton sampler is used most often for horizontal tows, but it also may be used for vertical tows (fig. 17B).

4.4 Desiccator, containing silica gel or anhydrous calcium sulfate.

4.5 Drying oven, thermostatically controlled for use at 105 °C.

4.6 Forceps, stainless steel, smooth tip, or tongs.

4.7 Graduated cylinders, plastic, of sufficient capacity (100 and 500 mL and 1 L are convenient sizes) for measuring known volumes of water samples.

4.8 Muffle furnace, for use at 500 °C.

4.9 Nylon monofilament screen cloth, 202-µm (or appropriate size for collecting microzooplankton) mesh opening.

4.10 Piston or Hensen-Stempel pipet, 4-mm diameter or 5-mL capacity, for obtaining subsamples from zooplankton samples.

4.11 Plankton nets, Wisconsin-type, open, or Birge-type, closing. The closing plankton nets have greater sampling flexibility in deep-water bodies because they can be closed at any selected depth (fig. 17A).

4.12 Plankton trap (Juday type), a 10-L closing box, attached plankton bucket (202-µm mesh openings or appropriate size for collecting microzooplankton), and messenger closing (fig. 17C), or transparent Plexiglas type that does not require messenger closing [Schindler-Patalas type (fig. 17D)].

4.13 Porcelain crucibles.

4.14 Sample containers, glass or plastic bottles, vials, or sealable plastic bags. However, bags are subject to leakage during prolonged storage.

4.15 Sampling tube or water core, a weighted thin-walled rubber or plastic tube that has a closing device for collecting a relatively large vertical column of water and its associated zooplankton (Edmondson and Winberg, 1971, p. 4).

4.16 Spatula, for stirring samples.

4.17 Water pump, and attached rubber or plastic hose. Water is pumped through a net that has a mesh size of 202 µm to retain the zooplankton (Committee on Oceanography, Biological Methods Panel, 1969, p. 48).

4.18 Water-sampling bottle, Van-Dorn type. Depth-integrating samplers are described in Guy and Norman (1970).

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Distilled or deionized water.

5.2 Dry ice, for freezing zooplankton samples onsite for transport back to the laboratory.

6. Analysis

Detailed information about various biomass-determination methods are presented by Beers (1976) and Ruttner-Kolisko (1977). Biomass determination by gravimetric methods is presented in the following paragraphs. Determinations need to be made on replicate samples when available or at least two subsamples if only one sample is available.

6.1 Place the zooplankton sample in a graduated cylinder, and if necessary, add distilled water to make up to a known volume. Pour the suspension into a beaker. Stir the contents using a spatula to ensure random distribution of the zooplankton.

6.2 Obtain the tare weight of a crucible that has been heated at 500 °C for 20 minutes and cooled to room temperature in a desiccator.

6.3 Place a known volume, using a large Hensen-Stempel pipet or equivalent, of the zooplankton suspension into the tared crucible and dry to a constant weight in an oven at a temperature no higher than 105 °C. Cool the crucibles containing dried zooplankton to room temperature in a desiccator before weighing. Weigh as rapidly as possible to decrease moisture uptake by the dry residue. Use these values to calculate dry weight.

6.4 Place the crucible containing the dried residue in a muffle furnace at 500 °C for 1 hour. Cool to room temperature.

6.5 Moisten the ash using distilled water and again ovendry at 105 °C to a constant weight as in 6.3. Use these weight values to calculate ash weight.

7. Calculations

7.1 Entire sample used:

$$\text{Dry weight of zooplankton (grams per cubic meter)} = \frac{\text{Dry weight of residue and crucible (grams) - tare weight of crucible (grams)}}{\text{Volume of water sample (liters)}} \times \frac{1{,}000\ \text{L}}{\text{Cubic meters}}.$$

7.2 If subsample used:

$$\text{Dry weight of zooplankton (grams per cubic meter)} = \frac{\left(\text{Dry weight of residue and crucible and subsample residue (grams) - tare weight of crucible (grams)}\right) \times \frac{\text{Volume of suspension (liters)}}{\text{Volume of subsample (liters)}}}{\text{Volume of water sample (liters)}} \times \frac{1{,}000\ \text{L}}{\text{Cubic meters}}.$$

7.3 Ash weight of zooplankton (grams per cubic meter)

$$= \frac{\text{Ash weight of residue and crucible (grams) - tare weight of crucible (grams)}}{\text{Volume of water sample (liters)}}$$

$$\times \frac{1{,}000\ \text{L}}{\text{Cubic meters}}$$

7.4 Ash-free, or organic weight, of zooplankton (grams per cubic meter) = dry weight of zooplankton (grams per cubic meter) - ash weight of zooplankton (grams per cubic meter).

8. Reporting of results

Report biomass of zooplankton to two significant figures.

9. Precision

No numerical precision data are available.

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SESTON (Total Suspended Matter)

Introduction

The weight of suspended matter in water (seston) is an important measurement in ecological studies. For example, this value has been shown to correlate with optical properties (Jerlov, 1968) and with temporal and spatial changes in aquatic environments (Maciolek and Tunzi, 1968; Moss, 1970; Reed and Reed, 1970). For some analyses, the sample may be prefiltered through a 150- to 350-μm mesh to eliminate large particles before filtration. The particulate residue remaining in the sample after sieving is designated microseston.

Collection

The sample-collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, seston abundance may vary transversely and with depth (Patten and others, 1966). To collect a sample of the seston at a particular depth, use a water-sampling bottle, Van-Dorn type (fig. 11). To collect a sample representative of the entire flow of a stream, use a depth-integrating sampler (Guy and Norman, 1970; Goerlitz and Brown, 1972). For small streams, a depth-integrated sample or a point sample at a single transverse position located at the centroid of flow may be adequate. Study design, collection, and sampling statistics for streams, rivers, and lakes are described in Federal Working Group on Pest Management (1974).

Seston samples need to be filtered immediately after collection. Record the mesh size of prefilter, if used. Record the volume of water filtered. The filters need to be thoroughly dried or stored in tightly closed plastic petri dishes at 1 to 4 °C (do not freeze) until ovendried. Samples that cannot be filtered without delay need to be preserved using 40 mg mercury per liter. Preservation will stabilize the seston content of samples for at least 8 days. However, the results of analyses of preserved samples are not necessarily the same as those obtained by immediate filtration.

The method described in this chapter is the glass-fiber filter adaptation by Strickland and Parsons (1968) of the method developed by Banse and others (1963).

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Glass-Fiber Filter Method
(B-3401-85)

Parameters and Codes:
Seston, dry weight (mg/L): 71100
Seston, ash weight (mg/L): 71101

1. Applications

The method is suitable for all water.

2. Summary of method

A known volume of water is prefiltered through a tared glass-fiber filter to remove the particulate matter. The increase in weight of the filter after drying at 105 °C is a measure of the dry weight of particulate matter in the sample. After ashing the residue at 500 °C, the difference between dry weight and ash weight is the weight of particulate organic matter in the sample.

3. Interferences

Although the method generally is free from interferences, bottles and sampling equipment need to be clean, and samples, filters, and funnels need to be protected from dust. Filtration needs to be at decreased pressure to avoid rupture and loss of cell contents of fragile organisms. Saline samples need to have the salts washed from the filter residues to prevent erroneous weight values.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Aluminum foil, laboratory grade.

4.2 Balance, capable of weighing to at least 0.1 mg.

4.3 Desiccator, containing silica gel or anhydrous calcium sulfate.

4.4 Drying oven, thermostatically controlled for use at 105 °C.

4.5 Filter flask, 1 L or 2 L. For onsite use, a polypropylene flask is appropriate.

4.6 Filter funnel, vacuum, 1.2-L capacity, stainless steel.

4.7 Forceps, stainless steel, smooth tip.

4.8 Glass filters, 47-mm diameter disks. For best results, all filters for a series of samples, including control filters, need to be from the same box and need to have a tare weight of 70- to 100-mg (±10 mg) weights.

4.9 Graduated cylinders, plastic, of sufficient capacity (100 and 500 mL and 1 L are convenient sizes) for measuring known volumes of water samples.

4.10 Manostat that contains mercury and calibration equipment to regulate the filtration suction at not more than 300 to 350 mm of mercury when filtering using an aspirator or an electric vacuum pump.

4.11 Muffle furnace, for use at 500 °C.

4.12 Plastic petri dishes and covers for filter storage.

4.13 Sample containers, plastic bottles, 1- to 5-L capacity.

4.14 Vacuum pump, water-aspirator pump or an electric vacuum pump for laboratory use; a hand-operated vacuum pump and gauge for onsite use.

4.15 Water-sampling bottle, Van-Dorn type. Depth-integrating samplers are described in Guy and Norman (1970).

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Distilled or deionized water. Filter if in doubt about whether water is particle free.

5.2 Mercuric chloride solution, 1 mL = 40 mg mercury (Hg^{2+}). Dissolve 55 g mercuric chloride ($HgCl_2$) in distilled water and dilute to 1 L.

6. Analysis

6.1 Arrange the required number of glass filters (do not overlap) on the shiny side of aluminum foil and heat in a muffle furnace at 450 to 500 °C for 30 minutes. Do not allow the temperature to exceed 500 °C. This preparation hardens the filters and removes any organic matter. About 20 filters is a convenient number with which to work.

6.2 Use at least 10 percent of the filters as controls. For large batches, use every 10th filter as a control; for small batches, use a filter at the beginning and one at the end as controls. The treatment of control filters is identical to that of the test filters except that no water is filtered through them.

6.3 Cool and transfer all filters, including the controls, to a shallow container of distilled water for 5 minutes. Use about 100 mL water for each filter. Handle the filters very carefully using clean, smooth-tip forceps to avoid fraying the filters.

6.4 Using the forceps, transfer the filters to the shiny side of the aluminum foil after gently shaking off excess water. Dry the filters in an oven at 105 °C for 30 minutes. Cool to room temperature in a desiccator (Note 1).

Note 1: Because of the difficulty of marking glass filters, the individual filters should be accounted for throughout the remaining steps. The filters should be placed on the aluminum foil in a definite sequence and, whenever possible, each filter should be kept in a numbered plastic petri dish.

6.5 Weigh each filter to the nearest 0.1 mg as rapidly as possible, and record this initial (tare) weight value. Close the desiccator tightly after each removal. Store the tared filters in numbered plastic petri dishes until needed.

6.6 When a sample is to be filtered, place a tared filter, wrinkled surface upward, on a filter funnel. A small slip of aluminum foil under the edge of the filter facilitates removal of the wet filters.

6.7 When vacuum is applied, wet the filter using distilled water to seat the disk on the filter funnel.

6.8 Measure out a suitable quantity of thoroughly mixed sample into a graduated cylinder. Complete mixing of the sample is essential prior to measuring. Pour the sample into the filter funnel and filter using a manostat or other suitable method to keep vacuum to 300 to 350 mm mercury (about 6 psi).

6.9 Maintaining vacuum, wash the funnel and filter three times using 5- to 10-mL volumes of distilled water, allowing the filter to suck "dry" between each wash.

6.10 Disconnect the vacuum and, using smooth-tip forceps, place the wet filter on the shiny side of aluminum foil. Store the filters at 1 to 4 °C in numbered petri dishes at this stage, if necessary.

6.11 Dry the filters in an oven at 105 °C for 1 hour. Include at least two control filters from 6.5 in this drying step for each batch of sample filters.

6.12 Place the filters in a desiccator, cool, and reweigh each filter rapidly to the nearest 0.1 mg as in 6.5. Include the control filters from 6.11. Use these values to calculate dry weight.

6.13 Again place the filters that have dried residue and the control filters on the shiny side of aluminum foil and heat in a muffle furnace at 500 °C to constant weight. Heat at least 30 minutes, but some samples may require longer times. Cool and rewet the filters using distilled water to restore the water of hydration of clays and other minerals that may have been lost.

6.14 Place the filters in a desiccator and reweigh each filter rapidly to the nearest 0.1 mg as in 6.5. Include the control filters from 6.13. These values are used to calculate ash weight.

7. Calculations

7.1 Dry weight of seston (milligrams per liter)

$$= \frac{\text{Dry weight of filter and residue (milligrams)} - \text{tare weight of filter (milligrams)}}{\text{Volume of water sample (liters)} - \text{blank correction (milligrams)},}$$

where blank correction (milligrams) = mean weight of control filters, in milligrams (from 6.12) - mean weight of control filters, in milligrams (from 6.5). The blank correction value may be positive or negative but should not exceed about 0.5 mg.

7.2 Ash weight of seston (milligrams per liter)

$$= \frac{\text{Ash weight of filter and residue (milligrams)} - \text{tare weight of filter (milligrams)}}{\text{Volume of water sample (liters)} - \text{blank correction (milligrams)},}$$

where blank correction (milligrams) = mean weight of control filters, in milligrams (from 6.14) - mean weight of control filters, in milligrams (from 6.5). The blank correction value may be positive or negative but should not exceed about 0.5 mg.

7.3 Ash-free or organic weight of seston (milligrams per liter) = dry weight of seston (milligrams per liter) - ash weight of seston (milligrams per liter).

8. Reporting of results

Report seston as follows: less than 1 mg/L, one significant figure; 1 mg/L or greater, two significant figures.

9. Precision

No numerical precision data are available.

10. References cited

Guy, H. P., and Norman, V. W., 1970, Field methods for measurement of fluvial sediment: U.S. Geological Survey Techniques of Water-Resources Investigations, bk. 3, chap. C2, 59 p.

PERIPHYTON

Introduction

Periphyton literally refers to aquatic plants growing around (on) solid surfaces. European investigators originated the term about 1924 to describe organisms growing on artificial substrates in water (Cooke, 1956). Recently, the term "periphyton" has been extended to include the entire community of micro-organisms that live attached to or on solid submerged surfaces, generally above the depth of light extinction (Young, 1945; Sladecek and Sladeckova, 1964; Wetzel, 1964). The term encompasses not only algae but associated bacteria, fungi, protozoans, rotifers, and other small organisms. Although some of the latter are more accurately benthos, they are invariably sampled as part of the community by most methods. Thus, the methods of periphyton estimation, which follow, include both autotrophic and heterotrophic components of the periphyton unless otherwise stated. Periphyton is synonymous with the term "Aufwuchs," as described by Ruttner (1963): "* * * all those organisms that are firmly attached to a substratum but do not penetrate into it." The complexity of the periphyton community has spawned an equally complex terminology based on substrate classification, and the reader is referred to Weitzel (1979) for a more complete account.

Collection

Most analyses of the periphyton community have been adopted from long-established methods of phytoplankton analyses. The attached benthic nature of periphyton, however, presents special collection problems that directly affect the success of various estimates. In fact, problems related to sampling are the principal sources of error in most methods. Major sampling problems include adherence of the periphyton to mineral substrates and the patchiness of their distribution, particularly in lotic systems. Gravel substrates, even those which seem smooth and uniform, actually have a complex and irregular texture. Methods have been developed for collecting periphyton from natural substrates (Douglas, 1958; Ertl, 1971; Stockner and Armstrong, 1971), which usually are restricted to taxonomic studies or community-structure analysis. However, biomass and production estimates are derived more commonly from artificial substrates (Nielson, 1953; Grzenda and Brehmer, 1960; Maciolek and Kennedy, 1964; Neal and others, 1967; Peters and others, 1968; Tilley and Haushild, 1975a, b; Busch, 1978; Clark and others, 1979; Hoffman and Horne, 1980). The decision to use natural or artificial substrates should be considered carefully based on the study objectives developed prior to beginning onsite investigations.

Careful sampling of natural substrates is likely to yield more complete information on species composition because irregularities of the microhabitat will be incorporated into the sample. Inability to remove tissue efficiently from natural substrates, however, may produce a large underestimate of biomass. Artificial substrates enable more efficient collecting of a large number of samples and partially overcome the problem of adherence. Lack of microhabitat diversity, however, may affect patterns of colonization and biomass accumulation. Artificial substrates standardize the physical environment in studies where surface uniformity is an important consideration.

Once the decision about substrate type has been made, the inherent patchiness of periphyton distribution still needs to be considered. Because periphyton colonization is affected by numerous variables (light, depth, current velocity, and substrate texture), variability on natural and artificial substrates generally is large. Tilley and Haushild (1975a,b) reported that 21 glass microscope slides exposed for 2 weeks at a single site in the Duwamish River, Wash., had chlorophyll concentrations ranging from 1.33 to 2.81 mg/m^2 and a mean of 1.97 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 0.74 mg/m^2. Twenty-two slides exposed for 3 weeks at a single site in the Duwamish River had chlorophyll concentrations ranging from 1.89 to 4.86 mg/m^2 and a mean of 3.44 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 1.44 mg/m^2. Similarly, Pryfogle and Lowe (1979) reported differences in periphyton cell counts as large as an order of magnitude between adjacent stones in Tymochtee Creek, Ohio.

Effort always should be made to minimize possible variance by sampling habitats that are representative of the site and needs to include depth, current velocity, and canopy cover. If specific habitats are selected for comparative studies (pools, riffles), care should be taken to duplicate this habitat type at all sites, and the habitat type should be reported as well as the results. Unless care is taken to standardize the habitat, the results will indicate differences in substrate placement and collection, rather than differences in water quality.

Sufficient colonization time is another important consideration, especially for studies assessing species composition, because incubated substrates may undergo algal succession (Busch, 1978). If there is sufficient colonization time, species composition on artificial substrates generally is similar to the natural community (Patrick and others, 1954; Castenholz, 1960; Weitzel and others, 1979; Hoffman and Horne, 1980), but large differences in biomass or chlorophyll concentrations may be measured (Grzenda and Brehmer, 1960; Castenholz, 1961; Sladeckova, 1962; Pieczynska and Spodniewska, 1963; Weitzel, 1979). Proper colonization time will depend on season, water temperature, light, and nutrient availability, and other factors. Neal and others (1967) reported that maximum accumulation of periphyton biomass on polyethylene strips occurred in about 2 weeks. Patrick and others (1954) reported a 2-week colonization period also maximized the number of species. For most circumstances, colonization period should be at least 14 days, but this will vary and must be determined for each season and water type.

Other mechanisms for overcoming the problems of patchiness are to increase the number of samples or to have larger composite samples representing a diversity of habitats at a single site. Vandalism is a common problem, so substrates need to be placed away from frequently visited areas.

Sampling from Natural Substrates

Natural submerged substrates commonly contain periphyton, and a known area can be sampled quantitatively. If the area is unknown, periphyton scraped from natural substrates may be used for species identification and for determination of relative abundance. Several devices for removing periphyton from a known area of natural substrates are shown in figure 18. The instrument used by Douglas (1958) consists of a broad-necked polyethylene bottle that has the bottom removed (fig. 18A). The neck of the bottle is held tightly against the surface to be sampled, and the periphyton inside the enclosed area is dislodged from the substrate using a stiff nylon brush. The loose periphyton is removed from the bottle using a pipet. Ertl's (1971) device consists of two concentric metal or plastic cylinders separated by spacers (fig. 18B). The space between the cylinders is filled with modeling clay, and the sampler is pressed firmly against the substrate to be sampled. Using a blunt stick or metal rod, the clay is forced down onto the substrate to isolate the sampling area of the inner circle. The periphyton within the inner circle is dislodged using a stiff brush and removed using a pipet. Stockner and Armstrong (1971) sampled periphyton using a plastic hypodermic syringe that has a toothbrush attached to the end of the syringe piston (fig. 18C). The barrel of the syringe is held tightly against the substrate, and the piston is pushed in until the brush contacts the periphyton. The piston then is rotated several times to dislodge the periphyton and then is withdrawn pulling the periphyton up with it. A glass plate is placed immediately under the end of the barrel and the syringe inverted. Four small holes at the base of the syringe enable free movement of water when procuring the sample.

Sampling from Artificial Substrates

Suitable artificial substrates are attached to supports and placed in a stream or lake (figs. 19, 20). The substrates must be submerged but may be near the surface or at any appropriate depth. In lakes, substrates commonly are suspended at several depths (fig. 19A, B, C) to provide a more realistic representation of the periphyton community. Substrates should be oriented similarly at all sites because settling of organic and inorganic detritus may increase depending on the orientation of the substrate (Castenholz, 1960; Liaw and MacCrimmon, 1978). Vertical orientation is preferred because it decreases the settling problem. In lakes and streams, substrates may be attached to natural objects, such as submerged trees, stumps (fig. 19D), logs or boulders, or they may be attached to stakes driven into the bottom (fig. 20A). Floating samplers also may be used (fig. 20B), but care should be taken to allow for overestimation when water levels vary. The sampler should be secured so it will not drift into any obstruction or become beached. In extremely shallow streams, it may be necessary to construct a weir to guarantee sufficient water to float the sampler. If such a weir is constructed, data from the sample should be compared only with data obtained from comparably placed samplers. A floating sampler should not be used for any area in which there is intermittent flow for any period during the exposure time.

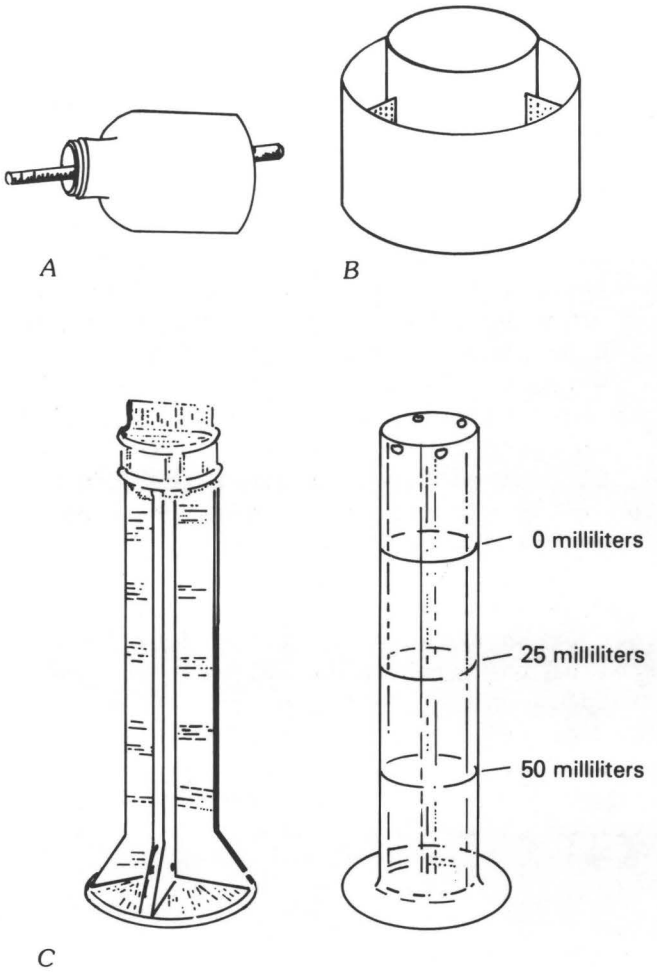


Figure 18.--Devices for collecting periphyton from natural substrates: (A) Brush and polyethylene-bottle device (modified from Douglas, 1958, p. 297; reproduced by permission of Duke University Press, Durham, N.C.). (B) Plastic or metal cylinder device (redrawn from Ertl, 1971, p. 576). (C) Plastic hypodermic syringe device (modified from Stockner and Armstrong, 1971, p. 218).

The artificial substrates should be placed in lighting conditions that typify the streams, rivers, or lakes being studied. For example, if most of the stream is shaded, an area that receives a great deal of sunlight should not be selected as being representative. In general, substrates collected from similar lighting conditions should be compared; but, depending on the study objective, this is not a requirement.

To ensure a continuous period of uniform colonization time of the artificial substrate, the substrate should be examined, periodically if possible, for any evidence of fouling or mechanical damage. If the substrate has been fouled or beached, the data for that sampling period should not be compared with data from any other substrate that has free, continuous, and uninterrupted exposure to the aquatic environment.

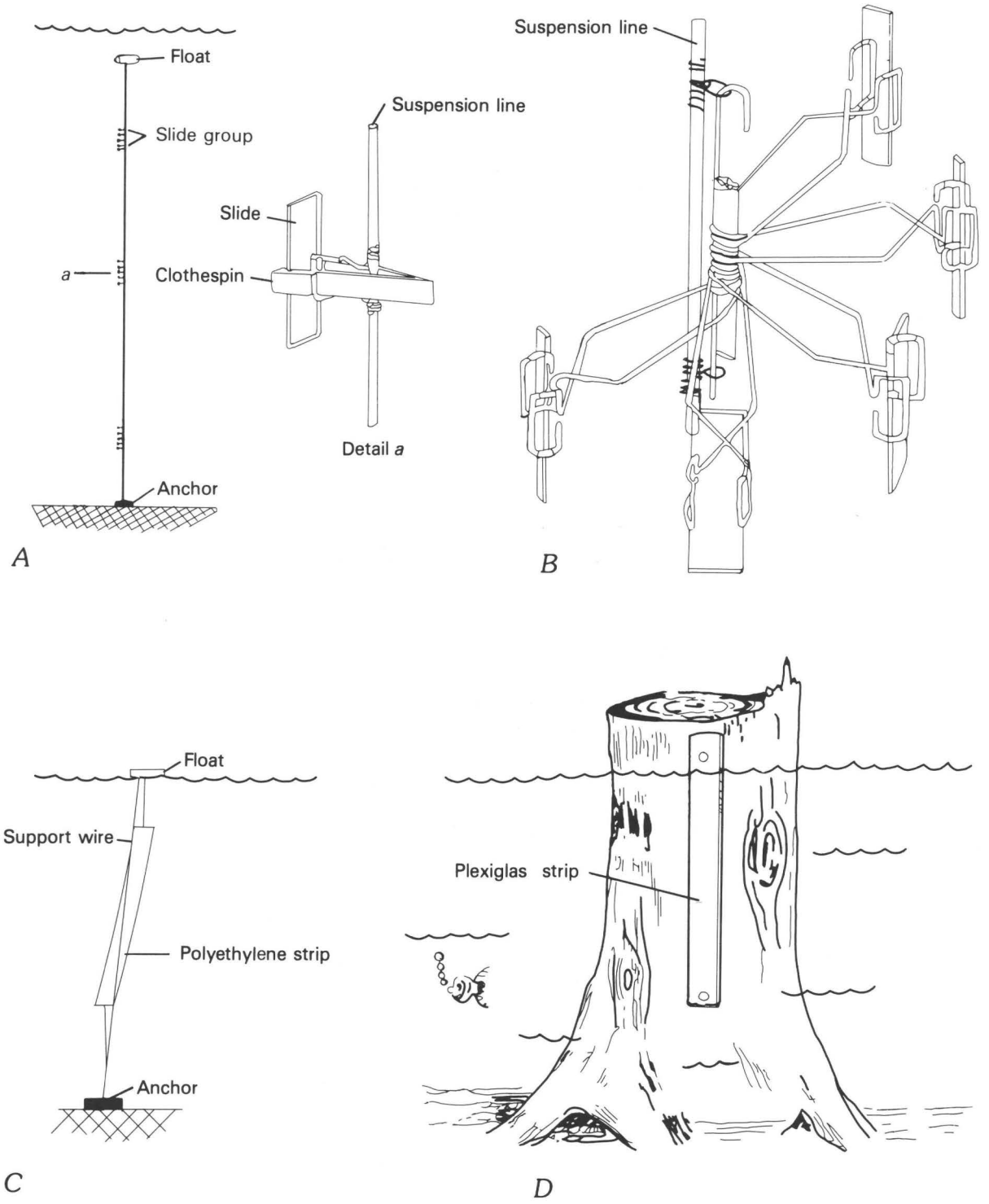
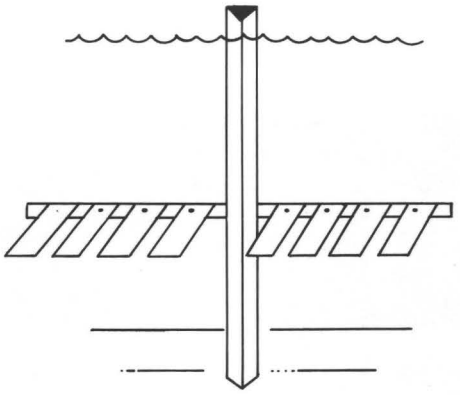
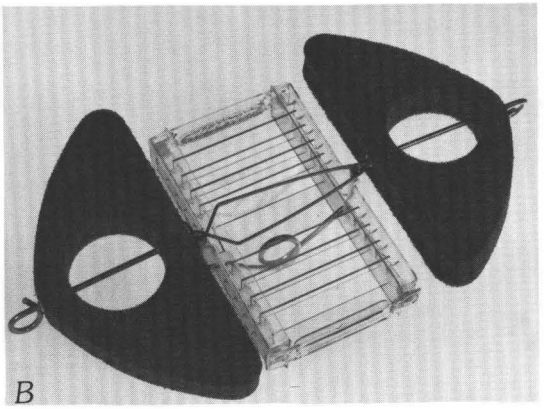


Figure 19.--Artificial-substrate sampling devices for periphyton: (A) Microscope slide-suspension device made from spring clothespins (from Nielson, 1953, p. 99). (B) Microscope slide-suspension device made from test-tube clamps (from Maciolek and Kennedy, 1964). (C) Polyethylene strip device. (D) Plexiglas strip attached to submerged object.



A



B

Figure 20.--Artificial-substrate sampling devices for periphyton: (A) Plexiglas plates attached to support (from Peters and others, 1968, p. 12). (B) Floating sampler, Periphytometer (photograph courtesy of Design Alliance, Inc., Cincinnati, Ohio).

The length of time required for colonization of the substrates by periphyton will depend on other environmental factors as well as water quality. Colonization times will vary and must be determined for each season and water type. The colonization period should be sufficiently long to enable the development of a microbial community large enough for measurement and, at the same time, avoid so much growth that sloughing would occur. Test samplers can be placed prior to the actual monitoring period to determine the most desirable colonization time for the prevailing (that is, seasonal and environmental) conditions. Suggested colonization periods for fresh to brackish water, mesotrophic to eutrophic, within the thermal range of 15 to 35 °C, is 14 days. Colonization periods during low productivity (that is, lack of nutrients or low temperature) or very high productivity may, by experience, be adjusted for the onsite conditions. Colonization periods should be identical for all sites in the entire study area.

After sufficient colonization of periphyton, indicated by visible green or brown growth, remove artificial substrates from the water. Periphyton may be scraped from the substrate onsite or in the laboratory, using razor blades, glass slides, or stiff brushes.

If the sample is to be examined within 2 or 3 hours after collection, no special treatment is necessary. A periphyton sample may be maintained at 3 to 4 °C for 24 hours, but for extended storage prior to identification and enumeration, preserve as follows: To each 100 mL of water and sample, add about 3 mL 40-percent formaldehyde solution (100 percent formalin), 0.5 mL 20-percent detergent solution, and 5 to 6 drops cupric sulfate ($CuSO_4$) solution (21 g $CuSO_4$ in 100 mL distilled water). This preservative maintains cell coloration and is effective indefinitely.

Many biologists consider Lugol's solution plus acetic acid to be the best algal preservative. The solution is prepared by dissolving 10 g iodine crystals and 20 g potassium iodide in 200 mL distilled water. Add 20 mL glacial acetic acid a few days prior to use (Vollenweider, 1974). Store in an amber glass bottle. Lugol's solution is effective for at least 1 year (Weber, 1968); it facilitates sedimentation of cells and maintains fragile cell structures, such as flagella. If Lugol's solution is used as the preservative, add 1 mL of solution to each 100 mL of water that has been added to the scraped periphyton sample. Store preserved samples in the dark, preferably in amber glass bottles.

For periphyton biomass determinations, freeze the sample if ovendrying cannot be started immediately. Storage should not exceed 2 weeks.

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Sedgwick-Rafter Method
(B-3501-85)

Parameter and Code:
Periphyton, total (cells/mm^2): 70945

1. Applications

The method quantifies the plant (autotrophic) part of the periphyton. It is suitable for all water.

2. Summary of method

Samples of the periphyton community are collected, preserved, and examined microscopically for types and numbers of algae. The periphyton samples may be from natural or artificial substrates, but the dimensions of the sample area must be known.

3. Interferences

3.1 Suspended or deposited sediment may interfere with collection procedures and with microscopic examination.

3.2 Strong adherence of periphyton to natural and artificial substrates may result in an underestimate of cell numbers per unit area.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Artificial substrates, glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.2 Collecting devices for the removal of periphyton from natural substrates. Three devices for collecting a sample of periphyton from natural substrates are shown in figure 18.

4.3 Microscope, conventional light microscope, or equivalent. Bright field condensor and objectives are required, and phase contrast is desirable, particularly for taxonomic examination. A series of objectives needs to be available (10X, 20X, and 40X), and 100X oil-immersion phase-contrast objectives need to be available for examination of ultraplankton. The microscope needs to be equipped with a movable mechanical stage that has vernier scales.

4.4 Pipet, transfer, 1 mL, large bore.

4.5 Sample containers, glass or plastic, suitable for the types and sizes of samples. Sturdy plastic bags are useful containers for artificial substrates or for pieces of natural substrate.

4.6 Scraping devices, razor blades, stiff brushes, spatulas, or glass slides are useful for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.7 Sedgwick-Rafter counting cell, 50×20×1 mm, and cover glass.

4.8 Whipple disc, placed in one ocular of the microscope.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Cupric sulfate solution, saturated. Dissolve 21 g cupric sulfate ($CuSO_4$) in 100 mL distilled water.

5.2 Detergent solution, 20 percent. Dilute 20 mL liquid detergent, phosphate free, to 100 mL using distilled water.

5.3 Distilled or deionized water.

5.4 Formaldehyde cupric sulfate solution. Mix 1 L 40-percent aqueous formaldehyde containing 10 to 15 percent methyl alcohol with 1 mL of cupric sulfate solution.

5.5 Lugol's solution plus acetic acid. Dissolve 10 g iodine (I_2) crystals and 20 g potassium iodide (KI) in 200 mL distilled water. Add 20 mL glacial acetic acid a few days prior to use; store in an amber glass bottle (Vollenweider, 1974).

6. Analysis

6.1 Remove periphyton from selected substrates for a representative sample. Document the type of habitat sampled.

6.2 Adjust the scraped periphyton sample to some convenient volume of suspension, such as 50 or 100±5 mL by adding preservative solution. If used to compare community composition between bodies of water or stream reaches, habitat type and substrate should be as identical as possible.

6.3 Place the Sedgwick-Rafter counting cell on a flat surface, and place the cover glass diagonally across the cell. Thoroughly mix the sample, remove a 1-mL aliquot using a large-bore pipet, and transfer the aliquot to the Sedgwick-Rafter counting cell. As the counting cell fills, the cover glass often rotates slowly and covers the inner part of the cell, but the cover glass must not float above the rim of the cell. Allow the counting cell to stand for 15 to 20 minutes until organisms settle.

6.4 Carefully place the Sedgwick-Rafter counting cell on the mechanical stage of a calibrated microscope. Because the method assumes a homogeneous distribution of periphyton, check quickly using low power for obviously uneven

distributions. If distribution appears reasonably uniform at 200X magnification, count the total number of algal cells enclosed by the Whipple disc. Consider any cell in the grid or touching two intersecting borders of the grid as being enclosed by the grid, but do not count those cells touching the opposite borders. Count and record the total number of cells in each of 20 random fields. When a 10X eyepiece and 20X objective are used, assume the total of the Whipple grid to be 0.5 mm on a side.

6.5 Some periphyton, particularly some blue-green algae, may not settle but, instead, may rise to the surface at the underside of the cover glass. When counting random fields, therefore, enumerate and record the total number of cells in the vertical column within the grid of the Whipple disc. Tabulate the number and lengths of trichomes of blue-green algae in each grid and determine the average number of cells per unit length of trichome. Consider empty diatom frustules as nonliving and do not include in calculations. Count frustules containing any part of a protoplast as having been living at the time of collection.

7. Calculations

7.1 Calibration factor = $$\frac{1{,}000 \text{ mm}^2}{\text{Area of Whipple grid at 200X magnification (square millimeters)}}$$.

7.2 Periphyton cells per milliliter of suspended scraping

$$= \frac{\text{Total cell count} \times \text{calibration factor}}{\text{Number of random fields x 1 mL}}$$.

7.3 Total periphyton cells per square millimeter of surface

$$= \frac{\text{Cells per milliliter of suspended scraping} \times \text{total volume of scrapings (millilters)}}{\text{Area of scraped surface (square millimeters)}}$$.

8. Reporting of results

Report periphyton density to two significant figures.

9. Precision

No numerical precision data are available.

10. References cited

Tilley, L. J., 1972, A method for rapid and reliable scraping of periphyton slides, in Geological Survey Research 1972: U.S. Geological Survey Professional Paper 800-D, p. D221-D222.

Vollenweider, R. A., ed., 1974, A manual on methods for measuring primary production in aquatic environments (2d ed.): Oxford and Edinburgh, Blackwell Scientific Publications, International Biological Programme Handbook 12, 225 p.

Additional Reading

McAlice, B. J., 1971, Phytoplankton sampling with the Sedgwick-Rafter cell: Limnology and Oceanography, v. 16, no. 1, p. 19-28.

Gravimetric Method for Biomass
(B-3520-85)

Parameters and Codes:
Periphyton, biomass, dry weight, total (g/m^2): 00573
Periphyton, biomass, ash weight (g/m^2): 00572

Gravimetric measurements are instantaneous; that is, they measure biomass at a moment in time in a community that is constantly changing. Because of large variability in biomass within a site, and because of control of periphyton growth by numerous physical (light, current velocity, storm frequency), chemical (nutrient regime), and biological (grazing) factors, comparisons between sites are impossible using casual sampling. To be used successfully, the gravimetric method should be employed with a specific objective in mind. To make comparisons between sites, samples should be collected from environments as nearly identical as possible. Application, as a mechanism to approximate the rate of biomass accumulation (net periphyton community productivity), is more valuable than a single estimate of biomass. The latter determination generally is done by incubating clean natural or artificial substrates in as nearly identical conditions as possible, and sampling on several dates for 2 to 4 weeks, or by incubating fresh substrates for specific periods (2-4 weeks) during different seasons (Castenholz, 1960; Sladecek and Sladeckova, 1964; Lyford and Gregory, 1975; Liaw and MacCrimmon, 1978; Rodgers and others, 1979). The equal and simultaneous time periods should be reported with the data.

1. Applications

The method quantifies all organic mass, autotrophic and heterotrophic, living and dead, associated with the periphyton community. Gravimetric determinations are suitable for all water.

2. Summary of method

Samples of the periphyton community are collected from known areas of natural or artificial substrates. The dry weight and ash weight are determined.

3. Interferences

3.1 Inorganic matter in the sample will cause erroneously large dry and ash weights.

3.2 Dead periphyton and organic detritus that settles on the substrate will cause an overestimate of living biomass.

3.3 Natural variability generally is large for biomass and may cause a problem when the method is used for comparison.

3.4 When used as an index of production of the net periphyton community, grazing can result in an underestimate, and detrital settling will result in an overestimate of production.

3.5 Colonization rates vary depending on orientation of substrates (horizontal or vertical) because orientation affects the settling of organic and inorganic detritus. Vertical orientation is preferred because it decreases the settling problem (Castenholz, 1960; Liaw and MacCrimmon, 1978).

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Artificial substrates, glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.2 Balance, capable of weighing to at least 0.1 mg.

4.3 Collecting devices for the removal of periphyton from natural substrates. Three devices for collecting a sample of periphyton from natural substrates are shown in figure 18.

4.4 Desiccator, containing silica gel or anhydrous calcium sulfate.

4.5 Drying oven, thermostatically controlled for use at 105 °C.

4.6 Filtration apparatus, non-metallic, and has a vacuum.

4.7 Forceps, stainless steel, smooth tip, or tongs.

4.8 Glass filters, 47-mm diameter disks.

4.9 Muffle furnace, for use at 500 °C.

4.10 Porcelain crucibles.

4.11 Sample containers, glass or plastic, suitable for the types and sizes of samples. Sturdy plastic bags are useful containers for artificial substrates or for pieces of natural substrate. Do not use glass containers for samples to be frozen.

4.12 Scraping devices, razor blades, stiff brushes, spatulas, or glass slides are useful for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

5. Reagents

5.1 Distilled or deionized water.

6. Analysis

6.1 Calculate the tare weight of a crucible containing a glass-fiber filter. Heat at 500 °C for about 20 minutes, cool to room temperature in a desiccator, and weigh to the nearest 0.1 mg.

6.2 Filter the water and the scrapings from the periphyton strip in the sample bottle through the tared glass-fiber filter. Place filter in crucible and dry at 105 °C to a constant weight. Cool crucibles containing dried periphyton to room temperature in a desiccator before weighing. Weigh as rapidly as possible to decrease moisture uptake by the dried residue. Use these weight values to calculate dry weight.

6.3 Place the crucible containing the dried residue in a muffle furnace at 500 °C for 1 to 4 hours. Cool to room temperature.

6.4 Moisten the periphyton ash using distilled water and again ovendry at 105 °C to constant weight as described in 6.2. Use these weight values to calculate ash weight.

7. Calculations

7.1 Dry weight of periphyton (grams per square meter)

$$= \frac{\text{Dry weight of crucible and residue (grams)} - \text{tare weight of crucible (grams)}}{\text{Area of scraped surface (square meters)}} .$$

7.2 Ash weight of periphyton (grams per square meter)

$$= \frac{\text{Ash weight of crucible and residue (grams)} - \text{tare weight of crucible (grams)}}{\text{Area of scraped surface (square meters)}} .$$

8. Reporting of results

Report periphyton biomass to three significant figures.

9. Precision

No numerical precision data are available.

10. References cited

Castenholz, R. W., 1960, Seasonal changes in the attached algae of freshwater and saline lakes in the Lower Grand Coulee, Washington: Limnology and Oceanography, v. 5, no. 1, p. 1-28.

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Lyford, J. H., and Gregory, S. V., 1975, The dynamics and structure of periphyton communities in three Cascade Mountain streams: Verhandlung Internationale Vereinigung Limnologie, v. 19, p. 1610-1616.

Rodgers, J. H., Jr., Dickson, K. L., and Cairns, John, Jr., 1979, A review and analysis of some methods used to measure functional aspects of periphyton, in Weitzel, R. L., ed., Methods and measurements of periphyton communities--A review: American Society for Testing and Materials Special Technical Publication 690, p. 142-167.

Sladecek, Vladimir, and Sladeckova, Alena, 1964, Determination of periphyton production by means of the glass slide method: Hydrobiologia, v. 23, no. 1, p. 125-158.

Tilley, L. J., 1972, A method for rapid and reliable scraping of periphyton slides, in Geological Survey Research 1972: U.S. Geological Survey Professional Paper 800-D, p. D221-D222.

Permanent-Slide Method for Periphytic Diatoms
(B-3540-85)

Parameter and Code: Not applicable

This procedure enables preparation of permanent mounts using a minimum of time and equipment. Numerous alternative methods for clearing diatom frustules (cell walls) and mounting exist in the literature. Alternative methods for clearing include nitric acid digestion of tissue on the slide (Knudsen, 1966), sulfuric acid and potassium permanganate (Hasle and Fryxell, 1970), hydrochloric acid (HCl) (Cupp, 1943), and potassium permanganate and HCl (Hasle, 1978). Hydrogen peroxide and potassium permanganate (Von der Webb, 1953), hydrogen peroxide and ultraviolet light (Swift, 1967), and hydrogen peroxide after mild heating (Wong, 1975) also have been used for tissue digestion. The reader is referred to the original papers for the details of these procedures.

1. Applications

This qualitative method is suitable for all water. Advantages of the method are that a permanent mount is prepared, and clearing of the cells enhances observation of frustule detail. The method, therefore, is important in the taxonomic study of diatoms.

2. Summary of method

The diatoms in a sample are concentrated, the cells are cleared, and a permanent mount is prepared. The mount is examined microscopically, and the number of diatom taxa is calculated from strip counts.

3. Interferences

3.1 Inorganic particulate matter, including salt crystals, interferes with mount preparation but can be decreased by sample washing.

3.2 The method does not distinguish living from dead diatoms. At certain seasons, particularly during low flow, more than one-half the cells may be dead (Pryfogle and Lowe, 1979). As a result, permanent mounts may provide an inaccurate estimate of community composition.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Artificial substrates, glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.2 Balance, that has an automatic tare.

4.3 Centrifuge, either swing-out type or fixed-head cup type, 3,000 to 4,000 r/min, 15- to 50-mL conical 100-mL pear-shaped centrifuge tubes, and simple siphoning or suction device to remove excess fluid after centrifugation.

4.4 Collecting devices for the removal of periphyton from natural substrates. Three devices for collecting a sample of periphyton from natural substrates are shown in figure 18.

4.5 Cover glasses, 18×18 or 22×22 mm, no. 1½, and microscope slides, glass, 76×25 mm.

4.6 Forceps, curved tip.

4.7 Graduated cylinders, plastic, of sufficient capacity (100 and 500 mL and 1 L are convenient sizes) for measuring known volumes of water samples.

4.8 Hotplate, thermostatically controlled to 538 °C. It is convenient to have a second hotplate for operation at about 93 to 121 °C as described in 6.10.

4.9 Microscope, conventional light microscope, or equivalent. Bright field condensor and objectives are required, and phase contrast is desirable, particularly for taxonomic examination. A series of objectives needs to be available (10X, 20X, and 40X), and 100X phase-contrast oil-immersion objectives need to be available for examination of ultraplankton. The microscope needs to be equipped with a movable mechanical stage that has vernier scales.

4.10 Pipets, 1-mL or 10-mL capacity, sterile.

4.11 Sample containers, glass or plastic, suitable for the types and sizes of samples. Sturdy plastic bags are useful containers for artificial substrates or for pieces of natural substrates.

4.12 Scraping devices, razor blades, stiff brushes, spatulas, or glass slides are useful for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.13 Whipple disc, placed in one ocular of the microscope.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Cupric sulfate solution, saturated. Dissolve 21 g cupric sulfate ($CuSO_4$) in 100 mL distilled water.

5.2 Detergent solution, 20 percent. Dilute 20 mL liquid detergent, phosphate free, to 100 mL using distilled water.

5.3 Distilled or deionized water.

5.4 Formaldehyde cupric sulfate solution. Mix 1 L 40-percent aqueous formaldehyde containing 10 to 15 percent methyl alcohol with 1 mL cupric sulfate solution.

5.5 Immersion oil, Cargille's nondrying type A.

5.6 Lugol's solution plus acetic acid. Dissolve 10 g iodine (I_2) crystals and 20 g potassium iodide (KI) in 200 mL distilled water. Add 20 mL glacial acetic acid a few days prior to use; store in an amber glass bottle (Vollenweider, 1974).

5.7 Mounting medium (table 13). Generally, mounting media should have a refractive index different than that of diatom frustules. Diatom frustules have a refractive index of approximately 1.15 (Reid, 1978).

6. Analysis

6.1 Remove the periphyton from the substrate using a suitable device.

6.2 By vigorous shaking, thoroughly disperse the periphyton in about 100 mL of preservative, or distilled water if working with unpreserved material.

6.3 If the sample contains great numbers of periphyton, as typically occurs in eutrophic water, dilute the sample. To dilute, thoroughly mix 50 mL sample with 50 mL distilled water (1:1 dilution) and proceed to 6.4. If microscopic examination reveals a concentration of periphyton still too numerous to count, thoroughly mix 50 mL 1:1 dilution with 50 mL distilled water (1:4 dilution). Make additional dilutions as appropriate.

6.4 If concentration is necessary, allow the sample to settle undisturbed in the sample container for 4 hours per centimeter of depth to be settled. After settling, weigh the sample container on an automatic tare balance.

6.5 Carefully siphon the supernatant to avoid disturbance of the settled material. Place sample container and remaining sample on balance and weigh. The decrease in weight (in grams) is equivalent to the number of milliliters of supernatant removed. Use the same method to obtain the volume of concentrate.

6.6 If the sample was collected from seawater or saline lakes, wash the periphyton, using distilled water, at least three times to ensure that the permanent mounts are not obscured by salt crystals. Add about 10 mL distilled water to the concentrate in the centrifuge tube, gently shake the tube to suspend the residue, fill the tube with distilled water, and centrifuge for 20 minutes. Decant the supernatant fluid and repeat the washing process two more times.

6.7 Place two or three drops of the concentrate on each of three or four cover glasses.

6.8 With the concentrate side up, place the cover glass on a hotplate and heat, slowly at first to prevent splattering, to about 538 °C (a higher temperature will melt diatom valves) for 30 minutes.

6.9 Remove cover glass from the hotplate and cool.

6.10 Place a drop of mounting medium (table 13) on a microscope slide and heat at about 93 to 121 °C for 3 to 4 minutes.

6.11 Invert the cover glass, concentrate side down, on the heated medium. Apply slight pressure to the cover glass (for example, with a pencil eraser) until visible air bubbles disappear. Remove slide from hotplate and allow to cool. If bubbles still are present under the cover glass, heat the slide and gently apply additional pressure to the cover glass. Label the slide to identify sample.

6.12 Examine the slide using the 100X objective (oil immersion). Count and identify all diatom taxa found in several lateral strips the width of the Whipple disc. Identify and tabulate 200 to 300 diatom cells, if possible. Generally, at least 100 individuals of the most common species should be enumerated. Ignore frustule fragments. Thin-walled forms, such as Rhizosolenia eriensis and Melosira crenulata, may be difficult to observe when using this method (Weber, 1966, p. 3). If a microscope that has a mechanical stage is used, recording of the x and y coordinates of lateral strips or individual cells enables investigators to later recheck and verify identification (Wong, 1975).

7. Calculations

Percent occurrence of each species

$$= \frac{\text{Number of diatoms of a given species}}{\text{Total number of diatoms tabulated}} \times 100 .$$

8. Reporting of results

Report percentage composition of diatoms to the nearest whole number. Report taxa and number of organisms per taxa.

9. Precision

No numerical precision data are available.

10. References cited

Cupp, E. E., 1943, Marine plankton diatoms of the west coast of North America: Bulletin of the Scripps Institute of Oceanography, University of California at La Jolla, v. 5, p. 1-238.

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Hasle, G. R., and Fryxell, G., 1970, Diatoms--Cleaning and mounting for light and electron microscopy: Transactions of the American Microscopical Society, v. 89, p. 469-474.

Knudsen, J., 1966, Biological techniques: New York, Harper and Row, 525 p.

Pryfogle, P. A., and Lowe, R. L., 1979, Sampling and interpretation of epilithic lotic diatom communities, in Weitzel, R. L., ed., Methods and measurements of periphyton communities--A review: American Society for Testing and Materials Special Technical Publication 690, p. 77-89.

Reid, F. M., 1978, Permanent slides, in Sournia, Alain, ed., Phytoplankton manual: Paris, UNESCO, Monographs on Oceanographic Methodology 6, p. 115-118.

Swift, Elijan, 1967, Cleaning diatom frustules with ultraviolet radiation and peroxide: Phycologia, v. 6, p. 161-163.

Tilley, L. J., 1972, A method for rapid and reliable scraping of periphyton slides, in Geological Survey Research 1972: U.S. Geological Survey Professional Paper 800-D, p. D221-D222.

Vollenweider, R. A., ed., 1974, A manual on methods for measuring primary production in aquatic environments (2d ed.): Oxford and Edinburgh, Blackwell Scientific Publications, International Biological Programme Handbook 12, 225 p.

Von Der Webb, 1953, A new method for concentrating and cleaning diatoms and other organisms: International Association Theoretical and Applied Limnology Proceedings, v. 1, p. 276-277.

Weber, C. I., 1966, A guide to the common diatoms at water pollution surveillance system stations: Cincinnati, Ohio, Federal Water Pollution Control Administration, Water Pollution Surveillance, 98 p.

Wong, R. L., 1975, Diatom flora of the phytoplankton of San Francisco Bay: San Francisco, San Francisco State University, M.S. thesis, 144 p.

Inverted-Microscope Method for the Identification and Enumeration of Periphytic Diatoms (B-3545-85)

Parameter and Code:
Diatoms, total, periphyton (number/mm^2): 81804

1. Applications

The method is suitable for all water. The diatoms are cleared, making identification of species possible. Reliable quantitative enumeration is possible after the diatoms are separated from one another and from extracellular organic matter.

2. Summary of method

Periphytic diatoms are collected by scraping them from their substrate. Organic components, including gelatinous stalks and matrices and cellular components in the diatoms, are decomposed by oxidation. The diatoms in a sample are concentrated, and a permanent mount is prepared from a 0.1-mL aliquot. The mount is examined microscopically for the purpose of identification and tabulation, and the cleared diatoms are placed in a counting cell for enumeration.

3. Interferences

Large quantities of sediment associated with the periphyton may obscure the diatoms in the counting cell. Sediment and other particulate matter, including salt crystals and carbonaceous residues, interfere with slide-mount preparation.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Artificial substrates, glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substances.

4.2 Collecting devices for the removal of periphyton from natural substrates. Three devices for collecting a sample of periphyton from natural substrates are shown in figure 18.

4.3 Counting cell, 26×76-mm glass slide that has 12-mm circular hole, covered by cementing no. 1½ cover glass to slide, and no. 1½ cover glass for top of cell.

4.4 Cover glasses, 18×18 or 22×22 mm, no. 1½, and microscope slides, glass, 76×25 mm.

4.5 Graduated cylinders, plastic, of sufficient capacity (100 and 500 mL and 1 L are convenient sizes) for measuring known volumes of water samples.

4.6 Hotplate, thermostatically controlled for operation at about 93 to 121 °C.

4.7 Inverted microscope.

4.8 Microspatula, 0.1 g.

4.9 Sample containers, glass or plastic, suitable for the types and sizes of samples. Sturdy plastic bags are useful containers for artificial substrates or for pieces of natural substrates.

4.10 Scraping devices, razor blades, stiff brushes, spatulas, or glass slides are useful for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.11 Vial, 10 mL, glass, disposable (for reference sample).

4.12 Water aspirator.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Cupric sulfate solution, saturated. Dissolve 21 g cupric sulfate ($CuSO_4$) in 100 mL distilled water.

5.2 Detergent solution, 20 percent. Dilute 20 mL liquid detergent, phosphate free, to 100 mL using distilled water.

5.3 Distilled or deionized water.

5.4 Formaldehyde cupric sulfate solution. Mix 1 L 40-percent aqueous formaldehyde containing 10 to 15 percent methyl alcohol with 1 mL of cupric sulfate solution.

5.5 Hydrogen peroxide (H_2O_2), 30 percent.

5.6 Immersion oil, Cargille's nondrying type A.

5.7 Lugol's solution plus acetic acid. Dissolve 10 g iodine (I_2) crystals and 20 g potassium iodide (KI) in 200 mL distilled water. Add 20 mL glacial acetic acid a few days prior to use; store in an amber glass bottle (Vollenweider, 1974).

5.8 Mounting medium (table 13). Generally, mounting media should have a refractive index different than that of diatom frustules. Diatom frustules have a refractive index of approximately 1.15 (Reid, 1978).

5.9 Potassium dichromate ($K_2Cr_2O_7$) or ammonium persulfate [$(NH_4)_2S_2O_8$].

6. Analysis

6.1 Place the scraped periphyton sample in a graduated cylinder (100-500 mL).

6.2 If formaldehyde solution perservatives have been added, wash (Note 1) the sample by filling the cylinder, to capacity, with distilled water and allow the periphyton to settle at a minimum rate of 2 hours per centimeter of depth. Although centrifugation accelerates sedimentation, it may damage fragile diatoms and, therefore, is not recommended. To determine when settling is complete, periodically examine the supernatant microscopically using the inverted microscope and the counting cells. When settling is completed, aspirate all but 5 to 10 percent of the supernatant, being careful not to disturb the sedimented material. Repeat the entire procedure several times.

Note 1: The washing procedure is important because samples concentrated for diatom analysis commonly contain dissolved materials, such as salts, preservatives, and detergents, that will leave interfering residues on a permanent-slide mount. Certain preservatives, such as formaldehyde solution, will produce extremely exothermic reactions when hydrogen peroxide is added.

6.3 To the rinsed, concentrated sample, add hydrogen peroxide in a volume approximately five times the concentrate volume and allow the sample to stand for 7 days. Ultraviolet radiation is an effective catalyst for hastening the oxidation process. Do not proceed to step 6.5 until all hydrogen peroxide has been reduced, as evidenced by the cessation of bubble formation.

6.4 If large quantities of extracellular organic matter are present, add a microspatula (approximately 0.1 g) of potassium dichromate (or ammonium persulfate) to the mixture inside a fume hood. This will initiate an exothermic reaction. After the reaction is completed (5-10 minutes), the potassium dichromate solution will change from purple to gold.

6.5 Fill the graduated cylinder with distilled water. Allow the mixture to stand for a minimum of 2 hours per centimeter of depth so that the cleared periphyton will settle to the bottom. Aspirate the mixture, carefully removing and discarding the liquid without disturbing the sediment on the bottom of the cylinder. Repeat this procedure until the supernatant is colorless.

6.6 Mix the concentrated sample well (but not vigorously), and place a small quantity onto each of three cover glasses and spread.

6.7 Place the cover glasses, concentrate side up, on a warm hotplate to increase the evaporation rate, but not enough to boil. Evaporate to dryness.

6.8 Using a glass rod, place several drops of mounting medium, diluted according to manufacturer's instructions, in the center of the cover glass. Commercially available mounting medium (table 13) ensures easily handled permanent mounts for examination during oil immersion. Medium that has high index of refraction (1.65+) is best for mounting diatoms. The greater the index of refraction, the greater the contrast of the microscopic image. Diatoms have a refractive index of about 1.15 and are invisible in medium of similar index.

6.9 Heat the cover glasses slowly, increasing the temperature until all the diluting solvent has been evaporated from the mounting medium. Cool and place the cover glass (concentrate side down) on the center of the slide, and reheat slowly until the medium has flowed to the edges of the cover glass. Remove from source of heat and cool. Ring the cover glass for permanence, if desired.

6.10 Examine the slides at 1,000X magnification (oil immersion) using a compound binocular microscope, and identify the diatom taxa.

6.11 If sediment does not interfere with the identification, adjust the volume of the concentrate in step 6.5 to obtain a frustule count of 5 to 10 frustules per field. Record this adjusted volume as the total (or final) volume. Mix the sample concentrate well (but not vigorously), and pipet sample into each of 10 counting cells. Slide cover glasses into place immediately.

6.12 Place the counting cell on the mechanical stage of a calibrated inverted microscope. Count and identify the diatoms in at least 50 randomly chosen fields at 300 to 500X magnification. Count a minimum of 100 diatom frustules, 300 to 500 if possible, distributing the count among cells using five fields per cell (Woelkerling and others, 1976). If broken or separated frustules are observed, count full half frustules (complete valves) and tabulate accordingly. If taxa that are not on the compiled taxa list are observed, identify them at 800 to 1,000X magnification.

7. Calculations

7.1 Diatoms per milliliter of suspended scraping

$$= \frac{\text{Total count}}{\text{(Number of fields) (chamber depth, in centimeters) (field area, in square centimeters)}} .$$

7.2 Total diatoms per square millimeter of surface

$$= \frac{\text{(Diatoms per milliliter of suspended scraping) (total volume of scraping, in milliliters)}}{\text{(Area of scraped surface, in square millimeters)}} .$$

7.3 Percent occurrence of each species

$$= \frac{\text{Number of diatoms of a given species}}{\text{Total number of diatoms tabulated}} \times 100 .$$

8. Reporting of results

Report diatom counts to two significant figures.

9. Precision

No precision data are available.

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MACROPHYTES

Introduction

Macrophytes include vascular plants, bryophytes, and algae that can be seen without magnification. The aquatic macrophytes referenced in this text are nonwoody macrophytes commonly found in wetlands or deep-water habitats (Cowardin and others, 1979). The characteristic vascular plant forms found in aquatic habitats are: (1) Emergent rooted aquatics, (2) floating-leaved rooted aquatics, (3) submersed rooted aquatics, and (4) free-floating aquatics. Some of these plants may form marginal mats or floating islands.

Bryophytes, the mosses and liverworts, generally are less conspicuous than the vascular plants. In swiftly flowing water, they generally grow attached to submerged or partly submerged rocks. In quiet water, mosses and liverworts may be attached to submerged rocks and mud substrata alone or may be among rooted vascular plants.

Algae are plants that lack true roots, stems, and leaves. They include the smallest of chlorophyll-bearing plants that consist of a single cell (commonly found in the phytoplankton or periphyton) as well as marine representatives ranging to several tens of meters in length. Freshwater species of algae occur as individual plants, colonies, or patches attached to rocks in flowing water. Such plants may be gray, green, blue-green, or olive, and may be slimy to the touch, such as Batrachospermum; or, they may be green and have a coarse filamentous structure and profuse lateral branching, such as Cladophora. In slow flowing or quiet water, algae that have stemlike and leaflike structures frequently are found. These plants commonly have a glistening or translucent appearance (Nitella), or they may be encrusted with lime, which gives rise to the common name stonewort (Chara). All of these types of algae also may be found in brackish coastal water or saline inland water.

Distribution and growth of aquatic macrophytes depend on depth of water, illumination, nutrient availability, water quality, substrate, and water velocity. Sometimes the rooted vascular plants are arranged in zones corresponding to successively greater water depths. The predominant vegetation in each deeper zone is composed of species more tolerant of water depth or decreasing illumination. These zones may be greatly compressed in turbid water. The processes of erosion and deposition and the resultant substrate composition partially control the extent to which plant zones develop. Free-floating aquatic plants may occur anywhere on the water surface; their distribution is controlled by water velocity and wind. The growth of aquatic macrophytes is related to the availability of nutrients. In some bodies of water, nutrient enrichment results in excessive growth of macrophytes that may become a major nuisance and may constitute an important water-quality problem. However, long-term nutrient enrichment may alter the macrophyte-phytoplankton-nutrient balance and may produce changes in species composition or to a decline in populations of aquatic macrophytes (Haslam, 1978). Tissue analysis of plants may provide information for evaluating nutrient supplies in natural water (Gerloff and Krombholz, 1966), for determining nutrient requirements for particular plant species (Fitzgerald, 1969), or for studying bioaccumulation of trace metals (Mayes and others, 1977).

Collection

Samples of macrophytes are collected by hand or with grappling hooks, rakes, oyster tongs, or dredges. Entire plants should be collected, including flowers and seeds, if present, and roots and rhizomes or tubers, if possible. During floral surveys, all habitats should be sampled in an effort to collect common and rare species. For some investigations, the relative abundance of plant species in the study area should be noted. For quantitative studies, a uniform sampling system for plant collection should be devised to provide some measure of abundance and productivity.

Plants to be placed in a herbarium or preserved for identification or further study should be pressed and mounted using standard techniques. Place emergent rooted aquatics and free-floating or floating-leaved rooted aquatics that have large coarse leaves (Nymphaea, or Pistia, for example) in a plant press for preservation. Use paper toweling or other absorbent material to soak up as much moisture from the specimens as possible before preparing them for the press. Carefully arrange each plant on one-half of a folded sheet of newspaper. Bend stems and leaves where necessary, but keep the plants as flat and as widely spread as possible. Label each plant for location collected, date collected, and species, if known. Fold the other one-half of the newspaper over each flattened plant, sandwich between two botanical driers, and place in a plant press. Many sheets that contain specimens may be added to the press, but each preparation must be separated by a botanical drier. Tie or strap the press securely.

Replace the damp botanical driers frequently (daily or weekly, depending on water content of plant material) until all plant parts are completely dry. This replacement is necessary if plant specimens are to be preserved satisfactorily. Plants being pressed should be kept cool to help control spoilage of the wet material, unless the press containing the plants is placed in a botanical drying rack to hasten drying using artificial heat. Before proceeding with the heat method of drying macrophytes, read the techniques described by Lawrence (1960, p. 241-243).

Submersed rooted aquatics, especially those with fine strap-like or dissected leaves, are limp and fragile and should not be handled in air. The same is true for algae. Wash thoroughly to remove epiphytes and debris, and float the specimen in water in a flat tray or sink. Arrange plant, slip mounting sheet under it, and remove from water, or drain water and allow plant to settle on paper. Good-quality herbarium paper can be used, or the plant can be floated on other paper and subsequently mounted on a herbarium sheet. For species that have emergent flowers (for example, Utricularia), remove flowering parts prior to floating and press separately by standard method. Place paper and plant on one-half of a folded sheet of newspaper and place a sheet of waxed paper directly on top of plant material. Fold the other one-half of the newspaper over the plant, sandwich between two botanical driers, and place in a plant press. Use of a drying rack and artificial heat is recommended.

Duckweed (Lemnaceae) should be floated onto index cards and placed between newspaper sheets in the plant press. The upper and lower sides of these plants should be visible when arranged on the index cards. When dry, the specimens will fall off the card and should be placed in a packet or mounted on a herbarium sheet.

After drying, glue or cloth tape should be used to affix specimens to herbarium paper. Packets of flowers, seeds, or small, delicate specimens should be mounted on the sheet with the remainder of the plant. Many algae have a natural muscilagenous coating that serves as a natural glue when dried.

Preserve small specimens of vascular plants and bryophytes in 70-percent ethyl alcohol, 2-percent solution of formalin, 2-percent oxyquinoline, or 8-hydroxyquinoline sulfate solution. Add a volume of preservative at least equal to the volume of plant material to ensure adequate preservation. Although this preservation is adequate for macrophytes in general, freshwater algae should be preserved as follows: to each 100 mL of sample, add about 3 mL 100-percent formalin (37- to 40-percent formaldehyde solution), 0.5 mL 20-percent detergent solution, and 5 to 6 drops cupric sulfate solution. For marine or brackish-water algae, use 4- to 5-percent final formalin solution made with the water in which the plant was collected. For large marine species, for example, Laminaria, use a mixture containing 10 percent phenol, 30 percent alcohol, 30 percent glycerine, and 30 percent water (Taylor, 1957). This will maintain flexibility and prevent specimens from becoming brittle.

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Floral Survey (Qualitative Method)
(B-4501-85)

Parameter and Code: Not applicable

1. Applications

The method is suitable for all water.

2. Summary of method

Specimens from each habitat are collected and identified using appropriate references and taxonomic keys. Specimens are preserved or pressed and mounted for herbarium collection or further study.

3. Interferences

Missing or incompletely developed plant parts (flowers, seeds, or other parts) or improperly preserved plant material may make identification of a specimen difficult.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Botanical driers. These driers are absorbent pads, measuring approximately 30×46 cm, for use in plant presses. When preserving submersed aquatics, artificial heat is needed with driers.

4.2 Collecting equipment, appropriate to the objectives of the study, the type of substrate, and the depth of water. Examples of suitable equipment are:

4.2.1 Dredge.

4.2.2 Oyster tongs that have steel blades welded across teeth and small cord attached across opening to control size of sample (Sincock and others, 1965; Kerwin and others, 1976; Carter and Haramis, 1980).

4.2.3 Plant grappling hook. A simple grappling hook may be fabricated by binding the shanks of several hooks from wire coathangers together using light-weight wire. Make a loop on an extra-long shank for attaching a line.

4.2.4 Steel garden rake.

4.3 Microscope, binocular, wide-field, dissecting-type, and fluorescent lamp.

4.4 Newspaper stock, folded to about 29×42 cm.

4.5 Plant press.

4.6 Sample containers, wide-mouth glass or plastic jars and leakproof caps or sealable plastic bags.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Cupric sulfate solution, saturated. Dissolve 21 g cupric sulfate ($CuSO_4$) in 100 mL distilled water.

5.2 Detergent solution, 20 percent. Dilute 20 mL liquid detergent, phosphate free, to 100 mL using distilled water.

5.3 Distilled or deionized water.

5.4 Ethyl alcohol, 70 percent.

5.5 Formaldehyde solution, 37 to 40 percent (formalin, 100 percent).

5.6 Oxyquinoline or 8-hydroxyquinoline sulfate, 2 percent. Dissolve 2 g 8-hydroxyquinoline sulfate in 50 mL distilled water and dilute to 100 mL. This preservative is used as a general substitute for either alcohol or formaldehyde solution for preserving macrophytes (Swingle, 1930; Lawrence, 1960, p. 255). This preservative lacks most of the objectionable features of formaldehyde solution and particularly is useful onsite because small envelopes or capsules of measured quantities of powder may be mixed with water as needed (Moore, 1950).

6. Analysis

Identify plant specimens using an appropriate taxonomic key, such as Muenscher (1944), Smith (1950), Conrad (1956), Wood (1967), Radford and others (1968), Fassett (1969), Britton and Brown (1970), Fernald (1970), Hotchkiss (1972), Correll and Correll (1975), and Beal (1977). A stereoscopic microscope may be required.

7. Calculations

None required.

8. Reporting of results

List the taxa of macrophytes identified.

9. Precision

No numerical precision data are available.

10. References cited

Beal, E. D., 1977, A manual of marsh and aquatic vascular plants of North Carolina with habitat data: North Carolina Agricultural Experiment Station Technical Bulletin 247, 298 p.

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Distribution and Abundance (Quantitative Method) (B-4520-85)

Parameter and Code:
Macrophytes, total (number/m^2): 70944

1. Applications

The method is suitable for all water.

2. Summary of method

The distribution of macrophytes is determined onsite and plotted on a map of the study area. The size of the subareas inhabited by different kinds of macrophytes or the size of the vegetated area can be determined by planimetry or dot grid if desired. Transect, grid, or quadrat sampling schemes are developed, and floral composition and relative abundance (percent cover, density, frequency of occurrence) are established.

3. Interferences

Physical factors, such as depth of water, may interfere with determination of macrophyte distribution and abundance. Missing or incompletely developed plant parts (flowers, seeds, or other parts) or improperly preserved plant material may make identification of a specimen difficult.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Aerial photographs at appropriate scales. Color infrared photographs are best for emergent rooted, floating-leaved rooted, or free-floating aquatic macrophytes; natural color or black-and-white photographs are preferred for submersed rooted aquatic macrophytes (Carter, 1977). Existing photographs can be obtained by contacting the National Cartographic Information Center (NCIC) in Reston, Va., or the EROS Data Center (EDC) in Sioux Falls, S. Dak.

4.2 Base map at appropriate scale. Scale-stable base maps may be obtained from the Water Resources Division Publications Office at standard scales (for example, 1:24,000, 1:250,000, 1:1,000,000).

4.3 Botanical driers. These driers are absorbent pads, measuring approximately 30×46 cm, for use in plant presses. When preserving submersed aquatics, artificial heat is needed with driers.

4.4 Collecting equipment, appropriate to the objectives of the study, the type of substrate, and the depth of water. Examples of suitable equipment are:

4.4.1 Dredge.

4.4.2 Oyster tongs that have steel blades welded across teeth and small cord attached across opening to control size of sample (Sincock and others, 1965; Kerwin and others, 1976; Carter and Haramis, 1980).

4.4.3 Plant grappling hook. A simple grappling hook may be fabricated by binding the shanks of several hooks from wire coathangers together using light-weight wire. Make a loop on an extra-long shank for attaching a line.

4.4.4 Steel garden rake.

4.5 Microscope, binocular, wide-field, dissecting-type, and fluorescent lamp.

4.6 Newspaper stock, folded to about 29×42 cm.

4.7 Plant press.

4.8 Polar planimeter, or dot grid at appropriate scale.

4.9 Sample containers, wide-mouth glass or plastic jars and leakproof caps or sealable plastic bags.

4.10 Surveying or other equipment, suitable for developing transect, grid, and quadrat sampling schemes (Cox, 1967; Mueller-Dombois and Ellenberg, 1974).

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Cupric sulfate solution, saturated. Dissolve 21 g cupric sulfate ($CuSO_4$) in 100 mL distilled water.

5.2 Detergent solution, 20 percent. Dilute 20 mL liquid detergent, phosphate free, to 100 mL using distilled water.

5.3 Distilled or deionized water.

5.4 Ethyl alcohol, 70 percent.

5.5 Formaldehyde solution, 37 to 40 percent (formalin, 100 percent).

5.6 Oxyquinoline or 8-hydroxyquinoline sulfate, 2 percent. Dissolve 2 g 8-hydroxyquinoline sulfate in 50 mL distilled water and dilute to 100 mL. This preservative is recommended as a general substitute for either alcohol or formaldehyde solution for preserving macrophytes (Swingle, 1930; Lawrence, 1960, p. 255). This preservative lacks most of the objectionable features of formaldehyde solution and particularly is useful onsite because small envelopes or capsules of measured quantity of powder may be mixed with water as needed (Moore, 1950).

6. Analysis

6.1 Identify plant specimens using an appropriate taxonomic key, such as Muenscher (1944), Smith (1950), Conrad (1956), Wood (1967), Radford and others (1968), Fassett (1969), Britton and Brown (1970), Fernald (1970), Hotchkiss (1972), Correll and Correll (1975), and Beal (1977). A stereoscopic microscope may be required.

6.2 Determine the mappable units (discrete vegetative communities, associations, or homogeneous stands) and choose the appropriate scale for mapping (Kuchler, 1967). This determination should be made after onsite observations and identification of mappable units using aerial photographs when available.

6.3 Determine the major floristic components of each map unit by onsite observation and sampling. If abundance is included, calculate percent cover, density, or frequency of occurrence, or all three, from transect or quadrat samples (Cox, 1967; Mueller-Dombois and Ellenberg, 1974).

6.4 Outline map units on map base or overlay material. Map legend or explanation should clearly identify each map unit and its symbol or color. Map also should include a scale and north arrow or latitude-longitude tick marks.

6.5 Determine the area (in square meters or square kilometers) covered by each vegetative community, association, or homogeneous stand, using a polar planimeter or dot grid.

7. Calculations

7.1 Percent cover

$$= \frac{\text{Area covered by community, association, or homogeneous stand (square meters or square kilometers)}}{\text{Total area (square meters or square kilometers)}} \times 100 .$$

7.2 Density

$$= \frac{\text{Number of individual plants}}{\text{Area sampled (square meters or square kilometers)}} .$$

7.3 Frequency of occurrence

$$= \frac{\text{Number of plots in which species occurs}}{\text{Total number of plots sampled}} .$$

8. Reporting of results

8.1 List the taxa of macrophytes identified.

8.2 The map shows distribution. Report the percent cover, density, or frequency of occurrence for each community, association, or homogeneous stand.

9. Precision

No numerical precision data are available.

10. References cited

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Wood, R. D., 1967, Charophytes of North America: West Kingston, R.I., Stellas' Printing, 72 p.

BENTHIC INVERTEBRATES

Introduction

The invertebrate animals inhabiting the bottom of lakes and streams and other water bodies perform essential consumer functions in aquatic ecosystems and serve as food for fish and other vertebrates including man. They are the most frequently used biological indicators of environmental quality. These organisms have the advantages of relatively large size, which facilitates identification; limited mobility, which restricts them to a particular environment; and a lifespan of months or years, which enables adaptation to conditions that have existed for a long period of time. Moreover, many benthic invertebrates inhabit specific types of environments that, if changed, result in changes in the composition of the benthic community (Hynes, 1970). In general, a varied benthic fauna, without excessively large numbers of any one group, is considered to be characteristic of good quality water. As conditions change (for example, in the presence of organic pollution), the number of species decreases, but the number of individuals of the remaining species may increase. Toxic pollutants may eliminate all benthic invertebrates. Thus, knowledge of the kinds and abundance of benthic invertebrates helps to indicate water-quality trends in the aquatic environment. The extensive literature about interpretation of benthic-invertebrate data and water quality has been reviewed by Hynes (1960, 1970), Warren (1971), Cairns and Dickson (1973), Hart and Fuller (1974), and Hellawell (1978).

Collection

Benthic invertebrates vary in size, and there is no clear distinction between the smallest benthic forms and the largest micro-organisms. Bottom-living invertebrates that are visible to the unaided eye commonly are included with the benthos. Because many early studies of the benthic invertebrates emphasized the quantity available for fishfood, the U.S. Standard no. 30 sieve (0.595-µm mesh openings), which retains most of the biomass, came into use (Davis, 1938; Welch, 1948). The no. 30 sieve also has been used in water-quality investigations, and the American Public Health Association and others (1985) states that the bottom-living invertebrates collected for study, termed "macroinvertebrates," are those which are retained on a no. 30 sieve.

The mesh openings of sampling nets and sieves ideally should be selected based on the needs of a particular study. If the mesh size is so large that the smaller invertebrates pass through the net, erroneous conclusions about life cycles or biomass result (Hynes, 1970). Mesh that is too fine clogs rapidly, resulting in loss of invertebrates by backwash. The results of sampling using a coarse and a fine net on the catch of different sizes of a particular benthic species are not easily predictable (Macan, 1963, p. 281). Jónasson (1955, 1958) reports that the diameter of the head determines whether or not a dipteran larva will pass through a given mesh. His data indicated a 640-percent increase in the number of invertebrates in lake samples as the sieve size decreased from 600 to 200 µm. Other investigators have reported similar results from various aquatic environments. Significant differences between retention of total individuals and total taxa in U.S. Standard no. 30 and no. 60 sieves were reported for reservoir silt substrates (Mason and others, 1975). Schwoerbel (1970) concluded that "* * * in quantitative

studies of the bottom, especially in problems of population dynamics in which immature larvae are of importance, a mesh size of less than 200 µm must be used, and in other respects the mesh width must be carefully adapted to the size of the animals selected." In a study of stream benthic sampling, Mundie (1971) reported that the younger (hence smaller) stages of invertebrates tend to predominate in a natural community. He concluded that even a mesh of 116 µm could enable 50 percent of the fauna to pass through, if the community contained large numbers of chironomid larvae and mayfly and stonefly nymphs. Mundie estimated that a net of 200- to 250-µm mesh would enable 70 to 80 percent of the fauna to pass through, but it still would be adequate for many purposes, such as general faunistic surveys and the estimation of biomass.

For these reasons, the U.S. Geological Survey has adopted the U.S. Standard no. 70 sieve (210-µm mesh opening) for retaining benthic-invertebrates collected as part of its water-quality investigations. Nets are to be 210±2-µm mesh-opening nylon or polyester monofilament screen cloth that has 35- to 44-percent open area. For uses requiring more rapid filtration, large-capacity screen cloth, made of 209-µm nylon monofilament, that has 56-percent open area may be used. These mesh sizes are small enough to retain many of the immature stages of the benthic invertebrates and, yet, are practical to use in flowing water. Special studies may require the use of the no. 30 sieve or other mesh sizes appropriate to the objectives. The size of mesh used always should be reported.

The mud usually should be washed from the sample, and this often results in prolonged immersion of the hands in water. During cold weather, wearing long-gauntlet rubber gloves can make this more bearable. To wash mud from the samples, put small quantities into a no. 70 or other appropriate sieve and agitate it gently ensuring that the mesh is submerged in the water. Washing samples by pouring water through the sieve must be done slowly to avoid forcing small invertebrates through the mesh.

Four methods for benthic-invertebrate sampling are described based on the type of sampling, and three methods for preparation of microscopic mounts needed for taxonomic identification of specific benthic groups are described. Recommended sampling equipment are listed in the "Apparatus" section for the first four methods. For additional information on benthic-invertebrate sampling methods, refer to Welch (1948), Hedgpeth (1957, p. 61-86), Macan (1958), Albrecht (1959), Barnes (1959), Needham and Needham (1962), Cummins (1962, 1966, 1975), Hynes (1964, 1970), Southwood (1966), Schwoerbel (1970), Edmondson and Winberg (1971), Holme and McIntyre (1971), Cairns and Dickson (1973), Weber (1973), Elliott and Tullett (1978), Hellawell (1978), Elliott and others (1980), Elliott and Drake (1981a,b), Cairns (1982), and American Public Health Association and others (1985).

Faunal Surveys

Qualitative faunal surveys determine the taxa present and may estimate the relative abundance of each taxon at each site. Because collection of rare taxa at each site is important, sampling should include a large area of bottom and as many habitats as feasible. Use of several collection methods at each site can increase the total number of taxa in the samples (Allan, 1975; Slack and others, 1976). Moreover, evidence indicates that the larger the sample

collected for qualitative analysis, the greater the number of taxa (Elliott and Drake, 1981b). A faunal survey of a large sampling area, such as a lake or river, usually precedes a quantitative investigation but may be an end in itself (Elliott, 1971a).

There is no universally accepted method for sampling benthic invertebrates. However, no habitat should be overlooked if the objective is to obtain a representative collection of the benthic invertebrates, and different habitats may require different collection methods. The success of the method will depend on the experience and skill of the collector. Sampling should include specimens from rocks, plant beds, logs and brush, clumps of decaying leaves, and deposits of mud, sand, and organic detritus. In streams, areas of fast current, slow current, backwater, near the banks, and in deeper parts should be sampled. Rocks may be lifted by hand and examined for invertebrates as the surface dries. Tufts of algae and moss should be collected and examined for animals. Invertebrates may be dislodged from floating vegetation or rooted plants using a dip net, or samples of the plants may be collected using grappling hooks or rakes and then the invertebrates removed. Methods for collecting plants are described in the "Macrophytes" section. More elaborate methods for sampling invertebrates living in or on plants involve enclosing a unit volume of the vegetation and surrounding water in a bag or box from which the invertebrates subsequently are removed (Welch, 1948; Gerking, 1957). Additional information on sampling is given in the "References Cited" at the back of this section.

Two types of collection devices are described: those using netting to concentrate the invertebrates dislodged from the substrate and those involving removal of the substrate. However, any collection method, including quantitative or hand methods, may be used for qualitative collection of benthic invertebrates.

Dip or hand net

The dip or hand net is the most useful general implement for collecting benthic invertebrates in wadable water and invertebrates living among floating plants in deeper water. The net can be used in water containing large concentrations of suspended sediment and among plants or large boulders to depths of 1 m or more. Macan (1958) described a method of working slowly upstream lifting rocks and holding the net to catch invertebrates swept into it. Clinging invertebrates were dislodged from rocks by vigorously swirling the rocks in the mouth of the net. Alternatively, the net may be held against the bottom, and the area immediately upstream disturbed by the hands or feet, enabling the current to carry invertebrates into the net. In still water, the net can be scraped rapidly along the bottom to catch easily dislodged invertebrates, or it can be swept through plant beds, probed into piles of brush, or used as a scoop to sample mud, silt, and deposits of leaves or other detritus. Additional information about dip-net sampling is given in the "Numerical Assessment" subsection.

Empty the net frequently either into a shallow, white tray, if the sample is to be sorted onsite, or into a wide-mouth container for transporting to the laboratory. Label and preserve each sample.

Dredges

As described by Hynes (1970, p. 237), dredges are instruments that are pulled across or through the bottom sediment and grabs are instruments that bite into the bottom from above. Grabs are considered to be quantitative sampling devices and are described in the "Distribution and Abundance" subsection.

Qualitative samples of benthic invertebrates from deep or swift rivers usually are collected using a dredge (Elliott and Drake, 1981b) (figs. 21, 22). The design varies, but often, large rocks are excluded whereas the smaller particles and the benthic invertebrates are retained in a mesh bag. The dredges developed by Usinger and Needham (1956) and Fast (1968) are examples. Dredges are lowered from a boat or bridge or even thrown from a high bank then pulled upstream along the bottom so the leading edge digs into and disturbs the sediment. The current from the flow of the stream plus the forward motion of the dredge carries invertebrates into the net. In still or slowly moving water, dredges should be pulled by a powered boat to prevent loss of active benthic invertebrates.

Figure 21.--Biological dredge (photograph courtesy of Wildlife Supply Co., Saginaw, Mich.).

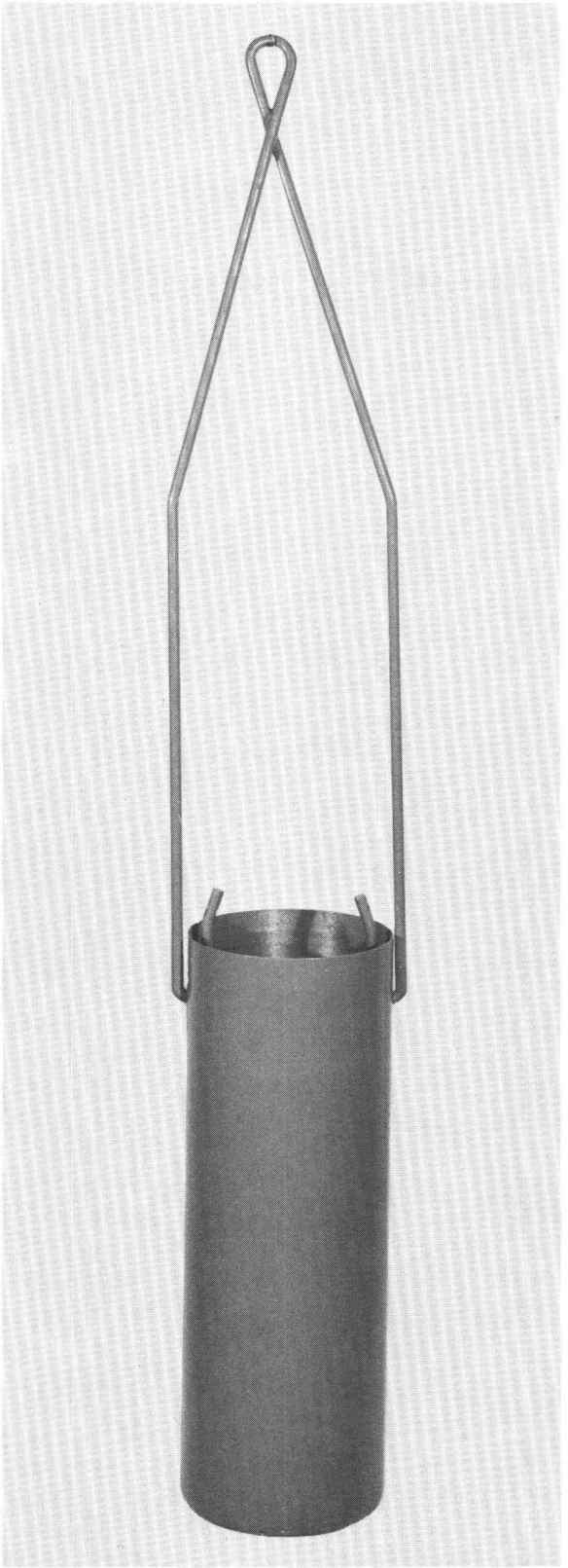


Figure 22.--Pipe dredge (photograph courtesy of Wildlife Supply Co., Saginaw, Mich.).

Elliott and Drake (1981b) compared four light-weight dredges for river sampling. Because of the variability between sampling units in the same sample, there was a lack of precision in estimates of the mean number of individuals indicating that the dredges are not suitable for quantitative sampling. Considerable variation also existed in their effectiveness as qualitative samplers for estimating the total number of taxa per sample. The largest efficiencies for a small sample (n=5) were for the medium (greater than 57 percent) and large (greater than 76 percent) dredges (called Naturalist's dredges) similar in design to that shown in figure 22. The mouth of the medium dredge was 45×17 cm and for the large version was 59×20 cm. Greater penetration depth into the substratum (range in modal particle sizes was 1-2 mm, 64-128 mm, and 128-256 mm) accounted for the superior performance of the Naturalist's dredges compared to the other types tested.

After collection, empty the dredge into a shallow tray or bucket, if the collection is to be sorted onsite, or into a wide-mouth container for transporting to the laboratory. Label and preserve each collection.

Numerical Assessment

Relative or semiquantitative surveys are conducted to compare benthic communities or populations at a specific site for different sampling times or at different sites for the same sampling time. That is, the objective is to make within- or between-site comparisons. Accurate measurements of the total benthos are not obtained, nor are the estimates of relative abundance of each species in the samples necessarily reliable. Sampling effort is limited and, if using artificial substrates, may be restricted to a small area at each site. Because different sampling methods will produce different results, the methods and sampling areas should be as uniform as possible throughout a study.

The statistical principles of benthic-invertebrate sampling are discussed by Elliott (1971a). The first requirement is a clear definition of the objectives of the study and the area to be sampled. The frequency of sampling may range from weekly, in detailed studies, to once a year, in general studies. When artificial substrates are used, sufficient time must be allowed for invertebrate colonization. Two sampling procedures using a dip net, one procedure involving collection of individual rocks, and three procedures using artificial substrates are described in the following subsections.

Dip or hand net

A dip or hand net is a mesh bag mounted on a metal rim that has an attached handle. It is a simple, effective sampling device for water less than 1 m deep and even may be effective in deeper water for sampling plant beds and other near-surface habitats. The dip net used in a standardized way will provide a numerical assessment of the differences between sampling sites in wadable water. Two general approaches are used, one in which the collector sweeps the net through the major aquatic habitats (Slack and others, 1976; Armitage and others, 1981) and one in which the net is held stationary while

the substratum is disturbed with the feet (Hynes, 1961; Morgan and Egglishaw, 1965; Frost and others, 1971; Armitage and others, 1974). The latter method is restricted to streams. The collecting approach used and the effort expended will depend on the size and variability of the sampling area and on the study objectives. Using the moving-net method, the most abundant species may be sampled adequately within 5 or 10 minutes by an experienced biologist. In a river study, Armitage and others (1981) reported that a 3-minute dip-net sample collected about 62 percent of the families and 50 percent of the species that were collected during an 18-minute sample. Slack and others (1976) reported that a 45-minute dip-net sample contained the largest percentage of taxa (78 percent) and the second largest percentage of individuals (41 percent) in a comparison of three collecting methods. Generally, collecting continues for at least 30 minutes in streams as much as 15 m wide and continues for an additional 30 minutes for each 15-m increase in width. Macan (1958) described a method of working slowly upstream, lifting rocks, and holding the net to catch invertebrates swept into it; clinging invertebrates were dislodged from rocks by vigorously swirling the rocks in the mouth of the net. In still water, the net can be scraped rapidly along the bottom to catch easily dislodged invertebrates, or it can be swept through beds of attached or floating plants, probed into piles of brush, or used as a scoop to sample mud, silt, and deposits of leaves or other detritus. The collecting effort and technique must be kept as uniform as possible during a particular study. Empty the dip net frequently to avoid clogging the mesh, which can cause a backwash that would result in loss of sample.

A rapid and versatile method for sampling consists of holding the flat side of a D- or triangular-shaped dip net firmly against the streambed, facing upstream and disturbing the stream bottom for a definite distance (about 0.5 m) just upstream from the net by vigorously kicking three or four times into the bed in an upstream direction (Hynes, 1961; Morgan and Egglishaw, 1965). A proportion of the dislodged invertebrates and detritus will be carried into the net by the current; the kicks should be separated by several seconds to enable this to occur. The method can be used for a variety of substrates from sand to rocks that have a diameter of 45 to 60 cm in weedbeds, or on bedrock using the boot as a scraper. The method has been evaluated by Frost and others (1971) and Armitage and others (1974). The minimum procedure, modified from Morgan and Egglishaw (1965), is to take three (four-kick) samples in a reach of stream: one in a riffle, one in a pool, and one in a position where conditions are intermediate between the other two sites. The minimum-procedure sites should not be near the banks and should be representative of the habitat; that is, select eroding areas in riffles and depositing areas in pools. Sampling may be increased or modified depending on the physical characteristics of the habitat and the study objectives, but it is important that the technique and net design be uniform throughout a study. Empty the dip net, after each series of kickings, into a shallow tray or bucket, if the collection is to be sorted onsite, or into a wide-mouth container for transporting to the laboratory. Label and preserve each collection.

Individual rocks

Because many benthic invertebrates from shallow streams or rocky shores of lakes live on or beneath rocks, a sampling method that involves lifting individual rocks and collecting the associated invertebrates was developed (Macan, 1958; Schwoerbel, 1970). The method consists of three procedures: selection of rocks, collection of rocks, and reporting of results. Because the number of benthic invertebrates per unit of rock area may vary with rock size (Lium, 1974), rocks of similar size should be collected for samples that are to be compared. In gravel-bed streams studied by Lium (1974), greatest invertebrate densities occurred on rocks between 45- and 90-mm mean diameter. As with other methods, the study objectives are decisive in selection of the sampling method and its application. Depending on the objectives, sampling may comprise 10, 20, or more individual rocks from a single habitat (for example, riffles) or from each of several habitats (for example, pools and riffles). Statistical techniques may be used to ensure random collection of rocks from each habitat.

The simplest collection procedure is to pick a rock at random, lift it gently off the substratum, quickly enclose the rock in a net of appropriate mesh size, and lift the net, rock, and associated invertebrates out of the water. This procedure is repeated until the desired number of rocks has been collected. A better method for rock collection is using the Lium sampler (fig. 23), which was designed to catch invertebrates that wash off a rock as it is lifted from the streambed. With the sampler opening facing upstream, approach the selected rock from the downstream side. Place the hood of the sampler over the rock, and press down to compress the flexible base against the streambed. The flexible base minimizes losses from around the edges of the sampler, and the hood minimizes outwash of invertebrates during rock removal. Invertebrates that are dislodged as the rock is lifted are carried by the current into the screen. Remove invertebrates trapped on the screen by inverting the sampler and washing them into a bucket. During each method of rock collection, scrub each rock thoroughly in a bucket of water using a soft-bristle brush to remove clinging invertebrates. Pour the contents of the bucket through a U.S. Standard no. 70 sieve. Empty the sieve into a shallow, white tray, if the sample is to be sorted onsite, or into a wide-mouth container for transporting to the laboratory. Label and preserve each collection.

If the results are to be reported as areal units, rock sizes must be determined. To report the population in terms of the projected area of rock, measure and record the two longest straight-line dimensions of each rock (A and B axes), in millimeters. To report the population in terms of total rock surface, measure each rock, in millimeters, across the B or intermediate axis (Leopold, 1970; Lium, 1974). The B axis, or breadth, is distinguished from the major axis (A, or length) and the minor axis (C, or width).

Figure 23.--Lium sampler.

Artificial substrates

An artificial substrate is defined by Cairns (1982) "* * * as a device placed in an aquatic ecosystem to study colonization by indigenous organisms. Although the device may be unnatural in composition, location, or both, most of the biological processes that occur on it appear to be quite similar to those occurring on natural substrates." Many types of standardized, reproducible surfaces are used as collection devices for colonization by benthic invertebrates (Beak and others, 1973; Hellawell, 1978; Cairns, 1982). The uniform shape and texture of artificial substrates greatly simplifies sampling when correctly used. Standardized sampling is especially desirable when the results from different investigators or from different environments are to be compared.

Artificial substrates have been used to investigate various problems in benthic population and community ecology, including organism-substrate relations, community structure and distribution, and island colonization. Artificial substrates also have been widely used in marine fouling studies and for sampling benthic invertebrates in stream-quality programs. Generally, the objectives are: (1) To determine the composition of the resident benthic community, (2) to collect representative and reproducible samples of benthic invertebrates for areal or temporal comparisons, or (3) to determine rates of species or biomass accrual.

Selection of an artificial substrate sampler and its method of exposure are determined by study objectives and the nature of the environment. Rosenberg and Resh (1982) distinguish between representative artificial substrates (RAS) and standardized artificial substrates (SAS). RAS are samplers that closely resemble the natural substrate over, on, or within which they are placed, such as a basket filled with rocks similar in size distribution to the natural stream bottom. SAS are samplers that differ from the natural substrate of the habitat in which they are placed, such as a multiple-plate sampler. If the objective is to relate the quality of flowing water to the composition of the benthic community, off-bottom exposure may be preferred. Suspension of the samplers within the water column eliminates the effects of bottom conditions that can mask the effects of water composition that serves as a control on benthic community structure (Mason and others, 1973). If the objective is to sample the resident fauna or to evaluate the effects of sediment properties on invertebrate communities, bottom exposure is necessary (Voshell and Simmons, 1977). Before deciding on an artificial-substrate method, onsite tests should be made to compare the relative effectiveness of different samplers and exposures in the habitat to be studied.

Colonization of artificial substrates, reported as biomass or numbers of individuals or species, normally increases rapidly at first then reaches a relatively stable or fluctuating equilibrium level (Rosenberg and Resh, 1982). Colonization rate and biomass vary seasonally, such as being slower in winter than in summer. For monitoring purposes, samplers should be retrieved during the equilibrium phase. The time required to reach equilibrium in 20 studies summarized by Rosenberg and Resh (1982) ranged from 3 to 49 days, but for most studies did not exceed 30 days. Until the colonization process is better understood, preliminary onsite tests should be made to determine optimum exposures for each study.

It is important to prevent losses of invertebrates during sampler retrieval. Many invertebrates leave artificial substrates as soon as they are disturbed. Rabini and Gibbs (1978) reported large losses of invertebrates from barbecue-basket samplers during removal by divers, and McDaniel (1974) reported some loss of invertebrates when retrieving multiple-plate samplers from deep water. Voshell and Simmons (1977) maintained that loss of invertebrates during sample collection and sampler retrieval was a factor contributing to variability among bottom samples in a reservoir. When retrieving a sampler from shallow water, approach from downstream and enclose the entire sampler in a net of appropriate mesh size to catch invertebrates that would be lost when the sampler is lifted from the water. Artificial substrates exposed in deep water should be designed to retain invertebrates that drop off the sampler during retrieval. When retrieved, empty or disassemble the sampler into a tub partially filled with water. Scrub all parts using a

soft-bristle brush to remove clinging invertebrates. Pour the contents of the tub through a sieve of appropriate mesh size and add the invertebrates detached from the sampler during recovery. The sampler also may be placed into a container of preservative and transported to the laboratory for cleaning. Cleaned samplers may be reused unless there is reason to believe that contamination by toxicants or oils has occurred (Weber, 1973). Do not reuse rocks or hardboard plates that have been exposed to preservative.

Multiple-plate sampler.--This sampler is a jumbo modification (Fullner, 1971) (fig. 24) and is the smallest and most adaptable of the artificial-substrate devices. These samplers are relatively inconspicuous by virtue of size and color, and the modest cost enables replication to further enhance the chances of recovery in small bodies of water where the samplers might be subject to vandalism. Attach multiple-plate samplers to floats, structures, weights, or rods driven into the streambed or lakebed. Install three samplers so they will remain submerged, and leave them to be colonized for the experimentally determined exposure period or for 4 to 5 weeks. Record the exposure time, which should be consistent among sites during a study.

The samplers may be installed in pools or riffles and on the bottom or suspended above it, but the macrohabitat should be as uniform as possible at all sites during a study. Usually samplers are installed on the bottom in riffles as much as 1 m deep. Make the collections as representative of the

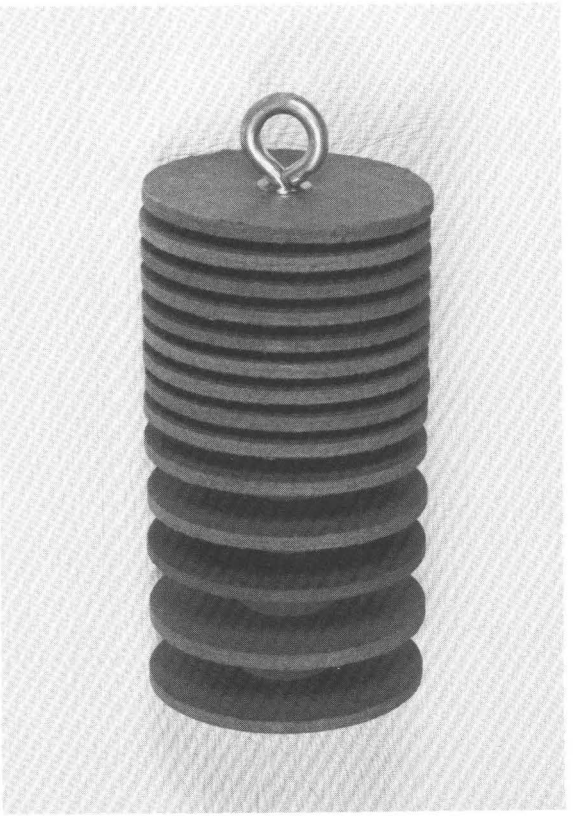


Figure 24.--Jumbo multiple-plate artificial-substrate sampler (photograph courtesy of Wildlife Supply Co., Saginaw, Mich.).

reach as possible by ensuring that the samplers are in eroding areas that are not close to the bank. In streams as much as a few meters in width, install the devices about midstream; in wider streams, install the devices about one-quarter of the total width from the nearest bank. In larger rivers or in lakes, the samplers usually are suspended from floats (fig. 25). When a float is used to suspend more than one sampler and the samples are to be kept separate, enclose each sampler in a retrieval net (fig. 26) to avoid loss of invertebrates when retrieving. It is necessary to reach into the water and gently pull a retrieval net over each sampler, securing the net by tightening the drawstring just above the top of the eyebolt that holds the sampler to the float rod. Enclose all multiple-plate samplers on the float before proceeding with substrate removal. When all the nets are in place, detach the samplers from the float. If only one sampler is used or if the results of multiple samplers are to be pooled, a dip net of appropriate size and mesh may be used to enclose the sampler(s) during recovery.

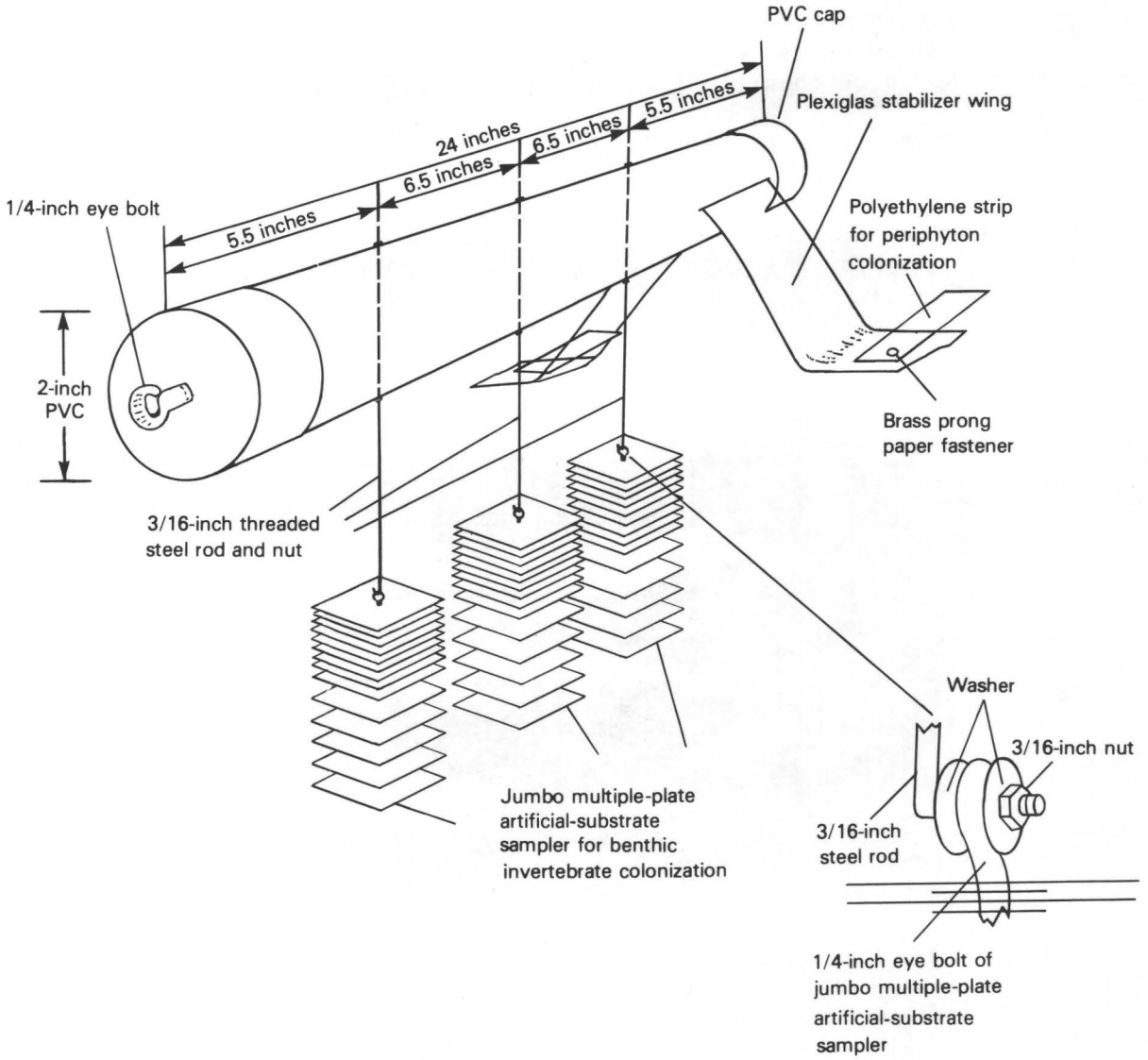


Figure 25.--Float for artificial substrates.

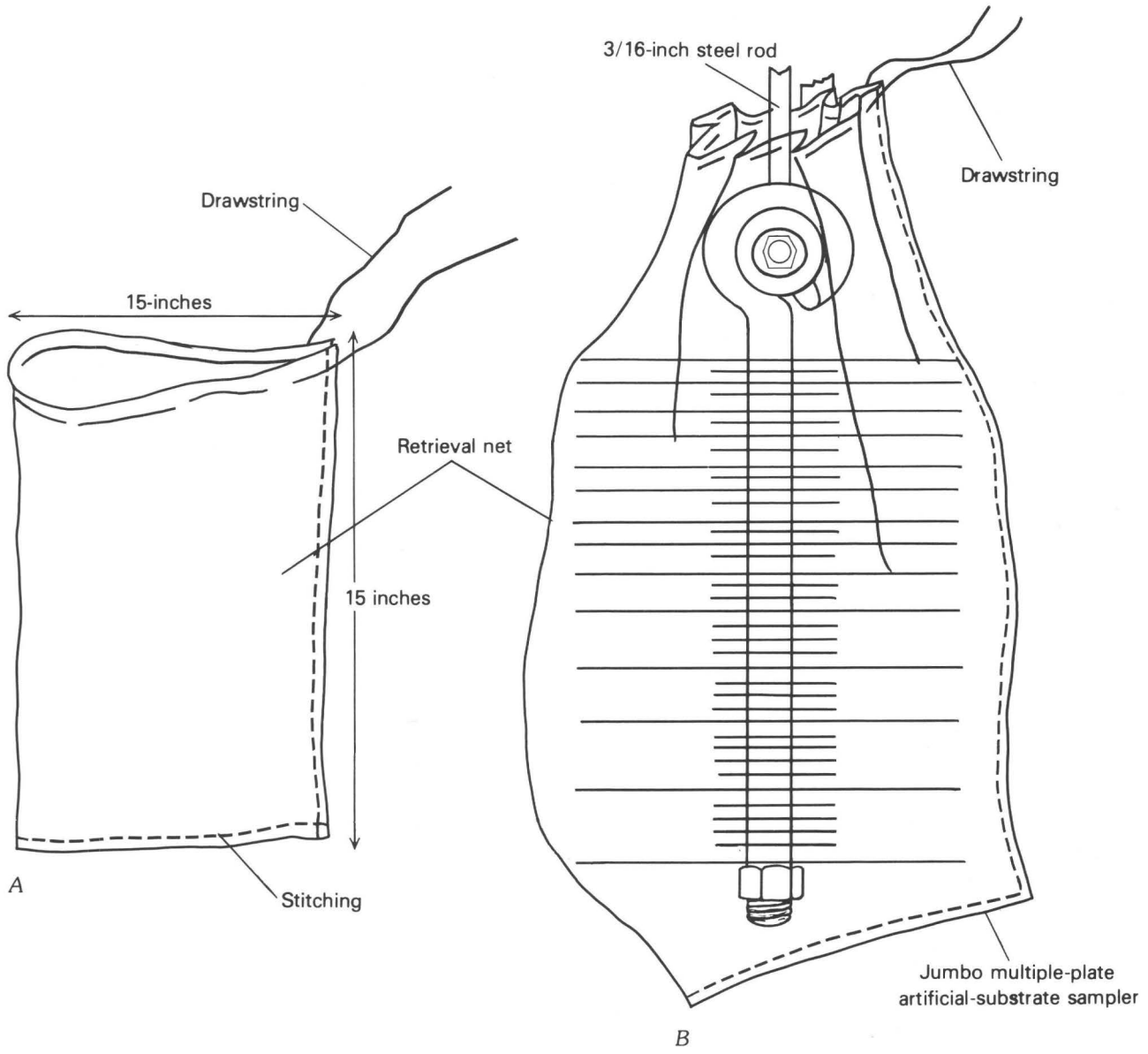


Figure 26.--Retrieval net.

Barbecue-basket sampler.--This sampler (fig. 27) is adapted for use in lakes and large rivers. Fill the basket with 30 rocks, 5 to 7.5 cm in diameter, and secure the sampler door using wire or small cable clamps. The rocks used to fill a series of samplers should be of the same general size, shape, and composition and should be cleaned by scrubbing with a brush before use. Angular limestone commonly is used in barbecue-basket samplers, although spheres of porcelain or concrete provide a more uniform substrate (Jacobi, 1971). Coniferous tree bark has been used as a lightweight substitute for rocks (Bergersen and Galat, 1975; Newlon and Rabe, 1977).

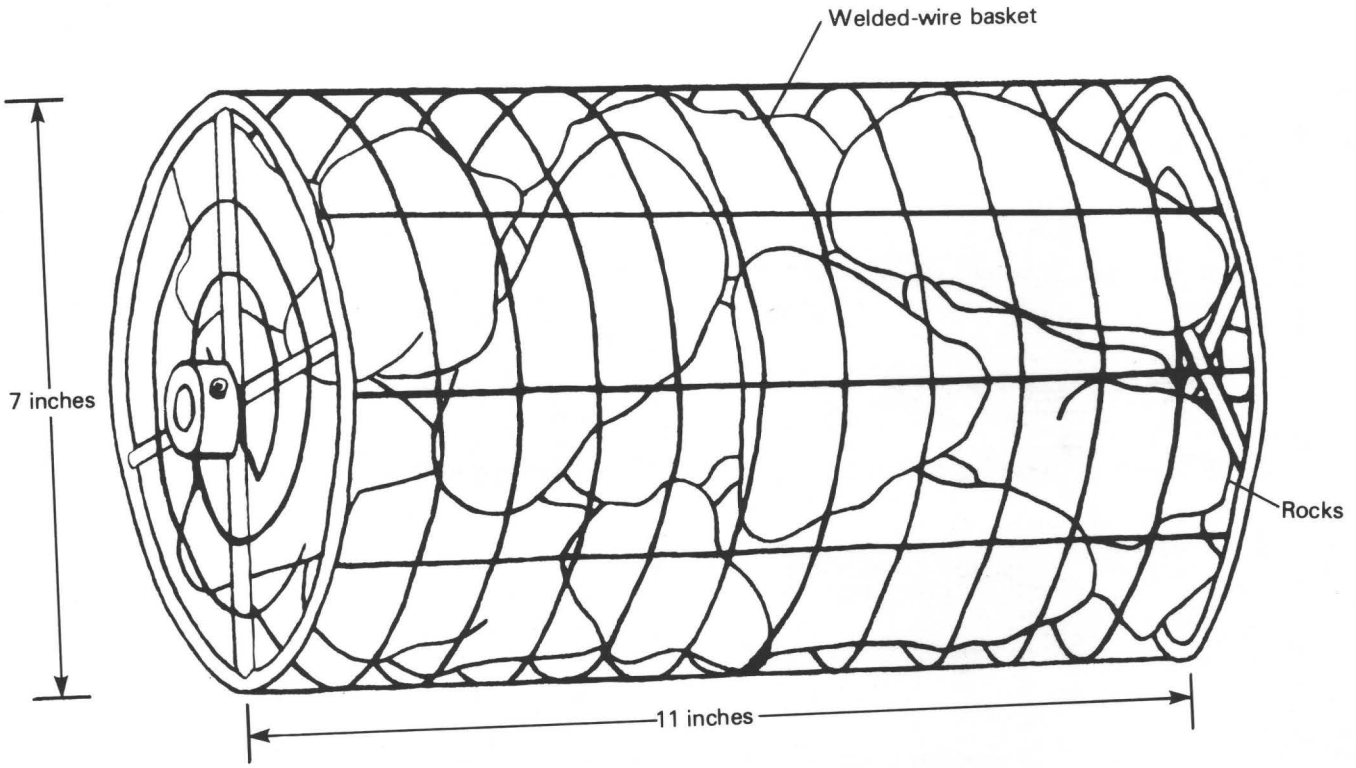


Figure 27.--Barbecue-basket artificial-substrate sampler.

If possible, suspend three samplers at a depth of 0.3 m below the surface for the experimentally determined exposure period or for 4 to 5 weeks. In environments of variable depth, suspend the samplers from a float. Barbecue-basket samplers also may be installed on the bottom in shallow or deep water, but the macrohabitat, depth, and exposure period must be uniform throughout a given study. Samplers must be protected from loss of invertebrates during retrieval. Samplers exposed in deep water may be enclosed in a retrieval net and brought to the surface by divers, or a net can be mounted on a rectangular frame so the net collapses on the natural substrate during colonization, but lifts to enclose the basket during retrieval.

Collapsible-basket sampler.--This sampler (fig. 28) is used if the objective is to compare sampler catches with the population of a surrounding rocky substrate. The basket can be loaded with materials simulating the natural bed on which it lies. This sampler is useful for lakes, shallow streams, or for deep, swift rivers. The sampler consists of a collapsible basket holding gravel or rocks and is surrounded by a nylon netting bag of appropriate mesh. A rim around the top helps retain the gravel. When lowered to the bottom, the basket collapses to form an area of gravel that is subsequently colonized. When raised off the bottom, the basket extends to its original hemispherical shape, and the surrounding net bag prevents loss of invertebrates during retrieval.

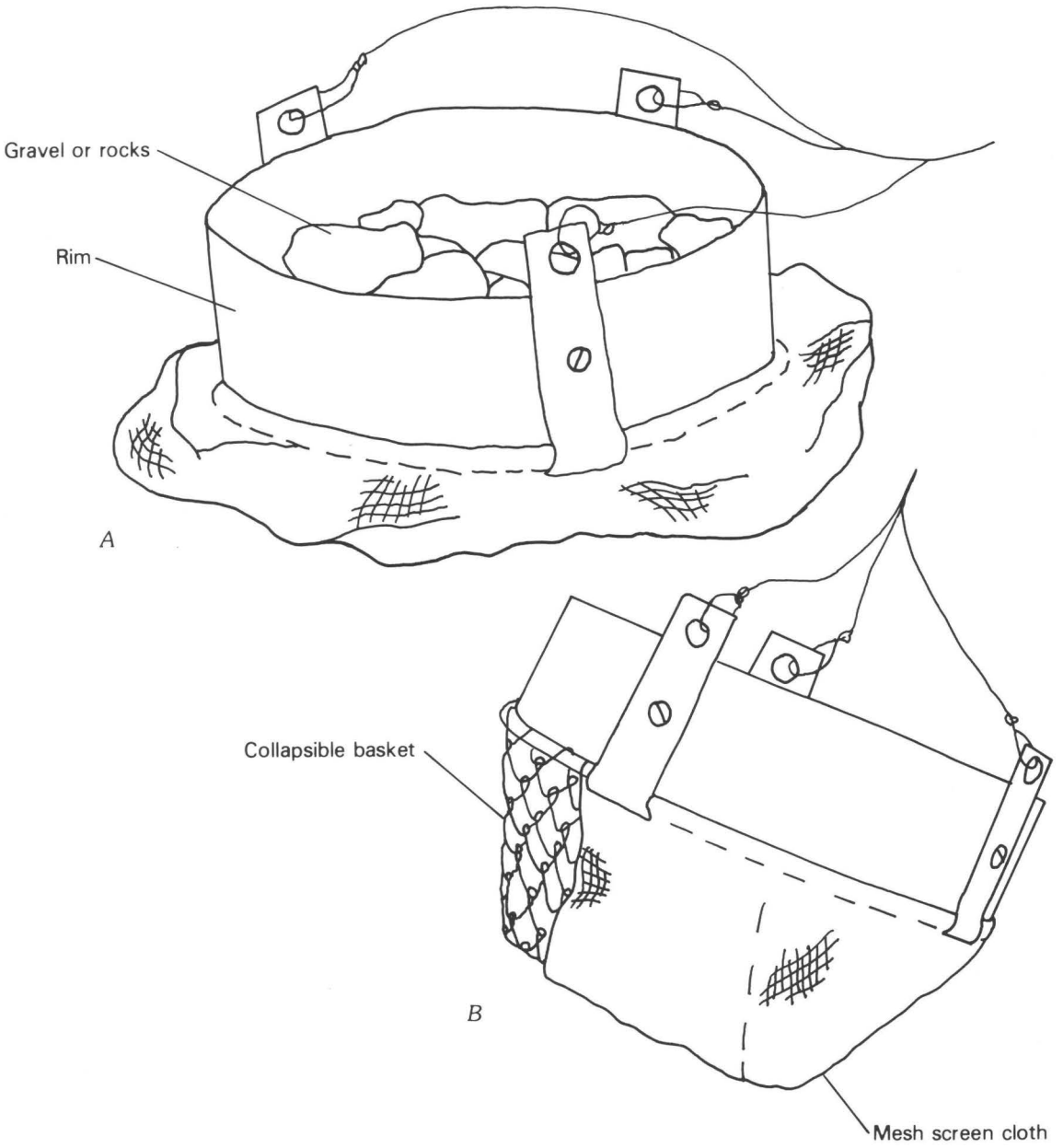


Figure 28.--Collapsible-basket artificial-substrate sampler: (A) Resting on streambed. (B) Being retrieved. (Redrawn from Bull, 1968.)

Expose the samplers in uniform macrohabitats at all sites during a study. If possible, install three samplers in a riffle in shallow streams. Make the collections as representative of the reach as possible by ensuring that the samplers are not close to the bank. In streams as much as a few meters in width, install the devices about midstream; in larger streams, install the devices about one-quarter of the total width from the nearest bank. Currents occasionally hinder the collapse of the sampler, but this can be overcome by connecting a strong rubberband to one side of the basket rim, extending it under the bottom of the wire basket, and attaching it to the other side of the

rim (Bull, 1968). The samplers are stable on the bottom at velocities as much as 0.9 m/s, but recovery often is easier if a line or light chain connects the sampler to an inconspicuous anchorage. At velocities greater than 0.9 m/s, the samplers should be anchored.

Distribution and Abundance

Absolute quantitative surveys are used to determine the numbers or biomass per unit area of streambed or lakebed and indicate changes in space and time. This type of sampling requires the greatest effort and, in many environments, the objectives cannot be achieved. Because all methods are somewhat selective, comparisons of the benthic invertebrates between sites or sampling dates should be based on uniform sampling methods.

The statistical principles of benthic-invertebrate sampling are discussed by Elliott (1971a). The first requirement is a clear definition of the objectives of the study and the area to be sampled.

When a knowledge of numbers or biomass per unit area is required, the major considerations are: (1) The size of the sampling units, (2) the number of sampling units in each sample, and (3) the location of sampling units in the sampling area. In general, the smaller the sampling units used, the more accurate and representative will be the results. Practical factors, such as particle size, will set a lower limit to the sampling-unit dimensions. Large numbers of sampling units in the total sample (greater than 50) are preferable, but usually impractical because of the labor involved in collection and analysis. The size of small samples can be calculated with a specified degree of precision (Elliott, 1971a, p. 128-131). The sampling units usually are randomly located in the sampling area, and all the available sites in the area must have an equal chance for selection. Stratified random sampling is preferable to simple random sampling.

A complete and accurate estimate of the numbers of all species in a large area of bottom often is impossible. Therefore, "* * * most quantitative investigations are restricted to a study of a small number of species in a large area, or a larger number of species in a small area * * *" (Elliott, 1971a, p. 127). This means that if the study objective is to compare the number and abundance of species at several sites or on different sampling dates, numbers or biomass per unit area may be needed only for a particular type of homogeneous substrate. However, the area of the substrate sampled must be clearly defined.

The literature about the quantitative study of benthic invertebrates in flowing water was reviewed by Hynes (1970) who concluded that quantitative data about the benthic invertebrates are extremely difficult to obtain and are, at best, very rough estimates. Nevertheless, if three or more samples are collected, a general idea of the abundance of the more common species can be obtained. Sampling in a long transect line, which parallels some obvious environmental gradient, such as from shallow to deep water, provides a greater probability that most species will be collected at least once (Elliott, 1971a, p. 127).

Sampling frequency must be based on study objectives. Waters (1969a) and Cummins (1975) emphasized that sampling for the estimation of benthic invertebrate production should be done during the period of maximum change in growth and survivorship. For populations having typical survivorship and maximum mortality during the early instars and having approximately exponential growth curves, initial sampling should be at short intervals and later sampling at decreased frequency. For a complete faunal study, short-interval sampling, weekly, or less, should be done during periods when most of the species are in early age classes. In the temperate zone, this period generally is late spring and late fall (Cummins, 1975).

Quantitative studies require the collection from the sampling unit of all benthic invertebrates within the selected size range. The area of the sampling unit is defined by the area of the sampling device, but the depth to which sampling should extend into the sediments remains a problem. The vertical distribution of invertebrates in soft sediments (Lenz, 1931; Cole, 1953; Ford, 1962; Brinkhurst and others, 1969) and in coarse sediment (Coleman and Hynes, 1970; Mundie, 1971; Bishop, 1973) has been studied. As a guide to the depth of sampling, Cummins (1975) proposed measuring the oxygen profile in the sediment to determine the depth of the oxygenated zone (Ericksen, 1963) or sampling at least to a depth at which the sediment seems anaerobic; 0.01 to 0.1 m in fine, homogeneous sediment and 0.1 to 0.3 m in coarse, heterogeneous sediment.

Brinkhurst (1967) listed the following theoretical specifications for a quantitative sampler:

1. Depth of penetration. Invertebrates are found deep in the sediment, and a true measurement of total standing crop or proportional representation of species requires that the sampler collect sediment from the surface to a depth of at least 20 cm.

2. Bite. The bite of a sampler should be deep enough so all depths are sampled equally in any one attempt. The bite characteristics should enable accurate estimation of the surface area that was sampled.

3. Closing mechanism. Complete closure is required, or some of the sample will be lost. The closing mechanism should be powerful enough to shear through twigs and other obstructions.

4. Internal pressure. The descent of a sampler should not cause a pressure wave that will disturb the topmost sediment or give a directional signal to invertebrates capable of retreating from the sample area.

Although a corer that is completely open during descent satisfies many of the theoretical requirements in still water, no sampler presently available satisfies all requirements, especially for rocky sediment and flowing water. One problem is that any solid object, such as a corer or box, lowered into a stream deflects the current downward and scours the bottom where the sample is to be collected (Macan, 1958). The devices listed in the following sections are those most commonly used or those that seem to be best suited to the work of the U.S. Geological Survey.

Box, drum, or stream-bottom fauna sampler

The box, drum, or stream-bottom fauna sampler (fig. 29), depending on its design, is used by pushing the bottom edge downward to seal a compressible edge or by rotating a cylinder back and forth into the substratum. In the latter design, teeth dig into the bed, and a flange of metal and foam rubber or plastic also isolates the enclosed area. In flowing water, mesh panels in the sides of the sampler decrease scour as it approaches the bottom. To remove the invertebrates from the sample area, begin by placing the large rocks into a bucket of water. Thoroughly disturb the remaining sediment by digging and stirring as deeply as possible using a garden trowel or fork, then stir the water vigorously using a small dip net to strain suspended material from the liquid. Some samplers have an attached bag net into which suspended invertebrates are carried by the current. Others require repeated sweeps. Empty the dip net into the bucket and continue the process until no additional invertebrates are collected. More sediment from the enclosed area may need to be removed as digging and stirring proceed. Remove the large rocks from the bucket and discard after scrubbing using a soft bristle brush. Pour the contents of the bucket through a U.S. Standard no. 70 sieve. Transfer the concentrated sample to a shallow, white tray, if the sample is to be sorted onsite, or into a wide-mouth container for transporting to the laboratory. Label and preserve each collection.

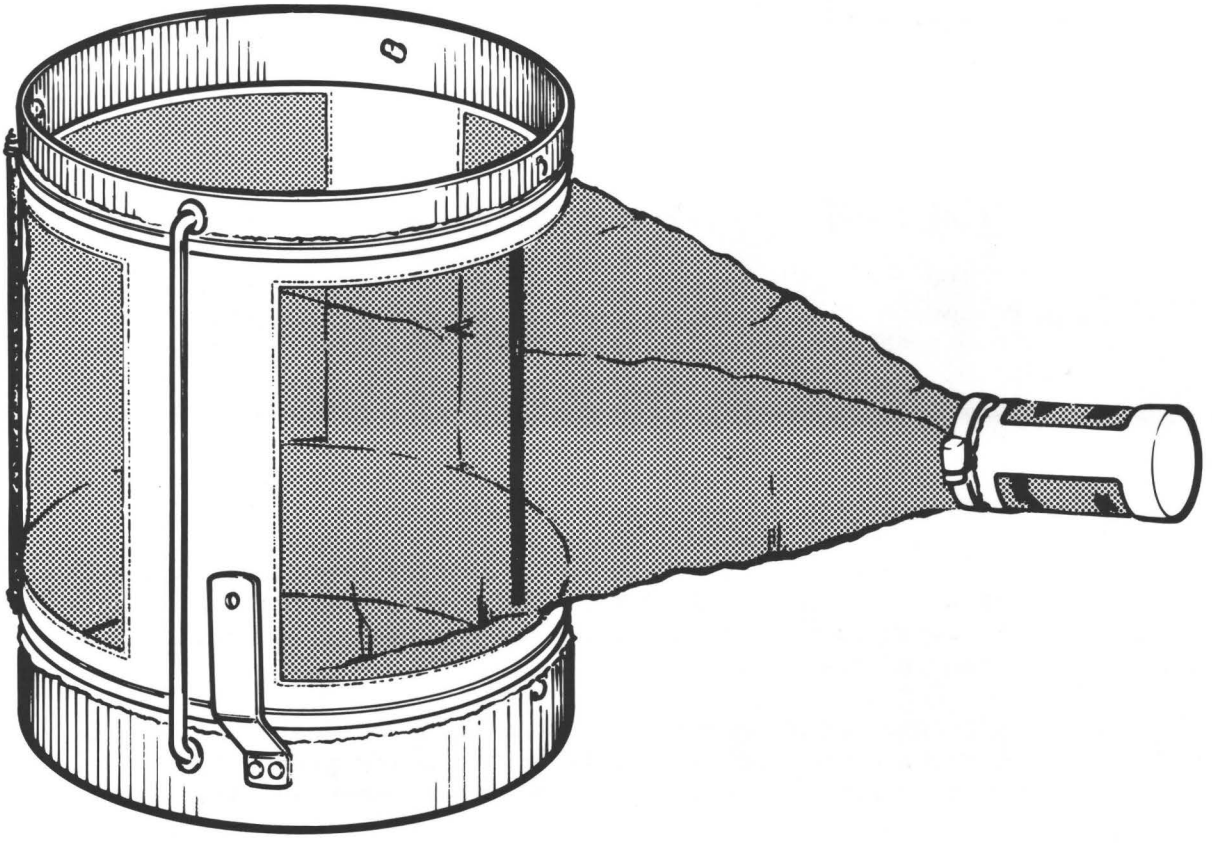


Figure 29.--Box, drum, or stream-bottom fauna sampler (sketch courtesy of Kahl Scientific Instrument Corp., El Cajon, Calif.).

Surber sampler

Press the bottom edge of the Surber sampler (fig. 30), or one of the modified samplers, firmly against the substrate to isolate the enclosed area as completely as possible. These samplers depend on the current to carry invertebrates into an attached net bag. Slack (1955) enclosed the sides and front of a Surber sampler with wire mesh and, in slowly moving water, used a rectangular fabric-covered paddle to produce a flow sufficient to sweep benthic invertebrates into the net. To remove the invertebrates from the area enclosed by the sampler, lift the larger rocks and scrub them into the mouth of the net. Thoroughly disturb the remaining sediment by repeatedly digging and stirring as deeply as possible, allowing the current to sweep the invertebrates and lighter detritus into the bag net. It is important, but difficult in practice, to avoid contamination of the sample by material from outside of the enclosed area. Empty the contents of the bag net into a shallow, white tray, if the sample is to be sorted onsite, or into a wide-mouth container for transporting to the laboratory. Label and preserve each collection.

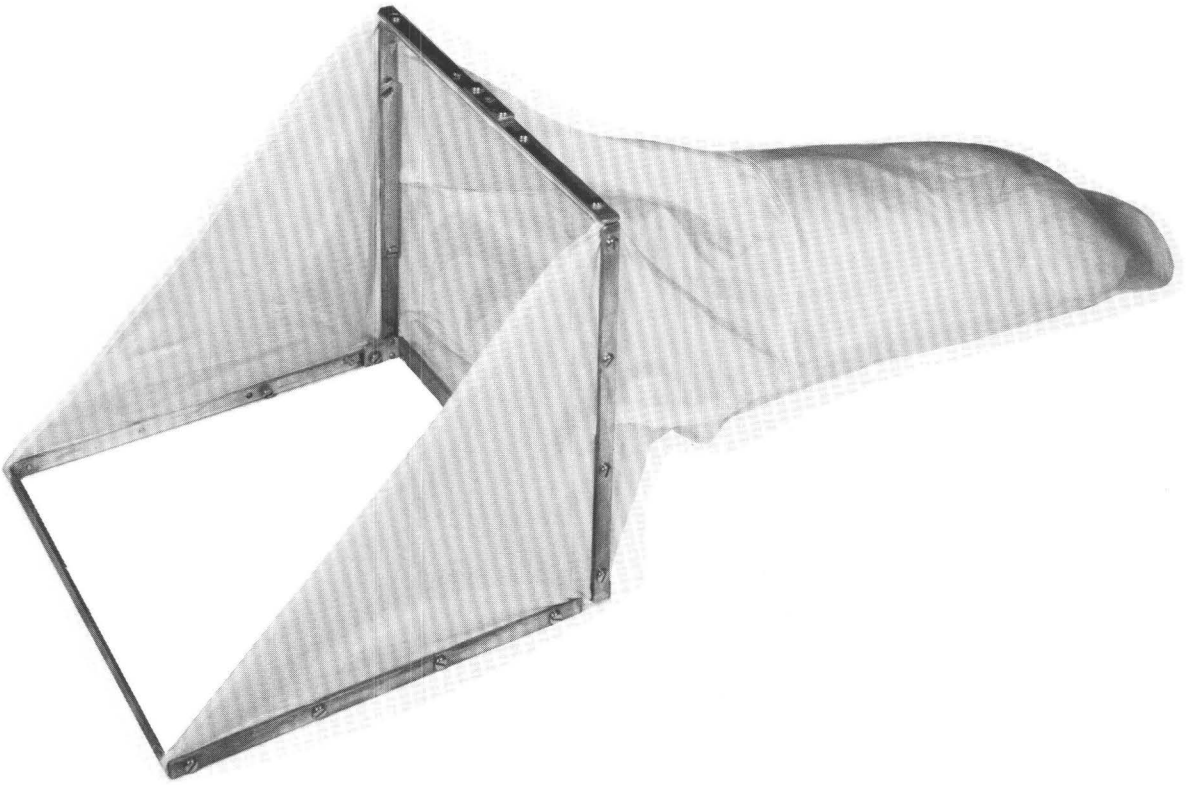


Figure 30.--Surber sampler (photograph courtesy of Wildlife Supply Co., Saginaw, Mich.).

Ekman grab

The preferred sampler for mud, silt, or fine sand is the Ekman grab (fig. 31). In shallow water, the sampler is operated manually, usually mounted on a pole. The Ekman grab can be used in this way to sample fairly hard sediment because the operator can force the sampler shut by exerting additional pressure on the upper edge of each jaw. In deep water, the sampler is lowered to the bottom, allowed to settle into the sediment, and then closed by dropping a messenger down the line.

In a tank and onsite comparison of seven grabs, Elliott and Drake (1981a) reported that the pole-operated Ekman grab performed well on a predominantly muddy bottom (particle size 0.004-0.06 mm) where the mean depth of penetration into the bottom was greater than 5 cm. In fine gravel of modal size (2-4 mm), efficiencies in terms of numbers per square meter were 54 percent, and the depth of penetration was less than 5 cm. The grab did not perform satisfactorily on a predominantly gravel bottom that had some rocks larger than 16 mm.

At the water surface, the sampler jaws are opened and the contents emptied into a tub, a large sieve, or a wide-mouth container for transporting to the laboratory. Label and preserve each collection.

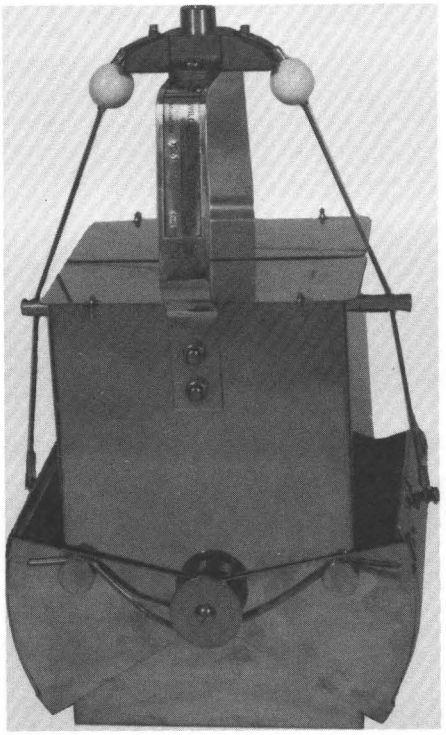


Figure 31.--Ekman grab, tall design (photograph courtesy of Wildlife Supply Co., Saginaw, Mich.).

Ponar and Van Veen grabs

Ponar and Van Veen grabs (figs. 32, 33) are heavy samplers that should be operated using a winch. They generally are used for deep-water sampling in gravel, hard sand, and clay, as well as in soft sediment. These instruments close on contact with the bottom; but, to operate effectively, they must bite vertically. This requirement poses little problem in lakes, but in river work, bottom sampling is especially difficult. When used from a drifting boat, the grab sometimes can be lowered nearly to the bottom, then dropped suddenly so it makes contact in an upright position.

In a tank and onsite comparison of seven grabs, Elliott and Drake (1981a) reported that the Ponar performed well on a predominantly muddy bottom (particle size 0.004-0.06 mm) where the mean depth of penetration into the mud was greater than 5 cm. In fine gravel of modal size (2-4 mm), and where the mean depth of penetration was greater than 5 cm, efficiencies in terms of numbers per square meter were 94 percent for the unweighted Ponar and 93 percent for the weighted Ponar. The only grab to operate adequately on a gravel bottom that had some rocks greater than 16 mm was the weighted Ponar.

In a tank and onsite comparison of seven grabs, Elliott and Drake (1981a) reported that the Van Veen grab had an efficiency of 71 percent in terms of numbers per square meter on a fine-gravel bottom (modal size 2-4 mm). The mean depth of penetration was greater than 5 cm. However, the Ekman and Ponar grabs performed better than the Van Veen grab on a predominantly muddy bottom.

Empty the sampler into a tub, and if mud is present, wash it from the sample. Pour the contents of the tub through a U.S. Standard no. 70 sieve. Transfer the concentrated sample to a shallow, white tray, if the sample is to be sorted onsite, or into a wide-mouth container for transporting to the laboratory. Label and preserve each collection.

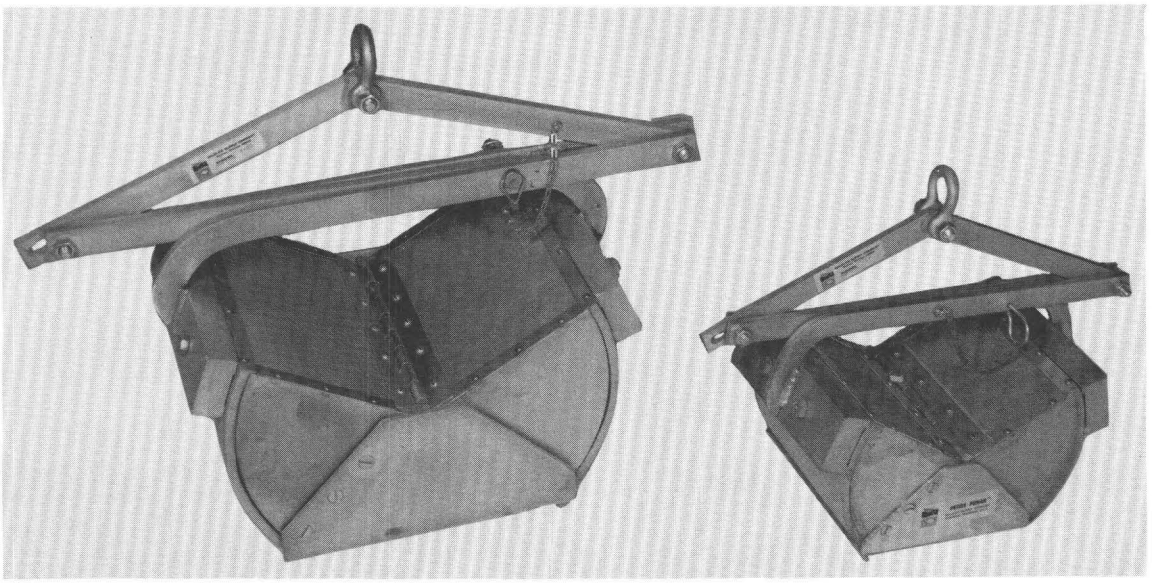


Figure 32.--Ponar grab (photograph courtesy of Wildlife Supply Co., Saginaw, Mich.).

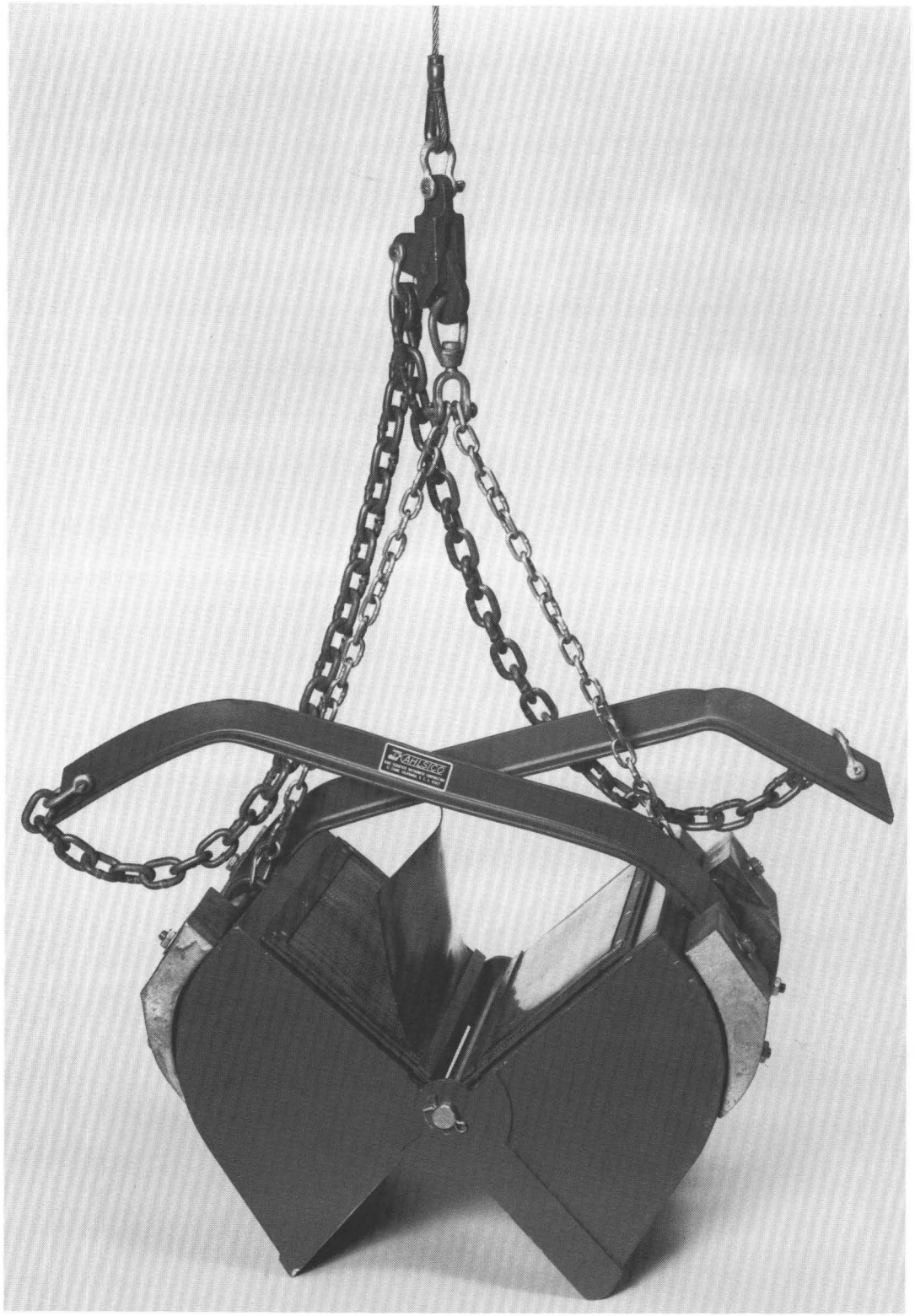


Figure 33.--Van Veen grab (photograph courtesy of Kahl Scientific Instrument Corp., El Cajon, Calif.).

Corers

These devices are used when an undisturbed sample of sediment is required. They are suitable especially for clay, silt, or sand bottom, and are used more widely in lakes than in streams. Hand corers designed for manual operation can be used in shallow water as much as several meters in depth. Deeper water requires devices such as the K.B.-type or Phleger corer (fig. 34), which depend on gravity to drive them into the sediment. All corers have been designed to retain the sample as the instrument is withdrawn from the sediment and returned to the surface. Follow the manufacturer's instructions for operating corers. Depending on the study objectives, sections of the core can be extruded and preserved separately, or the entire core may be retained in the tube. Intact cores are best preserved by freezing, but the sample can be sieved, labeled, and preserved.

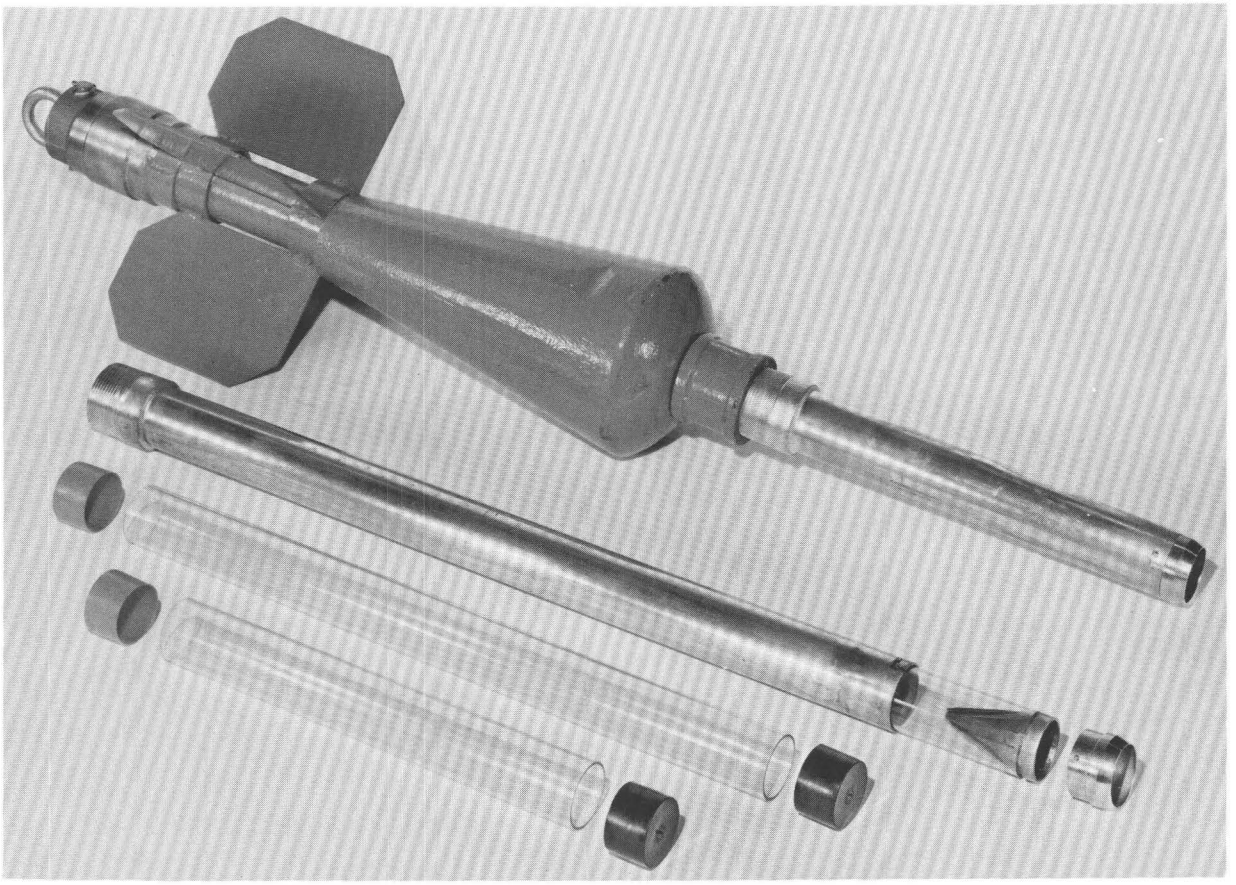


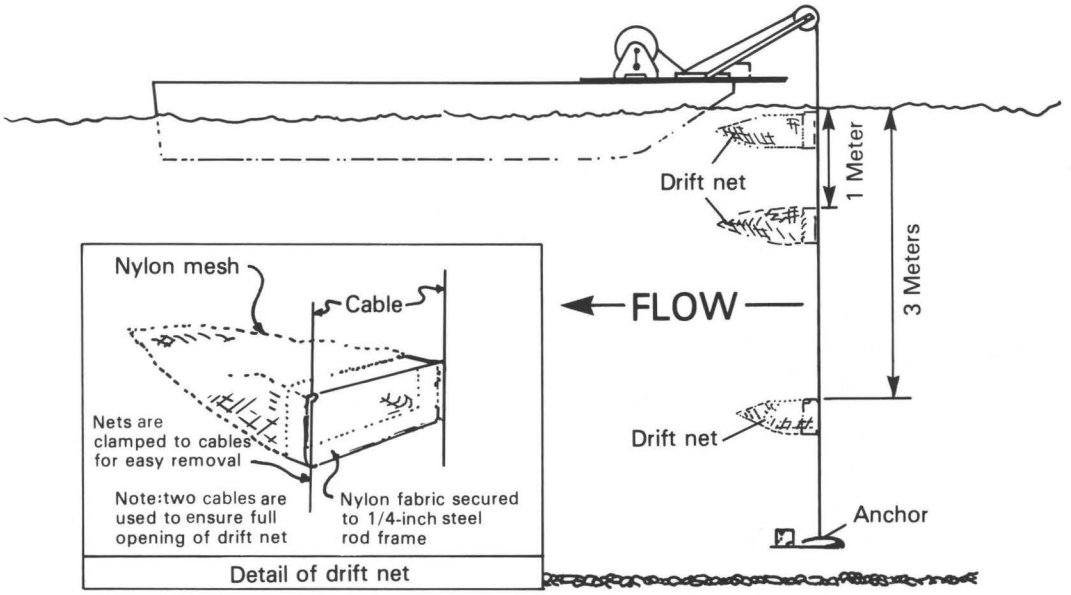
Figure 34.--Phleger corer (photograph courtesy of Kahl Scientific Instrument Corp., El Cajon, Calif.).

Invertebrate Drift

Studies have indicated that many kinds of benthic invertebrates become entrained in streamflow and that the resulting downstream drift of invertebrates is a regular feature of running water (Waters, 1969b, 1972; Müller, 1974). Because drifting invertebrates come from a variety of habitats, drift samples contain a relatively large variety of taxa (Waters, 1961; Larimore, 1974; Slack and others, 1976). The rate of invertebrate drift is affected by many factors, including light intensity, time of day, season of the year, stream discharge, and weather. The relation of invertebrate drift to water quality has been reported by Coutant (1964), Besch (1966), Wojtalik and Waters (1970), Wilson and Bright (1973), and Larimore (1974). Collections should be made upstream from any artificial disturbance of the streambed or banks. The distance that invertebrates drift varies with different species and with environmental conditions. Estimates of drift distances range from less than 1 m to more than 100 m (Hemsen, 1956; Waters, 1965; McLay, 1970), although McLay (1970) and Elliott (1971b) reported an exponential upstream decrease in the number of benthic invertebrates in the drift. Drift collections for impact assessment should be made; however, the fact that clean-water invertebrate species can be carried into stressed areas where they cannot survive needs to be emphasized.

Methods and equipment for collecting invertebrate drift are described by Elliott (1970). Drift samplers vary from simple nets to elaborate battery-powered devices capable of automatically collecting up to eight timed samples. A simple net of 210±2-μm or other appropriate mesh size on a square or rectangular frame is sufficient for making invertebrate drift collections (fig. 35). In shallow water, anchor the net with the opening upstream by driving steel rods into the streambed. Two types of deep-water exposures are shown in figure 36. Study objectives will determine the location, type, and duration of net exposure. Nets anchored downstream from riffles will catch more invertebrates than those downstream from pools, and the greater the volume of flow through the net, the larger the collection. The vertical position of drift nets in the water column is determined by water depth and study objectives. In water as much as 1 m deep, a mid-depth position commonly is used for a single drift net. Nets may be stacked, one above the other, to sample the entire water column from surface to bottom (Waters, 1969a). If the net opening is in contact with the stream bottom, nondrifting invertebrates may be collected. If the net opening extends above the water surface, the collection will include maximum numbers of floating adults, pupae, exuviae, and terrestrial species. If only aquatic invertebrates and life stages are of interest, the top of the net should be under water. In deep rivers, the net(s) may be near the stream bottom or near the surface, but the technique should be uniform if comparable collections are required. Because drift rates are faster at night than during the day, drift data are needed for at least 24 hours and collection periods commonly are 30 minutes, or 1-, 2-, or 3-hours, although collecting sometimes can last as much as 8 hours using properly designed nets. At the end of the collecting period, empty each net into a separate shallow, white tray, if the collection is to be sorted onsite, or into a wide-mouth container for transporting to the laboratory. Label and preserve each collection. Invertebrate drift can be collected as an adjunct to a faunal survey to determine drift density or to determine drift rate. Collection methods will vary depending on the study objectives.

Figure 35.--Stream drift nets (photograph courtesy of Wildlife Supply Co., Saginaw, Mich.).



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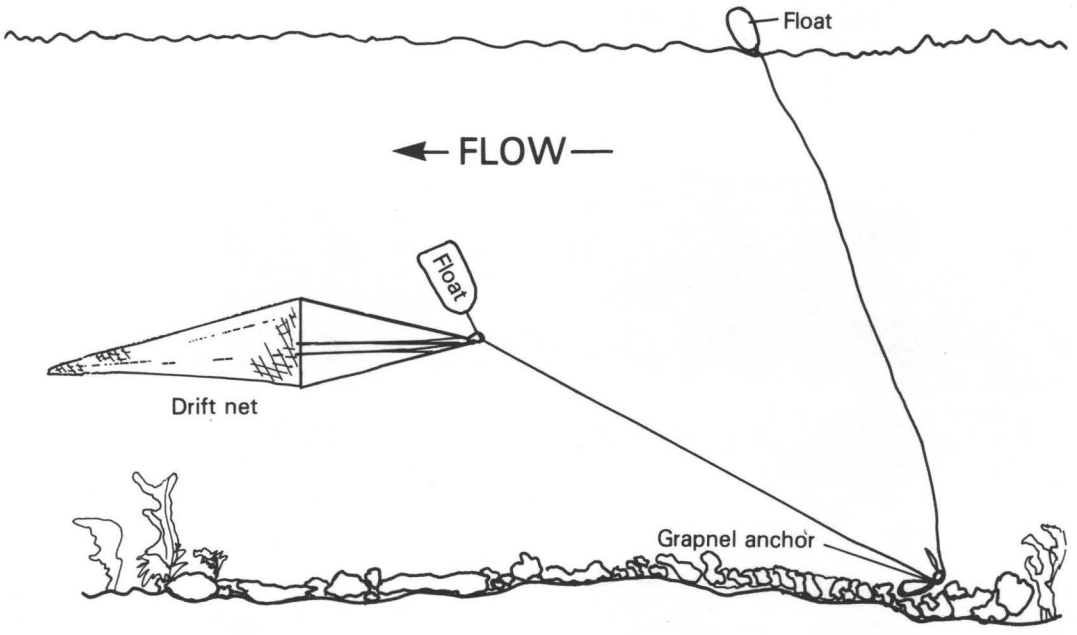

B

Figure 36.--Methods of exposing drift nets in deep rivers: (A) From an anchored boat (from Ferreira and Hoffman, 1978). (B) Float-supported net (from J. L. Barker, U.S. Geological Survey, written, commun., 1982).

Drift density

The nets, location, and exposure periods described in the preceding section are suitable for determination of invertebrate drift density (the quantity of invertebrates per unit volume of water) when the volume of water passing through the net during the collection period is known. Water volume can be determined from an average of the speed of the current measured in the mouth of the net at the beginning and the end of the collection period, multiplied by the area of the net opening and the length of the exposure period. A digital flowmeter mounted in the net opening can be used to determine the cumulative volume of water passing through the drift net. Drift density usually is assumed to be fairly uniform in the cross section at a given time (Waters, 1972), and results from a single drift net are assumed to be adequate. This can be checked by collecting using two or more nets exposed simultaneously at different points in the cross section.

Drift rate

The drift-density procedures also are suitable for determination of invertebrate drift rate (the quantity of invertebrates passing a given point per unit of time). Drift rate can be calculated from drift density if stream discharge is known. When drift density and discharge values are available for a 24-hour period, the total daily drift rate per instantaneous discharge or per total daily discharge can be calculated.

Sample Preparation

Samples for which only biomass will be determined need to be frozen, preferably freeze-dried, as soon as possible after collection. Samples for taxonomic determination need to be preserved in alcohol or formaldehyde. (Use of alcohol for preserving samples for biomass determinations will result in small values because of extraction of alcohol-soluble substances from the invertebrates.) To ensure adequate preservation of benthic-invertebrate collections, fill containers no more than one-half full with the sample so a volume of preservative can be added at least equal to the volume of organic material, including detritus. Preserve the invertebrates or the unsorted samples in 70-percent ethyl alcohol, 70-percent isopropyl alcohol, or 4-percent formaldehyde solution. If formaldehyde is used, replace with alcohol prior to identification and enumeration. Containers should be filled to the top to avoid excessive sloshing and damage to delicate specimens. If unsorted samples are to be stored for more than a few weeks, the preservative should be drained after 1 week and replaced with fresh preservative.

Label samples indicating the location, habitat, date and time of collection (local standard time) for drift collections, name of collector, and sample preparation (type of preservative, mesh size of sieves or nets, or other treatment). Soft black pencil may be used onsite, but use a water-proof carbon ink for permanent labels. Place labels inside the sample containers so they are visible from the outside, or place duplicate labels inside and outside the containers. Secure jar lids using tape to prevent loosening and subsequent loss of preservative by evaporation. This is especially important if samples are to be shipped or stored for more than a few weeks.

Sample Sorting

A requirement of all benthic-invertebrate methods is to separate the invertebrates from sediment and detritus in the samples. The following general apparatus, reagents, and procedures for sample sorting apply to all methods in this section.

Apparatus

A.1 Dishes, glass, petri, or Syracuse watchglasses.

A.2 Forceps that have fine or rounded points. Forceps that have fine points are useful for handling small invertebrates. Forceps that have rounded points are less likely to tear netting or puncture the mesh of sieves or other sampling equipment.

A.3 Hydrometer, plain form, range 1.000 to 1.220.

A.4 Ink, waterproof.

A.5 Labels, waterproof, or labels may be cut from sheets of plastic paper.

A.6 Microscope, stereoscopic variable power, 7X to 30X, and microscope illuminator.

A.7 Pipet, wide-bore.

A.8 Scoops, fine-mesh, made in various sizes and shapes, as needed, from pieces of brass or stainless-steel wire mesh attached to a handle. A convenient handle for the scoops is an X-Acto knife handle, or equivalent.

A.9 Sieves, U.S. Standard, 20-cm diameter, and mesh size appropriate to the study objectives. The no. 70 sieve (210-µm mesh opening) has been adopted for retaining benthic invertebrates collected as part of the water-quality programs of the U.S. Geological Survey. Sieves that have smaller or larger mesh may be more suitable for some studies. The no. 18 sieve (1,000-µm mesh opening) is useful for removing large rocks and sticks from samples. Stainless-steel mesh is recommended for all sieves because of its greater durability compared to brass.

A.10 Subsampler jar (Hynes, 1970, p. 244). Divide the bottom of a screw-topped jar into equal quadrants about 2 cm deep by embedding thin cardboard or plastic in paraffin.

A.11 Tape, plastic, or paraffin for sealing jar and vial lids.

A.12 Trays, white enamel. Useful sizes are 30×19×5 cm and 42×26×6 cm.

A.13 Vials that have poly seal screw lids. Convenient sizes are 7.5-, 15-, and 22-mL capacity.

Reagents

R.1 Rose Bengal biological stain.

R.2 Sucrose solution, specific gravity 1.12, for density separation of invertebrates from the debris in benthic samples. Dissolve 360 g granulated sugar per liter of water.

Procedure

P.1 If the study objectives require determination only of the most abundant benthic invertebrates, sorting often can be completed onsite. Wash the sample gently in a sieve of appropriate mesh size to remove mud and fine detritus. Pick the invertebrates directly from the sampled material; or, to enhance visibility of small invertebrates, cover the sample with water in a white enamel tray and stir repeatedly while removing the invertebrates using forceps or scoops.

P.2 Generally, sorting must be done in the laboratory. Pour small quantities of the sample into a shallow dish, covering the material with water, and scan the dish under low-power magnification (7X to 10X). Remove the invertebrates from the debris using forceps, fine-mesh scoops, or wide-bore pipets.

The sorting process is very time consuming for many types of collections. The optional steps described in the following paragraphs may be used to speed the work when the study objectives require complete analysis.

P.3 Density separation (optional). This step consists of treating the sample with a solution of such density that most of the invertebrates will float, and most of the unwanted detritus will sink. The recommended method employs a sucrose solution that has a specific gravity of 1.12 (Anderson, 1959; Lackey and May, 1971).

Drain the sample in a no. 70 or other appropriate sieve, discard the liquid, and transfer the residue to a white enamel tray. Flood the material in the tray with the sugar solution, and stir so the material is evenly spread over the bottom. Remove invertebrates quickly from the surface of the liquid using forceps, fine-mesh scoops, or wide-bore pipets. After removing all visible invertebrates, stir the material and remove any other invertebrates that appear. Pour the sugar solution through the sieve and cover the residue in the tray with water. Examine as described in P.2 looking carefully for oligochaete worms, for aquatic mites, and for heavier invertebrates, such as mollusks and caddisfly larvae. After this examination, pour the water through the sieve and repeat the sucrose treatment. Few invertebrates should be found but, if large numbers are seen, soak the sample in water and again treat with the sugar solution. Reuse the sugar solution by adjusting the specific gravity to 1.12 as determined using a hydrometer. However, the solution spoils rapidly and should not be stored for more than a few days.

P.4 Differential staining (optional). Separation of invertebrates, especially transparent forms, from detritus in the samples is facilitated by staining them red using 200 mg/L of Rose Bengal added to the preservative solution. Expose the invertebrates to the stain for at least 24 hours before examination. Prolonged contact with the stain may result in uptake of the red color by algae and plant detritus. If necessary to restore natural coloration for identification, remove the stain from the invertebrates by placing them in 95-percent ethyl alcohol (Mason and Yevich, 1967). A counterstaining technique in which Rose Bengal or Lugol's solution is counterstained with chlorazol black may be used to provide a definite color contrast between invertebrates and detritus (Williams and Williams, 1974).

P.5 Subsampling (optional). Some benthic samples are so large, or contain such large numbers of invertebrates, that sorting or counting the entire sample is impractical. Remove the larger invertebrates and pieces of detritus from the entire sample. Transfer the remainder of the sample to a screw-topped subsampler jar and add 70-percent alcohol to a depth of 10 to 12 cm. Close the jar and invert several times to mix thoroughly, then wait until the invertebrates have settled. Remove the contents of any two opposite quadrants using a wide-bore pipet to obtain one-half of the original sample. Repeat the process on one-half of the sample if further subsampling is required before sorting and counting.

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Faunal Survey (Qualitative Method)
(B-5001-85)

Parameter and Code: Not applicable

1. Applications

The method is applicable to all water.

2. Summary of method

Benthic invertebrates are collected by hand, dip net, dredge, or any other procedure appropriate to the environmental conditions and to the objectives of the study. The sampling equipment described in the following methods may be used to ensure that all habitats are sampled. Unsorted samples, usually containing varying quantities of sand, gravel, and plant detritus, are preserved onsite. In the laboratory, the benthic invertebrates are sorted from the extraneous material, identified, and counted. Results are reported as numbers of different kinds of benthic invertebrates (taxa) and the relative abundance of each taxon at different sites or times.

3. Interferences

Physical factors, such as stream velocity and depth of water, may interfere with sample collection. Most samples contain relatively large quantities of sediment and plant debris from which the benthic invertebrates must be sorted.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Biological dredge (fig. 21). The design depends on environmental conditions and study requirements.

4.2 Dip or hand nets are made in various shapes and sizes, are sturdy in design, and have a flat side for pressing the net closely against the streambed. Commercial nets are available in various materials and mesh sizes. The desired material and mesh opening should be specified when ordering. Dip nets for general use in the U.S. Geological Survey should have bags of 210±2-µm mesh-opening nylon or polyester monofilament screen cloth, unless otherwise indicated by the study objectives.

4.3 Forceps that have fine or rounded points. Forceps that have fine points are useful for handling small invertebrates. Forceps that have rounded points are less likely to tear netting or puncture the mesh of sieves or other sampling equipment. Forceps are less likely to be lost onsite if marked with bright paint or colored tape.

4.4 Gloves, waterproof, Trapper's, shoulder length.

4.5 Ink, waterproof.

4.6 Labels, waterproof, or labels may be cut from sheets of plastic paper.

4.7 Microscope, stereoscopic variable power, 7X to 30X, and microscope illuminator. A compound microscope of at least 200X magnification also is useful for taxonomic work.

4.8 Pipe dredge (fig. 22). This simple device, or a modification, is useful for collection of benthic invertebrates in swift, rocky rivers. Commercial dredges weigh 25 kg, but smaller and lighter versions can be made for special purposes. For collecting benthos, the dredge may be constructed without a bottom and with a sturdy mesh bag secured over the rear opening by a hose clamp.

4.9 Sample containers, plastic or glass, and plastic lids, for transporting unsorted samples to the laboratory. Wide-mouth jars of 120-, 240-, and 475-mL capacity are useful sizes. Sealable plastic bags also may be used for temporary storage of benthic-invertebrate samples.

4.10 Sieves, U.S. Standard, 20-cm diameter, and mesh size appropriate to the study objectives. The no. 70 sieve (210-µm mesh opening) has been selected for retaining benthic invertebrates collected as part of the water-quality programs of the U.S. Geological Survey. Sieves that have smaller or larger mesh may be more suitable for some studies. The no. 18 sieve (1,000-µm mesh opening) is useful for removing large rocks and sticks from samples. Stainless-steel mesh is recommended for all sieves because of its greater durability compared to brass.

4.11 Tape, plastic, or paraffin for sealing jar and vial lids.

4.12 Vials that have plastic poly seal screw lids. Convenient sizes are 7.5-, 15-, and 22-mL capacity.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Distilled or deionized water.

5.2 Glycerin.

5.3 Preservative solutions. Invertebrate samples may be preserved in 70-percent ethyl alcohol, 70-percent isopropyl alcohol, or 4-percent formaldehyde. A mixture of 70-percent ethyl alcohol and 5-percent glycerin is preferred for permanent storage. Prepare as follows:

5.3.1 Ethyl alcohol. Dilute 70 mL 95-percent alcohol to 95 mL using distilled water.

5.3.2 Ethyl alcohol and 5-percent glycerin. Dilute 70 mL 95-percent alcohol to 100 mL using 25 mL distilled water and 5 mL glycerin.

5.3.3 Isopropyl alcohol. Dilute 70 mL concentrated isopropyl alcohol to 100 mL using distilled water.

5.3.4 Formaldehyde. Dilute 10 mL 37- to 40-percent aqueous formaldehyde solution (formalin) to 100 mL using distilled water.

6. Analysis

Identify and count the benthic invertebrates in the sample according to taxonomic categories. The degree of identification required (species level is desirable) varies depending on the objectives of the study. A stereoscopic microscope is required; and, for some groups, dissections or microscopic mounts are needed to observe key characteristics. Appropriate reference books (Part 3, "Selected Taxonomic References" section of this report) should be available. The different categories of invertebrates can be placed in separate vials of 70-percent ethyl or 70-percent isopropyl alcohol, and can be labeled with the name of the invertebrate and the identification number, date, and origin of the sample. Add a few drops of glycerin or use the ethyl alcohol-glycerin preservative, and seal vial caps if the specimens are to be stored.

7. Calculations

7.1 When only part of the total sample is sorted or counted, project the results from the subsample to the number of specimens in the total sample:

$$\text{Total number of benthic invertebrates of a particular taxon in sample} = \frac{\text{Number of benthic invertebrates of the taxon in subsample}}{\text{Fraction of total sample in subsample}} .$$

7.2 Percent composition in sample

$$= \frac{\text{Number of benthic invertebrates of a particular taxon}}{\text{Total number of individuals of all taxa}} \times 100 .$$

8. Reporting of results

Report the number of taxa present, the percent composition of each taxon in the sample, and the type of sampling method(s) used.

9. Precision

No numerical precision data are available.

10. References cited

None.

Numerical Assessment (Relative or Semiquantitative Method) (B-5020-85)

Parameters and Codes:

Invertebrates, benthic, wet weight (g/m^2):	70940
Invertebrates, benthic, dry weight (g/m^2):	70941
Invertebrates, benthic, ash weight (g/m^2):	70942
Invertebrates, benthic, total ($organisms/m^2$):	70943

This method assumes that the objective is to compare the kinds and relative abundances of taxa in samples from several sites or on different sampling dates. The differences between samples are assumed to be directly proportional to differences between the sites or dates. The artificial-substrate method is recommended when collections must be made by persons inexperienced in biology. The procedures described in the "Distribution and Abundance (Quantitative Method)" section also are applicable to sample collection from homogeneous substrates.

1. Applications

The method is applicable to all water and especially is useful for indicating water-quality trends or differences between sites.

2. Summary of method

Benthic invertebrates are collected using uniform procedures throughout a wide area or collected from small, homogeneous areas at sites that are to be compared. Sampling methods include collecting samples, using a dip net, in a standardized manner or for a definite period of time, collecting samples from individual rocks, and using artificial substrates. Unsorted samples, usually containing varying quantities of sand, gravel, and plant detritus, are preserved onsite. In the laboratory, the benthic invertebrates are sorted from the extraneous material, identified, and counted. Biomass is determined if appropriate to the study objectives. Results are reported as numbers of different kinds of benthic invertebrates (taxa) and relative abundance of each taxon for the total collection or for a particular habitat or artificial substrate. Biomass is reported as wet, dry, ash, or ash-free weight.

3. Interferences

Physical factors, such as stream velocity, depth of water, and large rocks, may interfere with sampling in natural substrates. In these places, artificial substrates may provide adequate samples. However, because all sampling methods are selective, all the collections for a particular study must be done in a uniform way. Most samples contain sediment and plant debris from which the invertebrates must be separated. Losses of artificial-substrate samplers to environmental hazards or vandalism may preclude their use at some sites.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Artificial-substrate float, consisting of a 0.6-m length of polyvinylchloride (PVC) tubing that has a 5-cm inside diameter (ID) and ends sealed (fig. 25). Two clear Plexiglas stabilizer fins are attached near one end and an eyebolt at the other end. One to three multiple-plate samplers are suspended on rods below the float to a depth of 0.3 m measured from the water surface to the midpoint of each sampler.

4.2 Balance, capable of weighing to at least 0.1 mg.

4.3 Barbecue-basket artificial-substrate sampler (Mason and others, 1967), a cylindrical, welded-wire basket, about 18 cm in diameter and 28 cm long. The basket is filled with 30 rocks, 5 to 8 cm in diameter, or with porcelain spheres that provide interstices for invertebrate colonization and weight for stability (fig. 27). The basket may be placed on the bottom, or it may be suspended above the bottom from a fixed structure or a surface float. A suitable float is a 19-L metal container filled with polyurethane foam.

4.4 Brush, soft-bristle, for scrubbing invertebrates from rocks.

4.5 Collapsible-basket artificial-substrate sampler (Bull, 1968), consisting of a commercially manufactured basket of coiled wire, bolted to a metal or plastic rim made from 38×3.3 mm stock (fig. 28). The basket is filled with gravel or rock and is covered by a bag of 210±2-μm mesh-opening nylon or polyester monofilament screen cloth, unless otherwise indicated by the study objectives. The basket collapses when lowered onto the streambed but assumes its original shape when raised. The surrounding net prevents escape of invertebrates.

4.6 Desiccator, containing silica gel or anhydrous calcium sulfate.

4.7 Dip or hand nets are made in various shapes and sizes, are sturdy in design, and have a flat side for pressing the net closely against the streambed. Commercial nets are available in various materials and mesh sizes. The desired material and mesh opening should be specified when ordering. Dip nets for general use in the U.S. Geological Survey should have bags of 210±2-μm mesh-opening nylon or polyester monofilament screen cloth, unless otherwise indicated by the study objectives.

4.8 Drying oven, thermostatically controlled for use at 105 °C.

4.9 Forceps that have fine or rounded points. Forceps that have fine points are useful for handling small invertebrates. Forceps that have rounded points are less likely to tear netting or puncture the mesh of sieves or other sampling equipment. Forceps are less likely to be lost onsite if marked with bright paint or colored tape.

4.10 Gloves, waterproof, Trapper's, shoulder length.

4.11 Ink, waterproof.

4.12 Labels, waterproof, or labels may be cut from sheets of plastic paper.

4.13 Lium sampler for individual rocks (Lium, 1974; fig. 23). The sampler consists of a 16-gauge sheet metal hood and an attached conical screen of 210-µm stainless-steel mesh. The base of the hood is padded with flexible foam rubber encased in nylon. The overall dimensions of the sampler are 65 cm long and 45 cm high, including the handle and a base area of 929 cm^2.

4.14 Microscope, stereoscopic variable power, 7X to 30X, and microscope illuminator. A compound microscope of at least 200X magnification also is useful for taxonomic work.

4.15 Muffle furnace, for use at 500 °C.

4.16 Multiple-plate artificial-substrate sampler, jumbo modification (Fullner, 1971). The sampler consists of fourteen 7.6-cm square or circular plates of 3.3-mm thick tempered hardboard separated by one or more 2.54-cm square or circular spacers of the same material (fig. 24). Plates 1 to 9 are separated by a single hardboard spacer, plates 9 and 10 are separated by two spacers, plates 10 to 12 are separated by three spacers, and plates 12 to 14 are separated by four spacers. The plates and spacers are held together by a 6.4-mm diameter by 20-cm eyebolt that passes through a hole drilled in the center of each piece.

4.17 Porcelain crucibles.

4.18 Retrieval net for multiple-plate sampler (fig. 26). It is a rectangular bag made from a 38-cm square of 210±2-µm mesh-opening nylon or polyester monofilament screen cloth, unless otherwise indicated by the study objectives. The screen-cloth square is folded in half and stitched along two sides. A nylon drawstring serves to secure the top of the net around the eyebolt of the sampler.

4.19 Sample containers, plastic or glass, and plastic lids, for transporting unsorted samples to the laboratory. Wide-mouth jars of 120-, 240-, and 475-mL capacity are useful sizes. Sealable plastic bags also may be used for temporary storage of benthic-invertebrate samples.

4.20 Sieves, U.S. Standard, 20-cm diameter, and mesh size appropriate to the study objectives. The no. 70 sieve (210-µm mesh opening) has been selected for retaining benthic invertebrates collected as part of the water-quality programs of the U.S. Geological Survey. Sieves that have smaller or larger mesh may be more suitable for some studies. The no. 18 sieve (1,000-µm mesh opening) is useful for removing large rocks and sticks from samples. Stainless-steel mesh is recommended for all sieves because of its greater durability compared to brass.

4.21 Tub or bucket for washing samples or sampling equipment onsite.

4.22 Vials that have plastic poly seal screw lids. Convenient sizes are 7.5-, 15-, and 22-mL capacity.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Distilled or deionized water.

5.2 Glycerin.

5.3 Preservative solutions. Invertebrate samples may be preserved in 70-percent ethyl alcohol, 70-percent isopropyl alcohol, or 4-percent formaldehyde. A mixture of 70-percent ethyl alcohol and 5-percent glycerin is preferred for permanent storage. Prepare as follows:

5.3.1 Ethyl alcohol. Dilute 70 mL 95-percent alcohol to 95 mL using distilled water.

5.3.2 Ethyl alcohol and 5-percent glycerin. Dilute 70 mL 95-percent alcohol to 100 mL using 25 mL distilled water and 5 mL glycerin.

5.3.3 Isopropyl alcohol. Dilute 70 mL concentrated isopropyl alcohol to 100 mL using distilled water.

5.3.4 Formaldehyde. Dilute 10 mL 37- to 40-percent aqueous formaldehyde solution (formalin) to 100 mL using distilled water.

6. Analysis

6.1 Identify and count the benthic invertebrates in the sample according to taxonomic categories. The degree of identification required (species level is desirable) varies depending on the objectives of the study. A stereoscopic microscope is required; and, for some groups, dissections or microscopic mounts are needed to observe key characteristics. Appropriate reference books (Part 3, "Selected Taxonomic References" section of this report) should be available. The different categories of invertebrates can be placed in separate vials of 70-percent ethyl or 70-percent isopropyl alcohol and can be labeled with the name of the invertebrate and the identification number, date, and origin of the sample. Add a few drops of glycerin or use the ethyl alcohol-glycerin preservative, and seal vial caps if the specimens are to be stored.

6.2 The biomass of benthic invertebrates, expressed as wet, dry, ash, or ash-free weight, is best determined from samples that were frozen immediately after collection. Biomass determined from alcohol-preserved samples is much less satisfactory (Howmiller, 1972; Stanford, 1973; Donald and Patterson, 1977).

Although generally determined from a total sample, biomass may be determined for an individual taxon. Cases or houses, such as caddisfly larval cases, must be removed from the sample, but shells of mollusks and crustaceans can remain in the sample. If shelled animals constitute 50 percent of the total weight, their weights may be reported separately if only wet weight is

required. Separation of the shelled animals is not necessary if wet, dry, and ash weights are to be determined because the ash weight will include the weight of the shells.

6.3 To determine wet weight, remove external water from the invertebrates by blotting for 1 minute on filter paper. Subdivide clumps of invertebrates, but do not separate individuals during blotting. Weigh to 0.1 mg. An alternative method for removing excess liquid is the centrifuge method described by Stanford (1973).

6.4 To determine dry weight, place the invertebrates in a tared porcelain crucible, and dry in an oven at 105 °C to constant weight. Cool in a desiccator and weigh to 0.1 mg. Lower drying temperatures (60 °C) sometimes are used when there is danger of erroneously small values resulting from volatilization or decomposition of fats (Edmondson and Winberg, 1971).

6.5 To determine ash weight, heat the crucible and sample at 500 °C in a muffle furnace to constant weight. Allow at least 1 hour, but some samples will require longer times. Cool and rewet the ash using distilled water to restore the water of hydration of clays and other minerals that may have been lost. Dry at 105 °C to a constant weight. Cool in a desiccator and weigh to 0.1 mg.

7. Calculations

7.1 When only part of the total sample is sorted or counted, project the results from the subsample to the number of specimens in the total sample:

$$\text{Total number of benthic invertebrates of a particular taxon in sample} = \frac{\text{Number of benthic invertebrates of the taxon in subsample}}{\text{Fraction of total sample in subsample}} .$$

7.2 Percent composition in sample

$$= \frac{\text{Number of benthic invertebrates of a particular taxon}}{\text{Total number of individuals of all taxa}} \times 100 .$$

7.3 Wet weight of benthic invertebrates (grams per sample)

$$= \frac{\text{Wet weight of benthic invertebrates in all samples} + \text{weight of crucible (grams)} - \text{tare weight of crucible (grams)}}{\text{Number of samples}} .$$

7.4 Dry weight of benthic invertebrates (grams per sample)

$$= \frac{\text{Dry weight of benthic invertebrates in all samples} + \text{weight of crucible (grams)} - \text{tare weight of crucible (grams)}}{\text{Number of samples}} .$$

7.5 Ash weight of benthic invertebrates (grams per sample)

$$= \frac{\text{Ash weight of benthic invertebrates in all samples} + \text{weight of crucible (grams)} - \text{tare weight of crucible (grams)}}{\text{Number of samples}} .$$

7.6 Ash-free weight (loss on ignition) of benthic invertebrates (grams per sample)

= Dry weight (grams per sample) - ash weight (grams per sample) .

7.7 Results of sampling from individual rocks are expressed as benthic invertebrates per projected area (aspect) of rock or per total rock surface:

Benthic invertebrates per square meter of projected rock surface

$$= \frac{\text{Number of benthic invertebrates collected from rock}}{\text{Length of longest axis of rock (millimeters)} \times \text{length of intermediate axis of rock (millimeters)}} \times 10^6 ;$$

Benthic invertebrates per square centimeter of total rock surface

$$= \frac{\text{Number of benthic invertebrates collected from rock}}{\pi\,[\text{length of intermediate axis of rock (millimeters)}]^2} \times 100 .$$

8. Reporting of results

8.1 Report the number of taxa present, the percentage composition of each taxon in the sample, and the type of sampling method(s) used. Report biomass to two significant figures.

8.2 Report results in terms of the total sample collected at each sampling site, in a particular habitat, or from the artificial-substrate sampler(s).

9. Precision

No numerical precision data are available.

10. References cited

Bull, C. J., 1968, A bottom fauna sampler for use in stony streams: Progressive Fish Culturist, v. 30, p. 119-120.

Donald, G. L., and Patterson, C. G., 1977, Effect of preservation on wet weight biomass of Chironomidae larvae: Hydrobiologia, v. 53, no. 1, p. 75-80.

Edmondson, W. T., and Winberg, G. G., eds., 1971, A manual on methods for the assessment of secondary productivity in fresh waters: Oxford and Edinburgh, Blackwell Scientific Publications, International Biological Programme Handbook 17, 358 p.

Fullner, R. W., 1971, A comparison of macroinvertebrates collected by basket and modified multiple-plate samplers: Water Pollution Control Federation Journal, v. 43, no. 3, pt. 1, p. 494-499.

Howmiller, R. P., 1972, Effects of preservatives on weights of some common macrobenthic invertebrates: Transactions of the American Fisheries Society, v. 101, p. 743-746.

Lium, B. W., 1974, Some aspects of aquatic insect populations of pools and riffles in gravel bed streams in western United States: Journal of Research of the U.S. Geological Survey, v. 2, no. 3, p. 379-384.

Mason, W. T., Jr., Anderson, J. B., and Morrison, G. E., 1967, A limestone-filled, artificial substrate sampler-float unit for collecting macroinvertebrates in large streams: Progressive Fish Culturist, v. 29, p. 74.

Stanford, J. A., 1973, A centrifuge method for determining live weights of aquatic insect larvae, with a note on weight loss in preservative: Ecology, v. 54, p. 449-451.

Distribution and Abundance (Quantitative Method)
(B-5040-85)

Parameters and Codes:

Invertebrates, benthic, wet weight (g/m^2):	70940
Invertebrates, benthic, dry weight (g/m^2):	70941
Invertebrates, benthic, ash weight (g/m^2):	70942
Invertebrates, benthic, total ($organisms/m^2$):	70943

1. Applications

This method is used in studies of biological productivity of benthic-invertebrate populations or communities. It is applicable to all natural water.

2. Summary of method

Benthic invertebrates are collected from a defined area using a suitable procedure for removing samples of a known size. A sufficient number of samples is desired to ensure that most of the taxa present are included. Unsorted samples, usually containing varying quantities of sand, gravel, and plant detritus, are preserved onsite. In the laboratory, the benthic invertebrates are separated from the extraneous material, identified, and counted or weighed. Results are reported as numbers of different kinds of benthic invertebrates (taxa) and numbers of individuals in each taxon per unit area of bottom. Biomass is reported as wet, dry, ash, or ash-free weight per unit area of bottom.

3. Interferences

Physical factors, such as stream velocity, depth of water, and large rocks, may interfere with sampling. Most samples contain relatively large quantities of sediment and plant debris from which the invertebrates must be separated. The principal interference with quantitative sampling, however, is the heterogeneity of aquatic habitats and the temporal and spatial variability of the benthic-invertebrate populations (Hynes, 1970).

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Balance, capable of weighing to at least 0.1 mg.

4.2 Box, drum, or stream-bottom fauna sampler (Edmondson and Winberg, 1971, p. 69). This is a strong, metal cylinder open at the top and bottom that can be pushed into the sediment to isolate a definite area. The bottom of the cylinder may have a compressible edge to seal against the irregularities of the bed, or the edge may have triangular teeth about 4 cm long, which cut into the bed as the sampler is rotated. Cylindrical samplers can be lengths of stovepipe or 30-cm-diameter aluminum irrigation pipe (Weber, 1973), or they can be constructed to enclose any convenient area as defined by the study objectives and the size of the bed materials. A sample area of 900 to 1,000 cm^2 is common. The maximum practical height for the box is about

75 cm because the collector must be able to reach the bottom with the hands. One of various modifications of the solid cylinder is shown in figure 29. Other modifications are described by Welch (1948), Gerking (1957), Macan (1958), and Waters and Knapp (1961). Depending on the degree of resistance offered to water flow, these devices decrease the tendency for the sampler to cause scour as it approaches the bottom of a stream. Netting should be 210±2-μm mesh-opening nylon or polyester monofilament screen cloth, unless otherwise indicated by the study objectives.

4.3 Brush, soft-bristle, small dip net of appropriate mesh opening, and a garden trowel or small digging fork are needed for removing the invertebrates from the substrate enclosed by several of the samplers.

4.4 Corer, K.B.-type (fig. 34), or equivalent. Extra weights are available to increase the depth of penetration, and when so used, a winch may be required. These corers have been designed so water passes through during descent but are closed during ascent to prevent loss of sample. In shallow water, a hand corer may be used.

4.5 Desiccator, containing silica gel or anhydrous calcium sulfate.

4.6 Drying oven, thermostatically controlled for use at 105 °C.

4.7 Ekman grab, preferably the tall design (fig. 31), 15×15 cm square, 23 to 30 cm tall. Extra weights are available to increase the depth of penetration. In deep water, the grab is tripped using a messenger; whereas, in shallow water, the Ekman grab may be operated using a handle.

4.8 Forceps that have fine or rounded points. Forceps that have fine points are useful for handling small invertebrates. Forceps that have rounded points are less likely to tear netting or puncture the mesh of sieves or other sampling equipment. Forceps are less likely to be lost onsite if marked with bright paint or colored tape.

4.9 Gloves, waterproof, Trapper's, shoulder length.

4.10 Ink, waterproof.

4.11 Labels, waterproof, or labels may be cut from sheets of plastic paper.

4.12 Microscope, stereoscopic variable power, 7X to 30X, and microscope illuminator. A compound microscope of at least 200X magnification also is useful for taxonomic work.

4.13 Muffle furnace, for use at 500 °C.

4.14 Ponar grab (fig. 32), or screen-top sediment sampler. These grabs trip on contact with the bottom and have been designed so water passes through to lessen the shock wave (Flannagan, 1970; Hudson, 1970). Word and others (1976) reported improved performance when the fixed panels were replaced by hinged screen panels. Accessory weights may be used, and these grabs should be operated with a winch. When empty, the grab is about 23 kg without weights and about 32 kg with weights.

4.15 Porcelain crucibles.

4.16 Sample containers, plastic or glass, and plastic lids, for transporting unsorted samples to the laboratory. Wide-mouth jars of 120-, 240-, and 475-mL capacity are useful sizes. Sealable plastic bags also may be used for temporary storage of benthic-invertebrate samples.

4.17 Sieves, U.S. Standard, 20-cm diameter, and mesh size appropriate to the study objectives. The no. 70 sieve (210-µm mesh opening) has been selected for retaining benthic invertebrates collected as part of the water-quality programs of the U.S. Geological Survey. Sieves that have smaller or larger mesh may be more suitable for some studies. The no. 18 sieve (1,000-µm mesh opening) is useful for removing large rocks and sticks from samples. Stainless-steel mesh is recommended for all sieves because of its greater durability compared to brass.

4.18 Surber sampler (fig. 30). This sampler commonly has been used in stream studies, although the enclosed box-type samplers, such as the portable invertebrate box sampler are preferred, if available. Modifications of the surber sampler (Waters and Knapp, 1961; Withers and Benson, 1962; Mundie, 1971) eliminated many deficiencies of the original design. Netting used in the construction or operation of these samplers should be 210±2-µm mesh-opening nylon or polyester monofilament screen cloth, unless otherwise indicated by the study objectives.

4.19 Tape, plastic, or paraffin for sealing jar and vial lids.

4.20 Tub or bucket for washing samples or sampling equipment onsite.

4.21 Van Veen grab (fig. 33), weighs 48 kg and may be loaded with additional weights. The grab has a capacity of 40 L and samples an area of 1,500 cm^2. Screened panels enable water to flow through during descent to lessen the shock wave on the bottom. Rubber flaps cover the screened openings to prevent sediment washout during recovery.

4.22 Vials that have plastic poly seal screw lids. Convenient sizes are 7.5-, 15-, and 22-mL capacity.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Distilled or deionized water.

5.2 Glycerin.

5.3 Preservative solutions. Invertebrate samples may be preserved in 70-percent ethyl alcohol or 70-percent isopropyl alcohol. Formaldehyde solution is not recommended. A mixture of 70-percent ethyl alcohol and 5-percent glycerin is preferred for permanent storage. Prepare as follows:

5.3.1 Ethyl alcohol. Dilute 70 mL 95-percent alcohol to 95 mL using distilled water.

5.3.2 Ethyl alcohol and 5-percent glycerin. Dilute 70 mL 95-percent alcohol to 100 mL using 25 mL distilled water and 5 mL glycerin.

5.3.3 Isopropyl alcohol. Dilute 70 mL concentrated isopropyl alcohol to 100 mL using distilled water.

6. Analysis

6.1 Identify and count the benthic invertebrates in the sample according to taxonomic categories. The degree of identification required (species level is desirable) varies depending on the objectives of the study. A stereoscopic microscope is required; and, for some groups, dissections or microscopic mounts may be needed to observe key characteristics. Appropriate reference books (Part 3, "Selected Taxonomic References" section of this report) should be available. The different categories of invertebrates can be placed in separate vials of 70-percent ethyl or 70-percent isopropyl alcohol and can be labeled with the name of the invertebrate and the identification number, date, and origin of the sample. Add a few drops of glycerin or use the ethyl alcohol-glycerin preservative, and seal vial caps if the specimens are to be stored.

6.2 The biomass of benthic invertebrates, expressed as wet, dry, ash, or ash-free weight, is best determined from samples that were frozen immediately after collection. Biomass determined from alcohol-preserved samples is much less satisfactory (Howmiller, 1972; Stanford, 1973; Donald and Patterson, 1977).

Although generally determined from the total sample, biomass may be determined for an individual taxon. Cases or houses, such as caddisfly larval cases, must be removed from the sample, but shells of mollusks and crustaceans can remain in the sample. If shelled animals constitute 50 percent of the total weight, their weights may be reported separately if only wet weight is required. Separation of the shelled animals is not necessary if wet, dry, and ash weights are to be determined because the ash weight will include the weight of the shells.

6.3 To determine wet weight, remove external water from the invertebrates by blotting for 1 minute on filter paper. Subdivide large clumps of invertebrates, but do not separate individuals during blotting. Weigh to 0.1 mg. An alternative method for removing excess liquid is the centrifuge method described by Stanford (1973).

6.4 To determine dry weight, place the invertebrates in a tared porcelain crucible, and dry in an oven at 105 °C to a constant weight. Cool in a desiccator and weigh to 0.1 mg. Lower drying temperatures (60 °C) sometimes are used when there is danger of erroneously small values resulting from volatilization or decomposition of fats (Edmondson and Winberg, 1971).

6.5 To determine ash weight, heat the crucible and sample at 500 °C in a muffle furnace to a constant weight. Allow at least 1 hour, but some samples will require longer times. Cool and rewet the ash using distilled water to restore the water of hydration of clays and other minerals that may have been lost. Dry at 105 °C to a constant weight. Cool in a desiccator and weigh to 0.1 mg.

7. Calculations

7.1 When only part of the total sample is sorted or counted project the results from the subsample to the number of specimens in the total sample:

Total number of benthic invertebrates of a particular taxon in sample

$$= \frac{\text{Number of benthic invertebrates of the taxon in subsample}}{\text{Fraction of total sample in subsample}} .$$

7.2 Number of benthic invertebrates per square meter

$$= \frac{\text{Number of benthic invertebrates in all samples}}{\text{Area of sampler (square meters)} \times \text{number of samples}} .$$

7.3 Wet weight of benthic invertebrates (grams per square meter)

$$= \frac{\text{Wet weight of benthic invertebrates in all samples} + \text{weight of crucible (grams)} - \text{tare weight of crucible (grams)}}{\text{Area of sampler (square meters)} \times \text{number of samples}} .$$

7.4 Dry weight of benthic invertebrates (grams per square meter)

$$= \frac{\text{Dry weight of benthic invertebrates in all samples} + \text{weight of crucible (grams)} - \text{tare weight of crucible (grams)}}{\text{Area of sampler (square meters)} \times \text{number of samples}} .$$

7.5 Ash weight of benthic invertebrates (grams per square meter)

$$= \frac{\text{Ash weight of benthic invertebrates in all samples} + \text{weight of crucible (grams)} - \text{tare weight of crucible (grams)}}{\text{Area of sampler (square meters)} \times \text{number of samples}} .$$

7.6 Ash-free weight (loss on ignition) of benthic invertebrates (grams per square meter)

= Dry weight (grams per square meter) - ash weight (grams per square meter) .

8. Reporting of results

8.1 Report as follows: Less than 100 benthic invertebrates per square meter, nearest whole number; 100 benthic invertebrates or more, two significant figures. Report biomass to two significant figures.

8.2 Report results in terms of a unit area of the habitat sampled.

9. Precision

No numerical precision data are available.

10. References cited

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Invertebrate Drift
(B-5050-85)

Parameters and Codes: Not available

Because drifting invertebrates come from a variety of habitats, drift samples generally contain a large variety of taxa (Waters, 1961; Larimore, 1974; Slack and others, 1976). Benthic invertebrates respond to stresses of pollution, flood, drought, or insecticides by increased drifting; therefore, drift may be a useful indicator of water quality. Drift is a source of invertebrates for colonization of artificial-substrate samplers and for recolonization of depopulated areas of streams.

1. Applications

The method is applicable to all flowing water in which the velocity is at least 0.01 m/s.

2. Summary of method

Drifting invertebrates carried by flowing water are caught in a stationary net. Because the catch increases as the volume of water passing through the net increases, drift results are expressed as density (number of invertebrates or biomass per unit volume of water), as drift rate (number of invertebrates or biomass passing a sampling point in unit time), or as total daily drift rate (total number of invertebrates or biomass passing a given point in 24 hours).

3. Interferences

Drift nets may become clogged with ice, detritus, tree leaves, or sediment causing backflow and decreased sampling efficiency. If the opening of the net is in contact with the stream bottom, nondrifting invertebrates may be caught; if the opening extends above the surface, many adults and terrestrial invertebrates may be caught. Sufficient current must be present to carry the actively or passively drifting invertebrates into the net. If only naturally occurring drift rates are to be determined, nets should be installed upstream from disturbances caused by human activity, cattle, or other sources of artificially created invertebrate drift. Because drifting activity for many species varies greatly during a diel cycle, comparative collections should be made during similar time periods.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Balance, capable of weighing to at least 0.1 mg.

4.2 Current meter, pygmy, or digital flowmeter.

4.3 Desiccator, containing silica gel or anhydrous calcium sulfate.

4.4 Drift net (fig. 35) 30×30 cm, 15×30 cm, or 30×46 cm, that has anchor rods and clamps. Bag nets, 1 m or more in length, should have 210±2-µm mesh-opening nylon or polyester monofilament screen cloth, unless otherwise indicated by the study objectives. The percent open area of the netting should be as large as possible to facilitate flowthrough and decrease backflow. A net that is cylindrical for most of its length is less liable to clog than one that is tapered (Waters, 1969).

4.5 Drying oven, thermostatically controlled for use at 105 °C.

4.6 Forceps that have fine or rounded points. Forceps that have fine points are useful for handling small invertebrates. Forceps that have rounded points are less likely to tear netting or puncture the mesh of sieves or other sampling equipment. Forceps are less likely to be lost onsite if marked with bright paint or colored tape.

4.7 Ink, waterproof.

4.8 Labels, waterproof, or labels may be cut from sheets of plastic paper.

4.9 Microscope, stereoscopic variable power, 7X to 30X, and microscope illuminator. A compound microscope of at least 200X magnification also is useful for taxonomic work.

4.10 Muffle furnace, for use at 500 °C.

4.11 Porcelain crucibles.

4.12 Sample containers, plastic or glass, and plastic lids, for transporting unsorted collections to the laboratory. Wide-mouth jars of 120-, 240-, and 475-mL capacity are useful sizes. Sealable plastic bags also may be used for temporary storage of benthic-invertebrate samples.

4.13 Sieves, U.S. Standard, 20-cm diameter, and mesh size appropriate to the study objectives. The no. 70 sieve (210-µm mesh opening) has been selected for retaining benthic invertebrates collected as part of the water-quality programs of the U.S. Geological Survey. Sieves that have smaller or larger mesh may be more suitable for some studies. The no. 18 sieve (1,000-µm mesh opening) is useful for removing large rocks and sticks from samples. Stainless-steel mesh is recommended for all sieves because of its greater durability compared to brass.

4.14 Tape, plastic, or paraffin for sealing jar and vial lids.

4.15 Vials that have plastic poly seal screw lids. Convenient sizes are 7.5-, 15-, and 22-mL capacity.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Distilled or deionized water.

5.2 Glycerin.

5.3 Preservative solutions. Drift invertebrate samples may be preserved in 70-percent ethyl alcohol or 70-percent isopropyl alcohol. A mixture of 70-percent ethyl alcohol and 5-percent glycerin is preferred for permanent storage. Prepare as follows:

5.3.1 Ethyl alcohol. Dilute 70 mL 95-percent alcohol to 95 mL using distilled water.

5.3.2 Ethyl alcohol and 5-percent glycerin. Dilute 70 mL 95-percent alcohol to 100 mL using 25 mL distilled water and 5 mL glycerin.

5.3.3 Isopropyl alcohol. Dilute 70 mL concentrated isopropyl alcohol to 100 mL using distilled water.

6. Analysis

6.1 Identify and count the benthic invertebrates in the sample according to taxonomic categories. The degree of identification required (species level is desirable) varies depending on the objectives of the study. A stereoscopic microscope is required; and, for some groups, dissections or microscopic mounts are needed to observe key characteristics. Appropriate reference books (Part 3, "Selected Taxonomic References" section of this report) should be available. The different categories of invertebrates can be placed in separate vials of 70-percent ethyl or 70-percent isopropyl alcohol and can be labeled with the name of the invertebrate and the identification number, date, and origin of the sample. Add a few drops of glycerin or use the ethyl alcohol-glycerin preservative, and seal vial caps if the specimens are to be stored.

6.2 The biomass of drift invertebrates, expressed as wet, dry, ash, or ash-free weight, is best determined from samples that were frozen immediately after collection. Biomass determined from alcohol-preserved samples is much less satisfactory (Howmiller, 1972; Stanford, 1973; Donald and Patterson, 1977).

Although generally determined from the total sample, biomass may be determined for an individual taxon. Cases or houses, such as caddisfly larval cases, must be removed from the sample, but shells of mollusks and crustaceans can remain in the sample. If shelled animals constitute 50 percent of the total weight, their weights may be reported separately if only wet weight is required. Separation of the shelled animals is not necessary if wet, dry, and ash weights are to be determined because the ash weight will include the weight of the shells.

6.3 To determine wet weight, remove external water from the animals by blotting for 1 minute on filter paper. Subdivide large clumps of invertebrates, but do not separate individuals during blotting. Weigh to 0.1 mg. An alternative method of removing excess liquid is the centrifuge method described by Stanford (1973).

6.4 To determine dry weight, place the invertebrates in a tared porcelain crucible, and dry in an oven at 105 °C to a constant weight. Cool in a desiccator and weigh to 0.1 mg. Lower drying temperatures (60 °C) sometimes are used when there is danger of erroneously small values resulting from volatilization or decomposition of fats (Edmondson and Winberg, 1971).

6.5 To determine ash weight, heat the crucible and sample at 500 °C in a muffle furnace to a constant weight. Allow at least 1 hour, but some samples will require longer times. Cool and rewet the ash using distilled water to restore the water of hydration of clays and other minerals that may have been lost. Dry at 105 °C to a constant weight. Cool in a desiccator and weigh to 0.1 mg.

7. Calculations

7.1 When only part of the total sample is sorted or counted, project the results from the subsample to the number of specimens in the total collection:

$$\text{Number of drift invertebrates of a particular taxon in sample} = \frac{\text{Number of taxon in subsample}}{\text{Fraction of total sample in subsample}} .$$

7.2 Percent composition in sample

$$= \frac{\text{Number of drift invertebrates of a particular taxon}}{\text{Total number of drift invertebrates of all taxa}} .$$

Weight calculations may be on a sample basis or a daily (24 hour) basis depending on the study objectives.

7.3 Wet weight of drift invertebrates (grams)

= Wet weight of drift invertebrates + crucible (grams)
- tare weight of crucible (grams) .

7.4 Dry weight of drift invertebrates (grams)

= Dry weight of drift invertebrates + crucible (grams)
- tare weight of crucible (grams) .

7.5 Ash weight of drift invertebrates (grams)

= Ash weight of drift invertebrates + crucible (grams)
- tare weight of crucible (grams) .

7.6 Ash-free weight (loss on ignition) of drift invertebrates (grams)

$$= \text{Dry weight (grams)} - \text{ash weight (grams)} .$$

Invertebrate drift density and rate may be expressed on a sample basis or a daily (24 hour) basis depending on the study objectives (Waters, 1969, 1972; Elliott, 1970).

7.7 Drift density (number or grams per cubic meter)

$$= \frac{\text{Quantity of drift invertebrates (number or grams)}}{\text{Volume of water sampled (cubic meters)}} .$$

7.8 Drift rate (number or grams per time)

$$= \frac{\text{Quantity of drift invertebrates (number or grams)}}{\text{Volume of water sampled (cubic meter)} \times \text{stream discharge (cubic meters per time)}} .$$

7.9 Total daily drift rate (number or grams per 24 hours)

$$= \frac{\text{Total daily quantity of drift invertebrates (number or grams)}}{\text{Volume of water sampled (cubic meters)} \times \text{total stream discharge (cubic meters per 24 hours)}} .$$

8. Reporting of results

Report drift quantity, taxa, and methods of collection for daylight samples. If sampling was done for 24 hours, report drift quantity and taxa per unit volume and time to indicate any periodicity that occurred. Describe methods of collection.

9. Precision

No numerical precision data are available.

10. References cited

Donald, G. L., and Patterson, C. G., 1977, Effect of preservation on wet weight biomass of Chironomidae larvae: Hydrobiologia, v. 53, no. 1, p. 75-80.

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Permanent-Slide Method for Larvae of Chironomidae
(B-5200-85)

Parameter and Code: Not applicable

Chironomidae (midges) is a family of the insect Order Diptera (two-winged flies), and the immature stages are principally aquatic. The larvae, which are found in all kinds of water except the open ocean, make up a substantial part of most freshwater-invertebrate communities (Roback, 1957). They are important as a source of fishfood and are considered to be useful indicators of water quality. Chironomids are holometabolous (have complete metamorphosis). The larva, which is the feeding stage or most active phase of the chironomid life cycle, has a complete head capsule that is nonretractable within the thorax, and the mandibles are opposed (fig. 37). It has prolegs (not true insect legs) at both ends of the soft, wormlike body. The anterior prolegs are just behind the head capsule on the ventral side of the first thoracic segment and often are fused for their entire length. The posterior prolegs on the last abdominal segment are never fused. The larvae lack spiracles (respiratory openings in the abdominal walls). In some species, ventral gills, called blood gills, are just anterior to the posterior prolegs.

Some chironomid larvae move freely in water, but the larvae of many species live in tubes that they build from algae, fine sediment, and bits of plant debris bound or cemented together with a salivary secretion (fig. 38). Commonly, these structures have the appearance of sand tubes attached to rocks or other solid objects. Both ends of the tubes are open, and the larvae circulate water through them by undulating their bodies. The larvae feed on diatoms and other algae, organic detritus, microcrustaceans, and other midge larvae.

Adult chironomids are small, delicate, gnatlike, nonbiting flies (10 mm long) that are found in swarms by bodies of water, especially in the evening, and near lights at night. The life cycles of the insects are variable; some forms have only one generation every 2 years, while others have several generations during a year.

Identification of chironomid larvae is based mainly on the mouth parts that can be seen only through a microscope. The method described is a modification of procedures developed by Mason (1968, 1970) and Beck (1976) and is suitable for most chironomid larvae.

Some investigators, especially those who are working with chironomid systematics, dissect their larval specimens. They mount just the head capsules, and sometimes they dissect the head capsule and mount certain mouth parts separate from the head under one cover glass.

1. Applications

The method is suitable for all chironomid larvae.

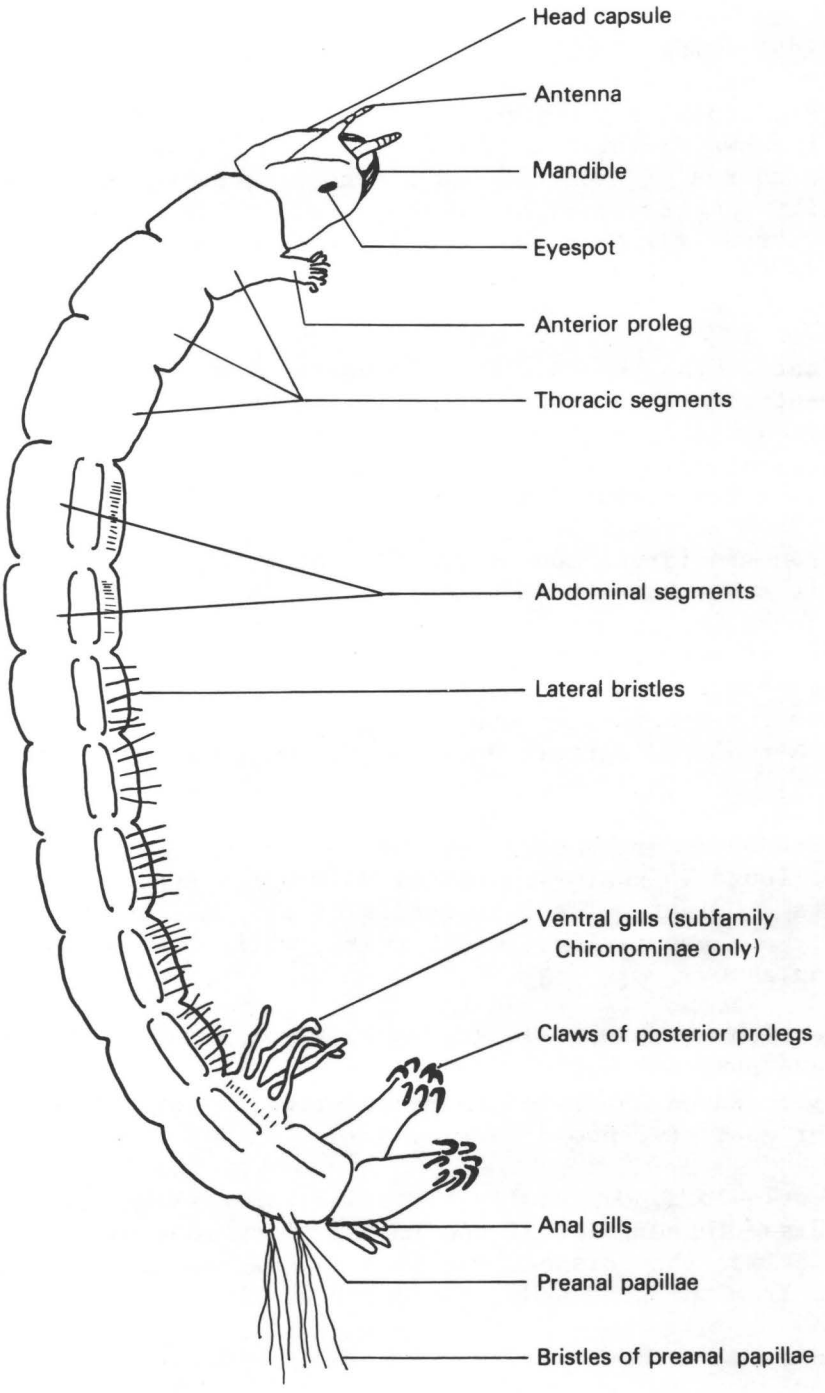


Figure 37.--Idealized external features of a larva of the Family Chironomidae. Features are from more than one subfamily.

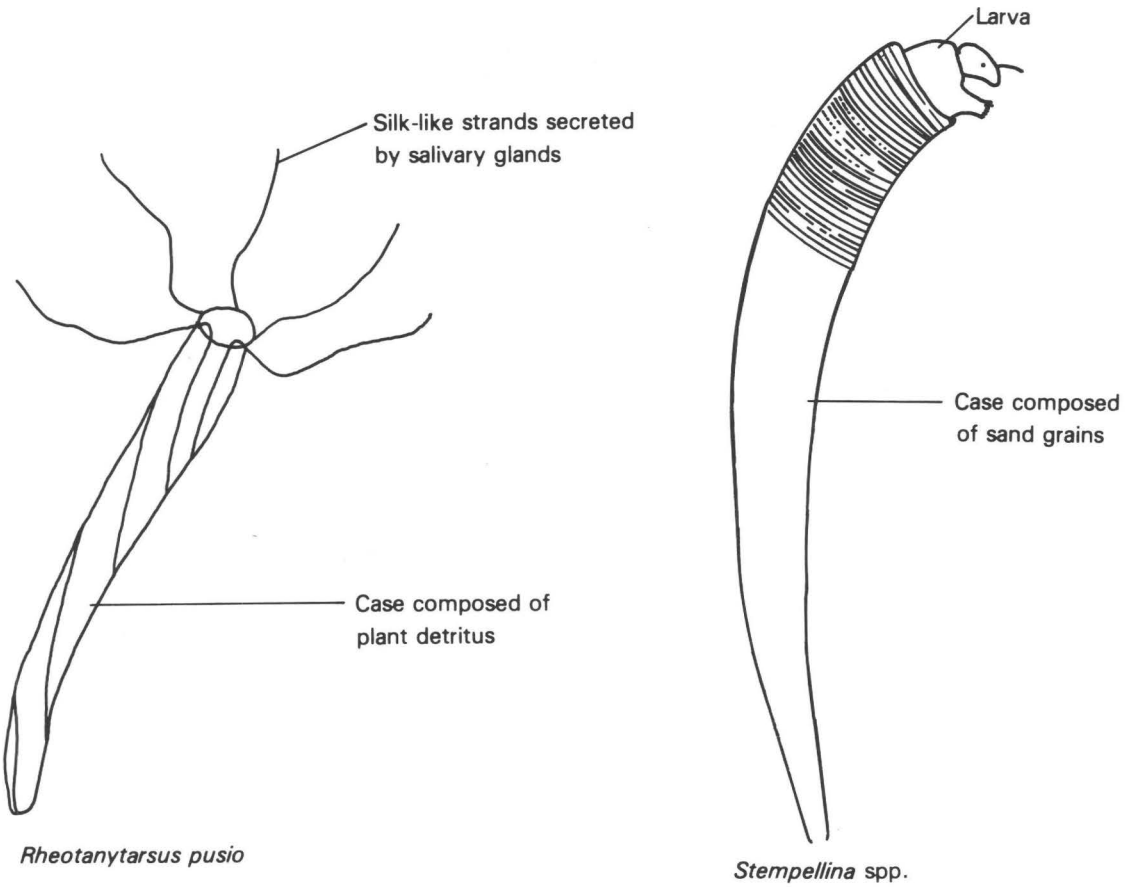


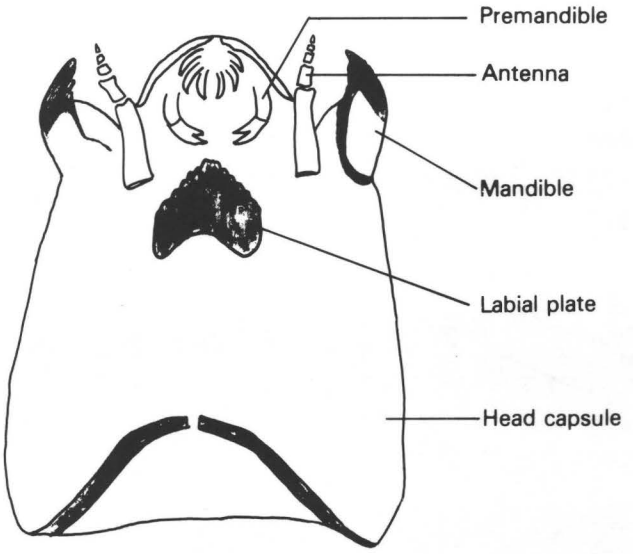
Figure 38.--Examples of tubes constructed by larvae of the Family Chironomidae.

2. Summary of method

Chironomidae larvae from a benthic-invertebrate sample are sorted into visually distinct groups. Representative specimens are heated in 10-percent potassium hydroxide solution to dissolve soft body tissues, placed ventral side up on a microscope slide in a mounting medium, and pressed under a cover glass. The mounted specimens are identified. The number of taxa and individuals in each taxon are tabulated and reported as a percentage of the benthic-invertebrate population or reported in other ways appropriate to the study objectives.

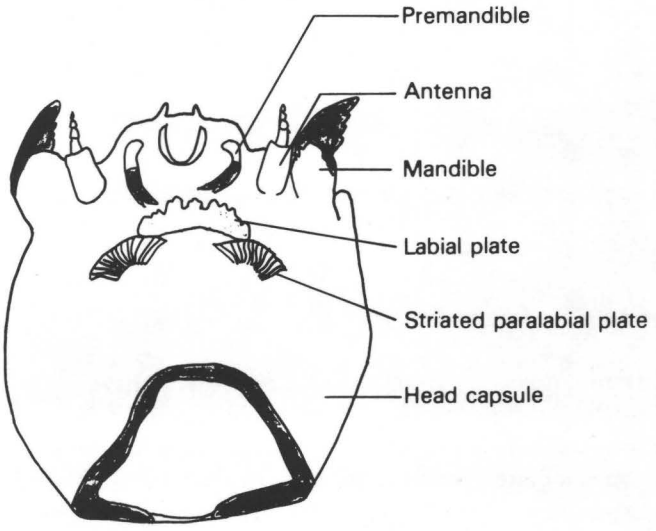
3. Interferences

Heating time is critical. If not heated long enough, the specimen may be too opaque for examination; if heated too long, the specimen will be too transparent and difficult to manipulate during mounting procedures. Sand and other material that cannot be removed by heating may be forced from the gut into the mouth when pressed, obscuring the mouth parts. Too much pressure during mounting may damage diagnostic features shown in figures 39 and 40.



Eukiefferiella spp.

Figure 39.--Ventral view of larval head capsule of the Subfamily Orthocladiinae, simplified.



Chironomus spp.

Figure 40.--Ventral view of larval head capsule of the Subfamily Chironominae, simplified. Notice that the left mandible is turned outward; changes in position of structures are common during mounting procedures.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Cover glasses, circular, no. 1 or 2, 12-mm diameter.

4.2 Crucibles, high-form, porcelain, 10-mL capacity.

4.3 Forceps, blunt curved tips, and microforceps, fine-tipped.

4.4 Hotplate, electric.

4.5 Labels for microscope slides. When many slides are prepared, information about the source of the sample can be typed on sheets of paper, photocopied and reduced one-half or two-thirds in size, cut out, and glued onto slides using white glue, or equivalent. Labels, waterproof, or labels may be cut from sheets of plastic paper.

4.6 Marking pen, permanent, waterproof, for labeling slides.

4.7 Microscope, compound, preferably having differential interference contrast capable of 1,000X magnification.

4.8 Microscope slides, glass, precleaned, 25×75 mm.

4.9 Needles, pins, and probes for manipulating specimens under a stereomicroscope.

4.10 Ocular micrometer, graduated to 5 μm.

4.11 Spot plates, white porcelain.

4.12 Stereoscopic zoom microscope (dissecting), capable of 80X magnification.

4.13 Vials, 4 mL, and poly seal screw lids.

4.14 White glue.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetic acid, glacial.

5.2 Distilled or deionized water.

5.3 Fingernail polish, clear.

5.4 Glycerin.

5.5 Mounting medium, CMC-10, or prepare medium as follows: In 50 mL distilled water, dissolve 30 g Gum arabic (amorphic), 200 g chloral hydrate, and 20 mL glycerin. Completely dissolve each solid ingredient before adding succeeding reagents. Filter final mixture through clean cheese cloth.

5.6 Potassium hydroxide solution, 10 percent. Dissolve 10 g potassium hydroxide (KOH) pellets in 100 mL distilled water.

5.7 Preservative solutions. Samples may be preserved in 70-percent ethyl alcohol or 70-percent isopropyl alcohol. A mixture of 70-percent ethyl alcohol and 5-percent glycerin is preferred for permanent storage. Prepare as follows:

5.7.1 Ethyl alcohol. Dilute 70 mL 95-percent alcohol to 95 mL using distilled water.

5.7.2 Ethyl alcohol and 5-percent glycerin. Dilute 70 mL 95-percent alcohol to 100 mL using 25 mL distilled water and 5 mL glycerin.

5.7.3 Isopropyl alcohol. Dilute 70 mL concentrated isopropyl alcohol to 100 mL using distilled water.

6. Analysis

Usually, time does not permit mounting all chironomids in a sample, so the results from a subsample are used to calculate the distribution of taxa and individuals in the original sample. The size of the subsample to be mounted for microscopic examination will depend on the size of the sample, the number of visually distinct groups, and the study objectives.

6.1 Using a stereoscopic microscope, separate the total sample into groups on the basis of general appearance and external features. Some morphological features most useful for separating specimens into groups are:

6.1.1 Body characteristics:

a. Length.
b. Color and color distribution.
c. Enlarged sections.
d. Presence or absence of blood gills.
e. Preanal papillae and bristles.

6.1.2 Head-capsule characteristics:

a. Length and width.
b. Color and darkened areas, such as mouth parts.
c. Number, shape, and arrangements of eyespots.
d. Shape and unusual appendages.

Individual depressions on porcelain spot plates are convenient compartments for separating the subsamples of larvae.

6.2 Randomly select representatives of each group for mounting. For small groups of 10 or fewer individuals, mount a subgroup of 5, or at least 50 percent. For larger groups, remove a subgroup by stratified random sampling and cluster or two-stage sampling. Store the unmounted specimens in vials of 70-percent ethyl alcohol containing one drop of glycerin.

6.3 Place subgroups in depressions of a spot plate filled with distilled water, and soak 10 minutes to remove the alcohol.

6.4 Transfer the subgroups to another spot plate or to crucibles containing 10-percent KOH (Note 1). Heat for 10 to 15 minutes or until the bodies are semitransparent and noticeably lighter in color. (CAUTION.--Excessive heating results in too much digestion of the soft parts, making the specimens too transparent and difficult to see and to manipulate). While heating, add distilled water to the KOH solution to compensate for evaporation.

Note 1: Use fresh KOH solution for each subgroup.

6.5 Transfer the specimens from the KOH solution to a clean spot plate of distilled water (Note 2) for at least 3 minutes to remove the KOH.

Note 2: Residual KOH can make the specimens too soft, thus interfering with the mounting medium. Instead of the water rinse, glacial acetic acid can be used to neutralize the KOH if residual KOH is a problem.

6.6 Transfer the specimens to another spot plate of 95-percent ethyl alcohol for 3 to 5 minutes. This treatment removes the water or acetic acid and makes the specimen crisp, which results in optimum distribution of mouth parts in the final preparation.

6.7 Place a small drop of mounting medium on a clean glass microscope slide. Position one specimen in the drop of medium, ventral side up, and if necessary, move the specimen using a dissecting needle and microforceps. Place a 12-mm diameter cover glass on the drop containing a specimen and, using a stereoscopic microscope, use the cover glass and the viscous mounting medium to roll, slide, or push each specimen so it lies flat, ventral side up. Apply additional pressure to spread the mouth parts. Allow preparation to dry for 1 week, keeping the slide horizontal (Note 3).

Note 3: With practice, this procedure can be effective for processing many specimens. Chironomids larger than the 12-mm cover glass should be cut in half and mounted under one or two cover glasses.

6.8 Specimens may dry after 2 or 3 years in the mounting medium unless the edges of the cover glass are sealed. To make the preparations more permanent, ring the slide by coating the edges of the cover glass and any exposed mounting medium with clear fingernail polish.

7. Calculations

7.1 When only part of the total sample of Chironomidae larvae is mounted and identified, project the results from those mounted to the total number of specimens:

$$\text{Total number of individuals of a particular taxon in sample} = \frac{\text{Number of individuals of the taxon in subsample}}{\text{Fraction of total sample in subsample}} .$$

7.2 Percent composition in sample

$$= \frac{\text{Number of individuals of a particular taxon}}{\text{Total number of individuals of all taxa}} \times 100 .$$

8. Reporting of results

Report the number of taxa present, the number and percentage of individuals in each taxon in the sample, and the method of collection.

9. Precision

No numerical precision data are available.

10. References cited

Beck, W. M., Jr., 1976, Biology of the larval chironomids: State of Florida, Department of Environmental Regulation Technical Series, v. 2, no. 1, 58 p.

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Method for Identification of Immature Simuliidae (B-5220-85)

Parameter and Code: Not applicable

Larvae and pupae of the insect Family Simuliidae (blackflies) commonly are abundant in swiftly flowing freshwater streams having cobble or gravel bottom. They occur in reaches that have smooth, relatively laminar flow as opposed to reaches that have pools, eddies, or turbulence (Hynes, 1970).

Simuliids are members of the insect Order Diptera (two-winged flies), and as adults can be a serious nuisance to man and animals, especially during the summer months when they emerge and swarm in great numbers. These humpbacked blackflies can inflict a stinging bite that may be followed by intense itching and sometimes bleeding. Severe attacks by blackflies have been known to cause the death of livestock from shock and loss of blood. Blackfly attacks also have been reported to cause a decrease in milk production at dairy farms. Some species of blackflies transmit human onchocerciasis, and other species transmit certain protozoan and other filarial organisms that cause diseases in birds.

Simuliids, like other dipterans, undergo complete metamorphosis (holometabolous). The adults are small and robust, usually dark-colored, and have broad wings, which have large anterior veins. An extensive taxonomic literature about the adults has been stimulated by the economic importance of blackflies. However, until recently, little research was done on the taxonomy of the immature forms.

The immature stages, larvae and pupae, are strictly aquatic. The pupae are enclosed in vaselike or slipperlike cases (fig. 41) attached to rocks, debris, or other solid objects. The pupae have a pair of conspicuous respiratory organs on the thorax and filaments numbering from 2 to 60 (fig. 42). The filaments protrude from the open end of the pupal case. Usually, a pair of prominent terminal hooks is on the last abdominal segment (fig. 42).

The larvae measure 3 to 15 mm in length and are attached to stones or other substrates. The larva is characterized by a soft body that is swollen posteriorly, a pair of mouth fans, one anterior proleg, and a posterior crochet ring composed of minute hooks (fig. 43) by which it adheres to the substrate. The larva moves in a looping manner by means of the posterior crochet ring and anterior proleg. A strand of sticky thread-like secretion (silk) from the head prevents the larva from being swept away by the current. The larval head capsule has many features used for identification. These include the arrangement of spots on the dorsal side, relative length and color of the antennae, shape of the occipital cleft located on the ventral surface (fig. 44), and the shape and tooth pattern of the submentum (fig. 44). The shape of the secondary mouth fan (fig. 45), used to filter food particles from the water, is an additional characteristic used for identification. The fan is exposed by grasping the larva firmly near the head, ventral side up, and lifting the primary fan up and out (Sommerman, 1953).

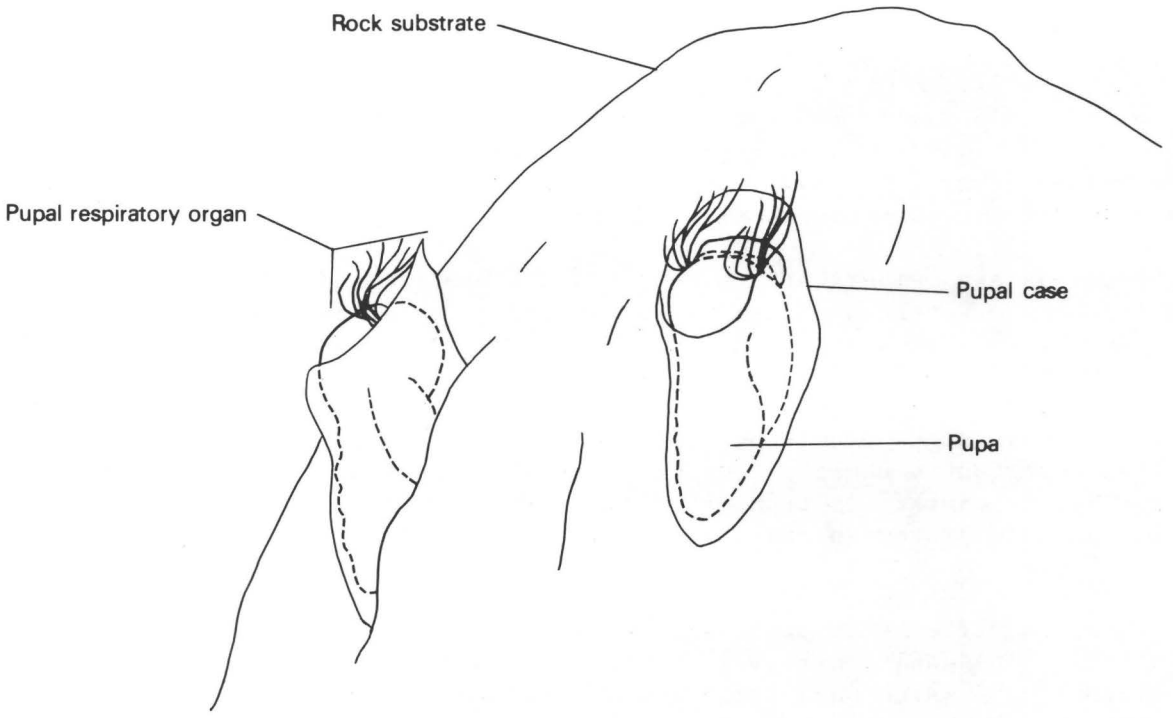

Figure 41.--One type of pupa of the Family Simuliidae enclosed in a slipperlike case attached to rocks in the water.

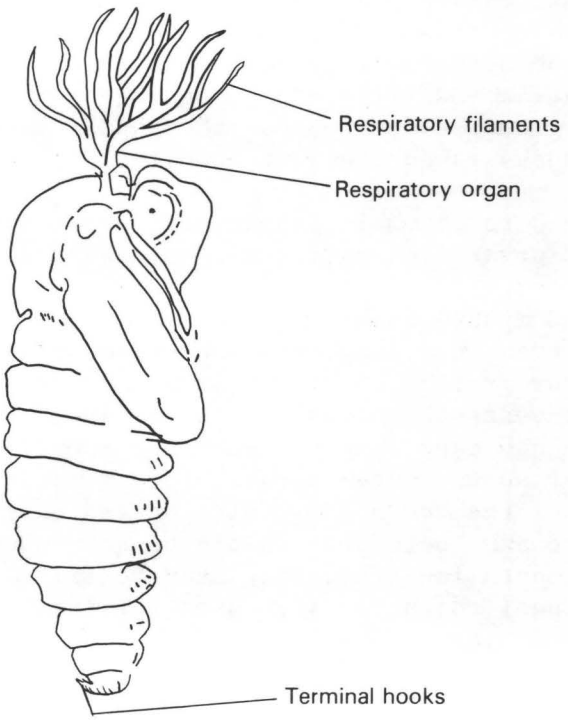

Figure 42.--Simplified features of a pupa of the Family Simuliidae, showing location and arrangement of the pupal respiratory filaments.

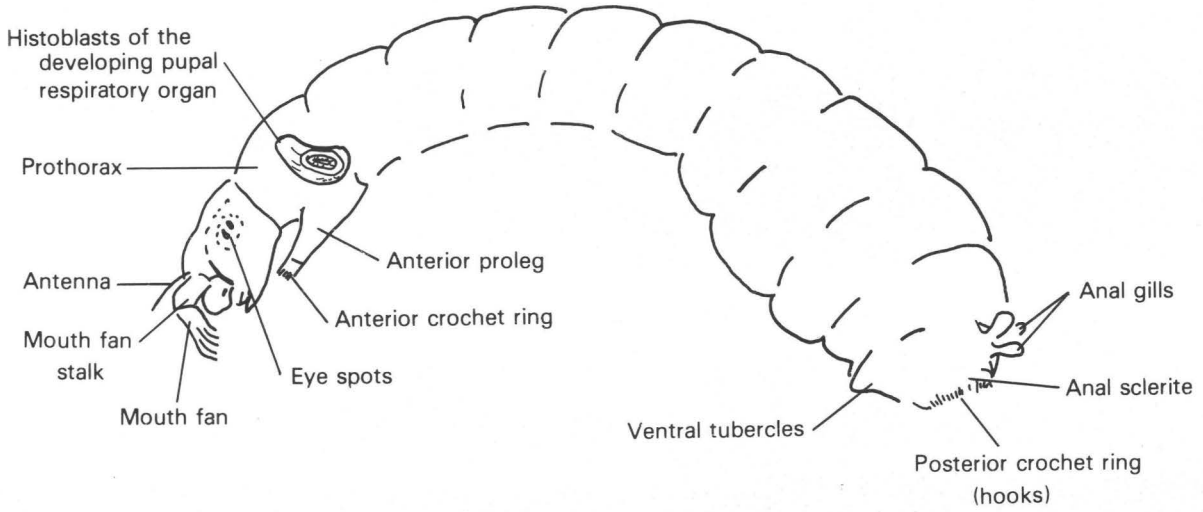

Figure 43.--Mature larva of the Family Simuliidae, simplified, showing most of the important external features needed for identification.

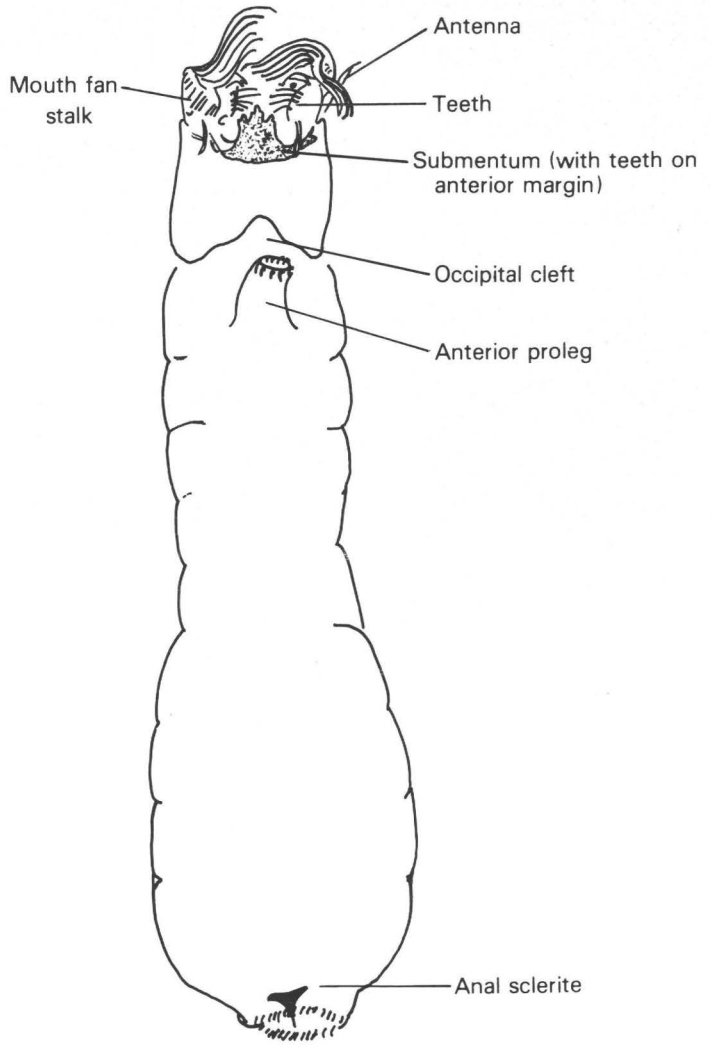

Figure 44.--A larva of the Family Simuliidae, simplified, showing the features that can be seen best after making a permanent mount.

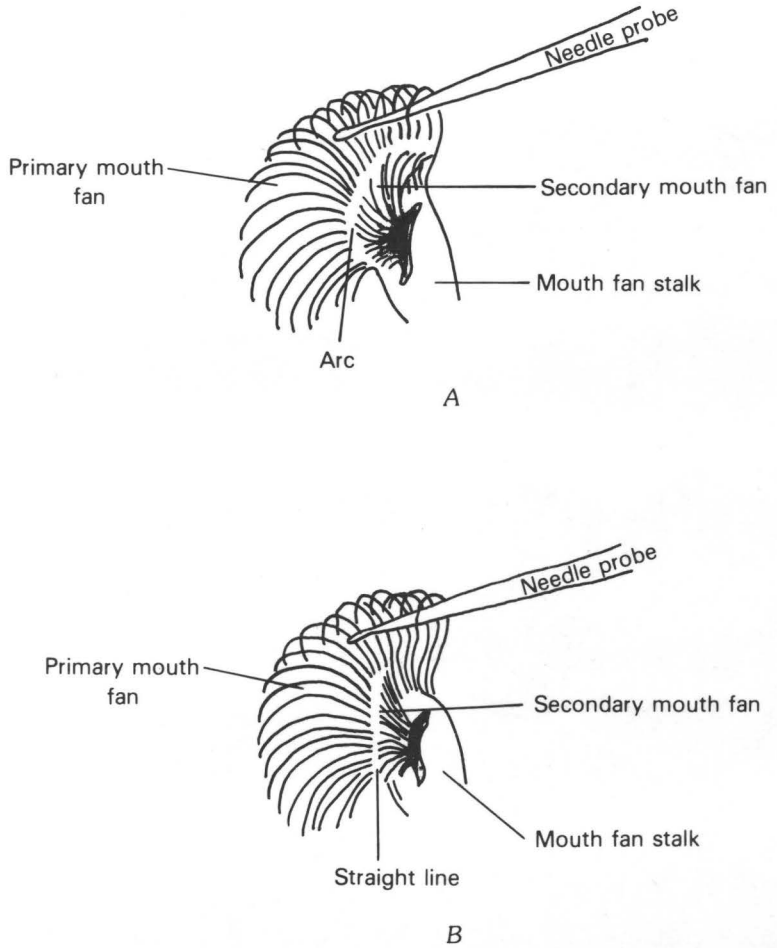

Figure 45.--Simuliidae larval mouth fans showing the two basic types of secondary fans, tips of the expanded secondary fan falling into: (A) an arc, and (B) a straight line.

On each side of the prothorax of a mature larva are histoblasts of the developing pupal respiratory organ (fig. 43). The number of filaments and their branching pattern are used for identification and to associate the larva with the pupa.

On the dorsal surface of the eighth abdominal segment are three simple or branched anal gills (fig. 43) that aid in respiration. These gills, which are useful for identifying genera, often are hidden in the rectal opening and may have to be exposed through dissection (Sommerman, 1953). In some genera, a pair of ventral tubercles is present just anterior to the posterior crochet ring (fig. 43).

Except for very small or mutilated specimens, most larvae and the pupae can be identified using a dissecting microscope without preparing a mount. Microscope slide mounts of the head region, however, are especially useful in identification of larvae to the species level.

1. Applications

The method is suitable for all immature Simuliidae.

2. Summary of method

The immature simuliids in a sample are examined and identified as precisely as possible without dissection or mounting. If necessary, dissection is performed and slide mounts are made. The taxa and numbers of individuals within each taxon are recorded and reported as a percentage of the total benthic-invertebrate population or reported in other ways appropriate to the study objectives.

3. Interferences

During slide preparation, overheating the larvae in 10-percent potassium hydroxide may result in brittleness, excessive transparency, or digestion of materials. The antennae are especially difficult to see if the specimen is overheated.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Cover glasses, circular, no. 1 or 2, 12-mm diameter.

4.2 Crucibles, high-form, porcelain, 10-mL capacity.

4.3 Forceps, blunt curved tips, and microforceps, fine-tipped.

4.4 Hotplate, electric.

4.5 Labels for microscope slides. When many slides are prepared, information about the source of the sample can be typed on sheets of paper, photocopied and reduced one-half or two-thirds in size, cut out, and glued onto slides using white glue, or equivalent. Labels, waterproof, or labels may be cut from sheets of plastic paper.

4.6 Marking pen, permanent, waterproof, for labeling slides.

4.7 Microscope, compound, preferably having differential interference contrast capable of 1,000X magnification.

4.8 Microscope slides, glass, precleaned, 25×75 mm.

4.9 Needles for manipulating and dissecting specimens under stereomicroscope.

4.10 Ocular micrometer, graduated to 5 μm.

4.11 Stereoscopic zoom microscope (dissecting), capable of 80X magnification.

4.12 Vials, 4 mL, and poly seal screw lids.

4.13 Watchglass, Syracuse type.

4.14 White glue.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetic acid, glacial.

5.2 Distilled or deionized water.

5.3 Fingernail polish, clear.

5.4 Glycerin.

5.5 Mounting medium, CMC-10, or prepare medium as follows: In 50 mL distilled water, dissolve 30 g Gum arabic (amorphic), 200 g chloral hydrate, and 20 mL glycerin. Completely dissolve each solid ingredient before adding succeeding reagents. Filter final mixture through clean cheese cloth.

5.6 Potassium hydroxide solution, 10 percent. Dissolve 10 g potassium hydroxide (KOH) pellets in 100 mL distilled water.

5.7 Preservative solutions. Samples may be preserved in 70-percent ethyl alcohol or 70-percent isopropyl alcohol. A mixture of 70-percent ethyl alcohol and 5-percent glycerin is preferred for permanent storage. Prepare as follows:

5.7.1 Ethyl alcohol. Dilute 70 mL 95-percent alcohol to 95 mL using distilled water.

5.7.2 Ethyl alcohol and 5-percent glycerin. Dilute 70 mL 95-percent alcohol to 100 mL using 25 mL distilled water and 5 mL glycerin.

5.7.3 Isopropyl alcohol. Dilute 70 mL concentrated isopropyl alcohol to 100 mL using distilled water.

6. Analysis

Usually, time does not permit mounting all the simuliids in a large sample, so the results from a subsample are used to calculate the distribution of taxa and number of individuals in the original sample. The size of the subsample for microscopic examination will depend on the size of the original sample, the number of visually distinct groups (see 6.2), and the study objectives.

6.1 Separate the pupae from the larvae and identify using a dissecting microscope. Identification of pupae is based primarily on the number and arrangement of respiratory filaments on the thorax. Slide mounts of pupae are not necessary because the filaments are clearly visible.

6.2 Using a dissecting microscope that has 7X or 20X magnification, separate the total larval group into subgroups on the basis of general external characteristics (for example, body color, presence or absence of ventral tubercles, color and length of antennae, size and shape of occipital cleft, and number and type of anal gills). Experience using taxonomic keys will aid in the selection of diagnostic characteristics for separating the subgroups.

6.3 Randomly select representatives of each subgroup for detailed microscopic examination and possible mounting. For small subgroups that have 10 or fewer individuals, select 5, or at least 50 percent. For larger subgroups, the subsampling should be by stratified random sampling and cluster or two-stage sampling. Store the remaining specimens in vials of 70-percent ethyl alcohol containing one or two drops of glycerin.

6.4 Place the selected larvae in a dish of 70-percent ethyl alcohol and examine using a stereoscopic microscope at a magnification of 10X to 70X. Identify the specimens using an appropriate taxonomic key. Examples of useful keys are Stone (1952), Sommerman (1953), Stone and Jamnback (1955), and Peterson (1970, 1978, and 1981).

6.5 In mature Simuliidae larvae, the histoblasts of the developing pupal respiratory filaments are well developed and can be used to identify the larvae with the pupal stage. The filaments are important key characteristics. Dissect them by piercing the integument around the entire filament, lift the filament, and cut it at the base. Record the number and pattern of the filament branches. Mount the filaments in a drop of mounting medium on a glass slide. Place a cover glass on the drop, and press firmly using a pair of curved-tip, blunt forceps.

If more information is needed to complete the larval identification, proceed to 6.6 through 6.10, which describe preparation of microscope slide mounts. Mounts facilitate identification of many small larvae by enabling the examination for submental teeth, mouth fan rays, and anal sclerites (fig. 45). Before mounting, be sure to record the important characteristics of the head specified in the keys, such as the anal gills, occipital cleft, ventral tubercles, and antennae, because they may be distorted when mounted.

6.6 Select eight larvae, and rinse each one in distilled water for 2 or 3 minutes. A Syracuse watchglass is a convenient vessel.

6.7 Place the larva in a high-form porcelain crucible containing 10-percent KOH, and heat on a hotplate for 8 to 15 minutes or until the body is noticeably lighter in color.

6.8 Rinse the larva in distilled water (Note 1) for 2 to 3 minutes, and rinse with 95-percent ethyl alcohol for at least 3 minutes to remove the residual water and KOH.

Note 1: Glacial acetic acid can be used to remove the KOH.

6.9 Place each larva in a drop of mounting medium on a clean glass slide and, using needles, position the specimen ventral side up. Place a circular cover glass on the preparation and press firmly using a pair of curved-tip, blunt forceps. Ensure that the larva remains ventral side up while pressing and that the antennae are clearly visible. Check the slide for clarity of diagnostic characteristics using a compound microscope. Allow preparation to dry for 1 week at room temperature, keeping the slide horizontal.

6.10 Specimens may dry after 2 or 3 years in the mounting medium unless the edges of the cover glass are sealed. To make the preparations more permanent, ring the slide by coating the edges of the cover glass and any exposed mounting medium with clear fingernail polish.

7. Calculations

7.1 When only part of the total sample of Simuliidae larvae is mounted and identified, project the results from the subsample to the total number of Simuliidae in the original sample:

$$\text{Total number of individuals of a particular taxon in sample} = \frac{\text{Number of individuals of the taxon in subsample}}{\text{Fraction of total sample in subsample}}.$$

7.2 Percent composition in sample

$$= \frac{\text{Number of individuals of a particular taxon}}{\text{Total number of individuals of all taxa}} \times 100 .$$

8. Reporting of results

Report the number of taxa present, the number and percentage of individuals in each taxon in the sample, and the method of collection.

9. Precision

No numerical precision data are available.

10. References cited

Hynes, H. B. N., 1970, The ecology of running waters: Toronto, University of Toronto Press, 555 p.

Peterson, B. V., 1970, The Prosimulium of Canada and Alaska: Memoirs of the Entomological Society of Canada, v. 69, 216 p.

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Stone, Alan, and Jamnback, H. A., 1955, The blackflies of New York: New York State Museum and Science Service Bulletin 349, 144 p.

Permanent- and Semipermanent-Slide Method for Aquatic Acari (B-5240-85)

Parameter and Code: Not Applicable

Water mites of the Order Acarina are found worldwide in almost all types of aquatic habitats, from the hot springs of Yellowstone National Park to the cold tundra pools of Alaska, and from swift, turbulent mountain streams to quiet lakes and stagnant ponds. Most species live in freshwater, although a few are strictly marine. Some species are subterranean. The adults and nymphs generally are free-living and predaceous, while the larvae primarily are parasitic on the immature and adult stages of Diptera, Hemiptera, Odonata, Plecoptera, and other aquatic and semiaquatic insects. The larvae also are known to parasitize the gills of crabs and mussels.

Water mites have little economic significance other than being food for fishes, such as the brook and rainbow trout (Marshall, 1933); however, this little-known group of arthropods may have unrecognized economic importance as a biological control agent of mosquitoes and other biting insects. Uchida and Miyazaki (1935) reported that an Anopheles mosquito infested with five or more mites cannot be induced to bite, thus interrupting the life cycle that is dependent on a blood meal. Abdel-Malek (1948) reported that Aedes adults infested with water mites produced fewer eggs than uninfected individuals.

Water mites may prove important in water-quality studies because of their acute sensitivity to environmental stress (Young, 1969) and their species, and even generic, specificity for particular habitats. The water-mite fauna found in a cold mountain stream is distinctively different from the fauna of a pond or lake or the fauna of a hot spring.

A water mite has four stages in its life cycle--egg, larva, nymph, and adult. The larva, the smallest stage, has three pairs of legs instead of four pairs as in the nymph and the adult stages. The nymph is larger than the larva and commonly is brightly colored with shades of red and orange, especially in stillwater forms. Stream mites frequently are a dull brown or greenish brown.

The adult water mite is ovoid to globular in shape and has an unsegmented, fused cephalothorax and abdomen. The sexes are separate. The dorsum may be thin and leathery, or may have sclerotized plates (fig. 46). The legs have short bristles and long swimming hairs, particularly in the pond and lake forms. The nymph differs from the adult by having an incomplete genital field; that is, it lacks a genital opening and has fewer genital acetabula (fig. 46).

The anterior end of the body has the mouth region or gnathosoma (fig. 46), which sometimes is lengthened anteriorly into a rostrum. At the base of the gnathosoma are two pairs of mouth parts that are key characteristics for identification, a pair of chelicerae (mandibles) and a pair of palps. The palps consist of five segments--P_1 through P_5 (fig. 47)--that may have a number of setae and spines and terminate in simple or scissorlike claws.

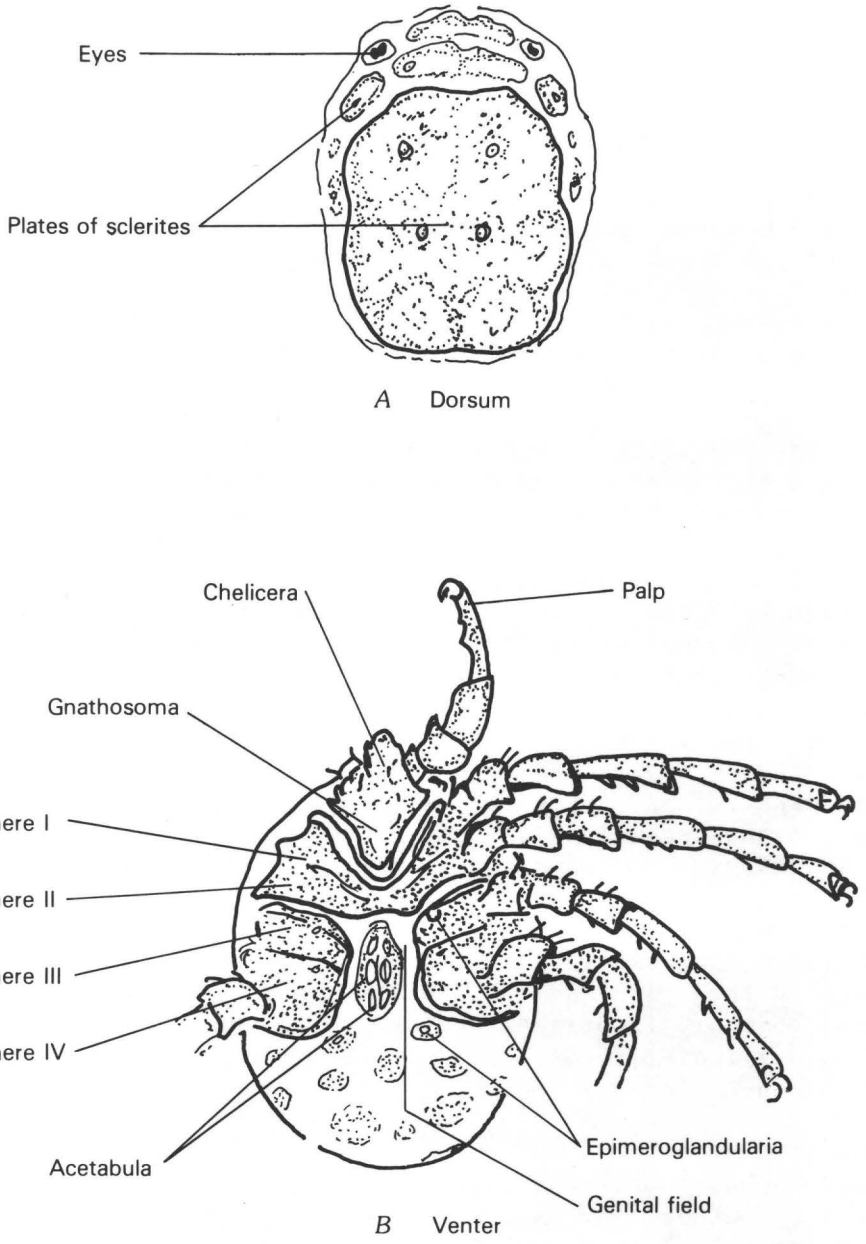

Figure 46.--Dorsal (A) and ventral (B) views of an adult water mite showing important morphological features used for identification.

The coxal parts of the legs, called epimeres (fig. 46), are on the underside or venter of the mite. There are four pairs of epimeres that vary in shape, position, and degree of fusion or separation. The genital field, consisting of a number of acetabula and a genital opening, is either between or behind the fourth epimere, or on the posterior margin of the venter.

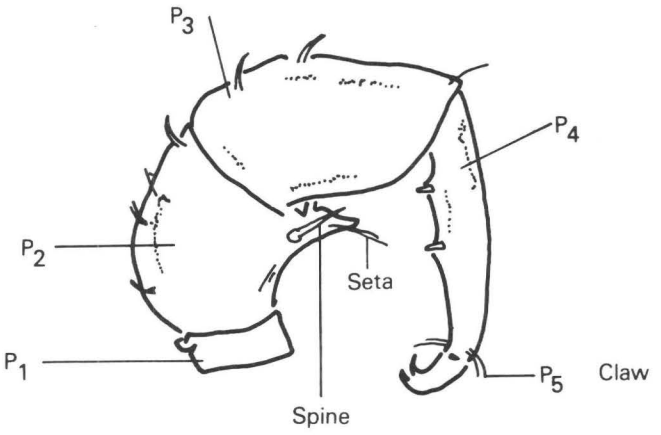

Figure 47.--Five-segmented palp (P_n) of a water mite.

Other diagnostic characteristics on the venter are three pairs of epimeroglandularia, each of which consists of a gland pore and a hair or seta. Epimeroglandularia I usually is found between epimere II and III, epimeroglandularia II is variable in position, but often is lateral to the genital opening, and epimeroglandularia III is behind epimere IV. The configuration of the epimeres, the number and arrangement of the acetabula in the genital field, and the relative position of the epimeroglandularia are important characteristics used in the identification of water mites.

Minimal information about water mites of streams exists. There are scattered descriptions of stream mites, but no single work exists that can be used for identifying them. In contrast, the water mites of ponds and lakes have been fairly well studied. Since the early 1900's, a few descriptive papers on North American water mites have appeared, particularly by researchers such as Marshall (1940, 1943), Cook (1954a,b, 1974), Crowell (1960), and Krantz (1975). Mitchell's (1954) checklist is a valuable source of information about reported American water-mite species and the relevant literature. To collect specifically for water mites, use the procedures described by Cook and Mitchell (1952).

To adequately identify water mites, mounts must be made for microscopic examination. The method described in this section is a modification of the double cover-glass glycerin method developed by Mitchell and Cook (1952) and Cook (1974).

1. Applications

This method is suitable for freshwater and marine mites, in the adult or nymph stage, that have been preserved in alcohol.

2. Summary of method

The water mites in a sample are dissected, cleared, and permanent-slide mounts are made for microscopic examination and identification. The kinds of taxa and the number of individuals in each taxon are recorded and reported as a percentage of the benthic-invertebrate population or reported in other ways appropriate to the study objectives.

3. Interferences

Failure to remove or digest the body contents of water mites will result in obscured mounts. Prolonged soaking in potassium hydroxide may damage the cuticle of mites. Unless the more time-consuming method is used, mounts will continue to clear and fade for a few days after slide preparation is complete, making specific identification difficult and sometimes impossible.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Cover glasses, circular, no. 1, 12 mm, and cover glasses, circular, no. 1, 22 mm.

4.2 Forceps, blunt curved tips, and microforceps, fine-tipped.

4.3 Hotplate, electric, or slide warmer.

4.4 Labels for microscope slides. When many slides are prepared, information about the source of the sample can be typed on sheets of paper, photocopied and reduced one-half or two-thirds in size, cut out, and glued onto slides using white glue, or equivalent. Labels, waterproof, or labels may be cut from sheets of plastic paper.

4.5 Marking pen, permanent, waterproof.

4.6 Microscalpel, capable of dissecting a specimen, 0.75 mm in diameter. A no. 1 insect pin, mounted on a wooden applicator stick and shaped into a microscalpel using a fine hone or emery cloth and a dissecting microscope, is satisfactory (Cook, 1974).

4.7 Microscope, compound, preferably having differential interference contrast capable of 1,000X magnification.

4.8 Microscope slides, glass, precleaned, 25×75 mm.

4.9 Needles, pins, or probes for manipulating specimens under a stereo-microscope.

4.10 Oven.

4.11 Spot plates, white porcelain.

4.12 Stereoscopic zoom microscope (dissecting), 30X to 70X magnification.

4.13 Vials, 4 mL, and poly seal screw lids.

4.14 Watchglass, Syracuse-type.

4.15 White glue.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Canada balsam, grade A.

5.2 Corrosive lactophenol. Add 50 mL lactic acid to 25 mL distilled water. Add 25 g phenol crystals and dissolve completely.

5.3 Distilled or deionized water.

5.4 Fingernail polish, clear.

5.5 Glycerin.

5.6 Glycerin jelly. Melt jelly in a dropper bottle or vial emersed in a beaker of hot water. Heat water just enough to liquefy the jelly.

5.7 Mounting medium, CMC-10, or prepare medium as follows: In 50 mL distilled water, dissolve 30 g Gum arabic (amorphic), 200 g chloral hydrate, and 20 mL glycerin. Completely dissolve each solid ingredient before adding succeeding reagents. Filter final mixture through clean cheese cloth.

5.8 Potassium hydroxide solution, 10 percent. Dissolve 10 g potassium hydroxide (KOH) pellets in 100 mL distilled water.

5.9 Preservative solutions. Samples may be preserved in 70-percent ethyl alcohol or 70-percent isopropyl alcohol. A mixture of 70-percent ethyl alcohol and 5-percent glycerin is preferred for permanent storage. Prepare as follows:

5.9.1 Ethyl alcohol. Dilute 70 mL 95-percent alcohol to 95 mL using distilled water.

5.9.2 Ethyl alcohol and 5-percent glycerin. Dilute 70 mL 95-percent alcohol to 100 mL using 25 mL distilled water and 5 mL glycerin.

5.9.3 Isopropyl alcohol. Dilute 70 mL concentrated isopropyl alcohol to 100 mL using distilled water.

6. Analysis

For samples containing few water mites, prepare mounts of all individuals. If the numbers are large, separate the mites into distinct groups (see 6.1) and take a subsample of each group (see 6.2). Use the results from the subsample to calculate the distribution of taxa and individuals in the original sample.

6.1 Using a dissecting microscope with 30X to 70X magnification, separate the water mites in a sample into groups on the basis of general external characteristics. Important characteristics include color, texture of the dorsum (for example, covered by a shield, small sclerites, or leathery), epimere configuration, number and arrangement of the acetabula, and position of the genital field (fig. 46).

6.2 Proceed to 6.3 if all water mites will be mounted. In large samples, randomly select representatives of each group for mounting on slides for microscopic examination. Subsampling should be done by stratified random sampling and cluster or two-stage sampling. Store remaining mites in vials of 70-percent ethyl alcohol containing one or two drops of glycerin.

6.3 Place the specimen to be examined in a watchglass containing 70-percent ethyl alcohol. Using a dissecting microscope, microscalpel, and fine-tipped microforceps, separate the dorsum from the venter, leaving a small section of the lateral body wall intact (fig. 48). The intact body wall prevents body parts and appendages from being lost. In large specimens from which the body contents can be removed using the tip of a needle, omit 6.4 and 6.5 for clearing, and proceed to 6.6 or 6.20. If the specimen is too small for dissection, pierce the body wall in the posterio-lateral area to facilitate the clearing process.

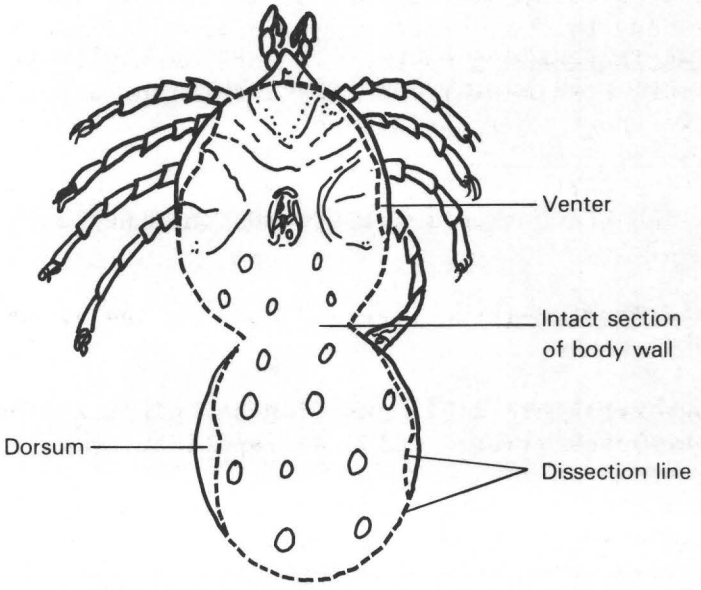

Figure 48.--A water mite showing the dorsum separated from the venter, leaving a small section of the lateral body wall intact (see 6.3).

6.4 Clear the specimen for 24 to 48 hours in a vial containing the corrosive lactophenol. Prolonged clearing has minimal damaging effect. If the specimen has a particularly hard cuticle, clear in 10-percent KOH for 1 to 2 hours. Care must be taken to avoid damage to the cuticle by prolonged soaking in KOH.

6.5 Remove the lactophenol or KOH corrosive by rinsing the specimen in three to four changes of distilled water (Note 1) followed by 70-percent ethyl alcohol.

Note 1: Two different methods of slide preparation are described based on the quality of the resulting mounts for taxonomic identification. The method described in 6.6 through 6.19 is more time consuming, but results in longer lasting slides suitable for species identification. The quicker, optional method described in 6.20 through 6.22 results in slides adequate for identification to family or genus. Selection of the method should be based on study objectives.

6.6 Transfer the specimen to glycerin. With weakly sclerotized specimens, distortion sometimes occurs when transferring directly to glycerin. For such specimens, proceed to 6.7 and 6.8.

6.7 Transfer the specimen to a depression in a spot plate containing two or three drops of alcohol-glycerin solution.

6.8 Place the spot plate and water mite in an oven at 55 °C for 30 to 40 minutes to evaporate the alcohol, leaving the mite in the glycerin.

6.9 Lift the specimen from the glycerin using the tip of a needle, and place on a 12-mm diameter circular cover glass.

6.10 Using a dissecting microscope, microforceps, and needle, separate the palps from the body by dissecting one palp from the gnathosoma or by removing the entire gnathosoma and palps. The dorsum may be severed from the venter. In very small specimens for which dissection is difficult, leave the specimen intact with the venter facing upward.

6.11 Arrange the parts on the cover glass so the original exterior surface of the venter and the dorsum faces upward, and the palps can be viewed as shown in figure 47.

6.12 Place a drop of melted glycerin jelly on the 12-mm cover glass and specimen.

6.13 Move the parts into final position and place a 22-mm circular cover glass on the smaller cover glass, jelly, and specimen.

6.14 Press large cover glass gently using curved-tip, blunt forceps to spread jelly evenly to edges of smaller cover glass, turn preparation over with smaller cover glass up, and continue pressing smaller cover glass enabling excess glycerin jelly to ooze from the edges.

6.15 Set preparation aside for at least 15 minutes to allow the glycerin jelly to set.

6.16 Place one drop of Canada balsam on a clean glass microscope slide, and place the double cover-glass preparation, 12-mm cover glass down, on the drop of balsam (fig. 49). Press lightly. If bubbles are present in the balsam under the cover glass, they may be removed by warming the slide preparation at 45 °C on a hotplate or on a slide warmer.

6.17 Label slide, using waterproof ink, and record the date, site, method of collection, identification number, or other information pertinent to the study.

6.18 Identify water mites using a compound microscope and appropriate taxonomic keys. Examples of keys for the nonspecialist are Newell (1959), Cook (1974), and Pennak (1978).

6.19 Allow slides to air-dry for at least 2 months before storing on edge.

6.20 Optional method. Place the specimen in a small drop of mounting medium on a clean glass microscope slide. Using a dissecting microscope, microforceps, and needle, dissect the specimen and arrange the parts as in 6.10 and 6.11. Ensure that the parts are pushed well into the medium and against the slide to prevent them from drifting away when the cover glass is applied.

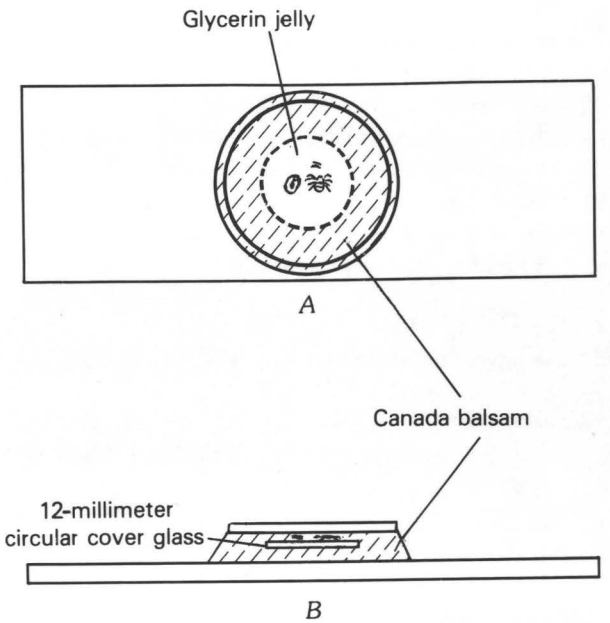

Figure 49.--Top (A) and side (B) views of the double cover-glass technique for mounting aquatic Acari (modified from Mitchell and Cook, 1952).

6.21 Place a 12-mm circular cover glass on the drop of mounting medium containing the specimen, and press cover glass gently using curved-tip, blunt forceps. Allow preparation to dry for 1 week at room temperature, keeping the slide horizontal.

6.22 Specimens may dry after 2 or 3 years in the mounting medium unless the edges of the cover glass are sealed. To make the preparations more permanent, ring the slide by coating the edges of the cover glass and any exposed mounting medium with clear fingernail polish.

7. Calculations

7.1 When only part of the total sample of Acari is mounted and identified, project the results from those mounted to the total number of specimens:

$$\text{Total number of individuals of a particular taxon in sample} = \frac{\text{Number of individuals of the taxon in subsample}}{\text{Fraction of total sample in subsample}} .$$

7.2 Percent composition in sample

$$= \frac{\text{Number of individuals of a particular taxon}}{\text{Total number of individuals of all taxa}} \times 100 .$$

8. Reporting of results

Report the number of taxa present, the number and percentage of individuals in each taxon in the sample, and the method of collection.

9. Precision

No numerical precision data are available.

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AQUATIC VERTEBRATES

Introduction

In most aquatic ecosystems, fish are the most common vertebrates. Because they are dependent on lesser life forms for food, the health of a local fish population commonly is used as an index for water quality and for the health of other aquatic organisms. Fish, however, are mobile animals and may avoid undesirable water quality (Whitmore and others, 1960). Moreover, they may exist for relatively long periods of time without food.

Although the investigation of fish populations is not a major interest of the U.S. Geological Survey, such investigations may at times provide valuable information about the aquatic environment. For example, length-weight relations can be used to compare fish from several streams, and changes in species composition with time may reveal water-quality trends, such as increased enrichment or a temperature increase of a particular aquatic environment. Stomach analyses reveal the organisms on which the fish feed; this information is essential to understanding the aquatic ecosystem.

The presence of dead or dying fish is indicative of lethal environmental conditions, unless it is a postspawning mortality or a delayed mortality resulting from cellular buildup of toxic materials. Onsite personnel can acquire valuable information by observing and collecting distressed fish. Pathological and histological examination of such fish may disclose the cause of death; however, on-the-spot observations of existing conditions, such as color of the water, floating material, effluent discharge, and the immediate collection of a water sample, are vital for a true explanation of the mortality (American Public Health Association and others, 1985).

In all States, some fish species and other aquatic vertebrates are protected by law, and the collection of others is regulated. Onsite personnel should ensure that they have complied with State laws before collecting samples of fish and other aquatic vertebrates. Hocutt (1978, p. 88) has prepared a listing, by specific year, for those States that require a permit or a license, or both, to collect fish. Czajka and Nickerson (1977) have prepared a similar list for the collection of reptiles and amphibians.

Although the methods described in this section are applicable to fish and other aquatic vertebrates, the emphasis generally will be on fish.

Collection

Collecting specimens for study requires a knowledge of the selectivity, limitations, and efficiency of the different types of sampling gear. Sampling gear and its use are discussed in Lagler (1956), Ramsey (1968), Weber (1973), Everhart and others (1975), Hocutt (1978), and American Public Health Association and others (1985).

Because of the nonrandom distribution of fish populations, the choice of sampling method, time of sampling, and frequency will depend on the objective of the particular investigation.

Active Sampling Gear

Active sampling gear, such as seines, trawls, electrofishing, chemical fishing, and hook and line, generally are less selective and commonly are preferred to passive techniques, such as gill, trap, hoop, and fyke nets.

If the data are to be used statistically (quantitatively), the method(s) of collection must be comparable numerically. Many fishery studies, for example, are concerned with determining yield biomass per unit area or estimating population densities in number per unit area based on a sample of the total population.

Ichthyocides (fish toxicants) provide the best method for collecting quantitative data; however, electrofishing often is the method of choice where chemicals cannot be used. While seines and other types of nets are basically qualitative gear, quantification of data is possible when the same experienced personnel do the collecting and all other factors are equal.

Seines

Seines consist of a length of strong netting material attached to a float line at the top and a heavily weighted lead line at the bottom. The ends of the seine are attached to a short stout pole or brail. If the net is large, hauling lines are attached to the top and bottom of the brail by a short bridle (fig. 50).

The sides, or wings, of the seine generally are of larger mesh than the middle, or bunt, part. The bunt may be in the form of a bag to confine the fish. Bag seines are most useful in ponds and lakes, and straight seines usually are used in streams and rivers. Small seines (50 ft or less) are adequate for capturing small fish. For capturing larger fish, especially in clear water, seines of 100 ft or more are necessary.

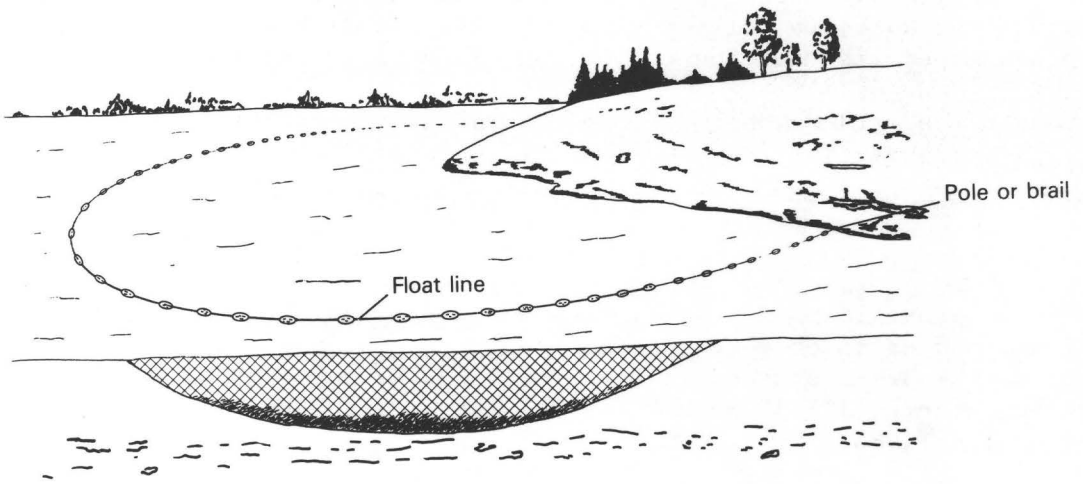

Figure 50.--Common haul seine (modified from Dumont and Sundstrom, 1961).

Bag seine.--The bag seine is most useful in small ponds or lakes but may be used in slow-flowing rivers. Select a shoreline section that is free of stumps and other obstructions. Secure or hold one end of the seine to the bank, and extend the seine into the water at right angles. Pull the extended end of the seine toward the bank so the seine forms the radius of a circle (Lagler, 1956, p. 8, fig. 2). With both ends of the seine beached, pull the remainder of the seine slowly into shore, keeping the lead line in contact with the bottom. Continue pulling until the opening of the bag reaches the shoreline. Remove the specimens, and process using the method selected based on the objectives of the study.

Straight seine.--Select a suitable area, usually a stream section having a smooth or relatively smooth bottom. Beginning at the downstream boundary of the area, pull the seine upstream into the current as rapidly as possible. Ensure that the bottom edge of the seine (lead line) is in contact with the stream bottom at all times. At the upstream boundary of the area, beach or bring the seine to the bank and quickly lift it from the water, forming a pocket in its center.

When using the larger seines in rivers and lakes, the usual method is to leave one end of the net, or hauling line, on shore while the net is played out by hand or boat perpendicular to the shore until the net is nearly extended. Direction then is changed (usually downstream) to lay out the remaining net parallel to the shore. When the net is fully extended, the end of the second haul line, or brail, is brought to the shore.

When fishing for pelagic or schooling species, one end of the net may be hauled first to form a hook against the shore. As soon as a school of fish enter the area, the second line is hauled. When fishing for nonschooling species, both ends of the net usually are hauled in at once.

With either type of net, be certain the lead line remains in contact with the bottom at all times. Continue pulling until the pocket, or bag, reaches the shoreline. Remove specimen(s) and process using the method selected based on the objective of the study.

Trawls

Trawls are specialized seines used in large, open-water areas where they are towed behind boats at sufficient speeds to overtake and enclose fish on the bottom or to collect schooling fish at various depths (figs. 51, 52). Beause of the size and weight of the equipment, trawls have limited usefulness in lakes and reservoirs. For more information, refer to Massman and others (1952), Rounsefell and Everhart (1953), and Dumont and Sundstrom (1961).

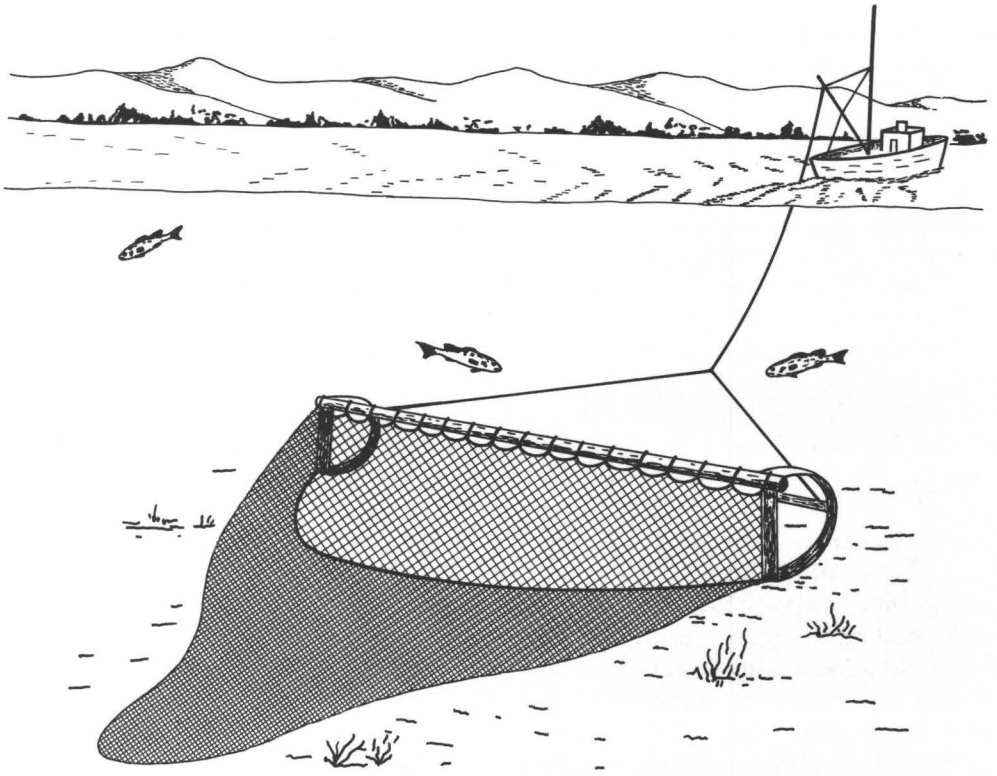

Figure 51.--Beam trawl (modified from Dumont and Sundstrom, 1961).

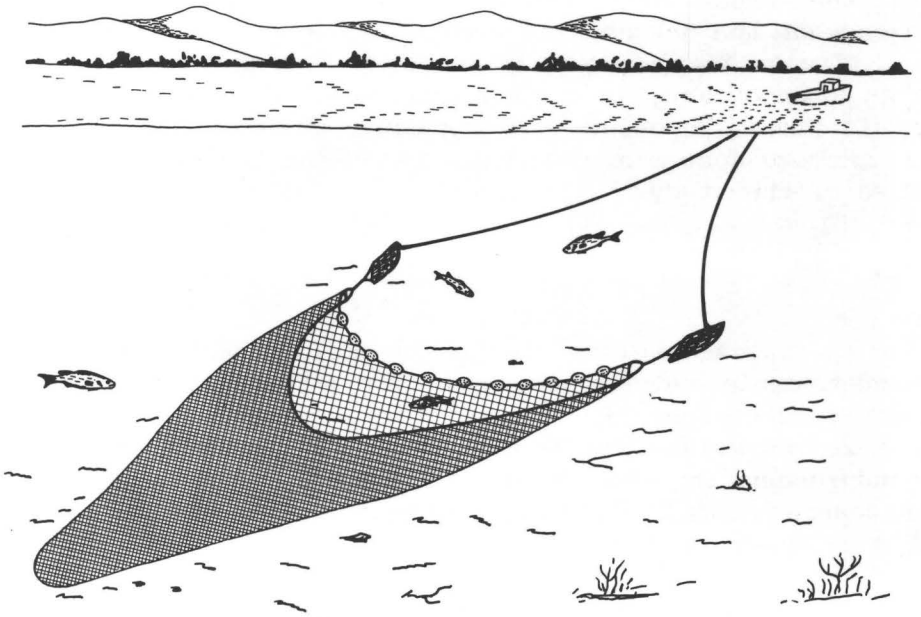

Figure 52.--Otter trawl (modified from Dumont and Sundstrom, 1961).

Electrofishing

Applying alternating or direct electrical current [at the specified (110 V ac or 220 V dc) output amperage] to water to induce subnarcosis or the temporary immobilization of fish is an efficient method of capturing fish. A pulsed direct current of 50 to 100 pulses per second, at the specified output amperage, includes electrotaxis of the fish and attracts it to the positive electrode, or anode, where it is netted (Sharpe and Burkhard, 1969). Alternating current is most useful in streams of very weak resistance.

Electrofishing can be hazardous and must be used with caution. All personnel engaged in electrofishing must wear protective rubber waders and low-voltage Trapper's gloves, and adhere strictly to safety precautions. Training of all crew members in first-aid for electrical shock and drowning is advisable. The method is best suited for small streams but is adaptable to lakes and slow-flowing rivers as described by Frankenberger (1960) and Sharpe (1964).

After selecting a suitable site, position the electrodes according to the manufacturer's instructions for the type of water being sampled. Electrofishing generally is done upstream from a natural barrier or block seine placed across the stream. Shock all areas that may have fish, such as brush, fallen trees, boulders, and undercut banks. When making population estimates, shock the same reach three or more times (Zippin, 1956). Capture efficiency varies with the species of fish, current velocity, turbidity, water conductivity, experience of personnel, and other variables (Cross and Stott, 1975). Friedman (1974) prepared a selected bibliography about the use of electrofishing that included the state of the art during 1974.

Captured fish should be placed in live cages for processing. When possible, identify specimens onsite, and release after processing. If onsite identification is not possible or only tentative, count the number of individuals in each taxa, and preserve about 20 representative specimens for laboratory examination. Processing of specimens will depend on study objectives but generally includes length, weight, sex, and scale samples for age-growth analysis. Lagler (1956) and Everhart and others (1975) are excellent sources for additional information about fishery science.

Ichthyocides

Ichthyocides, or fish toxicants, provide a good sampling method for making qualitative and quantitative studies of fish populations. Relative abundance, diversity, and biomass can be estimated more precisely using ichthyocides than using any other means. However, their use requires careful planning, and special permits from State conservation agencies usually are required.

Rotenone obtained as an emulsion, containing 5-percent active ingredient, is the most popular chemical because it is relatively safe to use, is not persistent in the environment, and is fairly easy to detoxify. A general review of the literature about ichthyocides was prepared by Lennon and others (1971) and about rotenone specifically by Schnick (1974).

Fish toxicants generally are used in areas such as small embayments of lakes and reservoirs or short reaches of streams or rivers. The concentration of active ingredient necessary to effect a good recovery of most fish is dependent on the species present and the alkalinity of the water. Alkaline water requires a larger concentration as do species of bullheads, carp, and eels. The successful use of rotenone is dependent on exposing the desired fish population to a lethal dose (generally 0.25 to 1 mg/L) for at least 15 minutes.

The use of rotenone in small streams is discussed by Lennon and Parker (1959) and Boccardy and Cooper (1963), in large rivers by Hocutt and others (1973), and in impoundment surveys by Eschmeyer (1939), Lambou (1959), and Bone (1970). Weber (1973) describes several methods of application.

To determine the quantity of rotenone to use, calculate the volume (acre-feet) of water to be treated. For lakes, the volume is simply the area times the mean depth, divided by 43,560 to obtain acre-feet. Because 1 acre-ft of water weighs 2,718,144 lb, an investigator would need approximately 2.7 lb of rotenone for a concentration of 1 (mg/L)/acre-ft. For streams, the quantity of rotenone is based on the cubic feet of water passing a point in the stream for the 15 minutes necessary for the exposure period. To calculate, multiply width times mean depth times velocity, which equals cubic feet of water per second. Cubic feet per second times 900 seconds (15 minutes) equals total cubic feet of water to treat. Total cubic feet divided by 43,560 equals acre-feet of water.

Potassium permanganate ($KMnO_4$) is used to detoxify the rotenone. To calculate the quantity of $KMnO_4$ necessary to detoxify the rotenone, calculate the weight of water treated and apply $KMnO_4$ at the same concentration that the rotenone was applied.

Hook and line

Although the method is too selective to be used for population studies, it is a useful technique for capturing small numbers of adult fish for metal or pesticide analyses when other methods are impractical.

Passive Sampling Gear

Gill nets and other entanglement and entrapment devices are used to passively sample fish communities in lakes, reservoirs, estuaries, and large slow-moving rivers. Gill nets hang vertically in the water and may be fished at the surface or at any depth. Because fish caught in the net die within a short period of time, the nets need to be checked at least once every 12 hours. Gill nets are set most successfully in the evening and recovered early the next morning. Gill nets generally are set perpendicular to the shoreline. Lackey (1968) and Jester (1977) describe the effective use of gill nets (fig. 53).

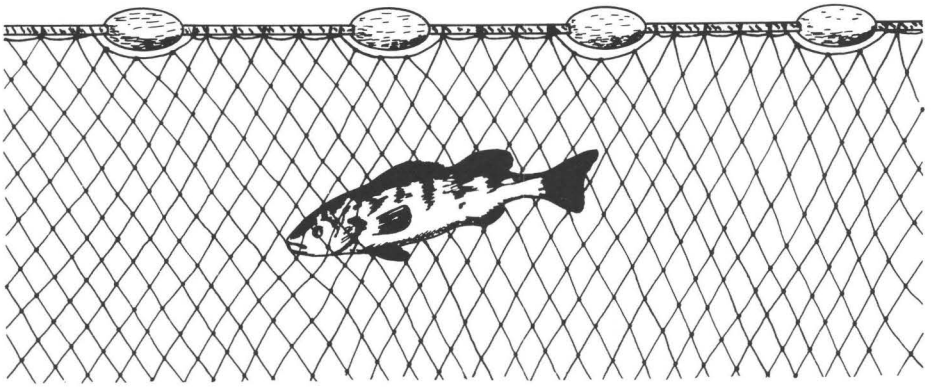

Figure 53.--Gill net (modified from Dumont and Sundstrom, 1961).

Drifting gill nets are set and fished the same way as stationary gill nets except they are allowed to drift with the current. Gill nets are selective in what they capture because of the size of the mesh of the net and because some species are more susceptible to nets than others (Berst, 1961).

Entrapment devices include a variety of nets and traps designed to lure and guide the fish through a series of funnels from which it cannot escape (Beamish, 1973; Yeh, 1977). The two most common devices are the hoop net (figs. 54, 55) and the trap net. These devices are easily set from a small boat. The nets are held in place by anchors or poles and are used in water less than 4 m deep. Fyke nets are a type of hoop net that has wings, or a lead, or both. They are used in lakes and reservoirs where fish movement is more random. Trap nets are similar to hoop nets except floats and weights instead of hoops are used to give the net shape. An adequate sample of fish often can be captured by using a combination of hoop and trap nets of various mesh sizes in the available habitat.

Investigation of Fish Kills

For investigation of fish kills, collect live or distressed specimens, if possible, because they are more suitable for pathological and histological examination. Specimens generally can be collected using a dip net. Specimens that have died recently are a second choice, but the fact that they were dead when collected should be noted clearly on the sample label. Collect about 0.5 kg of fish or other vertebrates and, if possible, about five individuals if the whole animal is to be ground for analysis. Collect a proportionally larger sample when individual tissues are to be analyzed. Generally, a sample of 5 kg will be adequate.

Collect specimens of the same type of organism as those affected from an area within the same body of water that had not been contaminated by the causative agent. These specimens should be handled separately. Collect 20 or more drops of blood from these specimens in a solvent-rinsed vial, seal with teflon or aluminum foil, cap, and freeze. Collection method will depend on the type of habitat to be sampled (Lagler, 1956).

Identify preserved specimens using the best available taxonomic keys or other appropriate means. Proper identification of species involved is necessary to assess the monetary loss due to the destruction of valuable fish and other animal life.

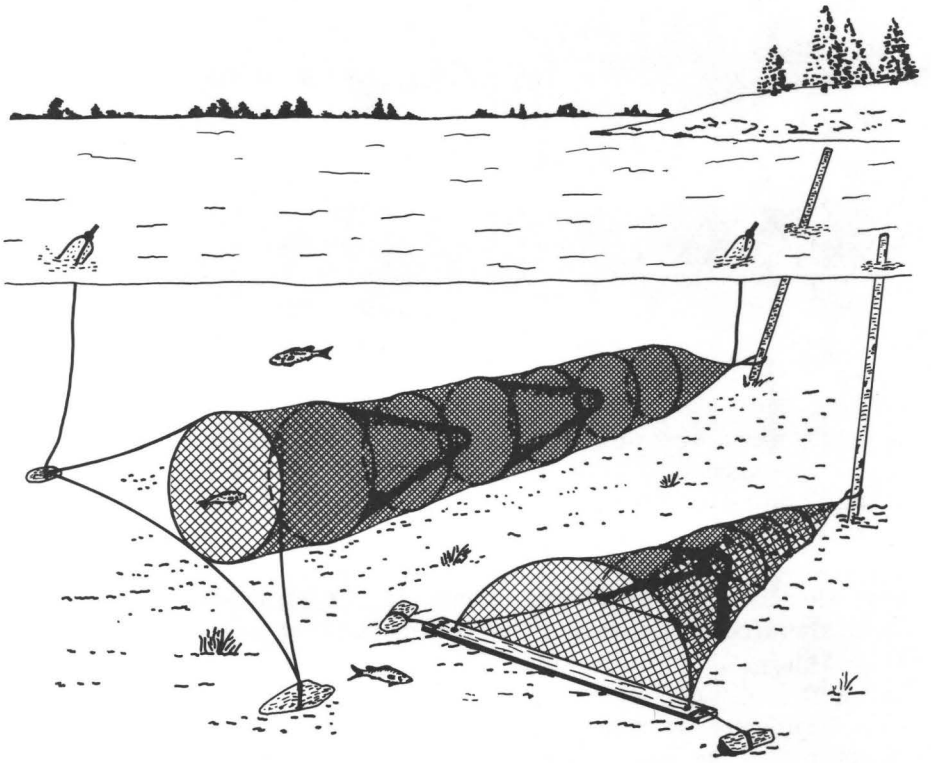

Figure 54.--Hoop net (modified from Dumont and Sundstrom, 1961).

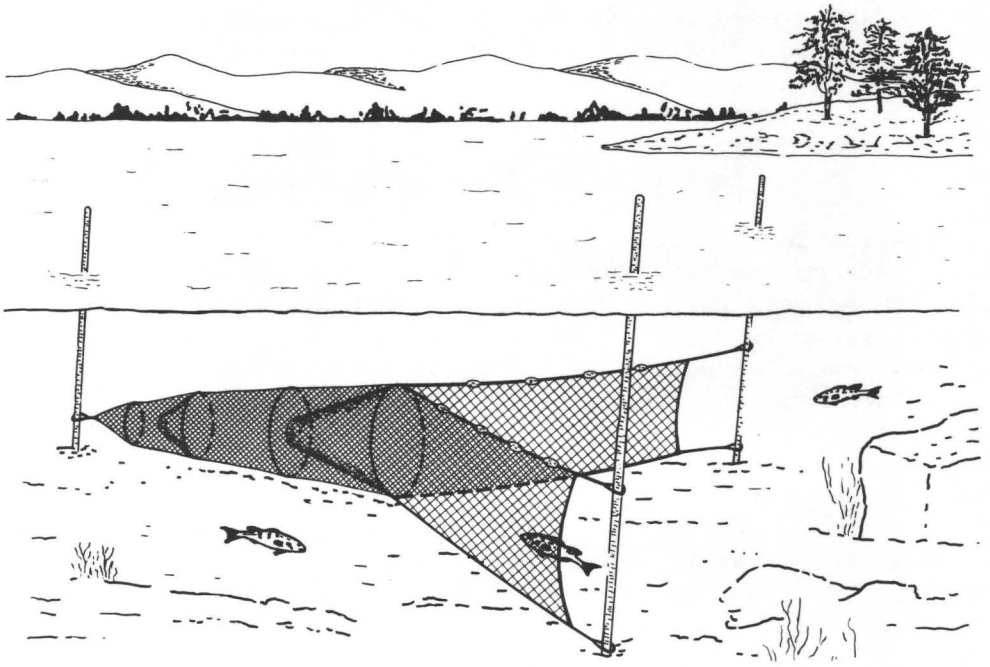

Figure 55.--Fyke net (modified from Dumont and Sundstrom, 1961).

Preparation and Storage

Package the fish in labeled polyethylene bags and freeze (Note 1). Samples may be packed in insulated cartons or chests and refrigerated using about 5 kg of dry ice per 5 to 8 kg of fish.

Note 1: Samples collected for polychlorinated biphenyl (PCB) or other organic-compound analysis should be stored in glass containers or wrapped in aluminum foil. If freezing facilities are not available, preserve the fish in ethyl alcohol (Cope, 1960; Wood, 1960).

Before placing in the preservative, slit each fish from the anus to the gills. Use at least five volumes of preservative for each volume of fish. To avoid contamination, package the fish collected dead separately from those that were collected alive. Labels placed in the same bag with wet fish may become illegible. Tie labels to the outside of the bag.

Estimate the intensity or degree of kill by counting the number of distressed or dead fish per unit length of shoreline, water-surface area, or number of fish passing a point per unit time. Record any factors at the site of the kill that will be useful in identifying the source of the kill. At a minimum, record the name and location of water, time, date, general characteristics of water (color, odor, and other characteristics), and present and previous weather conditions. Also, record name and telephone number of agency or individual reporting the kill, suspected causative agent(s), and suspected source(s).

Whenever possible, measure dissolved oxygen, temperature, pH, and specific conductance upstream and downstream from suspected source(s) of pollutant(s). Also, collect an adequate number of water samples (at least 1 L) upstream from and at the source(s) of suspected pollutant(s). The samples should be chilled to 4 °C.

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Faunal Survey (Qualitative Method)
(B-6001-85)

Parameter and Code: Not applicable

1. Applications

The methods are applicable to all water.

2. Summary of method

Fish and other aquatic vertebrates are collected, preserved, and identified using appropriate taxonomic keys.

3. Interferences

Physical factors, such as stream velocity, depth of water, and turbidity, may make collection difficult. Filamentous algae and macrophytes may interfere with the operation of nets and seines.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

Methods and equipment for the collection of fish are described by Lagler (1956), Needham and Needham (1962), Calhoun (1966), Weber (1973), Everhart and others (1975), Hocutt (1978), and American Public Health Association and others (1985). Hocutt (1978) also discussed methods and equipment for the collection of amphibians and reptiles. State conservation agencies, the U.S. Fish and Wildlife Service, and commercial fishermen are other sources of information for obtaining the proper collecting equipment. Weber (1973, p. 171) lists publications containing information about fishery sampling equipment.

4.1 Bag seine, about 25 to 50 ft × 6 or 8 ft. The mesh size should be 1/2 in. square for the wings and 1/4 in. square for the bag.

4.2 Dip net, about 15-in. bow, 45-in. handle, 18-in. depth knotless nylon net, and 3/8-in. square mesh.

4.3 Dissecting kit. Routine dissecting tools. Dissection of the fish for internal examination frequently is required.

4.4 Dissecting microscope, low power of about 7X and stronger, either rotary or stereozoom type of binocular microscope. A substage mirror is essential.

4.5 Divider, fine-pointed, or dial caliper, for measuring body proportions.

4.6 Electrofishing gear. The basic unit consists of a generator (110 V ac or 220 V dc), sufficient insulated electrical wire, and two or three electrodes.

4.7 Forceps, long, for removing specimens from jars, and fine-pointed forceps that meet at the tip, for proper grasping of fins of small fishes and for removal of pharyngeal teeth of small cyprinids.

4.8 Gill net, experimental, about 6×125 ft. Most nets are made in 25-ft panels joined into continuous lengths that have four to five panels of different mesh size. The mesh size should range from about 1/2 in. at one end to about 2 in. at the other end. When equipped with poly-foam float line and lead-core leadline, the nets are virtually tanglefree. Mesh combinations and hanging sequence may be varied to suit individual requirements.

4.9 Gloves, waterproof, low-voltage rubber, Trapper's, shoulder length, for use with electrofishing gear.

4.10 Light source that has very intense illumination. Many investigators favor a gooseneck lamp and a 100-W lightbulb; others favor the smaller lamps that project a concentrated beam of light. The important goal is to bring the light as close to the subject as possible.

4.11 Nylon-mesh cage, about 4×4×4 ft, and 1/4-in. mesh to hold fish after capture.

4.12 Rule, stainless steel, metric, and a divider for obtaining actual measurements.

4.13 Sample containers, plastic, wide-mouth jars, about 0.5-, 1-, and 2-L capacity. Lids should be of plastic if used for prolonged storage of preserved specimens.

4.14 Straight seine, 10×5 ft × 1/8-in. mesh, minnow type, and 25×6 ft × 1/4-in. square mesh.

4.15 Trawls, traps, and hoop nets, available through commercial fishing supply outlets.

4.16 Waders, chest-type, for use with electrofishing gear.

4.17 Waterproof ink.

4.18 Waterproof labels, or labels may be cut from sheets of plastic paper.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Alcohol, isopropyl, 40-percent solution. Dilute 40 mL concentrated isopropyl alcohol to 100 mL using distilled water.

5.2 Distilled or deionized water.

5.3 Formaldehyde solution, 4 percent. Dilute 10 mL 37- to 40-percent aqueous formaldehyde solution (formalin) to 100 mL using distilled water.

5.4 Household borax. Add about 3 g borax to 1 L 4-percent formaldehyde solution to prevent shrinkage of biological specimens.

6. Analysis

6.1 Preserve specimens in 4-percent formaldehyde solution (10-percent formalin) containing about 3 g borax per liter. Specimens more than 8 cm in length should be slit on the right side to ensure penetration of the preservative into the body cavity. After about a week in the formaldehyde solution, remove the specimens, wash thoroughly by several changes of tap water for at least 24 hours, and transfer the specimens to a 40-percent isopropyl alcohol solution. One change of alcohol is necessary to remove traces of formaldehyde before permanent preservation in 40-percent isopropyl alcohol solution (Needham and Needham, 1962).

6.2 Identify specimens using the best available taxonomic keys, such as Jordan and Everman (1890-1900) and Eddy (1978). Lagler (1956, p. 19-64) described the families of North American freshwater fish and listed local and regional publications about fish taxonomy. Weber (1973) also lists taxonomic references by region. Widely used regional fish keys include, for example, Schultz (1936), Hubbs and Lagler (1958), and Clemens and Wilby (1961). Examples of local keys are Simon (1946), Trautman (1957), and Cook (1959). The recognized common and scientific names of North America are reported in Bailey and others (1970). For the identification of other aquatic vertebrates, refer to Bishop (1947), Carr (1952), and Conant (1975).

6.3 When a tentative species identification has been made using a key, confirmation or rejection of the determination is based on: (1) A comparison with species characteristics listed in the key, (2) determination of correct geographic range, (3) comparison with photographs and drawings in various keys, and (4) identification by a specialist of individuals of questionable species.

7. Calculations

No calculations are necessary.

8. Reporting of results

Report the number of taxa and individuals of each taxon and the type of collection method used.

9. Precision

No numerical precision data are available.

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Life History (Quantitative Method)
(B-6020-85)

Parameter and Code: Not applicable

1. Applications

The method is applicable to all water.

2. Summary of method

Fish and other aquatic vertebrates are collected and identified. Fish studies commonly include the number of specimens captured per unit area or unit time. The fish also may be measured, weighed, sexed, and aged to provide comparative information between populations in the same environment or between populations in different environments. Methods used in the study of fish and fish populations are described by Lagler (1956), Ricker (1971), and Everhart and others (1975). Methods for the direct and indirect enumeration of populations are described in this section.

3. Interferences

Physical factors, such as stream velocity, depth of water, and turbidity, may make collection difficult. Filamentous algae and macrophytes may interfere with the operation of nets and seines.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

Methods and equipment for the collection of fish are described by Lagler (1956), Needham and Needham (1962), Calhoun (1966), Weber (1973), Everhart and others (1975), Hocutt (1978), and American Public Health Association and others (1985). Hocutt (1978) also discussed methods and equipment for the collection of amphibians and reptiles. State conservation agencies, the U.S. Fish and Wildlife Service, and commercial fishermen are other sources of information for obtaining the proper collecting equipment. Weber (1973, p. 171) lists publications containing information about fishery sampling equipment.

4.1 Bag seine, about 25 to 50 ft × 6 or 8 ft. The mesh size should be 1/2-in. square for the wings and 1/4-in. square for the bag.

4.2 Balance, capable of weighing to at least 1 g.

4.3 Container, for holding anesthesia.

4.4 Dip net, about 15-in. bow, 45-in. handle, 18-in. depth knotless nylon net, and 3/8-in. square mesh.

4.5 Dissecting kit. Routine dissecting tools. Dissections of the fish for internal examination frequently is required.

4.6 Dissecting microscope, low power of about 7X and stronger, either rotary or stereozoom type of binocular microscope. A substage mirror is essential.

4.7 Divider, fine-pointed, or dial caliper, for measuring body proportions.

4.8 Electrofishing gear. The basic unit consists of a generator (110 V ac or 220 V dc), sufficient insulated electrical wire, and two or three electrodes.

4.9 Forceps, long, for removing specimens from jars, and fine-pointed forceps that meet at the tip, for proper grasping of fins of small fishes and for removal of pharyngeal teeth of small cyprinids.

4.10 Gill net, experimental, about 6×125 ft. Most nets are made in 25-ft panels joined into continuous lengths that have four to five panels of different mesh size. The mesh size should range from about 1/2 in. at one end to about 2 in. at the other end. When equipped with poly-foam float line and lead-core leadline, the nets are virtually tanglefree. Mesh combinations and hanging sequence may be varied to suit individual requirements.

4.11 Gloves, waterproof, low-voltage rubber, Trapper's, shoulder length, for use with electrofishing gear.

4.12 Light source that has very intense illumination. Many investigators favor a gooseneck lamp and a 100-W lightbulb; others favor smaller lamps that project a concentrated beam of light. The important goal is to bring the light as close to the subject as possible.

4.13 Measuring board, or similar apparatus. A metric ruler that has a piece of wood at a right angle to the zero end is an adequate measuring device.

4.14 Nylon-mesh cage, about 4×4×4 ft, and 1/4-in. mesh to hold fish after capture.

4.15 Rule, stainless steel, metric, and a divider for obtaining actual measurements.

4.16 Sample containers, plastic, wide-mouth jars, about 0.5-, 1-, and 2-L capacity. Lids should be of plastic if used for prolonged storage of preserved specimens.

4.17 Scalpel or knife that has small sharp blade.

4.18 Small envelopes, 2⅛×3⅛ in., and bond typing-paper inserts for scale samples.

4.19 Straight seine, 10×5 ft × 1/8-in. mesh minnow type, and 25×6 ft × 1/4-in. square mesh.

4.20 Trawls, traps, and hoop nets, available through commercial fishing supply outlets.

4.21 Vials or small bottles, for stomach-content samples.

4.22 Waders, chest-type, for use with electrofishing gear.

4.23 Waterproof ink.

4.24 Waterproof labels, or labels may be cut from sheets of plastic paper.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Alcohol, isopropyl, 40-percent solution. Dilute 40 mL concentrated isopropyl alcohol to 100 mL using distilled water.

5.2 Anesthesia, MS 222 (tricanemethane sulfonate). Prepare a stock solution by dissolving 1 g MS 222 in 500 mL distilled water. Dilute the stock solution 1 part to 6 parts using distilled water before use.

5.3 Distilled or deionized water.

5.4 Formaldehyde solution, 4 percent. Dilute 10 mL 37- to 40-percent aqueous formaldehyde solution (formalin) to 100 mL using distilled water.

5.5 Household borax. Add about 3 g borax to 1 L 4-percent formaldehyde solution to prevent shrinkage of biological specimens.

6. Analysis

6.1 Preserve specimens in 4-percent formaldehyde solution (10-percent formalin) containing about 3 g borax per liter. Specimens more than 8 cm in length should be slit on the right side to ensure penetration of the preservative into the body cavity. After about a week in the formaldehyde solution, remove the specimens, wash thoroughly by several changes of tap water for at least 24 hours, and transfer the specimens to a 40-percent isopropyl alcohol solution. One change of alcohol is necessary to remove traces of formaldehyde before permanent preservation in 40-percent isopropyl alcohol solution (Needham and Needham, 1962).

6.2 Identify specimens using the best available taxonomic keys, such as Jordan and Everman (1890-1900) and Eddy (1978). Lagler (1956, p. 19-64) described the families of North American freshwater fish and listed local and regional publications about fish taxonomy. Weber (1973) also lists taxonomic references by region. Widely used regional fish keys include, for example, Schultz (1936), Simon (1946), Trautman (1957), and Hubbs and Lagler (1958). The recognized common and scientific names of North American fish are given in Bailey and others (1970). For the identification of other aquatic vertebrates, refer to Bishop (1947), Carr (1952), and Conant (1975).

6.3 When a tentative species identification has been made using a key, confirmation or rejection of the determination is based on: (1) A comparison with species characteristics listed in the key, (2) determination of correct geographic range, (3) comparison with photographs and drawings in various keys, and (4) identification by a specialist of individuals of questionable species.

6.4 Fish, amphibians, and other aquatic, cold-blooded animals can be handled easier and with less harm done to them if they are anesthetized. There also is less chance that the worker will be injured by sharp teeth or spines when the animal's reactions have been slowed. MS 222 (tricanemethane sulfonate), at the prescribed concentration, is the preferred anesthetic. Read label completely for directions and warnings about the use of this chemical.

6.5 Weigh each fish to the nearest gram after blotting dry using a paper towel or cheesecloth.

6.6 Measure the total length of each fish to the nearest millimeter. Fork length is preferred by some fisheries' biologists (fig. 56).

6.7 Food habits (optional). If the food habits of the fish are one of the study objectives, representative specimens usually must be killed. However, methods are available for removing food materials from the stomachs of living fish (Wales, 1962). Make a quantitative determination of the food present in the stomachs using a method appropriate to the study objectives. The usual methods are numerical, frequency of occurrence, percentage of bulk, gravimetric, and volumetric (Lagler, 1956, p. 120-128).

6.8 Age and growth by the length frequency method (optional). This method is based on the assumption that the lengths of individuals of a species of one age group will be normally distributed about the mean length, when collected at the same time. Accurate results using this method require fairly large samples of all age groups in the population (Carlander, 1969).

6.9 Age and growth by the scale-analysis method (optional). Using a knife blade or scalpel, remove a sample of scales from the left side of the fish (fig. 56). Place the scales in a folded piece of bond typing paper, and insert into an envelope. Record the following on the outside of the envelope: species, locality, method of capture, time, date, collector, length, weight, and sex (if known) of the fish. Using the collected scales, determine the age of the fish using the methods described in Lagler (1956, p. 131-158).

6.10 Population density (optional) is population size in relation to some unit of space. It generally is measured and expressed as the number of individuals or standing crop (biomass) per unit of area; for example, 53 brook trout per surface area, or 190 lb of fish per surface area.

The methods for determining population density can be divided into two general categories: (1) Direct or total count, and (2) indirect or sample count. The opportunity for total direct counting only occurs when the entire population can be concentrated, such as during a reclamation project or during a spawning run. More often the population must be estimated by sampling

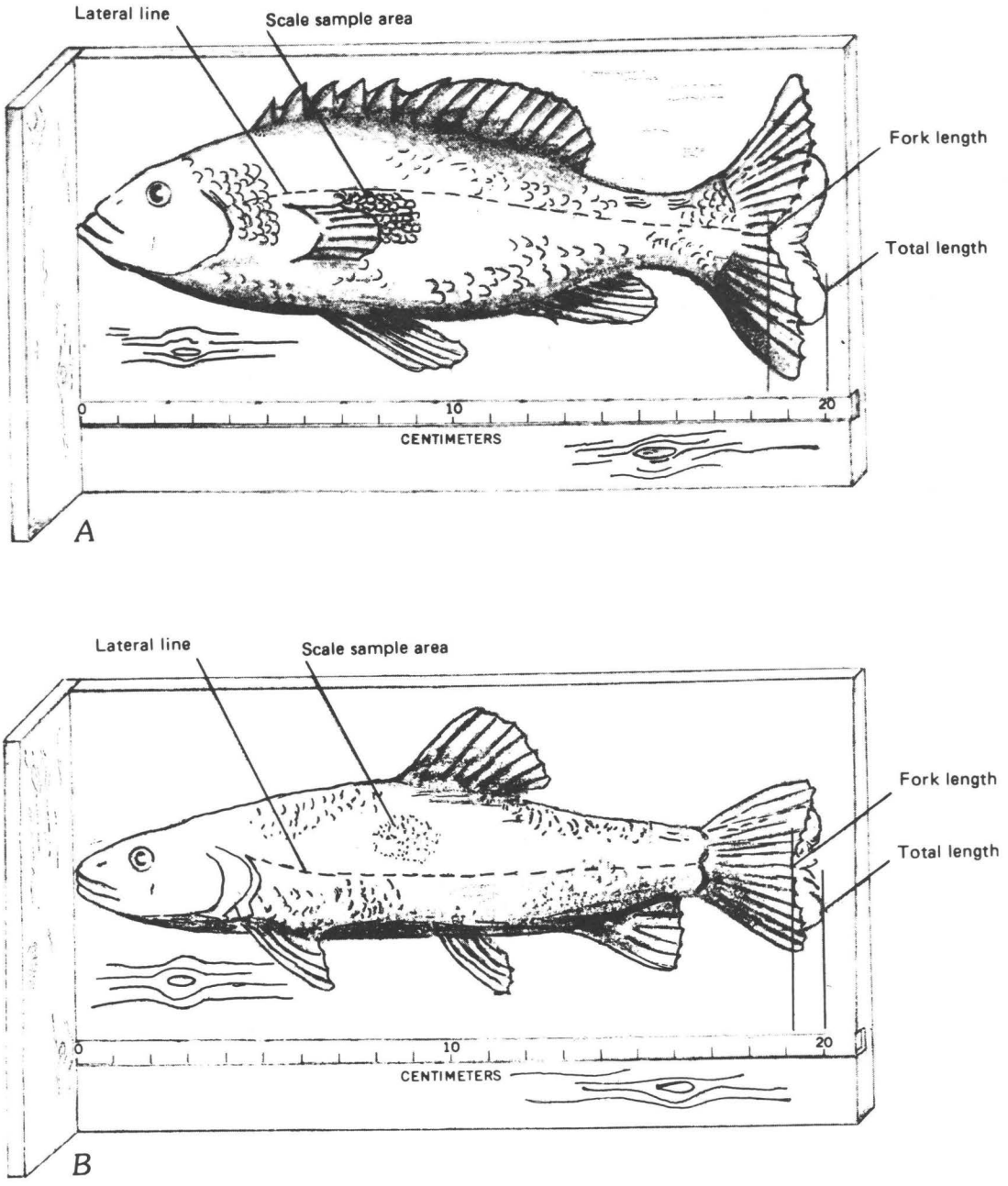

Figure 56.--Fish measurements and areas for scale collection on: (A) spiny-rayed and (B) soft-rayed fish.

methods. The three most commonly used sampling methods include: (1) The area-density method, (2) the mark and recapture method, and (3) the catch-per-unit-effort method. The methods are described in Cooper and Lagler (1956) and Everhart and others (1975).

6.10.1 The area-density method consists of counting the number of fish in a series of random or stratified plots or in areas that are representative of the total area whose population is to be estimated. The sample count then is expanded to an estimate of the population by multiplying the aggregate sample count by the fraction: total area (or time) divided by the sum of sample areas (Everhart and others, 1975).

6.10.2 The mark and recapture method of populations involves, first, the capture and release of a number of marked individuals into the population; and second, the subsequent recapture of marked individuals and the capture of unmarked individuals from the population.

6.10.3 The catch-per-unit-effort method requires a measurable decrease in the population by fishing and commonly is referred to as the DeLury (1947) regression method. The method of Moran (1951) and Zippin (1956, 1958) is appropriate when effort is constant. The DeLury (1947) and Leslie (1952) methods are appropriate when effort is variable. These methods are valid only if the population is closed, and the chance of capture is equal and remains constant from sample to sample. Examples of the application of data from the catch-per-unit-effort method to regression analyses are presented in Lagler (1956), Zippin (1956, 1958), and Everhart and others (1975).

Methods for measuring population density are numerous and too involved to go into detail here. The investigator should review the indicated literature and adapt proven techniques to fit a specific case.

7. Calculations

7.1 Percent species composition in sample

$$= \frac{\text{Number of individuals of a given species}}{\text{Total number of all fish collected}} \times 100 \ .$$

7.2 Plot weight as a function of length, as described in Lagler (1956, p. 159-166, figs. 47, 48).

7.3 Plot age as a function of length, as described in Lagler (1956, p. 149-158).

7.4 The calculations required for food-habit studies are determined by the methods of analysis. The usual methods are described in Lagler (1956, p. 120-130).

7.5 Calculate the population-density estimate from area-density data using the equation

$$\underline{N} = \frac{\underline{A}}{\underline{a}} \sum_{\underline{i}=1}^{\underline{a}} \underline{N}_{\underline{i}} \ ,$$

where N = the estimate of pouplation size;
A = the number of equal units of area (or time) occupied by the total population;
a = the number of units sampled; and
N_i = the number counted in the i^{th} sample area.

The estimated variance ($\hat{v}$) is

$$\hat{v}(N) = \frac{A^2 - aA}{a} \times \frac{a \sum_{i=1}^{a} N_i^2 - \sum_{i=1}^{a} N_i^2}{a(a-1)} .$$

7.6 Calculate the population-density estimate from mark and recapture data using the equation

$$N = MC/R$$

where N = the estimate of population size;
M = the number of individuals marked and released into the population;
C = the recapture sample size that includes both marked and unmarked individuals; and
R = the number of marked individuals that are recaptured.

If the population density is large enough for multiple marking and recapture periods, use Schnable's equation (1938)

$$N = \frac{\sum_{t=1}^{n} C_t M_t}{\sum_{t=1}^{n} R_t} .$$

7.7 Calculate the population-density estimate from catch-per-unit-effort data using the line or regression technique where catch-per-unit effort is plotted against cumulative catch. In such a graph, the catch-per-unit effort is the ordinate and the cumulative catch is the abscissa. Fit the straight regression line to its intercept with the x axis. The intercept value is the approximation of the population density (Lagler, 1956).

8. Reporting of results

8.1 Report percent species composition in sample to the nearest whole number.

8.2 Report weight to the nearest gram, and length to the nearest millimeter.

8.3 Report age to the nearest year.

8.4 Report food-habit analyses by the method used and by study objectives.

9. Precision

No numerical precision data are available.

10. References cited

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_____1958, The removal method of population estimation: Journal of Wildlife Management, v. 22, p. 82-90.

Methods for Investigation of Fish and Other Aquatic Vertebrate Kills (B-6040-85)

Parameter and Code: Not applicable

1. Applications

Methods of investigation and collection are applicable to all water.

2. Summary of method

2.1 Fish kills are an obvious and important event related to water quality. The methods in this section describe what important facts need to be documented when making an onsite investigation and how to properly preserve specimens for laboratory examination to determine the probable cause of death. The collection of fish and other vertebrates from a natural or man-caused kill generally is only one phase of a more comprehensive investigation that involves onsite and laboratory chemical tests.

2.2 Because fish-kill investigations normally are the responsibility of State and Federal enforcement agencies, the U.S. Geological Survey's involvement usually is that of a supportive role. However, because many fish kills are due to a slug of toxic material of short duration, personnel from the first agency on the scene should be prepared to collect the necessary samples and information.

2.3 For additional information about the investigation of fish kills, see Smith and others (1956), Burdick (1965), Federal Water Pollution Control Administration (1966, 1967), and American Public Health Association and others (1985).

3. Interferences

Physical factors, such as stream velocity and depth of water, may make collection difficult. Access to affected waters also is a common problem. Some pollutants are toxic or hazardous to humans and require special precautions.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Aluminum foil, heavy weight type.

4.2 Dip net, long handle, and 3/16-in. mesh.

4.3 Plastic bags, various sizes.

4.4 Waterproof ink.

4.5 Waterproof labels, or labels may be cut from sheets of plastic paper.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Distilled or deionized water.

5.2 Ethyl alcohol, 75 percent. Dilute 750 mL commercial 95-percent denatured ethyl alcohol to 950 mL using distilled water.

6. Analysis

Samples should be shipped to an appropriate laboratory for histological or pathological examination. The nearest laboratory can be located by contacting the local office of the State Fish and Game Department or State Department of Health.

7. Calculations

No calculations are necessary.

8. Reporting of results

Report estimated number of distressed or dead fish, or other observed aquatic vertebrates, followed with an appropriate qualifying statement such as estimation based on 1 hour of observation or number of specimens observed per unit length of shoreline. Degrees of severity of fish kills have been based on the number of dead or dying fish per length of shoreline (American Public Health Association and others, 1985).

9. Precision

No numerical precision data are available.

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CELLULAR CONTENTS

Introduction

Chlorophyll a is the primary photosynthetic pigment of all oxygen-producing photosynthetic organisms and is present in all algae (phytoplankton and periphyton). Thus, measurement of this pigment can indicate the quantity of algae present and provide an estimate of the primary productivity (Lorenzen, 1970). Because environmental and nutritional factors may affect the chlorophyll concentration without affecting the total algal biomass, this measurement is only an estimate. Green algae and euglenophytes also contain chlorophyll b (Wetzel, 1975). Certain other algae contain chlorophylls c and d. Ratios between the different types of chlorophyll may indicate the taxonomic composition of an algal community.

An estimate of the quantity of living micro-organisms (biomass) in an aquatic environment can be useful when assessing water quality. The universal occurrence and central function of adenosine triphosphate (ATP) in living cells and its chemical stability make it an excellent indicator of the presence of living material. The level of endogenous ATP (that is, the quantity of ATP per unit biomass) in bacteria (Allen, 1973), in algae (Holm-Hansen, 1970), and in zooplankton (Holm-Hansen, 1973) is relatively constant when compared to cellular organic-carbon content in several species of organisms. Furthermore, its concentration in all phases of a growth cycle remains relatively constant. In studies where cell viability was determined (Hamilton and Holm-Hansen, 1967; Dawes and Large, 1970), the concentration of ATP per viable cell remained relatively constant during periods of starvation. The quantity of ATP, therefore, can be used to estimate total living biomass.

Collection

The sites and methods used for phytoplankton and periphyton sampling should correspond as closely as possible to those selected for chemical and microbiological sampling. The sample-collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, phytoplankton abundance may vary transversely, with depth and width, and with time of day. To collect a sample representative of the phytoplankton concentration at a particular depth, use a water-sampling bottle. To collect a sample representative of the entire flow of a stream, use a depth-integrating sampler (Guy and Norman, 1970; Goerlitz and Brown, 1972). For small streams, a depth-integrated sample or a point sample at a single transverse position at the centroid of flow is adequate. For further information about collection of phytoplankton samples, see the "Phytoplankton" section.

After collection of the phytoplankton sample, place a 47-mm glass-fiber filter on a filter funnel. Filter a measured volume of water sample at a vacuum of no more than 250 mm of mercury. Rinse the sides of the filter funnel with a few milliliters of distilled water. For estuarine samples, use rinse water that is near the salinity of the sample.

Roll the filter so the plankton is on the inside and proceed with the prescribed method of determination or place the rolled filter in a glass vial, 22×85 mm, and store frozen in the dark. Storage should not exceed 2 weeks. Dry ice is used for preserving samples while in transit (samples must not thaw before analysis begins).

Most analyses of the periphyton community have been adapted from long-established methods of phytoplankton analyses. The attached benthic nature of periphyton, however, causes special collection problems that adversely affect the accuracy of various estimates. Methods have been developed for collecting periphyton from natural substrates and from artificial substrates.

Natural submerged substrates commonly contain periphyton that can be sampled quantitatively. The periphyton should be removed from a known area of substrate onsite. Several devices for removing periphyton from a known area of natural substrates are shown in figure 18. Stockner and Armstrong (1971) sampled periphyton using a plastic hypodermic syringe that had a toothbrush attached to the end of the syringe piston. Holding the barrel of the syringe tightly against the substrate, the piston is pushed in until the brush contacts the periphyton. The piston then is rotated several times to dislodge the periphyton and then is withdrawn, pulling the periphyton up with it. A glass plate is placed immediately under the end of the barrel, and the syringe inverted. Four small holes at the base of the syringe enable the water to move freely when procuring the sample.

The device used by Douglas (1958) consists of a broad-necked polyethylene flask that has the bottom removed. The neck of the flask is held tightly against the surface to be sampled, and the periphyton inside the enclosed area is dislodged from the substrate using a stiff nylon brush. The loose periphyton is removed from the flask using a pipet. Ertl's (1971) apparatus consists of two concentric metal, or plastic, cylinders separated by spacers. The space between the cylinders is filled with modeling clay, and the sampler is pressed firmly against the substrate to be sampled. Using a blunt stick or metal rod, the clay is forced down onto the substrate to isolate the sampling area of the inner circle. The periphyton within the inner circle is dislodged using a stiff brush and removed using a pipet.

Artificial substrates can be attached to a supporting object in a stream or lake (figs. 19, 20). The substrate must be submerged during the entire colonization period but may be near the surface of the water and can be suspended at several depths. The substrates may be attached to natural items, such as submerged trees, stumps, logs, or boulders, or they may be attached to stakes driven into the bottom. Floating samplers also may be used. The sampler should be secured so that it will not drift into any obstruction or become beached. In extremely shallow streams, a weir may have to be constructed to guarantee sufficient water to float the sampler. If such a weir is constructed, data from the sample should be compared only with data obtained from comparably placed samplers. A floating sampler is not recommended for any area that would have intermittent flow for any period during the exposure time.

The artificial substrates should be placed in areas of light that typify the streams, rivers, or lakes being studied. For example, if most of the stream is shaded, an area that receives a great deal of sunlight should not be selected as being representative. In general, substrate samples collected from similar lighting conditions need to be compared; but, depending on the study objective, this is not a requirement.

To ensure a continuous period of uniform substrate exposure to the environment being monitored, the sampler should be examined, periodically if possible, for any evidence of fouling or mechanical damage. If the sampler or substrate has been fouled or beached, the data for that sampling period should not be compared with data from any other substrate that has had free, continuous, and uninterrupted exposure to the aquatic environment.

The length of time required for colonization of the substrates by periphyton will depend on other environmental factors as well as water quality. Exposure times will vary and must be determined for each season and water type. The exposure period should be long enough to enable the development of a periphyton community large enough for measurement but, at the same time, should avoid so much growth that sloughing would occur. Test samplers can be used prior to the actual monitoring to determine the most desirable exposure time for the prevailing (that is, seasonal and environmental) conditions. The general exposure period for fresh to brackish waters, mesotrophic to eutrophic, within the thermal range of 15 to 35 °C, is 14 days. Exposure periods during special conditions of low productivity (that is, few nutrients, low temperature) or very high productivity may, by experience, be adjusted for the onsite conditions. Exposure periods should be identical for all sites in the entire study area.

The artificial substrates should be located so damage to the apparatus by floating debris is minimized. Vandalism is a common problem and placing the substrate away from frequently traveled areas is advisable. For further information on collection of periphyton samples, see the "Periphyton" section.

Place the detached periphyton from the natural substrate or the complete artificial substrate into a bottle containing water or preservative. Store frozen in the dark for no more than 2 weeks. Dry ice is used for preserving samples in transit.

References Cited

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Wetzel, R. G., 1975, Limnology: Philadelphia, W. B. Saunders, 743 p.

Chlorophyll in Phytoplankton by Spectroscopy
(B-6501-85)

Parameters and Codes:

Chlorophyll a, phytoplankton, spectrometric, uncorrected (µg/L):	32230
Chlorophyll b, phytoplankton, spectrometric (µg/L):	32231
Chlorophyll c, phytoplankton, spectrometric (µg/L):	32232
Chlorophyll, total, phytoplankton, spectrometric, uncorrected (µg/L):	32234

1. Applications

The method is suitable for all water.

2. Summary of method

Chlorophyll pigments are determined simultaneously without detailed separation. A water sample is filtered, and the phytoplankton cells retained on the filter are ruptured mechanically, using 90-percent acetone, to facilitate extraction of pigments. Concentrations of chlorophylls are calculated from measurements of absorbance of the extract at four wavelengths, corrected for a 90-percent acetone blank.

3. Interferences

Suspended materials in the sample may clog the membrane filter. Erroneously large values may result from the presence of fragments of tree leaves and other plant materials. Exposure to light or acid at any stage of storage and analysis can result in photochemical and chemical degradation of the chlorophylls. Large populations of photosynthetic bacteria will result in an overestimation of phytoplankton chlorophyll (Hussaing, 1973).

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Centrifuge, swing-out type, 3,000 to 4,000 r/min, and 15-mL graduated centrifuge tubes.

4.2 Filters, metricel, alpha-6, 0.45 µm, 25-mm diameter.

4.3 Filter flask, 1 or 2 L. Onsite, a polypropylene flask is used.

4.4 Filter funnel, vacuum, 1.2 L, stainless steel.

4.5 Filter holder, Pyrex microanalysis, frit support, 25 mm.

4.6 Manostat that has mercury and calibration equipment to regulate the filtration suction to not more than 250 mm of mercury when filtering using an aspirator or an electric vacuum pump.

4.7 Membrane filter, white, plain, 0.45-µm mean pore size, 47-mm diameter.

4.8 Source of vacuum for filtration: A water-aspirator pump or an electric vacuum pump for laboratory use; a handheld vacuum pump and gauge for onsite use.

4.9 Spectrometer (spectrophotometer; fig. 57) that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.10 Tissue grinder.

4.11 Water-sampling bottle. Depth-integrating samplers are described by Guy and Norman (1970).

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetone, 90 percent. Add nine volumes of acetone to one volume of distilled water.

5.2 Distilled or deionized water.

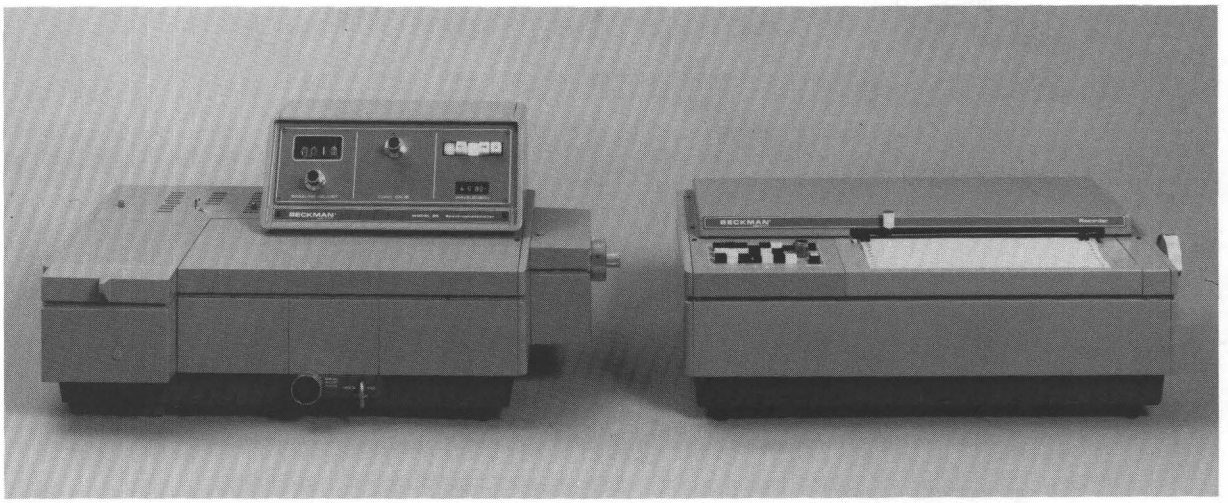

Figure 57.--Scanning spectrometer (spectrophotometer) (photograph courtesy of Beckman Instruments, Inc., Irvine, Calif.).

6. Analysis

6.1 If filter was frozen, allow it to thaw for 2 to 3 minutes at room temperature.

6.2 Place the filter in a tissue grinder. Add 3 to 4 mL of 90-percent acetone, and grind at 500 r/min for 3 minutes. If multiple filters are used, use a 40-mL grinder.

6.3 Transfer the sample to a 15-mL graduated centrifuge tube, and wash the pestle and grinder two or three times using 90-percent acetone. Adjust to some convenient volume, such as 10±0.1 mL. Store for 10 minutes in the dark at room temperature.

6.4 Centrifuge at 3,000 to 4,000 r/min for 10 minutes.

6.5 Carefully pour or pipet the supernatant into the spectrometer cell. Do not disturb the precipitate. If the extract is turbid, clear by making a twofold dilution using 90-percent acetone, or by filtering through an acetone-resistant filter.

6.6 Read the absorbances at 750, 664, 647, and 630 nm and compare to a 90-percent acetone blank. (Dilute the extract using 90-percent acetone if the absorbance is greater than 0.8.) If the 750-nm reading is greater than 0.005 absorbance unit per centimeter of light path, decrease the turbidity as in 6.5.

7. Calculations

7.1 Subtract the absorbance at 750 nm from the absorbance at each of the other wavelengths (that is, 664, 647, and 630 nm). Divide the differences by the light path of the spectrometer cell, in centimeters. The concentrations of chlorophylls in the extract, in micrograms per milliliter, are calculated by the following equations (Jeffrey and Humphrey, 1975):

Chlorophyll a, in micrograms per milliliter = $11.85e_{664}-1.54e_{647}-0.08e_{630}$;
Chlorophyll b, in micrograms per milliliter = $-5.43e_{664}+21.03e_{647}-2.66e_{630}$;
and
Chlorophyll c, in micrograms per milliliter = $-1.67e_{664}-7.60e_{647}+24.52e_{630}$;

where $e_{664} = \frac{\text{Absorbance at 664 nm - absorbance at 750 nm}}{\text{Light path, in centimeters}}$;

$e_{647} = \frac{\text{Absorbance at 647 nm - absorbance at 750 nm}}{\text{Light path, in centimeters}}$; and

$e_{630} = \frac{\text{Absorbance at 630 nm - absorbance at 750 nm}}{\text{Light path, in centimeters}}$.

7.2 Convert the values derived in 7.1 to the concentrations of chlorophylls, in micrograms per liter, in the originally collected sample. For example:

$$\text{Chlorophyll } \underline{a} \text{ (micrograms per liter)} = \frac{\text{Derived value (micrograms per milliliter)} \times \text{extract volume (milliliters)}}{\text{Sample volume (liters)}} .$$

8. Reporting of results

Report concentrations of chlorophyll a, b, or c, in micrograms per liter, as follows: less than 1 µg/L, one decimal; 1 µg/L and greater, two significant figures.

9. Precision

9.1 The precision of chlorophyll determinations is affected by the volume of water filtered, the range of chlorophyll values calculated, the volume of extraction solvent, and the light path of the spectrometer cells.

9.2 The following precision estimates were reported by Strickland and Parsons (1972).

Chlorophyll a precision at the 5 µg level. The correct value is in the range: Mean of n determinations $\pm 0.26/n^{\frac{1}{2}}$ µg chlorophyll a.

Chlorophyll b precision at the 0.5 µg level. The correct value is in the range: Mean of n determinations $\pm 0.21/n^{\frac{1}{2}}$ µg chlorophyll b.

9.3 The precision of chlorophyll c determinations is variable and very poor, anywhere between ±10 and ±30 percent of the quantity being measured; results are not accurate.

10. References cited

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Chlorophyll in Phytoplankton by Chromatography and Spectroscopy (B-6520-85)

Parameters and Codes:

Chlorophyll a, phytoplankton, chromatographic/spectrometric (µg/L): 70951
Chlorophyll b, phytoplankton, chromatographic/spectrometric (µg/L): 70952

1. Applications

The method is suitable for all water. The method is not suitable for the determination of chlorophyll c.

2. Summary of method

A plankton sample is filtered, and the chlorophylls are extracted from the algal cells. The chlorophylls are separated from each other and from chlorophyll degradation products by thin-layer chromatography. Chlorophylls are eluted and measured using a spectrometer.

3. Interferences

A substantial quantity of sediment may affect the extraction process. Exposure to light or acid at any stage of storage and analysis can result in photochemical and chemical degradation of the chlorophylls.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Air dryer.

4.2 Centrifuge.

4.3 Centrifuge tubes, graduated, screwcap, 15- and 40-mL capacity.

4.4 Chromatography sheet, thin-layer cellulose, 5×20 cm, 80-µm thick cellulose.

4.5 Developing tank and rack.

4.6 Evaporation device.

4.7 Filters, glass fiber, 47-mm diameter, capable of retaining particles having diameters of at least 0.45 µm.

4.8 Filter funnel, vacuum, 1.2 L, stainless steel.

4.9 Glass pipets, 10-mL capacity.

4.10 Glass vials, screwcap, 22×85 mm.

4.11 Gloves, long-service latex.

4.12 Grinding motor that has 0.1 horsepower.

4.13 Microdoser, and 50-µL syringe.

4.14 Pasteur pipets, disposable.

4.15 Propipet, or equivalent suction device.

4.16 Solvent-saturation pads, 13.4×22 cm.

4.17 Spectrometer (spectrophotometer; fig. 57) that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.18 Tissue grinder.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetone, 90 percent. Add nine volumes of acetone to one volume of distilled water.

5.2 Chlorophyll a, stock solution. Add 1 mL 90-percent acetone to 1 mg chlorophyll a (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2 and 5.3 are wrapped with aluminum foil as an added precaution.

5.3 Chlorophyll b, stock solution. Add 1 mL 90-percent acetone to 1 mg chlorophyll b.

5.4 Dimethyl sulfoxide (DMSO).

5.5 Distilled or deionized water.

5.6 Ethyl ether.

5.7 Methyl alcohol.

5.8 Nitrogen gas, prepurified.

5.9 Petroleum ether, 30 to 60 °C.

6. Analysis

6.1 If filter was frozen, allow it to thaw 2 to 3 minutes at room temperature.

6.2 Place the filter in a tissue grinder. Add 3 to 4 mL DMSO and grind at 500 r/min for 3 minutes. If multiple filters are used, use a 40-mL grinder.

CAUTION.--Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.3 Transfer the sample to a 15-mL graduated centrifuge tube, and wash the pestle and grinder twice using DMSO.

6.4 Add an equal volume of ethyl ether. Screw on cap and shake vigorously for 10 seconds. Wait 10 seconds and repeat shaking for 10 seconds more.

6.5 Remove cap and add slowly, almost dropwise, a volume of distilled water equal to 25 percent of the total volume of extractant (DMSO).

6.6 Cap and shake as in 6.4.

6.7 Centrifuge at 1,000 r/min for 10 minutes.

6.8 During centrifugation, prepare chromatography tank by pouring 294 mL petroleum ether and 6 mL methyl alcohol into the tank. Mix well. Prepare fresh before each use. Use two solvent-saturation pads and the developing rack to dry the chromatography sheet.

6.9 Remove the top ethyl ether layer containing chlorophyll using a pipet, and place in another 15-mL graduated centrifuge tube.

6.10 Add an equal volume of distilled water, and shake as in 6.4.

6.11 Centrifuge at 1,000 r/min for 5 minutes.

6.12 Remove the top ethyl ether layer using a capillary pipet, and place in the conical tube in the evaporation device. Evaporate to dryness by blowing nitrogen gas over the ethyl ether surface.

6.13 Immediately add 0.5 mL acetone. Mix. Wait 30 seconds and mix again. If all chlorophyll is not in solution, then repeat procedure.

6.14 Using microdoser, streak about 25 µL of the acetone-chlorophyll solution on the chromatography sheet, 15 mm from the bottom and 6 mm from each side, using the air dryer to speed evaporation of the solvent. If excessive trailing occurs during chromatography, the volume of the solvent should be decreased.

6.15 Develop chromatograph in the dark, using chlorophyll solution(s). Use enough chlorophyll (about 5 µL of the solutions as in 5.2 or 5.3, or both) to visually locate the spot of pigment. The time required for development is about 30 minutes. Remove strips when solvent has traveled within 2 to 3 cm from top of the strip.

6.16 Determine $\underline{R}_{\underline{f}}^{\wedge}$ values (Note 2) for pure chlorophylls.

Note 2: $\underline{R}_{\underline{f}}^{\wedge}$ value = distance traveled by the chlorophyll from the point of application divided by the distance traveled by the solvent from the point of application.

6.17 Locate the $\underline{R}_{\underline{f}}^{\wedge}$ value on the chromatography sheet; and, using a razor blade, scrape the cellulose off the sheet at the spot of the $\underline{R}_{\underline{f}}^{\wedge}$ value minus 0.07 for chlorophyll *a* (0.14 for chlorophyll *b*) × $\underline{R}_{\underline{f}}^{\wedge}$. Place the cellulose into a graduated centrifuge tube, and add acetone to a volume of 3 mL. This step should be done immediately after the chromatograph is removed from the tank. Shake the scraped cellulose and acetone vigorously for 10 seconds. Wait 1 minute and shake again vigorously for 10 seconds more.

6.18 Centrifuge at 1,000 r/min for 5 minutes.

6.19 Remove supernatant and read the absorbance on the spectrometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*.

7. Calculations

7.1 If the absorbance is greater than 0.01, determine concentrations using the specific absorptivities of 0.0877 L/mg × cm for chlorophyll *a* and 0.0514 L/mg × cm for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$\underline{C} = \frac{\underline{A}}{\underline{\alpha b}},$$

where $\underline{C}$ = concentration of chlorophyll, in milligrams per liter;
$\underline{A}$ = absorbance;
$\underline{b}$ = path length, in centimeters; and
$\underline{\alpha}$ = specific absorptivity.

If the absorbance is less than 0.01, use the fluorescence technique.

7.2 The concentration of chlorophyll obtained in 7.1 is corrected for the concentration step onsite and in the determination:

$$\text{Original sample (micrograms chlorophyll per liter)} = \frac{\text{Micrograms chlorophyll per milliliter (as in 6.19)} \times 3 \text{ mL} \times \dfrac{\text{Concentrate volume (microliters)}}{\text{volume streaked (microliters)}}}{\text{Volume filtered onsite (liters)}}.$$

8. Reporting of results

Report concentrations of chlorophylls *a* or *b* as follows: less than 1 µg/L, one decimal; 1 µg/L and greater, two significant figures.

9. Precision

No precision data are available.

10. Reference cited

Jeffrey, S. W., and Humphrey, G. F., 1975, New spectrophotometric equations for determining chlorophylls a, b, c_1, and c_2 in higher plants, algae, and natural phytoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

Chlorophyll in Phytoplankton by High-Pressure Liquid Chromatography (B-6530-85)

Parameters and Codes:

Chlorophyll a, phytoplankton, chromatographic/fluorometric (µg/L): 70953
Chlorophyll b, phytoplankton, chromatographic/fluorometric (µg/L): 70954

1. Applications

The method is suitable for the determination of chlorophylls a and b in phytoplankton in concentrations of 0.1 µg/L and greater and is suitable for all water.

2. Summary of method

A filtered phytoplankton sample is ruptured mechanically, and the chlorophylls pigments are separated from each other and degradation products by high-pressure liquid chromatography and determined by fluorescence spectroscopy (Shoaf and Lium, 1976, 1977).

3. Interferences

Exposure of the sample to heat, light, or acid can result in photochemical or chemical degradation of the chlorophylls. Large values will result from the presence of fragments of tree leaves or other plant materials that contain chlorophyll. Large populations of photosynthetic bacteria also will result in large values.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Auto-injector (recommended, but not required).

4.2 Centrifuge.

4.3 Centrifuge tubes, 15 and 50 mL, conical, screwcap, graduated.

4.4 Evaporation device.

4.5 Filters, glass fiber, 47-mm diameter, capable of retaining particles having diameters of at least 0.45 µm.

4.6 Fluorometer, equipped with excitation and emission filters.

4.7 Gloves, long-service latex.

4.8 High-pressure liquid chromatograph (HPLC), consisting of a solvent programmer, an isochromatic pump, an oven, and a column. (The column oven needs to be capable of maintaining a constant temperature in the 25 to 35 °C range.)

4.9 Pasteur pipets, disposable.

4.10 Separatory funnels, 125 mL.

4.11 Spectrometer (spectrophotometer; fig. 57) that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.12 Tissue homogenizer, 30-mL homogenizing flasks, and blades.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetone, 90 percent. Add nine volumes of acetone to one volume of distilled water and mix.

5.2 Chlorophyll a stock solution. Transfer 1 mg chlorophyll a to a 100-mL volumetric flask and fill to capacity using 90-percent acetone (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2, 5.3, 5.4, and 5.5 are wrapped with aluminum foil as an added precaution.

5.3 Chlorophyll b stock solution. Transfer 1 mg chlorophyll b to a 100-mL volumetric flask and fill to capacity using 90-percent acetone.

5.4 Chlorophyll standard solution. Mix 25 mL chlorophyll a stock solution with 25 mL chlorophyll b stock solution in a 50-mL centrifuge tube.

5.5 Chlorophyll working standard solutions. Use a 5-mL pipet to prepare the following mixtures.

5.5.1 High standard solution, chlorophylls a and b. Add 5 mL chlorophyll standard solution to 5 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.2 Mid-range standard solution, chlorophylls a and b. Add 3 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.3 Low standard solution, chlorophylls a and b. Add 1 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.6 Distilled or deionized water.

5.7 Diethyl ether, distilled in glass, unpreserved.

5.8 Dimethyl sulfoxide (DMSO).

5.9 Methyl alcohol, 96 percent. Pour 960 mL methyl alcohol, distilled in glass, into a 1-L graduated cylinder. Add distilled water to the mark and mix.

5.10 Nitrogen gas, prepurified.

6. Analysis

6.1 Sample preparation. Analyze only samples on glass-fiber filters. Record the volume of water filtered for the phytoplankton sample. [If a biomass determination is required, save the DMSO layer (see 6.1.7).]

6.1.1 Allow the frozen filter to thaw 2 to 3 minutes at room temperature.

CAUTION.--Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.1.2 Place the filter in a 30-mL tissue homogenizing flask. Add 15 mL DMSO and homogenize until the sample has been ruptured.

6.1.3 Transfer the sample to a 50-mL graduated centrifuge tube, and rinse the homogenizing flask and blade using 5 mL DMSO. Add the rinse to the centrifuge tube.

6.1.4 Add 20 mL diethyl ether to the centrifuge tube, screw on the cap, and shake vigorously for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.5 Remove the cap and slowly add, almost dropwise, 10 mL distilled water to the centrifuge tube. Secure the cap and shake gently. Vent, then shake for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.6 Centrifuge at 1,000 r/min for 10 minutes.

6.1.7 Transfer the top diethyl ether layer, using a disposable pipet, to a 125-mL separatory funnel. (If the DMSO layer appears green after diethyl ether extraction, repeat 6.1.4 through 6.1.7. There are, however, some green chlorophyll derivatives not extractable using diethyl ether.)

6.1.8 Add 15 mL distilled water to the separatory funnel, and shake vigorously for 10 seconds, venting often. Allow the layers to separate. (Break emulsions by adding 1 to 2 mL acetone and swirling the funnel gently.)

6.1.9 Drain and discard the bottom layer.

6.1.10 Rinse the upper part of the separatory funnel using 2 to 3 mL acetone. Remove the bottom layer that forms in the funnel and discard.

6.1.11 Decant the diethyl ether layer through the top of the separatory funnel into a centrifuge tube. Rinse the funnel using 5 mL diethyl ether, and add the rinse to the centrifuge tube.

6.1.12 Place the centrifuge tube on the evaporation device, and evaporate to 0.2 to 0.4 mL using a gentle stream of nitrogen gas.

6.1.13 Add sufficient acetone to the sample extract so the color intensity is between the color intensities of the high and low standards. If the color of the sample extract is not within the specified range after the addition of 20 mL acetone, take a 1-mL aliquot of the 20 mL extract, and dilute volumetrically until the desired color intensity is obtained.

6.2 High-pressure liquid-chromatographic analysis.

6.2.1 Measure the absorbance of the chlorophyll stock solutions using a spectrometer. Measure the absorbance at 664 nm for chlorophyll a and at 647 nm for chlorophyll b. Record the absorbance for three replicates of chlorophylls a and b. Average the three values for chlorophyll a and the three values for chlorophyll b, separately, and record each average separately for subsequent calculations.

6.2.2 Operate the HPLC system using 96-percent methyl alcohol as the mobile phase at a flow of 1.5 mL/min until the pressure stabilizes.

6.2.3 Calibrate the instrument by injecting 10 µL of the mid-range standard solution, and record the peaks of chlorophylls a and b.

6.2.4 Verify that the response of the fluorometer is linear by injecting the high and low standard solutions.

6.2.5 Analyze the sample by injecting 10 µL of the sample extract into the HPLC. Record the peaks of chlorophylls a and b, if any.

7. Calculations

7.1 Calculate the exact concentrations of the chlorophyll stock solutions from the equation:

$$C_s = \frac{A}{\alpha b},$$

where C_s = concentration of chlorophyll stock solution, in milligrams per liter;
A = average absorbance obtained in 6.2.1;
b = path length, in centimeters; and
α = specific absorptivity [0.0877 L/mg × cm for chlorophyll a and 0.0514 L/mg × cm for chlorophyll b (Jeffrey and Humphrey, 1975)].

7.2 Verify and correct the concentrations of the chlorophyll working standard solutions in 5.5 by using the chlorophyll stock solutions determined in 7.1.

7.3 Calculate the response factor for chlorophylls *a* and *b* in the chlorophyll working standard solution:

$$RF = \frac{V \times C_m}{I_s},$$

where RF = response factor of chlorophyll *a*, in milligrams per unit area;
V = volume of mid-range standard solution, injected, in milliliters;
C_m = concentration of chlorophyll *a* or *b* in the mid-range standard solution, in milligrams per liter; and
I_s = integrated area of the component peak.

7.4 Use the data from 6.2.5 to calculate the concentration of chlorophyll *a* or *b* in the original sample from the equation:

$$\text{Concentration (micrograms per liter)} = \frac{RF \times IV_e}{A_s \times V_i},$$

where RF = response factor of chlorophyll *a* or *b* from 7.3, in milligrams per unit area;
I = integrated area of the chlorophyll *a* or *b* peak in the sample as determined in 6.2.5;
V_e = final volume of the sample extract from 6.1.13, in milliliters;
A_s = volume of water filtered in 6.1, in liters; and
V_i = volume of sample extract injected in 6.2.5, in microliters.

8. Reporting of results

Report concentrations of chlorophylls *a* or *b* as follows: less than 1 µg/L, one decimal; 1 µg/L and greater, two significant figures.

9. Precision

No precision data are available.

10. References cited

Jeffrey, S. W., and Humphrey, G. F., 1975, New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1, and c_2 in higher plants, algae, and natural phytoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

Shoaf, W. T., and Lium, B. W., 1976, Improved extraction of chlorophyll a and b from algae using dimethyl sulfoxide: Limnology and Oceanography, v. 21, no. 6, p. 926-928.

_____1977, The quantitative determination of chlorophyll a and b from fresh water algae without interference from degradation products: Journal of Research of the U.S. Geological Survey , v. 5, no. 2, p. 263-264.

Chlorophyll in Phytoplankton by Chromatography and Fluorometry (B-6540-85)

Parameters and Codes:

Chlorophyll a, phytoplankton, chromatographic/fluorometric (µg/L): 70953
Chlorophyll b, phytoplankton, chromatographic/fluorometric (µg/L): 70954

1. Applications

The method is suitable for all water. The method is not suitable for determining chlorophyll c.

2. Summary of method

A plankton sample is filtered, and the chlorophylls are extracted from the algal cells. The chlorophylls are separated from each other and chlorophyll degradation products by thin-layer chromatography. Chlorophylls are eluted and measured using a spectrofluorometer.

3. Interferences

A substantial quantity of sediment may affect the extraction process. Exposure to light or acid at any stage of storage and analysis can result in photochemical and chemical degradation of the chlorophylls.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Air dryer.

4.2 Centrifuge.

4.3 Centrifuge tubes, graduated, screwcap, 15-mL capacity.

4.4 Chromatography sheet, thin-layer cellulose, 5×20 cm, 80-µm thick cellulose.

4.5 Developing tank and rack.

4.6 Evaporation device.

4.7 Filters, glass fiber, 47-mm diameter, capable of retaining particles having diameters of at least 0.45 µm.

4.8 Filter funnel, nonmetallic, that has vacuum or pressure apparatus.

4.9 Glass pipets, 5- and 10-mL capacity.

4.10 Glass vials, screwcap, 22×85 mm.

4.11 Gloves, long-service latex.

4.12 Grinding motor that has 0.1 horsepower.

4.13 Microdoser, and 50-μL syringe.

4.14 Pasteur pipets, disposable.

4.15 Propipet, or equivalent suction device.

4.16 Solvent-saturation pads, 13.4×22 cm.

4.17 Spectrofluorometer (fig. 58) that has redsensitive R446S photomultiplier, or equivalent. Use cells that have a light path of 1 cm.

4.18 Spectrometer (spectrophotometer; fig. 57) that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.19 Tissue grinder.

Figure 58.--Spectrofluorometer (photograph courtesy of AMINCO Division of SLM Instruments, Inc., Urbana, Ill.).

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetone, 90 percent. Add nine volumes of acetone to one volume of distilled water.

5.2 Chlorophyll a, stock solution. Add 1 mL 90-percent acetone to 1 mg chlorophyll a (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2 and 5.3 are wrapped with aluminum foil as an added precaution.

5.3 Chlorophyll b, stock solution. Add 1 mL 90-percent acetone to 1 mg chlorophyll b.

5.4 Dimethyl sulfoxide (DMSO).

5.5 Distilled or deionized water.

5.6 Ethyl ether.

5.7 Methyl alcohol.

5.8 Nitrogen gas, prepurified.

5.9 Petroleum ether, 30 to 60 °C.

6. Analysis

6.1 If filter was frozen, allow it to thaw 2 to 3 minutes at room temperature.

6.2 Place the filter in a tissue grinder. Add 3 to 4 mL DMSO and grind at 500 r/min for 3 minutes. If multiple filters are used, use a 40-mL grinder.

CAUTION.--Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.3 Transfer the sample to a 15-mL graduated centrifuge tube, and wash the pestle and grinder twice using DMSO.

6.4 Add an equal volume of ethyl ether. Screw on cap and shake vigorously for 10 seconds. Wait 10 seconds and repeat shaking for 10 seconds more.

6.5 Remove cap and add slowly, almost dropwise, a volume of distilled water equal to 25 percent of the total volume of extractant (DMSO).

6.6 Cap and shake as in 6.4.

6.7 Centrifuge at 1,000 r/min for 10 minutes.

6.8 During centrifugation, prepare chromatography tank by pouring 294 mL petroleum ether and 6 mL methyl alcohol into the tank. Mix well. Prepare fresh before each use. Use two solvent-saturation pads and the developing rack to dry the chromatography sheet.

6.9 Remove the top ethyl ether layer containing chlorophyll using a pipet, and place in another 15-mL graduated centrifuge tube.

6.10 Add an equal volume of distilled water, and shake as in 6.4.

6.11 Centrifuge at 1,000 r/min for 5 minutes.

6.12 Remove the top ethyl ether layer using a capillary pipet, and place in the conical tube in the evaporation device. Evaporate to dryness by blowing nitrogen gas over the ethyl ether surface.

6.13 Immediately add 0.5 mL acetone. Mix. Wait 30 seconds and mix again. If all chlorophyll is not in solution, then repeat procedure.

6.14 Using the microdoser, streak about 25 µL of the acetone-chlorophyll solution on the chromatography sheet, 15 mm from the bottom and 6 mm from each side, using the air dryer to speed evaporation of the solvent. If excessive trailing occurs during chromatography, the volume of the solvent should be decreased.

6.15 Develop chromatograph in the dark, using chlorophyll solution(s). Use enough chlorophyll (about 5 µL of the solutions as in 5.2 or 5.3, or both) to visually locate the spot of pigment. The time required for development is about 30 minutes. Remove strips when solvent has traveled within 2 to 3 cm from top of the strip.

6.16 Determine $\underline{R}_{\underline{f}}^{\wedge}$ values (Note 2) for pure chlorophylls.

Note 2: $\underline{R}_{\underline{f}}^{\wedge}$ value = distance traveled by the chlorophyll from the point of application divided by the distance traveled by the solvent from the point of application.

6.17 Locate the $\underline{R}_{\underline{f}}^{\wedge}$ value on the chromatography sheet; and, using a razor blade, scrape the cellulose off the sheet at the spot of the $\underline{R}_{\underline{f}}^{\wedge}$ value minus 0.07 for chlorophyll _a_ (0.14 for chlorophyll _b_) × $\underline{R}_{\underline{f}}^{\wedge}$. Place the cellulose into a graduated centrifuge tube, and add acetone to a volume of 3 mL. This step should be done immediately after the chromatograph is removed from the tank. Shake the scraped cellulose and acetone vigorously for 10 seconds. Wait 1 minute and shake again vigorously for 10 seconds more.

6.18 Centrifuge at 1,000 r/min for 5 minutes.

6.19 Determine the concentration of chlorophyll _a_ or _b_ using the spectrofluorometer as follows. Curves are prepared daily to standardize the spectrofluorometer. Five standard solutions of each chlorophyll should be

prepared at the concentrations of 0.25, 0.5, 1, 2, and 4 mg/L. These are prepared from the chlorophyll stock solutions by an appropriate dilution using 90-percent acetone. The absorbance then is read on a spectrometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*. Determine concentrations of standard solutions and samples using the specific absorptivities of 0.0877 L/mg × cm for chlorophyll *a* and 0.0514 L/mg × cm for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$C = \frac{A}{\alpha b},$$

where C = concentration of chlorophyll, in milligrams per liter;
A = absorbance;
b = path length, in centimeters; and
α = specific absorptivity.

6.20 These chlorophyll standard solutions are used to standardize the spectrofluorometer. For chlorophyll *a*, set the spectrofluorometer for an excitation wavelength of 430 nm and an emission wavelength of 670 nm. For chlorophyll *b*, the excitation wavelength is 460 nm and the emission wavelength is 650 nm. Set entrance and exit slits at 2 mm. Plot chlorophyll concentration versus relative fluorescence intensity. Determine unknown concentrations from the standard solution curve.

7. Calculations

The concentration of chlorophyll obtained in 6.20 is corrected for the concentration step onsite and in the determination:

$$\text{Original sample (micrograms chlorophyll per liter)} = \frac{\text{Micrograms chlorophyll per milliliter (as in 6.20)} \times 3\text{ mL} \times \dfrac{\text{Concentrate volume (microliters)}}{\text{volume streaked (microliters)}}}{\text{Volume filtered onsite (liters)}}.$$

8. Reporting of results

Report concentrations of chlorophylls *a* or *b* as follows: less than 1 µg/L, one decimal; 1 µ/L and greater, two significant figures.

9. Precision

No precision data are available.

10. Reference cited

Jeffrey, S. W., and Humphrey, G. F., 1975, New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1, and c_2 in higher plants, algae, and natural phytoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

Biomass/Chlorophyll Ratio for Phytoplankton
(B-6560-85)

Parameter and Code:
Biomass-chlorophyll ratio, phytoplankton: 70949

Plankton and periphyton communities normally are dominated by algae. As degradable, nontoxic organic materials enter a body of water, a frequent result is that a greater percentage of the total biomass is heterotrophic (nonchlorophyll-containing) organisms, such as bacteria and fungi. This change can be observed in the biomass to chlorophyll a ratio (or autotrophic index). Periphyton ratios for unpolluted water have been reported in the range of 50 to 100 (Weber, 1973); whereas, values greater than 100 may result from organic pollution (Weber and McFarland, 1969; Weber, 1973).

1. Applications

The method is suitable for the determination of chlorophylls a and b in concentrations of 0.1 µg/L and greater.

2. Summary of method

A filtered phytoplankton sample is ruptured mechanically, and the chlorophyll pigments are separated from each other and degradation products by high-pressure liquid chromatography and are determined by fluorescence spectroscopy (Shoaf and Lium, 1976, 1977). The dry weight and ash weight of the phytoplankton are determined to obtain the weight of organic matter (biomass). The biomass/chlorophyll a ratio is calculated from these values.

3. Interferences

3.1 A substantial quantity of sediment may affect the chlorophyll extraction process. Inorganic matter in the sample will cause erroneously large dry and ash weights; nonliving organic matter in the sample will cause erroneously large dry (and thus organic) weights.

3.2 Exposure of the sample to heat, light, or acid can result in photochemical or chemical degradation of the chlorophylls. Large values will result from the presence of fragments of tree leaves or other plant materials that contain chlorophyll. Large populations of photosynthetic bacteria also will result in large values.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Analytical balance, capable of weighing to at least 0.1 mg.

4.2 Auto-injector (recommended, but not required).

4.3 Centrifuge.

4.4 Centrifuge tubes, 15 and 50 mL, conical, screwcap, graduated.

4.5 Desiccator, containing anhydrous calcium sulfate.

4.6 Drying oven, thermostatically controlled for use at 105 °C.

4.7 Evaporation device.

4.8 Filters, glass fiber, 47-mm diameter, capable of retaining particles having diameters of at least 0.45 μm.

4.9 Filter funnel, nonmetallic, that has vacuum or pressure apparatus.

4.10 Fluorometer, equipped with excitation and emission filters.

4.11 Forceps or tongs.

4.12 Glass bottles, screwcap, smallest appropriate size for the sample.

4.13 Glass funnels.

4.14 Gloves, long-service latex.

4.15 High-pressure liquid chromatograph (HPLC), consisting of a solvent programmer, an isochromatic pump, an oven, and a column. (The column oven needs to be capable of maintaining a constant temperature in the 25 to 35 °C range.)

4.16 High-vacuum pump, capable of providing an absolute pressure of less than 1 torr.

4.17 Muffle furnace, for use at 500 °C.

4.18 Pasteur pipets, disposable.

4.19 Porcelain crucibles.

4.20 Separatory funnels, 125 mL.

4.21 Spectrometer (spectrophotometer; fig. 57) that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.22 Tissue homogenizer, 30-mL homogenizing flasks, and blades.

4.23 Vacuum flasks, stoppers, glass tubing, vacuum tubing, and a sintered glass tube.

4.24 Vacuum desiccator.

4.25 Vacuum oven.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetone, 90 percent. Add nine volumes of acetone to one volume of distilled water and mix.

5.2 Chlorophyll a stock solution. Transfer 1 mg chlorophyll a to a 100-mL volumetric flask and fill to capacity using 90-percent acetone (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2, 5.3, 5.4, and 5.5 are wrapped with aluminum foil as an added precaution.

5.3 Chlorophyll b stock solution. Transfer 1 mg chlorophyll b to a 100-mL volumetric flask and fill to capacity using 90-percent acetone.

5.4 Chlorophyll standard solution. Mix 25 mL chlorophyll a stock solution with 25 mL chlorophyll b stock solution in a 50-mL centrifuge tube.

5.5 Chlorophyll working standard solutions. Use a 5-mL pipet to prepare the following mixtures.

5.5.1 High standard solution, chlorophylls a and b. Add 5 mL chlorophyll standard solution to 5 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.2 Mid-range standard solution, chlorophylls a and b. Add 3 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.3 Low standard solution, chlorophylls a and b. Add 1 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.6 Distilled or deionized water.

5.7 Diethyl ether, distilled in glass, unpreserved.

5.8 Dimethyl sulfoxide (DMSO).

5.9 Methyl alcohol, 96-percent. Pour 960 mL methyl alcohol, distilled in glass, into a 1-L graduated cylinder. Add distilled water to the mark and mix.

5.10 Nitrogen gas, prepurified.

6. Analysis

6.1 Sample preparation. Analyze only samples on glass-fiber filters. Record the volume of water filtered for the phytoplankton sample. [If a biomass determination is required, save the DMSO layer (see 6.1.7).]

6.1.1 Allow the frozen filter to thaw 2 to 3 minutes at room temperature.

CAUTION.--Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.1.2 Place the filter in a 30-mL tissue homogenizing flask. Add 15 mL DMSO and homogenize until the sample has been ruptured.

6.1.3 Transfer the sample to a 50-mL graduated centrifuge tube, and rinse the homogenizing flask and blade using 5 mL DMSO. Add the rinse to the centrifuge tube.

6.1.4 Add 20 mL diethyl ether to the centrifuge tube, screw on the cap, and shake vigorously for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.5 Remove the cap and slowly add, almost dropwise, 10 mL distilled water to the centrifuge tube. Secure the cap and shake gently. Vent, then shake for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.6 Centrifuge at 1,000 r/min for 10 minutes.

6.1.7 Transfer the top diethyl ether layer, using a disposable pipet, to a 125-mL separatory funnel. (If the DMSO layer appears green after diethyl ether extraction, repeat 6.1.4 through 6.1.7. There are, however, some green chlorophyll derivatives not extractable using diethyl ether.)

6.1.8 Add 15 mL distilled water to the separatory funnel and shake vigorously for 10 seconds, venting often. Allow the layers to separate. (Break emulsions by adding 1 to 2 mL acetone and swirling the funnel gently.)

6.1.9 Drain and discard the bottom layer.

6.1.10 Rinse the upper part of the separatory funnel using 2 to 3 mL acetone. Remove the bottom layer that forms in the funnel and discard.

6.1.11 Decant the diethyl ether layer through the top of the separatory funnel into a centrifuge tube. Rinse the funnel using 5 mL diethyl ether, and add the rinse to the centrifuge tube.

6.1.12 Place the centrifuge tube on the evaporation device, and evaporate to 0.2 to 0.4 mL using a gentle stream of nitrogen gas.

6.1.13 Add sufficient acetone to the sample extract so the color intensity is between the color intensities of the high and low standard solutions. If the color of the sample extract is not within the specified range after the addition of 20 mL acetone, take a 1-mL aliquot of the 20 mL extract, and dilute volumetrically until the desired color intensity is obtained.

6.2 High-pressure liquid-chromatographic analysis.

6.2.1 Measure the absorbance of the chlorophyll stock solutions using a spectrometer. Measure the absorbance at 664 nm for chlorophyll a and at 647 nm for chlorophyll b. Record the absorbance for three replicates of chlorophylls a and b. Average the three values for chlorophyll a and the three values for chlorophyll b separately, and record each average separately for subsequent calculations.

6.2.2 Operate the HPLC system using 96-percent methyl alcohol as the mobile phase at a flow of 1.5 mL/min until the pressure stabilizes.

6.2.3 Calibrate the instrument by injecting 10 µL of the mid-range standard solution, and record the peaks of chlorophylls a and b.

6.2.4 Verify that the response of the fluorometer is linear by injecting the high and low standard solutions.

6.2.5 Analyze the sample by injecting 10 µL of the sample extract into the HPLC. Record the peaks of chlorophylls a and b, if any.

6.3 Dry weight and ash weight of organic matter.

6.3.1 Bake a porcelain crucible at 500 °C for 20 minutes. Cool to room temperature in a desiccator. Silica gel is not recommended. Measure the tare weight to the nearest 0.1 mg.

6.3.2 Remove the DMSO supernatant (6.1.7) using a disposable pipet. If biomass particles are visible in the supernatant, centrifuge first and then remove the supernatant. If the supernatant is still murky, filter through a tared glass-fiber filter, burn at 500 °C, and add filter ashes to sediment in crucible.

6.3.3 Quantitatively transfer the sediment to a 30-mL porcelain crucible using a microspoon or microspatula and rinses of distilled water.

6.3.4 Place the crucible in a 105 °C oven overnight to evaporate the water.

6.3.5 Place the crucible in a desiccated (preheated to 105 °C) vacuum oven. Lower the pressure in the oven to approximately 20 torr. Leave the crucible in the oven for 2 hours. Approximately every one-half hour or hour, redraw the vacuum (without reaching atmospheric pressure in the oven) to remove the DMSO fumes from the oven.

6.3.6 Cool cruicible in a vacuum desiccator to room temperature.

6.3.7 Weigh cruicible to the nearest 1 mg in a desiccated balance.

6.3.8 Reheat cruicible in the vacuum oven for 1 hour.

6.3.9 Cool cruicible in a vacuum desiccator and weigh. If the weight is not constant, reheat until constant weight within 5 percent is obtained. This value is used to calculate the dry weight.

6.3.10 Place the crucible containing the dried residue in a muffle furnace at 500 °C for 1 hour until a constant weight is obtained. This value is used to calculate the ash weight (Note 2).

Note 2: The ash is wetted to reintroduce the water of hydration of the clay and other minerals that, though not evaporated at 105 °C, is lost at 500 °C. This water loss may be as much as 10 percent of the weight lost during ignition and, if not corrected, will be interpreted as organic matter (American Public Health Association and others, 1985).

7. Calculations

7.1 Chlorophyll.

7.1.1 Calculate the exact concentrations of the chlorophyll stock solutions from the equation:

$$C_s = \frac{A}{\alpha b},$$

where C_s = concentration of chlorophyll stock solution, in milligrams per liter;
A = average absorbance obtained in 6.2.1;
b = path length, in centimeters; and
α = specific absorptivity [0.0877 L/mg × cm for chlorophyll *a* and 0.0514 L/mg × cm for chlorophyll *b* (Jeffrey and Humphrey, 1975)].

7.1.2 Verify and correct the concentrations of the chlorophyll working standard solutions in 5.5 by using the chlorophyll stock solutions determined in 7.1.1.

7.1.3 Calculate the response factor for chlorophylls *a* and *b* in the chlorophyll working standard solution:

$$RF = \frac{V \times C_m}{I_s},$$

where $\underline{RF}$ = response factor of chlorophyll a, in milligrams per unit area;
$\underline{V}$ = volume of mid-range standard solution injected, in milliliters;
$\underline{C_m}$ = concentration of chlorophyll a or b in the mid-range standard solution, in milligrams per liter; and
$\underline{I_s}$ = integrated area of the component peak.

7.1.4 Use the data from 6.2.5 to calculate the concentration of chlorophyll a or b in the original sample from the equation:

$$\text{Concentration (micrograms per liter)} = \frac{\underline{RF} \times \underline{IV_e}}{\underline{A_s} \times \underline{V_i}},$$

where $\underline{RF}$ = response factor of chlorophyll a or b from 7.1.3, in milligrams per unit area;
$\underline{I}$ = integrated area of the chlorophyll a or b peak in the sample as determined in 6.2.5;
$\underline{V_e}$ = final volume of the sample extract from 6.1.13, in milliliters;
$\underline{A_s}$ = volume of water filtered in 6.1, in liters; and
$\underline{V_i}$ = volume of sample extract injected in 6.2.5, in microliters.

7.2 Biomass.

$$\text{Organic weight (milligrams per liter)} = \frac{\text{Dry weight (milligrams)} - \text{ash weight (milligrams)}}{\text{Volume filtered onsite (liters)}}.$$

$$\text{7.3 Ratio} = \frac{\text{Biomass (milligrams per liter)} \times 1{,}000}{\text{Chlorophyll a or b (micrograms per liter)}}.$$

8. Reporting of results

8.1 Report concentrations of chlorophylls a and b as follows: less than 1 µg/L, one decimal; 1 µg/L and greater, two significant figures.

8.2 Report biomass as follows: less than 1 mg/L, one decimal; 1 mg/L and greater, two significant figures.

8.3 Report ratio to three significant figures.

9. Precision

No precision data are available.

10. References cited

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C. American Public Health Association, 1,268 p.

Jeffrey, S. W., and Humphrey, G. F., 1975, New spectrophotometric equations for determining chlorophylls a, b, c_1, and c_2 in higher plants, algae, and natural photoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

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Weber, C. I., 1973, Recent developments in the measurement of the response of plankton and periphyton to changes in their environment, in Glass, G., ed., Bioassay techniques and environmental chemistry: Ann Arbor Science, p. 119-138.

Weber, C. I., and McFarland, B., 1969, Periphyton biomass-chlorophyll ratio as an index of water quality: Cincinnati, Ohio, Federal Water Pollution Control Administration, Analytical Quality Laboratory, 19 p.

Chlorophyll in Periphyton by Spectroscopy
(B-6601-85)

Parameters and Codes:

Chlorophyll a, periphyton, spectrometric, uncorrected (mg/m^2):	32228
Chlorophyll b, periphyton, spectrometric, (mg/m^2):	32226
Chlorophyll c, periphyton, spectrometric, (mg/m^2):	32227
Chlorophyll, total, periphyton, spectrometric, uncorrected (mg/m^2):	32225

1. Applications

The method is suitable for all water and may be used for periphyton from natural or artificial substrates.

2. Summary of method

Chlorophyll pigments are determined simultaneously without detailed separation. The periphyton is scraped from a known area, suspended in water, and concentrated on a membrane filter. A water sample is filtered, and the periphyton cells retained on the filter are ruptured mechanically, using 90-percent acetone, to facilitate extraction of pigments. Concentrations of chlorophylls are calculated from measurements of absorbance of the extract at four wavelengths, corrected for a 90-percent acetone blank.

3. Interferences

Erroneously large values may result from the presence of fragments of tree leaves and other plant materials. Exposure to light or acid at any stage of storage and analysis can result in photochemical and chemical degradation of the chlorophylls.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Artificial substrates made of glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.2 Centrifuge, swing-out type, 3,000 to 4,000 r/min, and 15-mL graduated centrifuge tubes.

4.3 Collecting devices for the removal of periphyton from natural substrates. Three devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 18.

4.4 Filters, metricel, alpha-6, 0.45 µm, 25-mm diameter.

4.5 Filter flask, 1 or 2 L. Onsite, a polypropylene flask is used.

4.6 Filter funnel, vacuum, 1.2 L, stainless steel.

4.7 Filter holder, Pyrex microanalysis, frit support, 25 mm.

4.8 Glass pan, smallest appropriate size for scraping substrate.

4.9 Manostat that has mercury and calibration equipment to regulate the filtration suction to not more than 250 mm of mercury when filtering using an aspirator or an electric vacuum pump.

4.10 Membrane filter, white, plain, 0.45-µm mean pore size, 47-mm diameter.

4.11 Pasteur pipets, disposable.

4.12 Sample containers suitable for the type of sample. Glass bottles are useful containers for artificial substrates or for pieces of natural substrates.

4.13 Scraping device, razor blades, stiff brushes, spatulas, or glass slides, for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.14 Source of vacuum for filtration. A water-aspirator pump or an electric vacuum pump for laboratory use; a handheld vacuum pump and gauge for onsite use.

4.15 Spectrometer (spectrophotometer; fig. 57) that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.16 Tissue grinder, glass, pestle-type, 15-mL capacity. Homogenizer should be motor driven at about 500 r/min.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetone, 90 percent. Add nine volumes of acetone to one volume of distilled water.

5.2 Distilled or deionized water.

6. Analysis

6.1 If filter was frozen, allow it to thaw for 5 minutes at room temperature.

6.2 If an artificial substrate is used, scrape the periphyton off the substrate, using the scraping device, into a glass pan. Transfer all solid material to the tissue grinder.

6.3 Rinse the scraping device and substrate using 90-percent acetone. Store for 10 minutes in the dark at room temperature.

6.4 Grind at 400 r/min for 3 minutes.

6.5 Transfer the sample to a 15-mL graduated centrifuge tube, and wash the pestle and grinder two or three times using 90-percent acetone. Adjust to some convenient volume, such as 10±0.1 mL.

6.6 Centrifuge at 3,000 to 4,000 r/min for 10 minutes.

6.7 Carefully pour or pipet the supernatant into the spectrometer cell. Do not disturb the precipitate. If the extract is turbid, clear by making a twofold dilution using 90-percent acetone, or by filtering through an acetone-resistant filter.

6.8 Read the absorbances at 750, 664, 647, and 630 nm and compare to a 90-percent acetone blank. (Dilute the extract using 90-percent acetone if the absorbance is greater than 0.8.) If the 750-nm reading is greater than 0.005 absorbance unit per centimeter of light path, decrease the turbidity as in 6.7.

7. Calculations

7.1 Subtract the absorbance at 750 nm from the absorbance at each of the other wavelengths (that is, 664, 647, and 630 nm). Divide the differences by the light path of the spectrometer cell, in centimeters. The concentrations of chlorophylls in the extract, in micrograms per milliliter, are calculated by the following equations (Jeffrey and Humphrey, 1975):

Chlorophyll a, in micrograms per milliliter = $11.85e_{664}-1.54e_{647}-0.08e_{630}$;
Chlorophyll b, in micrograms per milliliter = $-5.43e_{664}+21.03e_{647}-2.66e_{630}$;
and
Chlorophyll c, in micrograms per milliliter = $-1.67e_{664}-7.60e_{647}+24.52e_{630}$;

where $e_{664} = \frac{\text{Absorbance at 664 nm - absorbance at 750 nm}}{\text{Light path, in centimeters}}$;

$e_{647} = \frac{\text{Absorbance at 647 nm - absorbance at 750 nm}}{\text{Light path, in centimeters}}$; and

$e_{630} = \frac{\text{Absorbance at 630 nm - absorbance at 750 nm}}{\text{Light path, in centimeters}}$.

7.2 Convert the values derived in 7.1 to the concentrations of chlorophylls, in milligrams per square meter, in the originally collected sample. For example:

$$\text{Chlorophyll } \underline{a} \text{ (milligrams per square meter)} = \frac{\text{Derived value (micrograms per milliliter)} \times \text{extract volume (milliliters)}}{\text{Area of scraped surface (square meters)} \times 1{,}000} .$$

8. Reporting of results

Report concentrations of chlorophyll a, b, or c, in milligrams per square meter, to three significant figures.

9. Precision

9.1 The precision of chlorophyll determinations is affected by the area scraped, the range of chlorophyll values calculated, the volume of extraction solvent, and the light path of the spectrometer cells.

9.2 Tilley and Haushild (1975a and b) reported that 21 glass microscope slides exposed for 2 weeks at a single site in the Duwamish River, Wash., had chlorophyll a concentrations that ranged from 1.33 to 2.81 mg/m^2 and had a mean of 1.97 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 7.4 mg/m^2. Twenty-two slides exposed for 3 weeks at a single site had chlorophyll a concentrations that ranged from 1.89 to 4.86 mg/m^2 and had a mean of 3.44 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 14.4 mg/m^2.

10. References cited

Jeffrey, S. W., and Humphrey, G. F., 1975, New spectrophotometric equations for determining chlorophylls a, b, c_1, and c_2 in higher plants, algae, and natural photoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

Tilley, L. J., 1972, A method for rapid and reliable scraping of periphyton slides, in Geological Survey Research 1972: U.S. Geological Survey Professional Paper 800-D, p. D221-D222.

Tilley, L. J., and Haushild, W. L., 1975a, Net primary productivity of periphytic algae in the intertidal zone, Duwamish River Estuary, Washington: Journal of Research of the U.S. Geological Survey, v. 3, no. 3, p. 253-259.

_____1975b, Use of productivity of periphyton to estimate water quality: Water Pollution Control Federation Journal, v. 47, no. 8, p. 2157-2171.

Chlorophyll in Periphyton by Chromatography and Spectroscopy (B-6620-85)

Parameters and Codes:

Chlorophyll a, periphyton, chromatographic/spectrometric (mg/m^2): 70955
Chlorophyll b, periphyton, chromatographic/spectrometric (mg/m^2): 70956

1. Applications

The method is suitable for all water. The method is not suitable for the determination of chlorophyll c.

2. Summary of method

A periphyton sample is obtained, and the chlorophylls are extracted from the algal cells. The chlorophylls are separated from each other and from chlorophyll degradation products by thin-layer chromatography. Chlorophylls are eluted and measured using a spectrometer.

3. Interferences

A substantial quantity of sediment may affect the extraction process. Exposure to light or acid at any stage of storage and analysis can result in photochemical and chemical degradation of the chlorophylls.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Air dryer.

4.2 Artificial substrates made of glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.3 Centrifuge.

4.4 Centrifuge tubes, graduated, screwcap, 15-mL capacity.

4.5 Chromatography sheet, thin-layer cellulose, 5×20 cm, 80-µm thick cellulose.

4.6 Collecting devices for the removal of periphyton from natural substrates. Three devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 18.

4.7 Developing tank and rack.

4.8 Evaporation device.

4.9 Filters, glass fiber, 47-mm diameter, capable of retaining particles having diameters of at least 0.45 µm.

4.10 Glass bottles, screwcap, smallest appropriate size for the sample.

4.11 Glass pan, smallest appropriate size for scraping substrate.

4.12 Gloves, long-service latex.

4.13 Grinding motor that has 0.1 horsepower.

4.14 Microdoser, and 50-µL syringe.

4.15 Pasteur pipets, disposable.

4.16 Scraping device, razor blades, stiff brushes, spatulas, or glass slides, for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.17 Solvent-saturation pads, 13.4×22 cm.

4.18 Spectrometer (spectrophotometer; fig. 57) that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.19 Tissue grinder, glass, pestle-type, 15-mL capacity. Homogenizer should be motor dirven at about 500 r/min.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetone, 90 percent. Add nine volumes of acetone to one volume of distilled water.

5.2 Chlorophyll a stock solution. Add 1 mL 90-percent acetone to 1 mg chlorophyll a (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2 and 5.3 are wrapped with aluminum foil as an added precaution.

5.3 Chlorophyll b stock solution. Add 1 mL 90-percent acetone to 1 mg chlorophyll b.

5.4 Dimethyl sulfoxide (DMSO).

5.5 Distilled or deionized water.

5.6 Ethyl ether.

5.7 Methyl alcohol.

5.8 Nitrogen gas, prepurified.

5.9 Petroleum ether, 30 to 60 °C.

6. Analysis

6.1 If filter was frozen, allow it to thaw 2 to 3 minutes at room temperature.

6.2 If an artificial substrate is used, scrape the periphyton off the substrate, using the scraping device, into a glass pan. Transfer all solid material into the tissue grinder.

6.3 Rinse the scraping device and substrate using DMSO.

CAUTION.--Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.4 Grind at 400 r/min for 3 minutes.

6.5 Transfer the sample to a 15-mL graduated centrifuge tube, and wash the pestle and grinder twice using DMSO.

6.6 Add an equal volume of ethyl ether. Screw on cap and shake vigorously for 10 seconds. Wait 10 seconds and repeat shaking for 10 seconds more.

6.7 Remove cap and add slowly, almost dropwise, a volume of distilled water equal to 25 percent of the total volume of extractant (DMSO).

6.8 Cap and shake as in 6.6.

6.9 Centrifuge at 1,000 r/min for 10 minutes.

6.10 During centrifugation, prepare chromatography tank by pouring 294 mL petroleum ether and 6 mL methyl alcohol into the tank. Mix well. Prepare fresh before each use. Use two solvent-saturation pads and the developing rack to dry the chromatography sheet.

6.11 Remove the top ethyl ether layer containing chlorophyll using a pipet, and place in another 15-mL graduated centrifuge tube.

6.12 Add an equal volume of distilled water, and shake as in 6.6.

6.13 Centrifuge at 1,000 r/min for 5 minutes.

6.14 Remove the top ethyl ether layer using a pipet, and place in conical tube in evaporation device. Evaporate to dryness by blowing nitrogen gas over the ethyl ether surface.

6.15 Immediately add 0.5 mL acetone. Mix. Wait 30 seconds and mix again. If all chlorophyll is not in solution, then repeat procedure.

6.16 Using microdoser, streak 25 µL of the acetone-chlorophyll solution on the chromatography sheet, 15 mm from the bottom and 6 mm from each side, using the air dryer to speed evaporation of the solvent. If excessive trailing occurs during chromatography, the volume of the solvent should be decreased.

6.17 Develop chromatograph in the dark, using chlorophyll solution(s). Use enough chlorophyll (about 5 µL of the solutions as in 5.2 or 5.3, or both) to visually locate the spot of pigment. The time required for development is about 30 minutes. Remove strips when solvent has traveled within 2 to 3 cm from top of strip.

6.18 Determine $\underline{R}_{\underline{f}}^{\wedge}$ values (Note 2) for pure chlorophylls.

Note 2: $\underline{R}_{\underline{f}}^{\wedge}$ value = distance traveled by the chlorophyll from the point of application divided by the distance traveled by the solvent from the point of application.

6.19 Locate the $\underline{R}_{\underline{f}}^{\wedge}$ value on the chromatography sheet; and, using a razor blade, scrape the cellulose off the sheet at the spot of the $\underline{R}_{\underline{f}}^{\wedge}$ value minus 0.07 for chlorophyll *a* (0.14 for chlorophyll *b*) × $\underline{R}_{\underline{f}}^{\wedge}$. Place the cellulose into a graduated centrifuge tube, and add acetone to a volume of 3 mL. This step should be done immediately after the chromatograph is removed from the tank. Shake the scraped cellulose and acetone vigorously for 10 seconds. Wait 1 minute and shake again vigorously for 10 seconds more.

6.20 Centrifuge at 1,000 r/min for 5 minutes.

6.21 Remove supernatant and read the absorbance on the spectrometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*.

7. Calculations

7.1 If the absorbance is greater than 0.01, determine concentrations using the specific absorptivities of 0.0877 L/mg × cm for chlorophyll *a* and 0.0514 L/mg × cm for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$\underline{C} = \frac{\underline{A}}{\underline{\alpha b}},$$

where $\underline{C}$ = concentration of chlorophyll, in milligrams per liter;
$\underline{A}$ = absorbance;
$\underline{b}$ = path length, in centimeters; and
$\underline{\alpha}$ = specific absorptivity.

If the absorbance is less than 0.01, use the fluorescence technique.

7.2 The concentration of chlorophyll obtained in 7.1 is corrected for the concentration step onsite and in the determination:

$$\begin{array}{l}\text{Original sample (milligrams chlorophyll per square meter)} \\ \qquad = \end{array} \frac{\begin{array}{c}\text{Micrograms chlorophyll per milliliter (as in 6.21)} \times \text{(3 mL)} \times \dfrac{500\ \mu L}{25\ \mu L} \\ \hline \text{Area of surface scraped (square meters)} \times 1{,}000\end{array}}{}.$$

8. Reporting of results

Report concentrations of chlorophylls *a* or *b*, in milligrams per square meter, to three significant figures.

9. Precision

Tilley and Haushild (1975a and b) reported that 21 glass microscope slides exposed for 2 weeks at a single site in the Duwamish River, Wash., had chlorophyll *a* concentrations that ranged from 1.33 to 2.81 mg/m^2 and had a mean of 1.97 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 7.4 mg/m^2. Twenty-two slides exposed for 3 weeks at a single site had chlorophyll *a* concentrations that ranged from 1.89 to 4.86 mg/m^2 and had a mean of 3.44 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 14.4 mg/m^2.

No other precision data are available.

10. References cited

Jeffrey, S. W., and Humphrey, G. F., 1975, New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1, and c_2 in higher plants, algae, and natural photoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

Tilley, L. J., and Haushild, W. L., 1975a, Net primary productivity of periphytic algae in the intertidal zone, Duwamish River Estuary, Washington: Journal of Research of the U.S. Geological Survey, v. 3, no. 3, p. 253-259.

_____1975b, Use of productivity of periphyton to estimate water quality: Water Pollution Control Federation Journal, v. 47, no. 8, p. 2157-2171.

Chlorophyll in Periphyton by High-Pressure Liquid Chromatography (B-6630-85)

Parameters and Codes:

Chlorophyll a, periphyton, chromatographic/fluorometric (mg/m^2): 70957
Chlorophyll b, periphyton, chromatographic/fluorometric (mg/m^2): 70958

1. Applications

The method is suitable for the determination of chlorophylls a and b in periphyton in concentrations of 0.1 mg/m^2 and greater and is suitable for all water.

2. Summary of method

A periphyton sample is ruptured mechanically, and the chlorophyll pigments are separated from each other and degradation products by high-pressure liquid chromatography and determined by fluorescence spectroscopy (Shoaf and Lium, 1976, 1977).

3. Interferences

Exposure of the sample to heat, light, or acid can result in photochemical or chemical degradation of the chlorophylls. Large values will result from the presence of fragments of tree leaves or other plant materials that contain chlorophyll. Large populations of photosynthetic bacteria also will result in large values.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Artificial substrates made of glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.2 Auto-injector (recommended, but not required).

4.3 Centrifuge.

4.4 Centrifuge tubes, 15 and 50 mL, conical, screwcap, graduated.

4.5 Centrifuge tubes, 50 mL, conical, pennyhead stopper, graduated.

4.6 Collecting devices for the removal of periphyton from natural substrates. Three devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 18.

4.7 Evaporation device.

4.8 Fluorometer, equipped with excitation and emission filters.

4.9 Glass pan, smallest appropriate size for scraping substrate.

4.10 Gloves, long-service latex.

4.11 High-pressure liquid chromatograph (HPLC), consisting of a solvent programmer, an isochromatic pump, an oven, and a column. (The column oven needs to be capable of maintaining a constant temperature in the 25 to 35 °C range.)

4.12 Pasteur pipet, disposable.

4.13 Scraping device, razor blades, stiff brushes, spatulas, or glass slides, for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.14 Separatory funnels, 125 mL.

4.15 Spectrometer (spectrophotometer; fig. 57) that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.16 Tissue homogenizer, 30-mL homogenizing flasks, and blades.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetone, 90 percent. Add nine volumes of acetone to one volume of distilled water and mix.

5.2 Chlorophyll a stock solution. Transfer 1 mg chlorophyll a to a 100-mL volumetric flask and fill to capacity using 90-percent acetone (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2, 5.3, 5.4, and 5.5 are wrapped with aluminum foil as an added precaution.

5.3 Chlorophyll b stock solution. Transfer 1 mg cholorphyll b to a 100-mL volumetric flask and fill to capacity using 90-percent acetone.

5.4 Chlorophyll standard solution. Mix 25 mL chlorophyll a stock solution with 25 mL chlorophyll b stock solution in a 50-mL centrifuge tube.

5.5 Chlorophyll working standard solutions. Use a 5-mL pipet to prepare the following mixtures.

5.5.1 High standard solution, chlorophylls a and b. Add 5 mL chlorophyll standard solution to 5 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.2 Mid-range standard solution, chlorophylls a and b. Add 3 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.3 Low standard solution, chlorophylls a and b. Add 1 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.6 Distilled or deionized water.

5.7 Diethyl ether, distilled in glass, unpreserved.

5.8 Dimethyl sulfoxide (DMSO).

5.9 Methyl alcohol, 96 percent. Pour 960 mL methyl alcohol, distilled in glass, into a 1-L graduated cylinder. Add distilled water to the mark and mix.

5.10 Nitrogen gas, prepurified.

6. Analysis

6.1 Sample preparation.

6.1.1 Allow the frozen sample to thaw 2 to 3 minutes at room temperature.

6.1.2 Scrape the periphyton off the substrate into a glass pan.

6.1.3 Use 15 mL DMSO to rinse the solid material into a 30-mL homogenizing flask. Homogenize the sample until the cells have been ruptured.

CAUTION.--Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.1.4 Transfer the sample to a 50-mL graduated centrifuge tube, and rinse the homogenizing flask and blade using 5 mL DMSO. Add the rinse to the centrifuge tube.

6.1.5 Add 20 mL diethyl ether to the centrifuge tube, screw on the cap, and shake vigorously for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.6 Remove the cap and slowly add, almost dropwise, 10 mL distilled water to the centrifuge tube. Secure the cap and shake gently. Vent, then shake for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.7 Centrifuge at 1,000 r/min for 10 minutes.

6.1.8 Transfer the top diethyl ether layer, using a disposable pipet, to a 125-mL separatory funnel. (If the DMSO layer appears green after diethyl ether extraction, repeat 6.1.5 through 6.1.8. There are, however, some green chlorophyll derivatives not extractable using diethyl ether.)

6.1.9 Add 15 mL distilled water to the separatory funnel, and shake vigorously for 10 seconds, venting often. Allow the layers to separate. (Break emulsions by adding 1 to 2 mL acetone and swirling the funnel gently.)

6.1.10 Drain and discard the bottom layer.

6.1.11 Rinse the upper part of the separatory funnel using 2 to 3 mL acetone. Remove the bottom layer that forms in the funnel and discard.

6.1.12 Decant the diethyl ether layer through the top of the separatory funnel into a centrifuge tube. Rinse the funnel using 5 mL diethyl ether, and add the rinse to the centrifuge tube.

6.1.13 Place the centrifuge tube on the evaporation device, and evaporate to 0.2 to 0.4 mL using a gentle stream of nitrogen gas.

6.1.14 Add sufficient acetone to the sample extract so the color intensity is between the color intensities of the high and low standards. If the color of the sample extract is not within the specified range after the addition of 20 mL acetone, take a 1-mL aliquot of the 20 mL extract, and dilute volumetrically until the desired color intensity is obtained.

6.2 High-pressure liquid-chromatographic analysis.

6.2.1 Measure the absorbance of the chlorophyll stock solutions using a spectrometer. Measure the absorbance at 664 nm for chlorophyll a and at 647 nm for chlorophyll b. Record the absorbance for three replicates of chlorophylls a and b. Average the three values for chlorophyll a and the three values for chlorophyll b separately, and record each average separately for subsequent calculations.

6.2.2 Operate the HPLC system using 96-percent methyl alcohol as the mobile phase at a flow of 1.5 mL/min until the pressure stabilizes.

6.2.3 Calibrate the instrument by injecting 10 µL of the mid-range standard solution, and record the peaks of chlorophylls a and b.

6.2.4 Verify that the response of the fluorometer is linear by injecting the high and low standard solutions.

6.2.5 Analyze the sample by injecting 10 µL of the sample extract into the HPLC. Record the peaks of chlorophylls a and b, if any.

7. Calculations

7.1 Calculate the exact concentrations of the chlorophyll stock solutions from the equation:

$$C_s = \frac{A}{\alpha b},$$

where C_s = concentration of chlorophyll stock solution, in milligrams per liter;
A = average absorbance obtained in 6.2.1;
b = path length, in centimeters; and
α = specific absorptivity [0.0877 L/mg × cm for chlorophyll *a* and 0.0514 L/mg × cm for chlorophyll *b* (Jeffrey and Humphrey, 1975)].

7.2 Verify and correct the concentrations of the chlorophyll working standard solutions in 5.5 by using the chlorophyll stock solutions determined in 7.1.

7.3 Calculate the response factor for chlorophylls *a* and *b* in the chlorophyll working standard solution:

$$RF = \frac{V \times C_m}{I_s},$$

where RF = response factor of chlorophyll *a*, in milligrams per unit area;
V = volume of mid-range standard solution injected, in milliliters;
C_m = concentration of chlorophyll *a* or *b* in the mid-range standard solution, in milligrams per liter; and
I_s = integrated area of the component peak.

7.4 Use the data from 6.2.5 to calculate the concentration of chlorophyll *a* or *b* on the original substrate:

$$\text{Concentration (milligrams per square meter)} = \frac{RF \times IV_e}{A_s \times V_i \times 1{,}000},$$

where RF = response factor of chlorophyll *a* or *b*, in milligrams per unit area;
I = integrated area of the chlorophyll *a* or *b* peak in the sample as determined in 6.2.5;
V_e = final volume of the sample extract from 6.1.14, in milliliters;
A_s = area of substrate, in square meters; and
V_i = volume of sample extract injected in 6.2.5, in microliters.

8. Reporting of results

Report concentrations of chlorophylls a or b as follows: less than 1 mg/m^2, one decimal; 1 mg/m^2 and greater, two significant figures.

9. Precision

No precision data are available.

10. References cited

Jeffrey, S. W., and Humphrey, G. F., 1975, New spectrophotometric equations for determining chlorophylls a, b, c_1, and c_2 in higher plants, algae, and natural photoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

Shoaf, W. T., and Lium, B. W., 1976, Improved extraction of chlorophyll a and b from algae using dimethyl sulfoxide: Limnology and Oceanography, v. 21, no. 6, p. 926-928.

_____1977, The quantitative determination of chlorophyll a and b from fresh water algae without interference from degradation products: Journal of Research of the U.S. Geological Survey, v. 5, no. 2, p. 263-264.

Chlorophyll in Periphyton by Chromatography and Fluorometry (B-6640-85)

Parameters and Codes:

Chlorophyll a, periphyton, chromatographic/fluorometric (mg/m^2): 70957
Chlorophyll b, periphyton, chromatographic/fluorometric (mg/m^2): 70958

1. Applications

The method is suitable for all water. The method is not suitable for the determination of chlorophyll c.

2. Summary of method

A periphyton sample is obtained and the chlorophylls are extracted from the algal cells. The chlorophylls are separated from each other and chlorophyll degradation products by thin-layer chromatography. Chlorophylls are eluted and measured using a spectrofluorometer.

3. Interferences

A substantial quantity of sediment may affect the extraction process. Exposure to light or acid at any stage of storage and analysis can result in photochemical and chemical degradation of the chlorophylls.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Air dryer.

4.2 Artificial substrates made of glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.3 Centrifuge.

4.4 Centrifuge tubes, graduated, screwcap, 15-mL capacity.

4.5 Chromatography sheet, thin-layer cellulose, 5×20 cm, 80-µm thick cellulose.

4.6 Collecting devices for the removal of periphyton from natural substrates. Three devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 18.

4.7 Developing tank and rack.

4.8 Evaporation device.

4.9 Filters, glass fiber, 47-mm diameter, capable of retaining particles having diameters of at least 0.45 µm.

4.10 Glass bottles, screwcap, smallest appropriate size for the sample.

4.11 Glass pan, smallest appropriate size for scraping substrate.

4.12 Gloves, long-service latex.

4.13 Grinding motor that has 0.1 horsepower.

4.14 Microdoser, and 50-mL syringe.

4.15 Pasteur pipets, disposable.

4.16 Scraping device, razor blades, stiff brushes, spatulas, or glass slides, for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.17 Solvent-saturation pads, 13.4×22 cm.

4.18 Spectrofluorometer (fig. 58) that has red-sensitive R446S photomultiplier, or equivalent. Use cells that have a light path of 1 cm.

4.19 Spectrometer (spectrophotometer; fig. 57) that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.20 Tissue grinder.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetone, 90 percent. Add nine volumes of acetone to one volume of distilled water.

5.2 Chlorophyll a stock solution. Add 1 mL 90-percent acetone to 1 mg chlorophyll a (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2 and 5.3 are wrapped with aluminum foil as an added precaution.

5.3 Chlorophyll b stock solution. Add 1 mL 90-percent acetone to 1 mg chlorophyll b.

5.4 Dimethyl sulfoxide (DMSO).

5.5 Distilled or deionized water.

5.6 Ethyl ether.

5.7 Methyl alcohol.

5.8 Nitrogen gas, prepurified.

5.9 Petroleum ether, 30 to 60 °C.

6. Analysis

6.1 If sample was frozen, allow it to thaw 2 to 3 minutes at room temperature.

6.2 If an artificial substrate is used, scrape the periphyton off the substrate, using the scraping device, into a glass pan. Transfer all solid material into the tissue grinder.

6.3 Rinse the scraping device and substrate using DMSO.

CAUTION.--Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.4 Grind at 400 r/min for 3 minutes.

6.5 Transfer the sample to a 15-mL graduated centrifuge tube, and wash the pestle and grinder twice using DMSO.

6.6 Add an equal volume of ethyl ether. Screw on cap and shake vigorously for 10 seconds. Wait 10 seconds and repeat shaking for 10 seconds more.

6.7 Remove cap and add slowly, almost dropwise, a volume of distilled water equal to 25 percent of the total volume of extractant (DMSO).

6.8 Invert the centrifuge tube gently, vent (to prevent tube from breaking from excess pressure), and then shake vigorously.

6.9 Centrifuge at 1,000 r/min for 10 minutes.

6.10 During centrifugation, prepare chromatography tank by pouring 294 mL petroleum ether and 6 mL methyl alcohol into tank. Mix well. Prepare fresh before each use. Use two solvent-saturation pads and the developing rack to dry the chromotography sheet.

6.11 Remove the top ethyl ether layer containing chlorophyll using a pipet, and place in another 15-mL graduated centrifuge tube.

6.12 Add an equal volume of distilled water, and shake as in 6.6.

6.13 Centrifuge at 1,000 r/min for 5 minutes.

6.14 Remove the top ethyl ether layer, using a pipet, and place in the conical tube in the evaporation device. Evaporate to dryness by blowing nitrogen gas over the ethyl ether surface.

6.15 Immediately add 0.5 mL acetone. Mix. Wait 30 seconds and mix again. If all chlorophyll is not in solution, then repeat procedure.

6.16 Using the microdoser, streak 25 μL of the acetone-chlorophyll solution on the chromotography sheet, 15 mm from the bottom and 6 mm from each side, using the air dryer to speed evaporation of the solvent. If excessive trailing occurs during chromatography, the volume of the solvent should be decreased.

6.17 Develop chromatograph in the dark, using chlorophyll solution(s). Use enough chlorophyll (about 5 μL of the solutions as in 5.2 or 5.3, or both) to visually locate the spot of pigment. The time required for development is about 30 minutes. Remove strips when solvent has traveled within 2 to 3 cm from top of strip.

6.18 Determine $\underline{R}_{\underline{f}}^{\wedge}$ values (Note 2) for pure chlorophylls.

Note 2: $\underline{R}_{\underline{f}}^{\wedge}$ value = distance traveled by the chlorophyll from the point of application divided by the distance traveled by the solvent from the point of application.

6.19 Locate the $\underline{R}_{\underline{f}}^{\wedge}$ value on the chromotography sheet; and, using a razor blade, scrape the cellulose off the sheet at the spot of the $\underline{R}_{\underline{f}}^{\wedge}$ value minus 0.07 for chlorophyll *a* (0.14 for chlorophyll *b*) × $\underline{R}_{\underline{f}}^{\wedge}$. Place the cellulose into a graduated centrifuge tube, and add acetone to a volume of 3 mL. This step should be done immediately after the chromotograph is removed from the tank. Shake the scraped cellulose and acetone vigorously for 10 seconds. Wait 1 minute and shake again vigorously for 10 seconds more.

6.20 Centrifuge at 1,000 r/min for 5 minutes.

6.21 Determine the concentration of chlorophyll *a* or *b* using the spectrofluorometer as follows. Curves are prepared daily to standardize the spectrofluorometer. Five standard solutions of each chlorophyll should be prepared at the concentrations of 0.5, 1, 2, 3, and 4 mg/L. These are prepared from the chlorophyll stock solutions by an appropriate dilution using 90-percent acetone.

6.22 For chlorophyll *a*, set the spectrofluorometer for an excitation wavelength of 430 nm and an emission wavelength of 670 nm. For chlorophyll *b*, the excitation wavelength is 460 nm and the emission wavelength is 650 nm. Set entrance and exit slits at 2 mm. Plot chlorophyll concentration versus relative fluorescence intensity. Determine unknown concentrations from the appropriate standard solution curve.

7. Calculations

7.1 The absorbance then is read on a spectrometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*. Determine concentrations of solutions and samples using the specific absorptivities of 0.0877 L/mg × cm for chlorophyll *a* and 0.0514 L/mg × cm for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$\underline{C} = \frac{\underline{A}}{\underline{\alpha b}},$$

where C = concentration of chlorophyll, in milligrams per liter;
A = absorbance;
b = path length, in centimeters; and
α = specific absorptivity.

7.2 The concentration of chlorophyll obtained in 6.22 is corrected for the concentration step onsite and in the determination:

$$\text{Original sample (milligrams chlorophyll per square meter)} = \frac{\text{Micrograms chlorophyll per milliliter (as in 6.22)} \times 3 \text{ mL} \times \frac{500\ \mu\text{L}}{25\ \mu\text{L}}}{\text{Area of surface scraped (square meters)} \times 1{,}000}.$$

8. Reporting of results

Report concentrations of chlorophylls a or b, in milligrams per square meter, to three significant figures.

9. Precision

Tilley and Haushild (1975a and b) reported that 21 glass microscope slides exposed for 2 weeks at a single site in the Duwamish River, Wash., had chlorophyll a concentrations that ranged from 1.33 to 2.81 mg/m^2 and had a mean of 1.97 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 7.4 mg/m^2. Twenty-two slides exposed for 3 weeks at a single site had chlorophyll a concentrations that ranged from 1.89 to 4.86 mg/m^2 and had a mean of 3.44 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 14.4 mg/m^2.

No other precision data are available.

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Biomass/Chlorophyll Ratio for Periphyton
(B-6660-85)

Parameter and Code:
Biomass-chlorophyll ratio, periphyton: 70950

Plankton and periphyton communities normally are dominated by algae. As degradable, nontoxic organic materials enter a body of water, a frequent result is that a greater percentage of the total biomass is heterotrophic (nonchlorophyll-containing) organisms, such as bacteria and fungi. This change can be observed in the biomass to chlorophyll a ratio (or autotrophic index). Periphyton ratios for unpolluted water have been reported in the range of 50 to 100 (Weber, 1973); whereas, values greater than 100 may result from organic pollution (Weber and McFarland, 1969; Weber, 1973).

1. Applications

The method is suitable for the determination of chlorophylls a and b in concentrations of 0.1 mg/m^2 and greater.

2. Summary of method

A periphyton sample is ruptured mechanically, and the chlorophylls are separated from each other and degradation products by high-pressure liquid chromatography and are determined by fluorescence spectroscopy (Shoaf and Lium, 1976, 1977). The difference between the ash weight and dry weight is the organic matter (biomass). The biomass/chlorophyll a ratio is calculated from these values.

3. Interferences

3.1 A substantial quality of sediment may affect the chlorophyll extraction process. Inorganic matter in the sample will cause erroneously large dry and ash weights; nonliving organic matter in the sample will cause erroneously large dry (and thus organic) weights.

3.2 Exposure of the sample to heat, light, or acid can result in photochemical or chemical degradation of the chlorophylls. Large values will result from the presence of fragments of tree leaves or other plant materials that contain chlorophyll. Large populations of photosynthetic bacteria also will result in large values.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Analytical balance, capable of weighing to at least 0.1 mg.

4.2 Artificial substrates made of glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.3 Auto-injector (recommended, but not required).

4.4 Centrifuge.

4.5 Centrifuge tubes, 15 and 50 mL, conical, screwcap, graduated.

4.6 Centrifuge tubes, 50 mL, conical, pennyhead stopper, graduated.

4.7 Collecting devices for the removal of periphyton from natural substrates. Three devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 18.

4.8 Desiccator, containing anhydrous calcium sulfate.

4.9 Drying oven, thermostatically controlled for use at 105 °C.

4.10 Evaporation device.

4.11 Filters, glass fiber, 47-mm diameter, capable of retaining particles having diameters of at least 0.45 µm.

4.12 Filter funnel, nonmetallic, that has vacuum or pressure apparatus.

4.13 Fluorometer, equipped with excitation and emission filters.

4.14 Forceps or tongs.

4.15 Glass bottles, screwcap, smallest appropriate size for the sample.

4.16 Glass funnels.

4.17 Glass pan, smallest appropriate size for scraping substrates.

4.18 Gloves, long-service latex.

4.19 High-pressure liquid chromotograph (HPLC), consisting of a solvent programmer, an isochromatic pump, an oven, and a column. (The column oven needs to be capable of maintaining a constant temperature in the 25 to 35 °C range.)

4.20 High-vacuum pump, capable of providing an absolute pressure of less than 1 torr.

4.21 Muffle furnace, for use at 500 °C.

4.22 Pasteur pipet, disposable.

4.23 Porcelain crucibles.

4.24 Scraping device, razor blades, stiff brushes, spatulas, or glass slides, for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.25 Separatory funnels, 125 mL.

4.26 Solvent-saturation pads, 13.4×22 cm.

4.27 Spectrometer (spectrophotometer; fig. 57) that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.28 Tissue homogenizer, 30-mL homogenizing flasks, and blades.

4.29 Vacuum flasks, stoppers, glass tubing, vacuum tubing, and a sintered glass tube.

4.30 Vacuum desiccator.

4.31 Vacuum oven.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acetone, 90 percent. Add nine volumes of acetone to one volume of distilled water.

5.2 Chlorophyll a stock solution. Transfer 1 mg chlorophyll a to a 100-mL volumetric flask and fill to capacity using 90-percent acetone (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2, 5.3, 5.4, and 5.5 are wrapped with aluminum foil as an added precaution.

5.3 Chlorophyll b stock solution. Transfer 1 mg chlorophyll b to a 100-mL volumetric flask and fill to capacity using 90-percent acetone.

5.4 Chlorophyll standard solution. Mix 25 mL chlorophyll a stock solution with 25 mL chlorophyll b stock solution in a 50-mL centrifuge tube.

5.5 Chlorophyll working standard solutions. Use a 5-mL pipet to prepare the following mixtures.

5.5.1 High standard solution, chlorophylls a and b. Add 5 mL chlorophyll standard solution to 5 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.2 Mid-range standard solution, chlorophylls a and b. Add 3 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.3 Low standard solution, chlorophylls a and b. Add 1 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.6 Distilled or deionized water.

5.7 Diethyl ether, distilled in glass, unpreserved.

5.8 Dimethyl sulfoxide (DMSO).

5.9 Methyl alcohol, 96 percent. Pour 960 mL methyl alcohol, distilled in glass, into a 1-L graduated cylinder. Add distilled water to the mark and mix.

5.10 Nitrogen gas, prepurified.

6. Analysis

6.1 Sample Preparation.

6.1.1 Allow the frozen sample to thaw 2 to 3 minutes at room temperature.

6.1.2 Scrape the periphyton off the substrate into a glass pan.

6.1.3 Use 15 mL DMSO to rinse the solid material into a 30-mL homogenizing flask. Homogenize the sample until the cells have been ruptured.

CAUTION.--Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.1.4 Transfer the sample to a 50-mL graduated centrifuge tube, and rinse the homogenizing flask and blade using 5 mL DMSO. Add the rinse to the centrifuge tube.

6.1.5 Add 20 mL diethyl ether to the centrifuge tube, screw on the cap, and shake vigorously for 10 seconds. Wait 10 seconds and shake another 10 seconds.

6.1.6 Remove the cap and slowly add, almost dropwise, 10 mL distilled water to the centrifuge tube. Secure the cap and shake gently. Vent, then shake for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.7 Centrifuge at 1,000 r/min for 10 minutes.

6.1.8 Transfer the top diethyl ether layer, using a disposable pipet, to a 125-mL separatory funnel. (If the DMSO layer appears green after diethyl ether extraction, repeat 6.1.5 through 6.1.8. There are, however, some green chlorophyll derivatives not extractable using diethyl ether.)

6.1.9 Add 15 mL distilled water to the separatory funnel, and shake vigorously for 10 seconds, venting often. Allow the layers to separate. (Break emulsions by adding 1 to 2 mL acetone and swirling the funnel gently.)

6.1.10 Drain and discard the bottom layer.

6.1.11 Rinse the upper part of the separatory funnel using 2 to 3 mL acetone. Remove the bottom layer that forms in the funnel and discard.

6.1.12 Decant the diethyl ether layer through the top of the separatory funnel into a centrifuge tube. Rinse the funnel using 5 mL diethyl ether, and add the rinse to the centrifuge tube.

6.1.13 Place the centrifuge tube on the evaporation device and evaporate to 0.2 to 0.4 mL using a gentle stream of nitrogen gas.

6.1.14 Add sufficient acetone to the sample extract so the color intensity is between the color intensities of the high and low standard solutions. If the color of the sample extract is not within the specified range after the addition of 20 mL acetone, take a 1-mL aliquot of the 20 mL extract, and dilute volumetrically until the desired color intensity is obtained.

6.2 High-pressure liquid-chromatographic analysis.

6.2.1 Measure the absorbance of the chlorophyll stock solutions using a spectrometer. Measure the absorbance at 664 nm for chlorophyll a and at 647 nm for chlorophyll b. Record the absorbance for three replicates of chlorophylls a and b. Average the three values for chlorophyll a and the three values for chlorophyll b, separately, and record each average separately for subsequent calculations.

6.2.2 Operate the HPLC system using 96-percent methyl alcohol as the mobile phase at a flow of 1.5 mL/min until the pressure stabilizes.

6.2.3 Calibrate the instrument by injecting 10 µL of the mid-range standard solution, and record the peaks of chlorophylls a and b.

6.2.4 Verify that the response of the fluorometer is linear by injecting the high and low standard solutions.

6.2.5 Analyze the sample by injecting 10 µL of the sample extract into the HPLC. Record the peaks of chlorophylls a and b, if any.

6.3 Dry weight and ash weight of organic matter.

6.3.1 Bake a porcelain crucible at 500 °C for 20 minutes. Cool to room temperature in a desiccator. Silica gel is not recommended. Measure the tare weight to the nearest 0.1 mg.

6.3.2 Remove the DMSO supernatant (6.1.8) using a disposable pipet. If biomass particles are visible in the supernatant, centrifuge first and then remove the supernatant. If the supernatant is still murky, filter through a tared glass-fiber filter, burn at 500 °C, and add filter ashes to sediment in crucible.

6.3.3 Quantitatively transfer the sediment to a 30-mL porcelain crucible using a microspoon or microspatula and rinses of distilled water.

6.3.4 Place the crucible in a 105 °C oven overnight to evaporate the water.

6.3.5 Place the crucible in a desiccated (preheated to 105 °C) vacuum oven. Lower the pressure in the oven to approximately 20 torr. Leave the crucible in the oven for 2 hours. Approximately every one-half hour or hour, redraw the vacuum (without reaching atmospheric pressure in the oven) to remove the DMSO fumes from the oven.

6.3.6 Cool crucible in a vacuum desiccator to room temperature.

6.3.7 Weigh crucible to the nearest 1 mg in a desiccated balance.

6.3.8 Reheat crucible in the vacuum oven for 1 hour.

6.3.9 Cool crucible in a vacuum desiccator and weigh. If the weight is not constant, reheat until constant weight within 5 percent is obtained. This value is used to calculate the dry weight.

6.3.10 Place the crucible containing the dried residue in a muffle furnace at 500 °C for 1 hour until a constant dry weight is obtained. This value is used to calculate the ash weight (Note 2).

Note 2: The ash is wetted to reintroduce the water of hydration of the clay and other minerals that, though not evaporated at 105 °C, is lost at 500 °C. This water loss may be as much as 10 percent of the weight lost during ignition and, if not corrected, will be interpreted as organic matter (American Public Health Association and others, 1985).

7. Calculations

7.1 Chlorophyll.

7.1.1 Calculate the exact concentrations of the chlorophyll stock solutions from the equation:

$$C_s = \frac{A}{\alpha b},$$

where C_s = concentration of chlorophyll stock solution, in milligrams per liter;
A = average absorbance obtained in 6.2.1;
b = path length, in centimeters; and
α = specific absorptivity [0.0877 L/mg × cm for chlorophyll *a* and 0.0514 L/mg × cm for chlorophyll *b* (Jeffrey and Humphrey, 1975)].

7.1.2 Verify and correct the concentrations of the chlorophyll working standard solutions in 5.5 by using the chlorophyll stock solutions determined in 7.1.1.

7.1.3 Calculate the response factor for chlorophylls *a* and *b* in the chlorophyll working standard solution:

$$RF = \frac{V \times C_m}{I_s},$$

where RF = response factor of chlorophyll *a*, in milligrams per unit area;
V = volume of mid-range standard solution injected, in milliliters;
C_m = concentration of chlorophyll *a* or *b* in the mid-range standard solution, in milligrams per liter; and
I_s = integrated area of the component peak.

7.1.4 Use the data from 6.2.5 to calculate the concentration of chlorophyll *a* or *b* on the original substrate:

$$\text{Concentration (milligrams per square meter)} = \frac{RF \times IV_e}{A_s \times V_i \times 1{,}000},$$

where RF = response factor of chlorophyll *a* or *b*, in milligrams per unit area;
I = integrated area of the chlorophyll *a* or *b* peak in the sample as determined in 6.2.5;
V_e = final volume of the sample extract from 6.1.14, in milliliters;
A_s = area of substrate, in square meters; and
V_i = volume of sample extract injected in 6.2.5, in microliters.

7.2 Biomass.

$$\text{Organic weight (milligrams per square meter)} = \frac{\text{Dry weight (milligrams)} - \text{ash weight (milligrams)}}{\text{Area of scraped surface (square meters)}}.$$

7.3 $$\text{Ratio} = \frac{\text{Biomass (milligrams per square meter)}}{\text{Chlorophyll } \underline{a} \text{ or } \underline{b} \text{ (milligrams per square meter)}} .$$

8. Reporting of results

8.1 Report concentrations of chlorophylls _a_ and _b_ as follows: less than 1 mg/m^2, one decimal; 1 mg/m^2 and greater, two significant figures.

8.2 Report biomass as follows: less than 1 mg/m^2, one decimal; 1 mg/m^2 and greater, two significant figures.

8.3 Report ratio to three significant figures.

9. Precision

No precision data are available.

10. References cited

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Adenosine Triphosphate (ATP)
(B-6700-85)

Parameter and Code:
Adenosine triphosphate (µg/L): 70998

Very sensitive methods of adenosine triphosphate (ATP) analysis have been developed because of McElroy's (1947) discovery that luminescence in fireflies has an absolute requirement for ATP. ATP is determined by measuring the intensity of light produced when ATP reacts with reduced luciferin (LH_2) and oxygen (O_2) in the presence of firefly luciferase and magnesium (Mg^{+2}), producing adenosine monophosphate (AMP), inorganic pyrophosphate (PPi), oxidized luciferin (L), water (H_2O), carbon dioxide (CO_2), and light (hv). The following equation shows this reaction:

$$ATP + LH_2 + O_2 \xrightarrow[Mg^{+2}]{\text{luciferase}} AMP + PPi + L + H_2O + CO_2 + hv.$$

The bioluminescent reaction is specific for ATP. The reaction rate is proportional to the ATP concentration, and 1 photon of light is emitted for each molecule of ATP hydrolyzed. When ATP is mixed with suitably buffered enzyme and substrates, a light flash follows that decays in an exponential fashion. Either the peak height of the light flash or the integration of the area under the decay curve can be used to prepare standard curves.

The sample-collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, phytoplankton abundance may vary transversely, with depth, and with time of day. To collect a sample representative of the phytoplankton concentration at a particular depth, use a water-sampling bottle. To collect a sample representative of the entire flow of a stream, use a depth-integrated sampler (Guy and Norman, 1970; Goerlitz and Brown, 1972). For small streams, a depth-integrated sample or a point sample at a single transverse position at the centroid of flow is adequate. Study design, collection, and statistics for streams, rivers, and lakes are described in Federal Working Group on Pest Management (1974).

The analysis section (6.1 through 6.16) in the method that follows describes the extraction of ATP from the living material (algae, bacteria, or fungi) in the sample. These extraction procedures ideally should be done immediately after collection. The sample may be stored 2 to 3 hours if necessary and if the temperature and lighting conditions are maintained; for example, do not put a warm sample from a well-lighted area into a cool, dark ice chest.

1. Applications

The method is suitable for all water.

2. Summary of method

A water sample is filtered, and the ATP is extracted from the living material. The extract from the living material (containing the ATP) is injected into a suitable buffered luciferin-luciferase enzyme solution. The intensity of light produced by the subsequent reaction is measured using an ATP photometer. The reaction rate is proportional to the ATP concentration, and 1 photon of light is emitted for each molecule of ATP hydrolyzed.

3. Interferences

In general, several metals (for example, mercury) and a large concentration of salts will inhibit the reaction; therefore, washing the filter using buffered distilled water, immediately after filtration to remove most of the dissolved salts is advisable. A substantial quantity of sediment may affect the extraction process.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Balance, analytical.

4.2 Constant-rate injector.

4.3 Cuvettes, 6×49 mm, quartz, 1-cm light-path length.

4.4 Cuvette caps.

4.5 Cuvette holder.

4.6 Distillation apparatus, glass.

4.7 Filter assemblies, 13-mm diameter, 0.45-μm mean pore size, self-supported filters (Note 1).

Note 1: These filters are resistant to the extracting agent, dimethyl sulfoxide.

4.8 Glass storage bottles, approximately 150-mL capacity, and autoclavable screwcaps.

4.9 Glass vials, approximately 15-mL capacity, and screwcaps, 22×85 mm.

4.10 Gloves, long-service latex.

4.11 Photometer, Chem-Glow photometer and integrator, ATP photometer, or luminescence biometer.

4.12 Pipet, 0.1, 0.2, and 1 mL that has disposable tips.

4.13 Sterilizer, horizontal steam autoclave or vertical steam autoclave.

4.14 Syringe, 50 µL, blunt-tipped (nonbeveled).

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.15 Tubes, graduated 12- or 15-mL centrifuge.

4.16 Vacuum-filter stand.

4.17 Vacuum pump, to provide at least 250 mm of mercury.

4.18 Volumetric flasks, 100-mL and 1-L sizes.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

All reagents are prepared using only freshly distilled water, which has an ATP value not greater than 0.1 µg/L.

5.1 Adenosine-5-triphosphate solutions, 1, 2.5, 10, 25, and 100 µg ATP per liter. Do the following steps rapidly because ATP is an unstable biochemical: Dissolve 119.3 mg $Na_2ATP \cdot 3H_2O$ (equivalent to 100 mg ATP) in 100 mL ATP diluent. Make two serial dilutions of 1:100 using the ATP diluent. Mix well between dilutions. The result is a 100-µg/L solution of ATP. Make 1:4, 1:10, 1:40, and 1:100 dilutions of the 100-µg/L solution using the ATP diluent to make ATP solutions of 25, 10, 2.5, and 1 µg/L concentrations. Pour small aliquots (approximately 100 µL) of the 1-, 2.5-, 10-, 25-, and 100-µg/L solutions into the cuvettes and cap using the cuvette caps. Quickfreeze the cuvettes immediately by immersing in a bath of acetone and dry ice; store at -20 °C or less.

5.2 ATP diluent. Dissolve 1.045 g morpholinopropane sulfonic acid (MOPS); 0.372 g ethylenediaminetetraacetic acid, disodium salt, dihydrate ($Na_2EDTA \cdot 2H_2O$); and 1.2 g magnesium sulfate ($MgSO_4$) in approximately 900 mL distilled water. Adjust the pH to 7.7 using sodium hydroxide and increase the final volume to 1 L using distilled water. If not used immediately, the solution should be autoclaved to prevent growth of micro-organisms and, thus, the production of ATP.

5.3 Distilled water.

5.4 Hydrochloric acid solution, 0.2N. Dilute 16.7 mL concentrated hydrochloric acid (HCl) to 1 L using distilled water.

5.5 Luciferin-luciferase buffer solution. The kit must be stored frozen at -20 °C or less. For daily use, dissolve one buffer-salt (MOPS and $MgSO_4$ at pH 7.4) tablet in 3 mL distilled water. Add the vial containing the lyophilized enzyme-substrate (luciferin-luciferase) powder to the buffer solution. Mix gently but completely. Do not allow the formation of bubbles because this may result in enzyme (luciferase) denaturation. Wait at least 15 minutes before using. Fresh solution must be prepared before each use, but it may be left at room temperature (20-24 °C) during the day. One tablet of buffer salt and one vial of enzyme-substrate powder provide enough solution for approximately 30 cuvettes.

5.6 Morpholinopropane sulfonic acid (MOPS) solution, 0.01M. Dissolve 2.09 g MOPS in approximately 900 mL distilled water. Adjust pH to 7.4 using sodium hydroxide. Increase final volume to 1 L using distilled water. Pour approximately 100 mL each into 150-mL glass bottles, cap loosely, and autoclave. After cooling, cap tightly and store at room temperature.

5.7 Dimethyl sulfoxide (DMSO) solution. Add nine volumes of DMSO to one volume 0.01M MOPS solution that was prepared in step 5.6. Mix well. Prepare fresh before each use.

CAUTION.--Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6. Analysis

6.1 Shake water sample and remove 25 mL. If sample obviously contains abundant living material (for example, algae, bacteria, or fungi), this aliquot may be decreased to a volume as small as 10 mL. Record the final volume.

6.2 Pour the sample aliquot into the filter assembly containing the membrane filter, which has a graduated centrifuge tube in place and a vacuum pump attached.

6.3 Apply a vacuum no greater than 250 mm mercury.

6.4 Release vacuum immediately when filtration is almost complete so sample does not dry.

6.5 Quickly add 5 mL distilled water and filter again, this time to dryness. Release vacuum immediately.

6.6 Replace graduated centrifuge tube with a clean and dry centrifuge tube.

6.7 Pipet 0.2 mL DMSO onto sample in filter assembly and distribute evenly by rotation of filter assembly. If the 0.2 mL does not cover the sample, it may be doubled; if so, the 1 mL volume in 6.10 also should be doubled to 2 mL. Record the change so that corrections for dilutions can be made.

6.8 Wait at least 20 seconds (not more than 30).

6.9 Apply vacuum until surface is dry.

6.10 Add 1 mL of MOPS solution.

6.11 Wait 10 seconds.

6.12 Apply vacuum until surface is dry.

6.13 Repeat 6.10 through 6.12.

6.14 Record final volume; this value should be 2.2 mL.

6.15 Mix contents of centrifuge tube.

6.16 Pour contents of the centrifuge tube into small screwcap vial (approximately 15-mL volume), and quickfreeze by immersing the bottom part in an acetone and dry-ice bath. The sample must be frozen until analyzed. Storage should not exceed 30 days.

6.17 Pipet 100 µL luciferin-luciferase solution into the cuvettes.

6.18 Rinse the syringe three times using 0.2N hydrochloric acid by drawing acid into the entire 50-µL length of the syringe; rinse three times using MOPS solution to neutralize any remaining acid; rinse three times using distilled water.

6.19 Thaw the ATP solutions at room temperature and mix well.

6.20 Test the photometer for response to the luciferin-luciferase solution (background luminescence) and 10 µL of the five ATP solutions. Follow specific instructions for the photometer used. This procedure prepares a standard curve and is linear for this analysis.

6.21 Rinse syringe as in 6.18.

6.22 Place cuvette in photometer.

6.23 Thaw sample prepared in 6.1 through 6.16 at room temperature for analysis. Mix well.

6.24 Rinse syringe three times using the sample.

6.25 Inject 10 µL sample into the cuvette, and record response. Analyze in duplicate.

6.26 If response is too great for photometer, the sample may be diluted. Dilutions using distilled water are linear.

7. Calculations

7.1 Prepare a standard curve from the five ATP solutions. The standard curve is linear and has a slope of 1. Compute the concentration of ATP in the injected sample in micrograms ATP per liter of sample.

7.2 This ATP value is corrected for the concentration step onsite using the following equation:

$$\text{Original sample (micrograms ATP per liter)} = \frac{\text{Micrograms ATP measured per liter}}{\text{Volume of sample filtered (liters)}} \times \frac{\text{Dilution}}{\text{Volume recovered after extraction (liters)}} .$$

If undiluted, the value for dilution equals 1; the volume recovered after extraction commonly is 2.2×10^{-3} L.

8. Reporting of results

Report ATP to the nearest 0.1 µg/L.

9. Precision

Reproducibility of analysis is approximately ±2 percent (single analyst).

10. References cited

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PRIMARY PRODUCTIVITY (Production Rate)

Introduction

Bodies of water differ greatly in their populations of plants and animals, and these differences may be used in the interpretation of water quality. Biological differences may be expressed qualitatively and quantitatively. For many purposes, however, the factor of greatest interest is the rate at which new organic matter is formed and accumulated in the system being studied. Organic matter can be produced by photosynthesis and chemosynthesis. In most environments, chemosynthesis is not an important component of primary productivity. Through photosynthesis, organic compounds are synthesized from water (H_2O) and carbon dioxide (CO_2) using energy absorbed from sunlight by chlorophyll. Light energy is used to convert carbon dioxide to reduced carbon compounds. This process can be summarized by

$$6\ CO_2 + 6\ H_2O + \text{light} \longrightarrow C_6H_{12}O_6 + 6\ O_2\ .$$

This implies that primary productivity could be determined by measuring any of the following parameters: (1) Uptake of carbon dioxide, (2) production of oxygen (O_2), or (3) increases in pH. In addition, changes in biomass or nutrient concentrations per unit time also can be a measure of primary productivity.

The underlying assumptions in the following methods are that the change in oxygen and dissolved carbon concentrations is a result of photosynthesis and respiration. As described in the preceding paragraph, photosynthesis involves uptake of carbon dioxide and production of oxygen. Respiration is the reverse of this process.

Two general approaches are described for the estimation of primary productivity. In the first, the organisms are isolated in suitable containers, and the production and respiration rates are estimated from changes in the dissolved-oxygen concentration or from changes in carbon dioxide concentration as measured by uptake of radioactive carbon [carbon 14 (^{14}C)]. If the rate of primary production is sufficient for accurate measurements to be made within 24 hours, the oxygen method is preferred. Vollenweider (1974) indicates that the oxygen method is impractical when there is less than a 7-mg (O_2/m^3)/hour photosynthetic rate for a 3-hour exposure. Alternatively, if the chlorophyll concentration is less than 1 mg/m^2, the oxygen method should not be used. Therefore, the ^{14}C method, which is of greater sensitivity, is preferred for use in oligotrophic (low-productivity) water. In the second approach, production and respiration rates for nonisolated natural communities are estimated from changes in the dissolved-oxygen concentration of the open water.

The metabolism of aquatic plants and animals may result in changes in the concentrations of dissolved substances in the environment. The diel (24-hour) rise and fall of dissolved oxygen or carbon dioxide has been used to determine the productivity of biological communities in streams (Odum, 1956, 1957; Hoskin, 1959; Edwards and Owens, 1962; Gunnerson and Bailey, 1963; Edwards, 1965; O'Connell and Thomas, 1965; Wright and Mills, 1967; Hornberger and Kelly, 1972, 1974) and in standing water (Talling, 1957; Odum and Hoskin, 1958; Park and others, 1958; Odum, 1959; Verduin, 1960; Odum and Wilson, 1962;

Lyford and Phinney, 1968; Welch, 1968; Eley, 1970; Cory, 1974; Hornberger and Kelly, 1974). The following methods use oxygen changes because of the ease with which they can be determined, but the principles are applicable as well to changes in total carbon dioxide (Vollenweider, 1974; Hall and Moll, 1975).

In the first approach, diel changes in the in-situ concentration of dissolved oxygen caused mainly by photosynthesis and respiration are used to estimate the primary productivity of the entire aquatic plant community. The advantages of this method are: (1) Unnatural effects of enclosures are eliminated, (2) phytoplankton and attached plants are included, and (3) observations can be of long duration or can be adapted for continuous monitoring. The disadvantages of the method are: (1) Limited sensitivity; (2) the unknown effects of transient conditions between sampling intervals; (3) the exchange of oxygen between the air and the water requiring calculation or measurement; and (4) in the graphical analysis, the necessity of assuming that the respiration rate is the same during the night as during the day. In standing water, unmeasured horizontal exchange (advection) may cause errors.

Changes in the dissolved-oxygen concentration in a reach of stream or in a standing body of water are results of photosynthesis, respiration, diffusion, and inflowing surface and ground water. If how these factors affect the oxygen concentration in the study area is known, a dissolved-oxygen curve can be drawn, and the primary productivity can be determined. The equation for the oxygen curve (Odum, 1956; Owens, 1965) is

$$\underline{Q} = \underline{P} - \underline{R} + \underline{D} + \underline{A} \ , \qquad (1)$$

where $\underline{Q}$ = rate of change (gain or loss) of dissolved oxygen per unit area;
$\underline{P}$ = rate of gross primary production per unit area;
$\underline{R}$ = rate of oxygen use (respiration) per unit area;
$\underline{D}$ = rate of oxygen uptake or loss by diffusion per unit area, depending on whether the water is undersaturated or oversaturated with oxygen when compared to the air; and
$\underline{A}$ = rate of supply of oxygen from drainage accrual.

If possible, select an area for study in which accrual has a negligible effect on the dissolved-oxygen concentration when compared with the other components.

The rate per unit area of the diffusion of oxygen into or out of the water, $\underline{D}$, is the product of the gas-transfer coefficient, $\underline{K}$, and the percentage-saturation deficit of oxygen between the water and air, $\underline{S}$, or

$$\underline{D} = \underline{K} \frac{\underline{S}}{100} \ , \qquad (2)$$

where $\underline{D}$ and $\underline{K}$ are in grams per square meter per hour. If equations 1 and 2 are divided by the depth, $\underline{z}$, in meters, then the terms are expressed as volume, or grams per cubic meter per hour. Conventionally, capital letters are used for quantities defined on an areal basis and lowercase letters are used for quantities defined volumetrically (Odum, 1956). Thus, $\underline{k}$ is the gas-transfer coefficient, in grams per cubic meter per hour.

Various equations for obtaining K and D, as well as example values, are described in Odum (1956), Odum and Hoskin (1958), Churchill and others (1962), Odum and Wilson (1962), and Owens and others (1964). Procedures for measuring and predicting the reaeration coefficient of open-channel flows are evaluated by Bennett and Rathbun (1972).

In the methods described in this section, the diffusion rate either is obtained directly by the plastic-dome technique (Copeland and Duffer, 1964) or is calculated from measurements of hydraulic (mean flow) parameters (Churchill and others, 1962). The determination of K and D during the study period by one of these methods is preferable, but if that is not possible, a value for K may be estimated from the following data (Odum and Hoskin, 1958, p. 20):

Water type	Gas-transfer coefficient, K (grams per square meter per hour at 0-percent saturation)
1. Quiet water less than 0.5 m deep or shallowly stratified	0.1-1
2. Bay and lakes that have gentle circulation and small waves	1-3
3. Rivers, streams, and open tidal water that have strong circulation and large waves	$\geq$3

The presence of sewage and surfactants in the water tends to decrease the K value when compared with the pure-water K value; whereas, winds tend to increase the K value when compared with the quiescent air K value (Bennett and Rathbun, 1972, p. 56-58).

A possible source of error when estimating gross primary productivity from changes in dissolved-oxygen concentration is the loss of oxygen to the atmosphere in the form of bubbles. Losses of 1 to 6.5 percent of the total oxygen production have been reported (Odum, 1957; Edwards and Owens, 1962). Although the rate of gas loss may be slow for many environments, estimates can be made of the quantity of oxygen produced during photosynthesis that is lost in this way (Owens, 1965).

The procedures for graphical analysis of the diel oxygen curve are described for streams (single-station and upstream-downstream methods) and for stratified water.

Collection

For oxygen light- and dark-bottle and ^{14}C methods, determine the depth of the euphotic zone (the region that receives 1 percent or more of the surface light) using an irradiance meter or submarine photometer. Quantum radiometers also are used for measurement of photosynthetically active radiation (Fee,

1976). If no other method is available, an estimate of the bottom limit of the euphotic zone is obtained by multiplying the Secchi disk depth by 2 (Dillon and Rigler, 1974; Vollenweider, 1974). Select sampling depths equivalent to 100-, 50-, 25-, 10-, 3-, and 1-percent light-penetration depths using the following equation:

$$\text{Depth at } (\underline{x})\text{-percent light} = \frac{\underline{\ln}(100/\underline{x})}{\underline{K}} ,$$

where, for example, depth at 25-percent light = $\underline{\ln}(100/25)/\underline{K}$; and $\underline{K}$ = extinction coefficient (Vollenweider, 1974) and is determined by

$$\underline{K} = \frac{\underline{\ln}(\underline{Is}/\underline{Iz})}{\underline{z}} ,$$

where $\underline{Is}$ = irradiance at the surface;
$\underline{Iz}$ = irradiance at depth, $\underline{z}$; and
$\underline{z}$ = photometer depth.

In-situ incubations for oxygen and ^{14}C should be no longer than 4 hours, and the incubation period should be at midday (1000-1400 hours). For further details, refer to Shindler and Holmgren (1971) or Hall and Moll (1975).

If a 4-hour incubation is too short to measure oxygen changes, then ^{14}C should be used. In studies where more than one site must be sampled in 1 day, an on-board incubation technique can be used for the ^{14}C method (Fee, 1973a and b, 1976). A similar technique for multistation investigations of primary productivity using the oxygen light- and dark-bottle method is described by Megard (1972).

Collect a water sample, using an opaque, nonmetallic sampler, from each preselected depth. The sample volume should be sufficient to rinse and fill three incubation [biochemical oxygen demand (BOD)] bottles and a sample bottle for determination of alkalinity. After collection, all samples should be kept in the dark at sample water temperature during the following procedures to avoid light injury to the organisms. Samples preferably should be collected in early morning. This procedure allows for measurements of light penetration and water sampling during daylight and for an incubation period from 1000 to 1400 hours (Schindler and Holmgren, 1971).

Oxygen Light- and Dark-Bottle Method for Phytoplankton

Transfer the water sample collected from each depth to an 8-L polyethylene bottle, and let it stand for 15 to 30 minutes (but not more than 1 or 2 hours) at a temperature slightly higher than the in-situ water temperature. Shake the bottles occasionally to eliminate oxygen supersaturation. Supersaturation is most likely to occur in extremely productive water or in samples that have warmed several degrees.

For each depth sampled, fill four light and two dark BOD bottles by letting the well-mixed sample flow gently through a rubber tube inserted to the bottom of the bottle. Allow the water to overflow for about three bottle volumes and slowly withdraw the filling tube while the water still is flowing into the bottle. Immediately stopper the bottle, taking care to avoid entrapment of bubbles. All bottles from each depth must have the same initial dissolved-oxygen concentration. This requirement can be met during filling by adding successive increments of sample to each of the bottles in rotation until all are filled and flushed about three times. Place all bottles in a dark storage box until used.

The sequence of the following two steps may be altered as required. The determination of the initial dissolved-oxygen concentration should be started as soon as incubation begins.

Immediately add the reagents for the azide modification of the Winkler method to two light BOD bottles from each depth. These samples, designated IB, are used for determination of the initial dissolved-oxygen concentration. Titration may be delayed several hours, if necessary, if the samples are kept cool and dark.

Secure the stoppers in the BOD bottles that are to be incubated. The method of securing may be part of the suspension system, or stainless-steel or aluminum wire may be wound around the neck of the bottle and looped over the stopper. Do not use copper wire. Cover the stopper and neck of the dark bottles with several layers of aluminum foil. Attach pairs of light and dark bottles to a bottle holder attached to a wire cable (fig. 59). Lower the holders to the depth corresponding to the original sample depth. The wire cable can be attached to a surface float or suspended from a supporting arm attached to a pier or similar structure. Care must be taken not to shade the bottles with opaque floats or nearby structures. Begin the incubation, and prepare any remaining IB samples for dissolved-oxygen determination. At the end of the incubation period, raise the bottles and place them in a darkened box.

Carbon-14 Method for Phytoplankton

Transfer the contents of ^{14}C bicarbonate stock ampoules to a 50-mL Erlenmeyer dispensing flask (see e in Analytical Problems in the "Supplemental Information" section for alternative method). Remove an ampoule of radioactive solution from storage. Carefully snap the ampoule neck. Using a clean, dry pipet, or syringe, that has a 7.5- or 10-cm needle, transfer the ^{14}C bicarbonate to the dispensing flask. The volume of ^{14}C bicarbonate in the dispensing flask should be sufficient to inoculate all BOD bottles and three inoculant standards. Swirl the contents to provide a homogeneous bicarbonate solution. Shake the sample thoroughly. Rinse each BOD bottle using a small volume of sample water.

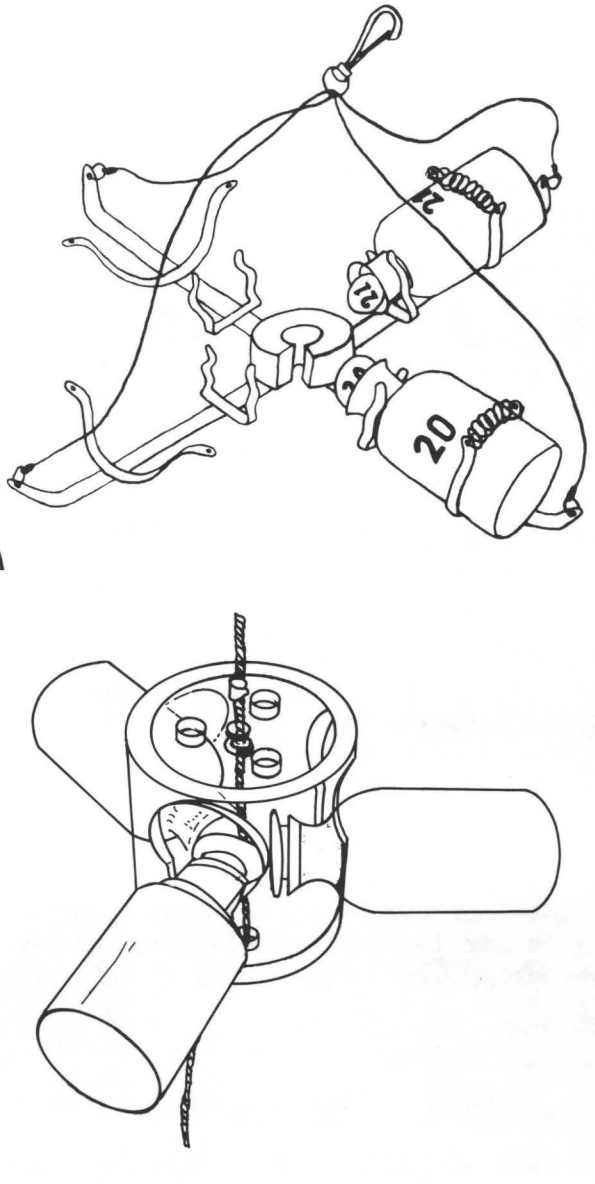

Figure 59.--Devices for holding light and dark bottles in a horizontal position: (A) Metal suspension frame (modified from Saunders and others, 1962); (B) polyethylene bottle holder (sketch based on photograph courtesy of Schindler and Holmgren, 1971).

Shake the sample thoroughly again. Fill one dark and two light BOD bottles with water from the sample depth. Also collect a sample for alkalinity determination from each depth. Place the light and dark BOD bottles in a plastic tray to confine possible spills and to minimize the potential for radioactive contamination of the working area. Alkalinity bottles that contain sample water should be capped and stored until analyzed in the lab-

oratory. Alkalinity determinations for the available carbon-12 (^{12}C) value used in primary productivity calculations are limited. Stainton (1973) describes the use of IR or gas-chromatographic techniques, especially for water that has small carbonate concentrations.

Inoculate each BOD bottle using ^{14}C bicarbonate, solution. The radioactivity of the sample after incubation is dependent on standing stock of the phytoplankton, growth rate, length of incubation, and volume of sample counted. Initially, the radioactivity of the sample should be increased by adding about 3 μCi ^{14}C bicarbonate per 100 mL of sample. With experience, one can decrease the strength of the inoculant so the resultant radioactivity is sufficiently high, but the natural alkalinity of the sample has not been altered unnecessarily.

Using a 1-mL precision volumetric pipet, dispense a 1-mL aliquot of ^{14}C bicarbonate inoculant into each light and dark BOD bottle. The tip of the pipet should be inserted well into the bottle. As the inoculant is added, the pipet tip is withdrawn from the bottle. Following inoculation, cap and shake each bottle well. Place the bottles in a darkened box until incubation begins. Cover the cap and neck of each dark bottle with black electrical tape.

The concentration of ^{14}C bicarbonate inoculant must be checked by preparing standards onsite. Using the precision volumetric pipet, dispense a 1-mL aliquot of ^{14}C bicarbonate inoculant into a clean volumetric flask, and dilute to 100 mL using distilled water. Transfer 0.1 mL of the diluted ^{14}C bicarbonate inoculant into each of three vials. Add 1 mL of liquid scintillation-grade phenethylamine to each vial of ^{14}C bicarbonate standard. Cap, shake well, and let stand for 5 minutes. To each vial of standard, add 10 mL Aquasol^{R} scintillation cocktail.

When all BOD bottles are ready for incubation, place one dark and two light bottles from each sampling depth into a bottle holder attached to a wire cable (fig. 59). Lower the holder to a depth corresponding to the original sample depth. The wire cable can be attached to a surface float or suspended from a supporting arm attached to a pier or similar structure. Care must be taken not to shade the bottles with opaque floats or nearby structures. At the end of the incubation period, raise the bottles and place them in a darkened box.

Oxygen Light- and Dark-Enclosure Method for Periphyton

Samples for periphyton primary-productivity determinations may be obtained either from natural or from artificial substrates. The best results will be from direct in-situ measurements of undisturbed periphyton.

Periphyton measurement sites should be selected on the basis of study objectives. If successive measurements are needed to determine primary-productivity changes with time for a selected reach of stream, each measurement must represent the same habitat. Similarly, if measurements are needed to compare periphyton among different reaches or different streams, the measurements must represent comparable habitats. Factors, such as water depth,

current speed, degree of sedimentation or erosion, and exposure to sunlight, must be similar if meaningful comparisons are to be made. The same attention to habitat applies to lake environments for which depth, sediment type, and presence of macrophyte beds are significant factors in site selection. The proximity of each measurement site to outfalls, marinas, bridges, or other effects of man must be considered.

Measurements of primary productivity of stream periphyton in static cultures may provide useful comparative values but undoubtedly are too small in absolute terms because of suppression of photosynthesis in the absence of current (Wetzel, 1964; Bombowna, 1972; Rodgers and others, 1978). To correct for the lack of current, methods have been developed for measuring primary productivity in plastic chambers in which water is circulated using a pump (McIntire and others, 1964; Thomas and O'Connell, 1966; Hansmann and others, 1971; Bombowna, 1972; Pfeifer and McDiffett, 1975; Rodgers and others, 1978; Gregory, 1980).

Circulating chambers are not available commercially; as a result, designs have varied. Three recent designs are shown in Gregory (1980), and Rodgers and others (1978), based on McIntire and others (1964). Some chambers have been miniaturized and use battery-operated pumps. The small size is convenient particularly in remote areas, but it has the disadvantage of collecting small samples; and the small pool volume may result in rapid oxygen supersaturation and nutrient depletion in water in the chamber. Large chambers that have large pool size are much more effective. The chambers made of Plexiglas are expensive to build and bulky to move. Because the most reliable pumps require line voltage, a generator usually is required. Because the chambers are submerged for temperature control, care is required when handling them because of the electrical hazard. Despite the many problems, the chamber (flowing enclosure) is a reliable method for obtaining estimates of primary productivity of periphyton.

Natural Substrates

Rocks or other substrate material of suitable size may be placed into circulating chambers, or the chambers may be constructed to enclose an undisturbed area of periphyton-covered substrate. If the periphyton is moved from its original depth, keep the samples in subdued light to avoid light injury.

Using a nonmetallic water-sampling bottle, collect a water sample from the same depth from which the periphyton was collected. The volume should be sufficient to rinse and fill all the circulating chambers and to determine the initial dissolved-oxygen concentration. For light-bottle and dark-bottle studies, samples preferably should be collected in the morning. This procedure allows for a 4-hour incubation period (Schindler and others, 1973).

Filter the required volume of water, and allow the filtrate to stand at a temperature slightly higher than the in-situ water temperature for 15 to 30 minutes. Shake the flask occasionally to eliminate oxygen supersaturation.

Enclose a known area of substrate containing living periphyton in a light and a dark circulating chamber containing a known volume of freshly filtered water. Fill the chambers and at least one BOD bottle so the chambers and the bottle(s) all have identical dissolved-oxygen concentrations. This requirement can be met during filling by adding successive increments of sample to each container in rotation until all are filled and flushed about three times. Keep all containers in the dark until used. Prevent entrapment of bubbles.

Place circulating chambers at the original depth from which the periphyton was collected, and incubate the samples for about 4 hours. In extremely productive water, where oxygen supersaturation is likely, an incubation period of 1 to 3 hours during midday may be sufficient.

Prepare the BOD bottle sample(s) for determination of the initial dissolved-oxygen concentration by using the methods of Skougstad and others (1979) or the American Public Health Association and others (1985). Titration may be delayed for several hours, if necessary, if the samples are kept cool and in the dark.

Diel Oxygen-Curve Method for Estimating Primary Productivity

The sample-collection method for estimating stream primary productivity will be determined by the type of environment being studied. In general, the objective is to determine the concentration of dissolved oxygen that is representative of the study area for each sampling interval. In well-mixed water, one or two determinations for each sampling period may be representative of the entire water mass. Even in well-mixed streams, the investigator must watch for spatial changes in dissolved-oxygen concentration. A consistent increase in dissolved oxygen toward the banks, when compared to the center of several rivers, was reported by Churchill and others (1962), and the effects of incompletely mixed tributary inflows can persist far downstream. Macrophytes frequently are distributed unevenly, which results in nonuniformity of water chemistry.

Sampling procedures are described for two types of stream conditions and for three methods of determining the diffusion rate, D. If the incoming water has metabolic characteristics similar to the outflowing water, follow the procedure for the single-station analysis. If the metabolic characteristics of the inflowing water are unknown or are not similar to the outflowing water, follow the procedure for the two-station analysis. Additional discussions of these methods are reported in Vollenweider (1974, p. 110-126) and Hall and Moll (1975).

Single-station analysis

Select a representative reach of stream in which surface- and groundwater accrual are negligible and in which similar conditions exist upstream. In such a stream, a second station would have a diel oxygen curve identical with that of the first station (Odum, 1956). Determine the cross-sectional mean velocity and the mean depth of flow to obtain stream discharge (Buchanan and Somers, 1969). Sufficient measurements must be made to determine the mean stream discharge for the 24-hour observation period.

Determine the dissolved-oxygen concentration, in milligrams per liter, and the temperature of the streamflow continuously, or at 1-, 2-, or 3-hour intervals for at least 24 hours. Make measurements at or near sunrise and sunset. Determine the barometric pressure.

If the Winkler method is used for dissolved-oxygen determination, collect duplicate or triplicate samples at each sampling time, and average the results from replicate samples. Collect the samples using a threefold-displacement sampler or using a water-sampling bottle to protect the water from contact with the air. If a water-sampling bottle is used, fill one or more BOD bottles by letting the sample flow gently through a rubber tube inserted into the bottom of the BOD bottle. Allow the water to overflow for about three bottle volumes, and slowly withdraw the filling tube while the water is still flowing into the bottle. Immediately stopper the BOD bottles, taking care not to entrap bubbles. Add the reagents for the azide modification of the Winkler method. Titration may be delayed several hours, if necessary, if the samples are kept cool and in the dark. Measure water temperature to ±0.5 °C at each sample time and location.

For small streams, a single sample at the centroid of flow may be adequate. For large streams, samples may be required from several verticals at centroids of equal flow (Guy and Norman, 1970; Goerlitz and Brown, 1972).

If an oxygen meter is used, determine the dissolved-oxygen concentration at the sampling times and locations described in the preceding paragraphs. When using a portable recording system, place the temperature sensor and electrode at the centroid of flow, and ensure that sufficient water current is maintained past the membrane of the oxygen electrode. For stream velocities less than 0.6 m/s at the electrode, increase flow to the membrane surface using a submersible stirrer. Many oxygen electrodes are photosensitive, and the membrane-covered surface needs to be protected from bright light during calibration and use. Determine the diffusion rate, $\underline{D}$, by one of the methods described in the "Diffusion Rate" section.

Two-station analysis

Select an upstream and a downstream station on a representative reach of stream in which surface- and ground-water accrual are negligible. Determine the cross-sectional mean velocity and the mean depth of flow to obtain stream discharge (Buchanan and Somers, 1969). Sufficient measurements must be made to determine the mean stream discharge for the 24-hour observation period. Measure the surface area, in square meters, and the mean depth, in meters, for the reach between the stations, and determine the average time required for water to travel between the stations. If the flow rate of the stream cannot be determined directly, it can be estimated from the time required for a spot of dye to pass from the upstream station to the downstream station and from the mean cross-sectional area of the reach.

Determine the dissolved-oxygen concentration, in milligrams per liter, and the water temperature at each station as described in the "Single-Station Analysis" section. Determine the diffusion rate, $\underline{D}$, by one of the methods described in the following section.

Diffusion rate

Determination of the rate at which oxygen enters or leaves the water when the concentration is not in equilibrium with the air is a critical step in the use of the oxygen-curve method for water. The rate at which oxygen diffuses in or out of the water increases as the degree of undersaturation or oversaturation increases. Moreover, in controlled streams that have open water or variable discharge, different gas-transfer coefficients, K, may need to be used at different times of day to explain changes in flow or in wind speed and direction (Odum and Wilson, 1962). The correction for wind does not need to be used for relatively protected areas.

Any of the following methods can be used for determining D. For the two-station analysis, D should be representative of the reach between the stations.

Hydraulic-parameter method.--A detailed study of reaeration of rivers downstream from Tennessee Valley Authority reservoirs indicated that water depth and velocity were the most important factors affecting K (Churchill and others, 1962). To calculate K and D, values are required for the cross-sectional mean velocity, the mean depth of flow, the water temperature, and the dissolved-oxygen concentration and percentage saturation continuously or at 1-, 2-, or 3-hour intervals for at least 24 hours. The measurements for these determinations are described in the "Single-Station Analysis" section.

Floating-diffusion-dome method.--D is determined directly by measuring changes in the concentration of oxygen in a plastic dome filled with air and floating on the water surface (Copeland and Duffer, 1964) (fig. 60). The changes in oxygen inside the dome with time are attributed to diffusion. Measurements of oxygen inside the dome are made at night to avoid errors resulting from greenhouse effects and to eliminate photosynthetic oxygen production.

Fill the dome with fresh air and float it on the water surface. Record the volume of air in the dome, the area of the dome in contact with the water, and the time of the initial measurements. At intervals of 2 to 5 hours during the night, measure the temperature and the fraction (percentage) of oxygen inside the dome using an oxygen meter capable of measuring gaseous oxygen. Record as in table 14. Simultaneously measure the dissolved-oxygen concentration and water temperature as described in the "Single-Station Analysis" section.

For lakes, the objectives of sampling are to determine the diel changes in the average concentration and percentage saturation of dissolved oxygen in the euphotic zone and the oxygen demand in the benthic zone. Total community metabolism of the water body then may be estimated on an areal basis.

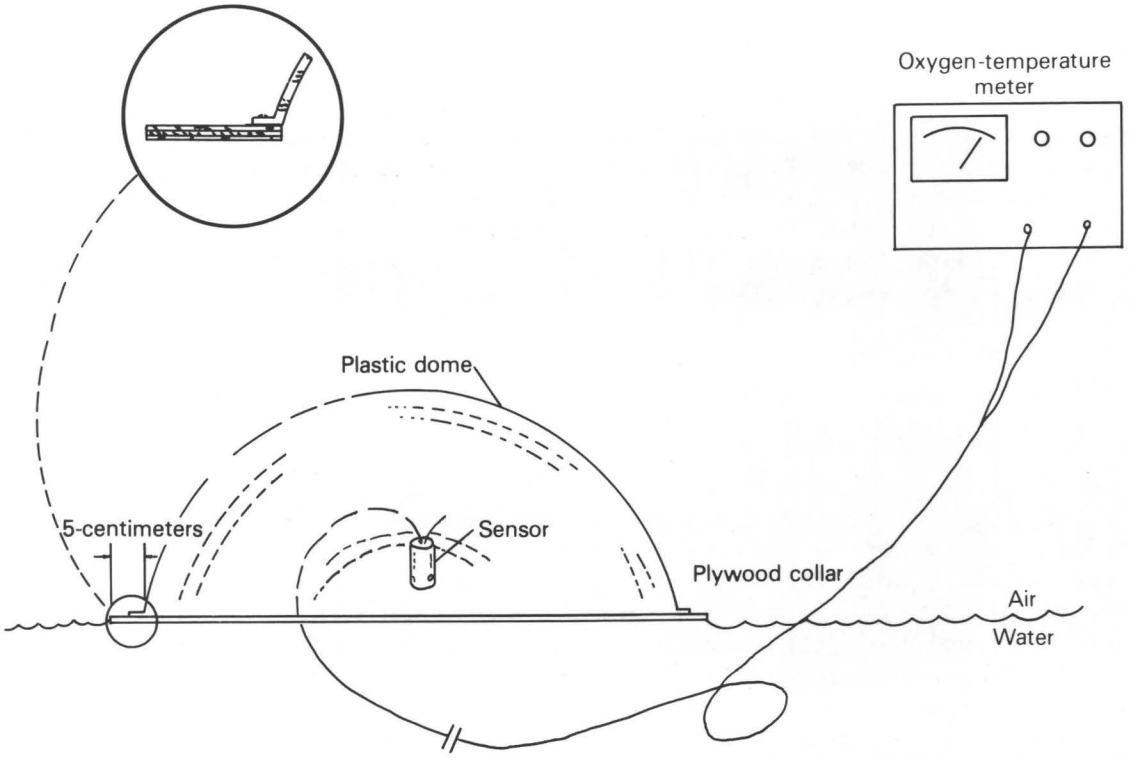

Figure 60.--Floating-diffusion-dome apparatus (modified from Hall, 1971).

Sampling stations should be located in areas representative of the water body if values are to be averaged to yield metabolism of the entire water body. Local hours of sunrise and sunset, as well as average barometric pressure during the study, are required; and phytoplankton standing crop and chlorophyll a are useful supportive data.

Determine the depth of the euphotic zone using a submersible photometer. If no other method is available, an estimate of the bottom limit of the euphotic zone is obtained by multiplying the Secchi disk depth by 2 (Dillon and Rigler, 1974; Vollenweider, 1974). Select sampling intervals equal to one-tenth of the depth of the euphotic zone. Respiration in the deepest part of the lake (hypolimnion) can be estimated by including one or more sampling depths between the euphotic zone and the bottom of the lake. A computer-analysis method requires that depth intervals be constant.

At 1-, 2-, or 3-hour intervals for each increment of depth, determine water temperature, dissolved-oxygen concentration, and if appropriate, salinity or conductivity. Determine D as described in the preceding paragraphs, or by the following method.

Table 14.--Hypothetical data for determining the diffusion rate, D, in a stream by the floating-diffusion-dome method

[The dome has a volume of 2.5 liters and an area of 0.038 square meter in contact with the water; ---, not applicable]

Time interval (hour)	Dome			Water			Gas-transfer coefficient, K (grams per square meter per hour at 0-percent saturation)
	Percent oxygen[1]	Temperature (degrees Celsius)	Volume oxygen (milliliters)	Temperature (degrees Celsius)	Average saturation deficit[2]	Oxygen diffusion rate, D (grams per square meter per hour)	
Beginning (0000)----	99.0	29.5	519.8	29.5	-26.6	0.82	3.1
End (0500)----	74.8	25.0	392.7	25.0			
Beginning (2000)----	99.4	30.0	521.8	30.0	-19.4	.64	3.3
End (2400)--	84.8	29.0	445.2	29.0			
Average K for study period----	---	---	---	---	---	---	3.2

[1]Fresh air = 100 percent.
[2]From table 15.

Nighttime rate-of-change method.--Odum (1956) and Odum and Hoskin (1958) developed this method to estimate reaeration gains or losses during darkness in the absence of photosynthesis. It assumes that there is no photosynthetic production of oxygen and that respiration is constant during the nighttime measurement interval.

Individual values for K corresponding to a nighttime measurement interval may be used to correct the surface-water layer value for nighttime diffusion. An arithmetic average of the nighttime values can be used to provide the daytime diffusion correction.

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Oxygen Light- and Dark-Bottle Method for Phytoplankton
(B-8001-85)

Parameters and Codes:	
Productivity, primary, gross [mg(O_2/m^3)/d]:	70959
Productivity, primary, gross [mg(O_2/m^2)/d]:	70960
Productivity, primary, net [mg(O_2/m^3)/d]:	70963
Productivity, primary, net [mg(O_2/m^2)/d]:	70964
Respiration [mg(O_2/m^3)/d]:	70967
Respiration [mg(O_2/m^2)/d]:	70968

1. Applications

The method is applicable to standing or slowly moving water. Best results are obtained in eutrophic water in which the production rate is about 3 to 200 mg(C/m^3)/h during the photoperiod (Strickland and Parsons, 1968, p. 263). The smaller limit for measurable oxygen production occurs when there is less than a 7-mg(O_2/m^3)/h photosynthetic rate for a 3-hour exposure (Vollenweider, 1974, p. 93).

2. Summary of method

Light (clear) and dark (blackened) bottles filled with water samples are suspended at several depths in the euphotic zone for a known period of time. The concentration of dissolved oxygen is measured at the beginning and at the end of the incubation period. Changes in the dissolved-oxygen concentrations of the enclosed samples are interpreted in terms of photosynthesis and respiration. Productivity is calculated on the basis of one carbon atom assimilated for each oxygen molecule released.

3. Interferences

3.1 The method uses isolated phytoplankton samples to indicate the response of the natural system. Care must be used when collecting the sample, handling the sample, and exposing the sample to light to prevent interference with the life requirements of the organisms. Water-sampling bottles or devices should be made of plastic or glass, and the essential metal parts should be made of stainless steel. Copper, brass, and bronze fittings on water-sampling bottles or on suspension equipment should not be used. The water-sampling bottles should be opaque to decrease the risk of light injury, and biochemical oxygen demand (BOD) bottle filling should be done in the shade or in an enclosure to avoid exposure of unadapted algae to full sunlight. Light leaks into the dark bottles must be prevented. The formation of bubbles in the BOD bottles results in errors during the determination of dissolved-oxygen changes; microbial activity and chemical oxygen demand cause losses of oxygen when incubation times exceed a few hours (Vollenweider, 1974; Hall and Moll, 1975).

3.2 Interferences with the chemical determination of dissolved oxygen were described by Skougstad and others (1979) and American Public Health Association and others (1985).

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies. All materials must be free of agents that inhibit photosynthesis and respiration.

4.1 BOD bottles, numbered, 300 mL, Pyrex or borosilicon glass, that have flared necks and pointed ground-glass stoppers. A supply of light and dark bottles is required. The dark bottles may be prepared by painting the bottles black and covering the paint with overlapping strips of black plastic tape. The exposed parts of the stoppers should be similarly blackened, and a hood of several layers of aluminum foil should cover the stopper and neck of the bottle during use (Note 1).

Note 1: To prepare the BOD bottles, fill with the acid cleaning solution and let stand for several hours. Rinse thoroughly using distilled water. Traces of iodine from the Winkler analysis should be removed by rinsing the bottles and stoppers using 0.01N sodium thiosulfate solution followed by thorough rinsing using distilled water. Do not use phosphorous-based detergents.

4.2 Dark box, preferably insulated, for storing filled BOD bottles until ready for incubation.

4.3 Equipment for determination of dissolved oxygen by the azide modification of the Winkler method (Skougstad and others, 1979; Golterman, 1982; American Public Health Association and others, 1985).

4.4 Polyethylene bottles, 8-L capacity that has cap and bottom tubulation.

4.5 Suspension system for holding light and dark bottles in a horizontal position at various depths (fig. 59).

4.6 Underwater light-measurement equipment. A quantum/radiometer/photometer measures photosynthetically active radiation (400-700 nm). If a submersible photometer is not available, a Secchi disk may be used.

4.7 Water-sampling bottle, Van-Dorn type or equivalent. If a clear acrylic bottle is used, care should be taken to avoid light shock to dark-adapted organisms. Depth-integrating samplers are described in Guy and Norman (1970).

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acid cleaning solution, 20 percent. Mix 20 mL concentrated hydrochloric acid (HCl) (specific gravity 1.19) with distilled water and dilute to 100 mL.

CAUTION.--Use rubber gloves, safety goggles, and protective clothing when handling concentrated HCl.

5.2 Distilled or deionized water.

5.3 Reagents for the azide modification of the Winkler method for dissolved oxygen (Skougstad and others, 1979; American Public Health Association and others, 1985).

5.4 Sodium thiosulfate solution, 0.01N. Dissolve 2.5 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in distilled water and dilute to 1 L.

6. Analysis

6.1 After suitable incubation, remove the BOD bottles from the suspension system; and, as quickly as possible, add the first two Winkler reagents to each bottle to arrest biological activity and to fix the dissolved oxygen. Complete the Winkler determination of dissolved oxygen for all samples; average the results from duplicate samples.

7. Calculations

Primary productivity is expressed as the quantity of oxygen released, or of carbon assimilated, per unit time. Adjust the following calculated values for the appropriate incubation period. Gross or net primary productivity is calculated on the assumption that one atom of carbon is assimilated for each molecule (two atoms) of oxygen released.

7.1 Gross primary productivity [$mg(O_2/m^3)/t$]

$$= \frac{LB - DB}{t} \times 1{,}000 \ ,$$

where LB = dissolved-oxygen concentration, in milligrams per liter, in the light bottle after incubation;
DB = dissolved-oxygen concentration, in milligrams per liter, in the dark bottle after incubation; and
t = incubation period, in hours or days, and 1,000 converts liters to cubic meters.

7.2 Gross primary productivity [$mg(C/m^3)/t$]

$$= \frac{LB - DB}{t} \times \frac{12}{32} \times 1{,}000 \ ,$$

where LB, DB, t, and 1,000 = as in 7.1;
12 = atomic weight of carbon; and
32 = molecular weight of oxygen.

7.3 Net primary productivity [$mg(O_2/m^3)/\underline{t}$]

$$= \frac{\underline{LB} - \underline{IB}}{\underline{t}} \times 1{,}000 \ ,$$

where $\underline{LB}$ = dissolved-oxygen concentration, in milligrams per liter, in the light bottle after incubation;
$\underline{IB}$ = initial dissolved-oxygen concentration, in milligrams per liter, in the light bottle before incubation; and
$\underline{t}$ = incubation period, in hours or days, and 1,000 converts liters to cubic meters.

7.4 Net primary productivity [$mg(C/m^3)/\underline{t}$]

$$= \frac{\underline{LB} - \underline{IB}}{\underline{t}} \times \frac{12}{32} \times 1{,}000 \ ,$$

where $\underline{LB}$, $\underline{IB}$, $\underline{t}$, and 1,000 = as in 7.3;
12 = atomic weight of carbon; and
32 = molecular weight of oxygen.

7.5 Respiration [$mg(O_2/m^3)/\underline{t}$]

$$= \frac{\underline{IB} - \underline{DB}}{\underline{t}} \times 1{,}000 \ ,$$

where $\underline{IB}$ = initial dissolved-oxygen concentration, in milligrams per liter, in the light bottle before incubation;
$\underline{DB}$ = dissolved-oxygen concentration, in milligrams per liter, in the dark bottle after incubation; and
$\underline{t}$ = incubation period, in hours or days, and 1,000 converts liters to cubic meters.

7.6 The gross or net primary productivity of a vertical column of water, 1 m^2 in cross section (milligrams oxygen per square meter per time or milligrams carbon per square meter per time), is determined by a summation of the productivities in successive cubic meter volumes, from top to bottom, in the euphotic zone at each study site. However, the maximum value in the euphotic zone for primary productivity, expressed on a cubic meter basis (pmax), has much more meaning for data interpretation than does an integrated square meter value (Megard, 1972). Therefore, the maximum cubic meter value should be reported in addition to the square meter integral value for primary productivity. On a graph of depth versus productivity (fig. 61), plot the experimentally determined productivity value for each incubation depth, and draw a line of best fit through the points. Integrate the area under the productivity-depth curve to obtain a total productivity value for the euphotic zone. An example of the vertical distribution of daily primary productivity in a lake is shown in figure 61.

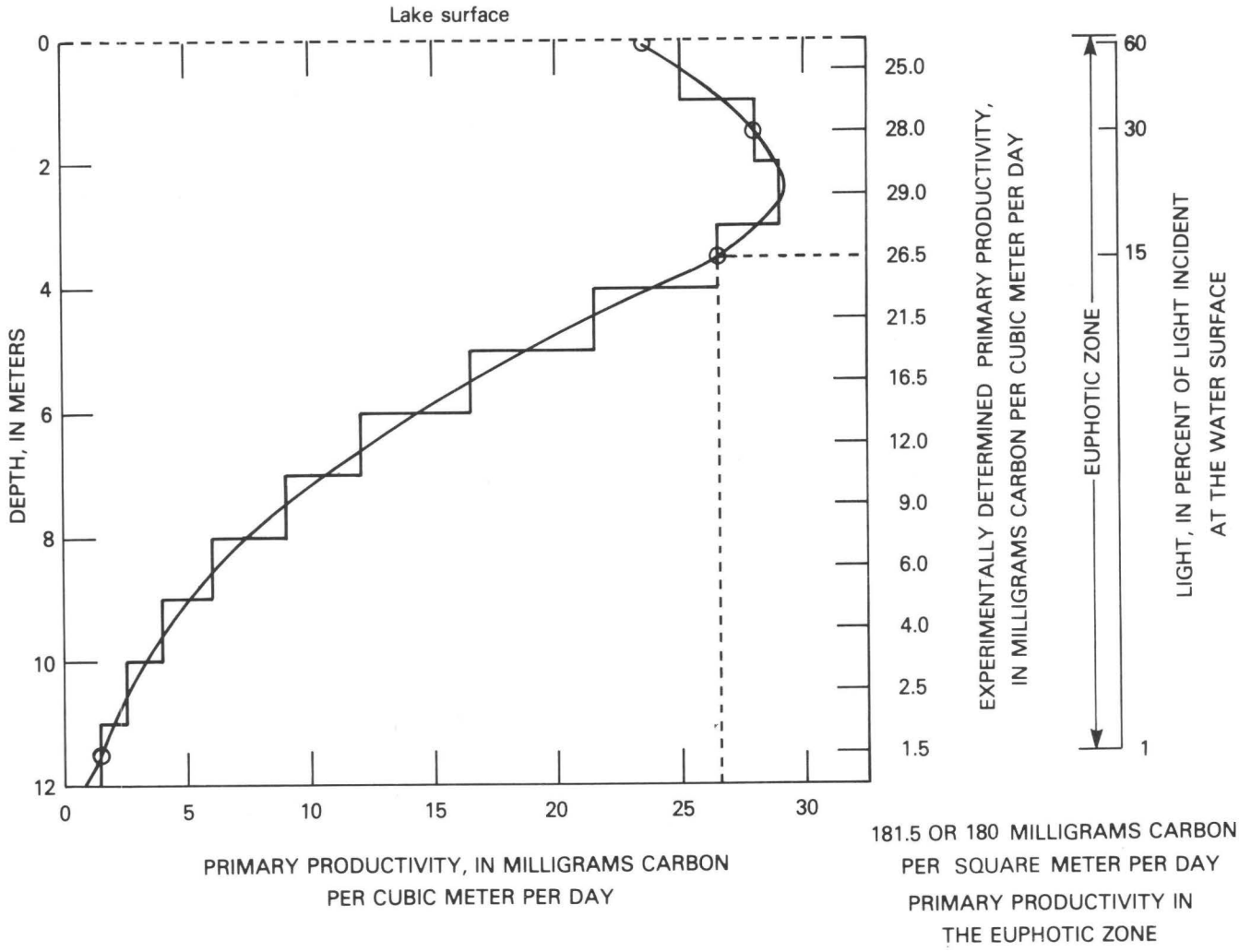

Figure 61.--Example of the vertical distribution of daily primary productivity in Koocanusa Reservoir, Mont. The circled points are values of primary productivity (milligrams carbon per cubic meter per day) calculated from contents of light and dark bottles suspended at those depths. The smooth curve was fitted by eye, and the area under the primary productivity-depth curve (milligrams carbon per square meter per day) was estimated by summing the values at 1-meter intervals through the euphotic zone (modified from Janzer and others, 1973).

8. Reporting of results

Report primary productivity as follows: less than 10 mg, one decimal; 10 mg and greater, two significant figures.

9. Precision

The following precision estimates were reported by Strickland and Parsons (1968, p. 263) for aliquots from a single, large sample and do not include variabilities from sampling. For precision at the 100-mg $(C/m^3)/h$ level, the correct value lies in the range: Mean of $\underline{n}$ determinations $\pm 15/\underline{n}^{\frac{1}{2}}$ mg(C/m^3)/hr

(6-hour incubation). For precision at the 10-mg (C/m^3)/h level, the correct value lies in the range: Mean of n determinations $\pm 1.5/n^{\frac{1}{2}}$ mg(C/m^3)/h (6-hour incubation).

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Carbon-14 Light- and Dark-Bottle Method for Phytoplankton (B-8020-85)

Parameters and Codes:

Productivity, primary, gross [mg(C/m^3)/d]:	70961
Productivity, primary, gross [mg(C/m^2)/d]:	70962
Productivity, primary, net [mg(C/m^3)/d]:	70965
Productivity, primary, net [mg(C/m^2)/d]:	70966

Phytoplankton primary productivity as determined by the ^{14}C light- and dark-bottle method measures the rate of assimilation of carbon dioxide (CO_2) into particulate organic material by contained algal populations. The ^{14}C method measures productivity by determining the rate of incorporation of a radioisotope tracer, $^{14}CO_2$, into organic material.

The ^{14}C method was used first by Steemann-Nielsen (1952). Originally, radioactivity of incorporated ^{14}C was measured using Geiger-Müller (GM) counters, but this measurement technique is rarely used because GM counters are susceptible to considerable back scatter and self-absorption and can have inaccurate counting efficiencies. Comparisons of the merits of GM measurements and liquid-scintillation measurements (Schindler, 1966; Wolfe and Schleske, 1967; Wallen and Geen, 1968) indicated that liquid-scintillation measurements do not have many of the drawbacks inherent with the use of GM counters. Pugh (1970, 1973) reported that counting efficiency as calculated by internal or external standardization can result in serious errors if applied to a heterogeneous sample, for example, a filter that has attached phytoplankton. High levels of self-absorption caused by dense layering of particulate material on filters can be corrected accurately only by using a filter standardization technique (Pugh, 1973). Many investigators proposed the use of solubilizers, emulsifiers, and bleaching to provide a homogeneous sample that has accurate counting efficiency. Schindler and others (1972) proposed acidification and bubbling of the sample to eliminate errors and uncertainties associated with filtration techniques (Arthur and Rigler, 1967). Further modifications of the acid bubbling method (Smith, 1975; Theodorsson and Bjarnason, 1975; Mague and others, 1980) have resulted in a technique that eliminates many problems inherent in ^{14}C-filtration methods (Goolsby, 1976; Gachter and Mares, 1979), particularly problems caused by filtration artifacts, accurate determination of counting efficiency, and excretion of dissolved organic material.

1. Applications

1.1 The ^{14}C method is applicable to standing or slowly moving eutrophic and oligotrophic water in freshwater or saline environments. In very eutrophic water, the rate of photosynthesis may be so rapid that adjustments in experimental procedure may be necessary (see "Supplemental Information" section). Lean and Burnison (1979) warn of possible insensitivity of acidification and bubbling techniques in water that has greater than 1,500 to 3,000 µm dissolved inorganic carbon.

1.2 Although radioisotope techniques seem to be straightforward, exactly what is being measured by ^{14}C techniques has never been determined precisely. Measures of gross or net productivity typically are of interest. But, because the technique cannot directly measure respiration, photorespiration, or the

rate of ^{14}C movement through the cellular carbon pool, accurate determinations of whether gross or net productivity is being measured cannot be made. Studies by Hobson and others (1976) and Gieskes and others (1979) indicate that incubations of 2 to 4 hours are needed to measure gross carbon uptake, whereas incubations of 24 hours are required to measure net productivity.

2. Summary of method

Measurements of primary productivity of organic matter using the ^{14}C method (Steemann-Nielsen, 1952) require adding radioactive bicarbonate, $NaH^{14}CO_3$, to an enclosed water sample. After incubation (either in situ or in an incubator), photosynthesis is stopped by chemical means before further processing. An aliquot of the fixed sample then is acidified and bubbled (Schindler and others, 1972) to separate the inorganic $^{14}CO_3^{-2}$ from the organic fraction. Following acidification and bubbling, an unfiltered subsample and a filtrate subsample are used for subsequent scintillation counting. After a volumetric subsample of the filtrate is acidified and bubbled, a known quantity is put into a scintillation vial and a light-sensitive scintillation fluor is added to the vial. As the ^{14}C atom decays, an energized β particle is emitted, which causes the scintillation solution to fluoresce pulses of light. Very sensitive photomultiplier tubes in a scintillation spectrometer record the light pulses. The ^{14}C activity in the sample is proportional to the frequency of light pulses. The uptake and reduction of CO_2 to organic matter is assumed to be proportional to the uptake of ^{14}C bicarbonate. Primary productivity, as the quantity of carbon fixed per unit time, is calculated from the proportion of ^{14}C fixed to ^{14}C available and total CO_2 in the sample.

3. Interferences

Some interferences are inherent in the ^{14}C method and cannot be avoided. A "Supplemental Information" section is included at the end of the description of this method to indicate commonly occurring problems and the types of procedures that minimize their effects.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies. All materials used must be free of agents that inhibit photosynthesis and respiration.

4.1 Bags, polyethylene, about 30×60 cm, for solid radioactive wastes.

4.2 Black tape, to cover cap and neck of dark bottles after inoculating using ^{14}C bicarbonate.

4.3 BOD bottles, numbered, 300 mL, Pyrex or borosilicon glass, that have flared necks and pointed ground-glass stoppers. A supply of light and dark bottles is required. The dark bottles may be prepared by painting the bottles black and covering the paint with overlapping strips of black plastic tape. The exposed parts of the stoppers should be similarly blackened, and a hood of several layers of aluminum foil should cover the stopper and neck of the bottle during use (Note 1).

Note 1: To prepare the BOD bottles, fill with the acid cleaning solution and let stand for several hours. Rinse thoroughly using distilled water. Traces of iodine from the Winkler analysis should be removed by rinsing the bottles and stoppers using 0.01N sodium thiosulfate solution followed by thorough rinsing using distilled water. Do not use phosphorous-based detergents.

4.4 Carboy, waste, 20 L, polyethylene.

4.5 Dark box, preferably insulated, for storing filled BOD bottles until ready for incubation.

4.6 Filtration assembly, 20-mL syringe that has the plunger removed, attached to a 25-mm filter unit. The sample is filtered through a 25-mm filter, and the filtrate is collected in a temporary holding vial.

4.7 Glass-fiber filters, 47-mm diameter disks, or membrane filters, white, plain, 0.45-µm mean pore size, 47-mm diameter.

4.8 Micropipet, automatic, precision volumetric, 1 mL.

4.9 Needles, hypodermic, 7.5 or 10 cm, Luer taper.

4.10 Pipet, automatic, adjustable, volumetric, 1 to 5 mL.

4.11 Pipet tips, disposable, 1-mL capacity.

4.12 Pipet tips, disposable, 5-mL capacity.

4.13 Repipettor.

4.14 Sample bubbler, for agitating the sample while stripping $^{14}CO_3^{-2}$ from the solution. A number of designs have been employed (Theodorsson and Bjarnason, 1975; Gachter and Mares, 1979). A system proven to be effective is shown in figure 62. After acid is added to the sample vial and the stopper is in place, air, which agitates the solution and mixes the sample and acid, is drawn through the inlet tube. The $^{14}CO_2$ is drawn away by vacuum and vented outside the laboratory.

4.15 Spectrometer (spectrophotometer; fig. 57) that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.16 Suspension system for holding light and dark bottles in a horizontal position at various depths (fig. 59).

4.17 Syringe, 10-mL Luer taper.

4.18 Underwater light-measurement equipment. A quantum/radiometer/photometer measures photosynthetically active radiation (400-700 nm). If a submersible photometer is not available, a Secchi disc may be used.

4.19 Vacuum pump.

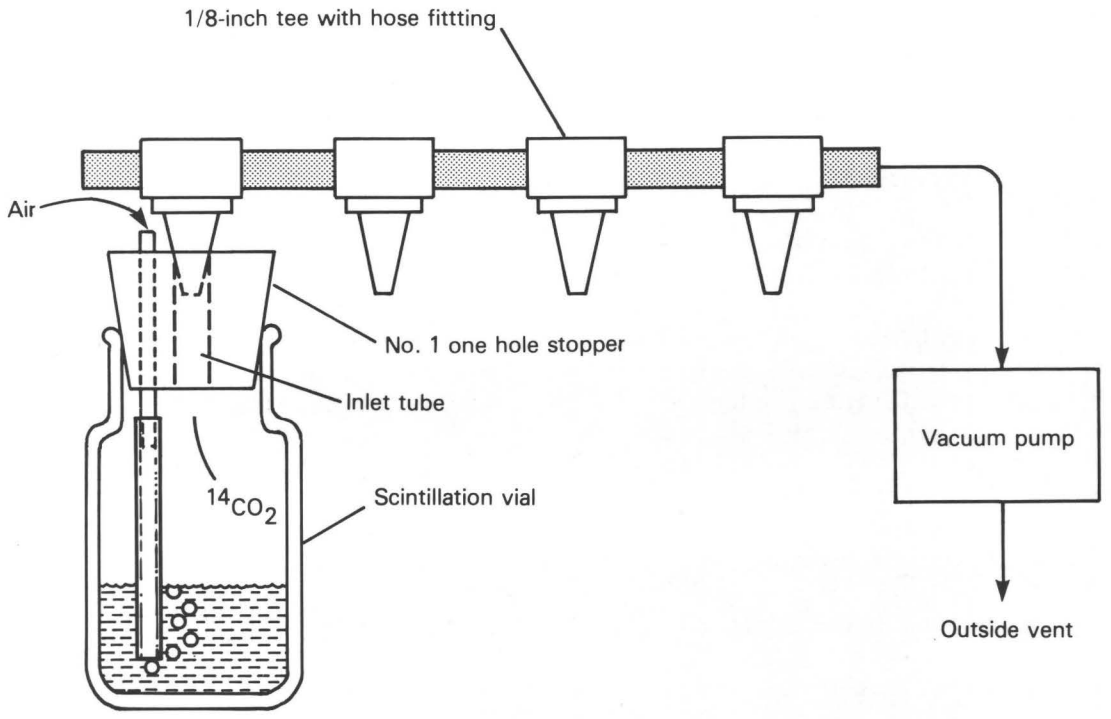

Figure 62.--Sample bubbler that has sample vial attached. The stopper is a no. 1 (one-hole stopper). An air vent is made from a 3-centimeter section of a no. 20 hypodermic needle to which is attached a short length of tygon tubing.

4.20 Vials, liquid scintillation, 20-mL capacity, that have plastic-lined screwcaps (Note 2).

Note 2: Place identifying marks on the caps and not on the sides of the vials.

4.21 Water-sampling bottle, Van-Dorn type or equivalent. If a clear acrylic bottle is used, care should be taken to avoid light shock to dark-adapted organisms. Depth-integrating samplers are described in Guy and Norman (1970).

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acid cleaning solution, 1N. Mix 82.6 mL concentrated HCl (specific gravity 1.19) per liter of distilled water.

CAUTION.--Use rubber gloves, safety goggles, and protective clothing when handling concentrated HCl.

5.2 Ammoniacal barium chloride solution. Dissolve 50 g $BaCl_2 \cdot 2H_2O$ in approximately 1 L lakewater or tapwater, add 75 to 100 mL concentrated NH_4OH (specific gravity 0.90), and place in the 20-L polyethylene waste carboy.

5.3 ^{14}C bicarbonate solution, $NaH^{14}CO_3$ or equivalent. Specific activity of 0.1 µCi/µg. Standard solutions of 1, 5, 10, or 20 µCi/mL are available. The activity necessary for a particular environment should be established by the researcher.

5.4 ^{14}C labeled toluene standard, certified calibration standard of toluene (^{14}C) that has a specific activity of 4×10^5 DPM/mL.

5.5 Distilled or deionized water.

5.6 Hydrochloric acid, 0.1N. Mix 8.3 mL concentrated hydrochloric acid (HCl) (specific gravity 1.19) with distilled water and dilute to 1 L in a repipettor that has 0.1-mL graduations.

5.7 Liquid-scintillation solution. Aquasol^R scintillation cocktail has been a satisfactory fluor. PCS Solubilizer premixed liquid-scintillation cocktail also has been satisfactory (Janzer and others, 1973).

5.8 Reagents for determining total alkalinity (CO_2, HCO_3^{-1}, and CO_3^{-2}) (Skougstad and others, 1979; American Public Health Association and others, 1985).

5.9 2-phenethylamine, scintillation grade. Phenethylamine is used to form carbonates, which are stable in Aquasol, to eliminate loss of radiocarbon from the acidic fluor.

6. Analysis

6.1 After incubation is completed, process the samples in a work area that has subdued lighting. After shaking the sample well, dispense a 3-mL aliquot of sample into a scintillation vial using a precision volumetric pipet. Add 0.2 mL of 0.1N HCl to decrease the pH to 2.5 to 3. Immediately insert a stopper (fig. 62) and attach the vial to the sample bubbler. Repeat in triplicate for each light and dark bottle.

6.2 Gravity filter 5 to 10 mL of each sample through a 0.45-µm glass-fiber filter. Pour the sample water into a 20-mL plastic syringe filtration unit. The filtrate is collected in a temporary holding vial from which a 3-mL subsample is dispensed into a scintillation vial. Add 0.2 mL of 0.1N HCl and bubble.

6.3 After aerating each sample for 10 to 15 minutes, remove the vial from the sample bubbler and replace the stopper with a scintillation vial cap. When convenient, add to each vial 10 mL liquid-scintillation solution, using a volume sufficient to produce a stable emulsion suitable for holding particulates dispersed throughout the medium.

6.4 Filter the remaining contents of all BOD bottles through a 0.45-µm glass-fiber filter. Dispose of the glass-fiber filters in the solid-waste disposal bag. Pour the collected filtrate into the 20-L polyethylene waste

carboy to react with the ammoniacal barium chloride solution; ^{14}C bicarbonate in solution will be precipitated as barium carbonate, which is allowed to settle ("Supplemental Information" subsection at the back of this section).

6.5 Temporary holding vials are reused after being washed, soaked in 1N HCl, rinsed, and dried.

6.6 When the vials are returned to the laboratory, wipe the outside of each vial using an acetone dampened tissue to remove dust and finger marks.

6.7 Dark adapt all vials until their activity drops to a consistent level. The time required for dark adaptation will vary but can be determined by counting a representative sample until little variation between successive counts is observed. Typically, a few hours is sufficient for dark adaptation.

6.8 Using a liquid-scintillation spectrometer, count each vial in series for 20 minutes. Repeat the counting procedure three times.

6.9 Determine the counting efficiency for each sample by internal standardization. After counting, add 100 µL of ^{14}C labeled toluene standard to two samples from each sampling depth. Repeat counting as described in 6.8.

6.10 Determine the counting efficiency for these spiked samples using the equation

$$\underline{E} = \frac{(\bar{\underline{R}}_{s'} - \bar{\underline{R}}_{s})}{\underline{S}} \times 100 \ ,$$

where $\underline{E}$ = the counting efficiency, in percent (Note 3);
$\bar{\underline{R}}_{s'}$ = the average counting rate of the sample, in counts per minute after the addition of the ^{14}C labeled toluene standard;
$\bar{\underline{R}}_{s}$ = the average counting rate of the sample, in counts per minute; and
$\underline{S}$ = the total activity of the ^{14}C labeled toluene standard added, in disintegrations per minute.

Note 3: Experience indicates that a variation of 2 percent in the counting efficiency is acceptable. If the variation is greater than 2 percent, the counting efficiency for all samples in light and dark bottles from the location(s) in question should be checked and count-rate corrections made, if necessary.

6.11 Activity of ^{14}C bicarbonate standards are determined in a similar manner. Because the activity of standard samples is intense, counting time should be decreased to 1 minute to prevent overloading the scintillation spectrometer's counting mechanism. After counting each standard three times, add 1 mL of ^{14}C labeled toluene standard to two samples. Repeat the counting procedure for the spiked samples. Counting efficiency for spiked standards is calculated as outlined in 6.10.

7. Calculations

7.1 Primary productivity is expressed as the quantity of carbon assimilated per unit time. Gross photosynthesis, based on incubations of 2 to 4 hours, should be reported as productivity per hour (milligrams carbon per cubic meter per hour). Net photosynthesis, based on 24-hour incubations, should be reported in milligrams carbon per cubic meter per day.

Net primary productivity = total $carbon_{fixed}$ - excreted $carbon_{fixed}$.

Gross primary productivity = total $carbon_{fixed}$.

$$Carbon_{fixed} = \frac{(\bar{B}_l - \bar{B}_d) \times W \times (V_i/V_a) \times 1.064}{\bar{S} \times D},$$

where $\bar{B}_l$ (DPM) = average light-bottle counting rate ($\bar{R}_x$) divided by sample counting efficiency (E) (see C in analytical problems in "Supplemental Information" section);

$\bar{B}_d$ (DPM) = average dark-bottle counting rate ($\bar{R}_s$) divided by sample counting efficiency (E);

W (mg/L) = alkalinity (actually ^{12}C-total inorganic carbon). Conversion of alkalinity data to inorganic carbon values is discussed in Vollenweider (1974);

V_i (mL) = volume incubated;

V_a (mL) = volume of aliquot acidified and bubbled;

1.064 = isotopic preference factor (Steemann-Nielsen, 1952);

$\bar{S}$ (DPM) = average ^{14}C bicarbonate standard counting rate ($\bar{R}_s$) × counting efficiency (E).

D = unit time;

Total $carbon_{fixed}$ = unfiltered sample fixation rate; and

Excreted $carbon_{fixed}$ = 0.45-μm filtrate sample fixation rate.

7.2 The primary productivity of a vertical column of water, 1 m^2 in cross section (milligrams carbon per square meter per time), is determined by a graphical summation of the productivity in successive cubic meter volumes, from top to bottom, in the euphotic zone at each study site. On a graph of depth versus productivity (fig. 61), plot the experimentally determined productivity value for each incubation depth, and draw a line of best fit through the points. Integrate the area under the productivity-depth curve to obtain a total productivity value for the euphotic zone. In addition, report the maximum cubic meter value of primary productivity (pmax) measured in the euphotic zone. LaBaugh (1979) and Smith (1979) have reported the usefulness of pmax in the interpretation of water-quality data related to primary productivity measured by the ^{14}C method. Kerekes (1975) describes why square-

meter primary-productivity data are less suitable for interpretive studies than cubic-meter primary-productivity data. An example of the vertical distribution of daily primary productivity in Koocanusa Reservoir is shown in figure 61.

8. Reporting of results

Report primary productivity as follows: two significant figures.

9. Precision

Estimates of precision of primary-productivity measurements based on replicate samples from in-situ incubations seldom are reported. Hager and others (1980) reported the precision of replicate ^{14}C samples to be 5 to 10 percent. Precision of the acid bubbling technique is reported by Gachter and Mares (1979) to range from 0.7 to 2.4 percent (n = 10).

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Interferences and limitations

Toxins.--Any substance on the collecting apparatus or BOD bottles that is foreign to the natural-water sample may have a deleterious effect on the productivity of the sample. All equipment and glassware must be cleaned between sampling. All traces of HCl cleaning solution must be rinsed from the BOD bottles to eliminate loss of the inoculant. Liquid-scintillation vials and preservatives, such as Lugol's and formalin, are very toxic. Such chemicals should be restricted from the sample preparation area.

Contamination of samples by bare metal may have detrimental (Doty and Oguri, 1959) and stimulatory (Goldman, 1963) effects on the sample. To decrease either effect, plastic, stainless-steel, or plastic-coated metal parts should be used when possible.

Analytical problems.--Since Steemann-Nielsen's (1952) description of the method, techniques for more accurate measurement of β-particle activity have led to many refinements in methods.

a. Counting methods. Originally, Geiger-Müller (GM) counters were used for measuring the frequency of β emissions. Although the equipment is less expensive than liquid-scintillation counters, the efficiency of GM counters is minimal (less than 20 percent), and there are serious errors that may be due to self-absorption and backscatter. GM counters require that the material be dried, a process that can result in a 30 to 50 percent loss in carbon (Wallen and Geen, 1968; Ward and Nakanishi, 1971). Liquid-scintillation counters have come into common use because of their more accurate counting efficiencies and ability to count wet filters and aqueous samples when a suitable fluor is used.

b. Quench. A decrease in the efficiency of a scintillation counter's detection of β emissions is caused by quenching of the sample. Of the three types of quench in liquid-scintillation samples--chemical, color, and physical--the last is the most difficult to correct when using phytoplankton samples. Large quantities of solid phytoplankton and filter material physically block the emission of light from the sample fluor.

c. Counting efficiency. Essential to an accurate estimation of the total activity of a sample is knowledge of the efficiency with which the scintillation spectrometer detects β emissions. Three common techniques for measuring counting efficiency are internal standardization, external standardization, and channels ratio. Specific techniques for implementing each of these methods are outlined in manuals supplied by manufacturers of scintillation spectrometers. These techniques for determining counting efficiency are limited in accuracy because they are suited ideally only for a homogeneous

solution, one without particulate matter. This is especially true for the external-standardization and channels-ratio techniques, which are based on efficiency curves of standard solutions that may not accurately represent the factors causing quench in a heterogeneous sample. Pugh (1970) has reported serious errors in measuring efficiencies using these techniques when attempts are made to count filters heavily laden with particulate material. Pugh (1970, 1973) developed a filter standardization technique for ^{14}C-sucrose incorporation onto membrane filters, as long as the weight of sample algae on the filters was small (less than 1 mg). Solubilizers have been used to dissolve the filter and attached algae, which results in a homogeneous sample whose counting efficiency can be determined by one of the standard techniques. The digests of such samples may be very dark and require bleaching with either peroxide (Gargas, 1975) or intense ultraviolet light to decrease color quenching. The efficiency of dissolution varies with the fluor used. Undissolved particles still may cause self-absorption and may require the addition of an emulsifier (Schindler, 1966) such as NCS or Protosol, to prevent settling of particulates.

d. Standardization of inoculant. Measurement of the activity of the ^{14}C bicarbonate inoculant can be inaccurate if the liquid-scintillation vial used is acidic. Iverson and others (1976) reported the loss of ^{14}C activity when $NaH^{14}CO_3$ was added to Aquasol^{R}, a xylene-based fluor. They advised the addition of an organic base, such as phenethylamine, to stabilize the ^{14}C and to achieve complete retention of the radioisotope in the scintillation vial. Other compounds that have been found suitable in toluene-based fluors include Bio-Sol, PCS tissue solubilizer, and monethylamine. The efficiency of retention of inorganic ^{14}C in any scintillation vial should be evaluated prior to onsite studies.

e. Commerical ^{14}C bicarbonate solutions. The purity of commercially supplied $NaH^{14}CO_3$ has been questioned by a number of investigators (Gargas, 1975). Large concentrations of silica, which might be stimulatory to diatom growth, have been reported (Gieskes and Van Bennekom, 1973). Contamination by known organics also has been noted (Sharp, 1977). Use of these inoculants might result in anomalously large excretion rates resulting in small estimates of net productivity. These dangers can be minimized by preparing the ^{14}C bicarbonate solution in one's own laboratory by dilution of a commercial solution using large specific concentrations (1-5 mCi/0.5-2 mL) or from solid $Ba^{14}CO_3$ (Gargas, 1975). Irradiation of the ^{14}C bicarbonate solution using intense ultraviolet light has been used to oxidize all of the organic material to $^{14}CO_2$.

f. Filtration. An integral component of the ^{14}C method as used by early investigators was filtration to concentrate the particulates, enabling the GM counter, which has questionable counting efficiency, to measure the level of sample activity. The process of filtration can cause cell rupture and loss of intracellular carbon if the differential pressure is too great. Although Nalewajko and Lean (1972) and McMahon (1973) attribute the filtration artifact reported by Arthur and Rigler (1967) to filter retention of unfixed radiotracer,

pressure differentials should be less than 100 mm of mercury to minimize cell breakage. The acid bubbling technique (Schindler and others, 1972) prevents the uncertainties due to possible absorption, cell rupture, and filtration corrections.

The presence of a filter in the scintillation vial adds to the difficulty of accurate determination of counting efficiency (Pugh, 1970, 1973). Solubilizers have been used to dissolve the filter. Unfortunately, the degree of dissolution attained depends on the filter and the fluor used (Schindler, 1966; Wallen and Geen, 1968; Pugh, 1973; Gargas, 1975). Solubilization of the filter can cause color quench that may be decreased by the addition of 1 to 2 drops of 30-percent hydrogen peroxide (Gargas, 1975) or by heating or suspending the samples in quartz tubes in strong ultraviolet light and adding peroxide (Schindler and others, 1974).

g. ^{14}C bicarbonate elimination. Decontamination of ^{14}C bicarbonate is necessary to remove residual inorganic ^{14}C from the sample. Steemann-Nielsen (1952) suggested exposing the filter to fumes of concentrated HCl. For greater speed, convenience, and safety, a few milliliters of dilute HCl were poured through the filter. The concentration of acid rinse ranged from 0.001N (Ryther and Vaccaro, 1954) to 1N (Smith and others, 1960). Which concentration is the most efficient is not clear. Williams and others (1972) and McMahon (1973) suggested simply washing the filter using nonradioactive, filtered sample water. Other investigators believed that the filters should not be washed with filtered sample water or dilute acids (McAllister, 1961; Gargas, 1975). Lean and Burnison (1979) suggested placing the filter in a scintillation vial, adding a few drops of 0.5N HCl, and fuming for 2 to 3 hours. Using acid bubbling techniques, ^{14}C bicarbonate is stripped from the aqueous sample after the addition of dilute acid. Efficiency of removal using acid bubbling is about 99.99 percent (Sharp, 1977; Mague and others, 1980) at pH 3.

Environmental variables.--Accurate measures of primary productivity and an evaluation of their significance is dependent on an understanding of how environmental variables may affect the measured results.

a. Light. Light preconditioning, adaptation, and shock can have a dramatic effect on primary productivity. When using population sites where the light is dim, light shock must be minimized (Steemann-Nielsen and Hansen, 1959; Goldman and others, 1963). Short-term incubation productivity measurements particularly are susceptible to light shock. A satisfactory way to minimize light shock is to make dawn-sunset incubations. Cells preconditioned to dim light and then exposed to bright light have increased excretion rates when compared with those kept under dim light (Nalewajko, 1966; Watt and Fogg, 1966; Ignatiades and Fogg, 1973). Hellebust (1965) suggests increased rate of excretion in bright light without dim-light preconditioning. Increases in excretion also are reported when samples are preconditioned to bright light and then are incubated in dim light.

An assumption made by many investigators is that for short incubation periods (for example, 2 hours) or long incubation periods (for example, 24 hours) the ^{14}C method measures the same type of productivity, gross or net. A second assumption is that for a specific incubation period, the method measures the same type of productivity, even when cells are exposed to varying irradiances (incubation depth). Neither assumption is correct. Hobson and others (1976) report that incubations for 24 hours are the minimum required for net productivity to be measured by ^{14}C techniques, and estimates of gross productivity can be calculated best after short exposure to ^{14}C. Their findings support those of McAllister and others (1961), Antia and others (1963), Bunt (1965), Ryther and Menzel (1965), and Paerl and MacKenzie (1977) that net productivity is measured in 24-hour experiments. Data from Hobson and others (1976) also indicate that the rate of passage of ^{14}C through the cellular carbon pool is dependent on irradiance. The incubation time required for measurement of net productivity is greater than 24 hours when samples are exposed to dim light. After 24 hours, productivity in the bright-light incubation bottle will more closely approximate net values while that in dim-light incubation bottles will approximate gross values. The integration of primary productivity when compared to depth, therefore, results in an overestimate of net production per unit area.

b. Temperature. Changes in temperature during sample handling or incubation can cause physiological stress on sensitive phytoplankton. All sample handling should be completed as quickly as possible after sample collection. Variation between the natural temperature of a sample and incubation temperature can seriously affect measured productivity. If it is necessary to incubate at a temperature different from the collection temperature, one can correct the data by application of Van't Hoff's law (Gargas, 1975)--an increase in temperature of 10 °C doubles the rate of an enzymatic process.

c. Nutrients. Nutrients may include carbon, trace minerals, chelators, and vitamins in addition to nitrogen, phosphorus, and silica. Primary productivity can be enhanced or inhibited depending on the concentrations of the nutrients involved. Samples from an oligotrophic system may be particularly sensitive to slight perturbations of the nutrient regime (Eppley and others, 1973). The concentration of a nutrient in a bottle may become limiting to photosynthesis during the course of incubation so the measured productivity does not represent accurately the natural system. Ambient nutrient concentrations may not be adequate evidence of the capacity of natural water to sustain intense productivity. Containment of a water sample for a prolonged period restricts interactions between the sample and the mixing and regeneration processes that normally replenish nutrients in the water. Although Eppley (1968) reported nutrient depletion in 36 samples contained for more than 24 hours, recent studies by Steemann-Nielsen (1978) and McCarthy and Goldman (1979) report that even in oligotrophic systems enough nutrients for rapid near-optimal growth are constantly available to phytoplankton by heterotrophic processes.

Nutrient contamination of sampling gear or incubation glassware can affect dramatically the results of an experiment. For example, Gieskes and Van Bennekom (1973) report dissolved silica in ^{14}C ampoules at concentrations of 800 to 1,000 µg-atoms/L caused by dissolution of silicate from the glassware wall during autoclaving. One could minimize this source of error by purchasing ^{14}C bicarbonate that has an intense specific activity (for example, 5 mCi/mL), and then diluting the ^{14}C bicarbonate to the desired activity (for example, 5 µCi/mL). Ultraviolet irradiation rather than autoclaving could be used to sterilize the solution.

Processes taking place in the sample bottle also may affect the speciation of a nutrient. In a very eutrophic system, photosynthesis by a contained population might enable the pH to increase to 9 to 10. As a result, NH_4^+ may be converted to the toxic form NH_3.

d. Zooplankton. At times, zooplankton can be so abundant that their grazing pressure might decrease the measured net primary productivity of a sample; therefore, productivity might be measured more accurately if the zooplankton are removed by filtering the sample through a screen. McCarthy and others (1974) reported that prescreening the sample to eliminate grazers had no effect on measured productivity, but production in 16 percent of the screened samples exceeded production in those not screened. They attribute the increased production in screened samples to heavy grazing pressure that occurred in unscreened ones. Venrick and others (1977) also could not attribute any decline in productivity to prefiltration. However, the phytoplankton population must not be decreased simultaneously with the zooplankton population. If the sizes of the algae and grazing population overlap, the researcher will have to decide whether inclusion of zooplankton in the sample or the exclusion of a part of the phytoplankton community from the sample will bias the results. Simultaneous incubation of screened and unscreened samples may be required.

e. Dark-bottle fixation. The effects of heterotrophic carbon fixation on primary productivity measured by the ^{14}C method are difficult to assess. Although phytoplankton can assimilate CO_2 independent of light energy (Kreb's Cycle), this is only 1 percent of the photosynthetic rate of CO_2 uptake. The incubation of a dark bottle is included in the ^{14}C method to correct for abiotic processes and heterotrophic uptake that will bias productivity calculations. Dark-bottle fixation, which is a biotic and an abiotic process (Petersen, 1978; Gieskes and others, 1979), is not related to light-bottle fixation, but to other factors and thus must be determined for each experiment. Although the processes involved in assimilation of CO_2 in the dark are not well understood, they account for 10 to 100 percent (Taguchi and Platt, 1977; Gieskes and others, 1979) of the assimilation measured in the light. Therefore, dark-bottle CO_2-uptake rates are subtracted from light-bottle CO_2-uptake rates when calculating productivity.

Sample containment.--The ^{14}C method assumes that enclosure of the water sample does not appreciably affect the response of the phytoplankton community to environmental variables, but confinement of the phytoplankton isolates them from many of the physical, chemical, and biological factors they normally encounter and increases their exposure to other variables. The effects of containment have not been investigated thoroughly.

The species composition of a contained population can change markedly during incubation. During incubations of 6 to 24 hours, Venrick and others (1977) noted a decrease in abundance of nearly all components of the phytoplankton and the complete disappearance of some ciliate groups. A tenfold decrease in production by contained samples compared to unenclosed populations is reported by Verduin (1960).

Enclosure in a bottle decreases circulation and turbulent mixing. Sedimentation of heavy cells and flotation of blue-green populations can result, altering the community structure (Goolsby, 1976). Incubation also maintains the organisms at specific depths or light intensity, rather than enabling them to mix vertically through the water column. Estimates of areal photosynthesis have been 19 to 87 percent larger using vertically cycled bottles rather than a series of specific depth samples (Marra, 1978).

Sheldon and others (1973) and Gieskes and others (1979) report that, although bottle volume may cause changes in contained populations, the results are not predictable. Sheldon and others (1973) report a significant increase in particles in small incubation bottles; whereas, no difference could be detected between 4-L bottle populations and the natural community. Gieskes and others (1979) reported little or no production in 30-mL bottles, but more than five times the production in 4-L bottles than that in 300-mL bottles. Although the most prudent approach is to use the largest practical bottle size, the question of optimum incubation bottle size and the effects of sample containment need to be evaluated further.

Respiration.--One of the principal limitations of the ^{14}C method is that the respiration rates in phytoplankton cannot be measured directly. Respiration takes place simultaneously with photosynthesis so, in time, some of the ^{14}C photosynthate will be respired back into $^{14}CO_2$ and H_2O. Because a large fraction of many aquatic systems is aphotic, realistic carbon budgets for a system are dependent on accurate estimation of respiration. The rate of heterotrophic ^{14}C fixation in dark bottles is not relevant to this process and, hence, cannot be used to calculate respiration rates (Holm-Hansen, 1974). Measurement of the time required for transfer of carbon through the cellular carbon pool is critical for accurate estimations of net primary productivity. Steemann-Nielsen and Hansen (1959) report respiration rate as the intercept of productivity (in milligrams carbon per hour) at zero irradiance. Until analytical methods are devised, a calculated respiration value rather than a directly measured value will have to suffice when using the ^{14}C method.

Excretion.--Estimates of the percent of photosynthate products that are released as extracellular material range from 0 to 75 percent (Sharp, 1977). Refinements in technique (Smith, 1975) have resulted in the conclusion that

extracellular products, although a minor component of production [less than 10 percent (Mague and others, 1980)], are real and must be accounted for in accurate estimates of primary productivity. Traditional filtration techniques used in the ^{14}C method hindered the measurement of these substances. Excreted organic material passed through the filter and was discarded with the filtrate. Acidification and bubbling of 0.45-μm filtrate enables measurement of this component of production.

Duration of incubation.--The question of the optimal duration for incubation that would result in the most accurate measure of primary productivity is fundamental to the method. The answer depends on many factors and cannot be absolutely prescribed. As evidenced by the preceding discussion, the researcher must decide which is the most suitable incubation period based on the information desired and the limitations with which one is faced. To ensure the standardization and reliability of the data, a 4-hour incubation at midday (1000-1400 hours) is suggested for in-situ light- and dark-bottle methods. The oxygen or ^{14}C method then is chosen on the basis of the limits of measuring oxygen production in the water body in question during that 4-hour incubation.

The most common measures of photosynthesis are gross primary productivity and net primary productivity. The rate of passage of ^{14}C through the carbon cellular pool is of critical importance in determining whether gross or net productivity is being measured. The ^{14}C method cannot measure both types of productivity simultaneously. For short periods, before significant losses by excretion and respiration, gross rates of production will be measured (Hobson and others, 1976; Savidge, 1978). Incubation periods of at least 24 hours at intense light are required for the ^{14}C method to measure net productivity (Hobson and others, 1976).

Extrapolation from short-term incubations to long-term results must include the diel variability in primary productivity by natural populations. Barnett and Hirota (1967) and Malone (1971) reported variability throughout a day in ^{14}C retention by different groups of phytoplankton. Paerl and Mackenzie (1977) report different diurnal patterns of carbon fixation and loss between net phytoplankton and nanoplankton communities; whereas, MacCaull and Platt (1977) were unable to distinguish a diel rhythm in the rate of photosynthesis of coastal marine phytoplankton. The lack of uniformity and predictability in ^{14}C assimilation during short-term incubations limits the suitability of assessing long-term trends based on short-term incubations. MacCaull and Platt (1977) report that differences in estimates of daily productivity based on early morning or midday productivities were as much as four times. However, Schindler and Holmgren (1971) reported midday incubations to be satisfactory.

If short-term incubations are necessary, a correction similar to that proposed by Vollenweider (1965) should be applied to decrease the magnitude of the error. He reported that if one divided the light day (sunrise to sunset) into 5 equal periods (I to V), then 10, 31, 30, 22, and 7 percent of daily productivity occurred during light periods I through V, respectively. Estimation of total daily productivity from partial-day incubations can be made using the graph shown in figure 63.

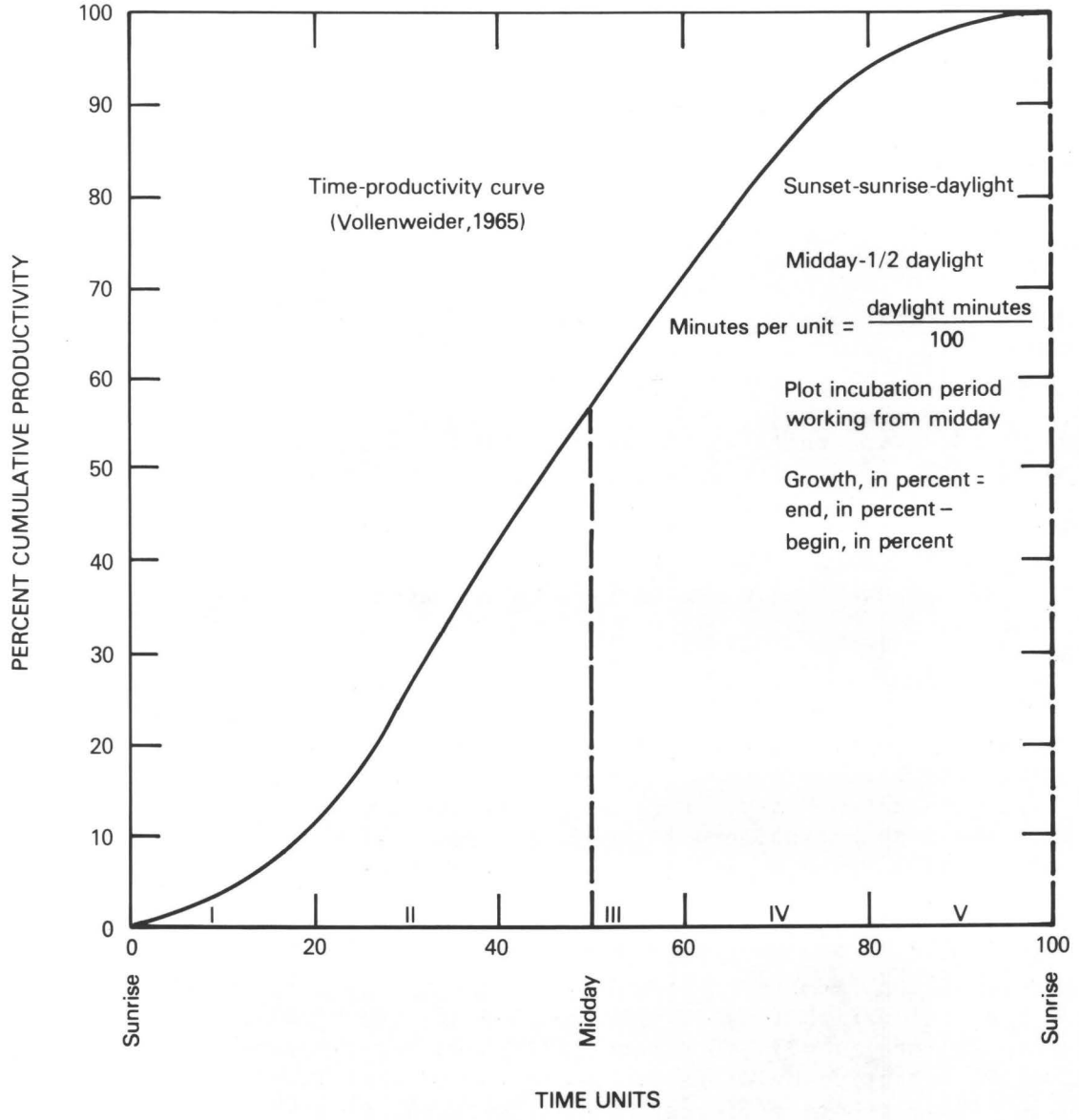

Figure 63.--Cumulative percentages for Vollenweider's five-period light day (modified from Janzer and others, 1973).

Example calculation:

Daylight period (sunrise to sunset):

0600 - 1800 hours = 12 hours = 720 minutes

$$\text{minutes per unit} = \frac{720}{100} = 7.2 \text{ minutes/time unit}$$

Incubation period, 1027 to 1427 hours:

0600 - 1027 hours = 4 hours 27 minutes = 267 minutes ÷ 7.2 = 37 time units
0600 - 1427 hours = 8 hours 27 minutes = 507 minutes ÷ 7.2 = 70 time units
37 time units = 38 percent cumulative productivity (from fig. 63)
70 time units = 85 percent cumulative productivity.

Growth, in percent = 85 percent - 38 percent = 47 percent. Alternatively, the correction proposed by Schindler and Holmgren (1971) that uses the ratio of solar radiation for the day to solar radiation during the incubation period is suggested.

Handling and disposal of radioactive wastes.--Radioactive ^{14}C (half-life 5,730 years) may be used in quantities as much as 100 µCi (1×10^{-6} Ci) specified by the license exempt provisions of Title 10, Part 30, Section 30.71 Schedule B, October 15, 1971, revision, "Rules of General Applicability to Licensing of Byproduct Materials," U.S. Atomic Energy Commission. Although the quantities used may be license exempt, all efforts should be made to minimize the release of ^{14}C to the environment and to avoid contamination of onsite and laboratory equipment.

The $^{14}CO_3$ and dissolved carbonate species remaining in solution after the phytoplankton have been removed by filtration are precipitated from the water as barium carbonate ($BaCO_3$) by mixing the filtrate with a solution of ammoniacal barium chloride ($BaCl_2 \cdot 2H_2O$) solution in a 20-L polyethylene waste carboy. After the waste solution has been added to the carboy, add 1N sodium carbonate (Na_2CO_3) solution to the waste to further scavenge $^{14}CO_3$ from solution. Calculate the maximum volume of 1N Na_2CO_3 needed using the following equation:

$$\text{Volume of } 1N\ Na_2CO_3 = 10.1\ [40.4 - (A_S \times V_W \times 0.00197)]\ ,$$

where 10 mL 1N Na_2CO_3 = 1 g $BaCO_3$;
40.4 g $BaCO_3$ = 50 g $BaCl_2 \cdot 2H_2O$ in polyethylene waste carboy;
A_S = sample alkalinity as calcium carbonate ($CaCO_3$), in milligrams per liter;
V_W = volume of waste in the carboy; and
0.00197 = factor to convert weight of $CaCO_3$, in milligrams, to grams $BaCO_3$.

Example: If a carboy contained 10 L of liquid waste that had an alkalinity of 85 mg/L, the volume, in milliliters of 1N Na_2CO_3 required to completely react with the 50 g $BaCl_2 \cdot 2H_2O$ added to the carboy, would be

Volume = 10.1 [40.4 - (85 × 10 × 0.00197)] = 391 mL required for total precipitation.

Scavenging of the ^{14}C from solution is more complete if the Na_2CO_3 solution is added in four or five volumes. The resulting $BaCO_3$ precipitate is allowed to settle before making the next addition of Na_2CO_3.

After settling, the $BaCO_3$ is separated by decantation of the supernatant. Add plaster of paris to the $BaCO_3$ slurry to form a solid block that is sent to the counting laboratory for disposal as radioactive waste. Retain the supernatant until a laboratory check of an aliquot by liquid-scintillation counting has indicated that the ^{14}C scavenge essentially was complete. The supernatant then may be discarded.

Oxygen Light- and Dark-Enclosure Method for Periphyton
(B-8040-85)

Parameters and Codes:

Productivity, primary, gross [mg(O_2/m^2)/d]:	70960
Productivity, primary, gross [mg(C/m^2)/d]:	70962
Productivity, primary, net [mg(O_2/m^2)/d]:	70964
Productivity, primary, net [mg(C/m^2)/d]:	70966
Respiration [mg(O_2/m^2)/d):	70968

1. Applications

The enclosure method of primary productivity is most suitable for shallow streams and for the littoral zones of lakes where light penetration is sufficient for photosynthesis. Best results are obtained in eutrophic water in which the production rate is about 3 to 200 mg(C/m^3)/h during the photoperiod (Strickland and Parsons, 1968, p. 263; Schindler and others, 1973).

2. Summary of method

Known areas of substrates containing living periphyton are isolated in sealed containers and filled with filtered stream or lake water of known dissolved-oxygen concentration. The samples are exposed in the euphotic zone, usually at the original depth, for a known period of time. Changes in the dissolved-oxygen concentrations of the enclosed samples are interpreted in terms of photosynthesis and respiration per unit area of periphyton.

3. Interferences

3.1 The method uses isolated periphyton samples to indicate the response of the natural system. Care must be used when collecting the sample, handling the sample, and exposing the sample to light to prevent interference with the life requirements of the organisms. Water-sampling equipment should be made of plastic or glass, and the essential metal parts should be made of stainless steel. Copper, brass, and bronze fittings should not be used. Samples of periphyton should be kept in the shade or in a circulating chamber before incubation to prevent exposure of unadapted algae to full sunlight. Light leaks into the dark chamber must be prevented.

3.2 The formation of bubbles in the experimental containers results in errors in the determination of dissolved-oxygen concentration changes. Air bubbles in circulating chambers result from two causes: (1) Incomplete filling of chambers, or (2) supersaturation. Extra care should be practiced initially to ensure that no trapped air bubbles are present in the chamber at the beginning of the experiment. Supersaturation also may be caused by warming of the sample between collection and filling or by excessive photosynthesis during the experiment. Supersaturation can be prevented by adjusting the length of the experimental period or by increasing the chamber size for light-bottle and dark-bottle studies.

3.3 Photosynthesis and respiration of phytoplankton in the water used to fill the circulating chambers can affect the results. This is prevented by filtering the water through a glass-fiber or membrane filter.

3.4 Microbial activity and chemical oxygen demand cause losses of dissolved oxygen when incubation times exceed a few hours. Interferences with the chemical determination of dissolved oxygen were described by Skougstad and others (1979) and the American Public Health Association and others (1985).

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies. All materials used must be free of agents that inhibit photosynthesis and respiration.

4.1 Artificial substrates made of glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.2 BOD bottles, numbered, 300 mL, Pyrex or borosilicon glass, that have flared necks and pointed ground-glass stoppers (Note 1).

Note 1: Before use, fill with acid cleaning solution and let stand for several hours. Rinse thoroughly using distilled water. Traces of iodine from the Winkler analysis should be removed by rinsing the bottles and stoppers using 0.01N sodium thiosulfate solution followed by thorough rinsing using distilled water. Do not use phosphorus-based detergents.

4.3 Collecting devices for the removal of periphyton from natural substrates. Three devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 18.

4.4 Dark box, preferably insulated, for storing filled BOD bottles until ready for incubation.

4.5 Equipment for determination of dissolved oxygen by the azide modification of the Winkler method (Skougstad and others, 1979; Golterman, 1982; American Public Health Association and others, 1985).

4.6 Filter flask, 1 or 2 L. For onsite use, a polypropylene flask is suggested.

4.7 Filter funnel, vacuum, 1.2-L capacity, stainless steel.

4.8 Glass-fiber filters, 47-mm diameter disks, or membrane filters, white, plain, 0.45-µm mean pore size, 47-mm diameter.

4.9 Light and dark circulating chambers of suitable size and shape, made of glass or plastic (McIntire and others, 1964; Wetzel, 1964, 1965; Thomas and O'Connell, 1966; Hansmann and others, 1971; Pfeifer and McDiffett, 1975; Rodgers and others, 1978; Gregory, 1980). Transparent containers can be made opaque by painting them black and covering the paint with overlapping strips of black plastic tape. The exposed parts of stoppers, if present, should be similarly blackened and covered with a hood of several layers of aluminum foil during use.

4.10 Polyethylene bottles, 8-L capacity that has cap and bottom tubulation.

4.11 Scraping devices, razor blades, stiff brushes, spatulas, or glass slides, for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.12 Vacuum pump, water-aspirator pump, or an electric vacuum pump for laboratory use; a hand-operated vacuum pump that has a gauge for onsite use.

4.13 Water-sampling bottle, Van-Dorn type. Depth-integrating samplers are described in Guy and Norman (1970).

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Acid cleaning solution, 20 percent. Mix 20 mL concentrated hydrochloric acid (HCl) (specific gravity 1.19) with distilled water and dilute to 100 mL.

5.2 Distilled or deionized water.

5.3 Filling water for the experimental circulating chambers. Prepare by filtering through a glass-fiber or a 0.45-μm membrane filter to remove plankton, unless it is known that plankton metabolism will be insignificant. Filter enough water to rinse and fill the chambers and to determine the initial concentration of dissolved oxygen. The water should be slightly undersaturated with dissolved oxygen. Dissolved oxygen may be decreased to 5 or 6 mg/L by passing the water through a sparging column (Hansmann and others, 1971) or by adding sodium sulfite with cobaltous chloride as a catalyst for the sulfite oxidation reaction (Pfeifer and McDiffett, 1975). For diel studies using large chambers, starting at dusk also will decrease the dissolved-oxygen concentration because periphyton metabolism occurs in the dark. This method requires continuous monitoring for dissolved-oxygen concentration because light and dark measurements are made sequentially in the same chamber.

5.4 Reagents for the azide modification of the Winkler method for dissolved oxygen (Skougstad and others, 1979; American Public Health Association and others, 1985).

5.5 Sodium thiosulfate solution, 0.01N. Dissolve 2.5 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in distilled water and dilute to 1 L.

6. Analysis

6.1 After suitable incubation, remove a sample of water from each circulating chamber and determine the dissolved-oxygen concentration. Average the results from duplicate samples.

7. Calculations

Primary productivity is expressed as the quantity of oxygen released or carbon assimilated per unit time. Respiration is expressed as the quantity of dissolved oxygen assimilated per unit time. Adjust the following calculated values for the appropriate incubation period. Gross or net primary productivity is calculated on the assumption that one atom of carbon is assimilated for each molecule (two atoms) of oxygen released. Average results from duplicate measurements.

7.1 Gross primary productivity $[mg(O_2/m^2)/\underline{t}]$

$$= \frac{(\underline{LC} - \underline{DC})\underline{V}}{\underline{tA}},$$

where $\underline{LC}$ = dissolved-oxygen concentration, in milligrams per liter, in the light circulating chamber after incubation;
$\underline{DC}$ = dissolved-oxygen concentration, in milligrams per liter, in the dark circulating chamber after incubation;
$\underline{V}$ = volume of water in the circulating chamber, in liters;
$\underline{t}$ = incubation period, in hours or days; and
$\underline{A}$ = area of periphyton-covered substrate, in square meters.

7.2 Gross primary productivity $[mg(C/m^2)/\underline{t}]$

$$= \frac{(\underline{LC} - \underline{DC})\underline{V}}{\underline{tA}} \times \frac{12}{32},$$

where $\underline{LC}$, $\underline{DC}$, $\underline{V}$, $\underline{t}$, and $\underline{A}$ = as in 7.1;
12 = atomic weight of carbon; and
32 = molecular weight of oxygen.

7.3 Net primary productivity $[mg(O_2/m^2)/\underline{t}]$

$$= \frac{(\underline{LC} - \underline{IC})\underline{V}}{\underline{tA}},$$

where $\underline{LC}$, $\underline{DC}$, $\underline{V}$, $\underline{t}$, and $\underline{A}$ = as in 7.1; and
$\underline{IC}$ = initial dissolved-oxygen concentration, in milligrams per liter, in the light circulating chamber before incubation.

7.4 Net primary productivity $[mg(C/m^2)/\underline{t}]$

$$= \frac{(\underline{LC} - \underline{IC})\underline{V}}{\underline{tA}} \times \frac{12}{32},$$

where $\underline{LC}$, $\underline{V}$, $\underline{t}$, and $\underline{A}$ = as in 7.1;
$\underline{IC}$ = as in 7.3; and
12 and 32 = as in 7.2.

7.5 Respiration $[mg(O_2/m^2)/\underline{t}]$

$$= \frac{(\underline{IC} - \underline{DC})\underline{V}}{\underline{tA}}$$

where $\underline{DC}$, $\underline{V}$, $\underline{t}$, and $\underline{A}$ = as in 7.1; and
$\underline{IC}$ = as in 7.3.

8. Reporting of results

Report primary productivity as follows: less than 10 mg, one decimal; 10 mg and greater, two significant figures.

9. Precision

No numerical precision values are available.

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Diel Oxygen-Curve Method for Estimating Primary Productivity and Community Metabolism in Streams
(B-8120-85)

Parameters and Codes:

Productivity, primary, gross [mg(O_2/m^3)/d]:	70959
Productivity, primary, gross [mg(O_2/m^2)/d]:	70960
Productivity, primary, net [mg(O_2/m^3)/d]:	70963
Productivity, primary, net [mg(O_2/m^2)/d]:	70964
Respiration [mg(O_2/m^3)/d]:	70967
Respiration [mg(O_2/m^2)/d]:	70968

Two analytical approaches are described for evaluating oxygen metabolism in streams. The graphical approach, developed for a hypothetical stream, provides an estimate of gross primary productivity, or the total quantity of oxygen produced during a diel (24-hour) period, and of total community respiration, or the total quantity of oxygen consumed during a diel period. Diel net primary productivity, or the oxygen that was not consumed, is calculated as the difference between gross productivity and total respiration. The graphical approach assumes that daytime respiration is constant or that it varies only linearly with time. This is the major limitation to the graphical approach.

The alternative analytical approach consists of data processing using a Fortran computer program (Program designation: Primary production, J330). A complete description of the program is in the user manual by Stephens and Jennings (1976). The program will calculate daytime net oxygen production and nighttime oxygen respiration for the single-station or the two-station analysis. The arithmetic difference between these is a 24-hour community metabolism that is equivalent to diel net primary productivity and should be entered into the computer using parameter code 70964. Other parameter codes are not compatible for any calculations made by program J330. Gross productivity is not calculated. Program J330 functions by assuming that production occurs only during daylight hours, and any change in dissolved oxygen that occurred during this period, after correcting for diffusion, is due to production. Any change in dissolved oxygen that occurred during hours of darkness, after correcting for diffusion, is due to respiration.

1. Applications

The method is applicable to streams in which the biological productivity is relatively intense. If the incoming water has a metabolic history similar to the outflowing water, the single-station analysis may be made. If the metabolic characteristics of the inflowing water are unknown or are not similar to the outflowing water, the two-station analysis should be made.

2. Summary of method

Dissolved-oxygen concentration and water temperature are determined in the open water continuously or at 1- to 3-hour intervals for at least 24 hours. Community primary productivity and respiration are estimated from rates of oxygen change after correction for the exchange of oxygen between the water and the atmosphere.

3. Interferences

3.1 Undetected advection, accrual of surface or ground water, and loss of oxygen from the water in the form of bubbles are possible sources of error. The limited sensitivity of this diel oxygen-curve method precludes its use in unproductive water. Limitations of dissolved-oxygen meters are that oxygen changes can be greater than 0.1 mg/L. Corresponding changes when using the Winkler method require a minimum of 0.02 mg/L. The diel oxygen-curve method should be used in water of comparative homogeneity.

3.2 In shallow, turbulent streams, the rate at which equilibrium is achieved between the water and the atmosphere is too rapid for the diel oxygen-curve method to be used. In these instances, a method based on the equililbrium between carbon dioxide, bicarbonate, and pH has been developed to measure photosynthesis and respiration (Wright and Mills, 1967).

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies. All materials used must be free of agents that inhibit photosynthesis and respiration.

4.1 Barometer for measuring local barometric pressure.

4.2 Floating-diffusion dome, clear Plexiglas, approximately 22 cm in diameter, or larger. Suitable domes are available from restaurant equipment suppliers. The device described by Hall (1971) consists of a 40.5-cm-diameter dome sealed onto a floating collar of 1-cm marine plywood (fig. 60). The oxygen and temperature sensors can be inserted from below into a support inside the dome or through holes in the dome. The dome is painted silver to decrease the greenhouse effect on the inside temperature.

4.3 Equipment for determination of dissolved oxygen by the azide modification of the Winkler method (Skougstad and others, 1979; Golterman, 1982; American Public Health Association and others, 1985).

4.4 Graph paper, 1-mm squares.

4.5 Recorder, portable, for continuous measurements of dissolved oxygen or for use with oxygen meters.

4.6 Stirrer, submersible, battery operated, for use with membrane-electrode oxygen instruments.

4.7 Thermistor or thermometer for determining water temperature and gas temperature in the diffusion dome. Most oxygen meters include thermistors suitable for making these measurements.

4.8 Water-sampling bottle, Van-Dorn type. Depth-integrating samplers are described in Guy and Norman (1970).

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Reagents required for the azide modification of the Winkler method for dissolved oxygen (Skougstad and others, 1979; American Public Health Association and others, 1985).

5.2 Sodium thiosulfate solution, 0.01N. Dissolve 2.5 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in distilled water, and dilute to 1 L.

6. Analysis

6.1 Single-station analysis. Using the data collected and following the procedures in the "Single-Station Analysis" subsection of the "Primary Productivity" section, tabulate time versus temperature and dissolved-oxygen concentration as listed in table 15, columns 1 through 3, and plot curves as in figure 64A and B . Graph paper that has 1-mm squares is convenient to use for these plots.

6.2 Determine the percentage saturation for each dissolved-oxygen value using tables indicating oxygen solubility at various temperatures, pressures, and salinities (Mortimer, 1981; American Public Health Association and others, 1981). Tabulate the values in table 15, column 6, and plot a curve of time versus measured percentage of dissolved-oxygen saturation as shown in figure 64.

6.3 Using the measured dissolved-oxygen-concentration data (table 15, col. 3), determine the hourly rate of change in dissolved oxygen (milligrams per liter per hour) by subtracting successive pairs of dissolved-oxygen values. Tabulate the values, and plot the rate curve from the values in table 15, column 4, and as shown in figure 64D (curve labeled "Before correction for diffusion").

6.4 Subtract each percentage-saturation value determined in 6.2 from 100 percent, recording values less than 100 as negative. List these percentage-saturation deficits as in table 15, column 7. Proceed to 6.9 or 6.10 depending on the method used to determine the diffusion rate. If area-based gas transfer coefficient, K, is estimated, proceed to 6.12.

6.5 Two-station analysis. Using the data collected and following the procedures in the "Two-Station Analysis" subsection of the "Primary Productivity" section, determine the average dissolved-oxygen concentration and average temperature for the reach between stations for each sample interval. Tabulate time versus average temperature and time versus average dissolved-oxygen concentration as listed in table 15, columns 1 through 3. Plot curves as in figure 64A and B. Graph paper that has 1-mm squares is convenient to use for these plots.

Table 15.--Hypothetical data for determining community primary productivity of a stream by the oxygen-curve method

[The mean depth of flow is 1.2 meters; the gas transfer coefficient on a volume basis, $\underline{k}$, is 2.67 grams per cubic meter per hour at 100-percent saturation deficit; h, hours; °C, degrees Celsius; mg/L, milligrams per liter; (mg/L)/h, milligrams per liter per hour; $(g/m^3)/h$, grams per cubic meter per hour]

		Dissolved oxygen						
1	2	3	4	5	6	7	8	9
Time (h)	Temperature (°C)	Measured (mg/L)[1]	Rate of change [(mg/L)/h]	Concentrations at saturation (mg/L)	Measured saturation (percent)	Average saturation deficit, $\underline{S}$ (percent)	$\frac{\underline{S} \times \underline{k}}{100}$ $[(g/m^3)/h]$	Corrected rate of change $[(g/m^3)/h]$
0000	29.5	6.00		7.7	78			
			-0.05			-23.0	-0.614	-0.664
0100	29.0	5.95		7.8	76			
			-.05			-24.5	-.654	-.704
0200	28.0	5.90		7.9	75			
			-.05			-26.5	-.708	-.758
0300	27.0	5.85		8.1	72			
			-.05			-29.0	-.774	-.824
0400	25.5	5.80		8.3	70			
			+.10			-30.0	-.801	-.701
0500	25.0	5.90		8.4	70			
			.00			-28.5	-.761	-.761
0600	27.0	5.90		8.1	73			
			+.40			-23.5	-.627	-.227
0700	28.0	6.30		7.9	80			
			+.55			-15.0	-.400	+.150
0800	30.0	6.85		7.6	90			
			+1.00			-7.5	-.200	+.800
0900	31.0	7.85		7.5	105			
			+.95			+11.5	+.307	+1.257
1000	31.5	8.80		7.4	118			
			+.60			+22.5	+.601	-1.201
1100	32.0	9.40		7.4	127			
			+.65			+32.0	+.854	-1.504
1200	32.5	10.05		7.4	137			
			+.45			+41.0	+1.095	+1.545
1300	33.5	10.50		7.2	145			
			+.10			+45.0	+1.202	+1.302
1400	33.0	10.60		7.3	145			
			-.15			+43.5	+1.161	+1.011
1500	32.5	10.45		7.4	142			
			-.25			+38.5	+1.028	+.778

Table 15.--Hypothetical data for determining community primary productivity of a stream by the oxygen-curve method--Continued

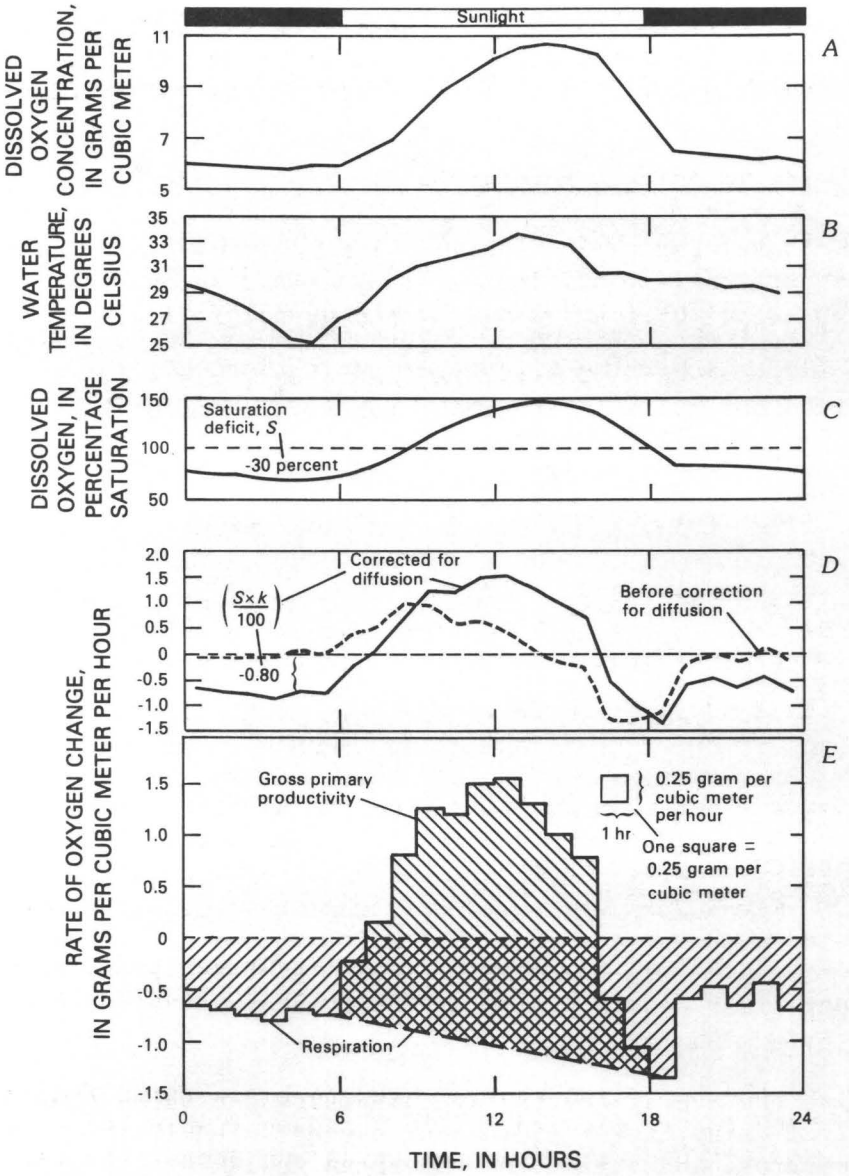
		Dissolved oxygen						
1	2	3	4	5	6	7	8	9
Time (h)	Temperature (°C)	Measured (mg/L)[1]	Rate of change [(mg/L)/h]	Concentrations at saturation (mg/L)	Measured saturation (percent)	Average saturation deficit, S (percent)	$\frac{S \times k}{100}$ [(g/m^3)/h]	Corrected rate of change [(g/m^3)/h]
1600	30.5	10.20		7.6	135			
			-1.30			+26.5	+0.708	-0.592
1700	30.5	8.90		7.6	118			
			-1.30			+9.0	+.240	-1.060
1800	30.0	7.60		7.6	100			
			-1.15			-7.5	-.200	-1.350
1900	30.0	6.45		7.6	85			
			-.15			-16.0	-.427	-.577
2000	30.0	6.30		7.6	83			
			.00			-17.5	-.467	-.467
2100	29.5	6.30		7.7	82			
			-.15			-19.0	-.507	-.657
2200	29.5	6.15		7.7	80			
			+.10			-20.0	-.534	-.434
2300	29.0	6.25		7.8	80			
			-.15			-21.0	-.561	-.711
2400	29.0	6.10		7.8	78			

[1]Milligrams per liter equals grams per cubic meter.

6.6 Determine the average percentage of dissoved-oxygen saturation for each sample interval using tables indicating oxygen solubility at various temperatures, pressures, and salinities (American Public Health Association and others, 1985). Tabulate the values in table 15, column 6, and plot a curve of time versus average percentage of dissolved-oxygen saturation as shown in figure 64C.

6.7 Using the average dissolved-oxygen-concentration data for the reach (table 15, col. 3), determine the average hourly rate of change in dissolved oxygen (milligrams per liter per hour) by subtracting successive pairs of oxygen values. Tabulate the values, and plot the rate curve from the values in table 15, column 4, and as shown in figure 64D (curve labeled "Before correction for diffusion").

6.8 Subtract each average percentage-saturation value determined in 6.6 from 100 percent, recording values less than 100 as negative. List these average percentage-saturation deficits as in table 15, column 7. Proceed to 6.9, 6.10, or 6.13 depending on the method used to determine the diffusion rate. If K is estimated, proceed to 6.12.



Diffusion correction calculations:

K = 3.2 grams per square meter per hour

$$k = \frac{K}{z} = \frac{\text{3.2 grams per square meter per hour}}{\text{1.2 meters}} = \text{2.67 grams per cubic meter per hour}$$

$$\frac{S \times k}{100} = \frac{-30 \times 2.67}{100} = \text{-0.80 gram per cubic meter per hour}$$

$$\text{Gross productivity} = \frac{(81.3 \text{ squares})}{\text{day}} \text{ (0.25 gram per cubic meter) (1.2 meters)} = \text{24.4 grams per square meter per day}$$

$$\text{Community respiration} = \frac{(84.1 \text{ squares})}{\text{day}} \text{ (0.25 gram per cubic meter) (1.2 meters)} = \text{25.2 grams per square meter per day}$$

Figure 64.--Diel oxygen curve and supported data (from tables 14 and 15) for determining community primary productivity and community respiration of a stream by the oxygen-curve method. The mean depth of flow is 1.2 meters, the gas transfer coefficient on an area basis, K, is 3.2 grams per square meter per hour, and on a volume basis, k, is 2.67 grams per cubic meter per hour at 100-percent saturation deficit (modified from Odum and Hoskin, 1958).

6.9 Determine the volume-based gas transfer coefficient, k, for each sample interval from measurements of the hydraulic parameters. The following procedure is adapted from Hall (1971) for k derived from volume-based gas transfer coefficient per day, k_2. Thus, from Churchill and others (1962),

$$k_2 \text{ (at 20 °C)} = 5.026 V^{0.969} R^{-1.673} ,$$

where k_2 = volume-based gas transfer coefficient per day;
V = cross-sectional mean velocity, in feet per second; and
R = hydraulic radius (approximately the depth of flow), in feet.

Using a known dissolved-oxygen-saturation value for a specific time, Hall (1971) obtained the following equation for k in terms of k_2:

$$k = \frac{2.3\ (k_2 C_s)}{24} ,$$

where k = volume-based gas transfer coefficient, in grams per cubic meter per hour, and is for a 100-percent saturated deficit; and
C_s = the 100-percent saturation deficit, in grams per cubic meter.

The 2.3 converts the k_2 defined in terms of $\log_{10}$ to k defined in terms of $\log_e$.

For temperatures other than 20 °C, correct to k_2 at a rate of 2.41-percent increase or decrease per degree above or below 20 °C. Estimate k for the study period by averaging the k values determined for each sampling interval (Note 1). Proceed to 6.14.

Note 1: Some situations require use of different gas transfer coefficients at different times of day as explained in the "Diffusion Rate" subsection.

6.10 Determine the diffusion rate, D, for each nighttime sample interval from measurements made in the floating-diffusion dome (table 14). Calculate the volume of oxygen in the dome at the beginning and end of the sample interval as follows:

$$V_t = V_d (0.21) \frac{F_t}{100} ,$$

where V_t = volume of oxygen, in milliliters, in the dome at a specific time, t;
V_d = volume of atmospheric gases, in milliliters, in the dome;
F_t = percentage oxygen saturation in the dome atmosphere at time, t, when fresh air equals 100-percent oxygen saturation; and
0.21 = fractional volume of oxygen in the air.

Indicate the concentration of oxygen in the floating-diffusion dome in terms of standard temperature and pressure for each sample interval using the equation

$$\Delta\underline{V} = \frac{273\underline{V}_0}{273 + \underline{T}_0} - \frac{273\underline{V}_1}{273 + \underline{T}_1} ,$$

where $\Delta\underline{V}$ = change in volume of oxygen, in milliliters, in the dome at standard temperature and pressure;
$\underline{V}_0$ = volume of oxygen, in milliliters, in the dome at the beginning of the interval;
$\underline{T}_0$ = temperature, in degrees Celsius, in the dome at the beginning of the interval;
$\underline{V}_1$ = volume of oxygen, in milliliters, in the dome at the end of the interval;
$\underline{T}_1$ = temperature, in degrees Celsius, in the dome at the end of the interval; and
273 = factor for converting to absolute temperature.

Oxygen weighs 0.00143 g/mL at standard temperature and pressure. Therefore, $\underline{D}$ may be computed from

$$\underline{D} = \frac{(\Delta\underline{V})(0.00143)}{\underline{A}(\Delta\underline{t})} ,$$

where $\underline{D}$ = rate of diffusion of oxygen into the water, in grams per square meter per hour;
$\underline{A}$ = area of the dome, in square meters, that is in contact with the water surface; and
$\Delta\underline{t}$ = time interval, in hours, between the two measurements.

6.11 Using the following equation, convert the area-based rate of diffusion for each sampling interval to a value at 0-percent saturation of the water (rate of diffusion if the water contained no oxygen) by dividing $\underline{D}$ by the average percentage-saturation deficit during the time of measurement, or

$$\underline{K} = \frac{\underline{D}\ (100)}{\underline{S}} ,$$

where $\underline{K}$ = area-based gas transfer coefficient, in grams per square meter per hour, at 0-percent saturation (100-percent saturation deficit); and
$\underline{S}$ = average percentage-saturation deficit between the water and the air during the sample interval (derived from 6.4 to 6.8).

6.12 Convert each area value to a volume value by dividing by the mean depth of water, in meters, or

$$\underline{k} = \frac{\underline{K}}{\underline{z}} ,$$

where k = volume-based gas transfer coefficient, in grams per cubic meter per hour, at 0-percent saturation; and
z = mean depth, in meters.

Estimate k for the study period by averaging the k values determined for each sampling interval (Note 2). Proceed to 6.14.

Note 2: Some situations require use of different diffusion constants at different times of day.

6.13 Determine the average k for each sample interval from measurements of the nighttime average rate of oxygen change. This can be estimated by calculating k values for each nighttime sampling interval using the Odum (1956) method as presented by Eley (1970):

$$k = \frac{q_n - q_n + 1}{S_n - S_n + 1},$$

where q_n = average rate of change in oxygen, in grams per cubic meter, for the reach at nighttime, n;
$q_n + 1$ = average rate of change in oxygen, in grams per cubic meter, for the reach at nighttime, $n + 1$;
S_n = average oxygen-saturation deficit for the reach at nighttime, n; and
$S_n + 1$ = average oxygen-saturation deficit for the reach at nighttime, $n + 1$.

Proceed to 6.14.

6.14 Determine the quantity of oxygen (grams per cubic meter) gained or lost by diffusion during each sampling interval. To adjust for atmospheric reaeration, multiply the average k (from 6.9, 6.12, or 6.13) by each percentage oxygen-saturation deficit value (from 6.4 or 6.8), and divide by 100 to convert percentage to fractional values. List these values as in table 15, column 8.

6.15 Using figure 64D, the hourly rate-of-change graph plotted as directed in 6.3 or 6.7, prepare a corrected rate-of-change curve by adding or subtracting, graphically, the quantity of oxygen, in grams per cubic meter, gained or lost by diffusion during each sampling interval (from 6.14). Draw the curve as in figure 64D (curve labeled "Corrected for diffusion"). The corrected rate-of-change curve is replotted as a step function to facilitate graphical integration as shown in figure 64E.

6.16 Connect a line between the presunrise and postsunset negative rate-of-change points on the corrected rate-of-change curve as shown in figure 64E (Odum and Wilson, 1962). This line is an estimate of daytime respiration (Note 3).

Note 3: The maximum rate of respiration often occurs immediately after sunset, and the rate declines to a minimum before sunrise. Where presunrise and postsunset respiration differ, connect the line diagonally from the dawn-

respiration rate to the sunset-respiration rate on the corrected rate-of-change graph. The values for respiration and gross primary productivity are affected by the placement of the respiration line. The accuracy of the method probably is limited by this step (Odum and Hoskin, 1958, p. 22). Graphs in which the rates of change are very irregular enable more subjectivity of choice of the respiration line than do smooth curves.

7. Calculations

The following volume- or concentration-based calculations, in grams per cubic meter per day, can be converted to area-based calculations, in grams per square meter per day, by multiplying by the average water depth of the study area, in meters.

7.1 An estimate of gross primary productivity, P_g, in grams oxygen per cubic meter per day, is the area above the daytime respiration line and below the daytime rate-of-change line (fig. 64E). The area may be determined from the plot by counting the graph-paper squares and multiplying by the value, in grams per cubic meter, of one square.

7.2 An estimate of community respiration, R_t, in grams oxygen per cubic meter per day, is the area above the nighttime negative rate-of-change line and the daytime respiration line and below the zero rate-of-change line (fig. 64E). The area may be determined from the plot by counting the graph-paper squares and multiplying by the value, in grams per cubic meter, of one square. The graphical procedure integrates the hourly values during a 24-hour period; hence, the respiration rate is on a per-day basis.

7.3 An estimate of net primary productivity, P_n, in grams oxygen per cubic meter per day, is the difference between P_g and R_t.

7.4 An index of the trophic nature of the community may be calculated as the ratio of photosynthetic productivity to respiration, P:R. Communities having a P:R ratio less than 1 have an excess of respiration compared to productivity. They are heterotrophic; that is, they degrade organic compounds through oxygen metabolism at a greater rate than they fix carbon in photosynthesis. Autotrophic communities have a P:R ratio greater than 1 and release more oxygen through photosynthesis than they consume through respiration.

8. Reporting of results

Report community primary productivity and respiration, in milligrams, as follows: less than 10 mg, one decimal; 10 mg or more, two significant figures.

9. Precision

No numerical precision data are available.

10. References cited

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Diel Oxygen-Curve Method for Estimating Primary Productivity and Community Metabolism in Stratified Water
(B-8100-85)

Parameters and Codes:

Productivity, primary, gross [mg(O_2/m^3)/d]:	70959
Productivity, primary, gross [mg(O_2/m^2)/d]:	70960
Productivity, primary, net [mg(O_2/m^3)/d]:	70963
Productivity, primary, net [mg(O_2/m^2)/d]:	70964
Respiration [mg(O_2/m^3)/d]:	70967
Respiration [mg(O_2/m^2)/d]:	70968

If complete vertical mixing occurs in the water body, a series of single-station analyses may be sufficient to characterize the oxygen regime in the water. However, in many places, the water may be stratified, and a vertical dissolved-oxygen variation from near saturation at the surface to near zero concentration at the bottom may exist. If these conditions do exist, production of oxygen may be limited to the euphotic zone, and an oxygen deficit could exist in the lower or hypolimnetic water.

Two analytical approaches for evaluating oxygen metabolism in stratified water are described and contrasted using synthetic data for a hypothetical lake. The graphical approach provides an estimate of gross primary productivity, or the total quantity of oxygen produced during a diel (24-hour) period, and of total community respiration, or the total quantity of oxygen consumed during a diel period. Diel net primary productivity, or the oxygen that was not consumed, is calculated as the difference between gross productivity and total respiration. The graphical approach assumes that daytime respiration is constant or that it varies only linearly with time. This is the major limitation to the graphical approach.

The alternative analytical approach consists of data processing using a Fortran computer program (Program designation: Primary production, J330). A complete description of the program is in the user manual by Stephens and Jennings (1976). The program will calculate daytime net oxygen production and nighttime oxygen respiration for the single-station or two-station analysis. The arithmetic difference between these is a 24-hour community metabolism that is equivalent to diel net primary productivity and should be entered into the computer using parameter code 70964. Other parameter codes are not compatible for any calculations made by program J330. Gross productivity is not calculated. Program J330 functions by assuming that production occurs only during daylight hours, and any change in dissolved oxygen that occurred during this period, after correcting for diffusion, is due to production. Any change in dissolved oxygen that occurred during hours of darkness, after correcting for diffusion, is due to respiration. The program also enables exchange between the horizontal segments of a stratified water body using estimated or measured vertical-dispersion coefficients.

1. Applications

The method is applicable to eutrophic estuaries, lakes, and other stratified bodies of water in which a vertical variation in dissolved oxygen exists. The lower limit for measurable oxygen production occurs when phytoplankton densities, expressed as chlorophyll _a_, are less than 1 mg/m^3 (Talling, 1974).

2. Summary of method

From average values for temperature, dissolved oxygen, and, if appropriate, salinity, an average rate of change in dissolved oxygen is calculated for the entire water body. Average dissolved-oxygen values for the surface-water layer are corrected for diffusion. The resulting curve of diel changes in the in-situ concentration of dissolved oxygen, mainly due to photosynthesis and respiration, is used to estimate the primary productivity of the entire aquatic-plant community.

3. Interferences

Undetected advection, accrual of surface or ground water, and loss of oxygen from the water in the form of bubbles are possible sources of error. The limited sensitivity of this diel oxygen-curve method precludes its use in unproductive water. Limitations of dissolved-oxygen meters are that oxygen changes can be greater than 0.1 mg/L. Corresponding changes when using the Winkler method require a minimum of 0.02 mg/L. The method should be used in water of comparative horizontal homogeneity.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies. All materials used must be free of agents that inhibit photosynthesis and respiration.

4.1 Barometer for measuring local barometric pressure.

4.2 Floating-diffusion dome, clear Plexiglas, approximately 22 cm in diameter, or larger. Suitable domes are available from restaurant equipment suppliers. The device described by Hall (1971) consists of a 40.5-cm-diameter dome sealed onto a floating collar of 1-cm marine plywood (fig. 60). The oxygen and temperature sensors can be inserted from below into a support inside the dome or through holes in the dome. The dome is painted silver to decrease the greenhouse effect on the inside temperature.

4.3 Equipment for determination of dissolved oxygen by the azide modification of the Winkler method (Skougstad and others, 1979; Golterman, 1982; American Public Health Association and others, 1985).

4.4 Equipment for determination of salinity by titration (Strickland and Parsons, 1968) or by electrical conductivity, if appropriate.

4.5 Graph paper, 1-mm squares.

4.6 Polar planimeter and maps appropriate to the study (see 6.1).

4.7 Thermistor or thermometer for determining water temperature and gas temperature in the diffusion dome. Most oxygen meters include thermistors suitable for making these measurements.

4.8 Underwater light-measurement equipment. A quantum/radiometer/photometer measures photosynthetically active radiation (400-700 nm). If a submersible photometer is not available, a Secchi disc may be used.

4.9 Water-sampling bottle, Van-Dorn type. Depth-integrating samplers are described in Guy and Norman (1970).

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Reagents required for the azide modification of the Winkler method for dissolved oxygen (Skougstad and others, 1979; American Public Health Association and others, 1985).

5.2 Sodium thiosulfate solution, 0.01N. Dissolve 2.5 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$ in distilled water, and dilute to 1 L.

5.3 Reagents for determination of salinity (Strickland and Parsons, 1968), if appropriate.

6. Analysis

6.1 Lake morphometry. The volume of water contained in a lake may be calculated from measurements of each depth contour on a good topographic or bathymetric map. An accurate, scaled map and planimeter are required. Winter (1981) describes errors in bathymetric map drawing. Determine the area enclosed within each contour interval using a planimeter. Typically, the planimeter will indicate area, in square inches (or centimeters), that then must be converted to actual area using the map scale. A small lake (fig. 65) was planimetered to obtain the morphometric data in table 16. Using the map scale of 1:250,000, the actual area represented by 1 in^2 of map was calculated to be 6.25×10^{10} in^2. This value, when divided by the number of square inches in a square mile (4.01×10^9), provides the factor (15.59) used to calculate the actual surface area of each contour. Conversion to metric units is made using the relation 1 mi^2 equals 2.59×10^6 m^2 (table 16, col. 3).

The volume of each contour (table 16, col. 4) is calculated as

$$V_{n-m} = 1/3\ (A_m + A_n + A_m A_n)(n - m)\ ,$$

where V_{n-m} = the volume of a given element between contour n and contour m, in cubic meters;

A_m = the area at contour m, in square meters;

A_n = the area at contour n, in square meters; and

$n - m$ = the interval between contour n and contour m, in meters.

Total lake volume is the summation of all element volumes.

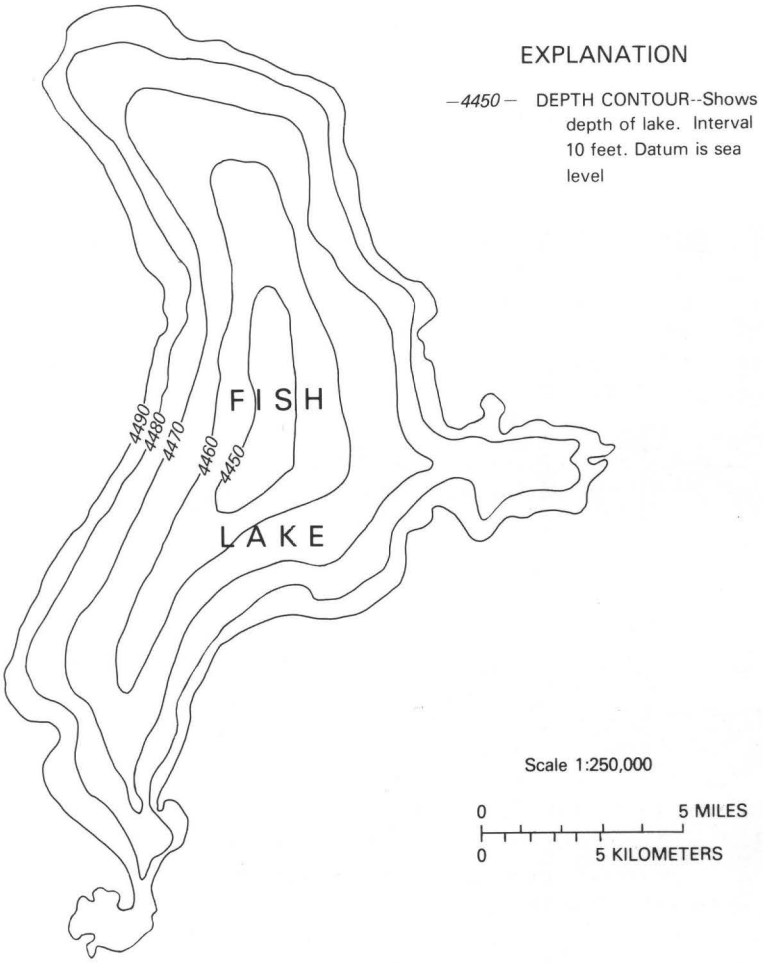

Figure 65.--Fish Lake, used in morphometric analysis.

6.2 From the data collected, average the temperature, dissolved oxygen, and, if appropriate, salinity values at each depth interval (table 17) for several stations to eliminate the effects of horizontal heat and solute exchange. Tabulate time versus surface dissolved-oxygen concentration and temperature. These surface dissolved-oxygen values are to be corrected for diffusion as described below. Tabulate average dissolved-oxygen values for each remaining depth interval as in table 17, column 3. These values are not corrected for diffusion. Proceed from 6.3 through 6.12 for the graphical-analysis procedure.

6.3 Graphical analysis. Determine the percentage saturation for each average surface dissolved-oxygen value using tables indicating oxygen solubility at various temperatures, pressures, and salinities (American Public Health Association and others, 1985). Tabulate the values in table 17, column 6, and plot a curve of time versus measured percentage surface dissolved-oxygen saturation as shown in figure 64C.

Table 16.--Morphometric data and results of graphical analysis of community primary productivity and respiration for Fish Lake

[Area values: gross primary productivity, 78.98 grams per square meter per day; respiration, 81.29 grams per square meter per day; net primary productivity, -2.31 grams per square meter per day; productivity/respiration, 0.972; ----, not applicable]

1 Lake slice (depth interval equals 3 meters)	2 Elevation (feet)	3 Area ($\times 10^8$ square meters)	4 Volume ($\times 10^8$ cubic meters)	5 Gross primary productivity (grams per cubic meter per day)	6 Lake slice gross primary productivity ($\times 10^8$ grams per cubic meter per day)	7 Respiration (grams per cubic meter per day)	8 Lake slice respiration ($\times 10^8$ grams per cubic meter per day)
Surface	4,490	3.83	----	----	----	----	----
1	4,480	2.81	9.37	20.33	190.49	21.03	197.05
2	4,470	1.82	6.89	9.13	62.91	9.18	63.25
3	4,460	.75	4.24	9.00	38.16	10.05	42.61
4	4,450	.15	1.88	5.30	9.96	4.48	8.42
Total	----	----	----	----	301.5	----	311.33

Table 17.--Hypothetical data for determining community primary productivity for each individual depth in a lake by the oxygen-curve method

[The gas transfer coefficient on an area basis, K, is 3.2 grams per square meter per hour, and on a volume basis, k, is 2.67 grams per cubic meter per hour at 100-percent saturation deficit; h, hours; °C, degrees Celsius; mg/L, milligrams per liter; (mg/L)/h, milligrams per liter per hour; $(g/m^3)/h$, grams per cubic meter per hour]

		Dissolved oxygen						
1	2	3	4	5	6	7	8	9
Time (h)	Temperature (°C)	Measured (mg/L)[1]	Rate of change [(mg/L)/h]	Concentrations at saturation (mg/L)	Measured saturation (percent)	Average saturation deficit, S (percent)	$\frac{S \times k}{100}$ [$(g/m^3)/h$]	Corrected rate of change [$(g/m^3)/h$]
0000	29.5	6.00		7.7	78			
			-0.05			-23.0	-0.614	-0.664
0100	29.0	5.95		7.8	76			
			-.05			-24.5	-.654	-.704
0200	28.0	5.90		7.9	75			
			-.05			-26.5	-.708	-.758
0300	27.0	5.85		8.1	72			
			-.05			-29.0	-.774	-.824
0400	25.5	5.80		8.3	70			
			+.10			-30.0	-.801	-.701
0500	25.0	5.90		8.4	70			
			.00			-28.5	-.761	-.761
0600	27.0	5.90		8.1	73			
			+.40			-23.5	-.627	-.227
0700	28.0	6.30		7.9	80			
			+.55			-15.0	-.400	+.150
0800	30.0	6.85		7.6	90			
			+1.00			-7.5	-.200	+.402
0900	31.0	7.85		7.5	105			
			+.95			+11.5	+.307	+1.257
1000	31.5	8.80		7.4	118			
			+.60			+22.5	+.601	+1.201
1100	32.0	9.40		7.4	127			
			+.65			+32.0	+.854	+1.504
1200	32.5	10.05		7.4	137			
			+.45			+41.0	+1.095	+1.545
1300	33.5	10.50		7.2	145			
			+.10			+45.0	+1.202	+1.302
1400	33.0	10.60		7.3	145			
			-.15			+43.5	+1.161	+1.011
1500	32.5	10.45		7.4	142			
			-.25			+38.5	+1.028	+.778

Table 17.--Hypothetical data for determining community primary productivity for each individual depth in a lake by the oxygen-curve method--Continued

		Dissolved oxygen						
1	2	3	4	5	6	7	8	9
Time (h)	Temperature (°C)	Measured (mg/L)[1]	Rate of change [(mg/L)/h]	Concentrations at saturation (mg/L)	Measured saturation (percent)	Average saturation deficit, S (percent)	$\frac{S \times k}{100}$ [(g/m^3)/h]	Corrected rate of change [(g/m^3)/h]
1600	30.5	10.20		7.6	135			
			-1.30			+26.5	+0.708	-0.592
1700	30.5	8.90		7.6	118			
			-1.30			+9.0	+.240	-1.060
1800	30.0	7.60		7.6	100			
			-1.15			-7.5	-.200	-1.350
1900	30.0	6.45		7.6	85			
			-.15			-16.0	-.427	-.577
2000	30.0	6.30		7.6	83			
			.00			-17.5	-.467	-.467
2100	29.5	6.30		7.7	82			
			-.15			-19.0	-.507	-.657
2200	29.5	6.15		7.7	80			
			+.10			-20.0	-.534	-.434
2300	29.0	6.25		7.8	80			
			-.15			-21.0	-.561	-.711
2400	29.0	6.10		7.8	78			

[1]Milligrams per liter equals grams per cubic meter.

6.4 Using the surface dissolved-oxygen-concentration data (table 17) determine the hourly rate of change in dissolved oxygen (milligrams per liter per hour) by subtracting successive pairs of dissolved-oxygen values. Tabulate the values, and plot the rate curve from the values in table 17, column 4, and as shown in figure 64D (curve labeled "Before correction for diffusion").

6.5 Subtract each percentage-saturation value determined in 6.3 from 100 percent, recording values less than 100 as negative. List these percentage-saturation deficits as in table 17, column 7. Proceed to 6.6 or 6.7 depending on the method used to determine the diffusion rate. If area-based transfer coefficient, K, is estimated, proceed to 6.8.

6.6 Determine the diffusion rate, $\underline{D}$, for each nighttime sample interval from measurements made in the floating-diffusion dome (table 14). Calculate the volume of oxygen in the dome at the beginning and end of the sample interval as follows:

$$\underline{V}_{\underline{t}} = \underline{V}_{\underline{d}}(0.21)\frac{\underline{F}_{\underline{t}}}{100},$$

where $\underline{V}_{\underline{t}}$ = volume of oxygen, in milliliters, in the dome at a specific time, $\underline{t}$;
$\underline{V}_{\underline{d}}$ = volume of atmospheric gases, in milliliters, in the dome;
$\underline{F}_{\underline{t}}$ = percentage oxygen saturation in the dome atmosphere at time, $\underline{t}$, when fresh air equals 100-percent oxygen saturation; and
0.21 = fractional volume of oxygen in the air.

Indicate the concentration of oxygen in the floating-diffusion dome in terms of standard temperature and pressure for each sample interval using the equation

$$\Delta\underline{V} = \frac{273\underline{V}_0}{273 + \underline{T}_0} - \frac{273\underline{V}_1}{273 + \underline{T}_1},$$

where $\Delta\underline{V}$ = change in volume of oxygen, in milliliters, in the dome at standard temperature and pressure;
$\underline{V}_0$ = volume of oxygen, in milliliters, in the dome at the beginning of the interval;
$\underline{T}_0$ = temperature, in degrees Celsius, in the dome at the beginning of the interval;
$\underline{V}_1$ = volume of oxygen, in milliliters, in the dome at the end of the interval;
$\underline{T}_1$ = temperature, in degrees Celsius, in the dome at the end of the interval; and
273 = factor for converting to absolute temperature.

Oxygen weighs 0.00143 g/mL at standard temperature and pressure. Therefore, $\underline{D}$ may be computed from

$$\underline{D} = \frac{(\Delta\underline{V})(0.00143)}{\underline{A}(\Delta\underline{t})},$$

where $\underline{D}$ = rate of diffusion of oxygen into the water, in grams per square meter per hour;
$\underline{A}$ = area of the dome, in square meters, that is in contact with the water surface; and
$\Delta\underline{t}$ = time interval, in hours, between the two measurements.

Proceed to 6.8.

6.7 Determine the volume-based gas transfer coefficient, $\underline{k}$, for each sample interval from measurements of the nighttime rate of oxygen change. This can be estimated by calculating $\underline{k}$ values for each nighttime surface sampling interval using the Odum (1956) method as presented by Eley (1970):

$$\underline{k} = \frac{\underline{q}_{\underline{n}} - \underline{q}_{\underline{n}} + 1}{\underline{S}_{\underline{n}} - \underline{S}_{\underline{n}} + 1},$$

where $\underline{k}$ = volume-based gas transfer coefficient for oxygen, in grams per cubic meter per hour, at 0-percent saturation;
$\underline{q}_{\underline{n}}$ = rate of change of the surface oxygen, in grams per cubic meter, at nighttime, $\underline{n}$;
$\underline{q}_{\underline{n}} + 1$ = rate of change of the surface oxygen, in grams per cubic meter, at nighttime, $\underline{n} + 1$;
$\underline{S}_{\underline{n}}$ = oxygen-saturation deficit for the surface water at nighttime, $\underline{n}$; and
$\underline{S}_{\underline{n}} + 1$ = oxygen-saturation deficit for the surface water at nighttime, $\underline{n} + 1$.

Proceed to 6.9.

6.8 Using the following equation, convert the area-based rate of diffusion for each sampling interval to a value at 0-percent saturation of the water (rate of diffusion if the water contained no oxygen) by dividing $\underline{D}$ by the average percentage-saturation deficit during the time of measurement, or

$$\underline{K} = \frac{\underline{D}(100)}{\underline{S}},$$

where $\underline{K}$ = area-based gas transfer coefficient, in grams per square meter per hour, at 0-percent saturation (100-percent saturation deficit); and
$\underline{S}$ = average percentage-saturation deficit between the water and the air during the sample interval (derived from 6.5).

6.9 Convert each area value to a volume value by dividing by the depth of water, in meters, for the surface interval, or

$$\underline{k} = \frac{\underline{K}}{\underline{z}},$$

where $\underline{z}$ = depth, in meters, of the surface interval.

Estimate $\underline{k}$ for the study period by averaging the $\underline{k}$ values determined for each sampling interval (Note 1). Proceed to 6.10.

Note 1: Some situations require use of different diffusion constants at different times of day.

6.10 Determine the quantity of oxygen (grams per cubic meter) gained or lost by diffusion at the surface during each sampling interval. To adjust for atmospheric reaeration, multiply the average k (from 6.9) by each percentage oxygen-saturation deficit value (from 6.5), and divide by 100 to convert percentage to fractional values. List these values as in table 17, column 8.

6.11 Using figure 64D, the hourly rate-of-change graph plotted as directed in 6.4, prepare a corrected rate-of-change curve by adding or subtracting, graphically, the quantity of oxygen, in grams per cubic meter, gained or lost by diffusion during each sampling interval (from 6.10). Draw the curve as in figure 64D (curve labeled "Corrected for diffusion"). The corrected rate-of-change curve is replotted as a step function to facilitate graphical integration as shown in figure 64E. Dissolved-oxygen values for each remaining depth interval are tabulated as in table 17, column 3, but not corrected for diffusion, and their hourly rates of change (col. 4) are plotted as was done for the surface interval in figure 64E.

6.12 Connect a line between the presunrise and postsunset negative rate-of-change points on the corrected rate-of-change curve as shown in figure 64E (Odum and Wilson, 1962). This line is an estimate of daytime respiration (Note 2).

Note 2: The maximum rate of respiration often occurs immediately after sunset, and the rate declines to a minimum before sunrise. Where presunrise and postsunset respiration differ, connect the line diagonally from the dawn-respiration rate to the sunset-respiration rate on the corrected rate-of-change graph. The values for respiration and gross primary productivity are affected by the placement of the respiration line. The accuracy of the method probably is limited by this step (Odum and Hoskin, 1958, p. 22). Graphs in which the rates of change are very irregular enable more subjectivity of choice of the respiration line than do smooth curves.

7. Calculations

7.1 An estimate of gross primary productivity, in grams oxygen per cubic meter per day, for each depth increment is the area above the daytime respiration line and below the daytime rate-of-change line (fig. 64E, for the surface interval). The area may be determined from the plot by counting the graph-paper squares and multiplying by the value, in grams per cubic meter, of one square. Total gross productivity of each lake slice, in grams oxygen per cubic meter per day, is obtained by multiplying the lake-slice volumetric-productivity value, in grams oxygen per cubic meter per day, by the total water volume of the lake-slice interval, in cubic meters. Total productivity of the entire water body, in grams oxygen per cubic meter per day, is the summation of all lake-slice-interval productivity values. Total productivity of the water divided by the surface area, in square meters, of the water body will provide an areal value, in grams oxygen per square meter per day, useful when comparing primary-productivity values from diverse water bodies.

7.2 An estimate of community respiration, in grams oxygen per cubic meter per day, for each depth increment is the area above the nighttime negative rate-of-change line and below the zero rate-of-change line (fig. 64E, for the surface interval). The area may be determined from the plot by

counting the graph-paper squares and multiplying by the value, in grams per cubic meter, of one square. Total community respiration of each lake slice, in grams oxygen per cubic meter per day, is calculated by multiplying the lake-slice volumetric respiration, in grams oxygen per cubic meter per day, by the total water volume of the lake-slice interval, in cubic meters. Total respiration of the entire water body, in grams oxygen per cubic meter per day, is the summation of all lake-slice-interval respiration values. Total respiration of the water divided by the surface area, in square meters, of the water body will provide an areal value, in grams oxygen per square meter per day, useful when comparing respiration from diverse water bodies.

7.3 An estimate of primary productivity for each lake-slice interval or the entire water body may be calculated by subtracting the appropriate gross primary-productivity value from the corresponding respiration value.

7.4 An index of the trophic nature of the community may be calculated as the ratio of photosynthetic productivity to respiration, P:R. Communities having a P:R ratio less than 1 have an excess of respiration compared to productivity. They are heterotrophic; that is, they degrade organic compounds through oxygen metabolism at a greater rate than they fix carbon in photosynthesis. Autotrophic communities have a P:R ratio greater than 1 and release more oxygen through photosynthesis than they consume through respiration.

8. Reporting of results

Report community primary productivity and respiration, in milligrams, as follows: less than 10 mg, one decimal; 10 mg or more, two significant figures.

9. Precision

Mean coefficients of variation among substations within four stations in Keystone Reservoir, Okla., were reported by Eley (1970). The coefficient of variation for gross primary productivity ranged from 2.72 to 9.36 percent, and the coefficient of variation for community respiration ranged from 1.71 to 11.67 percent. Average coefficients of variation among replicate observations in eight laboratory microcosms containing water from Keystone Reservoir were 1.8 percent for gross primary productivity and 5.7 percent for community respiration.

Replications of the diurnal-curve method at three similar stations in the upper Laguna Madre, Tex., were within 20 percent of the mean (Odum and Hoskin, 1958).

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BIOASSAY

Introduction

The abundance and composition of algae are related to water quality and are affected by the availability of growth substances, the major components of which are phosphorus and nitrogen. The significance of measuring algal growth potential (AGP) in water samples is that a distinction can be made between the growth substances of a sample determined by chemical analysis and the quantity of algal growth that the water can support. The AGP test that has no spikes does not identify the substances that limit or stimulate growth, nor does it indicate the presence of toxic or inhibitory substances in the water. The test does, however, enable the comparison of growth responses of test water from different sources or from the same source at different times.

Determination of AGP on a sample filtered at the time of collection measures the growth response elicited by dissolved nutrients. Samples that are autoclaved and then filtered measure a growth response that results from nutrients that are present in living organisms and organic matter as well as from dissolved nutrients.

A series of AGP bioassays, using phosphorus and nitrogen spikes, will indicate one of three conditions in a body of water: phosphorus limitation, nitrogen limitation, and the absence of phosphorus or nitrogen limitation. If phosphorus or nitrogen are not limiting--that is, there is no stimulation of growth in the spiked culture flasks--then one of several conditions may exist in the test water: minor element (micronutrient) limitation, limitation by an organic growth factor, or limitation by the presence of a toxic substance. This test will not differentiate between these possibilities; however, autoclaving does remove some biologically produced inhibitors.

In very productive water where the natural concentration of phosphorus and nitrogen exceeds the concentration of phosphorus and nitrogen in the spiked media, the concentration of the spikes may have to be increased. The limiting nutrient in a body of water also may change with time. A system that is phosphorus limited in June may be limited by some other nutrient in August. Consequently, any conclusions based on samples collected at one or two sampling times must be qualified accordingly. In addition, positive results for phosphorus or nitrogen limitation do not imply that those are the only limiting factors. There may be simultaneous micronutrients, light, or other limitations.

The minimum chemical data that must be collected to evaluate the assay response and define nutrient limitation are: initial pH, and concentrations of total phosphorus, orthophosphate, nitrite, nitrate, and total ammonia plus organic nitrogen.

Collection

To ensure maximum correlation of results, water collected for the AGP tests need to be subsampled for chemical and other biological analyses. The sample-collection method and sample size will be specified by study objec-

tives. Use a nonmetallic sampler. Do not reuse containers when toxic or nutrient contamination is suspected. Collection of samples intended for AGP analysis for dissolved substances only must be filtered at the time of collection.

Prepare the sample for analysis by autoclaving or filtering (0.22-µm pore size membrane, low-water extractable, membrane filter), or both. Autoclaving will solubilize additional nutrients, including many of those contained in filterable organisms. If a sample is collected during an algal bloom, it especially may be important to autoclave the sample. The autoclaving will oxidize algal excretions that would inhibit algal growth and result in erroneous data (Boyd, 1973). If autoclaving is desired, the length of time at 121 °C and 1.1 kg/cm^2 should be 10 to 30 minutes per liter. After autoclaving, the sample needs to be cooled to room temperature and then bubbled with a mixture of 1-percent carbon dioxide in air until the original pH is restored, or bubbled for about 5 minutes. The bubbling will minimize loss by resolubilizing some precipitates that might have formed during autoclaving. In very hard water or water containing large concentrations of suspended particulate matter, autoclaving may cause irreversible precipitation of certain constituents in the sample; therefore, the pH before and after autoclaving and carbon dioxide equilibration should be reported. Allow the sample to equilibrate in air at 24 °C. Shaking will speed the equilibration.

Changes can occur in a sample during storage regardless of conditions, so keep the storage time to a minimum. Store the sample in the dark at 0 to 4 °C and have a minimum of air space over the sample. If storage for more than 1 week is necessary, autoclave or filter, or both, the sample before storage.

Algal Growth Potential (AGP), Spikes for Nutrient Limitation (B-8502-85)

Parameters and Codes:

Algal growth potential, filtered (mg/L): 85209
Algal growth potential, filtered and spiked with 0.05 mg/L P
Algal growth potential, filtered and spiked with 1.0 mg/L N
Algal growth potential, filtered and spiked with 1.0 mg/L N and 0.05 mg/L P

Algal growth potential, unfiltered (mg/L): 70988
Algal growth potential, unfiltered and spiked with 0.05 mg/L P
Algal growth potential, unfiltered and spiked with 1.0 mg/L N
Algal growth potential, unfiltered and spiked with 1.0 mg/L N and 0.05 mg/L P

1. Applications

The method is suitable for all freshwater and is similar to the original method developed by Oswald and Golveke (1966) and the method developed by the U.S. Environmental Protection Agency (1978).

2. Summary of method

2.1 A water sample is autoclaved or filtered, or both, and placed in a covered Erlenmeyer flask. This sample is inoculated with the test algal species and incubated under constant temperature and light intensity until the rate of growth is less than 5 percent per day. The number of algal cells and the mean cell volume are determined using an electronic particle counter (fig. 66), and these values are used to determine the maximum standing crop.

2.2 The electronic particle counter has been used for counting and sizing nonfilamentous unialgal species (Hastings and others, 1962; El-Sayed and Lee, 1963). Operation of the counter is as follows: The algal cells, which are relatively poor electrical conductors, are suspended in an electrolyte solution, and as they pass through a small aperture, each cell causes a voltage drop that is recorded as a count. The height of the pulse resulting from the voltage drop is proportional to cell volume. The knowledge of the cell number per unit volume of sample and the change in mean cell volume enable standing crop to be measured reproducibly and accurately.

3. Interferences

3.1 Particles in the counting medium (for example, dust or lint) may block the aperture of the electronic particle counter or may cause false counts. These interferences are eliminated by passing all media and water samples through a 0.22-μm pore size, low-water extractable, membrane filter. Samples for the analysis should be collected in a nonmetallic sampler because certain metals in a metallic sampler may affect results.

3.2 Autoclaving may cause precipitation of certain constituents in the sample and increase the pH. These precipitates may not be irreversible. The sample often may be clarified by exposing it to 1 percent carbon dioxide plus air until the original pH is restored.

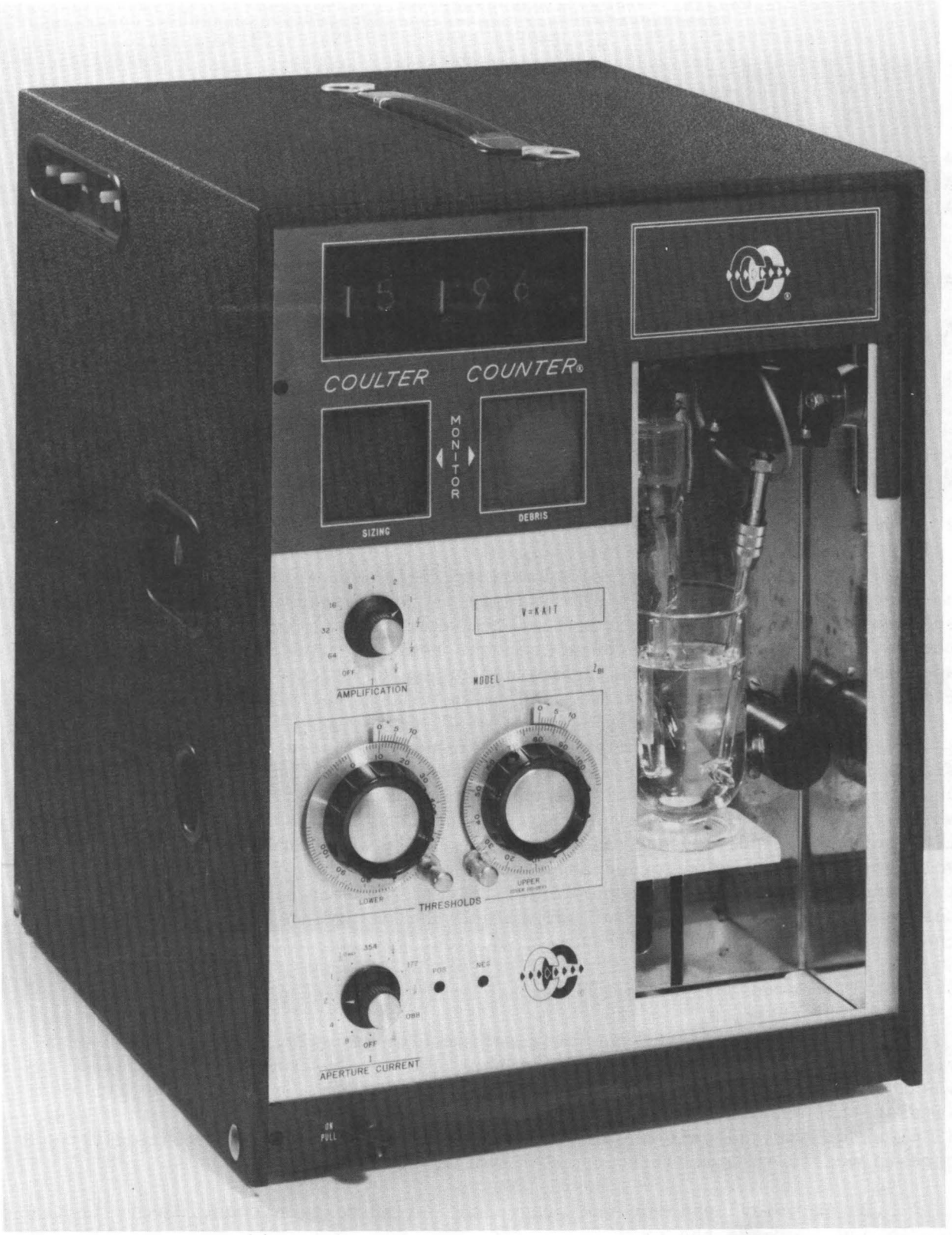

Figure 66.--Electronic particle counter (photograph courtesy of Coulter Electronics, Inc., Hialeah, Fla.).

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 Centrifuge, either swing-out or fixed-head cup-type, 3,000 to 4,000 r/min, 15- to 50-mL conical or 100-mL pear-shaped centrifuge tubes, and simple siphoning or suction device to remove excess fluid after centrifugation.

4.2 Electronic particle counter and mean cell volume accessory that has 100-µm aperture tube and a 500-µL manometer.

4.3 Environmental chamber (walk-in), that has temperature control (24±2 °C) and illumination (cool, white fluorescent that provides 4,300 lumens/m^2).

4.4 Onsite filtration apparatus, nonmetallic, and vacuum apparatus.

4.5 Flasks, Erlenmeyer, 250 mL, covered with 50-mL beakers, both glass, and prepared as follows. Wash using detergent and rinse thoroughly using tap water. Rinse using a 10-percent hydrochloric acid (HCl) solution by swirling the HCl solution so the entire inner surface of the flask is coated. The flasks then are rinsed thoroughly using particle-free distilled or deionized water (filtered through a 0.22-µm membrane filter) and covered with the 50-mL beakers. Autoclave at 1.05 kg/cm^2 (15 psi) for 20 minutes, and dry in an oven at 50 °C. Sterilized flasks and beakers must be stored in closed cabinets until used.

4.6 Laboratory filtration apparatus, sterile, disposable.

4.7 Membrane filters, 0.22-µm pore size, 47-mm diameter, low-water extractable.

4.8 Oven, for use at 50 °C.

4.9 pH meter.

4.10 Pipets and disposable tips, 0.1- and 1-mL capacities.

4.12 Refrigerator(s), without circulation blower.

4.12 Sample container, linear polyethlene bottles, 1 L.

4.13 Shaker, rotatory, capable of 120 oscillations per minute.

4.14 Sterilizer, horizontal steam autoclave, or vertical steam autoclave.

CAUTION.--If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal

eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.15 Vacuum pump.

4.16 Water-sampling bottle, Van-Dorn type. Depth-integrating samplers are described in Guy and Norman (1970).

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 Aperture cleaner. Bleach or nitric acid may be used, but aperture tube should be removed when these are used.

5.2 Calcium chloride solution. Dissolve 2.205 g calcium chloride ($CaCl_2 \cdot H_2O$) in 500 mL distilled water.

5.3 Cultures of test alga, Selenastrum capricornutum Printz. The culture medium is prepared in the following manner. Add 1 mL each of sodium nitrate ($NaNO_3$), magnesium sulfate ($MgSO_4$), magnesium chloride ($MgCl_2$), sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), micronutrient, and potassium phosphate (K_2HPO_4) solutions, in the order listed, to 900 mL distilled water, and then dilute to 1 L. Filter the medium through a membrane filter (0.22-μm mean pore size) at 25 cm mercury. Place about 100 mL in 250-mL Erlenmeyer flasks rinsed with filtered culture medium and cover with a 50-mL beaker. Autoclave the prepared flasks at 121 °C at 1.05 kg/cm^2 (15 psi) for 30 minutes and allow to equilibrate for 12 hours in the environmental chamber. Store extra culture medium at 0 to 5 °C until used.

The cultures used for inoculum are maintained by weekly transferring an aliquot of a 7- to 10-day-old culture to new media. The quantity of culture maintained depends on the conditions necessary to provide an adequate supply of algal cells at the proper growth stage for the AGP test. Extreme care must be used to prevent contamination of stock cultures.

Media that contain 1-percent agar are used to maintain stock cultures for a long period of time. Cultures on agar should be prepared every 6 to 8 weeks. The algal transfer should be streaked on the agar to isolate colonies. A clean colony should be transferred every 6 weeks to culture medium that is five times the strength 1-percent agar, and this (5X) culture should be transferred to the (1X) medium in about 2 weeks to reestablish fresh inoculum. Seven- to ten-day-old liquid cultures always should be used to provide inoculum for the AGP test.

5.4 Distilled or deionized water. Filter if in doubt about the water being particle free.

5.5 Hydrochloric acid (HCl), 10 percent.

5.6 Magnesium chloride solution. Dissolve 6.082 g $MgCl_2 \cdot 6H_2O$ in 500 mL distilled water.

5.7 Magnesium sulfate solution. Dissolve 3.593 g $MgSO_4$ in 500 mL distilled water.

5.8 Micronutrient solution. Dissolve 92.76 mg H_3BO_4, 207.69 mg $MnCl_2 \cdot 4H_2O$, 1.64 mg $ZnCl_2$, 79.88 mg $FeCl_3 \cdot 6H_2O$, 150 mg $Na_2EDTA \cdot 2H_2O$ (ethylenediaminetetraacetate) 0.39 mg $CoCl_2$, 3.63 mg $NaMoO_4 \cdot 2H_2O$, and 5.7 µg $CuCl_2 \cdot 2H_2O$ in 500 mL distilled water.

5.9 Potassium phosphate solution (particle free). Dissolve 0.522 g K_2HPO_4 in 500 mL distilled water. Filter the solution.

5.10 Potassium phosphate solution. Dissolve 143 mg K_2HPO_4 in 500 mL distilled water (for spike).

5.11 Saline solution (diluent), particle-free.

5.12 Sodium bicarbonate solution. Dissolve 7.5 g $NaHCO_3$ in 500 mL distilled water.

5.13 Sodium nitrate solution (particle free). Dissolve 12.75 g $NaNO_3$ in 500 mL distilled water.

5.14 Sodium nitrate solution. Dissolve 303.4 mg $NaNO_3$ in 500 mL distilled water (for spike). Filter the solution.

6. Analysis

6.1 Depending on type of analysis requested, AGP for dissolved substances with or without spikes or AGP for digested sample (autoclaved) with or without spikes, filter 100-mL aliquots of sample to provide each test, 6.3 to 6.6, with three replicate flasks. (Prepare filter by filtering 100 mL through each filter to saturate filter; use filtrate to wash replicate flasks. Filter vacuum should not exceed 25 cm mercury.)

6.2 Prepare one replicate for each sample to be used as an uninoculated batch control to determine particle background of sample.

6.3 Prepare three flasks to be used as controls for the following spikes or to provide the basic AGP test.

6.4 Add 1 mL potassium phoshate solution to three of the flasks.

6.5 Add 1 mL sodium nitrate solution to three more of the flasks.

6.6 Add 1 mL sodium nitrate solution and 1 mL potassium phosphate solution to each of three more flasks.

6.7 Place the covered flasks in the environmental chamber for temperature equilibration at 24 °C for at least 12 hours.

6.8 Rinse algal inoculum (see 5.3) free of culture medium using the following procedure: Place 30 mL in two 50-mL centrifuge tubes, cover, and centrifuge at 5,000 r/min for 5 minutes. Decant the supernatant and add 30 mL of filtered distilled water and resuspend the cells. Repeat the centrifugation and decantation step. Add 10 mL filtered distilled water and resuspend the cells. Combine tube contents. Mix.

6.9 Determine the concentration of the algal particles using the electronic particle counter. (Final concentration should be about 10×10^6 cells/mL).

6.10 Pipet a volume of the cell suspension into each of the sets of test samples in the flasks to make a final concentration in the test water of about 10,000 particles (cells) per milliliter.

6.11 Place the flasks (inoculated replicates plus uninoculated control) in the environmental chamber on a rotatory shaker at 120 oscillations per minute and expose to constant illumination of 4,300 lumens/m^2 produced by cool, white fluorescent tubes.

6.12 Incubate 3 to 4 days counting the number of cells in the flasks each day; thereafter, count until the growth rate is less than or equal to 5 percent per day.

7. Calculations

Maximum standing crop is determined when the increase in algal density (cells per unit volume) is less than 5 percent per day and is defined as milligram(s) dry weight algae per liter by the following equation:

$$\text{cells/mL} \times \underline{\text{MCV}} \times 2.5\times10^{-7} \times \text{dilution factor}$$

$$= \frac{\text{micrograms dry weight per liter}}{1000} = \text{milligrams dry weight per liter ,}$$

where cells/mL = coincident corrected cell count per milliliter (determined by the electronic particle counter);

$\underline{\text{MCV}}$ = mean cell volume (determined by mean cell volume accessory), in cubic micrometers;

2.5×10^{-7} = factor to convert maximum standing crop to dry weight of algal biomass (determined gravimetrically). The 2.5×10^{-7} conversion factor was determined by dividing the known total cell volume of Selenastrum capricornutum Printz culture in artificial media into the gravimetric dry weight measured from the corresponding cell suspension. The factors should be determined for each laboratory performing the analysis. As a maintenance function, recompute these factors every 6 months. Question calculations and experimental procedure if the new factor is not within ±2 to 3×10^{-7}; and

Dilution factor = dilution of algal cells from pure culture using particle-free saline solution for proper counting.

This equation is valid only when MCV has been determined using an electronic particle counter calibrated using an appropriate reference particle.

8. Reporting of results

Report maximum standing crop, in milligram(s) dry weight algae per liter, as follows: two significant figures.

9. Precision

The precision is dependent on the biomass of Selenastrum capricornutum produced. For typical samples, the precision is approximately ±10 percent.

Examples of growth responses of Selenastrum capricornutum and chemical analyses in nitrogen- and phosphorus-limited water are listed in tables 18 to 21 in the "Supplemental Information" subsection at the back of this section.

Table 18.--Growth responses representative of phosphorus limitation

[Adapted from the U.S. Environmental Protection Agency, 1978]

Sample treatment	Maximum standing crop (milligrams dry weight per liter)
Control	2.16
Control + 0.05 milligrams per liter phosphorus	5.81
Control + 1.0 milligrams per liter nitrogen	2.30
Control + 0.05 milligrams per liter phosphorus and 1.0 milligrams per liter nitrogen	23.69

Table 19.--Chemical analysis of phosphorus-limited control test water and predicted phosphorus and nitrogen yields of Selenastrum capricornutum

[Adapted from the U.S. Environmental Protection Agency, 1978]

Nutrient	Predicted yield[1] (milligrams per liter)
0.021 milligrams per liter total phosphorus .006 milligrams per liter orthophosphorus	= 0.006 × [2]430 = 2.58 ±20 percent
.368 milligrams per liter total nitrogen .120 milligrams per liter nitrate plus nitrite as nitrogen .040 milligrams per liter ammonia as nitrogen .160 milligrams per liter nitrite plus nitrate plus ammonia as nitrogen	= 0.160 × [2]38 = 6.10 ±20 percent
27:1 N:P ratio	

[1]Predicted yield of Selenastrum capricornutum based on soluble inorganic phosphorus or nitrogen concentrations in the test water if all other essential nutrients are present in excess.

[2]Yield coefficients of 430 and 38 determined experimentally by Miller and others (1978) and the U.S. Environmental Protection Agency (1978).

Table 20.--Growth responses representative of nitrogen limitation

[Adapted from the U.S. Environmental Protection Agency, 1978]

Sample treatment	Maximum standing crop (milligrams dry weight per liter)
Control	4.06
Control + 0.05 milligrams per liter phosphorus	4.21
Control + 1.0 milligrams per liter nitrogen	12.68
Control + 1.0 milligrams per liter phosphorus and 1.0 milligrams per liter nitrogen	34.52

Table 21.--Chemical analysis of nitrogen-limited control test water and predicted phosphorus and nitrogen yields of Selenastrum capricornutum

[Adapted from the U.S. Environmental Protection Agency, 1978]

Nutrient	Predicted yield[1] (milligrams per liter)
0.072 milligrams per liter total phosphorus .030 milligrams per liter orthophosphorus .160 milligrams per liter total nitrogen .055 milligrams per liter nitrate plus nitrite as nitrogen .020 milligrams per liter ammonia as nitrogen .075 milligrams per liter nitrite plus nitrate plus ammonia as nitrogen 2.5:1 N:P ratio	= 0.030 × [2]430 = 12.90 ±20 percent = 0.075 × [2]38 = 2.85 ±20 percent

[1]Predicted yield of Selenastrum capricornutum based on soluble inorganic phosphorus or nitrogen concentrations in the test water if all other essential nutrients are present in excess.

[2]Yield coefficients of 430 and 38 determined experimentally by Miller and others (1978) and the U.S. Environmental Protection Agency (1978).

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Supplemental Information

The kind of responses that can be expected when phosphorus and nitrogen are limiting are listed in tables 18 and 20. There is no significant increase in maximum standing crop (MSC) when nitrogen is added alone; however, the phosphorus spike produced more than double the MSC of the control. The combined spike of phosphorus and nitrogen increased growth even more, indicating that the phosphorus spike was large enough that, when added alone, it caused nitrogen to become the limiting nutrient in the medium.

The yield coefficients 430 and 38 listed in table 19 to predict the MSC were developed by the U.S. Environmental Protection Agency (1978). The ratio of these factors is about 11:1 and is considered to be the optimum N:P ratio. A ratio of greater than 11:1 indicates probable phosphorus limitation, and a ratio of less than 11:1 indicates probable nitrogen limitation. The ratio of total soluble inorganic nitrogen to orthophosphorus is 27:1 (table 19), a strong indication of phosphorus limitation. The assay response confirms this prediction.

Comparing the predicted results in table 19 and the growth response in table 18 indicates that the growth of the control (2.16 MSC) corresponds with (within the stipulated confidence limits) the predicted result of 2.58 MSC. The control and phosphorus spikes (5.81 MSC) correspond with the predicted results based on total soluble inorganic nitrogen (6.10 MSC), again clearly indicating phosphorus limitation and indicating that by adding 0.05 mg/L of phosphorus, the system was nitrogen limited.

A representative growth response and chemical analysis for a system that is nitrogen limited is listed in tables 20 and 21. The N:P ratio is less than 11:1 (2.5:1). The predicted yield based on the orthophosphorus concentration is 12.90 MSC and 2.85 MSC based on the total soluble inorganic nitrogen. No significant increase occurs in the sample when a phosphorus spike is added. The nitrogen spike produces an MSC that corresponds with that predicted by the phosphorus concentration, and the combined spike produces a threefold increase in the MSC, indicating that, by adding the nitrogen spike, the system has been changed to one that is phosphorus limited.

When a test water does not attain the predicted yields and nutrient spikes do not cause an increase in MSC, one of the following causes should be investigated: (1) Some other nutrient instead of phosphorus or nitrogen was limiting; (2) chemical analysis for orthophosphorus and total soluble nitrogen was inaccurate; or (3) toxicants were present.

Phosphorus limitation is the most usual case. Nitrogen limitation is not as common. Trace-element limitation is rare but has been documented (Goldman, 1972). The U.S. Environmental Protection Agency (1978) indicates that less than 2 percent of all water is trace-element limited. This method does not describe trace-element limitation, nor does it describe toxicity. With modification, this method can be used to detect trace-element limitation and the presence of toxic substances.

PART 2: GLOSSARY

[n, noun; pl, plural; adj, adjective; v, verb; sing, singular]

Acarina, acari (n, pl).--An Order of Arachnoidea that includes mites and ticks.

Accuracy (n).--A measure of the degree of conformity of a value generated by a specific procedure for the true value. The concept of accuracy includes precision and bias (American Society for Testing and Materials, 1980).

Aerobe (n), aerobic (adj).--An organism living or growing only in the presence of free oxygen.

Agar (n).--A gelatinous substance derived from seaweed and used as a base for culture media.

AGP (n).--Abbreviation for algal growth potential, the maximum quantity of algae that a water body can sustain.

Alga, algae (n), algal (adj).--A group of plants, mostly aquatic, single-celled, colonial, or multicelled, containing chlorophyll and lacking roots, stems, and leaves.

Algal bloom (n).--A large number of a particular algal species.

Allochthonous (adj).--Originating outside the area being studied. Also see autochthonous.

Amino acid (n).--A class of nitrogen-containing organic compounds, large numbers of which become linked together to form proteins.

Anaerobe (n), anaerobic (adj).--An organism living or growing in the absence of free oxygen.

Aquatic (adj).--Pertaining to water; aquatic organisms, such as phytoplankton or fish, live in or on water.

Assimilation (n).--The total rate of organic matter used by heterotrophs; secondary productivity plus respiration and other losses. Also see secondary productivity.

ATP (n).--Abbreviation for adenosine triphosphate, an organic, phosphate-rich compound, important in the transfer of energy in organisms.

Autochthonous (adj).--Originating within the area. Also see allochthonous.

Autotroph (n), autotrophic (adj).--An organism, such as an alga, in which organic matter is synthesized from inorganic substances, commonly by the process of photosynthesis.

Bacterium, bacteria (n), bacterial (adj).--Microscopic unicellular organisms, typically spherical, rod-like, or spiral and threadlike in shape, often clumped into colonies. Some bacteria cause disease, and others perform an essential role in the recycling of materials; for example, by decomposing organic matter into a form available for reuse by plants.

Benthic invertebrate (n).--An invertebrate of the benthos.

Benthos (n), benthic (adj).--The community of organsims living in or on the bottom of an aquatic environment.

Bias (n).--A persistent positive or negative deviation of the average value of the method from the true value (American Society for Testing and Materials, 1980).

Bioassay (n).--The use of living organisms to test the effects of a substance. Also see toxicity bioassay.

Biology (n), biological (adj).--The science or study of life.

Biomass (n).--The quantity of living matter present at any given time, expressed as the number or weight per unit area or volume of habitat. Same as standing crop.

Biotic community (n).--All the plant and animal populations living together in a habitat and functioning as a unit by virtue of food and other relations.

Blackfly (n).--See simuliidae.

Bloom (n).--See algal bloom.

Botany (n).--The science or study of plants.

Broth medium (n).--A liquid mixture of defined composition used to provide nourishment for the growth of micro-organisms in culture.

Bryophyta (n, pl), bryophyte (n).--The division of the plant kingdom containing mosses and liverworts.

Carnivore (n).--An organism that obtains its nourishment by consuming animals; includes many types of fish and aquatic insects.

Chemosynthesis (n), chemosynthetic (adj).--A chemical synthesis of organic compounds in bacteria by energy derived from oxidation-reduction reactions of mineral compounds.

Chironomidae (n, pl), chironomid (n).--A family of the insect Order Diptera that includes midges.

Chlorophyll (n).--The green pigments of plants.

Class (n).--The taxonomic category below phylum, consisting of orders. Also see taxonomy.

Coliform bacteria (n).--A particular group of bacteria used as indicators of possible sewage pollution. They formally are characterized as aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose and form gas at 35 °C within 48 hours.

Community (n).--Any naturally occurring group of different organisms inhabiting a common environment and interacting with one another through food relations.

Compensation level or depth (n).--The depth of water at which gross photosynthesis (oxygen production) balances respiration (oxygen uptake) during a 24-hour period.

Concentration (n).--The weight or number per unit volume or area of a water-quality constituent or characteristic.

Culture (n, v).--Cultivation of or act of cultivating living material, such as micro-organisms, in nutrient medium; any inoculated nutrient medium whether or not it contains living organisms.

Culture medium (n).--See nutrient medium.

Denitrification (n).--The biochemical reduction of nitrates and nitrites during the oxidation of organic matter and the evolution of gaseous nitrogen.

Detritivore (n).--An animal that obtains its nourishment by consuming organic detritus; includes many types of aquatic insects.

Detritus (n).--Fragmented material of inorganic or organic origin.

Diatom (n).--A unicellular or colonial alga having a siliceous shell.

Diel (adj).--Relating to a 24-hour period that usually includes a day and the adjoining night.

Diurnal (adj).--Relating to daytime or something recurring every day, commonly used as a synonym for diel.

Division (n).--The primary taxonomic category of the plant kingdom, consisting of classes. Also see taxonomy.

Dorsum (n), dorsal (adj).--The upper surface of an organism. Also see ventrum.

Dredge (n).--An instrument pulled across or through the bottom of a lake or stream to sample the benthos. Also see grab.

Ecology (n), ecologic(al) (adj).--The science or study of the relation of organisms or groups of organisms to their environment.

Ecosystem (n).--The community of plants and animals interacting together and with the physical and chemical environment.

Emersed plant (n).--A rooted, aquatic plant that has leaves or other structures extending above the water surface (sometimes called emergent plant).

Environment (n).--The sum of all the external physical, chemical, and biological conditions that affect the life and development of an organism.

Epilimnion (n).--The upper, relatively warm, circulating zone of water in a thermally stratified lake. Also see hypolimnion, metalimnion, and thermocline.

Euphotic zone (n).--That part of the aquatic environment in which the light is sufficient for photosynthesis; commonly considered to be that part of a water body in which the intensity of underwater light equals or exceeds 1 percent of the intensity of surface light.

Eutrophication (n), eutrophic (adj).--Enrichment of water, a natural process that may be accelerated by the activities of man; pertaining to water in which primary production is intense as a consequence of a large supply of available nutrients. Also see oligotrophic.

Facultative (adj).--Able to live and grow in many different environments. Also see obligate.

Family (n).--The taxonomic category below order consisting of genera. Also see taxonomy.

Fauna (n), faunal (adj).--A collective term for all the kinds of animals in an area. Also see flora.

Fecal coliform bacteria (n).--That part of the coliform group that is present in the gut or the feces of warm-blooded animals; they are indicators of possible sewage pollution.

Fecal streptococcal bacteria (n).--A particular group of bacteria found in the gut of warm-blooded animals; their presence in natural water verifies fecal pollution. They are formally characterized as gram-positive, cocci bacteria that are capable of growth in brain-heart infusion broth either at 45 °C and 10 °C (the enterococci species) or at 45 °C only (Streptococcus bovis and S. equinus).

Flagellum, flagella (n).--A fine, long, thread-like structure having lashing or undulating movement, projecting from a cell; it is used for locomotion.

Flora (n), floral (adj).--A collective term for all the kinds of plants in an area. Also see fauna.

Food chain (n).--The transfer of food energy from the source in plants through a series of organisms through repeated eating and being eaten (Odum, 1971). Also see food web.

Food web (n).--The interconnecting pattern of food chains. Also see food chain.

Formalin (n).--A clear, aqueous solution containing about 37 percent formaldehyde by volume and 5 to 10 percent methyl alcohol; when diluted with water, it is used as a general biological preservative.

Fungus, fungi (n).--Plants lacking chlorophyll, including molds, yeast, mildews, rusts, and mushrooms. Fungi derive their nourishment directly from other organisms (parasitic fungi) or from dead organic matter (saprophytic fungi).

Genus, genera (n), generic (adj).--The taxonomic categories below family, consisting of species; the first part of the scientific name of organisms. Also see taxonomy.

Generation (n).--A group of organisms about the same age.

Generation time (n).--The period of time between the origin of a generation of organisms and the origin of their offspring.

Grab (n).--An instrument designed to bite into the bottom sediment of a lake or stream to sample the benthos. Also see dredge.

Greenhouse effect (n).--An increase in temperature within a glass or plastic enclosure ascribed to entrance of short-wave radiation into the enclosure; whereas, long-wave radiation from heated objects within the enclosure is absorbed by the glass or plastic. Thus, solar energy enters but is unable to leave.

Grid (n).--An imaginary or measured, usually rectangular, arrangement of lines used to delineate an area for sampling.

Grid sampling (n).--A sampling scheme in which the area to be investigated is subdivided into equal-size units and from which the units to be sampled are selected randomly.

Gross primary productivity (n).--The total rate at which organic matter is formed by photosynthesis, including the organic matter used in respiration during the period of measurement. The term is synonymous with gross primary production, total photosynthesis, and total assimilation.

Growth (n).--The increase in biomass by synthesis of living matter.

Growth medium (n).--See nutrient medium.

Habitat (n).--The place where an organism lives.

Hemacytometer (n).--A thin-walled glass chamber used for counting very small cells or organisms using a high-power microscope objective.

Herbivore (n).--An organism that obtains its nourishment by consuming plants.

Heterotroph (n), heterotrophic (adj).--An organism that requires organic material as a source of nutrition; this includes all types of animals and many types of bacteria.

Holdfast (n).--A structure by which an organism attaches to a substrate.

Hydrobiology (n).--The science or study of life in water.

Hypolimnion (n).--The lower, relatively cold, noncirculating water zone in a thermally stratified lake. Also see epilimnion, metalimnion, and theromocline.

Incubation (n).--Maintenance of organisms in conditions favorable for growth and development.

Interpretive (adj).--A type of sampling program or study designed to collect information useful when describing a system and cause-and-effect relations within the system.

Invertebrate (n).--An animal that does not have a backbone. Common aquatic examples include worms, insects, snails, and crayfish.

Kingdom (n).--The highest biological classification category. Also see taxonomy.

Larva, larvae (n), larval (adj).--An active, immature stage of an animal during which its bodily form differs from that of the adult. Also see nymph.

Lentic (adj).--Of or pertaining to nonflowing water; for example, a lake or pond.

Life history (n).--The environmental relations of an organism, including distribution, morphology, growth, reproduction, and behavior.

Light injury (n).--Physiological damage resulting from exposure of an organism, usually a plant, to a light intensity greater than that to which the organism was adapted.

Limnetic zone (n).--The open-water zone of a water body above the compensation level.

Limnology (n).--The science or study of inland water; the ecology of inland water.

Littoral (n, adj).--Pertaining to the shallow zone of a body of water where light penetrates to the bottom.

Liverwort (n).--See bryophyta.

Lotoc (adj).--Of or pertaining to flowing water; for example, a river or creek.

Macroinvertebrate (n).--An invertebrate, usually a benthic organism, that is retained on a U.S. Standard no. 30 sieve (0.595-mm mesh opening).

Macrophyte (n).--Large plants that can be seen without magnification; includes mosses and seed plants.

Medium (n).--See nutrient medium.

Membrane filter (n).--A thin, microporous material of specific pore size used to filter bacteria, algae, and other very small particles from water.

Metabolism (n).--The chemical processes of living cells by which energy is derived and material is assimilated.

Metalimnion (n), metalimnetic (adj).--The middle layer of water in a thermally stratified lake in which temperature decreases rapidly with increasing depth. Also see epilimnion, hypolimnion, and thermocline.

Metamorphosis (n), metamorphic (adj).--The period of rapid transformation from larval to adult form.

Microseston (n).--The suspended matter in water that will pass through a 150- to 350-µm mesh. Also see seston.

Midge (n).--See chironomidae.

Mite (n).--See acari.

Monitoring (n).--A type of sample or program designed to determine time trends.

Morphology (n), morphological (adj).--The study of a life form; the physical attributes of an organism.

Morphometry (n), morphometric (adj).--The measurement of external form.

Moss (n).--See bryophyta.

Nekton (n).--Actively swimming aquatic organisms, such as fish.

Net community productivity (n).--The rate of storage of organic matter not used by the organisms in the environmental area being studied during the period of measurement; net primary productivity minus heterotrophic use.

Net primary productivity (n).--The rate of storage of photosynthetically produced organic matter in plant tissues in excess of the respiratory use by the plants during the measurement period. The term is synonymous with apparent photosynthesis, net photosynthesis, and net assimilation.

Neuston (n).--Organisms living on or under the surface film of water.

Niche (n).--The location and ecological function of an organism in the environment.

Nitrification (n).--The biological formation of nitrate or nitrite from compounds containing reduced nitrogen.

Nutrient (n).--Any chemical element, ion, or compound that is required by an organism for the continuation of growth, for reproduction, and for other life processes.

Nutrient medium, nutrient media (n).--A chemical mixture of defined composition used to provide nourishment for the growth of micro-organisms in culture. The medium may be in liquid form, called broth, or may be solidified using agar.

Nymph (n), nymphal (adj).--An immature stage of an insect that resembles the adult stage in bodily form. Also see larvae.

Obligate (adj).--Restricted to living and growing in a single environment. Also see facultative.

Oligotrophic (adj).--Pertaining to water in which primary production is small as a consequence of a small supply of available nutrients. Also see eutrophic.

Order (n).--The taxonomic category below class consisting of families. Also see taxonomy.

Organism (n).--Any living entity.

Pathogen (n), pathogenic (adj).--A disease-causing organism.

Periphyton (n), periphytic (adj).--The community of micro-organisms that are attached to or live on submerged surfaces.

Phaeopigment (n).--The degradation product of chlorophyll.

Photoperiod (n).--The duration of daylight during a 24-hour period.

Photosynthesis (n), photosynthetic (adj).--A biochemical synthesis of carbohydrates from water and carbon dioxide in the chlorophyll-containing tissues of plants in the presence of light.

Phylum, phyla (n).--The primary taxonomic category of the animal kingdom, consisting of classes. Also see taxonomy.

Phytoplankter (n).--An individual phytoplanktonic organism.

Phytoplankton (n), phytoplanktonic (adj).--The plant part of the plankton.

Plankter (n).--An individual planktonic organism.

Plankton (n), planktonic (adj).--The community of suspended or floating organisms that drift passively with water currents.

Poikilothermic organism (n).--An animal whose body temperature approximates that of the environment; commonly called cold blooded.

Pollution (n).--"***an undesirable change in the physical, chemical, or biological characteristics of our air, land, and water that may or will harmfully affect human life or that of other desirable species, our industrial process, living conditions, and cultural assets; or that may or will waste or deteriorate our raw material resources***" (National Academy of Sciences--National Research Council, Committee on Pollution, 1966, p. 3). Also see water pollution.

Population (n).--A group of interacting and interbreeding individuals of the same type living in a common habitat and having little reproductive contact with other groups of the same species.

Precision (n).--The degree of conformity of repeated measurements of the same parameter expressed quantitatively as the standard deviation computed from the results of a series of controlled determinations (American Society for Testing and Materials, 1980).

Primary productivity (n).--The rate at which radiant energy is stored by photosynthetic and chemosynthetic activity of producer organisms (chiefly green plants) in the form of organic substances that can be used as food materials (Odum, 1971, p. 43). Also see gross primary productivity, net primary productivity, net community productivity, and secondary productivity.

Production (n).--The total quantity of living matter produced in an area per unit time. Also see primary productivity and secondary productivity.

Profundal (adj).--Referring to the deep-water zone of a water body in which plant growth is limited by the absence of light.

Protein (n).--A complex nitrogenous substance of plant or animal origin formed from amino acids; essential constituent of all living cells.

Protista (n).--A biological kingdom consisting of unicellular (single-celled) organisms.

Protoplast (n).--The living contents of a cell; the nucleus, cytoplasm, and plasma membrane that constitute a living unit.

Protozoa (n, pl), protozoan (n).--Single-celled microscopic organisms of the phylum Protozoa.

Pupa, pupae (n), pupal (adj).--The inactive stage of certain insects during which the larva transforms into the adult. Also see larvae.

Random (n, adj).--The nonuniform, haphazard distribution of organisms in the environment.

Random sample (n).--A sample collected from a population or an area using an unbiased procedure so every part of the population or area has an equal chance of being sampled.

Reconnaissance (n, adj).--A type of sample or program designed to determine the present status of something; a preliminary survey.

Respiration (n).--A life process in which carbon compounds are oxidized to carbon dioxide and water, and the released energy is used in metabolic processes.

Rotifera (n, pl), rotifer (n).--The phylum containing microscopic organisms that swim and feed by means of a ciliated hand; also known as the wheel.

Sample (n).--A small, separated part of something that is representative of the whole.

Saproplankton (n).--The bacteria and fungi of the plankton.

Secondary productivity (n).--The rate of increase of organic matter in the heterotrophs of the community; assimilation minus respiration and other losses. Also see assimilation and primary productivity.

Sediment (n).--Fragmental material, mineral and organic, that is in suspension or is transported by the water mass or has been deposited on the bottom of the aquatic environment.

Seine (n).--A net used for collecting fish and other large aquatic animals.

Sessile (adj).--Pertaining to an organism that is attached to an object.

Seston (n).--The total particulate matter suspended in water.

Simuliidae (n, pl), simuliid (n).--A family of the insect Order Diptera that includes blackflies.

Species (n. sing., n. pl.).--The basic unit for the classification of organisms; the taxonomic category below genus, and the second part of the scientific name of an organism. Also see taxonomy. The biological concept of species, in contrast to the purely taxonomic concept, has been defined by Mayr (1940) as "***a group of actually or potentially interbreeding organisms reproductively isolated from other such groups of interbreeding organisms."

Specimen (n).--A part or individual used as a sample of a whole or group; an organism used for study.

Standing crop (n).--The quantity of living matter present at any given time, reported as the number or weight per unit area or volume of habitat. Same as biomass.

Statistical population (n).--The whole aggregate of something in an area being sampled.

Stratified water (n).--A body of water having a series of horizontal strata. Also see thermal stratification.

Submersed plant (n).--An aquatic macrophyte that completes its life cycle and lives entirely below the surface of the water (sometimes called submerged or submergent).

Substrate (n).--The physical surface on which something lives.

Suspended sediment (n).--Fragmental material, mineral and organic, that is maintained in suspension in water by turbulence and currents or by colloidal suspension.

Taxon, taxa (n).--Any classification category of organisms, such as phylum, class, order, or species.

Taxonomy (n).--The division of biology concerned with the classification and naming of organisms; synonymous with systematic biology. The classification of organisms is based on a hierarchical scheme beginning with the species at the base. The higher the classification level, the fewer features the organisms have in common. Also see species. As an example, the taxonomy of the common stonefly, Pteronarcys californica is as follows:

Kingdom	Animal
Phylum	Arthropoda
Class	Insecta
Order	Piecovtera
Family	Pteronarcidae
Genus	Pteronarcys
Species	californica
Scientific name	Pteronarcys californica

Thermal stratification (n).--A temperature distribution characteristic of many lakes in which the water is separated into three horizontal layers: a warm epilimnion at the surface, a metalimnion in which the temperature gradient is steep, and a cold hypolimnion at the bottom.

Thermocline (n).--The plane of maximum rate of temperature decrease in a thermally stratified lake, sometimes used as a synonym for metalimnion. See also epilimnion and hypolimnion.

Toxicity bioassay (n).--Determination of the potency of a toxic substance by measuring the intensity of a biological response. Also see bioassay.

Transect sampling (n).--A sampling scheme in which a longitudinal or transverse section of a stream or other area is marked off in equally spaced divisions, and samples are collected at predetermined division sites.

Vascular plant (n).--A multicellular macrophyte that possesses conductive tissues, including ferns and similar plants and seed plants; aquatic representatives may be rooted or may float in or on the water.

Ventrum (n), ventral (adj).--The bottom surface of an organism. Also see dorsum.

Vertebrate (n).--An animal that has a backbone enclosing a nerve cord; aquatic examples include fish and amphibians.

Water pollution (n).--Variously defined as "***any thing which brings about a reduction in the diversity of aquatic life and eventually destroys the balance of life in a stream***" (Patrick, 1953, p. 33); "***the addition of something to water which changes its natural qualities so that the riparian owner does not get the natural qualities of the stream transmitted to him***" (quoted in Hynes, 1960, p. 1); "***any impairment of the suitability of water for any of the beneficial uses, actual or potential, for man-caused changes in the quality of water***" (Warren, 1971, p. 14). Also see pollution.

Water quality (n).--Kinds and quantities of matter dissolved and suspended in natural water, the physical characteristics of the water, and the ecological relations between aquatic organisms and the environment.

Water weed (n).--A popular term for an aquatic plant, usually one of the macrophytes.

Yield (n).--The quantity (weight or number) of biomass removed from a given aquatic area in a given time.

Zoology (n), zoological (adj).--The science or study of animals.

Zooplankter (n).--An individual zooplanktonic organism.

Zooplankton (n), zooplanktonic (adj).--The animal part of the plankton.

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PART 3: SELECTED TAXONOMIC REFERENCES

This section consists of references for the identification of aquatic organisms. The lists are not intended to be complete but rather to provide an introduction to the literature for the various taxonomic groups. Two types of references are included: (1) Keys and morphological descriptions for particular groups of organisms, mostly at the generic or higher taxonomic level; and (2) descriptions or lists of taxa for the various States or other geographic areas. North American freshwater taxa are emphasized.

Except for the general reference works, the listings are arranged by systematic or taxonomic category rather than by habitat or biological community. The analytical methods and their taxonomic groups, presented in Part 1 of this chapter, are listed in table 22.

Table 22.--Taxonomic group(s) of greatest significance for the methods in Part 1

Method	Taxonomic group(s)
Bacteria	Bacteria and fungi
Phytoplankton	Algae
Zooplankton	Protozoa (including flagellates) Coelenterata Rotifera Smaller crustacea
Periphyton	Bacteria and fungi Algae Protozoa (includes flagellates) Coelenterata Gastrotricha Rotifera Tardigrada
Macrophytes	Macrophyton Algae
Benthic invertebrates	Porifera Turbellaria Nemertea (Phynchocoela) Nematoda (Nemata) Gordiida Bryozoa Annelida Crustacea Aquatic Insecta Aquatic Acari Mollusca
Aquatic vertebrates	Aquatic vertebrates

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PROTOZOA (Including Flagellates)

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