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AN IMPROVED METHOD FOR THE DETERMINATION OF ADENOSINETRIPHOSPHATE IN ENVIRONMENTAL SAMPLES

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Abstract—An improved method has been developed for the analysis of the adenosinetriphosphate (ATP) content of environmental samples with the luciferin–luciferase assay. Factors affecting the recovery and stability of the ATP as well as the enzymatic reaction were optimized. The most versitile method utilizes procedure which can be used for natural waters, sediments and sludges. This involves homogenization at room temperature in 10^{-2} M trisodium phosphate, pH 11.7, containing 23% chloroform. This method is more effective than other methods tested and results in minimal interference with the enzymatic reaction. It also provides improved ATP recovery and long term stability of extracted ATP. Another method that can be used on natural waters and sludges is by boiling for 5 min in 10^{-2} M glycine buffer, 5×10^{-3} M Mg-EDTA, pH 10, which resulted in complete extraction and no hydrolytic loss of ATP. However, it is not satisfactory for sediments containing high humic acid substances as well as high metal loadings.

In addition to the direct comparison of endogenous ATP extraction by various methods, actual recoveries of spiked radioactive ATP was measured. ATP adsorption, hydrolysis and recoveries in each method were determined by the use of [8-^{14}C] ATP and [γ-^{32}P] ATP.

INTRODUCTION

The determination of biomass in water, wastewater and sediments has become an important aspect of the environmental sciences. Estimates of the quantity of biota are required in surveillance, monitoring, management, toxicology, ecology and laboratory studies. Of the many methods used for this purpose, one of the most promising is the use of adenosine-5'-triphosphate (ATP) as an estimator of biomass. ATP is an ubiquitous component of living matter, is degraded rapidly upon cell death, is relatively stable with regard to biomass of many studied organisms (Hamilton & Holm-Hansen, 1967; Patterson *et al.*, 1969; D'Eustachio *et al.*, 1968; Holm-Hansen, 1970). In addition, the quantitative measurement of ATP, at micro-level, is relatively simple and can easily be carried out in laboratory or in field operations. The method most often used in the quantification of ATP is the ATP-coupled oxidation of luciferin by luciferase with measurement of the bioluminescence produced. Several articles have been written on the mechanism and kinetics of this reaction (McElroy, 1947; Seliger & McElroy, 1960; Plant *et al.*, 1968).

In spite of inherent advantages of using the bioluminescence method, utilizing luciferin–luciferase, it has not received complete acceptance. This is mainly due to the fact that in literature, numerous and varying conditions are described for extraction and assay of ATP. In addition, there is lack of agreement with regard to instability of ATP, interference by coextractants, lack of reproducibility and the requirement for internal standards in some methods.

We have examined ATP extraction, optimum conditions for bioluminescence, stability, interferences and adsorption problems. One method is described which is suitable for water, wastewater and sediment samples and an alternate choice is also presented which can be used for all except sediment samples. A brief outline of these techniques has been described elsewhere (Afghan *et al.*, 1976).

MATERIALS AND METHODS

Reagents and water

All chemicals were reagent grade. Solutions and dilutions were made with low response water (LRW) obtained from a Milli-Q2 system (Millipore Corp.). All solutions were sterilized by passing through 0.45 μm membrane filters and were stored at 4°C.

Extraction procedure for natural waters

One litre of the sample was prefiltered through a 200 μm plastic screen. Subsampling was performed while stirring to maintain uniformity of composition. Fifty milliliter aliquots were filtered through 24 mm diameter Whatman 984H glass-fiber filters, using a vacuum of 250 mm mercury. Care was taken to prevent drying of the pad during filtration. The filter pad was then transferred to a scintillation vial in a heating block at 110–115°C (Pierce Reacti-Therm Heating Block No. 18800 and blank Reacti-Block, model K, No. 18810, milled with five holes 1⅛ in. deep and suitable diameters for a snug fit with scintillation vials) which contained 5 ml of boiling 10^{-2} M alkaline glycine–Mg EDTA buffer (10^{-2} M glycine, 5×10^{-3} M magnesium ethylenediaminetetraacetate, pH 10). It is essential that the

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filter pad is completely in the extracting solution, during boiling, to ensure complete lysis and quantitative extraction of ATP. The cap was replaced loosely and the heating block agitated periodically for 5 min. The vial was then placed in an ice bath to cool, the contents transferred to a centrifuge tube and centrifuged for 5 min at 3000 rpm. The supernatant was decanted into a centrifuge tube and stored at 4°C.

Extraction procedure for sediments

Ten milliliters of 10^{-2} M Na_3PO_4 was placed in a 30 ml beaker and 3 ml of distilled chloroform was added. The tip of a Polytron homogenizer (Brinkman Instruments, equipped with a PT-10 tip) was placed in the beaker and turned to full speed just prior to the addition of sediment. A 0.6 cm^3 aliquot of sediment was added by means of a fabricated sediment subsampler and homogenized for 2 min. The sediment subsampler was a 1 cm^3 plastic tuberculin syringe cut off at the zero cm^3 mark and the new tip sharpened with a cork borer sharpener. The contents of the beaker were centrifuged for 10 min at 3000 rpm, the supernatant was filtered through a 13 mm 0.45 μm membrane filter using 720 mm mercury vacuum for clarification and removal of dissolved chloroform. The clarified extractant was diluted 0.5 ml to 5.0 ml 10^{-2} M glycine pH 10.0 plus 5×10^{-3} M Mg-EDTA buffer and stored at 4°C.

Replicate 0.6 c samples were taken for dry weight determinations by drying to constant weight.

ATP Determination

The DuPont 760 Luminescence Biometer (E. I. DuPont Instrument Co.) coupled with a fast response recorder (Hewlett Packard model 7101 B/Br) was used for quantification of ATP in extracts. The DuPont Reagent Kit (DuPont No. 760145–901) was used to prepare the reagent solution following the instructions in the DuPont Manual (DuPont, 1970). The reagent was prepared daily and is stable for at least 8 h at room temperature and 24 h at 4°C.

Electronic stability of the instrument, dark current measurement and inherent light intensity of the bioluminescence reagent were determined as described (DuPont, 1970). The biometer was calibrated for each reagent kit with 10 μl injections of 100 $\mu g\,l^{-1}$ ATP standard solution. Then a calibration curve was prepared by triplicate 10 μl injections of standard solutions of 10, 50 100 $\mu g\,l^{-1}$ ATP in the glycine-Mg EDTA buffer as well as blanks of 10 μl of water and the extraction solution. For the determination of ATP in samples, 10 μl of sample is used, in triplicate, with periodic standards to check biometer stability. The coefficient of variation for sediment and sludge determinations was about 5.1%.

RESULTS AND DISCUSSION

Reaction conditions

One of the primary requirements for a sensitive and reproducible ATP assay is the availability of low response water for making solutions, dilutions and rinsing glassware. The conventional method of treating distilled water by acidification, boiling, cooling and neutralization and filtering through a 0.45 μm membrane filter was time consuming and the water had a varying residual response. The Milli-Q2 water system was a good source of low response water. The luminescence of 10 μl of water from the Milli-Q2 system was found to be consistent at $0.1 \pm 0.05 \times 10^5$ as compared with 8×10^7 for 0.1 $\mu g\,l^{-1}$ ATP.

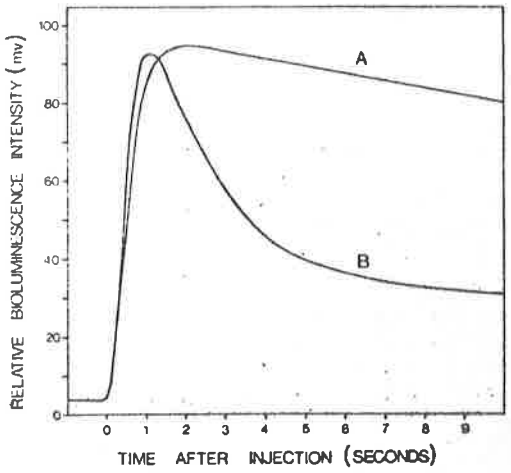

Fig. 1. Bioluminescence decay with two different luciferin–luciferase preparations. A fast-response recorder was used to follow the decay kinetics of A. Sigma reagents with a 10 μl injection of 60 $\mu g\,l^{-1}$ ATP. B. Dupont reagents with a 10 μl injection of 0.8 $\mu g\,l^{-1}$ ATP.

The luciferin–luciferase reaction with ATP proceeds at a rapid rate and accurate quantification depends upon fast, efficient and reproducible mixing of sample and reagents (Johnson *et al.*, 1974). The Biometer system depends upon the force of the sample injection into the reaction mixture to mix the solutions. A flat-ended needle is used in conjunction with a Shandon injector to give a reproducible response. A fast-response recorder is used during the injection–reaction period to ensure a good injection and normal reaction. The peaktime and decay curve are very characteristic for the ATP–bioluminescence reaction as shown in Fig. 1A, therefore, the graphs serve as a permanent record of the injections and a diagnostic for poor injections. In case of improper mixing, the decay curve is uncharacteristic and that assay must be repeated.

The firefly luciferin and luciferase reagents are important variables in the determinations (Lundin & Thore, 1975). Three commercial luciferase preparations were tested: the DuPont reagent kit and Sigma and Worthington lyophilized preparations. The DuPont purified reagent was found to be 50–75 times more sensitive than the others and to have a slower reaction decay. Figure 1 illustrates the reaction peak of the DuPont reagents to be between 2–4 s and the Sigma reagents to be at 1 s. While the Sigma luminescence intensity decays at about $2\%\,s^{-1}$.

ATP is usually the major, but not the only nucleotide present in significant concentrations in microbial cells (Bagnare & Finch, 1972). Therefore, a number of other ribonucleotides were also tested for their response in the luciferin–luciferase assay (Table 1). With the exception of ADP trace-to-nil responses were obtained with all nucleotides tested. Further, the peak times of all ribonucleotides other than ADP were much greater than ATP and would pose more of an interference problem for luminescence meters using

Table 1. Bioluminescence of ribonucleotides

Ribonucleotide 2×10^{-7} M	Peak time, min	Response at 0.05 min mV	%	Response at peak mV	%	Integrated area 0.25–1.25 min area. cm^2	%
ATP	0.05	68	100	68	100	585	100
ADP	0.05	0.8	1.2	0.8	1.2	6.9	1.2
AMP	Nil	Nil	Nil	Nil	Nil	Nil	Nil
CTP	9	Nil	Nil	0.4	0.6	1.7	0.3
GTP	3	<0.1	<0.1	3.2	4.7	19.2	3.3
ITP	3	<0.1	<0.1	2.1	3.1	12.9	2.2
UTP	9	≪0.1	≪0.1	0.9	1.3	4.5	0.8

an integration mode than machines using a peak height mode (Table 1). The ADP used (Sigma Chemical Co.) gave 1.2% of the ATP response and had a peak time equal to that of ATP. This probably reflects the level of ATP contamination of the ADP preparation as shown previously (Holmson *et al.*, 1966).

The hydrogen ion concentration strongly influences the rate of the luciferin–luciferase reaction with ATP. A wide variety of buffers and a range of pH values have been cited as optimum for the reaction. Holm-Hansen and co-workers (Holm-Hansen & Booth, 1966; Hamilton & Holm-Hansen, 1967) used pH 7.75 while others (McElroy, 1963; Van Dyke *et al.*, 1969; Stanley & Williams, 1969 and Kimmich *et al.*, 1975) have used pH 7–8. The pH-dependence of the reaction was not symmetrical (Fig. 2) gradually increasing to pH 7.2 and falling off rapidly after pH 7.7. The activity changed little between pH 7.2–7.6 and the optimum was selected as pH 7.4 ± 0.2. Ten buffers at 10^{-2} M and pH 7.4 were tested for their ability to support the reaction (Table 2). The highest responses were obtained with glycine, HEPES, MOPS, TES and Tris buffers whereas the other buffers gave a response which varied from 66 to 82% relative to MOPS morpholinopropane sulfonic acid buffer.

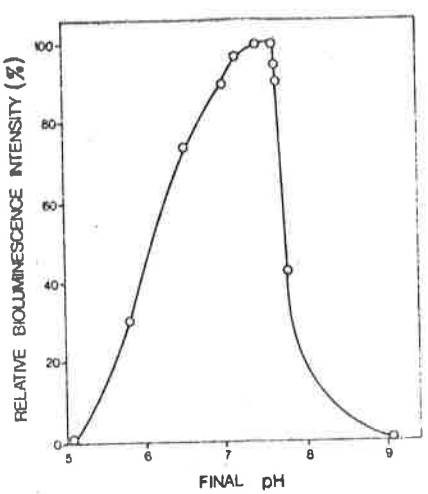

Fig. 2. Effect of hydrogen ion concentration on the bioluminescence reaction. The MOPS buffer used in the reaction was adjusted with either HCl or NaOH to the final pH indicated.

In general, the increased ionic strength in the sample has an adverse affect on the reaction. The degree of inhibition varies among salts and is dependent upon both the anions and cations. The effect of salts on the reaction became apparent even at low concentrations (Fig. 3) and were not due to heavy metal contaminants. Ionic strengths (I) as low as I = 0.1 of potasium nitrate gave a 22% decrease in the luminescence intensity. Potassium salts were slightly more active in decreasing luminescence intensity and the corresponding sodium salts and the order of activity of the anions were $ClO_4^- > NO_3^- > Cl^- > SO_4^-$.

Factors affecting quantification of ATP

ATP and many other organic phosphate compounds can be unstable in solution (Kalben & Koch, 1967; Leninger, 1970). Early in these studies, ATP standards varied from one time to another even though frozen immediately after preparation. Rapid freezing of ATP standards in liquid nitrogen and storage at −35°C largely eliminated variability. Some other factors which influence the stability of ATP in solution were investigated. ATP stability was tested in 14 different buffer and pH conditions. Hydrolysis occurred relatively fast in 2×10^{-2} M Tris, pH 7.4 or 10^{-2} M MOPS, pH 7.5 as shown in (Table 3). No hydrolysis was found in glycine, carbonate and phosphate buffers at pH 10 or above. ATP standards made up in 10^{-2} M glycine, pH 10 were stable for over 6 months at room temperature. This buffer was selected for all further preparation and storage of ATP solutions.

As boiling has been commonly used in cell lysis and liberation of ATP (Holm-Hansen & Booth, 1966; Cheer *et al.*, 1974 Levin *et al.*, 1975; Brezonik *et al.*, 1975), the effect of boiling on ATP stability was studied. No decomposition was observed in the alkaline glycine, carbonate or phosphate buffers when they were boiled for up to 15 min, whereas 60% of the ATP was lost by boiling ATP solutions for 10 min in 10^{-2} M MOPS, pH 7.4 (Afghan *et al.*, 1977).

The presence of organic and inorganic materials in samples is also known to interfere with the analyses (McElroy, 1963; Lundin & Thore, 1975). Interference can be due to both inhibition of the luciferase activity or by catalysis of non-enzymatic hydrolysis of ATP.

Table 2. Effect of various buffers on the luciferin–luciferase assay

Buffer*	ATP added $\mu g\,l^{-1}$	RLI mV	Recovery %
Arsentate	50	26	72
	100	49	69
S-Collidine	50	28	78
	100	55	77
Glycine†	50	37	103
	100	72	101
Glycyl glycine	50	26	72
	100	48	68
HEPES	50	37	103
	100	70	99
MOPS	50	36	100
	100	71	100
Phosphate	50	24	67
	100	47	66
TES	50	35	97
	100	72	101
Tris	50	36	100
	100	72	101
Tris maleate	50	29	81
	100	58	82

* All buffers were at 10^{-2} M and pH 7.4.

Abbreviations—HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; MOPS, 3-(*N*-morpholino) propanesulfonate; TES, *N*-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulfonate; Tris, Tris-(hydroxymethyl)-aminomethane.

† Glycine pH decreased with addition of luciferin–luciferase mixture.

Several cations were tested for their action on the enzymatic determination of $50\,\mu g\,l^{-1}$ ATP. The luminescence intensity was decreased from 82% of control by 2×10^{-4} M Fe^{3+} (Table 4) to 2% of control by 2.5×10^{-4} M Mn^{2+}. The decay curve shape differences were not influenced by the addition of metal ions to the solutions even though the luminescence intensity decreased, except in the case of Cu^{2+} where the decay curve was slower and took about three times longer to peak. This inhibition was not overcome by the addition of 10^{-2} M Mg-EDTA after the extracts were stored for 1 week, except in the case of Cu^{2+} and Mn^{2+} (unpublished results). The inhibition was removed, however, by inclusion of 10^{-2} M Mg-EDTA in the glycine buffer before extraction of the sample or addition of the ATP spike. Because these and other metal ions are extremely common in environmental samples, the Mg-EDTA was always included in the 10^{-2} M glycine, pH 10 extraction buffer.

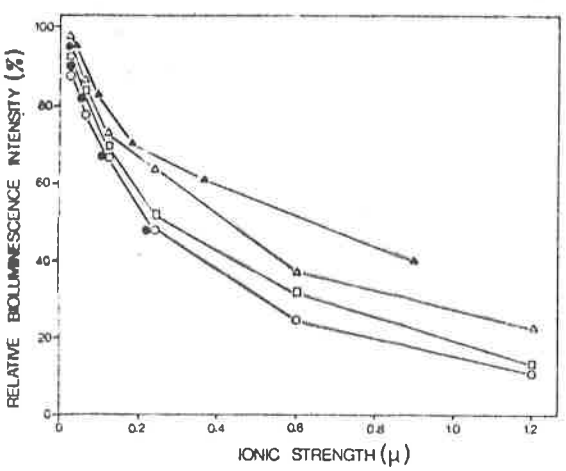

Fig. 3. Effect of various salts on the bioluminescence reaction. The salts were added at the ionic strengths indicated in the sample. A 10 μl sample of $83\,\mu g\,l^{-1}$ ATP was injected into the 100 μl of reagent in the normal manner. ▲ = Na_2SO_4, △ = NaCl, □ = $NaNO_3$, ○ = KNO_3, ● = $KClO_4$.

The high concentrations of proteins and other organic compounds in biological materials and environmental samples suggested that they be tested for their effect on the assay. No interference was encountered with up to $1500\,mg\,l^{-1}$ bovine serum albumin. Other organic compounds tested were amino acids, carbohydrates, fatty acids, amino polycarboxylic acid, humic substances and lignosulfonic acid salts. None of these compounds interfered except fulvic acid and lignosulfonic acid salts when present above $25\,mg\,l^{-1}$. Even this interference was not significant to the assay as employed here because a 5–10 dilution of samples is performed before analysis.

ATP Extractions

(a) *Water samples.* Most water samples have a low biomass such that concentration is required for adequate sensitivity. The most common method of concentration is by filtration through fine glass-fibre filters or membrane filters followed by cell lysis in a boiling buffer at a pH between 7.4 and 7.8. In our

Table 3. The stability of 100 $\mu g\,l^{-1}$ ATP standards in various buffers

Buffer constituents*	pH	RLI† %	% of initial bioluminescence 15 min	30 min
Phosphate‡ (Na_3PO_4)	11.7	95.2	100.0	101.0
Carbonate‡ (Na_2CO_3)	10.8	98.1	96.2	98.1
Glycine	10.5	98.2	100.1	99.0
Glycine	9.9	100.0	98.8	98.2
Borate‡	9.8	97.6	97.6	101.9
Glycine	9.0	97.3	97.4	90.1
Pyridine	8.7	66.7	76.5	47.2
Tris§	7.8	92.5	96.7	91.3
MOPS	7.5	66.7	68.3	25.2
MOPS + 10^{-3} M Mg-EDTA	7.5	83.1	96.8	85.7
Tris	7.4	65.6	65.6	15.4
Tris + 10^{-2} M Mg-EDTA	7.3	79.0	90.6	73.2
Citrate‡	4.7¶	85.0	100.0	99.4
Glycine‡	2.4	92.7	98.6	99.0

* 10^{-2} Molar buffers except 2×10^{-2} M for Tris.
† Relative Luminescence Intensity.
‡ ATP solutions prepared from stock of 1% ATP in 10^{-2} M glycine and were never frozen.
§ ATP prepared fresh in buffer and frozen 1 month in liquid nitrogen.
‖ pH adjusted to 9.9 prior to assay.
¶ pH adjusted to 7.3 prior to assay.

procedure, lysis was accomplished in boiling glycine buffer, pH 10. A heating block was a convenient and reproducible means of heating the vials containing extraction buffer. A block temperature of 110–115°C gave best results, while temperatures greater than this resulted in evaporation of the buffer and non-reproducible results. A fry-pan sand bath is commonly used (Cheer *et al.*, 1974) for heating the extraction buffer but was found to be less effective than the temperature-controlled block as shown in (Table 5). Recoveries of ATP from the fry-pan sand bath averaged about 76% of those from the heating block when sludge was injected directly and only 47% when material on filters was used. Similarly, testing of a block that contained long narrow tubes which could subsequently be used for centrifugation did not give a satisfactory performance and gave low recoveries of ATP as compared to Pierce Reacti-Therm Heating Block.

A comparison was made between the alkaline glycine extraction—DuPont Luminescence Biometer method and a boiling Tris-Lab-Line ATP Photometer system. Boiling 2×10^{-2} M Tris, pH 7.75, in a sand

Table 4. Effect of metals on ATP bioluminescence and removal of interference

Composition of solution	% recovery of 50 $\mu l\,l^{-1}$ ATP 10^{-2} M glycine, pH 10	10^{-2} M glycine + 10^{-2} M Mg-EDTA, pH 10
10^{-2} M Ca^{2+}	57	85
10^{-2} M Mg^{2+}	91	93
10^{-3} M Al^{3+}	57	96
2.5×10^{-4} M Fe^{2+}	60	95
2×10^{-4} M Fe^{3+}	82	93
4×10^{-3} M Cu^{2+}	11	88
10^{-4} M Pb^{2+}	32	98
2.5×10^{-4} M Mn^{2+}	2	96

Table 5. ATP extraction using different boiling techniques

Sample	ATP Content of extract, $\mu g\,l^{-1}$* Heating block	Fry-pan sand bath
Pilot plant activated sludge†	91.7 ± 2.1	74.3 ± 7.0
Bench-scale activated sludge†	57.6 ± 2.0	40.6 ± 3.3
Lake Ontario water‡	7.7 ± 1.1	2.7 ± 0.5
Lake simulator column water‡	16.2 ± 2.2	9.4 ± 1.8

* Mean ± standard error of three replicate samples.
† Direct injection of sample into boiling buffer.
‡ Samples were collected on glass fibre filters and immersed in boiling buffer.

Table 6. Comparison of methods for analysis of ATP in water samples

Sample number	Lab-Line Photometer				Dupont Biometer			
	Tris, uncentrifuged		Tris, centrifuged		Tris, centrifuged		Glycine, centrifuged	
	Found	Spike recovery*	Found	Spike recovery	Found	Spike recovery	Found	Spike recovery
	$\mu g\,l^{-1}$	%	$\mu g\,l^{-1}$	%	$\mu g\,l^{-1}$	%	$\mu g\,l^{-1}$	%
1	2.4	49	4.2	87	5.4	97	7.4	99
49	2.3	72	3.7	88	4.7	96	14.4	97
81	2.6	34	5.7	86	7.4	101	10.7	100
93	1.7	65	2.5	86	2.9	100	8.3	102
109	5.4	63	8.3	93	9.5	99	13.0	97
117	3.3	62	4.9	87	6.3	101	9.6	101

* Spike with 10 $\mu g\,l^{-1}$ ATP

bath was used to release the ATP from the samples and they were analyzed, without centrifugation, by injecting a 200 μl sample into a 100 μl purified firefly reagent in the Lab-Line ATP Biometer using an integration of 15–75 s. Although both methods gave similar trends, the results using an alkaline glycine extraction were 30–150% higher. There could be many reasons contributing to this difference, viz. the integration versus peak height method of analysis, the removal of debris by centrifugation, ATP hydrolysis or differences due to the different sample/solvent ratios used in these two systems for analysis.

To compare these two techniques, a study was made of several water samples with both luminescence meters, both extractions, with and without centrifugation of the extract before analysis (Table 6). Matrix interference in the uncentrifuged extract adversely affected the Tris extracts analyzed in the Lab-Line ATP Photometer. Results increase by about 1.6 times after clarification by centrifugation. The results with the centrifuged Tris extracts were slightly lower when measured with the Lab-Line Photometer as compared with the DuPont Biometer. Highest results were obtained with alkaline glycine extractions, measured with the DuPont Biometer. No matrix interference was observed in any samples measured in the DuPont Biometer.

(b) *Sludge samples.* Sludge samples, high in biomass, do not require concentration and can be injected directly into an extraction medium. Seven different extraction media (Holm-Hansen & Booth, 1966; Kalbhen & Koch, 1967; DuPont, 1970; Dhople & Hanks, 1973) were tested for their extraction ability and interference with the enzymatic assay for ATP (Table 7). Boiling glycine, pH 10 and Polytron homogenization in 23% chloroform in Na_3PO_4, pH 11.7 gave the highest apparent ATP concentration and also 100% recovery of an internal spike. All other combinations gave lower ATP content and lower recoveries of ATP spikes; none of these methods except perchloric acid gave ATP recoveries equal to those above even when corrected for matrix interference. Times of 5 min boiling in glycine buffer or 2 min homogenization in $CHCl_3$–Na_3PO_4 were selected as they were greater than the minimum time required for complete extraction and were less than times where losses occurred. The chloroform trisodium phosphate was later tested on water samples by introducing the glass-fibre filter containing the biological material right under the Polytron tip in the solution

Table 7. Comparison of ATP extraction procedures on activated sludge (bench-scale)

Extraction treatment	Apparent concentration of ATP for 0.2 ml sludge sample, $mg\,l^{-1}$	Recovery of spiked ATP, %	Corrected ATP concentration of sample, $mg\,l^{-1}$	Relative extraction efficiency, %
Boiling Tris	2.9	96	3.0	88
Boiling glycine	3.2	100	3.2	94
Vortexing cold 6% $HClO_4$	1.6	48	3.3	97
Vortexing acetone	1.9	100	1.9	56
Vortexing *n*-BuOH* in glycine	2.1	73	2.9	85
Vortexing 90% DMSO† in glycine	0.7	61	1.1	32
Vortexing 23% $CHCl_3$ in glycine	2.0	91	2.2	65
Homogenizing 23% $CHCl_3$ in Na_3PO_4	3.4	100	3.4	100

* *n*-Butanol.
† Dimethyl sulfoxide.

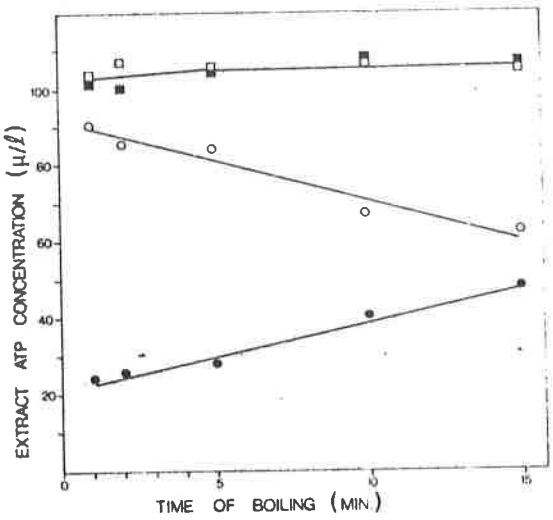

Fig. 4. Effect of Mg-EDTA on the apparent ATP concentration in activated sludge. 10^{-2} M glycine, pH 10 in the presence or absence of 5×10^{-3} M Mg-EDTA was used to liberate ATP from activated sludge by boiling for various times. ○ = No Mg-EDTA; ● = no Mg-EDTA, analyzed after 3 days; □ = Mg-EDTA; ■ = Mg-EDTA, analyzed after 16 days.

while homogenizing. Recoveries were slightly higher and more reproducible than the boiling glycine method but the method may not be as amenable to field usage. Complete and rapid killing of cells was necessary to prevent phosphatases degrading ATP. This necessitated addition of the sludge directly to the boiling glycine or to the $CHCl_3$–Na_3PO_4 while homogenizing. Addition of the extractant to the sludge or sediment gave lower recoveries which is believed to be due to incomplete lysis and/or enzymatic degradation of ATP. In both methods, the addition of 0.1–1.0 ml of sludge gave linear ATP values with increasing volumes of sludge.

As with water samples, Mg-EDTA addition to the alkaline glycine buffer was only effective in removing heavy metal interference when it was included in the extraction buffer and was not effective if added later (Fig. 4). Boiling times up to 15 min in alkaline glycine buffer containing Mg-EDTA gave uniformly high recoveries and these extracts were stable for over 2 weeks. In the absence of Mg-EDTA, recoveries were lower and decreased with boiling time, probably due to precipitation and non-enzymatic hydrolysis of ATP.

The $CHCl_3$–Na_3PO_4 extraction method was tested against the conventional boiling Tris buffer pH 7.75 (Levin *et al.*, 1975) using activated sludge from a pilot-scale sewage treatment plant. After extraction, the Tris extracts were frozen in liquid nitrogen and stored at −35°C to minimize hydrolysis while the alkaline phosphate extracts were stored at 4°C. The analysis of the extracts on the DuPont Bioluminescence Biometer showed the Tris extracts to contain only 89% of the ATP content of the trisodium phosphate extracts.

Stabilities of the extracts were tested under various conditions. The centrifuged $CHCl_3$–Na_3PO_4 extracts were extremely stable with 96% recovery of the ATP after storage at room temperature for 2 weeks. The centrifuged alkaline glycine extracts gave 93% recovery after 2 weeks at 4 C or 78% after 2 weeks at room temperature. The recoveries were about 20% lower if the debris was not removed by centrifugation before storage.

(c) *Sediment ATP extractions*

Sediment samples for ATP analysis presents several special problems. Sediment composition can vary widely depending upon the geographical location and sedimentation conditions. The composition of sediment is above all a function of the supply of undissolved substances such as minerals, vegetation residues, organic colloids, organic decomposition products, amorphous diatomaceous silicic acid and biogenically precipitated lime. It is known that phosphates can exchange with other ions or natural sediment constituents such as aluminum and iron hydroxides and oxides and clay minerals. Since this exchange varies with buffer constituents and pH, the alkaline buffers were tested for interference with the ATP assay by a number of sediment inorganic constituents and sterilized sand and clay (Table 8). These materials were leached with the buffers, centrifuged and then spiked with 20 and 50 $\mu g\ l^{-1}$ ATP. Of the three alkaline buffers tested, the trisodium phosphate gave quantitative recoveries. This is partially because the solubility of many of the mineral constituents in strongly alkaline phosphate medium is low and the leaching of these ions is kept at a minimum. It should be noted that in some cases, as with clays, organic material is leached from the sample and results in quenching of the bioluminescence. This does not present a problem here, however, as the dilution of the Na_3PO_4 extract with glycine–Mg EDTA solution prior to analysis resulted in quantitative recovery.

These same materials were used to determine ATP loss on particulate material. ATP can be lost by sorption or chemical interaction on cellular debris or insoluble sedimental material. ATP was added to the insoluble material suspended in one of the alkaline buffers and mixed for 1 h. After centrifugation, the supernatants were analyzed for ATP (Table 8). There was no loss of ATP on particulates in the alkaline phosphate buffer while large and variable losses occurred with the glycine and Na_2CO_3 buffers depending upon the nature of the particulate material.

The most commonly used method for extraction of ATP from sediments are based on Lee's method (Lee *et al.*, 1971) of extraction with cold 0.6 N H_2SO_4. Recently a method using H_2SO_4 extraction followed by charcoal adsorption to eliminate inorganic interferences has been published (Hodson *et al.*, 1976). These methods were compared with the $CHCl_3$–Na_3PO_4 extraction technique. Nine surface sediments for all three Lake Erie basins were analyzed by the

Table 8. Matrix effects and loss of ATP on particulates from mineral constituents of sediments and sediments with varying chemical composition

Material	ATP added $\mu g\,l^{-1}$	% Recovery in 10^{-2} M extracts: Glycine pH 10 Spike*	Glycine pH 10 Supernatant†	Na_2CO_3 pH 10.8 Spike*	Na_2CO_3 pH 10.8 Supernatant†	Na_3PO_4 pH 11.7 Spike*	Na_3PO_4 pH 11.7 Supernatant†
Extractant	20	100	100	100	100	100	100
	50	100	100	100	100	100	100
5×10^{-2} M $CaCO_3$	20	80	27	103	21	89	104
	50	76	26	98	20	93	102
5×10^{-2} M $Fe(OH)_3$	20	70	30	101	25	82	96
	50	71	34	101	25	78	99
5×10^{-2} M $Al(OH)_3$	20	56	0	49	0	88	100
	50	53	0	52	0	92	97
4% Sterilized sand	20	97	96	95	83	99	98
	50	92	95	102	88	100	103
4% Sterilized clay	20	72	53	51	60	45	99
	50	65	56	46	57	50	97

* Materials were leached with buffer and then centrifuged, spiked with 20 or 50 $\mu g\,l^{-1}$ ATP and analyzed.
† Materials were mixed with buffer containing 20 or 50 $\mu g\,l^{-1}$ ATP for 1 h at room temperature, centrifuged and the supernatant analyzed.

$CHCl_3$–Na_3PO_4 method (Table 9). Sediments from stations 1, 4 and 8 were extracted by Lee's procedure (Lee *et al.*, 1971) with recoveries of 8.5, 13 and 41% respectively. The H_2SO_4 extraction and charcoal cleanup (Hodson *et al.*, 1976) yielded 8.5 and 10% on samples from stations 1 and 4, and could be increased to 15% on both samples by the additional step of Polytron homogenization. The reasons for these low recoveries were investigated by the use of [8-^{14}C] ATP and [γ-^{32}P] ATP. In parallel experiments, labelled ATP was added to the sediments and an extraction was made with either cold 0.6 N H_2SO_4 or 10^{-2} M Na_3PO_4, 23% $CHCl_3$ (Table 9). Recoveries of [^{14}C] ATP with the alkaline phosphate medium ranged from 94.0 to 98.5%. With 0.6 N H_2SO_4, recovery was variable with a range from 8.5 to 62.8%. This compares with Lee's data (Lee *et al.*, 1971) of 20–85% recovery in the sediments used in that study. A small proportion of the ATP loss in the H_2SO_4 extraction was due to hydrolysis of the γ-PO_4 as shown by the higher [^{32}P] recovery than [^{14}C] recovery in some sediments, notably stations 3, 4 and 6.

Various concentrations of nitric acid which has recently been used as an extractant for water samples (Deming, J. W., Hehnemaun Hospital and Medical Centre, Philadelphia, PA, personal communication) were tested for their ability to extract ATP from sediments. Labelled ATP was extracted with one of 0.1, 0.6, 1 and 2 N NHO_3 with recoveries of 1.3, 2.4, 5.1 and 8.3% respectively using sediment from station 1.

Application to environmental studies

These methods are being applied in our laboratories for routine analyses in sediment biomass and good correlations have been obtained with dehydrogenase and DNA-synthetic activity (Tobin *et al.*, 1976*b*). Other activities include water monitoring, surveillance and monitoring sludge biomass in pilot- and bench-scale activated sludge systems. In one study to determine the biodegradability of nonionic surfactants, information on build-up of the sludge biomass and the degradation of total organics versus polyethylene glycol ($\bar{M}w \simeq 400$) was required. Analysis of the sludge over the 42-day test (Fig. 5) revealed a parallel in the biodegration of polyethylene glycol with biomass until 21 days when a steady-state was reached. After that time, the degradation rate of polyethylene glycol continued to increase, presumably due to acclimation of the micro-organisms and/or population

Table 9. Recovery of endogenous and spiked labelled ATP in Lake Erie sediments

Sampling station	Endogenous ATP recovery ($\mu g\,g^{-1}$)	Recovery in extracts %: 0.6 N H_2SO_4 -[^{14}C]ATP	0.6 N H_2SO_4 [γ-^{32}P]ATP	10^{-2} M Na_3PO_4, 23% $CHCl_3$ [8-^{14}C]ATP
1	1.17 ± 0.05	13.7	21.8	95.0
2	0.39 ± 0.01	19.4	21.5	94.7
3	0.53 ± 0.02	5.2	13.2	98.5
4	1.00 ± 0.08	18.4	30.0	96.2
5	0.30 ± 0.02	16.2	20.5	95.4
6	0.16 ± 0.01	8.5	17.5	94.0
7	0.37 ± 0.04	24.1	35.7	94.6
8	0.22 ± 0.02	62.8	65.3	97.1
9	0.20 ± 0.03	32.9	37.6	96.5

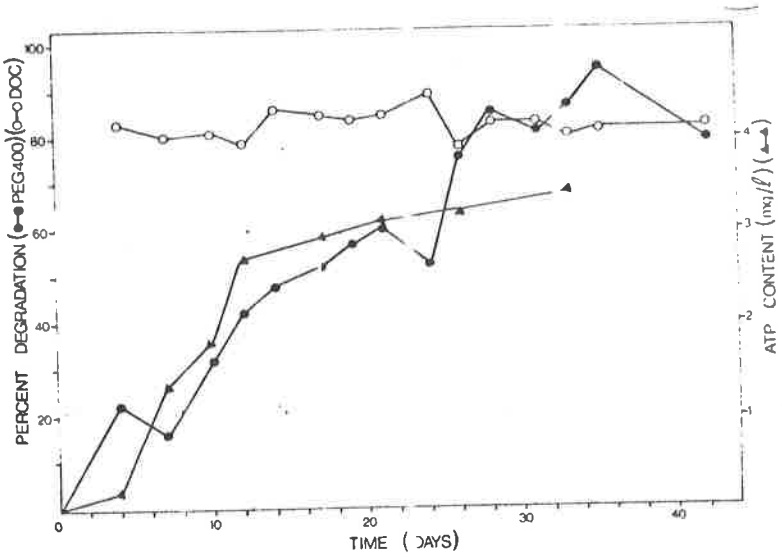

Fig. 5. Analysis of ATP content of a bench-scale activated sludge plant used for biodegradability studies.

shifts (Tobin *et al.*, 1976*a*). Overall degradation of organic material was greater than 95% throughout the course of the experiment and did not vary with biomass.

Potential applications

A number of avenues of further investigation in the application of these methods are being pursued in our laboratories. The ATP measurements can be used to distinguish between categories of microbiota, viz. bacterial versus algal cells, by means of size, selective lysis or in concert with chlorophyll measurements on duplicate samples. The methodology may be useful in the monitoring and control of sludge biomass in sewage treatment plants. Effluents may also be monitored so that the minimum amount of chlorination required to obtain near-zero viability in the effluent is used and could greatly reduced the potential for excess chlorine to produce chlorinated hydrocarbons.

Studies on immobilized luciferase reactors in an automated bioluminescence system are being initiated. The potential for automated extraction, cleanup and analysis could greatly reduce the time and cost of large numbers of analyses. Such developments can extend the applicability of the method to a great many new areas in environmental and industrial endeavours.

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