# EXTRACTION AND ANALYSIS OF ADENOSINE TRIPHOSPHATE FROM AQUATIC ENVIRONMENTS

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U.S. GEOLOGICAL SURVEY

Water-Resources Investigations 81-5

50272-101

| REPORT DOCUMENTATION PAGE | 1. REPORT NO. | 2. | 3. Recipient's Accession No. |
|---|---|---|---|

**4. Title and Subtitle**
EXTRACTION AND ANALYSIS OF ADENOSINE TRIPHOSPHATE FROM AQUATIC ENVIRONMENTS

**5. Report Date**
April 1981

**6.**

**7. Author(s)**
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**8. Performing Organization Rept. No.**
USGS/WRI-81-5

**9. Performing Organization Name and Address**
U.S. Geological Survey, Water Resources Division
Gulf Coast Hydroscience Center
National Space Technology Laboratories
NSTL Station, MS 39529

**10. Project/Task/Work Unit No.**

**11. Contract(C) or Grant(G) No.**
(C)
(G)

**12. Sponsoring Organization Name and Address**
U.S. Geological Survey, Water Resources Division
Gulf Coast Hydroscience Center
National Space Technology Laboratories
NSTL Station, MS 39529

**13. Type of Report & Period Covered**
Progress Report

**14.**

**15. Supplementary Notes**

**16. Abstract (Limit: 200 words)**

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**17. Document Analysis a. Descriptors**
*Bacteria, *Bioluminescence, *Periphyton, *Sediments, Analysis, Aquatic microbiology, Biomass

**b. Identifiers/Open-Ended Terms**
*Adenosine triphosphate, Extraction, Acid extractants

**c. COSATI Field/Group**

**18. Availability Statement**
No restriction on distribution

**19. Security Class (This Report)**
UNCLASSIFIED

**20. Security Class (This Page)**
UNCLASSIFIED

**21. No. of Pages**
33

**22. Price**

(See ANSI-Z39.18) *See Instructions on Reverse*

**OPTIONAL FORM 272** (4-7)
(Formerly NTIS-35)
Department of Commerce

UNITED STATES DEPARTMENT OF THE INTERIOR

JAMES G. WATT, Secretary

GEOLOGICAL SURVEY

Doyle G. Frederick, Acting Director

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# EXTRACTION AND ANALYSIS OF ADENOSINE TRIPHOSPHATE FROM AQUATIC ENVIRONMENTS

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## ABSTRACT

A variety of adenosine triphosphate (ATP) extraction procedures have been investigated for their applicability to samples from aquatic environments. The cold sulfuric-oxalic acid procedure was best suited to samples consisting of water, periphyton, and sediments. Due to cation and fulvic acid interferences, a spike with a known quantity of ATP was necessary to estimate losses when sediments were extracted. Variable colonization densities for periphyton required that several replicates be extracted to characterize accurately the periphyton community. Extracted samples were stable at room temperature for one to five hours, depending on the ATP concentration, if the pH was below 2. Neutralized samples which were quick frozen and stored at -30°C were stable for months.

## INTRODUCTION

Adenosine triphosphate (ATP) is present in all living cells where it is an essential component in energy transfer and metabolism processes. It is rapidly degraded to mono- and di-phosphate nucleotides in dead or ruptured cells (Holm-Hansen and Booth, 1966). This property makes it a useful specific indicator of living cells, allowing quantification of biomass in aquatic systems.

Over the past few years, ATP has been used to determine the biomass of bacteria (Hamilton and Holm-Hansen, 1967), zooplankton (Traganza and Graham, 1977), fungi (Qureshi and Patel, 1976), and algae (Holm-Hansen, 1970), as a method for characterizing sediments and soils in environmental samples (Lee and others, 1971; Hersman and Temple, 1978) and as a natural tracer for dissolved organic matter in seawater (Hodson and Azam, 1977).

Adenosine triphosphate measurements have a wide range of application in water quality studies and have been used specifically to monitor standing crop and trophic level in reservoirs (Stanford, 1977; Robinson, 1975), nutrient limitation (Cavari, 1976), distribution of size classes of plankton in lakes (Rudd and Hamilton, 1973), and as a bioassay response factor (Brezonik and others, 1975). A principle problem in ATP methodology is extracting it from environmental samples. The compound is very labile and extraction procedures must rupture cells, release the ATP,

inactivate normally present ATP'ase enzymes, and preserve the compound in such a state as to prevent hydrolysis. Due to these requirements and the diverse nature of the samples, many extraction techniques have been proposed.

The following paper will compare and evaluate various specific extraction procedures for:

1) Water samples
2) Sediment samples
3) Periphyton samples.

Our criteria in evaluating extraction techniques was to find the technique for each of the above types of samples that was:

1) Quantitative
2) Precise
3) Adaptable to a field sampling procedure.

Several extraction techniques were evaluated for each category of sample, methods were modified where necessary, and a working procedure for extraction, storage, and analysis of ATP was established.

## EXTRACTION OF SAMPLES

Sample extractions generally fall into one of three categories: boiling buffer; acid; and organic solvents. Historically, boiling Tris buffer (pH 7.75) has been the most popular method (Holm-Hansen and Booth, 1966; Qureshi and Patel, 1976; Brezonik and others, 1975; Paerl and Williams, 1976). However, bicarbonate buffer ($NaHCO_3$, pH 8.5) has been used on soils (Hersman and Temple, 1978), and marine sediments (Bancraft and others, 1976), and glycine extraction has been proposed by Afghan and others (1977). Sodium phosphate buffer with citric acid at pH 7.70 has also been used on sediments (Bulleid, 1978). Boiling buffers work well for liquid samples but may not be effective for particulates. Cooling of the boiling buffer below 90-95°C by addition of the sample has been shown to greatly reduce the efficiency of the extraction (Patterson and others, 1970). Karl and LaRock (1975b) also reported reduced recoveries of an ATP spike in boiling Tris extractions relative to sulfuric acid extraction. In an attempt to overcome the problems of boiling buffer extraction of particulate matter such as sediments, several cold acid extraction techniques have been developed, many following the procedure of Lee and others (1971) using 0.6 N sulfuric acid. Perchloric and nitric acids (.1 and 1 N) were evaluated as successful for bacterial populations by Knust and others (1975) and perchloric acid has been used on sediments (Bancroft and others, 1976). Several problems are encountered when acid extraction methods are used, particularly on sediments: (1) solubilization of metal cations such as $Ca^{++}$ occurs which interferes in the analysis; (2) fulvic acids are also solubilized which then bind to the extracted ATP making it unavailable for assay; (3) the pH is lowered below the narrow range at which the luciferin-luciferase assay reaction

will occur; and (4) carbonates in the sediments neutralize the acid. The problem with solubilization of metals has been overcome by use of exchange resins (Lee and others, 1971), chelation with EDTA (Karl and LaRock, 1975a), or precipitation with oxalic acid (Lee and others, 1971). Binding of fulvic acids was investigated by Cunningham and Wetzel (1978) and found to range from 10 percent ATP loss at 0.1 g/L fulvic acid to 80 percent loss at 1 g/L. This problem necessitates the addition of a known quantity of ATP to allow estimation of the degree of binding. Neutralization of the acid extracted ATP to pH 7.75 with ammonium hydroxide provides a ready solution to the third problem of acid extractions, and increasing the normality of the acid will overcome the problems with high-carbonate containing sediments. A procedure also exists (Hodson and others, 1976) which utilizes activated charcoal columns to remove acid-extracted ATP from a sample. This method removes the interferences caused by dissolved ions, eliminates the neutralization step, and allows concentration of dilute quantities of ATP. Its principal disadvantages are the increased time and manipulations required for extraction.

Techniques using organic or inorganic solvents have been developed to meet the need for a single extraction step for several compounds. The use of dimethylsulfoxide (DMSO), formamide, n-butanol, chloroform, ethanol, methanol, acetone, nitric and perchloric acids, and methylene chloride was investigated by Knust and others (1975) using bacterial systems. It was concluded that acetone performed equally as well as nitric and perchloric acids but must be completely evaporated prior to assay. The 40 minutes required to evaporate 5 ml of acetone greatly lengthened total extraction time. Clark and others (1978) found that a modified acetone extraction of periphyton could also be used to extract chlorophyll. Shoaf and Lium (1976) reported DMSO extraction of algal cells to be quantitative and more convenient than boiling Tris or butanol-octanol extractions. Trisodium phosphate (pH 11.7) combined with chloroform has been used on sediments and organic sludges by Tobin and others (1978).

Several disadvantages are evident in solvent extraction methods. In addition to the increased time required for the removal of the organic solvent prior to analysis, many of the organic solvents are very flammable, produce toxic vapors, and are hazardous to handle, particularly under field conditions.

## LIQUID PHASE EXTRACTION PROCEDURES

The extraction of ATP from water samples or growth media represents the least difficult procedure in ATP methodology. Generally, interfering substances such as metal cations are present in low concentrations and there is a minimum of particulate material. Under these conditions, most extraction methods are applicable. A boiling Tris buffer extraction followed by cold-quenching the sample and freezing in liquid nitrogen is effective for some samples. A satisfactory procedure consists of

bringing 10 ml of .05 M Tris buffer (pH 7.75), containing a glass bead, to boil in a 15 ml centrifuge tube, adding 1 ml of liquid sample and boiling for 5 minutes. The tube is removed from the hot sand bath, quenched in cold water, and water lost through evaporation is replaced with ultra-pure water. The sample is then made to 15 ml with Tris buffer. A small aliquot of the sample is placed in a 1.5-ml snap-cap plastic vial and frozen in liquid nitrogen. However, when samples are concentrated through centrifugation or filtration a boiling extraction may not be applicable. Azam and Hodson (1977) showed that use of filtration to concentrate ATP from seawater samples followed by immersion of the filter in boiling buffer resulted in lower recoveries of ATP than an acid extraction of the filter.

During ATP analysis of natural stream samples, light emission peaks were observed to be rounded and peak areas were reduced in some samples extracted using boiling Tris buffer. The initial procedure for boiling Tris extractions involved use of a marble boiling chip (Boileezer,[1/] Fisher Scientific) to prevent bumping in the extraction tube. These rock chips are relatively inert, but do contain calcium. Several experiments were conducted to determine if material leached from the chip interfered with the ATP assay and the extent of the interference.

The highest recovery of an ATP spike was observed in the non-boiled Tris extraction using no boiling chip and in both boiled Tris and boiled Glycine-EDTA buffers which used glass beads as boiling chips. Apparently, a sufficient amount of calcium leaches from the marble Boileezer chip and binds with the ATP rendering it unreactive with the luciferin-luciferase substrate. Calcium has been observed to be inhibitory to the ATP light reaction by Aledort and others (1966) and Karl and LaRock (1975b) found that a 100 millimolar calcium solution inhibits 97-100 percent of the ATP light reaction.

It was observed that boiling Tris extractions of bacteria from a rich growth media did not exhibit reductions in peak area or peak rounding when Boileezer chips were used. An ATP spike was added to complete growth media and a phosphate-free media and extracted using boiling Tris with marble chips and also with glass beads. The mean recovery for marble chip treated complete media was 103 percent, for phosphate-free media it was only 27 percent. Recovery for phosphate-free media using a glass bead was 107 percent and for spiked distilled water using a glass bead was 96 percent. Clearly, the phosphate in the growth media reacted with the calcium released from the boiling chip and prevented it from interfering with the ATP light reaction. Few environmental systems have abundant phosphate so routine boiling extractions should be done using glass beads or Teflon boiling chips instead of rock mineral chips which may leach calcium.

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[1/] The use of the brand names used in this report are for identification purposes only and does not imply endorsement by the U.S. Geological Survey.

Quantitative extractions of liquid samples are also possible using a cold sulfuric-oxalic acid extraction method modified from the procedure of Cunningham and Wetzel (1978). The addition of a cold liquid sample to a boiling buffer lowers the temperature (cold quenching) and may prevent rapid destruction of ATP hydrolyzing enzymes. A cold acid extraction circumvents this problem but requires that the pH and ionic strength be controlled. A comparison was made between boiling Tris buffer extraction and the sulfuric-oxalic acid method in one experiment.

Several liquid samples were extracted for 10 minutes in boiling Tris buffer and also using the cold sulfuric-oxalic acid procedure. The acid extraction resulted in 13 and 18 percent greater ATP values for two concentrations of a bacterial culture and 77 percent greater for a local stream sample. Student's t test on the means of triplicate extractions indicated there was a significant difference ($\alpha = .05$) in the ATP results for each extraction category. This indicates that the boiling Tris extraction is useable for laboratory cultures if care is exercised to prevent cold quenching, but the sulfuric-oxalic acid method is more appropriate for environmental samples where inhibitory substances such as calcium may be present. Figure 1 is a flow chart of the suggested cold sulfuric-oxalic acid extraction method.

## COMPARISON OF SEDIMENT EXTRACTION METHODS

Boiling extraction methods have been reported to be less efficient on particulate material such as sediments or periphyton because the addition of the sample cold quenches the buffer and allows hydrolysis of the ATP (Karl and LaRock, 1975b). In an earlier experiment the authors noted that boiling Tris extraction of particulate algal material resulted in one-third less ATP than a cold acid extraction. Further evaluation of sediment processing methods were therefore limited to cold acids and solvents.

The cold trisodium phosphate-chloroform extraction procedure of Afghan and others (1977) was applied to six 2-gram splits of a homogenized organic-rich clay sediment from a local stream. Several problems were encountered in the extraction. Vacuum filtration (> 720 mm Hg) is used to clarify the extractant and boil off the chloroform. It was found that (1) the chloroform was difficult to boil off and caused considerable foaming and splashing in the filtration flask; (2) the light cellulose plant debris material in the sediment failed to spin down during centrifugation and subsequently plugged the glass fiber filter; and (3) there was considerable brown coloring in the extracts, even after filtering through 0.2 µm filters. Three of the six splits were spiked with a 21 ng ATP standard prior to extraction. All samples were made to a constant volume with 0.1 M glycine and 0.1 M EDTA and frozen in liquid nitrogen.

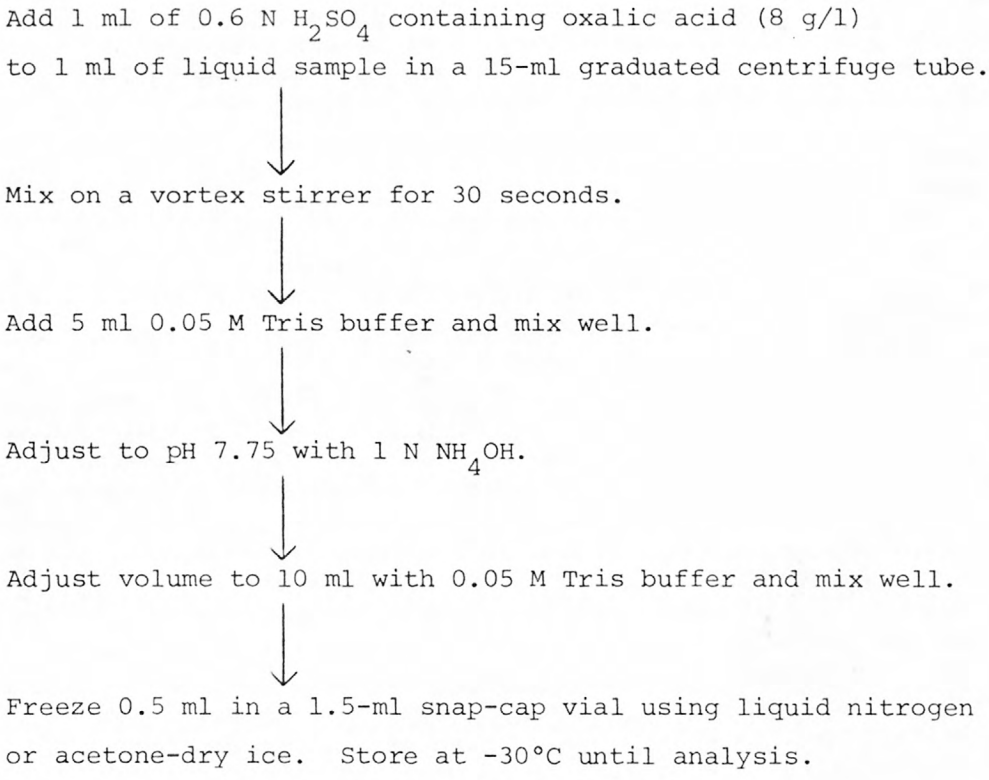


Figure 1.--Suggested procedure for the extraction of ATP from liquid samples using the sulfuric-oxalic acid method.

Upon analysis, the sediment samples averaged 1 ng/ml (4.5 ng/g) and the average recovery from the spike was only 0.3 percent. The near complete loss of ATP from the sediment samples was believed due to fulvic materials binding with the ATP as reported by Cunningham and Wetzel (1978).

The trisodium phosphate-chloroform extraction method was repeated using another set of six splits of sediment with an organic content of 1.8 percent which had been homogenized using a Polytron (Brinkman Instruments). In this experiment, each split was divided into two samples after the filtration step and a 36 ng ATP standard was added to one sample of each pair in an attempt to quantify the effects of the fulvic material. The sediment samples averaged 39 ng ATP per g dry weight with a coefficient of variation (standard deviation/mean) of 27 percent and a range of 28.01 to 52.02 ng/g. Recovery of the spike ranged from 43 percent to 75 percent with a mean of 62 percent. The recovery showed a linear inverse relationship to the dry weight of the extracted sediment (fig. 2) with a correlation coefficient of -0.97 and a standard error of y on x of 2.9 percent. It is evident that some form of spiking must be used to estimate the loss of ATP from sediment extractions.

An alternative to solvent extraction procedures appeared to be desirable to eliminate the solvent removal step and decrease the variability. The use of an acid extraction method was investigated for sediments.

Five 0.5-g aliquots of a sterile, dry, stream sediment were each spiked with 200 ng of ATP standard and extracted using the sulfuric-oxalic acid procedure of Cunningham and Wetzel (1978). Samples were adjusted to pH 7.75 with 1 N ammonium hydroxide and frozen in liquid nitrogen. The recovered ATP ranged from 27 ng (14 percent recovery) to 45 ng (25 percent recovery) with a mean of 37.2 ng (19 percent recovery) and a coefficient of variation of 18 percent. An unspiked, sterile sediment blank contained less than 0.5 ng ATP. The low recoveries are believed to be the result of fulvic acid interferences which were also observed for the trisodium phosphate-chloroform method. An additional experiment was run to investigate the relationship between weight of the sediment extracted and recovery of an ATP spike. Three replicates each of four weights (0.1, 0.4, 0.8, and 1.0 g) of sterile, natural sediment were spiked with a 200 ng ATP standard and extracted using the sulfuric-oxalic acid procedure. Again, the greater the weight of sediment extracted, the lower the recovery of ATP spike (fig. 3). Recovery averaged 55 percent for 0.1 g of sediment to 14 percent for 1.5 g of sediment. A 200 ng spike in the absence of sediment was 100 percent recovered. Hodson and others (1976) also reported a similar decrease in ATP recovery from sediments with increasing sediment to extractant ratios.

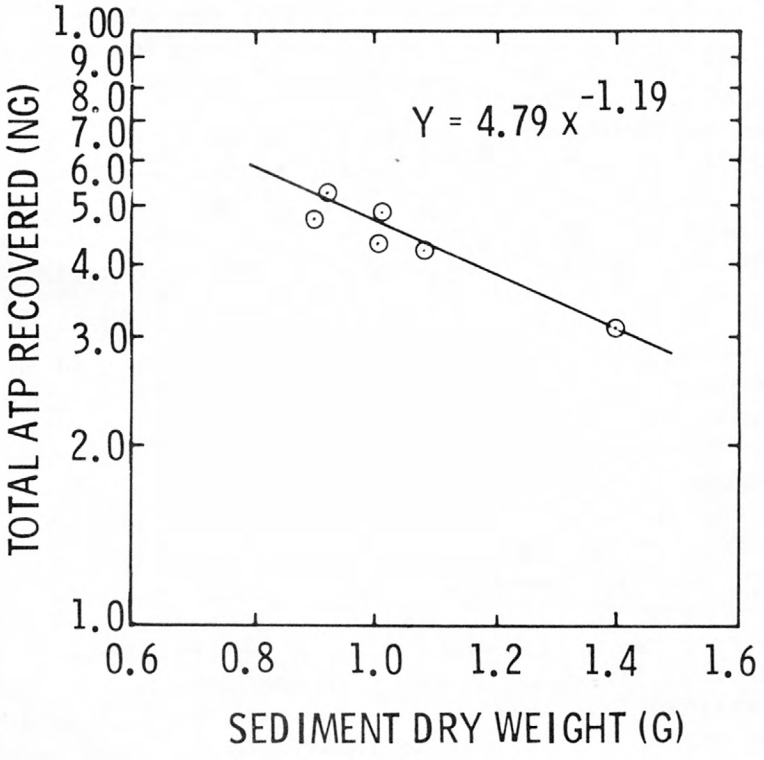


Figure 2.--Recovery of a 36-ng ATP spike as a function of the weight of an organic sediment extracted using the trisodium phosphate-chloroform procedure. Each point is representative of a single determination.

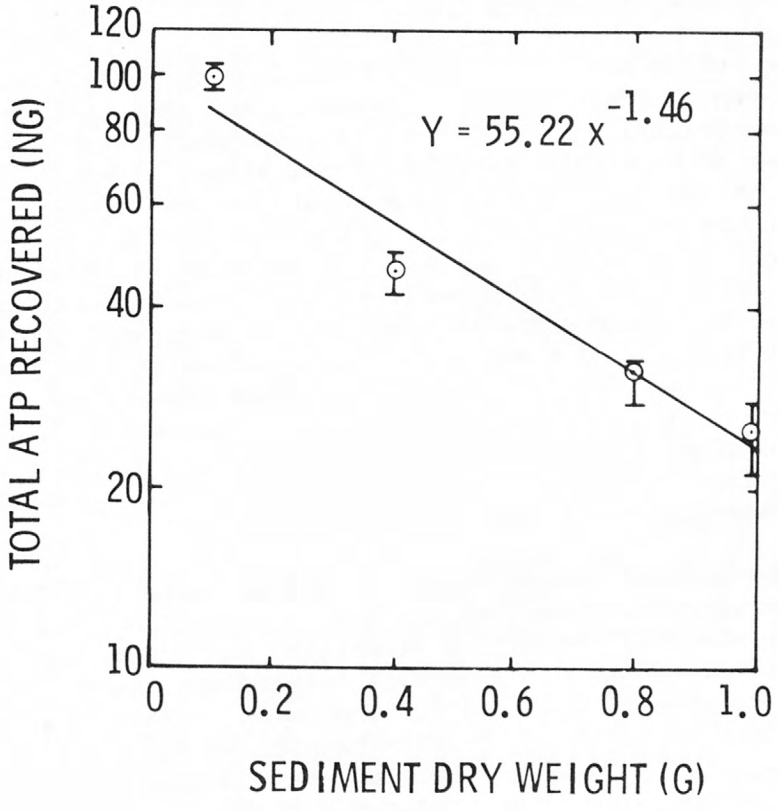


Figure 3.--Recovery of a 200-ng ATP spike as a function of the dry weight of a sterile sediment extracted using the sulfuric-oxalic acid method. Points represent the means of three replicates with the range indicated.

The relationship between sediment dry weight and recovery of ATP using the sulfuric-oxalic acid method fit an equation of the form

$$y = y_o e^{-kS}$$

where

$y$ = amount of the ATP spike recovered,
$y_o$ = amount of spike added,
$S$ = sediment dry weight,
$k$ = slope of a semi-logarithmic line.

The correlation coefficient was 0.96.

A boiling buffer extraction method was investigated in an attempt to reduce the amount of fulvic acid extracted and thus minimize the interference associated with this material. Extraction of ATP from six splits of about 50 mg of a homogenized local stream sediment was investigated using boiling McIlvaine buffer (0.04 M $Na_2HPO_4$ to pH 7.7 with citric acid) as suggested by Bulleid (1978). Three of the six samples were spiked with 36 ng ATP prior to boiling to determine ATP loss due to procedure. Analysis indicated a variable recovery of the spike ranging from 52 percent to 72 percent with a mean of 61 percent. The sediments were assayed and contained a mean ATP concentration of 58 ng/mg sediment dry weight, with a range of 47 to 68 ng/mg. The greatest recovery of the spike occurred in the sample containing the least amount of sediment indicating the presence of interfering substances or sorption by the sediment. The lack of a linear relationship may indicate sorptive losses due to surface area related phenomena.

Sediments represent the most difficult substrates for ATP extraction due to the high content of cations, anions, and humic materials which co-extract with the ATP, reduce its stability, and interfere with the analytical light reaction. A summary of comparisons are given in table 1. Acceptable recoveries of added ATP and lower variability were obtained with the trisodium phosphate-chloroform method and the sulfuric-oxalic acid method. Due to the tedious procedure of removing the chloroform from the samples, the authors adopted the sulfuric-oxalic acid extraction procedure for routine use. It is mandatory that an internal standard of ATP be added to one split following centrifugation to estimate losses due to fulvic acid interferences (Cunningham and Wetzel, 1978). The suggested procedure for sediment extractions appears in figure 4. It is desirable to use the least amount of sediment that will provide sufficient ATP for analysis to minimize the quantity of interfering substances extracted with the ATP.

## COMPARISON OF PERIPHYTON EXTRACTION METHODS

The extraction of ATP from a laboratory culture of the alga Chlorella using dimethyl sulfoxide (DMSO)-Tris buffer as an extractant (Shoaf and Lium, 1976) was investigated. Three 1-ml samples of an actively growing

Table 1.--Summary of ATP recoveries from sediment using various extraction methods

| Extraction method | Sediment dry weight (mg) | Sediment type | ATP present | Number of replicates | Spike recovery | |
|---|---|---|---|---|---|---|
| | | | | | $\bar{x}$ (percent) | Coefficient of variation (percent) |
| Boiling McIlvaine buffer | 50 | Natural, sand-clay | 47-68 ng/mg | 6 | 61 | 17 |
| Cold tri-sodium phosphate chloroform | 1000 | Natural, sandy | .03-.05 ng/mg | 6 | 62 | 18 |
| Cold tri-sodium phosphate chloroform | 500 | Sterile natural, clay | 200 ng spike | 5 | 81 | 7 |
| Cold sulfuric-oxalic acid | 100 | Sterile natural, clay | 200 ng spike | 3 | 55 | 8 |
| Cold sulfuric-oxalic acid | 400 | Sterile natural, clay | 200 ng spike | 3 | 25 | 16 |

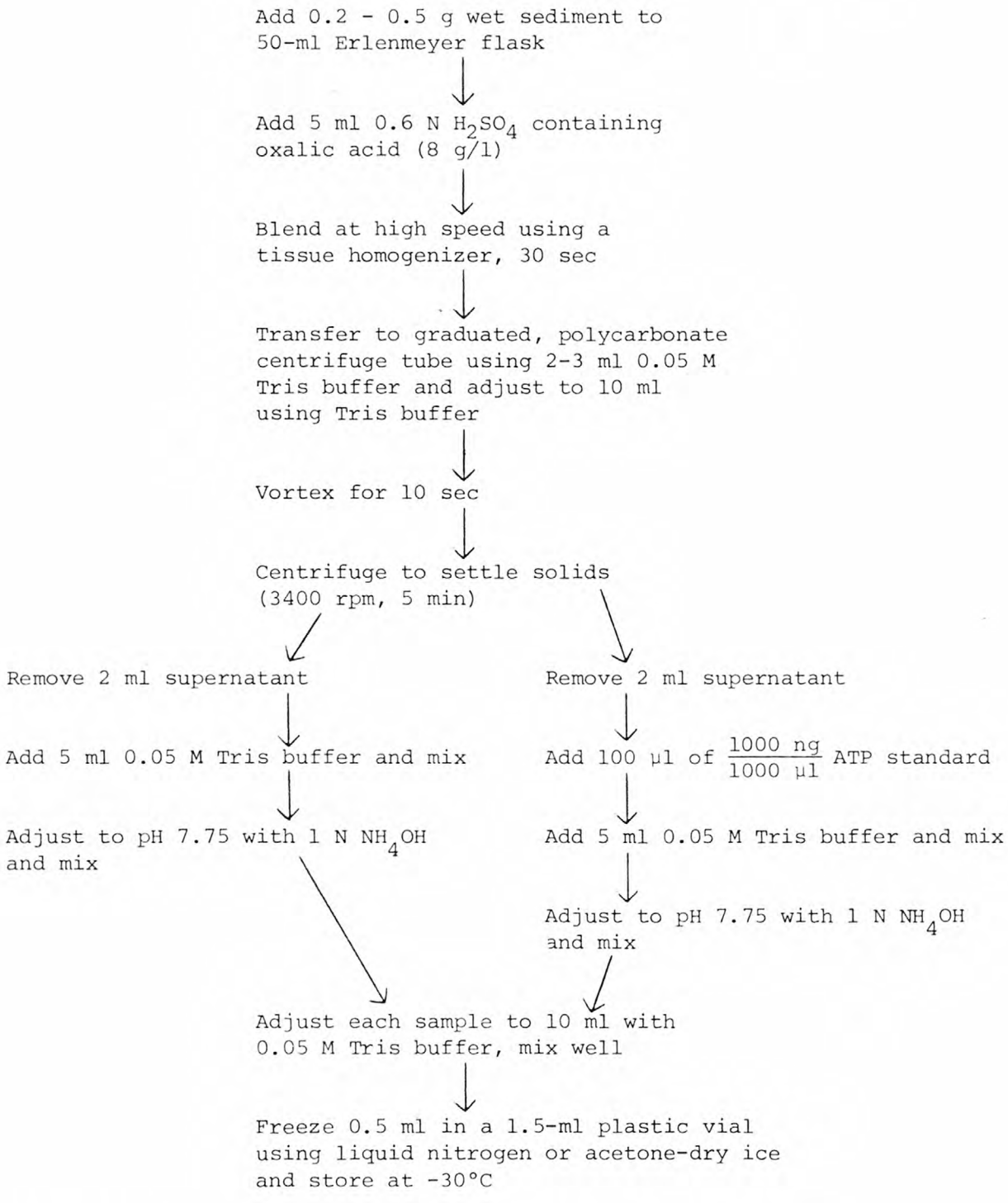


Figure 4.--Suggested ATP extraction procedure for sediments. (Adapted from Cunningham and Wetzel, 1978.)

culture were extracted to give an indication of precision of the technique. An additional three samples with one-half the cell concentration were extracted as an indicator of the quantitative nature of the method. Three additional samples were spiked with 10 ng of ATP standard. The mean ATP content of the first set of samples was 22.6 ng/ml with a coefficient of variation of 8 percent. The second set had an ATP content of 13.1 ng/ml with a coefficient of variation of 16 percent. Therefore, the method appeared to be quantitative because the observed mean of 13.1 ng/ml was not significantly different (t test, 5 percent level, 1 tail) from the expected mean of 11.3.

This technique required filtration to concentrate the cells, a procedure which may lead to substantial losses in ATP (Holm-Hansen, 1975). The extraction efficiency of the DMSO-Tris method was compared to boiling Tris buffer extractions of 1-ml liquid samples of bacteria and of stream water. The Tris extractions averaged 43 percent greater ATP for two bacterial extractions and from four to ten times greater for four stream water samples compared to the DMSO-Tris method. Recovery of a series of ATP spikes of varying concentration in five stream water samples using the DMSO-Tris procedure was variable, ranging from 61 percent to 153 percent. It was concluded that the boiling Tris method was superior to the DMSO-Tris method for the extraction of algal material.

The glycine-EDTA procedure of Afghan and others (1977) utilizing 0.1 M glycine adjusted to pH 10 with NaOH and containing 0.01 M Mg-EDTA as a boiling extractant was investigated. Triplicate extractions of 1 ml each of a lab culture of Chlorella or of a bacterial culture taken from a respirometer were added directly to 13 ml of boiling buffer and extracted 5 minutes. The same type of samples were also extracted using boiling 0.05 M Tris buffer (pH 7.75) for comparative purposes. In addition, one ml of Chlorella was spotted on a 0.2 μm, 25 mm-diameter filter which was then extracted in boiling glycine-EDTA to simulate a filtered sample. The results are summarized in table 2. A one-way analysis of variance test indicated there was a significant difference ($\alpha = 0.05$) among both the Chlorella extracts and the bacterial extracts. Total ATP extracted from the Chlorella and bacteria was greater with the Tris extractions than either of the other two methods. The coefficient of variation for the bacterial extractions was very low. Variation within either the Tris or direct glycine extractions of Chlorella is within an acceptable range for particulate samples. The filtered samples extracted with glycine had considerable variation and lower extraction of ATP relative to direct extraction in boiling Tris.

The sulfuric-oxalic acid extraction procedure was used on replicate periphyton scrapings from a glass slide colonized in a local stream. Extractions of 6.5 $cm^2$, 25.8 $cm^2$, and 58.1 $cm^2$ all yielded comparable ATP values when expressed per unit area extracted. The mean for all data was 409 $ng/cm^2$ with a range of 387 to 430 and a coefficient of variation of 4 percent.

Table 2.--Comparison of replicates of three ATP extractions of algal and bacterial cultures using Tris buffer and glycine-EDTA

| Organism | Extraction method | Mean ATP extracted (ng/ml) | Coefficient of variation (percent) |
|---|---|---|---|
| Chlorella | Tris (pH 7.75) | 63 | 12 |
| Chlorella | Glycine-EDTA (pH 10) | 45 | 14 |
| Chlorella | Filtered-glycine EDTA (pH 10) | 24 | 59 |
| Bacteria | Tris (pH 7.75) | 81 | 2 |
| Bacteria | Glycine-EDTA (pH 10) | 75 | 3 |

In another experiment triplicate areas of a periphyton strip were extracted, then rinsed in distilled water and re-extracted. Initial extraction appeared to be 99 percent efficient in removing the ATP from the strips, again indicating a quantitative technique. The linear relationship between area extracted and total ATP leads us to believe the method is quantitative, at least over the range tested.

Establishing the precision of a periphyton extraction method is difficult due to the lack of uniformly colonized surfaces necessary for replicate extractions. Spatial differences in current, light, and nutrients interact to produce patchiness in surface growth on all substrates which is visable even to the casual observer. Consequently, estimates of reproducibility for a periphyton extraction method actually represent a combination of extraction precision and the variable colonization of the substrate. Several estimates of this overall precision are given for the sulfuric-oxalic acid procedure in table 3. Over-all precision may be inferred from the results of extractions on a large data set as given for the Little Lost Man Creek study where extractions of three randomly chosen segments from four periphyton strips at each of three sites were done over a three-day period. Variability in the periphyton ATP content at any one time and site is reflected in the range of the coefficient of variation values of 4 to 77 percent. The mean of all coefficient of variation values was 56 percent, the median was 35 percent.

Under field conditions it may be necessary to maintain the extracted ATP for a period in the absence of refrigeration. One experiment was conducted to investigate the stability of the acid extracted ATP over various time intervals at room temperature. Triplicate extractions were made of a 6.5-$cm^2$ periphyton area and one aliquot of the extract was immediately adjusted to pH 7.75 and frozen in liquid nitrogen. The remaining portion was left at room temperature (20°C) in an acid (pH < 2) condition. At various intervals, aliquots were removed, adjusted to pH 7.75 with 1 N $NH_4OH$, and frozen in liquid nitrogen. Storage at room temperature under acid conditions was effective for at least 5 hours for samples with a concentration of about 15 ng/ml of extract. This concentration was obtained from periphyton having 200 ng/$cm^2$. Storage intervals in excess of 5 hours resulted in considerable losses, with samples losing 45 percent of the ATP within 21 hours and 61 percent within 28 hours (table 4).

In a related experiment, three sections of a single, heavily colonized strip were extracted and stored at room temperature and at a pH below 2. At various time intervals, aliquots were removed, adjusted to pH 7.75, and quick frozen. The initial concentration of ATP of 154.2 ng/ml represents the extract concentration from a periphyton strip with an ATP content of 1300 ng/$cm^2$, considerably higher than in previous experiments. Analysis of the means (table 5) indicates there was a significant loss (14 percent) of ATP after 3.5 hours of storage. Within 26.5 hours, 52 percent of the initial ATP was lost. The loss followed first order

Table 3.--Range of estimates of precision for periphyton strips extracted using the sulfuric-oxalic acid method

| Experiment number | Substrate | Size of sample ($cm^2$) | Number of replicates | Mean ATP ($ng/cm^2$) | Coefficient of variation (percent) |
|---|---|---|---|---|---|
| 4-153A | Plastic strip | 4.5 | 3 | 650 | 31 |
| 4-153B | Plastic strip (re-extraction of 4-153A) | 4.5 | 3 | 8 | 18 |
| 4-157 | Plastic strip | 4.5 | 3 | 1285 | 5 |
| 4-140 | Plastic strip | 12.9 | 4 | 360 | 24 |
| 4-128 | Glass slide | 12.9<br>25.8<br>58.1 | 1<br>1<br>1 | 409 | 4 |
| Little Lost Man Creek (3 sites, 9 times each, over 3-day period) | Plastic strip | 2.25 | 3 | 120 | 4-77 with a mean of 56 |

Table 4.--Effect of storage time at room temperature and pH < 2 on ATP extracted from periphyton, experiment 4-147. Adjacent underlined means are not significantly ($\alpha$ = .05) different from each other when analyzed with Keuls Multiple Range test

| Strip number | ATP concentrations in ng/ml after indicated storage times (hours) | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1.8 | 3.8 | 5.3 | 21.3 | 26.3 | 27.8 |
| 1 | 14.0 | 15.7 | 11.0 | 11.3 | 6.9 | 5.7 | 4.6 |
| 2 | 15.2 | 15.4 | 16.4 | 13.7 | 9.9 | 7.9 | 7.6 |
| 3 | 11.9 | 10.3 | 10.3 | 13.4 | 6.1 | 5.2 | 3.7 |
| Mean (ng/ml) | 13.7 | 13.8 | 12.6 | 12.8 | 7.6 | 6.3 | 5.3 |
| Coefficient of variation (percent) | 12 | 22 | 27 | 10 | 26 | 23 | 39 |

Table 5.--Effect of storage time at room temperature and pH < 2 on ATP extracted from periphyton, experiment 4-157.

| Strip number | ATP concentrations in ng/ml after indicated storage times (hours) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 3.5 | 4.7 | 5.9 | 22.5 | 24 | 26.5 |
| 1 | 146.0 | 149.3 | 142.3 | 144.0 | 129.6 | 86.2 | 90.6 | 82.1 |
| 2 | 159.6 | 145.1 | 131.3 | 130.2 | 113.1 | 83.8 | 91.1 | 71.5 |
| 3 | 156.9 | 144.4 | 125.4 | 125.1 | 126.1 | 82.5 | 80.3 | 70.0 |
| Mean (ng/ml) | 154.2 | 146.3 | 133.0 | 133.1 | 122.9 | 84.2 | 87.3 | 74.5 |
| Coefficient of variation (percent) | 5 | 2 | 6 | 7 | 7 | 2 | 7 | 9 |

reaction kinetics (fig. 5) with the greatest loss in the first six hours when ATP concentrations were highest. This is contrasted with data (table 4) which indicate lower concentrations of ATP are stable for up to five hours without appreciable degradation.

It is likely that acid extracted ATP from sediments and liquid samples would exhibit the same trend. Afghan and others (1977) reported hydrolysis of extracted ATP to be greatest at neutral pH ranges. Karl and LaRock (1975a) reported acid extracted sediment ATP to be stable at least 1.5 months when neutralized and frozen, and acidified standards (< 11 ng/ml) were stable at least 65 minutes at room temperature. The authors have not experienced noticeable problems in samples frozen immediately after extraction but a delay of up to 4 hours may be tolerable for extracted samples having an ATP content less than 15 ng/ml if stored at an acid pH. Sample extracts with higher ATP content, near a concentration of 150 ng/ml range, should not be stored at room temperatures for over one hour. Periphyton extracts have been stored for months at -30°C with no significant losses, if the samples were quick-frozen immediately following neutralization.

## SUGGESTED PERIPHYTON EXTRACTION METHOD

The method used to extract ATP from periphyton (fig. 6) is a modification of the sediment procedure of Cunningham and Wetzel (1978). Typically, artificial substrates such as plastic strips are used to provide uniformity, but natural substrates may also be extracted with this method. A small segment (about 2 $cm^2$) of the substrate is cut from the original plastic strip and placed in a 50 ml beaker with a solution of 5 ml of 0.6 N $H_2SO_4$ containing 8 g oxalic acid per liter. The oxalic acid binds with any calcium in the system. Both sides of the segment are then scraped using a flat plastic or glass rod. The mixture is transferred using 1-2 ml 0.05 M Tris buffer to a 25-ml Erlenmeyer flask and homogenized for 30 sec using a high speed tissue homogenizer. Samples are transferred to 15-ml polycarbonate centrifuge tubes, made to 10 ml with 0.05 M Tris and centrifuged 5 minutes at 3400 rpm. Polycarbonate tubes or suitable high speed glass tubes are used to prevent breakage during centrifugation. A suitable aliquot (usually 2 ml) is removed, adjusted to pH 7.70 to 7.75 with 1 N $NH_4OH$ and made to 10 ml with 0.05 M Tris. Approximately 0.5 ml of sample is placed in a plastic snap-cap vial, frozen in liquid nitrogen, and stored in a freezer at -30°C. The remaining pellet in the centrifuge tube may be used for biomass determination such as dry weight and ash free weight.

## SAMPLE ANALYSIS

ATP analyses were made using a Chem-Glow photometer, Model J4-7441, and an integrator-timer, Model J4-7462 (American Instrument Co.). The integrator-timer was set to integrate the light-emission curve for 30 seconds after initiation of the reaction. A strip-chart recorder was also attached to the photometer to give a visable record of each analysis.

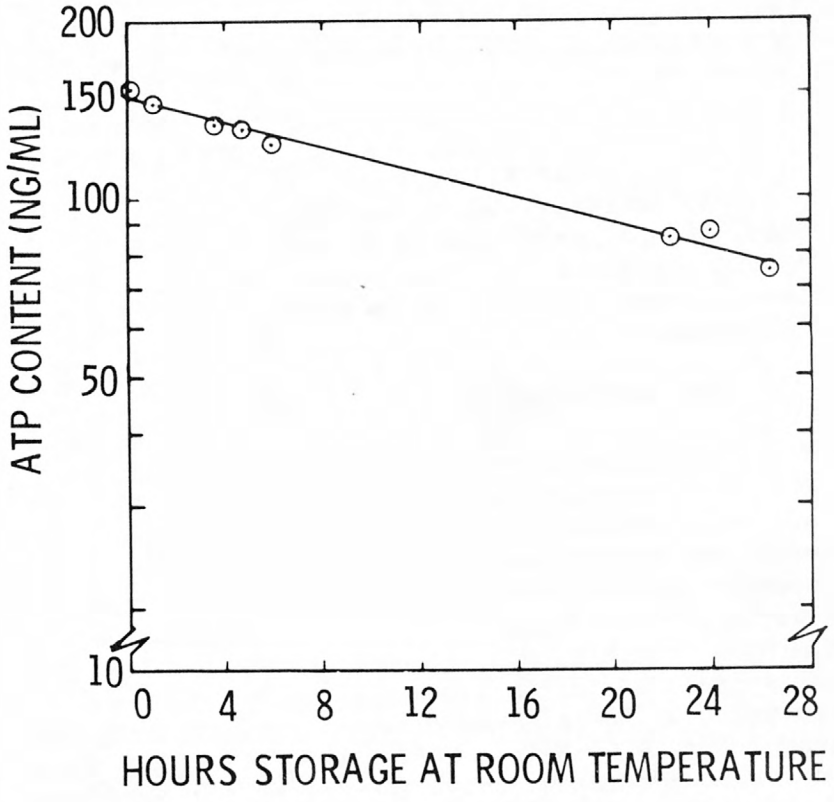


Figure 5.--The effect of storage time on ATP content of periphyton extracts stored at pH < 2 and room temperature. Initial mean concentration of ATP in the samples was 154.2 ng/ml. Points represent the mean of three replicates with the coefficient of variation never greater than 9%. The coefficient of determination for the regression was .986.

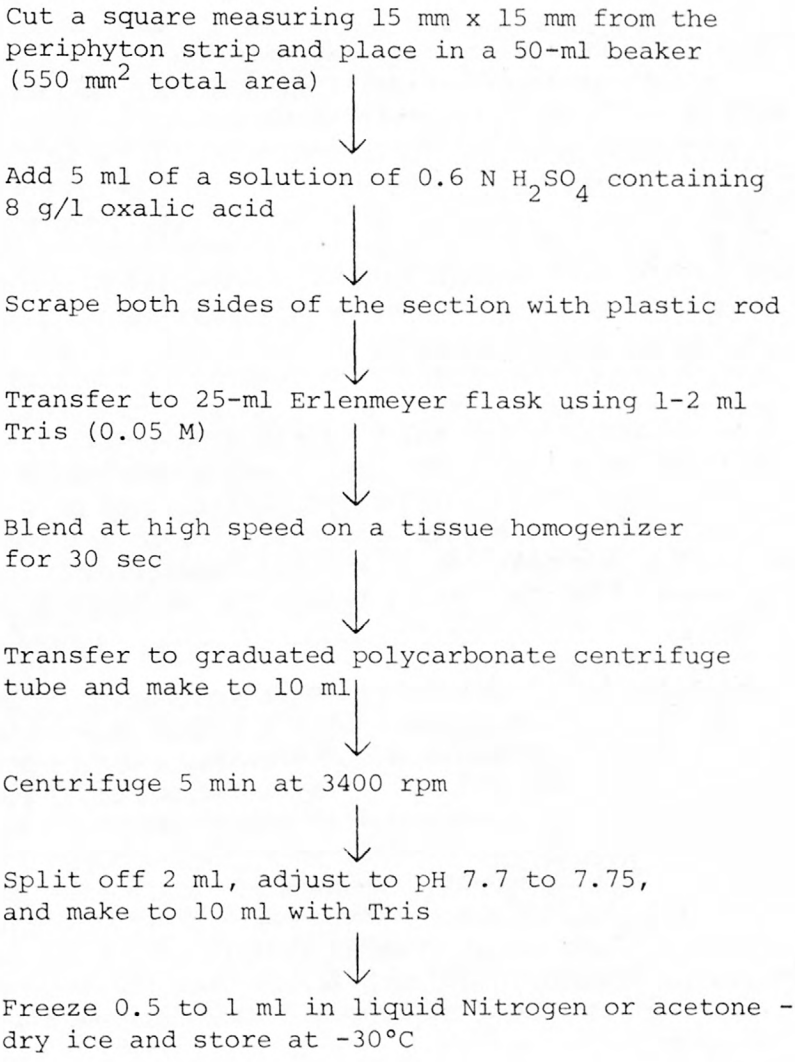


Figure 6.--Suggested periphyton ATP extraction method using sulfuric-oxalic acid.

This proved to be a very useful ancillary tool because the peaktime and decay curve are very characteristic for the ATP bioluminescence reaction. Any problems with the injection or reaction produces uncharacteristic decay curves and the sample can be immediately re-run. The strip chart trace also serves as a permanent record of the assay. Finally, line-voltage noise is easily monitored from the recorder trace and analyses can be interrupted until the baseline is quiet. Normally, all instruments were allowed to warm up at least one hour before analysis.

All buffers, ATP standards, and enzyme solutions were prepared using Milli Q Reagent Grade Water (Millipore Corp.) which is an ultra-pure water having an 18 mega ohm resistivity.

The DuPont Biometer reagent kit was used to prepare the reaction mixture. Aliquots of 100 µl of the hydrated enzyme reagent were dispensed with a digital pipette into disposable 6 X 50 mm glass cuvettes. The enzyme reagent in the cuvettes was stored at room temperature along with the samples until analysis. All samples were analyzed within 90 minutes of hydratation of the enzyme. Tobin and others (1978) report that the DuPont enzyme is stable for at least 8 hours at room temperature.

A 1 mg/L stock ATP solution was prepared from crystalline adenosine 5 - triphosphate disodium salt (ATP - $Na_2 \cdot 3H_2O$) obtained from Sigma Chemical Co. and diluted with pH 7.75 Tris buffer (0.05 M). Ten-milliliter aliquots were transferred to sterile serum vials with red rubber septums and stored at -30°C. A comparison of stored versus freshly prepared stock solution showed that the ATP is stable for at least 2 years when stored at -30°C.

It has been shown by other workers (Karl and LaRock, 1975a; Tobin and others, 1978) that the ion content of the sample affects the light emission reaction of the ATP analysis. Therefore, dilute ATP standards were made from the stock ATP solution using a diluent having the same ionic strength and composition as a sample would have for a given extraction scheme. Our basic procedure was to make a range of dilute standards (1, 5, 10, and 20 ng/ml), transfer 0.5 milliliter aliquots to 6 X 50 mm glass culture tubes with plastic caps, freeze immediately in liquid nitrogen, and store at -30°C until analysis. Our experience has been that the dilute standards are very stable when stored in this manner which also has been reported by other workers (Cheer and others, 1974).

Prior to the analysis of a sample, the cuvette containing the enzyme reagent was placed in the photometer counting chamber. The background luminescence of the enzyme reagent was counted for 30 seconds at the photometer and integrator setting to be used for the sample. This background luminescence was recorded and later subtracted from the value obtained for the sample.

According to Johnson and others (1974), quantification of the luciferin-luciferase reaction with ATP is highly dependent upon fast, efficient, and reproducible mixing of sample and enzyme. A Hamilton CR700-20 pushbutton syringe was used for injection of the sample into the enzyme. This syringe was developed for carbon analysis instruments and injects the sample with enough force to thoroughly mix it with the enzyme. The pushbutton feature provided reproducible injection rates and eliminated any operator effects. The needle tip has to extend below the level of the enzyme or foaming occurs which typically gives abnormally low results. It was necessary to order special needles long enough to reach the bottom of the cuvette.

An injection of 10 µl of sample into 100 µl of enzyme provided ample enzyme-substrate conditions even for high ATP (500 ng/ml) containing samples. Mixture of the 10 µl sample in the 100 µl enzyme was instantaneous.

To minimize cross contamination between samples during analyses, the syringe was rinsed five times with ultra-pure water and five times with the sample prior to injection of the sample into the enzyme. When possible, samples with similar expected ATP concentrations were run as a group. Standards were analyzed from the lowest concentration to the highest.

## SUMMARY

Adenosine triphosphate (ATP) is a useful indicator of living organisms in aquatic samples. Successful extraction and stabilization of the ATP represent the most critical aspects of its use.

A variety of extraction procedures have been proposed but evaluation of several procedures used on liquid samples, sediments, and periphyton demonstrated that the cold sulfuric-oxalic acid procedure was the most suited for all types of samples. The procedure consists of extraction of a sample using 0.6 N sulfuric acid containing oxalic acid to precipitate calcium, centrifugation to settle debris, adjustment to pH 7.75 with ammonium hydroxide, dilution with Tris buffer, and quick freezing.

Few problems are encountered when liquid samples are processed, but with sediment samples, interferences due to solubilized cations and fulvic acids necessitate use of spiking a split of the original extract with an ATP standard to determine losses. With periphyton samples, the inherent variability of colonization of substrates makes it difficult to obtain reproducible data for replicate extractions. A number of replicate extractions of a single substrate are therefore needed to characterize accurately the periphyton community.

Extracted samples were found to be stable at room temperature for 1 to 5 hours, depending on the ATP concentration, if the pH is below 2. Samples which are quick frozen and stored at -30°C are stable for months.

In the analytical stage, reproducible results were obtained using a photometer-integrator system with a chart recorder for visual inspection of the curve of the light reaction. High purity enzyme was used for its sensitivity and low background. Injection of a 10-µl quantity of sample or standard into a 100-µl volume of enzyme produced adequate mixing for the light reaction and reproducible results. A general summary of suggested extraction procedures for various types of samples is presented in table 6.

Table 6.--Summary of suggested extraction procedures for various types of environmental samples. Precision estimated by coefficient of variation.

| Sample type | Extraction procedures | Sample size | Observed precision for replicate extractions (percent) | Comments |
|---|---|---|---|---|
| Liquid | Boiling Tris $H_2SO_4$-oxalic acid | 1 ml | 11 | Use only inert glass or Teflon boiling chips if Tris buffer is used. |
| Sediment | $H_2SO_4$-oxalic acid | .5-1 g (wet) | 18 | Use minimal quantity of sediment and spike a split to estimate losses. |
| Periphyton | $H_2SO_4$-oxalic acid | 1-7 $cm^2$ | 4-31 | Several replicate extractions are necessary because of variable colonization rates on substrates. |

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