ADENOSINE TRIPHOSPHATE CONCENTRATION IN RELATION
TO MICROBIAL BIOMASS IN AQUATIC SYSTEMS

By

Hunter Wilson Cunningham, Jr.

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1977

MASTER

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ABSTRACT

ADENOSINE TRIPHOSPHATE CONCENTRATION IN RELATION TO MICROBIAL BIOMASS IN AQUATIC SYSTEMS

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Analyses of adenosine triphosphate (ATP) extracted from a sediment community by the sulfuric acid method are complicated by inhibitions from inorganic and organic compounds. Inhibitions by inorganic compounds are reversible while those by organic compounds are irreversible. The primary inhibition by organic compounds results by complexing with acid-soluble fulvic acids which will prevent the detection of as much as 80% of the ATP present in a sample by the luciferin-luciferase reaction. Analytical techniques were developed to partially circumvent such interferences.

Biomass interpretations from ATP concentrations in aquatic systems are complicated by the diversity of the microbiota and by the variability in the carbon to ATP ratio caused by environmental conditions. However, when levels of ATP are considered as a physiological condition of a sedimentary community, this data provide a means to interpret community metabolism not available hitherto.

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LIST OF ABBREVIATIONS

ADP	=	Adenosine diphosphate
AMP	=	Adenosine monophosphate
ATP	=	Adenosine triphosphate
BSA	=	Bovine serum albumin
E	=	Enzyme (luciferase)
EDTA	=	Sodium ethylenediamine tetraacetate
FA	=	Fulvic acid
HA	=	Humic acid
HEPES	=	N-2-hydroxyethylpiperazine-N ⁺ 2-ethansulfonic acid
L	=	Luciferin (oxidized)
LH ₂	=	Luciferin (reduced)
OD	=	Optical density
PPi	=	Pyrophosphate
PVP	=	Polyvinylpyrrolidone
SE	=	Standard Error
TRIS	=	Tris (Hydroxy methyl) amino methane

INTRODUCTION

Investigations of the degradation of organic material in the aquatic environment require an assessment of the biomass and metabolic activity of various microorganisms involved in the process. Evaluation of microbial biomass and metabolic activity are difficult because of the small size and diversity of the microbiota. The traditional method for the assessment of microbial biomass is by direct microscopic enumeration. Microscopic enumeration is laborious, subject to large error, and provides limited information about the metabolic activity of the various microfloral components. An alternate method is measurement of a specific cellular constituent which is ubiquitous to living cells. By relating an average cellular content of this constituent to measured levels in the environment, a rapid means of estimating the microbial biomass is possible.

Of many possible cellular constituents, the ubiquitous cellular component, adenosine triphosphate(ATP), has been shown to provide a reasonable estimate of microbial biomass in aquatic systems (Holm-Hansen and Booth, 1966; Holm-Hansen, 1969). Microbial populations of planktonic systems are usually defined as the fraction passing through a 150 to 250 μm porosity mesh and retained by a 0.4 μm porosity filter (cf. Holm-Hansen and Booth, 1966; Rudd and Hamilton, 1973). A similar fractionation for sedimentary populations is not practical.

The validity of estimates of microbial biomass from ATP levels requires three assumptions (Holm-Hansen and Paerl, 1972):

1. Measured levels of ATP must originate only from living cells and only insignificant levels are associated with non-living detrital material.
2. Submicrogram quantities of ATP are capable of precise and rapid detection.
3. Cellular levels of ATP are similar among different taxonomic groups and levels remain constant regardless of environmental conditions.

An accumulation of ATP in the particulate organic fraction in aquatic systems has not been found. Biomass estimates of microflora in ocean profiles based on chlorophyll a and ATP were in agreement with estimates made by direct microscopic counts. However, biomass estimates from an equally ubiquitous compound, DNA, were erroneous because of extracellular DNA accumulation in the detrital fraction (Holm-Hansen, 1969). If extracellular ATP accumulated in the detrital fraction, a pattern similar to DNA would be expected. Indirect evidence indicates that accumulation of ATP in aquatic sediments is prevented by action of bacterial phosphatase activity (Lee et al., 1971b).

Methods for the Detection of ATP

The luciferin-luciferase complex obtained from the firefly (Photinus pyralis) is a specific enzyme for ATP which allows measurement of submicrogram quantities of ATP when ATP (substrate) is the rate limiting entity (Strehler and Totter, 1952).

Quantification is possible because one photon of light is emitted per molecule of ATP by the following reaction (Seliger and McElroy, 1964):

Emitted light of 560 to 580 nm is quantified by a photomultiplier cathode tube and ATP concentrations are determined by comparing the area of the light emission curve from unknown quantities of ATP to those of known concentrations of ATP. Using purified luciferin-luciferase allows detection of $1 \times 10^{-13} \text{ g ml}^{-1}$ while crude extracts will detect $1 \times 10^{-10} \text{ g ml}^{-1}$ (Holm-Hansen and Paerl, 1972). Precision of an average ATP sample encountered in natural samples (4-9 μg) is $\pm 10\%$ of the mean value (Holm-Hansen and Booth, 1966). The precision declines rapidly if the concentration of ATP in the extract approaches the detection limit ($1.5 \times 10^{-7} \text{ g ml}^{-1}$) (Lee et al., 1971a).

Use of the luciferin-luciferase complex for the determination of ATP is subject to potential errors caused by the chemical nature of the extract. Common errors are depression of luminescence from non-ATP sources (Strehler and Totter, 1952).

Ionic interferences can severely suppress the luminescence to the point of extinction. For example, a 10 mM concentration of cations commonly present in environmental samples will cause as much as a 40% decrease in the bioluminescence; anionic species are equally inhibitory. Relative effectiveness of inhibition by some cation is $\text{Ca}^{++} > \text{K}^+ \geq \text{Na}^+ > \text{Mg}^{++}$, while anionic effects are

$\text{CO}_3^{=}$ > $\text{PO}_4^{=}$ > $\text{SO}_4^{=}$ > Cl^- (Karl and LaRock, 1975). Circumvention of cationic effects in natural extracts by exchange resins and chelation with EDTA have proven effective (Lee et al., 1971a; Karl and LaRock, 1975) while anionic interferences can be partially circumvented by preparation of the ATP standards in the presence of the major anion of the extraction agent (discussed later).

Suppression of bioluminescence by binding of ATP with substances in the extract has not proven to be a severe problem in analyses of particulate matter from planktonic microflora (Holm-Hansen and Booth, 1966). However, interferences of organic compounds extracted from particulate organic matter may be quite inhibitory. Inhibitions of bioluminescence from 11 to 76% are caused by inactivation of luciferin-luciferase by polyphenolic substances extracted from plant material. These inhibitions were reversed by removal of the phenolic compounds on insoluble polyvinylpyrrolidone (PVP) columns and protection of the enzyme complex by additions of soluble PVP and bovine serum albumin to the luciferin-luciferase preparation (Guinn and Eidenbock, 1972). The binding of ATP to phenolic substances was not investigated and inhibitions induced by chemically analogous humic substances in ecological samples have not been investigated hitherto.

Light emissions from compounds such as other nucleotide triphosphates (NTP) and ADP in the test solution or ATP present in crude luciferin-luciferase preparations are potential sources of error (Holm-Hansen and Booth, 1966; Karl and LaRock, 1975; Laake, 1976). Errors produced from NTP and ADP are caused by the presence of

transphosphorylase enzymes in crude luciferin-luciferase preparations. Transphosphorylase enzymes are responsible for the conversion of $\text{NTP} \rightarrow \text{ATP}$ and $2 \text{ ADP} \rightarrow \text{ATP} + \text{AMP}$. Contributions to light emissions by ADP are less than 1% (Holm-Hansen and Booth, 1966). However, conversion of $\text{NTP} \rightarrow \text{ATP}$ produces an abnormal light decay curve by forming a second peak after the initial ATP peak height and decay. This secondary peak introduces a significant error when ATP calculations are determined by the area method. NTP effects can be circumvented by calculating ATP concentrations from initial peak height (less precise) or by using a purified luciferin-luciferase preparation (Laake, 1976). A serious error for samples with low concentration of ATP is light emissions from endogenous ATP in crude enzyme preparations. Background emissions can be reduced by incubation of the enzyme at room temperature for several hours before use (Lee et al., 1971a; Karl and LaRock, 1975).

Evaluation of various inhibitions of bioluminescence caused by chemical species in the extract requires an ATP internal standard if accurate estimates of environmental ATP levels are desired. The relative efficiency of an extraction method from a given substratum can be evaluated by comparison of the percentage recovery by the various extraction methods (Table 1). The choice of Na_2ATP or live bacterial cells as an internal standard appears to be quite arbitrary. Bacterial cells are claimed to circumvent enzymatic and chemical losses of ATP encountered with Na_2ATP (Lee et al., 1971a). However, Na_2ATP provides a good estimate of the inhibition of bioluminescence when enzymatic activity is inactivated before the addition of Na_2ATP .

Table 1. Literature values for the efficiency of various ATP extraction methods from various substrata

Method	Substratum	Internal std*	% Recovery	Source
ORGANIC				
+Butanol	soil	A	78.9-98%	Asmus, 1973
+Butanol-Octanol	soil	A	73-100%	Anderson; Davies, 1973
Ethanol	plant tissue	A	24-89%	Guinn; Eidenbock, 1972
BOILING BUFFER				
0.02 M TRIS pH 7.75	plankton	-	100%	Holm-Hansen; Booth, 1966
+0.02 M TRIS pH 7.80	sediment	A	3-6%	Karl; LaRock, 1975
0.02 M TRIS pH 7.80	sediment	B	3%	Lee et al., 1971
0.10 M NaHCO ₃ pH 8.50	sediment	B	64-100%	Bancroft et al., 1976
COLD ACID				
0.6 N H ₂ SO ₄	sediment	B	20-85%	Lee et al., 1971
"	sediment	A	81-94%	Karl; LaRock, 1975
"	soil	A	77-99%	Asmus, 1973
"	peat	B	27-100%	Greaves et al., 1973
30% HClO ₄	bacteria	A	100%	Cole et al., 1967

*A = Na₂ATP; B = live bacteria

+ artificial systems

(Guinn and Eidenbock, 1972). Since a bacterial internal standard is not subjected to the same physical protection as a natural bacterial population (cf. Karl and LaRock, 1975), claims that bacterial standards provide an estimate of extraction efficiency of ATP from the endogeneous population (Bancroft et al., 1976) are not totally correct. The accuracy of a Na_2ATP internal standard is superior to the bacterial internal standard since rapid changes in ATP pool sizes of bacterial culture can occur during manipulations (Cole et al., 1967).

ATP Extraction Procedures

An adequate, consistent procedure for the extraction of ATP from a natural substratum requires that the extracting agent inactivates all phosphatase enzymes immediately and the ATP is quantitatively extracted from the organisms without producing a significant chemical inhibition of the luciferin-luciferase reaction (Laake, 1976). Most ATP extractions can be classified into one of three general treatments, those employing either: 1) organic solvents (Asmus, 1973; Guinn and Eidenbock, 1972; Anderson and Davies, 1973); 2) boiling neutral or slightly alkaline buffer (Holm-Hansen and Booth, 1966; Bancroft et al., 1976; Lundin and Thore, 1975); or 3) cold acid (Lee et al., 1971a; Karl and LaRock, 1975). The choice of an extracting method is dependent on the nature of the substratum and the number of samples which are being processed (Table 1).

Wide application of organic solvent extraction of ATP to environmental studies has been limited because the time required

to remove the organic solvent from the extract limits the number of samples which can be processed. Organic solvents, however, have proven to be effective extracting agents because they rapidly inactivate degradative enzymes. Efficiency of extraction ranges from 73.7 to 100% for soils of various sand and clay composition (Anderson and Davies, 1973); $89.2\% \pm 6.2$ for organic forest litter (Asmus, 1973); and 29 to 84% for higher plant material (Guinn and Eidenbock, 1972). Organic solvents do not prevent the chemical binding of ATP to clays (Anderson and Davies, 1973), and they may extract compounds from the substratum which severely inhibit the luciferin-luciferase reaction (Guinn and Eidenbock, 1972).

Extraction of ATP from planktonic samples and laboratory cultures of microorganisms with boiling buffer has proven quite effective, with little or no chemical interference. The general procedure is to filter the water sample through a 0.4 μ m porosity filter and with the immediate immersion of the filter into boiling TRIS buffer (0.02 M pH 7.75) for 5 to 30 minutes. Since extraction efficiency declines rapidly as the temperature approaches 80° to 90°C, the temperature of the extracting buffer is a critical factor (Holm-Hansen and Booth, 1966). When large quantities of suspended particulate matter are present, volumes filtered should be less than 0.5 liters since the relationship between the filtered volume and amount of ATP extracted becomes inconsistent thereafter (Sutcliffe et al., 1976).

Application of the boiling buffer extraction method to sediments provide inconclusive data on the efficiency of the method. Boiling TRIS buffer (0.02 M pH 7.8) was ineffective in extracting

ATP from freshwater and marine sediments; less than 5% of ATP added as live cells was recovered (Lee et al., 1971a; Karl and LaRock, 1975). Apparently, sediment particles protect adhering bacteria from extraction by producing thermal gradients which lower the effective temperature of the buffer (Karl and LaRock, 1975). However, extraction with 16 ml of boiling 0.1 M NaHCO_3 (pH 8.5) for 30 seconds followed by dilution with 40 ml of 0.1 M TRIS (pH 7.8) produced extraction efficiencies from 64 to 100% for sediments of various chemical composition. ATP values obtained were statistically similar to ATP values determined on replicate samples by the acid extraction method (Bancroft et al., 1976). Clearly, more comparative methodological research is required before more definite statements about boiling buffer extraction of ATP from sediments can be made.

For ATP extraction from particulate organic material, cold acid extraction provide the most satisfactory results (Asmus, 1973). Acids commonly used are perchloric, trichloroacetic, or sulfuric at concentration of 0.1 N or greater (cf. Lundin and Thore, 1975). Normality of the acid has little effect on the recovery of ATP as long as the final pH of the extract is less than 1.0 and the temperature is maintained ca. 0°C during extraction to prevent degradation (Lee et al., 1971a). Although sulfuric acid is commonly used in ecological studies (Table 1), considerable variability between replicate determination make sulfuric acid less than an ideal extracting agent (cf. Bancroft et al., 1976; Lundin and Thore, 1975). Trichloroacetic acid which provides highly reproducible results and limited inhibitions with laboratory bacterial cultures (Lundin and Thore, 1975) has not been used for natural samples.

Inhibitory effects inherent to acid extractions reduces the desirability of the method. Solubilization of cationic species in environmental samples requires the addition of time consuming cation removal steps and the dilution of the sample. Cation removal steps such as exchange resin (Lee et al., 1971a), chelation (Karl and LaRock, 1975), and charcoal adsorption (Hodson et al., 1976) are associated with loss of ATP and sample dilution which often approaches the detection limit of luciferin-luciferase complex. Anionic inhibitions produced by the major anion of the extracting acid can be as severe as the cationic induced inhibitions. For example with perchloric acid extractions, considerable quantities of ATP are removed with $KClO_4$ precipitation during the neutralization of the acid (Lundin and Thore, 1975; Guinn and Eidenbock, 1972). Similarly, sulfate ion is strongly inhibitory to bioluminescence of the luciferin-luciferase reaction. These anionic inhibitions introduced by the extracting acid can be circumvented if the ATP standards contain the same concentration of the anion as the final ATP extract (Karl and LaRock, 1975). Inhibitory effects of organic compounds solubilized by acid extraction of ATP determinations have not been investigated.

Interpretation of ATP Data

Extrapolation from ATP levels to microbial biomass requires that the ratio of ATP to cellular carbon remains constant over a wide range of physiological conditions (Holm-Hansen and Paerl, 1972). Intuitively, environmental levels of ATP represent the total community; therefore, validity of biomass estimates requires that the ratio of

ATP to cellular carbon is similar among taxonomic groups which compose the community (Hamilton and Holm-Hansen, 1968). As seen in Table 2, either assumption is valid. ATP:cellular carbon ratio ranges from 0.2 to 0.7% for bacteria and algae, respectively and as high as 0.92% for zooplankton. ATP:cellular carbon ratio is influenced by physiological conditions of the environment (cf. Asmus, 1973; Forrest, 1965). In addition, ATP:cellular carbon values in Table 2 were derived from monospecies cultures. In bacterial cultures, ATP production per unit glucose metabolized decreases when competition for the common energy source exists between two different species (Kao et al., 1973). A similar variance in ATP:carbon ratio was observed for natural phytoplankton populations which were competing for a limited phosphorus source (Cavari, 1976).

For ecological studies, environmental levels of ATP should be only interpreted when ATP levels are coupled with the energy metabolism of the community (Forrest, 1965). Since ATP levels represent the amount of physiological energy available for metabolism, ATP levels when measured simultaneously with physiological parameters provide a means of delineating regions of metabolic activity (Rudd and Hamilton, 1973; Hobbie et al., 1972; Holm-Hansen, 1973). ATP interpreted as a physiological parameter, therefore, offers a means to evaluate metabolic conditions of microbial communities not available hitherto.

Table 2. ATP values determined for various laboratory cultures of microorganisms

Taxonomic Group	ATP Expressed As	Growth Phase	Mean	Variability	Source
Marine Bacteria 5 Species	% carbon	life cycle	0.70	0.3-1.1	Hamilton + Holm-Hansen 1968
Bacteria 5 Species	% carbon	log lag death	0.20 0.06* 0.06*	+ 0.02 (SE) + 20% + 20%	Asmus, 1973
Fungi 8 Species	% carbon	log lag death	0.43 0.16* 0.14*	+ 0.04 (SE) + 6% + 9%	Asmus, 1973
Actinomycetes 6 species	% carbon	log lag death	0.46 0.22* 0.22*	+ 0.06 (SE) + 5% + 5%	Asmus, 1973
Algae 6 Species	% carbon	log lag death	0.70	+ 0.08 (SE) + 23% + 25%	Asmus, 1973
Bacteria 3 Species	% dry weight	life cycle	—	0.072- 0.22	Holm-Hansen + Booth, 1966
Algae 8 Species	% dry weight	life cycle	—	0.002- 0.17	Holm-Hansen + Booth, 1966

Table 2. (continued)

Taxonomic Group	ATP Expressed As	Growth Phase	Mean	Variability	Source
<u>Calanus finarchicus</u> (Zooplankton)	% carbon	life cycle	—	0.86-0.92	Balch, 1972
<u>Escherichia coli</u>	% dry weight	life cycle	—	0.33-0.68*	Cole et al., 1967
<u>Streptococcus faecalis</u>	% dry weight	life cycle	—	0.09-0.675*	Forrest, 1965

*value calculated from data given

METHODS

Assessment of the various ATP extraction techniques were performed with a mixture of 1 ml of 16-hr culture of bacteria (Achromonas sp.) and 0.4 g of autoclaved, oven-dry marl sediment of the littoral zone of Lawrence Lake (Barry Co., Michigan). The following methods were compared: (1) Boiling 0.025 M HEPES buffer, pH 7.5 (M. J. Klug, personal communication); (2) Boiling 0.1 M NaHCO_3 , pH 8.5 (Christian et al., 1975); (3) H_2SO_4 extraction with cation removal by exchange resins (Lee et al., 1971a); (4) H_2SO_4 extraction with Ca^{++} chelation by EDTA (Karl and LaRock 1975); (5) H_2SO_4 extraction with Ca^{++} removal by precipitation with oxalic acid; (6) Freezing at -15°C and extraction in 0.025 M HEPES, pH 7.5; and (7) Freezing at -60°C and extraction in 0.025 M HEPES, pH 7.5. Initial evaluations of percentage recovery of ATP by these seven methods in sediments revealed that the most promising were H_2SO_4 extraction with cation removal by exchange resin and by oxalate precipitation and the two freezing techniques. Only these extraction methods were examined further.

H_2SO_4 -Cation Exchange

7 ml of cold, 1.2 N H_2SO_4 were added to approximately 0.4 g of sediment-bacteria mixture. The samples were vortexed for a total of 60 seconds at 10-sec intervals. The particulate materials was removed by filtration or refrigerated centrifugation at 12,000 g for 10 minutes. The supernatant was mixed with 2 g of cation exchange resin (Dowex 50, H^+ form), vortexed, and filtered (Whatman No. 1) under reduced pressure. The filtrate was neutralized to

pH 7.5 with 1 N NH_4OH and diluted to 15 ml with glass-distilled water. 1.2 N H_2SO_4 was used instead of the conventional 0.6 N H_2SO_4 because the highly calcareous sediment neutralized the low normality acid and resulted in a pH > 6. Experiments showed that more ATP was recovered by 1.2 N H_2SO_4 . NH_4OH was shown to be a superior neutralizing agent over NaOH.

H_2SO_4 -Oxalate Precipitation

7 ml of cold, 1.2 N H_2SO_4 plus 1 ml of 0.5 M oxalic acid were added to 0.4 g of sediment-bacteria mixture. Samples were then treated similarly to the above-discussed method except that the cation exchange resin was excluded.

Freezing Buffer

After 15 ml of 0.025 M HEPES buffer (pH 7.5) were added to 0.4 g of sediment-bacteria mixture, the mixture was vortexed for 15 seconds and immediately frozen at -15°C in a conventional freezer or at -60°C in an ultrafreezer. Before assaying, the samples were thawed slowly to room temperature with 5-second vortexing every 10 minutes. Particulate material was removed by filtration or refrigerated centrifugation.

The normal procedure adopted for natural sediments was as follows. Approximately 1 gram (wet weight, approximately 0.1 gram dry weight) was placed into a 50-ml centrifuge tube. Then 5 ml of cold 1.2 N H_2SO_4 containing oxalic acid (8 g l^{-1}) was added to the sample and the sample was ground for 15 seconds with a teflon tissue grinder and mixed by a 15-second vortexing. Then 5 ml of 0.03 M HEPES was added, mixed, and the particulate material

removed by refrigerated centrifugation (12,000 g, 10 min). The supernatant was removed and its volume measured. Two 4-ml subsamples were pipetted into acid-washed scintillation vials. To one of the subsamples, 0.2 μ g of ATP was added as an internal standard. The samples were held on ice until titration with 1.0 N NH_4OH to pH 7.5. The final volume was adjusted to 10 ml with glass distilled water, giving a dilution of 6-8 fold from the original extract. Further dilution produced concentrations of ATP to levels below the detection of the luciferin-luciferase reaction for sediments low in organic matter. This extraction procedure was chosen because the time required to process a sample was less than the cation resin method and the precision of the method was superior. Although the time requirement to process a given sample is increased, the results of our study indicate that the addition of a Na_2ATP internal standard to a subsample of each extract was necessary to circumvent population heterogeneity.

ATP Assay Procedure

The luciferin-luciferase complex obtained a 25 mg of lyophilized firefly lanterns (Sigma Chemical Co. FLE-250) was reconstituted with 25 ml of 0.025 M HEPES buffer, pH 7.5 containing 5 mM MgCl_2 . The enzyme was kept at room temperature for 3-4 hr to reduce endogenous ATP and then centrifuged (12,000 g, 10 min) to remove particulate material. The enzyme was either used immediately or frozen at -15°C . The frozen enzyme retained sufficient activity under these conditions for over a month.

Bioluminescence was assayed using an Aminco ATP Photometer on 0.5 ml samples into which 0.1 ml of enzyme was injected via a Hamilton syringe. The area of the initial 30-second response curve was determined by automatic digital integration (Columbia Model CSI-208). The amount of ATP of the samples was evaluated by comparison of responses to known amounts of ATP Na₂ (1×10^{-9} g to 5×10^{-6} g).

Phosphatase Activity

The sediment sample was prepared in a manner similar to the freezing buffer extraction except only 10 ml of buffer was employed. After thawing the sample, 2×10^{-9} g of 3-O-methyl fluorescein phosphate was added and the sample vortexed. The sample was immediately centrifuged (7000 g, 5 min) and fluorescence of the supernatant was measured (excitation 430 nm, emission > 510 nm). The sample was vortexed and held at 25°C for 2 hours. At the end of the incubation period, the sample was centrifuged and the fluorescence of the supernatant measured. The phosphatase activity was calculated by comparing the net change in fluorescence to the fluorescence of a known quantity of fluorescein, the product of the hydrolysed substrate. The results are expressed as ng product hour⁻¹.

Protease Activity

Approximately 1 g (wet weight; ca. 0.1 g dry weight) of sediment was added to a screw-cap test tube. Immediately, 5 ml of 0.2 M TRIS (pH 7.4), 0.1 g of Azocoll (Calbiochem), and 2 drops of toluene were added to the sample. Then the sample was incubated for 2 hours at 25°C on a Reciprocal shaker (60 oscillations minute⁻¹). The

enzymatic activity of the sample was destroyed by immersion in a boiling water bath for 10 minutes. Then the sample was centrifuged for 5 minutes at 6500 g and the optical density (OD) of the supernatant measured at 520 nm. Blanks were prepared by incubating replicate samples without Azocoll until immediately before immersion into the boiling water bath. Relative enzymatic activity was calculated by $OD(\text{sample}) - OD(\text{blank})$, with the activity being proportional to the ΔOD , and expressed as $\Delta OD \text{ h}^{-1} \text{ g dry wt}^{-1}$.

Fulvic Acids

The fulvic acid fraction of two different calcareous sediment was extracted following the procedures for highly calcareous sediments (Figure 1) after Schnitzer and Khan (1972). After lyophilization, the fulvic acids were resolubilized to a concentration of 10 g l^{-1} in glass distilled water and the pH was adjusted to 7.5 with $1 \text{ N NH}_4\text{OH}$. Lower concentrations were obtained by dilution.

The effects of fulvic acids upon the luciferin-luciferase reaction were evaluated by mixing 1:1 v/v ratios of known concentrations of fulvic acids (1×10^{-2} to 1 g l^{-1}) and ATP- Na_2 ($0.25 \times 10^{-6} \text{ g}$). To evaluate the possibility that fulvic acids were complexing ATP, 50 μl of $\text{U-}^{14}\text{C-ATP}$ ($582 \text{ } \mu\text{Ci mM}^{-1}$, $51 \text{ } \mu\text{Ci ml}^{-1}$; Amersham Searle) were added to 100 ml of fulvic acids at a concentration of 1 g l^{-1} . The fulvic acids were then fractionated into molecular weight classes of greater than 10,000, less than 10,000 but greater than 1000, and less than 1000 via Amicon ultrafiltration. One ml of each molecular weight class was suspended in 10 ml of Instagel (Packard Co.) and radioassayed by liquid scintillation (Beckman LSC-150). The efficiency of radioassay was determined by the external standard ratio method.

Figure 1. Outline of procedure used to separate humic acid (HA) and fulvic acid (FA) fractions from sediments

RESULTS AND DISCUSSION

The effects of different extraction methods on the percentage recovery of ATP from laboratory bacterial cultures are compared in Table 3. The percentage recovery was calculated as:

$$\frac{\text{ATP value of bacteria plus oven-dry sediment}}{\text{ATP value of bacteria}} \times 100.$$

By this means the percentage recovery is independent of completeness of extraction and reflects the relative losses of ATP inherent to a given technique and sediment. The decrease of ATP capable of reacting with the luciferin-luciferase complex cannot be ascribed to the presence of Ca^{++} ions in the extract since the concentration of Ca^{++} was reduced to non-inhibitory levels by cation exchange resins or oxalate precipitation. Similarly, Ca^{++} levels extracted by the freezing buffer method were non-inhibitory. However, the percentage recovery for the acid extraction was influenced by the amount of organic matter in the sediment and was related directly to the quantity of humic substances present (as measured by fluorescence at 460 nm). The freezing extractions were independent of the organic matter content of the sediment and the percentage recovery was inversely related to the amount of humic substances present.

The effectiveness of extraction of ATP from a natural microbial population of highly organic freshwater marsh sediment by cold 1.2 N H_2SO_4 and freezing HEPES buffer is also shown in Table 4. Similar to Lee, et al. (1971a), acid extractions with cation removal by exchange resins and oxalate precipitation are characterized by a high degree of variability and a loss of a significant portion of

the ATP. Lower percentage recovery for the oxalate precipitation technique was caused by ATP removal with the oxalate precipitate, but a portion of the humic substance was also removed. The percentage recovery for the natural populations was similar to those observed for a laboratory culture in organic sediments by the cation exchange method but was lowered considerably by the oxalate precipitation technique. The causal mechanism of this difference is not understood.

The freezing buffer extraction, which appeared to be a rapid technique with low variability (Table 3), produced a percentage recovery of less than 1% for natural populations (Table 4). Differences between the percentage recovery observed for the laboratory population and the natural population can be ascribed to the phosphatase activity of the two populations. The natural population had a phosphatase activity of 20 ng product hour⁻¹ whereas the laboratory population possessed a phosphatase activity of 1 ng product hour⁻¹. When the phosphatase activity was expressed as activity hour⁻¹ μg ATP⁻¹, the natural population had approximately 5 times more activity per unit biomass (6.1 ng product hour⁻¹ μg ATP⁻¹) than the laboratory culture with values of 1.2 ng product hour⁻¹ μg ATP⁻¹. The bacterial culture utilized for the laboratory populations possessed a phosphatase activity of 57.8 ng product hour⁻¹ μg ATP⁻¹ before the addition of oven dry sediment. Inactivation of phosphatase enzymes released during freeze lysis of the Achromonas culture by humic substances explained the inverse relationship found between the amount of humic substances which fluoresce at 460 nm and the percentage recovery of the freezing buffer extraction (Table 3). Phosphatase activity in the natural population was greater than the inactivation effect

Table 3. Efficiency of different extraction methods for ATP of sediments

Method	% Organic Matter of Sediments	% Recovery (+S.E.)*			Fluorescence Units ¹		mg Ca ⁺⁺ l ⁻¹ of extracts
		Exp. 1 (n=6)	Exp. 2 (n=8)	Exp. 3 (n=14)	Exp. 2	Exp. 3	
H ₂ SO ₄	4.28	56.52±2.08	52.5±5.1	--	890±	-	--
Cation Exchange	8.48	40.39±3.47*	39.8±3.9*	28.4±2.8	1076±5	880	8.72
H ₂ SO ₄	4.28	38.57±0.81	78.5±3.3	--	855±18	-	--
Oxalate pptn.	8.48	21.68±1.18*	64.6±2.3	75.4±8.5	1015±43	855	3.12
Freezing (-15°C)	4.28	49.26±1.71	42.7±1.7	--	750±30	-	--
0.025M HEPES	8.48	50.85±0.60	46.0±1.0	32.2±0.5	1065±21	782	74.15
Freezing (-60°C)	4.28	72.90±1.09	56.1±1.0	--	693±37	-	--
0.025M HEPES	8.48	76.98±1.79	51.4±1.7	42.6±0.2	933±33	802	82.63

*Values between the two sediment types are significantly different ($p < 0.05$) by Mann-Whitney U Test

¹Fluorescence values in Experiment 2 were determined by dilution (1:2) with GDW of the sample. Values in Experiment 3 were determined by decreasing the intensity of the exciting wavelength 3x.

Table 4. Three different extraction procedures on a highly organic lake sediment. February 1976.

Method	ug ATP g dry wt ⁴	Coef. of Variation	% Recovery	Fluorescence
H ₂ SO ₄ Cation exchange	0.71 ± 0.11	57.1%	36.7%	447
H ₂ SO ₄ Oxalate pptn.	0.84 ± 0.17	31.5%	28.2%	85.5
Freezing 0.025M HEPES -60°	0.07	15.2%	< 1%	-

of the humic substances found in natural sediments. This phosphatase activity would prevent the accumulation of extracellular ATP in the sediment.

Lee et al. (1971a) reported that recovery efficiencies of ATP from lake sediments were not dependent on the amount of organic matter present, CaCO_3 levels, or the phosphorus-binding capacity but was dependent on an unresolved chemical component. In our experiments, dependency of the ATP recovery on the concentration of acid-soluble organic matter (fulvic acids) extracted from two marl sediments was demonstrated (Figure 2). Sediment 1 was a marl sediment collected from a marl bench devoid of aquatic macrophytes with a 4.3% organic matter content. Sediment 2 was collected from a marl bench densely populated by Scirpus subterminalis and Najas flexilis with an organic matter content of 8.5%. Cation analysis by atomic absorption of the fulvic acid fractions revealed that $2.8 \text{ mg l}^{-1} \text{ Na}^+$ was present and Ca^{++} , Mg^{++} , and K^+ were absent. Therefore, cation inhibition does not explain the observed effects of the fulvic acids. The fulvic acids had a marked effect at a concentration greater than $1 \times 10^{-1} \text{ g l}^{-1}$ with 70 to 80% of the reaction being inhibited at a concentration of 1 g l^{-1} . The fluorescence at this concentration was 63 and 170 for Sediment 1 and Sediment 2, respectively. The percentage recovery was inversely correlated to fluorescence of the fulvic acids ($r = -0.89$, $p < 0.01$) and ultraviolet absorption (Wetzel and Otsuki 1974) at 250 nm ($r = -0.74$, $p < 0.01$). The percentage recovery was not correlated to the light absorption at 550 nm, the major wavelength emitted by the luciferin-luciferase reaction (Seliger and McElroy 1964).

Figure 2. Recovery of ATP in relation to increasing concentration of extracted fulvic acids.

Values are expressed as the mean ($n=6$) $\pm$ standard error. ----- = Sediment 1,

———— = Sediment 2

Figure 2.

The degree of inhibition was not significantly reversed whether the dilutions of the fulvic acids were made with glass distilled water, 0.025 M HEPES (pH 7.5), 0.0125 M HEPES, 0.0125 M HEPES (containing 5mM MgCl_2), or 5mM MgCl_2 . These responses indicate that the available magnesium required for the luciferin-luciferase reaction was not affected by the presence of fulvic acids.

Guinn and Eidenbock (1972) reported that the percentage recovery of ATP in the presence of polyphenolic substances could be increased by the addition of 50 mg of bovine serum albumin and 50 mg of polyvinylpyrrolidone (PVP) to the luciferin-luciferase enzyme mixture. The elucidated mechanism was a competitive binding of the polyphenolic substances by the protein and PVP, thus releasing the enzyme for the reaction. The protein and PVP may have functioned also as a disruptor of the humic-like polyphenol-ATP complex formed in the extract. Experimental assays were undertaken in an attempt to reverse the inhibitory effect of fulvic acids by adsorption to protein and PVP before addition of Na_2ATP (Figure 3). Clearly, the protein and PVP did not disrupt the inhibitory effect of the fulvic acids but produced further inhibition. The presence of protein (2 mg ml^{-1} extract) during the extraction and titration of ATP from natural sediments did not enhance the recovery of ATP while PVP (2 mg ml^{-1} extract) decreased the reactive ATP present (Table 5). The concentration dependency and the low percentage recovery for H_2SO_4 -protein extracted ATP suggested that the protein inactivated a portion of the ATP. Attempts to reverse the fulvic acid interference by the addition of EDTA and Mg^{++} ($3 \times 3 \times 3$ factorial) failed to produce a significant response as did increasing the ionic strength of the HEPES buffer in

Figure 3. Recovery of ATP in relation to increasing concentrations of extracted fulvic acids (FA) with polyvinylpyrrolidone (PVP) and bovine serum albumin (BSA). Values are expressed as the mean (n=6) $\pm$ standard error

Figure 3.

Table 5. Effects of Bovine serum albumin (2 mg ml⁻¹) and polyvinylpyrrolidone (2 mg ml⁻¹) on the acid extraction of ATP from natural sediment

	$\mu\text{g ATP}$ $\text{g dry wt}^{-1} \pm \text{SE}$	% Recovery
No protein or PVP	2.11 ± 0.41	29
Protein	2.51 ± 0.31	4.2
PVP	0.83 ± 0.10	11
Protein and PVP	0.98 ± 0.10	12

the extract. The non-competitive nature of the fulvic acid-ATP complex to protein, chelator, and ionic strength suggested that the fulvic acid moiety binds ATP by either strong hydrogen or covalent bonds (cf. Schnitzer and Kahn, 1972).

The ability of fulvic acids to complex ATP is demonstrated in Table 6. A significant relationship exists between the amount of U- 14 C-ATP bound and the concentration of the various molecular weight classes of fulvic acids at a concentration of 1 g l^{-1} . The majority of the ATP is complexed by the $< 10,000$ and > 1000 molecular weight class which represents the major fraction of the fulvic acids. Some adsorption of the ATP to the filters accounted for the discrepancies observed between the amount of ATP complexed and the amount of fulvic acids removed by filtration through the 1000 molecular weight filter. When the amount of ATP complexed by a given fraction was corrected for adsorption to the filter, the value of ATP complexed by the fulvic acids corresponded to the amount of inhibition observed in Figure 2. Thus, the main mechanism of inhibition by the fulvic acids is the formation of a fulvic acid-ATP complex incapable of reacting with the luciferin-luciferase system.

Complex formation appears to be unaffected by simple procedural steps such as increasing the ionic strength of the extract or dilution. Thus, fulvic acids represent a non-competitive loss of ATP. Because of the universal presence of fulvic acids in all sedimentary systems, fulvic acid inhibitions will affect all acid-extracted ATP determinations on particulate organic material or sediments. The extent of inhibition will be a variable quantity dependent on the chemical nature and concentration of the fulvic

Table 6. The percentage of ATP complexed with various molecular weight fractions of fulvic acids

	Molecular Weight	%Radioactivity retained on filter	% FA removed
Sediment 1	> 10000	3	-
	< 10000 but > 1000	86.75	-
	< 1000 (unbound)	10.25	-
Sediment 2	> 10000	9	10
	< 10000 but > 1000	83.6	80
	< 1000 (unbound)	5.4	10

acids extracted for a given sediment.

Validity of direct biotic carbon estimates from in situ ATP measurements is questionable because of variability of carbon to ATP ratios induced by physiological conditions of the environment and the diverse community of organisms contributing to measured levels of ATP. For example, carbon to ATP ratios which remain relatively constant in laboratory cultures of algae (Holm-Hansen, 1970) will fluctuate by an order of magnitude for natural phytoplankton populations experiencing phosphorus limitations (Cavari, 1976). Similar variations between laboratory and natural populations of bacteria may occur under natural conditions of substrate limitation. Since ATP extraction techniques are non-selective, sedimentary ATP emanates from bacteria as well as from zooplankton, algae, protozoans, and nematodes (Christian et al. 1975). Large zooplankton are known to have different carbon to ATP ratios than bacteria (Balch, 1976) while carbon to ATP ratio for sedimentary protozoans and anaerobic bacteria have not been examined hitherto. Thus, measured levels of sedimentary ATP represent relative estimates of total community biomass from which conversion to biotic carbon should be approached with caution.

ATP level within a single organism is regulated by the physiological conditions imposed by its environment and ATP is a known regulator of cell metabolism. Expanded to a community level, in situ ATP data, when combined with measured physiological parameters, designate areas of metabolic activity (Rudd and Hamilton, 1973). Thus, monitoring relative fluctuations of community ATP levels provides an indication of changes in physiological energy available

in community metabolism. Expression of metabolic data normalized with respect to ATP present should provide an estimate of the metabolic activity of a community. For example, alkaline phosphatase activity of Achromonas does not demonstrate a significant increase until 24 hours of growth when examined directly (Table 7). Normalization with respect to cellular ATP levels revealed that the 12-hour population exhibited approximately twice as much phosphatase activity as the 10-hour population.

Similar analyses of community metabolism in a freshwater marsh sediment of protease activity demonstrated the advantages of data normalization in relation to community ATP levels (Table 8). Protease activity represent ability of the microbial population to degrade large proteinaceous molecules in the environment into simpler polymers for utilization by the microbe as a carbon or nitrogen source. Direct analysis reveals that sites A and B were similar and had significantly less metabolic activity than site C. After normalization, the metabolism of sites B and C per unit total biomass was similar and exhibited 3 times more protease activity than site A. Therefore, communities at sites B and C were more physiologically active per unit biomass than the community at site A at this time. Similar patterns emerged from simultaneous measurements of β -glucosidase and phosphatase activity.

Table 7. Changes in alkaline phosphatase activity as a function of growth for Achromonas sp.
grown in 0.8% nutrient broth

Hours of Growth	$\mu\text{g ATP}$ ml culture^{-1}	Phosphatase	
		$\text{ng product h}^{-1}\text{ml}^{-1}$	ng product h^{-1} $\mu\text{g ATP}^{-1}$
8	0.896	27.1	30
10	0.784	28.5	36
12	0.528	34.9	66
14*	0.464	34.4	74
16	0.576	37.1	64
24	0.005	53.3	107

* End of logarithmic growth

Table 8. Estimates of protease activity of inlet sediments (Lawrence Lake, Barry Co., Michigan), July 1976

Site	$\mu\text{g ATP}$ g dry wt^{-1}	$\Delta \text{OD h}^{-1}$ g dry wt^{-1}	$\Delta \text{OD h}^{-1}$ $\mu\text{g ATP}^{-1}$
A	16.37	0.85 ± 0.21	0.05 ± 0.01
B	5.26	0.79 ± 0.14	0.15 ± 0.03
C	11.96	1.53 ± 0.48	0.13 ± 0.04

[Values are expressed as means $\pm$ limits of the range; N = 3 per site.

Optical Density = OD.]

CONCLUSIONS

Measurement of environmental levels of ATP in sediment communities is a complex problem because of various chemical interferences associated with extraction of ATP from particulate materials. The major chemical interferences emanating from sediment particles is the acid-soluble organic fraction, the fulvic acids. Fulvic acids bind in a non-competitive fashion with ATP. Fulvic acid-ATP complex is not capable of reacting with the luciferin-luciferase complex and can inhibit detection of as much as 80% of the ATP present in an environmental sample. Although the mechanism of binding of ATP by fulvic acids has not been elucidated totally, evidence in this study indicates that a covalent bond or strong hydrogen bond occurs between the fulvic acid moiety and ATP.

Traditional interpretations of environmental levels of ATP as an estimate of microbial biomass are not satisfactory. Two necessary conditions for this interpretation to be true are that cellular levels of ATP remain constant under physiological variations and that a similarity exists between ATP:cellular carbon ratios for different taxonomic groups; both are questionable. However, environmental ATP levels used as a measurement of the physiological energy available to community metabolism may be a valuable tool in comparing metabolism between different communities.

Table 9 demonstrates four theoretical physiological states possible in a sediment community. ATP levels represent physiological size of the population and also represent the amount of physiological energy available for community metabolism. Metabolism represents some physiological function of the community such as CO₂ evolution

Table 9. Theoretical relationships between ATP levels and physiological condition of a sediment community

	Low ATP	High ATP
Low metabolic rate	small population metabolic steady state	large population metabolically inactive
High metabolic rate	small population with high metabolic activity	large population metabolic steady state

(respiration), enzymatic rate of degradative enzymes, or photosynthesis. As a result, both the size and physiological function of the community are defined, and interpretations are possible while assumptions as to the meaning of ATP are eliminated. For example, a microbial community with a low ATP value and high metabolic rate reflects a potential for growth, such as, in response to a new nutrient source. A microbial community with a high ATP value and low metabolic rate reflects an inactive population which is in an inactive phase of diel metabolism or a population which has utilized all available resources and senescence has been initiated.

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