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

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

Chapter A4

METHODS FOR COLLECTION AND ANALYSIS OF AQUATIC BIOLOGICAL AND MICROBIOLOGICAL SAMPLES

Edited by

Task Group on Biology and Microbiology
P. E. Greeson, Chairman
T. A. Ehlke
G. A. Irwin
B. W. Lium
K. V. Slack

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V. E. McKelvey, Director

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PREFACE

The series of manuals on techniques describes methods used by the 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 manual was prepared by many 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. The looseleaf format of this methods manual is designed to permit flexibility in revision and publication. Supplements, to be prepared as the need arises, will be issued to purchasers at no charge as they become available.

Reference to trade names, commercial products, manufacturers, or distributors in this manual does not constitute endorsement by the U.S. Geological Survey nor recommendation for use.

This manual supersedes "Methods for Collection and Analysis of Aquatic Biological and Microbiological Samples" by K. V. Slack, R. C. Averett, P. E. Greeson, and R. G. Lipscomb (U.S. Geol. Survey Techniques Water-Resources Inv., book 5, chap. A4, 1973).

CONTRIBUTORS

BIOLOGICAL AND MICROBIOLOGICAL SAMPLING AND STATISTICS

R. C. Averett

BACTERIA

T. A. Ehlke
G. G. Ehrlich
P. E. Greeson

PHYTOPLANKTON

P. E. Greeson
H. V. Leland
R. G. Lipscomb
B. W. Lium
W. T. Shoaf
L. J. Tilley

ZOOPLANKTON

L. J. Britton
R. F. Ferreira
P. E. Greeson

SESTON

P. E. Greeson
K. V. Slack

PERIPHYTON

R. C. Averett
P. E. Greeson
G. A. Irwin
B. W. Lium
W. T. Shoaf

MACROPHYTES

P. E. Greeson
R. G. Lipscomb

BENTHIC INVERTEBRATES

S. S. Hahn
K. V. Slack
L. J. Tilley

AQUATIC VERTEBRATES

R. C. Averett
G. A. Irwin

CELLULAR CONTENTS

P. E. Greeson
B. W. Lium
W. T. Shoaf

PRODUCTIVITY

V. J. Janzer
J. R. Knapton
L. J. Schroder, II
K. V. Slack
D. W. Stephens

BIOASSAY

B. W. Lium
W. T. Shoaf

SELECTED TAXONOMIC REFERENCES

K. V. Slack

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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

Abstract

Chapter A4 contains methods used by the U.S. Geological Survey to collect, preserve, and analyze waters to determine their biological and microbiological properties. Part 1 discusses biological sampling and sampling statistics. The statistical procedures are accompanied by examples. Part 2 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 application, interferences, apparatus, reagents, collection, analysis, calculations, reporting of results, precision and references are given. Part 3 consists of a glossary. Part 4 is a list of taxonomic references.

Introduction

The Department of the Interior has the basic responsibility for the appraisal, conservation, and efficient utilization 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 Interior agencies, the U.S. Geological Survey's primary responsibility in relation to water is to assess its availability and utility as a national 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 groundwater resources. Included, also, is the need for supporting research to increase the effectiveness of these studies.

As part of its mission the 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 manual was prepared to provide accurate and precise methods for the collection and analysis of aquatic biological and microbiological samples. Although excellent and authoritative manuals on aquatic biological analyses are available, their methods and procedures are often diverse. The purpose of this manual is to set forth in a single chapter 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 basic information from field 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 basic data. Because these data comprise an irreplaceable fund of information, the future uses of which cannot now be foreseen, it has seemed advisable to depart occasionally from established practices in water-quality techniques. These departures are clearly described, adequately justified, and increase the validity of the results.

It is clear from the accelerating rate of publication of reports on this subject that new and improved methods are being developed in response to man's increasing awareness of his environmental role. A technique which 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 to a degree to integrate the experience of the past and to select the most appropriate methods from an ever-growing population.

A methods manual is only one of several tools available to the investigator. At best it can tell him "how to," it can never tell him "what to;" nor can it tell him what a specific numerical value means. Entire volumes have been written on subjects, for example, primary productivity, to which this manual 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.

Part 1. Biological Sampling and Statistics

Introduction

The organisms that live in water occupy a variety of habitat types. Benthic invertebrates that inhabit lakes and ponds may resemble the types found in the pools of streams, but not those in the riffles. Similarly, the phytoplankton of lakes and reservoirs has a different species composition than that of streams.

A riffle may be considered a macrohabitat, but in it are numerous microhabitats which vary in current speed, size of substrate material, depth of water, exposure to the sun, and other environmental factors. Each microhabitat may be occupied by different kinds, as well as numbers of organisms, a complexity that makes biological sampling both challenging and difficult.

This section discusses some of the environmental, as well as biological, considerations in the sampling of aquatic organisms. The first part describes distribution patterns of organisms and the design of simple reconnaissance and monitoring programs. The second part describes distribution models and sampling statistics.

Aquatic organisms may be grouped into the broad categories of plankton, benthos, and nekton (Welch, 1952, p. 221). Plankton are organisms of relative small size that have either weak powers of locomotion, or none at all. They are free floating and drift passively with the movement of the water. Organisms included in the plankton are the phytoplankton or plant plankton, the zooplankton or animal plankton, and some bacteria. Plankton may be collected with the same methods used for the collection of water samples for chemical or suspended-sediment analysis. That is, to collect a sample of plankton at a given 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). Further discussion is given in Part 2, "Phytoplankton."

The benthos are those organisms that live in or on the bottom of lakes, streams, or estuaries. Although many of them possess powers of locomotion, they generally do not move great distances. Examples of benthic organisms are worms, mollusks, and the juvenile forms (larvae and nymphs) of most aquatic insects. Various types of nets, grabs, dredges, and artificial substrates are used to collect these organisms. A discussion of collecting devices and their use is found in Part 2, "Benthic Invertebrates."

The nekton are larger organisms which swim freely. Their distribution is usually unaffected by the movement of the surrounding water. Examples include most fishes, other aquatic vertebrates, and some zooplankters. These organisms are captured with traps, nets, and other more specialized equipment. A detailed discussion of sampling equipment and its use for collecting nekton is found in Part 2, "Zooplankton" and "Aquatic Vertebrates."

One of the important and fascinating problems of biology, one that has held the attention of scientists for centuries, is that of defining the patterns, as well as the causative factors for the distribution and abundance of plants and animals in time and space (Andrewartha and Birch, 1954). Although our knowledge is still meager, sufficient information is available for the design of sampling programs that provide inferences about organisms and their distribution patterns.

We now know that organisms are seldom distributed randomly except possibly when the population density is very low. Usually, organisms in nature are underdispersed or clumped or "patchy" (the contagious distribution of Elliot, 1971) in their distribution (fig. 1). This clumped distribution pattern is brought about by the behavior and habitat requirements of the organisms. For example, many aquatic insects deposit eggs in clusters, and the resulting larvae or nymphs often remain in the area after hatching. Burrowing organisms may find only small sections of stream bottom, where fine sediment has deposited, as a suitable habitat. Consequently, they tend to cluster in these

areas. In other instances, the clumped distribution of the food supply influences the distribution of aquatic organisms.

A combination of anatomical, physiological, and behavioral factors often restricts or controls the distribution of organisms to particular habitats. Some organisms such as blackfly larvae (Simuliidae) have holdfast structures by which they attach to solid surfaces in the fast-flowing water of streams. Other organisms, such as some midge larvae (Chironomidae) are specialized for burrowing in soft mud and feeding on organic matter and, thus, are restricted to lake bottoms or to the pools of streams. Some caddisfly larvae such as Hydropsychidae are restricted to flowing water where they spin nets to catch organic particles carried by the current. Other groups of organisms are less specialized and consequently occur in a variety of habitats.

Although some areas in a stream may be densely populated with organisms, other areas of similar appearance may be almost devoid of life. This illustrates the subjectivity of habitat classification schemes based upon human judgment. A relatively large number of samples must be collected from a large number of habitat types if the goal of a sampling program is to describe the flora and fauna of an area with a high degree of accuracy. Biological data often provide the most useful basis for assessing water-quality conditions and changes. Because biological samples, in general, are far more time consuming to collect and to analyze than are chemical samples, a biological sampling program must be carefully designed if it is to yield useful results.

Biological surveys and simplified sampling methods

Although sampling programs based on statistical design provide the most reliable information, it is not always necessary or feasible to employ them. General faunal and floral reconnaissance surveys and some surveillance and monitoring programs can be conducted successfully with simplified sampling techniques. In this regard, the species list and other simple and subjective methods are still useful ways to present data (Hynes, 1964). In the following discussion, benthic invertebrates are used to illustrate biological sampling although the principles apply as well to other aquatic plant and animal communities.

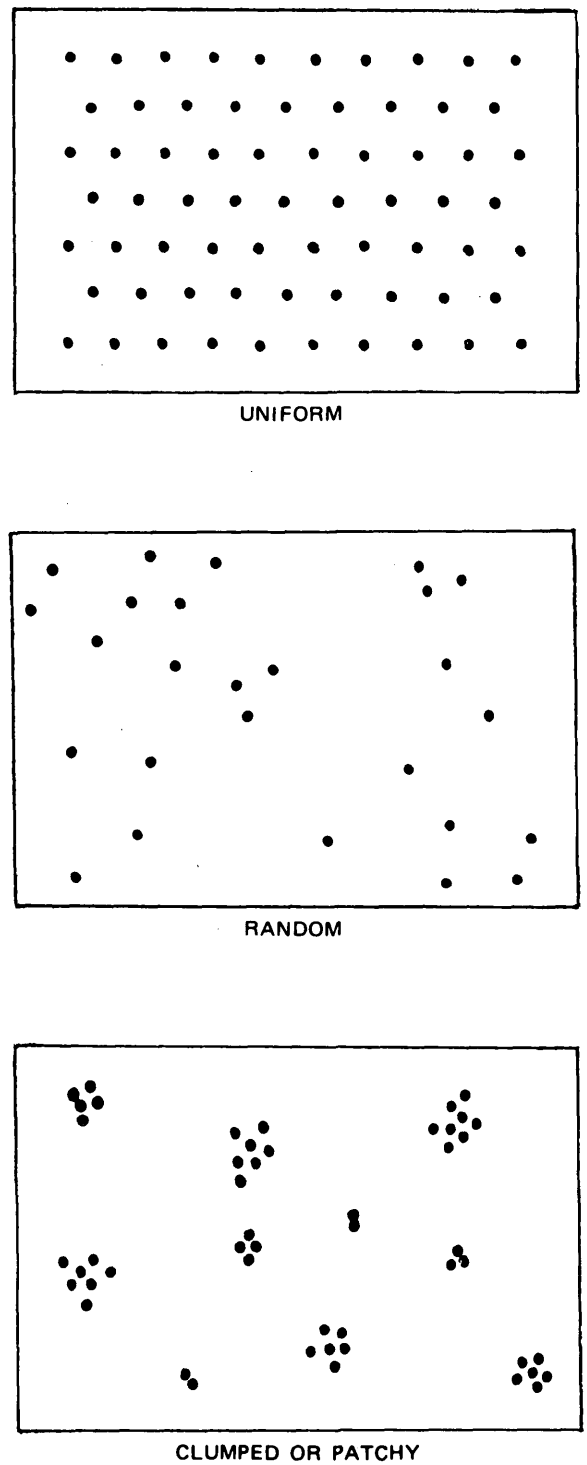

Figure 1.—Distribution patterns of organisms. In nature most organisms occur in the clumped or patchy distribution. (Modified from Odum, 1971, with permission.)

Determination of the various habitat types in the area to be sampled is the first step in the design of a faunal survey. The physical substrate (boulders, rubble, sand, and mud), velocity of flow, exposure to the sun, width, and depth of water should be considered. After the various habitat types have been noted, the investigation can proceed by collecting samples from each habitat. Although not always necessary, it is generally useful to determine the proportion of the area of the stream or lake bottom sampled, even when making a general faunal survey.

The number of samples to collect in each habitat type (stream riffle, for example) is a subjective matter if quantitative techniques are not used. Needham and Usinger (1956) found that two samples, each measuring 1 square foot in area (0.093m^2), taken from a Surber (1937) sampler were sufficient to be reasonably certain of obtaining representatives of the principal organisms in a riffle in Prosser Creek, Calif. Gaufin, Harris, and Walter (1956) found in Lytle Creek, Ohio, that, on the average, as many as 10–15 percent of the species were not discovered until at least eight Surber samples were collected. However, their findings showed that three samples contained at least half and sometimes two-thirds of the species observed after 10 samples. Chutter and Noble (1966) also found that three Surber samples collected at least one individual of all the more common animals in a South African stream. In addition, it was shown that the more carefully the sampling site is defined the more reliable will be the sample data.

In general, three 1-square-foot (0.2787m^2) samples per habitat type are considered sufficient for a faunal survey in a stream (Cairns and Dickson, 1971, p. 762). If the investigation is to measure or show changes in biomass, more intensive sampling is required (Hynes, 1970, p. 27). See Part 2 for additional discussion of quantitative sampling of benthic invertebrates.

Many water-quality investigations require monitoring or surveillance of an aquatic area over a long period of time. Such systematic resampling usually involves using either a transverse or a longitudinal transect system, or a grid or quadrant system.

Transect

In a stream, transect sampling consists of collecting samples either along a section of its length or along a line across the stream (fig. 2A). In a lake or reservoir, it consists of collecting samples along a line which may be delineated by buoys. Samples may be collected at uniform intervals along the transect line or at random

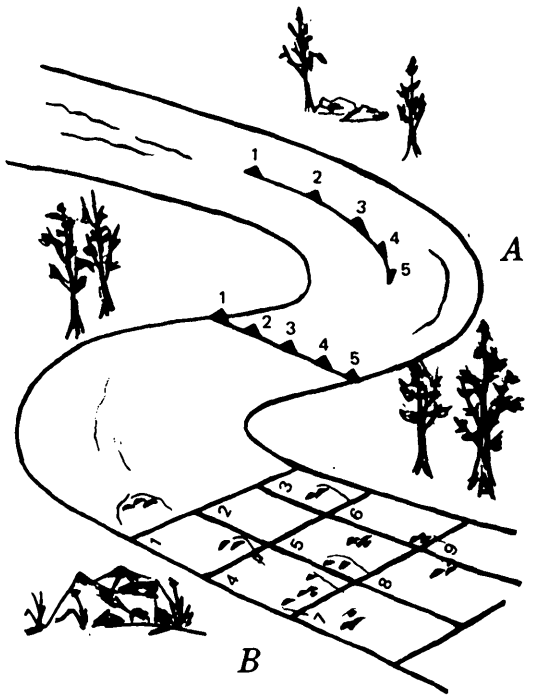

Figure 2.—Examples of transect- and grid-sampling schemes. *A*, Longitudinal and transverse transects. *B*, A grid of nine sampling sites.

locations selected with the aid of a table of random numbers.

If the transect line is longitudinal with a stream and includes pools and riffles, each habitat type should be considered as a separate entity. That is, the number of samples to be collected in the pool area should be independently chosen from the number to be collected in the riffles. Essentially this is the stratified method of sampling. The method insures that both pools and riffles are sampled equally.

A transect sampling program for lakes and reservoirs can be designed in the same manner as for streams, except as previously mentioned, the type of bottom material often cannot be determined until the sample is brought to the surface.

Grid or quadrant

A sampling grid or quadrant consists of an imaginary or physical rectangular arrangement of lines, covering all or part of a given habitat (fig. 2B). For example, assume that a riffle measuring 5 by 20 meters is to be sampled for benthic organisms. If a 0.5-m^2-sampling device is used, there are potentially 200 sampling units, each of which could be assigned a number. If 10 sampling units are to be sampled, they may be selected by number taken from a table of

random numbers (Snedecor, 1956, p. 10–13; Snedecor and Cochran, 1967, p. 543–546) or in some other randomized fashion as described in the section on "Statistical Sampling." A grid or quadrant sampling scheme should, as with the transect scheme, give equal consideration to the various habitat types.

In lakes, reservoirs, and deep rivers, grid boundaries may be established with the aid of buoys. Sampling within the area delineated by the buoys can be carried out similarly to that described for streams. If the sampling is for organisms suspended in the water (phytoplankton and zooplankton, for example) several samples from different depths may be required.

The simplified sampling techniques described above are useful for faunal and floral surveys, general reconnaissance surveys, and for some surveillance and monitoring programs. In many instances they are adequate to fulfill the objectives of detailed studies, although most interpretive studies will require more intensive sampling and a well-planned experimental design. In addition, interpretive studies usually require that the experimenter know something about the inherent error in his sampling equipment, the variability within samples collected in the same area or habitat, and the variability between samples collected in different habitats. Consequently, the sampling design for interpretive studies is often based upon distribution models, sampling statistics, and expressions of biological diversity.

Statistical techniques in biological sampling

Regardless of the type or purpose of the study, sample collection should be designed on a meaningful basis. A large number of samples collected at the wrong time or place have less value than a few samples carefully selected as to time and place of collection. The frequency of sample collection will depend on the variability of environmental and biological factors and on the study objectives. The greater the habitat variability, the more intensive the sampling program must be. Life history events of the organisms also must be considered in the design of a sampling program. Some organisms have two or more generations a year; others have but one. Moreover, many aquatic insects spend only the juvenile period of their lives in water, emerging just prior to the adult transformation. In lakes and reservoirs, phytoplankton and zooplankton succeed one another in a somewhat rhythmic fashion; one may be abundant while the other is scarce. Vertical and horizontal movements of phytoplankton and zooplankton also are common in lakes and reservoirs. These facts, plus many others increase the variability of the results of biological investigations.

In spite of the temporal and spatial variability of aquatic populations, statistical techniques are available for the design of sampling programs and for the evaluation of biological data. References on the subject of statistical sampling and analysis are Snedecor (1956), Steel and Torrie (1960), Stanley (1963), and Snedecor and Cochran (1967). Specific work on sampling statistics in aquatic biology was reported by Needham and Usinger (1956), Gaufin, Harris, and Walter (1956), Ricker (1958, 1971), Chutter and Noble (1966), Elliott (1971), Gulland (1971), and Chutter (1972). Although it is beyond the scope of this manual to describe statistical sampling techniques in detail, the application and shortcomings of some mathematical models for the distribution of organisms, and some simple random-sampling techniques will be described briefly. Additional discussion of the methods given here is provided in the cited references.

For the purposes of the discussion to follow, certain terms and concepts must be defined. A clear distinction should be made at the onset between sampling statistics and test statistics. Sampling statistics are designed to determine the number of samples needed to make valid inferences about the population being sampled. Sampling statistics also provide a framework for the design of experiments and of data-collection programs. Test statistics are designed to determine if a significant difference exists between the means of several samples at a specified probability level. That is, test statistics help determine whether the different samples can be considered statistically as taken from the same or from different populations.

The purpose of statistical sampling is to gather quantitative information about some attributes of the population under consideration. The samples are used to represent the population, defined for statistical purposes as the whole aggregate of something within an area being studied. The population may be, for example, all the mayflies on the rocks of a particular riffle. An attribute or characteristic is some measurable or descriptive quality of the population. For example, it may be the number of mayflies of a particular species in the stream riffle or their dry weight per unit area. When the riffle is sampled, some of the mayflies are removed, and their kinds, numbers, weights, or other attributes are determined or measured and used to estimate these same attributes for all the remaining mayflies, that is, for the entire population in the riffle.

A statistic of a sample is used to estimate a parameter of the population. Standard symbols for distinguishing between the statistics of a sample and the parameter of a population (modified from Elliott, 1971, p. 13) are:

	Statistics of sample	*Parameters of population*
Arithmetic mean	$\bar{x}$	μ
Variance	s^2	σ^2
Standard deviation	s	σ
Standard error of mean	$s_{\bar{x}}$	σ_μ
Number of sampling units	n	N

A variable is a changeable feature of the population. Counts or measurements, such as number or weight of mayflies per unit area of stream are the variables that are recorded and are subject to statistical treatment.

A variable may be continuous or discontinuous (discreet). A continuous variable may assume any value within a given range. Measurements such as lengths or weights are, in theory, continuous variables because within their range they can have any value. Discontinuous variables can only assume integral (whole number) values. One of the most common discreet variables is the count; for example the number of mayflies per unit area of stream riffle. In the discussion which follows, the symbol x will be used to denote the variables. The symbol $\bar{x}$ (x-bar) denotes the mean or average of a group of sample variables.

A sampling unit is an area where a sample is collected. The number of sampling units in a particular aquatic environment will depend, for example, on the size of the area to be sampled (stream riffle, for example), the size of the sampling apparatus used, and the objectives of the study.

Before discussing statistical sampling, some mention must be made of the models used to describe the various distribution patterns of organisms in nature, and how discontinuous variables (count data) are transformed to approximate the normal distribution. Although the sampling statistics that will be given later can be used without direct application to distribution models, their ultimate success in estimating the number of organisms in the population or other discontinuous attributes depends upon an understanding and application of the statistics involved in the distribution models.

Distribution models

It was mentioned earlier that organisms are seldom distributed randomly or uniformly in nature, but rather that a clumped or patchy (contagious) distribution is usual (fig. 1). Nevertheless, there are examples where the individuals in a population form groups of a particular size and these groups closely approach a random distribution (Odum, 1971, p. 205). Moreover, although the organisms may be clumped within a riffle or other large area, the individuals within the clump may be distributed randomly (Odum, 1971, p. 206).

It is necessary to determine the type of distribution and the degree of clumping of organisms if meaningful statements are to be made about the population. Statistical methods designed for random or uniform distribution are not applicable for strongly clumped distributions (Elliott, 1971, p. 37; Odum, 1971, p. 205). Also the number of samples (sampling units) depends upon the type of distribution. If the organisms are truly randomly dispersed, only a few samples may be needed to gather useful information about the population. If the population is strongly clumped, however, many more samples will be needed. The size of the sampling units in the area to be sampled also is dependent upon the distribution of the organisms as will be shown later. Because the distribution patterns of organisms in a particular stream or lake are usually unknown, intensive sampling is often needed in the initial phases of a distribution or population estimation study.

If the region to be studied is divided into sampling units, then for the density of organisms per sampling unit, the three distribution patterns in figure 1 can be expressed as mathematical models based upon the relationship of the population variance (σ^2) to the population mean (μ). Elliott (1971, p. 16) lists the following three mathematical distributions and their uses:

1. Positive binomial: This model is appropriate (but only approximate) when the variance is significantly less than the mean ($\sigma^2<\mu$). It describes the uniform or regular distribution of figure 1.
2. Poisson: This model is used when the variance is approximately equal to the mean ($\sigma^2=\mu$). It describes the random distribution of figure 1.
3. Negative binomial: This is the model used when the variance is significantly greater than the mean ($\sigma^2>\mu$). It describes the clumped or patchy distribution of figure 1.

It is beyond the scope of this manual to describe the mathematical basis and calculations for each of these distributions, but some of their characteristics will be given. For additional discussion see Stanley (1963, p. 21–62), Snedecor and Cochran (1967, p. 199–227), and Elliott (1971, p. 14–79). Much of that which

follows is from Elliott (1971), which should be consulted for a more detailed treatment.

Positive binomial distribution

This model is used when the organisms are somewhat evenly distributed or spaced in their environment ($\sigma^2<\mu$). This uniform distribution may result when the individuals in a population are crowded and move away from one another such as young salmonid fish (Chapman, 1966). Territorial behavior may also produce a uniform distribution of sedentary invertebrates over a small area of stream or lake bottom (Elliott, 1971, p. 46).

Although the distribution of organisms in an aquatic environment may not be uniform over a large area (riffle bottom, for example), it may be uniform over a smaller area of a square meter or less. Thus, the regular distribution will often be useful in describing the dispersion of a population of organisms in a small area. The sampling unit must be chosen large enough so that $\mu>1$, but small enough so that sufficient samples can be collected in the area of interest.

Poisson series distribution

This model is used when the organisms are randomly distributed in space ($\sigma^2=\mu$). Generally, this is the first distribution of organisms to be considered. However, too often a random distribution is assumed without being determined. In a random distribution there is equal chance of an individual occupying any point in an area being sampled, and the presence of an individual at one point does not influence the position of other individuals nearby (Elliott, 1971, p. 38). There is, in fact, no system in a Poisson distribution—some individuals appear in groups and others as more widely dispersed individuals.

Thus, although a Poisson distribution is often accepted after agreement with a statistical test, the possibility still exists that the population has a nonrandom distribution. Consequently, the possible reasons for a random distribution must be considered. Elliott (1971, p. 39) mentions that random distribution could result from the influence of a single factor whose values are themselves randomly distributed, or from chance effects. He further mentions that if the first possibility is rejected, the second must be considered with the conclusions that environmental factors have either no effect or a minor effect on the dispersion of the population or there is no tendency for the individuals of the population to move toward each other.

Elliott ends by mentioning that these explanations may be difficult to accept and concludes that, while nonrandomness may be present, it is difficult to detect by field-sampling techniques.

Obviously the size of the individual sampling unit must be considered. If the size of a sampling unit is much larger or much smaller than the average size of the clumps of individuals in the sampled area, and the clumps are regularly or randomly distributed, then the population is apparently random. However, even a properly sized sample will not detect a clumped or patchy distribution if there are but a few individuals per sample. When the population dispersion is low, a random distribution is usually a suitable hypothesis. For a discussion see Stanley (1963, p. 57–62), MacArthur and Connell (1966, p. 44–57), and Elliott (1971, p. 38–45 and 68–71).

When the population dispersion is random, the variance of the sample (s^2) decreases steadily with the size of the sampling unit, so that the sample variance approaches the sample mean ($s^2 \doteq \bar{x}$) as the sample mean approaches zero. Whereas such results (that is, an appearance of equality of s^2 and $\bar{x}$) may seem to be strong evidence for random dispersion, Elliott (1971, p. 69) gives two reasons for caution: (1) The largest sampling unit may contain a sample smaller than the mean size of the clumps— that is, the dispersion is clumped or patchy with very large groups of individuals, or (2) the smallest sampling unit may be larger than the mean size of the clumps which are themselves regularly distributed.

Although there are pitfalls in assuming that organisms in a population are randomly distributed, it is still a useful assumption in many instances. When a large number of sampling units are sampled ($n\geqslant 30$), randomness often is a valid assumption, and statistical inferences usually can be made (Elliott, 1971, p. 81).

Negative binomial distribution

This is frequently the only model that truly describes the distribution pattern of organisms in nature. It is the clumped, patchy, or contagious distribution of Elliott (1971) ($\sigma^2>\mu$). Although there often are definite clumps or patches of organisms in nature, the distribution patterns of the clumps may vary greatly from habitat to habitat. The actual dispersion pattern will depend upon the size and spatial distribution of the clumps and, of course, on the spatial distribution of the individual organisms within the clumps (Elliott, 1971, p. 50). Consequently, there are various patterns for a clumped distribution, and though several mathematical models have been proposed to describe them, the negative binomial is probably the most useful.

The negative binomial distribution has two parameters, the mean and the exponent k, and is described in Elliott (1971, p. 23–29). Because the distribution can be derived from several models, it is useful in describing several types of clumped or patchy distributions. Elliott (1971, p. 51) lists the following models as being appropriate to benthic samples:

1. True contagion: When the presence of one individual increases the chance that another will occur in the same sampling unit.
2. Constant birth-death-immigration rates: If each clump of a population has constant rates of birth and death per individual and of immigration rate per unit of time, then a negative binomial will result.
3. Randomly distributed clumps: If the clumps of individual organisms are distributed randomly and the individuals are in a logarithmic distribution within the clumps, then a negative binomial will result.

For a complete discussion of the negative binomial distribution as applied to organisms in nature, see Elliott (1971, p. 50–79).

Test statistics and data transformation

The discussion of the distribution of organisms in nature has been concerned with count data or discontinuous variables; that is, the number of organisms inhabiting a unit area or volume. Counts are important in biological studies, but in many instances the variables measured are continuous; that is, they may assume any value within a given range. Lengths, weights, and ages of organisms are examples of continuous variables. These types of measurements generally follow the normal distribution. Although the normal distribution is not a useful model for count data, it is the distribution often associated with statistical tests of significance between sample means. These tests are not included in this section although they are the next logical step in the analysis of biological data after adequate samples have been collected.

Statistical tests based upon the normal distribution are called parametric tests, and they involve evaluating the null hypothesis which assumes that a particular distribution is a useable model for the samples. Nonparametric tests are available (Snedecor and Cochran, 1967, p. 120–134; Elliott, 1971, p. 112–125), but most statistical testing has traditionally been based upon the estimates of parameters of the normal distribution.

The requirements for the use of the normal distribution for statistical inference (Snedecor, 1956, p. 35–65; Stanley, 1963, p. 21–31; Snedecor and Cochran, 1967, p. 32–65; and Elliott, 1971, p. 94–112) are as follows:

1. The variance of the sample must be independent of the mean.
2. The components of the variance must be additive.
3. The frequency of the counts must approximate a normal distribution.

Because these conditions are often not realized with count data, it is often necessary to transform the data to normalize their frequency and to fulfill the other requirements of a normal distribution (Elliott, 1971, p. 30 and 98). Snedecor (1956, p. 314–328), Snedecor and Cochran (1967, p. 325–338), and Elliott (1971, p. 30–36) discuss the transformation of discontinuous (count) data. Although no single transformation is useable for all distributions, Elliott (1971, p. 33) suggests the following empirical transformations for the several distributions discussed above:

Original distribution	Distribution not known	Transformation	Special conditions
Poisson	$s^2=\bar{x}$	Replace x by $\sqrt{x}$.	No counts <10.
Poisson	$s^2=\bar{x}$	Replace x by $\sqrt{x+0.5}$.	Some counts <10.
Negative binomial	------	Replace x by $\sinh^{-1}\sqrt{\frac{x+0.375}{k+2(0.375)}}$.	$k>5$.
Negative binomial	------	Replace x by $\log(x+k/2)$.	$2<k<5$.
	$s^2>\bar{x}$	Replace x by $\log x$.	No zero counts.
	$s^2>\bar{x}$	Replace x by $\log(x+1)$.	Some zero counts.

Statistical sampling

Statistical sampling techniques permit some estimate, within a given range, to be made about the number of organisms in a population or some other attribute associated with the organisms in the population. They are not without their pitfalls, however, and not the solution to all biological sampling problems. As Elliott (1971, p. 9) has warned, "Statistical methods should not be used as a salvage operation!" They must be used carefully and with an understanding of their limitations.

Sample size

Determination of the number of sampling units (n) that must be sampled in order to yield meaningful results is of paramount importance in the practical problems of designing and financing a biological study program. Unfortunately, no specific answer can be given to the question of sample size because the number of samples needed depends upon: (1) The size (area or volume) of the sampling unit, (2) the number of sampling units in each area or volume to be sampled, (3) the location of the selected sampling units in the area to be sampled (Elliott, 1971, p. 128), and (4) the cost of collecting and analyzing samples.

Generally, a sampling unit of small size (area or volume) is the most suitable for determining the distribution pattern of a population. If the population has a truly random distribution, then all sampling units should be equally useful in the estimation of the population parameter. A small sampling unit is also more efficient (efficiency being measured in terms of the relative sample sizes needed to give estimates of equal precision) than a larger unit when the dispersion of the population is clumped or patchy. The advantages of a small sampling unit over a larger one are: (1) More small sampling units can be taken for the same amount of effort, (2) many small sampling units have more degrees of freedom than a few larger sampling units (degrees of freedom will be discussed later), and (3) many small sampling units will cover a wider range of habitat types than a few larger units. A disadvantage in the use of the small sampling unit is that the sampling error at the edge of the unit is greater. Thus, the size of the sampling unit must be a compromise between statistical accuracy and practical requirements (Elliott, 1971, p. 128).

Elliott (1971, p. 81–93) lists the total sample sizes needed (n = number of sampling units required) for the various types of distribution models for count data previously discussed. In general, he suggests that with rare exceptions at least 30 sampling units be sampled, but preferably 50. In reality a sampling of even 30 sampling units often cannot be realized. Nevertheless, the experimenter should be aware of the number of sampling units needed for accurate estimates of the population or its attributes. More will be said about the required number of sampling units under the various statistical sampling methods that follow. In the samples that illustrate the calculations of the several techniques, the sample number has been kept purposefully low to illustrate the application of the formula.

Simple random sampling

Random sampling requires that the sampling units be selected without bias and that the samples collected be representative of the entire population (Elliott, 1971, p. 131). Consequently, every sampling unit in the population must have an equal chance of being selected. This is often accomplished with a table of random numbers such as in Snedecor (1956, p. 10–13), Arkin and Colton (1962, p. 158–161), and Snedecor and Cochran (1967, p. 543–546).

As an example, a grid system established on a stream riffle revealed that there were 200 sampling units. The investigator wished to select randomly 10 of these units for the purpose of collecting samples. A table of random numbers was consulted and the first 10 three-digit numbers between 001 and 200 that appeared either in the columns or rows were taken as the units to be sampled. For example, in Snedecor and Cochran (1967, p. 543), starting with row 00, sampling units 085, 058, 186, 015, 030, 149, 079, 002, 095, and 070 would be chosen. Here the 10 numbers were selected between row numbers 00 and 06. However, it is not necessary to start at the top of the table of random numbers; any row or column can be used. The 10 sampling units also could have been drawn from 200 numbered and well mixed cards or key tags, placed in a container.

Simple random sampling is popular because it is easy to carry out, is unbiased, and provides an estimate of the error of sampling, or how much the sample statistics may be expected to vary from the population parameters. A random sample usually will have a relatively large error when applied to a natural population. It is for this reason that stratified random sampling (to be discussed later) is preferred. Nevertheless, there are numerous occasions in which simple random sampling will provide adequate results; for example, in phytoplankton sampling in well-mixed lakes, rivers, or estuaries, or even for benthic invertebrate sampling in areas of uniform substrate.

The mean ($\bar{x}$) of a simple random sample can be estimated as

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} \quad (1)$$

where x_i is an individual observation and n is the sample size, or the number of sampling units sampled. The mean of a group of data represents the value that, on the average, would be expected to occur. Expected upper and lower values or confidence limits for the mean can be calculated, and when this is done, the mean becomes a more useful statistic because expected deviations from it are known.

The first step in placing limits around the sample mean consists of calculating the sample variance. The variance of a sample is the sum of the squared deviations of the individual variables ($\bar{x}$) from the mean of all the variables ($\bar{x}$). It is calculated as

$$s^2 = \frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1} = \frac{\sum_{i=1}^{n} x_i^2 - \frac{\left(\sum_{i=1}^{n} x\right)^2}{n}}{n-1} \quad (2)$$

where s^2 is the sample variance, and x is the arithmetic mean (as above). The term $\sum_{i=1}^{n} x_i^2$ is the sum of the squared individual variables or observations, whereas $\left(\sum_{i=1}^{n} x\right)^2$ is the square of the sum of the individual variables. The sample size is denoted by n. Note that instead of dividing the sum of the squared deviations by n, as was done in determining the mean, the value $n-1$ is used. The notation $n-1$ refers to the degrees of freedom of the sample. Though the reason for this is long and involved, it is an important distinction. See Snedecor and Cochran (1967, p. 45–46) for a discussion of degrees of freedom.

The standard error of the mean ($s_{\bar{x}}$) is a measure of how the sample mean varies. It provides information on how much error is inherent in an estimate of the mean and how reliable the sample mean may be when it is used to estimate the population mean.

The standard error of the mean of a simple random sample is computed as

$$s_{\bar{x}} = \sqrt{\frac{s^2}{n}} \quad (3)$$

where s^2 is the sample variance as before.

The standard deviation of a random sample (s), calculated as the square root of the variance, is a measure of the spread of the individual measurements about the mean (Snedecor and Cochran, 1967, p. 32–34) and should not be confused with the standard error of the mean.

The standard error of the mean provides useful information, but it is often desirable to place some confidence limits about the mean. The confidence limits define the upper and the lower values that the mean may have at a given probability level. For example, if confidence limits are calculated for the sample mean at the 95-percent probability level, it means that the probability is 95 to 5 (19 to 1), that the population mean (μ) lies somewhere between the calculated limits placed on the sample mean ($\bar{x}$).

The confidence limits about the mean are calculated as

$$\bar{x} \pm (t)(s_x) \quad (4)$$

where t is from Student's t distribution and has $n-1$ degrees of freedom, and s_x is the standard error of the mean as given in equation 3. Values for t are given in Snedecor (1956, p. 46), Arkin and Colton (1962, p. 121), Snedecor and Cochran (1967, p. 549), and in other statistical texts. The table is used by noting the degrees of freedom ($n-1$ in equation 2) in the left-hand column, and reading the t-value in the body of the table under the appropriate probability level. Note that the probability level (P) appears at the top of the table as the probability that a value will fall outside the probability limits; for example, the t values for the 95-percent probability value are to be found in the column 0.05 (that is, $1-0.95$). Although the value of t is usually taken at the 95-percent probability level ($P=0.05$) in biological sampling, the experimenter need not be bound by this general rule.

From a preliminary sample, it is possible to calculate the number of additional samples (n) needed in order to determine the mean within some preselected percentage error

$$n = \frac{t^2 s^2}{L^2} \quad (5)$$

where L^2 is a preselected allowable error of the sample mean and t^2 and s^2 are as defined above.

The degrees of freedom for t are not known for this equation because n is unknown. As a result, the value of t is usually approximated as 2. This is sufficiently accurate because the value of t at the 95-percent probability level ($P=0.05$) ranges only from 2.042 at 30 degrees of freedom to 1.960 at ∞ degrees of freedom.

The value for L is selected by the experimenter on the basis of program objectives and operational constraints. Too great a level of accuracy, however, may be expensive or unattainable because the allowable error in sampling is squared. Thus, a 5-percent allowable error would require not twice, but four times as many samples as a 10-percent allowable error. An illustration of this formula applied to a biological problem may be found in Snedecor and Cochran (1967, p. 516–519), Edmondson and Winberg (1971, p. 193–194), and in the example that follows.

Example 1. Calculations involving a simple random sample

A total of 150 multiple-plate artificial-substrate samplers (see Part 2, "Benthic Invertebrates, Numerical Assessment") were placed in a single riffle. After 4 weeks, 10 of the samplers were removed, and the number of caddisfly larvae (Trichoptera) were counted on each of the 10 samplers. The counts were as follows:

Sampler	Number of caddisfly larvae (x_i)	x_i^2
1	15	225
2	12	144
3	12	144
4	8	64
5	13	169
6	11	121
7	14	196
8	12	144
9	10	100
10	13	169
Total	120	1,476

From these counts the mean was calculated as in equation 1:

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} = \frac{120}{10} = 12.0.$$

In this instance, because the data are discontinuous, whole numbers are used. Thus, on the average, each multiplate sampler would be expected to yield 12 caddisfly larvae.

From equation 2, the variance (s^2) of the sample becomes:

$$s^2 = \frac{\sum_{i=1}^{n} x_i^2 - \frac{\left(\sum_{i=1}^{n} x\right)^2}{n}}{n-1} = \frac{1{,}476 - \frac{120^2}{10}}{9} = 4.0.$$

The standard error of the mean ($s_{\bar{x}}$) from equation 3 is

$$s_{\bar{x}} = \sqrt{\frac{s^2}{n}} = \sqrt{\frac{4.0}{10}} = \sqrt{0.4} = 0.63.$$

In this example, for a standard deviation of one, the mean value would be expected to vary by one caddisfly larva (12 ± 1), that is, range between 11 and 13 larvae. If probability statements about the mean are to be made, it is necessary to calculate a confidence limit of the mean (equation 4). For this example, the value of t, at the 95-percent probability level ($P=0.05$) and at $n-1$ or nine degrees of freedom is 2.262. The confidence limits about the mean thus become

$$\bar{x} \pm (t)(s_{\bar{x}}) = 12 \pm (2.262)(0.63) = 12 \pm 1.42.$$

Therefore, from the preliminary sample, there is a 95-percent probability that the population mean (μ) number of caddisfly larvae lies between 12 ± 2, or 10 and 14.

With equation 5 it is possible to calculate the number of samples needed to keep the final mean at a given probability level, within some preselected percentage error. Considering the value of t to be 2, at the 95-percent probability level ($P=0.05$), the number of samples needed for an allowable 10-percent error of the sample mean would be

$$n = \frac{t^2 s^2}{L^2} = \frac{(4)\ (4)}{(12 \times 0.1)^2} = 11.$$

Thus, if a 10-percent error is allowable, 11 samples must be collected. If the allowable error is 20 percent, 16/5.6 or 3 samples must be collected. A 30-percent error would require 16/13 or only 1 sample.

Stratified random sampling

Stratified random sampling is useful when the strata are distinct, have known sizes, and when an individual stratum is more homogeneous than the population as a whole. The method is particularly useful in that it provides information on the relative sample sizes needed to equalize the variances among the several strata. Stratified random sampling has obvious applicability in reservoir and lake sampling, where sampling stations are established at several locations horizontally, but the samples are collected at various depths. In this application, the method determines the depth at which the variance of a given constituent is greatest and the number of samples needed to reduce the variance to a particular level.

In practice, the entire sampling area is divided into various strata and each stratum is sampled independently. A stratum is a subdivision either by area or volume. It may refer to the layering of water masses in a thermally stratified lake or reservoir or to horizontal stratification, such as morphological changes in the type of bottom material in a stream section.

If prior information is available about the relation between the variable of interest and other controllable variables, then stratified random sampling increases sampling efficiency by dividing the population into several more or less homogeneous strata and is preferred over simple random sampling (Elliott, 1971, p. 132). Moreover, stratified random sampling increases the accuracy of population estimates because it attempts to sample so that the strata or subdivisions of the population are adequately represented.

If the strata are unequal in size (area, for example) the number of sampling units allocated is made proportional to the area of each stratum; that is, to the total number of available sampling units in the stratum. The actual sampling units (areas to be sampled) within each stratum are located randomly.

When the units in a sample have been proportionally allocated, the sampling fraction in each stratum is the same (Elliott, 1971, p. 132); that is,

$$\frac{n_1}{N_1}=\frac{n_2}{N_2}=\cdots=\frac{n_k}{N_k}=\frac{n}{N} \tag{6}$$

where n_1 is the number of samples collected within stratum 1, and N_1 is the total sample units within stratum 1. That is, random sampling units of n_1, n_2, . . . n_k units are selected from the k strata containing N_1, N_2, . . . N_k sampling units. Note that n and N refer to the total number of sampling units in the sample and in the population, respectively. These are calculated as

$$\begin{aligned}\sum_{i=1}^{k} n &= n_1+n_2+\cdots+n_k \\ \sum_{i=1}^{k} N &= N_1+N_2+\cdots+N_k\end{aligned} \tag{7}$$

therefore N_1/N, N_2/N are the relative weights attached to each stratum. The sample thus becomes self-weighting, and the arithmetic mean of the whole sample is the best estimate of the population mean (Elliott, 1971, p. 133). The number of sampling units to sample in each stratum (n_i) is calculated as

$$n_i=\left(\frac{N_i}{N}\right)(n). \tag{8}$$

The simplest allocation is to make the sampling fraction the same in each stratum, as given in equations 6 and 8, but the optimum allocation is to make n_i proportional to $N_i s_i$. That is,

$$n_i=\frac{N_i s_i}{\sum_{i=1}^{k} N_i s_i} \tag{9}$$

where N_i is the total number of sampling units in the ith stratum and s_i is the standard deviation (square root of the variance) for the ith stratum (Snedecor and Cochran, 1967, p. 523–526). The stratum variance s_i^2 is determined from a preliminary sample in each stratum. The optimum sample allocation requires that the sample units be selected randomly.

Stratified random samples have an overall mean $\bar{x}$ calculated as

$$\bar{x}=\frac{\sum_{i=1}^{k} n_i\bar{x}_i}{\sum_{i=1}^{k} n}=\frac{n_1\bar{x}_1+n_2\bar{x}_2+\cdots+n_k\bar{x}_k}{n} \tag{10}$$

where n_i is the number of sampling units sampled in the ith stratum, and $\bar{x}_i$ is the sample mean from these sampling units. Again, n designates the number of sampling units sampled from all the strata (Snedecor and Cochran, 1967, p. 520; Elliott, 1971, p. 133).

The variance of the mean ($s_{\bar{x}}^2$) of a stratified random sample is calculated as

$$\begin{aligned}s_{\bar{x}}^2 &= \sum_{i=1}^{k}\left(\frac{N_i}{N}\right)^2\left(\frac{s_i^2}{n_i}\right)\left(1-\frac{n_i}{N_i}\right) \\ &=\left(\frac{N_1}{N}\right)^2\left(\frac{s_1^2}{n_1}\right)\left(1-\frac{n_1}{N_1}\right) \\ &\quad+\left(\frac{N_2}{N}\right)^2\left(\frac{s_2^2}{n_2}\right)\left(1-\frac{n_2}{N_2}\right) \\ &\quad+\cdots+\left(\frac{N_k}{N}\right)^2\left(\frac{s_k^2}{n_k}\right)\left(1-\frac{n_k}{N_k}\right)\end{aligned} \tag{11}$$

where s_i^2 refers to the sample variance of the ith stratum calculated as in equation 2 for a simple random sample and N_i and n_i and N are the total number of sampling units in the ith stratum, the number of sampling units sampled in the ith stratum, and the total number of sampling units in all strata (ΣN_i), respectively.

If the sampling units actually sampled in a stratum exceed 10 percent of the total sampling units in the stratum, that is, if $n_i/N_i>0.1$, then a finite correction

factor, $1-(n_i/N_i)$, is needed (Snedecor and Cochran, 1967, p. 522; Elliott, 1971, p. 133). The finite correction factor can be computed only when the total number of sampling units in each stratum is known. With a simple random sample, the total number of possible sampling units is either seldom known, or n_i/N_i does not exceed 0.1.

The standard error of the mean ($s_{\bar{x}}$) of a stratified random sample is

$$s_{\bar{x}} = \sqrt{\sum_{i=1}^{k} \left(\frac{N_i}{N}\right)^2 \left(\frac{s_i^2}{n_i}\right) \left(1-\frac{n_i}{N_i}\right)} \qquad (12)$$

which is the square root of the variance of the mean as given in equation 11. Once again, the finite correction factor may or may not be used.

An approximate confidence interval for the strata mean can be calculated from equation 4, with t having $n-1$ degrees of freedom.

Example 2. Calculations involving a stratified random sample

Consider the stream riffle discussed under "Grid or Quadrant" in "Biological Surveys and Simplified Sampling Methods." In the example the riffle measured 5 by 20 m and was to be sampled with a 0.5-m^2 sampler. Consequently, there were 200 sampling units ($N=200$) in the riffle. Consider that further observations revealed four distinct strata based upon the type of bed material in the riffle. Upon measurement these four strata contained approximately the following number of sampling units: Rubble and gravel (N_1), 50 sampling units; gravel and sand (N_2) , 60 sampling units; gravel and mud (N_3), 60 sampling units; and sand (N_4), 30 sampling units.

If a total of 40 sampling units (n) are to be sampled from all the strata, then using proportional allocation (eq 8), the following calculations are made to determine the number of sampling units to be sampled in each stratum:

Stratum habitat type	Stratum No.	$\frac{N_i}{N}n$	Number of sampling units (n_i) to sample in each stratum
Rubble and gravel	1	$\frac{50}{200}\times 40$	10
Gravel and sand	2	$\frac{60}{200}\times 40$	12
Gravel and mud	3	$\frac{60}{200}\times 40$	12
Sand	4	$\frac{30}{200}\times 40$	6

From this simple proportional allocation of sampling units among the four strata, 10 sampling units would be sampled in stratum 1, 12 each in strata 2 and 3, and 6 in stratum 4.

Let one objective of the study be to determine the number of Chironomidae (midge) larvae found in the samples taken in each stratum as well as in the riffle as a whole. To do this, the 10 sampling units in stratum 1 are sampled, the samples sorted, and the number of Chironomidae larvae counted. The same procedure is followed for the remaining three strata. From these data the mean ($\bar{x}$), as given in equation 1, and the variance (s^2) from equation 2, as given for simple random sampling, and the sample standard deviation (square root of the variance) are computed for each stratum. The results of these calculations are as follows:

Stratum habitat type	Sampling units		Chironomidae per sampling unit		
	Per stratum (N_i)	Sampled per stratum (n_i)	Mean ($\bar{x}_i$)	Variance (s_i^2)	Standard deviation (s_i)
Rubble and gravel (n_1)	50	10	10	3	1.73
Gravel and sand (n_2)	60	12	5	5	2.24
Gravel and mud (n_3)	60	12	12	3	1.73
Sand (n_4)	30	6	2	1	1.00
Total (Σ)	200	40	--	--	----

Note that the standard deviation is simply the square root of the stratum variance. This is the sample variance as given in equation 2 and should not be confused with the standard error of the mean as given in equation 3. The standard deviation values will be used later in calculating the optimum allocation.

Using the results tabulated above, the overall mean of the stratified random sample (mean number of Chironomidae) can be calculated from equation 10 as follows:

$$\bar{x} = \frac{\sum_{i=1}^{k} n_i \bar{x}_i}{\sum_{i=1}^{k} n} = \frac{(10)\,(10)}{40} + \frac{(12)\,(5)}{40} + \frac{(12)\,(12)}{40} + \frac{(6)\,(2)}{40} = 7.9;$$

or, because these are discontinuous (discrete) variables, the mean would be eight Chironomidae per sampling unit.

A simple random mean as given in equation 1 would have been 29/4 or 7.25.

The overall variance of this stratified sample mean must consider the finite correction factor, because $n_1/N_1=0.2$, or is greater than 0.1. Consequently, the variance of the mean must be calculated as in equation 11 as follows:

$$s_{\bar{x}}^2=\sum_{i=1}^{k}\left(\frac{N_i}{N}\right)^2\left(\frac{s_i^2}{n_i}\right)\left(1-\frac{n_i}{N_i}\right)$$

$$=\left(\frac{50}{200}\right)^2\left(\frac{3}{10}\right)\left(1-\frac{10}{50}\right)$$

$$+\left(\frac{60}{200}\right)^2\left(\frac{5}{12}\right)\left(1-\frac{12}{60}\right)$$

$$+\left(\frac{60}{200}\right)^2\left(\frac{3}{12}\right)\left(1-\frac{12}{60}\right)$$

$$+\left(\frac{30}{200}\right)^2\left(\frac{1}{6}\right)\left(1-\frac{6}{30}\right)=0.066.$$

The standard error of the sample mean is calculated as in equation 12 as follows:

$$s_{\bar{x}}=\sqrt{\sum_{i=1}^{k}\left(\frac{N_i}{N}\right)^2\left(\frac{s_i^2}{n_i}\right)\left(1-\frac{n_i}{N_i}\right)}$$

$$=\sqrt{0.066}=0.26.$$

Using the standard error of the mean, it is possible to calculate an estimated confidence limit for the overall mean of the stratified random sample as given in equation 4. The finite value of t, at the 95-percent probability level ($P=0.05$) is 1.96, which may be used. Consequently, the approximate confidence limits about the mean at the 95percent probability level become

$$\bar{x}\pm(t)(s_{\bar{x}})=7.9\pm(1.96)(0.26)=7.9\pm0.51.$$

Thus, the mean number of Chironomidae in the riffle would be expected to vary between 1,580±102, or between 1,478 and 1,682 with a confidence of 95 percent; that is, 7.9×200, the mean times the total number of sampling sites, ±0.51×200, the standard error times the number of sampling sites. In practice, the mean 7.9 would be rounded to the nearest integer, or 8.

Although this simple method of proportional allocation of sampling units is useful, the best allocation of the sampling units to be sampled among the various strata is to chose n_1 proportional to N_1s_1, that is, proportional to the standard deviation of the sample multiplied by the total number of sampling units in the stratum under consideration. When this is done, the total number of sampling units to be sampled in each stratum is a function of the standard deviation (square root of the variance) of the preliminary sample. Consequently, the final allocation will be to collect more samples from those strata with a high standard deviation and fewer samples from those strata with a lower standard deviation.

Optimum allocation can be determined by taking a subsample, estimating the variance, and then applying these results in determining the number of samples to be collected within each stratum. For example, using the results in the previous table, let the overall objective be td sample 80 sampling units in the four strata, and let the previous 40 sampling units be the preliminary sample from which the variance and standard deviation were determined. When this is done, the optimum allocation of the 80 sampling units would be as follows:

Stratum habitat type	Sampling units per stratum (N_i)	Standard deviation of each stratum sample (s_i)	N_is_i	Relative sample size $\left(\frac{N_is_i}{\Sigma N_is_i}\right)$	Sampling units to sample per stratum $\left(\frac{N_is_i}{\Sigma N_is_i}\times 80\right)$
Rubble and gravel (n_1)	50	1.73	86.5	0.24	19
Gravel and sand (n_2)	60	2.24	134.4	.38	30
Gravel and mud (n_3)	60	1.73	103.8	.29	23
Sand (n_4)	30	1.00	30.0	.08	6
Total (Σ)	200	----	354.7	----	--

The estimated optimum allocation for the riffle under consideration would be to sample 19 sampling units (0.24×80) in the rubble and gravel stratum, 30 units (0.38×80) in the gravel and sand stratum, 23 units (0.29×80) in the gravel and mud stratum, and six units (0.08×80) in the sand stratum.

The preceeding examples are concerned with the sampling of stream or lake beds. The stratified-random-sampling technique obviously has much wider applicability in biological study design. For example, phytoplankton (as well as other materials in suspension or solution) may be sampled in rivers as a function of discharge, using the stratified random sampling technique. The long-term average discharge of the river at a given sampling site is plotted as a function of time and the resulting hydrograph is then partitioned into various strata based upon the discharge. The number of samples to be collected at a given discharge is determined using the proportional or optimum allocation schemes as described above.

Thermal and chemical stratification may occur in lakes, reservoirs, or deep rivers, resulting in well-

defined water layers or masses. The distribution of organisms within the various strata may be quite different, making stratified random sampling the most effective technique. In the case of summer thermal stratification in a lake, the strata normally would be the epilimnion, metalimnion and the hypolimnion. The size of each stratum would be either the depth or volume of the specific thermal zone. The number of samples to collect, for example, of phytoplankton, would be based upon proportional or optimum allocation, using depth or volume as strata boundaries.

When designing sampling programs, physiological requirements of the organisms should be considered. It would be useless for example, to sample for fish or even most invertebrates in an anoxic layer of a lake. The experimenter must always take these factors into consideration in the design of sampling programs.

Cluster or two-stage sampling

When the sampling units fall into obvious groups or clusters, the sampling scheme can be based upon cost factors. The first requirement is that the primary units (n_1) be selected. These may be, for example, the numbers of riffles in a particular stream section. The second requirement is to select the number of secondary or subunits (n_2) within each primary unit. After the number of n_1, or primary groups, have been chosen, the n_2, or secondary groups, are randomly selected and the samples collected.

The objectives of cluster sampling are to determine how many samples are needed to reduce the sample variance without exceeding a given cost (Snedecor and Cochran, 1967, p. 528–534). For example, it may be found that the establishment of more sampling stations, with the subsequent collection of fewer samples per station, will provide a lower variance, at less cost, than a reduction in stations with an increase in the number of samples per station. Needed is an estimate of the costs (c_1 and c_2) for the primary (n_1) and secondary (n_2) units. For example, c_1 might be the cost incurred in order to visit a site, and c_2 might be the cost for collecting and processing the sample once the observer is at the site.

The estimated mean ($\bar{x}$) of a cluster sample is:

$$\bar{x} = \frac{\sum_{i=1}^{n_1}\sum_{j=1}^{n_2} x_{ij}}{\sum_{i=1}^{n_1} n_2} \qquad (13)$$

where x_{ij} is the value of the variable recorded or measured at the jth secondary unit of the ith primary unit. The term n_1 refers to the number of primary units sampled (for example riffles in a stream section) and the term n_2 to the number of secondary units sampled (for example sampling units sampled in each riffle).

Before calculating the overall variance of the mean, it is necessary to calculate the within-sample variance (s_2^2) and the between-sample variance (s_1^2) from the preliminary samples. The within-sample variance is calculated as follows:

$$s_2^2 = \frac{\sum_{i=1}^{n_1}\sum_{j=1}^{n_2}(x_{ij}-\bar{x}_i)^2}{\sum_{i=1}^{n_2}(n_2-1)} = \frac{\sum_{i=1}^{n_1}\sum_{j=1}^{n_2} x_{ij}^2 - \frac{\sum_{i=1}^{n_1}\left(\sum_{j=1}^{n_2} x_{ij}\right)^2}{\sum_{i=1}^{n_1} n_2}}{N-n_1}. \qquad (14)$$

This is the same as equation 2, except that the x_{ij} refers to all the variables (x's) in all the samples collected in all the primary (n_1) units. The term x_i is the mean as calculated in equation 1 under simple random sampling and is the mean for all samples taken at the ith primary unit, for example within a given riffle. The term N is the total number of samples collected at all n_1 plus n_2 sites.

The second expression for equation 14 is easier to use. The term $\sum_{i=1}^{n_1}\sum_{j=1}^{n_2} x_{ij}^2$ is the sum of the squares of all the individual observations from all the primary or n_1 groups. The term $\sum_{i=1}^{n_1}\left(\sum_{j=1}^{n_2} x_{ij}\right)^2 \Big/ \sum_{i=1}^{n_1} n_2$ is the square of the sums of all the secondary (n_2) groups, divided by the number of sampling units sampled in each secondary group. Note that the equation is divided by $N-n_1$ degrees of freedom. For example, if 30 is the total number of sampling units sampled in a group of three primary units, the degrees of freedom ($N-n_1$) would be $30-3=27$.

The between-sample variance (s_1^2) is calculated as follows:

$$s_1^2 = \sum_{i=1}^{n_1}\frac{(\bar{x}_i-\bar{x})}{n_1-1} = \frac{\frac{\sum_{i=1}^{n_1}\left(\sum_{j=1}^{n_2} x_{ij}\right)^2}{\sum_{i=1}^{n_1} n_2} - \frac{\sum_{i=1}^{n_1}\sum_{j=1}^{n_2}(x_i)^2}{\sum_{i=1}^{n_1}\sum_{j=1}^{n_2} n}}{n_1-1} \qquad (15)$$

where $\bar{x}_i$ is the mean among samples in a given area, and $\bar{x}$ is the mean of all samples from all areas. The term $\sum_{i=1}^{n_1}\left(\sum_{j=1}^{n_2} x_{ij}\right)^2$ is, as before, from the second expression for equation 14, whereas the term $\sum_{i=1}^{n_1}\sum_{j=1}^{n_2}(x_i)^2$ is the square of the sum of all observations from all sampling units. Note that equation 15 is the variance between sampling units. These two equations provide estimates of the within-site variance (that is, the variance between samples within a given site) and the between-site variance (that is, the variance between samples collected at different sites).

According to Snedecor and Cochran (1967, p. 280 and 529) it is necessary to calculate the component of variance for the between-site variance. This estimated component of the between-site variance is calculated as

$$\hat{s}_1^2 = \frac{s_1^2 - s_2^2}{n_0} \tag{16}$$

where s_1^2 and s_2^2 are, as before, the between- and within-sample variances. The term n_0 is the number of secondary units taken per primary (n_1) units if the number of primary units are equal (Snedecor and Cochran, 1967, p. 281 and 530).

With these two variances ($\hat{s}_1^2$ and s_2^2) calculated, the variance of the mean ($s_{\bar{x}}^2$) of a cluster or two-stage sample is calculated as

$$s_{\bar{x}}^2 = \frac{\hat{s}_1^2}{\sum_{i=1}^{n} n_1} + \frac{s_2^2}{\sum_{i=1}^{n_1} n_2} \tag{17}$$

where n_1 and n_2 are, as before, the number of primary and secondary units, respectively.

If the total cost (C) of sampling the primary and secondary units is

$$C = c_1 n_1 + c_2 n_1 n_2 \tag{18}$$

where c_1 and c_2 are the cost of sampling n_1 and n_2, respectively, then the best allocation, as estimated from a preliminary sample, is to choose the number of secondary units to sample in each primary unit as

$$n_2 = \sqrt{\left(\frac{c_1}{c_2}\right)\left(\frac{s_2^2}{\hat{s}_1^2}\right)}. \tag{19}$$

From equation 18 the number of primary units to be sampled can be calculated as

$$n_1 = \frac{C}{c_1 + n_2 c_2}. \tag{20}$$

Note the similarity between the cluster or two-stage sampling scheme and the optimum allocation method given under stratified random sampling. Both methods are based upon the variance or standard deviation from preliminary samples. Consequently, rather than being subjective, the number of samples to collect is based upon a sampling statistic.

The following examples illustrate the use and calculation of cluster or two-stage sampling.

Example 3. Calculations involving cluster or two-stage sampling

Consider a stream section having a series of pools. The bottom sediment in each pool is similar, consisting of sand mixed with clay and organic debris. As a preliminary effort, four pools were selected for sampling. In each pool, three Ekman dredge samples were collected. (See Part 2, "Benthic Invertebrates.") From these preliminary samples the wet weights of chironomid (midge) larvae per Ekman dredge haul were determined. An analysis of the preliminary samples revealed a cost ratio of n_1 to n_2 of 30; that is, $c_1 = 30c_2$.

The data for the four pools were arranged as follows in which x is the chironomid weight (in milligrams) from each Ekman dredge haul:

Dredge sample	Pool 1		Pool 2		Pool 3		Pool 4	
	x	x^2	x	x^2	x	x^2	x	x^2
1	3	9	1	1	2	4	4	16
2	4	16	1	1	1	1	2	4
3	5	25	2	4	3	9	3	9
Total	12	50	4	6	6	14	9	29

From these values, the overall mean can be calculated from equation 13 as follows:

$$\bar{x} = \frac{\sum_{i=1}^{n_1}\sum_{j=1}^{n_2} x_{ij}}{\sum_{i=1}^{n_1} n_2} = \frac{12+4+6+9}{12} = 2.58.$$

The next step is to compute the within-sample variance (s_2^2) as given in equation 14:

$$s_2^2 = \frac{\sum_{i=1}^{n_1}\sum_{j=1}^{n_2} x_{ij}^2 - \frac{\sum_{i=1}^{n_1}\left(\sum_{j=1}^{n_2} x_{ij}\right)^2}{\sum_{i=1}^{n_1} n_2}}{N-n_1}$$

$$= \frac{50+6+14+29 - \frac{12^2}{3}+\frac{4^2}{3}+\frac{6^2}{3}+\frac{9^2}{3}}{8}$$

$$= \frac{99-92.3}{8} = 0.84.$$

The first calculation for the between-sample variance (s_1^2) is made as in equation 15:

$$s_1^2 = \frac{\frac{\sum_{i=1}^{n_1}\left(\sum_{j=1}^{n_2} x_{ij}\right)^2}{\sum_{i=1}^{n_1} n_2} - \frac{\sum_{i=1}^{n_1}\sum_{j=1}^{n_2}(x_i)^2}{\sum_{i=1}^{n_1}\sum_{j=1}^{n_2} n}}{n_1-1}$$

$$= \frac{92.3 - \frac{(31)^2}{14}}{4-1} = 7.9.$$

Note that the value 92.3 is part of the within-sample variance calculation. The value 31 is simply the sum of all the variables (x).

Before computing the number of n_1 and n_2 units to be sampled, it is necessary to calculate an estimate of the component of the between-site variance ($\hat{s}_1^2$) using equation 16. Thus

$$\hat{s}_1^2 = \frac{s_1^2 - s_2^2}{n_0} = \frac{7.9-0.84}{3} = 2.35.$$

As a result, the variance components are $\hat{s}_1^2=2.35$ and $s_2^2=0.84$.

From these two variance values, the overall variance of the mean (eq 17) is calculated:

$$s_{\bar{x}}^2 = \frac{\hat{s}_1^2}{\sum_{i=1}^{n} n_1} + \frac{s_2^2}{\sum_{i=1}^{n_1} n_2} = \frac{2.35}{4} + \frac{0.84}{12} = 0.66.$$

Variance cost ratios can now be established to calculate the number of sampling units to sample in each pool (n_2) from equation 19 as follows:

$$n_2 = \sqrt{\left(\frac{c_1}{c_2}\right)\left(\frac{s_2^2}{\hat{s}_1^2}\right)} = \sqrt{\left(\frac{30}{1}\right)\left(\frac{0.84}{2.35}\right)} = 3.27.$$

Thus, it is determined that three Ekman dredge hauls should be collected from each pool. The next step is to determine how many pools or n_1 units to sample.

A c_1/c_2 ratio of 30 means it costs 30 times as much to sample an entire pool as to collect a single sample. The total cost of the sampling can be computed as in equation 18 as follows:

$$C=c_1n_1+c_2n_1n_2=(30c_2)(4)+(c_2)(12)=132c_2.$$

Therefore,

$$132c_2=n_1(30c_2)+n_1(3c_2)=n_1(33c_2).$$

Finally, from equation 20,

$$n_1 = \frac{C}{c_1+n_2c_2} = \frac{132}{33} = 4.0.$$

On the basis of the preliminary sample information, the minimum variance would be obtained by collecting three Ekman dredge samples in four pools if the present level of effort is to be maintained (or not exceeded) and all n_1 are equal.

Systematic sampling

Systematic sampling was discussed, in a general way, in the section "Biological Surveys and Simplified Sampling Methods." At this point it is appropriate to discuss the advantages and disadvantages of the method in relation to the previously described statistical sampling methods. If the objectives of a study are only to determine the numbers or types of organisms in relation to time or space, systematic sampling may be useful. One technique of systematic sampling is to select randomly the position or time of collection of the first sample and to collect the others at some predetermined space or time interval. Figure 2 illustrates how sample sites may be spaced for systematic sampling. The first site to be sampled is randomly chosen from a set of random numbers and the other sites are selected at equal distance or time intervals from it. From Snedecor and Cochran (1967, p. 519–520) and Elliott (1971, p. 134) the advantages of systematic sampling are:

1. It is easy to draw a sample, since only one random number is required.
2. The units in the sample are distributed evenly throughout the population.

The disadvantages are:

1. The sample may be biased when the interval between units in the sample coincides with a periodic variation in the population.

2. There is no reliable method for estimating the standard error of the mean.

From a statistical standpoint, the disadvantages are far too serious to recommend systematic sampling for use in intensive studies of the distribution and abundance of organisms in nature. The fact that the sample may be biased is in itself a serious limitation. Moreover, without some knowledge of the sample standard error of the mean, it is impossible to make quantitative statements about the data collected. The method is not recommended if the data are to receive statistical treatment.

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Part 2. Description of Methods

BACTERIA

Bacteria can be collected, observed, and counted directly, using the highest resolution of the light microscope. These methods are difficult and are seldom used except in research. 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 to the number of bacteria in the original sample. The first procedure 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.

The membrane filter (MF) technique has attained widespread application in microbiology principally because it is simple and quick to perform and is statistically more reliable than the multiple tube (MPN) determination. A brief discussion of the merits and limitations of the MF technique 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 (micrometers) 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 (American Public Health Assoc. and others, 1976; Green and others, 1975; Sladek and others, 1975) are the most common size used in microbiology because the type of bacteria most often enumerated are larger than 0.5 μm. Membranes with pore size less than 0.45 μm are available but are less commonly used in microbiology because of their susceptibility to clogging. Filters are manufactured in many sizes from about 13 mm to 293 mm in diameter but only the 47-mm diameter size is commonly used in microbiology.

Bacterial analysis begins with sample collection and media and equipment preparation all of which are discussed with each specific method. At some point in each method, however, a sample aliquot is passed through a filter. Membrane filters have a high flow rate initially due to the large void volume, but the filter will clog very quickly if the sample is significantly turbid. For this and other reasons, the MF method cannot be used for turbid waters. Even with relatively clear waters sample filtration generally is limited to about 100–250 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 either solid (agar) or liquid (broth) medium. Incubation is allowed to proceed for 24 or 48 hours at 35°C for total coliform and fecal streptococci bacteria or 44.5°C for fecal coliforms. It is very important that the temperature be held within the limits established for each method. Recent work (Green and others, 1975) indicates that many more cells are retained on the surface of the membrane than actually grow. Because optimum cell growth depends on an adequate nutrient supply, solid (agar) media have been found to yield higher colony counts than broth grown cultures. This is due to the larger volume (6.5 ml vs 1.8 ml) of medium used in the agar technique. During incubation there is a tendency for the petri dishes to lose moisture and dry. This is particularly true of dry (air) incubators at 44.5°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 grown to visible colonial size (24 or 48 hr 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 100 ml of sample.

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. The useful shelf life of membrane filters is 1 year (American Public Health Assoc. and others, 1976).

Standard plate count (membrane filter method)
(B–0001–77)

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

1. Application

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. The test described herein may also be performed by the agar plate method (American Public Health Association and others, 1976, p. 908–913).

The method is applicable for all waters with a dissolved-solids content of less than 20,000 mg/l (milligrams per liter).

2. Summary of method

The sample is filtered in the field immediately after collection, and the filter is placed on tryptone glucose extract (TPC) agar or broth medium. The colonies are counted after appropriate incubation. A staining procedure is used to enhance the contrast between the bacterial colonies and the filter.

3. Interferences

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

Some species of bacteria 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 bacteriological testing must be free of agents which inhibit bacterial growth.

The following apparatus list assumes the use of a field kit for bacteriological water tests such as the Portable Water Laboratory, Millipore (XX63 001 50), 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 below are included in the Portable Water Laboratory (fig. 3).

4.1 *Water-sampling bottle.* Samplers for obtaining water samples under sterile conditions are marketed by General Oceanics, Inc., Hydro Products, Kahl Scientific Instrument Corp., and others. A metallic water sampler, lowered at a speed of 1 m/s (meter per second), may be effective for sterile collection of water samples (Kriss and others, 1966). Metallic water-sampling bottles are available from Wildlife Supply Co. (1050 or 1200); Kahl Scientific Instrument Corp. (130WA100); InterOcean Systems, Inc. (206); Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type); or equivalent.

4.2 *Filter-holder assembly.* Millipore (XX53 001 20*), or equivalent, and *syringe and two-way valve,* Millipore (XX62 000 35*), or equivalent.

4.3 *Membrane filters,* white, grid, sterile packed, 0.45-μm (micrometer) pore size, 47-mm (millimeter) diameter, Millipore (HAWG 047 SO), or Gelman (63068), or equivalent; *absorbent pads,* Millipore (APIO 047 SO), or equivalent.

4.4 *Plastic petri dishes* with *covers,* disposable, sterile, 50×12 mm, Millipore (PD10 047 00*I*), or equivalent.

Figure 3.—Portable water laboratory. (Photograph, courtesy of Millipore Corp., Bedford, Mass.)

4.5 *Forceps,* stainless steel, smooth tip, Millipore (XX62 000 06*I*), or equivalent.

4.6 *Incubator* for operation at a temperature of 35 ±0.5°C. A portable incubator as provided in the Portable Water Laboratory, Millipore (XX63 000 00*I* or XX63 004 00), or equivalent, which operate on either 110 volts a.c. or 12 volts d.c., is convenient for field use. A larger incubator with a more precise temperature regulation, National Appliance (320), or equivalent, is satisfactory for laboratory use.

4.7 *Microscope,* binocular wide-field dissectingtype, Bausch & Lomb (31–26–29–73), or equivalent, with fluorescent lamp, Bausch & Lomb (31–33–63) or equivalent.

4.8 *Sterilizer,* steam autoclave, Curtin Matheson Scientific (209–536) or Market Forge Sterilmatic, or equivalent.

4.9 *Bottles,* milk dilution, APHA (American Public Health Assoc.), Pyrex or Kimax with screwcaps.

4.10 *Pipets, 1.0-ml capacity,* presterilized, disposable, glass or plastic with cotton plugs, Millipore (XX63 001 35*), or equivalent, or sterile, disposable, 1.0-ml (milliliter) hypodermic syringes.

4.11 *Pipets, 11.0-ml capacity,* Corning (7057), or equivalent. Wrap the pipets in kraft paper and sterilize in the autoclave, or place in a pipet box, Curtin Matheson Scientific, or equivalent, and heat in an oven at 170°C for 2 hours. Presterile, disposable, 10.0-ml pipets may be used.

4.12 *Propipet* for use with 1.0-, 10.0-, and 11.0-ml pipets.

4.13 *Thermometer,* with range of at least 40° –100°C, Brooklyn Thermometer Co. (6410Y) or equivalent.

5. Reagents

5.1 *Tryptone glucose extract broth.*—Prepare medium according to manufacturer's instructions, using Difco Bacto M-Plate Count Broth (0751) or BBL M-Standard Methods Broth (11369), or equivalent. If agar is desired, add 1.5 percent Difco Bacto agar (0140), or equivalent, to the broth.

5.2 *Buffered dilution water:* Dissolve 34.0 g (grams) potassium dihydrogen phosphate (KH_2PO_4) in 500 ml distilled water. Adjust to pH 7.2 with 1 N sodium hydroxide (NaOH). Dilute to 1 liter with distilled water. Sterilize in dilution bottles for 20 minutes at 121°C at 1.05 kg per cm^2 or 15 psi (pounds per square inch). After opening a bottle of stock solution, refrigerate the unused part. Discard contaminated solutions, indicated by slight turbidity or precipitate accumulation. Add 1.2 ml of this stock phosphate buffer solution to 1 liter of distilled water containing 0.1 percent Difco peptone (0118), or equivalent. Dispense in milk dilution bottles in amounts that will provide 99 ml±2.0 after autoclaving at 121°C at 1.05 kg per cm^2 (15 psi) for 20–30 minutes. Loosen caps or stoppers prior to sterilizing and tighten when bottles have cooled.

5.3 *Ethyl alcohol:* 95 percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methanol may be used for this purpose.

5.4 *Methyl alcohol:* Absolute, for sterilizing filter holder assembly.

5.5 *Methylene blue staining solution:* Add 3.0 g methylene blue dye to 300 ml of 95 percent ethyl alcohol. Dissolve 0.1 g of potassium hydroxide (KOH) in 1,000 ml of distilled water. Add to the alcoholic methylene blue solution and mix well.

6. Collection

Samples for bacteriologic examination must be collected in bottles that have been carefully cleansed and autoclaved for 20 minutes at 121°C at 1.05 kg per cm^2 (15psi). Sterilized milk dilution bottles are ideal sample containers. When the sample is collected, ample air space must be left in the bottle to facilitate mixing of the sample by shaking. Care must be taken to avoid contamination of the sample and sample bottle at the time of collection and in the period prior to analysis.

To insure maximum correlation of results, the sample sites and methods used for bacteria should correspond as closely as possible to those selected for chemical and plankton sampling. However, sampling for bacteria at various depths is complicated by the requirement to avoid possible contamination of the deeper water layers by bacteria carried from shallower depths on the inner walls of the sampler.

The sample collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, bacterial abundance may vary transversely, with depth and with time of day. To collect a surface sample from a stream or lake, open a sterile milk dilution bottle, grasp it near its base, and plunge it, neck downward, below the water surface. Allow the bottle to fill by slowly turning 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 the case of a lake, a current should be artificially created by pushing the bottle horizontally forward in a direction away from the hand (American Public Health Association and others, 1976, p. 905).

To collect a sample representative of the bacterial concentration at a particular depth, use one of the water-sampling bottles discussed in 4.1 above. For

small streams, a point sample at a single transverse position located at the centroid of flow may be adequate (Goerlitz and Brown, 1972).

As soon as possible after collection, preferably within 1 hour and not more than 6 hours, filter the sample and place the membrane filter on growth medium as described in 7.5–7.10 below. Samples must be kept cool during the time between collection and filtration. If filtration is delayed, chill or refrigerate the sample, but do not freeze.

The size of the sample to be filtered depends on the expected bacterial density of the water being tested. 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 waters: Ground waters: 10- and 50-ml samples. Unpolluted surface waters: 0.001-, 0.01-, 0.1-, and 1.0-ml samples.

7. Analysis

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

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

7.2 Saturate each pad with about 2 ml of tryptone glucose extract broth and tip the petri dish to expel excess liquid. If agar is used, pour liquid medium at 45°–50°C into the bottom dish to a depth of about 4 mm (6–7 ml). Pads are not used. Replace petri dish tops and allow agar to solidify.

7.3 Sterilize filter apparatus. In the laboratory the funnel and filter base may be wrapped separately in kraft paper packages and sterilized in the autoclave for 15 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Cool to room temperature before use.

Field sterilization of filter apparatus should be in accordance with the manufacturer's instructions. Autoclave sterilization in the laboratory prior to the field trip is preferred.

7.4 Assemble filtration equipment and, using sterilized forceps, place a sterile membrane filter over the porous plate of the apparatus, grid side up. Place funnel on filter with care to avoid tearing or creasing the membrane.

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

If the volume of sample is between 1.0 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 organisms.

If the volume of original water sample is less than 1.0 ml, proceed as above after preparing appropriate dilutions by adding the sample to a sterile milk dilution bottle in the following amounts:

Dilution	Volume of sample added to 99 ml dilution bottle	Filter this volume
1:10	11.0 ml original sample	11.0 ml of 1:10 dilution.
1:100	1.0 ml original sample	1.0 ml of 1:100 dilution.
1:1,000	1.0 ml of 1:10 dilution	1.0 ml of 1:1,000 dilution.
1:10,000	1.0 ml of 1:100 dilution	1.0 ml of 1:10,000 dilution.

Note: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer between bottles, close and shake the bottle vigorously at least 25 times. Diluted samples should be filtered within 20 minutes after preparation.

7.6 Apply vacuum and filter the sample. When vacuum is applied with a syringe fitted with a two-way valve, proceed as follows: Attach the filter 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 airlock before the filter 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.

7.7 Rinse sides of funnel twice with 20–30 ml of sterile buffered dilution water while applying vacuum.

7.8 Release vacuum and remove funnel from receptacle and place upside down on a clean surface.

7.9 With flame-sterilized forceps remove the membrane filter from the filter 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.

7.10 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.

7.11 Clearly mark the lid of each plastic dish indicating location, time of collection, time of incubation, sample number, and sample volume. Use a waterproof felttip marker or grease pencil.

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

7.13 Seal the plastic petri dish by firmly pressing down on the top.

7.14 Incubate the filters in the tightly closed petri dishes in an inverted position (pad and filter at the top) for 24 ±2 hours at 35°±0.5°C.

7.15 After incubation, saturate a fresh absorbent pad with 1.8 ml of methylene blue staining solution.

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

7.17 Enumerate colonies that are dark blue against a lighter colored background. The counts are best made with the aid of ×10 to ×15 magnification. Illumination is not critical.

7.18 Autoclave all cultures at 121°C for 15 minutes at 1.05 kg per cm^2 (15 psi) before discarding.

8. Calculations

$$\text{Colonies/ml of water} = \frac{\text{number of colonies counted}}{\text{vol. of original sample filtered (ml)}}.$$

9. Report

The number of colonies per milliliter should be reported to two significant figures and designated as "standard plate count at 35°C".

If the number of colonies on the highest dilution filter exceeds 150, report the result as greater than 150 times the dilution factor. If no filters contain colonies, report the result as less than 1 per the volume of the largest sample filtered.

10. Precision

No numerical precision data are available.

References

American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater [14th ed.]: New York, Am. Public Health Assoc., 1193 p.

Goerlitz, D. F., and Brown, Eugene, 1972, Methods for analysis of organic substances in water: U. S. Geol. Survey Techniques Water-Resources Inv., book 5, chap. A3, 40 p.

Green, B. L., Clausen, Elizabeth, and Litsky, Warren, 1975, Comparison of the new Millipore HC with conventional membrane filters for the enumeration of fecal coliform bacteria: Appl. Microbiol., v. 30, p. 697–699.

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Sladek, K. J., Suslavich, R. V., Sohn, B. I., and Dawson, F. W., 1975, Optimum membrane structures for growth of coliform and fecal coliform organisms: Appl. Microbiol., v. 30, p. 685–691.

Total coliform bacteria (membrane filter method)

Immediate incubation test (B–0025–77)

Parameter and code: Coliform membrane filter, immediate M-endo medium (colonies100 ml) 31501

1. Application

The standard test for presence of members of the coliform group may be carried out by the following membrane filter technique or by the multiple-tube fermentation technique described in "Presumptive Test," "Presumptive Field Test," "Confirmation Test," or in American Public Health Association and others (1976, p. 916–918).

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

The method is applicable to fresh and to saline waters.

2. Summary of method

The sample is filtered in the field immediately after collection, and the filter is placed on a nutrient medium designed to promote the growth of members of the coliform group and to suppress the growth of most noncoliform colonies. After incubating at 35°C for an appropriate time, the colonies are counted.

3. Interferences

Suspended materials may not permit testing of sample volumes sufficient to produce significant results. Coliform colony formation on the filter may be inhibited by high numbers of noncoliform colonies, by the presence of algal filaments and detritus, or by toxic substances.

Water samples with a high suspended-solids content may be divided between two or more membrane filters. The multiple-tube method will give the most reliable results under conditions of high suspended-solids content when coliform counts are low.

4. Apparatus

All materials used in bacteriological testing must be free of agents which inhibit bacterial growth.

The following apparatus list assumes the use of a field kit for bacteriological water tests such as the Portable Water Laboratory, Millipore (XX63 001 50), 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 below are included in the Portable Water Laboratory (fig. 3).

4.1 *Water-sampling bottle.* Samplers for obtaining water samples under sterile conditions are marketed by General Oceanics, Inc., Hydro Products, Kahl Scientific Instrument Corp., and others. A metallic water sampler, lowered at a speed of 1 m/s, may be effective for sterile collection of water samples (Kriss and others, 1966). Metallic water-sampling bottles are available from Wildlife Supply Co. (1050 or 1200); Kahl Scientific Instrument Corp. (130WA100); InterOcean Systems, Inc. (206); Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type); or equivalent.

4.2 *Filter-holder assembly,* Millipore (XX63 001 20*) or equivalent, and *syringe and two-way valve,* Millipore (XX62 000 35*) or equivalent.

4.3 *Membrane filters,* white, grid, sterile packed, 0.45- or 0.7-μm mean pore size, 47-mm diameter,

Millipore (HAWG 047 SO or HCWG 047 S1), or equivalent; *absorbent pads,* Millipore (APIO 047 SO) or equivalent.

4.4 *Plastic petri dishes* with *covers,* disposable, sterile, 50×12 mm, Millipore (PD10 047 00*) or equivalent.

4.5 *Forceps,* stainless steel, smooth tips, Millipore (XX62 000 06*) or equivalent.

4.6 *Incubator* for operation at a temperature of 35±0.5°C. A portable incubator as provided in the Portable Water Laboratory, Millipore (XX63 000 00* or XX63 004 00), or equivalent, which operates on either 110 volts a.c. or 12 volts d.c., is convenient for field use. A larger incubator with a more precise temperature regulation, National Appliance (320), or equivalent, is satisfactory for laboratory use.

4.7 *Microscope,* binocular wide-field dissecting-type, Bausch & Lomb (31–26–29–73) or equivalent, with fluorescent lamp, Bausch & Lomb (31–33–63) or equivalent.

4.8 *Sterilizer,* steam autoclave, Curtin Matheson Scientific (209-536) or Market Forge Sterilmatic or equivalent.

4.9 *Bottles,* milk dilution, APHA, Pyrex or Kimax with screwcaps.

4.10 *Pipets, 1.0 ml capacity,* presterilized, disposable, glass or plastic with cotton plugs, Millipore (XX63 001 35*) or equivalent, or sterile, disposable, 1.0-ml hypodermic syringes.

4.11 *Pipets, 11.0-ml capacity,* Corning (7057), or equivalent. Wrap the pipets in kraft paper and sterilize in the autoclave, or place in a pipet box, Curtin Matheson Scientific or equivalent, and heat in an oven at 170°C for 2 hours. Presterile, disposable 10.0-ml pipets may be used.

4.12 *Propipet* for use with 1.0-, 10.0-, and 11.0-ml pipets.

4.13 *Thermometer,* with range of at least 40° –100°C, Brooklyn Thermometer Co. (6410Y) or equivalent.

5. Reagents

5.1 *M-Endo broth:* Add 4.8 g of M-Endo broth MF, Difco (0749), or equivalent, to 100 ml of 2 percent nondenatured ethyl alcohol in a beaker and stir for 3 minutes. Place the beaker on a hotplate and heat to boiling, stirring constantly. When the medium reaches the boiling point, promptly remove from heat and cool to below 45°C. Do not sterilize by autoclaving. Store the finished medium in the dark at 2°–10°C for a maximum period of 4-5 days.

5.2 *M-Endo agar:* Add 4.8 g of M-Endo broth MF, Difco (0749), or equivalent, to 100 ml of 2 percent nondenatured ethyl alcohol, then add 1.5 g agar, Difco (0140), or equivalent. Stir well and place the beaker containing the medium on a hot plate and heat to 96°C, stirring constantly. Do not autoclave or boil. When the temperature reaches 96°C promptly remove from heat and cool to 45°–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°–10°C for a maximum period of 4–5 days.

5.3 *Buffered dilution water:* Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 ml distilled water. Adjust to pH 7.2 with 1 *N* sodium hydroxide (NaOH). Dilute to 1 liter with distilled water. Sterilize in dilution bottles for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). After opening a bottle of stock solution, refrigerate the unused part. Discard contaminated solutions, indicated by slight turbidity or precipitate accumulation.

Add 1.2 ml of this stock phosphate buffer solution to 1 liter of distilled water containing 0.1 percent Difco peptone (0118), or equivalent. Dispense in milk dilution bottles in amounts that will provide 99 ml±2.0 after autoclaving at 121°C at 1.05 kg per cm^2 (15 psi) for 20–30 minutes. Loosen caps or stoppers prior to sterilizing, and tighten when bottles have cooled.

5.4 *Ethyl alcohol:* 95 percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methanol may be used for sterilization.

5.5 *Methyl alcohol:* Absolute, for sterilizing filter holder assembly.

6. Collection

Samples for bacteriologic examination must be collected in bottles that have been carefully cleansed and autoclaved for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Sterilized milk dilution bottles are ideal sample containers. When the sample is collected, ample air space must be left in the bottle to facilitate mixing of the sample by shaking. Care must be taken to avoid contamination of the sample and sample bottle at the time of collection and in the period prior to analysis.

To insure maximum correlation of results, the sample sites and methods used for bacteria should correspond as closely as possible to those selected for chemical and plankton sampling. However, sampling for bacteria at depth is complicated by the necessity to avoid contamination of the deeper water layers by bacteria carried from shallower depths on the inner walls of the sampler.

The sample collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers,

and estuaries, bacterial abundance may vary transversely, with depth, and with time of day. To collect a surface sample from a stream or lake, open a sterile milk dilution bottle, grasp it near its base, and plunge it neck downward, below the water surface. Allow the bottle to fill by slowly turning 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 the case of a lake, a current should be artificially created by pushing the bottle horizontally forward in a direction away from the hand (American Public Health Association and others, 1976).

To collect a sample representative of the bacterial concentration at a particular depth, use one of the water-sampling bottles discussed in 4.1 above. For small streams, a point sample at a single transverse position located at the centroid of flow may be adequate (Goerlitz and Brown, 1972).

As soon as possible after collection, preferably within 1 hour and not more than 6 hours, filter the sample and place the membrane filter on growth medium as described in 7.5–7.10 below. Samples must be kept cool during the time between collection and filtration. If filtration is delayed, chill or refrigerate the sample, but do not freeze.

The volumes of sample to be filtered should be such that, after incubation, one of the membrane filters will contain from 20 to 80 coliform colonies and not more than 200 of all types (total coliform plus noncoliform colonies). It is extremely important that the limitation on total colonies be observed, otherwise the medium used in the method may not be capable of supporting development of the characteristic metallic sheen. If the upper limit of 80 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 coliform colonies per membrane filter is arbitrarily set as a number below which statistical validity becomes questionable. However, the bacterial population in some samples is such that even with 200 or fewer total colonies (coliform plus noncoliform) per 100 ml of sample, fewer than 20 coliform colonies will be present on the filter membrane.

The following sample volumes are suggested for filtration:

1. Unpolluted raw surface water: 0.1-, 0.4-, 1.6-, 6.4-, 25.6-, and 100.0-ml samples will cover a range of 20 to 80,000 coliforms per 100 ml using the criterion of 20 to 80 coliform colonies on a filter for an ideal determination.
2. Polluted raw surface water: 0.002-, 0.006-, 0.025-, 0.1-, 4.0-, and 1.6-ml samples will cover a range of 1,200 to 4,000,000 coliform colonies per 100 ml.

7. Analysis

7.1 If M-Endo agar is to be used, use plates prepared in 5.2 and proceed to 7.3. If M-Endo broth is to be used, proceed as follows. Place a sterile absorbent pad in the bottom (larger half) of each sterile plastic petri dish using flame sterilized forceps.

Note: Dip forceps in ethyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

7.2 Saturate each pad with about 2 ml of M-Endo broth and tip the petri dish to expel excess liquid. Replace petri dish tops.

7.3 Sterilize filter apparatus. In the laboratory the funnel and filter base may be wrapped separately in kraft paper packages and sterilized in the autoclave for 15 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Cool to room temperature before use.

Field sterilization of filter apparatus should be in accordance with the manufacturer's instructions. Autoclave sterilization in the laboratory prior to the field trip is preferred.

7.4 Assemble filtration equipment and, using sterilized forceps, place a sterile membrane filter over the porous plate of the apparatus, grid side up. Place funnel on filter with care to avoid tearing or creasing the membrane.

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

If the volume of sample is between 1.0 ml and 10 ml, pour about 20 ml of sterilized buffer dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of organisms.

If the volume of original water sample is less than 1.0 ml, proceed as above after preparing appropriate dilutions by adding the sample to a sterile milk dilution bottle in the following amounts:

Dilution	Volume of sample added to 99 ml dilution bottle	Filter this volume
1:10	11.0 ml original sample	11.0 ml of 1:10 dilution.
1:100	1.0 ml original sample	1.0 ml of 1:100 dilution.
1:1,000	1.0 ml of 1:10 dilution	1.0 ml of 1:1,000 dilution.
1:10,000	1.0 ml of 1:100 dilution	1.0 ml of 1:10,000 dilution.

Note: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer between bottles, close

and shake the bottle vigorously at least 25 times. Diluted samples should be filtered within 20 minutes after preparation.

7.6 Apply vacuum and filter the sample. When vacuum is applied with a syringe fitted with a two-way valve, proceed as follows: Attach the filter 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 airlock before the filter 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.

7.7 Rinse sides of funnel twice with 20–30 ml of sterile buffered dilution water while applying vacuum.

7.8 Release vacuum and remove funnel from receptacle and place upside down on a clean surface.

7.9 With flame-sterilized forceps remove the membrane filter from the filter base and place it on the broth-soaked absorbent pad or 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.

7.10 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.

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

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

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

7.14 Incubate the filters in the tightly closed petri dishes in an inverted position (filter at the top) for 22–24 hours at 35°±0.5°C.

7.15 Remove the filters and allow to dry for at least 1 minute on an absorbent surface.

7.16 Count the number of coliform sheen colonies, that is, dark colonies with 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 coliform colonies. The counts are best made with the aid of a × 10 to × 15 magnification. The illuminator (fluorescent) should be placed as directly above the filter as possible.

7.17 Autoclave all cultures at 121°C for 15 minutes at 1.05 kg per cm^2 (15 psi) before discarding.

8. Calculations

8.1 For colony counts between the ideal of 20 and 80, use the formula:

$$\text{Coliform colonies/100 ml} = \frac{\text{coliform colonies counted} \times 100}{\text{vol. of original sample filtered (ml)}}.$$

8.2 Counts less than the ideal of 20 colonies or greater than 80 colonies per filter should be reported as number per 100 ml, followed by the statement, "Estimated count based on nonideal colony count."

8.3 If no filters develop characteristic coliform colonies, calculate assuming that the largest sample volume filtered had one coliform colony. Report as less than that calculated number per 100 ml.

8.4 If all filters bear colonies too numerous to count, a minimum estimated value can be reported. Assume a count of 80 coliform colonies on the smallest filtered volume, then calculate according to the formula in 8.1. Report as greater than (>) the calculated value.

8.5 Sometimes two or more filters of a series will produce colony counts within the recommended counting range. Colony counts should be made on all such filters. The method for calculating and averaging is as follows:

Volume filter 1	Colony count filter 1
+ Volume filter 2	+ Colony count filter 2
Volume sum	Colony count sum

$$\text{Coliform colonies/100 ml} = \frac{\text{colony count sum} \times 100}{\text{vol. sum (ml)}}.$$

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

9. Report

The coliform concentration is reported as coliform colonies per 100 ml, M-Endo immediate incubation at 35°C. Values less than 10, report whole numbers; 10 or more, report two significant figures.

10. Precision

No numerical precision data are available.

References

American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater [14th ed.]: New York, Am. Public Health Assoc., 1193 p.

Goerlitz, D. F., and Brown, Eugene, 1972, Methods for analysis of organic substances in water: U.S. Geol. Survey Techniques Water-Resources Inv., book 4, chap. A3, 40 p.

Kriss, A. E., Lebedeva, M. N., and Tsiban, A. V., 1966, Comparative estimate of a Nansen and microbiological water bottle for sterile collection of water samples from depths of seas and oceans: Deep-Sea Research, v. 13, p. 205–212.

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

Total coliform bacteria (membrane filter method)

Delayed incubation test (B–0030–77)

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

1. Application

The method is applicable to fresh and to saline waters. It is used where 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.

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

2. Summary of method

The sample is filtered in the field immediately after collection; 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°C for the stipulated time, and counting the typical coliform colonies.

3. Interferences

Suspended materials may not permit testing of sample volumes sufficient to produce significant results. Coliform colony formation on the filter may be inhibited by high numbers of noncoliform colonies, by the presence of algal filaments and detritus, or by toxic substances.

Water samples with a high suspended-solids content may be divided between two or more membrane filters.

4. Apparatus

All materials used in bacteriological testing must be free of agents which inhibit bacterial growth.

The following apparatus list assumes the use of a field kit for bacteriological water tests such as the Portable Water Laboratory, Millipore (XX63 001 50), 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 below are included in the Portable Water Laboratory (fig. 3).

4.1 *Water-sampling bottle.* Samplers for obtaining water samples under sterile conditions as marketed by General Oceanics, Inc., Hydro Products, Kahl Scientific Instrument Corp., and others. A metallic water sampler, lowered at a speed of 1 m/s, may be effective for sterile collection of water samples (Kriss and others, 1966). Metallic watersampling bottles are available from Wildlife Supply Co. (1050 or 1200); Kahl Scientific Instrument Corp. (130WA100); InterOcean Systems, Inc. (206); Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type); or equivalent.

4.2 *Filter-holder assembly,* Millipore (XX63 001 20*) or equivalent, and *syringe and two-way valve,* Millipore (XX62 000 35*) or equivalent.

4.3 *Membrane filters,* white, grid, sterile packed, 0.45- or 0.7-μm mean pore size, 47-mm diameter,

Millipore (HAWG 047 SO or HCWG 047 SI), or equivalent; *absorbent pads,* Millipore (APIO 047 SO) or equivalent.

4.4 *Plastic petri dishes* with *covers,* disposable, sterile, 50×12 mm, Millipore (PD10 047 00*) or equivalent.

4.5 *Forceps,* stainless steel, smooth tips, Millipore (XX62 000 06*) or equivalent.

4.6 *Incubator* for operation at a temperature of 35°±0.5°C. A portable incubator as provided in the Portable Water Laboratory, Millipore (XX63 000 00* or XX63 004 00), or equivalent, which operates on either 110 volts a.c. or 12 volts d.c., is convenient for field use. A larger incubator with a more precise temperature regulation, National Appliance (320) or equivalent, is satisfactory for laboratory use.

4.7 *Microscope,* binocular wide-field dissection-type, Bausch & Lomb (31–26–29–73) or equivalent, with fluorescent lamp, Bausch & Lomb (31–33–63) or equivalent.

4.8 *Sterilizer,* steam autoclave, Curtin Matheson Scientific (209-536) or Market Forge Sterilmatic or equivalent.

4.9 *Bottles,* milk dilution, APHA, Pyrex or Kimax with screwcaps.

4.10 *Pipets, 1.0-ml capacity,* presterilized, disposable, glass or plastic with cotton plugs, Millipore (XX63 001 35*) or equivalent, or sterile, disposable, 1.0-ml hypodermic syringes.

4.11 *Pipets, 11.0-ml capacity,* Corning (7057) or equivalent. Wrap the pipets in kraft paper and sterilize in the autoclave, or place in a pipet box, Curtin Matheson Scientific or equivalent, and heat in an oven at 170°C for 2 hours. Presterile, disposable, 10.0-ml pipets may be used.

4.12 *Propipet* for use with 1.0-, 10.0-, and 11.0-ml pipets.

4.13 *Thermometer,* with range of at least 40°–100°C, Brooklyn Thermometer Co. (6410Y) or equivalent.

5. Reagents

5.1 *M-Endo broth:* Add 4.8 g of M-Endo broth MF, Difco (0749) or equivalent, to 100 ml of 2 percent nondenatured ethyl alcohol solution in a beaker and stir for 3 minutes. Place the beaker on a hotplate and heat to boiling, stirring constantly. When the medium reaches the boiling point, promptly remove from heat and cool to below 45°C. Do not sterilize by autoclaving. Store the finished medium in the dark at 2°–10°C for a maximum period of 4–5 days.

5.2 *M-Endo agar:* Add 4.8 g of M-Endo broth MF, Difco (0749) or equivalent, to 100 ml of 2 percent nondenatured ethyl alcohol, then add 1.5 g agar, Difco (0140), or equivalent. Stir well and place the beaker containing the medium on a hotplate and heat to 96°C, stirring constantly. Do not autoclave or boil. When the temperature reaches 96°C, promptly remove from heat and cool to 45°–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°–10°C for a maximum period of 4–5 days.

5.3 *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. Discard unused solution after 6 months.

5.4 *M-Endo preservative medium:* To 100 ml of M-Endo broth described in 5.1, add 3.2 ml of 12 percent sodium benzoate solution.

5.5 *Cyclohexamide:* Dissolve 500 mg of cyclohexamide (Upjohn actidione, Sigma actidone, or equivalent) in 10 ml of distilled water. The cyclohexamide solution should be refrigerated; storage should not exceed 6 months. Cyclohexamide is a powerful skin irritant and should be handled with caution according to the manufacturer's directions. Add 1 ml of cyclohexamide solution to 100 ml of M-Endo preservative medium described in 5.3.

5.6 *Buffered dilution water:* Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 ml distilled water. Adjust to pH 7.2 with 1 *N* sodium hydroxide (NaOH). Dilute to 1 liter with distilled water. Sterilize in dilution bottles for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). After opening a bottle of stock solution, refrigerate the unused part. Discard contaminated solutions, indicated by slight turbidity or precipitate accumulation.

Add 1.2 ml of this stock phosphate buffer solution to 1 liter of distilled water containing 0.1 percent Difco peptone (0118), or equivalent. Dispense in milk dilution bottles in amounts that will provide 99 ml±2.0 after autoclaving at 121°C at 1.05 kg per cm^2 (15psi) for 20–30 minutes. Loosen caps or stoppers prior to sterilizing, and tighten when bottles have cooled.

5.7 *Ethyl alcohol:* 95 percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methanol may be used for sterilization.

5.8 *Methyl alcohol:* Absolute, for sterilizing filter holder assembly.

6. Collection

Samples for bacteriologic examination must be collected in bottles that have been carefully cleansed and

autoclaved for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Sterilized milk dilution bottles are ideal sample containers. When the sample is collected, ample air space must be left in the bottle to facilitate mixing of the sample by shaking. Care must be taken to avoid contamination of the sample and sample bottle at the time of collection and in the period prior to analysis.

To insure maximum correlation of results, the sample sites and methods used for bacteria should correspond as closely as possible to those selected for chemical and plankton sampling. However, sampling for bacteria at depth is complicated by the requirement to avoid contamination of the deeper water layers by bacteria carried from shallower depths on the inner walls of the sampler.

The sample-collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, bacterial abundance may vary transversely, with depth, and with time of day. To collect a surface sample from a stream or lake, open a sterile milk dilution bottle, grasp it near its base, and plunge it, neck downward, below the water surface. Allow the bottle to fill by slowly turning 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 the case of a lake, a current should be artificially created by pushing the bottle horizontally forward in a direction away from the hand (American Public Health Association and others, 1976, p. 905).

To collect a sample representative of the bacterial concentration at a particular depth, use one of the water-sampling bottles discussed in 4.1 above. For small streams, a point sample at a single transverse position located at the centroid of flow may be adequate (Goerlitz and Brown, 1972).

As soon as possible after collection, preferably within 1 hour and not more than 6 hours, filter the sample and place the membrane filter on the preservative medium as described in 7.5–7.10 below. Samples must be kept cool during the time between collection and filtration. If filtration is delayed, chill or refrigerate the sample but do not freeze.

The volumes of sample to be filtered should be such that, after incubation, one of the membrane filters will contain from 20 to 80 coliform colonies and not more than 200 of all types (total coliform plus noncoliform colonies). It is extremely important that the limitation on total number of colonies be observed, otherwise the medium used in the method may not be capable of supporting development of the characteristic metallic sheen. If the upper limit of 80 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 coliform colonies per membrane filter is arbitrarily set as a number below which statistical validity becomes questionable. However, the bacterial population in some samples is such that even with 200 or fewer total colonies (coliform plus noncoliform) per 100 ml of sample, fewer than 20 coliform colonies will be present on the filter membrane.

The following sample volumes are suggested for filtration:

1. Unpolluted raw surface water: 0.1-, 0.4-, 1.6-, 6.4-, 25.6-, and 100.0-ml samples will cover a range of 40 to 80,000 coliforms per 100 ml using the criteria of 20 to 80 coliform colonies on a filter for an ideal determination.
2. Polluted raw surface water: 0.002-, 0.006-, 0.025-, 0.1-, 0.4-, and 1.6-ml samples will cover a range of 1,200 to 4,000,000 coliform colonies per 100 ml.

7. Analysis

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

Note: Dip forceps in ethyl alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

7.2 Saturate each pad with about 2 ml of M-Endo preservative medium and tip the petri dish to expel excess liquid. Replace petri dish tops.

7.3 Sterilize filter apparatus. In the laboratory, the funnel and filter base may be wrapped separately in kraft paper packages and sterilized in the autoclave for 15 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Cool to room temperature before use.

Field sterilization of filter apparatus should be in accordance with the manufacturer's instructions. Autoclave sterilization in the laboratory prior to the field trip is preferred.

7.4 Assemble filtration equipment, and using sterilized forceps, place a sterile membrane filter over the porous plate of the apparatus, grid side up. Place funnel on filter with care to avoid tearing or creasing the membrane.

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

If the volume of sample is between 1.0 ml 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 organisms.

If the volume of original water sample is less than 1.0 ml, proceed as above after preparing appropriate dilutions by adding the sample to a sterile milk dilution bottle in the following amounts:

Dilution	Volume of sample added to 99 ml dilution bottle	Filter this volume
1:10	11.0 ml original sample	11.0 ml of 1:10 dilution.
1:100	1.0 ml original sample	1.0 ml of 1:100 dilution.
1:1,000	1.0 ml of 1:10 dilution	1.0 ml of 1:1,000 dilution.
1:10,000	1.0 ml of 1:100 dilution	1.0 ml of 1:10,000 dilution.

Note: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer between bottles, close and shake the bottle vigorously at least 25 times. Diluted samples should be filtered within 20 minutes after preparation.

7.6 Apply vacuum and filter the sample. When vacuum is applied with a syringe fitted with a two-way valve, proceed as follows: Attach the filter 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 airlock before the filter assembly fills with water. Hold plunger to pull the sample through the filter assembly. 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.

7.7 Rinse sides of funnel twice with 20–30 ml of sterile buffered dilution water while applying vacuum.

7.8 Remove funnel from receptacle and place upside down on a clean surface.

7.9 With flame-sterilized forceps remove the membrane filter from the filter 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.

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

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

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

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

7.14 Place the petri dish containing the membrane filter in a shipping container and mail so as to arrive in the laboratory within 72 hours. Limited bacterial growth sometimes occurs on the preservative medium when high temperatures are encountered.

7.15 In the laboratory, transfer the membrane from the petri dish in which it was shipped to a fresh sterile petri dish containing either M-Endo agar or to an absorbent pad saturated with M-Endo broth or agar. Use sterile forceps and insure a good contact between the filter and medium.

7.16 Incubate the filters in the tightly closed petri dishes in an inverted position (filter at the top) for 20–22 hours at 35°±0.5°C.

7.17 Remove the filters and allow to dry for at least 1 minute on an absorbent surface.

7.18 Count the number of coliform sheen colonies, that is, dark colonies with 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 coliform colonies. The counts are best made with the aid of a × 10 to × 15 magnification. The illuminator (fluorescent) should be placed as directly above the filter as possible.

7.19 Autoclave all cultures at 121°C for 15 min at 1.05 kg per cm^2 (15 psi) before discarding.

8. Calculations

8.1 For colony counts between the ideal of 20 and 80 use the formula:

$$\text{Coliform colonies/100 ml} = \frac{\text{coliform colonies counted} \times 100}{\text{vol. of original sample filtered (ml)}}.$$

8.2 Counts less than the ideal of 20 colonies or greater than 80 colonies per filter should be reported as number per 100 ml, followed by the statement, "Estimated count based on non-ideal colony count."

8.3 If no filters develop characteristic coliform colonies, calculate assuming that the largest sample volume filtered had one coliform colony. Report as less than that calculated number per 100 ml.

8.4 If all filters bear colonies too numerous to count, a minimum estimated value can be reported. Assume a count of 80 coliform colonies on the smallest filtered volume, then calculate according to the formula in 8.1. Report as greater than (>) the calculated value.

8.5 Sometimes two or more filters of a series will produce colony counts within the recommended counting range. Colony counts should be made on all such filters. The method for calculating and averaging is as follows:

Volume filter 1	Colony count filter 1
+ Volume filter 2	+ Colony count filter 2
Volume sum	Colony count sum

$$\text{Coliform colonies/100 ml} = \frac{\text{colony count sum} \times 100}{\text{vol. sum (ml)}}.$$

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

9. Report

The coliform concentration is reported as coliform colonies per 100 ml, M-Endo delayed incubation at 35°C. Values less than 10, report whole number; 10 or more, report two significant figures.

10. Precision

No numerical precision data are available.

References

American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater [14th ed.]: New York, Am. Public Health Assoc., 1193 p.

Goerlitz, D. F., and Brown, Eugene, 1972, Methods for analysis of organic substances in water: U.S. Geol. Survey Techniques Water-Resources Inv., book 5, chap. A3, 40 p.

Kriss, A. E., Lebedeva, M. N., and Tsiban, A. V., 1966, Comparative estimate of a Nansen and microbiological water bottle for sterile collection of water samples from depths of seas and oceans: Deep-Sea Research, v. 13, p. 205–212.

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

Total coliform bacteria (most probable number, MPN, method)

Presumptive test (B–0035–77)

Parameter and code: Coliform, presumptive (MPN) 31507

1. Application

This method is applicable to all types of fresh and saline waters. It is applicable to waters with large suspended solids content and waters with high counts of noncoliform bacteria.

2. Summary of method

Decimal dilutions of multiple sample aliquots are inoculated into lauryl tryptose broth. The cultures are incubated at 35°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.

3. Interferences

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

4. Apparatus

All materials used in bacteriological testing must be free of agents which inhibit bacterial growth.

4.1 *Water-sampling bottle.* Samples for obtaining water samples under sterile conditions as marketed by General Oceanics, Inc., Hydro Products, Kahl Scientific Instrument Corp., and others. A metallic water sampler, lowered at a speed of 1 m/s, may be effective for sterile collection of water samples (Kriss and others, 1966). Metallic water-sampling bottles are available from Wildlife Supply Co. (1050 or 1200); Kahl Scientific Instrument Corp. (130WA100); InterOcean Systems, Inc. (206); Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type); or equivalent.

4.2 *Durham (fermentation) tubes and serum vials.* Serum vials of any type may be used. The size of the vial and durham tube, used for detection of gas production, should be such that the tube is completely filled with medium and at least partly submerged in the vial. The specific choice of fermentation tubes and serum vials depends on the volume of water to be tested and whether the test is to be run in the laboratory or in the field.

The following combinations have been found to be satisfactory for the stated use.

4.2a For testing 50-ml aliquots, use screwcap milk dilution bottles, APHA, Pyrex or Kimax serum vials; use flint glass culture tubes, 10×75 mm, Kimble (73500) or equivalent, as inner, inverted fermentation tubes.

4.2b For laboratory testing of 10 ml or smaller aliquots, use culture tubes, flint glass, 20×150 mm, Kimble (73500) or equivalent, and culture tubes, flint glass 10×75 mm, Kimble (73500) or equivalent, and test tube caps, 20 mm, Scientific Products (T13990-20) or equivalent.

4.3 *Culture-tube rack,* galvanized for 20 mm tubes, Thomas-Kolmer or equivalent.

4.4 *Incubator* for operation at a temperature of 35°±0.5°C. National Appliance (320) or equivalent, is satisfactory for laboratory use.

4.5 *Sterilizer,* steam autoclave, Curtin Matheson Scientific (209-536) or Market Forge Sterilmatic or equivalent.

4.6 *Bottles,* milk dilution, APHA, Pyrex or Kimax with screwcaps.

4.7 *Pipets* 1.0-ml capacity, presterilized, disposa-

ble, glass or plastic with cotton plugs, Millipore (XX63 001 35) or equivalent, or sterile, disposable 2.5-ml hypodermic syringes, Becton Dickinson (5610) or equivalent.

4.8 *Pipets* 11.0-ml capacity, Corning (7057) or equivalent. Wrap the pipets in kraft paper and sterilize in the autoclave, or place in a pipet box, Curtin Matheson Scientific or equivalent, and heat in an oven at 170°C for 2 hours. Presterile, disposable, 10.0-ml pipets may be used.

4.9 *Propipet* for use with 1.0-,, 10.0-, and 11 ml pipets.

5. Reagents

5.1 *Lauryl Tryptose Broth.* Difco Bacto Lauryl Tryptose Broth (0241) or BBL Lauryl Sulfate Broth (11338) or equivalent. Prepare according to American Public Health Association and others (1976, p. 893) or according to directions on bottle label.

Place 50 ml of medium containing 71.2 g/l (grams per liter) of Difco Bacto Lauryl Tryptose Broth or BBL Lauryl Sulfate Broth in a milk dilution bottle for each 50 ml aliquot of sample to be tested.

Place 10 ml of medium containing 71.2 g/l of Difco Bacto Lauryl Tryptose Broth or BBL Lauryl Sulfate Broth in a 20×150 mm culture tube for each 10 ml aliquot of sample to be tested.

Place 10 ml of medium containing 35.6 g/l of Difco Bacto Lauryl Tryptose Broth or BBL Lauryl Sulfate Broth in 20×150 ml culture tube for each 1 ml or smaller aliquot of sample to be tested.

In each milk dilution bottle or culture tube place, mouth downward (inverted), one 10×75 mm durham tube (fig. 4). Sterilize bottles in upright position at 121°C at 1.05 kg per cm^2 (15 psi) for 15 minutes. Air will be expelled from the inverted, inner durham tube during heating; each will fill completely with medium during cooling. Before using check to see that there are no air bubbles in the inverted durham tubes.

5.2 *Buffered dilution water.* Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 ml distilled water. Adjust to pH 7.2 with 1 *N* sodium hydroxide (NaOH). Dilute to 1 liter with distilled water. Sterilize in dilution bottles for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). After opening a bottle of stock solution, refrigerate the unused part. Discard contaminated solutions, indicated by slight turbidity or precipitate.

Add 1.2 ml of this stock phosphate buffer solution to 1 liter of distilled water containing 0.1 percent Difco peptone (0118), or equivalent. Dispense in milk dilution bottles in amounts that will provide 99 ml±2.0 after autoclaving at 121°C at 1.05 kg per cm^2 (15 psi) for 20–30 minutes. Loosen caps or stoppers prior to sterilizing and tighten when bottles have cooled.

6. Collection

Samples for bacteriologic examination must be collected in bottles that have been carefully cleaned and autoclaved for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Sterilized milk dilution bottles are ideal sample containers. When the sample is collected, ample air space must be left in the bottle to facilitate mixing of the sample by shaking. Care must be taken to avoid contamination of the sample and sample bottle at the time of collection and in the period prior to analysis.

To insure maximum correlation of results, the sample sites and methods used for bacteria should correspond as closely as possible to those selected for chemical and plankton sampling. However, sampling for bacteria at depth is complicated by the requirement to avoid contamination of the deeper water layers by bacteria carried from shallower depths on the inner walls of the sampler.

The sample collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, bacterial abundance may vary transversely, with depth, and with time of day. To collect a surface sample from a stream or lake, open a sterile milk dilution bottle, grasp it near its base, and plunge it, neck downward, below the water surface. Allow the bottle to fill by slowly turning 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 the case of a lake, a current should be artificially created by pushing the bottle horizontally forward in a direction away from the hand (American Public Health Association and others, 1976, p. 965).

To collect a sample representative of the bacterial concentration at a particular depth, use one of the water-sampling bottles discussed in 4.1 above. For small streams a point sample at a single transverse position located at the centroid of flow may be adequate (Goerlitz and Brown, 1972).

As soon as possible after collection prefereably within 1 hour and not more than 6 hours, inoculate the decimal dilutions of the sample into the lauryl tryptose broth serum vials. Samples must be kept cool during the time between collection and inoculation. If inoculation is delayed, chill or refrigerate the sample but do not freeze.

The volumes of decimal dilutions should be such

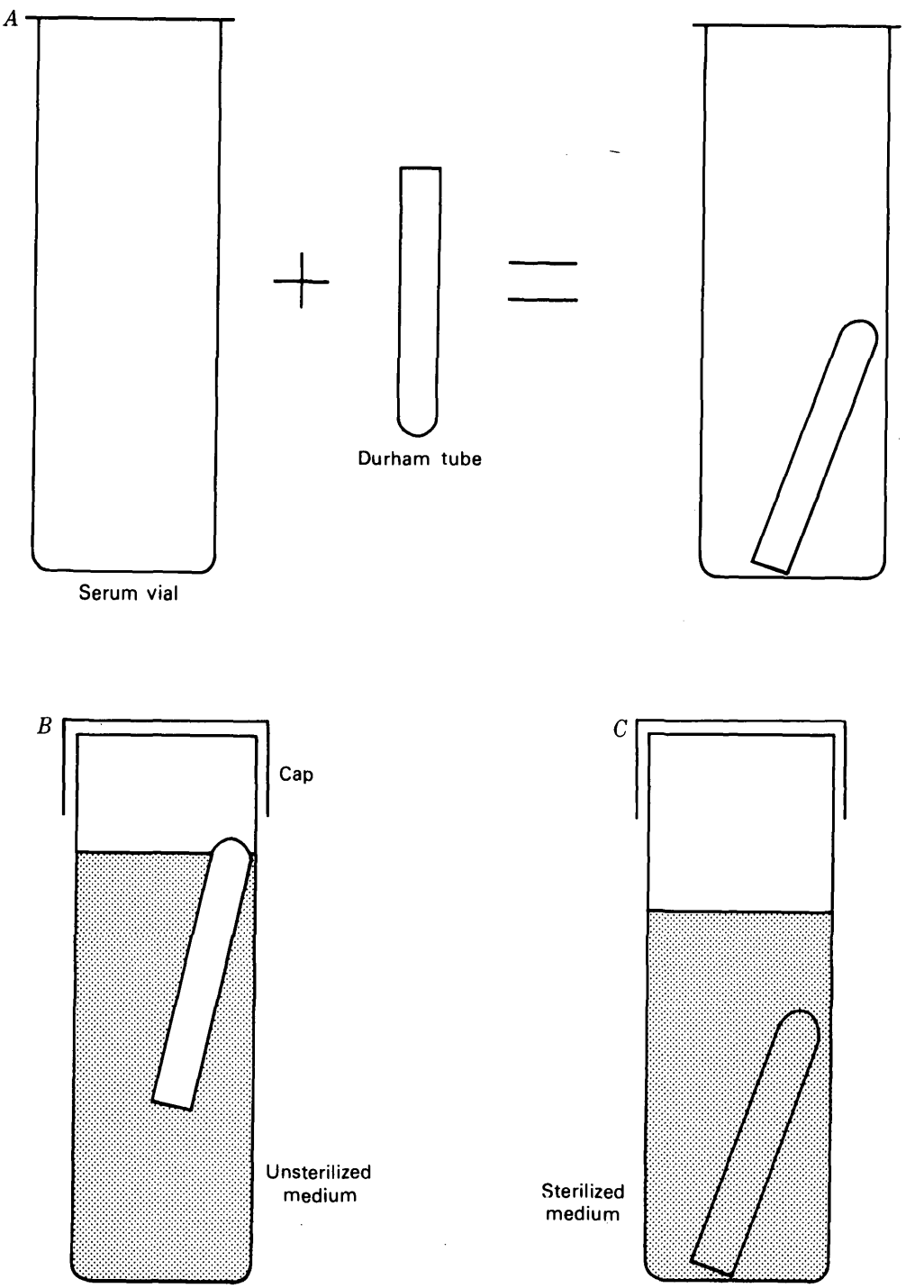

Figure 4.—Preparation of culture tube (step 5.1). *A.* Invert durham tube inside serum vial. *B.* Add unsterilized medium and cap. *C.* Durham tube fills with medium following sterilization.

that, after incubation, both positive and negative results are obtained among the range of volumes. The method fails if only positive or only negative results are obtained with all volumes tested.

The following sample volumes are suggested:

1. Unpolluted raw surface water: 0.1-, 1.0-, and 10.0- and 50.0-ml samples will cover a MPN range

of < 1 to ≥ 240 coliform organisms per 100 ml.

2. Polluted raw surface water: 0.001-, 0.01-, 0.1-, and 1.0-ml will cover a MPN range of 20 to 2.4×10^6 coliform organisms per 100 ml.

7. Analysis

7.1 Set up five vials of lauryl tryptose broth for each sample volume to be tested.

7.2 If 0.1 ml or more is to be inoculated transfer the measured samples directly to the serum vials using sterile pipets or presterilized disposable hypodermic syringes.

If the volume of original water sample is less than 0.1 ml, proceed as above after preparing appropriate dilutions by adding the sample to a sterile milk dilution bottle in the following amounts:

Dilution	Volume of sample added to 99 ml dilution bottle	Filter this volume
1:100	1.0 ml original sample	1.0 ml of 1:100 dilution.
1:1,000	1.0 ml of 1:10 dilution	1.0 ml of 1:1,000 dilution.
1:10,000	1.0 ml of 1:100 dilution	1.0 ml of 1:10,000 dilution.

Note: Use a sterile pipet or hypodermic syringe for each vial. After each transfer between bottles, close and shake the bottle vigorously 25 times. Diluted samples should be inoculated within 20 minutes after preparation.

7.3 Clearly mark each set of serum vials indicating location, time of collection, sample number, and sample volume. Code each vial for easy identification.

7.4 Place the inoculated vials in the test-tube or other appropriate rack and incubate at 35°C±0.5 °C for 24 ±2 hours. Culture vials must be maintained in an upright position.

7.5 Remove vials from incubator and examine. Gas in any amount in the durham tube, even a pinhead size bubble, constitutes a positive test (fig. 5). The appearance of an air bubble must not be confused with actual gas production. The broth medium will become cloudy with actual fermentation and small bubbles of gas may appear in the medium outside the durham tube when the serum vial is shaken gently (American Public Health Association and others, 1976, p. 916).

7.6 Autoclave all gas-positive vials for 15 minutes at 121°C at 1.05 kg per cm^2 (15 psi) before discarding.

7.7 Replace all gas-negative vials in incubator and incubate for an additional 24±2 hours at 35°C ±0.5°C.

7.8 Remove vials from incubator and examine for gas formation. Autoclave all remaining vials of lauryl tryptose broth as in 7.6 before discarding.

8. Calculations

Record the number of gas-positive vials occurring over all sample volumes tested. When more than three volumes are tested, the results from only three of these are used in computing the MPN. To select the three dilutions for the MPN index, proceed as follows: Take as the first member the smallest sample volume in which all tests are positive (no larger sample volume giving any negative results) and the two next succeed-

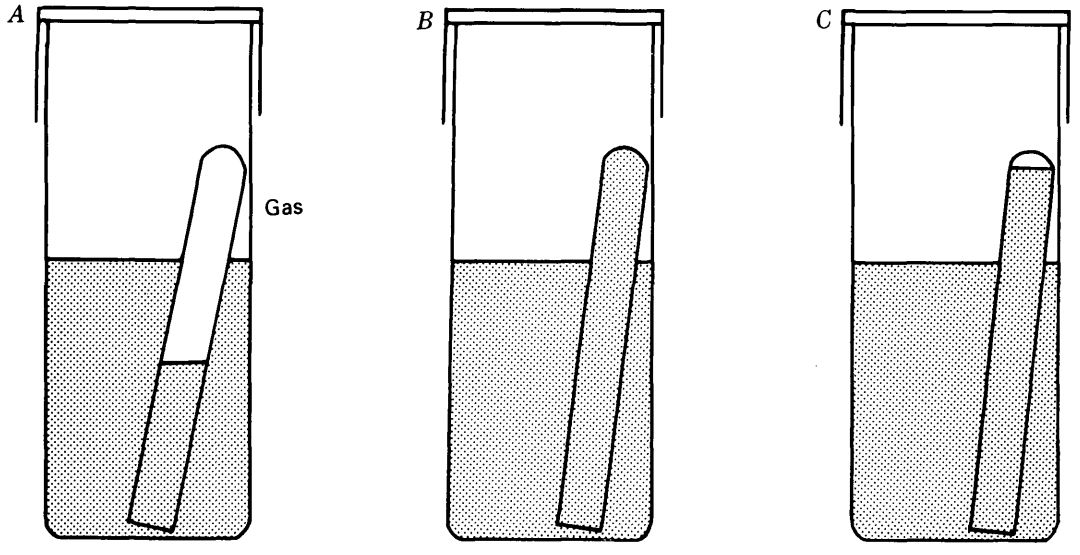

Figure 5.—Examination for gas formation (steps 7.5 and 7.8). *A.* Positive. *B.* Negative. *C.* Positive.

ing smaller sample volumes (American Public Health Association and others, 1976 p. 923–926).

In the examples given below, the number in the numerator represents positive tubes; the denominator represents the total number of tubes inoculated:

Example	Decimal dilutions 1 ml	0.1 ml	0.01 ml	0.001 ml	Combination of positives
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	

In example c, the first three dilutions should be taken to place the positive results in the middle dilution. When a positive occurs in a dilution higher than the three chosen according to the guideline, as in d, it should be placed in the result for the highest chosen dilution as in e.

A table giving MPN for various combinations of positive and negative results when five 10 ml dilutions, five 1.0 ml and five 0.1 ml dilutions are tested is shown in table 1. If a series of decimal dilutions other than 10.0-, 1.0-, and 0.1-ml is used, record the MPN as the value from the table multiplied by a factor of 10 divided by the volume in which all tests were positive. MPN tables for other combinations of sample volumes and number of tubes at each level of inoculation are given by the American Public Health Association and others (1976, p. 924–925).

Table 1.—MPN index and 95 percent confidence limits for various combinations of positive and negative results when five 10-ml, five 1-ml, and five 0.1-ml dilutions are used

[American Public Health Association and others, 1976, p. 924-925]

Number of tubes giving positive reaction out of:			MPN Index per 100 ml	95 percent confidence limits		Number of tubes giving positive reaction out of:			MPN Index per 100 ml	95 percent confidence limits	
5 of 1 ml each	5 of 0.1 ml each	5 of 0.01 ml each		Lower	Upper	5 of 1 ml each	5 of 0.1 ml each	5 of 0.01 ml each		Lower	Upper
0	0	0	< 2								
0	0	1	2	< 0.5	7	4	2	1	26	9	78
0	1	0	2	< .5	7	4	3	0	27	9	80
0	2	0	4	< .5	11	4	3	1	33	11	93
						4	4	0	34	12	93
1	0	0	2	< .5	7						
1	0	1	4	< .5	11	5	0	0	23	7	70
1	1	0	4	< .5	11	5	0	1	31	11	89
1	1	1	6	< .5	15	5	0	2	43	15	110
1	2	0	6	< .5	15	5	1	0	33	11	93
						5	1	1	46	16	120
2	0	0	5	< .5	13	5	1	2	63	21	150
2	0	1	7	1	17						
2	1	0	7	1	17	5	2	0	49	17	130
2	1	1	9	2	21	5	2	1	70	23	170
2	2	0	9	2	21	5	2	2	94	28	220
2	3	0	12	3	28	5	3	0	79	25	190
						5	3	1	110	31	250
3	0	0	8	1	19	5	3	2	140	37	340
3	0	1	11	2	25	5	3	3	180	44	500
3	1	0	11	2	25	5	4	0	130	35	300
3	1	1	14	4	34	5	4	1	170	43	490
3	2	0	14	4	34	5	4	2	220	57	700
3	2	1	17	5	46	5	4	3	280	90	850
3	3	0	17	5	46	5	4	4	350	120	1,000
4	0	0	13	3	31	5	5	0	240	68	750
4	0	1	17	5	46	5	5	1	350	120	1,000
4	1	0	17	5	46	5	5	2	540	180	1,400
4	1	1	21	7	63	5	5	3	920	300	3,200
4	1	2	26	9	78	5	5	4	≥1,600	640	5,800
4	2	0	22	7	67	5	5	5	≥2,400		

9. Report

The coliform concentration is reported as MPN coliforms per 100 ml. Values less than 10, report whole numbers; 10 or more, report two significant figures.

10. Precision

Unless large numbers of sample aliquots are used for each decimal dilution, the precision of the MPN procedure is poor. Table 1 shows the 95 percent confidence limits for various combination of positive and negative results when five 10-ml, five 1-ml, and five 0.1-ml dilutions are used.

References

American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater (14th ed.): New York, Am. Public Health Assoc., 1193 p.

Goerlitz, D. F., and Brown, Eugene, 1972, Methods for the investigation of organic substances in water: U.S. Geol. Survey Techniques Water-Resources Inv., book 5, chap. A3, 40 p.

Kriss, A. E., Lebedeva, M. N., and Tsiban, A. V., 1966, Comparative estimate of a Nansen and microbiological water bottle for sterile collection of water samples from depths of seas and oceans: Deep-Sea Research, v. 13, p. 205– 212.

Total coliform bacteria (most probable number, MPN, method)

Presumptive field test (B–0040–77)

Parameter and code: Coliform, presumptive (MPN) 31507

1. Application

This method is applicable to all types of fresh and saline waters. It is applicable to waters with large suspended-solids content and waters with high counts of non-coliform bacteria. It is suitable for application at the sample 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°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. The method described herein is similar to the "Total Coliform MPN Method (presumptive test)", except that provision is made for the incubation of samples under field conditions. Certain noncoliform organisms can ferment lactose with gas formation.

3. Interferences

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

4. Apparatus

All materials used in bacteriological testing must be free of agents which inhibit bacterial growth.

4.1 *Water-sampling bottle.* Samplers for obtaining water samples under sterile conditions as marketed by General Oceanics, Inc., Hydro Products, Kahl Scientific Instrument Corp., and others. A metallic water sampler, lowered at a speed of 1 m/s, may be effective for sterile collection of water samples (Kriss and others, 1966). Metallic water-sampling bottles are available from Wildlife Supply Co. (1050 or 1200); Kahl Scientific Instrument Corp. (130WA100); InterOcean Systems, Inc. (206); Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type); or equivalent.

4.2 *Durham (fermentation) tubes and serum vials*

4.2a For testing 50 or 100 ml aliquots use milk dilution bottles, APHA, Pyrex or Kimax with screwcaps as serum vials; use flint glass culture tubes, 10×75 mm, Kimble (73500) or equivalent as fermentation tubes.

4.2b *Serum bottles,* 10-ml capacity, Wheaton (223739) or equivalent.

4.2c *Rubber stoppers,* 13×20 mm, Wheaton (224183) or equivalent.

4.2d *Aluminum seals,* one piece, 20 mm, Wheaton (224183) or equivalent.

4.2e *Fermentation tubes,* 25×16 mm test tubes, Thomas (9185-R12) or equivalent.

4.2f *Crimper,* for attaching aluminum seals, Wheaton (224303) or equivalent.

4.2g *Decapper,* for removing aluminum seals from spent tubes, Wheaton (224183) or equivalent.

4.3 *Incubator* for operation at a temperature of 35°±0.5°C. A portable incubator as provided in the Portable Water Laboratory, Millipore (XX63 001 50) or equivalent, which operates on either 110 volts a.c. or 12 volts d.c., is convenient for field use. A larger incubator with a more precise temperature regulation, National Appliance (320) or equivalent, is satisfactory for laboratory use.

4.4 *Sterilizer,* steam autoclave, Curtin Matheson Scientific (209–536) or Market Forge Sterilmatic or equivalent.

4.5 *Bottles,* milk dilution, APHA, Pyrex or Kimax with screwcaps.

4.6 *Hypodermic Syringes,* 2.5 ml, Becton Dickinson (5610) or equivalent.

4.7 *Pipets,* 1.0-ml capacity, presterilized, disposable, glass or plastic with cotton plugs, Millipore (XX63 001 35) or equivalent.

4.8 *Pipets,* 11.0-ml capacity, Corning (7057) or equivalent. Wrap the pipets in kraft paper and sterilize in the autoclave, or place in a pipet box, Curtin Matheson Scientific or equivalent, and heat in an oven at 170°C for 2 hours. Presterile, disposable, 10.0-ml pipets may be used.

4.9 *Propipet* for use with 1.0-, 10.0-, and 11.0-ml pipets.

5. Reagents

5.1 *Lauryl tryptose broth,* Difco Bacto Lauryl Tryptose Broth (0241) or BBL Lauryl Sulfate Broth (11338) or equivalent. Prepare according to American Public Health Association and others (1976, p. 965) or according to directions on bottle label.

Place 50 ml of medium containing 71.2 g/l of Difco Bacto Lauryl Tryptose Broth or BBL Lauryl Sulfate Broth in a milk dilution bottle for each 50 ml aliquot of water to be tested.

Place 9 ml of medium containing 35.6 g/l of Difco Bacto Lauryl Tryptose Broth or BBL Lauryl Sulfate Broth in each 10 ml serum bottle for each 1 ml or smaller aliquot of sample to be tested.

Place one fermentation tube, mouth downward (inverted) in each bottle of broth. Place screwcaps on milk dilution bottles. Loosen caps prior to sterilizing and tighten when bottles have cooled.

Place one fermentation tube, mouth downward (inverted) in each serum bottle. Place rubber stopper in mouth and attach aluminum seal using crimper.

Sterilize in upright position at 121°C at 1.05 kg per cm^2 (15 psi) for 15 minutes. Air will be expelled from the inverted, inner durham tube during heating; each will fill completely with medium during cooling. Before using check to see that there are no bubbles in the inverted durham tubes.

5.2 *Buffered dilution water:* Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 ml distilled water. Adjust to pH 7.2 with 1 *N* sodium hydroxide (NaOH). Dilute to 1 liter with distilled water. Sterilize in dilution bottles for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). After opening a bottle of stock solution, refrigerate the unused part. Discard contaminated solution, indicated by slight turbidity or precipitate.

Add 1.2 ml of this stock phosphate buffer solution to 1 liter of distilled water containing 0.1 percent Difco peptone (0118), or equivalent. Dispense in milk dilution bottle in amounts that will provide 99 ml±2.0 after autoclaving at 121°C at 1.05 kg per cm^2 (15 psi) for 20-30 minutes. Loosen caps or stoppers prior to sterilizing, and tighten when bottles have cooled.

6. Collection

Samples for bacteriological examination must be collected in bottles that have been carefully cleaned and autoclaved for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Sterilized milk dilution bottles are ideal sample containers. When the sample is collected, ample air space must be left in the bottle to facilitate mixing of the sample by shaking. Care must be taken to avoid contamination of the sample and sample bottle at the time of collection and in the period prior to analysis.

To insure maximum correlation of results, the sample sites and methods used for bacteria should correspond as closely as possible to those selected for chemical and plankton sampling. However, sampling for bacteria at depth is complicated by the requirement to avoid contamination of the deeper water layers by bacteria carried from shallower depths on the inner walls of the sampler.

The collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, estuaries, bacterial abundance may vary transversely, with depth and with time of day. To collect a surface sample from a stream or lake, open a sterile milk dilution bottle, grasp it near its base, and plunge it, neck downward, below the water surface. Allow the bottle to fill by slowly turning 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 the case of 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, 1976, p. 965).

To collect a sample representative of the bacterial concentration at a particular depth, use one of the

water-sampling bottles discussed in 4.1 above. For small streams, a point sample at a single transverse position located at the centroid of flow may be adequate (Goerlitz and Brown, 1972).

As soon as possible after collection, preferably within 1 hour and not more than 6 hours, inoculate the decimal dilutions of the sample into the lauryl tryptose broth serum vials. Samples must be kept cool during the time between collection and inoculation. If inoculation is delayed, chill or refrigerate the sample but do not freeze.

The volumes of decimal dilutions should be such that, after incubation, both positive and negative results are obtained among the range of volumes. The method fails if only positive or only negative results are obtained with all volumes tested.

The following sample volumes are suggested:

1. Unpolluted raw surface waters: 0.1-, 1.0-, 10.0-, and 50.0-ml samples will cover a MPN range of <1 to ≥ 240 coliform organisms per 100 ml.
2. Polluted raw surface water: 0.0001-, 0.001-, 0.01-, and 0.1-ml will cover a MPN range of 20 to 2.4×10^6 coliform organisms per 100 ml.

7. Analysis

7.1 Set out five vials of lauryl tryptose broth for each volume to be tested.

7.2 If the volume to be tested is 0.1 ml or more, transfer the measured samples directly to the serum vials using either sterile pipets, presterilized disposable hypodermic syringes, or other sterile measuring device such as a graduated cylinder.

If the volume of original water sample is less than 0.1 ml, proceed as above after preparing appropriate dilutions by adding the sample to a sterile milk dilution bottle in the following amounts:

Dilution	Volume of sample added to 99 ml dilution bottle	Filter this volume
1:100	1.0 ml original sample	1.0 ml of 1:100 dilution.
1:1,000	1.0 ml of 1:10 dilution	1.0 ml of 1:1,000 dilution.
1:10,000	1.0 ml of 1:100 dilution	1.0 ml of 1:10,000 dilution.

Note: Use a sterile pipet or hypodermic syringe for each vial. After each transfer between bottles, close and shake the bottle vigorously 25 times. Diluted samples should be inoculated within 20 minutes after preparation.

7.3 When using serum bottles with rubber stoppers, proceed as follows: Remove the inserts from the metal caps and swab the exposed area of the rubber septum with a bit of cotton saturated with 70 percent ethanol or isopropanol.

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

Carefully return bottle to normal, upright position with stopper at top. Make sure that inverted vial is completely filled with medium and no residual bubbles remain in the vial.

7.4 Clearly mark each set of serum vials indicating location, time of collection, sample number, and sample volume. Code each vial for easy identification.

7.5 Place the inoculated vials in the incubator and incubate at $35° \pm 0.5°C$ for 24 ± 2 hours. Culture vials must be maintained in an upright position.

7.6 Remove vials from incubator and examine. Gas in any amount in the inverted vial, even a pinhead size bubble, constitutes a positive test. The appearance of an air bubble must not be confused with actual gas production. The broth medium will become cloudy with actual fermentation and small bubbles of gas may appear in the medium outside the durham tube when serum vial is shaken gently (American Public Health Association and others, 1976, p. 916).

7.7 Autoclave all gas-positive vials for 15 minutes at 121°C at 1.05 kg per cm^2 (15 psi) before discarding.

7.8 Replace all gas-negative vials in incubator and incubate for an additional 24 ± 2 hours at $35°C \pm 0.5°C$.

7.9 Remove vials from incubator and examine for gas formation. Autoclave all vials of lauryl tryptose broth as in 7.7 before discarding.

8. Calculations

Record the number of gas-positive vials occurring over all sample volumes tested. When more than three volumes are tested, the results from only three of these are used in computing the MPN. To select the three dilutions for the MPN index, proceed as follows: Take as the first member the smallest sample volume in which all tests are positive (no larger sample volume giving any negative results) and the two next succeeding smaller sample volumes (American Public Health Association and others, 1976, p. 923–926).

In the examples given below, the number in the

numerator represents positive tubes; the denominator represents the total number of tubes inoculated:

Example	1 ml	Decimal dilutions 0.1 ml	0.01 ml	0.001 ml	Combination of positives
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	

In example c, the first three dilutions should be taken to place the positive results in the middle dilution. When a positive occurs in a dilution higher than the three chosen according to the guideline, as in d, it should be placed in the result for the highest chosen dilution as in e.

A table giving MPN for various combinations of positive and negative results when five 10-ml, five 1.0-ml, and five 0.1-ml dilutions are tested is shown in table 1. If a series of decimal dilutions other than 10.0-, 1.0-, and 0.1-ml is used record the MPN as the value from the table multiplied by a factor of 10 divided by the volume in which all tests were positive. MPN tables for other combinations of sample volumes and number of tubes at each level of inoculation are given by the American Public Health Association and others (1976, p. 924–925).

9. Report

The coliform concentration is reported as MPN coliforms per 100 ml. Values less than 10, report whole numbers; 10 or more, report two significant figures.

10. Precision

Unless large numbers of sample aliquots are used for each decimal dilution, the precision of the MPN procedure is poor. Table 1 shows the 95 percent confidence limits for various combinations of positive and negative results when five 10-ml, five 1-ml, and five 0.1-ml dilutions are used.

References

American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater (14th ed.): New York, Am. Public Health Assoc., 1193 p.

Goerlitz, D. F., and Brown, Eugene, 1972, Methods for the investigation of organic substances in water: U.S. Geol Survey Techniques Water-Resources Inv., Book 5, Chap. A3, 40 p.

Kriss, A.E., Lebedeva, M.N., and Tsiban, A.V., 1966, Comparative estimate of a Nansen and microbiological water bottle for sterile collection of water samples from depths of seas and oceans: Deep-Sea Research, v. 13, p. 205–212.

Confirmation of total coliform bacteria (B–0045–77)

Parameter and code: Coliform, confirmed (MPN) 31505

1. Application

Because some members of the coliform group may react atypically and not produce the characteristic sheen colonies, the identity of suspected coliform colonies should be verified. Geldreich, Jeter, and Winter (1967) discussed verification and other aspects of the membrane filter procedure.

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

The confirmation test is applicable to coliform 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

Material from selected colonies on the membrane filters is placed in tubes of sterile lactose broth and incubated at 35°C for 48 hours. Material from tubes showing gas formation within 48 hours is placed in tubes of sterile brilliant green lactose bile broth. Gas production in the brilliant green lactose bile broth within 48 hours at 35°C confirms the presence of coliform bacteria.

The confirmation procedure is compatible with the procedure described by the American Public Health Association and others (1976, p. 916–918).

3. Interferences

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

4. Apparatus

4.1 *Inoculating loop,* platinum-iridium wire, 3 mm, Brown & Sharpe gage 26, A. H. Thomas Co. (7012-E20) or equivalent.

4.2 *Bunsen burner,* for sterilizing inoculating loop.

4.3 *Culture tubes,* flint glass, 16×150 mm, Kimble (73500) or equivalent, and *culture tubes,* flint glass, 6×50 mm, Corning (9820) or equivalent, and *test tube caps,* 16 mm, Scientific Apparatus (9468) or equivalent.

4.4 *Culture-tube rack,* for 16-mm tubes, Thomas-Kolmer or equivalent.

4.5 *Incubator,* capable of operating at a temperature of 35°C±0.5°C, or water bath, capable of operating at a temperature of 35°C±0.5°C, Curtin Matheson Scientific or equivalent.

4.6 *Sterilizer,* steam autoclave, Matheson Scientific (59827–20) or Market Forge Sterilmatic or equivalent.

5. Reagents

5.1 *Lauryl tryptose broth,* prepackaged lauryl tryptose broth in 16×125 mm tubes with fermentation shell, Hyland (056–143) or equivalent. Medium also may be prepared according to American Public Health Association and others (1976).

5.2 *Brilliant green lactose broth,* prepackaged brilliant green lactose broth in 16×125 mm tubes with fermentation shell, Hyland (056–039) or equivalent. The medium also may be prepared according to American Public Health Association and others (1976).

6. Collection

No sample collections are necessary.

7. Analysis

7.1 The membrane filter method for total coliform bacteria should be conducted according to procedures described in this manual.

7.2 From the incubated membrane filter, select a colony or colonies to be confirmed for coliform bacteria.

7.3 Sterilize the inoculating loop by flaming in the burner. The long axis of the wire should be held parallel to the cone of the flame so that the entire end of the wire and loop are heated to redness.

7.4 Remove from flame and allow the wire to cool for about 10 seconds. Touch the loop lightly to the colony. Part of the colony material will remain on the wire.

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

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

7.7 Remove tubes from incubator and examine. Gas in any amount in the inverted vial constitutes a positive test.

7.8 Using a sterile inoculating loop, transfer one loopful of broth from each lauryl tryptose broth tube showing gas to a series of tubes of sterile brilliant green lactose broth. Sterilize the loop after each transfer.

7.9 Sterilize all gas-positive lauryl tryptose broth tubes in the autoclave for 15 minutes at 121°C at 1.05 kg per cm^2 (15 psi) before discarding.

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

7.11 Examine the remaining lauryl tryptose tubes. Transfer one loopful of material from each tube producing gas to a tube of brilliant green lactose broth as in 7.8 and continue as in 7.10.

If no gas appears in the lauryl tryptose broth tube within 48±3 hours, the original colony was not of the coliform group.

Autoclave all tubes of lauryl tryptose broth as in 7.9 before discarding.

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

7.13 Tubes of brilliant green lactose broth should be autoclaved as in 7.9 before discarding.

8. Calculations

No calculations are necessary.

9. Report

Results of the coliform confirmation test are included in the colony counts for total coliform bacteria.

10. Precision

No precision data are available.

References

American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater [13th ed.]: New York, Am. Public Health Assoc., 1193 p.

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

Fecal coliform bacteria (membrane filter method)
(B–0050–77)

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

1. Application

Fecal coliforms are that part of the coliform group that is present in the intestines and feces of warmblooded 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 in this manner (American Public Health Association and others, 1976, p. 875–877).

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

The method is applicable to fresh and saline waters.

2. Summary of method

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

3. Interferences

Suspended materials may not permit testing of sample volumes sufficient to produce significant results. Fecal coliform colony formation on the filter may be inhibited by high numbers of noncoliform colonies, by the presence of algal filaments and detritus, or by toxic substances.

Water samples with a high suspended-solids content may be split between two or more membrane filters. The multiple-tube method described by the American Public Health Association and others (1976, p. 922) will give the most reliable results under conditions of high suspended solids content, when fecal coliform counts are low.

4. Apparatus

All materials used in bacteriological testing must be free of agents which inhibit bacterial growth.

4.1 *Water-sampling bottle.* Samplers for obtaining water samples under sterile conditions are marketed by General Oceanics, Inc., Hydro Products, Kahl Scientific Instrument Corp., and others. A metallic water sampler, lowered at a speed of 1 m/s, may be effective for sterile collection of water samples (Kriss and others, 1966). Metallic water-sampling bottles are available from Wildlife Supply Co. (1050 or 1200); Kahl Scientific Instrument Corp. (130WA100); InterOcean Systems, Inc. (206); Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type); or equivalent.

4.2 *Incubator* with temperature of 44.5°±0.2°C is required. A Portable Heaterblock Incubator (fig. 6), Millipore (XX63 004 00) or equivalent, may be used both in the field and in the laboratory.

4.3 *Filter-holder assembly,* Millipore (XX63 001 20) or equivalent, and all-metal *syringe and two-way valve,* Millipore (XX62 000 35) or equivalent.

4.4 *Membrane filters,* white, grid, sterile packed, 0.7-μm pore size, 47-mm diameter Millipore (HCWG 047 Sl) or equivalent; absorbent pads, Millipore (APIO 047 SO) or equivalent.

4.5 *Plastic petri dishes* with *covers,* disposable, sterile, 50×12 mm, Millipore (PD10 047 00) or equivalent.

4.6 *Forceps,* stainless steel, smooth tips, Millipore (XX62 000 06) or equivalent.

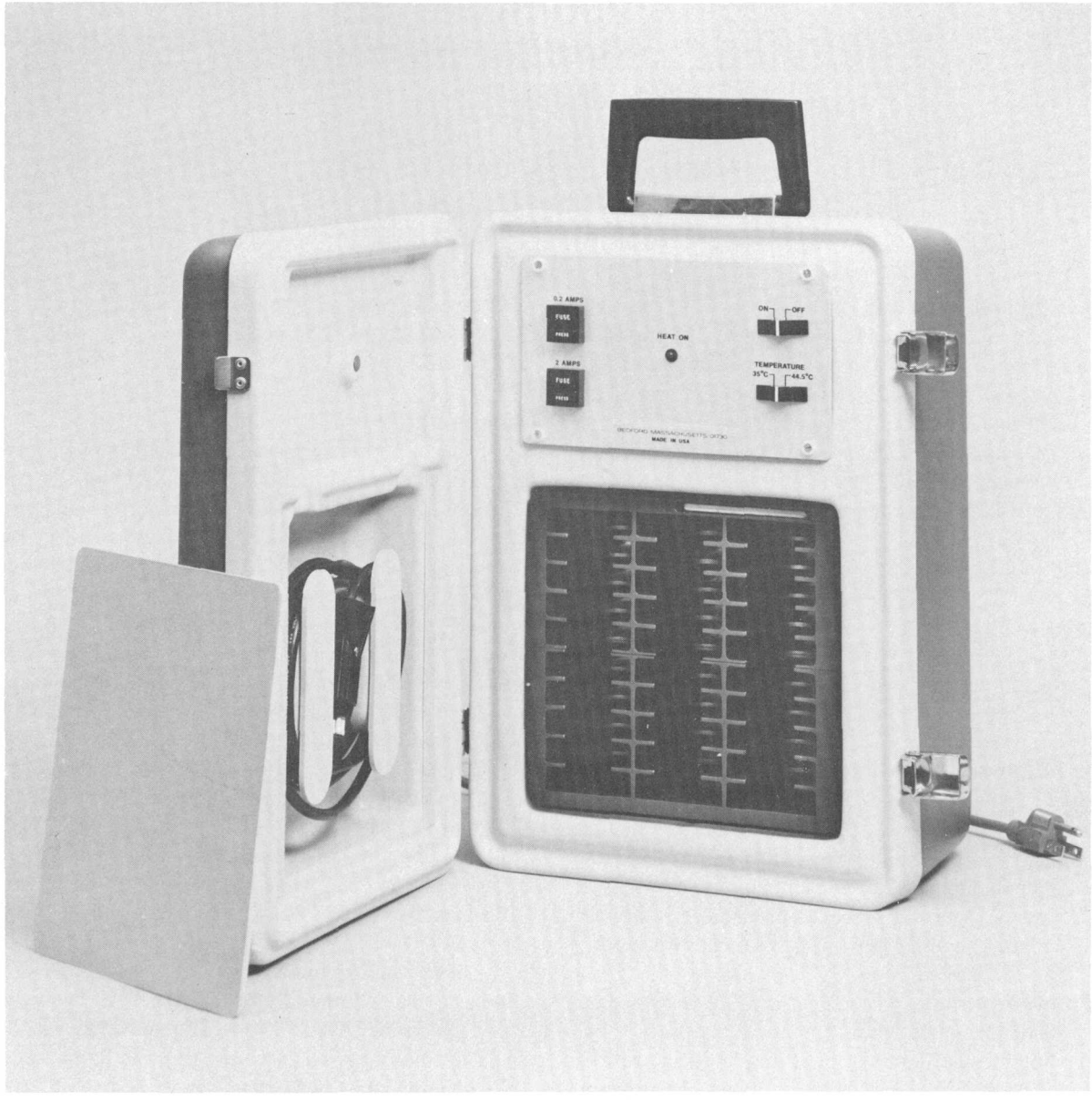

Figure 6.—Portable heaterblock incubator. (Photograph courtesy of Millipore Corp., Bedford, Mass.)

4.7 *Microscope,* binocular wide-field dissecting-type, Bausch & Lomb (31–26–29–73) or equivalent, with fluorescent lamp, Bausch & Lomb (31–33–63) or equivalent.

4.8 *Sterilizer,* steam autoclave, Curtin Matheson Scientific (209–536) or Market Forge Sterilmatic or equivalent.

4.9 *Bottles,* milk dilution, APHA, Pyrex or Kimax, with screwcaps.

4.10 *Pipets, 1.0-ml capacity,* sterilized, disposable, glass or plastic with cotton plugs, Millipore (XX63 001 35) or equivalent, or sterile, disposable, 1.0-ml hypodermic syringes.

4.11 *Pipets, 11.0-ml capacity,* Corning (7057) or

equivalent. Wrap the pipets in kraft paper and sterilize in the autoclave, or place in a pipet box, Curtin Matheson Scientific or equivalent, and heat in an oven at 170°C for 2 hours. Presterile, disposable, 10.0-ml pipets may be used.

4.12 *Propipet* for use with 1.0-, 10.0-, and 11.0-ml pipets.

4.13 *Plastic bags,* sealable, waterproof, Nasco, Whirl-Pak (B736N), or equivalent.

4.14 *Thermometer,* with range of at least 40°–100°C, Brooklyn Thermometer Co. (6410Y) or equivalent.

5. Reagents

5.1 *M-FC broth:* Add 0.74 g M-FC broth, Difco (0883) or equivalent, to 20 ml distilled water. Add 0.2 ml rosolic acid solution to the M-FC broth and stir. Place beaker containing broth in boiling water bath for 3 minutes, or less. If medium begins to boil, promptly remove from heat and cool.

Refrigerate the finished medium until used; storage should not exceed 72 hours; preferably the media should not be stored for more than 24 hours.

5.2 *M-FC agar:* Add 5.2 g M-FC agar, Difco 0677 or equivalent, to 100 ml distilled water. Heat to boiling with constant stirring, 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. When the medium solidifies, store the prepared petri dishes at 2°–10°C for a maximum of 72 hours; preferably the medium should not be stored for more than 24 hours.

5.3 *Rosolic acid solution:* Add 10 ml of 0.2 *N* sodium hydroxide (NaOH) to 0.10 g of rosolic acid crystals, Difco (3228), or equivalent. Stir vigorously to dissolve. Do not heat. Store in the dark at room temperature for a maximum of 3 weeks. Discard if color changes from deep red to orange.

5.4 *Buffered dilution water:* Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_2) in 500 ml distilled water. Adjust to pH 7.2 with 1 *N* sodium hydroxide (NaOH). Dilute to 1 liter with distilled water. Sterilize in dilution bottles for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). After opening a bottle of stock solution, refrigerate the unused part. Discard contaminated solutions, indicated by slight turbidity or precipitate accumulation.

Add 1.2 ml of this stock phosphate buffer solution to 1 liter of distilled water containing 0.1 percent Difco peptone (0118), or equivalent. Dispense in milk dilution bottles in amounts that will provide 99 ml±2.0 after autoclaving at 121°C at 1.05 kg per cm^2 (15 psi) for 20–30 minutes. Loosen caps or stoppers prior to sterilizing, and tighten when bottles have cooled.

5.5 *Ethyl alcohol:* 95 percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methanol may be used for sterilization.

5.6 *Methyl alcohol:* Absolute, for sterilizing filter holder assembly.

6. Collection

Samples for bacteriologic examination must be collected in bottles that have been carefully cleansed and autoclaved for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Sterilized milk dilution bottles are ideal sample containers. When the sample is collected, ample air space must be left in the bottle to facilitate mixing of the sample by shaking. Care must be taken to avoid contamination of the sample and sample bottle at the time of collection and in the period prior to analysis.

To insure maximum correlation of results, the sample sites and methods used for bacteria should correspond as closely as possible to those selected for chemical and plankton sampling. However, sampling for bacteria at depth is complicated by the necessity to avoid contamination of the deeper water layers by bacteria carried from shallower depths on the inner walls of the sampler.

The sample collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, bacterial abundance may vary transversely, with depth, and with time of day. To collect a surface sample from a stream or lake, open a sterile milk dilution bottle, grasp it near its base, and plunge it, neck downward, below the water surface. Allow the bottle to fill by slowly turning 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 the case of a lake, a current should be artificially created by pushing the bottle horizontally forward in a direction away from the hand (American Public Health Association and others, 1976, p. 965).

To collect a sample representative of the bacterial concentration at a particular depth, use one of the water-sampling bottles discussed in 4.1 above. For small streams, a point sample at a single transverse position located at the centroid of flow may be adequate (Goerlitz and Brown, 1972).

As soon as possible after collection, preferably within 1 hour and not more than 6 hours, filter the sample and place the membrane filter on growth

medium as described in 7.5–7.10 below. Samples must be kept cool during the time between collection and filtration. If filtration is delayed, chill or refrigerate the sample, but do not freeze.

The volume of sample to be filtered should be such that after incubation 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 waters: 0.1-, 0.3-, 0.9-, 2.7-, 8.1-, 24.3-, and 72.9-ml samples;
2. Polluted raw surface waters: 0.01-, 0.03-, 0.1-, 0.3-, 0.9-, and 2.7-ml samples.

7. Analysis

7.1 If using M-FC broth, place a sterile absorbent pad on the bottom (larger half) of each sterile plastic petri dish using sterilized forceps. Proceed to 7.3 if M-FC agar is to be used.

Note: Dip forceps in alcohol, pass through flame to ignite alcohol, and allow to burn out. Do not hold forceps in flame.

7.2 Saturate each pad with about 2 ml of M-FC broth and tip the petri dish to expel excess liquid. Replace petri dish tops.

7.3 Sterilize filter apparatus. In the laboratory, the funnel and filter base may be wrapped separately in kraft paper packages and sterilized in the autoclave for 15 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Cool to room temperature before use.

Field sterilization of filter apparatus should be in accordance with the manufacturer's instructions. Autoclave sterilization in the laboratory prior to the field trip is preferred.

7.4 Assemble filtration equipment, and, using sterilized forceps, place a sterile membrane filter over the porous plate of the apparatus, grid side up. Place funnel on filter with care to avoid tearing or creasing the membrane.

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

If the volume of sample is between 1.0 ml and 10 ml, pour about 20 ml of sterilized buffer dilution water into the funnel before transferring the measured sample onto the membrane. This facilitates distribution of organisms.

If the volume of original water sample is less than 1.0 ml, proceed as above after preparing appropriate dilutions by adding the sample to a sterile milk dilution bottle in the following amounts:

Dilution	Volume of sample added to 99 ml dilution bottle	Filter this volume
1:10	11.0 ml original sample	11.0 ml of 1:10 dilution.
1:100	1.0 ml original sample	1.0 ml of 1:100 dilution.
1:1,000	1.0 ml of 1:10 dilution	1.0 ml of 1:1,000 dilution.
1:10,000	1.0 ml of 1:100 dilution	1.0 ml of 1:10,000 dilution.

Note: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer between bottles, close and shake the bottle vigorously at least 25 times. Diluted samples should be filtered within 20 minutes after preparation.

7.6 Apply vacuum and filter sample. When vacuum is applied with a syringe fitted with a two-way valve, proceed as follows: Attach the filter 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 filter 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.

7.7 Rinse sides of funnel twice with 20–30 ml of sterile buffered dilution water while applying vacuum.

7.8 Remove funnel from receptacle and place upside down on a clean surface.

7.9 With flame-sterilized forceps remove the membrane filter from the filter base and place it on the agar surface or 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.

7.10 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.

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

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

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

7.14 Place each petri dish in a waterproof plastic bag or seal the dish with waterproof plastic tape if using a waterbath incubator.

7.15 Incubate the filters in the petri dishes in an inverted position in an incubator at 44.5°±0.2°C for

22±2 hours. Begin incubation within 20 minutes after placing membrane filter on the M-FC medium.

7.16 Remove the filters and 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 with the aid of a × 10 to × 15 magnification.

7.17 Autoclave all cultures at 121°C for 15 minutes at 1.05 kg per cm^2 (15 psi) before discarding.

8. Calculations

8.1 For colony counts between the ideal of 20 and 60, use the formula:

$$\text{Fecal coliform colonies/100 ml} = \frac{\text{fecal coliform colonies counted} \times 100}{\text{vol. of original sample filtered (ml)}}.$$

8.2 Counts less than the ideal of 20 colonies or greater than 60 colonies per filter should be reported as number per 100 ml, followed by the statement, "Estimated count based on nonideal colony count."

8.3 If no filters develop characteristic fecal coliform colonies, calculate assuming that the largest sample volume filtered had one fecal coliform colony. Report as less than that calculated number per 100 ml.

8.4 If all filters bear colonies too numerous to count, a minimum estimated value can be reported. Assume a count of 60 coliform colonies on the smallest filtered volume, then calculate according to the formula in 8.1. Report as greater than (>) the calculated value.

8.5 Sometimes two or more filters of a series will produce colony counts within the recommended counting range. Colony counts should be made on all such filters. The method for calculating and averaging is as follows:

Volume filter 1	Colony count filter 1
+ Volume filter 2	+ Colony count filter 2
Volume sum	Colony count sum

$$\text{Fecal coliform colonies/100 ml} = \frac{\text{colony count sum} \times 100}{\text{vol. sum (ml)}}.$$

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

9. Report

The fecal coliform concentration is reported as fecal coliform colonies per 100 ml. Values less than 10, report whole numbers; 10 or more, report two significant figures.

10. Precision

No numerical precision data are available. However, the method gives 93 percent accuracy for differentiating between coliforms of warm-blooded animals and coliforms from other sources (American Public Health Association and others, 1976, p. 937).

References

American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater [14th ed.]: New York, Am. Public Health Assoc., 1193 p.

Goerlitz, D. F., and Brown, Eugene, 1972, Methods for analysis of organic substances in water: U.S. Geol. Survey Techniques Water-Resources Inv., book 5, chap. A3, 40 p.

Kriss, A. E., Lebedeva, M. N., and Tsiban, A.V., 1966, Comparative estimate of a Nansen and microbiological water bottle for sterile collection of water samples from depths of seas and oceans: Deep-Sea Research, v. 13, p. 205-212.

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

Fecal streptococcal bacteria (membrane filter method)
(B–0055–77)

Parameter and code: Streptococci, fecal, MF, KF agar (colonies/100 ml) 31673

1. Application

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

The method is applicable to most types of waters.

2. Summary of method

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

3. Interferences

Suspended materials may not permit testing of sample volumes sufficient to produce significant results. Streptococcal colony formation on the filter may be inhibited by high numbers of nonstreptococcal colonies, by the presence of algal filaments and detritus, or by toxic substances.

Water samples with a high suspended-solids content may be divided between two or more membrane filters. The multiple tube method described by the American Public Health Association and others (1976, p. 942–944) will give the most reliable results under conditions of high suspended-solids content, when streptococcal counts are low.

4. Apparatus

All materials used in bacteriological testing must be free of agents which inhibit bacterial growth.

The following apparatus list assumes the use of a field kit for bacteriological water tests such as the Portable Water Laboratory, Millipore (XX63 001 50), 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 below are included in the Portable Water Laboratory (fig. 3).

4.1 *Water-sampling bottle.* Samplers for obtaining water samples under sterile conditions are marketed by General Oceanics, Inc., Hydro Products, Kahl Scientific Instrument Corp., and others. A metallic water sampler, lowered at a speed of 1 m/s, may be effective for sterile collection of water samples (Kriss and others, 1966). Metallic water-sampling bottles are available from Wildlife Supply Co. (1050 or 1200); Kahl Scientific Instrument Corp. (130WA100); InterOcean Systems, Inc. (206); Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer type); or equivalent.

4.2 *Filter-holder assembly,* Millipore (XX63 001 20*) or equivalent, and *syringe and two-way valve,* Millipore (XX62 000 35*) or equivalent.

4.3 *Membrane filters,* white, grid, sterile packed, 0.45- or 0.7-μm mean pore size, 47-mm diameter, Millipore (HAWG 047 SO or HCWG 047 SI), or equivalent.

4.4 *Plastic petri dishes* with *covers,* disposable, sterile 50×12 mm, Millipore (PD10 047 00*) or equivalent.

4.5 *Forceps,* stainless steel, smooth tips, Millipore (XX62 000 06*) or equivalent.

4.6 *Incubator* for operation at a temperature of 35°±0.5°C. A portable incubator as provided in the Portable Water Laboratory, Millipore (XX63 001

50*), or equivalent, which operates on either 110 volts a.c. or 12 volts d.c., is convenient for field use. A larger incubator with a more precise temperature regulation, National Appliance (320) or equivalent, is satisfactory for laboratory use.

4.7 *Microscope,* binocular wide-field dissection-type, Bausch & Lomb (31–26–29–73) or equivalent, with fluorescent lamp, Bausch & Lomb (31–33–63) or equivalent.

4.8 *Sterilizer,* steam autoclave, Curtin Matheson Scientific (209–536) or Market Forge Sterilmatic or equivalent.

4.9 *Bottles,* milk dilution, APHA, Pyrex or Kimax, with screwcaps.

4.10 *Pipets, 1.0-ml capacity,* presterilized, disposable, glass or plastic with cotton plugs, Millipore (XX63 001 35*) or equivalent, or sterile, disposable, 1.0-ml hypodermic syringes.

4.11 *Pipets, 11.0-ml capacity,* Corning (7057) or equivalent. Wrap the pipets in kraft paper and sterilize in the autoclave, or place in a pipet box, Matheson Scientific (55930-20) or equivalent, and heat in an oven at 170°C for 2 hours. Presterile, disposable, 10.0-ml pipets may be used.

4.12 *Propipet* for use with 1.0-, 10.0-, and 11.0-ml pipets.

4.13 *Thermometer,* with range of at least 40° –100°C, Brooklyn Thermometer Co. (6410Y) or equivalent.

5. Reagents

5.1 *KF Streptococcus agar:* Suspend 7.64 g KF Streptococcus agar, Difco (0496), BBL (11312), or equivalent in a 125-ml screw cap erlenmeyer flask containing 100 ml of distilled water. Stir and heat to boiling. Once boiling starts, heat at this temperature an additional 5 minutes. Remove from heat and cool to 50°–60°C. Add 1 ml of 1 percent TTC solution after the medium has cooled below 60°C. If commercially obtained 1 percent *sterile* TTC solution is to be used, swab the rubber septum atop the vial with 95 percent ethanol. Remove 1.0 ml with a sterile, disposable 2.5-ml hypodermic syringe equipped with a 20–24 gauge×38-mm needle. When the medium has cooled to approximately 50°C, it should be poured into 12×50-mm petri dishes to a depth of 4 mm (6–7 ml). After solidification occurs, the prepared plates should be stored in a refrigerator for no longer than 2 weeks if sterile TTC was used. If unsterilized TTC was used, the prepared plates cannot be stored over 24 hours prior to use.

5.2 *Buffered dilution water:* Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 ml distilled water. Adjust to pH 7.2 with 1 *N* sodium hydroxide (NaOH). Dilute to 1 liter with distilled water. Sterilize in dilution bottles for 20 minutes at 121°C at 15 psi. After opening a bottle of stock solution, refrigerate the unused part. Discard contaminated solutions, indicated by slight turbidity or precipitate accumulation.

Add 1.2 ml of this stock phosphate buffer solution to 1 liter of distilled water containing 0.1 percent Difco peptone (0118), or equivalent. Dispense in milk dilution bottles in amounts that will provide 99 ml±2.0 after autoclaving at 121° at 1.05 kg per cm^2 (15 psi) for 20–30 minutes. Loosen caps or stoppers prior to sterilizing, and tighten when bottles have cooled.

5.3 *Ethyl alcohol:* 95 percent denatured or absolute ethyl alcohol for sterilizing equipment. Absolute methanol may be used for sterilization.

5.4 *Methyl alcohol:* absolute, for sterilizing filter holder assembly.

5.5 *TTC solution:* 1 percent sterile solution is prepared by dissolving 0.1 g triphenyltetrazolium chloride Difco (0643) or equivalent in 10 ml of distilled water. The solution is aseptically filtered through a 0.45-μm membrane filter. Sterile 1 percent TTC solution also is available from commercial sources, Difco (3112), BBL (11924), or equivalent. Store sterilized TTC solution at 2°–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, *freshly prepared* unsterilized TTC solution may be substituted if the KF medium will be used promptly. TTC solution cannot be sterilized by heat.

6. Collection

Samples for bacteriologic examination must be collected in bottles that have been carefully cleansed and autoclaved for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Sterilized milk dilution bottles are ideal sample containers. When the sample is collected, ample air space must be left in the bottle to facilitate mixing of the sample by shaking. Care must be taken to avoid contamination of the sample and sample bottle at the time of collection and in the period prior to analysis.

To insure maximum correlation of results, the sample sites and methods used for bacteria should correspond as closely as possible to those selected for chemical and plankton sampling. However, sampling for bacteria at depth is complicated by the requirement to avoid contamination of the deeper water layers by

bacteria carried from shallower depths on the inner walls of the samplers.

The sample collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, bacterial abundance may vary transversely, with depth, and with time of day. To collect a surface sample from a stream or lake, open a sterile milk dilution bottle, grasp it near its base, and plunge it, neck downward, below the water surface. Allow the bottle to fill by slowly turning 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 the case of a lake, a current should be artificially created by pushing the bottle horizontally forward in a direction away from the hand (American Public Health Association and others, 1976, p. 905).

To collect a sample representative of the bacterial concentration at a particular depth, use one of the water-sampling bottles discussed in 4.1 above. For small streams, a point sample at a single transverse position located at the centroid of flow may be adequate (Goerlitz and Brown, 1972). As soon as possible after collection, preferably within 30 minutes and not more than 6 hours, filter the sample and place the membrane filter on growth media as described in 7.5–7.10 below. Samples must be kept cool during the time between collection and filtration. If filtration is delayed, ice or refrigerate the sample, but do not freeze.

Fecal streptococci generally are present in fewer numbers than coliform bacteria; therefore, the filtered volume of sample must be larger than that used for other bacterial determinations. When filtering water of unknown quality, the following sample volumes are suggested: 0.05, 0.25, 1.25, 6.25, 31.25 and 100.0 ml.

7. Analysis

7.1 Pour agar medium at 45°–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.

7.2 Sterilize filter apparatus. In the laboratory, the funnel and filter base may be wrapped separately in kraft paper packages and sterilized in the autoclave for 15 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Cool to room temperature before use.

Field sterilization of filter apparatus should be in accordance with the manufacturer's instructions. Autoclave sterilization in the laboratory prior to the field trip is preferred.

7.3 Assemble filtration equipment and, using flamed forceps, place a sterile membrane filter over the porous plate of the apparatus, grid side up. Place funnel on filter with care to avoid tearing or creasing the membrane.

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

If the volume of sample is between 1.0 ml 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 organisms.

If the volume of original water sample is less than 1.0 ml, proceed as above after preparing appropriate dilutions by adding the sample to a sterile milk dilution bottle in the following amounts:

Dilution	Volume of sample added to 99 ml dilution bottle	Filter this volume
1:10	11.0 ml original sample	11.0 ml of 1:10 dilution.
1:100	1.0 ml original sample	1.0 ml of 1:100 dilution.
1:1,000	1.0 ml of 1:10 dilution	1.0 ml of 1:1,000 dilution.
1:10,000	1.0 ml of 1:100 dilution	1.0 ml of 1:10,000 dilution.

Note: Use a sterile pipet or hypodermic syringe for each bottle. After each transfer between bottles, close and shake the bottle vigorously at least 25 times. Diluted samples should be filtered within 20 minutes after preparation.

7.5 Apply vacuum and filter the sample. When vacuum is applied with a syringe fitted with a two-way valve, proceed as follows. Attach the filter 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 airlock before the filter 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.

7.6 Rinse sides of funnel twice with 20–30 ml of sterile buffered dilution water while applying vacuum.

7.7 Remove funnel from receptacle and place upside down on a clean surface.

7.8 With flame-sterilized forceps remove the membrane filter from the filter base and place it on the agar medium 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.

7.9 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.

7.10 Clearly mark the lid of each plastic dish indicating location, time of collection, time of incubation,

sample number, and sample volume. Use a waterproof felt-tip marker or grease pencil.

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

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

7.13 Incubate the petri dishes with filters in an inverted position (agar and filter at the top) for 48±2 hours at 35°±0.5°C.

7.14 Remove filters and 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 with the aid of × 10 to × 15 magnification. Illumination is not critical.

7.15 Autoclave all cultures at 121°C for 15 minutes at 1.05 kg per cm^2 (15 psi) before discarding.

8. Calculations

8.1 For colony counts between the ideal of 20 and 100, use the formula:

$$\text{Fecal streptococcal colonies/100 ml} = \frac{\text{fecal streptococcal colonies counted} \times 100}{\text{vol. of original sample filtered (ml)}}.$$

8.2 Counts less than the ideal of 20 colonies or greater than 100 colonies per filter should be reported as number per 100 ml, followed by the statement, "Estimated count based on nonideal colony count."

8.3 If no filters develop characteristic fecal streptococcal colonies, calculate assuming that the largest sample volume filtered had one fecal streptococcal colony. Report as less than that calculated number per 100 ml.

8.4 If all filters bear colonies too numerous to count, a minimum estimated value can be reported. Assume a count of 100 fecal streptococci colonies on the smallest filtered volume, then calculate according to the formula in 8.1. Report as greater than (>) the calculated value.

8.5 Sometimes two or more filters of a series will produce colony counts within the recommended counting range. Colony counts should be made on all such filters. The method for calculating and averaging is as follows:

Volume filter 1	Colony count filter 1
+ Volume filter 2	+ Colony count filter 2
Volume sum	Colony count sum

$$\text{Fecal streptococcal colonies/100 ml} = \frac{\text{colony count sum} \times 100}{\text{vol. sum (ml)}}.$$

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

9. Report

The fecal streptococcal concentration is reported as fecal streptococcal colonies per 100 ml. Values less than 10, report whole numbers; 10 or more, report two significant figures.

10. Precision

No numerical precision data are available.

References

American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater [14th ed.]: New York, Am. Public Health Assoc., 1193 p.

Goerlitz, D. F., and Brown, Eugene, 1972, Methods for analysis of organic substances in water: U.S. Geol Survey Techniques Water-Resources Inv., book 5, chap. A3, 40 p.

Kriss, A. E., Lebedeva, M. N., and Tsiban, A. V., 1966, Comparative estimate of a Nansen and microbiological water bottle for sterile collection of water samples from depths of seas and oceans: Deep-Sea Research, v. 13, p. 205–212.

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

Confirmation of fecal streptococcal bacteria (B-0060-77)

Parameter and code: Not applicable

1. Application

KF agar medium is selective for the growth of fecal streptococci. A few other types of bacteria, chiefly non-fecal streptococci, may appear occasionally on this medium. Colonies of non-fecal streptococci are typically very small, but exhibit the characteristic red or pink coloration and would be counted as fecal streptococci in the membrane filter method. In case of doubt, identity of material from suspected colonies may be confirmed according to this procedure.

The fecal streptococcal bacteria are distinguished from other bacteria by having the following three characteristics: (1) they lack the enzyme catalase; (2) they can grow at 45°C; (3) they grow in 40 percent bile. The confirmation procedure uses these three characteristics as criteria for identification. The procedure is similar to that given by the American Public Health Association and others (1976, p. 945).

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°C and in the presence of 40 percent bile. Absence of catalase, growth at 45°C and in 40 percent bile broth constitute a positive test for fecal streptococci. Presence of catalase or failure to grow at 45°C or in bile broth 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 below.

4. Apparatus

4.1 *Inoculating loop,* platinum-iridium wire, 3mm, Brown and Sharpe gage 26, A. H. Thomas Co. (7012-E20) or equivalent.

4.2 *Bunsen burner,* for sterilizing inoculating loops.

4.3 *Culture tubes,* flint glass, 16×150 mm, Kimble (73500) or equivalent and *test tube caps,* 16 mm, Scientific Apparatus (9468) or equivalent.

4.4 *Culture tube rack,* for 16-mm tubes, Thomas-Kolmer or equivalent.

4.5 *Incubator,* capable of maintaining temperature of 35°–45°±0.5°C, National Appliance (320) or equivalent.

4.6 *Sterilizer,* steam autoclave, Market Forge Sterilmatic or equivalent.

4.7 *Microscope slides,* glass, 76×25 mm.

5. Reagents

5.1 *Brain-heart infusion agar:* Add 52.0 g of brain-heart infusion agar, Difco (0418) or equivalent, to 1,000 ml of distilled water. Heat with vigorous stirring until solution becomes clear. Remove from heat immediately upon clearing. Place 5 ml of hot solution in each of about 12 16×150 mm 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 per 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. 7). Allow to cool until solid.

5.2 *Brain-heart infusion broth:* Add 37 g of brain-heart infusion, Difco (0037) or equivalent, to 1,000 ml of distilled water. Stir until dissolved. Place 6 ml of broth in each of another 12 16×150 mm tubes. Cap each tube. Sterilize at 121°C at 1.05 kg per cm^2 (15 psi) for 15 minutes.

5.3 *Brain-heart infusion-40 percent bile broth:* Add 37 g brain-heart infusion to 1,000 ml of water. Stir until dissolved. Place 6 ml of brain-heart infusion broth in each of another 12 16×150 mm stainless steel

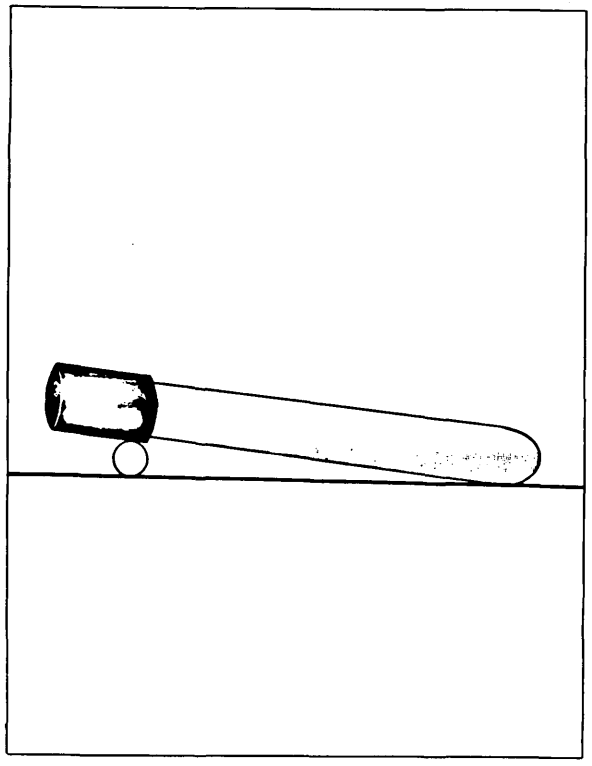

Figure 7—Preparation of agar slant.

capped culture tubes. Sterilize at 121°C at 1.05 kg per cm^2 (15 psi) for 15 minutes.

Add 100 g of oxgall, Difco (0128) or equivalent, to 1,000 ml of water. Stir until dissolved. Place 4 ml of 10 percent oxgall solution in each of another 12 16×150 mm stainless steel capped culture tubes. Sterilize at 121°C at 1.05 kg per cm^2 (15 psi) for 15 minutes.

Remove the 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 *Hydrogen peroxide solution,* 3 percent.

5.5 *Potassium iodide,* crystals.

6. Collection

No sample collections are necessary

7. Analysis

7.1 The membrane filter method for fecal streptococcal bacteria should be conducted.

7.2 From the incubated membrane filter select a colony or colonies to be confirmed for fecal streptococcal bacteria.

7.3 Sterilize the inoculating loop by flaming in the burner. The long axis of the wire should be held parallel to the cone of the flame so that the entire end of the wire and loop are heated to redness. Remove from flame and allow the wire to cool for about 10 seconds. Do not allow the loop to contact any foreign surface during the cooling period. When cool, touch the loop lightly to a single colony. Part of the colony material will adhere to the wire.

7.4 Uncap a brain-heart infusion agar slant and hold it at an angle of about 45° with the flat surface of the slant upward (fig. 8). Insert the loop with colony material into the tube. Starting at the base of the slant, lightly rub the loop against the agar working toward the top in a zig-zag pattern (fig. 8).

7.5 Recap the tube. Flame the loop and inoculate additional tubes as above until all colonies to be tested have been placed on agar in separate tubes. Place the inoculated tubes in the test tube rack and incubate at 35°±0.5 °C for 24–48 hours.

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

7.7 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

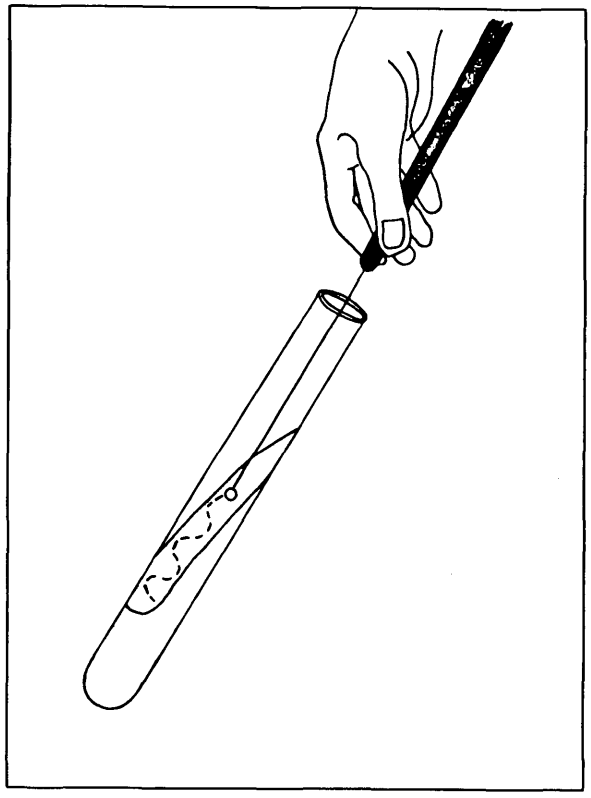

Figure 8—Method of streaking on an agar slant.

indicates that the hydrogen peroxide solution is acceptable for use. If otherwise, discard and obtain a fresh hydrogen peroxide solution.

7.8 Flame the loop and allow to cool. Immediately uncap a tube of brain-heart infusion agar having growth. Remove a loopful of growth from the culture 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 observe 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 confirmation should be continued. The presence of bubbles constitutes a positive catalase test indicating the presence of a non-streptococcal bacteria and the test may be terminated at this point.

7.9 Proceed as follows with all catalase negative cultures. Uncap one tube each of brain-heart infusion broth and brain-heart infusion-40 percent bile broth. Using a flamed loop, transfer one loopful of material from the 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.

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

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

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

7.13 Remove tubes from incubator and examine. Appearances of turbidity in the inoculated tubes as compared to the controls constitutes a positive test for growth.

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

7.14 All inoculated tubes and smeared slides should be autoclaved at 121°C at 1.05 kg per cm^2 (15 psi) for 15 minutes before discarding.

8. Calculations

No calculations are necessary.

9. Report

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

10. Precision

No precision data are available.

References

American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater (14th ed.): New York, Am. Public Health Assoc., 1193 p.

Sulfate-reducing bacteria (most probable number, MPN, method) (B–0400–77)

Parameter and code: Sulfate-reducing bacteria (MPN) 31855

1. Application

Sulfate-reducing bacteria are commonly found in environments where reducing conditions prevail such as ground waters, the hypolimnion of stratified lakes, saturated soils, and muds from lake bottoms and stream bottoms.Their implications for geochemistry have been discussed by Kuznetsov and others (1963). Many species of bacteria reduce sulfate during the synthesis of sulfur containing amino acids but only two genera of obligate anaerobic bacteria utilize sulfate reduction as a major energy yielding reaction with the production of hydrogen sulfide. These are *Desulfovibrio* and *Desulfotomaculum*.

The method described here is similar to the sulfate-reducing bacteria test given by the American Petroleum Institute (1965). The method is applicable for all waters including brines of high salt content.

2. Summary of method

Samples are collected and handled using techniques that minimize exposure to oxygen. Serial decimal dilutions are prepared. Several portions of each of at least three consecutive decimal dilutions are inoculated into suitable culture media. The tubes are incubated 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 resulting from the distribution of the test specimen and dilution thereof among a number of tubes of suitable culture medium.

Desulfovibrio sp. and *Desulfotomaculum nigricans* can be 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 taken as a positive response to the presence of sulfate-reducing bacteria.

3. Interferences

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.

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 only traces of oxygen will render the medium unsuitable.

4. Apparatus

All materials used in bacteriological testing must be free of agents which inhibit bacterial growth.

4.1 *Water-sampling bottle or coring apparatus.* As appropriate follow guidelines given in section 6.

4.2 *Hypodermic syringes.* 2.5-ml capacity equipped with 20 gauge×38 mm (1½ in.) needle (Becton Dickinson No. 5618 or equivalent).

4.3 *Cotton balls.* Obtain from local pharmacy.

4.4 *Pipets.* 1.0-ml capacity, presterilized, disposable, glass or plastic with cotton plugs, Falcon (7506) or equivalent (optional procedure only).

4.5 *Pipets.* 10-ml capacity, presterilized disposable, glass or plastic with cotton plugs, Falcon (7551) or equivalent (optional procedure only).

4.6 *Test tubes.* 16×100 mm, glass disposable, Kimble (73500) or equivalent (optional procedure only).

4.7 *Test tube caps.* 16 mm, Bacti Capall or equivalent (optional procedure only).

4.8 *Sterilizer.* Steam autoclave, Curtin Matheson Scientific (209-536) or equivalent (optional procedure only).

4.9 *Anaerobic incubator.* BBL (60465) or equivalent (optional procedure only).

4.10 *Culture tube rack.* For 16-mm test tubes, Thomas-Kolmer or equivalent (optional procedure only).

4.11 *Bottles.* Milk dilution, APHA, Pyrex or Kimax with screwcaps (optional procedure only).

4.12 *Propipet* for use with 1.0- and 10-ml pipets.

5. Reagents

5.1 *Sulfate API broth.* Ready to use presterilized media packed in 10-ml serum bottles such as Difco (0500-86-2) or equivalent, are recommended. If field preparation of medium is opted, add 14.5 g of Sulfate API broth, Difco (0500) or equivalent to 1,000 ml of distilled water and warm gently to dissolve. Place 6 ml of Sulfate API broth in 16×100 mm test tubes and cap. Sterilize at 121°C for 10 minutes at 1.05 kg per cm^2 (15 psi).

5.2 *70 percent ethanol.* Dilute 74 ml of 95 percent ethyl alcohol to 100 ml with distilled water or undiluted isopropanol (ordinary rubbing alcohol).

5.3 *Anaerobic gas charges.* Disposable, BBL (70304) or equivalent (optional procedure only).

5.4 *Catalyst replacement pellets for anaerobic jar.* BBL (70303) or equivalent (optional procedure only).

5.5 *Buffered dilution water.* Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 ml distilled water. Adjust to pH 7.2 with 1 *N* sodium hydroxide (NaOH). Dilute to 1 liter with distilled water. Sterilize in dilution bottles for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). After opening a bottle of stock solution, refrigerate the unused part. Discard contaminated solutions, indicated by slight turbidity or precipitate. Add 1.2 ml of this stock phosphate buffer solution to 1 liter of distilled water containing 0.1 percent Difco peptone (0118), or equivalent. Dispense in milk dilution bottles in amounts that will provide 99 ml±2.0 after autoclaving at 121°C at 1.05 kg per cm^2 (15 psi) for 20–30 minutes. Loosen caps or stoppers prior to sterilizing and tighten when bottles have cooled.

6. Collection

Samples for bacteriologic examination should be collected in clean, sterile containers. The sample should be taken in such a manner as to preclude contamination from external sources. Maintaining the integrity of a specimen taken from a reduced environment is a difficult task. Many types of specialized devices for obtaining liquid and solid samples from reduced environments have been described.

6.1 *Water sampling.* Two techniques for sampling water may be distinguished. In the subsurface sampling technique a bottom-hole sampler is lowered down a well or into a body of water to a preselected depth and a sample of the fluid at that depth is trapped in a pressure tight section of the sampler. The sampler is brought to the surface where the sample is processed. The surface-sampling technique consists of taking samples of liquid from flowing or pumped wells. The choice of technique is influenced by the type of reservoir fluid, the producing characteristics and mechanical conditions of the well, and the presence or absence of mechanical equipment in the well. Instructions for collecting samples from closed systems are given by the American Society for Testing and Materials (1966) and American Petroleum Institute (1966). Often a satisfactory sample from a flowing well may be obtained by attaching a piece of gumrubber tubing to a tap and allowing the stream of water to move upward through the tubing. After about 5 minutes sterilize a section of tubing near the base by swabbing with a piece of cotton moistened with 70 percent ethanol solution or isopropanol. Puncture the tubing with a hypodermic needle and draw a sample of water into a syringe. Inject this part of water into a sealed serum bottle containing properly reduced medium.

6.2 *Sediment sampling.* Sediment sampling devices suitable for use in anaerobic environments are available. The simplest device, applicable in soft muds and mucks, consists of a length of thin-wall plastic or metal tubing. This is pushed into the soil to the desired depth and the open end is then tightly stoppered with a rubber stopper. The entire assembly is then 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., 2400) may be required. With either instrument, fine grained material may be sampled by inserting large bore hypodermic needles or cannulae through holes drilled through the side of the coring tube. These samples may be treated in the same way as water samples.

7. Analysis

Two factors must be decided when planning a multiple-tube test:

1. What volumes of water should be tested?

2. How many tubes of each volume should be tested?

Choose a range of volumes so that both positive and negative results are obtained over the range tested. The method fails if only positive or only negative results are obtained with all volumes tested. The number of 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 are also increased. For general use the three tube series is recommended and is described below. Order-of-magnitude estimates can be made with a one-tube series. Increased precision can be obtained using a five-tube series. Sulfate reducing bacteria may be cultivated using either 9-ml presterilized medium in vials and hypodermic syringe or in ordinary test tubes using field prepared API sulfate broth. The presterilized medium procedure requires less equipment and operator time, so is recommended for most studies. However, the procedure using field prepared medium is more economical if large numbers of samples are anticipated, and uses equipment more easily obtained locally. Soil can be tested by the latter procedure also. For water samples use volumes of 1, 0.1, 0.01, 0.001 and 0.0001 ml. For soil samples use dilutions of 10^{-2} to 10^{-6}.

Procedure using presterilized medium and hypodermic syringes.

7.1 Remove the inserts from the metal caps and swab the exposed part of the rubber septa with a bit of cotton saturated with 70 percent ethanol or isopropanol.

7.2 Using a sterile, disposable 2.5 ml syringe equipped with a sterile 20 gauge needle 38 mm (1½ in.) in length obtain 1 ml of sample.

7.3 Invert a serum bottle so that the rubber septum is at the bottom. Inoculate the medium by carefully puncturing the septum with the needle and inserting the needle only until the beveled tip is inside the bottle. Discharge the contents of the syringe into the bottle and withdraw the syringe. Agitate the bottle vigorously.

7.4 Using a new sterile syringe withdraw 1.0 ml from the previously inoculated bottle and then inoculate a fresh serum bottle as in 7.3.

7.5 In order 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 parts of a given water sample.

Step 1 Lay out 10 bottles of culture medium.
Step 2 Prepare them as in 7.1.
Step 3 Obtain 1 ml of sample as in 7.2 and inoculate one bottle of medium as in 7.3.
Step 4 Using the 10^{-1} dilution prepared in step 3 inoculate three fresh bottles of culture medium as in 7.4.
Step 5 Using one of the 10^{-2} dilutions prepared in step 4 inoculate 3 fresh bottles of culture medium as in 7.4.
Step 6 Using one of the 10^{-3} dilutions prepared in step 5 inoculate 3 fresh bottles of culture medium as in 7.4.

Similar protocols can be established for other combinations using any number of tubes per dilution level.

7.6 Clearly mark each inoculated serum bottle indicating location, time of collection, sample number, and sample volume. Code each bottle, for easy identification when recording results.

7.7 Incubate tubes at room temperature (18°–25°C) for 28 days. Tubes which turn black within 2 hours are not to be considered positive since this will probably be due to the presence of sulfide ion in the sample. Subcultures of these false positives may be made after 1 week as in 7.1–7.3.

7.8 Examine tubes after 28 days. Record as positive all tubes which have significant amounts of black precipitate. Upon shaking, the tubes should assume an inky black appearance. Record as negative turbid tubes which are only slightly grayish.

Optional procedure using field prepared API sulfate broth.

7.9 Set up a suite of 1, 3, or 5 tubes of Sulfate API broth for each sample volume to be tested.

7.10 Inoculate the culture medium using either 0.1 or 1.0 ml of an appropriate sample volume, or a weighed aliquot of soil (such as 1.0 g). Dilutions of the original sample are necessary when inoculation of 0.1 ml (g) or less is anticipated. The dilution scheme shown below may be used in these cases.

Dilution	Volume of sample added to 99 ml dilution bottle	Size of inoculum
1:10	----------	0.1 ml or 0.1g of original sample
1:100	1.0 ml of original sample (or 1.0g)	1.0 ml of 1:100 dilution
1:1,000	----------	0.1 ml of 1:100 dilution
1:10,000	1.0 ml of 1:100 dilution	1.0 ml of 1:10,000 dilution
1:100,000	----------	0.1 ml of 1:10,000 dilution

7.11 Mark each inoculated tube indicating sample location, date, and sample volume.

7.12 Place inoculated tubes upright in an anaerobic incubator at room temperature (18°–25°C) for 28 days. Mason jars or other large tight sealing glass jars may be used in place of an anaerobic incubator if disposable gas generator envelopes such as BBL 70308 and replaceable catalyst charges BBL 70303 are used. Throwaway gas generators enable the user to use any tight sealing container as an anaerobic incubator.

7.13 Inoculated tubes are observed for growth in the same manner as in 7.7 and 7.8.

7.14 Autoclave all cultures at 121°C for 15 minutes at 1.05 kg per cm^2 before discarding.

8. Calculation

Record the number of positive results from the inoculated tubes and select the three dilutions for the MPN index using the following rule (American Public Health Association and others, 1976). Take as the first number the least concentrated (smallest sample volume) dilution in which all tests are positive and the two next succeeding higher dilutions. Using this sequence of three numbers refer to table 2 or 3 for MPN indices for 3 and 5 tube multiple-tube series as appropriate. Confidence limits at the 95 percent level are also given.

If only one tube is inoculated at each decimal dilution level record the highest dilution showing a positive response as compared to the lowest dilution showing a negative response. Record the results as a range of numbers, for example 100–1,000 sulfate-reducing bacteria per ml. If all tubes are positive record the result as a number greater than that indicated by the value of the lowest 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.

8.1 The results of a test were recorded as follows:

Volume (ml)	Tube number 1	2	3	Result
0.1	+	+	+	3/3
0.01	+	+	+	3/3
0.001	+	+	-	2/3
0.0001	-	-	-	0/3

Table 2.—MPN index and 95 percent confidence limits for various combinations of positive and negative results when three 1.0-ml, three 0.1-ml, and three 0.01-ml dilutions are used

[From: American Public Health Association and others, 1976]

Number of tubes giving positive reaction out of: 3 of 1 ml each	3 of 0.1 ml each	3 of 0.01 ml each	MPN Index per ml	95 percent confidence limits 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		

Following the rule given above and selecting 0.01, 0.001, and 0.0001 sample volumes, a sequence of 3–2–0 is found. From this an MPN of 9.3 is found in table 2. Dividing by 10^{-2} to correct for the effect of dilution the MPN of the sample is found to be 930 sulfate-reducing organisms per milliliter. The 95 percent confidence limits are 150 and 3,800.

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

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

Selecting 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 11.0. Dividing by 10^{-6} the MPN is computed to be 11×10^{6} sulfate-reducing bacteria per milliliter with 95 percent confidence limits of 3.1×10^{6} and 25×10^{6} sulfate-reducing bacteria per milliliter.

8.3 The following results were observed with a three-tube series:

Volume (ml)	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 with confidence limits of 0 and 1.5.

The various combinations recorded in tables 2 and 3 represent those most likely to be observed. Other combinations are statistically unlikely. If unlikely combinations are observed it is probable either that the multiple-tube technique is inapplicable, or that errors of manipulation have occurred.

9. Report

For one-tube series the data are reported as a range of numbers.

For multiple-tube tests report results as MPN of sulfate-reducing bacteria per milliliter for water sam-

Table 3.—MPN index and 95 percent confidence limits for various combinations of positive and negative results when five 1.0-ml, five 0.1-ml, and five 0.01-ml dilutions are used

[From: American Public Health Association and others, 1976]

Number of tubes giving positive reaction out of:			MPN Index per ml	95 percent confidence limits		Number of tubes giving positive reaction out of:			MPN Index per ml	95 percent confidence limits	
5 of 1 ml each	5 of 0.1 ml each	5 of 0.01 ml each		Lower	Upper	5 of 1 ml each	5 of 0.1 ml each	5 of 0.01 ml each		Lower	Upper
0	0	0	< 0.2								
0	0	1	.2	< 0.05	0.7	4	2	1	2.6	0.9	7.8
0	1	0	.2	< .05	.7	.4	3	0	2.7	.9	8.0
0	2	0	.4	< .05	1.1	4	3	1	3.3	1.1	9.3
						4	4	0	3.4	1.2	9.3
1	0	0	.2	< 0.05	.7						
1	0	1	.4	< .05	1.1	5	0	0	2.3	.7	7.0
1	1	0	.4	< .05	1.1	5	0	1	3.1	1.1	8.9
1	1	1	.6	< .05	1.5	5	0	2	4.3	1.5	11.0
1	2	0	.6	< .05	1.5	5	1	0	3.3	1.1	9.3
						5	1	1	4.6	1.6	12.0
2	0	0	.5	< .05	1.3	5	1	2	6.3	2.1	15.0
2	0	1	.7	.1	1.7						
2	1	0	.7	.1	1.7	5	2	0	4.9	1.7	13.0
2	1	1	.9	.2	2.1	5	2	1	7.0	2.3	17.0
2	1	1	.9	.2	2.1	5	2	2	9.4	2.8	22.0
2	2	0	.9	.2	2.1	5	3	0	7.9	2.5	19.0
2	3	0	1.2	.3	2.8	5	3	1	11.0	3.1	25.0
3	0	0	.8	.1	1.9	5	3	2	14.0	3.7	34.0
3	0	1	1.1	.2	2.5	5	3	3	18.0	4.4	50.0
3	1	0	1.1	.2	2.5	5	4	0	13.0	3.5	30.0
3	1	1	1.4	.4	3.4	5	4	1	17.0	4.3	49.0
3	2	0	1.4	.4	3.4	5	4	2	22.0	5.7	70.0
3	2	1	1.7	.5	4.6	5	4	3	28.0	9.0	85.0
3	3	0	1.7	.5	4.6	5	4	4	35.0	12.0	100.0
4	0	0	1.3	.3	3.1	5	5	0	24.0	6.8	75.0
4	0	1	1.7	.5	4.6	5	5	1	35.0	12.0	100.0
4	1	0	1.7	.5	4.6	5	5	2	54.0	18.0	140.0
4	1	1	2.1	.7	6.3	5	5	3	92.0	30.0	320.0
4	1	2	2.6	.9	7.8	5	5	4	160.0	64.0	580.0
4	2	0	2.2	.7	6.7	5	5	5	⩾ 240.0		

ples or as MPN per g for soil samples. The method of reckoning unit weight (wet, dry and so forth) of soil samples should also be reported. Report values less than 10 with one significant figure and other values with two significant figures.

10. Precision

The MPN inherently has a low order of precision. For precise estimates, large number of tubes must be inoculated. Precision increases rapidly as the number of tubes is increased from 1 to 5, but then increases at a much less rapid rate so that the gain in using 10 tubes instead of 5 is 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 10-fold dilution series is given below:

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

Tables 2 and 3 show the 95 percent confidence limits for various combinations of numbers of tubes and results.

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Nitrifying Bacteria (most probable number, MPN, method) (B–0420–77)

Parameter and code: Nitrifying bacteria (MPN) 31854

1. Application

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 occurs in two distinct steps, each mediated by a specific group of bacteria. The *Nitrosomonas* group, which includes several genera of bacteria, is able to oxidize ammonium (NH_4^+) only to nitrite (NO_2^-) as shown:

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

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

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

Hydrogen ions produced in the oxidation of ammonium to nitrite may be of some geochemical significance because the excess acid can dissolve minerals and participate in exchange reactions on clays. Nitrification is important in soils because the process controls the supply of nitrate used by higher plants. In surface waters, nitrification contributes to oxygen demand.

The responsible organisms, *Nitrosomonas* and *Nitrobacter*, are autotrophic bacteria. They obtain their energy from the inorganic oxidations indicated above and use carbon dioxide as a cellular carbon source. The media for enumerating these bacteria are assumed to be free of organic carbon. This assumption is valid to the extent that initially only nitrifiers will grow on the media. Later, as the autotrophs grow and release cell substances to the media, heterotrophs will develop.

The medium for enumerating *Nitrosomonas* contains NH_4^+. Appearance of NO_2^- in the inoculated cultures, but not in 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 enumerating *Nitrobacter* contains NO_2^-; disappearance of NO_2^- from the inoculated cultures, but not from control cultures, presumptively indicates the presence of *Nitrobacter*. The method described is similar to that described by Alexander and Clark (1965) and is applicable to all types of fresh and saline waters and soils.

2. Summary of method

Decimal dilutions of multiple sample aliquots are inoculated into organic-free media containing ammonium ions for *Nitrosomonas* enumeration or nitrite ions for *Nitrobacter* enumeration. The inoculated cultures are incubated at 28°C for 3 weeks, following which the inoculated cultures and control cultures are tested for the presence of nitrite. The most-probable-number (MPN) of each group of nitrifying bacteria is determined from the distribution of positive and negative responses among the inoculated tubes.

3. Interferences

No interferences are known for the procedure.

4. Apparatus

All materials used in bacteriological testing must be free of agents which inhibit bacterial growth.

4.1 *Water-sampling bottle*, samplers for obtaining water samples under sterile conditions are marketed by General Oceanic, Inc., Hydro Products, Kahl Scientific Instrument Corp., and others. A metallic water sampler, lowered at a speed of 1 m/s, may be effective for sterile collection of water samples (Kriss and others, 1966). Metallic water-sampling bottles are

available from Wildlife Supply Co. (1050 or 1200); Kahl Scientific Instrument Corp. (130WA100); Inter Ocean Systems, Inc. (206); Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type); or equivalent.

4.2 *Culture tubes and caps,* flint glass tubes, 16×125 mm, Kimble (73500), Corning (9805), or equivalent; tube caps, 16 mm, Scientific Products (T1390-16) or equivalent.

4.3 *Culture tube rack,* for 16-mm tubes, Thomas-Kolmer or equivalent.

4.4 *Incubator* with temperature range from 5°C above ambient to 60°C. National Appliance (320) or equivalent, or water bath capable of maintaining a temperature of 28±1°C, Matheson (65310-10) or equivalent.

4.5 *Sterilizer,* steam autoclave, Curtin Matheson Scientific (209-536), Market Forge Sterilmatic, or equivalent.

4.6 *Bottles,* milk dilution, APHA, Pyrex or Kimax with screwcaps.

4.7 *Glass beads,* solid, 3mm, Fisher Scientific (11-312A) or equivalent.

4.8 *Sieve,* 10 mesh, Fisher (408816) or equivalent.

4.9 *Pipets,* 1.0-ml capacity, presterilized, disposable, glass or plastic with cotton plugs, Millipore (XX63 001 35) or equivalent.

4.10 *Pipets,* 11.0-ml capacity, Corning (7057) or equivalent. Wrap the pipets in kraft paper and sterilize in the autoclave, or place in pipet box, Curtin Matheson Scientific or equivalent, and heat in an oven at 170°C for 2 hours. Presterile, disposable, 10.0-ml pipets may be used.

4.11 *Propipet* for use with 1.0-, 10.0-, and 11.0-ml pipets.

5. Reagents

5.1 *Ammonium-calcium carbonate medium* for most-probable-number (MPN) of *Nitrosomonas.* To 1,000 ml of distilled water, add 0.5 g of ammonium sulfate [$(NH_4)_2SO_4$], 1.0 g of potassium phosphate dibasic (K_2HPO_4), 0.03 g of ferrous sulfate ($FeSO_4 \cdot 7H_2O$), 0.3 g of sodium chloride (NaCl), 0.3 g of 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 per cm^2 (15 psi) for 15 minutes.

5.2 *Nitrite-calcium carbonate medium* for most-probable-number (MPN) of *Nitrobacter.* To 1,000 ml of distilled water, add 0.006 g of potassium nitrite (KNO_2), 1.0 g of potassium phosphate dibasic (K_2HPO_4), 0.3 g of sodium chloride (NaCl), 0.1 g of magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 1.0 g of calcium carbonate ($CaCO_3$), and 0.3 g of calcium chloride ($CaCl_2$). Place 3 ml of medium in each culture tube, cap, and autoclave at 121°C at 1.05 kg per cm^2 (15 psi) for 15 minutes.

5.3 *Griess-Ilosvay reagent:* (a)Dissolve 0.6 g sulfanilic acid in 70 ml hot (90+°C) distilled water; cool the solution, add 20 ml of concentrated hydrochloric acid (HCl), dilute the mixture to 100 ml with distilled water, and mix; (b) dissolve 0.6 g of alpha naphthylamine in 10 to 20 ml of distilled water containing 1 ml of concentrated hydrochloric acid (HCl); dilute to 100 ml with distilled water and mix; and (c) dissolve 16.4 g of sodium acetate ($CH_3COONa \cdot 3H_2O$) in distilled water; dilute to 100 ml with distilled water and mix. Store the solutions separately in dark bottles in a refrigerator. Stability of the solutions is unknown; however, storage should not exceed 1 month.

5.4 *Zinc-copper-manganese dioxide mixture:* Mix together 1 g of powdered zinc metal (Zn), 1 g of powdered manganese dioxide (MnO_2), and 0.1 g of powdered copper (Cu).

5.5 *Buffered dilution water:* Dissolve 34.0 g potassium dihydrogen phosphate (KH_2PO_4) in 500 ml distilled water. Adjust to pH 7.2 with 1 *N* sodium hydroxide (NaOH). Dilute to 1 liter with distilled water. Sterilize in dilution bottles at 121°C at 1.05 kg per cm^2 (15 psi) for 20 minutes. After opening a bottle of stock solution, refrigerate the unused part. Discard contaminated solution, indicated by slight turbidity or precipitate accumulation. For water sample dilution blanks, add 1.2 ml of sterile stock phosphate buffer solution to 1 liter of distilled water containing 0.1 percent Difco peptone (0118), or equivalent. Dispense in milk dilution bottles in amounts that will provide 99 ml±2 after autoclaving at 121°C at 1.05 kg per cm^2 (15 psi) for 20 minutes. Loosen caps prior to sterilizing and tighten when bottles have cooled.

For soil sample dilution blanks, place 95 ml of distilled water and about three dozen, 3-mm diameter, glass beads in a milk dilution bottle. For each 95 ml dilution blank, prepare also 5 dilution blanks of 90 ml distilled water in milk dilution bottles. Omit the glass beads from the 90 ml blanks. Autoclave at 121°C at 1.05 kg per cm^2 (15 psi) for 20 minutes. Loosen caps prior to sterilizing and tighten when bottles have cooled.

6. Collection

Water samples for bacteriologic examination must be collected in containers that have been sterilized in

an autoclave for 20 minutes at 121°C at 1.05 kg per cm^2 (15 psi). Sterilized milk dilution bottles are ideal sample containers. When the sample is collected, ample air space must be left in the bottle to facilitate mixing of the sample by shaking. Care must be taken to avoid contamination of the sample and sample bottle at the time of collection and in the period prior to analysis.

To insure maximum correlation of results, the sample sites and methods used for nitrifying bacteria should correspond to those selected for chemical and other biological sampling. Sampling for bacteria at depth is complicated by the requirement to avoid contamination of the deeper water layers by bacteria carried from shallower depths on the inner walls of the sampler.

The sample collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, bacterial abundance may vary transversely, with depth, and with time of day. To collect a surface sample from a stream or lake, open a sterile milk dilution bottle, grasp it near its base, and plunge it, neck downward, below the water surface. Allow the bottle to fill slowly turning 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 the case of a lake, a current should be artificially created by pushing the bottle horizontally forward in a direction away from the hand (American Public Health Association and others, 1976, p. 905). To collect a sample representative of the bacterial concentration at a particular depth, use one of the water sampling bottles discussed in 4.1 above. For small streams, a point sample at a single transverse position located at the centroid of flow may be adequate (Goerlitz and Brown, 1972).

As soon as possible after collection, preferably within 4 hours and not more than 6 hours, inoculate the decimal dilutions of the sample into tubes of ammonium-calcium carbonate medium and nitrite-calcium carbonate medium. Samples must be kept cool during the time between collection and inoculation. If inoculation is delayed, chill or refrigerate the sample but do not freeze.

Collect soil samples in a sterile manner and place in polyethylene bags or waxed cardboard containers. 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 1-2 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 and mix thoroughly before taking an aliquot for analysis. If desired, a separate subsample may be taken for determination of dry weight (Clark, 1965).

The sizes of inoculums should be such that, after incubation, both positive and negative results are obtained. The method fails if only positive or only negative results are obtained with all volumes tested. The following sample 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}.

7. Analysis

7.1 Before starting the analysis, clear an area of the laboratory bench and swab it with a bit of cotton moistened with 70 percent ethyl alcohol or undiluted isopropanol.

7.2 Set out four tubes of ammonium-calcium carbonate medium and four tubes of nitrite-calcium carbonate medium for each volume to be tested. Three of the tubes will be inoculated with a decimal dilution; the fourth tube will be a control tube.

7.3 If the volume of water sample to be tested is greater than 0.1 ml, transfer the measured samples directly to the culture tubes using sterile pipets. Take care when removing caps from sterile culture tubes so as to avoid contamination.

When testing water, if the volume of the desired sample aliquot is less than 0.1 ml, proceed as above after preparing appropriate dilutions by adding the sample to a sterile milk dilution bottle in the following amounts:

Dilution	Volume of sample added to 99 ml dilution bottle	Size of inoculum
1:100	1.0 ml original sample	1.0 ml of 1:100 dilution
1:1,000		0.1 ml of 1:100 dilution
$1:10^4$	1.0 ml 1:100 dilution	1.0 ml of $1:10^4$ dilution
$1:10^5$		0.1 ml of $1:10^4$ dilution
$1:10^6$	1.0 ml $1:10^4$ dilution	1.0 ml of $1:10^6$ dilution
$1:10^7$		0.1 ml of $1:10^6$ dilution

Note: Use a sterile pipet for each bottle. After each transfer between bottles, close and shake the bottle vigorously 25 times. Diluted samples should be inoculated within 20 minutes after preparation.

To prepare a decimal dilution series of a soil sample, proceed by transferring 10 g of moist soil to a sterile water blank containing 95 ml of water and glass beads. Cap the bottle tightly and shake vigorously 25 times. Immediately after shaking, transfer 10 ml from the center of the suspension to a sterile 90 ml water blank. Shake vigorously 25 times and continue the dilutions

until a sufficiently dilute sample is obtained. Using this dilution series proceed until all tubes are inoculated.

7.4 Clearly mark each set of inoculated tubes and uninoculated control tubes indicating location, time of collection, sample number, and sample volume. Code each tube for easy identification when recording results.

7.5 Place the inoculated tubes and control tubes in a rack and incubate at 28°C for 21 days. Clearly defined results will occur *only* if the bacteria consume *all* the nitrite (or convert all ammonium to nitrite). For this reason incubation should always proceed for 21 days.

7.6 After incubation, test each inoculated tube and control tube for nitrite using the Griess-Ilosvay reagent. Immediately prior to the test, mix together in equal parts the sulfanilic acid reagent, the alpha naphthylamine reagent, and the sodium acetate reagent. Add one drop *only* of this mixture to each tube. Observe the contents of each tube for the development of a reddish color within 5 minutes.

7.7 Growth of *Nitrosomonas* is usually evidenced by a brick red color at the bottom of a tube and a purplish-red coloration in the overlying liquid. Control tubes and inoculated tubes without nitrite may turn faintly pink; thus it is imperative that uninoculated control tubes be used in color comparison.

7.8 To all tubes of ammonium-calcium carbonate medium *(Nitrosomonas)* that do not develop a purplish red color within 5 minutes, add a small pinch of the Zn-Cu-MnO_2 mixture. If a reddish color develops, record the tube as positive for *Nitrosomonas* on the basis that the initial negative reading for nitrite indicated that the nitrite found by *Nitrosomonas* was oxidized to nitrate by *Nitrobacter*.

7.9 Record as positive for *Nitrobacter* all tubes of nitrite-calcium carbonate medium that *do not* develop the characteristic purplish red color formed by the reaction of nitrite with the Griess-Ilosvay reagent.

7.10 A positive result in a control tube indicates a contamination of the medium and results of the test, therefore, are invalid.

7.11 Autoclave all cultures at 121°C for 15 min at 1.05 kg per cm^2 (15 psi) before discarding.

8. Calculations

Record the number of positive inoculated tubes occurring over all sample volumes tested. When more than three volumes are tested, the results from only three of these are used in computing the MPN. To select the three dilutions for the MPN index, proceed as follows: Take as the first member the smallest sample volume in which all tests are positive (no larger sample volume giving any negative results) and the two next succeeding smaller sample volumes (American Public Health Association and others, 1976, p. 923-926).

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

Table 4—MPN index and 95 percent confidence limits for various combinations of positive and negative results when three 10-ml, three 1-ml, and three 0.1-ml dilutions are used [American Public Health Association and others, 1976, p. 924]

Number of tubes giving positive reaction out of:			MPN index per 100 ml	95% confidence limits	
3 of 10 ml each	3 of 1 ml each	3 of 0.1 ml each		Lower	Lower
0	0	0	<3	--	--
0	0	1	3	<0.5	9
0	1	0	3	<0.5	13
1	0	0	4	<0.5	20
1	0	1	7	1	21
1	1	0	7	1	23
1	1	1	11	3	36
1	2	0	11	3	36
2	0	0	9	1	36
2	0	1	14	3	37
2	1	0	15	3	44
2	1	1	20	7	89
2	2	0	21	4	47
2	2	1	28	10	150
3	0	0	23	4	120
3	0	1	39	7	130
3	0	2	64	15	380
3	1	0	43	7	210
3	1	1	75	14	230
3	1	2	120	30	380
3	2	0	93	15	380
3	2	1	150	30	440
3	2	2	210	35	470
3	3	0	240	36	1,300
3	3	1	460	71	2,400
3	3	2	≥1,100	150	4,800
3	3	3	>2,400	--	--

Example	1 ml	Decimal dilutions 0.1 ml	0.01 ml	0.001 ml	Combination of positives
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	

In b the three dilutions should be chosen to place the positive result in the middle dilution. When a positive result occurs in a dilution higher than the three chosen according to the rule, as in c, it should be placed in the result for the highest chosen dilution as in d.

A table giving MPN for various combinations of positive and negative results when three 10.0-ml, three 1.0-ml, and three 0.1-ml dilutions are used is given in table 4. If a series of decimal dilutions other than 1.0, 0.1, and 0.01 ml is used, record the MPN as the value from the table multiplied by a factor of 10 divided by the volume in which all tests were positive. MPN tables for other combinations of sample volumes and number of tubes are given by the American Public Health Association and others (1976, p. 924-926).

9. Report

The concentration of nitrifying bacteria is reported as MPN *Nitrosomonas* and MPN *Nitrobacter* per 100 ml for water samples or as MPN per 100 g for soil samples. The method of expressing unit weight (wet or dry) of soil samples should be indicated. Values less than 10, report whole numbers; 10 or more, report two significant figures.

10. Precision

The MPN inherently has a low order of precision. Precision increases as the number of tubes is increased. It increases rapidly as the number of tubes increases from 1 to 5 but then increases at a slower rate so that the gain in using 10 tubes instead of 5 is 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 decimal dilution series is given below.

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

Table 4 shows the 95 percent confidence limits for various combinations of positive and negative results, when three 10-ml, three 1-ml, and three 0.1-ml dilutions are used.

References

Alexander, Martin, and Clark, F. E., 1965, Nitrifying bacteria, *in* Blach, C. A., ed., Methods of soil analysis: Madison, Wisc., Am. Soc. of Agronomy, part 2, p. 1477–1483.

American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater (14th ed): New York, Am. Public Health Assoc., 1193 p.

Clark, F. E., 1965, Agar-plate method for total microbial count, *in* Blach, C. A., ed., Methods of soil analysis: Madison, Wisc., Am. Soc. of Agronomy, part 2, p. 1463.

Goerlitz, D. T. and Brown, Eugene, 1972, Methods for the analysis of organic substances in water: U.S. Geol Survey Techniques Water-Resources Inv., book 5, chap. A3, 40 p.

Kriss, A. E., Lebedeva, M. N., and Tsiban, A. V., 1966, Comparative estimate of a Nansen and microbiological water bottle for sterile collection of water samples from depths of seas and oceans: Deep-Sea Research, v. 13, p. 205–212.

Salmonella and *Shigella* (diatomaceous-earth and membrane filter method) (B–0100–77)

Parameter and code: Not applicable

1. Application

Pathogenic bacteria of the genera *Salmonella* and *Shigella may be isolated from water by similar methods. The genus Salmonella* comprises over 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 highly specific for a particular type. The members of the genus *Shigella* are all potentially pathogenic and are similar to *Salmonella* in many respects. *Shigella* causes acute bacillary 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 together with fecal coliforms. The pathogens in water form an extremely small part of the total bacterial population because of overwhelming numbers of coliforms. Geldreich (1970) reported isolation of *Salmonella* in less than 27.6 percent of freshwater samples when the fecal coliform concentration was under 200 colonies per 100 ml. *Salmonella* was isolated in 85.2 percent of water samples having fecal coliform densities between 200 and 2,000 colonies per 100 ml and was isolated in 98.1 percent of samples having fecal coliform densities exceeding 20,000 colonies per 100 ml.

Because of the low incidence of pathogenic bacteria in most waters, large volumes (several liters) 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.

The method is applicable for all fresh and estuarine waters. Very few reports of the occurrence of *Salmonella* and *Shigella* in marine environments are available except to indicate that sediments may be an important source.

2. Summary of method

Samples are collected in a sterile manner to avoid contamination, while minimizing exposure of field personnel to possible pathogens. Several liters of water are filtered through either diatomaceous earth or a membrane filter. The bacteria-laden membrane filter or diatomaceous earth is divided into parts for inoculation into suitable enrichment media. Selenite and tetrathionate broth media are recommended for all *Salmonella* and most *Shigella* determinations.

After incubation at 41.5°C, selective solid media plates are streaked at 24-hour intervals for up to 5 days. Colonies showing typical *Salmonella* or *Shigella* characteristics that appear on the selective media are purified and further classified by biochemical reactions. Several non-pathogenic organisms share some important biochemical characteristics with the *Salmonella-Shigella* group. For this reason, a large battery of differential biochemical tests is necessary for presumptive identification of the pathogenic Enterobacteriaceae. Identification cannot be considered confirmed until verified serologically. A diagramatic identification scheme is shown in Figure 9.

3. Interferences

The membrane-filter method may not work with waters having a large suspended-solids content. Additionally, many bacteria, other than *Salmonella* and

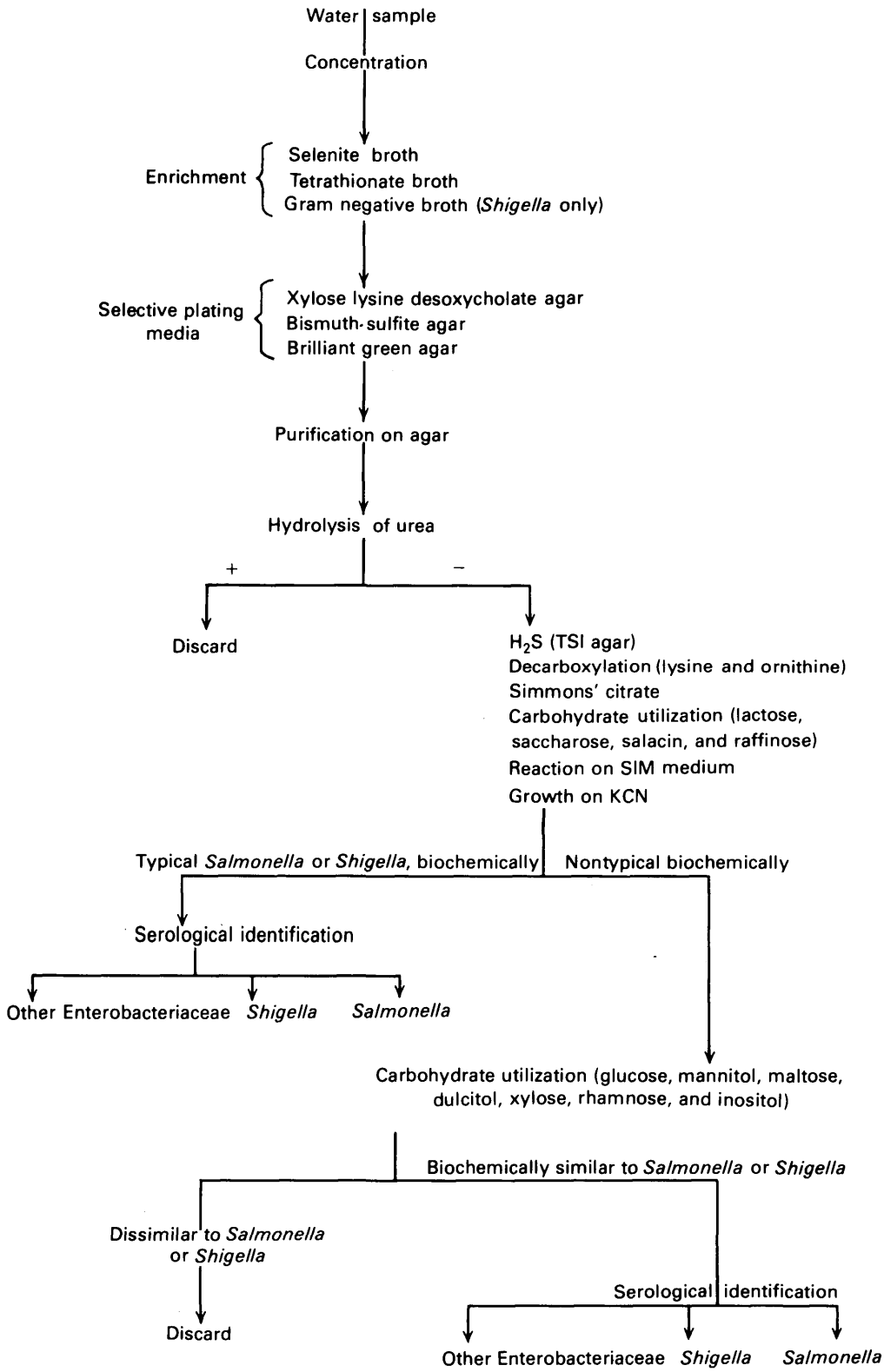


Figure 9—Identification scheme for *Salmonella* and *Shigella*.

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 bacteriological testing must be free of agents that inhibit bacterial growth.

4.1 *Water-sampling bottle:* Samplers for obtaining water samples under sterile conditions are marketed by General Oceanics, Inc., Hydro Products, Kahl Scientific Instrument Corp., and others. A metallic water sampler, lowered at a speed of 1 m/s, may be effective for sterile collection of water samples (Kriss and others, 1966). Metallic water-sampling bottles are available from Wildlife Supply Co. (1050 or 1200); Kahl Scientific Instrument Corp. (130WA100); InterOcean Systems, Inc. (206); Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type); or equivalent. An autoclavable 1-liter bottle, Nalgene 2006 or equivalent, is needed for sample storage.

4.2 *Filter-holder assembly*, Millipore (XX63 001 20) or equivalent, and syringe and two-way valve, Millipore (XX62 000 35) or equivalent.

4.3 *Membrane filters and absorbent pads*, white, grid, sterile packed, 0.45-μm mean pore size, 47-mm diameter, Millipore (HAWG 047 S1), Gelman (63068), or equivalent; Millipore (AP10 047 SO) or equivalent.

4.4 *Plastic petri dishes* with cover, disposable, sterile, 100×15 mm, GSA stock (6640-051-9495) or equivalent.

4.5 *Forceps*, stainless steel, smooth tips, Millipore (XX62 000 06) or equivalent.

4.6 *Incubator* for operation at a temperature of 35°C and 41.5°C. A portable incubator as provided in the Portable Water Laboratory, Millipore (XX63 000 00) or equivalent, which operates on either 110 volts a.c. or 12 volts d.c., is convenient for field use. A large incubator, with more precise temperature regulation, National Appliance (320) or equivalent, is satisfactory for laboratory use.

4.7 *Sterilizer*, steam autoclave, Curtin Matheson Scientific (209-536), Market Forge Sterilmatic, or equivalent.

4.8 *Bottles*, milk dilution, APHA, Pyrex or Kimax, with screwcaps.

4.9 *Laboratory balance*, Ohaus model 310 or equivalent, with sensitivity to 0.01 g.

4.10 *Hotplate* or kitchen stove.

4.11 *Bacteriological transfer loops and needles.*

4.12 *Flasks*, 125-ml, screwcap, erlenmeyer (borosilicate glass).

4.13 *Scissors*, autoclavable.

4.14 *Spatula*, laboratory, 120×20 mm.

4.15 *Test tubes*, screwcaps, 16×150 mm.

4.16 *Test tubes*, with loose-fitting caps, 16×150 mm.

4.17 *Durham tubes*, 6×50 mm, Corning No. 9820 or equivalent.

4.18 *Diatomaceous earth*, Johns-Manville "Celite" or equivalent.

5. Reagents

5.1 *Selenite broth*, Difco No. 0275-02-6 or equivalent.

5.2 *Tetrathionate broth*, Difco No. 0104-02-3 or equivalent.

5.3 *G. N. (gram negative) broth*, Difco No. 0486-02-1 or equivalent.

5.4 *Brilliant green agar*, Difco No. 0285-02-4 or equivalent.

5.5 *Bismuth sulfite agar*, Difco No. 0073-02-0 or equivalent.

5.6 *Veal infusion broth*, Difco No. 0344-02-3 or equivalent.

5.7 *XLD (xylose-lysine desoxycholate) agar*, Difco No. 0788-02-6 or equivalent.

5.8 *Urea agar base*, Difco No. 0283-02-6 or equivalent.

5.9 *Agar*, Difco No. 0140-02-9 or equivalent.

5.10 *Triple sugar iron agar (TSI)*, Difco No. 0265-02-8 or equivalent.

5.11 *Decarboxylase base Moeller*, Difco No. 0890-02-1 or equivalent.

5.12 *Lysine*, Difco No. 0705-11-5 or equivalent.

5.13 *L-ornithine*, Difco No. 0293-11-3 or equivalent.

5.14 *Purple broth base*, Difco No. 0227-02-5 or equivalent.

5.15 *Lactose*, Difco No. 0156-15-5 or equivalent.

5.16 *Sucrose*, Difco No. 0176-15-1 or equivalent.

5.17 *Salicin*, Difco No. 0177-11-4 or equivalent.

5.18 *KCN (potassium cyanide) broth base*, Difco No. 0647-02-7 or equivalent.

5.19 *Potassium cyanide (KCN)*, powdered, reagent grade.

5.20 *SIM (sulfide-indole-motility) medium*, Difco No. 0271-02-0 or equivalent.

5.21 *Raffinose*, Difco No. 0174-13-5 or equivalent.

5.22 *Salmonella O Antiserum Kit*, Difco No. 2892-32-9 or equivalent.

5.23 *Salmonella H. Antiserum Kit,* Difco No. 2328-32-3 or equivalent.

Note: It is important that manufacturer's instructions be followed closely in the preparation and storage of all media. If field inoculation is intended, discretion is advised in the final dispensing of selenite and tetrathionate broth. The container must allow room for membrane filters or diatomaceous earth and must fit in a field incubator.

6. Collection

Samples should be collected in clean, sterile containers. Great care is advised to preclude the possibility of contamination of the sample or the collector. Sterile disposable gloves are recommended. A minimum of 2 liters of samples is necessary for filtration. As this procedure will be used for qualitative determinations, samples representative of mean flow of a stream generally are not required.

If filtration and incubation are not to be done immediately, the samples must be chilled until time of processing. Maximum allowable storage time is 6 hours.

7. Analysis

7.1 Concentration. The sample must be concentrated before inoculation into selective media. Two procedures are available for concentration—membrane filtration and diatomaceous earth filtration.

Membrane filter procedure: Filter 2 liters (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 holders are available. When filtration is complete, remove the filter from the filtering apparatus and transfer equal-sized pieces of the filter to selective growth media. Record volume of sample that was filtered.

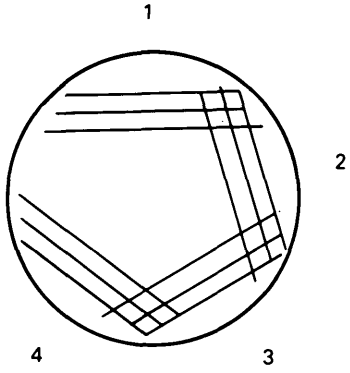
Diatomaceous earth procedure: Place a sterile 47-mm diameter absorbent pad in the filtering funnel and fill the neck halfway with diatomaceous earth. Pour 2 liters of sample *slowly* into the filtration apparatus and apply vacuum. When the sample has been completely filtered, transfer equal parts of the diatomaceous earth to the selective growth media. Note: Not all bacteria are retained; the filtrate will contain some bacteria and possibly pathogens.

7.2 If isolation of *Salmonella* is desired, transfer one-half of the membrane filter(s) or diatomaceous earth 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 filter(s) or diatomaceous earth to GN broth. GN broth cannot be used to isolate *Salmonella*.

7.3 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 (see 7.5)

7.4 After arrival at the laboratory, transfer primary culture flasks to a laboratory incubator prewarmed to 41.5°C and prepare selective plating media. For *Salmonella,* brilliant green agar, bismuth sulfite agar, and XLD agar, are recommended. XLD agar is recommended also for *Shigella*. One to four 100-mm plates of each plating medium will be needed for every primary (broth) culture.

7.5 After suitable (see below) incubation, streak broth cultures having evidence of bacterial growth onto plating media prepared in 7.4. Selenite broth cultures with growth become turbid and develop orange-red coloration. Optimum recovery of *Salmonella* from selenite broth is obtained after 24 hours incubation at 41.5°C, 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 up to 5 days to recover all *Salmonella*. Streak the GN broth after 24 hours incubation only. Streak with care and precision so that isolated colonies will grow in a discrete pattern. Note: The streak pattern shown below should give good results, if care is taken to flame the needle after streaking each section:



7.6 Incubate inoculated plates in an inverted (upside down) position at 41.5°C. XLD agar plates should be incubated for 24 hours. All other plates are incubated for 48 hours.

7.7 After incubation, inspect the plates for *Sal-*

monella or *Shigella* colonies. The plates usually have luxuriant bacterial growth, so great care and discretion is necessary in the selection of possible colonies of pathogens.

On brilliant green agar, *Salmonella* typically forms pinkish white colonies with a red background *(if well isolated)*. If the plate is overgrown with colonies, *Salmonella* may be indistinguishable from the usually more numerous non-pathogens. On bismuth sulfite agar, *Salmonella* develops black colonies with or without 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. Some green colored colonies, therefore, should be isolated. On XLD agar, *Shigella* forms red colonies and *Salmonella* produces black-centered red colonies.

7.8 Carefully transfer all suspected *Salmonella* or *Shigella* colonies with a sterile loop to fresh agar plates. Incubate at 41.5°C for 48 hours. Repeated examination, streaking, and incubation of suspected *Salmonella* and *Shigella* must be continued until pure cultures are obtained.

7.9 After the suspected *Salmonella* or *Shigella* cultures have been developed in pure culture, they must be subjected 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, the scheme in figure 9 may be used.

There are many published identification schemes for *Salmonella* and *Shigella*. Publications by Edwards and Ewing (1972), Brezenski and Russomanno (1969), Presnell and Miescier (1971), and Claudon and others (1971) show various approaches to the identification procedure. The manufacturers of bacteriological media also provide useful leaflets covering certain testing procedures. Difco Laboratories publications (1968, 1969a, 1969b, 1971a, and 1971b) are available upon 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 by the Christensen method (Difco Laboratories, 1969b). Screen urease negative cultures for biochemical action as follows: Lysine and ornithine decarboxylation by Moeller method (Difco Laboratories 1969a), citrate utilization by Simmons method (Difco Laboratories, 1953), H_2S production on TSI, fermentation of lactose, saccharose, salicin, and raffinose, growth in KCN broth, and action on SIM medium. Procedural details are shown in table 5.

If biochemical tests (Table 6) indicate the isolated culture is likely to be *Salmonella* or *Shigella,* identify serologically.

7.10 American Public Health Association and others (1976) states "serological identification of *Salmonella* or *Shigella* involves complex, highly specialized procedures which, if called for, should be carried out as described by Edwards and Ewing, 1972." Difco Laboratories (1971b) presents one procedure for the serological identification of *Salmonella*.

A brief description of the serological process may enhance the understanding of the non-serologist. 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 × 100 magnification. The serological process is so specific that more than 1,000 different *Salmonella* types (serotypes) have been identified.

A foreign body which 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* with minimal effort. The O antigen is first identified using *Salmonella* O antiserum. If the results are positive (clumping occurs), the culture is 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 should be determined using *Salmonella* H antiserum. With this step, most *Salmonella* can be classified into a

Table 5—Biochemical test procedure for *Salmonella* and *Shigella*

Test	Media requirements	Media preparation	Inoculation and incubation	Typical result
Urease	Urea agar base (Difco 0283) agar (Difco 0140)	Prepare medium in slants with generous butts.	Make 1 streak along along entire length. Don't inoculate butt. Incubate at 37°C, 24 hours.	*Salmonella* and *Shigella* are negative (no color change). Others turn medium pink within 24 hours.
Decarboxylation of lysine and ornithine	Decarboxylase base (Difco 0872) L–lysine (Difco 0705) L–ornithine (Difco 0293).	Use amino acids at 0.5 percent, added to base medium. Ornithine must be adjusted to pH 6.5 with ION NaOH. Dispense in 5 ml amounts in screw cap tubes.	Inoculate with a 24 hour agar slant culture. Screw caps on tightly and incubate at 37°C, 24 hours.	Reddish violet if positive, yellow if negative. *Salmonella* usually +, *Shigella* – on lysine, variable on ornithine–see table 6.
Citrate	Simmons citrate agar (Difco 0091).	Prepare medium in slants with generous butts.	Make 1 streak along entire length, and stab the butt using a needle. Incubate 37°C, 24 to 48 hours.	*Shigella* is negative (green color). Most *Salmonella* are + (deep blue).
H_2S production	TSI agar (Difco 0265)	Prepare medium in slants with generous butts.	Streak slant heavily along entire length and stab the butt. Incubate at 37°C, 24 hours.	*Salmonella:* Red slant, yellow butt, H_2S^+ (blackening), gas variable. *Shigella:* Red slant, yellow butt, H_2S^-(no blackening).
Carbohydrate utilization	Purple broth base (Difco 0227) lactose (Difco 0156) saccarose (sucrose), (Difco 0176) salicin (Difco 0177) raffinose (Difco 0174).	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 vials.	Inoculate from 24 hours, 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 (Difco 0271). Indole test strips (Difco 1627).	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 culture. Stab in center to ½ depth. Incubate at 37°C, 24 to 48 hours.	If indole is produced, paper turns pink. Medium blackens if H_2S is produced. *Salmonella* negative for indole, may produce H_2S.
KCN	KCN broth base (Difco 0647). 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 heavily from 24 hour KCN broth culture without KCN. Incubate at 37°, 48 hours.	*Salmonella* and *Shigella* will not grow. Other enterobacteriaceae may, see table 6.

specific serotype. A diagrammatic serological scheme for *Salmonella* is shown in figure 10.

All cultures not retained for serological testing should be autoclaved at 121°C for 15 minutes at 1.05 kg per cm^2 (15 psi) before discarding.

If Difco reagents are used for serological identification, the procedure is as follows:

7.10.1 Somatic O Antigen Analysis (Difco Laboratories, 1971b).

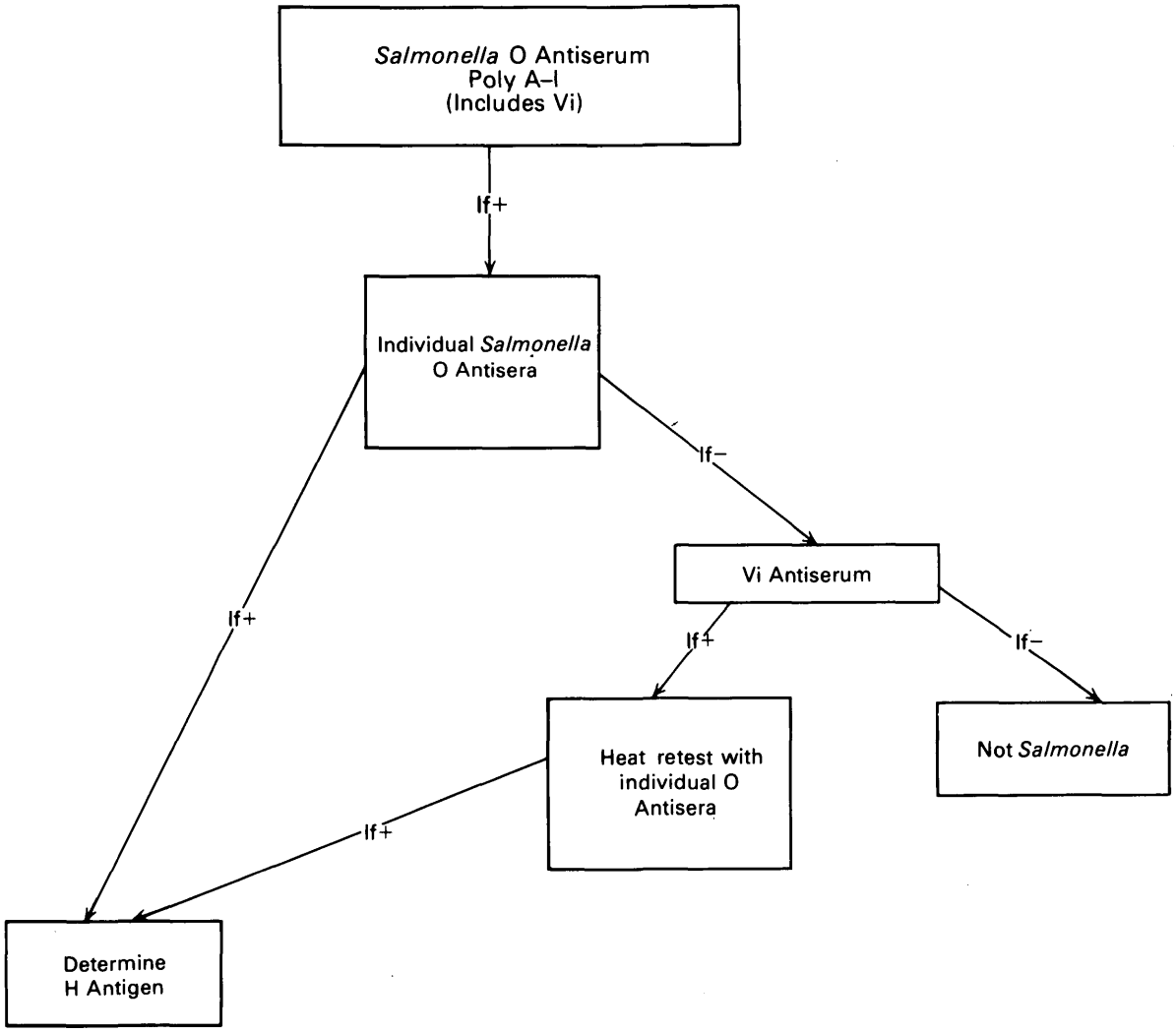


Figure 10—*Salmonella* serology (from Difco, 1971b).

1. Only microorganisms that give typical *Salmonella* reactions culturally and biochemically should be tested.
2. Colonies growing on triple sugar iron (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 standardo. 3. Care must be taken to insure an even suspension.
4. Using a wax pencil, mark a micro 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:

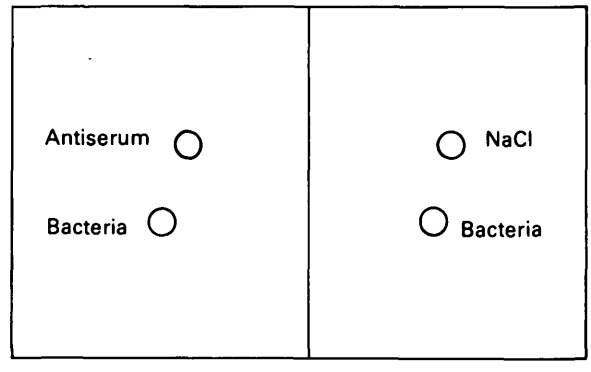


Table 6—Differentiation of Enterobacteriaceae by biochemical tests

[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
KCN	−	−	−	−	−	+
MOTILITY	+ or −	−	+	+	+	+
GELATIN (22°C)	−	−	−	−	(+)	−
LYSINE DECARBOXYLASE	d	−	+	+	+	−
ARGININE DIHYDROLASE	d	− or (+)	−	(+) or +	+ or (+)	d
ORNITHINE DECARBOXYLASE	d	d	+	+	+	d
PHENYLALANINE DEAMINASE	−	−	−	−	−	−
MALONATE	−	−	−	−	+	d
GAS FROM GLUCOSE	+	−	+	+	+	+
LACTOSE	+	−	−	−	d	d
SUCROSE	d	−	−	−	−	d
MANNITOL	+	+ or −	−	+	+	+
DULCITOL	d	d	−	d	−	d
SALICIN	d	−	−	−	−	d
ADONITOL	−	−	−	−	−	−
INOSITOL	−	−	−	d	−	−
SORBITOL	+	d	−	+	+	+
ARABINOSE	+	d	−	+	+	+
RAFFINOSE	d	d	−	−	−	d
RHAMNOSE	d	d	−	+	+	+

6. Place one drop of 0.85 percent sodium chloride solution to the square adjacent to the one containing the antiserum. This will serve as a negative control of the bacterial suspension.
7. Using a clean inoculation loop, transfer a loopful (0.05 ml) of the bacterial suspension (step 3) to the square containing salt solution. Mix bacterial and salt solutions thoroughly to obtain an even mixture.
8. Transfer a second loopful of bacterial suspension

Table 6—Differentiation of Enterobacteriaceae by biochemical tests—Continued

KLEBSIELLEAE									PROTEEAE					
Klebsiella	Enterobacter						Serratia	Pecto-bacterium	Proteus				Providencia	
	cloacae	aerogenes	hafniae		liquefaciens				vulgaris	mirabilis	morganii	rettgeri	alcalifaciens	stuartii
			37°C	22°C	37°C	22°C		25°C						
− or +	−	−	−	−	−	−	−	− or +	+	−	+	+	+	+
−	−	−	+ or −	−	+ or −	− or +	− or +	+ or −	+	+	+	+	+	+
+	+	+	+ or −	+	− or +	+ or −	+	− or +	−	− or +	−	−	−	−
+	+	+	(+) or −	d	+	+	+	d	d	+ or (+)	−	+	+	+
−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
+	+ or −	−	−	−	d	−	d[w]	d[w]	+	+	+	+	−	−
+	+	+	+	+	+	+	+	+ or −	+	+	+	+	+	+
−	+	+	+	+	d	+	+	+ or −	+	+	+	+	+	+
−	(+) or −	− or (+)		−		+	+	+ or (+)	+ or (+)	+	−	−	−	−
+	−	+	+	+	+ or −	+	+	−	−	−	−	−	−	−
−	+	−	−	−	−	−	−	− or +	−	−	−	−	−	−
−	+	+	+	+	+	+	+	−	−	+	+	−	−	−
−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
+	+ or −	+ or −	+ or −	+ or −	−	−	−	− or +	−	−	−	−	−	−
+	+	+	+	+	+	+	+ or −	− or +	+ or −	+	d	− or +	+ or −	−
+	+	+	− or (+)	− or (+)	d	(+)	− or (+)	d	−	−	−	−	−	−
+	+	+	d	d	+	+	+	+	+	d	−	d	d	d
+	+	+	+	+	+	+	+	+	−	−	−	+ or −	−	d
− or +	− or +	−	−	−	−	−	−	−	−	−	−	−	−	−
+	+ or (+)	+	d	d	+	+	+	+	d	d	−	d	−	−
+ or −	− or +	+	−	−	d	d	d	−	−	−	−	d	+	−
+	d	+	−	−	+	+	d	−	−	−	−	+	−	+
+	+	+	−	−	+	+	+	−	−	−	−	d	−	d
+	+	+	+	+	+	+	−	+	−	−	−	−	−	−
+	+	+	−	−	+	+	−	+ or (+)	−	−	−	−	−	−
+	+	+	+	+	−	−	−	d	−	−	−	+ or −	−	−

(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, proceed further to identify the group to which the organism belongs by using the desired individual *Salmonella* O antisera groups in the same manner

as described above 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 with *Salmonella* Vi antiserum by the method described above. *If there is no agglutination with Salmonella Vi antiserum at this point, the culture may be regarded as being 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 with the desired individual *Salmonella* O antisera groups and the *Salmonella* Vi antiserum. If the organism does not react with the Vi antiserum after heating, but reacts with *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 organism may be classified as a member of the *Citrobacter (Escherichia freundii)* Group. Edwards and Ewing (1972) recommended resubmitting for further biochemical tests all cultures giving 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 organisms.
13. Organisms giving positive agglutination with *Salmonella* O antiserum groups may be analyzed further for their H antigens using the appropriate *Salmonella* H antisera, if necessary.

7.10.2 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 organism. The tube test procedure of Edwards and Bruner (1947) is recommended. It is necessary to have a motile organism when testing for H antigens. Usually TSI broth cultures of fresh isolates are satisfactory for use as antigens for this purpose. Occasionally, it is necessary to increase the motility of the test organisms by making several consecutive transfers in SIM medium. This is a semi-solid medium which permits visual determination of bacterial movement. If the organism grows well on SIM medium, this can be used in the manner described in table 6. Inoculate the 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 organisms 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 tube with the motile organisms 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 +8.5 g sodium chloride in 1 liter distilled water).
3. Dilutions with *Salmonella* H antisera depend on which sera are to be employed. 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 of 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 of the rehydrated antiserum to 16 ml of 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 part of the rehydrated polyvalent antiserum to 33 parts of 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 amount 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 seriological tubes.
5. Add 0.5 ml of the antigen and incubate in a water bath at 50°C for 1 hour.
6. Observe for agglutination and record. Autoclave all cultures at 121°C for 15 min at 1.05 kg per cm^2 (15 psi) before discarding.

8. Calculation

Not applicable.

9. Report

Report results only as positive or negative for *Salmonella* or *Shigella* in the sample. Record the sample volume if it is known.

10. Precision

No precision data are available.

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PHYTOPLANKTON

The assemblage of organisms that drift passively with the currents are collectively called plankton. Because of the direct association with the water and particularly with masses of water that move in response to wind- or gravity-generated currents, the species composition and abundance of plankton are related to water quality. Moreover, the phytoplankton or plant part of the plankton directly affect water composition, notably the dissolved oxygen, pH, concentration of certain solutes, and optical properties. At times the abundance or presence of particular species of phytoplankton result in nuisance conditions.

There is no standard method for collecting and enumerating the micro-organisms comprising a phytoplankton sample. The organisms may be so abundant that a dilution of the sample must be made for enumeration. There may be so few of them in a sample that many field counts must be made. Morphological differences between the phytoplankton groups and the preservation of samples which cannot be examined immediately are additional factors which influence the selection of a phytoplankton counting method.

Sedgwick-Rafter method
(B–1501–77)

Parameter and code: Phytoplankton, total (cells/ml) 60050

1. Application

The Sedgwick-Rafter method is one of several procedures for determining the concentration of phytoplankton. The method is easily performed and provides reasonably reproducible information when used with a calibrated microscope equipped with an eyepiece measuring device such as the Whipple ocular micrometer (American Public Health Association and others, 1976, p. 1024–1026).

The method is much less time consuming than the membrane filter method. The disadvantage of the method is that the Sedgwick-Rafter counting cell does not permit use of a high-power microscope objective. However, the kinds of organisms present in a phytoplankton sample may be determined under high-power magnification, prior to using the counting cell. The method is suitable for all waters, both fresh and saline.

2. Summary of method

A 1-ml aliquot of a thoroughly mixed phytoplankton sample is placed in a Sedgwick-Rafter counting cell and examined microscopically with the aid of a Whipple ocular micrometer. The number of algal cells present in random fields are counted. The density of phytoplankton in the sample, as cells per milliliter, is calculated.

3. Interferences

Large concentrations of suspended sediment may obscure the algae in a plankton sample.

4. Apparatus

4.1 *Water-sampling bottle,* Wildlife Supply Co. (1510 or 1920) (figs. 11 and 12); Scott Instruments, Seattle, Wash.; or Foerst Mechanical Specialties Co., Improved Water Sampler, Kemmerer-type; or equivalent. Depth integrated samplers are discussed in Guy and Norman (1970).

4.2 *Sample containers,* plastic bottles, 1,000-ml capacity.

4.3 *Microscope,* binocular, American Optical A.O. Series 20 Advanced Microstar (×200) or equivalent.

4.4 *Ocular micrometer,* Whipple grid, A. H. Thomas Co. (6585-H10) or equivalent.

4.5 *Sedgwick-Rafter counting cell,* APHA, 50×20 ×1 mm. A. H. Thomas Co. (9851-C20), Wildlife

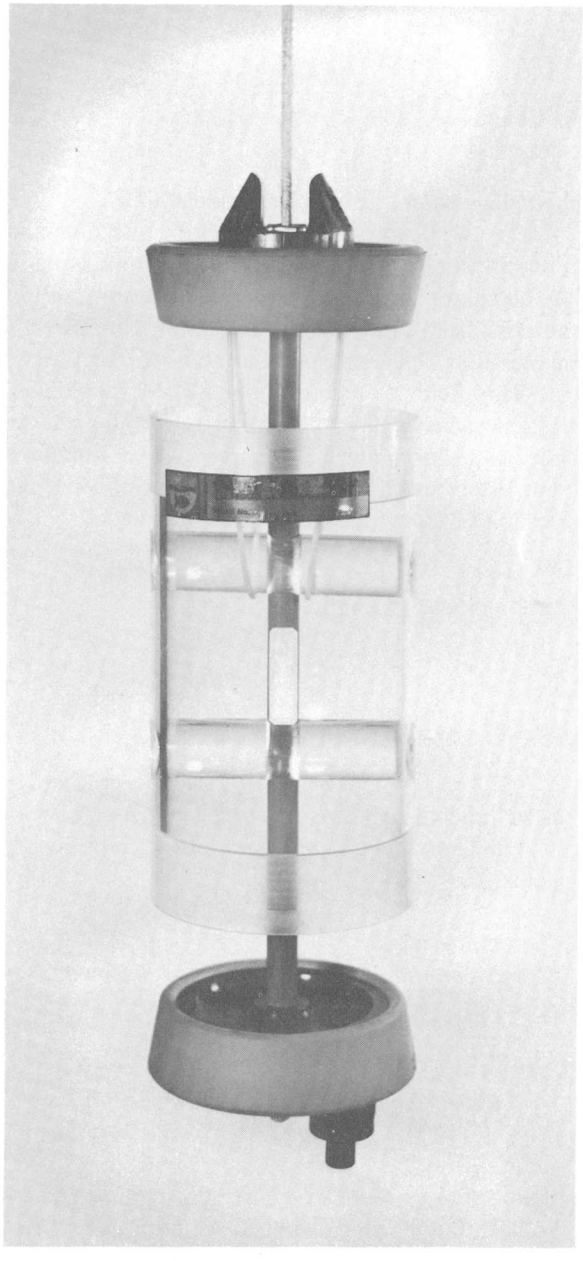

Figure 11.—Kemmerer water bottle. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

Supply Co. (1801) (fig. 13), or equivalent, with cover glass, A. H. Thomas Co. (9851-C25) or equivalent.

4.6 *Pipet,* transfer, 1 ml, large bore.

5. Reagents

5.1 *Cupric sulfate solution,* saturated, dissolve 21 g $CuSO_4$ in 100 ml distilled water.

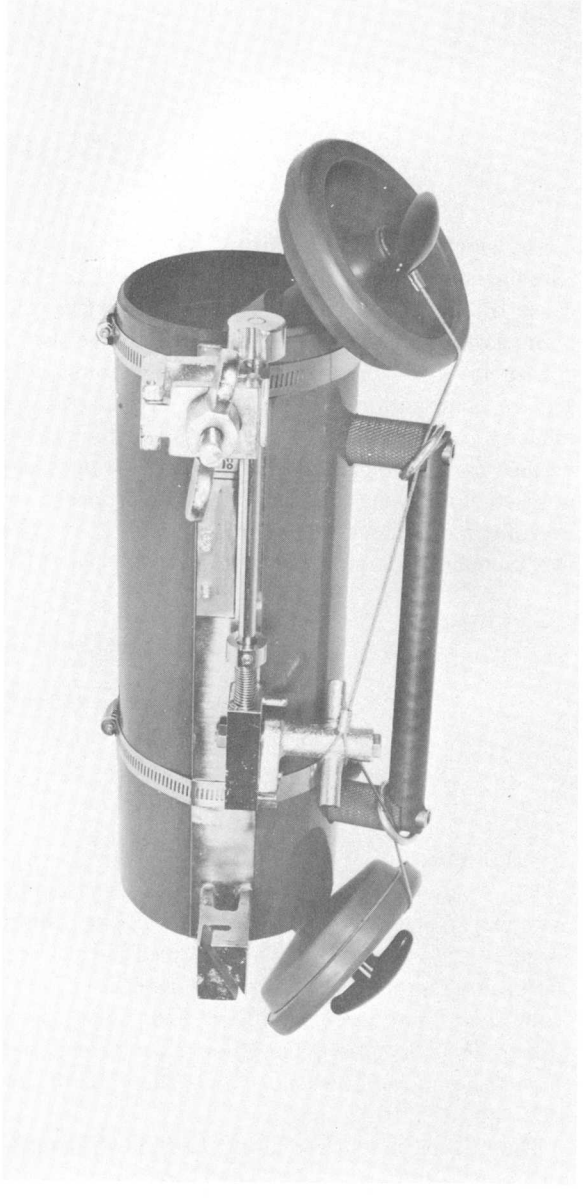

Figure 12.—Van Dorn water bottle. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

5.2 *Formaldehyde-cupric sulfate solution,* mix 1 liter of 40 percent aqueous formaldehyde containing 10–15 percent methanol, Fisher Scientific No. F–78, or equivalent, with 1 ml of solution 5.1.

5.3 *Detergent solution,* 20 percent, dilute 20 ml liquid detergent (Liqui-Nox, Catalog No. C6308–2, phosphate free, or equivalent) to 100 ml with distilled water.

5.4 *Lugol's solution:* Dissolve 10 g iodine crystals and 20 g potassium iodide in 200 ml distilled water.

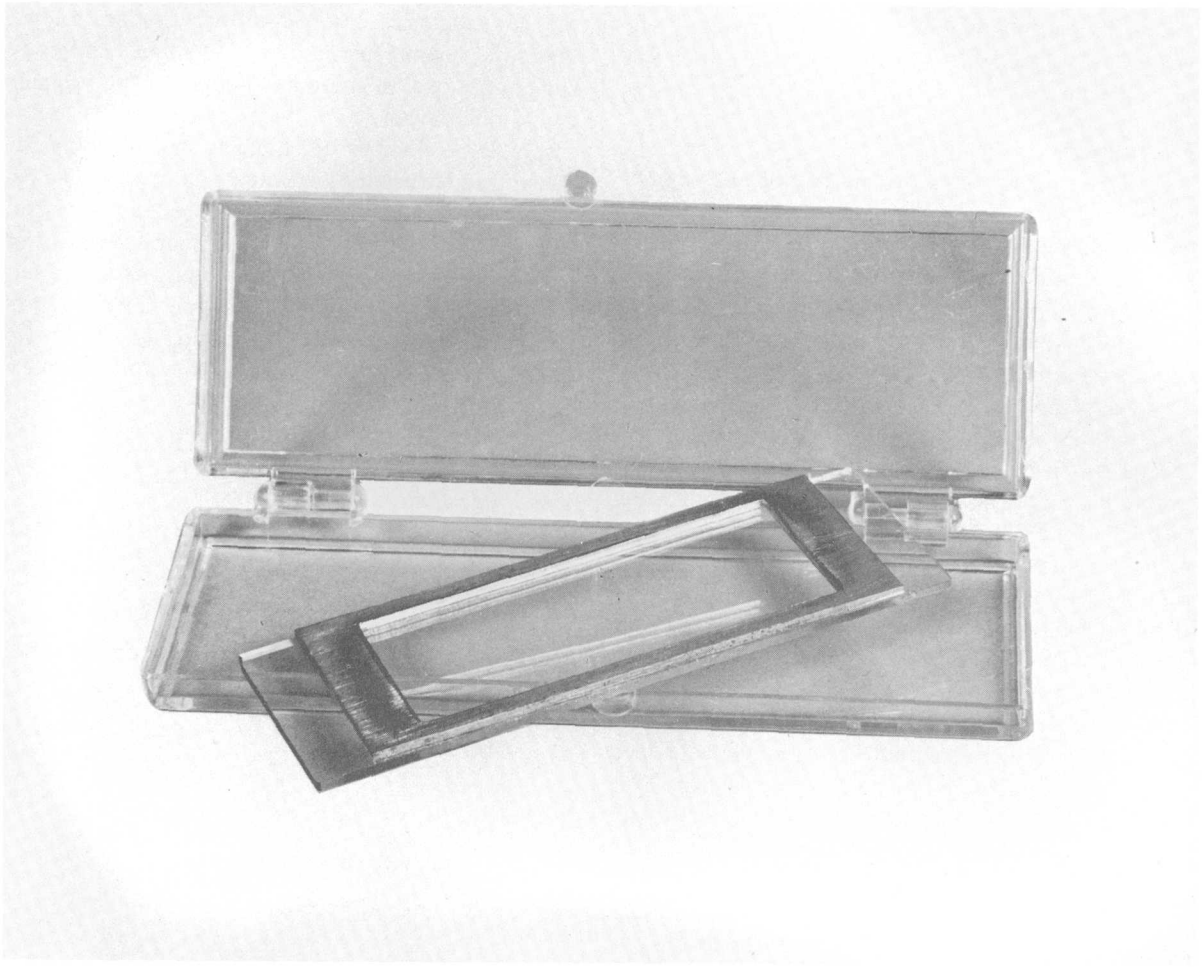

Figure 13—Sedgwick-Rafter counting cell. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

6. Collection

A phytoplankton sample consists of a volume of water, usually 1 liter. To insure maximum correlation of results, the sample sites and methods used for phytoplankton should correspond as closely as possible to those selected for chemical and bacteriological 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 with time of day. To collect a sample representative of the phytoplankton density 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 located 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).

If the sample is to be examined within 2 or 3 hours after collection, no special treatment is necessary. A phytoplankton sample may be maintained for 24 hours at 3°–4°C, but for extended storage, preserve as follows: To each 1,000 ml of sample, add 30 ml of 37–40 percent aqueous formaldehyde solution (100 percent Formalin), 5 ml of 20 percent detergent solution, and 1 ml of cupric sulfate solution. This preservative maintains cell coloration and is effective indefinitely.

Many biologists consider Lugol's solution to be the best phytoplankton preservative. It has been found to be 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 pre-

ferred as a preservative, add 1 ml Lugol's solution to each 100 ml of sample. Store the preserved samples in the dark.

7. Analysis

7.1 Most fresh waters contain phytoplankton concentrations that can be enumerated microscopically without dilution or concentration. If neither dilution nor concentration is needed, proceed to 7.2.

If the phytoplankton sample contains great numbers of organisms, as typically occurs in eutrophic waters, the sample must be diluted. To dilute, thoroughly mix 50 ml of sample with 50 ml distilled water (1:1 dilution) and proceed to 7.2. If microscopic examination reveals a density of organisms still too numerous to count, thoroughly mix 50 ml of 1:1 dilution with 50 ml distilled water (1:4 dilution). Additional dilutions may be made as appropriate.

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. Carefully siphon the supernatant to avoid disturbance of the settled material. Place sample container with remaining sample on balance and weigh. The reduction in weight (in grams) is equivalent to the number of milliliters of supernatant removed. The same method can be used to obtain the volume of concentrate.

7.2 With the Sedgwick-Rafter counting cell on a flat surface, place the cover glass diagonally across the cell. Thoroughly mix the sample, remove a 1-ml aliquot with a large-bore pipet and transfer the aliquot to the Sedgwick-Rafter counting cell. As the counting cell fills, the cover glass often will rotate slowly and cover the inner part of the cell. The cover glass must not float above the rim of the cell. Allow the counting cell to stand for 15–20 minutes or until the organisms have settled.

7.3 Carefully place the counting cell on the mechanical stage of a calibrated microscope. Count and identify the total number of algal cells (× 200 magnification) enclosed by the ocular grid in a randomly chosen field. In making the count, enumerate all forms that touch two intersecting borders of the grid, but do not count those that touch the opposite borders. Count a minimum of 5 fields or 100 organisms or a maximum of 300 fields (at × 200), whichever is obtained first. Note: Moving the counting cell in a vertical plane can prevent motion sickness for the observer.

Some phytoplankton, particularly some blue-green algae, may not settle but instead rise to the underside of the cover glass. When counting random fields, therefore, enumerate and record the total number of cells in the vertical column delimited by the Whipple ocular micrometer.

If a large number of colonies appear within the ocular grid, determine the average number of cells per colony and multiply by the number of colonies present. Similarly, tabulate the numbers and lengths of trichomes of blue-green algae in each grid and determine the average number of cells per unit length of trichome. Count frustules containing any part of a protoplast as having been living at the time of collection.

8. Calculations

8.1 Calibration factor

$$= \frac{1{,}000\ \text{mm}^2}{\text{area of Whipple disk at} \times 100\ \text{magnification}\ (\text{mm}^2)}.$$

8.2 For samples neither diluted nor concentrated:

$$\text{Phytoplankton cells/ml} = \frac{\text{total cell count}}{\text{number of random fields} \times 1\ \text{ml}} \times \text{calibration factor.}$$

8.3 For diluted samples:

$$\text{Phytoplankton cells/ml} = \frac{\text{total cell count} \times \text{vol. of final dilution (ml)}}{\text{number of random fields} \times \text{vol. of original sample (ml)} \times 1\ \text{ml}} \times \text{calibration factors.}$$

8.4 For concentrated samples:

$$\text{Phytoplankton cells/ml} = \frac{\text{total cell count} \times \text{vol. of conc. (ml)}}{\text{number of random fields} \times \text{vol. of original sample (ml)} \times 1\ \text{ml}} \times \text{calibration factors.}$$

9. Report

Report phytoplankton densities to two significant figures.

10. Precision

No precision data are available.

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Inverted microscope method
(B–1520–77)

Parameter and code: Phytoplankton, total (cells/ml) 60050

1. Application

The method is suitable for all waters.

2. Summary of method

Taxonomic and numerical assessment of natural populations of phytoplankton require direct microscopic examination. The inverted microscope method permits the observation of the phytoplankton in an aliquot of water at high-power magnification without disrupting or crushing the delicate organisms.

The phytoplankton are concentrated by settling to the bottom of a sample container or a vertical-tube sedimentation apparatus (Utermohl, 1931, 1936, 1958; Lovegrove, 1960). Lund, Kipling, and LeCren (1958) reported that all known algae can be settled.

An aliquot of a phytoplankton sample is poured into a plankton chamber or a sedimentation apparatus. The algae settle onto a microscope cover glass which forms the bottom of the chamber or apparatus, and the settled algae are observed from beneath using an inverted microscope. Because this method permits use of the high dry and oil-immersion objectives on the microscope, very small forms can be identified and enumerated.

3. Interferences

The method is generally free of interferences. Suspended sediment may obscure microorganisms in a sample. Previously used sample bottles and parts of the sedimentation apparatus must be scrubbed thoroughly to remove adherent diatoms and other material, especially from the bottom surfaces. Convection currents and air bubbles in the apparatus can interfere with sedimentation.

4. Apparatus

4.1 *Inverted microscope,* Zeiss Invertoscope D, Nikon (MS-76560), Tiyoda (2020), or equivalent.

4.2 *Ocular micrometer,* Whipple grid, Bausch & Lomb (31-16-13) or equivalent.

4.3 *Plankton chamber,* 26×76 mm glass slide with 12-mm circular hole covered by cementing no. 1½ cover slip to slide.

4.4 *Sedimentation apparatus* of the type described by Lovegrove (1960) (fig. 14), 8-cm high, 25-ml capacity, Scott Instruments, Seattle, Wash., or equivalent. Other sizes may be needed for some types of samples (see 7.3 below).

4.5 *Coverglass,* 22-mm diameter, No. 1 and No. 1½.

4.6 *Rubber cement* for attaching cover glass to the counting chamber.

4.7 *Sample containers,* plastic bottles, 1,000-ml capacity.

4.8 *Water-sampling bottle,* Wildlife Supply Co. (1510 or 1920) (figs. 11 and 12); Scott Instruments, Seattle, Wash., Foerst Mechanical Specialties Co., Improved Water Sampler, Kemmerer-type; or equivalent. Depth-integrated samplers are discussed by Guy and Norman (1970).

4.9 *Cotton swabs.*

4.10 *Vacuum grease.*

4.11 *Pipet,* serological, 1 ml.

4.12 *Balance,* with automatic tare, Sartorius or equivalent.

5. Reagents

5.1 *Cupric sulfate solution,* saturated, dissolve 21 g $CuSO_4$ in 100 ml distilled water.

5.2 *Formaldehyde*-cupric sulfate solution, mix 1 liter of 40 percent aqueous formaldehyde containing 10–15 percent methanol, Fisher Scientific No. F–78, or equivalent, with 1 ml of solution 5.1.

5.3 *Detergent solution,* 20 percent, dilute 20 ml liquid detergent (Liqui-Nox, Catalog C6308–2, phos-

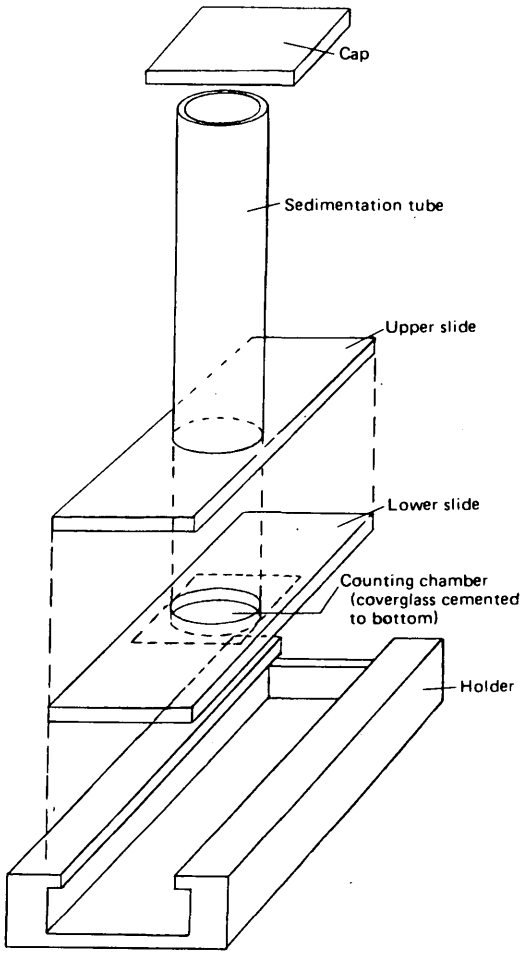

Figure 14.—Sedimentation apparatus. (Modified from Lovegrove, 1960).

phate free, or equivalent) to 100 ml with distilled water.

5.4 *Lugol's solution:* Dissolve 10 g iodine crystals and 20 g potassium iodide in 200 ml distilled water.

6. Collection

A phytoplankton sample consists of a volume of water, usually 1 liter. To insure maximum correlation of results, the sample sites and methods used for phytoplankton should correspond as closely as possible to those selected for chemical and bacteriological 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 with time of day. To collect a sample representative of the phytoplankton density 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 located at the centroid of flow may be adequate. Study design, collection, and statistics for streams, rivers, and lakes are described in Federal Working Group on Pest Management (1974).

Preserve sample as follows: To each 1,000 ml of sample add 40 ml of 37–40 percent aqueous formaldehyde solution (100 percent Formalin), 5 ml of 20 percent detergent solution, and 1 ml of cupric sulfate solution. This preservative maintains cell coloration and is effective indefinitely.

Many biologists consider Lugol's solution to be the best plankton preservative. It has been 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 preferred as a preservative, add 1 ml Lugol's solution to each 100 ml of sample. Store the preserved samples in the dark.

7. Analysis

7.1 If using the sedimentation apparatus (fig. 14), proceed to 7.5. If using the plankton chamber, 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, tare the sample container on an automatic balance.

7.2 Carefully siphon the supernatant to avoid disturbance of the settled material. Place sample container with remaining sample on balance and weigh. The reduction in weight (in grams) is equivalent to the number of milliliters of supernatant removed. The same method can be used to obtain the volume of concentrate.

7.3 Mix the concentrated sample well (but not vigorously) and pipet an appropriate volume into each of two plankton chambers. Slide cover slip into place.

7.4 Place the plankton chamber on the mechanical stage of a calibrated microscope. Proceed to 7.10.

7.5 To prepare the sedimentation apparatus, cement a No. 1 glass cover glass to the bottom of the lower slide to form the bottom of the counting chamber (fig. 14). When dry, remove the excess rubber cement from the inside of the counting chamber with a knife.

7.6 Test for leaks: Coat the underside of the upper slide (fig. 14) with vacuum grease, and press onto the lower slide to form a watertight seal. Assemble the apparatus and fill with distilled water so that the meniscus bulges slightly above the top of the sedimen-

tation tube. Slide the cap over the top to seal the tube. Let stand overnight and check for water loss in the morning.

7.7 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 7.2. Allow 4 hours settling time per 1 cm of sedimentation tube length. The volume of sample is dependent on the density of algae. In plankton-poor waters, 100 ml of sample may be required; in more fertile waters, 25 ml or less of sample may be sufficient. The 25 ml volume is most commonly used. The samples may be diluted, if necessary.

Note: Air bubbles on the sides of the chamber tube can be prevented if the water sample and the sedimentation apparatus are at the same temperature when the sample is introduced. The apparatus should be maintained at a constant temperature to avoid convection currents which can interfere with settling.

7.8 After settling, isolate the algae in the counting chamber from the remainder of the apparatus. To separate the sedimentation tube and upper slide from the lower slide and counting chamber (fig. 14), move the sedimentation tube to one side splitting the water column. Remove the tube cap and siphon or pipet off the supernatant. Remove the empty sedimentation tube.

7.9 Remove the lower slide with the counting chamber from the holder (fig. 14). Place the cap over the top of the counting chamber to form a closed cell. If an air bubble remains under the cap, tease it to one side of the chamber and carefully add distilled water to fill the void. Replace the tube cap and put the slide on the inverted microscope.

7.10 Count and identify the total number of algal cells (at × 200–300 magnification) in randomly chosen fields. In making the counts, enumerate all forms that intersect two of the borders of the grid, but do not count those that intersect the opposite borders. If a large number of colonies appear within the field, determine the average number of cells in an average size colony and multiply by the number of colonies present. Similarly, tabulate the numbers and lengths of trichomes of blue-green algae in each grid and determine the average number of cells per unit length of trichome. Count all algae containing any part of a protoplast as having been living at the time of collection. Count a minimum of 100 units (unit - one filament, one colony, or one unicellular algae) or 250 fields (at × 200–300) whichever is obtained first. For concentrated samples count a minimum of 10 fields.

8. Calculations

$$\frac{(\text{chamber area, mm}^2) \times (\text{number of fields})}{(0.96)^* \times (\text{field area, mm}^2)}$$

$$\frac{\times\ (\text{total count})}{\times\ (\text{initial volume, ml})}$$

$$\frac{\times\ (\text{volume of concentrate, ml})}{\times\ (\text{chamber volume, ml})}$$

*Compensates for addition of formaldehyde-detergent preservative.

9. Report

Report phytoplankton concentrations to two significant figures. Report values for each of the three groups: diatoms, green algae, and blue-green algae.

10. Precision

No precision data are available.

References

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Hemacytometric method
(B–1540–77)

Parameter and code: Phytoplankton, total (cells/ml) 60050

The Sedgwick-Rafter cell is too thick to use with high-power microscope objectives. Observation of fine structure necessary for identification of some organisms is thus not possible. Furthermore, the ability to count individual cells, especially in filamentous species, is limited. Thinner walled counting chambers which can be used with high-power objectives are commercially available. Most common is the biomedical hemacytometer, a single piece of thermal-and shock-resistant glass with 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 reduce the possibility of accidentally scratching the viewing area and 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.

1. Application

The method is suitable for all waters, but samples generally must be concentrated.

2. Summary of method

Phytoplankton counts cannot be made with good precision in most waters without prior sample concentration. Concentration may be accomplished by sedimentation or centrifugation of the sample. Careful attention to the manufacturer's instructions in use of the hemacytometer and knowledge of number of counts needed for statistical reliability are important in its routine use. Depending on the number of phytoplankton cells in the sample, procedures described by the manufacturer for counting either leucocytes or erythrocytes in random fields are employed. The cell density of phytoplankton in the sample, as cells per milliliter, is calculated.

3. Interferences

The method is generally free of interferences. Suspended sediment may obscure organisms in the sample. Dead phytoplankton may appear similar to living cells. The disadvantages of this counting cell is that the sample must have a very high density of plankton to yield statistically reliable data (American Public Health Assoc. and others, 1976).

4. Apparatus

4.1 *Water-sampling bottle,* Kemmerer-type or Van Dorn-type, Wildlife Supply Co. (1510 or 1920) (figs. 11 and 12), Kahl Scientific Instrument Corp. (135 WA), or equivalent. Depth-integrated samplers are discussed in Guy and Norman (1970).

4.2 *Sample containers,* glass or plastic bottles, 500- or 1,000-ml capacity.

4.3 *Microscope,* standard, bright field or phase contrast objectives and condensers, with × 10 and × 45 (or × 43) objectives and × 10 eyepiece (Zeiss Standard RA 34, American Optical Series 150, Nikon F-KE, or equivalent).

4.4 *Hemacytometer,* counting chamber with cover glasses. American Optical (1492), or equivalent.

4.5 *Pipet,* transfer, 10 ml.

4.6 *Pipet,* Pasteur, disposable.

5. Reagents

5.1 *Cupric sulfate solution,* saturated, dissolve 21 g $CuSO_4$ in 100 ml distilled water.

5.2 *Formaldehyde-cupric sulfate solution,* mix 1 liter of 40 percent aqueous formaldehyde containing 10–15 percent methanol, Fisher Scientific No. F–78, or equivalent, with 1 ml of solution 5.1.

5.3 *Detergent solution,* 20 percent, dilute 20 ml liquid detergent (Liqui-Nox, Catalog No. C6308-2, phosphate free, or equivalent), to 100 ml with distilled water.

5.4 *Lugol's solution:* Dissolve 10 g iodine crystals and 20 g potassium iodide in 200 ml distilled water.

6. Collection

A phytoplankton sample consists of a volume of water, usually 1 liter. To insure maximum correlation of results, the sample sites and methods used for phytoplankton should correspond as closely as possible to those selected for chemical and bacteriological 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 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 located at the centroid of flow may be adequate. Study design, collection, and statistics for streams, rivers, and lakes are described in Federal Working Group on Pest Management (1974).

If the sample is to be examined within 2 or 3 hours after collection, no special treatment is necessary. A phytoplankton sample may be maintained for 24 hours at 3°–4°C, but for extended storage, preserve as follows: To each 1,000 ml of sample, add about 40 ml of 37–40 percent formaldehyde solution (100 percent Formalin), 5 ml of 20 percent detergent solution, and 1 ml of cupric sulfate solution. This preservative maintains cell coloration and is effective indefinitely.

Many biologists consider Lugol's solution to be the best plankton preservative. It has been found to be 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 preferred as a preservative, add 1 ml Lugol's solution to each 100 ml of sample. Store the preserved samples in the dark.

7. Analysis

7.1 Phytoplankton densities in many waters cannot be enumerated with precision using the hemacytometer unless the sample is first concentrated. Such high cell densities are found in very eutrophic waters and in laboratory cultures. The sample to be analyzed should be sufficiently concentrated to insure that at least 50 to 100 cells are present when the hemacytometer is filled.

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. Carefully siphon the supernatant to avoid disturbance of the settled material. Place sample container with remaining sample on balance and weigh. The reduction in weight (in grams) is equivalent to the number of milliliters of supernatant removed. The same method can be used to obtain the volume of concentrate.

7.2 Clean chambers, cover glasses, and pipets are requisite to accurate cell counts. The counting chamber should be cleaned with water, alcohol, or a mild soap and wiped dry with lens tissue. Place a clean cover glass onto the counting chamber, rubbing it into close contact with the supporting ribs of the chamber. Using the Pasteur pipet, place a drop of 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 slide. A little practice soon indicates the proper size drop to fill the chamber without overflow. Any overflow will draw phytoplankton into the moat and it will be necessary to clean the chamber and refill it.

Let the preparation stand for one or two minutes or until the cells settle onto the bottom of the counting chamber. Find the ruled area and examine the preparation at × 100 magnification to make certain the cells are evenly distributed. Any irregularities will not permit accurate estimation of the number of phytoplankton.

7.3 To determine the density of phytoplankton examine cells at × 100 or × 450 magnification. Count the number of cells in a sufficient number (see below) of divisions of the gridded area and multiply by the appropriate dilution factor. Carefully follow the manufacturer's instructions that come with the hemacytometer. If the cell density is low or moderate (this is a judgment factor), use the manufacturer's procedure for counting white corpuscles (leucocytes). If the cell density is high, use the manufacturer's procedure for counting red blood cells (erythrocytes).

8. Calculations

Count the cells in the four corner 1-mm squares and in the central ruled area on both sides of the hemacytometer (10 mm^2 in all). Disregard cells touching two of the boundary lines. Divide the number of cells counted by the concentration factor (c) and the fraction of the chamber volume examined (1/10) to

obtain the number of phytoplankton in 1 mm^3 of sample.

$$\text{Number of cells per } mm^3 = \frac{\text{number of cells counted} \times 100}{\text{number of 1 } mm^2 \text{ areas counted} \times c}$$

Multiply number of cells per cubic millimeter by 1,000 to obtain the number of cells per milliliter.

If the density of phytoplankton is low, it will be necessary to examine more 1-mm^2 areas. Counts of forty 1-mm^2 areas to achieve good precision in estimates of individual taxon densities are common.

If cell density is fairly high, use the procedure for counting red blood cells (erythrocytes). Count phytoplankton in the 5 groups of 16 small squares located at the center and 4 corners of the central square millimeter. To avoid counting a cell twice, those on a line are counted only when on the top and left lines. The same rule applies when counting a column of four squares. Within the group, counts may be made by rows or by columns as convenient.

Divide the number of cells counted by the concentration factor (c) and the fraction of the chamber volume examined (1/4000) to obtain the number of phytoplankton in 1 mm^3 of sample.

$$\text{Number of cells per } mm^3 = \frac{\text{number of cells counted} \times 4{,}000}{\text{number of small squares counted} \times c}$$

Multiply number of cells per cubic millimeter by 1,000 to obtain the number of cells per milliliter.

9. Report

Report phytoplankton counts to two significant figures and record as cells/ml.

10. Precision

No precision data are available.

References

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Membrane filter method
(B–1560–77)

Parameter and code: Phytoplankton, total (cells/ml) 60050

1. Application

An advantage of the membrane filter method is that the phytoplankton sample is reduced to a permanent microscope slide which can be retained for later examination. The method is suitable for all waters. The method given is modified from McNabb (1960).

2. Summary of method

A fresh or preserved sample is filtered. The organisms retained on the membrane from a sample are fixed with Formalin. The filter is rendered transparent, mounted on a microscope slide, and the density of cells is calculated from random area counts.

3. Interferences

If the slide is not properly prepared, air bubbles and nonuniform thickness of mounting medium may interfere with examination of the sample. A disadvantage of the membrane filter method is that soft cells may be distorted, making identification difficult or impossible. Organisms with rigid cell walls are not distorted by the method.

Large amounts of inorganic sediment, detritus, or organic and inorganic precipitates in the sample may clog the filter.

4. Apparatus

4.1 *Filtration equipment,* Millipore, Pyrex microanalysis filter holder (XX10 025 00) (fig. 15), or equivalent, with vacuum filtering flask.

4.2 *Membrane filter,* white, grid, 0.45-μm mean pore size, 25-mm diameter, Millipore (HAWG 024 00) or equivalent.

4.3 *Vacuum-pressure pump,* Millipore (XX60 000 00) or *syringe and two-way valve,* Millipore (XX62 000 05) or equivalent.

4.4 *Forceps,* stainless, smooth tip, Millipore (XX62 000 06) or equivalent.

4.5 *Water-sampling bottle,* Wildlife Supply Co. (1510 or 1920) (figs. 11 and 12); Scott Instruments, Seattle, Wash.; or Foerst Mechanical Specialties Co., Improved Water Sampler, Kemmerer-type; or equivalent. Depth-integrated samplers are discussed in Guy and Norman (1970).

4.6 *Sample containers,* plastic bottles, 1,000-ml capacity.

4.7 *Laboratory oven or incubator,* Millipore (XX63 500 00) or equivalent for operation at 40°C.

4.8 *Cover-glass squares,* 25×25 mm, No. 1, and *microscope slides,* glass, 76×25 mm (3×1 in.).

4.9 *Microscope,* binocular, flat field, American Optical A.O. Series 20 Advanced Microstar or equivalent.

4.10 *Water trap,* second filtering flask.

4.11 *Graduated cylinder,* 100 ml.

5. Reagents

5.1 *Cupric sulfate solution,* saturated: Dissolve 21 g $CuSO_4$ in 100 ml distilled water.

5.2 *Formaldehyde-cupric sulfate solution:* Mix 1 liter of 40 percent aqueous formaldehyde containing 10–15 percent methanol, Fisher Scientific No. F-78, or equivalent, with 1 ml of solution 5.1.

5.3 *Detergent solution,* 20 percent: Dilute 20 ml liquid detergent (Liqui-Nox, Catalog No. C6308-2, phosphate free, or equivalent) to 100 ml with distilled water.

5.4 *Lugol's solution:* Dissolve 10 g iodine crystals and 20 g potassium iodide in 200 ml distilled water.

5.5 *Mounting medium,* Permount, Scientific Products (M7640), or equivalent.

5.6 *Immersion oil,* Cargille's nondrying type A, Scientific Products (M6002-1), or equivalent.

5.7 *Fingernail polish,* clear, thin.

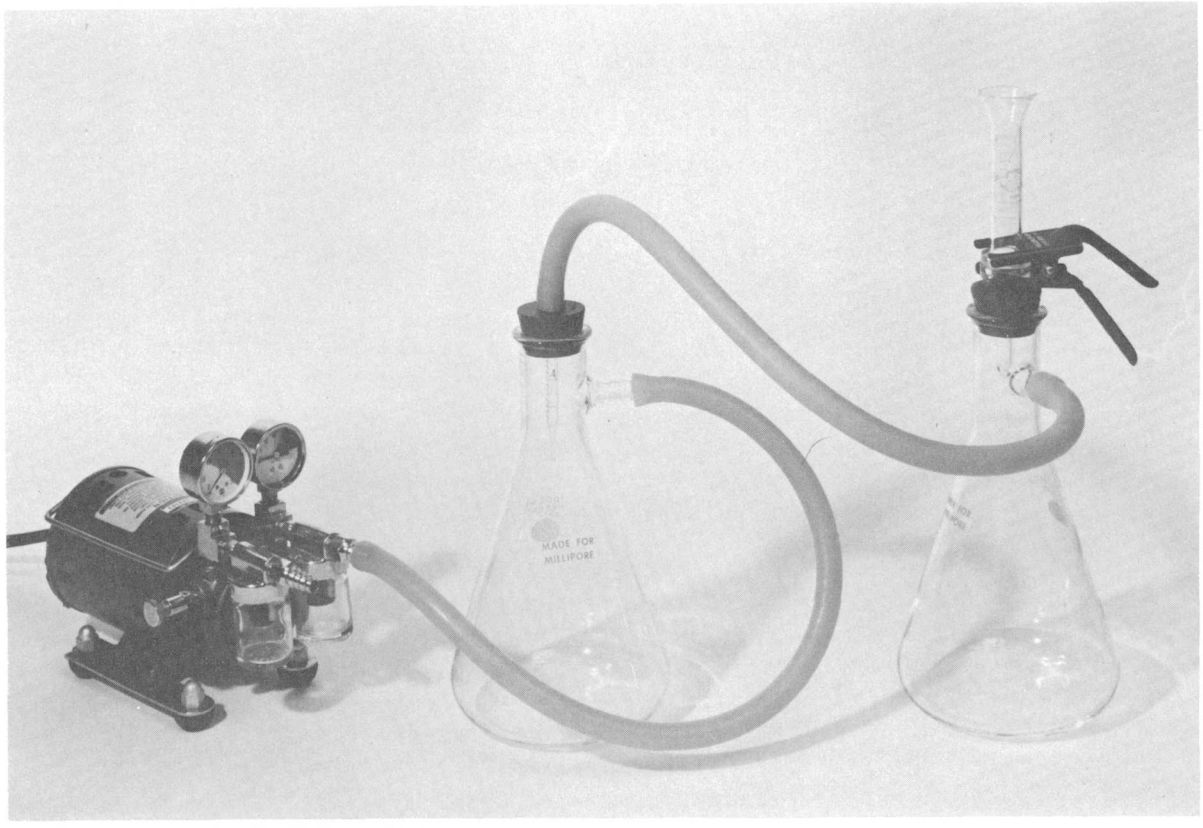

Figure 15.—Membrane filtration device. (Photograph courtesy of Millipore Corp., Bedford, Mass.)

6. Collection

A phytoplankton sample consists of a volume of water, usually 1 liter. To insure maximum correlation of results, the sample sites and methods used for phytoplankton should correspond as closely as possible to those selected for chemical and bacteriological 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 with time of day. To collect a sample representative of the phytoplankton density 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 located at the centroid of flow may be adequate. Study design, collection, and statistics for streams, rivers, and lakes are described in Federal Working Group on Pest Management (1974).

If the sample is to be examined within 2 or 3 hours after collection, no special treatment is necessary. A phytoplankton sample may be maintained for 24 hours at 3°–4°C, but for extended storage, preserve as follows: To each 1,000 ml of sample add 40 ml of 37–40 percent aqueous formaldehyde solution (100 percent Formalin), 5 ml of 20 percent detergent solution, and 1 ml of cupric sulfate solution. This preservative maintains cell coloration and is effective immediately.

Many biologists consider Lugol's solution to be the best plankton preservative. It 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 preferred as a preservative, add 1 ml Lugol's solution to each 100 ml of sample. Store the preserved samples in the dark.

7. Analysis

7.1 The optimum density of plankton for counting on a membrane filter is determined by experimentation. Depending on the quantity of plankton in the sample, an aliquot of 25 to 100 ml, measured with a

graduated cylinder, is poured into the tube of the filtration device (fig. 15).

7.2 Attach pyrex microanalysis filter holder to the vacuum pump. Include a second filtering apparatus (water trap).

7.3 Place a 25-mm gridded filter on the holder using smooth tipped forceps.

7.4 Using the graduated cylinder, measure out an appropriate sample volume and with the vacuum on, pour it into the filter holder.

7.5 During filtration, maintain vacuum at about 130 mm (5 in.) of mercury to minimize selective drawdown of particles suspended in the water. If the sample has not been preserved, near the end of filtration add about 3 ml of 37–40 percent aqueous formaldehyde solution to preserve the cells collected on the filter. After the final mixture passes through the filter, carefully remove the vacuum hose from the filtering flask before turning off the vacuum.

7.6 Place the filter on a labeled microscope slide and place a few drops of immersion oil on the filter to render it transparent. The oil may be added either to a wet or to a dry filter. Place the slide in an oven or incubator at 40°C until the filter clears (about 45 minutes).

7.7 When the filter is transparent, complete the permanent mount. Place about 2 drops of warmed Permount in the center of the filter. Drop a cover glass carefully over the filter on the slide so as to minimize entrapment of bubbles. Apply slight pressure to the cover glass with a pencil-end eraser as the slide cools. If bubbles appear or persist under the cover glass, warm the slide and apply additional pressure to the cover glass. Seal the edges of the cover glass with two or more applications of clear fingernail polish.

7.8 Examine the slides using the desired microscope and lens combination. Enumerate at least 100 organisms within the random grids on the filter. If a large number of colonies appear, determine the average number of cells per colony and multiply by the number of colonies present. Similarly, tabulate the numbers and lengths of trichomes of blue-green algae in each grid and determine the average number of cells per unit length of trichome. Count frustules containing any part of a protoplast as having been living at the time of collection.

8. Calculations

8.1 Phytoplankton cells/ml

$$= \frac{\text{total cell count}}{\text{number of random grids X area of grid (mm}^2\text{)}}$$

$$X \quad \frac{\text{effective filter areas (mm}^2\text{)}}{\text{vol. of filtered sample (ml)}}$$

9. Report

Report phytoplankton densities to two significant figures.

10. Precision

No precision data are available.

References

Federal Working Group on Pest Management, 1974, Guidelines on sampling and statistical methodologies for ambient pesticide monitoring: Washington, D.C., Federal Working Group on Pest Management, 59 p.

Goerlitz, D. F., and Brown, Eugene, 1972, Methods for analysis of organic substances in water: U.S. Geol. Survey Techniques Water-Resources Inv., book 5, chap. A3, 40 p.

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Permanent slide method for planktonic diatoms
(B–1580–77)

Parameter and code: Not applicable.

1. Application

The method is suitable for all waters. Advantages of the method are that a permanent mount is prepared and clearing of the cells enhances observation of frustule (cell wall) detail. The method, therefore, is important in the taxonomic study of diatoms.

The following methods are similar to that of Weber (1966) and of American Public Health Association and others (1976).

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

Particulate matter including salt crystals interferes with mount preparation.

4. Apparatus

4.1 *Water-sampling bottle,* Wildlife Supply Co. (1510 or 1920); Scott Instruments, Seattle, Wash.; or Foerst Mechanical Specialties Co., Improved Water Sampler, Kemmerer-type; or equivalent. Depth-integrated samplers are discussed in Guy and Norman (1970).

4.2 *Sample containers,* plastic bottles, 1,000-ml capacity.

4.3 *Microscope,* binocular, American Optical A.O. Series 20 Advanced Microstar or equivalent.

4.4 *Ocular micrometer,* Whipple grid, Bausch & Lomb (31-16-13), or equivalent.

4.5 *Hotplate,* thermostatically controlled to 538°C (1,000°F), Corning (PC–35) or equivalent. It is convenient to have a second hotplate for operation at about 93°C–121°C (200°F–250°F) as described in 7.7.

4.6 *Cover glass squares,* 18×18 or 22×22 mm, No. 1½ and *microscope slides,* glass, 76×25 mm (3×1 in.).

4.7 *Forceps,* cover glass, curved tip, Scientific Products (F7020) or equivalent.

5. Reagents

5.1 *Cupric sulfate solution,* saturated: Dissolve 21 g $CuSO_4$ in 100 ml distilled water.

5.2 *Formaldehyde-cupric sulfate solution:* Mix 1 liter of 40 percent aqueous formaldehyde containing 10–15 percent methanol, Fisher Scientific No. F–78, or 70 percent ethanol, or equivalent, with 1 ml of solution 5.1.

5.3 *Detergent solution,* 20 percent: Dilute 20 ml liquid detergent (Liqui-Nox, Catalog No. C6308-2, phosphate free, or equivalent) to 100 ml with distilled water.

5.4 *Lugol's solution:* Dissolve 10 g iodine crystals and 20 g potassium iodide in 200 ml distilled water.

5.5 *Caedex,* Ward's Natural Science Establishment (37W9600) or equivalent.

5.6 *Immersion oil,* Cargille's nondrying type A, Scientific Products (M6002–1) or equivalent.

6. Collection

A sample of planktonic diatoms consists of a volume of water, usually 1 liter. To insure maximum correlation of results, the sample sites and methods used for planktonic diatoms should correspond as closely as possible to those selected for chemical and bacteriological 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 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 located at the centroid of flow may be adequate. Study design, collection, and statistics for streams, rivers, and lakes are described in Federal Working Group on Pest Management (1974).

If the sample is to be examined within 2 or 3 hours after collection, no special treatment is necessary. A diatom sample may be maintained for 24 hours at 3°–4°C, but for extended storage, preserve as follows: to each 1,000 ml of sample add about 30 ml of 37–40 percent formaldehyde solution (Formalin), 5 ml of 20 percent detergent solution, and 1 ml of cupric sulfate solution. This preservative maintains cell coloration and is effective indefinitely.

Many biologists consider Lugol's solution to be the best phytoplankton preservative. It is effective for at least 1 year (Weber, 1968); it facilitates sedimentation of cells and maintains fragile cell structures. If Lugol's solution is preferred as a preservative, add 1 ml Lugol's solution to each 100 ml of sample. Store the preserved samples in the dark.

7. Analysis

7.1 If the phytoplankton sample contains great numbers of organisms, as typically occurs in eutrophic waters, the sample must be diluted. To dilute, thoroughly mix 50 ml of sample with 50 ml distilled water (1:1 dilution) and proceed to 7.2. If microscopic examination reveals a concentration of organisms still too numerous to count, thoroughly mix 50 ml of 1:1 dilution with 50 ml distilled water (1:4 dilution). Additional dilutions may be made as appropriate.

7.2 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. Carefully siphon the supernatant to avoid disturbance of the settled material. Place sample container with remaining sample on balance and weigh. The reduction in weight (in grams) is equivalent to the number of milliliters of supernatant removed. The same method can be used to obtain the volume of concentrate.

7.3 If the sample was collected from sea water or saline lakes, the diatoms should be washed with distilled water at least three times to insure that the permanent mounts will not be obscured with 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.

7.4 Place two or three drops of the concentrate on each of three or four cover glasses.

7.5 With the concentrate side up, place the cover glass on a hotplate and heat, slowly at first to prevent splattering, to about 538°C (1,000°F) and incinerate for 30 minutes.

7.6 Remove cover glass from the hotplate and cool.

7.7 Place a drop of Caedex on a microscope slide and heat for 3–4 minutes at about 93°–121°C (200°–250°F).

7.8 Invert the cover glass, concentrate side down, on the heated Caedex. Apply slight pressure to the cover glass (for example, with a pencil eraser). Remove slide from hotplate and allow to cool. If bubbles are present under the cover glass, heat the slide and apply additional pressure to the cover glass. Label slide to identify sample.

7.9 Examine the slide with the × 1000 objective lens (oil immersion). Count and identify diatom taxa found in several lateral strips the width of the Whipple grid. Identify and tabulate 200–300 diatom cells, if possible. Generally, at least 100 individuals of every dominant 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).

8. Calculations

Percent occurrence of each taxa

$$= \frac{\text{number of diatoms of a given taxa}}{\text{total number of diatoms tabulated}} \times 100.$$

9. Report

Report percentage composition of diatoms to the nearest whole number. Report number of taxa and number of organisms per taxa.

10. Precision

No precision data are available.

References

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ZOOPLANKTON

The zooplankton are the animal part of the plankton. Although many species are restricted to standing bodies of water, some forms, such as rotifers and ostracods, also live in streams. Zooplankton are secondary consumers which feed upon bacteria and phytoplankton and are, in turn, consumed by fish. Because they are the grazers in the aquatic environment, the zooplankton are a vital part of the aquatic food web.

Several characteristics of zooplankton affect the sampling methods used in their study. They range widely in size and are capable of extensive movements within the water column, requiring the use of a variety of sampling techniques. Many zooplankton, such as copepods and cladocerans, migrate vertically, approaching the surface at night and sinking to lower depths at dawn. They often maintain relatively constant depths during daylight hours. This vertical movement, as well as the ability of zooplankton to avoid sampling devices, must be considered in their investigation. No single method can conclusively and accurately sample the entire zooplankton community. A number of methods for the collection of zooplankton, as well as procedures for determining their grazing rates and production, are found in the references.

The size of mesh and the other design attributes of a zooplankton net depend on the abundance of organisms in the water and on the expected towing speed of the net. See Barkley (1972) for a discussion of the selectivity of towed-net samplers. Additional considerations in the selection of nets for various applications are given in Edmondson and Winberg (1971) and Unesco Press (1968). In general, when fine-mesh nets are used with a Clark-Bumpus sampler, the ratio of the net mouth opening to the length of the filtering area (effective net length) should be about 1:15. A mesh finer than 0.363 mm should not be used in highly productive (eutrophic) waters, but in unproductive (oligotrophic) waters, nets of 0.076-mm mesh opening may be used. However, frequent clogging of the net is to be expected, and the horizontal towing speed may have to be reduced to about 2.9 km/hr (1.8 miles/hr). In heavy densities of zooplankton, both the towing speed and the length of tow must be reduced.

Nets of 0.202-mm mesh are recommended for general use in Geological Survey studies. The Committee on Oceanography, Biological Methods Panel (1969, p. 47–49) considered microzooplankton as that part of the zooplankton too small to be retained by a 0.202-mm mesh. Such zooplankton are usually sampled by water-sampling bottles or pump. These organisms may be counted using the Sedgwick-Rafter method as given for phytoplankton. Zooplankton retained by a 0.202-mm mesh are considered smaller mesoplankton. Methods for the study of these organisms are given in this section.

Although the collector need not be restricted to the use of the 0.202-mm mesh size, it is important to report the mesh size used when presenting zooplankton results.

Counting chamber method
(B–2501–77)

Parameter and code: Zooplankton, total (organisms /m^3) 70946

1. Application

The method is suitable for all waters.

2. Summary of method

Samples of the zooplankton community are collected, preserved, and examined microscopically for numbers and types of organisms per unit volume of water sampled.

3. Interferences

Suspended materials in the water may interfere with

the collection and microscopic examination of zooplankton.

4. Apparatus

Methods and equipment for the collection and examination of zooplankton are described in Edmondson and Winberg (1971); American Public Health Association and others (1976); Schwoerbel (1970); Pennak (1953); Unesco Press (1968); and Welch (1948). Some common types of equipment used for the collection of zooplankton are listed below:

4.1 *Water-sampling bottle,* Wildlife Supply Co. (1510 or 1920); Scott Instruments, Seattle, Wash.; or Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type); or equivalent. Depth-integrated samplers are discussed in Guy and Norman (1970).

4.2 *Sampling tube or water core,* a weighted thin-walled rubber or plastic tube having a closing device for collecting a relatively large vertical column of water and its associated zooplankton (Edmondson and Winberg, 1971, p. 4).

4.3 *Water pump,* with attached rubber or plastic hose. Water is pumped through nets of various mesh openings to retain the zooplankton (Committee on Oceanography, Biological Methods Panel, 1969, p. 48). If nets finer than No. 16 (0.086-mm openings) are used, phytoplankton also will be retained in the sample and may interfere with the counting of the zooplankton (Schwoerbel, 1970, p. 47–48).

4.4 *Plankton trap* (Juday), a 10-liter closing metal box with attached plankton bucket (0.202-mm mesh openings), Wildlife Supply Co. (33) or equivalent (fig. 16*A*).

4.5 *Plankton nets,* open, or remote-closing types with 0.202-mm mesh openings, Wildlife Supply Co. (40 and 21) or equivalent. The remote-closing nets have greater sampling flexibility because they can be closed at any selected depth (figs. 16*B* and *C*).

4.6 *Clarke-Bumpus plankton sampler,* with 0.202-mm mesh netting, Wildlife Supply Co. (37), Kahl Scientific Instrument Corp. (012WA300), or equivalent. An impeller at the net opening registers the volume of water filtered through the net. The Clarke-Bumpus sampler is most often used for horizontal tows, but it also may be used for vertical tows (fig. 16*D*).

4.7 *Nylon monofilament screen cloth,* 0.202-mm mesh opening. Tobler, Ernst, and Traber, Inc., Nitex, or equivalent.

4.8 *Graduated cylinders,* plastic, Bel-Art Products, Nalgene Labware, or equivalent, of sufficient capacity (100, 500, and 1,000 ml are convenient sizes) for measuring known volumes of water samples.

4.9 *Sample containers,* glass or plastic bottles, or sealable plastic bags, Nasco, Whirl-Pak, or equivalent, are satisfactory.

4.10 *Beaker,* 250-ml capacity, for use as a mixing vessel for zooplankton samples.

4.11 *Piston or Hensen-Stempel pipet,* 4-mm diameter, Wildlife Supply Co. (1805) or equivalent, for taking subsamples from zooplankton samples.

4.12 *Spatula,* for stirring samples.

4.13 *Counting chambers, Sedgwick-Rafter counting cell,* APHA, 50×20×1 mm, A. H. Thomas Co. (9851–C20) or equivalent, with *cover glass,* A. H. Thomas Co. (9851–C25) or equivalent, is used in counting small samples. For sample aliquots greater than 1 ml, *open counting cells,* InterOcean Systems, Inc., Plankton Sorting Tray (304), or equivalent, are used. The construction of large volume cells is discussed in Edmondson and Winberg (1971, p. 131–132). Thinner counting cells, such as the Palmer-Maloney cell, Wildlife Supply Co. (1803), or equivalent, may be needed for organisms smaller than 10 μm (Edmondson and Winberg, 1971, p. 135).

4.14 *Microscope,* binocular, flat-field, zoom lens, with illuminator, Bausch & Lomb (PB-252) or equivalent, for the smaller organisms. For the larger zooplankton a binocular, wide-field dissecting microscope, Bausch & Lomb (31–26–29–73) or equivalent, is adequate.

4.15 *Ocular micrometer,* Whipple grid, Bausch & Lomb (31–16–13) or equivalent.

5. Reagents

5.1 *Formaldehyde solution,* 2 percent: 5 ml of 37–40 percent aqueous formaldehyde solution (Formalin) diluted to 100 ml with distilled water.

Note: Commercial formaldehyde solution is slightly acid and may be neutralized by maintaining a small deposit of sodium or calcium carbonate in the stock bottle.

5.2 *Glycerin,* used to prevent drying of stored zooplankton samples.

6. Collection

Zooplankton collection methods are discussed in Edmondson and Winberg (1971, p. 1–20) and Schwoerbel (1970, p. 37–52). Several of the commonly used techniques are described below. The study objectives must be considered in selecting appropriate methods of collection. However, to insure maximum correlation of results, the sample sites and methods

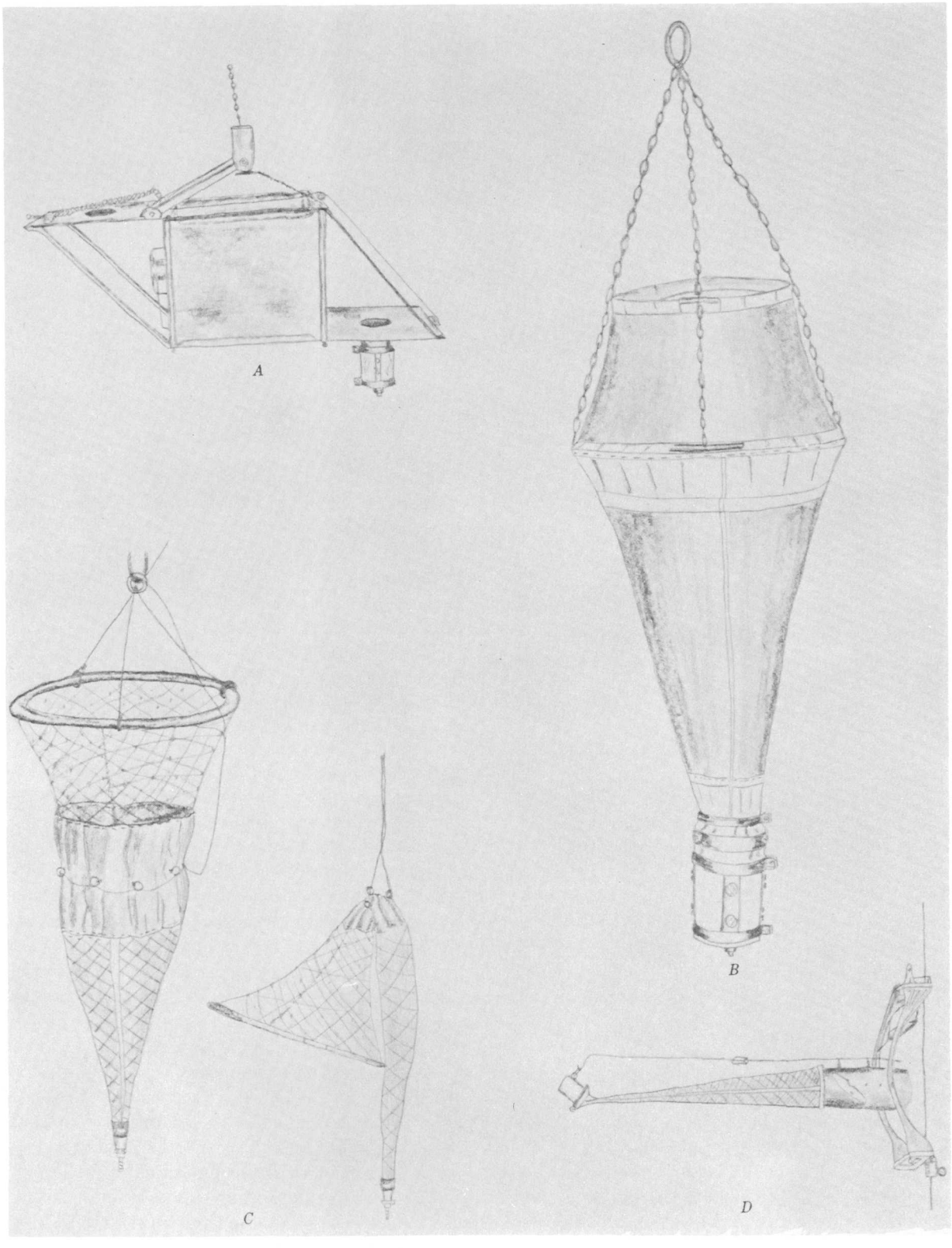

Figure 16.—Examples of zooplankton-collecting devices. *A,* Plankton trap (Juday) in open position. *B,* Standard "*Wisconsin*"-*type* plankton net. *C,* Closing plankton net shown in open and closed positions. *D,* Clarke-Bumpus plankton sampler.

used for zooplankton should correspond as closely as possible to those selected for other biological, bacteriological, and chemical sampling.

6.1 *Water-sampling bottle.* In lakes, reservoirs, deep rivers, and estuaries, zooplankton abundance may vary transversely, with depth, and with time of day. To collect a sample representative of the zooplankton density 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 located at the centroid of flow, may be adequate.

Pour the contents of the sampling bottle through a 0.202-mm monofilament screen cloth which retains the zooplankton. Proceed to 7.1 below.

6.2 *Sampling tube or water core.* This method of sampling is appropriate when information on the vertical distribution of zooplankton is not required. An additional limitation of the method is that strong-swimming zooplankton may avoid capture. To take a sample, lower the flexible tube to the desired depth enclosing a core of water. Retrieve the sampling tube by pulling on a rope that is connected between two rings about 10 cm apart at the base of the tube. This action closes the tube (Edmondson and Winberg, 1971, p. 4). Empty the tube through a 0.202-mm nylon monofilament screen cloth which retains the zooplankton. Proceed to 7.1 below.

6.3 *Water pump.* The water pump method is widely used for quantitative studies (Schwoerbel, 1970, p. 47–48; Edmondson and Winberg, 1971, p. 12). It has the advantage of easily collecting large volumes of water from various depths.

Submerge a flexible tube attached to a pump to a preselected depth. Flush the tube with an amount of water equal to three times its volume to eliminate water that entered when the tube was lowered. Pump a measured volume of water through a 0.202-mm nylon monofilament screen cloth that retains the zooplankton. Proceed to 7.1 below.

6.4 *Plankton trap.* Lower an open plankton trap to a predetermined depth and close by dropping a messenger. Retrieve the trap and allow the water to drain through the attached plankton bucket. Wash the zooplankton from the plankton bucket into a sample container and proceed to 7.1 below.

6.5 *Plankton net.* Plankton nets are particularly useful for qualitative studies of zooplankton. Lower the net to a known depth and retrieve to sample a vertical column of water. Concentrate the zooplankton in the removable bucket attached to the end of the net by repeated washing with water. Wash the zooplankton from the plankton bucket into a sample container and proceed to 7.1 below. Open plankton nets may become clogged and lose sampling efficiency during long retrieval. Nets that can be closed at a preselected depth by dropping a messenger are advantageous in these conditions. In general, a large ratio of filtering surface to mouth-opening area reduces clogging. Therefore, long nets are more efficient than short nets.

6.6 *Clarke-Bumpus plankton sampler.* This device consists of a net and flow meter mounted on a horizontal frame. The net is opened and closed with a messenger. By knowing the initial and final reading on the counter of the flow meter, the volume of water that has passed through the net can be determined (Edmondson and Winberg, 1971, p. 6–12; Schwoerbel, 1970, p. 45).

To take a sample, record the initial reading on the flow-meter dials. Lower the sampler to the selected depth and open the net by dropping a messenger. After towing the sampler for a known interval of time or distance, close the net with another messenger and retrieve. Record the final reading on the flow-meter dials. Concentrate the zooplankton in the removable bucket by repeated washings. Wash the zooplankton from the plankton bucket into a sample container and proceed to 7.1 below.

7. Analysis

7.1 If the zooplankton have been concentrated on monofilament screen cloth, transfer the cloth with the sample to a sample container. Preserve the samples with 2 percent formaldehyde solution (5 percent Formalin). Add several drops of glycerin to the sample to prevent drying during storage. Label the sample with the volume of water filtered or with the information needed to determine this value. For example, record the length of a vertical net tow and the diameter of the net opening.

Count the zooplankton using one of the methods below (7.3 or 7.6). The taxonomic keys in Edmondson (1959), Pennak (1953), and Needham and Needham (1962) are used to identify the different taxa of zooplankton for qualitative analysis and for the calculations of percent species composition.

7.2 Adjust the zooplankton sample to some convenient volume of suspension, such as 50 or 100 ml $\pm$5, by adding or removing preservative solution.

7.3 Sedgwick-Rafter method. With the counting cell on a flat surface, place the cover glass diagonally

across the cell. Thoroughly mix the sample, remove a 1-ml aliquot with a Hensen-Stempel pipet, and transfer the aliquot to the Sedgwick-Rafter counting cell. As the counting cell fills, the cover glass will often 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 counting cell to stand for 15–20 minutes so that the organisms will settle.

7.4 Carefully place the counting cell on the mechanical stage of a calibrated microscope.

7.5 At 100 × magnification, count the total number of zooplankton enclosed by the Whipple ocular grid. Consider any organism touching two intersecting borders of the grid as being enclosed by the grid, but do not count those that touch the opposite borders. Count and record the total number of organisms in each of 20 random fields. When counting random fields, enumerate and record the total number of organisms in the vertical column delimited by the Whipple ocular micrometer.

Note: Moving the counting cell in a vertical plane can prevent motion sickness for the observer.

7.6 Open-counting-chamber method. With this method, the entire contents can be counted or, with the aid of etched or painted guidelines on the bottom, the zooplankton can be counted in random sections of the chamber to determine an average density. An advantage of the open chamber is the accessibility of the contents during counting. The Whipple ocular micrometer is not needed since sections of the cell are delineated. Several drops of liquid detergent can be added to the cell to decrease the surface tension and prevent floating of the zooplankton. A binocular microscope is adequate to count the organisms.

8. Calculations

8.1 Percent species composition in sample

$$= \frac{\text{number of individuals of a particular taxon}}{\text{total number of individuals of all taxa}} \times 100.$$

8.2 For Sedgwick-Rafter method:

$$\text{Calibration factor} = \frac{1{,}000\ \text{mm}^2}{\text{area of Whipple disk at} \times 100\ \text{magnification}\ (\text{mm}^2)}.$$

8.3 For Sedgwick-Rafter method:

$$\text{Zooplankton/ml of concentrated sample} = \frac{\text{total count}}{\text{number of random fields} \times 1\ \text{ml}} \times \text{calibration factor}.$$

8.4 For Sedgwick-Rafter method:

$$\text{Total zooplankton/m}^3 = \frac{\text{zooplankton/ml of conc. sample} \times \text{total vol. of conc. sample (ml)}}{\text{vol. of water sampled (liters)}} \times \frac{1{,}000\ \text{(liters)}}{(\text{m}^3)}.$$

8.5 For open-counting chamber method:

$$\text{Total zooplankton/m}^3 = \frac{\text{average count/section} \times \text{number of sections} \times \text{total vol. of conc. sample (ml)}}{\text{vol. of chamber (ml)} \times \text{vol. of water sampled (liters)}} \times \frac{1{,}000\ \text{(liters)}}{(\text{m}^3)}.$$

9. Report

Report zooplankton densities as total number of organisms/m³ to two significant figures.

10. Precision

No numerical precision data are available.

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American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater [14th ed.]: New York, Am. Public Health Assoc., 1193 p.

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Gravimetric method for biomass
(B–2520–77)

Parameters and codes:
Zooplankton, dry weight (g/m³) 70947
Zooplankton, ash weight (g/m³) 70948

1. Application

The method is suitable for all waters.

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 organic matter 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 high dry and ash weights; nonliving organic matter in the sample will cause erroneously high dry and organic weights.

4. Apparatus

Methods and equipment for the collection and examination of zooplankton are described in Edmondson and Winberg (1971); American Public Health Association and others (1976); Schwoerbel (1970); Pennak (1953); Unesco Press (1968); and Welch (1948). Some common types of equipment used for the collection of zooplankton are listed below:

4.1 *Water-sampling bottle,* Wildlife Supply Co. (1510 or 1920); Scott Instruments, Seattle, Wash.; or Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type); or equivalent. Depth-integrated samplers are discussed in Guy and Norman (1970).

4.2 *Sampling tube or water core,* a weighted, thin-walled rubber or plastic tube with a closing device for collecting a relatively large vertical column of water with its associated zooplankton (Edmondson and Winberg, 1971, p. 4).

4.3 *Water pump,* with attached rubber or plastic hose. Water is pumped through nets of various mesh openings to retain the zooplankton (Committee on Oceanography, Biological Methods Panel, 1969, p. 48). If nets finer than No. 16 (0.086-mm openings) are used, phytoplankton also will be retained in the sample and may interfere with the counting of the zooplankton (Schwoerbel, 1970, p. 47–48).

4.4 *Plankton trap* (Juday), a 10-liter closing metal box with attached plankton bucket (0.202-mm mesh openings), Wildlife Supply Co. (33) or equivalent (fig. 16*A*).

4.5 *Plankton nets,* open, or remote-closing types with 0.202-mm mesh openings, Wildlife Supply Co. (40 and 21) or equivalent. The remote closing nets have greater sampling flexibility because they can be closed at any selected depth (fig. 16*B* and *C*).

4.6 *Clarke-Bumpus plankton sampler* with 0.202-mm mesh netting, Wildlife Supply Co. (37), Kahl Scientific Instrument Corp. (012WA300), or equivalent. An impeller at the net opening registers the volume of water filtered through the net. The Clarke-Bumpus sampler is most often used for horizontal tows, but it also may be used for vertical tows (fig. 16*D*).

4.7 *Nylon monofilament screen cloth,* 0.202-mm mesh opening, Tobler, Ernest, and Traber, Inc., Nitex, or equivalent.

4.8 *Graduated cylinders,* plastic, Bel-Art Products, Nalgene Labware, or equivalent, of sufficient capacity (100, 500, and 1,000 ml are convenient sizes) for measuring known volumes of water samples.

4.9 *Sample containers,* plastic, bottles, vials or sealable bags, Nasco, Whirl-Pak, or equivalent.

Note: Do not use glass containers for samples to be frozen.

4.10 *Beaker,* 250-ml capacity, for use as a mixing vessel for zooplankton samples.

4.11 *Piston or Hensen-Stempel pipet,* 4-mm diameter, Wildlife Supply Co. (1805) or equivalent, for taking subsamples from zooplankton samples.

4.12 *Homogenizer,* VirTis (23), or equivalent.

4.13 *Drying oven,* thermostatically controlled for use at 105°C.

4.14 *Porcelain crucibles.*

4.15 *Desiccator* containing silica gel.

4.16 *Forceps or tongs.*

4.17 *Muffle furnace* for use at 500°C.

4.18 *Balance* capable of weighing at least 0.1 mg.

5. Reagents

5.1 *Distilled water.*

5.2 *Formaldehyde solution,* 2 percent (5 percent Formalin).

6. Collection

Zooplankton collection methods are discussed in Edmondson and Winberg (1971, p. 1–20) and Schwoerbel (1970, p. 37–52). Several of the commonly used techniques are described below. The study objectives must be considered in selecting appropriate methods of collection. However, to insure maximum correlation of results, the sample sites and methods used for zooplankton should correspond as closely as possible to those selected for other biological, bacteriological, and chemical sampling.

6.1 Water-sampling bottle. In lakes, reservoirs, deep rivers, and estuaries, zooplankton abundance may vary transversely, with depth, and with time of day. To collect a sample representative of the zooplankton density 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 located at the centroid of flow may be adequate.

Pour the contents of the sampling bottle through a 0.202-mm monofilament screen cloth which retains the zooplankton. Proceed to 7.1 below.

6.2 Sample tube or water core: This method of sampling is appropriate when information on the vertical distribution of zooplankton is not required. An additional limitation of the method is that strong-swimming zooplankton may avoid capture. To take a sample, lower the flexible tube to the desired depth enclosing a core of water. Retrieve the sampling tube by pulling on a rope that is connected between two rings about 10 cm apart at the base of the tube. This action closes the tube (Edmondson and Winberg, 1971, p. 4). Empty the tube through a 0.202-mm nylon monofilament screen cloth which retains the zooplankton. Proceed to 7.1 below.

6.3 Water pump. The water pump method is widely used for quantitative studies (Schwoerbel, 1970, p. 47–48; Edmondson and Winberg, 1971, p. 12). It has the advantage of easily collecting large volumes of water from various depths.

Submerge a flexible tube attached to a pump to a preselected depth. Flush the tube with an amount of water equal to three times its volume to eliminate water that entered when the tube was lowered. Pump a measured volume of water through a 0.202-mm nylon monofilament screen cloth which retains the zooplankton. Proceed to 7.1 below.

6.4 Plankton trap. Lower an open plankton trap to a predetermined depth and close by dropping a messenger. Retrieve the trap and allow the water to drain through the attached plankton bucket. Wash the zooplankton from the plankton bucket onto a 0.202-mm monofilament screen cloth which retains the zooplankton. Proceed to 7.1 below.

6.5 Plankton net. Plankton nets are particularly useful for qualitative studies of zooplankton. Lower the net to a known depth and retrieve to sample a vertical column of water. Concentrate the zooplankton in the removable bucket attached to the end of the net by repeated washing with water. Wash the zooplankton from the plankton bucket onto a 0.202-mm monofilament screen cloth which retains the zooplankton. Proceed to 7.1 below. Open plankton nets may become clogged and lose sampling efficiency during long retrievals. Nets that can be closed at a preselected depth by dropping a messenger are advantageous. In general, a large ratio of filtering surface to mouth-opening area reduces clogging. Therefore, long nets are more efficient than short nets.

6.6 Clarke-Bumpus plankton sampler. This device consists of a net and flowmeter mounted on a horizontal frame. The net is opened and closed with 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 (Edmondson and Winberg, 1971, p. 6–12; Schwoerbel, 1970, p. 45).

To take a sample, record the initial reading on the flowmeter dials. Lower the sampler to the selected depth and open the net by dropping a messenger. After towing the sampler for a known interval of time or distance, close the net with another messenger and retrieve. Record the final reading on the flowmeter

dials. Concentrate the zooplankton in the removable bucket by repeated washings. Wash the zooplankton from the plankton bucket onto a 0.202-mm monofilament screen cloth which retains the zooplankton. Proceed to 7.1 below.

7. Analysis

7.1 Wash the screen cloth containing the zooplankton by dipping several times in distilled water, place in a plastic bag, and preserve in the field by freezing with dry ice. Keep frozen until gravimetric determinations can be made (Committee on Oceanography, Biological Methods Panel, 1969, p. 57). If the samples cannot be kept frozen, preserve in 2 percent neutralized formaldehyde solution (5 percent Formalin), but use the same preservation method consistently throughout a study. Label the sample with the volume of water filtered or with the information needed to determine this value. For example, record the length of a vertical net tow and the diameter of the net opening.

7.2 Place the zooplankton samples in a beaker and thaw if necessary. Add a measured volume of distilled water to bring the contents of the beaker above the blades of a homogenizer and homogenize the sample (Committee on Oceanography, Biological Methods Panel, 1969, p. 57–58).

7.3 Obtain the tare weight of a crucible that has been held at 500°C for 20 minutes and cooled to room temperature in a desiccator.

7.4 Place the zooplankton suspension from a known volume of water sample into the tared crucible and dry in an oven to constant weight at a temperature no higher than 105°C.

Note: 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 dried residue. These values are used to calculate dry weight.

7.5 Place the crucible containing the dried residue in a muffle furnace for 1 hour at 500°C. Cool to room temperature.

7.6 Moisten the ash with distilled water and again ovendry at 105°C to constant weight as described in 7.4. These weight values are used to calculate ash weight.

8. Calculations

8.1 Dry weight of zooplankton (g/m^3)

$$= \frac{\text{dry wt of crucible and residue (g)} - \text{tare wt of crucible (g)}}{\text{vol. of water sampled (liters)}} \times \frac{1{,}000\,\text{(liters)}}{(m^3)}.$$

8.2 Ash weight of zooplankton $(g/m)^3$

$$= \frac{\text{dry wt of crucible and residue (g)} - \text{tare wt of crucible (g)}}{\text{vol. of water sampled (liters)}} \times \frac{1{,}000\,\text{(liters)}}{(m^3)}.$$

8.3 Volatile or organic weight of zooplankton (g/m^3) = dry weight (g/m^3) − ash weight (g/m^3).

9. Report

Report biomass to two significant figures.

10. Precision

No numerical precision data are available.

References

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SESTON
(total suspended matter)

The weight of suspended matter in water is a significant 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 purposes the sample may be prefiltered through a 150- to 350-μm mesh to eliminate large particles. The particulate residue remaining in the sample after prefiltration is designated microseston.

The method described below is the glass-fiber-filter adaptation by Strickland and Parsons (1968) of the method of Banse, Falls, and Hobson (1963).

Glass-fiber filter method
(B–3401–77)

Parameters and codes:
Seston, dry weight (mg/l) 71100
Seston, ash weight (mg/l) 71101

1. Application

The method is suitable for all waters.

2. Summary of method

A known volume of water is passed 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 material in the sample. After ashing the residue at 500°C, the difference between dry weight and ash weight is taken as the weight of particulate organic matter in the sample.

3. Interferences

Although the method is generally free from interferences, it is essential that bottles and sampling equipment be clean and that samples, filters, and funnels be protected from dust. Filtration should be at reduced pressure to avoid rupture and loss of cell contents of fragile organisms. Saline samples must have the salts washed from the filter residues to prevent erroneous weight values.

4. Apparatus

4.1 *Glass filters,* Whatman, GF/C grade, or equivalent, 47-mm-diameter disks. For best results all filters for a series of samples, including control filters, should be from the same box and should have a tare weight within about 10 mg on 70- to 100-mg weights.

4.2 *Filter funnel,* vacuum, 1,200-ml capacity, stainless steel, Gelman Instrument Co. (Parabella) or equivalent.

4.3 *Filter flask,* 1,000 or 2,000 ml. For field use a polypropylene flask, Bel-Art Products (H–38941), Nalgene Labware (4101), or equivalent is suggested.

4.4 *Source of vacuum* for filtration: a water-aspirator pump or an electric vacuum pump for use in the laboratory; a hand-held vacuum pump with gauge, Edmund Scientific Co. (71,301) or equivalent, for use in the field.

4.5 *Manostat* with mercury and calibration equipment to regulate the filtration suction at not more than 300 to 350 mm of mercury when filtering with an aspirator or an electric vacuum pump.

4.6 *Forceps,* stainless steel, smooth tip, Millipore (XX62 000 06) or equivalent.

4.7 *Balance* capable of weighing to at least 0.1 mg.

4.8 *Plastic petri dishes* with *covers* for filter storage, Millipore (PD10 047 00) or equivalent.

4.9 *Desiccator* containing silica gel.

4.10 *Aluminum foil,* laboratory grade.

4.11 *Drying oven,* thermostatically controlled for use at 105°C.

4.12 *Muffle furnace,* for use at 500°C.

4.13 *Graduated cylinders* of a size suitable to the volume of water to be filtered. Plastic cylinders, Bel-Art Products, Nalgene Labware, or equivalent of 500- and 1,000-ml capacity are convenient for field use.

4.14 *Water-sampling bottle,* Wildlife Supply Co. (1510 or 1920); Scott Instruments, Seattle, Wash.; or Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type), or equivalent. Depth-integrated samplers are discussed in Guy and Norman (1970).

4.15 *Sample containers,* plastic bottles, 1-liter capacity.

5. Reagents

5.1 *Mercuric chloride solution,* 1 ml containing 40 mg Hg^{2+}: Dissolve 55.0 g $HgCl_2$ in distilled water and dilute to 1,000 ml.

5.2 *Distilled water.* Filter if in doubt as to the freedom from particles.

6. 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 representative of the seston at a particular depth, use a water-sampling bottle (figs. 11 and 12). 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 statistics for streams, rivers, and lakes are described in Federal Working Group on Pest Management (1974).

Adjust the sample volume to the amount of suspended material present. Very clear waters will require 3 or 4 liters of sample; waters with a high sediment or phytoplankton content may require only 500 ml or less. Filter the maximum volume that will not clog the filter.

Seston samples should be filtered as described in sections 7.9 and 7.10 below, immediately after collection. Record the mesh size of prefilter, if used. Record the volume of water filtered. The filters should be thoroughly dried or stored in tightly closed plastic petri dishes at 1°–4°C (do not freeze) until ovendrying. Samples that cannot be filtered without delay should be preserved with 40 mg Hg^{2+}/1 (1 ml of the mercuric chloride solution per liter of sample). This method for seston 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.

7. Analysis

7.1 Arrange the required number of glass filters without overlap onto the shiny side of aluminum foil and heat to 450°–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.

7.2 Designate 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.

7.3 Handle the cooled filters very carefully using clean, smooth tip forceps to avoid fraying the fibers. Transfer the filters including the controls to a shallow container of distilled water for 5 minutes. Allow about 100 ml of water for each filter.

7.4 With forceps, transfer the filters to 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: Because of the difficulty of marking glass filters, it is necessary to keep track of individual filter disks throughout the remaining steps. The disks should be placed on the aluminum foil in a definite sequence and, whenever possible, each disk should be kept in a numbered container.

7.5 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.

7.6 When a sample is to be filtered, place a tared filter disk, wrinkled surface upward, on a membrane-filter apparatus. A small slip of aluminum foil under

the edge of the disk facilitates removal of the wet filters.

7.7 With vacuum applied, wet the filter with distilled water to seat the disk on the filter base plate.

7.8 Measure out a suitable amount 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 control vacuum to 300–350 mm (about 12 in.) of mercury (about 6 psi).

7.9 With vacuum on, wash the filter and funnel three times with 5–10 ml volumes of distilled water allowing the filter to suck "dry" between each wash.

7.10 Disconnect the vacuum and, with smooth tip forceps, remove the wet filter to the shiny side of aluminum foil. The filters may be stored at 1°–4°C in numbered petri dishes at this stage, if necessary.

7.11 Dry the filters in an oven at 105°C for 1 hour. Include at least two control filters from 7.5 above in this drying step for each batch of sample filters.

7.12 Place the filters in a desiccator, cool, and reweigh each disk rapidly to the nearest 0.1 mg as in 7.5 above. Include the control filters from 7.11. These values are used to calculate dry weight.

7.13 Again place the filters with their dried residue and the control filters on the shiny side of aluminum foil and heat in a muffle furnace at 500°C for 30 minutes.

7.14 Place the filters in a desiccator, cool, and reweigh each filter rapidly to the nearest 0.1 mg as in 7.5 above. Include the control filters from 7.13. These values are used to calculate the ash weight.

8. Calculations

8.1 Dry weight of seston (mg/l)

$$= \frac{\text{dry weight of filter and residue (mg)} - \text{tare weight of filter (mg)}}{\text{volume of sample (liters)}}$$

where blank correction (mg) = mean weight of control filters in mg (from 7.12) − mean weight of control filters in milligrams (from 7.5).

The blank correction value may be positive or negative, but should not exceed about 0.5 mg.

8.2 Ash weight of seston (mg/l)

$$= \frac{\text{ignition weight of filter and residue (mg)} - \text{tare weight of filter (mg)}}{\text{volume of sample (liters)}}$$

where blank correction (mg) = mean weight of control filters in mg (from 7.14) − mean weight of control filters in milligrams (from 7.5).

The blank correction value may be positive or negative, but should not exceed about 0.5 mg.

8.3 Volatile or organic weight of seston (mg/l) = dry weight of seston (mg/l) − ash weight of seston (mg/l).

9. Report

Report seston as follows: Less than 1 mg/l, one significant figure; 1 mg/l and above, two significant figures.

10. Precision

No numerical precision data are available.

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PERIPHYTON

Periphyton literally refers to plants growing around (upon) solid surfaces. However, the term has been extended to include the entire community of microorganisms that are attached to or live upon submerged solid surfaces (Young, 1945; Sladecek and Sladeckova, 1964; Wetzel, 1964). The term, therefore, encompasses not only attached plants (algae) but the associated bacteria, fungi, protozoans, rotifers, and other small organisms. As used here, periphyton is synonymous with the term "Aufwuchs" as given by Ruttner (1963, p. 183).

Although methods have been developed for collecting periphyton samples from natural substrates (Douglas, 1958; Ertl, 1971; Stockner and Armstrong, 1971), biomass and production measurements more commonly have been made using artificial substrates (Nielson, 1953; Grzenda and Brehmer, 1960; Maciolek and Kennedy, 1964; Neal and others, 1967; Peters and others, 1968; Tilley and Haushild, 1975a, 1975b). The artificial substrate standardizes the physical environment.

The following methods describe the collection and measurement of periphyton on both natural and artificial substrates. Quantitative measurements of periphyton are usually made with the use of artificial substrates, but periphyton collected from known areas of natural substrates also may be used. Periphyton collected from natural or artificial substrates may be used for qualitative studies.

Sedgwick-Rafter method
(B–3501–77)

Parameter and code: Periphyton, total (cells/mm^2) 70945

1. Application

The method quantifies the plant part of the periphyton. It is suitable for all waters.

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

Suspended or deposited sediment and excessive growth may interfere with collection procedures and with microscopic examination.

4. Apparatus

4.1 *Artificial substrates* made of glass slides, Plexiglas, polyethylene strips, or other materials, Kahl Scientific Instrument Corp. (003WA250, 003WA260, 003WA270), Craftsman Designers, Inc. (Periphytometer), or equivalent. See figures 17 and 18 for selected types of artificial substrates.

4.2 *Collecting devices* for the removal of periphyton from natural substrates. Three such devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 19.

4.3 *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).

4.4 *Sample containers* of glass or plastic suitable for the types of samples. Sturdy plastic bags are useful containers for artificial substrates or for pieces of natural substrate.

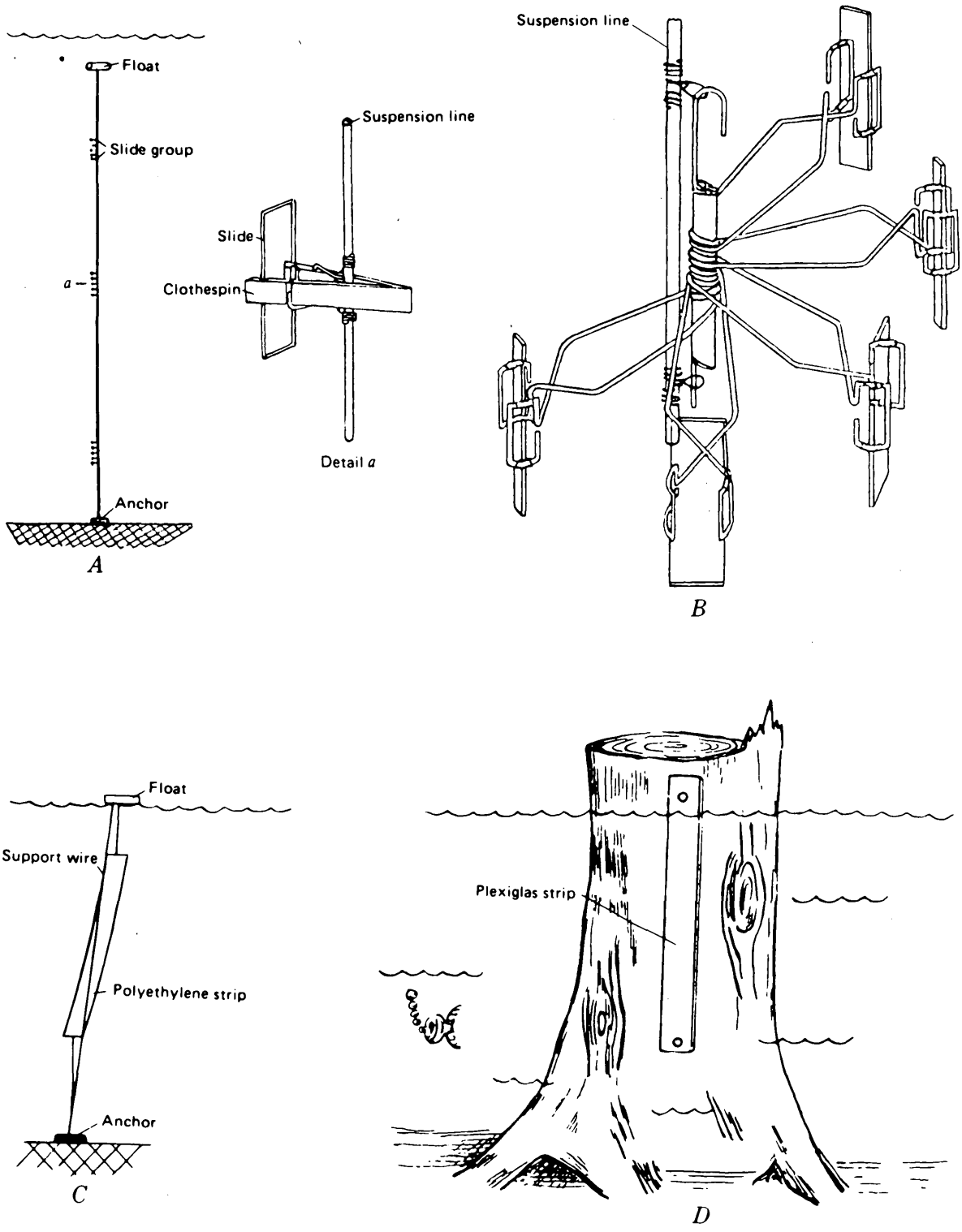

Figure 17.—Artificial-substrate sampling devices for periphyton. *A,* Microscope slide-suspension apparatus made of spring clothespins. (From Nielson, 1953, p. 99) *B,* Microscope slide-suspension apparatus made of test-tube clamps. (With permission from U.S. Bureau of Sport Fisheries and Wildlife.) *C,* Polyethylene strip apparatus. (Modified from Neal and others, 1967.) *D,* Plexiglas strip attached to submerged object.

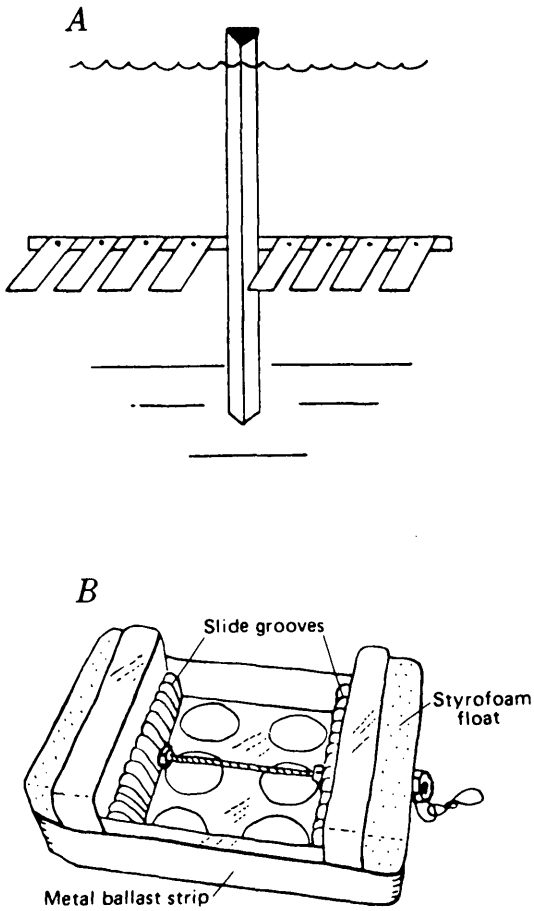

Figure 18—Artificial-substrate sampling devices for periphyton. *A*, Plexiglas plates attached to support. (From Peters and others, 1968, p. 12.) *B*, Floating sampler made from a plastic microscope-slide box, 25-slide capacity.

4.5 *Microscope,* binocular, flat-field, zoom lens with illuminator, Bausch & Lomb (PB–252) or equivalent.

4.6 *Ocular micrometer,* Whipple grid, Bausch & Lomb (31–16–13) or equivalent.

4.7 *Sedgwick-Rafter counting cell,* APHA, 50201 mm, A. H. Thomas Co. (9851–C20) or equivalent, with *cover glass,* A. H. Thomas Co. (9851–C25) or equivalent.

4.8 *Pipet,* transfer, 1 ml, large bore.

5. Reagents

5.1 *Cupric sulfate solution,* saturated: Dissolve 21 g $CuSO_4$ in 100 ml distilled water.

5.2 *Formaldehyde-cupric sulfate solution:* Mix 1 liter of 40 percent aqueous formaldehyde containing 10–15 percent methanol, Fisher Scientific No. F–78, or equivalent, with 1 ml of solution 5.1.

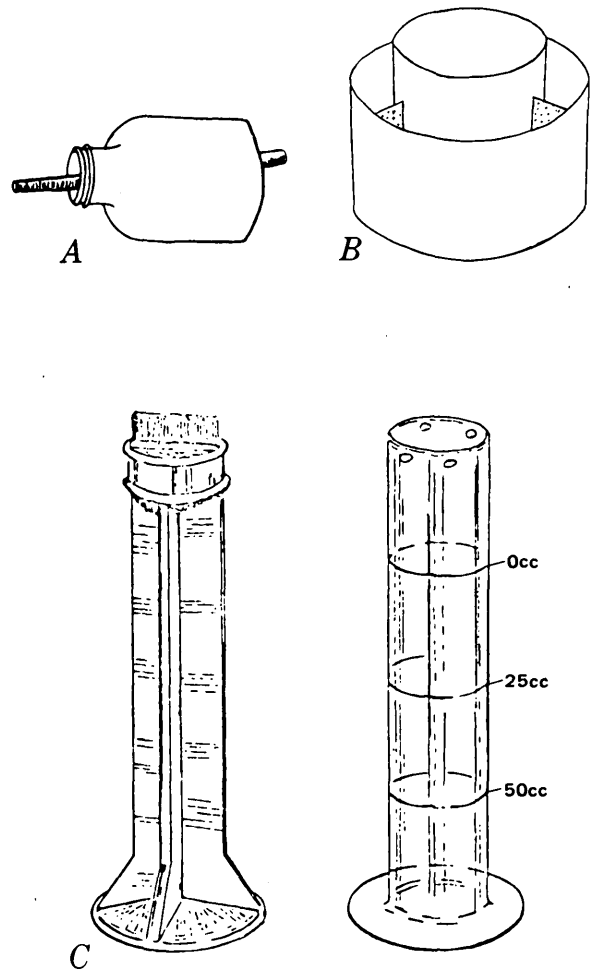

Figure 19—Examples of devices for collecting periphyton from natural or artificial substrates. *A*, Brush and polyethylene-bottle device. (Modified from Douglas, 1958, p. 297) *B*, Plastic or metal cylinder device. (Redrawn from Ertl, 1971, p. 576.) *C*, Plastic hypodermic syringe device. (Redrawn from Stockner and Armstrong, 1971, p. 218.)

5.3 *Detergent solution,* 20 percent: Dilute 20 ml liquid detergent (LiquiNox, Catalog No. C6308–2, phosphate free, or equivalent) to 100 ml with distilled water.

5.4 *Lugol's solution:* Dissolve 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 using; store in amber glass bottles (Vollenweider, 1969).

6. Collection

6.1 Artificial substrates. Place a suitable artificial substrate in the stream or lake and attach it to a supporting object. Figures 17 and 18 illustrate several types of artificial substrates. The substrates must be submerged

but may be near the surface of the water or at any other appropriate depth. In lakes, the substrates usually are suspended at several depths (fig. 17*A*, *B*, *C*). In lakes and streams the substrates may be attached to natural objects such as submerged trees, stumps (fig. 17*D*), logs or boulders, or they may be attached to stakes driven into the bottom (fig. 18*A*). Floating samplers may be used (fig. 18*B*). The artificial substrates must be exposed to the light so that photosynthesis can take place, and they should be located so that damage to the apparatus by floating debris is minimized. Vandalism is a common problem and placing the substrates away from frequently visited areas is advisable. The length of time required for colonization of the substrates by periphyton will depend upon the season, water temperature, light and nutrient availability and other factors. Neal, Patten, and DePoe (1967) found that the maximum accumulation of periphyton biomass on polyethylene strips occurred in about 2 weeks. Exposure probably should be at least 14 days, but this will vary and must be determined for each season and water type.

Tilley and Haushild (1975a, b) found in the Duwamish River, Wash., that 21 glass microscope slides exposed for 2 weeks at a single site ranged in chlorophyll *a* from 13.3 to 28.1 mg/m^2, with a mean of 19.7 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 ranged in chlorophyll *a* from 18.9 to 48.6 mg/m^2, with a mean of 34.4 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 14.4 mg/m^2.

After sufficient colonization of periphyton, indicated by visible green or brown growth, remove the artificial substrate from the water. Periphyton may be scraped from the substrate, in the field or in the laboratory, as described in 4.3 above. 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 for 24 hours at 3°–4°C, but for extended storage, preserve as follows: To each 100 ml of water and sample, add about 3 ml of 40 percent formaldehyde solution (100 percent Formalin), 0.5 ml of 20 percent detergent solution, and 5–6 drops of cupric sulfate solution. This preservative maintains cell coloration and is effective indefinitely.

Many biologists consider Lugol's solution to be the best algal preservative. It 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 the solution to each 100 ml of water added to the scraped periphyton sample. Store the preserved samples in the dark.

6.2 Natural submerged substrates often contain periphyton, a known area of which 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 19. The instrument used by Douglas (1958) consists of a broad-necked polyethylene bottle with the bottom removed (fig. 19*A*). 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 with a stiff nylon brush. The loose periphyton is removed from the bottle with a pipet. Ertl's (1971) apparatus consists of two concentric metal or plastic cylinders separated with spacers (fig. 19*B*). The space between the cylinders is filled with modeling clay, and the sampler is pressed firmly against the substrate to be sampled. With a blunt stick or metal rod the clay is forced down onto the substrate so as to isolate the sampling area of the inner circle. The periphyton within the inner circle is dislodged with a stiff brush and removed with a pipet. Stockner and Armstrong (1971) sampled periphyton with a plastic hypodermic syringe which had a toothbrush attached to the end of the syringe piston (fig. 19*C*). With the barrel of the syringe held tightly against the substrate, the piston is pushed in until the brush contacts the periphyton. The piston is then rotated several times to dislodge the periphyton and then is withdrawn, pulling the periphyton up with it. A glass plate is immediately placed under the end of the barrel and the syringe inverted. Four small holes at the base of the syringe allow for free movement of water when procuring the sample (J. C. Stockner, written commun., March 1972).

Immediately proceed to 7.1 below or preserve as follows: To each 100 ml of water and sample, add about 3 ml of 2 percent formaldehyde solution (5 percent Formalin), 0.5 ml of 20 percent detergent solution, and 5–6 drops of cupric sulfate solution. This preservative maintains cell coloration and is effective indefinitely.

Many biologists consider Lugol's solution to be the best algal preservative. It 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 the solution to each 100 ml of water added to the scraped periphyton sample. Store the preserved samples in the dark.

7. Analysis

7.1 Adjust the scraped periphyton sample to some convenient volume of suspension, such as 50 or 100 ml±5, by adding or removing preservative solution.

7.2 With the Sedgwick-Rafter counting cell on a flat surface, place the cover glass diagonally across the cell. Thoroughly mix the sample, remove a 1-ml aliquot with a large-bore pipet and transfer the aliquot to the Sedgwick-Rafter counting cell. As the counting cell fills, the cover glass will often 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 counting cell to stand for 15–20 minutes so that the organisms will settle.

7.3 Carefully place the counting cell on the mechanical stage of a calibrated microscope. At × 200 magnification, count the total number of algal cells enclosed by the Whipple ocular grid. Consider any cell 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.

Note: Moving the counting cell in a vertical plane can prevent motion sickness for the observer.

Some algae, particularly some blue-green algae, may not settle but instead 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 delimited by the Whipple ocular micrometer. 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. County empty diatom frustules as nonliving. Count frustules containing any part of a protoplast as having been living at the time of collection.

8. Calculations

8.1 Calibration factor

$$= \frac{1{,}000 \text{ mm}^2}{\text{area of Whipple disk at} \times 100 \text{ magnification (mm}^2\text{)}}.$$

8.2 Periphyton cells/ml of suspended scraping

$$= \frac{\text{total cell count} \times \text{calibration factor}}{\text{number of random fields} \times 1 \text{ ml}}.$$

8.3 Total periphyton cells/mm² of surface

$$= \frac{\text{cells/ml of suspended scraping} \times \text{total vol. of scrapings (ml)}}{\text{area of scraped surface (mm}^2\text{)}}.$$

9. Report

Report cell counts to two significant figures.

10. Precision

No numerical precision data are available.

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Gravimetric method for biomass
(B–3520–77)

Parameters and codes:
Periphyton, biomass, dry weight, total (g/m²) 00573
Periphyton, biomass, ash weight (g/m²) 00572

1. Application

The method quantifies all organisms in the periphyton community. It is suitable for all waters.

2. Summary of method

Samples of the periphyton community are collected from known areas of artificial or natural substrates. The dry weight and ash weight are determined, and the weight of organic matter per unit area is calculated.

3. Interferences

Inorganic matter in the sample will cause erroneously high dry and ash weights; nonliving organic matter in the sample will cause erroneously high dry and organic weights.

4. Apparatus

4.1 *Artificial substrates* made of glass slides, Plexiglas, polyethylene strips, or other materials (figs. 17 and 18).

4.2 *Collecting devices* for the removal of periphyton from natural substrates. Three such devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 19.

4.3 *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).

4.4 *Sample containers* of glass or plastic suitable for the types 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.

4.5 *Porcelain crucibles.*

4.6 *Balance* capable of weighing to at least 0.1 mg.

4.7 *Drying oven,* thermostatically controlled for use at 105°C.

4.8 *Muffle furnace,* for use at 500°C.

4.9 *Desiccator* containing silica gel.

4.10 *Forceps* or *tongs.*

5. Reagents

5.1 *Distilled water.*

6. Collection

6.1 Artificial substrates. Place a suitable artificial substrate in the stream or lake and attach it to a supporting object. Figures 17 and 18 illustrate several types of artificial substrates. The substrates must be submerged but may be near the surface of the water or at any other appropriate depth.

In lakes, the substrates are usually suspended at various depths (fig. 17*A, B, C*). In lakes and streams, the substrates may be attached to natural objects such as submerged trees, stumps (fig. 17*D*) logs, or boulders or they may be attached to stakes driven into the bottom (fig. 18*A*). Floating samplers may be used (fig. 18*B*). The artificial substrates must be exposed to the light so that photosynthesis can take place, and they should be located so that damage to the apparatus by floating debris is minimized. Vandalism is a common problem and placing the substrates away from frequently traveled areas is advisable. The length of time required for colonization of the substrates by periphyton will depend upon the season, water temperature, light and nutrient availability, and other factors. Neal, Patten, and DePoe (1967) found that the maximum accumulation of periphyton biomass on polyethylene strips occurred in about 2 weeks. Nielson (1953) exposed his slides for 20–30 days. Exposure probably

should be at least 14 days, but this will vary and must be determined for each season and water type.

After sufficient colonization of periphyton, indicated by visible green or brown growth, remove the artificial substrate from the water. Periphyton may be scraped from the substrate in the field or in the laboratory, as described in 4.3 above. Immediately proceed to 7.2 below, or if ovendrying cannot be started, air-dry or freeze the sample. Begin ovendrying as soon as possible.

6.2 Natural submerged substrates often contain periphyton, a known area of which can be sampled quantitatively. Several devices for removing periphyton from a known area of natural substrates are shown in figure 19. The instrument used by Douglas (1958) consists of a broad-necked polyethylene bottle with the bottom removed (fig. 19*A*). 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 with a stiff nylon brush. The loose periphyton is removed from the bottle with a pipet. Ertl's (1971) apparatus consists of two concentric metal or plastic cylinders separated with spacers (fig. 19*B*). The space between the cylinders is filled with modeling clay and the sampler is pressed firmly against the substrate to be sampled. With a blunt stick or metal rod the clay is forced down onto the substrate so as to isolate the sampling area of the inner circle. The periphyton within the inner circle is dislodged with a stiff brush and removed with a pipet. Stockner and Armstrong (1971) sampled periphyton with a plastic hypodermic syringe which had a toothbrush attached to the end of the syringe piston (fig. 19*C*). With the barrel of the syringe held tightly against the substrate, the piston is pushed in until the brush contacts the periphyton. The piston is then rotated several times to dislodge the periphyton and then is withdrawn, pulling the periphyton up with it. A glass plate is immediately placed under the end of the barrel and the syringe inverted. Small holes drilled through the base of the barrel facilitate periphyton collection (J. G. Stockner, written commun., March 1972).

Immediately proceed to 7.1 below, or if ovendrying cannot be started, freeze the sample. Storage should not exceed 2 weeks.

7. Analysis

7.1 Obtain the tare weight of a crucible that has been held at 500°C for about 20 minutes and cooled to room temperature in a desiccator.

7.2 Place the periphyton scraped from a known surface area into the tared crucible and dry in an oven at 105°C to constant weight, that is, until further drying produces no change in weight.

Note: Cool the 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. These values are used to calculate dry weight.

7.3 Place the crucible containing the dried residue in a muffle furnace for 1 hour at 500°C. Cool to room temperature.

7.4 Moisten the periphyton ash with distilled water and again ovendry at 105°C to constant weight as described in 7.2. These weight values are used to calculate ash weight.

8. Calculations

8.1 Dry weight of periphyton (g/m^2)

$$= \frac{\text{dry wt of crucible and residue (g)} - \text{tare wt of crucible (g)}}{\text{area of scraped surface } (m^2)}.$$

8.2 Ash weight of periphyton (g/m^2)

$$= \frac{\text{ash wt of crucible and residue (g)} - \text{tare wt of crucible (g)}.}{\text{area of scraped surface } (m^2)}.$$

8.3 Volatile or organic weight of periphyton (g/m^2) = dry weight (g/m^2) − ash weight (g/m^2).

9. Report

Report biomass as grams per square meter to three significant figures.

10. Precision

No numerical precision data are available.

References

Douglas, Barbara, 1958, The ecology of the attached diatoms and other algae in a small stony stream: Jour. Ecology, v. 46, p. 295–322.

Ertl, Milan, 1971, A quantitative method of sampling periphyton from rough substrates: Limnology and Oceanography, v. 16, no. 3, p. 576–577.

Neal, E. C., Patten, B. C., and DePoe, C. E., 1967, Periphyton growth on artificial substrates in a radioactively contaminated lake: Ecology, v. 48, no. 6, p. 918–923.

Nielson, R. S., 1953, Apparatus and methods for the collection of attached materials in lakes: Progressive Fish-Culturist, v. 15, no. 2, p. 87–89.

Stockner, J. G., and Armstrong, F. A. J., 1971, Periphyton of the experimental lakes area, Northwestern Ontario: Fisheries Research Board Canada Jour., v. 28, p. 215–229.

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Permanent slide method for periphytic diatoms
(B–3540–77)

Parameter and code: Not applicable

1. Application

The method is suitable for all waters. Advantages of the method are that a permanent mount is prepared, and clearing of the cells enhances observation of frustule (cell wall) 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 diatoms is calculated from strip counts.

3. Interferences

Particulate matter including salt crystals interferes with mount preparation.

4. Apparatus

4.1 *Artificial substrates* made of glass slides, Plexiglas, polyethylene strips, or other materials, Kahl Scientific Instrument Corp. (003WA250, 003WA260, 003WA270), Craftsman Designers, Inc. (Periphytometer), or equivalent. See figures 17 and 18 for selected types of artificial substrates.

4.2 *Collecting devices* for the removal of periphyton from natural substrates. Three such devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 19A–C.

4.3 *Sample containers* of glass or plastic suitable for the types of sample. Sturdy plastic bags are useful containers for artificial substrates or for pieces of natural substrates.

4.4 *Scraping devices.* Razor blades, stiff brushes, spatulas, or glass slides are useful devices for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard flat surfaces (Tilley, 1972).

4.5 *Centrifuge,* swing-out type, 3,000 to 4,000 rpm, with 50-ml graduated centrifuge tubes, Savegard (CT–1140) or equivalent.

4.6 *Hotplate,* thermostatically controlled to 538°C (1,000°F) Corning PC–35 electric hotplate or equivalent. It is convenient to have a second hotplate for operation at about 93°–121°C (200°–250°F) as described in 7.9.

4.7 *Microscope,* binocular, flat-field, zoom lens, with illuminator, Bausch & Lomb (PB-252) or equivalent.

4.8 *Ocular micrometer,* Whipple grid, Bausch & Lomb (31–16–13) or equivalent.

4.9 *Cover glass squares,* 18×18 or 22×22 mm, No. 1½and *microscope slides,* glass, 76×25 mm (3×1 in.).

4.10 *Forceps,* cover-glass, curved tip, Scientific Products (F7020) or equivalent.

5. Reagents

5.1 *Cupric-sulfate solution,* saturated: Dissolve 21 g $CuSO_4$ in 100 ml distilled water.

5.2 *Formaldehyde-cupric sulfate solution:* Mix 1 liter of 40 percent aqueous formaldehyde containing 10–15 percent methanol, Fisher Scientific No. F–78, or equivalent, with 1 ml of solution 5.1.

5.3 *Detergent solution,* 20 percent: Dilute 20 ml liquid detergent (LiquiNox, Catalog No. C6308–2, phosphate free, or equivalent) to 100 ml distilled water.

5.4 *Lugol's solution:* Dissolve 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 using; store in amber glass bottles (Vollenweider, 1969).

5.5 *Caedex,* Ward's Natural Science Establishment (37W9600) or equivalent.

5.6 *Immersion oil,* Cargille's nondrying type A, Scientific Products (M6002–1), or equivalent.

6. Collection

6.1 Artificial substrates. Place a suitable artificial substrate in the stream or lake and attach it to a supporting object. Figures 17 and 18 illustrate several types of artificial substrates. The substrate must be submerged but may be near the surface of the water or at any other appropriate depth. In lakes, the substrates are usually suspended at several depths (fig. 17*A, B, and C*). In lakes and streams the substrates may be attached to natural items such as submerged trees, stumps (fig. 17*D*), logs or boulders, or they may be attached to stakes driven into the bottom (fig. 18*A*). Floating samplers also may be used (fig. 18*B*). The artificial substrates must be exposed to the light so that photosynthesis can take place, and they should be located so that 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. The length of time required for colonization of the substrates by periphyton will depend upon the season, water temperature, light and nutrient availability, and other factors. Neal, Patten, and DePoe (1967) found that the maximum accumulation of periphyton biomass on polyethylene strips occurred in about 2 weeks. Exposure probably should be at least 14 days, but this will vary and must be determined for each season and water type.

Tilley and Haushild (1975a, 1975b) found in the Duwamish River, Wash., that 21 glass microscope slides exposed for 2 weeks at a single site ranged in chlorophyll *a* from 13.3 to 28.1 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 ranged in chlorophyll *a* from 18.9 to 48.6 mg/m^2, with a mean of 34.4 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 14.4 mg/m^2.

After sufficient colonization of periphyton, indicated by visible green or brown growth, remove the artificial substrate from the water. Scrape the periphyton from the substrate into a bottle containing about 100 ml of water, or the entire substrate may be placed into a container for laboratory processing. If the periphyton in a sample is to be examined within 2 or 3 hours after collection, no special treatment is necessary. A periphyton sample may be maintained for 24 hours at 3°–4°C, but for extended storage, preserve as follows: To each 100 ml of sample, add about 3 ml of 2 percent formaldehyde solution (5 percent Formalin), 0.5 ml of 20 percent detergent solution, and 5–6 drops of cupric sulfate solution. This preservative maintains cell coloration and is effective indefinitely.

Many biologists consider Lugol's solution to be the best algal preservative. It 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 preferred as a preservative, add 1 ml Lugol's solution to each 100 ml of sample. Store the preserved samples in the dark.

6.2 Natural submerged substrates often contain periphyton and may be used for qualitative studies. The most convenient collection method consists of removing entire substrates, such as rocks, leaves, or wood, to the laboratory for processing. Often, however, the periphyton must be removed from the substrate in the field, and figure 19A, B, and C illustrates several devices for collecting periphyton from natural substrates. The instrument used by Douglas (1958) consists of a broad-necked polyethylene bottle with the bottom removed (fig. 19A). 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 with a stiff nylon brush. The loose periphyton is removed from the bottle with a pipet. Ertl's (1971) apparatus consists of two concentric metal or plastic cylinders separated with spacers (fig. 19B). The space between the cylinders is filled with modeling clay, and the sampler is pressed firmly against the substrate to be sampled. With a blunt stick or metal rod the clay is forced down onto the substrate so as to isolate the sampling area of the inner circle. The periphyton within the inner circle is dislodged with a stiff brush and removed with a pipet. Stockner and Armstrong (1971) sampled periphyton with a plastic hypodermic syringe which had a toothbrush attached to the end of the syringe piston (fig. 19*C*). With the barrel of the syringe held tightly against the substrate, the piston is pushed in until the brush contacts the periphyton. The piston is then rotated several times to dislodge the periphyton and then is withdrawn, pulling the periphyton up with it. A glass plate is immediately placed under the end of the barrel and the syringe inverted. Four small holes at the base of the syringe allow for free movement of water when procuring the sample (J. G. Stockner, written commun., March 1972).

Preserve the periphyton as described in 6.1.

7. Analysis

7.1 Remove the periphyton from the substrate with a suitable device as outlined in 4.7, 6.1 and 6.2.

7.2 By vigorous shaking, thoroughly disperse the scrapings in about 100 ml of preservative or of distilled water if fresh material is being examined.

7.3 If the periphyton sample contains great numbers of diatoms, as typically occurs in eutrophic waters, the sample must be diluted. To dilute, thoroughly mix 50 ml of sample with 50 ml distilled water (1:1 dilution) and proceed to 7.4. If microscopic examination reveals a concentration of organisms still too numerous to count, thoroughly mix 50 ml of 1:1 dilution with 50 ml distilled water (1:4 dilution). Additional dilutions may be made as appropriate.

7.4 Diatom samples should be concentrated by sedimentation. Place the dispersed sample in a graduated cylinder of appropriate size. The organisms usually settle at a rate of 2 cm per 4 hours. Carefully pipet or siphon off the supernatant fluid. Wash the concentrate into 50 ml centrifuge tubes. Centrifuge for about 20 minutes at 3,000 to 4,000 rpm.

7.5 If the sample was collected from sea water or saline lakes, the diatoms should be washed with distilled water at least three times to insure that the permanent mounts will not be obscured with 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. Extract the supernatant fluid and repeat the washing process two more times.

7.6 Place 2 or 3 drops of the concentrate on each of three or four cover glasses.

7.7 With the concentrate side up, place the cover glass on a hotplate and heat, slowly at first to prevent splattering, to about 538°C (1,000°F) and incinerate for 30 minutes.

7.8 Remove cover glass from the hotplate and cool.

7.9 Place a drop of Caedex on a microscope slide and heat 3-4 minutes at about 93°–121°C (200°–250°F).

7.10 Invert the cover glass, concentrate side down, on the heated Caedex. Apply slight pressure to the cover glass (for example, with a pencil eraser). Remove slide from hotplate and allow to cool. If bubbles are present under the cover glass, heat the slide and apply additional pressure to the cover glass. Label the slide to identify sample.

7.11 Examine the slide with the × 97 objective lens (oil immersion). Count and identify all diatoms found in several lateral strips the width of the Whipple grid. Identify and tabulate 200–300 diatom cells, if possible. Generally, at least 100 individuals of every important species should be enumerated. Ignore frustule fragments.

8. Calculations

Percent occurrence of each species

$$= \frac{\text{number of diatoms of a given species}}{\text{total number of diatoms tabulated}} \times 100.$$

9. Report

Report percentage composition of diatoms to the nearest whole number. Report taxa and number of organisms per taxa.

10. Precision

No numerical precision data are avilable.

References

Douglas, Barbara, 1958, The ecology of the attached diatoms and other algae in a small stony stream: Jour. Ecology, v. 46, p. 295–322.

Ertl, Milan, 1971, A quantitative method of sampling periphyton from rough substrates: Limnology and Oceanography, v. 16, no. 3, p. 576–777.

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Tilley, L. J., and Haushild, W. L., 1975a, Net primary productivity of periphytic algae in the intertidal zone, Duwamish River Estuary, Washington: Jour. Research, U.S. Geol. Survey, v. 3, p. 253–259.

_____ 1975b, Use of productivity of periphyton to estimate water quality: Jour. Water Pollution Fed., v. 47, p. 2157–2171.

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MACROPHYTES

The most commonly occurring macrophytes are the rooted vascular plants that usually are arranged in zones or belts corresponding closely to successively greater depths in shallow water. Each deeper zone has its dominant vegetation composed of species more tolerant of decreasing illumination. The processes of erosion and deposition are constantly controlling the extent to which these plant zones develop. The characteristic plant forms that dominate these environmental gradients (in order of decreasing depth) are (1) submersed rooted aquatics, (2) floating-leaved rooted aquatics, (3) emersed rooted aquatics, and (4) marginal mats. Communities of vascular plants also may live unattached in the water, and because depth of water is of no consequence, these plants may occur anywhere on the water surface.

Bryophytes, the mosses and liverworts, are less conspicuous than the vascular plants. They generally grow in mats attached to submerged or partly submerged rocks in swiftly flowing water. In shallow quiet water, particularly along the edges, mosses and liverworts may grow attached to submerged rocks and mud substrata among rooted vascular plants.

Algae are plants that lack true roots, stems, and leaves. They include the smallest of the chlorophyll-bearing plants consisting of a single cell (commonly found in the plankton) as well as marine representatives ranging to several tens of meters (hundreds of feet) in length. Fresh-water species of algae, which range greatly in size within these two extremes occur as individual plants or in large patches attached to rocks in flowing water. Such plants may be gray, green, blue green, or olive and slimy to the touch, such as *Batrachospermum,* or, they may be green and have a coarse filamentous structure with profuse lateral branching, such as *Cladophora.*

In deeper slow-flowing or quiet water, algae with stemlike and leaflike structures are frequently found. These plants often have a glistening or translucent appearance *(Nitella),* or they may be encrusted with lime, which gives rise to the common name "stonewort" *(Chara).*

Growth of aquatic macrophytes depends on the availability of nutrients. In some bodies of water, nutrient enrichment results in excessive growth of macrophytes, and this acceleration of productivity is a major nuisance condition and an important water-quality problem. Tissue analysis of plants may provide information for evaluating nutrient supplies in natural waters (Gerloff and Krombholz, 1966) and for determining the nutrient requirements for particular plant species (Fitzgerald, 1969).

Floral survey (qualitative method) (B–4501–77)

Parameter and code: Not applicable

1. Application

The method is suitable for all waters.

2. Summary of method

Specimens from each habitat are collected and identified using appropriate references and taxonomic keys.

3. Interferences

Missing or incompletely developed plant parts, or

improperly preserved plant material may make identification of a specimen difficult.

4. Apparatus

4.1 *Collecting equipment,* appropriate to the objectives of the work, the type of substrate, and the depth of water. Examples of useful equipment are:

(a) Plant grappling bar, Ward's Natural Science Establishment (10W0890) or equivalent. A simple grappling hook may be fabricated by binding with lightweight wire the shanks of several hooks removed from wire coathangers. Provide a loop from an extra long shank for attaching a line.
(b) Steel garden rake.
(c) Dredge.

4.2 *Sample containers,* wide-mouth glass or plastic jars with leak-proof caps, or sealable plastic bags, Nasco, Whirl-Pak, or equivalent.

4.3 *Plant press,* such as CCM: General Biological, Inc. (120A20) or equivalent.

4.4 *Botanical driers,* such as CCM: General Biological, Inc. (120A25) or equivalent. These driers are absorbent pads, measuring approximately 30×46 cm (12×18 in.), for use in plant presses.

4.5 *Newspaper stock,* folded to about 29×42 cm (11.5×16.5 in.), CCM: General Biological, Inc. (120A28) or equivalent.

4.6 *Microscope,* binocular, wide-field, dissecting-type, Bausch & Lomb (31–26–29–73) or equivalent, with *fluorescent lamp,* Bausch & Lomb (31–33–63) or equivalent.

5. Reagents

5.1 *Oxyquinoline or 8-hydroxyquinoline sulfate,* 2 percent: Dissolve 2 g 8-hydroxyquinoline sulfate, Aldrich Chemical Co. (10,807–3) or equivalent, 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). The recommended preservative lacks most of the objectional features of formaldehyde solution, and it is particularly useful in fieldwork because small envelopes or capsules of measured quantities of powder may be mixed with water as needed (Moore, 1950).

5.2 *Formaldehyde solution,* 2 percent: 5 ml of 37–40 percent aqueous formaldehyde solution (Formalin) diluted to 100 ml with distilled water.

5.3 *Detergent solution,* 20 percent: 20 ml liquid detergent diluted to 100 ml with distilled water.

5.4 *Cupric sulfate solution,* saturated: Dissolve 21 g cupric sulfate in 100 ml distilled water.

6. Collection

Samples of macrophytes are collected by hand, with grappling devices, rakes, or with dredges. Collect entire plants including flowers and seed pods, if present, and roots, rhizomes, or tubers, if possible. All habitats should be sampled in an effort to collect both common and rare species. For some investigations, the relative abundance of plant species in the study area should be noted.

Preserve small specimens of vascular plants and bryophytes in 2 percent oxyquinoline or 8-hydroxyquinoline sulfate. Add a volume of preservative at least equal to the volume of plant material to insure adequate preservation. Although this preservative is effective for macrophytes in general, it is recommended that algae be preserved as follows: to each 100 ml of water add about 3 ml of 2 percent formaldehyde solution (5 percent Formalin), 0.5 ml of 20 percent detergent solution, and 5–6 drops of cupric sulfate solution.

Place large plants in a plant press for preservation. Use paper toweling or other absorbent material to blot as much moisture from the specimens as possible before preparing them for the press. Carefully arrange each plant on one-half of a single-folded sheet of newspaper. Bend stems and leaves where necessary but keep the plants as flat and as widely spread as possible. Fold the other half of the newspaper over each flattened plant, sandwich between two botanical driers, and place in a plant press. Many sheets with 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 drying pads daily 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 can be placed in a botanical drying rack to hasten drying of the plant material with the aid of artificial heat. Before proceeding with the heat method of drying macrophytes, read the techniques described by Lawrence (1960, p. 241–243).

7. Analysis

7.1 Identify plant specimens using an appropriate taxonomic key such as Conrad, 1956; Fassett, 1968; Hotchkiss, 1972; Muenscher, 1944; Smith, 1950. A stereoscopic microscope may be required.

8. Calculations

None required.

9. Report

List the taxa of macrophytes identified.

10. Precision

No numerical precision data are available.

References

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Distribution and abundance (quantitative method)
(B–4520–77)

Parameter and code: Macrophyte, total (no./m^2) 70944

1. Application

The method is suitable for all waters.

2. Summary of method

The distribution of macrophytes is determined in the field and plotted on a map of the study area. The size of subareas inhabited by the various kinds of macrophytes is determined by planimetry (American Public Health Association and others, 1976). Transect- or grid-sampling schemes are established, and the density or number of individual plants per unit area is determined. The method is modified from Oosting (1956, p. 30–55), and Daubenmire (1968, p. 79–92).

3. Interferences

Physical factors such as depth of water may interfere with determination of macrophyte distribution and abundance. Missing or incompletely developed plant parts or improperly preserved plant material may make identification of a specimen difficult.

4. Apparatus

4.1 *Collecting equipment* appropriate to the objectives of the work, the type of substrate, and the depth of water: Examples of suitable equipment are:

(a) Plant grappling bar, Ward's Natural Science Establishment (10W0890) or equivalent. A simple grappling hook may be fabricated by binding with lightweight wire the shanks of several hooks removed from wire coathangers. Provide a loop from an extra long shank for attaching a line.
(b) Steel garden rake.
(c) Dredge.

4.2 *Surveying or other equipment* suitable for establishing transect and grid-sampling schemes.

4.3 *Sample containers,* wide-mouth glass or plastic jars with leak-proof caps, or sealable plastic bags, Nasco, Whirl-Pak, or equivalent.

4.4 *Plant press,* such as CCM: General Biological, Inc. (120A20), or equivalent.

4.5 *Botanical driers,* such as CCM: General Biological, Inc. (120A25), or equivalent. These are absorbent pads, measuring approximately 30×46 cm (12×18 in.), for use in plant presses.

4.6 *Newspaper stock,* folded to about 29×42 cm (11.5×16.5 in.), CCM: General Biological, Inc. (120A28), or equivalent.

4.7 *Microscope,* binocular, wide-field dissecting-type, Bausch & Lomb (31–26–29–73) or equivalent, with *fluorescent lamp,* Bausch & Lomb (31–33–63) or equivalent.

4.8 *Polar planimeter.*

5. Reagents

5.1 *Oxyquinoline or 8-hydroxyquinoline sulfate,* 2 percent: Dissolve 2 g 8-hydroxyquinoline sulfate, Aldrich Chemical Co. (10,807–3) or equivalent, 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). The recommended preservative lacks most of the objectional features of formaldehyde solution, and it is particularly useful in field work because small envelopes or capsules of measured quantity of powder may be mixed with water as needed (Moore, 1950).

5.2 *Formaldehyde solution,* 37–40 percent.

5.3 *Detergent solution,* 20 percent: 20 ml liquid detergent diluted to 100 ml with distilled water.

5.4 *Cupric sulfate solution,* saturated: Dissolve 21 g cupric sulfate in 100 ml distilled water.

6. Collection

The intensity of sampling should be commensurate with the objective(s) of the study.

Samples of macrophytes are collected by hand, with grappling devices, rakes, or with dredges. Collect entire plants including flowers and seed pods, if present, and roots, rhizomes, or tubers, if possible.

Preserve small specimens of vascular plants and bryophytes in 2 percent oxyquinoline or 8-hydroxyquinoline sulfate. Add a volume of preservative at least equal to the volume of plant material to insure adequate preservation. Although this preservative is effective for macrophytes in general, it is recommended that algae be preserved as follows: To each 100 ml of water add about 3 ml of 40 percent formaldehyde solution (100 percent Formalin), 0.5 ml of 20 percent detergent solution, and 5–6 drops of cupric sulfate solution.

Place large plants in a plant press for preservation. Use paper toweling or other absorbent material to blot as much moisture from the specimens as possible before preparing them for the press. Carefully arrange each plant on one-half of a single-folded sheet of newspaper. Bend stems and leaves where necessary but keep the plants as flat and as widely spread as possible. Fold the other half of the newspaper over each flattened plant, sandwich between two botanical driers, and place in a plant press. Many sheets with 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 drying pads daily 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 can be placed in a botanical drying rack to hasten drying of the plant material with the aid of artificial heat. Before proceeding with the heat method of drying macrophytes, read the techniques described by Lawrence (1960, p. 241–243).

7. Analysis

7.1 Outline on a map of the study area the distribution of each type of macrophyte.

7.2 Supplement onsite observations with results from aerial photography or other airborne sensors, if possible (Wilson, 1969).

7.3 Determine the density of macrophytes in each subarea.

7.4 Identify plant specimens using an appropriate taxonomic key such as Conrad, 1956; Fassett, 1968; Hotchkiss, 1972; Muenscher, 1944; Smith, 1950. A stereoscopic microscope may be required.

7.5 Determine the area (in square meters) inhabited by each type of macrophyte using a map of the study area and a polar planimeter.

8. Calculations

8.1 Number of macrophytes/m^2

$$= \frac{\text{number of macrophytes}}{\text{number of samples} \times \text{area of one sample } (m^2)}$$

9. Report

9.1 List the taxa of macrophytes identified.

9.2 Report distribution as number of macrophytes per square meter.

9.3 Report the density of macrophytes as follows: Less than 10 individuals/m^2, to the nearest whole number; 10 individuals and above, two significant figures.

10. Precision

No numerical precision data are available.

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BENTHIC INVERTEBRATES

The invertebrate animals inhabiting the bottoms of lakes and streams and other water bodies 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 allows for response to conditions that have prevailed over a long period of time. Moreover, many benthic invertebrates inhabit specific types of environments which, 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 organisms. Thus, knowledge of the kinds and abundance of benthic invertebrates helps to indicate trends in the condition of the aquatic environment. The extensive literature on interpretation of benthic invertebrate data with regard to water quality has been reviewed by Hynes (1960, 1970), Warren (1971), Cairns and Dickson (1973), and Hart and Fuller (1974).

Benthic organisms vary widely in size, and there is no clear distinction between the smallest benthic forms and the largest microorganisms. Bottom living invertebrates that are visible to the unaided eye are usually included within the benthos. Because many early studies of the benthic fauna emphasized the quantity available for fishfood, the U.S. Standard No. 30 sieve, 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 in fact, the American Public Health Association and others (1976) states that the stream-bottom inhabiting organisms collected for study, termed "macroinvertebrates," are those which are retained on a U.S. Standard No. 30 sieve (0.595-mm spaces).

The mesh openings of sampling nets and sieves ideally should be selected on the basis of need for the purposes of a particular study. If the mesh size is so large that the smaller specimens 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 organisms by backwash. The results of sampling with 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). Jońasson (1955, 1958) found that the diameter of the head determines whether or not a dipteran larva will pass through a given mesh. His data demonstrated a 640-percent increase in the numbers of organisms in lake samples as the sieve size decreased from 600 μm to 200 μm. Other investigators have reported similar results from various aquatic environments. Significant differences between retention of total individuals and total taxa in No. 30 and No. 60 sieves was 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 width of less than 200 μm must be used, and in other respects the mesh width must be carefully adapted to size of the animals selected." In a study of stream benthic sampling, Mundie (1971) found 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 allow 50 percent of the fauna to pass through if the community contained high proportions of chironomid larvae and mayfly and stonefly nymphs. Mundie estimated that a net of 200- to 250-μm mesh would allow 70–80 percent of the animals to pass through, but would be adequate for many purposes, such as the estimation of biomass and for general faunistic surveys.

In view of the foregoing evidence, the Geological Survey has adopted the U.S. Standard Sieve No. 70 (210-μm mesh opening) for retaining benthic invertebrate organisms collected as part of its water-quality

programs. Nets are to be of 210-μm nylon monofilament screen cloth (Tobler, Ernst, and Traber, Inc., Nitex, or equivalent) with about 43-percent open area. In applications requiring more rapid filtration, 216-μm nylon monofilament high-capacity screen cloth (Tobler, Ernst, and Traber, Inc., Nitex, or equivalent) with 57-percent open area may be used. These mesh sizes are sufficiently small to retain many of the immature stages of the benthic fauna 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 should always be reported.

Three types of sampling for benthic invertebrates are described:

1. Faunal surveys determine the taxa present and estimate the relative abundance of each taxon at each station. As it is important to collect the rare taxa at each station, sampling should cover a large area of bottom and as many habitats as feasible. Use of several collection methods at each station can increase the total number of taxa in the samples (Slack, and others, 1976). 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).
2. Relative or semiquantitative surveys demonstrate changes in space and time. Accurate measurements of the total benthos are not obtained, nor are the estimates of relative abundance of each species within the samples necessarily reliable. Sampling effort is limited, and as in the case of artificial substrates, may be restricted to a small area at each station. As different sampling methods will give different results, the methods and sampling areas should be as uniform as possible throughout a study.
3. Absolute quantitative surveys determine the numbers or biomass per unit area of streambed or lakebed and demonstrate changes in space and time. This type of sampling requires the greatest amount of effort and in many environments the objectives cannot be achieved. Comparisons of the benthic fauna between stations or sampling dates should be based upon uniform sampling methods, because all methods are somewhat selective.

Faunal survey (qualitative method) (B–5001–77)

Parameter and code: Not applicable

1. Application

The method is applicable to all waters.

2. Summary of method

Benthic invertebrates are collected by hand, with a dip net, or in any other manner appropriate to the environmental conditions and to the objectives of the study. All habitats are sampled. Unsorted samples, usually containing varying amounts of sand, gravel, and plant detritus, are preserved in the field. In the laboratory the animals are sorted from the extraneous material, identified, and counted. Results are reported as numbers of different kinds of organisms (taxa) and the relative abundance of each taxon at different stations or times.

3. Interferences

Physical factors such as stream velocity and depth of water may interfere with sampling. Most samples contain relatively large amounts of sediment and plant debris from which the organisms must be sorted.

4. Apparatus

Methods and equipment for the collection of benthic invertebrates are described in Welch (1948), Hedgpeth (1957, p. 61–86), Barnes (1959), Needham and Needham (1962), Southwood (1966), Schwoerbel (1970), American Public Health Association and others (1976), Holme and McIntyre (1971), and Edmondson and Winberg (1971). Some common types of equipment used for faunal surveys are listed below.

4.1 *Dip nets,* are made in various shapes and sizes, and any sturdy design is acceptable. Some nets have a flat edge which is held against the streambed during use. Suitable dip nets are Turtox/Cambosco (73–407,

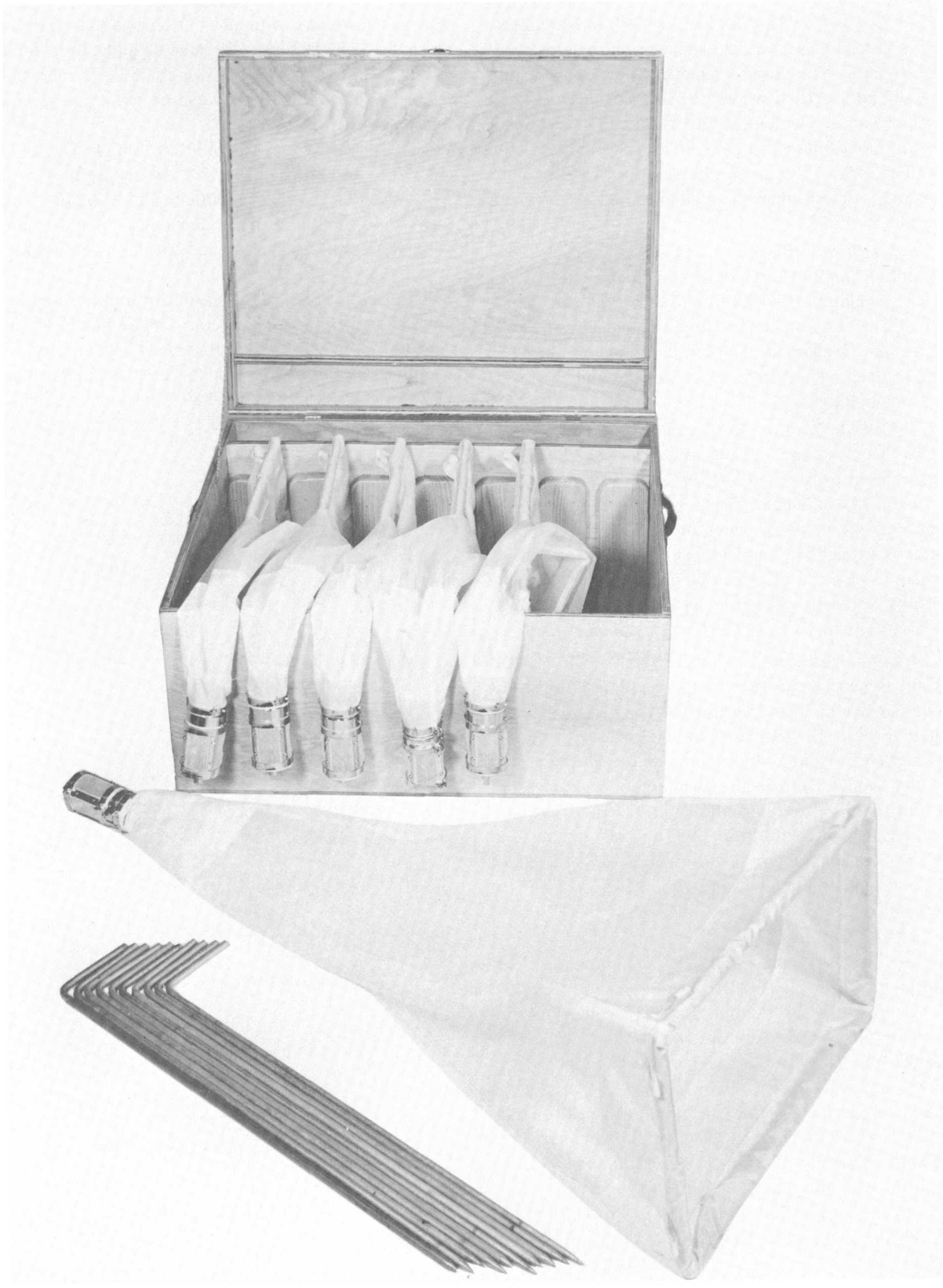

Figure 20.—Stream drift nets. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

73–408, 73–412, 73–422, and 73–440) or equivalent. 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 Geological Survey should have bags of nylon monofilament screen cloth (Tobler, Ernst, and Traber, Inc., Nitex, or equivalent) 216-μm mesh opening, unless otherwise dictated by the study objectives.

4.2 *Drift net,* 30×30 cm, 15×30 cm, or 30×46 cm, Wildlife Supply Co. (15), or equivalent, with anchor rods and clamps (fig. 20). Bags, 1 m or more in length, should be of nylon monofilament screen cloth (Tobler, Ernst and Traber, Inc., Nitex, or equivalent) 216-μm mesh opening, or other mesh size appropriate to the study objectives.

4.3 *Ekman grab,* preferably the tall design (fig. 21), 15×15 cm square, 23–30 cm high: Kahl Scientific Instrument Corp. (214WA170), Wildlife Supply Co. (196T), or equivalent. Extra weights are available to increase the depth of penetration. In deep water, the grab is tripped with a messenger, whereas in shallow water the Ekman grab may be operated with a handle (Kahl Scientific Instrument Corp. (214WA172), Wildlife Supply Co. (196H) or equivalent.

4.4 *Petersen grab,* Wildlife Supply Co. (1750), Kahl Scientific Instrument Corp. (214WA195), or equivalent (fig. 22). This grab trips on bottom contact. It may be used with or without accessory weights. Generally it is advisable to operate the Petersen grab with a winch. The weight when empty is about 18 kg (39 lb) without weights and about 32 kg (70 lb) with weights for the Wildlife Supply Co. instrument. The Kahl Scientific Corp. instrument is constructed of heavier material and weighs about 45 kg (100 lb) without accessory weights.

4.5 *Ponar grab,* Wildlife Supply Co. (1725), *Screen-Top Sediment Sampler,* Kahl Scientific Instrument Corp. (214WA010), or equivalent (fig. 23). This grab trips on bottom contact like the Petersen design, but has provision for water to pass through to lessen the shock wave and may be superior in performance (Flannagan, 1970; Hudson, 1970). Accessory weights may be used, and it is advisable to operate these grabs with a winch. The weight when empty is about 23 kg (45 lb) without weights and about 32 kg (70 lb) with weights.

4.6 *Pipe dredge,* Wildlife Supply Co. (170), Kahl Scientific Instrument Corp. (215WA123), or equivalent (fig. 24). This simple device is useful for sampling swift, rocky rivers. 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. Commercial dredges weigh 25 kg (55 lb), but smaller and lighter versions can be made for special purposes.

4.7 *Biological dredge,* Wildlife Supply Co. (171 and 175), Kahl Scientific Instrument Corp. (012WA570, 215WA100, 215WA200, or 215WA400), or equivalent, depending upon the sampling requirements (figs. 25 and 26). A powered boat is needed to operate these dredges.

4.8 *Sample containers,* plastic for transporting unsorted samples to the laboratory. Widemouth jars of 120-, 240- and 475-ml (4-, 8- and 16-oz) capacity are useful sizes. Jar lids should be of plastic. Sealable plastic bags (Nasco, Whirl-Pak, or equivalent) may also be used for temporary storage of benthic samples.

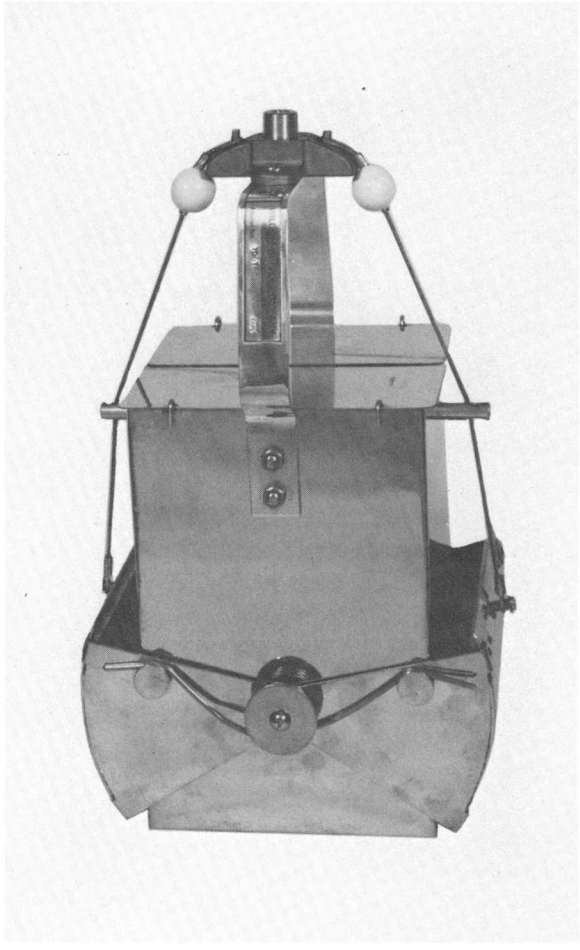

Figure 21.—Ekman grab, tall design. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

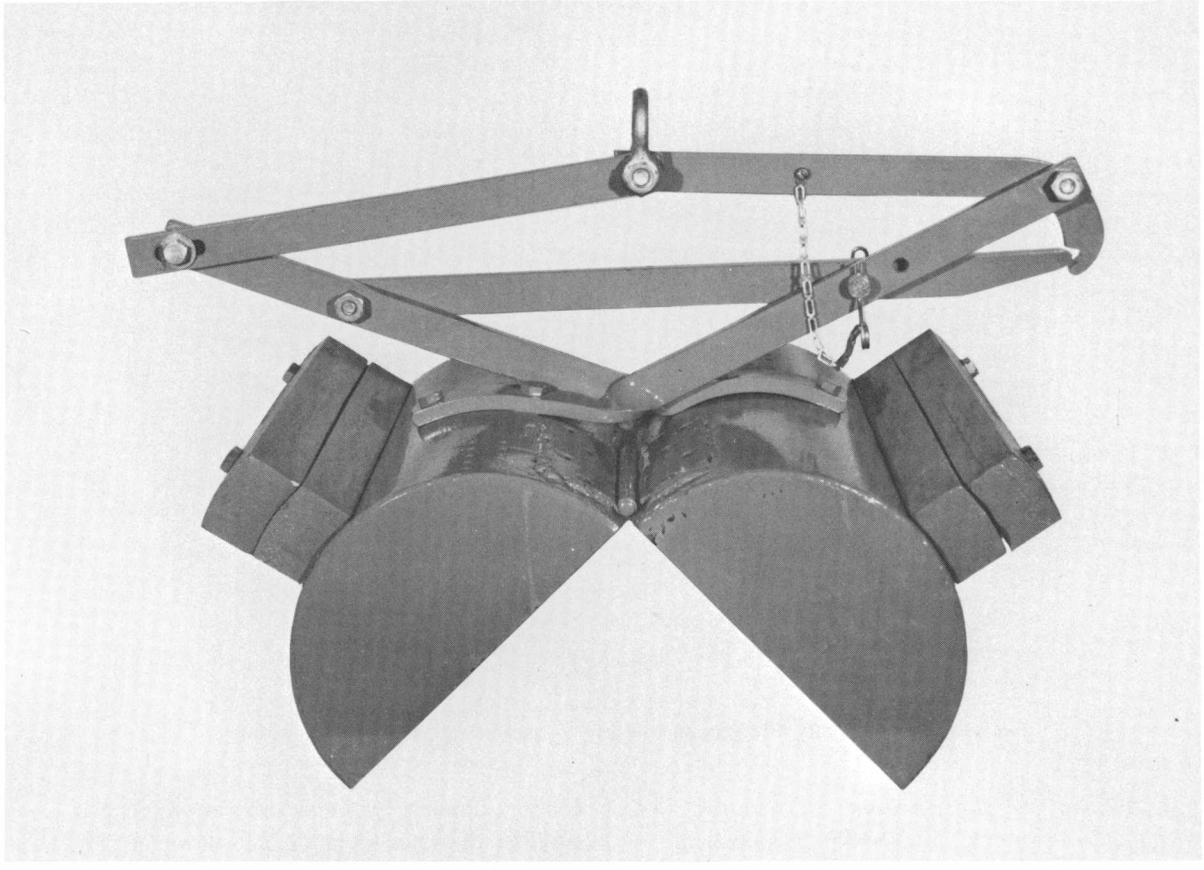

Figure 22.—Petersen grab. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

4.9 *Vials* with plastic screw lids. Convenient sizes are 7.5-, 15-, and 22-ml (2-, 4-, and 6-dram) capacity.

4.10 *Waterproof labels,* Turtox/Cambosco (376A182), or equivalent; or labels may be cut from sheets of plastic paper, Nalgene Labware (6304-0811) or equivalent.

4.11 *Waterproof ink,* Higgins Eternal Ink or equivalent.

4.12 *Plastic tape,* Scotch (33) or equivalent, for sealing jar and vial lids.

4.13 *Forceps* with fine or rounded points. Fine points are useful for handling small organisms. Rounded points are less likely to tear netting or puncture the mesh of sieves or other sampling equipment. These are less likely to be lost in the field if marked with bright paint or colored tape.

4.14 *U.S. Standard sieves,* 20-cm (8-in) diameter, with mesh size appropriate to the study objectives. The No. 70 sieve, 210-μm mesh opening, has been adopted for retaining benthic invertebrate organisms collected as part of the water-quality programs of the Geological Survey. Sieves with smaller or larger mesh, such as U.S. No. 30 (595-μm openings) may be more suitable for some studies. The No. 18 sieve (1,000-μm openings) 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.

4.15 *Microscope,* stereoscopic variable power, × 7 to × 30, Bausch & Lomb (BVB–73) or equivalent, with *microscope illuminator,* Bausch & Lomb (31–33–24–01) or equivalent. A compound microscope of at least × 100 magnification also is useful for taxonomic work.

4.16 *Trays,* white enamel. Useful sizes are 30×19×5 cm (12×7.5×2 in.) and 42×26×6 cm (16.5×10×2.25 in.).

4.17 *Dishes,* glass petri or Syracuse watchglasses.

4.18 *Hydrometer,* plain form, range 1.000-1.220, Scientific Products (H8750-1) or equivalent.

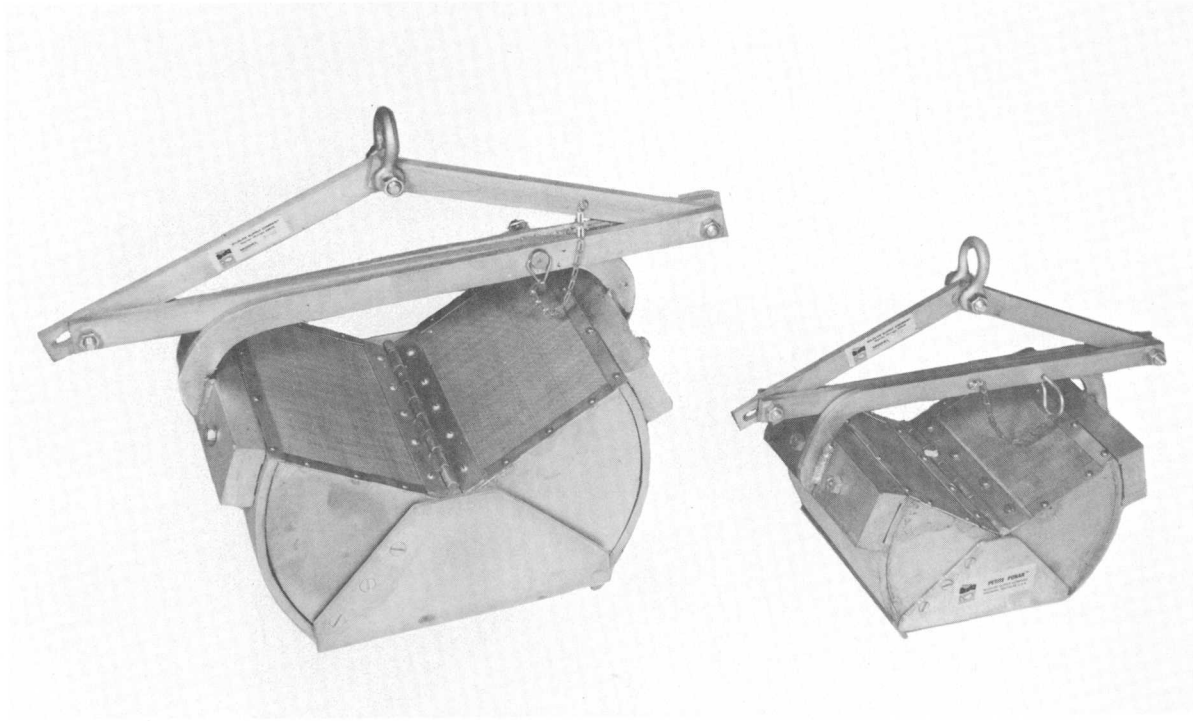

Figure 23.—Ponar grab. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

4.19 *Pump, air,* aquarium-type or *pump, pressure-vacuum,* Millipore (XX60 000 00) or equivalent.

4.20 *Air diffuser stones,* porous aquarium type.

4.21 *Fine-mesh scoops,* 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.

4.22 *Beaker,* 15 ml or small *measuring cup* to be used for withdrawing subsamples. Alternately a *wide-bore pipet* of suitable capacity can be made by cutting the tip from a volumetric pipet.

4.23 *Beakers* and *graduated cylinders,* large sizes as needed for the types of samples in process.

5. Reagents

5.1 *Preservative solution.* Invertebrate samples may be preserved in 70 percent ethyl alcohol or 40 percent isopropyl alcohol. Formaldehyde solution is not recommended. Ethyl alcohol is preferred for permanent storage. Prepare as follows:

Ethyl alcohol: 70 ml of 95 percent alcohol diluted to 95 ml with distilled water.

Isopropyl alcohol: 40 ml of concentrated alcohol diluted to 100 ml with distilled water.

5.2 *Sucrose solution,* specific gravity 1.12, for density separation of invertebrates from the debris in benthic samples. Dissolve 360 g of granulated sugar per liter of water (about 2.5 lb of sugar per gal of solution).

5.3 *Rose Bengal biological stain,* Matheson, Coleman, and Bell (RX155) or equivalent.

5.4 *Glycerin.*

6. Collection

There is no universally accepted method for sampling the benthos. However, no habitat should be overlooked if the objective is to obtain a representative collection of the aquatic organisms, and different habitats may require different methods. The success of the method will depend on the experience and skill of the collector. Sampling should include 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, and backwaters, near the banks and in deeper parts, should be sampled. Rocks may be lifted by hand and examined for organisms as the surface dries. Tufts of algae and moss should be collected and examined for animals. Invertebrate animals may be dislodged from floating vegetation or rooted plants with a dip net, or samples of the plants may be collected with hooks or rakes followed by removal of the animals. Methods for collecting plants are described in the section on "Mac-

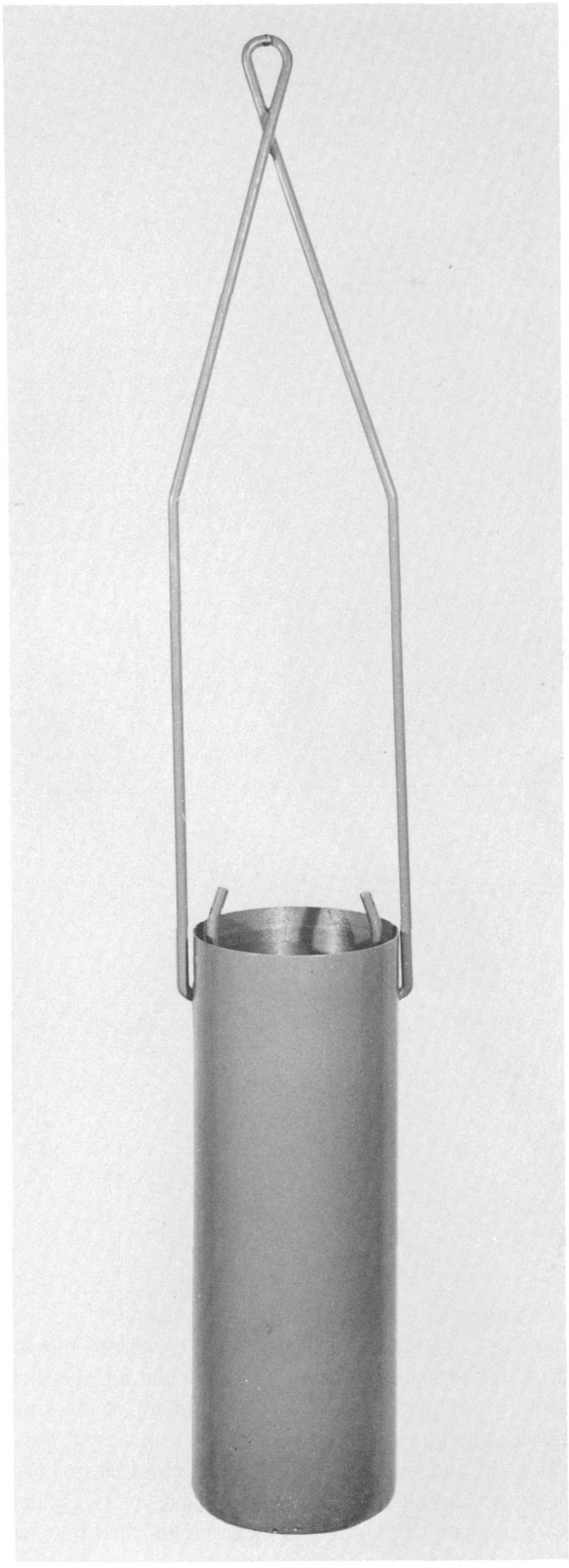

Figure 24.—Pipe dredge. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

rophytes''. 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 animals are subsequently removed (Welch, 1948; Gerking, 1957). Additional information on sampling is given in the references listed at the end of this section.

Two types of collecting devices are described, those using netting for concentrating the organisms dislodged from the substrate (6.1, 6.2, and 6.3, some biological dredges) and those involving removal of the substrate (6.3, grabs).

6.1 Dip net. This is the most useful general collecting implement for wadable waters. It can be used in water with high concentrations of suspended sediment and among plants or large boulders to depths of a meter or more. Macan (1958) described a method of working slowly upstream lifting rocks and holding the net so as to catch animals swept into it. Clinging animals were dislodged from rocks by vigorous swirling in the mouth of the net. Alternately, the net may be held against the bottom and the area immediately upstream disturbed with the hands or feet, allowing the current to carry animals into the net. In still water the net can be scraped rapidly along the bottom to catch easily dislodged animals, 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.

Empty the net frequently either into a shallow white tray if the sample is to be sorted in the field or into a wide-mouth container for transporting to the laboratory. Label and preserve the catch as described in 6.4 and 6.5 below.

6.2 Drift net. Studies have shown that many kinds of benthic invertebrates become entrained in streamflow and that the resulting downstream drift of animals is a regular feature of running waters (Waters, 1969, 1972; Müller, 1974). Organisms in the draft are not carried from far upstream; as the distance from the sampling point increases, each unit area of bottom contributes a progressively smaller proportion of drifting invertebrates to the total catch (McLay, 1970; Elliott, 1971b). Because drifting organisms 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 demonstrated by Coutant (1964), Besch (1966), Wojtalik and Waters (1970), Wilson and Bright (1973), and Larimore (1974).

A simple net of appropriate mesh on a square or

Figure 25.—Biological dredge. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

rectangular frame is sufficient for qualitative sampling (fig. 20). Anchor the net with the opening upstream using steel rods driven into the streambed. Study objectives will determine the location and type of net exposure. Sampling below riffles will catch more organisms than below pools, and the greater the volume of flow through the net the larger the sample. If a sample of the natural drift is required, the net should be installed upstream from any disturbance caused by other sampling activity. If the drift sample is intended to supplement the faunal list, the streambed can be disturbed upstream from the net to increase the rate of drift. If the net extends above the water surface, the sample will include maximum numbers of floating adults, pupae, exuviae, and terrestrial species. If only aquatic organisms and life stages are required, the top of the net should be under water. In deep rivers, the net may be near the bottom or near the surface but the technique should be uniform throughout a study. For best results, drift samples should be collected over a

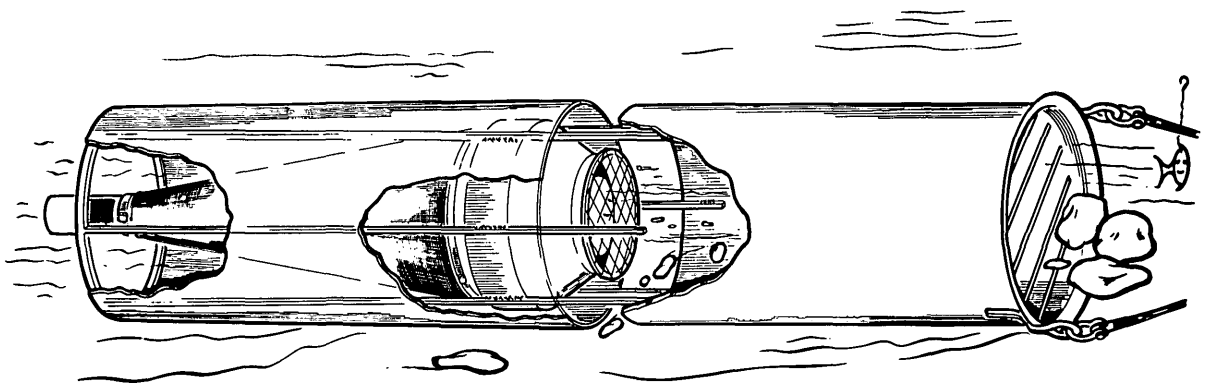

Figure 26.—Biological dredge. (Photograph courtesy of Kahl Scientific Instrument Corp., El Cajon, Calf.)

24-hour period. Because drift rates usually are higher at night than during the day, with a maximum shortly after sunset, Larimore (1974) recommended a 30- or 60-minute collection during the second hour after sunset. For the purposes of many qualitative faunal surveys, a daylight sample is adequate.

At the end of the specified sampling period, empty the net into a shallow white tray if the sample is to be sorted in the field or into a wide-mouth container for transporting to the laboratory. Label and preserve the catch as described in 6.4 and 6.5 below.

6.3 Grabs and dredges. As discussed by Hynes (1970, p. 237) these terms should be applied, respectively, to instruments which bite into the bottom from above and instruments which are pulled across or through the bottom sediment. Grabs are usually considered to be quantitative sampling devices because a defined area of substrate is enclosed and removed, they also are suitable for qualitative collection. The Ekman grab (fig. 21) is designed for soft sediments in the absence of current. The Petersen grab and its modifications (figs. 22 and 23) can sample firmer sediments, but neither works well in fast currents because of the failure to contact the bottom perpendicularly.

Qualitative samples of benthic invertebrates from deep or swift rivers are usually collected with some type of dredge (figs. 24, 25, and 26). The design varies, but often, as in the dredges developed by Usinger and Needham (1956), and Fast (1968), large rocks are excluded whereas the smaller particles and the benthic animals are retained in a mesh bag. Dredges are lowered from a boat or bridge or even thrown from a high bank then pulled upstream along the bottom so that 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 organisms into the net. In still or slowly moving water, dredges should be pulled with a powered boat to prevent loss of active benthic animals.

Dredges should be emptied at the end of each retrieval, either into a white tray if the sample is to be sorted in the field, or into a wide-mouth container for transporting to the laboratory. In either case label and preserve the catch as described in 6.4 and 6.5 below.

6.4 To insure adequate preservation, fill containers no more than half full with the sample so that a volume of preservative can be added at least equal to the volume of organic material, including detritus. Preserve the organisms or the unsorted samples in 70 percent ethyl alcohol or 40 percent isopropyl alcohol. Containers should be filled nearly to the top to avoid excessive sloshing and damage to delicate specimens.

Note: If unsorted samples are to be stored for more than a few weeks, the preservative should be drained after about 1 week and replaced with fresh preservative.

6.5 Label the sample with the location, habitat, date, and time of collection (local standard time), name of collector, and sample treatment (type of preservative, mesh size of sieves or nets, or other treatment). Soft black pencil may be used in the field, but

use a waterproof carbon ink for permanent labels. Place labels inside the sample containers so that they are visible from the outside, or place duplicate labels inside and outside the containers. Secure jar lids with tape to prevent their working loose and the subsequent loss of preservative by evaporation. This is especially important if samples are to be shipped or stored for more than a few weeks.

7. Analysis

7.1 If the study objectives require only determination of the most abundant benthic forms, sorting often can be completed in the field. Wash the sample gently in a net or sieve of appropriate mesh size to remove mud and fine detritus. Pick the organisms directly from the sampled material, or, to enhance visibility of small forms, cover the collected sample with water in a white enamel tray and stir repeatedly while removing the organisms with forceps or scoops.

7.2 Generally, sorting must be done in the laboratory. This may be done by pouring small portions of sample into a shallow dish, covering the material with water, and scanning the dish under low-power magnification (× 3 to × 10). Remove the organisms from the debris with forceps or with fine-mesh scoops.

The sorting process is very time consuming for many types of samples. The optional steps described in 7.4 through 7.6 below may be used to speed the work when the study objectives require complete analysis.

7.3 Identify and enumerate the benthic invertebrates in the sample according to taxonomic categories. The degree of identification required varies with the objectives of the study. Most identifications will be performed by a biologist with specialized training in this work. A stereoscopic microscope is required and, for some groups, dissections or microscopic mounts are needed to observe key characters. Appropriate reference books (Part 4, "Selected Taxonomic References") should be available. It is convenient to place the different categories of organisms in separate vials of 70 percent ethyl or 40 percent isopropyl alcohol, labeled with the name of the organisms and the identification number, date, and origin of the sample. Add a few drops of glycerin and seal vial caps with tape if the specimens are to be stored.

7.4 Density separation (optional). This step consists of treating the sample with a solution of such a density that most of the invertebrates will float and most of the unwanted detritus will sink. The recommended method employs a 1.12-specific-gravity sucrose solution (Anderson,1959; Lackey and May, 1971).

Drain the sample on a No. 70 or other appropriate sieve, discard the liquid, and transfer the residue to a white enamel pan. Flood the material in the pan with the sugar solution and stir so that the material is evenly spread over the bottom. Remove organisms from the surface of the liquid with forceps or with fine-mesh scoops. After removing all visible organisms, stir the material, and remove any specimens that appear. Pour the sugar solution through the sieve and cover the residue in the pan with water. Examine as described in 7.2, looking especially carefully for oligochaete worms, aquatic mites, and for the heavier organisms such as mollusks and caddisfly larval cases. After this examination, pour the water through the sieve and repeat the sucrose treatment. Few organisms should be found and, if large numbers are recovered, the sample should be soaked in water and again treated with the sugar solution. The sugar solution may be reused by adjusting the specific gravity to 1.12 as determined with a hydrometer. However, the solution spoils rapidly and should not be stored for more than a few days.

7.5 Differential staining (optional). Separation of invertebrates, especially transparent forms, from samples is facilitated by staining them red with 200 mg/l of Rose Bengal added to the preservative solution. Expose the organisms 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 in the sample. If necessary to restore natural coloration for identification, remove the stain from the organisms by placing them in 95 percent ethyl alcohol (Mason and Yevich, 1967). A counterstaining technique in which Rose Bengal or Lugol's iodine is counterstained with chlorazol black may be used to provide a high color contrast between invertebrates and detritus (Williams and Williams, 1974).

7.6 Subsampling (optional). Some benthic samples are so large, or contain such large numbers of organisms, that it is impractical to sort or count the entire sample. Remove the larger organisms and pieces of detritus from the entire sample as described in 7.2. Make the remaining sample up to definite volume and pour into a beaker about one-third larger than the volume of the sample. Mix the sample so as to distribute the organisms randomly throughout the fluid, but not so violently that delicate specimens are fragmented. Bubbles from an aquarium air diffuser are a gentle, effective method of mixing. While the sample is thoroughly agitated, remove a subsample with a small dipper or with a wide-bore pipet, keeping the tip of the pipet in motion during filling. The subsample

should be at least one-quarter to one-third of the sample volume, although if necessary the subsample may be divided again before sorting or counting.

8. Calculations

8.1 When only part of the total sample is sorted or counted as described in 7.6 above, extrapolate the results from the subsample to the number of specimens in the total sample. Total number of individuals of a particular taxon in sample

$$= \frac{\text{number of individuals of the taxon in subsample}}{\text{volume of subsample (ml)}} \times \text{volume of total sample (ml)}$$

8.2 Percent composition in sample

$$= \frac{\text{number of individuals of a particular taxon}}{\text{total number of individuals of all taxa}} \times 100.$$

9. Report

Report the number of taxa present, the percent composition of each taxon in the sample, and the type of collection method(s) used.

10. Precision

No numerical precision data are available.

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Numerical assessment (relative or semiquantitative method) (B–5020–77)

Parameters and codes:
Invertebrates, benthic, wet weight (g/m²) 70940
Invertebrates, benthic, dry weight (g/m²) 70941
Invertebrates, benthic, ash weight (g/m²) 70942
Invertebrates, benthic, total (organisms/m²) 70943

1. Application

The method is applicable to all waters and is especially suited to demonstrating water-quality trends or differences between stations. This method assumes that the objective is to compare the kinds and relative abundances of taxa in samples (catches) at several stations or on different sampling dates. The differences between samples (catches) are assumed to be directly proportional to differences between the stations or times. The artificial-substrate method is recommended when collections must be made by persons inexperienced in field biology. The procedures given in "Distribution and Abundance (Quantitative Method)" also are applicable to sample collection from homogeneous substrates.

2. Summary of Method

Benthic invertebrates are collected in a uniform way over a broad area or from small, homogeneous areas at stations that are to be compared. Sampling methods include collecting with a dip net in a standardized manner or for a definite period of time, sampling individual rocks, and using artificial substrates. Unsorted samples, usually containing varying amounts of sand, gravel, and plant detritus, are preserved in the field. In the laboratory the animals 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 organisms (taxa) and relative abaundance of each taxon in the total collection or for a particular habitat or artificial substrate. Biomass is reported as wet, dry, ash, or organic weight.

3. Interferences

Physical factors such as stream velocity and depth of water may interfere with sampling. However, because the sampling methods are selective, it is important that all the collections for a particular study be made in a uniform way. Most samples contain sediment and plant debris from which the organisms must be separated. Losses of artificial-substrate samplers because of vandalism may preclude their use at some stations.

4. Apparatus

Methods and equipment for the numerical assessment of benthic invertebrates are described in Macan (1958), Albrecht (1959), Needham and Needham (1962), Cummins (1962, 1966, 1975), Hynes (1964, 1970), Southwood (1966), Schwoerbel (1970), American Public Health Association and others (1976), and Cairns and Dickson (1973).

4.1 *Dip nets* are made in various shapes and sizes, and a sturdy design with a flat side for pressing closely against the streambed is recommended. Suitable dip nets are Turtox/Cambosco (73–412, 73–422, and 73–440) or equivalent. 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 Geological Survey should have bags of nylon monofilament screen cloth (Tobler, Ernst, and Traber, Inc., Nitex, or equivalent) 216-μm mesh opening, unless otherwise dictated by the study objectives.

4.2 *Lium sampler* for individual rocks (Lium, 1974, and fig. 27). The sampler consists of a 16-gage sheet metal hood with an attached conical screen of 210-μm

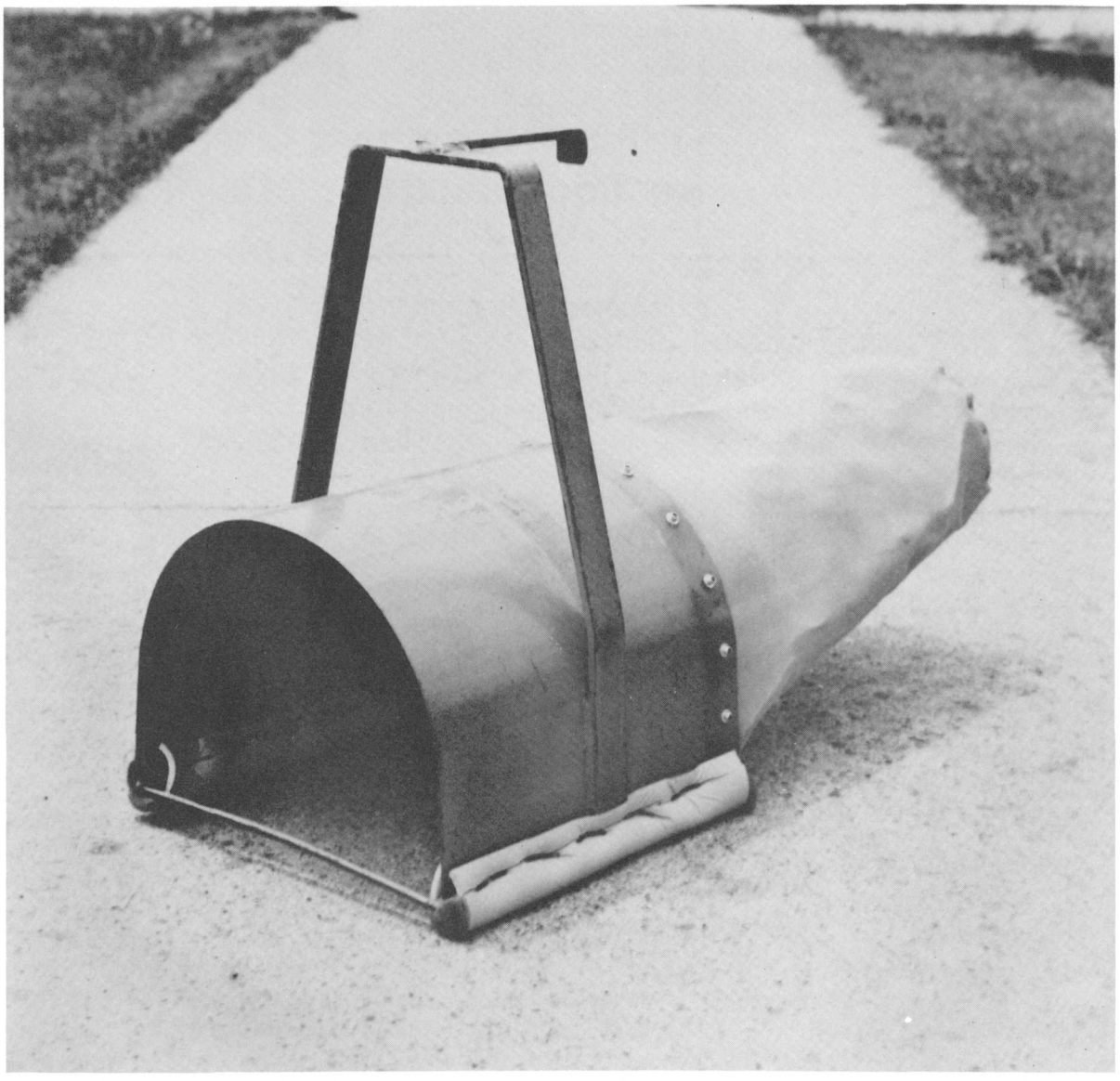

Figure 27.—Lium sampler.

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 (26 in.) long, 45 cm (18 in.) high including the handle, and the base of the hood is 929 cm^2 (144 $in.^2$).

4.3 *Multiple-plate sampler,* ''jumbo'' modification (Fullner, 1971). The sampler consists of 14 7.6-cm (3-in.) square or round plates of 3.3-mm (⅛-in.) thick tempered hardboard (Masonite or equivalent) separated by one or more 2.54-cm (1-in.) square spacers of the same material (fig. 28). Plates 1–9 are separated by a single hardboard spacer, plates 9 and 10 are separated by two spacers, plates 10–12 are separated by three spacers, and plates 12–14 are separated by four spacers. The plates and spacers are held together with a 6.4-mm (¼-in.) diameter by 20-cm (8-in.) eyebolt which passes through a hole drilled in the center of each piece.

4.4 *Retrieval net* for multiple-plate sampler (fig. 29): A rectangular bag made from a 38-cm (15-in.) square of nylon monofilament screen cloth (Tobler, Ernst, and Traber, Inc., Nitex, or equivalent), 210-μm mesh opening, unless otherwise dictated 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.

4.5 *Artificial-substrate float* consisting of a 0.6-m (2-ft) length of polyvinylchloride (PVC) tubing 5-cm (2-in.) inside diameter (ID) with the ends sealed (fig. 30). 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 at a depth of 0.3 m (1 ft) measured from the water surface to the midpoint of each sampler.

4.6 *Barbecue-basket sampler* (Mason and others, 1967), a cylindrical, welded-wire basket, about 18 cm (7 in.) in diameter and 28 cm (11 in.) long (Androck, Inc., Worcester, Mass., or equivalent). The basket is filled with 30 rocks, 5 to 8 cm in diameter, which provide interstices for organism colonization and weight for stability (fig. 31). 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-liter (5-gal) metal container filled with polyurethane foam.

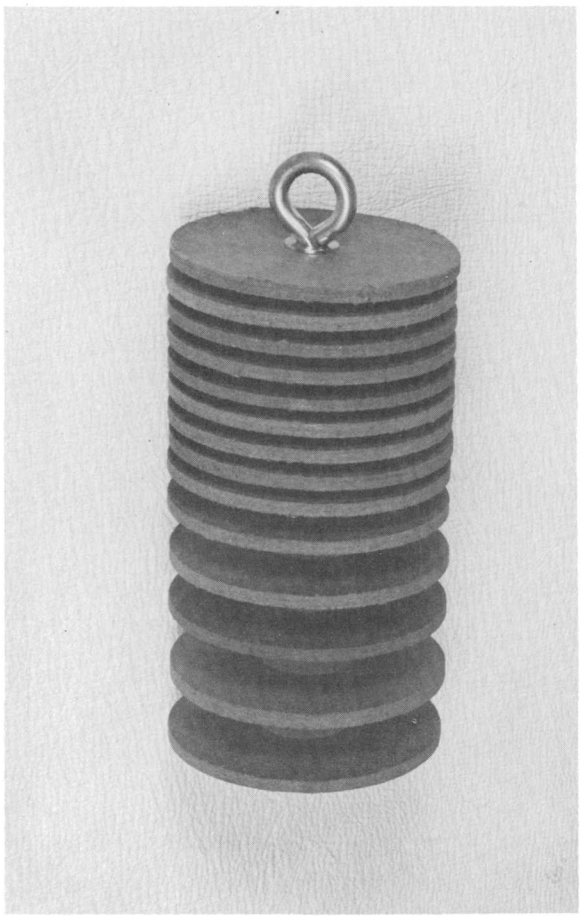

Figure 28.—Jumbo multiplate artificial substrate. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

4.7 *Collapsible-basket 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 (1½×⅛ in.) stock (fig. 32). The basket is filled with gravel and is surrounded by a bag of nylon monofilament screen cloth (Tobler, Ernst, and Traber, Inc., Nitex, or equivalent), 210- or 216-μm mesh opening unless otherwise dictated 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 organisms.

4.8 *Sample containers* of plastic for transporting unsorted samples to the laboratory. Wide-mouth jars of 120-, 240-, and 475-ml (4-, 8-, and 16-oz) capacity are useful sizes. Jar lids should be of plastic. Sealable plastic bags (Nasco, Whirl-Pak, or equivalent) also may be used for temporary storage of benthic samples.

4.9 *Vials* with plastic screw lids. Convenient sizes are 7.5-, 11-, and 22-ml (2-, 4-, and 6-dram) capacity.

4.10 *Waterproof labels,* Turtox/Cambosco (376A182) or equivalent; or labels may be cut from sheets of plastic paper, Nalgene Labware (6304–0811) or equivalent.

4.11 *Waterproof ink,* Higgins Eternal Ink or equivalent.

4.12 *Plastic tape,* Scotch (33) or equivalent, for sealing jar and vial lids.

4.13 *Forceps* with fine points and rounded points. Forceps with fine points are useful for handling small organisms. Forceps with rounded points are less likely to tear netting or pucture the mesh of sieves or other sampling equipment. These are less likely to be lost in the field if marked with bright paint or colored tape.

4.14 *Soft-bristle brush* for scrubbing organisms from rocks.

4.15 *Tub* or *bucket* for washing samples or sampling equipment in the field.

4.16 *U.S. Standard Sieves,* 20-cm (8-in.) diameter, with mesh appropriate to the study objectives. The No. 70 sieve, 210-μm mesh opening, has been adopted for retaining benthic invertebrate organisms collected as part of the water-quality programs of the Geological Survey. Sieves with smaller or larger mesh, such as U.S. No. 30 (595-μm openings) may be more suitable for some studies. The No. 18 sieve (1,000-μm openings) 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.

4.17 *Microscope,* stereoscopic variable power, × 7 to × 30, Bausch & Lomb (BVB–73) or equivalent, with *microscope illuminator,* Bausch & Lomb (31–33–24–01) or equivalent. A compound microscope of at least × 100 magnification also is useful for taxonomic work.

4.18 *Trays,* white enamel. Useful sizes are 30×19×5 cm (12×7.5×2 in.) and 42×26×6 cm (16.5×10×2.25 in.).

4.19 *Dishes,* glass petri or Syracuse watchglasses.

4.20 *Hydrometer,* plain form, range 1.000–1.220, Scientific Products (H8750–1) or equivalent.

4.21 *Pump, air,* aquarium-type, or *pump, pressure-vacuum,* Millipore (XX60 000 00) or equivalent.

4.22 *Air-diffuser stones,* porous aquarium type.

4.23 *Fine-mesh scoops,* 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.

4.24 *Beaker,* 15-ml, or small *measuring cup* to be used for withdrawing subsamples. Alternately, a

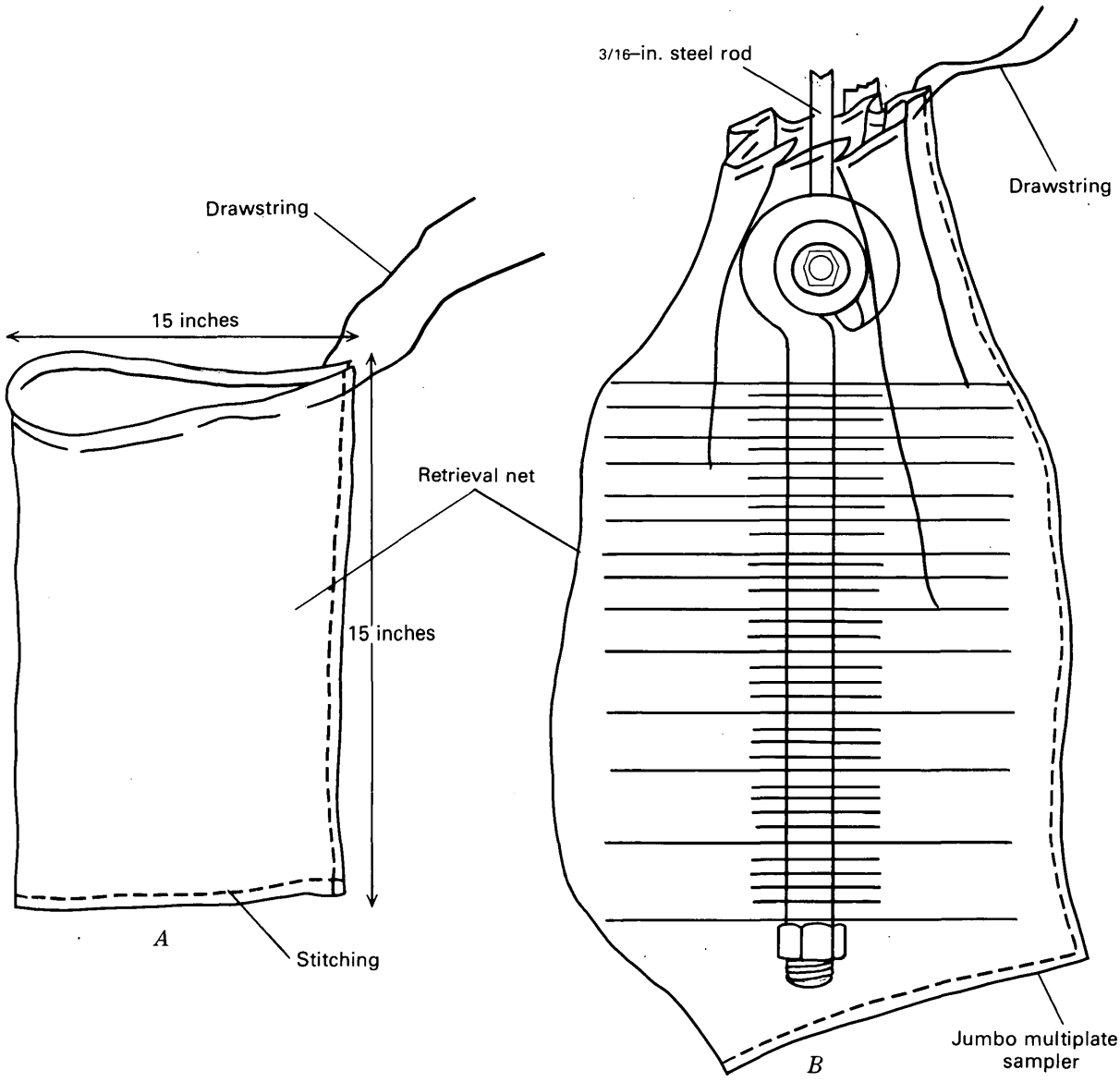

Figure 29.—Retrieval net.

wide-bore pipet of suitable capacity can be made by cutting the tip from a volumetric pipet.

4.25 *Beakers and graduated cylinders,* large sizes as needed for the types of samples in process.

4.26 *Balance* capable of weighing to 0.1 mg.

4.27 *Porcelain crucibles.*

4.28 *Drying oven,* thermostatically controlled for use at 105°C.

4.29 *Muffle furnace,* for use at 500°C.

4.30 *Desiccator* containing dry silica gel.

5. Reagents

5.1 *Preservative solution.* Invertebrate samples may be preserved in 70 percent ethyl alcohol or 40 percent isopropyl alcohol. Formaldehyde solution is not recommended. Ethyl alcohol is preferred for permanent storage. Prepare as follows: Ethyl alcohol: 70 ml of 95 percent alcohol diluted to 95 ml with distilled water. Isopropyl alcohol: 40 ml of concentrated alcohol diluted to 100 ml with distilled water.

5.2 *Sucrose solution,* specific gravity 1.12, for den-

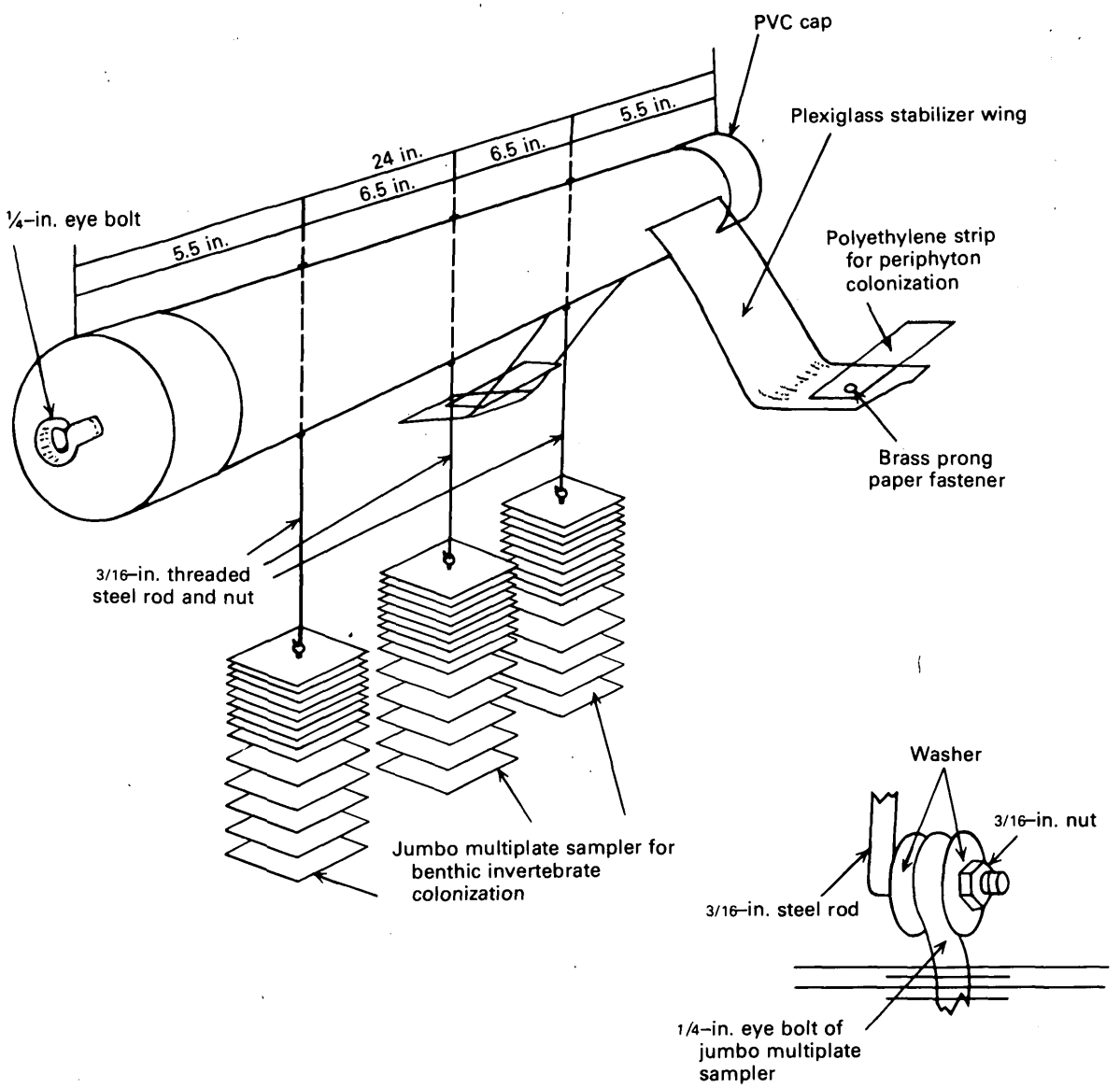

Figure 30.—Float for artificial substrates.

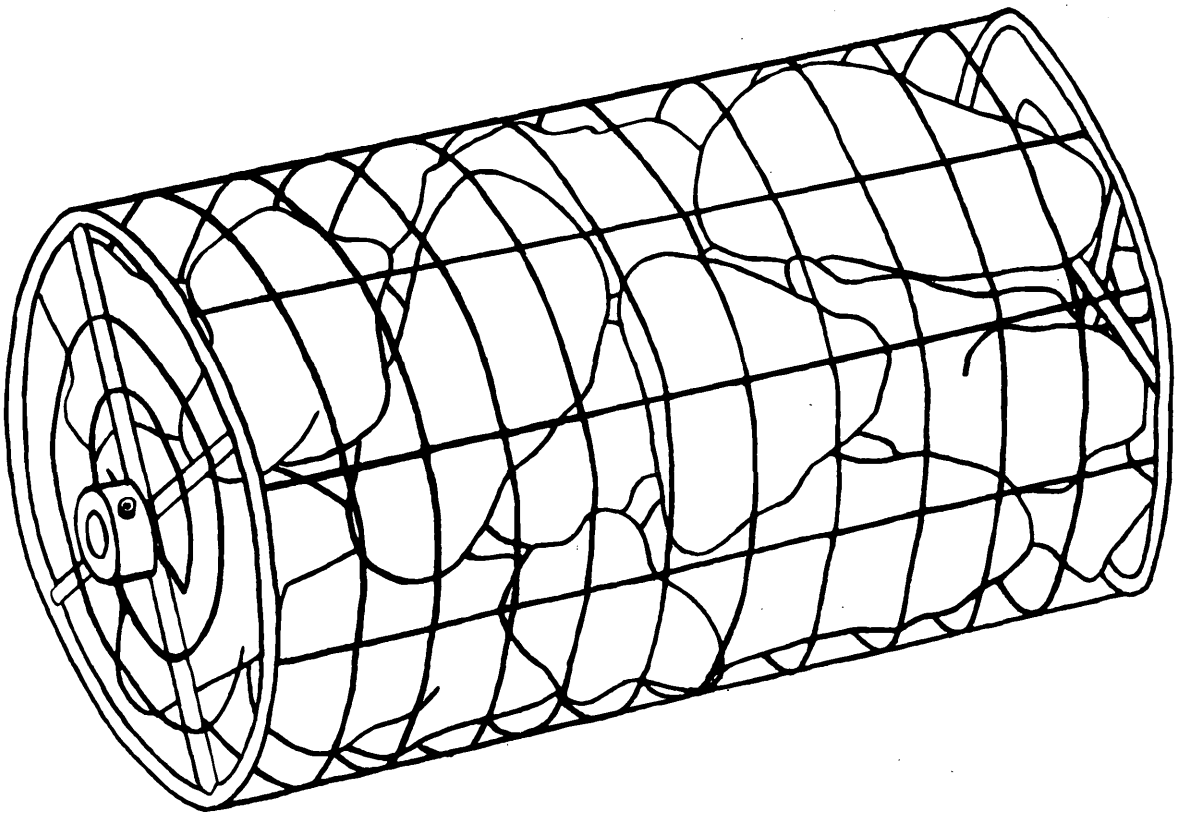

Figure 31.—Barbecue-basket artificial-substrate sampler.

sity separation of invertebrates from debris in benthic samples: Dissolve 360 g of granulated sugar per liter of water (about 2.5 lb of sugar per gal of solution).

5.3 *Rose Bengal biological stain.* Matheson, Coleman, and Bell (RX155) or equivalent.

5.4 *Glycerin.*

6. Collection

The statistical principles of benthic invertebrate sampling, discussed by Elliott (1971), are summarized in "Part 1, Biological Sampling and Statistics." 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 intervals in detailed studies to once a year in general surveys. When artificial substrates are used, sufficient time must be allowed for organism colonization, usually 4 to 6 weeks. Two sampling procedures using a dip net (6.1 and 6.2), one involving collection of individual rocks (6.3), and three using artificial substrates (6.4, 6.5, and 6.6) are described below.

6.1 The dip net used for a standardized period of time will provide a numerical assessment of the differences between stations in wadable waters. The collecting period will depend on the size and variability of the sampling area and on the study objectives. The most abundant species may be adequately sampled within a 5- or 10-minute period by an experienced biologist. Generally, however, collecting should continue for at least 30 minutes in streams up to 15-m wide and 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 animals swept into it; clinging animals were dislodged from rocks by vigorous swirling in the mouth of the net. In still water the net can be scraped rapidly along the bottom to catch easily dislodged animals, 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. It is important to keep the collecting effort and technique as uniform as possible during a particular study. Empty the dip net frequently into a shallow white tray if the sample is to be sorted in the field, or into a wide-mouth container for transporting to the laboratory. Label and preserve the catch as described in 6.7 and 6.8 below.

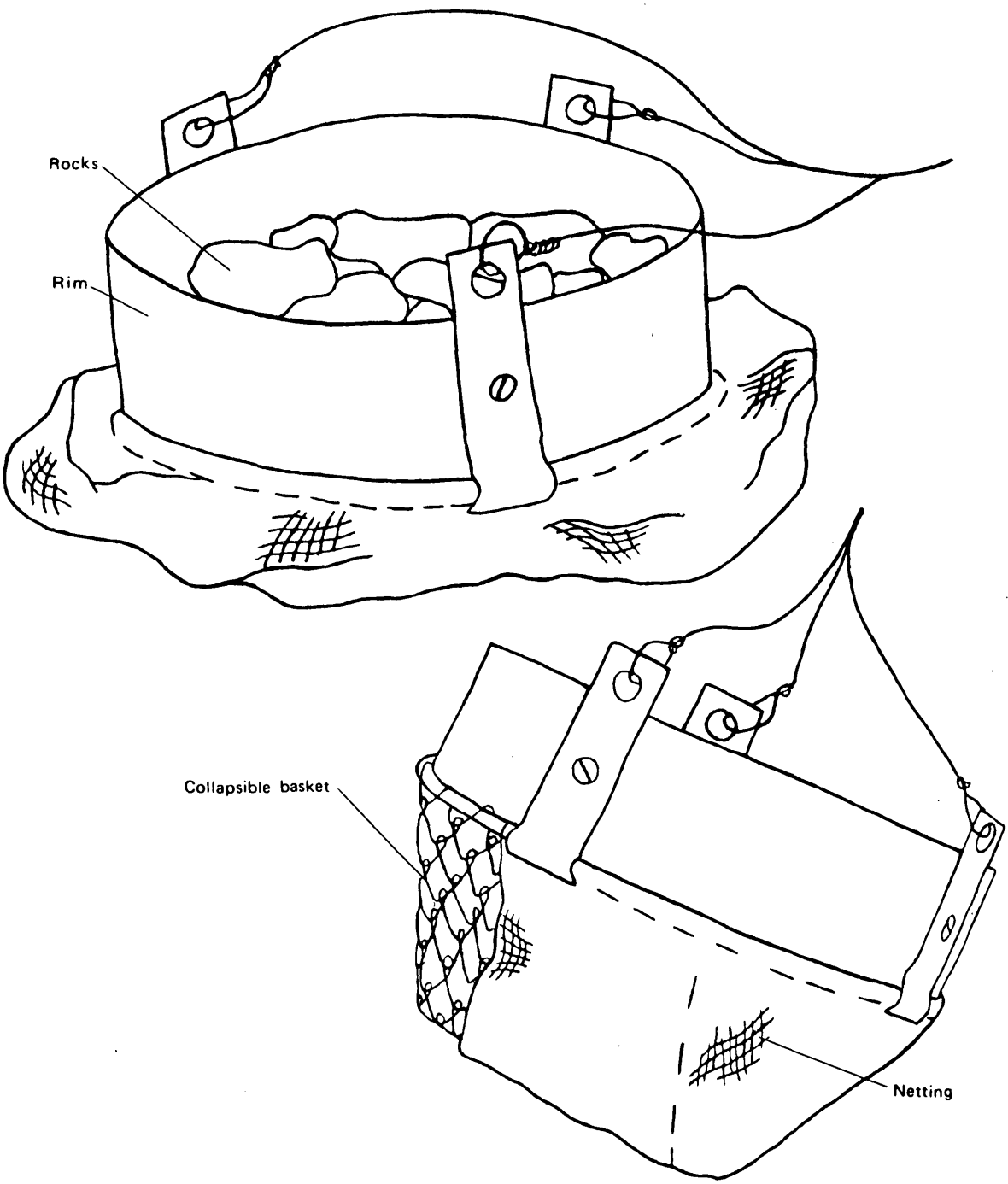

Figure 32.—Collapsible-basket artificial-substrate sampler. Upper: Resting on streambed. Lower: Being retrieved. (Redrawn from Bull, 1968.)

6.2 A rapid and versatile method of dip-net sampling consists of holding the flat side of the 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 (Morgan and Egglishaw, 1965). A proportion of the dislodged organisms and detritus will be carried into the net by the current; the kicks should be separated by several seconds to allow this to occur. The method can be used on a wide variety of substrates from sand to rocks 45–60 cm (1.5–2 ft) across, in weedbeds, or in

bedrock using the boot as a scraper. The recommended 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 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 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 white tray if the sample is to be sorted in the field, or into a wide-mouth container for transporting to the laboratory. Label and preserve the catch as described in 6.7 and 6.8 below.

6.3 The fact that many benthic invertebrates of shallow streams or rocky shores of lakes live on or beneath rocks is the basis for a sampling method involving lifting individual rocks and collecting the associated organisms (Macan, 1958; Schwoerbel, 1970). The method consists of three operations: selection of rocks, collection of rocks, and expression of results. As with other methods, the study objectives are decisive in selection of the sampling method and its application. The simplest 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 organisms out of the water. This operation is repeated until 10 or 20 rocks have been collected. Because the number of benthic invertebrates per unit of rock area may vary with the size of the rock (Lium, 1974), rocks of similar size should be collected for samples that are to be compared. In gravel-bed streams studied by Lium (1974), highest insect densities occurred on rocks between 45- and 90-mm mean diameter. Depending on the objectives, rock sampling may consist of 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 insure random collection of rocks from each habitat.

The Lium sampler (fig. 27) was designed to catch the organisms that wash off a rock as it is picked from the streambed. With the front 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 conforms to the streambed, and the hood minimizes outwash of organisms during rock removal. Organisms that are dislodged as the rock is lifted are carried by the current into the screen. Remove organisms trapped on the screen by inverting the sampler and washing them into a bucket. Regardless of the method of rock collection, scrub each rock thoroughly with a soft-bristle brush in a bucket of water to remove clinging organisms. Pour the contents of the bucket through an appropriate sieve. Empty the sieve into a shallow white tray if the sample is to be sorted in the field, or into a wide-mouth container for transporting to the laboratory. Label and preserve the catch as described in 6.7 and 6.8 below.

If the results are to be expressed in areal units (8.7 below), rock sizes must be determined. To express the population in terms of the projected area of rock, measure the two longest straight-line dimensions of each rock (A and B axes) in millimeters and multiply them together. Complete calculations as in 8.7 below. To express 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). Complete calculations as in 8.7 below.

6.4 Artificial substrates consist of standardized, reproducible surfaces for colonization by aquatic organisms. Their uniform shape and texture compared to natural substrates greatly simplifies the problem of sampling. Standardized sampling is especially desirable when the results from different investigators or from different environments are to be compared. A basic assumption in the use of artificial substrates is that the kinds and relative abundance of colonizing organisms are generally representative of the composition of the communities at the sampled location. In actual fact, the devices are selective for species adapted to hard surfaces. Because of the inherent bias of the artificial substrates, the method is sometimes supplemented with other types of sampling.

The multiple-plate sampler, "jumbo" modification, (fig. 28) is the smallest and most adaptable of the recommended artificial-substrate devices. The samplers are relatively inconspicuous by virtue of size and color, and the modest cost permits replication to further enhance the chances of recovery in small bodies of water where vandalism is a problem. Attach multiple-plate samplers to floats, structures, weights, or rods driven into the streambed or lakebed. Install three samplers, and leave them in place for 4 to 6 weeks to allow for organism colonization. Record the

exposure time, which should be consistent among sites throughout a study.

The samplers may be installed in pools or riffles, on the bottom or suspended above it. The recommended practice is to install samplers on the bottom in riffles up to 1 m deep. Make the collections as representative of the reach as possible by insuring that the samplers are in eroding areas not close to the bank. In streams up to a few meters in width, install the devices at about midstream; in wider streams, install the devices at about one-quarter of the total width from the nearest bank. In large rivers or in lakes, the samplers are usually suspended from floats as described in 4.5 above (fig. 30).

Many animals leave the samplers as soon as they are disturbed. To minimize losses, approach from downstream. In shallow water, lift the sampler off the bottom, and quickly slip a net or a sieve of appropriate mesh size under the sampler as it is raised out of the water. When a float is used to suspend more than one sampler and the samples are to be kept separate, approach from downstream and, without removing the float from the water, enclose each sampler in a retrieval net (fig. 29) to avoid loss of organisms. 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 which 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.

The organisms may be removed in the field by disassembling the sampler and scrubbing the plates with a soft-bristle brush in a bucket of water. Pour the contents of the bucket through a sieve of appropriate mesh size. To the sample on the sieve, add the organisms detached from the sampler during recovery. The sampler can be reassembled and used again unless there is reason to believe that it has been contaminated by toxicants or oils (Weber, 1973). If the organisms are not removed in the field, place the multiple-plate sampler and the detached portion of sample into a wide-mouth container or sturdy plastic bag for transporting to the laboratory. Label and preserve the samples as described in 6.7 and 6.8 below. Samplers that have been placed in preservative must be discarded.

6.5 The barbecue-basket sampler (fig. 31) is adapted to use in lakes and large rivers. Fill the basket with 30 rocks, 5–7.5 cm (2–3 in.) in diameter, and secure the sampler door with wire or small cable clamps. The rocks used to fill a series of samplers should be of the same general size, shape, and composition, if possible, and they should be scrubbed with a brush before use. Angular limestone is often used in barbecue-basket samplers.

If possible, suspend three samplers at a depth of 0.3 m (1 ft) below the surface for a period of 4 to 6 weeks. In environments of variable depth, suspend the samplers from a float. Barbecue-basket samplers also may be installed on the bottom in deep or shallow water, but it is important that the depth and exposure period be uniform throughout a given study (American Public Health Association and others, 1976).

When collecting a sampler, enclose the basket in a net or tub to prevent escape of animals. Slip a large dip net (Turtox/Cambosco, 73–422, or equivalent) under the basket to catch organisms that would be lost when the sampler is lifted from the water.

Empty the sampler into a tub partially filled with water. Scrub the rocks and basket with a soft-bristled brush to remove clinging organisms. Pour the contents of the tub through a sieve of appropriate mesh size. To the residue on the sieve, add the organisms detached from the sampler during recovery. Load the basket with the cleaned rocks for reuse unless there is reason to believe that the sampler has been contaminated by toxicants or oils (Weber, 1973). Alternately, the basket may be emptied into a container of preservative and the rocks transported to the laboratory for cleaning. Label and preserve the sample as described in 6.7 and 6.8 below. Do not reuse rocks that have been exposed to preservative.

6.6 The collapsible-basket sampler (fig. 32) is the artificial-substrate sampler recommended if the objective is to compare sampler catches with the population of the surrounding substrate. The basket can be loaded with materials simulating the natural bed on which it lies. This sampler is especially useful for shallow streams or for deep, swift rivers. The sampler consists of a collapsible basket holding gravel and 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 which is subsequently populated. When raised off the bottom, the basket extends to its original hemispherical shape, and the surrounding net bag prevents loss of organisms.

If possible, install three samplers in a riffle. Make

the collections as representative of the reach as possible by insuring that the samplers are not close to the bank. In streams up to a few meters in width, install the devices at about midstream; in larger streams, install the devices at about one-quarter of the total width from the nearest bank. Currents occasionally hinder the collapse of a sampler, but this can be overcome by connecting a strong rubberband on one side of the basket rim, under the bottom of the wire basket, and up to the other side of the rim (Bull, 1968). The samplers are stable on the bottom at velocities up to about 0.9 m/s (3 ft/s) (C. J. Bull, written commun., Dec. 1969), but recovery is often 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.

Many animals leave artificial-substrate samplers as soon as they are disturbed. To minimize losses, approach the sampler from downstream, and lift the sampler off the bottom with a single quick motion. Allow the water to drain through the net bag, empty the gravel into a tub partially filled with water, and scrub the rocks and basket with a soft-bristled brush to remove clinging organisms. Rinse the net in the water, and pour contents of the tub through a sieve of appropriate mesh size. To the residue on the sieve, add the organisms detached from the sampler during recovery. Load the basket with cleaned rocks for reuse unless there is reason to believe that it has been contaminated by toxicants or oils (Weber, 1973). Alternately, the basket may be emptied into a container of preservative and the rocks transported to the laboratory for cleaning. Label and preserve the sample as described in 6.7 and 6.8 below. Do not reuse rocks that have been exposed to preservative.

6.7 Samples for which only biomass will be determined should be frozen as soon as possible after collection. Samples for taxonomic determination should be preserved in alcohol. Use of alcohol for preserving samples for biomass determination will result in low values because of extraction of alcohol-soluble substances from the organisms. To insure adequate preservation, fill containers no more than half full with the sample so that a volume of alcohol can be added at least equal to the volume of organic material, including detritus. Preserve the organisms or the unsorted samples in 70 percent ethyl alcohol or 40 percent isopropyl alcohol. Containers should be filled nearly to the top to avoid excessive sloshing and damage to delicate specimens. Note: If unsorted samples are to be stored for more than a few weeks, the preservative should be drained after about 1 week and replaced with fresh preservative.

6.8 Label the sample with the location, habitat, date and time of collection (local standard time), name of collector, method of sampling, and sample treatment (type of preservative, mesh size of sieves or nets, or other treatment). Soft black pencil may be used in the field, but use a waterproof carbon ink for permanent labels. Place labels inside the sample containers so that they are visible from the outside, or place duplicate labels inside and outside the containers. Secure jar lids with tape to prevent their working loose and the subsequent loss of preservative by evaporation. This is especially important if samples are to be shipped or stored for more than a few weeks.

7. Analysis

7.1 If the study objectives require determination of only the most abundant benthic forms, sorting often can be completed in the field. Wash the sample gently in a net or sieve of appropriate mesh size to remove mud and fine detritus. Pick the organisms directly from the sampled material or, to enhance visibility of small forms, cover the collected sample with water in a white enamel tray, and stir repeatedly while removing the organisms with forceps or scoops.

7.2 Generally, sorting must be done in the laboratory. This may be done by pouring small portions of sample into a shallow dish, covering the material with water, and scanning the dish under low-power magnification (× 3 to × 10). Remove the organisms from the debris with forceps or with fine-mesh scopps. If taxonomic determination is required, proceed to 7.3. If only biomass determination is required, proceed to 7.4.

The sorting process is very time consuming for many samples. The optional steps described in 7.8 through 7.10 below may be used to speed the work when the study objectives require complete analysis.

7.3 Identify and enumerate the benthic invertebrates in the sample according to taxonomic categories. The degree of identification required varies with the objectives of the study. Most identifications will be performed by a biologist with specialized training in this work. A stereoscopic microscope is required, and, for some groups, dissections or microscopic mounts may be needed to observe key characteristics. Appropriate reference books (see "Part 4, Selected Taxonomic References") should be available. It is convenient to place the different categories of organisms in separate vials of 70 percent ethyl or 40 percent isopropyl alcohol, labeled with the name of the organism and the

identification number, date, and origin of the sample. Add a few drops of glycerin, and seal vial caps with tape if the specimens are to be stored.

7.4 The biomass of benthic invertebrates, expressed as wet, dry, ash, or organic (ash-free) weight, is best determined on samples that were frozen immediately after collection. Biomass determined on alcohol-preserved samples is less satisfactory. Biomass values for a particular study should be determined on samples treated in the same way.

Although generally determined on a total sample, biomass may be determined for an individual taxon. It is recommended that cases or "houses," such as caddisfly larval cases, be removed from the sample, but that shells of mollusks and crustaceans be included. If shelled animals constitute a major part of the total weight, their weights may be reported separately.

7.5 To determine wet weight, remove external water or preservative from the animals by blotting for 1 minute on filter paper. Subdivide clumps of organisms, but do not separate individuals during blotting. Weigh to 0.1 mg.

7.6 To determine dry weight, place the organisms 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. Higher drying temperatures are sometimes used, but there is danger of erroneously low values resulting from volatilization or decomposition of fats (Edmondson and Winberg, 1971).

7.7 To determine ash weight, ignite the crucible and sample at 500°C to constant weight. Allow at least 1 hour, but some samples will require longer times. Cool in a desiccator and weigh to 0.1 mg.

7.8 Density separation (optional). This consists of treating the sample with a solution of such a density that most of the invertebrates will float, and most of the unwanted detritus will sink. The recommended method employs a sucrose solution of specific gravity 1.12 (Anderson, 1959; Lackey and May, 1971).

Drain the sample on a No. 70 or other appropriate sieve, discard the liquid, and transfer the residue to a white enamel pan. Flood the material in the pan with the sugar solution, and stir so that the material is evenly spread over the bottom. Remove organisms from the surface of the liquid with forceps or with fine-mesh scoops. After removing all visible organisms, stir the materials, and remove any specimens that appear. Pour the sugar solution through the sieve and cover the residue in the pan with water. Examine as described in 7.2, looking especially carefully for oligochaete worms, aquatic mites, and for the heavier organisms such as mollusks and caddisfly larvae in cases. After this examination, pour the water through the sieve, and repeat the sucrose treatment. Few organisms should be found, but, if large numbers are recovered, the sample should be soaked in water and again treated with the sugar solution. The sugar solution may be reused by adjusting the specific gravity to 1.12 as determined with a hydrometer. However, the solution spoils rapidly and should not be stored for more than a few days.

7.9 Differential staining (optional). Separation of invertebrates, especially transparent forms, from samples is facilitated by staining them red with 200 mg/l of Rose Bengal added to the preservative solution. Expose the organisms 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 in the sample. If necessary to restore natural coloration for identification, remove the stain from the organisms by placing them in 95 percent ethyl alcohol (Mason and Yevich, 1967). A counterstaining technique in which Rose Bengal or Lugol's iodine is counterstained with chlorazol black may be used to provide a high color contrast between invertebrates and detritus (Williams and Williams, 1974).

7.10 Subsampling (optional). Some benthic samples are so large or contain such large numbers of organisms that it is impractical to sort or count the entire sample. Remove the larger organisms and pieces of detritus from the entire sample as described in 7.2. Make the remaining sample up to a definite volume, and pour into a beaker about one-third larger than the volume of the sample. Mix the sample to distribute the organisms uniformly throughout the fluid, but not so violently that delicate specimens are fragmented. Bubbles from an aquarium air diffuser are a gentle, effective method of mixing. While the sample is thoroughly agitated, remove a subsample with a small dipper or with a wide-bore pipet, keeping the tip of the pipet in motion during filling. The subsample should be at least one-quarter to one-third of the sample volume, although if necessary the subsample may be divided again before sorting or counting.

8. Calculations

8.1 When only part of the total sample is sorted or counted as described in 7.10 above, extrapolate the results from the subsample to the number of specimens

in the total sample. Total number of individuals of a particular taxon in sample

$$= \frac{\text{number of individuals of the taxon in subsample}}{\text{volume of subsample (ml)}} \times \text{volume of total sample (ml).}$$

8.2 Percent composition in sample

$$= \frac{\text{number of individuals of a particular taxon}}{\text{total number of individuals in collection}} \times 100.$$

8.3 Wet weight of benthic invertebrates (g/sample)

$$= \frac{\text{wet wt of organisms in all samples (g)}}{\text{number of samples}}$$

8.4 Dry weight of benthic invertebrates (g/sample)

$$= \frac{\text{dry wt of organisms in all samples (g)}}{\text{number of samples}}$$

8.5 Ash weight of benthic organisms (g/sample)

$$= \frac{\text{ash wt of organisms in all samples (g)}}{\text{number of samples}}$$

8.6 Organic weight (loss on ignition) of benthic invertebrates (g/sample)

= dry wt (g or mg/sample) − ash wt (g or mg/sample.

8.7 Results of individual rock sampling (6.3 above) are expressed as benthic organisms/projected area (aspect) of rock or organisms/total rock surface:

Benthic invertebrates/m^2 of projected rock surface

$$= \frac{\text{number of organisms collected from rock}}{\text{length of longest axis of rock (mm)} \times \text{length of intermediate axis of rock (mm)} \times 10^6}$$

Benthic invertebrates/cm^2 of total rock surface

$$= \frac{\text{number of organisms collected from rock}}{\pi\,[\text{length of intermediate axis of rock (mm)}]^2} \times 100.$$

9. Report

Report the number of taxa present, the percentage composition of each taxon in the sample, and the type of collection methods used. Report biomass to two significant figures.

Results are expressed in terms of the total collection at each sampling station, of a particular habitat sampled, or of the artificial-substrate sampler(s).

10. Precision

No numerical precision data are available.

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Distribution and abundance (quantitative method)
(B–5040–77)

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. Application

This method must be used in studies of biological productivity of benthic communities. It is applicable to all natural waters.

2. Summary of method

Benthic invertebrates are collected from a defined area using a suitable method 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 amounts of sand, gravel, and plant detritus, are preserved in the field. In the laboratory the animals are separated from the extraneous material, identified, and counted or weighed. Results are reported as numbers of different kinds of organisms (taxa) and of individuals in each taxon per unit area. Biomass is reported as wet, dry, ash, or organic weight per unit area of bottom.

3. Interferences

Physical factors such as stream velocity and depth of water may interfere with sampling. Most samples contain relatively large amounts of sediment and plant debris from which the organisms must be separated. The principal interference with quantitative sampling, however, is the heterogeneity of many streambeds and lakebeds and the temporal and spatial variability of the invertebrate populations.

4. Apparatus

Methods and equipment for quantitative sampling of benthic invertebrates are described in Welch (1948), Macan (1958), Albrecht (1959), Cummins (1962, 1966, 1975), Schwoerbel (1970), Hynes (1970), American Public Health Association and others (1976), Holme and McIntyre (1971), and Edmondson and Winberg (1971). Brinkhurst (1967) listed the following theoretical specifications for a quantitative sampler:

(*a*) Depth of penetration. Animals are found deep in the sediment, and a true measure of total standing crop or proportional representation of species requires that the sampler take sediment from the surface to a depth of at least 20 cm.

(*b*) Bite. The "bite" of a sampler should be such that all depths are sampled equally in any one attempt. The "bite" characteristics should allow the surface area sampled to be estimated accurately.

(*c*) 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.

(*d*) Internal pressure. The descent of a sampler should not create a pressure wave that will blow off the topmost sediments or give a directional signal to organisms 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 sediments and

flowing waters. 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 it is desired to sample (Macan, 1958). The devices listed below are those most commonly used or those that appear to be best suited to the work of the Geological Survey.

4.1 *Cylindrical box or drum sampler,* Edmondson and Winberg (1971, p. 69). This is a strong metal cylinder open at top and bottom which 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 (1.5 in.) long which cut into the bed as the sampler is rotated. Cylindrical samplers can be lengths of stovepipe, 30-cm-(12-in.) diameter aluminum irrigation pipe (Weber, 1973), or constructed to enclose any convenient area in keeping with the study objectives and the size of the bed materials. A sample area of 900 to 1,000 cm^2 (about 1 ft^2) is common. The maximum practical height for the box is about 75 cm (29.5 in.) because of the necessity to reach the bottom with the hands.

A *soft-bristle brush,* a small *dip net* of nylon monofilament screen cloth (Tobler, Ernst and Traber, Inc., Nitex, or equivalent), 210- or 216-μm or other approximate mesh opening, and a *garden trowel* or small *digging fork* are needed for removing the organisms from the substrate enclosed by the sampler.

4.2 *Stream-bed fauna sampler*, Kahl Scientific Instrument Corp. (215WA305) (fig. 33), or equivalent, is one of various modifications of the solid cylinder. Others 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.

4.3 *Surber stream-bottom sampler*, Wildlife Supply Co. (12), Kahl Scientific Instrument Corp. (215WA340), or equivalent (fig. 34). This sampler has been widely used in stream studies, although the enclosed box-type samplers are preferred, if available. Modifications of the stream-bottom sampler (Waters and Knapp, 1961; Withers and Benson, 1962; Mundie, 1971) eliminated many deficiencies of the original

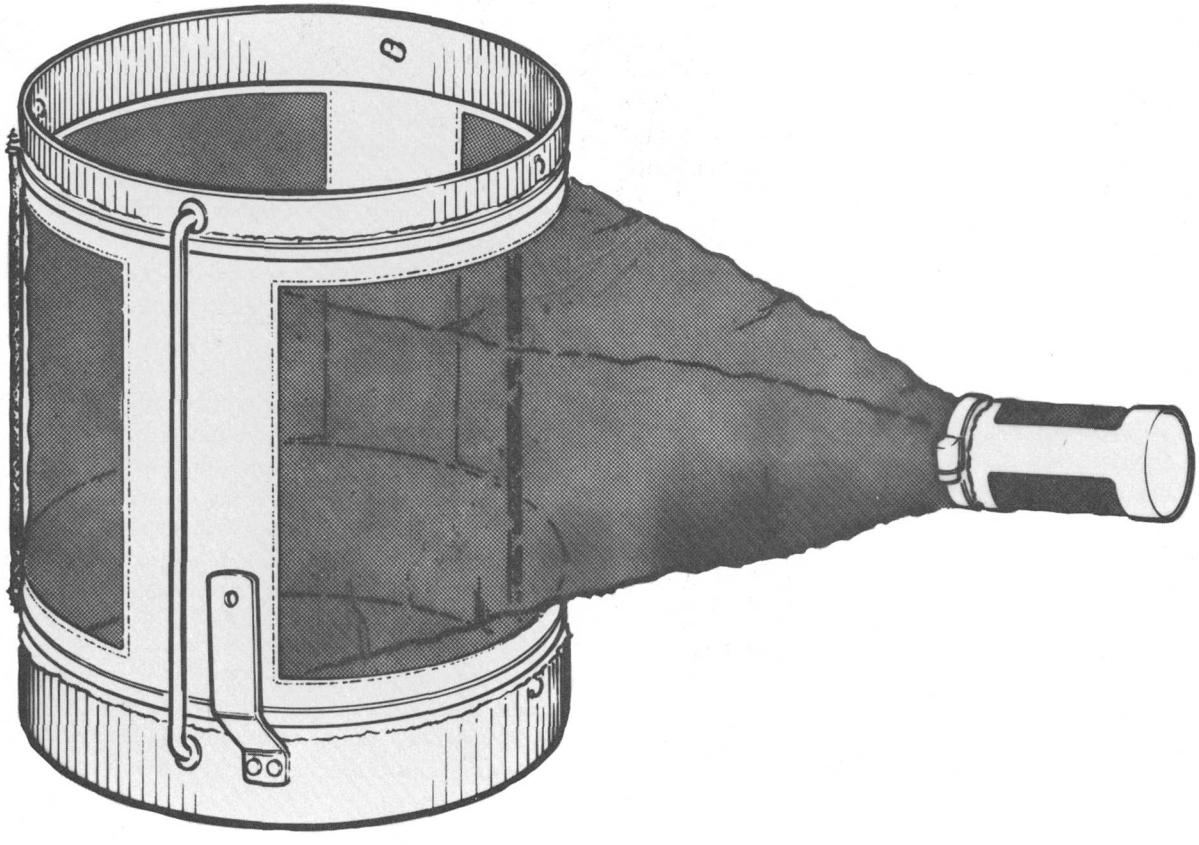

Figure 33.—Fauna sampler. (Photograph courtesy of Kahl Scientific Instrument Corp., El Cajon, Calif.)

design. Netting used in the construction or operation of these samplers should be nylon monofilament screen cloth (Tobler, Ernst, and Traber, Inc., Nitex, or equivalent) 210- or 216-μm mesh opening, unless otherwise dictated by the study objectives.

4.4 *Ekman grab*, preferably the tall design (fig. 21), 15×15 cm square, 23–30 cm high, Kahl Scientific Instrument Corp. (214WA170), Wildlife Supply Co. (196T), or equivalent. Extra weights are available to increase the depth of penetration. In deep water the grab is tripped with a messenger, whereas in shallow water the Ekman grab may be operated with a handle: Kahl Scientific Instrument Corp. (214WA172), Wildlife Supply Co. (196H), or equivalent.

4.5 *Ponar grab*, Wildlife Supply Co. (1725), *Screen-top sediment sampler*, Kahl Scientific Instrument Corp. (214WA010) or equivalent (fig. 23). This grab trips on bottom contact and has provision for water to pass through to lessen the shock wave (Flannagan, 1970; Hudson, 1970). Accessory weights may be used, and it is advisable to operate these grabs with a winch. The weight when empty is about 23 kg (45 lb) without weights and about 32 kg (70 lb) with weights.

4.6 *Van Veen grab*, Kahl Scientific Instrument Corp. (214WA265) (fig. 35), weighs 48 kg (107 lb) and may be loaded with additional weights. The grab has a capacity of 40 liters (10.5 gal) and samples an area of 1,500 cm^2(1.6 ft^2). Screened panels allow 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.

4.7 *Core sampler*, K.B.-type, Wildlife Supply Co. (2400), Phleger corer, Kahl Scientific Corp. (217WA200) (fig. 36), or equivalent. Extra weights are available to increase the depth of penetration, and, when so used, a winch may be required. These corers have provision for water to pass through during descent but are closed to prevent loss of sample during ascent. In shallow water a *hand corer* (Kahl Scientific Instrument Corp. 217WA100; Wildlife Supply Co. 2420, 2422, or 2424; or equivalent) may be used.

4.8 *Gloves, waterproof*, Hudson Bay trappers gloves, 79-cm (31-in.) gauntlet, Herter's, Inc., (YB4A) or equivalent.

4.9 *Sample containers* of plastic for transporting

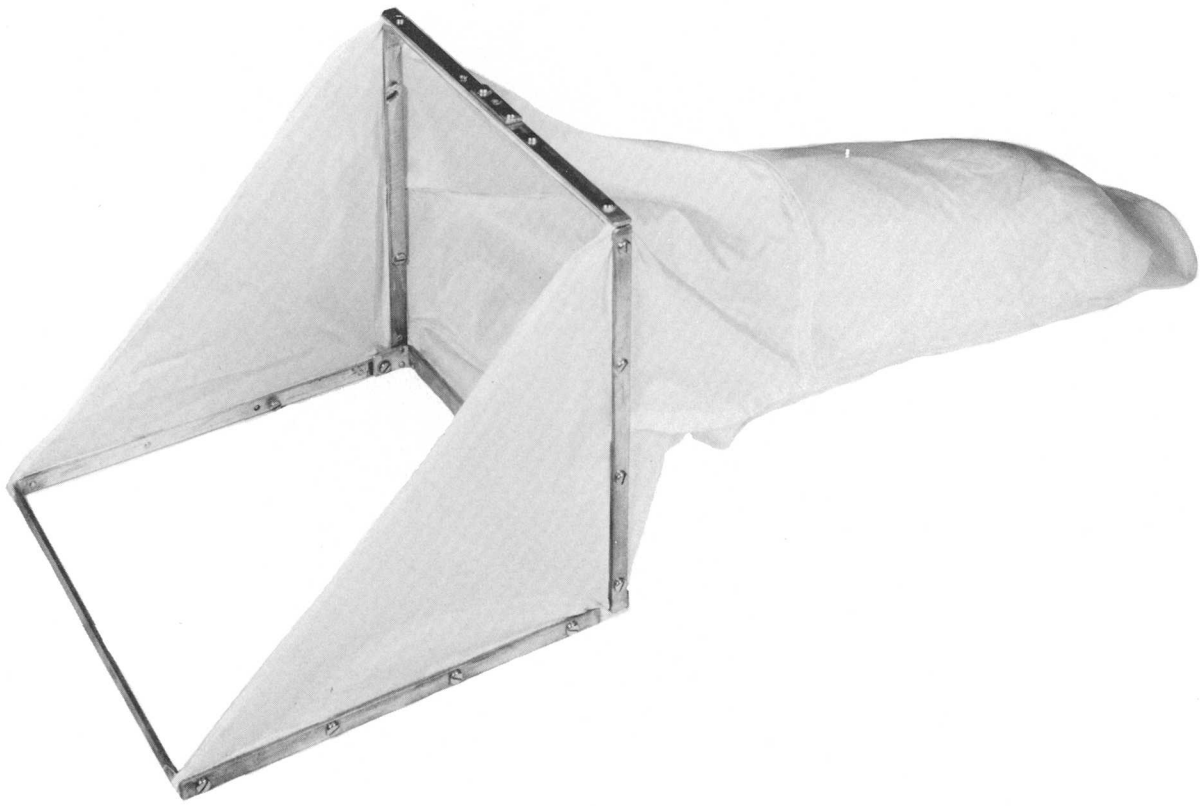

Figure 34.—Surber stream-bottom sampler. (Photograph courtesy of Wildlife Supply Co., Saginaw, Mich.)

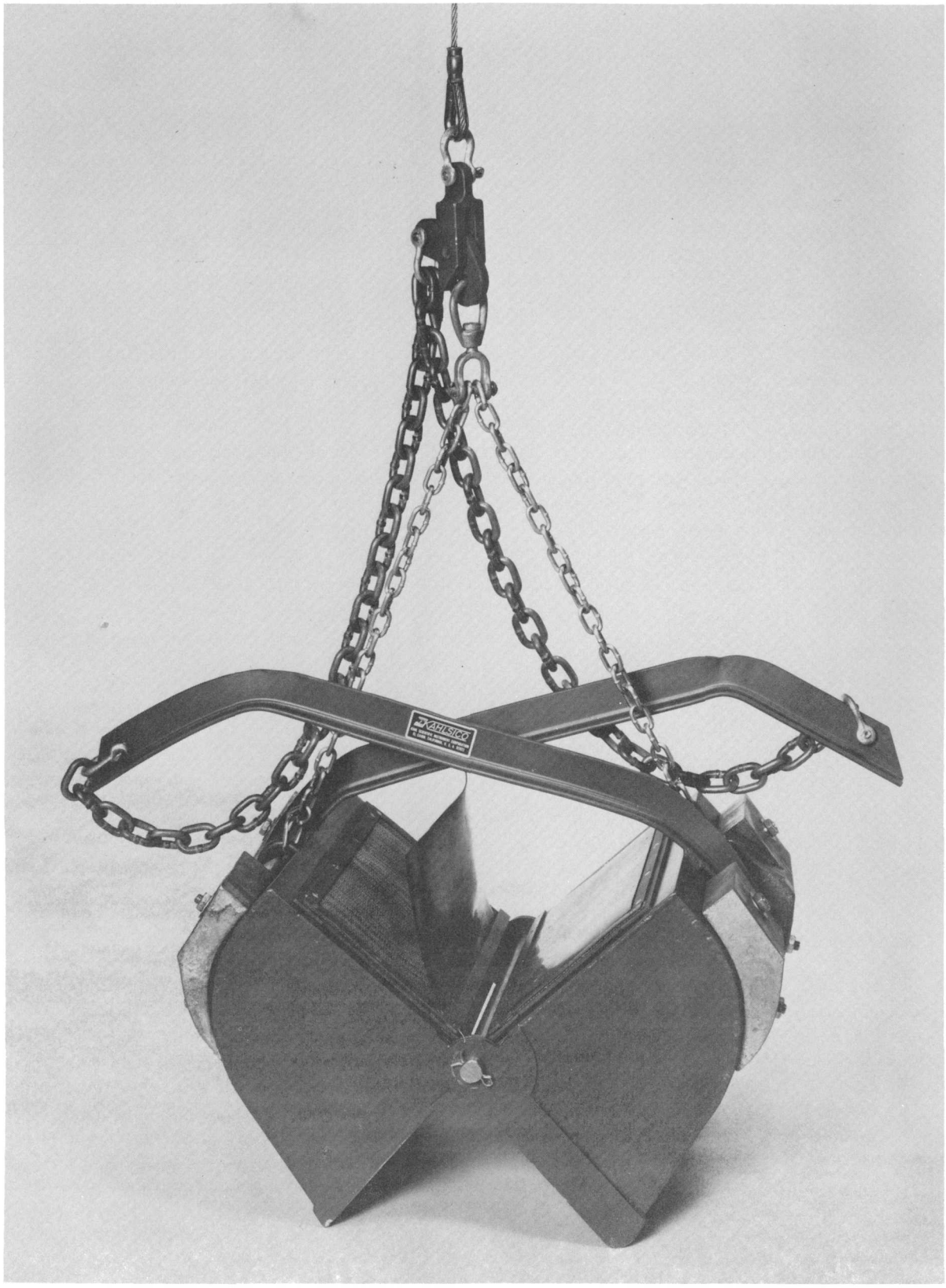

Figure 35.—Van Veen grab. (Photograph courtesy of Kahl Scientific Instrument Corp., El Cajon, Calif.)

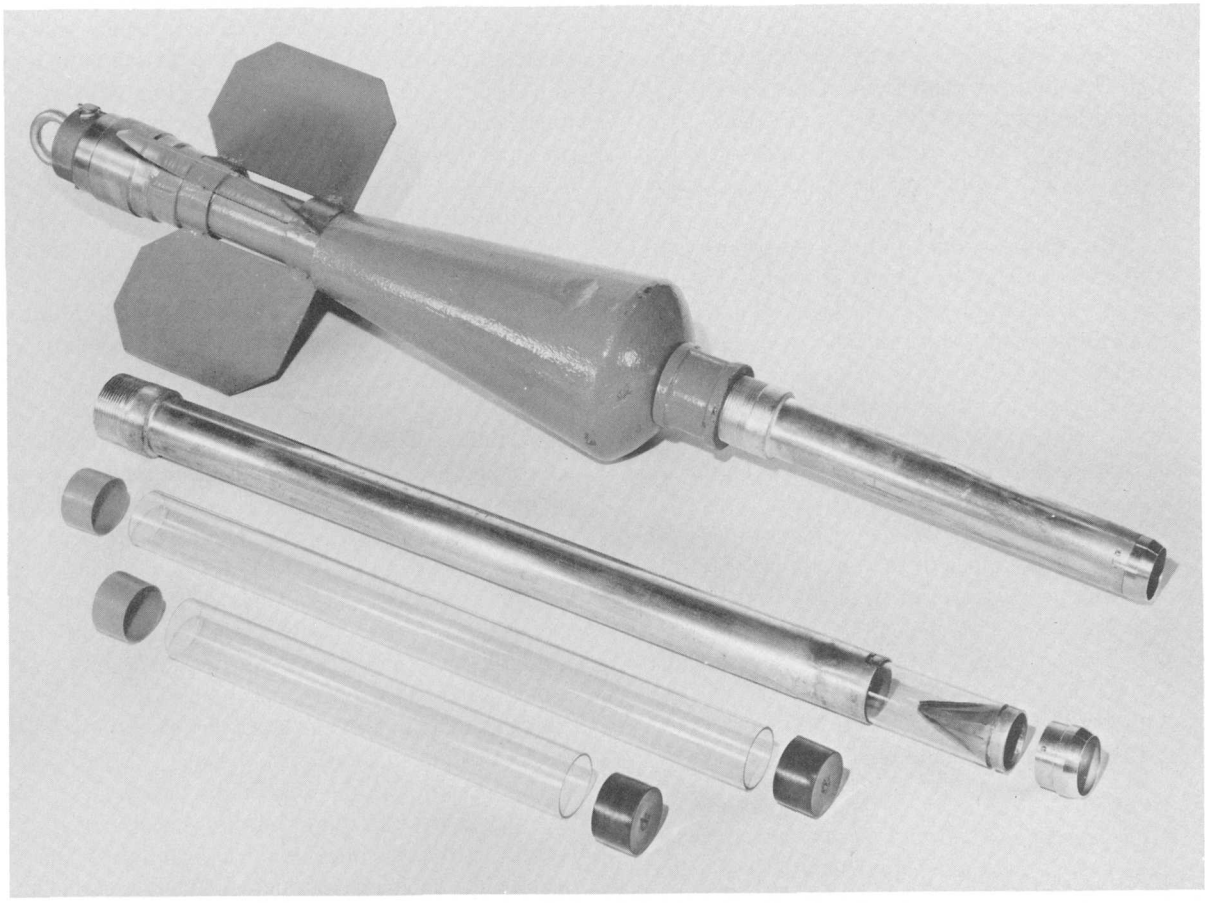

Figure 36.—Phleger corer. (Photograph courtesy of Kahl Scientific Instrument Corp., El Cajon, Calif.)

unsorted samples to the laboratory. Wide-mouth jars of 120-, 240-, and 475-ml (4-, 8-, and 16-oz) capacity are useful sizes. Jar lids should be of plastic if samples are to be stored before processing. Sealable plastic bags (Nasco, Whirl-Pak, or equivalent) also may be used for temporary storage of benthic sample.

4.10 *Vials* with plastic screw lids. Convenient sizes are 7.5-, 15-, and 22-ml (2-, 4-, and 6-dram) capacity.

4.11 *Waterproof labels*, Turtox/Cambosco (376A182) or equivalent; or labels may be cut from sheets of plastic paper, Nalgene Labware (6304–0811) or equivalent.

4.12 *Waterproof ink*, Higgins Eternal Ink or equivalent.

4.13 *Plastic tape*, Scotch (33) or equivalent, for sealing jar and vial lids.

4.14 *Forceps* with fine points and rounded points. Forceps with fine points are useful for handling small organisms. Forceps with rounded points are less likely to tear netting or puncture the mesh of sieves or other sampling equipment. These are less likely to be lost in the field if marked with bright paint or colored tape.

4.15 *U.S. Standard Sieve*, 20-cm (8-in.) diameter, with mesh appropriate to the study objectives. The No. 70 sieve, 210-μm mesh opening, has been adopted for retaining benthic invertebrate organisms collected in the water-quality programs of the Geological Survey. Sieves with smaller or larger mesh, such as U.S. No. 30, 595-μm mesh openings, may be more suitable for some studies. The No. 18 sieve, 1,000-μm mesh, 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.

4.16 *Tub or bucket* for washing samples or sampling equipment in the field.

4.17 *Microscope*, stereoscopic variable power, x 7 to x 30, Bausch and Lomb (BVB–73) or equivalent, with microscope illuminator, Bausch and Lomb (31–33–24–01) or equivalent. A compound microscope of at least × 100 magnification also is useful for taxonomic work.

4.18 *Trays*, white enamel. Useful sizes are

30×19×5 cm (12×7.5×2 in.) and 42×26×6 cm (16.5×10×2.25 in.).

4.19 *Dishes*, glass petri or Syracuse watchglasses.

4.20 *Hydrometer*, plain form, range 1.000–1.220, Scientific Products (H8750–1) or equivalent.

4.21 *Pump, air*, aquarium-type, or *pump, pressure-vacuum*, Millipore (XX60 000 00) or equivalent.

4.22 *Air-diffuser stones*, porous aquarium-type.

4.23 *Fine-mesh scoops*, 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.

4.24 *Beaker*, 15-ml, or small *measuring cup* to be used for withdrawing subsamples. Alternately, a wide-bore pipet of suitable capacity can be made by cutting the tip from a volumetric pipet.

4.25 *Beakers and graduated cylinders*, large sizes as needed for the types of samples in process.

4.26 *Balance*, capable of weighing to 0.1 mg.

4.27 *Porcelain crucibles*.

4.28 *Drying oven*, thermostatically controlled for use at 105°C.

4.29 *Muffle furnace*, for use at 500°C.

4.30 *Desiccator* containing silica gel.

5. Reagents

5.1 *Preservative solution*. Invertebrate samples may be preserved in 70 percent ethyl alcohol or 40 percent isopropyl alcohol. Formaldehyde solution is not recommended. Ethyl alcohol is preferred for permanent storage. Prepare as follows. Ethyl alcohol: 70 ml of 95 percent alcohol diluted to 95 ml with distilled water. Isopropyl alcohol: 40 ml of concentrated alcohol diluted to 100 ml with distilled water.

5.2 *Sucrose solution*, specific gravity 1.12, for density separation of invertebrates from debris in benthic samples: Dissolve 360 g of granulated sugar per liter of water (about 2.5 lb of sugar per gal of solution).

5.3 *Rose Bengal biological stain*. Matheson, Coleman and Bell (RX155) or equivalent.

5.4 *Glycerin*.

6. Collection

The statistical principles of benthic invertebrate sampling are discussed by Elliott (1971) and are summarized in "Part 1, Biological Sampling and Statistics." 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 employed, 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 ($n > 50$) are preferable, but usually impractical because of the labor involved in collection and analysis. The size of small samples can be calculated for a specified degree of precision (Elliott, 1971, p. 128–131). The sampling units are usually located at random in the sampling area, and all the available sites in the area must have an equal chance of selection for the sample. Stratified random sampling is preferable to simple random sampling.

It is often impossible to make a complete and accurate estimate of the numbers of all species in a large area of bottom. 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, 1971, p. 127). This means that if the study objective is to compare the number and abundance of species at several stations or on different sampling dates, numbers or biomass per unit area may be needed only for a particular type of homogeneous substrate. It is important, however, to define clearly the area of the substrate sampled.

The literature on the quantitative study of benthic invertebrates in flowing waters was reviewed by Hynes (1970). He concluded that quantitative data on the benthic fauna are extremely difficult to obtain and are at best very rough estimates. Nevertheless, if three or more samples are collected, a general picture of the abundance of the more common species can be expected. Sampling in a long transect line which parallels some obvious environmental gradient, such as from shallow to deep water, provides a high probability that most species will be taken at least once (Elliott, 1971, p. 127).

Sampling frequency must be based on study objectives. Waters (1969) and Cummins (1975) emphasized that sampling for the estimation of benthic production should be adjusted to the period of maximum change in growth and survivorship. For populations having typical survivorship with maximum mortality in the early instars and with 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 at weekly intervals or less should be conducted during periods when most of the species are in early age classes. In the temperate zone this period is generally late spring and late fall (Cummins, 1975).

Quantitative studies require the collection of all benthic invertebrates within the selected size range

from the sampling unit. 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 organisms has been studied in soft sediments (Lenz, 1931; Cole, 1953; Ford, 1962; Brinkhurst and others, 1969) and in coarse sediments (Coleman and Hynes, 1970; Mundie, 1971; Bishop, 1973). As a guide to the depth of sampling, Cummins (1975) proposed measuring the oxygen profile in the sediments to determine the depth of the oxygenated zone (Ericksen, 1963) or sampling at least to a depth at which the sediments appear anaerobic; 0.01–0.1 m in fine, homogeneous sediments and 0.1–0.3 m in coarse, heterogeneous sediments.

6.1 The cylindrical box or drum sampler, depending on its design, is used by pushing the bottom edge downward to seal a compressible edge or by rotating the cylinder back and forth into the substratum. In the latter, teeth dig into the bed, and a flange of metal and foam rubber or plastic also helps to isolate the enclosed area. In flowing water, use one of the samplers with mesh panels to decrease scour as it approaches the bottom.

To remove the fauna from the sample area, begin by placing the large rocks into a bucket of water. In cold weather this work is more bearable if long-gauntlet rubber gloves are worn. Thoroughly disturb the remaining sediment by digging and stirring as deeply as possible with a trowel or fork, then stir the water vigorously with the small dip net while straining suspended material from the liquid. Empty the net into the bucket and continue the process until no additional animals are collected. Samplers for streams usually have a net into which suspended organisms and detritus are carried by the current. It may be necessary to remove more sediment from the enclosed area as digging and stirring proceed. Remove the large rocks from the bucket and discard after scrubbing with a brush. Pour the contents of the bucket through a sieve of appropriate mesh size. Transfer the concentrated sample to a shallow white tray if the sample is to be sorted in the field or into a wide-mouth container for transporting to the laboratory. Label and preserve the sample as described in 6.6 and 6.7 below.

6.2 Press the bottom edge of the Surber sampler (fig. 34), or one of the recommended modified samplers, firmly against the substrate to isolate the enclosed area as completely as possible. These samplers depend on the current to carry organisms 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 create a flow sufficient to sweep benthic organisms into the net.

To remove the fauna from the area enclosed by the sampler, lift the larger rocks and scrub them into the mouth of the net. In cold weather this work is more bearable if long-gauntlet rubber gloves are worn. Thoroughly disturb the remaining sediment by repeatedly digging and stirring as deeply as possible, allowing the current to sweep the organisms 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 in the field or into a wide-mouth container for transporting to the laboratory. Label and preserve the catch as described in 6.6 and 6.7 below.

6.3 The Ekman grab (fig. 21) is the preferred sampler for mud, silt, or fine sand. In deep water the sampler is lowered to the bottom, allowed to settle into the sediment, and then tripped closed by dropping a messenger down the line. On arrival at the surface, the sampler jaws are opened and the contents emptied into a tub, a sieve, or a wide-mouth container for transporting to the laboratory. 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 sediments because the operator can force the sampler shut by exerting additional pressure on the upper edge of each jaw.

It usually is desirable to wash mud from the sample. This is best done by putting small amounts of a sample into a No. 70 or other appropriate sieve and agitating it gently with the mesh submerged in water. Washing samples by pouring water through the sieve must be done gently to avoid forcing small organisms through the mesh. Label and preserve the sample as described in 6.6 and 6.7 below.

6.4 The Ponar, Screen-top, and Van Veen grabs (figs. 23 and 35) can be used for deep-water sampling in gravel, hard sand, and clay, as well as in soft sediments. These instruments trip on bottom contact, 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 can sometimes be lowered nearly to the bottom, then dropped suddenly so that it makes contact in an upright position. Empty the sampler into a tub, and wash the sample free from mud, if present. This is best done by putting small amounts of sample in a No. 70 or other appropriate sieve and agitating it gently with the mesh submerged in water. Washing samples by pouring water through the sieve must be done gently to avoid

forcing small organisms through the mesh. Label and preserve the sample as described in 6.6 and 6.7 below.

6.5 Corers are used when an undisturbed sample of sediment is required. They are especially suitable for clay, silt, or sand bottoms, and are more widely used in lakes than in streams. Hand corers designed for manual operation can be used in shallow water up to several meters in depth. Deeper waters require devices such as the K.B.-type or Phleger corer (fig. 36) which depend on gravity to drive them into the sediment. All corers have some provision for retaining the sample as the instrument is withdrawn from the sediment and returned to the surface. Follow the manufacturer's instructions carefully 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 as in 6.6 and 6.7 below.

6.6 Samples for which only biomass will be determined should be frozen as soon as possible after collection. Samples for taxonomic determination should be preserved in alcohol. To insure adequate preservation, fill containers no more than half full with the sample so that a volume of alcohol can be added at least equal to the volume of organic material, including detritus. Preserve the organisms or the unsorted sample in 70 percent ethyl alcohol or 40 percent isopropyl alcohol. Containers should be filled nearly to the top to avoid excessive sloshing and damage to delicate specimens.

Note: If unsorted samples are to be stored for more than a few weeks, the preservative should be drained after about 1 week and replaced with fresh preservative.

6.7 Label the sample with the location, habitat, date and time of collection (local standard time), name of collector, sampling method, and sample treatment (type of preservative, mesh size of screens or nets, or other treatment). Soft black pencil may be used in the field, but use a waterproof carbon ink for permanent labels. Labels should be placed inside the sample containers so that they are visible from the outside, or duplicate labels should be placed inside and outside. Secure jar lids with tape to prevent their working loose and the subsequent loss of preservative by evaporation. This is especially important if samples are to be stored or shipped.

7. Analysis

7.1 If the study objectives require determination of only the most abundant benthic forms, sorting often can be completed in the field. Wash the sample gently in a net or sieve of appropriate mesh size to remove mud and fine detritus. Pick the organisms directly from the sampled material or, to enhance visibility of small forms, cover the collected sample with water in a white enamel tray, and stir repeatedly while removing the organisms with forceps or scoops.

7.2 Generally, sorting must be done in the laboratory. This may be done by pouring small portions of sample into a shallow dish, covering the material with water, and scanning the dish under low-power magnification (× 3 to × 10). Remove the organisms from the debris with forceps or with fine-mesh scoops. If taxonomic determination is required, proceed to 7.3. If only biomass determination is required, proceed to 7.4.

The sorting process is very time consuming for many samples. The optional steps described in 7.8 through 7.10 below may be used to speed the work when the study objectives require complete analysis.

7.3 Identify and enumerate the benthic invertebrates in the sample according to taxonomic categories. The degree of identification required varies with the objectives of the study. Most identifications will be performed by a biologist with specialized training in this work. A stereoscopic microscope is required, and, for some groups, dissections or microscopic mounts may be needed to observe key characters. Appropriate reference books (See "Part 4. Taxonomic References") should be available. It is convenient to place the different categories of organisms in separate vials of 70 percent ethyl or 40 percent isopropyl alcohol, labeled with the name of the organism and the identification number, date, and origin of the sample. Add a few drops of glycerin to the vials, and seal caps with tape if the specimens are to be stored.

7.4 The biomass of benthic invertebrates, expressed as wet, dry, ash, or organic (ash-free) weight, is best determined on samples that were frozen immediately after collection. Biomass determined on alcohol-preserved samples is less satisfactory, but biomass values for a particular study should be determined on samples treated in the same way. Although generally determined on the total sample, biomass may be determined for the individual taxa. It is recommended that cases or "houses", such as caddisfly larval cases, be removed from the sample, but shells of mollusks and crustaceans be included. If shelled animals constitute a major part of the total weight, their weights may be reported separately.

7.5 To determine wet weight, remove external water or preservative from the animals by blotting for 1 minute on filter paper. Subdivide large clumps of or-

ganisms, but do not separate individuals during blotting. Weigh to 0.1 mg.

7.6 To determine dry weight, place the organisms 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. Higher drying temperatures are sometimes used, but there is danger of erroneously low values resulting from volatilization or decomposition of fats (Edmondson and Winberg, 1971).

7.7 To determine ash weight, ignite the sample in the tared crucible at 500°C to constant weight. Allow at least 1 hr, but some samples will require longer times. Cool in a desiccator, and weigh to 0.1 mg.

7.8 Density separation (optional). This step consists of treating the sample with a solution of such a density that most of the invertebrates will float, and most of the unwanted detritus will sink. The recommended method employs a sucrose solution of specific gravity 1.12 (Anderson, 1959; Lackey and May, 1971).

Drain the sample on a No. 70 or other appropriate sieve, discard the liquid, and transfer the residue to a white enamel pan. Flood the material in the pan with the sugar solution, and stir so that the material is evenly spread over the bottom. Remove organisms from the surface of the liquid with forceps or with fine-mesh scoops. After removing all visible organisms, stir the material, and remove any specimens that appear. Pour the sugar solution through the sieve, and cover the residue in the pan with water. Examine as described in 7.2, looking especially carefully for oligochaete worms, aquatic mites, and for the heavier organisms such as mollusks and caddisfly larvae in cases. After this examination, pour the water through the sieve, and repeat the sucrose treatment. Few organisms should be found, but, if large numbers are recovered, the sample should be soaked in water and again treated with the sugar solution. The sugar solution may be reused by adjusting the specific gravity to 1.12 as determined with a hydrometer. However, the solution spoils rapidly and should not be stored for more than a few days.

7.9 Differential staining (optional). Separation of invertebrates, especially transparent forms, from samples is facilitated by staining them red with 200 mg/l of Rose Bengal added to the preservative solution. Expose the organisms 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 in the sample. If necessary to restore natural coloration for identification, remove the stain from the organisms by placing them in 95 percent ethyl alcohol (Mason and Yevich, 1967). A counterstaining technique in which Rose Bengal or Lugol's iodine is counterstained with chlorazol black may be used to provide a high color contrast between invertebrates and detritus (Williams and Williams, 1974).

7.10 Subsampling (optional). Some benthic samples are so large or contain such large numbers of organisms that it is impractical to sort or count the entire sample. Remove the larger organisms and pieces of detritus from the entire sample as described in 7.2. Make the remaining sample up to a definite volume, and pour into a beaker about one-third larger than the volume of the sample. Mix the sample to distribute the organisms randomly throughout the fluid, but not so violently that delicate specimens are fragmented. Bubbles from an aquarium air diffuser are a gentle, effective method of mixing. While the sample is thoroughly agitated, remove a subsample with a small dipper or with a wide-bore pipet, keeping the tip of the pipet in motion during filling. The subsample should be at least one-quarter to one-third of the sample volume, although if necessary the subsample may be divided again before sorting or counting.

8. Calculations

8.1 When only part of the total sample is sorted or counted as described in 7.10 above, extrapolate the results from the subsample to the number of specimens in the total sample. Total number of individuals of a particular taxon in sample

$$= \frac{\text{number of individuals of the taxon in subsample}}{\text{volume of subsample (ml)}} \times \text{volume of total sample (ml).}$$

8.2 Number of benthic invertebrates/m^2

$$= \frac{\text{number of organisms in all samples}}{\text{area of sampler } (m^2) \times \text{ number of samples.}}$$

8.3 Wet weight of benthic invertebrates (g/m^2)

$$= \frac{\text{wet wt of organisms in all samples (g)}}{\text{area of sampler } (m^2) \times \text{ number of samples.}}$$

8.4 Dry weight of benthic invertebrates (g/m^2)

$$= \frac{\text{dry wt of organisms in all samples (g)}}{\text{area of sampler } (m^2) \times \text{ number of samples.}}$$

8.5 Ash weight of benthic invertebrates (g/m^2)

$$= \frac{\text{ash wt of organisms in all samples (g)}}{\text{area of sampler } (m^2) \times \text{ number of samples.}}$$

8.6 Organic weight (loss on ignition) of benthic invertebrates (g/m^2)

= dry wt (g/m^2) − ash wt (g/m^2).

9. Report

Report as follows: Less than 100 individuals/m^2, to the nearest whole number; 100 individuals and above, two significant figures. Report biomass to two significant figures.

Results are expressed in terms of a unit area of the habitat sampled.

10. Precision

No numerical precision data are available.

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Permanent slide method for larvae of Chironomidae (B–5200–77)

Parameter and code: Not applicable

Chironomidae (midges) is a family of the insect Order Diptera (2-wing 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 significant 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 which 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), but instead have anal gills on the last segment. 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 which they build from algae, fine sediment, and bits of plant debris bound or cemented together with a salivary secretion (fig. 38). Often 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 upon diatoms and other algae, organic detritus, microcrustaceans, and other midge larvae.

Adult chironomids are small, delicate, gnat-like nonbiting flies (< 10 mm long) which 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 in 2 years, while others have several generations in 1 year.

Identification of chironomid larvae is based mainly on the mouth parts which can be seen only with a microscope. The method described is a modification of procedures given by Mason (1968, 1970) and Beck (1968).

1. Application

The method is suitable for all chironomid larvae.

2. Summary of method

Chironomidae larvae from a benthic invertebrate sample are sorted into visually distinct groups. The specimens are heated in 10 percent KOH solution to dissolve all soft body tissues, placed ventral side up on a microscope slide in a mounting medium, and pressed under a cover glass. The mounts are identified. The number of taxa and individuals within each taxon are tabulated and expressed as a percentage of the benthic invertebrate population.

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.

4. Apparatus

4.1 *Compound light microscope*, Tiyoda (20049) or equivalent, capable of × 400 to ×500 magnification.

4.2 *Stereoscopic zoom microscope*, (dissecting) Nikon SMZ or equivalent, capable of × 80 magnification.

4.3 *Microscope slides*, glass, precleaned, 25×75 mm.

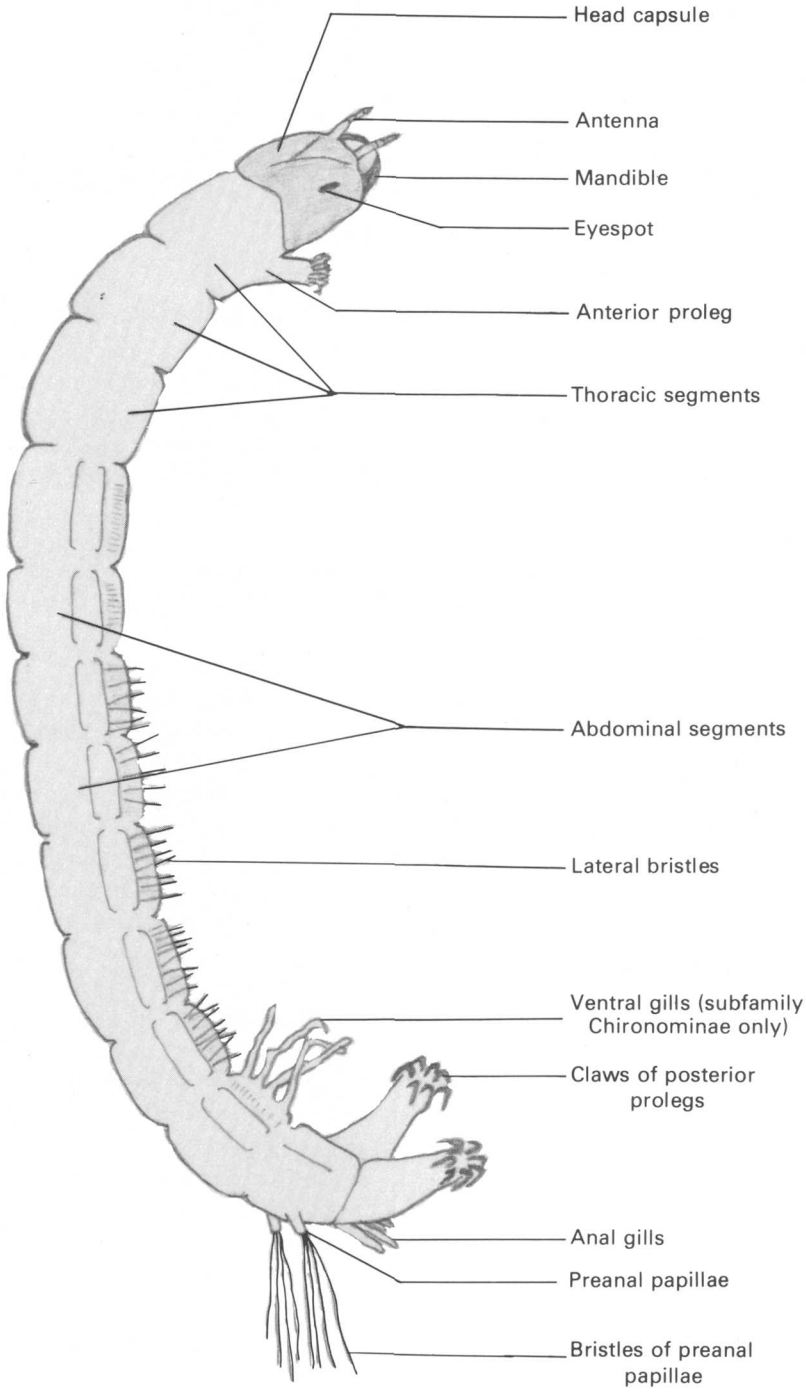


Figure 37.—Idealized external features of a larva from the Family Chironomidae. Features are taken from more than one subfamily.

4.4 *Cover glass*, round, No. 1 or 2, 12-mm diameter.

4.5 *Needles, pins, and probes* for manipulating specimens under stereomicroscope.

4.6 *Vials*, 4-ml (1-dram).

4.7 *Crucible*, high-form, porcelain, 10-ml capacity.

4.8 *Hotplate*, electric.

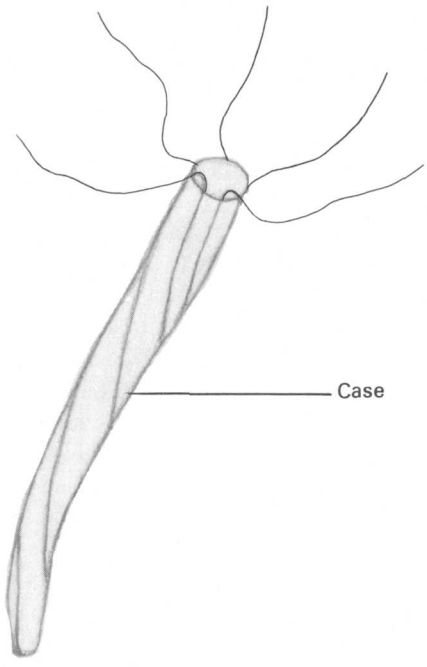
4.9 *Ocular micrometer*, graduated to 5 μm.

4.10 *Forceps*, blunt curved tips.

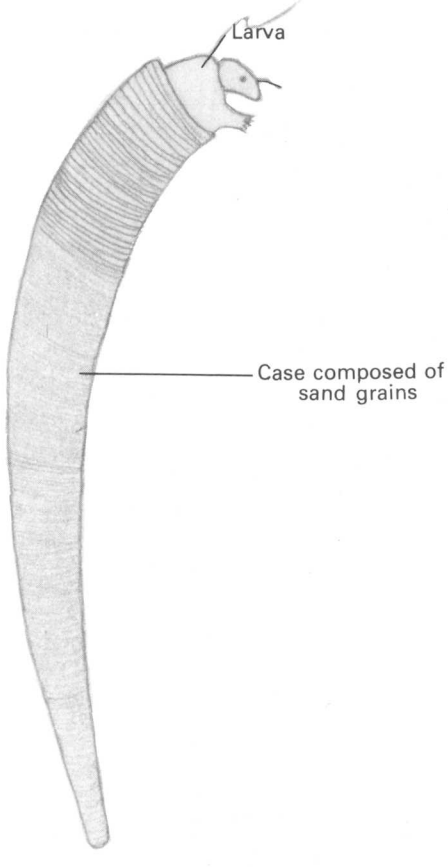
4.11 *Microforceps*, fine-tipped, Trident or equivalent.

4.12 *Marking pen*, permanent waterproof, Sanford's Sharpie or equivalent, for labeling slides.

4.13 *Spot plates*, white porcelain.

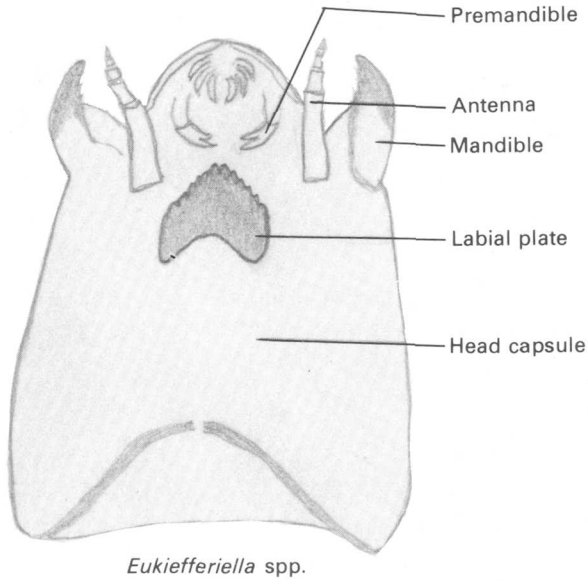


Rheotanytarsus pusio



Stempellina spp.

Figure 38.—Two examples of cases constructed by Family Chironomidae larvae.



Eukiefferiella spp.

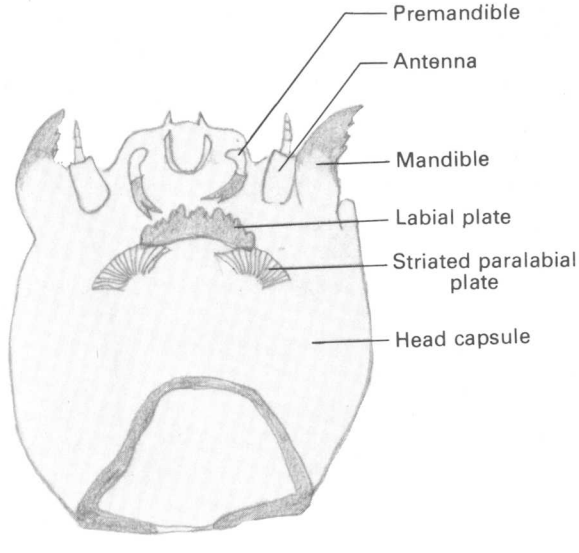
Figure 39.—Ventral view of larval head capsule from the Subfamily Orthocladiinae, simplified.

5. Reagents

5.1 *Alcohol, ethyl*, 70 percent: 70 ml of 95 percent alcohol diluted to 95 ml with distilled water; or *alcohol, isopropyl*, 40 percent: 40 ml of concentrated alcohol diluted to 100 ml with distilled water.

5.2 *Alcohol, ethyl*, 95 percent.

5.3 *Mounting medium*, CMCP–10, CCM: General



Chironomus spp.

Figure 40.—Ventral view of larval head capsule from the Subfamily Chironomidae, simplified. Notice that the left mandible is turned outward; changes in position of structures are common during mounting procedures.

Biological, Inc., or equivalent.

5.4 *Potassium hydroxide solution*, 10 percent: Dissolve 10 grams KOH pellets in 100 ml of distilled water.

5.5 *Glycerin*.

5.6 *Acetic acid*, glacial.

5.7 *Fingernail polish*.

6. Collection

Chironomid larvae are collected by any of the methods described for benthic invertebrates appropriate to the study objectives.

7. Analysis

Usually, time does not permit mounting of 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 original sample size, the number of visually distinct groups, and the study objectives.

7.1 Separate the total sample into visually distinct groups on the basis of general appearance and external features (for example, total length, color, size and shape and angle of attachment of head capsule, presence or absence of "blood gills," banding, and other peculiarities of the body). The individual depressions of porcelain spot plates are a convenient means of separating groups of larvae while processing.

7.2 Randomly select representatives of each visually distinct 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 in accordance with stratified random sampling and cluster or two-stage sampling. Store the unmounted specimens in vials of 70 percent ethyl alcohol or 40 percent isopropyl alcohol containing one drop of glycerin.

7.3 Place subgroups in depressions of a spot plate filled with distilled water and soak 3 to 5 minutes to remove the alcohol.

7.4 Transfer the subgroups to another spot plate or crucible filled with 10 percent KOH. Heat for 10–15 minutes or until the bodies are semitransparent and noticeably lighter in color. Caution: Excessive heating will result in too much digestion, making the specimens transparent and difficult to see and to manipulate. Add distilled water to the KOH solution while heating to compensate for evaporation. Note: Use fresh KOH solution for each subgroup.

7.5 Transfer the specimens from the KOH solution to a clean spot plate of distilled water for at least 3 minutes to remove the KOH.

Note: 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 serious problem.

7.6 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.

7.7 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, manipulate the specimen with 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 high viscosity mounting medium to roll, slide, or push each specimen so that it lies flat. Apply additional pressure to spread the mouth parts. Allow preparation to dry to 1 week, keeping the slide horizontal. Note: With practice, this procedure can be effective in processing many specimens. Chironomids larger than the 12-mm cover glass should be cut in half and mounted under one or two cover glasses.

7.8 Specimens may dry out after 2 or 3 years in the CMCP–10 mounting medium unless the edges of the cover glass are sealed. To make the preparations more permanent, ring the edges with fingernail polish. To ring a slide, coat the edges of the cover glass and any exposed mounting medium with the fingernail polish.

8. Calculations

8.1 When only part of the total number of Chironomidae larvae in a sample is mounted and identified, extrapolate the results from those mounted to the total number of specimens. Total number of individuals in a taxon of a sample

$$= \frac{\text{number of individuals of the taxon in subsample}}{\text{volume of subsample (ml)}} \times \text{volume of total sample (ml).}$$

8.2 Percent composition in sample

$$= \frac{\text{number of individuals of a given taxon}}{\text{total number of individuals of all taxa}} \times 100.$$

9. Report

Report the number of taxa present, the number and percentage of individuals in each taxon in the sample, and the method of collection.

10. Precision

No numerical precision data are available.

References

Beck, W. M., Jr., 1968, Chironomidae, *in* Parrish, F. K., ed., Keys to water quality indicative organisms (Southwestern United States): Federal Water Pollution Control Administration, U.S. Dept. of Interior, p. v1–v22.

Mason, W. T., Jr., 1968, An introduction to the identification of chironomid larvae: Div. of Pollution Surveillance, Federal Water Pollution Control Administration, U.S. Dept. of Interior, 89 p.

——— 1970, Preparing adult Chironomidae for identification: Newsletter, Analytical Quality Control Laboratory, Federal Water Quality Administration, U.S. Dept. of Interior, no. 6, p. 10.

Roback, S. S., 1957, The immature tendipedids of the Philadelphia area: Monographs of the Academy of Natural Sciences of Philadelphia, no. 9, 152 p.

Permanent slide method for immature Simuliidae
(B–5220–77)

Parameter and code: Not applicable

Larvae and pupae of the insect Family Simuliidae (blackflies) are often abundant in swiftly flowing freshwater streams having cobble or gravel bottoms. They occur in reaches with smooth, relatively laminar flow as opposed to reaches with pools, eddies, or turbulence (Hynes, 1970).

Simuliids are members of the insect Order Diptera (2-wing 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. Heavy 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 Simuliidae transmit human onchocerciasis, and others 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 with heavy anterior veins. An extensive taxonomic literature on 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 encased in a vaselike or slipperlike case (fig. 41) attached to rocks, debris, or other solid objects. The pupae have a pair of conspicuous respiratory organs on the thorax with 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 3–15 mm in length and are found 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 occiptal 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 important character. 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).

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) which aid in respiration. These gills, which are useful in separating genera, are often 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 mutiliated specimens, the pupae and most larvae can be identified using a dissecting microscope without preparing a mount. Microscope slide mounts of the head region, however, are a great aid in identification of larvae.

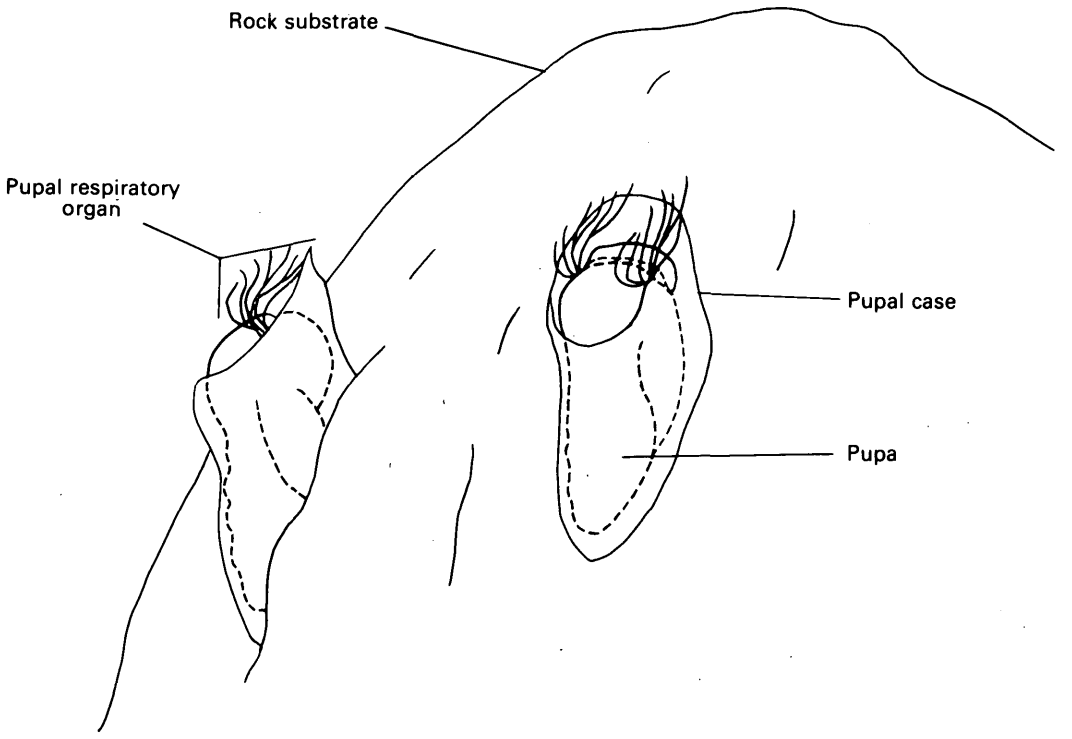


Figure 41.—Simplified drawing of one type of Simuliidae pupa encased in a slipperlike case attached to rocks in the water.

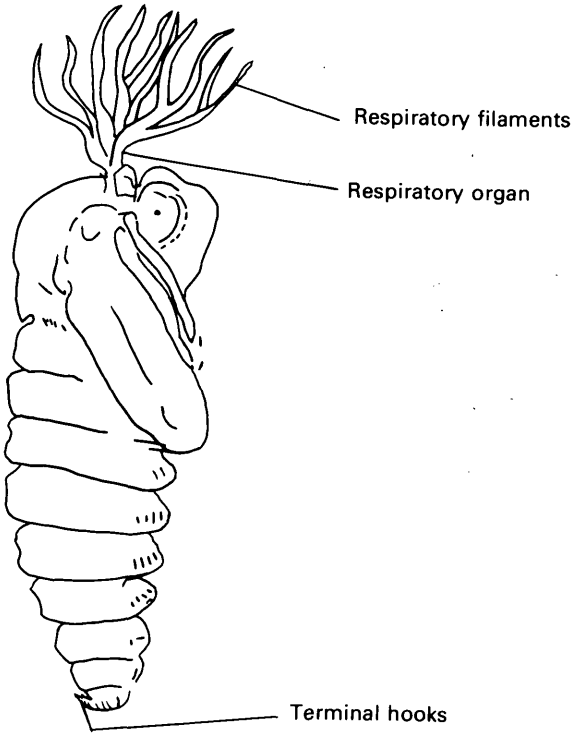


Figure 42.—Simplified features of a Simuliidae pupa, showing location and arrangement of the pupal respiratory filaments.

1. Application

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 expressed as a percentage of the total benthic invertebrate population, or in other ways appropriate to the study objectives.

3. Interferences

In slide preparation, overheating the larvae in 10 percent KOH may result in brittleness, excessive transparency, or digestion of materials. The antennae are especially difficult to see if the specimen is overheated.

4. Apparatus

4.1 *Stereoscopic zoom microscope* (dissecting), Nikon SMZ or equivalent, capable of × 80 magnification.

4.2 *Compound light microscope*, Tiyoda (20049) or equivalent, capable of × 400 to ×500 magnification.

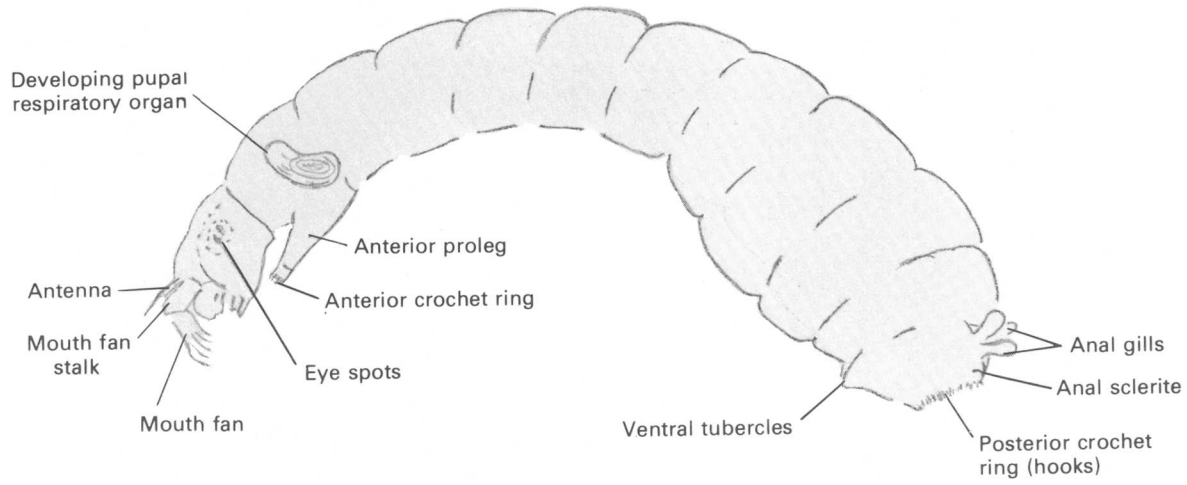


Figure 43.—Mature larva of the Family Simuliidae, simplified, showing most of the important external features needed for identification.

4.3 *Microscope slides*, glass, precleaned, 25×75 mm.

4.4 *Cover glass*, round, No. 1, 12-mm diameter.

4.5 Several *needles* and one pair fine-tipped straight *forceps* for manipulating and dissecting specimens under stereomicroscope.

4.6 *Forceps*, with blunt curved tips.

4.7 *Crucible*, high-form, porcelain, 10-ml capacity.

4.8 *Watchglass*, Syracuse type.

4.9 *Hotplate*, electric.

4.10 *Vials*, 4-ml (1-dram).

4.11 *Marking pen*, permanent waterproof, Sanford's Sharpie or equivalent, for labeling slides.

4.12 *Ocular micrometer*, graduate to 5 μm.

5. Reagents

5.1 *Alcohol, ethyl*, 70 percent: 70 ml of 95 percent alcohol diluted to 95 ml with distilled water; or *alcohol, isopropyl*, 40 percent: 40 ml of concentrated alcohol diluted to 100 ml with distilled water.

5.2 *Alcohol, ethyl*, absolute or 95 percent.

5.3 *Mounting medium*, CMCP–10, CCM: General Biological, Inc., or equivalent.

5.4 *Potassium hydroxide solution*, 10 percent: Dissolve 10 grans KOH pellets in 100 ml of distilled water.

5.5 *Glycerin*.

5.6 *Acetic acid, glacial*.

5.7 *Fingernail polish*.

6. Collection

Simuliidae larvae are collected by any of the methods described for benthic invertebrates appropriate to the study objectives.

7. Analysis

Usually, time does not allow for examining 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 original sample size, the number of visually distinct groups (see 7.2), and the study objectives.

7.1 The pupae are separated from the larvae and identified with the aid of 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.

7.2 Using a dissecting microscope with × 7 or × 20 magnification, separate the total larval sample into visually distinct groups on the basis of general external features (for example, color, presence or absence of ventral tubercles, length of antennae, shape of occipital cleft and number and type of anal gills). Experience with taxonomic keys will aid in the selection of diagnostic characters for separating the groups.

7.3 Randomly select representatives of each visually distinct group for detailed microscopic examination and possible mounting. For small groups of 10 or fewer individuals select 5, or at least 50 percent. For larger groups, the subsampling should be in accordance with stratified random sampling and cluster or two-stage sampling. Store the remaining

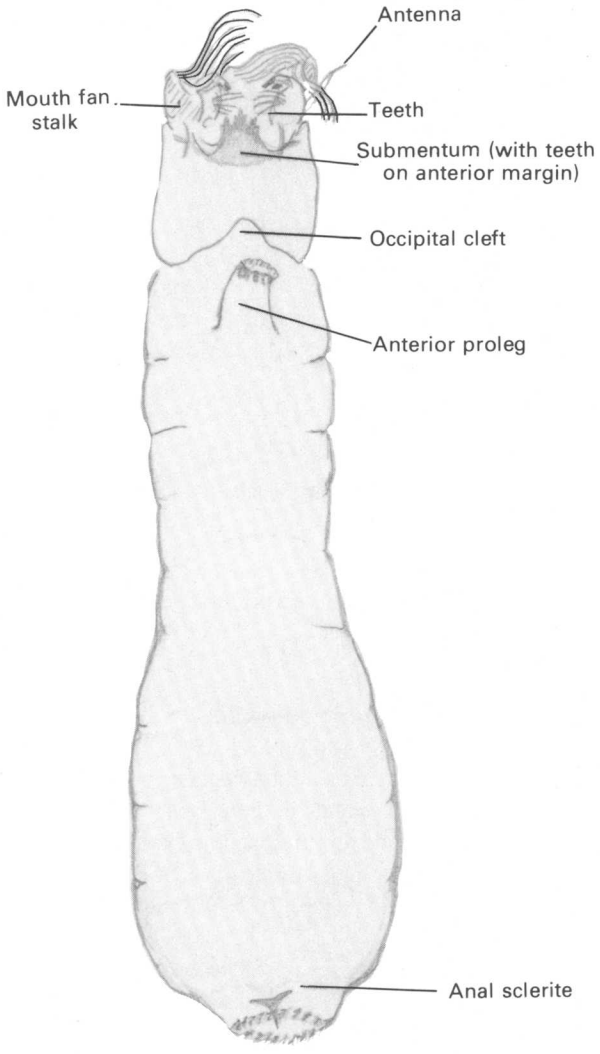


Figure 44.—View of the Simuliidae larva, simplified, showing the location of features that can be seen best after making a permanent mount.

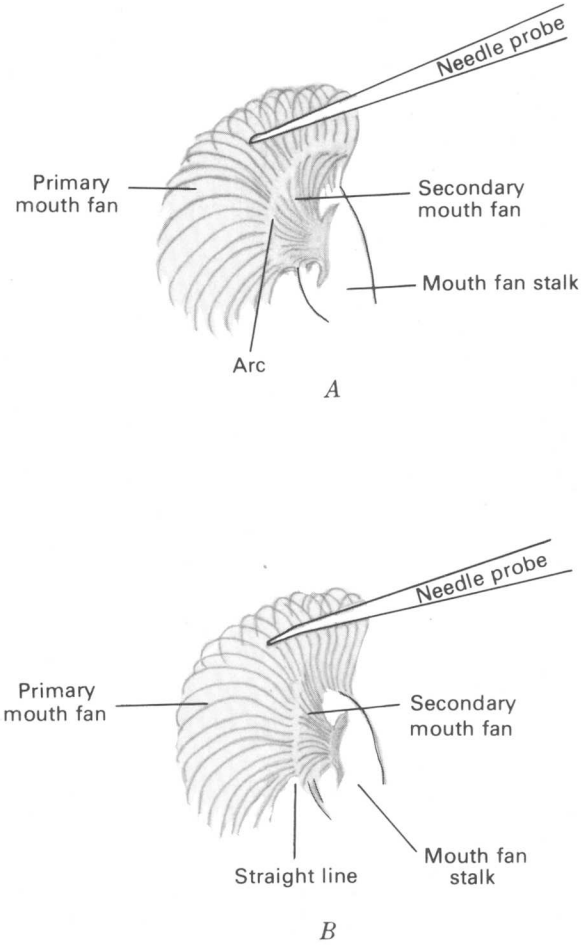


Figure 45.—Simuliidae larval mouth fans showing the two basic types of secondary fans, tips of the expanded secondary fan falling into (*A*) an arc, and (*B*) a straight line.

specimens in vials of 70 percent ethyl alcohol or 40 percent isopropyl alcohol containing a drop or two of glycerin.

7.4 Place the selected larvae in a dish of 70 percent ethyl alcohol and examine with a stereoscopic microscope at a magnification of × 10 to × 70. Identify the specimens using an appropriate taxonomic key. Examples of useful keys are Sommerman (1953), Peterson (1970), Stone and Jamnback (1955), and Stone (1952).

7.5 In mature Simuliidae larvae, the histoblasts of the developing pupal respiratory filaments are well developed and can aid in associating the larvae with the pupal stage. The filaments are important key characters. Dissect them by piercing the integument around the entire filament, lift the filament, and cut it at the base. Note the number and pattern of the filament branches. Mount the filaments in a drop of CMCP–10 mounting medium on a glass slide. Place a cover glass on the drop, and press firmly with a pair of curved blunt forceps.

If more information is needed to complete the larval identification, proceed to sections 7.6 and 7.10 which describe preparation of microscope slide mounts. Mounts facilitate identification of many small larvae by allowing for the examination of submental teeth, mouth fan rays, and anal sclerites (fig. 44). Before mounting, be sure to note the important characters of the head region specified in the keys because they may be distorted in the mount.

7.6 Working with about eight larvae, rinse each one

in distilled water for 2 or 3 minutes. A Syracuse watchglass is a convenient vessel.

7.7 Place the larva in a high-form porcelain crucible containing 10 percent KOH, and heat on a hotplate for 8–15 minutes or until the body is noticeably lighter in color.

7.8 Rinse the larva in distilled water for 2–3 minutes, and rinse with 95 percent ethyl alcohol for at least 3 minutes to remove the residual water and KOH. Note: Glacial acetic acid can be used to remove the KOH.

7.9 Place each larva in a drop of CMCP–10 mounting medium on a clean glass slide and, using needles, position the specimen ventral side up. Place a round cover glass on the preparation and press firmly with a pair of curved blunt forceps. Insure that the larva remains ventral side up while pressing and that the antennae are clearly visible. Check the slide for clarity of diagnostic features with a compound microscope. Allow preparation to dry for 1 week at room temperature, keeping the slide horizontal.

7.10 Specimens may dry out after 2 or 3 years in the CMCP–10 mounting medium unless the edges of the cover glass are sealed. To make the preparations more permanent, ring the edges with fingernail polish. To ring a slide, coat the edges of the cover glass and any exposed mounting medium with the fingernail polish.

8. Calculations

8.1 When only part of the total number of Simuliidae larvae in a sample are identified, extrapolate the results from the subsample to the total number of specimens. Total number of individuals in a taxon of a sample

$$= \frac{\text{number of individuals of the taxon in subsample}}{\text{volume of subsample (ml)}} \times \text{volume of total sample (ml).}$$

8.2 Percent composition in sample

$$= \frac{\text{number of individuals of a given taxon}}{\text{total number of individuals of all taxa}} \times 100.$$

9. Report

Report the number of taxa present, the number and percentage of individuals in each taxon in the sample, and the method of collection.

10. Precision

No numerical precision data are available.

References

Hynes, H. B. N., 1970, The ecology of running waters: Toronto, Toronto Univ. Press, 55 p.

Peterson, B. V., 1970, The *Prosimulium* of Canada and Alaska: Memoirs Entomological Soc. Can., v. 69, 216 p.

Sommerman, K. M., 1953, Identification of Alaskan blackfly larvae Simuliidae: Diptera: Proc. Ent. Soc., Washington, v. 55, p. 258–273.

Stone, Alan, 1952, The Simuliidae of Alaska: Proc. Ent. Soc., Washington, v. 54, p. 69–96.

Stone, Alan and Jamnback, H. A., 1955, The blackflies of New York: New York St. Museum Bull. 349, 144 p.

Permanent slide method for aquatic Acari
(B–5240–77)

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 are generally free-living and predaceous, while the larvae are primarily parasitic on the immature and adult stages of Chironomidae, Plecoptera, Odonata, Hemiptera, 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). 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) demonstrated that an *Anopheles* mosquito infested with five or more mites cannot be induced to bite, thus interrupting the life cycle which is dependent on a blood meal. Abdel-Malek (1948) reported that *Aedes* adults infested with water mites produced fewer eggs than uninfested individuals.

Water mites may prove important in water quality 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 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. The nymph is larger than the larva and often is brightly colored with shades of red and orange, especially in still-water forms. Stream mites are frequently 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, leathery, or bear sclerotized plates (fig. 46*a*). The legs bear 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 suckers or acetabula (fig. 46*b*).

The anterior end of the body bears the mouth region or gnathosome (fig. 46*b*) which sometimes is lengthened anteriorly into a rostrum. At the base of the gnathosoma are two pairs of mouthparts which are key characters in identification, a pair of chelicerae (mandibles) and a pair of palps. The palps consist of five segments—P_1–P_5 (fig. 47)—which may bear a number of setae and spines and terminate in simple or scissorslike claws.

The coxal parts of the legs, called epimeres (fig. 46*b*) are on the underside or venter of the mite. There are four pairs of epimeres which 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.

Other diagnostic features on the venter are three pairs of epimeroglandularia, each of which consists of a gland pore and a hair or seta. Epimeroglandularia I (epg. I) is usually found between epimere II and III, epg. II is variable in position, but is often lateral to the genital opening, and epg. 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 features used in the identification of water mites.

The mite fauna of streams is poorly known. There are scattered descriptions of stream mites, but no single work exists that can be used for identifying mites of streams.

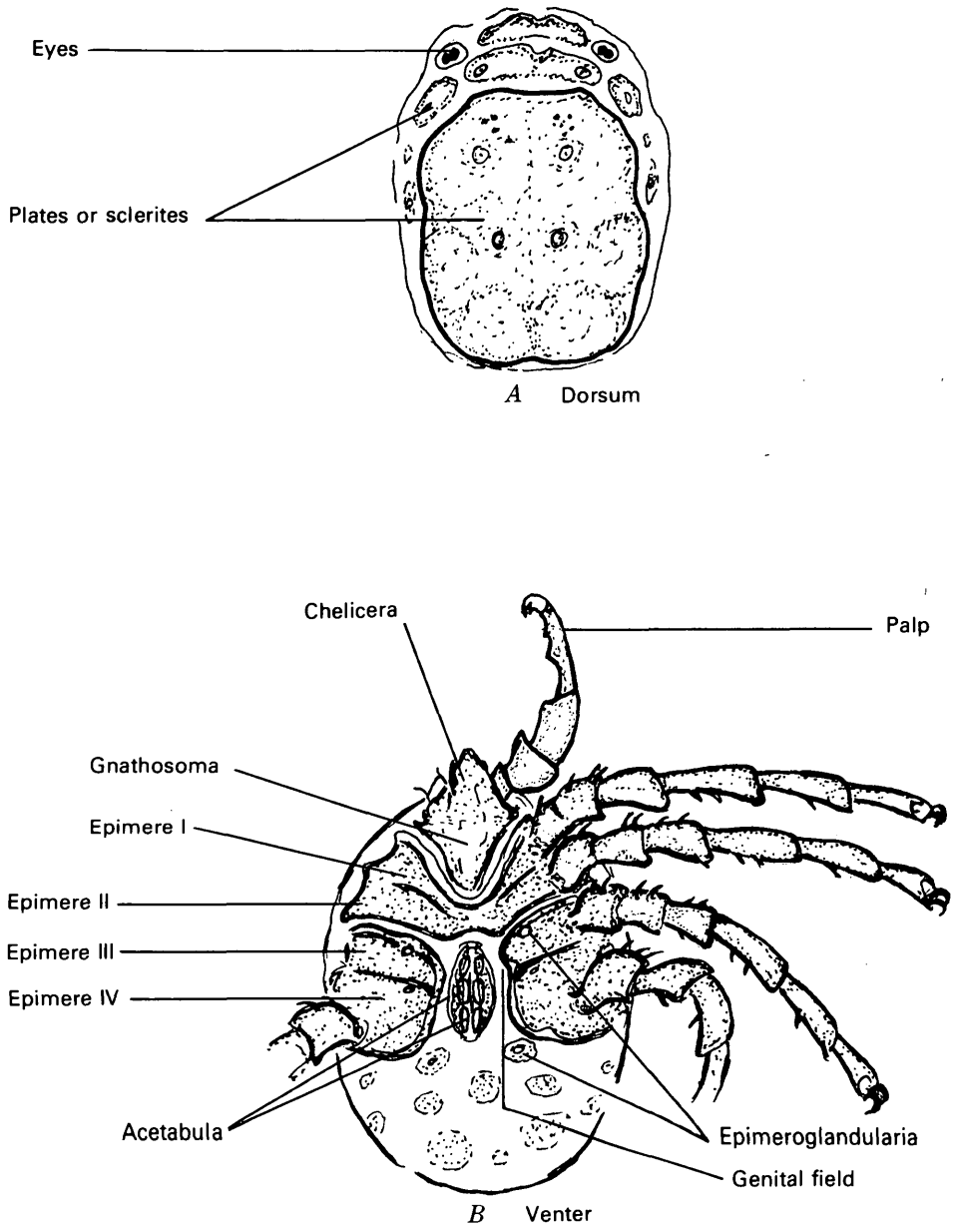


Figure 46.—Dorsal (*a*) and ventral (*b*) view of an adult water mite showing important morphological features used in identification.

The mite faunas of ponds and lakes, on the other hand, are fairly well known. Since the early 1900's, only a few descriptive papers on North American water mites have appeared, particularly by researchers such as Marshall (1940, 1943), Crowell (1960), Cook (1954a,b), and Krantz (1975). Mitchell's check list (1954) is a valuable source of information on reported American species and the relevant literature.

To adequately identify water mites, mounts must be made for microscopic examination. The method described is a modification of the double cover glass glycerin method of Mitchell and Cook (1952).

1. Application

This method is suitable for freshwater and marine mites, in the adult or nymph stage, which 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.

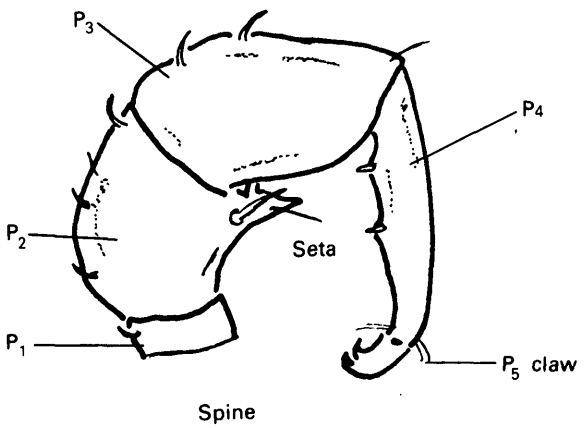


Figure 47.—Enlarged drawing of the 5-segmented palp (P_n) of a water mite.

3. Interferences

3.1 Failure to remove or digest the body contents of mites will result in unclear mounts.

3.2 Do not boil the glycerin jelly mounting medium during heating to avoid bubbles between the cover glasses of the mount.

3.3 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

4.1 *Stereoscopic zoom microscope* (dissecting), × 30 to × 70 magnification, Bausch and Lomb or equivalent.

4.2 *Compound light microscope*, × 400 magnification, Tiyoda (model No. 20049); Nikon Sukt, or equivalent.

4.3 *Microscope slides*, glass, precleaned, 25×75 mm.

4.4 *Cover glasses*, circular, No. 1, 12 mm.

4.5 *Cover glasses*, circular, No. 1, 22 mm.

4.6 *Oven*, Lab Line or equivalent.

4.7 *Hotplate*, electric.

4.8 *Surface thermometer*, 540°C (1,000°F) for a hotplate.

4.9 *Needles, pins, or probes* for manipulation of specimens.

4.10 *Microforceps*, fine-tipped, Trident or equivalent.

4.11 *Microscalpel*, Trident or a similar instrument capable of dissecting a specimen, 0.75 mm in diameter.

4.12 *Spot plates*, glazed, porcelain, 12 depressions.

4.13 *Vials*, 4-ml (1-dram) with screwcaps.

4.14 *Forceps*, blunt curved tips.

4.15 *Watchglass*, Syracuse-type.

4.16 *Index cards*, 7.6×12.7 cm (3×5 in.)

4.17 *Marking pen*, permanent waterproof, Sanford's Sharpie or equivalent.

5. Reagents

5.1 *Glycerin jelly*, thin sheet: Melt glycerin jelly in a covered petri dish at 95°C until a sheet of jelly forms, 0.5–1.0 mm in depth. Cool. Cut in 6-mm squares.

5.2 *Alcohol, ethyl*, 70 percent: 70 ml of 95 percent alcohol diluted to 95 ml with distilled water; or *alcohol, isopropyl*, 40 percent: 40 ml of concentrated alcohol diluted to 100 ml with distilled water.

5.3 *Mounting medium*, CMCP–10, CCM: General Biological, Inc., or equivalent.

5.4 *Corrosive lactophenol*: To 25 ml distilled water, add 50 ml lactic acid and dissolve 25 g phenol crystals.

5.5 *Glycerin-alcohol* solution: Mix 5 ml of glycerin in 95 ml of 70 percent ethyl alcohol.

5.6 *Glycerin*.

5.7 *Canada balsam*, grade A.

5.8 *Fingernail polish*.

6. Collection

6.1 Water mites are collected by any of the methods described for benthic invertebrates appropriate to the study objectives.

6.2 For collections specifically for water mites, use the procedures described by Cook and Mitchell (1952).

7. Analysis

For samples containing few mites, prepare mounts of all individuals. If the numbers are large, separate the mites into distinct groups (see 7.1 below) and take a subsample of each group (see 7.2 below). Use the results from the subsample to calculate the distribution of taxa and individuals in the original sample.

7.1 Using a dissecting microscope with × 30 to × 70 magnification, separate the water mites in a sample into distinct groups on the basis of general external features. Important features 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 genital field (fig. 46*b*).

7.2 Proceed to 7.3 if all mites will be mounted. In large samples, randomly select representatives of each visually distinct group for mounting on slides for mi-

croscopic examination. Subsampling should be done in accordance with stratified random sampling and cluster or two-stage sampling. Store remaining mites in vials of 70 percent ethyl alcohol or 40 percent isopropyl alcohol containing a drop or two of glycerin.

7.3 Place the specimen to be examined in a watch-glass containing 70 percent ethyl alcohol or 40 percent isopropyl 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.

7.4 Carefully remove the contents of the body with tip of the scalpel or needle. It is necessary to remove most of the body contents in this manner because alcohol-preserved specimens clear poorly in acid or basic corrosives.

7.5 Clear specimen of any remaining body contents in a vial containing the corrosive lactophenol for 24–48 hours. Prolonged clearing has little damaging effect.

7.6 Remove the lactophenol corrosive by rinsing the specimen in three to four changes of distilled water followed by 70 percent ethyl alcohol.

Note: Two different methods of slide preparation are given and are based on the quality of the resulting mounts for taxonomic identification. The method given in steps 7.7 through 7.21 is more time consuming, but results in longer lasting slides suitable for species identification. The quicker optional method given in steps 7.22 through 7.24 results in slides adequate for identification to family or genus. Selection of the method should be based on study objectives.

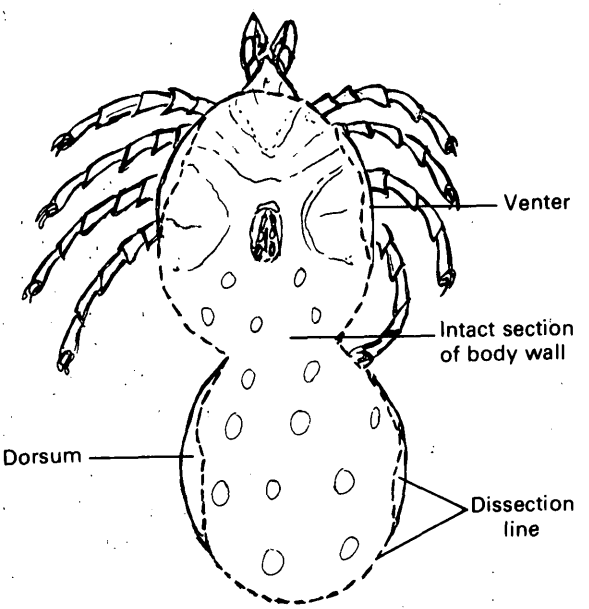


Figure 48.—Simplified drawing of a mite showing how the dorsum is separated from the venter, leaving a small section of lateral body wall intact (see step 7.3).

7.7 Transfer the specimen to a depression in a spot plate containing two or three drops of glycerin-alchohol solution.

7.8 Place the spot plate and mite in a 55°C oven for about 30–40 minutes to evaporate the alcohol, leaving the mite in the glycerin.

7.9 Lift the specimen from the glycerin with the tip of a needle, and place on a 12-mm diameter circular cover glass.

7.10 Using a dissecting microscope, microforceps, and needle, separate the palps from the body by dissecting one palp from the gnathosoma or removing the entire gnathosoma and palps. The dorsum may be severed from the venter.

7.11 Arrange the parts on the cover glass so that the original exterior surface of the venter and dorsum face upward and the palps can be viewed as shown in (fig. 47).

7.12 With forceps, place the flat, smooth side of a 6-mm square of glycerin jelly over the specimen prepared in 7.11 above. A smooth surface on the glycerin jelly helps to prevent air bubbles.

7.13 Heat the cover glass with specimen and jelly on a hotplate (60°–65°C) to allow jelly to melt slightly (15–20 seconds). An index card under the cover glass preparation on the hotplate assists in handling and controlling heat (fig. 49).

7.14 Check preparation with dissecting microscope and, using a needle, remove any bubbles in the jelly.

7.15 Heat a 22-mm diameter circular cover glass on the hotplate (about 60°C) for 1 or 2 minutes.

7.16 Place the hot 22-mm cover glass on the smaller cover glass, jelly, and specimen. Press the large cover glass gently with curved forceps to spread jelly evenly to edges of smaller cover glass. If jelly hardens too quickly, rewarm preparation on hotplate, larger cover glass down, and press smaller cover glass with curved forceps.

Note: Avoid overheating to prevent bubble formation in the jelly between the cover glasses.

7.17 Set preparation aside for at least 15 minutes to allow the glycerin jelly to set.

7.18 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. 50). Press lightly. If bubbles are present in the balsam under the cover glass, they may

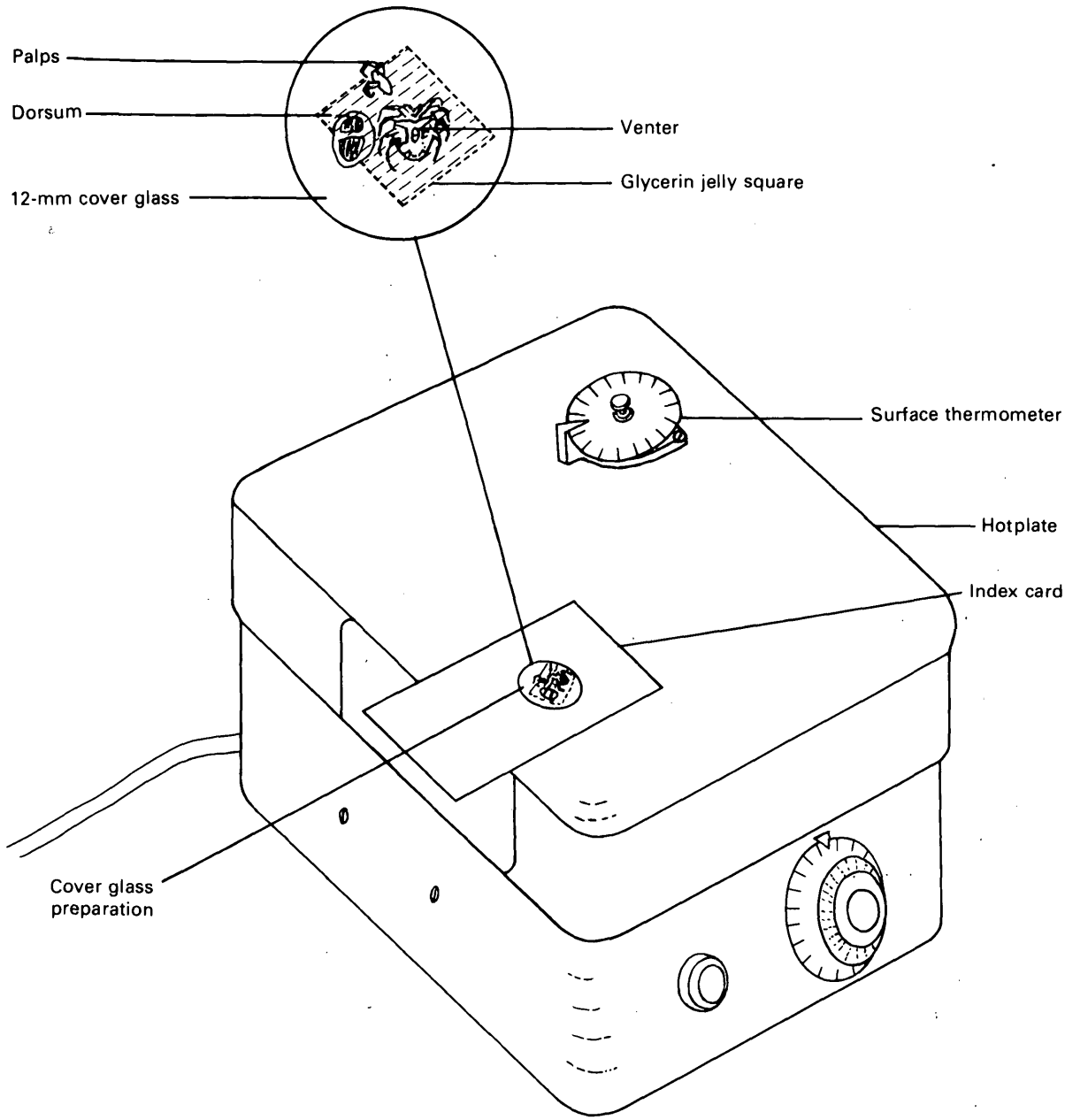


Figure 49.—Step 7.13 in the preparation of a permanent slide mount for water mites. The enlarged inset shows the arrangement of mite parts on the 12-mm cover glass and the glycerin jelly square.

be removed by warming the slide preparation on a hotplate at 45°C.

7.19 Label slide with waterproof ink, recording the date, site, method of collection, identification number, or other information pertinent to the study.

7.20 Identify water mites using a compound microscope and appropriate taxonomic keys. Examples of keys for the nonspecialist are Edmondson (1959), Baker and Wharton (1952), and Pennak (1953).

7.21 Allow slides to air-dry for at least 2 months before storing on edge.

7.22 Optional method. Place the specimen in a small drop of CMCP–10 mounting medium on a clean glass microscope slide. With the aid of a dissecting mi-

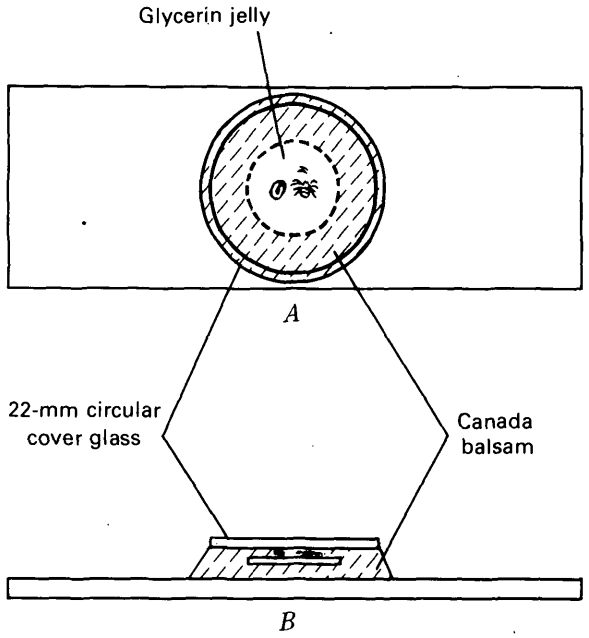


Figure 50.—Top (*a*) and side (*b*) view of the double cover glass technique for mounting aquatic Acari (modified from Mitchell and Cook, 1952).

croscope, microforceps, and needle, dissect the specimen and arrange the parts as in steps 7.10 and 7.11 above. Insure 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.

7.23 Place a 12-mm cover glass on the drop of mounting medium containing the specimen, and press cover glass gently with curved forceps. Allow preparation to dry for 1 week at room temperature, keeping the slide horizontal.

7.24 Specimens may dry out after 2 or 3 years in the CMCP–10 mounting medium unless the edges of the cover glass are sealed. To make the preparations more permanent, ring the edges with fingernail polish. To ring a slide, coat the edges of the cover glass and any exposed mounting medium with the fingernail polish.

8. Calculations

8.1 Determine the number of individuals in a given taxon in a sample from the number of individuals of that taxon in a subsample (see 7.2 above): Number of a given taxon in original sample

$$= \frac{\text{number of the given taxon in subsample}}{\text{total number of individuals in subsample}} \times \text{total number of individuals of all taxa in original sample.}$$

8.2 Percent composition in a sample

$$= \frac{\text{number of individuals of a given taxon}}{\text{total number of individuals of all taxa}} \times 100.$$

9. Report

Report the number of taxa present, the number and percentage of individuals in each taxon in the sample, and the type of collection method(s) used.

10. Precision

No numerical precision data are available.

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AQUATIC VERTEBRATES

In most aquatic ecosystems, fish are the most common vertebrates. Because they are dependent upon the life forms below them for food, the well-being of a local fish population often is used as an index to water quality and to the well-being of other aquatic organisms. Fish, however, are mobile animals, and may avoid undesirable water-quality conditions (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 activity of the Geological Survey, it may at times provide valuable information about the aquatic environment. For example, length-weight relationships 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 upon which the fish feed, essential information in understanding the aquatic ecosystem.

The presence of dead or dying fish, unless it is a postspawning mortality or a delayed mortality resulting from cellular buildup of toxic materials, is indicative of lethal environmental conditions. Field personnel can perform an important function by observing and collecting distressed fish. Pathological 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, 1976).

In all States, some fish species and other aquatic vertebrates are protected by law, and others have their collection regulated. Field personnel should insure that they have complied with State laws before making collections of fish and other aquatic vertebrates.

Although the methods given here are applicable to both fish and other aquatic vertebrates, the emphasis will generally be on fish.

Faunal survey (qualitative method) (B–6001–77)

Parameter and code: Not applicable

1. Application

The methods are applicable to all waters.

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 and depth of water may make collection difficult. Filamentous algae and floating macrophytes may interfere with the operation of nets and seines.

4. Apparatus

Methods and equipment for the collection of fish are discussed in American Public Health Association and others (1976), Lagler (1956, p. 7–15), and Needham and Needham (1962, p. 94–97). State conservation agencies are another source of information for obtain-

ing instructions on the use of fish-collecting equipment. Some common types of equipment used for the collection of fish and other aquatic vertebrates are described below.

4.1 *Straight seine*, about 1.8×3.8 m (6×12 ft), Sterling Net and Twine Co., Inc., or equivalent, with wood, aluminum, or plastic-tubing handles. The mesh size of the seine should be about 6.5 mm (¼ in.) square to insure capture of small or young specimens (Lagler, 1956, p. 7).

4.2 *Bag seine*, about 3 m (10 ft) by 7.6 m (25 ft) to 15 m (50 ft), Sterling Net and Twine Co., Inc., or equivalent. The mesh size of the seine should be about 13 mm (½ in.) square for the wings and about 6.5 mm (¼ in.) square for the bag (Lagler, 1956, p. 7).

4.3 *Gill net*, experimental, about 1.8 m (6 ft) by about 38 m (125 ft), Sterling Net and Twine Co., Inc., or equivalent. The mesh size should range from about 6.5 mm (¼ in.) square at one end to about 50.8 mm (2 in.) square at the other end (Lagler, 1956, p. 11).

4.4 *Electrofishing gear*. The basic electrofishing unit consists of a generator (110 volts a.c. or 220 volts d.c.), sufficient insulated electrical wire, 61–152 m (200–500 ft), two electrodes (or electrodes and an anode, in the case of direct current), and a dip net with an insulated handle (Lagler, 1956, p. 7; Sharpe and Burkhard, 1969). Many types of electrofishing gear are available commercially. Safety regulations and procedures for the use of electrofishing equipment should be thoroughly understood and practiced.

4.5 *Wire or nylon mesh cages*, 6.5-mm (¼ in.) mesh, to hold fish after capture.

4.6 *Waders*, chest-type for use with electrofishing gear.

4.7 *Gloves*, waterproof, Herter's, Inc., Hudson Bay trapper gloves (YB4A) or equivalent, for use with electrofishing gear.

4.8 *Sample containers* of plastic. Wide-mouth jars of about 0.5-, 1-, and 2-liter (pt, qt, and ½-gal) capacity are useful sizes. Lids should be of plastic if used for prolonged storage of preserved specimens.

4.9 *Waterproof labels*, Turtox/Cambosco (376A182) or equivalent; or labels may be cut from sheets of plastic paper, Nalgene Labware (6304–0811) or equivalent.

4.10 *Waterproof ink*, Higgins Eternal Ink or equivalent.

5. Reagents

5.1 *Formaldehyde solution*, 4 percent: 10 ml of 37–40 percent aqueous formaldehyde solution (Formalin) diluted to 100 ml with water.

5.2 *Household borax*: Add about 3 g per liter of 4 percent formaldehyde solution to prevent shrinkage of biological specimens.

5.3 *Alcohol, isopropyl*, 40 percent solution: 40 ml of concentrated isopropyl alcohol diluted to 100 ml with distilled water.

6. Collection

American Public Health Association and others (1976) and Lagler (1956, p. 7–11) discussed fish collection methods in detail.

6.1 Straight seines. 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. Insure 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. Remove the specimens, and process in accordance with the objectives of the study.

6.2 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 with the seine forming 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 in accordance with the objectives of the study.

6.3 Gill nets. These nets may be used in slow-flowing rivers, ponds, or lakes where they depend upon the fish moving into them. Gill nets hang vertically in the water and may be of the floating or sinking type. Fish captured with gill nets usually die within a short period of time. Set gill nets at right angles to the shoreline with the small mesh end nearest the bank. In most instances, success of capture is enhanced by leaving them set overnight. Remove the specimens, and process in accordance with the objectives of the study.

6.4 Electrofishing.[1] This method requires two operators or more, depending upon the type of gear and size of water body. All personnel engaged in electrofishing should wear protective waders and rubber

[1] Local permit required for obtaining scientific specimens in this manner.

gloves. The method is best suited for small streams but has been used in slow-flowing rivers and lakes. After selecting a suitable site, position the electrodes in accordance with the manufacturer's instructions for the type of water being sampled. Electrofishing often proceeds in an upstream direction (Sharpe and Burkhard, 1969). Shock all areas likely to have fish, such as brush piles, boulders, and undercut banks, and repeat the procedures two or three times. Collect the stunned fish with a dip net, and place in cages for processing. Air-breathing vertebrates also may be held in cages, but must have access to the atmosphere. Process the specimens in accordance with the objectives of the study.

6.5 When possible, identify specimens in the field, and release after counting. If field identification is not possible or only tentative, count the number of individuals in each taxon, and preserve representative samples for laboratory examination.

7. Analysis

7.1 Preserve specimens in 4 percent formaldehyde solution (10 percent Formalin) containing about 3 g of borax per liter. Specimens more than 8 cm (about 3 in.) in length should be slit on the right side to insure 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 a period of 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 (Needham and Needham, 1962).

7.2 Identify specimens using the best available taxonomic keys, such as Eddy (1957) and Jordan and Everman (1890–1900). Lagler (1956, p. 19–64) described the families of North American freshwater fish and listed local and regional publications on fish taxonomy. Widely used regional fish keys include, for example, Schultz (1936), Hubbs and Lagler (1958) and Clemens and Wilby (1961). Examples of local keys are Cook (1959), Trautman (1957), and Simon (1946). The recognized common and scientific names of North American fishes are given in "American Fisheries Society, Committee on Names of Fishes" (1960). For the identification of other aquatic vertebrates, refer to Bishop (1947), Carr (1952), and Conant (1958).

8. Calculations

No calculations are necessary.

9. Report

Report the number of taxa and individuals of each taxon and the type of collection method used.

10. Precision

No numerical precision data are available.

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Life history (quantitative method) (B–6020–77)

Parameter and code: Not applicable

1. Application

The method is applicable to all waters.

2. Summary of method

Fish and other aquatic vertebrates are collected and identified. Fish studies often include the number of specimens captured per unit area or unit time. The fish may also be measured and weighed to provide comparative information between populations in the same aquatic environment or between populations in different aquatic environments.

Methods used in the study of fish and fish populations are described in Rounsefell and Everhart (1953), Lagler (1956, p. 120–210), and Ricker (1971). The methods of length-weight relationships, age and growth, and food habit analysis are presented.

3. Interferences

Physical factors such as stream velocity and depth of water may make collection difficult. Filamentous algae and floating macrophytes may interfere with the operation of nets and seines.

4. Apparatus

Methods and equipment for the collection of fish are discussed in American Public Health Association and others (1976), Lagler (1956, p. 7–15), and Needham and Needham (1962, p. 94–97). State conservation agencies are another source of information for obtaining instructions on the use of fish-collecting equipment. Some common types of equipment used for the collection of fish and other aquatic vertebrates are described below.

4.1 *Straight seine*, about 1.8×3.8 m (6×12 ft) Sterling Net and Twine Co., Inc., or equivalent, with wood, aluminum, or plastic-tubing handles. The mesh size of the seine should be about 6.5 mm (¼ in.) square to insure capture of small or young specimens (Lagler, 1956, p. 7).

4.2 *Bag seine*, about 3 m (10 ft) by 7.6 m (25 ft) to 15 m (50 ft), Sterling Net and Twine Co., Inc., or equivalent. The mesh size of the seine should be about 13 mm (½ in.) square for the wings and about 6.5 mm (¼ in.) square for the bag (Lagler, 1956, p. 7).

4.3 *Gill net*, experimental, about 1.8×38 m (6×125 ft), Sterling Net and Twine Co., Inc., or equivalent. The mesh size should range from about 6.5 mm (¼ in.) square at one end to about 50.8 mm (2 in.) square at the other end (Lagler, 1956, p. 11).

4.4 *Electrofishing gear*. The basic electrofishing unit consists of a generator (110 volt a.c. or 220 volt d.c.), sufficient insulated electrical wire 61–152 m (200–500 ft), two electrodes (or two electrodes and an anode, in the case of direct current) and a dip net with an insulated handle (Lagler, 1956, p. 7; Sharpe and Burkhard, 1969). Many types of electrofishing gear are available commercially. Safety regulations and procedures for the use of electrofishing equipment should be thoroughly understood and practiced.

4.5 *Wire or nylon mesh cages*, 6.5-mm (¼ in.) mesh, to hold fish after capture.

4.6 *Waders*, chest-type for use with electrofishing gear.

4.7 *Gloves*, waterproof, Herter's, Inc., Hudson Bay trapper gloves (YB4A) or equivalent, for use with electrofishing gear.

4.8 *Container* for holding anesthesia.

4.9 *Measuring board* or apparatus, Wildlife Supply Company (151) or equivalent. A metric ruler with a piece of wood at right angle to the zero end is an adequate measuring device.

4.10 *Balance*, accurate to 1.0 g.

4.11 *Scalpel or knife* with small sharp blade.

4.12 *Small envelopes* (coin envelopes) with bond

typing-paper inserts for scale samples (Lagler, 1956, p. 138–141).

4.13 *Vials or small bottles* for stomach-content samples.

4.14 *Sample containers* of plastic. Wide-mouth jars of about 0.5-, 1-, and 2-liter (pt, qt, and ½-gal) capacity are useful sizes. Lids should be of plastic if used for prolonged storage of preserved specimens.

4.15 *Waterproof labels*, Turtox/Cambosco (376A182) or equivalent; or labels may be cut from sheets of plastic paper, Nalgene Labware (6304–0811) or equivalent.

4.16 *Waterproof ink*, Higgins Eternal Ink or equivalent.

5. Reagents

5.1 *Formaldehyde solution*, 4 percent: 10 ml of 37–40 percent aqueous formaldehyde solution (Formalin) diluted to 100 ml with water.

5.2 *Household borax:* Add about 3 g per liter of 4 percent formaldehyde solution to prevent shrinkage of biological specimens.

5.3 *Alcohol, isopropyl*, 40 percent solution: 40 ml of concentrated isopropyl alcohol diluted to 100 ml with distilled water.

5.4 *Anesthesia, MS 222 (tricanemethanesulfonate)*: Prepare a stock solution of 1.0 g MS 222 per 500 ml of water. Dilute the stock solution 1 to 6 with water before use. MS 222 may be purchased at most chemical supply houses.

6. Collection

American Public Health Association and others (1976) and Lagler (1956, p. 7–11) discussed fish-collection methods in detail.

6.1 Straight seines. 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. Insure 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. Remove the specimen, and process in accordance with the objectives of the study.

6.2 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 with the seine forming 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 in accordance with the objectives of the study.

6.3 Gill nets. These nets may be used in slow-flowing rivers, ponds, or lakes where they depend upon the fish moving into them. Gill nets hang vertically in the water and may be of the floating or sinking type. Fish captured with gill nets usually die within a short period of time. Set gill nets at right angles to the shoreline with the small mesh end nearest the bank. In most instances, success of capture is enhanced by leaving them set overnight. Remove the specimens and process in accordance with the objectives of the study.

6.4 Electrofishing.[1] This method requires two operators or more, depending upon the type of gear and size of water body. All personnel engaged in electrofishing should wear protective waders and rubber gloves. The method is best suited for small streams but has been used in slow-flowing rivers and lakes. After selecting a suitable site, position the electrodes in accordance with the manufacturer's instructions for the type of water being sampled. Electrofishing often proceeds in an upstream direction (Sharpe and Burkhard, 1969). Shock all areas likely to haeve fish, such as brush piles, boulders, and undercut banks, and repeat the procedure two or three times. Collect the stunned fish with a dip net, and place in cages for processing. Air-breathing vertebrates also may be held in cages, but must have access to the atmosphere. Process the specimens in accordance with the objectives of the study.

6.5 When possible, identify specimens in the field and release after counting. If field identification is not possible or only tentative, count the number of individuals in each taxon, and preserve representative samples for laboratory examination.

7. Analysis

7.1 Preserve specimens in 4 percent formaldehyde solution (10 percent Formalin) containing about 3 g of borax per liter. Specimens more than 8 cm (about 3 in.) in length should be slit on the right side to insure 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 tapwater for a period of at least 24 hours,

[1] Local permit required for obtaining scientific specimens in this manner.

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 (Needham and Needham, 1962).

7.2 Identify specimens using the best available taxonomic keys such as Eddy (1957) and Jordan and Everman (1890–1900). Lagler (1956, p. 19–64) described the families of North American freshwater fish and listed local and regional publications on fish taxonomy. Widely used regional fish keys include, for example, Schultz (1936), Hubbs and Lagler (1958), and Clemens and Wilby (1961). Examples of local keys are Cook (1959), Trautman (1957), and Simon

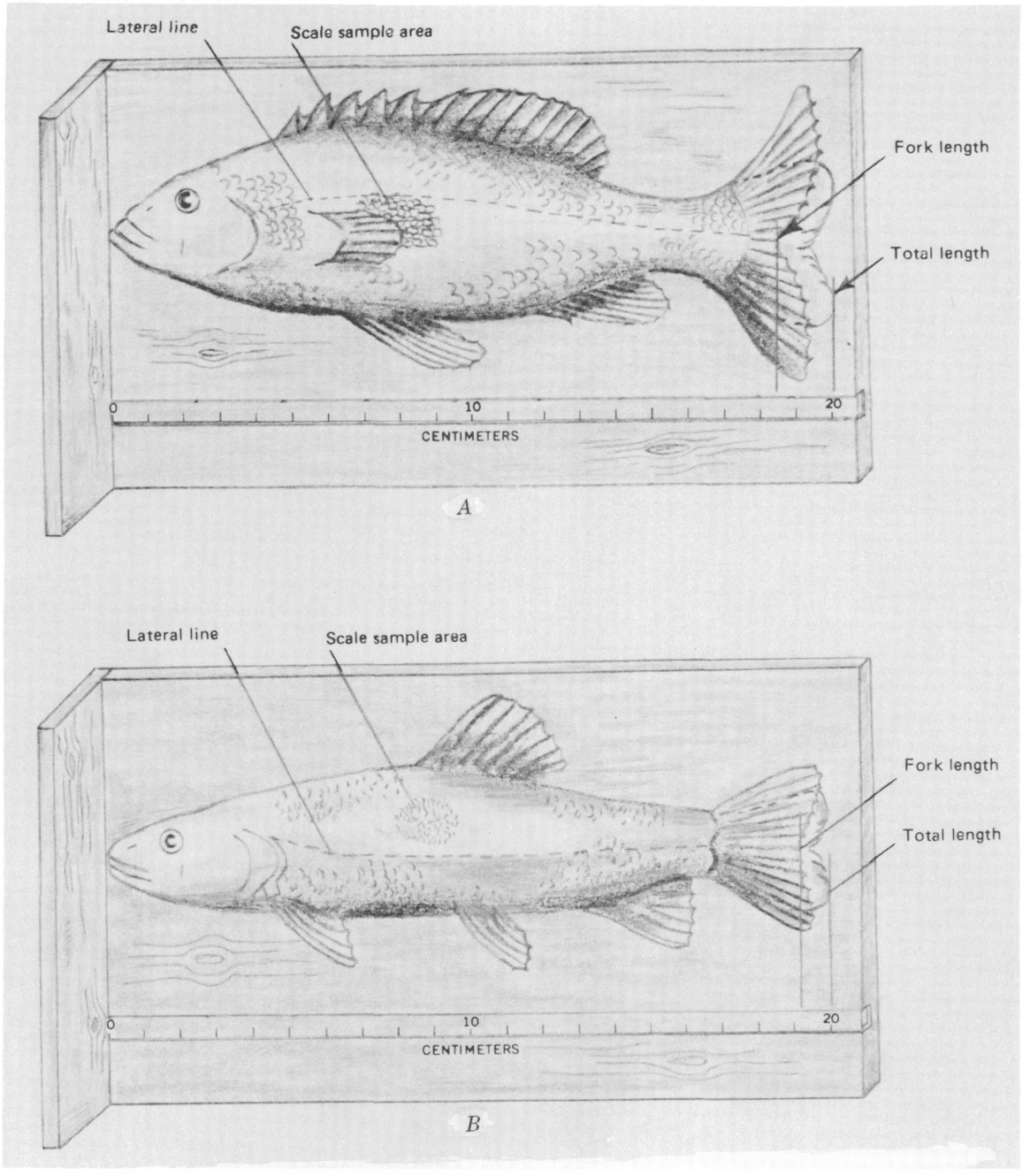

Figure 51.—Fish measurements and areas for scale collection on spiny-rayed (*A*) and soft-rayed (*B*) fish.

(1946). The recognized common and scientific names of North American fishes are given in American Fisheries Society, Committee on Names of Fishes (1960). For the identification of other aquatic vertebrates, refer to Bishop (1947), Carr (1952), and Conant (1958).

7.3 Anesthetize living fish by placing them in a solution of MS 222 as prepared under 5.4.

7.4 Weigh each fish to the nearest gram after blotting dry with a paper towel or cheesecloth.

7.5 Measure the length of each fish to the nearest millimeter, and return the fish to the stream or lake.

7.6 Food habits (optional). If the food habits of the fish are one of the study objectives, representative specimens usually must be sacrificed. 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 utilizing 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.).

7.7 Age and growth by the scale-analysis method (optional). With a knife blade or scalpel remove a sample of scales from the left side of the fish (fig. 51). Place the scales in a folded piece of bond typing paper, and insert into the coin envelope. Record the following on the outside of the coin envelope: species, locality, method of capture, time, date, collector, and length, weight, and sex (if known) of fish. Using the collected scales, determine the age of the fish using the methods described in Lagler (1956, p. 131–158).

8. Calculations

8.1 Calculate the percentage species composition as:

$$\text{Percent species composition in sample} = \frac{\text{number of individuals of a given species}}{\text{total number of all fish collected}} \times 100.$$

8.2 Plot weight as a function of length as described in Lagler (1956, p. 159–166, figs. 47 and 48).

8.3 Plot age as a function of length as described in Lagler (1956, p. 162–163, figs. 47 and 48).

8.4 The calculations required for food-habit studies are determined by the methods of analysis. The usual methods are described in Lagler (1956, p. 120–128).

9. Report

9.1 Report percentage species composition to the nearest whole number.

9.2 Report weight to the nearest gram, and length to the nearest millimeter.

9.3 Report age to the nearest year.

9.4 Report food-habit analyses in accordance with the method used and study objectives.

10. Precision

No numerical precision data are available.

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Methods for investigation of fish and other aquatic vertebrate kills
(B–6040–77)

Parameter and code: Not applicable

1. Application

Fish kills are an obvious and important event related to water quality. The methods given here describe how to record significant facts and obtain properly preserved specimens for laboratory examination. Live specimens provide the most useful histochemical information. But the collection and preservation of dead fish also provide useful information on the species and relative sizes of the affected organisms, and possibly on the cause of death.

The method is applicable to all waters.

2. Summary of method

Distressed fish and other aquatic vertebrates are collected, preserved, and shipped to an appropriate laboratory for examination.

3. Interferences

Physical factors such as stream velocity and depth of water may make collection difficult. Filamentous algae and floating macrophytes may interfere with the operation of nets and seines.

4. Apparatus

4.1 *Hand net*, long handle, with 4.8-mm (3/16-in.) mesh size, Sterling Net and Twine Co., Inc., or equivalent.

4.2 *Straight seine*, about 1.8×3.8m (6×12 ft), Sterling Net and Twine Co., Inc., or equivalent, with wood, aluminum, or plastic-tubing handles. The mesh size of the seine should be about 6.5 mm (¼ in.) square to insure capture of small or young specimens (Lagler, 1956, p. 7.).

4.3 *Plastic bags*, various sizes, sold for household use. The heavier bags intended for freezing or storage of large objects are especially useful.

4.4 *Waterproof labels*, Turtox/Cambosco (376A182) or equivalent; or labels may be cut from sheets of plastic paper, Nalgene Labware (6304–0811) or equivalent.

4.5 *Waterproof ink*, Higgins Eternal Ink or equivalent.

5. Reagents

5.1 *Ethyl alcohol*, 75 percent: Dilute 750 ml of commercial 95 percent ethyl alcohol to 950 ml with distilled water.

5.2 *Bouin's fluid*: To 75 ml of saturated aqueous picric acid, add 25 ml of 37 percent formaldehyde and 5 ml of glacial acetic acid. Samples may be stored in the fluid for several weeks.

6. Collection

For additional information on the collection and investigation of fish kills, see American Public Health Association and others (1976).

6.1 Collect live specimens if possible (Cope, 1960). Dead specimens are a second choice, but the fact that they were dead upon collection should be clearly noted on the sample label.

6.2 Distressed fish often may be collected with a hand net or by hand.

6.3 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. Insure 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. Remove the specimens, and process in accordance with the objectives of the study.

6.4 Collect about ½ kilogram (approximately 1 lb)

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. The amount of fish that can be collected will depend upon a number of circumstances. Generally, a sample of 5 kg (about 10 lb) will be adequate.

6.5 Package the fish in labeled polyethylene bags and freeze. Samples may be packed in insulated cartons or chests and refrigerated with about 5 kg (10 lb) of dry ice per 5–8 kg (10–15 lb) of fish. Note: Samples collected for polychlorinated biphenyl (PCB) determinations should be stored in glass containers.

If freezing facilities are not available, preserve the fish in ethyl alcohol. Bouin's solution is a suitable preservative for specimens collected for histological analysis (Cope, 1960; Wood, 1960).

Before placing in the preservative, slit each fish from the anus to the gills. Use at least 5 volumes of preservative for each volume of fish.

Package the fish collected dead separately from those that were collected alive to avoid contamination. Labels placed in the same bag with wet fish may become illegible. Tie labels to the outside of the bag.

6.6 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.

6.7 Record any circumstances appearing 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, water temperature, general appearance of water (color, foam, oil slick, floating debris, continuous flow), and present weather and previous weather conditions, if known. Whenever possible, measure dissolved oxygen, pH, specific conductance, and collect at least a 1-liter sample of water for chemical analysis.

7. 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 or State Department of Health.

8. Calculations

No calculations are necessary.

9. Report

Report estimated number of distressed or dead fish, or other observed aquatic vertebrates, followed with an appropriate qualifying statement such as estimation based upon 1 hour of observation or number of specimens observed per unit length of shoreline. Degrees of severity of fish kills have been established on the basis of numbers of dead or dying fish per length of shoreline (American Public Health Association and others, 1976).

10. Precision

No numerical precision data are available.

References

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CELLULAR CONTENTS

Chlorophyll *a* is the primary photosynthetic pigment of all oxygen-producing photosynthetic organisms and is present in all algae. Thus, measurement of this pigment can give some insight into the amount 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 then give some indication of the taxonomic composition of an algal community.

An estimate of the quantity of living microorganisms (biomass) in an aquatic environment can be useful in assessing water quality. The universal occurrence and central role of adenosine triphosphate (ATP) in living cells and its chemical stability allow it to be an excellent indicator of the presence of living material. The level of endogenous ATP (that is, the amount of ATP per unit biomass) in bacteria (Allen, 1973), algae (Holm-Hansen, 1970), and 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.

Chlorophyll in phytoplankton by spectroscopy (B—6501–77)

Parameters and codes:

Chlorophyll *a*, phytoplankton, spectrophotometric, uncorrected (μg/l) 32230
Chlorophyll *b*, phytoplankton, spectrophotometric (μg/l) 32231
Chlorophyll *c*, phytoplankton, spectrophotometric (μg/l) 32232
Chlorophyll, total, phytoplankton, spectrophotometric, uncorrected (μg/l) 32234

1. Application

The method is suitable for all waters.

2. Summary of method

Chlorophyll pigments are determined simultaneously without elaborate separation. A water sample is filtered, and the phytoplankton cells retained on the filter are mechanically disrupted to facilitate extraction of pigments by 90 percent acetone. 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 high 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 the phytoplankton chlorophyll (Hussaing, 1973).

4. Apparatus

4.1 *Water-sampling bottle*, Wildlife Supply Co. (1510 or 1920); Scott Instruments, Seattle, Wash.; or Forest Mechanical Specialties Co. (Improved Water Sampler, Kemmerer-type); or equivalent. Depth-integrating samplers are discussed in Guy and Norman (1970).

4.2 *Filter funnel*, vacuum, 1,200 ml, stainless-steel, Gelman Instrument Co. (Parabella) or equivalent.

4.3 *Filter flask*, 1,000 or 2,000 ml. For field use a polypropylene flask, Bel-Art Products (H–38941), Nalgene Labware (4101), or equivalent is suggested.

4.4 *Source of vacuum for filtration*: A water-aspirator pump or an electric vacuum pump for laboratory use; a hand-held vacuum pump with gauge, Edmund Scientific Co. (71,301) or equivalent, for field use.

4.5 *Manostat* with mercury and calibration equipment to regulate the filtration suction at not more than 250 mm (10 in.) of mercury when filtering with an aspirator or an electric vacuum pump.

4.6 *Membrane filter*, white, plain, 0.45-μm mean pore size, 47-mm diameter, Millipore (HAWP 047 00) or equivalent.

4.7 *Tissue homogenizer* (grinder), Teflon pestle-type, 15-ml capacity. Homogenizer should be motor driven at about 500 rpm (revolutions per minute).

4.8 *Centrifuge*, swing-out type, 3,000 to 4,000 rpm, with 15-ml graduate centrifuge tubes, Saveguard (CT–1140) or equivalent.

4.9 *Spectrophotometer*, with a bandwidth of 2.0 nm (nanometers) or less allowing absorbance to be read to $\mp$ 0.001 units, Beckman (model 25) (fig. 52) or equivalent. Use cells with a light-path of 1 cm.

4.10 *Filters*, metricel, alpha-6, 0.45-μm, 25-mm diameter.

4.11 *Filter holder*, Pyrex microanalysis, frit support, 25 mm, Millipore (XX1002500) or equivalent.

5. Reagents

5.1 *Acetone*, 90 percent: Dilute 90 ml acetone, spectrophotometric grade, to 100 ml with distilled water.

6. Collection

The sample sites and methods used should correspond as closely as possible to those selected for chemical and bacteriological 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-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.

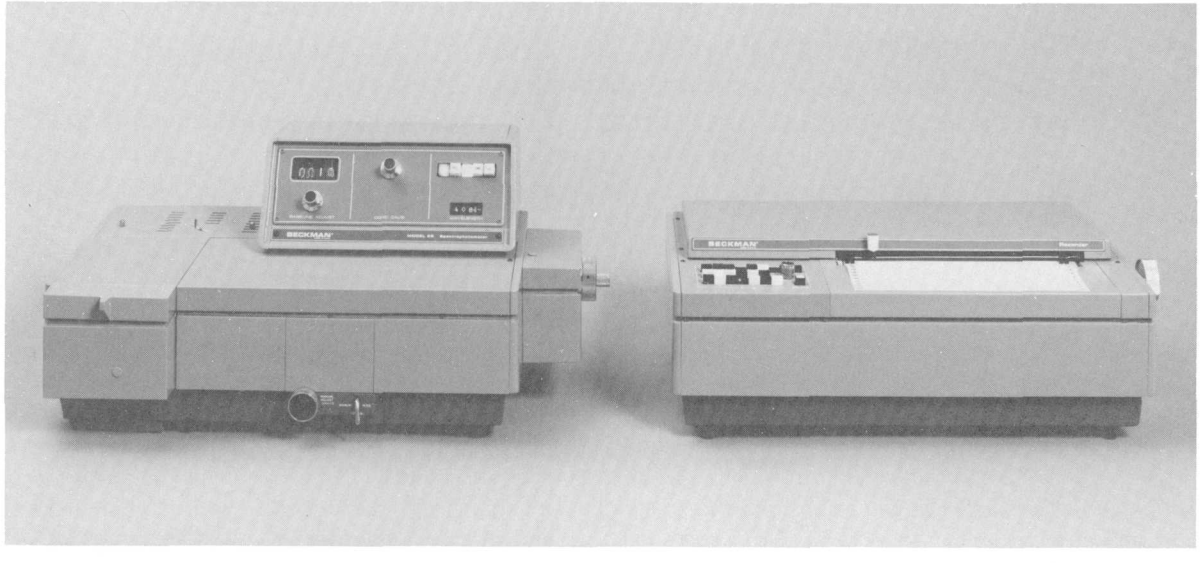

Figure 52.—Scanning spectrophotometer. (Photograph courtesy of Beckman Instruments, Inc., Irvine, Calif.)

7. Analysis

7.1 Place a membrane filter on the filtration apparatus.

7.2 Filter the sample at a vacuum of no more than 250 mm (10 in.) of mercury (about 5 psi). Rinse the sides of the filter funnel with a few milliliters of distilled water.

7.3 Roll the filter with the plankton on the inside and proceed immediately with the analysis described below. Extraction should be completed immediately, but if the sample must be stored, place the rolled filter in a 15-mm (4-dram) glass vial with cap, and freeze. Storage should not exceed 15 days.

Dry ice is recommended for freezing samples in transit.

7.4 Place the filter in a tissue homogenizer. Add 3–4 ml of 90 percent acetone, and grind 3 minutes at about 500 rpm.

7.5 Transfer the sample to a 15-ml graduated centrifuge tube, and wash the pestle and homogenizer two or three times with 90 percent acetone. Adjust to some convenient volume such as 10 ml $\mp$ 0.1. Keep for 10 minutes in the dark at room temperature.

7.6 Centrifuge for 10 minutes at 3,000 to 4,000 rpm.

7.7 Carefully pour or pipet the supernatant into the spectrophotometer cell. Do not disturb the precipitate. If the extract is turbid, clarify by making twofold dilution of acetone, of by filtering through acetone-resistant filter (4.10 and 4.11).

7.8 Read the absorbances at 750, 664, 647, and 630 nm against a 90 percent acetone blank. (Dilute the extract with 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, reduce the turbidity as in 7.7 above.

8. Calculations

8.1 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 spectrophotometer cell in centimeters. The concentrations of chlorophylls in the extract, as μg/ml are given by the following equations (Jeffrey and Humphrey, 1975):

chlorophyll *a* in μg/ml
$$= 11.85e_{664} - 1.54e_{647} - 0.08e_{630}$$

chlorophyll *b* in μg/ml
$$= -5.43e_{664} + 21.03e_{647} - 2.66e_{630}$$

chlorophyll *c* in μg/ml
$$= -1.67e_{664} - 7.60e_{647} + 24.52e_{630}$$

where:

e_{664} = absorbance at 664 nm − absorbance at 750 nm

e_{647} = absorbance at 647 nm − absorbance at 750 nm

e_{630} = absorbance at 630 nm − abosrbance at 750 nm

8.2 Convert the values derived in 8.1 to the concentrations of chlorophylls, as μg/l, in the originally collected sample. To do so, multiply the derived value, in μg/ml, by the volume of the extract, in milliliters, and devide by the volume of the original samples, in liters. For example:

$$\text{chlorophyll a } (\mu\text{g/l}) = \frac{\text{derived value } (\mu\text{g/ml}) \times \text{extract vol. (ml)}}{\text{sample vol. (liters)}}$$

9. Report

Report chlorophyll *a*, *b*, or *c* in micrograms per liter (to three significant figures) of original water sample.

10. Precision

The precision of chlorophyll determinations is influenced by the volume of water filtered, the range of chlorophyll values found, the volume of extraction solvent, and the light-path of the spectrophotometer cells.

The following precision estimates are given by Strickland and Parsons (1968, p. 187):

Chlorophyll *a* precision at the 5 μg level. The correct value lies in the range: Mean of η determinations $\mp 0.26/\eta^{1/2}$ μg chlorophyll *a*.

Chlorophyll *b* precision at the 0.5 μg level. The correct value lies in the range: Mean of η determinations $\mp 0.21/\eta^{1/2}$ μg chlorophyll *b*.

The precision of chlorophyll *c* determinations is variable and very poor, anywhere between $\mp$10 and $\mp$30 percent of the amount being measured; results are not accurate, almost always being too high.

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Chlorophyll in phytoplankton by chromatography and spectroscopy (B–6520–77)

Parameters and codes:
Chlorophyll *a*, phytoplankton, chromato/spectro (μg/l) 70951
Chlorophyll *b*, phytoplankton, chromato/spectro (μg/l) 70952

1. Application

The method is suitable for all waters. 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 with a spectrophotometer.

3. Interferences

A substantial amount 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

4.1 *Spectrophotometer*, Beckman model 25 (fig. 52) or equivalent, with slit width 2 nm or less.

4.2 *Filters*, glass-fiber, 47-mm diameter, Gelman 61694, type A, or equivalent, capable of retaining particles having a diameter of at least 0.45 micrometers.

4.3 *Developing tank and rack*, Scientific Products No. 21432–740 or equivalent.

4.4 *Solvent saturation pads*, Gelman No. 51334 or equivalent, 13.4 cm ×22 cm.

4.5 *Centrifuge*, IEC Model HN–S, with IEC 221 rotor and IEC 302 shield, or equivalent.

4.6 *Centrifuge tubes*, graduated, screwcap, 15-ml capacity.

4.7 *Tissue grinder*, Thomas No. 3431–E15 or equivalent.

4.8 *Evaporation device*, Organomation No. 11151 or equivalent.

4.9 Grinding motor, Curtin Matheson No. 214–700 or equivalent, with 0.1 horsepower.

4.10 *Chromatography sheet*, thin-layer cellulose, Baker No. 0–4468 or equivalent, 5×20 cm, 80-micrometer thick cellulose.

4.11 *Microdoser*, with 50-μl syringe, Brinkman Instruments No. 25–20–000–4 or equivalent.

4.12 *Air dryer*, Oster model No. 202 or equivalent.

4.13 *Spotting template*, Camag or equivalent.

4.14 *Disposable Pasteur pipets*, Scientific Products No. P5200–1 or equivalent.

4.15 *Filtration apparatus*, nonmetallic, with vacuum apparatus.

4.16 *Glass vials*, screwcap, 22×85 mm.

4.17 *Cuvettes*, 1-cm light-path length.

5. Reagents

5.1 *Methanol*, Burdick and Jackson or equivalent purity.

5.2 *Dimethyl sulfoxide*, Burdick and Jackson or equivalent purity.

5.3 *Ethyl ether*, Burdick and Jackson or equivalent purity.

5.4 *Acetone*, Burdick and Jackson or equivalent purity.

5.5 *Petroleum ether*, 30°–60°C, Baker No. 2–9268 or equivalent purity.

5.6 *Chlorophyll a*, solution: Add to 1 milligram Sigma Chemical Co. No. C5753 or equivalent purity, 1 ml of acetone (5.4).

5.7 *Chlorophyll b*, solution: Add to 1 milligram Sigma Chemical Co. No. C5878 or equivalent purity, 1 ml of acetone (5.4).

5.8 *Acetone*, 90 percent: Add 9 volumes of acetone (5.4) to 1 volume of distilled water, until the volume is 1 liter.

5.9 *Distilled water*.

5.10 *Nitrogen gas*, prepurified.

6. Collection

6.1 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).

6.2 Place a 47-mm glass fiber filter on the filtration apparatus.

6.3 Filter a measured quantity of water sample at a vacuum of no more than 250 mm (10 in.) of mercury. Rinse the sides of the filter funnel with a few milliliters of distilled water.

6.4 Roll the filter with the plankton on the inside and proceed with the analysis described below, 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 recommended for preserving samples in transit. Samples should be kept in the dark.

7. Analysis

7.1 Allow the frozen filter to thaw 1 minute at room temperature.

7.2 Place the filter in a tissue homogenizer. Add 3 to 4 ml of dimethyl sulfoxide, and grind 3 minutes at about 500 rpm.

7.3 Transfer the sample to a 15-ml graduated centrifuge tube, and wash the pestle and homogenizer twice with dimethyl sulfoxide.

7.4 Add an equal volume of diethyl ether. Screw on cap, and shake vigorously for 10 seconds. Wait 10 seconds, and repeat shaking for 10 seconds.

7.5 Remove cap and add slowly, almost dropwise, an amount of distilled water equal to 25 percent of the total volume of extractant.

7.6 Cap and shake as in 7.4.

7.7 Centrifuge at 1,000 × *g* for 10 minutes.

7.8 During centrifugation, prepare chromatography tank by placing 294 ml petroleum ether (5.5) and 6 ml methanol (5.1) into tank with two solvent pads and rack. Mix well. Prepare fresh daily.

7.9 Remove upper ethyl ether layer containing chlorophyll with a capillary pipet, and place in another 15-ml graduated screwcap tube.

7.10 Add an equal volume of distilled water, and shake as in 7.4.

7.11 Centrifuge at 1,000 × *g* for 5 minutes.

7.12 Remove upper ethyl ether layer with a capillary pipet, and place in conical tube in evaporation device (4.8). Evaporate to dryness by blowing nitrogen over the ether surface.

7.13 Immediately add 0.5 ml acetone. Mix. Wait 30 seconds. Mix. If all chlorophyll is not in solution then repeat procedure.

7.14 Using microdoser, streak 25 microliters of the acetone-chlorophyll solution on the cellulose thin layer sheet (4.10) 15 mm from bottom and 6 mm from each side, using the air dryer to speed evaporation of solvent. If excessive trailing occurs during chromatography, this amount should be decreased.

7.15 Develop chromatograph in dark with chlorophyll standard(s) prepared in same manner. Use enough chlorophyll to visually locate the spot (about 5 μl of the standard solutions as in 5.6 and (or) 5.7). Time required for development is about 30 minutes. Remove strips when solvent has traveled to approximately 2–3 centimeters from top of strip.

7.16 Determine $R\hat{f}$ values for pure chlorophylls. (Note: $R\hat{f}$ *value* = distance traveled by the chlorophyll from the point of application divided by the distance traveled by the solvent from the point of application.)

7.17 Locate the $R\hat{f}$ value on the unknown sheet, and with a razor blade scrape the cellulose off from the $R\hat{f}$ value minus 0.07 for chlorophyll *a* (0.14 for chlorophyll *b*) ×$R\hat{f}$. Place the cellulose into a graduated centrifuge tube and add acetone to a volume of 3 ml. This step should be completed immediately after removal from the tank. Mix the scraped cellulose and acetone vigorously 10 seconds. Wait 1 minute. Mix again vigorously for 10 seconds.

7.18 Centrifuge at 1,000 × *g* for 5 minutes.

7.19 Remove supernatant and read the absorbance on the spectrophotometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*. If the absorbance is greater than 0.01, determine concentrations using the specific absorptivities of 0.0877 l/mg for chlorophyll *a*

and 0.0514 l/mg for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$A = \propto cb,$$

where

A = absorbance,

c = concentration in milligrams per liter,

b = path-length in centimeters, and

$\propto$ = specific absorptivity.

If the absorbance is less than 0.01, use fluorescence technique.

8. Calculations

The value obtained from the solution in the cuvette is then corrected for the concentration step in the field and in the analysis:

$$\mu \text{ g chlorophyll/l (original sample)} = \frac{\mu \text{ g chlorophyll/ml (in covette)} \times (3 \text{ ml}) \times \frac{500\mu \text{ l}}{25\mu \text{ l}}}{\text{(volume filtered in field, liters)}}$$

9. Report

Report chlorophyll *a* or *b* in micrograms per liter (to three significant figures) of original water sample.

10. Precision

No precision data are available.

References

Federal Working Group on Pest Management, 1974, Guidelines on sampling and statistical methodologies for ambient pesticide monitoring: Washington, D.C., Fed. Working Group on Pest Management, 59 p.

Goerlitz, D. F., and Brown, Eugene, 1972, Method for analysis of organic substances in water: U.S. Geol. Survey Technique Water-Resources Inv., book 5, chap. A3, 40 p.

Guy, H. P., and Norman, V. W., 1970, Field methods for the measurement of fluvial sediments: U.S. Geol. Survey Technique Water-Resources Inv., book 3, chap. C2, 59 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 phytoplankton: Biochem. Physiol. Pflanzen, v. 167, p. 191–194.

Chlorophyll in phytoplankton by chromatography and fluorometry (B–6540–77)

Parameters and codes:
Chlorophyll *a*, phytoplankton, chromato/fluoro (μg/l) 70953
Chlorophyll *b*, phytoplankton, chromato/fluoro (μg/l) 70954

1. Application

The method is suitable for all waters. 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 from chlorophyll degradation products by thin layer chromatography. Chlorophylls are eluted and measured with a spectrofluorometer.

3. Interferences

A substantial amount 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

4.1 *Spectrofluorometer*, American Instrument Aminco-Bowman (fig. 53) or equivalent, with red-sensitive R446S photomultiplier.

4.2 *Fluorescence cuvettes*, 1-cm light-path length.

4.3 *Filters*, glass fiber, 47-mm diameter, Gelman 61694, type A, or equivalent, capable of retaining particles having a diameter of at least 0.45 micrometers.

4.4 *Developing tank and rack*, Scientific Products No. 21432–740 or equivalent.

4.5 *Solvent saturation pads*, Gelman No. 51334 or equivalent, 13.4×22 cm.

4.6 *Centrifuge*, IEC model HN–S, with IEC 221 rotor and IEC 302 shield, or equivalent.

4.7 *Centrifuge tubes*, graduated, screwcap, 15-ml capacity.

4.8 *Tissue grinder*, Thomas No. 3431–E15 or equivalent.

4.9 *Evaporation device*, Organomation No. 11151 or equivalent.

4.10 *Grinding motor*, Curtin Matheson No. 214-700 or equivalent, with 0.1 horsepower.

4.11 *Chromatography sheet*, thin-layer cellulose, Baker No. 0-4468, or equivalent, 5×20 cm, 80-micrometer thick cellulose.

4.12 *Microdoser*, with 50-μl syringe, Brinkmann Instruments No. 25–20–000–4 or equivalent.

4.13 *Air dryer*, Oster model No. 202 or equivalent.

4.14 *Spotting template*, Camag or equivalent.

4.15 *Disposable Pasteur pipets*, Scientific Products No. P5200–1 or equivalent.

4.16 *Filtration apparatus*, nonmetallic, with vacuum apparatus.

4.17 *Glass vials*, screwcap, 22×85 mm.

5. Reagents

5.1 *Methanol*, Burdick and Jackson or equivalent purity.

5.2 *Dimethyl sulfoxide*, Burdick and Jackson or equivalent purity.

5.3 *Ethyl ether*, Burdick and Jackson or equivalent purity.

5.4 *Acetone*, Burdick and Jackson or equivalent purity.

5.5 *Petroleum ether*, 30°–60°C, Baker No. 2–9268, or equivalent purity.

5.6 *Chlorophyll a*, solution: Add to 1 milligram Sigma Chemical Co. No. C5753 or equivalent purity, 1 ml of acetone (5.4).

5.7 *Chlorophyll b*, solution: Add to 1 milligram

Figure 53.—Spectrofluorometer. (Photograph courtesy of AMINCO Division of Travenol Laboratories, Inc., Silver Spring, Md.).

Sigma Chemical Co. No. C5878 or equivalent purity, 1 ml of acetone (5.4).

5.8 *Acetone,* 90 percent: Add 9 volumes of acetone (5.4) to 1 volume of distilled water, until the volume is 1 liter.

5.9 *Distilled water.*

5.10 *Nitrogen gas,* prepurified.

6. Collection

6.1 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).

6.2 Place a 47-mm glass fiber filter on the filtration apparatus.

6.3 Filter a measured quantity of water sample at a vacuum of no more than 250 mm (10 in.) of mercury. Rinse the sides of the filter funnel with a few milliliters of distilled water.

6.4 Roll the filter with the plankton on the inside, and proceed with the analysis described below, 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 recommended for preserving samples in transit. Samples should be kept in the dark.

7. Analysis

7.1 Allow the frozen filter to thaw 1 minute at room temperature.

7.2 Place the filter in a tissue homogenizer. Add 3 to 4 ml of dimethyl sulfoxide, and grind 3 minutes at about 500 rpm.

7.3 Transfer the sample to a 15-ml graduated centrifuge tube, and wash the pestle and homogenizer twice with dimethyl sulfoxide.

7.4 Add an equal volume of diethyl ether. Screw on

cap, and shake vigorously for 10 seconds. Wait 10 seconds, and repeat shaking for 10 seconds.

7.5 Remove cap and add slowly, almost dropwise, an amount of distilled water equal to 25 percent of the total volume of extractant.

7.6 Cap and shake as in 7.4.

7.7 Centrifuge at 1,000 × g for 10 minutes.

7.8 During centrifugation, prepare chromatography tank by placing 294 ml petroleum ether (5.5) and 6 ml methanol (5.1) into tank with two solvent pads and rack. Mix well. Prepare fresh daily.

7.9 Remove upper ethyl ether layer containing chlorophyll with a capillar pipet, and place in another 15-ml graduated screwcap tube.

7.10 Add an equal volume of distilled water, and shake as in 7.4.

7.11 Centrifuge at 1,000 × g for 5 minutes.

7.12 Remove upper ethyl ether layer with a capillary pipet, and place in conical tube in evaporation device (4.9). Evaporate to dryness by blowing nitrogen over the ether surface.

7.13 Immediately add 0.5 ml acetone. Mix. Wait 30 seconds. Mix. If all chlorophyll is not in solution then repeat waiting and mixing.

7.14 Using microdoser, streak 25 microliters on the cellulose thin layer sheet (4.11) 15 mm from bottom and 6 mm from each side, using the air dryer to speed evaporation of solvent. If excessive trailing occurs during chromatography, this amount should be decreased.

7.15 Develop chromatograph in dark with chlorophyll standard(s) prepared in same manner. Use enough chlorophyll to visually locate the spot (about 5 μl of 5.6 and (or) 5.7). Time required for development is about 30 minutes. Remove strips when solvent has traveled to approximately 2–3 centimeters from top of strip.

7.16 Determine $R\hat{f}$ values for pure chlorophylls. (Note: $R\hat{f}$ value = distance traveled by the chlorophyll from the point of application divided by the distance traveled by the solvent from the point of application.)

7.17 Locate the $R\hat{f}$ value on the unknown sheet, and with a razor blade scrape the cellulose off from the $R\hat{f}$ value minus 0.07 for chlorophyll *a* (0.14 for chlorophyll *b*) × $R\hat{f}$ to the $R\hat{f}$ value plus 0.07 (for chlorophyll *a*; 0.14 for chlorophyll *b*) × $R\hat{f}$. Place the cellulose into a graduated centrifuge tube and add acetone to a volume of 3 ml. This step should be completed immediately after removal from the tank. Mix the scraped cellulose and acetone vigorously 10 seconds. Wait 1 minute. Mix again vigorously for 10 seconds.

7.18 Centrifuge at 1,000 × g for 5 minutes.

7.19 Determine the concentration of chlorophyll *a* or *b* with the spectrofluorometer as follows. Standard curves are prepared on a daily basis to standardize the spectrofluorometer. Five standards of each chlorophyll should be prepared at the approximate concentrations of 0.5, 1, 2, 3, 5 mg/l. These are prepared from the standard chlorophyll solutions (5.6, 5.7) by an appropriate dilution into 90 percent acetone. The absorbance is then read on a spectrophotometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*. Determine concentrations of standards and samples using the specific absorptivities of 0.0877 l/mg for chlorophyll *a* and 0.0514 l/mg for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$A = \propto cb,$$

where

A = absorbance,

c = concentration in milligrams per liter,

b = path-length in centimeters, and

$\propto$ = specific absorptivity

These solutions are then used to standardize the spectrofluorometer. For chlorophyll *a*, set the spectrofluorometer for an excitation wavelength of 430 nm and an emission wavelength 670 nm. For chlorophyll *b*, the excitation wavelength is 460 nm and 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 curve.

8. Calculations

The value obtained from the cuvette is then corrected for the concentration step in the field and in the analysis:

$$\mu\text{g chlorophyll/l (original sample)} = \frac{\mu\text{g chlorophyll/ml (in cuvette)} \times (3\text{ ml}) \times \frac{500\ \mu\text{l}}{25\ \mu\text{l}}}{\text{(volume filtered in field, liters)}}$$

9. Report

Report chlorophyll *a* or *b* in micrograms per liter (to three significant figures) of original water sample.

10. Precision

No precision data are available.

References

Federal Working Group on Pest Management, 1974, Guidelines on sampling and statistical methodologies for ambient pesticide monitoring: Washington, D.C., Fed. Working Group on Pest Management, 59 p.

Goerlitz, D. F., and Brown, Eugene, 1972, Method for analysis of organic substances in water: U.S. Geol. Survey Tech. Water-Resources Inv., book 5, chap. A3, 40 p.

Guy, H. P., and Norman V. W., 1970, Field methods for the measurement of fluvial sediments: U.S. Geol. Survey Tech. Water-Resources Inv., book 3, chap. C2, 59 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 phytoplankton: Biochem. Physio. Pflanzen, v. 167, p. 191–194.

Biomass/chlorophyll ratio for plankton
(B–6560–77)

Parameter and code: Biomass-chlorophyll ratio, plankton (ratio) 70949

1. Application

Plankton and periphyton communities are normally dominated by algae. As degradable nontoxic organic materials are introduced to a body of water, a frequent result is that a greater percentage of the total biomass is from heterotrophic (nonchlorophyll containing) organisms such as bacteria and fungi. Such a change can be observed in the biomass to chlorophyll *a* ratio (or autotrophic index). Periphyton ratios for unpolluted waters have been reported to be in the range of 50–100 (Weber, 1973), while values greater than 100 may result from organic pollution (Weber and McFarland, 1969; Weber, 1973).

The method is suitable for all waters. 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 with a spectrophotometer or spectrofluorometer. The dry weight and ash weight of the plankton are determined to obtain the weight of organic matter (biomass). The biomass/chlorophyll *a* ratio is calculated from these values.

3. Interferences

A substantial amount of sediment may affect the chlorophyll extraction process. Inorganic matter in the sample will cause erroneously high dry and ash weights; nonliving organic matter in the sample will cause erroneously high dry (and thus organic) weights. Exposure to light or acid at any stage of storage and analysis can result in photochemical and chemical degradation of the chlorophylls.

4. Apparatus

4.1 *Spectrophotometer*, Beckman Model 25 (fig. 52) or equivalent, with slit width 2 nm or less.

4.2 *Spectrofluorometer*, American Instrument Aminco-Bowman (fig. 53) or equivalent, with red-sensitive R446S photomultiplier.

4.3 *Fluorescence cuvettes*, 1-cm light-path length.

4.4 *Filters*, glass-fiber, 47-mm diameter, Gelman 61694, type A, or equivalent, capable of retaining particles having a diameter of at least 0.45 micrometer.

4.5 *Developing tank and rack*, Scientific Products No. 21432-740 or equivalent.

4.6 *Solvent saturation pads*, Gelman No. 51334 or equivalent, 13.4×22 cm.

4.7 *Centrifuge*, IEC model HN–S, with IEC 221 rotor and IEC 302 shield, or equivalent.

4.8 *Centrifuge tubes*, graduated, screwcap, 15-ml capacity.

4.9 *Tissue grinder*, Thomas No. 3431-E15 or equivalent.

4.10 *Evaporation device*, Organomation No. 11151 or equivalent.

4.11 *Grinding motor*, Curtin Matheson No. 214–700 or equivalent, with 0.1 horsepower.

4.12 *Chromatography sheet*, thin-layer cellulose, Baker No. 0–4468 or equivalent, 5×20 cm, 80-micrometer thick cellulose.

4.13 *Microdoser*, with 50-μl syringe, Brinkmann Instruments No. 25–20–000–4 or equivalent.

4.14 *Air dryer*, Oster model No. 202 or equivalent.

4.15 *Spotting template*, Camag or equivalent.

4.16 *Disposable Pasteur pipets*, Scientific Products No. P5200–1 or equivalent.

4.17 *Filtration apparatus*, nonmetallic, with vacuum apparatus.

4.18 *Glass vials*, screwcap, 22×85 mm.

4.19 *Porcelain crucibles*.

4.20 *Analytical balance*, capable of weighing to at least 0.1 mg.

4.21 *Drying oven*, thermostatically controlled for use at 105°C.

4.22 *Muffle furnace*, for use at 500°C.

4.23 *Desiccator*, containing silica gel.

4.24 *Forceps or tongs*.

5. Reagents

5.1 *Methanol*, Burdick and Jackson or equivalent purity.

5.2 *Dimethyl sulfoxide*, Burdick and Jackson or equivalent purity.

5.3 *Ethyl ether*, Burdick and Jackson or equivalent purity.

5.4 *Acetone*, Burdick and Jackson or equivalent purity.

5.5 *Petroleum ether*, 30°–60°C, Baker No. 2–9268 or equivalent purity.

5.6 *Chlorophyll a*, solution: Add to 1 milligram Sigma Chemical Co. No. C5753 or equivalent purity, 1 ml of acetone (5.4).

5.7 *Chlorophyll b*, solution: Add to 1 milligram Sigma Chemical Co. No. C5878 or equivalent purity, 1 ml of acetone (5.4).

5.8 *Acetone*, 90 percent: Add 9 volumes of acetone (5.4) to 1 volume of distilled water, until the volume is 1 liter.

5.9 *Distilled water*.

5.10 *Nitrogen gas*, prepurified.

6. Collection

6.1 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).

6.2 Place a 47-mm glass-fiber filter on the filtration apparatus.

6.3 Filter a measured quantity of water sample at a vacuum of no more than 250 mm (10 in.) of mercury. Rinse the sides of the filter funnel with a few milliliters of distilled water.

6.4 Roll the filter with the plankton on the inside, and proceed with the analysis described below, 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 recommended for preserving samples in transit.

7. Analysis

7.1 Allow the frozen filter to thaw 1 minute at room temperature.

7.2 Place the filter in a tissue homogenizer. Add 3 to 4 ml of dimethyl sulfoxide, and grind 3 minutes at about 500 rpm.

7.3 Transfer the sample to a 15-ml graduated centrifuge tube, and wash the pestle and homogenizer twice with dimethyl sulfoxide.

7.4 Add an equal volume of diethyl ether. Screw on cap, and shake vigorously for 10 seconds. Wait 10 seconds, and repeat shaking for 10 seconds.

7.5 Remove cap and add slowly, almost dropwise, an amount of distilled water equal to 25 percent of the total volume of extractant.

7.6 Cap and shake as in 7.4.

7.7 Centrifuge at 1000 × g for 10 minutes.

7.8 During centrifugation, prepare chromatography tank by placing 294 ml petroleum ether (5.5) and 6 ml methanol (5.1) into tank with two solvent pads and rack. Mix well. Prepare fresh daily.

7.9 Remove upper ethyl ether layer containing chlorophyll with a capillary pipet, and place in another 15-ml graduated screwcap tube.

7.10 Add an equal volume of distilled water, and shake as in 7.4.

7.11 Centrifuge at 1,000 × g for 5 minutes.

7.12 Remove upper ethyl ether layer with a capillary pipet, and place in conical tube in evaporation device (4.10). Begin evaporating to dryness with nitrogen by blowing nitrogen over the ether surface. Save pelleted material and dimethyl sulfoxide-water supernatant for biomass measurement (7.22).

7.13 When almost dry, immediately add 0.5 ml acetone. Mix. Wait 30 seconds. Mix. If all chlorophyll is not in solution, then repeat waiting and mixing.

7.14 Using microdoser, streak 25 microliters of the acetone-chlorophyll solution on the cellulose thin layer sheet (4.12) 15 mm from bottom and 6 mm from each side, using the air dryer to speed evaporation of solvent. If excessive trailing occurs during chromatography, this amount should be decreased.

7.15 Develop chromatograph in dark with chlorophyll standard(s) prepared in same manner. Use

enough chlorophyll to visually locate the spot (about 5 μl of the standard solution as in 5.6 and (or) 5.7). Time required for development is about 30 minutes. Remove strips when solvent has traveled to approximately 2–3 centimeters from top of strip.

7.16 Determine $R\hat{f}$ values for pure chlorophylls. (Note: $R\hat{f}$ value = distance traveled by the chlorophyll from the point of application, divided by the distance traveled by the solvent from the point of application).

7.17 Locate the $R\hat{f}$ value on the unknown sheet and with a razor blade scrape the cellulose off from the $R\hat{f}$ value minus 0.07 for chlorophyll *a* (0.14 for chlorophyll *b*) × $R\hat{f}$ to the $R\hat{f}$ value plus 0.07 (for chlorophyll *a;* 0.14 for chlorophyll *b*) × $R\hat{f}$. Place the cellulose into a graduated centrifuge tube, and add acetone to a volume of 3 ml. This step should be completed immediately after removal from the tank. Mix the scraped cellulose and acetone vigorously 10 seconds. Wait 1 minute. Mix again vigorously for 10 seconds.

7.18 Centrifuge at 1,000 × g for 5 minutes.

7.19 Remove supernatant and read the absorbance on the spectrophotometer at 664 nm for chlorophyll *a* and 644 nm for chlorophyll *b*. If the absorbance is greater than 0.01, determine concentrations using the specific absorptivities of 0.0877 l/mg for chlorophyll *a* and 0.0574 l/mg for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$A = \propto cb,$$

where

A = absorbance,

c = concentration in milligrams per liter,

b = path-length in centimeters, and

$\propto$ = specific absorptivity.

If the absorbance is less than 0.01 proceed to 7.20, otherwise proceed to 7.21.

7.20 Determine the concentration of chlorophyll *a* or *b* with the spectrofluorometer as follows. Standard curves are prepared on a daily basis to standardize the spectrofluorometer. Five standards of each chlorophyll should be prepared at the approximate concentrations of 0.5, 1, 2, 3, 5 mg/l. These are prepared from the standard chlorophyll solutions (5.6, 5.7) by an appropriate dilution into 90 percent acetone. The absorbance is then read on a spectrophotometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*. Determine concentrations of standards and samples using the specified absorptivities of 0.0877 l/mg for chlorophyll *a* and 0.0514 l/mg for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$A = \propto cb$$

where

A = absorbance,

c = concentration in milligrams per liter,

b = path-length in centimeters, and

$\propto$ = specific absorptivity.

These solutions are then used to standardize the spectrofluorometer. For chlorophyll *a*, set the spectrofluorometer for an excitation wavelength of 430 nm and an emission wavelength 670 nm. For chlorophyll *b*, the excitation wavelength is 460 nm and 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 curve.

7.21 Obtain the tare weight of a crucible that has been held at 500°C for about 20 minutes and cooled to room temperature in a desiccator.

7.22 Place the pelleted material (7.12) and supernatant (7.12) into the tared crucible, and dry in an oven at 105°C to constant weight; that is, until further drying produces no change in weight.

Note: Cool the crucible containing dried material to room temperature in a desiccator before weighing. Weigh as rapidly as possible to decrease moisture uptake by the dried residue. These values are used to calculate dry weight.

7.23 Place the crucible containing the dried residue in a muffle furnace for 1 hour at 500°C. Cool to room temperature.

7.24 Moisten the ash with distilled water, and again ovendry at 105°C to constant weight as described in 7.22. These weight values are used to calculate ash weight. Note: The ash is wetted to reintroduce the water of hydration of the clay and other minerals that, though not driven off at 105°C, is lost at 500°C. This water loss may amount to as much as 10 percent of the weight lost during ignition and, if not corrected for, will be interpreted as organic matter (American Public Health Association and others, 1976).

8. Calculations

Chlorophyll: The value obtained from the solution

in the cuvette is corrected for the concentration step in the field and in the analysis.

$$\mu g \text{ chlorophyll/l (original sample)} = \frac{\mu g \text{ chlorophyll/ml (in cuvette)} \times (3 \text{ ml}) \times \frac{500\ \mu l}{25\ \mu l}}{\text{(volume filtered in field, liters)}}$$

Biomass:

$$\text{organic weight, mg/l} = \frac{\text{(dry weight, mg)} - \text{(ash weight, mg)}}{\text{(volume filtered in field, liters)}}$$

Ratio:

$$= \frac{\text{biomass (mg/l)}}{\text{chlorophyll } a\ (\mu g/l) \times 1000}$$

9. Report

Report chlorophyll *a* or *b* in micrograms per liter (to three significant figures) of original water sample. Report biomass in milligrams per liter (to three significant figures). Report ratio to three significant figures.

10. Precision

No precision data are available.

References

American Public Health Association and others, 1976, Standard methods for the examination of water and wastewater (14th ed.): New York, Am. Public Health Assoc., 1193 p.

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Weber, C. L., 1973, Recent developments in the measurement of the response of plankton and periphyton to changes in their environment, in bioassay techniques and environmental chemistry, G. Glass, ed., Ann Arbor Science Publishers, Inc., p. 119–138.

Weber, C. I., and McFarland, B., 1969, Periphyton biomass-chlorophyll ratio as an index of water quality, Presented at the 17th Annual Meeting, Midwest Benthological Society, Gilbertsville, Ky., April, 1969.

Chlorophyll in periphyton by spectroscopy
(B–6601–77)

Parameters and codes:
Chlorophyll *a*, periphyton, spectrophotometric, uncorrected (mm/m²) 32228
Chlorophyll *b*, periphyton, spectrophotometric (mg/m²) 32226
Chlorophyll *c*, periphyton, spectrophotometric (mg/m²) 32227
Chlorophyll, total, periphyton, spectrophotometric, uncorrected (mg/m²) 32225

1. Application

The method is suitable for all waters and may be used for periphyton from natural or artificial substrates.

2. Summary of method

Chlorophyll pigments are determined simultaneously without elaborate separation. The periphyton is scraped from a known area, suspended in water, and concentrated on a membrane filter. The algal cells are mechanically disrupted to facilitate extraction of pigments by 90 percent acetone. 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 high 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

4.1 *Artificial substrates* made of glass slides, Plexiglas, polyethylene strips, or other materials, Kahl Scientific Instrument Corp. (003WA250, 003WA260, 003WA270), Craftsman Designers, Inc. (Periphytometer), or equivalent. See figures 17 and 18 for selected types of artificial substrates.

4.2 *Collecting devices* for the removal of periphyton from natural substrates. Three such devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 19.

4.3 *Sample containers* suitable for the type of sample. Glass bottles are useful containers for artificial substrates or for pieces of natural substrates.

4.4 *Scraping devices*. Razor blades, stiff brushes, spatulas, or glass slides are useful devices for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.5 *Filter funnel*, vacuum, 1,200-ml, stainless-steel, Gelman Instrument Co. (Parabella) or equivalent.

4.6 *Filter flask*, 1,000-ml or 2,000-ml. For field use a polypropylene flask, Bel-Art Products (H-38941), Nalgene Labware (4101), or equivalent, is suggested.

4.7 *Source of vacuum* for filtration: A water-aspirator pump or an electric vacuum pump for laboratory use; a hand-held vacuum pump with gage, Edmund Scientific Co. (71,301) or equivalent, for field use.

4.8 *Manostat* with mercury and calibration equipment to regulate the filtration suction at not more than 250 mm (10 in.) of mercury when filtering with an aspirator or an electric vacuum pump.

4.9 *Membrane filter*, white, plain, 0.45-μm mean pore size, 47-mm diameter, Millipore (HAWP 047 00) or equivalent.

4.10 *Tissue homogenizer* (grinder), glass pestle-type 15-ml capacity, Corning (7725) or equivalent. Homogenizer should be motor driven at about 500 rpm.

4.11 *Centrifuge*, swing-out type, 3,000- to 4,000-

rpm, with 15-ml graduated centrifuge tubes, Savegard (CT–1140) or equivalent.

4.12 *Spectrophotometer,* with a bandwidth of 2 nm or less allowing absorbance to be read to ± 0.001 units, Beckman (model 25) (fig. 52) or equivalent. Use cells with a light-path of 1 cm.

4.13 *Filters,* metricel, alpha-6, 0.45 μm, 25-mm diameter.

4.14 *Filter holder,* Pyrex microanalysis, frit support, 25 mm, Millipore (XX1002500) or equivalent.

5. Reagents

5.1 *Acetone,* 90 percent: Add 9 volumess of acetone to 1 volume of distilled water.

6. Collection

6.1 Artificial substrates. Place a suitable artificial substrate in the stream or lake, and attach it to a supporting object. Figures 17 and 18 illustrate several types of artificial substrates. The substrate must be submerged but may be near the surface of the water at any other appropriate depth. In lakes, the substrates are usually suspended at several depths (fig. 17*A*, *B*, and *C*). In lakes and streams the substrates may be attached to natural items such as submerged trees, stumps (fig. 17*D*), logs, or boulders, or they may be attached to stakes driven into the bottom (fig. 18*A*). Floating samplers also may be used (fig. 18*B*). The artificial substrates must be exposed to the light so that photosynthesis can take place, and they should be located so that 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. The length of time required for colonization of the substrates by periphyton will depend upon the season, water temperature, light and nutrient availability, and other factors. Neal, Patten, and DePoe (1967) found that the maximum accumulation of periphyton biomass on polyethylene strips occurred in about 2 weeks. Nielson (1953) exposed his slides for 20–30 days. Exposure probably should be at least 14 days, but this will vary and must be determined for each season and water type.

After sufficient colonization of periphyton, indicated by visible green or brown growth, remove the artificial substrate from the water.

6.2 Natural submerged substrates often contain periphyton which can be sampled quantitatively. The most convenient collection method consists of removing entire substrates, such as rocks, leaves, or wood, to the laboratory for processing. Usually the periphyton must be removed from a known area of substrate in the field, and figure 19*A*, *B*, and *C* illustrate several devices for collecting periphyton from natural substrates. The instrument used by Douglas (1958) consists of a broad-necked polyethylene flask with the bottom removed (fig. 19*A*). 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 with a stiff nylon brush. The loose periphyton is removed from the flask with a pipet.

Ertl's (1971) apparatus consists of two concentric metal or plastic cylinders separated with spacers (fig. 19*B*). The space between the cylinders is filled with modeling clay, and the sampler is pressed firmly against the substrate to be sampled. With 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 with a stiff brush and removed with a pipet. Stockner and Armstrong (1971) sampled periphyton with a plastic hypodermic syringe which had a toothbrush attached to the end of the syringe piston (fig. 19*C*). With the barrel of the syringe held tightly against the substrate, the piston is pushed in until the brush contacts the periphyton. The piston is then rotated several times to dislodge the periphyton and then is withdrawn, pulling the periphyton with it. A glass plate is immediately placed under the end of the barrel and the syring inverted. Four small holes at the base of the syringe allow for free movement of water when procuring the sample (J. G. Stockner, written commun., March 1972).

6.3 Place the detached periphyton from the natural substrate or the complete artificial substrate into a bottle containing no water or preservative. Store frozen in the dark for not more than 2 weeks. Dry ice is recommended for preserving samples in transit.

7. Analysis

7.1 Allow the frozen sample to thaw 5 minutes at room temperature.

7.2 If an artificial substrate is used, scrape the periphyton off the substrate with the scraping device into a glass pan. Transfer all solid material into the tissue-grinding vessel.

7.3 Rinse the scraping vessel and substrate with 90 percent acetone. Keep for 10 minutes in the dark at room temperature.

7.4 Grind 3 minutes at about 400 rpm.

7.5 Transfer the sample to a 15-ml graduated centrifuge tube, and wash the pestle and homogenizer two or three times with 90 percent acetone. Adjust to some convenient volume such as 10 ml ± 0.1.

7.6 Centrifuge for 10 minutes at 3,000 or 4,000 rpm.

7.7 Carefully pour or pipet the supernatant into the spectrophotometer cell. Do not disturb the precipitate. If extract is turbid, clear by making twofold dilution of 90 percent acetone, or by filtering through acetone resistant filter (4.13 and 4.14).

7.8 Read the absorbances at 750, 664, 647, and 630 nm against a 90-percent acetone blank. (Dilute the extract with 90 percent acetone if the absorbance is greater than 0.8.) If the 750 nm reading is greater than 0.005 per centimeter of light-path, reduce the turbidity as in 7.7 above.

8. Calculations

8.1 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 spectrophotometer cell in centimeters. The concentrations of chlorophylls in the extract, as μg/ml are given by the following equations (Jeffrey and Humphrey, 1975):

chlorophyll *a* in μg/ml

$$= 11.85e_{664} - 1.54e_{647} + 0.08e_{630}.$$

chlorophyll *b* in μg/ml

$$= -5.43e_{664} + 21.03e_{647} - 2.66e_{630}.$$

chlorophyll *c* in μg/ml

$$= -1.67e_{664} - 7.60e_{647} + 24.52e_{630}.$$

where

$$^{e}664 = \frac{\text{absorbance at 664 nm} - \text{absorbance at 750 nm,}}{\text{light-path in cm}}$$

$$^{e}647 = \frac{\text{absorbance at 647 nm} - \text{absorbance at 750 nm, and}}{\text{light-path in cm}}$$

$$^{e}630 = \frac{\text{absorbance at 630 nm} - \text{absorbance at 750 nm}}{\text{light-path in cm}}$$

8.2 Convert the values derived in 8.1 to the concentrations of chlorophylls, as mg/m^2, in the originally collected sample. To do so, multiply the derived value, in μg/ml, by the volume of the extract, in ml; divide by the area of scraped surface, in m^2, multiplied by 1,000 to convert μg to mg. For example:

$$\text{chlorophyll } a \text{ (mg/m}^2\text{)} = \frac{\text{derived value } (\mu\text{g/ml}) \times \text{extract volume (ml)}}{\text{area of scraped surface (m}^2\text{)} \times 1{,}000}$$

9. Report

Report chlorophyll *a*, *b*, or *c* in milligrams per square meter (to three significant figures) of original water sample.

10. Precision

The precision of chlorophyll determinations is influenced by the area scraped, the range of chlorophyll values found, the volume of extraction solvent, and the light-path of the spectrophotometer cells.

Tilley and Haushild (1975a, 1975b) found in the Duwamish River, Wash., that 21 glass microscope slides exposed for 2 weeks at a single site ranged in chlorophyll *a* from 13.3 to 28.1 mg/m^2, with a mean of 19.7 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 ranged in chlorophyll *a* from 18.9 to 48.6 mg/m^2, with a mean of 34.4 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 14.4 mg/m^2.

References

Douglas, Barbara, 1958, The ecology of the attached diatoms and other algae in a small stony stream: Jour. Ecology, v. 46, p. 295–322.

Ertl, Milan, 1971, A quantitative method of sampling periphyton from rough substrates: Limnology and Oceanography, v. 16, p. 576–577.

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Chlorophyll in periphyton by chromatography and spectroscopy (B–6620–77)

Parameters and codes:
Chlorophyll *a*, periphyton, chromato/spectro (mg/m²) 70955
Chlorophyll *b*, periphyton, chromato/spectro (mg/m²) 70956

1. Application

The method is suitable for all waters. 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 with a spectrophotometer.

3. Interferences

A substantial amount 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 chlorophyll.

4. Apparatus

4.1 *Spectrophotometer*, Beckman model 25 (fig. 52) or equivalent, with slit width 2 nm or less.

4.2 *Cuvettes*, 1-cm light-path length.

4.3 *Filters*, glass fiber, 47-mm diameter, Gelman 61694, type A, or equivalent, capable of retaining particles having a diameter of at least 0.45 micrometers.

4.4 *Developing tank and rack*, Scientific Products No. 21432–740 or equivalent.

4.5 *Solvent saturation pads*, Gelman No. 51334 or equivalent, 13.4×22 cm.

4.6 *Centrifuge*, IEC Model HN–S, with IEC 221 rotor and IEC 302 shield, or equivalent.

4.7 *Centrifuge tubes*, graduated, screwcap, 15-ml capacity.

4.8 *Tissue grinder*, Thomas No. 3431–E15 or equivalent.

4.9 *Evaporation device*, Organomation No. 11151 or equivalent.

4.10 *Grinding motor*, Curtin Matheson No. 214–700 or equivalent, with 0.1 horsepower.

4.11 *Chromatography sheet*, thin-layer cellulose, Baker No. 0–4468 or equivalent, 5×20 cm, 80-micrometer thick cellulose.

4.12 *Microdoser*, with 50-μl syringe, Brinkmann Instruments, No. 25–20–000–4, or equivalent.

4.13 *Air dryer*, Oster model No. 202 or equivalent.

4.14 *Spotting template*, Camag or equivalent.

4.15 *Disposable Pasteur pipets*, Scientific Products No. P5200–1 or equivalent.

4.16 *Glass bottles*, screwcap, smallest appropriate size for the sample.

4.17 *Scraping device*, glass microscope slide for flat substrates. Razor blades, stiff brush, or spatulas, are also useful devices for removing periphyton from different types of substrates.

4.18 *Glass pan*, smallest appropriate size for scraping substrate.

4.19 *Gloves*, H. T. Rubber, Edmont Wilson, or equivalent.

5. Reagents

5.1 *Methanol*, Burdick and Jackson or equivalent purity.

5.2 *Dimethyl sulfoxide*, Burdick and Jackson or equivalent purity.

5.3 *Ethyl ether*, Burdick and Jackson or equivalent purity.

5.4 *Acetone*, Burdick and Jackson or equivalent purity.

5.5 *Petroleum ether*, 30°–60°C, Baker No. 2–9268 or equivalent purity.

5.6 *Chlorophyll a*, solution: Add to 1 milligram Sigma Chemical Co. No. C5753 or equivalent purity, 1 ml of acetone (5.4).

5.7 *Chlorophyll b*, solution: Add to 1 milligram Sigma Chemical Co. No. C5878 or equivalent purity, 1 ml of acetone (5.4).

5.8 *Acetone*, 90 percent: Add 9 volumes of acetone (5.4) to 1 volume of distilled water, until the volume is 1 liter.

5.9 *Distilled water*.

6. Collection

6.1 Artificial substrates. Place a suitable artificial substrate in the stream or lake, and attach it to a supporting object. Figures 17 and 18 illustrate several types of artificial substrates. The substrate must be submerged but may be near the surface of the water or at any other appropriate depth. In lakes, the substrates are usually suspended at several depths (fig. 17*A*, *B*, and *C*). In lakes and streams the substrates may be attached to natural items such as submerged trees, stumps (fig. 17*D*), logs, or boulders, or they may be attached to stakes driven into the bottom (fig. 18*A*). Floating samplers also may be used (fig. 18*B*). The artificial substrates must be exposed to the light so that photosynthesis can take place, and they should be located so that 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. The length of time required for colonization of the substrate by periphyton will depend upon the season, water temperature, light and nutrient availability, and other factors. Neal, Patten, and DePoe (1967) found that the maximum accumulation of periphyton biomass on polyethylene strips occurred in about 2 weeks. Nielson (1953) exposed his slides for 20–30 days. Exposure probably should be at least 14 days, but this will vary and must be determined for each season and water type.

After sufficient colonization of periphyton, indicated by visible green or brown growth, remove the artificial substrate from the water.

6.2 Natural submerged substrates often contain periphyton which can be sampled quantitatively. The most convenient collection method consists of removing entire substrates, such as rocks, leaves, or wood, to the laboratory for processing. Usually the periphyton must be removed from a known area of substrate in the field, and figure 19*A*, *B*, and *C* illustrate several devices for collecting periphyton from natural substrates. The instrument used by Douglas (1958) consists of a broad-necked polyethylene flask with the bottom removed (fig. 19*A*). 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 with a stiff nylon brush. The loose periphyton is removed from the flask with a pipet. Ertl's (1971) apparatus consists of two concentric metal or plastic cylinders separated with spacers (fig. 19*B*). The space between the cylinders is filled with modeling clay, and the sampler is pressed firmly against the substrate to be sampled. With 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 with a stiff brush and removed with a pipet. Stockner and Armstrong (1971) sampled periphyton with a plastic hypodermic syringe which had a toothbrush attached to the end of the syringe piston (fig. 19*C*). With the barrel of the syringe held tightly against the substrate, the piston is pushed in until the brush contacts the periphyton. The piston is then rotated several times to dislodge the periphyton and then is withdrawn, pulling the periphyton with it. A glass plate is immediately placed under the end of the barrel and the syringe inverted. Four small holes at the base of the syringe allow for free movement of water when procuring the sample (J. G. Stockner, written commun., March 1972).

6.3 Place the detached periphyton from the natural substrate or the complete artificial substrate into a bottle containing no water or preservative. Store frozen in the dark for not more than 2 weeks. Dry ice is recommended for preserving samples in transit.

7. Analysis

7.1 Allow the frozen sample to thaw 5 minutes at room temperature.

7.2 If an artificial substrate is used, then scrape the periphyton off the substrate with the scraping device into a glass pan. Transfer all solid material into the tissue-grinding vessel. *Caution:* Rubber gloves should be worn during scraping and next step.

7.3 Rinse the scraping vessel and substrate with dimethyl sulfoxide.

7.4 Grind 3 minutes at about 400 rpm.

7.5 Transfer the sample to a 15-ml graduated centrifuge tube, and wash the pestle and homogenizer twice with dimethyl sulfoxide.

7.6 Add an equal volume of diethyl ether. Screw on

cap, and shake vigorously for 10 seconds. Wait 10 seconds, and repeat shaking for 10 seconds.

7.7 Remove cap and add slowly, almost dropwise, an amount of distilled water equal to 25 percent of the total volume of extractant.

7.8 Cap and shake as in 7.6.

7.9 Centrifuge at 1,000 × g for 10 minutes.

7.10 During centrifugation, prepare chromatography tank by placing 294 ml petroleum ether (5.5) and 6 ml methanol (5.1) into tank with two solvent pads and rack. Mix well. Prepare fresh daily.

7.11 Remove upper ethyl ether layer containing chlorophyll with a capillary pipet, and place in another 15-ml graduated screwcap tube.

7.12 Add an equal volume of distilled water, and shake as in 7.6.

7.13 Centrifuge at 1,000 × g for 5 minutes.

7.14 Remove upper ethyl ether layer with a capillary pipet, and place in conical tube in evaporation device (4.9). Evaporate to dryness by blowing nitrogen over the ether surface.

7.15 Immediately add 0.5 ml acetone. Mix. Wait 30 seconds. Mix. If all chlorophyll is not in solution, then repeat waiting and mixing.

7.16 Using microdoser, streak 25 microliters on the cellulose thin layer sheet (4.11) 15 mm from bottom and 6 mm from each side, and use the air dryer to speed evaporation of solvent. If excessive trailing occurs during chromatography, this amount should be decreased.

7.17 Chromatograph in dark with chlorophyll standard(s) prepared in same manner. Use enough chlorophyll to visually locate the spot (about 5 μl of the standard solution as in 5.6 and (or) 5.7). Time required for development is about 30 minutes. Remove strips when solvent has traveled to approximately 2–3 centimeters from top of strip.

7.18 Determine $R\hat{f}$ values for pure chlorophylls (Note: $R\hat{f}$ value = distance traveled by the chlorophyll from the point of application, divided by the distance traveled by the solvent from the point of application).

7.19 Locate the $R\hat{f}$ value on the unknown sheet and with a razor blade scrape the cellulose off from the $R\hat{f}$ value minus 0.07 for chlorophyll *a* (0.14 for chlorophyll *b*) × $R\hat{f}$ to the $R\hat{f}$ value plus 0.07 (for chlorophyll *a*; 0.14 for chlorophyll *b*) × $R\hat{f}$. Place the cellulose into a graduated centrifuge tube, and add acetone to a volume of 3 ml. This step should be completed immediately after removal from the tank. Mix the scraped cellulose and acetone vigorously 10 seconds. Wait 1 minute. Mix again vigorously for 10 seconds.

7.20 Centrifuge at 1,000 × g for 5 minutes.

7.21 Remove supernatant and read the absorbance on the spectrophotometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*. If the absorbance is greater than 0.01, determine concentrations using the specific absorptivities of 0.0877 l/mg for chlorophyll *a* and 0.0514 l/mg for chlorophyll *b* from the following equation (Jeffrey and Humphrey 1975):

$$A = \propto cb,$$

where

A = absorbance,

c = concentration in milligrams per liter,

b = path-length in centimeters, and

$\propto$ = specific absorptivity.

If the absorbance is less than 0.01 use fluorescence technique.

8. Calculations

The value obtained from the cuvette is then corrected for the concentration step in the field and in the analysis:

$$\text{mg chlorophyll/m}^2 \text{ (original sample)} = \frac{\mu\text{g chlorophyll/ml (in cuvette)} \times (3\text{ ml}) \times \frac{500\mu\text{l}}{25\mu\text{l}}}{\text{(area of surface scraped, square meters)} \times 1{,}000}$$

9. Report

Report chlorophyll *a* or *b* in milligrams per square meter (to three significant figures) of original substrate.

10. Precision

Tilley and Haushild (1975a, 1975b) found in the Duwamish River, Wash., that 21 glass microscope slides exposed for 2 weeks at a single site ranged in chlorophyll *a* from 13.3 to 28.1 mg/m^2, with a mean of 19.7 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 ranged in chlorophyll *a* from 18.9 to 48.6 mg/m^2, with a mean of 34.4 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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Douglas, Barbara, 1958, The ecology of the attached diatoms and other algae in a small stony stream: Jour. Ecology, v. 46, p. 295–322.

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Tilley, L. S., and Haushild, W. D., 1975a, Net primary productivity of periphytic algae in the intertidal zone, Duwamish River Estuary, Washington: U.S. Geol. Survey Jour. Research, v. 3, p. 253–259.

_____ 1975b, Use of productivity of periphyton to estimate water quality: Jour. Water Pollution Control Fed., v. 47, p. 2,157–2,171.

Chlorophyll in periphyton by chromatography and fluorometry (B–6640–77)

Parameters and codes:
Chlorophyll *a*, periphyton, chromato/fluoro (mg/m²) 70957
Chlorophyll *b*, periphyton, chromato/fluoro (mg/m²) 70958

1. Application

The method is suitable for all waters. 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 with a spectrofluorometer.

3. Interferences

A substantial amount 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 chlorophyll.

4. Apparatus

4.1 *Spectrofluorometer*, American Instrument Aminco-Bowman (fig. 53) or equivalent, with red-sensitive R446S photomultiplier.

4.2 *Fluorescence cuvettes*, 1-cm light-path length.

4.3 *Filters*, glass fiber, 47-mm diameter, Gelman 61694, type A, or equivalent, capable of retaining particles having a diameter of at least 0.45 micrometers.

4.4 *Developing tank and rack*, Scientific Products No. 21432–740, or equivalent.

4.5 *Solvent saturation pads*, Gelman No. 51334 or equivalent, 13.4×22 cm.

4.6 *Centrifuge*, IEC model HN–S, with IEC 221 rotor and IEC 302 shield, or equivalent.

4.7 *Centrifuge tubes*, graduated, screwcap, 15-ml capacity.

4.8 *Tissue grinder*, Thomas No. 3431–E15 or equivalent.

4.9 *Evaporation device*, Organomation No. 11151 or equivalent.

4.10 *Grinding motor*, Curtin Matheson No. 214–700 or equivalent, with 0.1 horsepower.

4.11 *Chromatography sheet*, thin-layer cellulose, Baker No. 0–4468 or equivalent, 5×20 cm, 80-micrometer thick cellulose.

4.12 *Microdoser*, with 50-μl syringe, Brinkman Instruments No. 25–20–000–4 or equivalent.

4.13 *Air dryer*, Oster model No. 202 or equivalent.

4.14 *Spotting template*, Camag or equivalent.

4.15 *Disposable Pasteur pipets*, Scientific Products No. P5200–1 or equivalent.

4.16 *Glass bottles*, screwcap, smallest appropriate size for the sample.

4.17 *Scraping device*, glass microscope slide for flat substrates. Razor blades, stiff brushes, or spatulas are also useful devices for removing periphyton from different types of substrates.

4.18 *Glass pan*, smallest appropriate size for scraping substrate.

4.19 *Gloves*, H. T. Rubber, Edmont Wilson, or equivalent.

5. Reagents

5.1 *Methanol*, Burdick and Jackson or equivalent purity.

5.2 *Dimethyl sulfoxide*, Burdick and Jackson or equivalent purity.

5.3 *Ethyl ether*, Burdick and Jackson or equivalent purity.

5.4 *Acetone*, Burdick and Jackson or equivalent purity.

5.5 *Petroleum ether*, 30°–60°C, Baker No. 2–9268 or equivalent purity.

5.6 *Chlorophyll a*, solution: Add to 1 milligram Sigma Chemical Co. No. C5753 or equivalent purity, 1 ml of acetone (5.4).

5.7 *Chlorophyll b*, solution: Add to 1 milligram Sigma Chemical Co. No. C5878 or equivalent purity, 1 ml of acetone (5.4).

5.8 *Acetone*, 90 percent: Add 9 volumes of acetone (5.4) to 1 volume of distilled water.

5.9 *Distilled water*.

5.10 *Nitrogen gas*, prepurified.

6. Collection

6.1 Artificial substrates. Place a suitable artificial substrate in the stream or lake, and attach it to a supporting object. Figures 17 and 18 illustrate several types of artificial substrates. The substrate must be submerged but may be near the surface of the water or at any other appropriate depth. In lakes, the substrates are usually suspended at several depths (fig. 17*A*, *B*, and *C*). In lakes and streams the substrates may be attached to natural items, such as submerged trees, stumps (fig. 17*D*), logs, or boulders, or they may be attached to stakes driven into the bottom (fig. 18*A*). Floating samplers also may be used (fig. 18*B*). The artificial substrates must be exposed to the light so that photosynthesis can take place, and they should be located so that 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. The length of time required for colonization of the substrates by periphyton will depend upon the season, water temperature, light and nutrient availability, and other factors. Neal, Patten, and DePoe (1967) found that the maximum accumulation of periphyton biomass on polyethylene strips occurred in about 2 weeks. Nielson (1953) exposed his slides for 20–30 days. Exposure probably should be at least 14 days, but this will vary and must be determined for each season and water type.

After sufficient colonization of periphyton, indicated by visible green or brown growth, remove the artificial substrate from the water.

6.2 Natural submerged substrates often contain periphyton which can be sampled quantitatively. The most convenient collection method consists of removing entire substrates such as rocks, leaves, or wood, to the laboratory for processing. Usually the periphyton must be removed from a known area of substrate in the field, and figure 19*A*, *B*, and *C* illustrate several devices for collecting periphyton from natural substrates. The instrument used by Douglas (1958) consists of a broad-necked polyethylene flask with the bottom removed (fig. 19*A*). 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 with a stiff nylon brush. The loose periphyton is removed from the flask with a pipet. Ertl's (1971) apparatus consists of two concentric metal or plastic cylinders separated with spacers (fig. 19*B*). The space between the cylinders is filled with modeling clay, and the sampler is pressed firmly against the substrate to be sampled. With 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 with a stiff brush and removed with a pipet. Stockner and Armstrong (1971) sampled periphyton with a plastic hypodermic syringe which had a toothbrush attached to the end of the syringe piston (fig. 19*C*). With the barrel of the syringe held tightly against the substrate, the piston is pushed in until the brush contacts the periphyton. The piston is then rotated several times to dislodge the periphyton and then is withdrawn, pulling the periphyton with it. A glass plate is immediately placed under the end of the barrel and the syringe inverted. Four small holes at the base of the syringe allow for free movement of water when procuring the sample (J. G. Stockner, written commun., March 1972).

6.3 Place the detached periphyton from the natural substrate or the complete artificial substrate into a bottle containing no water or preservative. Store frozen in the dark for not more than 2 weeks. Dry ice is recommended for preserving samples in transit.

7. Analysis

7.1 Allow the frozen sample to thaw 5 minutes at room temperature.

7.2 If an artificial substrate is used, scrape the periphyton off the substrate with the scraping device into a glass pan. Transfer all solid material into the tissue-grinding vessel. *Caution:* Rubber gloves should be worn during scraping and next step.

7.3 Rinse the scraping vessel and substrate with dimethyl sulfoxide.

7.4 Grind 3 minutes at about 400 rpm.

7.5 Transfer the sample to a 15-ml graduated centrifuge tube, and wash the pestle and homogenizer twice with dimethyl sulfoxide.

7.6 Add an equal volume of diethyl ether. Screw on cap, and shake vigorously for 10 seconds. Wait 10 seconds, and repeat shaking for 10 seconds.

7.7 Remove cap and add slowly, almost *dropwise*,

an amount of distilled water equal to 25 percent of the total volume of extractant.

7.8 Cap and shake as in 7.6.

7.9 Centrifuge at 1,000 × g for 10 minutes:

7.10 During centrifugation, prepare chromatography tank by placing 294 ml petroleum ether (5.5) and 6 ml methanol (5.1) into tank with two solvent pads and rack. Mix well. Prepare fresh daily.

7.11 Remove upper ethyl ether layer containing chlorophyll with a capillary pipet, and place in another 15-ml graduated screwcap tube.

7.12 Add an equal volume of distilled water, and shake as in 7.6.

7.13 Centrifuge at 1,000 × g for 5 minutes.

7.14 Remove upper ethyl ether layer with a capillary pipet, and place in conical tube in evaporation device (4.9). Evaporate to dryness by blowing nitrogen over the ether surface.

7.15 Immediately add 0.5 ml acetone. Mix. Wait 30 seconds. Mix. If all chlorophyll is not in solution, then repeat waiting and mixing.

7.16 Using microdoser, streak 25 microliters on the cellulose thin layer sheet (4.11) 15 mm from bottom and 6 mm from each side, using the air dryer to speed evaporation of solvent. If excessive trailing occurs during chromatography, this amount should be decreased.

7.17 Chromatograph in dark with chlorophyll standard(s) prepared in same manner. Use enough chlorophyll to visually locate the spot (about 5 μl of the standard solution as in 5.6 and (or) 5.7). Time required for development is about 30 minutes. Remove strips when solvent has traveled to approximately 2–3 centimeters from top of strip.

7.18 Determine $R\hat{f}$ values for pure chlorophylls. (Note: $R\hat{f}$ value = distance traveled by the chlorophyll from the point of application, divided by the distance traveled by the solvent from the point of application.)

7.19 Locate the $R\hat{f}$ value on the unknown sheet and with a razor blade scrape the cellulose off from the $R\hat{f}$ value minus 0.7 for chlorophyll *a* (0.14 for chlorophyll *b*) × $R\hat{f}$ to the $R\hat{f}$ value plus 0.07 (for chlorophyll *a*; 0.14 for chlorophyll *b*) × $R\hat{f}$. Place the cellulose into a graduated centrifuge tube, and add acetone to a volume of 3 ml. This step should be completed immediately after removal from the tank. Mix the scraped cellulose and acetone vigorously 10 seconds. Wait 1 minute. Mix again vigorously for 10 seconds.

7.20 Centrifuge at 1,000 × g for 5 minutes.

7.21 Determine the concentration of chlorophyll *a* or *b* with the spectrofluorometer as follows. Standard curves are prepared on a daily basis to standardize the spectrofluorometer. Five standards of each chlorophyll should be prepared at the approximate concentrations of 0.5, 1, 2, 3, 5 mg/l. These are prepared from the chlorophyll solutions (5.6, 5.7) by an appropriate dilution into 90 percent acetone. The absorbance is then read on a spectrophotometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*. Determine concentrations of standards and samples using the specific absorptivities of 0.0877 l/mg for chlorophyll *a* and 0.0514 l/mg for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$A = \alpha c b,$$

where

A = absorbance,

c = concentration in milligrams per liter,

b = path-length in centimeters, and

α = specific absorptivity.

For chlorophyll *a*, set the spectrofluorometer for an excitation wavelength of 430 nm and an emission wavelength 670 nm. For chlorophyll *b*, the excitation wavelength is 460 nm and 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 curve.

8. Calculations

The value obtained from the cuvette is then corrected for the concentration step in the field and in the analysis:

$$\text{mg chlorophyll/m}^2 \text{ (original sample)} = \frac{\mu\text{g chlorophyll/ml (in cuvette)} \times (3\text{ ml}) \times \frac{500\ \mu\text{l}}{25\ \mu\text{l}}}{(\text{area of surface scraped, square meters}) \times 1{,}000}.$$

9. Report

Report chlorophyll *a* or *b* in milligrams per square meter (to three significant figures) of original substrate.

10. Precision

Tilley and Haushild (1975a, 1975b) found in the Duwamish River, Wash., that 21 glass microscope slides exposed for 2 weeks at a single site ranged in chlorophyll *a* from 13.3 to 28.1 mg/m^2, with a mean of 19.7 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 ranged in chlorophyll *a* from 18.9 to 48.6 mg/m^2, with a mean of 34.4 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–6660–77)

Parameter and code: Biomass-chlorophyll ratio, periphyton (ratio) 70950

1. Application

The method is suitable for all waters. 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 with a spectrophotometer or spectrofluorometer. The dry weight and ash weight of the periphyton are determined to obtain the weight of organic matter (biomass). The biomass/chlorophyll *a* ratio is calculated from these values.

3. Interferences

A substantial amount of sediment may affect the chlorophyll extraction process. Inorganic matter in the sample will cause erroneously high dry and ash weights; nonliving organic matter in the sample will cause erroneously high dry (and thus organic) weights. Exposure to light at any stage of storage and analysis can result in the photochemical and chemical degradation of the chlorophylls.

4. Apparatus

4.1 *Spectrophotometer*, Beckman model 25 (fig. 52) or equivalent, with slit width 2 nm or less.

4.2 *Spectrofluorometer*, American Instrument Aminco-Bowman (fig. 53) or equivalent, with red-sensitive R4468 photomultiplier.

4.3 *Fluorescence cuvettes*, 1-cm light-path length.

4.4 *Filters*, glass-fiber, 47-mm diameter, Gelman 61694, type A, or equivalent, capable of retaining particles having a diameter of at least 0.45 micrometer.

4.5 *Developing tank and rack*, Scientific Products No. 21432–740 or equivalent.

4.6 *Solvent saturation pads*, Gelman No. 51334 or equivalent, 13.4×22 cm.

4.7 *Centrifuge*, IEC model HN–S, with IEC 221 rotor and IEC 302 shield, or equivalent.

4.8 *Centrifuge tubes*, graduated, screwcap, 15-ml capacity.

4.9 *Tissue grinder*, Thomas No. 3431-E15 or equivalent.

4.10 *Evaporation device*, Organomation No. 11151 or equivalent.

4.11 *Grinding motor*, Curtin Matheson No. 214–700 or equivalent, with 0.1 horsepower.

4.12 *Chromatography sheet*, thin-layer cellulose, Baker No. 0–4468 or equivalent, 5×20 cm, 80-micrometer thick cellulose.

4.13 *Microdoser*, with 50-µl syringe, Brinkman Instruments No. 25–20–000–4 or equivalent.

4.14 *Air dryer*, Oster model No. 202 or equivalent.

4.15 *Spotting template*, Camag or equivalent.

4.16 *Disposable Pasteur pipets*, Scientific Products No. P5200–1 or equivalent.

4.17 *Glass bottles*, screwcap, smallest appropriate size for the sample.

4.18 *Scraping device*, glass microscope slide.

4.19 *Glass pan*.

4.20 *Porcelain crucibles*.

4.21 *Analytical balance*, capable of weighing to at least 0.1 mg.

4.22 *Drying oven*, thermostatically controlled for use at 105°C.

4.23 *Muffle furnace*, for use at 500°C.

4.24 *Desiccator*, containing dry silica gel.

4.25 *Forceps or tongs*.

4.26 *Rubber gloves*.

5. Reagents

5.1 *Methanol*, Burdick and Jackson or equivalent purity.

5.2 *Dimethyl sulfoxide*, Burdick and Jackson or equivalent purity.

5.3 *Ethyl ether*, Burdick and Jackson or equivalent purity.

5.4 *Acetone*, Burdick and Jackson or equivalent purity.

5.5 *Petroleum ether*, 30°–60°C, Baker No. 2–9268 or equivalent purity.

5.6 *Chlorophyll a*, solution: Add to 1 milligram Sigma Chemical Co. No. C5753 or equivalent purity, 1 ml of acetone (5.4).

5.7 *Chlorophyll b*, solution: Add to 1 milligram Sigma Chemical Co. No. C5878 or equivalent purity, 1 ml of acetone (5.4).

5.8 *Acetone*, 90 percent: Add 9 volumes of acetone (5.4) to 1 volume of distilled water.

5.9 *Distilled water*.

5.10 *Nitrogen gas*, prepurified.

6. Collection

6.1 Artificial substrates. Place a suitable artificial substrate in the stream or lake and attach it to a supporting object. Figures 17 and 18 illustrate several types of artificial substrates. The substrate must be submerged but may be near the surface of the water or at any other appropriate depth. In lakes, the substrates are usually suspended at several depths (fig. 17*A*, *B*, and *C*). In lakes and streams the substrates may be attached to natural items, such as submerged trees, stumps (fig. 17*D*), logs, or boulders, or they may be attached to stakes driven into the bottom (fig. 18*A*). Floating samplers also may be used (fig. 18*B*). The artificial substrates must be exposed to the light so that photosynthesis can take place, and they should be located so that 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. The length of time required for colonization of the substrates by periphyton will depend upon the season, water temperature, light and nutrient availability, and other factors. Neal, Patten, and DePoe (1967) found that the maximum accumulation of periphyton biomass on polyethylene strips occurred in about 2 weeks. Nielson (1953) exposed his slides for 20–30 days. Exposure probably should be at least 14 days, but this will vary and must be determined for each season and water type.

After sufficient colonization of periphyton, indicated by visible green or brown growth, remove the artificial substrate from the water.

6.2 Natural submerged substrates often contain periphyton which can be sampled quantitatively. The most convenient collection method consists of removing entire substrates such as rocks, leaves, or wood, to the laboratory for processing. Usually the periphyton must be removed from a known area of substrate in the field, and figures 19*A*, *B*, and *C* illustrate several devices for collecting periphyton from natural substrates. The instrument used by Douglas (1958) consists of a broad-necked polyethylene flask with the bottom removed (fig. 19*A*). 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 with a stiff nylon brush. The loose periphyton is removed from the flask with a pipet. Ertl's (1971) apparatus consists of two concentric metal or plastic cylinders separated with spacers (fig. 19*B*). The space between the cylinders is filled with modeling clay, and the sampler is pressed firmly against the substrate to be sampled. With 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 with a stiff brush, and removed with a pipet. Stockner and Armstrong (1971) sampled periphyton with a plastic hypodermic syringe which had a toothbrush attached to the end of the syringe piston (fig. 19*C*). With the barrel of the syringe held tightly against the substrate, the piston is pushed in until the brush contacts the periphyton. The piston is then rotated several times to dislodge the periphyton and then is withdrawn, pulling the periphyton with it. A glass plate is immediately placed under the end of the barrel and the syringe inverted. Four small holes at the base of the syringe allow for free movement of water when procuring the sample (J. G. Stockner, written commun., March 1972).

6.3 Place the detached periphyton from the natural substrate or the complete artificial substrate into a bottle containing no water or preservative. Store frozen in the dark for not more than 2 weeks. Dry ice is recommended for preserving samples in transit.

7. Analysis

7.1 Allow the frozen strip to thaw 5 minutes at room temperature.

7.2 Scrape the periphyton off the strip with glass slide into a glass pan. Transfer all solid material into the tissue-grinding vessel. Always wear rubber gloves in this step and step 7.3.

7.3 Rinse the scraping vessel and the strip with dimethyl sulfoxide.

7.4 Grind 3 minutes at about 500 rpm.

7.5 Transfer the sample to a 15-ml graduated cen-

trifuge tube, and wash the pestle and homogenizer twice with dimethyl sulfoxide.

7.6 Add an equal volume of diethyl ether. Screw on cap, and shake vigorously for 10 seconds. Wait 10 seconds, and repeat shaking for 10 seconds.

7.7 Remove cap and add slowly, almost *dropwise*, an amount of distilled water equal to 25 percent of the total volume of extractant.

7.8 Cap and shake as in 7.6.

7.9 Centrifuge at 1,000 × g for 10 minutes.

7.10 During centrifugation, prepare chromatography tank by placing 294 ml petroleum ether (5.5) and 6 ml methanol (5.1) into tank with two solvent pads and rack. Mix well. Prepare fresh daily.

7.11 Remove upper ethyl ether layer containing chlorophyll with a capillary pipet, and place in another 15-ml graduated screwcap tube.

7.12 Add an equal volume of distilled water, and shake as in 7.6.

7.13 Centrifuge at 1,000 × g for 5 minutes.

7.14 Remove upper ethyl ether layer with a capillary pipet and place in conical tube in evaporation device (4.10). Begin evaporating to dryness with nitrogen by blowing nitrogen over the ether surface. Save pelleted material and dimethyl sulfoxide-water supernatant for biomass measurement (7.24).

7.15 When nearly dry, immediately add 0.5 ml acetone. Mix. Wait 30 seconds. Mix. If all chlorophyll is not in solution, then repeat waiting and mixing.

7.16 Using microdoser, streak 25 microliters on the cellulose thin layer sheet (4.12) 15 mm from bottom and 6 mm from each side, using the air dryer to speed evaporation of solvent. If excessive trailing occurs during chromatography, this amount should be decreased.

7.17 Chromatograph in dark with chlorophyll standard(s) prepared in same manner. Use enough chlorophyll to visually locate the spot (about 5 μl of the standard solution as in 5.6 and (or) 5.7). Time required for development is about 30 minutes. Remove strips when solvent has traveled to approximately 2–3 centimeters from top of strip.

7.18 Determine $R\hat{f}$ values for pure chlorophylls. (Note: $R\hat{f}$ value = distance traveled by the chlorophyll from the point of application, divided by the distance traveled by the solvent from the point of application.)

7.19 Locate the $R\hat{f}$ value on the unknown sheet and with a razor blade scrape the cellulose off from the $R\hat{f}$ value minus 0.07 for chlorophyll *a* (0.14 for chlorophyll *b*) × $R\hat{f}$ to the $R\hat{f}$ value plus 0.07 (for chlorophyll *a*; 0.14 for chlorophyll *b*) × $R\hat{f}$. Place the cellulose into a graduated centrifuge tube, and add acetone to a volume of 3 ml. This step should be completed immediately after removal from the tank. Mix the scraped cellulose and acetone vigorously 10 seconds. Wait 1 minute. Mix again vigorously for 10 seconds.

7.20 Centrifuge at 1,000 × g for 5 minutes.

7.21 Remove supernatant and read the absorbance on the spectrophotometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*. If the absorbance is greater than 0.01, determine concentrations using the specific absorptivities of 0.0877 l/mg for chlorophyll *a* and 0.0514 l/mg for chlorophyll *b*, from the following equation (Jeffrey and Humphrey, 1975):

$$A = \propto cb,$$

where

A = absorbance,

c = concentration in milligrams per liter,

b = path-length in centimeters, and

$\propto$ = specific absorptivity.

If the absorbance is less than 0.01 proceed to 7.22; otherwise, proceed to 7.23.

7.22 Determine the concentration of chlorophyll *a* or *b* with the spectrofluorometer as follows. Standard curves are prepared on a daily basis to standardize the spectrofluorometer. Five standards of each chlorophyll should be prepared at the approximate concentrations of 0.5, 1, 2, 3, 5 mg/l. These are prepared from the standard chlorophyll solutions (5.6, 5.7) by an appropriate dilution into 90 percent acetone. The absorbance is then read on a spectrophotometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*. Determine concentrations of standards and samples using the specific absorptivities of 0.0877 l/mg for chlorophyll *a* and 0.0514 l/mg for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$A = \propto cb,$$

where

A = absorbance,

c = concentration in milligrams per liter,

b = path length in centimeters, and

$\propto$ = specific absorptivity

These solutions are then 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 emission wavelength is 650 nm. Set the entrance and exit slits at 2 mm. Plot chlorophyll concentration versus relative fluorescence intensity. Determine unknown concentrations from the appropriate standard curve.

7.23 Obtain the tare weight of a crucible that has been held at 500°C for about 20 minutes and cooled to room temperature in a desiccator.

7.24 Place the pelleted material (7.14) and supernatant (7.14) into the tared crucible and dry in an oven at 105°C to constant weight; that is, until further drying produces no change in weight.

Note: Cool the crucible containing dried material to room temperature in a desiccator before weighing. Weigh as rapidly as possible to decrease moisture uptake by the dried residue. These values are used to calculate dry weight.

7.25 Place the crucible containing the dried residue in a muffle furnace for 1 hour at 500°C. Cool to room temperature.

7.26 Moisten the ash with distilled water, and again oven dry at 105°C to constant weight as described in 7.24. These weight values are used to calculate ash weight.

Note: The ash is wetted to reintroduce the water of hydration of the clay and other minerals that, though not driven off at 105°C, is lost at 500°C. This water loss may amount to as much as 10 percent of the weight lost during ignition and, if not corrected for, will be interpreted as organic matter (American Public Health Association and others, 1976).

8. Calculation

Chlorophyll: The value obtained from the cuvette is then corrected for the concentration step in the field and in the analysis.

$$\text{mg chlorophyll/m}^2 \text{ (original sample)} = \frac{\mu\text{g chlorophyll/ml (in cuvette)} \times (3\text{ ml}) \times \frac{500\ \mu\text{l}}{25\ \mu\text{l}}}{(\text{area of surface scraped, square meters}) \times 1{,}000}$$

Biomass:

$$\frac{\text{Organic weight, mg}}{\text{area scraped, m}^2} = \frac{(\text{dry weight, mg}) - (\text{ash weight, mg})}{(\text{area of scraped surface, m}^2)}$$

Ratio:

$$\frac{\text{Biomass (mg/m}^2)}{\text{Chlorophyll } a \text{ (mg/m}^2)}$$

9. Report

Report chlorophyll *a* or *b* in milligrams per square meter (to three significant figures) of original water sample. Report biomass in milligrams per square meter (to three significant figures).

10. Precision

Tilley and Haushild (1975a, 1975b) found in the Duwamish River, Wash., that 21 glass microscope slides exposed for 2 weeks at a single site ranged in chlorophyll *a* from 13.3 to 28.1 mg/m^2, with a mean of 19.7 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 ranged in chlorophyll *a* from 18.9 to 48.6 mg/m^2, with a mean of 34.4 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 14.4 mg/m^2.

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Adenosine triphosphate (ATP)
(B–6700–77)

Parameter and code: Adenosine triphosphate 70998

Very sensitive methods of ATP analysis have been developed from McElroy's (1947) finding that luminescence in fireflies has an absolute requirement for ATP. ATP is determined by measuring the amount of light produced when ATP reacts with reduced luciferin (LH_2) and oxygen (O_2) in the presence of firefly luciferase and magnesium, producing adenosine monophosphate (AMP), inorganic pyrophosphate (PPi), oxidized luciferin (L), water (H_2O), carbon dioxide (CO_2) and light (hv), by the following reaction:

$$ATP + O_2 + LH_2 \xrightarrow[Mg^{+2}]{\text{luciferase}}$$

$$AMP + PPi + L + H_2O + CO_2 + hv$$

The bioluminescent reaction is specific for ATP, and the reaction rate is proportional to the ATP concentration with 1 photon of light emitted for each molecule of ATP hydrolyzed. When ATP is introduced to suitably buffered enzyme and substrates, a light flash follows that decays in an exponential fashion. Either the peak height of the light flash or integration of the area under the decay curve can be used to form standard curves.

1. Application

The method is suitable for all waters.

2. Summary of method

A water sample is filtered and the ATP is extracted from the cells. The cell extract (containing the ATP) is injected into a suitably buffered luciferin-luciferase enzyme solution. The amount of light produced in the subsequent reaction is measured with an ATP photometer. The reaction rate is proportional to the ATP concentration, with 1 photon of light being emitted for each molecule of ATP hydrolyzed.

3. Interferences

Several metals (for example, Hg^{+2}, Cd^{+2}) and high concentration of salts, in general, will inhibit the reaction. It is advisable to wash the filter with buffered distilled water immediately after filtration to remove most of the dissolved salts. A substantial amount of sediment may affect the extraction process.

4. Apparatus

4.1 *Vacuum filter stand,* Dupont No. 760308 or equivalent.

4.2 *Filter assemblies,* 13-mm diameter, 0.45-micrometer mean pore size, self-supported filters, Dupont No. 760312 or equivalent. (Note: These filters are resistant to the extracting agent, dimethyl sulfoxide.)

4.3 *Vacuum pump,* to provide at least 250 mm (10 in.) of mercury, Millipore No. XX60–000–00 or equivalent.

4.4 *Tubes,* graduated 12- or 15-ml centrifuge, Corning No. 8140 or equivalent.

4.5 *Photometer,* Chem-Glow photometer with integrator, American Instrument Co., models J4–7441 and J4–7462; ATP photometer, JRB Inc., model 2000; luminescence biometer, Dupont Instrument Co., model 760; or equivalent.

4.6 *Cuvettes,* 6×49mm, Dupont No. 760140 or equivalent.

4.7 *Cuvette holder,* Dupont No. 760151 or equivalent.

4.8 *Microliter syringe,* 50-microliter, blunt-tipped (nonbeveled), Hamilton No. 705–N, point style 3, or equivalent.

4.9 *Constant-rate injector,* Shandon Repro-jector, model SAA 1350, or equivalent.

4.10 *Microliter pipet,* 0.1, 0.2 and 1 milliliter, Eppendorf or equivalent, with disposable tips.

4.11 *Distillation apparatus,* glass.

4.12 *Balance,* analytical.

4.13 *Autoclave.*

4.14 *Volumetric flasks,* 100- and 1,000-ml sizes.

4.15 *Cuvette caps,* Dupont No. 201907 or equivalent.

4.16 *Glass storage bottles,* approximately 150-ml capacity, with autoclavable screwcaps.

4.17 *Glass vials,* approximately 15-ml capacity, with screwcaps.

5. Reagents

(All reagents are prepared with freshly distilled, not just deionized, water which gives a response not greater than 0.1 μg/l, when measured for ATP.)

5.1 *Luciferin-Luciferase buffer solution,* Dupont No. 760145–902 or equivalent. The kit must be stored frozen at −20°C or less. For daily use, dissolve one buffer-salt (morpholinopropane sulfonic acid and magnesium sulfate at pH 7.4) tablet in 3 milliliters of low-response water. Add the vial containing the lyophilized enzyme-substrate (luciferase-luciferin) powder to the buffer solution. Mix gently but completely. Avoid the formation of bubbles, as this may result in enzyme (luciferase) denaturation. Wait at least 15 minutes before using. Fresh solution must be prepared daily, 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.

5.2 *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 of low-response distilled water. Adjust the pH to 7.7 with sodium hydroxide, and bring the final volume to 1 liter. The final concentrations are 10 m*M* MOPS, 1 m*M* EDTA, and 10 m*M* $MgSO_4$. If not used immediately, the solution should be autoclaved to prevent growth of microorganisms and thus the production of ATP.

5.3 *Adenosine-5-triphosphate solutions,* 1, 2.5, 10, 25 and 100 micrograms ATP (Sigma Chemical Co., No. A3127, or equivalent) per liter. Complete 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 of ATP diluent (step 5.2 above). Make two serial dilutions of 1:100 using the ATP diluent (Step 5.2 above). 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 (step 5.2 above) to make solutions of 25, 10, 2.5, and 1 μg/l concentrations. Place small aliquots (approximately 100 μl) of the 1-, 2.5-, 10-, 25-, and 100-μg/l solutions in the reaction cuvettes, and cap with cuvette caps. Quickfreeze the cuvettes immediately by immersing in a bath of acetone and dry ice; store at −20°C or less.

5.4 *Morpholinopropane sulfonic acid (MOPS) solution,* 0.01 *M*: Dissolve 2.09 g MOPS (Dupont No. 202021 or equivalent) in approximately 900 ml of low-response distilled water. Adjust pH to 7.4 with sodium hydroxide. Bring final volume to 1,000 ml. Place approximately 100 ml each into 150-ml glass bottles, cap loosely, and autoclave. After cooling, cap tightly and store at room temperature.

5.5 *Neutral dimethyl sulfoxide (DMSO) solution:* Add 9 volumes of DMSO (Eastman No. 13096 or equivalent) to 1 volume 0.01 *M* MOPS solution (step 5.4 above). Mix well. Prepare fresh daily. Exercise caution because DMSO is harmful if absorbed through skin.

5.6 *Hydrochloric acid solution,* 0.2 *N:* dilute concentrated hydrochloric acid 1:56.

6. Collection

6.1 The sample collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, phytoplankton abundance may vary transversley, 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 remaining steps describe the extraction of ATP from the living material (algae, bacteria, fungi) in the sample. These extraction procedures ideally should be performed immediately after collection. The sample may be stored 2 to 3 hours if necesary and if the temperature and lighting conditions are maintained; for example, do not place a warm sample from a well-lighted area into a cool, dark ice chest.

6.2 Mix contents of the sampler well and remove 25 milliliters. (Note: If sample obviously contains abundant living material (for example, algae, bacteria, fungi), then this volume may be reduced substantially (as low as approximately 10 ml).) The volume filtered must be recorded.

6.3 Pour the sample aliquot into the filter assembly containing the membrane filter and with graduated tube in place (and vacuum pump attached).

6.4 Apply vacuum no greater than 250 mm (10 in.) mercury.

6.5 Release vacuum immediately when filtration is almost complete so that sample does not dry.

6.6 Quickly add 5 ml of low-response distilled water and filter again, this time to dryness. Release vacuum immediately.

6.7 Replace graduated centrifuge tube with a clean and dry graduated tube.

6.8 Pipet 0.2 ml of neutral dimethyl sulfoxide (step 5.5 above) 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 step 6.11 should also be doubled to 2 ml. The change must be recorded so that corrections for dilutions can be made.

6.9 Wait at least 20 seconds (not more than 30).

6.10 Apply vacuum until surface is dry.

6.11 Add 1 ml of MOPS solution (step 5.4 above).

6.12 Wait 10 seconds.

6.13 Apply vacuum until surface is dry.

6.14 Repeat step 6.11 to 6.13.

6.15 Record final volume; this value should be approximately 2.2 ml.

6.16 Mix contents of graduated centrifuge tube.

6.17 Pour tube contents 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 assayed. Storage should not exceed 30 days.

7. Analysis

7.1 Pipet 100 microliters of the luciferin-luciferase solution into the reaction cuvettes.

7.2 Rinse microliter syringe three times with 0.2 *N* hydrochloric acid by drawing acid into the entire 50 microliters; rinse three times with the MOPS buffer solution to neutralize any remaining acid; rinse three times with low-response distilled water.

7.3 Thaw the ATP standards at room temperature, and mix well.

7.4 Determine the response of the instrument for this luciferin-luciferase preparation (background luminescence) and 10 microliters of the five standard ATP solutions. Follow specific instructions for the instrument used. This procedure is the preparation of a standard curve and is linear for this assay.

7.5 Rinse microliter syringe as in 7.2.

7.6 Place reaction cuvette in instrument.

7.7 Thaw sample at room temperature for analysis. Mix well.

7.8 Rinse microliter syringe three times with the sample.

7.9 Inject 10 microliters of sample into reaction cuvette, and record response. Assay in duplicate.

7.10 If response is too great for instrument, the sample may be diluted. Dilutions with low-response distilled water are linear.

8. Calculations

8.1 A standard curve is prepared with ATP. The standard curve is linear with a slope of 1. The concentration of ATP in the injected sample is determined in micrograms ATP per liter of sample.

8.2 This value is corrected for the concentration step in the field by the following equation:

$$\begin{array}{l}\mu g\ ATP/l \\ \text{(original sample)}\end{array} = \frac{(\mu g\ ATP\ measured/l)}{\begin{array}{c}\text{(volume of sample)} \\ \text{(filtered, in liters)}\end{array}} \times \frac{(dilution)^*}{\begin{array}{c}\text{(volume recovered)}^{**} \\ \text{(after extraction,)} \\ \text{(in liters)}\end{array}}$$

* If undiluted, this value equals one.

** Usually 2.2×10^{-3} liter.

9. Report

Report adenosine triphosphate in micrograms per liter of original water sample to the nearest 0.1 microgram per liter.

10. Precision

Reproducibility of assay is approximately ±2 percent (single operator).

References

Federal Working Group on Pest Management, 1974, Guidelines on sampling and statistical methodologies for ambient pesticide monitoring: Washington, D.C., Fed. Working Group on Pest Management, 59 p.

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PRIMARY PRODUCTIVITY (PRODUCTION RATE)

Bodies of water differ widely in their populations of plants and animals, and these differences may be used in the interpretation of water quality. Biological differences may be expressed both qualitatively and quantitatively; that is, as the abundance of individuals of all kinds or as diversity of kinds. For many purposes, however, the factor of greatest interest is the rate at which new organic matter is formed and accumulated within the system under study. The basic process involved is that of photosynthesis which may be summarized as:

$$6CO_2 + 6H_2O \xrightarrow{\text{light}} C_6H_{12}O_6 + 6O_2.$$

The rate of photosynthesis customarily is determined by measuring the amount of oxygen produced or the amount of carbon dioxide consumed.

The underlying assumption in the following methods is that the change in oxygen concentration in an illuminated volume of water containing plants and animals is a result of oxygen production in photosynthesis by chlorophyll-containing plants and of oxygen consumption in respiration by both plants and animals. In the dark, only respiration occurs. Therefore, the amount of oxygen produced in the light is an estimate of net photosynthesis or net primary productivity. The amount of oxygen consumed in the dark, added to the amount of oxygen produced in the light, is an estimate of gross photosynthesis or gross primary productivity. (This assumes that the rate of respiration is the same in the light and in the dark.)

Two general approaches are described for the estimation of primary productivity of waters. 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 content as measured by uptake of radioactive carbon (carbon-14). If the rate of primary production is sufficient for accurate measurements to be made within 24 hours, the oxygen method is preferred. The carbon-14 method is of greater sensitivity and is preferred for use in oligotrophic waters. 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.

Oxygen light- and dark-bottle method for phytoplankton (B–8001–77)

Parameters and codes:
Productivity, primary, gross (mg O_2/m^3/day) 70959
Productivity, primary, gross (mg O_2/m^2/day) 70960
Productivity, primary, gross (mg C/m^3/day) 70961
Productivity, primary, gross (mg C/m^2/day) 70962
Productivity, primary, net (mg O_2/m^3/day) 70963

Productivity, primary, net (mg O_2/m^2/day) 70964
Productivity, primary, net (mg C/m^3/day) 70965
Productivity, primary, net (mg C/m^2/day) 70966
Respiration (mg O_2/m^3/day) 70967
Respiration (mg O_2/m^2/day) 70968

1. Application

The method is applicable to standing or slowly moving waters. Best results are obtained in eutrophic waters in which the production rate is between about 3 and 200 mg C/m^3/hr of photoperiod (Strickland and Parsons, 1968, p. 263). The lower limit for measurable oxygen production occurs when phytoplankton densities, expressed as chlorophyll *a*, fall below 1 mg/m^3 (Vollenweider, 1969, p. 74).

2. Summary of method

Light (clear) and dark (blackened) containers filled with water samples are suspended at several depths within 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 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

The method uses isolated plankton samples to indicate the response of the natural system. Care must be used in sampling, sample handling, and light exposure not to interfere with the life requirements of the organisms. Water-sampling bottles or devices should be constructed of plastic or glass with essential metal parts of stainless steel. Copper, brass, and bronze fittings on sampling bottles or on suspension equipment should not be used. The water-sampling bottles should be opaque to reduce the risk of light injury, and 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 experimental bottles results in errors in the determination of dissolved oxygen changes; microbial activity and chemical oxygen demand cause losses of oxygen when incubation times exceed a few hours.

Interferences with the chemical determination of dissolved oxygen were described by Brown, Skougstad, and Fishman (1970) and by the American Public Health Association and others (1976).

4. Apparatus

All materials must be free of agents which inhibit photosynthesis and respiration.

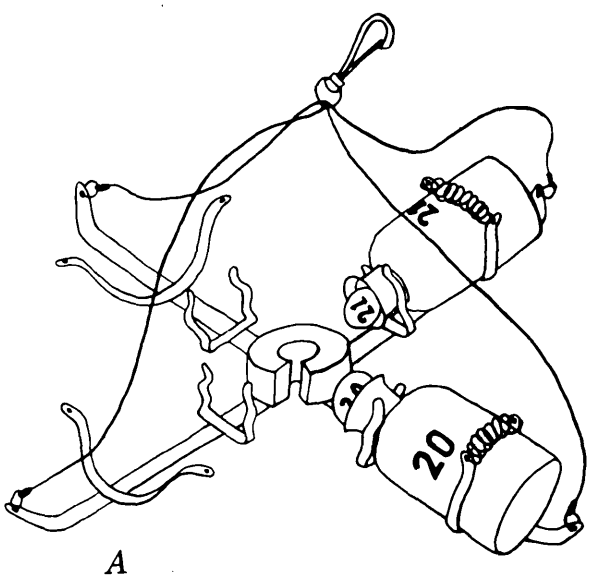
4.1 *Water-sampling bottle,* Van Dorn-type, opaque acrylic-plastic with stainless-steel metal parts, 4- to 6-liter capacity. A similar sampling bottle constructed of polyvinyl chloride, Wildlife Supply Co. (1140, 1160); Scott Instruments, Seattle, Wash.; Kahl Scientific Instrument Corp. (135WA); General Oceanics, Inc. (1010); or equivalent may be used if the opaque acrylic-plastic bottle is not available.

4.2 *BOD (biochemical oxygen demand) bottles,* numbered, 300-ml, Pyrex or borosilicon glass, with flared necks and pointed ground-glass stoppers. A supply of both light (clear) and dark (blackened) bottles is required. Blackened bottles are available from Kahl Scientific Instrument Corp., or may be prepared by painting the bottles black and covering the paint with overlapping strips of black plastic tape, Scotch (33) or equivalent. 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.

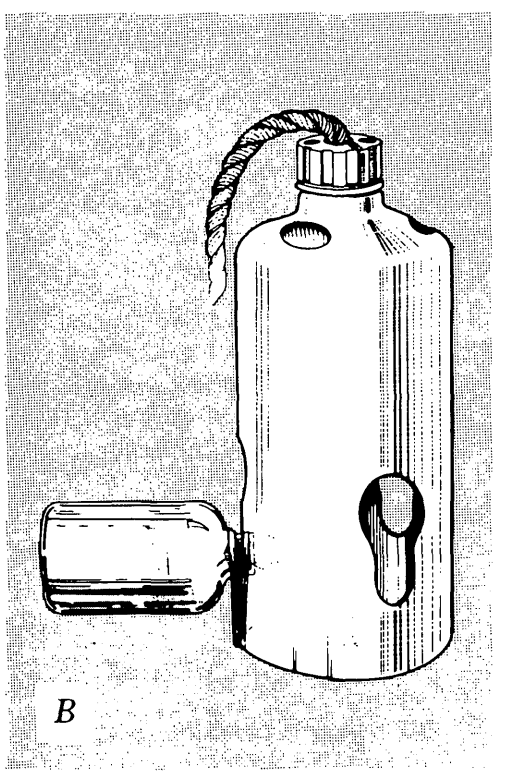
To prepare the BOD bottles, fill with the acid cleaning solution and let stand for several hours. Rinse thoroughly with distilled water. Traces of iodine from the Winkler analysis should be removed by rinsing the bottles and stoppers with 0.01 *N* sodium thiosulfate solution followed by thorough rinsing with distilled water. Do not use phosphorus-based detergents.

4.3 *Equipment for determination of dissolved oxygen* by the azide modification of the Winkler method (Brown and others, 1970; American Public Health Association and others, 1976).

4.4 *Suspension system* for holding light and dark bottles in a horizontal position at various depths (fig. 54). The BOD bottle holder shown in figure 54*B* is made from a thick-walled 2-liter Boston-round polyethylene bottle. Three "keyholes" are cut into the sides of the bottle, the bottom is cut out, and air vents



A



B

Figure 54—Devices for holding light and dark bottles in a horizontal position. *A*, Metal suspension frame. (Modified from Saunders and others, 1962.) *B*, Holder made from a 2-liter polyethylene bottle (Janzer and others, 1973).

are cut into the top to prepare a holder similar to that of Schindler and Holmgren (1971). Each stoppered BOD bottle is held in an essentially horizontal position with a heavy rubberband stretched over the neck of the bottle inside the holder, pulled under the bottom edge of the holder, and then over the neck of the BOD bottle outside the holder. Each holder, with BOD bottles attached as described, is then secured to a buoy by a length of nylon cord run through a hole in the cap of the bottle holder.

4.5 *Polyethylene bottles*, 8-liter capacity with cap and bottom tubulation, Bel-Art Products (F–11842 or H–11873), Nalgene Labware (2318), or equivalent.

4.6 *Dark box*, preferably insulated, for storing filled BOD bottles until ready for incubation.

4.7 *Underwater light-measurement equipment*, Kahl Scientific Instrument Corp. (268WA310), Hydro Products (620–S), InterOcean (510), or equivalent. If a submersible photometer is not available, a Secchi disk, Kahl Scientific Instrument Corp. (281WA100), Wildlife Supply Co. (59), or equivalent, may be used as described in 6.1 below.

5. Reagents

5.1 *Reagents for the azide modification of the Winkler method* for dissolved oxygen (Brown and others, 1970; American Public Health Association and others, 1976).

5.2 *Acid cleaning solution*, 20 percent: Mix 20 ml of concentrated HCl with distilled water, and dilute to 100 ml.

5.3 *Sodium thiosulfate solution*, 0.01 *N:* Dissolve 2.5 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in distilled water and dilute to 1 liter.

6. Collection

6.1 Determine the depth of the euphotic zone (the region that receives 1 percent or more of the surface light) with a submersible photometer. If no other method is available, an estimate of the lower limit of the euphotic zone is obtained by multiplying the Secchi disk depth (Welch, 1948) by 5 (Verduin, 1956). Select sampling intervals equal to one-tenth of the depth of the euphotic zone; fewer depth intervals are permissible in shallow euphotic zones.

6.2 Collect a water sample with a nonmetallic sampler from each of the preselected depths. The sample volume should be sufficient to fill three, preferably six, BOD bottles in a set as described in 6.4 below. Keep the samples in subdued light at all times during the following procedures to avoid light injury to the organisms.

Note: Samples should preferably be taken after 10 a.m. and as shortly before noon (local standard time) as practical. This procedure allows for measurements of light penetration and water sampling during daylight and an incubation period from noon to sunset (Committee on Oceanography, Biological Methods Panel, 1969).

6.3 Transfer the water sample from each depth to an 8-liter polyethylene bottle, and let stand for 15–30 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 highly productive water or in samples that have warmed several degrees.

6.4 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 BOD bottles. 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 to avoid entrapment of bubbles. It is important that all bottles have the same initial oxygen content. 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 oxygen concentration should be started as nearly as possible to the time that incubation begins.

6.5 Immediately add the reagents for the azide modification of the Winkler method to two clear 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.

6.6 Secure the stoppers in the BOD bottles that are to be incubated. The method used may be arranged as 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 (*LB*) and dark (*DB*) bottles to the suspension apparatus so that each set of bottles is incubated at the depth from which the filling water was originally taken. Begin the incubation and fix any remaining *IB* samples for oxygen determination.

The incubation period may vary from 1 to 24 hours but is usually one-half the photoperiod, preferably from noon until dusk. In highly productive waters where oxygen supersaturation is likely, an incubation period of 1–3 hours, preferably during midday, may be sufficient.

7. Analysis

7.1 After a suitable incubation period, remove the BOD bottles from the suspension apparatus, 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.

8. 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. That is, double the values obtained for half photoperiod exposure, and express as primary productivity per day. However, because the rate of photosynthesis varies during the day, short-period incubation results should be reported as primary productivity per hour. Net or gross primary productivity is calculated on the assumption that one atom of carbon is assimilated for each molecule (two atoms) of oxygen released.

8.1 Gross primary productivity

$$(\text{mg } O_2/m^3/\text{time}) = \frac{LB - DB}{t} \times 1{,}000$$

where

LB is the dissolved-oxygen concentration in mg/l in the light bottle after incubation,

DB is the dissolved-oxygen concentration in mg/l in the dark bottle after incubation,

t is the incubation period in hours or days, and 1,000 converts liters to cubic meters.

Gross primary productivity

$$(\text{mg } C/m^3/\text{time}) = \frac{LB - DB}{t} \times \frac{12}{32} \times 1{,}000$$

where *LB*, *DB*, *t*, and 1,000 are as defined above, 12 is the atomic weight of carbon, and 32 is the molecular weight of oxygen.

8.2 Net primary productivity

$$(\text{mg } O_2/m^3/\text{time}) = \frac{LB-IB}{t} \times 1{,}000$$

where

LB is the dissolved-oxygen concentration in mg/l in the light bottle after incubation,

IB is the initial dissolved-oxygen concentration in mg/l in the bottle before incubation (see 6.5 above),

t is the incubation period in hours or days, and 1,000 converts liters to cubic meters.

Net primary productivity (mg C/m^3/time) =

$$= \frac{LB-IB}{t} \times \frac{12}{32} \times 1{,}000$$

where *LB*, *IB*, *t* and 1,000 are as defined above, 12 is the atomic weight of carbon, and 32 is the molecular weight of oxygen.

8.3 Respiration (mg O_2/m^3/time) =

$$= \frac{IB-DB}{t} \times 1{,}000$$

where

IB is the initial dissolved-oxygen concentration in mg/l in the bottle before incubation (see 6.5 above),

DB is the dissolved-oxygen concentration in mg/l in the dark bottle after incubation,

t is the incubation period in hours or days, and 1,000 converts liters to cubic meters.

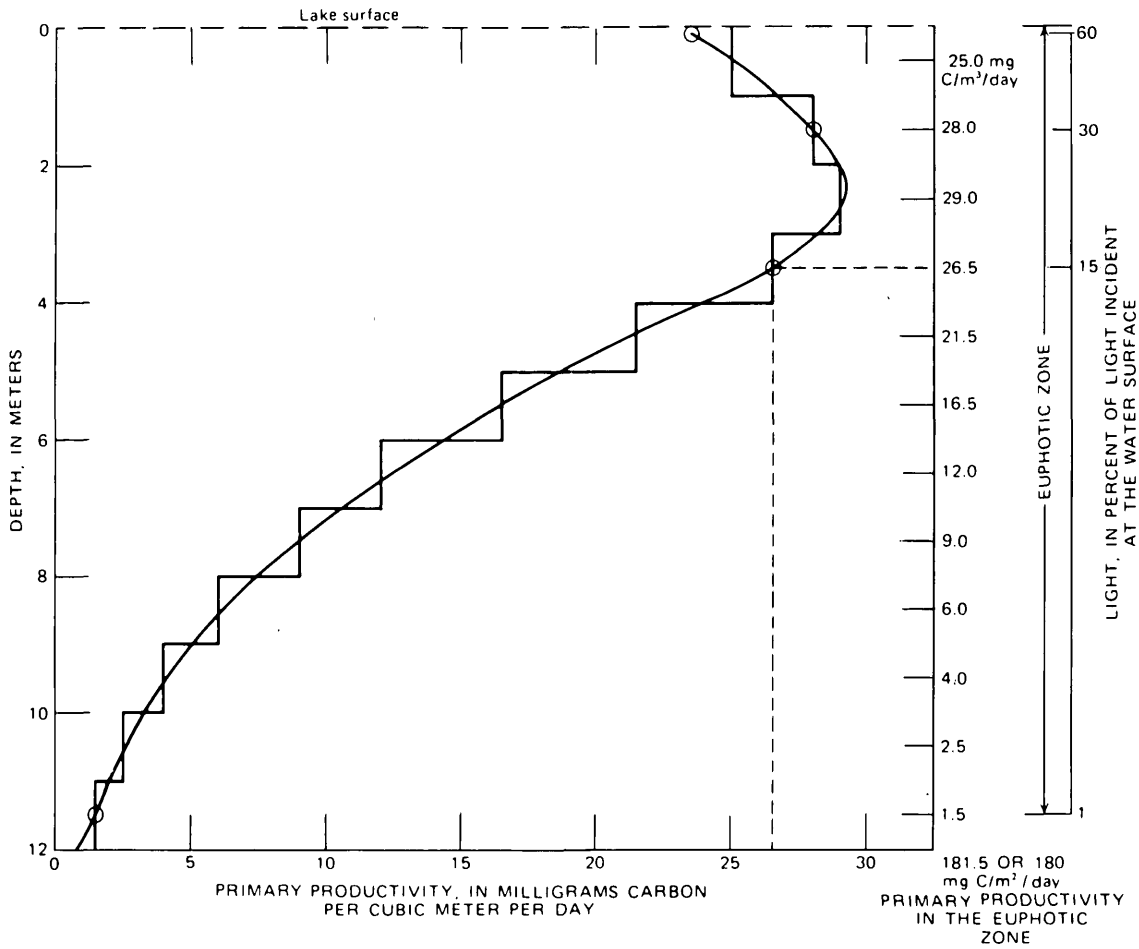


Figure 55.—Example of the vertical distribution of daily primary productivity in Koocanusa Reservoir, Mont. The circled points are values of primary productivity (mg C/m^3/day) calculated from results of light and dark bottles suspended at those depths. The smooth curve was fitted by eye, and the area under the primary productivity-depth curve (mg C/m^2/day) was estimated by summing the values at 1-meter intervals through the euphotic zone (from Janzer and others, 1973).

8.4 The gross or net primary productivity of a vertical column of water 1 m^2 in cross section (mg O_2/m^2/time or mg C/m^2/time) is determined by a summation of the productuvities 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. 55), 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 55.

9. Report

Report primary productivity as follows: Less than 10 mg, one decimal; 10 mg and above, two significant figures.

10. Precision

The following precision estimates were given by Strickland and Parsons (1968, p. 263) for aliquots from a single large sample and do not include variabilities from sampling: Precision at the 100-mg-C/m^3/hr level. The correct value lies in the range: Mean of n determinations $\pm 15/n^{½}$ mg C/m^3/hr (6-hr incubation). Precision at the 10-mg-C/m^3/hr level. The correct value lies in the range: Mean of n determinations $\pm 1.5/n^{½}$ mg C/m^3/hr (6-hr incubation).

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Carbon-14 light- and dark-bottle method for phytoplankton
(B–8020–77)

Parameters and codes:
Productivity, primary, gross (mg C/m³/day) 70961
Productivity, primary, gross (mg C/m²/day) 70962
Productivity, primary, net (mg C/m³/day) 70965
Productivity, primary, net (mg C/m²/day) 70966

1. Application

Radioactivity associated with the phytoplankton filtered from the incubated samples may be determined by any of several techniques including (1) planchet counting using an end-window Geiger tube or thin-window proportional counter, (2) total-combustion and gas-phase counting of the carbon dioxide produced, or (3) liquid-scintillation counting. Liquid-scintillation counting, the technique used in this method, was shown by Wolfe and Schelske (1967) to have numerous advantages over Geiger and thin-window counting techniques. In addition, liquid-scintillation counters are more common and easier to use than gas-phase counting systems. Loss of labeled carbon dioxide from the samples may occur on drying the filters for planchet counting. This loss is eliminated when using liquid-scintillation counting by the addition of 0.10 ml of quaternary ammonium base to each liquid-scintillation vial prior to adding the wet plankton-containing filter.

The method is applicable to standing or to slowly moving oligotrophic waters. The range of the carbon-14 method is from about 0.05 to 100 mg C/m³/hr of photoperiod (Strickland and Parsons, 1968, p. 267).

This method is based on the in situ incubation and radioactivity-counting techniques used by the U.S. Geological Survey in determining primary productivity at Lake Koocanusa near Libby, Mont. (Janzer and others, 1973).

2. Summary of method

A known amount of radioactive carbonate ($^{14}CO_3^{-2}$) is added to water samples in light and dark bottles, and the bottles are suspended at several depths within the photosynthetic zone for a known period of time. The total inorganic carbonate content is determined on an aliquot of each sample. After incubation, the samples are filtered through membrane filters, and the retained plankton cells are washed. Inorganic carbon-14 is removed by treating the filters with dilute hydrochloric acid, and the radioactivity retained by the cells is measured by liquid scintillation. The quantity of carbon fixed in the organic matter of the phytoplankton is a measure of the net primary productivity.

3. Interferences

The method uses isolated samples of water and plankton to indicate the response of the natural system. Consequently, care must be used in sampling, sample handling, and light exposure not to interfere with the life requirements of the organisms. Water-sampling bottles or devices should be constructed of plastic or glass with essential metal parts of stainless steel. Copper, brass, and bronze fittings on sampling bottles or on suspension equipment should not be used. The water-sampling bottles should be opaque to reduce the risk of light injury, and 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 incubation bottles must be prevented.

Vollenweider and Nauwerck (1961) concluded that the incubation period of in situ carbon-14 experiments should not exceed 4–6 hours, to avoid carbon-14 losses due to cell respiration and the leaching and exchange of soluble organic materials through the cell walls.

Loss of labeled carbon dioxide from the filters dur-

ing drying or exposure to air may result in low productivity values being obtained. Wallen and Geen (1968) reported as much as 50 percent loss of activity resulting from the drying of filters, with most of the loss occurring during the first 24 hours. Retention of dissolved compounds by membrane filters may result in errors when relatively small sample volumes (10–25 ml) are filtered for analysis (Nalewajko and Lean, 1972). In the method described here, the total volume of each BOD bottle is filtered for each determination.

4. Apparatus

All materials used must be free of agents which inhibit photosynthesis and respiration.

4.1 *Water-sampling bottle*, Van Dorn-type, opaque acrylic-plastic with stainless-steel metal parts, 4- or 5-liter capacity. A similar sampling bottle constructed of polyvinyl chloride, Wildlife Supply Co. (1140); Scott Instruments, Seattle, Wash.; Kahl Scientific Instrument Corp. (135WA); General Oceanics, Inc. (1010); or equivalent, may be used if the opaque acrylic-plastic bottle is not available.

4.2 *BOD (biochemical oxygen demand) bottles*, numbered, 300-ml, Pyrex or borosilicate glass, with flared necks and pointed ground-glass stoppers. A supply of both light (clear) and dark (blackened) bottles is required. Opaque bottles are available from Kahl Scientific Instrument Corp., or may be prepared by painting the bottles black and covering the paint with overlapping strips of black plastic tape, Scotch (33) or equivalent. 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.

To prepare the BOD bottles, fill with the acid cleaning solution and let stand for several hours. Rinse thoroughly with distilled water. Traces of iodine from the Winkler analysis should be removed by rinsing the bottles and stoppers with 0.01 *N* sodium thiosulfate solution followed by thorough rinsing with distilled water. Do not use phosphorus-based detergents.

4.3 *Suspension system* for holding light and dark bottles in a horizontal position at various depths (fig. 54). The BOD bottle holder shown in figure 54*B* is made from a thick-walled 2-liter Boston round polyethylene bottle. Three "keyholes" are cut into the sides of the bottle, the bottom is cut out, and air vents are cut into the top to prepare a holder similar to that of Schindler and Holmgren (1971). Each stoppered BOD bottle is held in an essentially horizontal position with a heavy rubberband stretched over the neck of the bottle inside the holder, pulled under the bottom edge of the holder, and then over the neck of the BOD bottle outside the holder. Each holder, with BOD bottles attached as described, is then secured to a buoy by a length of nylon cord run through a hole in the cap of the bottle holder.

4.4 *Dark box*, preferably insulated, for storing filled BOD bottles until ready for incubation.

4.5 *Underwater light-measurement equipment*, Kahl Scientific Instrument Corp. (268WA310), Hydro Products (620–S), InterOcean (510), or equivalent. If a submersible photometer is not available, a Secchi disk, Kahl Scientific Instrument Corp. (281WA100), Wildlife Supply Co. (59), or equivalent, may be used as described in 6.1 below.

4.6 *Tray*, Fiberglas, about 50×75 cm by 5 cm deep (20×30×2 in.), Fisher Scientific Co. (15–239–2) or equivalent.

4.7 *Flasks, filter*, polyethylene, 1-liter size; two required.

4.8 *Ring stands and clamps* or *support base* to secure filter flasks.

4.9 *Filter assembly*, Millipore Sterifil, (XX11 047 00) or equivalent, and all-metal *syringe and two-way valve*, Millipore (XX62 000 05) or equivalent. A small *vacuum gage* such as is provided with a hand-held vacuum pump, Nalgene Labware (6130–0010), Kahl Scientific Instrument Corp. (270WA100), or equivalent, may be used for manual control of filtration vacuum (see 6.13 below).

4.10 *Forceps*, stainless-steel, smooth-tip, Millipore (XX62 000 06) or equivalent.

4.11 *Filters*, membrane, Gelman GA–6 47-mm, 0.45 μm, or equivalent.

4.12 *Carboy*, waste, 20-liter (5-gal), polyethylene.

4.13 *Syringe*, 1- and 5-ml, Luer taper, Becton-Dickenson Plastipak disposable syringe or equivalent.

4.14 *Needles*, hypodermic, 2.5- and 7.5- or 10-cm (1- and 3- or 4-in.), Luer taper.

4.15 *Bags*, polyethylene, about 30×60 cm (12×24 in.), for solid radioactive wastes.

4.16 *Tissue*, absorbent.

4.17 *Vials*, liquid-scintillation, 22-ml capacity, with plastic-lined screwcaps, Amersham/Searle or equivalent. Prior to use, 0.10 ml of NCS quaternary ammonium base is added to each vial (see 5.7 below). Note: Place identifying marks on the caps, not on the sides of the vials.

4.18 *Aluminum foil*.

4.19 *Wash bottles*.

4.20 *Pipets, 100-microliter*.

4.21 *Pipet filler*, rubber-bulb-type.

4.22 *Rubber policemen* on a bent, 15-cm (6-in.) heavy glass rod (see 6.13 below).

4.23 *Liquid-scintillation spectrometer.*

4.24 *Apparatus for determining total alkalinity,* (Brown and others, 1970, or American Public Health Association and others, 1976.)

5. Reagents

5.1 *Reagents for determining total alkalinity* (CO_2, HCO_3^{-1} and CO_3^{-2}), following the methods of Brown and others (1970) or American Public Health Association and others (1976).

5.2 *Chrome-sulfuric acid cleaning solution:* Dissolve 63 g sodium (or potassium) dichromate by heating with 35 ml distilled water in a 2-liter beaker. Add 1 liter concentrated H_2SO_4. Caution! Use rubber gloves, safety goggles, and protective clothing in handling this cleaning agent (American Public Health Association and others, 1976).

5.3 *Radioactive carbonate solution,* ($Na_2{}^{14}CO_3$), prepared for phytoplankton studies, approximately 1 μCi/ml (microcurie per milliliter) in sealed 10-ml glass ampoules, specific activity $\geq$ 0.1 μCi/μg, New England Nuclear Corp., or equivalent. Store ampoules in individual polyethylene bags in refrigerator or cooler, but do not freeze. Note: Use of ampoules containing more than the volume of solution required for a single inoculation eliminates the step of rinsing the individual ampoules to obtain the full volume.

5.4 *Ammoniacal barium chloride solution:* Dissolve 50 g of $BaCl_2 \cdot 2H_2O$ in approximately 1 liter of lakewater or tapwater, add 75–100 ml of concentrated NH_4OH, and place in the 20-liter (5-gal) polyethylene waste bottle.

5.5 *Hydrochloric acid, 0.1 N:* Mix 8.3 ml concentrated HCl (sp gr 1.19) with distilled water, and dilute to 1 liter.

5.6 *Liquid-scintillation solution:* Weigh 32.0 g of scintillation-grade 2,5-diphenyl oxazole (PPO) and 3.2 g of 1,4-bis-2-(4-methyl-5-phenyloxazole)-benzene (POPOP) scintillator fluors, and place in a 4-liter argon-flushed volumetric flask. Dissolve in a small amount of the following v/v mixture and then dilute to the 4-liter mark: 10 percent Triton X–100, 14 percent Triton X–114 (Rohm and Haas Co., Philadelphia, Pa.) and 76 percent reagent-grade *p*-xylene. Store under argon in a glass bottle. Amersham/Searle PCS Solubilizer premixed liquid scintillation cocktail also has been found to be satisfactory.

5.7 *NCS quaternary ammonium hydroxide base in toluene.* Available from Amersham/Searle Corp., Arlington Heights, Ill.

5.8 *Formaldehyde solution,* neutral, reagent-quality, 37–40 percent aqueous formaldehyde solution (Formalin), in screwcapped vials.

5.9 *Acetone* for cleaning the exterior of counting vials.

5.10 *Carbon-14 labeled toluene standard:* Weigh 1.0000 g of a radiocarbon toluene compound having a known specific activity, Packard Instrument Co. (6004062) or equivalent. Using spectrograde toluene, dilute to a concentration to obtain a specific activity of 20,000 to 30,000 disintegrations/min/ml. Store in a tightly capped glass container.

6. Collection

6.1 To prepare the BOD bottles, carefully fill with the acid cleaning solution and let stand for several hours. Remove the cleaning solution, and rinse with at least four changes of tapwater and two of distilled water; dry and stopper until needed. Do not use phosphorus-based detergents.

6.2 Determine the depth of the euphotic zone (the region that receives 1 percent or more of the surface light) with an irradiance or illuminance meter. If no other method is available, an estimate of the lower limit of the euphotic zone is obtained by multiplying the Secchi disk depth by 5 (Verduin, 1956). Select sampling intervals equal to one-tenth of the depth of the euphotic zone; fewer depth intervals are permissible in shallow euphotic zones. In Lake Koocanusa, samples from the 60, 30, 15, 3, and 1 percent light penetration depths were sufficient to define the productivity-depth curve.

6.3 Collect a water sample with a nonmetallic sampler from each of the preselected depths. The sample volume should be sufficient to fill three BOD bottles in a set as described in 6.4 and to determine alkalinity. Keep the samples in subdued light at all times during the following procedures to avoid light injury to the organisms.

Note: Vollenweider (1965) noted that

> It has been suggested that day rate estimates from short exposures would be facilitated if the duration of standard exposures, instead of being constant, was chosen proportional to the day length, for example, by dividing the light day (sunrise to sunset) into five equal periods, and exposures were made over the periods II and III . . . Accordingly, the uncertainty introduced in estimating daily productions would not be larger than about ± 10 percent; this is less than any other procedure.

He reported the productivity occurring during periods I through V as 9.95, 30.8, 30.4, 21.85, and 6.95 percent, respectively. During normal field operations, it is usually difficult, if not impossible, to start and stop

incubations at the exact times corresponding to the selected periods, usually, combination of II, III, or IV. Determination of the total daily productivity occurring during the actual incubation time interval selected, however, can be determined readily by the use of a graph as shown in figure 56.

6.4 Fill one dark and two light BOD bottles with water from each level. Place bottles in the Fiberglas tray to confine possible spills and to minimize the potential for radioactive contamination of the working area and supplies.

6.5 Establish the amount of radioactive solution to be added to each BOD bottle. Initial productivity determinations should be made using relatively high levels of carbon-14 spike to insure adequate counting rates if productivity rates are low. A concentration of 3 μCi of carbon-14 in each BOD bottle should be adequate. If laboratory counting rates obtained on the filters are sufficiently high, subsequent samples may be spiked at lower concentrations or incubation periods may be reduced (See also 6.8 below.)

6.6 Remove an ampoule of radioactive solution from the cooler and, leaving it in the plastic bag, carefully snap the ampoule neck. Using a clean dry syringe with a 7.5- or 10-cm (3- or 4-in.) needle, carefully draw slightly in excess of amount of radioac-

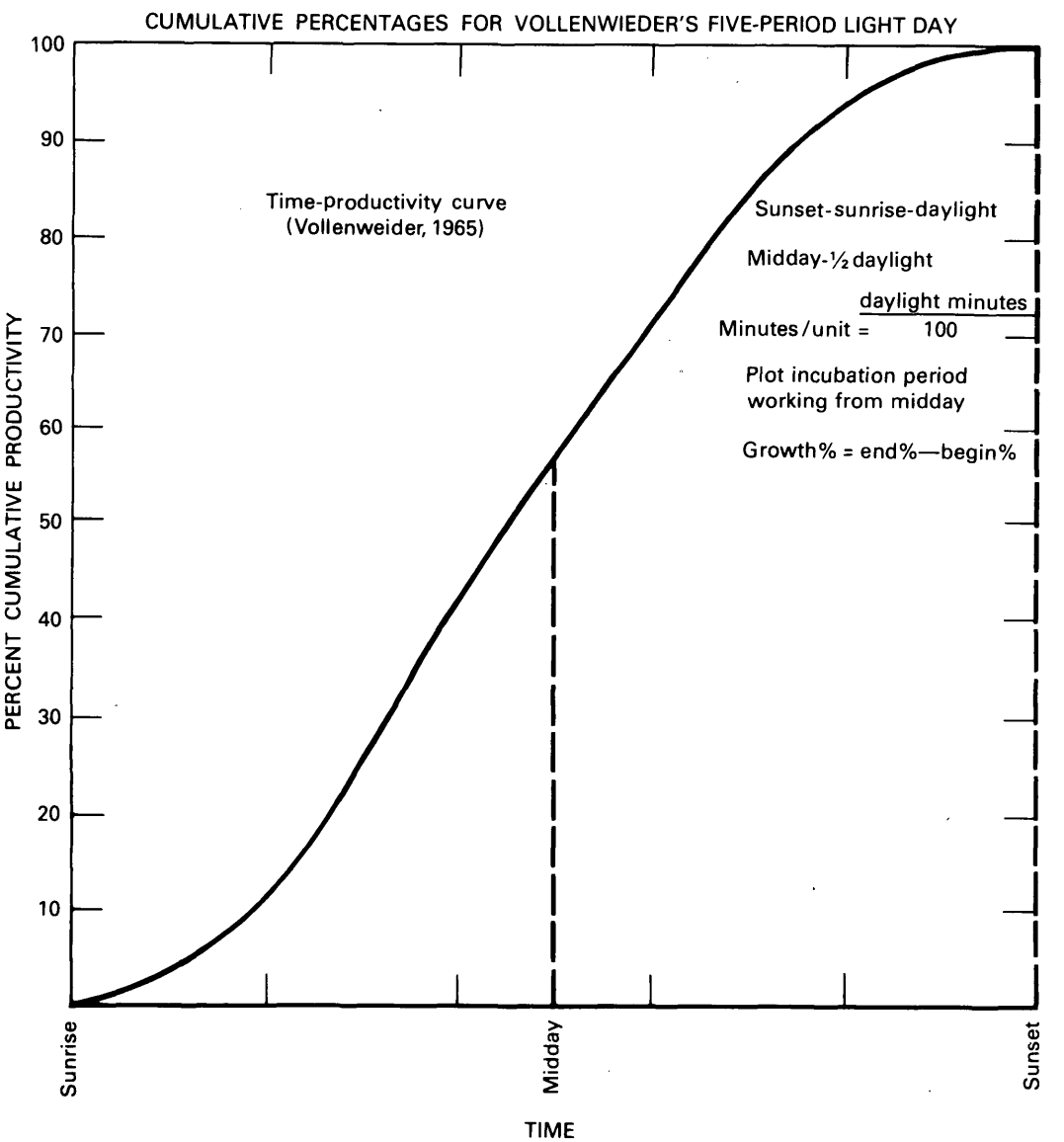

Figure 56.—Cumulative percentages for Vollenweider's five-period light day (see step 6.3).

tive solution to be used into the syringe. Remove the needle from the ampoule, and wrap the needle tip with several thicknesses of absorbent tissue. Invert syringe and expel air and a small amount of radioactive solution into the tissue wad to retain the exact volume desired in the syringe (±0.01 ml). Place the contaminated tissue in the solids-waste bag, and then inject the radioactive solution into the bottom half of the BOD bottle to be incubated. Stopper the BOD bottle securely, and mix thoroughly by repeated inversion. Store in a dark box until all bottles at the site are ready for incubation.

Wipe the needle with tissue, and place the tissue in the waste bag. The syringe and needle are used to inoculate subsequent BOD bottles at the site without further washing or drying. Note: Use the same ampoule to inoculate all three bottles in a set and the standard (described in 6.8 below), and make a record of the ampoule used. Place identifying marks on the caps, not on the sides of the liquid-scintillation vials.

6.7 When all bottles are ready for incubation, place the one dark and two light bottles from each sample into a bottle holder, attach the holder to a buoy, immerse the holder to the original depth for incubation and record the time. Note: The buoys with bottles attached are anchored or secured to a larger buoy to reduce drifting during the incubation period.

6.8 The concentration of the radioactive carbonate solution in each ampoule used in the study should be checked by preparing standards in the field. One vial with scintillation solution already added will be supplied by the counting laboratory for each ampoule. Using the pipet filler, carefully fill a 100-microliter pipet to the mark with radioactive solution. Drain the pipet into a liquid-scintillation vial containing scintillation solution supplied by the counting laboratory, and blow out as much solution as possible. Rinse pipet twice with distilled water adding both washes to the liquid-scintillation vial; use the same technique as for the radioactive solution. Send the vial to the laboratory for counting. When through using the pipet, rinse it several times with distilled water followed by an acetone wash; dry and replace in storage vial. Note: Volume measurements should be as accurate as possible because 1 microliter of solution contains 1 nCi (nanocurie) of carbon-14 yielding approximately 2,000 disintegrations per minute. Consequently, only trace amounts of contamination on the filter membranes completely obscure the low count rates that may be obtained for phytoplankton-fixed carbon-14.

6.9 After all BOD bottles have been inoculated, wash the contaminated syringe and needle several times with distilled water, transferring washes to the waste carboy. Place the glass ampoules and remaining radioactive carbonate solution in the waste carboy. Dry and store syringe and needle. Contaminated tissues on tray should be placed in solids-waste bag for later disposal.

6.10 Determine the alkalinity for each set of incubated samples from the initial water samples collected in 6.3 above (American Public Health Association and others, 1976) ; Brown and others, 1970).

6.11 At the end of the incubation period, record the time, raise the bottles, wipe them dry, and place in the Fiberglas tray.

6.12 Remove the stopper (contaminated) from a BOD bottle, and place it on tissue in the tray. Using a separate syringe from that used for radioactive carbonate solution, add 1 ml of neutral formaldehyde solution (Formalin) to the bottle to kill the organisms and to stop photosynthesis. Do not insert the needle into the radioactive solution in the BOD bottle. Carefully insert the stopper, and pour the liquid displaced by the formaldehyde solution into the waste carboy. Wipe the area around the bottle cap with absorbent tissue, and place the contaminated tissue in the solids-waste bag.

6.13 Filter the entire contents or a measured aliquot from each BOD bottle through a 47-mm, 0.45-μm membrane filter using the plastic-filter apparatus and vacuum-flask 1. If the entire sample is filtered, police the sample-bottle sides with a bent glass rod fitted with a rubber policeman. Rinse the bottle several times with 5- to 10-ml portions of distilled water to transfer all plankton to the filter. With the syringe and two-way valve, or the hand-held vacuum pump, apply sufficient vacuum to initiate and maintain flow through the filter. Note: Apply a vacuum of not more than 150–200 mm (6–8 in.) of mercury to avoid rupture of phytoplankton cells and loss of radioactive material through the filters. Filtered lake water may be substituted for distilled water in this and subsequent collection steps.

6.14 When essentially all the liquid has passed through the filter, wash the filter and funnel three times with 5to 10-ml portions of distilled water from a wash bottle to remove excess radioactive solution adhering to the filter. Vacuum the filter "dry" each time between washings so that no visible liquid remains on the filter surface although the filter itself is wet.

6.15 Remove the intact filter assembly from filter-flask 1 and place it in flask 2. Add the contents of flask 1 to the waste carboy (see 6.18 below). Wash the filter and residue with 5-10 ml of 0.1 *N* hydrochloric acid, retaining the filtrate in flask 2. Note: A second flask (2) is used for the acid wash to prevent volatilization of the

radioactive carbonate in flask 1. The contents of filter-flask 2 are discarded as any other dilute acid waste.

6.16 Allow the acid to remain in contact with the filter for 1 or 2 minutes, vacuum the filter "dry", and wash several times with 5- to 10-ml portions of distilled water. Again vacuum the filter dry, disassemble the filter holder and, without touching the algae-coated surface, roll the filter into a loose cylinder, algae side inward, and place the filter into a liquid-scintillation vial. Cap the vial securely, and ship to the counting laboratory. Note: It is recommended that the filters not be touched by hand (Strickland and Parsons, 1968, p. 272). Plastic gloves can be worn to handle the filters, but with practice the filters can be manipulated with two pairs of smooth-tip forceps. Grasp the edge of a filter membrane with one pair of forceps, roll the edge inward toward the center of the disk, and hold this initial roll with the second pair of forceps. Then, using the two pairs of forceps alternately, complete the rolling operation and insert the filter into the vial. To minimize contamination of the filters with the relatively high levels of carbon-14 activity present in the inoculated bottles, wash hands and forceps frequently. The wastewater from this washing may be discarded.

6.17 Rinse the filter assembly successively in 0.1 *N* hydrochloric acid and then with distilled water prior to filtering the next sample. Discard these rinse liquids.

6.18 Pour the filtrate collected in flask 1 into the 20-liter polyethylene waste carboy to react with the ammoniacal barium chloride solution. Carbon-14 in solution as HCO_3^{-1} or CO_3^{-2} will be precipitated as barium carbonate, which is allowed to settle. (See "Appendix" following the description of this method.)

7. Analysis

7.1 Prepare a laboratory blank by filtering 15 ml of lake water, and place the moist filter membrane in the counting vial; pipet 0.10 ml of NCS base reagent into the vial; add 18.0 ml of scintillator solution, cap securely, and mix. Prepare in triplicate. Using a fresh ampoule of carbon-14 carbonate, prepare triplicate laboratory standards in the same manner as described in section 6.8.

7.2 When the vials containing the sample filters and the field standards are received in the counting laboratory, add 18.0 ml of the scintillation solution to each vial, and cap securely.

7.3 Clean the outside of each vial which is to be counted by liquid scintillation. Hold each vial by the cap, and wipe the outside walls with an acetone-impregnated tissue to remove dust and finger marks.

7.4 Dark-adapt all vials from 7.1 and 7.2 above by storing them in the dark for 24 hours before counting. This procedure will reduce or eliminate spurious counts from chemiluminescence or fluorescence.

7.5 With a liquid-scintillation spectrometer, count each vial in series for 20 minutes, and repeat the counting procedure five times so that each vial is counted for a total of 100 minutes. Do not count the vials for 100 consecutive minutes.

7.6 Average the counting rates for each sample and field standard. Average the counting rates for all three laboratory standards and for all three laboratory blanks. Subtract the average laboratory blank value from each of the other average count values to obtain the average net counting rate.

7.7 After counting, add 100 microliters of carbon-14 labeled toluene standard (reagent 5.10) to 20 percent of the vials containing sample filters, and repeat counting as described in section 7.4 and 7.5. Errors in determining liquid scintillation counting efficiencies for two-phase systems with intact algae on membrane filters were reviewed by Stephens (1976), who described a procedure for dissolving algae retained on alkali resistant membrane filters.

7.8 Determine the counting efficiency for these spiked samples using the equation

$$E = \frac{(\bar{R}_s' - \bar{R}_s)}{S} \times 100$$

where

E = the counting efficiency in percent,

$\bar{R}_s'$ = the average net counting rate of the sample in counts per minute after the addition of the labeled toluene standard,

$\bar{R}_s$ = the average net counting rate of the sample in counts per minute, and

S = the total activity of the labeled toluene standard added in disintegrations per minute.

Note: This efficiency measurement is a check for possible self-absorption in the samples. Experience indicates that a range of 2 percent or less in the counting efficiency is acceptable. If the range is greater than 2 percent, the counting efficiency for all samples in both light and dark BOD bottles from the location(s) in question should be checked and count-rate corrections made, if necessary.

8. Calculations

The radiocarbon-measured primary productivity is

expressed as the quantity of carbon assimilated per unit time. Adjust the calculated values at each incubation level for the appropriate incubation period. That is, double the values obtained for half-photoperiod exposures, and express as primary productivity per day. However, because the rate of photosynthesis varies during the day, short-period incubation results should be reported as primary productivity per hour. In either situation, the incubation time should be presented with the results of the calculated productivity.

8.1 Radiocarbon-measured primary productivity (mg C/m^3/day)

$$= \frac{(\bar{R}_l - \bar{R}_d) \times W \times 1.064}{\bar{R} \times D}$$

where

$\bar{R}_l$ = average net light-bottle counting rate in counts/min,

$\bar{R}_d$ = average net dark-bottle counting rate in counts/min,

$\bar{R}$ = calculated average counting rate in counts/min for the total quantity of the radioactive carbonate added to the BOD bottle; determine from the net counting rate of the field standard and the total volume of radioactive solution added to the BOD bottle. (See example below.)

W = alkalinity in mg/l as $CaCO_3$ ×120 to convert to mg C/m^3/mg $CaCO_3$ and is the amount of carbon present in the water, including dissolved CO_2, HCO_3^{-1} and CO_3^{-2} species, capable of being assimilated by the phytoplankton,

D = decimal equivalent of the total daily productivity which occurred during the incubation period, according to Vollenweider's calculation model (1965),

V_i = volume of the sample which was spiked and incubated,

V_f = volume of the sample which was filtered, and

1.064 = correction factor for isotope effect (American Public Health Association and others, 1976).

Example—One dark and two light BOD bottles each were inoculated with 3 ml of radioactive carbonate solution having about 1 μCi/ml, and a field standard was prepared from 100 microliters of the same solution. The bottles were incubated from 1010 to 1430 hours in a reservoir having an alkalinity of 84 mg/l as $CaCO_3$. After filtration of the samples, the following count data were obtained in the laboratory by liquid scintillation:

		Average net counts/min
Light bottle	2,088	2,332
Light bottle	2,577	
Dark bottle		146
Field standard		69.7×10^3

Multiply the field standard value in counts/min by 30 to obtain the amount of radioactivity added to each BOD bottle in 3 ml of the radioactive carbonate solution. Then, radiocarbon-measured primary productivity

$$= \frac{(\bar{R}_l - \bar{R}_d) \times W \times 1.064}{\bar{R} \times t}$$

$$= \frac{(2{,}332 - 146) \times 84 \times 120 \times 1.064}{69.7 \times 10^3 \times 30 \times 4.3}$$

$$= \frac{2.186 \times 10.080 \times 10^6 \times 1.064}{8.987 \times 10^6}$$

$$= \frac{23.445}{8.987} = 2.6 \text{ mg C/m}^3\text{/hr}$$

8.2 The primary productivity of a vertical column of water, 1 m^2 in cross section (mg C/m^2/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. 55), 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 Koocanusa Reservoir is shown in figure 55.

9. Report

Report radiocarbon-measured primary productivity as follows: less than 10 mg, one decimal; 10 mg and above, two significant figures.

10. Precision

Strickland and Parsons (1968, p. 267) gave the following precision values for a generally similar method: At the 25-mg-C/m^3/hr level, ±3 mg (5-hr incubation, 1 μCi added); at the 1.5-mg-C/m^3/hr level, ±0.15 mg (7-hr incubation, 5 μCi added).

Appendix
Handling and disposal of radioactive waste

Radioactive carbon-14 (half-life 5,730 yr) may be used in quantities up to a maximum of 100 μCi (1×10^{-6} Ci) under 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 taken to minimize the release of radioactive carbon to the environment; also, avoid contamination of field and laboratory equipment.

Radioactive carbonate and dissolved carbonate species remaining in solution after the phytoplankton have been removed by filtration are precipitated from the water as barium carbonate by mixing the filtrate with a solution of ammoniacal-barium chloride solution in a 20-liter (5-gal) waste carboy. When all the waste solution has been added to the carboy, add 1.0 *N* sodium carbonate solution to the waste to further scavenge radioactive carbonate from the solution. Calculate the maximum volume of 1.0 *N* sodium carbonate needed using the following equation:

$$\text{Volume of } 1.0N\ Na_2CO_3 = 10.1\ [40.4 - (As \times Vw \times 0.00197)]$$

where

10.1 ml of 1.0 *N* Na_2CO_3 is equivalent to 1 g of $BaCO_3$,

40.4 g $BaCO_3$ is equivalent to 50 g $BaCl_2$ in waste carboy,

As is the sample alkalinity as $CaCO_3$ in milligrams per liter,

Vw is the volume of waste in the carboy, and

0.00197 is a factor to convert weight of $CaCO_3$ in milligrams to grams of $BaCO_3$.

Example:—If a carboy contained 10 liters of liquid waste with an alkalinity of 85 mg/l, the volume in milliliters of 1.0 *N* Na_2CO_3 required to completely react with the 50 g of $BaCl_2 \cdot 2H_2O$ added to the carboy would be

$$\text{Vol} = 10.1\ [40.4 - (85\times10\times0.00197)]$$
$$= 391 \text{ ml required for total precipitation.}$$

Scavenging of the carbon-14 from solution has been found to be more complete if the sodium carbonate solution is added in four or five portions. The resulting barium concarbonate precipitate is allowed to settle before making the next addition of sodium carbonate.

After settling, the barium carbonate is separated by decantation of the supernate. Add plaster of paris to the barium carbonate slurry to form a solid block that is sent to the counting laboratory for disposal as radioactive waste. Retain the supernate until a laboratory check of an aliquot by liquid-scintillation counting has shown that the carbon-14 scavenge was essentially complete. The supernate may then be discarded.

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Oxygen light- and dark-enclosure method for periphyton
(B–8040–77)

Parameters and codes:
Productivity, primary, gross (mg O_2/m^2/day) 70960
Productivity, primary, gross (mg C/m^2/day) 70962
Productivity, primary, net (mg O_2/m^2/day) 70964
Productivity, primary, net (mg C/m^2/day) 70966
Respiration (mg O_2/m^2/day) 70968

1. Application

The method is most suitable for shallow streams and for the littoral zones of lakes where light penetration is sufficient for photosynthesis. Best results will be in eutrophic waters in which the production rate is equivalent to about 3–200 mg C/m^3/hr of photoperiod (Strickland and Parsons, 1968, p. 263). Measurements of productivity of stream periphyton in static cultures may provide useful comparative values, but are undoubtedly too low in absolute terms because of suppression of photosynthesis in the absence of current (Wetzel, 1964).

Methods have been described for measuring primary productivity in plastic chambers in which water is circulated with a pump (Thomas and O'Connell, 1966; McIntire and others, 1964; Hansmann and others, 1971; Pfeifer and McDiffett, 1975). However, the equipment is not generally available, and the reader is referred to the original papers for details of construction and use.

2. Summary of method

Known areas of substrates containing living periphyton are isolated in sealed light and dark containers filled with filtered lake or stream water of known oxygen content. The samples are exposed in the euphotic zone, usually at the original depth, for a known period of time. Changes in the oxygen concentration of the enclosed water samples are interpreted in terms of photosynthesis and respiration per unit area of periphyton.

3. Interferences

The method uses isolated samples of periphyton to indicate the response of the natural system. Care must be used in collection, sample handling, and light exposure not to interfere with the life requirements of the organisms. Sampling equipment should be constructed of plastic or glass with essential metal parts of stainless steel. Copper, brass, and bronze fittings should be avoided. Samples of periphyton should be kept in the shade or in an enclosure before incubation to avoid exposure of unadapted algae to full sunlight. Light leaks into the dark incubation enclosures must be prevented.

The formation of bubbles in the experimental containers results in errors in the determination of dissolved oxygen changes. Bubbles result from entrapped air or incomplete filling of the enclosures, or from dissolved-oxygen supersaturation, especially in samples that warm several degrees between collection and filling. Photosynthesis and respiration of plankton in the water used to fill the chambers may affect the results under some conditions. This may be avoided by filtering the water through a glass fiber or membrane filter. Microbial activity and chemical oxygen demand cause losses of oxygen when incubation times exceed a few hours.

Interferences with the chemical determination of dissolved oxygen were described by Brown and others (1970) and by the American Public Health Association and others (1976).

4. Apparatus

All materials used must be free of agents which inhibit photosynthesis and respiration.

4.1 *Water-sampling bottle,* Van Dorn-type, opaque acrylic-plastic with stainless-steel metal parts, 4- or 6-liter capacity. A similar sample bottle constructed of polyvinyl chloride, Wildlife Supply Co. (1140, 1160); Scott Instruments, Seattle, Wash.; Kahl Scientific Instrument Corp. (135WA); General Oceanics, Inc. (1010); or equivalent, may be used if the opaque acrylic-plastic bottle is not available.

4.2 *Light and dark enclosures* of suitable size and shape, constructed of glass or plastic (Wetzel, 1964, 1965; McIntire and others, 1964; Thomas and O'Connell, 1966; Hansmann and others, 1971; Pfeifer and McDiffett, 1975). Transparent containers can be made opaque by painting them black and covering the paint with overlapping strips of black plastic tape, Scotch (33) or equivalent. The exposed parts of stoppers, if present, should be similarly blackened, and covered with a hood of several layers of aluminum foil during use.

4.3 *Equipment for determination of dissolved oxygen* by the azide modification of the Winkler method (Brown and others, 1970; American Public Health Association and others, 1976).

4.4 *Polyethylene bottles,* 8-liter capacity with cap and bottom tubulation, Bel-Art Products (F–1184 or H–11872), Nalgene Labware (2318), or equivalent.

4.5 *Dark box,* preferably insulated for storing filled containers until ready for incubation.

4.6 *Filter funnel,* vacuum, 1,200-ml capacity, stainless-steel, Gelman Instrument Co. (Parabella) or equivalent.

4.7 *Filter flask,* 1,000- or 2,000-ml. For field use, a polypropylene flask, Bel-Art Products (H–38941), Nalgene Labware (4101), or equivalent.

4.8 *Vacuum pump,* water-aspirator pump or an electric vacuum pump for laboratory use; a hand operated vacuum pump, Nalgene Labware (6130–0010), Kahl Scientific Instrument Corp. (270WA100), or equivalent, for field use.

4.9 *Glass filter,* Whatman, GF/C grade, or equivalent, or *membrane filters,* white, plain, 0.45-μm mean pore size, 47-mm diameter, Millipore (HAWP 047 00) or equivalent.

4.10 *Periphyton-collecting equipment* appropriate to the study objectives. The periphyton may be attached either to natural or to artificial substrates. A known area of substrate with the intact periphyton growth is required for each enclosure.

4.11 *BOD bottles,* numbered, 300-ml, Pyrex or borosilicon glass, with flared necks and pointed ground-glass stoppers. Before use, fill with acid cleaning solution, and let stand for several hours. Rinse thoroughly with distilled water. Traces of iodine from the Winkler analysis should be removed by rinsing the bottles and stoppers with 0.01 *N* sodium thiosulfate solution followed by thorough rinsing with distilled water. Do not use phosphorus-based detergents.

5. Reagents

5.1 *Reagents for the azide modification of the Winkler method* for dissolved oxygen (Brown and others, 1970; American Public Health Association and others, 1976).

5.2 *Acid cleaning solution,* 20 percent: Mix 20 ml of concentrated HCl with distilled water, and dilute to 100 ml.

5.3 *Sodium thiosulfate solution,* 0.01 *N:* Dissolve 2.5 g sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in distilled water and dilute to 1 liter.

5.4 *Filling water* for the experimental enclosures. Prepare by filtering water from the study source through a glass-fiber filter 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 enclosures and to determine the initial concentration of dissolved oxygen. The water should be slightly under saturation with dissolved oxygen. Dissolved oxygen may be lowered to 5 or 6 mg/l by passing the water through a spraying column (Hansmann and others, 1971) or by adding sodium sulfite with cobaltous chloride serving as a catalyst for the sulfite oxidation reaction (Pfiefer and McDiffett, 1975).

6. Collection

Samples for primary productivity determination 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 productivity changes with time for a selected reach of stream, each measurement must represent the same microhabitat. Similarly, if measurements are needed to compare periphyton productivity among different reaches or different streams, the measurements must represent comparable microhabitats. 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 microhabitat applies to lake environments for which depth, sediment type, fetch, 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 always be considered.

6.1 *Artificial substrates.* Place a suitable artificial substrate in the stream or lake, and attach it to a supporting object. Figure 17*A–D* illustrates several types of artificial substrates. The substrate must be submerged, but may be near the surface of the water or at any other appropriate depth. In lakes, the substrates are usually suspended at several depths (fig. 17*A, B,* and *C*). In lakes and streams, the substrates may be attached to natural items such as submerged trees, stumps (fig. 17*D*), logs, or boulders, or they may be attached to stakes driven into the bottom (fig. 18*A*). Floating samplers also may be used (fig. 18*B*). The artificial substrate must be exposed to the light so that photosynthesis can take place, and it should be located so that damage to the apparatus by floating debris is minimized. Vandalism is a common problem, and placing the substrate away from frequently visited areas is advisable. The length of time required for colonization of the substrates by periphyton will depend upon the season, water temperature, light and nutrient availability, and other factors. Neal, Patten, and DePoe (1967) found that the maximum accumulation of periphyton biomass on polyethylene strips occurred in about 2 weeks. Nielson (1953) exposed slides for 20–30 days. Exposure probably should be at least 14 days, but the time will vary and must be determined for each season and water type. After sufficient colonization of periphyton, indicated by visible green or brown growth, transfer the artificial substrates to the incubation chambers, preferably without removal from water to avoid entrapment of air bubbles. Keep the periphyton samples in subdued light to avoid light injury.

6.2 *Natural substrates.* Rocks or other substrate material of suitable size may be placed into incubation 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.

6.3 With 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 incubation chambers and to determine the initial dissolved-oxygen concentration.

Note: Samples preferably should be taken after 10 a.m. and as shortly before noon (local standard time) as practical. This procedure allows for an incubation period from noon to sunset (Committee on Oceanography, Biological Methods Panel, 1969).

6.4 Filter the required volume of water, and allow the filtrate to stand for 15–30 minutes at a temperature slightly higher than the in situ water temperature. Shake the container occasionally to eliminate oxygen supersaturation, or use one of the methods listed in step 5.4.

6.5 Enclose a known area of substrate containing living periphyton in each of a light and a dark incubation chamber containing a known volume of freshly filtered water. Fill the incubation chambers and at least one BOD bottle so that the chambers and the bottle(s) all have the same initial dissolved-oxygen content. 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. Avoid entrapment of bubbles.

6.6 Place the chambers at the original depth from which the periphyton was collected, and incubate the samples for 1–24 hours. The incubation period is usually one-half the photoperiod, preferably from noon until dusk. In highly productive waters where oxygen supersaturation is likely, an incubation period of 1–3 hours during midday may be sufficient.

6.7 Fix the BOD bottle sample(s) for determination of the initial dissolved-oxygen concentration following the methods of Brown, Skougstad, and Fishman (1970) or the American Public Health Association and others (1976). Titration may be delayed for several hours, if necessary, for samples kept cool and dark.

7. Analysis

7.1 After a suitable incubation period, remove a sample of water from each incubation chamber and determine the dissolved-oxygen concentration as in 6.7. Average the results from duplicate samples.

8. Calculations

Primary productivity is expressed as the quantity of oxygen released or of carbon assimilated per unit time. Respiration is expressed as the quantity of oxygen assimilated per unit time. Adjust the following calculated values for the appropriate incubation period. That is, double the values obtained for half-photoperiod

exposures, and express as primary productivity per day. However, because the rate of photosynthesis varies during the day, short-period incubation results should be reported as primary productivity per hour. Net or gross primary productivity is calculated on the assumption that one atom of carbon is assimilated for each molecule (2 atoms) of oxygen released. Average results from duplicate measurements.

8.1 Gross primary productivity

$$(\text{mg } O_2/m^2/\text{time}) = \frac{(LC-DC)V}{tA}$$

where

LC = dissolved-oxygen concentration in mg/l in the light chamber after incubation,

DC = dissolved-oxygen concentration in mg/l in the dark chamber after incubation,

V = volume of water in the chamber in liters,

t = incubation period in hours or days, and

A = area of the periphyton-covered substrate in square meters.

Gross primary productivity

$$(\text{mg } C/m^2/\text{time}) = \frac{(LC-DC)V}{tA} \times \frac{12}{32}$$

where LC, DC, V, t, and A are as defined above, 12 is the atomic weight of carbon, and 32 is the molecular weight of oxygen.

8.2 Net primary productivity

$$(\text{mg } O_2/m^2/\text{time}) = \frac{(LC-IC)V}{tA}$$

where

LC = dissolved-oxygen concentration in mg/l in the light chamber after incubation,

IC = initial dissolved-oxygen concentration in mg/l in the chamber before incubation,

V = volume of water in the chamber in liters,

t = incubation period in hours or days, and

A = area of the periphyton-covered substrate in square meters.

Net primary productivity (mg C/m^2/time) =

$$= \frac{(LC-IC)V}{tA} \times \frac{12}{32}$$

where LC, IC, V, t, and A are as defined above, 12 is the atomic weight of carbon, and 32 is the molecular weight of oxygen.

8.3 Respiration (mg O_2/m^2/time)

$$= \frac{(IC-DC)V}{tA}$$

where

IC = initial dissolved-oxygen concentration in mg/l in the chamber before incubation,

DC = dissolved-oxygen concentration in mg/l in the dark chamber after incubation,

V = volume of water in the chamber in liters.

t = incubation period in hours and days, and

A = area of the periphyton-covered substrate in square meters.

9. Report

Report primary productivity rate as follows: less than 10 mg, one decimal; 10 mg and above, two significant figures.

10. Precision

No numerical precision values are available.

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Diel oxygen curve method for estimating primary productivity and community metabolism

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 of carbon dioxide has been used to determine the productivity of biological communities in streams (Odum, 1956, 1957; Hoskin, 1959; Edwards, 1965; Edwards and Owens, 1962; Gunnerson and Bailey, 1963; O'Connell and Thomas, 1965; Wright and Mills, 1967; Hornberger and Kelly, 1972, 1974) and in standing waters (Talling, 1957; Odum, 1959; Odum and Hoskin, 1958; Park and others, 1958; Verduin, 1960; Odum and Wilson, 1962; Lyford and Phinney, 1968; Welch, 1968; Eley, 1970; Hornberger and Kelly, 1974; Cory, 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, 1969, p. 98).

In this approach, diel changes in the in situ concentration of dissolved oxygen due mainly to photosynthesis and respiration are used to estimate the primary productivity of the entire aquatic plant community. The advantages of this method are that unnatural effects of enclosures are eliminated, both phytoplankton and attached plants are included, and observations can be of long duration or can be adapted for continuous monitoring. The disadvantages of the method are limited sensitivity, the unknown effects of transient conditions between sampling intervals, the exchange of oxygen between the air and the water requiring calculation of measurement, and, in the graphical analysis, the necessity of assuming that the respiration rate is the same at night as during the day. In standing waters, 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 a result of photosynthesis, respiration, diffusion, and inflowing surface and ground water. If it is known how these factors affect the oxygen concentration in the study area, a dissolved-oxygen curve can be established, and the primary productivity can be determined. From Odum (1956) and Owens (1965) the formula for the oxygen curve may be given as:

$$Q = P - R \pm D + A, \qquad (1)$$

where

Q = rate of change (gain or loss) of dissolved oxygen per unit area,

P = rate of gross primary productivity per unit area,

R = rate of oxygen utilization (respiration) per unit area,

D = rate of oxygen uptake or loss by diffusion per unit area, depending on whether the water is undersaturated or oversaturated with oxygen with respect to the air, and

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 oxygen concentration as compared with the other components.

The rate per unit area of the diffusion of oxygen into or out of the water, D, is the product of the gas transfer coefficient, K, and the percentage-saturation deficit of oxygen between the water and air, S, or

$$D = K\frac{S}{100} \qquad (2)$$

where D and K are in g/m^2/hr. If equations 1 and 2 are divided by the depth, z, in meters, then the terms are expressed on a volume basis, or g/m^3/hr. Conventionally, capital letters are used for quantities defined on a volume basis (Odum, 1956). Thus k is the gas transfer coefficient in g/m^3/hr.

Various formulas for obtaining K and D, as well as example values, are given in Odum (1956), Odum and

Hoskin (1958), Odum and Wilson (1962), Churchill, Elmore and Buckingham (1962), and Owens, Edwards, and Gibbs (1964). Procedures for measuring and predicting the reaeration coefficient of open-channel flows are evaluated by Bennett and Rathbun (1972).

In the methods given here, 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). It is always preferable to determine K and D during the study period by one of these methods, 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, ($g/m^2/hr$ at 0 percent saturation)*
1. Quiet water less than ½ m deep or shallowly stratified	0.1–1
2. Bay and lakes with gentle circulation and small wave action	1–3
3. Rivers, streams, and open tidal waters with strong circulation and large waves	≧3

The presence of sewage and surfactants in the water tends to reduce the gas transfer coefficient compared with the pure-water value, whereas winds tend to increase K with respect to the value existing for quiescent air conditions (see Bennett and Rathbun, 1972, p. 56–58).

A possible source of error in the estimation of gross primary productivity from changes in dissolved-oxygen concentration is the loss of oxygen to the atmosphere in the form of bubbles. Losses of 1–6.5 percent of the total oxygen production have been reported (Odum, 1957; Edwards and Owens, 1962). Although the rate of gas loss may be small for many environments, estimates can be made of the amount of oxygen produced during photosynthesis which is lost in this way (Owens, 1965).

The procedures for graphical analysis of the diel oxygen curve are given for streams (single-station and upstream-downstream methods) and for stratified waters.

Diel oxygen curve method for estimating primary productivity and community metabolism in streams

(B–8120–77)

Parameters and codes:

Productivity, primary, gross (mg O_2/m^3/day) 70959
Productivity, primary, gross (mg O_2/m^2/day) 70960
Productivity, primary, net (mg O_2/m^3/day) 70963
Productivity, primary, net (mg O_2/m^2/day) 70964
Respiration (mg O_2/m^3/day) 70967
Respiration (mg O_2/m^2/day) 70968

1. Application

Two analytical approaches are presented for evaluating oxygen metabolism in streams. The graphical method, illustrated for a hypothetical stream, provides an estimate of gross production, or the total amount of oxygen produced over a diel period and total community respiration, or the total amount of oxygen consumed over a diel period. Diel net productivity, or the excess of oxygen produced over that consumed, is calculated as the difference between gross production and total respiration. The graphical method assumes that daytime respiration is constant or that it varies only linearly with time. This is the major limitation to the graphical method.

The alternative analytical approach consists of data processing using a FORTRAN computer program (Program designation: Primary production, J330). A complete reference to the program is found 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 case. The arithmetic difference between daytime net production and night respiration is given as 24-hour community metabolism which is equivalent to diel net production. Gross production is not calculated. Program J330 operates by assuming production may occur only during daylight hours, and any change in dissolved oxygen, after correcting for diffusion, during this period is due to production. Any change in dissolved oxygen during hours of darkness, after correcting for diffusion, is attributed to respiration.

The method is applicable to streams in which the biological productivity is relatively high. 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

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 the method precludes its use in unproductive waters. The method should be used in waters of comparative homogeneity.

In shallow, turbulent streams the rate of equilibrium of oxygen between water and the atmosphere is too rapid for the method to be used. In these cases, a method based on the carbon dioxide-bicarbonate-pH equilibrium has been proposed to measure photosynthesis and respiration (Wright and Mills, 1967).

4. Apparatus

All materials used must be free of agents which inhibit photosynthesis and respiration.

4.1 *Equipment for determination of dissolved oxygen* by the azide modification of the Winkler method (Brown and others, 1970; or American Public Health Association and others, 1976). A properly calibrated *oxygen meter* with electrode may be used in place of the Winkler method for dissolved-oxygen measurements. Examples of suitable battery-operated, membrane-electrode instruments are Beckman Instruments, Inc. (100801), Martek Instruments, Inc. (DOA), Weston and Stack, Inc. (300), Yellow Springs Instrument Co. (54 and 51A), or equivalent.

4.2 *Portable dissolved-oxygen recording system,* Delta Scientific Corp. (3310–01 or 3410–01) or equivalent, for continuous measurements. Satisfactory recording systems may be assembled from any of the membrane-electrode instruments listed in section 4.1 which have a recorder output. A suitable *portable recorder* for use with the oxygen meters is Cole-Parmer Instrument Co. Mark VII portable potentiometer recorder (8341–3) or equivalent.

4.3 *Battery-operated submersible stirrer* for use with membrane-electrode oxygen instruments. Suitable examples are Delta Scientific Corp. (1010–10), Yellow Springs Instrument Co. (5517–1), Weston and Stack (A–15), Martek Instruments, Inc. (120–30), or equivalent.

4.4 *Water-sampling bottle,* Wildlife Supply Co. (1120 or 1200) Scott Instruments, Seattle, Wash., or Foerst Mechanical Specialties Co. (improved water sampler, Kemmerer-type), or equivalent, or a threefold displacement sampler, Precision Scientific Co. (69770) or equivalent. Depth-integrated samplers are discussed in Guy and Norman (1970).

4.5 *Thermistor* or *thermometer* for determining water temperature and gas temperature in the diffusion dome. Most oxygen meters include thermistors suitable for making these measurements.

4.6 *Diffusion dome* made of clear Plexiglas approximately 22 cm in diameter, or larger. Suitable domes are obtainable 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 (3/8 in.) marine plywood (fig. 57). The oxygen and temperature sensors are inserted either through holes in the dome or from below into a support inside the dome. The dome is painted silver to reduce the greenhouse effect on the inside temperature.

4.7 *Barometer* for measuring local barometric pressure.

4.8 *Graph paper,* 10×10 squares to the centimeter, Keuffel & Esser (46 1510) or equivalent.

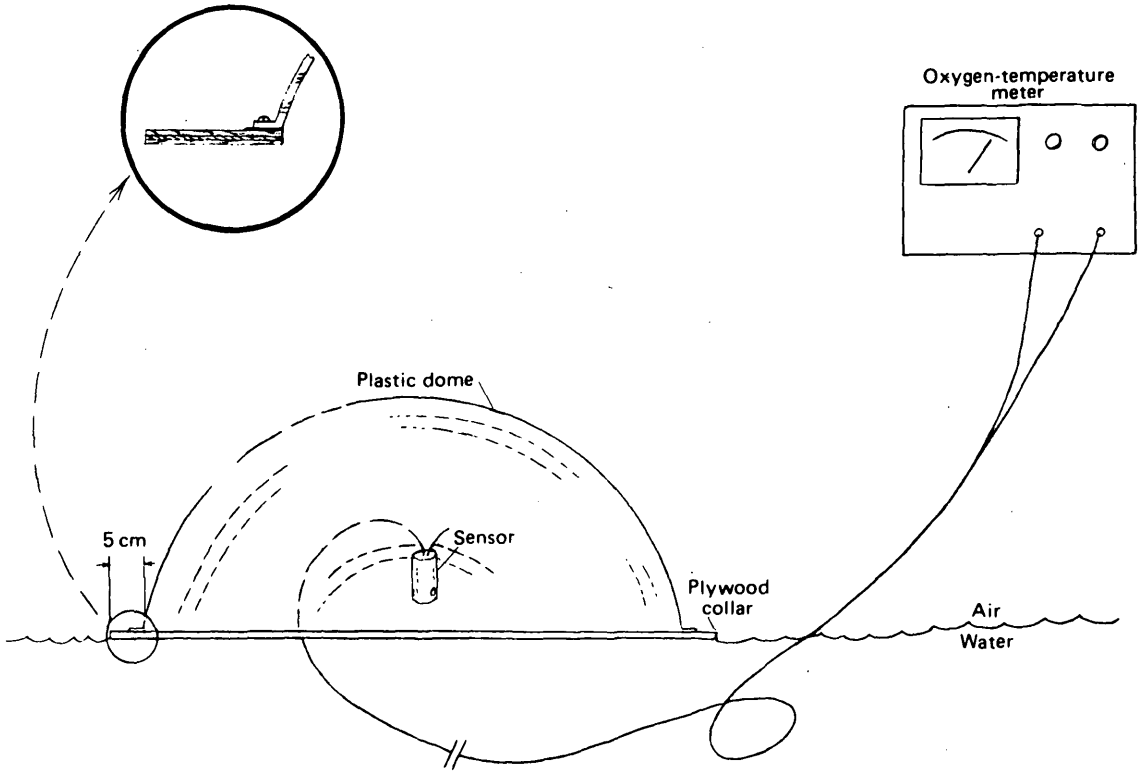

Figure 57.—Floating-diffusion-dome apparatus (modified from Hall, 1971).

5. Reagents

5.1 *Reagents for the azide modification of the Winkler method* for dissolved oxygen (Brown and others, 1970; American Public Health Association and others, 1976).

6. Collection

The sample-collection method will be determined primarily by the type of environment under study. In general, the objective is to determine the concentration of oxygen that is representative of the study area for each sampling interval. In well-mixed waters, one or two determinations for each sampling period may be representative of the entire water mass. Even in supposedly well-mixed streams, the investigator must watch for spatial changes in oxygen concentration. A consistent increase in dissolved oxygen toward the banks relative 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 exhibit uneven distribution, which results in nonuniformity of water chemistry.

Sampling procedures are described for two types of stream conditions and three methods for determining the diffusion rate, D. If the incoming water has a metabolic history similar to the outflowing water, follow the procedure for the single-station analysis, steps 6.1 and 6.2. 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, steps 6.3 and 6.4.

6.1 Single-station analysis. Select a representative reach of stream in which surface- and ground-water accrual are negligible and in which similar conditions exist upstream. In such a stream a second station would reveal 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 discharge for the 24-hour observation period.

6.2 Determine the dissolved-oxygen concentration (mg/l) and the temperature of the streamflow continuously, or at 1-, 2-, or 3-hour intervals for at least 24 hours. Include samples for times at or near sunrise and sunset. Tabulate barometric pressure.

If the Winkler method is used for oxygen determination, collect duplicate or triplicate samples at each sampling time, and average the results from replicate samples. Collect the samples with a threefold displacement sampler or with 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 to 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 dark. Measure water temperature to ±0.5°C at each sample time and location.

For small streams, a single sample at the position of 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 above. When using a portable recording system, mount the temperature sensor and electrode at the centroid of flow, and insure that sufficient water current is maintained past the membrane of the oxygen electrode. For stream velocities less than about 0.6 meters per second (2 ft/s) at the electrode, increase flow to the membrane surface with a submersible stirrer. Note: The Beckman (100801) dissolved-oxygen system reportedly requires minimal stirring for in situ measurements. Many oxygen electrodes are photosensitive; protect the membrane-covered surface from bright light during calibration and use.

Determine the diffusion rate, D, by one of the methods described in 6.5 below.

6.3 Two-station analysis. Select an upstream and a downstream station on a representative reach of streams 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 (m^3/hr) (Buchanan and Somers, 1969). Sufficient measurements must be made to determine the mean discharge for the 24-hour observation period. Measure the surface area (m^2) and the mean depth (m) for the reach between the stations, and determine the average time required for water to travel between the stations. If it is not possible to determine the flow rate of the stream directly, it can be estimated from the time taken 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.

6.4 Determine the dissolved-oxygen concentration (mg/l) and the water temperature at each station as described in 6.2 above. Determine the diffusion rate, D, by one of the methods described in 6.5 below.

6.5 Diffusion rate, D. Determination of the rate at which oxygen enters or leaves the water when the content is not in equilibrium with the air is a critical step in the use of the oxygen curve method in many waters. 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 with highly variable discharge or open waters, it may be necessary to use different gas transfer coefficients, K, at different times of day to account for changes in flow or in wind speed and direction (Odum and Wilson, 1962). The correction for wind effects can be neglected in relatively protected areas.

Either 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 below Tennessee Valley Authority reservoirs determined that water depth and velocity were the most important factors affecting the area-based gas transfer coefficient (Churchill and others, 1962). To compute the gas transfer coefficient and the diffusion rate of oxygen, 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 6.1 through 6.4 above. Complete the calculations as directed below.

Floating-dome method. The diffusion rate, D, is determined directly by measuring changes in the content of oxygen in a plastic dome filled with air and floating on the water surface (Copeland and Duffer, 1964; and fig. 57). The changes in oxygen inside the dome with time are attributed to diffusion. Dome measurements 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 periods of darkness, determine the temperature and the fraction (percentage) of oxygen in the dome atmosphere with an oxygen meter capable of measuring gaseous oxygen. Record as in table 7. Simultaneously measure the oxygen concentration and temperature of the water as described in 6.2 above. Complete the calculations as directed below.

7. Analysis

7.1 *Single-station analysis.* From the data collected in 6.2 above, tabulate time versus dissolved-oxygen concentration and temperature as shown in table 8, columns 1–3, and plot curves as in figure 58*A* and *B*. Graph paper with 1-mm squares is convenient to use for these plots.

7.2 Determine the percentage saturation for each oxygen value using tables of oxygen solubility at various temperatures, pressures, and salinities (American Public Health Association and others, 1976). Tabulate and plot a curve of time versus percentage oxygen saturation as shown in table 8, column 6, and figure 58*C*.

7.3 From the observed oxygen-concentration data (table 8, col. 3) determine the hourly rate of change in dissolved oxygen (mg/l/hr) by subtracting successive pairs of oxygen values. Tabulate the values and plot the rate curve as shown in table 8, column 4, and in figure 58*D* (curve labeled "Before correction for diffusion").

7.4 Subtract each percentage-saturation value determined in 7.2 above from 100 percent, recording values less than 100 as negative. List these percentage-saturation deficits as in table 8, column 7. Proceed to 7.9 or 7.10 depending on the method used to determine the diffusion rate. If K is estimated, proceed to 7.12 below.

7.5 *Two-station analysis.* From the data collected in 6.4 above, determine the average dissolved-oxygen concentration and average temperature for the reach between stations for each sample interval. Tabulate time versus average dissolved-oxygen concentration and temperature as shown in table 8, column 1–3, and plot curves as in figure 58*A* and *B*. Graph paper with 1-mm squares is convenient to use for these plots.

7.6 Determine the average percentage saturation for each sample interval using tables of oxygen solubility at various temperatures, pressures, and salinities (American Public Health Association and others, 1976). Tabulate and plot a curve of time versus average percentage oxygen saturation as shown in table 8, column 6, and figure 58*C*.

7.7 From the average observed oxygen-concentration data for the reach (table 8, col. 3) determine the average hourly rate of change in dissolved oxygen (mg/l/hr) by subtracting successive pairs of oxygen values. Tabulate the values and plot the rate curve as shown in table 8, column 4, and in figure 58*D* (curve labeled "Before correction for diffusion").

7.8 Subtract each average percentage-saturation value determined in 7.6 above from 100 percent, recording values less than 100 as negative. List these average percentage-saturation deficits as in table 8, column 7. Proceed to 7.9, 7.10, or 7.13 depending on the method used to determine the diffusion rate. If K is estimated, proceed to 7.12 below.

7.9 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 k_2. Thus, from Churchill, Elmore, and Buckingham (1962):

$$k_2(\text{at } 20°\text{C}) = 5.026V^{0.969}R^{-1.673} \qquad (3)$$

Table 7.—Example data for determining the diffusion rate, D, *in a stream by the floating-dome method*

[The dome has a volume of 2.5 liters and an area of 0.038 m² in contact with the water]

Time interval (hr)	Dome			Water			Gas transfer coefficient, K, (g/m²/hr at 0 percent saturation)
	Percent oxygen [1]	Temperature (°C)	Volume oxygen (ml)	Temperature (°C)	Average saturation deficit [2]	Oxygen diffusion rate, D (g/m²/hr)	
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 8.

where

k_2 = gas transfer coefficient per day,

V = cross-sectional mean velocity in feet per second (ft/s), and

R = hydraulic radius (approximately the depth of flow) in feet.

With a known oxygen saturation value for a specific time, Hall (1971) obtained the following expression for k in terms of k_2:

$$k = \frac{2.3(k_2 C_s)}{24} \quad (4)$$

where C_s is the 100-percent saturation deficit expressed as g/m^3, and k is in g/m^3/hr and is for a 100-percent saturated deficit. The 2.3 converts the k_2 defined in terms of $\log_{10}$ to k defined in terms of $\log_e$.

Table 8.—Example data for determining community primary productivity of a stream by the oxygen curve method

[The mean depth of flow is 1.2 m; the gas transfer coefficient on an area basis, K, is 3.2 g/m^2/hr, and on a volume basis, k, is 2.67 g/m^3/hr at 100-percent saturation deficit]

Time (hrs)	Temperature (°C)	Dissolved oxygen					
		Observed (mg/l) [1]	Rate of change (mg/l/hr)	Conc. at saturation (mg/l)	Observed saturation (percent)	Average saturation deficit, S (percent)	$\frac{S \times k}{100}$ (g/m^3/hr)
0000	29.5	6.00		7.7	78		
			−0.05			−23.0	−0.614
0100	29.0	5.95		7.8	76		
			−.05			−24.5	−.654
0200	28.0	5.90		7.9	75		
			−.05			−26.5	−.708
0300	27.0	5.85		8.1	72		
			−.05			−29.0	−.774
0400	25.5	5.80		8.3	70		
			+.10			−30.0	−.801
0500	25.0	5.90		8.4	70		
			0			−28.5	−.761
0600	27.0	5.90		8.1	73		
			+.40			−23.5	−.627
0700	28.0	6.30		7.9	80		
			+.55			−15.0	−.400
0800	30.0	6.85		7.6	90		
			+1.00			−7.5	−.200
0900	31.0	7.85		7.5	105		
			+.95			+11.5	+.307
1000	31.5	8.80		7.45	118		
			+.60			+22.5	+.601
1100	32.0	9.40		7.4	127		
			+.65			+32.0	+.854
1200	32.5	10.05		7.35	137		
			+.45			+41.0	+1.095
1300	33.5	10.50		7.25	145		
			+.10			+45.0	+1.202
1400	33.0	10.60		7.3	145		
			−.15			+43.5	+1.161
1500	32.5	10.45		7.35	142		
			−.25			+38.5	+1.028
1600	30.5	10.20		7.55	135		
			−1.30			+26.5	+.708
1700	30.5	8.90		7.55	118		
			−1.30			+9.0	+.240
1800	30.0	7.60		7.6	100		
			−1.15			−7.5	−.200
1900	30.0	6.45		7.6	85		
			−.15			−16.0	−.427
2000	30.0	6.30		7.6	83		
			0			−17.5	−.467
2100	29.5	6.30		7.7	82		
			−.15			−19.0	−.507
2200	29.5	6.15		7.7	80		
			+.10			−20.0	−.534
2300	29.0	6.25		7.8	80		
			−.15			−21.0	−.561
2400	29.0	6.10		7.8	78		

[1] Milligrams per liter = grams per cubic meter.

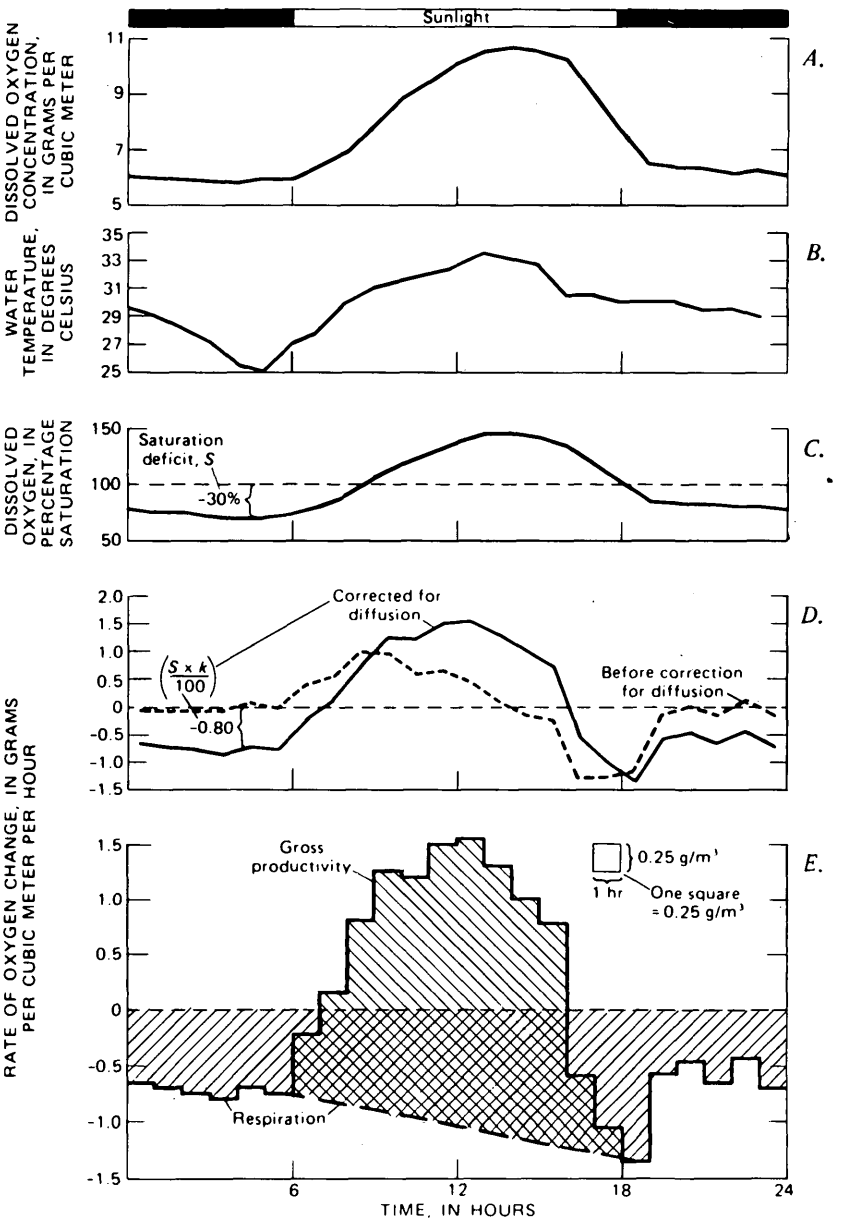

Figure 58.—Example of diel oxygen curve and supporting data (from tables 7 and 8) for determining community primary productivity and community respiration of a stream by the oxygen curve method. The mean depth of flow is 1.2 m, the gas transfer coefficient on an area basis, K, is 3.2 g/m²/hr, and on a volume basis, k, is 2.67 g/m³/hr at 100-percent saturation deficit. (Modified from Odum and Hoskin, 1958.)

For temperatures other than 20°C, apply a correction to k_2 at the rate of 2.41-percent increase or decrease per degree above or below 20°C. Estimate the gas transfer coefficient, k, for the study period by averaging the k values determined for each sampling interval. Proceed to 7.14 below. Note: Some situations require use of different gas transfer coefficients at different times of day as explained in 6.5 above.

7.10 Determine the diffusion rate, D, for each nighttime sample interval from measurements in the floating dome (table 7). Calculate the volume of oxygen in the dome at the beginning and end of the sample interval as follows:

$$V_t = V_d(0.21)\frac{F_t}{100} \tag{5}$$

where

V_t = volume of oxygen in milliliters in the dome at a specific time t,

V_d = the 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.

Express the concentration of oxygen gas in the dome in terms of standard temperature and pressure for each sample interval using the equation

$$\Delta V = \frac{273V_0}{273+T_0} - \frac{273V_1}{273+T_1} \tag{6}$$

where

ΔV = change in volume of oxygen in milliliters in the dome at standard conditions of temperature and pressure,

V_0 = volume of oxygen in milliliters in the dome at the beginning of the interval,

V_1 = volume of oxygen in milliliters in the dome at the end of the interval,

T_0 = temperature in degrees Celsius in the dome at the beginning of the interval,

T_1 = temperature in degrees Celsius in the dome at the end of the interval, and

273 = factor for converting to absolute temperature.

Oxygen gas weighs 0.00143 g/ml at standard temperature and pressure. Therefore, the rate of oxygen diffusion D, (g/m²/hr) may be computed from

$$D = \frac{(\Delta V)(0.00143)}{A(\Delta t)} \tag{7}$$

where

A = area of the dome in square meters in contact with the water surface, and

Δt = time interval in hours between the two measurements.

7.11 Using equation 8, 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 D by the average percentage-saturation deficit during the time of measurement, or

$$K = \frac{D(100)}{S} \tag{8}$$

where

K = gas transfer coefficient in g/m²/hr at 0-percent saturation (100-percent saturation deficit),

D = rate of diffusion of oxygen into the water in g/m²/hr, as before, and

S = average percentage-saturation deficit between the water and the air during the sample interval (from 7.4 or 7.8 above).

7.12 Convert each area value to a volume value by dividing by the mean depth of water in meters, or

$$k = \frac{K}{z} \tag{9}$$

where k is the gas transfer coefficient in g/m³/hr at 0-percent saturation, and z is the mean depth in meters.

Estimate the gas transfer coefficient, k, for the study period by averaging the k values determined for each sampling interval. Proceed to 7.14 below. Note: Some situations require use of different diffusion constants at different times of day as explained in 6.5 above.

7.13 Determine the average volume-based gas transfer coefficient, 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):

$$k = \frac{q_n - q_{n+1}}{S_n - S_{n+1}} \quad (10)$$

where

k = g O_2/m^3/hr at 0-percent saturation,

q_n = average rate of change (g O_2/m^3) for the reach at nighttime n,

q_{n+1} = average rate of change (g O_2/m^3) 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 7.14 below.

7.14 Determine the amount of oxygen (g/m^3) gained or lost by diffusion during each sampling interval. To obtain the correction for atmospheric reaeration, multiply the average volumetric gas transfer coefficient, k (from 7.9, 7.12, or 7.13 above), by each percentage oxygen-saturation deficit value (from 7.4 or 7.8 above), and divide by 100 to convert percentage to fractional values. List these correction values as in table 8, column 8.

7.15 Refer to figure 58*D*, the hourly rate-of-change graph plotted as directed in 7.3 or 7.7 above. Now prepare a corrected rate-of-change curve by adding or subtracting graphically the amount of oxygen (g/m^3) gained or lost by diffusion during each sampling interval (from 7.14 above). Draw the curve as in figure 58*D* (curve labeled "Corrected for diffusion"). In figure 58*E* of the example, the corrected rate-of-change curve is replotted as a step function to facilitate graphical integration.

7.16 Connect a line between the presunrise and postsunset negative rate-of-change points on the corrected rate-of-change curve as shown in figure 58*E* (Odum and Wilson, 1962). This line is an estimate of daytime respiration. Note: 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 both respiration and productivity are affected by the placement of the respiration line. The accuracy of the method is probably limited by this step (Odum and Hoskin, 1958, p. 22). Graphs in which the rates of change are very irregular introduce more subjectivity into the choice of the respiration line than do smooth curves.

8. Calculations

The following calculations on a volume or concentration basis (g/m^3/day) can be converted to an area basis (g/m^2/day) by multiplying by the average water depth of the study area in meters.

8.1 An estimate of gross productivity, P_g, in g O_2/m^3/day is the area above the daytime respiration line and below the daytime rate-of-change line (fig. 58*E*). The area may be determined from the plot by counting the graph-paper squares and multiplying by the g/m^3 value of one square.

8.2 An estimate of community respiration, R_t, in g O_2/m^3/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. 58*E*). The area may be determined from the plot by counting the graph-paper squares and multiplying by the g/m^3 value of one square. Note that the graphical procedure integrates the hourly values over a 24-hour period; hence, the respiration rate is now on a per-day basis.

8.3 An estimate of net productivity, P_n, in g O_2/m^3/day is the difference between P_g and R_t.

8.4 An index of the trophic nature of the community may be calculated as the ratio of photosynthetic production to respiration, P/R. Communities having a P/R ratio less than 1 exhibit an excess of respiration over production. 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 act to release more oxygen through photosynthesis than they consume through respiration.

9. Report

Report community productivity and respiration (in milligrams) as follows: less than 10 mg O_2/day, one decimal; 10 mg and above, two significant figures.

10. Precision

No numerical precision data are available.

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Diel oxygen curve method for estimating primary productivity and community metabolism in stratified waters (B–8100–77)

Parameters and codes:
Productivity, primary, gross (mg O_2/m^3/day) 70959
Productivity, primary, gross (mg O_2/m^2/day) 70960
Productivity, primary, net (mg O_2/m^3/day) 70963
Productivity, primary, net (mg O_2/m^2/day) 70964
Respiration (mg O_2/m^3/day) 70967
Respiration (mg O_2/m^2/day) 70968

1. Application

If complete vertical mixing occurs within the water body, a series of single station analyses may be sufficient to characterize the oxygen regime within the water. However, in many cases, 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. Under these conditions, production of oxygen will be limited to the euphotic zone, and an oxygen deficit will exist in the lower or hypolimnetic water.

Two analytical approaches for evaluating oxygen metabolism in stratified waters are presented and contrasted using synthetic data for a hypothetical lake. The graphical method provides an estimate of gross production, or the total amount of oxygen produced over a diel period and total community respiration, or the total amount of oxygen consumed over a diel period. Diel net productivity, or the excess of oxygen produced over that consumed, is calculated as the difference between gross production and total respiration. The graphical method assumes that daytime respiration is constant or that it varies only linearly with time. This is the major limitation to the graphical method.

The alternative analytical approach consists of data processing using a FORTRAN computer program (Program designation: Primary production, J330). A complete reference to the program is found in the user manual by Stephens and Jennings (1976). The program will calculate daytime net oxygen production and nighttime oxygen respiration. The arithmetic difference between these is given as 24-hr community metabolism which is equivalent to diel net production. Gross production is not calculated. Program J330 operates by assuming production may only occur during daylight hours and any change in dissolved oxygen, after correcting for diffusion, during this period is due to production. Any change in dissolved oxygen during hours of darkness, after correcting for diffusion, is attributed to respiration. The program also allows for exchange between the horizontal segments of a stratified water body using estimated or measured vertical dispersion coefficients.

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*, fall below 1 mg/m^3 (Talling, 1969).

2. Summary of method

From average values for temperature, dissolved oxygen, and, if appropriate, salinity, an average rate of change of 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, due mainly 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 the method precludes its use in unproductive waters. The method should be used in waters of comparative horizontal homogeneity.

4. Apparatus

All materials used must be free of agents which inhibit photosynthesis and respiration.

4.1 *Equipment for determination of dissolved oxygen* by the azide modification of the Winkler method (Brown and others, 1970; or American Public Health Association and others, 1976). A properly calibrated oxygen meter with electrode and stirrer may be used in place of the Winkler method for dissolved-oxygen measurements. Examples of suitable battery-operated, membrane-electrode instruments are Beckman Instruments, Inc. (100801), Martek Instruments, Inc. (DOA), Weston and Stack, Inc. (300), Yellow Springs Instrument Co., (54 and 51A), or equivalent.

4.2 *Underwater light-measurement equipment*, Kahl Scientific Instrument Corp. (268WA310), Hydro Products (620–S), InterOcean (510), or equivalent. If a submersible photometer is not available, a *Secchi disk*, Kahl Scientific Instrument Corp. (281WA100), Wildlife Supply Co. (59), or equivalent may be used as described in 6.1 below.

4.3 *Equipment for determination of salinity* by titration (Strickland and Parsons, 1968) or by electrical conductivity, Beckman Instruments (RS5–3), Yellow Springs Instrument Co. (33–S–C–T), or equivalent, if appropriate.

4.4 *Water-sampling bottle*, Wildlife Supply Co. (1120 or 1200), Scott Instruments, Seattle, Wash., or Foerst Mechanical Specialties Co. (Improved Water Sampler, Kemerer-type), or equivalent or a threefold displacement sampler, Precision Scientific Co. (69770), or equivalent. Depth-integrated samplers are discussed in Guy and Norman (1970).

4.5 *Thermistor* or *thermometer* for determining water temperature and gas temperature in the diffusion dome. Most oxygen meters include thermistors suitable for making these measurements.

4.6 *Diffusion dome* made of clear Plexiglas approximately 22 cm in diameter or larger. Suitable domes are obtainable 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 (⅜-in) marine plywood (fig. 57). The oxygen and temperature sensors are inserted either through holes in the dome or from below into a support inside the dome. The dome is painted silver to reduce the greenhouse effect on the inside temperature.

4.7 *Barometer* for measuring local barometric pressure.

4.8 *Graph paper*, 10×10 to the centimeter, Keuffel & Esser (46 1510), or equivalent.

4.9 *Polar planimeter* and *maps* appropriate to the study (see 7.1 below).

5. Reagents

5.1 *Reagents for the azide modification of the Winkler method* for dissolved oxygen (Brown and others, 1970; American Public Health Association and others, 1976).

5.2 *Reagents for determination of salinity* (Strickland and Parsons, 1968), if appropriate.

6. Collection

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 of the benthic zone. Total community metabolism of the water body may then be estimated on an areal basis.

Sampling stations should be located in areas representative of the water body if values are to be averaged to yield metabolism of the entire 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.

6.1 Determine the depth of the euphotic zone (the region that receives 1 percent or more of the surface light) with submersible photometer. If no other method is available, an estimate of the lower limit of the euphotic zone is obtained by multiplying the Secchi disk depth (Welch, 1948) by 5 (Verduin, 1956). Select sampling intervals equal to one-tenth of the depth of the euphotic zone. Respiration within the deepest portion of the lake (hypolimnion) can be estimated by including one or more sampling depths between the euphotic zone and the bottom. The computer analysis method requires that depth intervals be constant.

6.2 At 1-, 2-, or 3-hr intervals at each increment of depth, determine temperature, dissolved-oxygen concentration, and, if appropriate, salinity or conductivity. Determine the diffusion rate as described in 6.3.

6.3 Diffusion rate, D. Determination of the rate at which oxygen enters or leaves the water when the content is not in equilibrium with the air is a critical step in the use of the oxygen curve method in many waters. The rate at which oxygen diffuses in or out of the water increases as the degree of undersaturation or oversaturation increases. In some open waters, it may be necessary to use different gas transfer coefficients, K, at different times of day to account for changes in currents and in wind speed and direction (Odum and Wilson, 1962). The correction for wind effects can be neglected in relatively protected areas.

Either of the following methods can be used for determining D:

Floating-dome method. The diffusion rate, D, is determined directly by measuring changes in the content of oxygen in a plastic dome filled with air and floating on the water surface (Copeland and Duffer, 1964; and fig. 57). The changes in oxygen inside the dome with time are attributed to diffusion. Dome measurements are made at night to avoid temperature 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 2–5 hr during periods of darkness, determine the temperature and the fraction (percentage) of oxygen in the dome atmosphere with an oxygen meter capable of measuring gaseous oxygen. Simultaneously measure the oxygen concentration and temperature of the water.

Complete the calculations as directed below.

Night-time rate of change method. Odum (1956) and Odum and Hoskin (1958) proposed this method to estimate reaeration gains or losses during darkness in the absence of photosynthesis. It assumes there is no photosynthetic production of oxygen and that respiration is constant during the nighttime measurement interval.

Individual values for the gas transfer coefficient (k) corresponding to a nighttime sampling interval may be used to correct the surface water layer for nighttime diffusion. An arithmetic average of the nighttime values can be used to provide the daytime diffusion correction. Complete the calculations as directed below.

7. Analysis

7.1 *Lake Morphometry.* The volume of water contained within a lake may be calculated from measurements of each depth contour taken from a good topographic or bathymetric map. An accurate, scaled map and planimeter are required. Determine the area enclosed within each contour interval using a planimeter. Typically, the planimeter will indicate area in square inches (or centimeters) which must then be converted to actual area using the map scale. Figure 59 represents a small lake which was planimetered to obtain the morphometric data in table 9. 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 ($250{,}000^2$). 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 (table 9, col. 3). Conversion to metric units is made using the relationship 1 mi^2 equals 2.59×10^6 m^2.

Table 9.—Morphometric data and results of graphical analysis of community production and respiration for Fish Lake

[Area values: Gross production = 78.98 $g/m^2/day$
Respiration = 81.29 $g/m^2/day$
Net production = -2.31 $g/m^2/day$
P/R = 0.972]

Lake slice (depth interval = 3m)	Elevation (ft)	Area (X $10^8 m^2$)	Volume (X $10^8 m^3$)	Gross production ($g/m^3/day$)	Slice gross production (X $10^8/m^3$/ day)	Respiration ($g/m^3/day$)	Slice respiration (X $10^8 g/m^3$/ day)
Surface	4490	3.83	–	–	–	–	–
1	4480	2.81	9.37	20.33	190.49	21.03	197.05
2	4470	1.82	6.89	9.13	62.91	9.18	63.25
3	4460	.75	4.24	9.00	38.16	10.05	42.61
4	4450	.15	1.88	5.30	9.96	4.48	8.42
Total	---	---	---	---	302.5 X 10^8 g/day		311.33 X 10^8 g/day

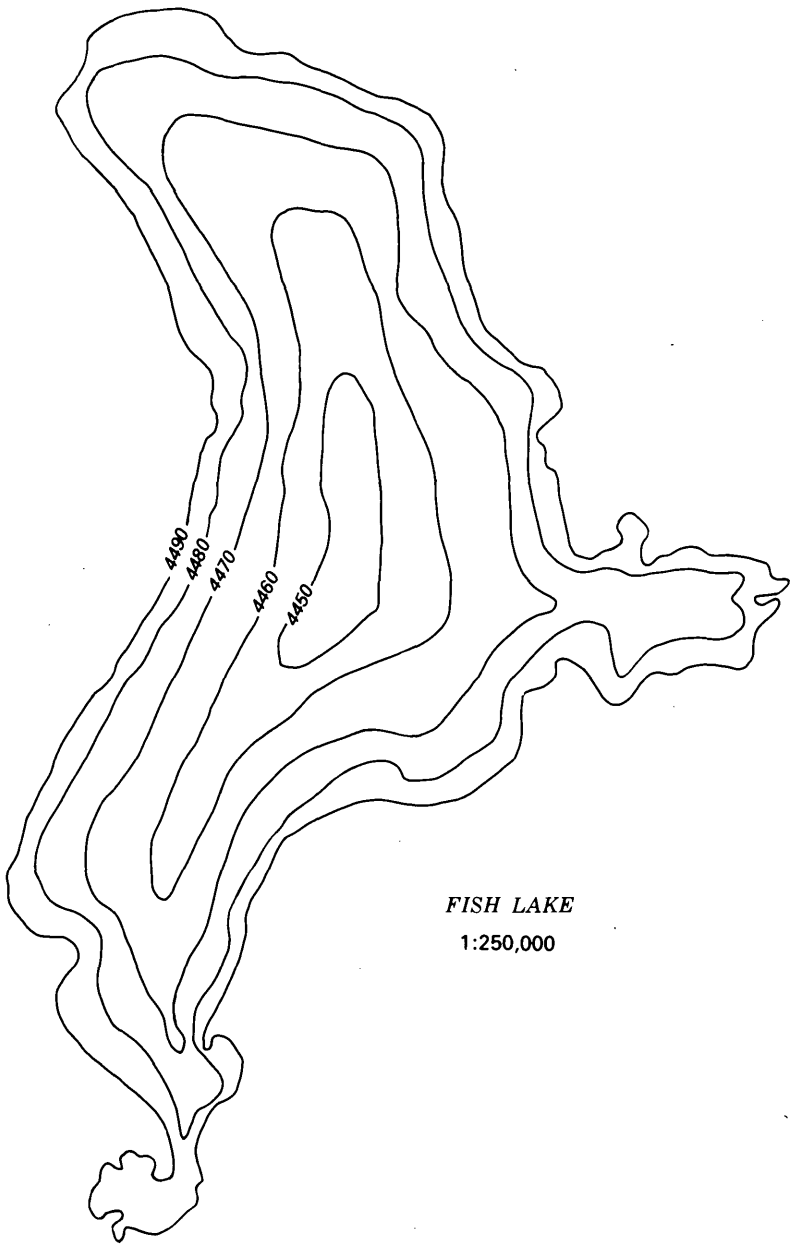

Figure 59.—Map of hypothetical Fish Lake used in morphometric analysis.

The volume of each contour (table 9, col. 4) is calculated as

$$V_{n-mm} = 1/3\,(A_m + A_n + \sqrt{A_m A_n})\,(n\text{–m}) \quad (1)$$

where

V_{n-m} is the volume of a given element between contour n and contour m (in cubic meters),

A_m is the area at contour m (in square meters),

A_n is the area at contour n (in square meters), and

n–m is the interval between contour n and contour m (in meters).

Total lake volume is the summation of all element volumes.

7.2 From the data collected in 6.2 above, average the temperature, dissolved oxygen, and, if appropriate, salinity values at each depth interval for several stations to eliminate the effects of horizontal heat and solute exchange. Tabulate time versus average surface

Table 10.—Example data for determining community primary productivity of a lake by the oxygen curve method

[The depth of flow is 1.2 m; the gas transfer coefficient on an area basis, K, is 3.2 g/m^2/hr, and on a volume basis, k, is 2.67 g/m^3/hr at 100-percent saturation deficit]

		Dissolved oxygen						
1	2	3	4	5	6	7	8	9
Time (hrs)	Temperature (°C)	Observed (mg/l)[1]	Rate of change ((mg/l/hr)	Conc. at saturation (mg/l)	Observed saturation (percent)	Average saturation deficit, S (percent)	$S \times k$ 100 (g/m^3/hr)	Corrected rate of change (g/m^3/hr)
0000	29.5	6.00		7.7	78			
			- 0.05			- 23.0	- 0.614	- .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			
			0			- 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	+ .402
1000	31.5	8.80		7.45	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		7.35	137			
			+.45			+ 41.0	+ 1.095	+ 1.545
1300	33.5	10.50		7.25	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		7.35	142			
			-.25			+ 38.5	+ 1.028	+ .778
1600	30.5	10.20		7.55	135			
			- 1.30			+ 26.5	+.708	- .592
1700	30.5	8 90		7.55	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			
			0			- 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 = grams per cubic meter.

dissolved-oxygen concentration and temperature as shown in table 10, columns 1–3. 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 10, column 3. These values are not corrected for diffusion. Proceed to 7.3 through 7.12 for the graphical analysis procedure.

7.3 Graphical analysis. Determine the percentage saturation for each average surface oxygen value using tables of oxygen solubility at various temperatures, pressures, and salinities (American Public Health Association and others, 1976). Tabulate and plot a curve of time versus percentage surface oxygen saturation as shown in table 10, column 6, and figure 58*C*. Graph paper with 1-mm squares is convenient to use for these plots.

7.4 From the surface oxygen-concentration data (table 10) determine the hourly rate-of-change in dissolved oxygen (mg/l/hr) by subtracting successive pairs of oxygen values. Tabulate the values and plot the rate curve as shown in table 10, column 4, and in figure 58*D* (curve labeled "Before correction for diffusion").

7.5 Subtract each percentage-saturation value determined in 7.3 above from 100 percent, recording values less than 100 as negative. List these percentage-saturation deficits as in table 10, column 7. Proceed to 7.6 or 7.7 depending on the method used to determine the diffusion rate. If K is estimated, proceed to 7.8 below.

7.6 Determine the diffusion rate, D, for each nighttime sample interval from measurements in the floating dome. Calculate the volume of oxygen in the dome at the beginning and end of the sample interval as follows:

$$V_t = V_d(0.21)\frac{F_t}{100} \qquad (2)$$

where

V_t is the volume of oxygen in milliliters in the dome at a specific time t,

V_d is the volume of atmospheric gases in milliliters in the dome,

F_t is the percentage oxygen saturation in the dome atmosphere at time t when fresh air equals 100-percent oxygen saturation, and 0.21 is the fractional volume of oxygen in the air.

Express the concentration of oxygen gas in the dome in terms of standard temperature and pressure for each sample interval using the equation

$$\Delta V = \frac{273V_0}{273+T_0} - \frac{273V_1}{273+T_1} \qquad (3)$$

where

ΔV is the change in volume of oxygen in milliliters in the dome at standard conditions of temperature and pressure,

V_0 is the volume of oxygen in milliliters in the dome at the beginning of the interval,

V_1 is the volume of oxygen in milliliters in the dome at the end of the interval,

T_0 is the temperature in degrees Celsius in the dome at the beginning of the interval,

T_1 is the temperature in degrees Celsius in the dome at the end of the interval, and

273 is the factor for converting to absolute temperature.

Oxygen gas weighs 0.00143 g/ml at standard temperature and pressure. Therefore, the rate of oxygen diffusion, D, (g/m^2/hr) may be compared from

$$D = \frac{(\Delta V)(0.00143)}{A(\Delta t)} \qquad (4)$$

where

A is the area of the dome in square meters in contact with the water surface and

Δt is the time interval in hours between the two measurements. Proceed to 7.8 below.

7.7 Determine the volume-based gas transfer coefficient, k, for each sample interval from measurements of the nighttime rate of oxygen change. This can be estimated by calculating k values for each nighttime surface sampling interval using the Odum (1956) method as presented by Eley (1970):

$$k = \frac{q_n - q_n + 1}{S_n - S_n + 1} \qquad (5)$$

where

k is g O_2/m^3/hr at 0-percent saturation,

q_n is the rate-of-change of the surface g O_2/m^3 at nighttime n,

q_{n+1} is the rate-of-change of the surface g O_2/m^3 at nighttime $n+1$,

S_n is the oxygen saturation deficit of the surface water at nighttime n, and

S_{n+1} is the oxygen saturation deficit of the surface water at nighttime $n+1$.

Proceed to 7.9 below.

7.8 Using equation 6, 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 D by the average percentage-saturation deficit during the time of measurement, or

$$K = \frac{D(100)}{S} \quad (6)$$

where

D is the rate of diffusion of oxygen into the water in g/m²/hr as before,

K is the gas transfer coefficient in g/m²/hr at 0-percent saturation (100-percent saturation deficit), and

S is the average percentage-saturation deficit between the water and the air during the sample interval (from 7.5 above).

7.9 Convert each area value to a volume value by dividing by the depth of water in meters for the surface interval, or

$$k = \frac{K}{z} \quad (7)$$

where

k is the gas transfer coefficient in g/m³/hr at 0-percent saturation, and

z is the thickness in meters of the surface interval.

Estimate k for the study period by averaging the k values determined for each sampling interval. Proceed to 7.10 below. Note: Some situations require use of different diffusion constants at different times of day as explained in 6.3 above.

7.10 Determine the amount of oxygen (g/m³) gained or lost by diffusion at the surface during each sampling interval. To obtain the correction for atmospheric reaeration, multiply the average k (from 7.9 above) by each percentage oxygen-saturation deficit value (from 7.5 above) and divide by 100 to convert percentage to fractional values. List these correction values as in table 10, column 8.

7.11 Refer to figure 58*D*, the hourly rate-of-change graph plotted as directed in 7.4 above. Now prepare a corrected rate-of-change curve by adding or subtracting graphically the amount of oxygen (g/m³) gained or lost by diffusion during each sampling interval (from 7.10 above). Draw the curve as in figure 58*D* (curve labeled "Corrected for diffusion"). In figure 58*E* of the example, the corrected rate-of-change curve is replotted as a step function to facilitate graphical integration. Dissolved-oxygen values for each remaining depth interval are tabulated as in table 10, 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 58*E*.

7.12 Connect a line between the presunrise and postsunset negative rate-of-change points on the corrected rate-of-change curve as shown in figure 58*E* (Odum and Wilson, 1962). This line is an estimate of daytime respiration. Note: 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 both respiration and productivity are affected by the placement of the respiration line. The accuracy of the method is probably limited by this step (Odum and Hoskin, 1958, p. 22). Graphs in which the rate-of-change are very irregular introduce more subjectivity into the choice of the respiration line than do smooth curves.

8. Calculations

8.1 An estimate of gross productivity in g O_2/m³/day for each depth increment is the area above the daytime respiration line and below the daytime rate-of-change line (fig. 58*E*, for the surface interval). The area may be determined from the plot by counting the graph-paper squares and multiplying by the g/m³ value of one square. Total gross productivity of each slice in g O_2/m³/day is obtained by multiplying the slice volumetric productivity value in g O_2/m³/day by the total water volume of the slice interval in cubic meters. Total productivity of the entire water body in g O_2/day is the summation of all slice interval production values. Total productivity of the water divided by the surface area in square meters of the water body will provide an areal value, in g O_2/m²/day, useful in comparing productivities from diverse water bodies.

8.2 An estimate of community respiration in g O_2/m³/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. 58*E*, for the surface interval). The area may be determined from the plot by

counting the graph-paper squares and multiplying by the g/m^3 value of one square. Total community respiration of each slice in g O_2/m^3/day is obtained by multiplying the slice volumetric respiration in g O_2/m^3/day by the total water volume of the slice interval in cubic meters. Total respiration of the entire water body in g O_2/day is the summation of all slice interval production values. Total respiration of the water divided by the surface area in square meters of the water body will provide an areal value, in g O_2/m^2/day, useful in comparing respiration from diverse water bodies.

8.3 An estimate of productivity for each slice increment or the entire waterbody may be calculated by taking the difference between the appropriate gross production value and the corresponding respiration value.

8.4 An index of the trophic nature of the community may be calculated as the ratio of photosynthetic production to respiration, *P/R*. Communities having a *P/R* ratio less than one exhibit an excess of respiration over production. 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 act to release more oxygen through photosynthesis than they consume through respiration.

9. Report

Report community productivity and respiration (in milligrams) as follows: less than 10 mg O_2/day, one decimal; 10 mg and above, two significant figures.

10. 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 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 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
Algal growth potential (AGP)
(B–8501–77)

Parameter and code: Algal growth potential (mg/l) 85209

1. Application

The knowledge of algal growth potential (AGP) is important in water quality studies. Both the abundance and composition of algae are related to water quality, because algal growth is primarily influenced by the availability of growth substances. The significance of measuring algal growth potential in water samples is that differentiation can be made between the growth substances of a sample determined by chemical analysis and the growth substances that are actually available for algal growth. The addition of growth substances to the sample can give an indication of which growth substance(s) is(are) limiting for algal growth.

The electronic particle counter has been used for counting and sizing nonfilamentous unialgal species (Hasting and others, 1962; El-Sayed and Lee, 1963). The principle of operation is as follows: The algal cells, which are relatively poor electrical conductors, are suspended in an electrolyte 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 as an algal cell passes through the aperture is proportional to cell volume. The knowledge of both the particles (cells or colonies) per unit volume of sample and the change in mean particle (cell or colony) volume allow growth rates to be measured reproducibly and accurately.

The method is suitable for all fresh waters. The method is similar to that of the U.S. Environmental Protection Agency (1969, 1971).

2. Summary of method

A 100-ml aliquot of a water sample is filtered and placed in a covered Erlenmeyer flask. This sample is inoculated with an appropriate number of algal cells of a known species and incubated under constant temperature and light intensity until the rate of growth is less than 5 percent per day. The number of particles (cells or colonies) per unit volume of sample and the mean particle (cell or colony) volume are determined with an electronic particle counter, and these values are used to determine the maximum standing crop.

3. Interferences

Particles in the counting medium (for example, dust or lint) may block the aperture of the counting cell or give false counts. Interferences are eliminated by passing all media and water samples through a 0.22-μm filter. Samples for analysis should be collected in a nonmetallic sampler because certain metals in a metallic sampler may affect results.

4. Apparatus

4.1 *Field filtration apparatus*, nonmetallic, with vacuum apparatus.

4.2 *Sample container*, linear polyethylene bottles, 1,000 ml.

4.3 *Centrifuge*, International Equipment Co. (Model 428) or equivalent, with rotor for 15-ml tubes.

4.4 *Environmental chamber*, walk-in, with temperature control (24°±2°C), and illumination (cool white fluorescent) which provides 4,300 lm/m^2, Forma Scientific or equivalent.

4.5 *Shaker*, rotatory, capable of 120 oscillations per minute, New Brunswick Scientific (G10 Gyrotary) or equivalent.

4.6 *Electronic particle counter and mean cell volume accessory*, Coulter Model ZBI (fig. 60) or equivalent, with 100-μm aperture tube and 500-μl manometer.

4.7 *Flasks*, Erlenmeyer, 250 ml, covered with 50-ml beakers, both glass.

4.8 *Vials*, glass, 21×70 mm.

Figure 60.—Electronic particle counter. (Photograph courtesy of Coulter Electronics, Inc., Hialeah, Fla.).

4.9 *Tubes*, glass graduated centrifuge, 15 ml.

4.10 *Pipets*, Eppendorf or equivalent, with disposable tips, 0.1- and 1.0-ml capacities.

4.11 *Laboratory filtration apparatus*, sterile, disposable, Falcon filter (7103) 0.22 μm without grid.

4.12 *Membrane filters*, 0.22-μm pore size, 47-mm

diameter, low water extractable, Millipore No. GSTF or equivalent.

4.13 *Distillation apparatus*, glass.

4.14 *Balance*, analytical, Mettler (H35) or equivalent.

4.15 *Autoclave*, steam, Curtin Matheson Scientific (209–536) or equivalent.

5. Reagents

5.1 *Isoton* (Coulter Electronics) or equivalent particle-free saline solution.

5.2 *Aperture cleaner*, Isoterge (Coulter Electronics) or equivalent; bleach or nitric acid may be used, but aperture tube should be removed when these are used.

5.3 *Sodium nitrate solution*: Dissolve 12.75 g $NaNO_3$ in 500 ml distilled water.

5.4 *Magnesium sulfate solution*: Dissolve 3.593 g $MgSO_4$ in 500 ml distilled water.

5.5 *Magnesium chloride solution*: Dissolve 6.082 g $MgCl_2 \cdot 6H_2O$ in 500 ml distilled water.

5.6 *Sodium bicarbonate solution*: Dissolve 7.5 g $NaHCO_3$ in 500 ml distilled water.

5.7 *Calcium chloride solution*: Dissolve 1.66 g $CaCl_2$ in 500 ml distilled water.

5.8 *Micro nutrient solution*: Dissolve 92.76 mg H_3BO_4, 207.69 mg $MnCl_2 \cdot 4H_20$, 16.35 mg $ZnCl_2$, 79.88 mg $FeCl_3 \cdot 6H_20$, 150 mg Na_2EDTA (ethylendiaminetetraacetate) $\cdot 2H_20$, 0.39 mg $CoCl_2$, 3.63 mg $NaMoO_4 \cdot 2H_20$, and 5.7 μg $CuCl_2 \cdot 2H_20$ in 500 ml distilled water.

5.9 *Potassium phosphate solution*: Dissolve 0.522 g K_2HPO_4 in 500 ml distilled water.

5.10 *Test algae: Selenastrum capricornutum* Printz.

6. Collection

6.1 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 representatitive 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 located 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).

6.2 Filter the sample through a 0.22-μm mean pore size (low water extractable) filter immediately after collection to remove indigenous algae, bacteria, fungi, and other oganisms which are capable of utilizing the available nutrients in the sample. A maximum vacuum of 25 cm (10 in) is recommended to prevent damage to delicate algal cells and thus possible release of additional nutrients into the sample. The filtration apparatus should be rinsed thoroughly before the next sample is filtered. Initial filtering of the sample through a large pore-sized filter may be utilized when suspended sediment concentration is high. The filtered sample should be placed in the dark and immediately shipped to the laboratory chilled at 0–4°C with minimum of air space over the sample.

7. Analysis

7.1 Filter approximately 400 ml of the sample in the laboratory with a vacuum no greater than 25 cm (10 in) mercury using the sterile Falcon filter (7103) of 0.22-μm mean pore size.

7.2 Place 100 ml of filtered sample in each of three autoclaved 250-ml Erlenmeyer flasks (rinsed with some of filtered sample) and cover with a 50-ml beaker.

7.3 Place the covered flasks in the environmental chamber for temperature equilibration at 24°C for at least 12 hr.

7.4 Rinse algal innoculum (see Appendix) free of culture medium in the following manner: Add 1 ml of 7–10 day stock culture to 10 ml of filtered (0.22μm) distilled water and centrifuge at 5,000 rpm for 5 min. Decant the supernatant and add 10 ml of filtered distilled water, and resuspend the cells. Repeat the centrifugation and decantation step as previously described. Add 10 ml of filtered distilled water and resuspend the cells.

7.5 Determine the concentration of the algal particles (cells or colonies of cells) with the electronic particle counter.

7.6 Pipet a volume of the cell suspension into two of the test water flasks to give a final particle cell concentration in the test water of approximately 10,000 particles (cells) per milliliter.

7.7 Place the flasks (duplicate innoculated samples plus uninoculated control) in the environmental chamber on a rotatory shaker at 120 oscillations per minute exposed to a constant illumination of 4,300 lm/m^2, produced by cool-white fluorescent tubes.

7.8 Determine particle counts and mean particle volumes daily (if growth is rapid) or every other day (if growth is slow), until increase in algal concentration (cells per unit volume) is less than 5 percent per day. Refer to the electronic particle counter and mean cell

volume accessory manuals for operation and calibration procedures.

8. Calculations

8.1 Maximum standing crop is determined when the increase in algal concentration (cells per unit volume) is less than 5 percent per day and is defined as milligram(s) dry weight algae per liter at that time by the following equation:

$$\frac{\text{cells}}{\text{ml}} \times \frac{\mu\text{m}^3}{\text{cell}} \times \frac{2.5\times 10^{-7}\mu\text{g dry wt}}{\mu\text{m}^3} \times$$

$$\text{dilution factor} =$$

$$\frac{\mu\text{g dry wt}}{\text{ml}} = \frac{\text{mg dry wt}}{\text{l}}$$

where

cells/ml is the coincident corrected cell count per milliliter (determined by electronic particle counter).

μm^3/cell is the volume of cell in cubic micrometers (determined by mean cell volume accessory),

μg dry wt/μm^3 is the dry weight (gravimetric) cells per cubic micrometer. Dilution factor is the dilution of algal cells from pure culture with particle free saline solution for proper counting range. Results are expressed as milligram(s) dry weight per liter.

9. Report

Report maximum standing crop in milligram(s) dry weight algae per liter to two significant figures.

10. Precision

Precision of result is ±10 percent.

Appendix
Culturing Techniques for *Selenastrum capricornutum*

Culture medium is prepared in the following manner. Add 1 ml of each solution in 5.3 through 5.9 in the order given to approximately 900 ml of distilled water and then dilute to 1 l. Filter the medium through a membrane filter (0.22 μm mean pore size) at 25 cm (10 in) mercury. Place 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 min and allow to equilibrate with carbon dioxide and temperature for 12 hr in the environmental chamber.

The culture is maintained by transferring a 1-ml sample of a 7 to 10-day-old culture to a fresh media flask (as described above). The transfer can be as often as necessary to provide an adequate supply of algal cells at the proper growth stage for the algal growth potential test. Extreme care should be exercised to avoid contamination of stock cultures.

To retain a pure culture over a long period of time, it is advantageous to prepare media with 1-percent agar and transfer algae onto fresh plates every 6–8 weeks and to start fresh liquid cultures from a single colony at 6 to 8-week intervals. For regular inoculation, liquid cultures are superior as agar cultures as usually not uniform because the cell layers on the agar surface are differently supplied with light and nutrients (as a result of shading and diffusion).

Preparation of Culture Flasks

The following is the recommended procedure for culture flask preparation. Wash with detergent and rinse thoroughly with tap water. Rinse with a ten percent solution (by volume) of reagent grade hydrochloric acid (HCl) by swirling the HCl solution so that the entire inner surface is covered. The glassware should be rinsed very well with distilled water. Rinse the flasks with particle-free distilled water (that is, filtered through a 0.22-μm membrane filter), and cover with 50-ml beakers. Autoclave for 20 min at 1.05 kg/cm^2 (15 psi) and 121°C, and dry in oven at 50°C. Cooled flasks and beakers are stored in closed cabinets until used.

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Part 3. Glossary

Acarina, acari (n, pl). An Order of Arachnida 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 both precision and bias. (Am. Soc. Testing and Materials, 1975).

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 amount of algae that a water is capable of sustaining.

Alga, algae (n), **algal** (adj). A group of plants, mostly aquatic, single-celled, colonial, or multi-celled, containing chlorophyll and lacking roots, stems and leaves.

Algal bloom (n). A large number of a particular algal species, often amounting to 0.5 to 1 million cells per liter.

Allochthonous (adj). Originating outside the area under consideration. *See also* **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 utilization by heterotrophs without regard to its fate; secondary productivity plus respiration and other losses. *See also* **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 under consideration. *See also* **Allochthonous.**

Autotroph (n), **autotrophic** (adj). An organisms, such as in 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, 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 organisms living in or on the bottom of an aquatic environment.

Bias (n). A persistent positive or negative deviation of the method average value from the true value (Am. Soc. Testting and Materials, 1975).

Bioassay (n). The use of living organisms to test the effects of a substance. *See also* **Toxicity bioassay.**

Biology (n), **biological** (adj). The science or study of organisms.

Biomass (n). The amount 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 relationships.

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 microorganisms 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 fishes and aquatic insects.

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. *See also* **Taxonomy.**

Coliform bacteria (n). A particular group of bacteria used as indicators of possible sewage pollution. They are formally characterized as aerobic, and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria which ferment lactose with gas formation within 48 hours at 35°C.

Community (n). Any naturally occurring group of different organisms inhabiting a common environment and interacting with one another through food relationships.

Compensation level or depth (n). The depth of water at which gross photosynthesis (oxygen production) balances respiration (oxygen uptake) over a 24-hour period.

Concentration (n). The weight or number per unit volume or area of a water-quality constituent or characteristic.

Contagious (adj). A clumped or patchy pattern of distribution. *See also* **Negative binomial.**

Culture (n, v). Cultivation of or act of cultivating living material such as microorganisms 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 in the oxidation of organic matter with the evolution of gaseous nitrogen.

Detritivore (n). An animal that obtains its nourishment by consuming organic detritus; includes many 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, often used as a synonym for diel.

Division (n). The primary taxonomic category of the plant kingdom, consisting of classes. *See also* **Taxonomy.**

Dorsum (n), **dorsal** (adj). The upper surface of an organism. *See also* **Ventrum.**

Dredge (n). An instrument pulled across or through the bottom of a lake or stream to sample the benthos. *See also* **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 with the physical and chemical environment.

Emersed plant (n). A rooted aquatic plant with 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 and influences that affect the life and development of an organism.

Epilimnion (n). The upper, relatively warm, circulating zone of water in a thermally stratified lake.

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 waters in which primary production is high as a consequence of a large supply of available nutrients. *See also* **Oligotrophic.**

Facultative (adj). Able to live and grow under more than one set of conditions. *See also* **Obligate.**

Family (n). The taxonomic category below order consisting of genera. *See also* **Taxonomy.**

Fauna (n), **faunal** (adj). A collective term for all the kinds of animals in an area. *See also* **Flora.**

Fecal coliform bacteria (n). That part of the coliform group that is present in the gut or the feces of warmblooded animals; they are indicators of possible sewage pollution.

Fecal streptococcal bacteria (n). A particular group of bacteria found in the gut of warmblooded animals; their presence in natural waters is considered to verify fecal pollution. They are formally characterized as gram-positive, cocci bacteria which 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. *See also* **Fauna.**

Food chain (n). The transfer of food energy from the source in plants through a series of organisms with repeated eating and being eaten (Odum, 1971). *See also* **Food web.**

Food web (n). The interconnecting pattern of food chains. *See also* **Food chain.**

Formalin (n). A clear aqueous solution containing about 37 percent formaldehyde by volume and 5–10 percent methanol; when diluted with water, it is used as a general biological preservative.

Fungus, fungi (n). Plants lacking chlorophyll including molds, yeasts, 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. *See also* **Taxonomy.**

Generation (n). A group of organisms of 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. *See also* **Dredge.**

Grab sample (n). A sample of something collected by any method without regard to time.

Greenhouse effect (n). An increase in temperature within a glass or plastic enclosure ascribed to the fact that short-wave solar radiation enters 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, from among 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 up in respiration within 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 under 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 animals and many 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, non-circulating water zone in a thermally stratified lake.

Incubation (n). Maintenance of organisms under conditions favorable for growth and development.

Interpretive (adj). A type of sampling program or study designed to collect information useful in describing a system and cause and effect relationships within the system.

Invertebrate (n). An animal without a backbone. Common aquatic examples include worms, insects, snails, and crayfish.

Kingdom (n). The highest biological classification category. *See also* **Taxonomy.**

Larva, larvae (n), **larval** (adj). An active immature stage of an animal during which its bodily form differs from that of the adult. *See also* **Nymph.**

Lentic (adj). Of or pertaining to nonflowing water, for example, lake or pond.

Life history (n). The environmental relationships 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 inalnd waters; the ecology of inland waters.

Littoral (n, adj). Pertaining to the shallow zone of a body of water where light penetrates to the bottom.

Liverwort (n). *See* **Bryophyta.**

Lotic (adj). Of or pertaining to flowing water, for example, 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. *See also* **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. *See also* **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 life form; the physical attributes of an organism.

Morphometry (n), **morphometric** (adj). The measurement of external form.

Moss (n). *See* **Bryophyta.**

Negative binomial (n). A mathematical expression which represents the occurrence of organisms in clumps, separated by less densely populated areas. *See also* **Contagious.**

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 under study during the period of measurement; *net primary productivity* minus *heterotrophic* utilization.

Net primary productivity (n). The rate of storage of photosynthetically produced organic matter in plant tissues in excess of the respiratory utilization 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 microorganisms in culture. The medium may be in liquid form, called broth, or may be solidified with agar.

Nymph (n), **nymphal** (adj). An immature stage of an insect that resembles the adult stage in bodily form. *See also* **Larvae.**

Obligate (adj). Restricted to living and growing under a single life condition. *See also* **Facultative.**

Oligothrophic (adj). Pertaining to waters in which primary production is low as a consequence of a small supply of available nutrients. *See also* **Eutrophic.**

Order (n). The taxonomic category below class consisting of families. *See also* **Taxonomy.**

Organism (n). Any living entity.

Pathogen (n), **pathogenic** (adj). A disease-causing organism.

Periphyton (n), **periphytic** (adj). The community of microorganisms that are attached to or live upon 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 bio-chemical 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. *See also* **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 which drift passively with water currents.

Poikilothermic organism (n). An animal whose body temperature approximates that of the environment; often 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). *See also* **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 agreement of repeated measurements of the same property, expressed in terms of dispersion of

test results about the mean result obtained by repetitive testing of a homogeneous sample(s) under specific conditions (Am. Soc. Testing and Materials, 1975).

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 which can be used as food materials (Odum, 1971, p. 43). *See also* **Gross primary productivity, Net primary productivity, Net community productivity, and Secondary productivity.**

Production (n). The total amount of living matter produced in an area per unit time regardless of the fate of the living matter. *See also* **Primary productivity** and **Secondary productivity.**

Profundal (adj). Referring to the deepwater 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). The biological kingdom consisting of unicellular (single-celled) organisms.

Protoplast (n). The living contents of a cell; the nucleous, cytoplasm, and plasma membrance which 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 tranforms into the adult. *See also* **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 in an unbiased manner so that every part of the population or area has an equal chance of being selected.

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 liberated 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, 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. *See also* **Assimilation** and **Primary productivity.**

Sediment (n). Fragmental material, both mineral and organic, that is in suspension or is being 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. and 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. *See also* **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 amount of living matter present at any given time, expressed as the number or weight per unit area or volume of habitat. Same as *biomass*.

Statistical population (n). The whole aggregate of something within an area being sampled.

Stratified water (n). A body of water having a series of horizontal strata. *See also* **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 upon which something lives.

Suspended sediment (n). Fragmental material, both mineral and organic, that is maintained in suspension in water by the upward components of 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 upon a hierarchical scheme beginning with the species at the base. The higher the classification level, the fewer features the organisms have in common. *See also* **Species.** As an example, the taxonomy of the common stonefly, *Pteronarcys californica* is as follows:

Kingdom	*Animal*
Phylum	*Arthropodal*
Class	*Insecta*
Order	*Plecoptera*
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 metallimnion.

Toxicity bioassay (n). Determination of the potency of a toxic substance by measuring the intensity of a biological response. *See also* **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 their allies and seed plants; aquatic representatitves may be rooted or may float in or on the water.

Ventrum (n), **ventral** (adj). The lower surface of an organism. *See also* **Dorsum.**

Vertebrate (n). An animal with a backbone enclosing a nerve cord; aquatic examples include fishes 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 water 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) *See also* **Pollution.**

Water quality (n). Kinds and amounts of matter dissolved and suspended in natural waters, the physical characteristics of the waters, and the ecological relationships between aquatic organisms and the environment.

Water weed (n). A popular term for an aquatic plant, usually one of the macrophytes.

Yield (n). The amount (weight or number) of *biomass* removed from a given aquatic area in a given time. (Same as crop or harvest.)

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 4.
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. Table 11 relates the taxonomic groups to the methods presented in part 2 of this chapter.

Table II—Taxonomic group(s) of greatest significance for the methods in Part 2

Method	Taxonomic group(s)
Bacteria	Bacteria and fungi
Phytoplankton	Algae
Zooplankton	Protozoa (including Flatellates) Coelenterata Rotifera "Smaller" Crustacea Diptera
Periphyton	Bacteria and fungi Algae Protozoa (including 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

Table II—Taxonomic group(s) of greatest significance for the methods in Part 2—Continued

GENERAL TAXONOMIC REFERENCES—Marine

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