Environment Canada
Environnement Canada

Canada Centre For Inland Waters
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Biomass Estimation by Measurement of Adenosine Triphsphate (ATP)

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MANUSCRIPT No. 14-AMD-3-81-BA

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International Biological Program (IBP)
Handbook No. 12

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INTRODUCTION

The determination of biomass in water, wastewater and sediments has become an important aspect of environmental studies. In biological studies of the aquatic environment it is often necessary to relate the measured metabolic rates to the living cells present[A-1]. Biological activity in sediments and aquatic environment is also considered in nutrient cycling, transformation of metals, degradation and modification of organics and in hypolimnetic oxygen depletion[A-2]. The measurement of viable biomass is also required in wastewater technology and effluent treatment during secondary treatment of wastes to reduce biodegradable organic contamination.

Numerous publications have indicated that the ATP assay can provide a rapid measure of total biomass[A-3,A-4]. The ATP assay appears to satisfy the characteristics of an ideal biomass indicator better than other available parameters. This has led to widespread application of ATP assay to estimate biomass. For example, ATP has been used to estimate biomass in natural waters, process waters,

soils, sediments, activated sludge, foods, etc. ATP assay is also used for rapid assessment of water quality and for monitoring the disinfection of wastewater streams[A-5 to A-10]. Potential application of ATP measurement is also advocated to categorize and distinguish between microbiota viz bacteria versus algal cells, by means of size, selective lysis or in concert with chlorophyll measurements on duplicated samples[A-11].

The method most often used for the quantification of ATP is the ATP-coupled oxidation of Luciferin by Luciferase with the measurement of the bioluminescence produced. Inspite of inherent advantages of using 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, lysis efficiency, interferences by coextractants, lack of reproducibility and the requirement of internal standards. Afghan et al[A-2, A-5, A-6] examined the bioluminescence reaction and the lysis techniques and proposed two methods to quantitatively analyse ATP content in environmental samples. These methods are being adopted and/or being considered for adaptation as standard methods of test for determination of ATP content and estimation of biomass.

GENERAL OUTLINE OF THE TECHNIQUES

These methods describe the lysis of aquatic microorganisms and biota and the extraction of ATP prior to determination by the firefly bioluminescence method. Two extraction procedures are recommended and the choice of the extraction technique will depend upon the type of instrumentation available in the laboratory as well as the nature of sample. Extraction technique "A" utilizes polytron homogenization of sample in trisodium phosphate containing chloroform. Extraction "B" employs boiling alkaline glycine buffer containing magnesium disodium ethylenediamine. The extracts are centrifuged to remove debris and in case of extraction "A" chloroform is completely removed prior to quantitative analysis. Quantitative analysis is carried out using ATP-coupled oxidation of luciferin by luciferase which produces a selective bioluminescence and is proportional to ATP content in the sample. The bioluminescence reaction can be described as follows[1]:

$$\underset{\text{(Luciferase)}}{E} + \underset{\text{(Luciferin)}}{LH_2} + ATP \xrightarrow{Mg^{++}} \underset{\substack{\text{(Luciferyl}\\ \text{aldenylate}\\ \text{complex)}}}{E \cdot LH_2 \cdot AMP} + \underset{\substack{\text{(pyro-}\\ \text{phos-}\\ \text{phate)}}}{PP}$$

$$E \cdot LH_2 \cdot AMP + O_2 \longrightarrow E + \text{Product} + CO_2 + AMP + \underline{\text{Light}}$$

ANALYTICAL PROCEDURE

1. Scope

1.1 This method describes the extraction for the lysis of aquatic microorganisms and the extraction of ATP prior to determination by the firefly bioluminescence method.

1.1.1 Extraction technique "A" is more versatile and can be used for laboratory cultures, waters, wastewaters, activated sludges and sediments.

1.1.2 Extraction technique "B" is more practical for field applications. It can be used for laboratory cultures, waters, wastewaters and activated sludges but is not recommended for samples with high solid or metal content such as sediments and raw waste effluents (see 5.3.1 to 5.3.3).

1.2 This method covers the measurement of ATP in microorganisms in concentrations normally found in laboratory cultures, waters, wastewaters, activated sludges and sediments.

1.2.1 The sensitivity and range of the method depends upon the type of luminescence photometer, its mode of operation (peak height or integrated area) and the quality of the firefly reagent and extraction solution.

1.2.2 Under optimum conditions the sensitivity of the bioluminescence assay is 0.05 µg/litre of ATP in the final extract.

1.2.3 The practical detection limit based on the above (1.2.2) is 1 μg/litre for aqueous samples using direct extraction and 0.005 μg/litre using a 10-fold preconcentration. Similarly, the detection limit for sediment ATP is 0.015 μg/g of dry sediment.

1.3 Knowledge of the concentration of ATP can be related to viable biomass or metabolic activity, or by utilizing an average concentration (or amount) of ATP per cell (see 4.3.3). An estimated count of microorganisms can be obtained in the case of unispecies cultures.

1.4 This method offers a high degree of sensitivity, rapidity, accuracy and reproducibility. However, extreme care must be taken at each step in the analysis to ensure meaningful and reliable results.

1.4.1 The coefficient of variation for extraction technique "A" ±5% and for extraction technique "B" is ±7%.

2. Summary of Method

2.1 Determine the biomass in the sample by direct extraction when cell counts are greater than 10^7 cells per millilitre or when the endogenous concentration of ATP in the sample is greater than 5 μg/litre, as in the case of sediments, activated sludge, laboratory cultures and waste effluents high in nutrients. When the cell counts are less than 10^7

cell per millilitre, as in the case of natural waters, the microorgansims are concentrated on glass fiber filters by vacuum filtration of an appropriate volume of sample. The degree of concentration is dependent upon the turbidity of the sample or the suspended solid content of the sample.

2.2 Extraction technique "A" -- the ATP is extracted from the sample by homogenizing in trisodium phosphate solution containing chloroform ($CHCl_3$). The extracts of sediment samples are centrifuged. The extracts of aqueous samples and the supernate of sediment extracts are filtered under vacuum for clarification and the removal of dissolved chloroform. The clarified sediment extracts are diluted prior to analysis to prevent the interference of soluble humic substances with the bioluminescence assay.

2.3 Extraction technique "B" -- the ATP is extracted in boiling alkaline glycine buffer containing magnesium disodium ethylenediamine tetraacetate (Mg-EDTA). The extracts are cooled and then centrifuged to remove debris.

2.4 A carefully measured aliquot of the final extract is mixed with a standard quantity of buffered luciferin-luciferase reaction mixture and the light produced is measured with an appropriate luminescence photometer.

2.5 The instrument response is compared to that obtained for calibration standards and expressed in terms of ATP content per unit volume of aqueous sample or per unit weight of dry sediment.

3. Significance and Use

3.1 A rapid and routine procedure for determining biomass of the living microorganisms in cultures, waters, wastewaters, activated sludges and sediments is frequently of vital importance. However, classical techniques such as direct microscope counts, turbidity, organic chemical analyses, cell tagging, and plate counts are expensive, time-consuming, or tend to underestimate total numbers. In addition, some of these methods do not distinguish between living and non-living cells.

3.2 The ATP firefly (luciferin-luciferase) method is a rapid, sensitive determination of viable microbial biomass. ATP is the primary energy donor for life processes, does not exist in association with non-living detrital material, and the amount of ATP per unit of biomass (expressed in weight) is relatively constant. (ATP per cell varies with species and physiological state of the organism).

3.3 This method can be used to:

3.3.1 Estimate viable microbial biomass in cultures, waters, wastewaters, activated sludges, sediments, and other systems inhabited by microorganisms.

3.3.2 Estimate the amount of total viable biomass in plankton and periphyton samples.

3.3.3 Estimate the number of viable cells in a unispecies culture if the ATP content (or if the average amount

of ATP) per cell is known. For example, an average value of 5×10^{-10} μg ATP/bacteria cell has been proposed in the literature.

3.3.4 Estimate and differentiate between zooplanktonic, phytoplanktonic, and planktonic ATP by size fractionation, and simultaneous chlorophyll measurements.

3.3.5 Measure the mortality rate of microorganisms in toxicity tests in entrainment studies, and in other situations where populations of microorganisms are placed under stress.

3.3.6 Monitor biomass of activated sludge and waste effluents.

4. Terminology

4.1 Description of terms.

4.1.1 Biomass — the total quantity of living organisms in a sample or system.

5. Interferences

5.1 Reagents must be of high purity so that background light emission is held to a minimum for the measurement of ATP.

5.2 ATP-free glassware, prepared by the procedure in 6.10 is required for the determination of ATP.

5.3 Luciferase is an enzyme and as such can be inhibited or denatured by the presence of heavy metals, high salt concentrations, and organic solvents in the sample.

5.3.1 Calcium and heavy metals interfere with the ATP bioluminescence reaction if they are present in the extracts of technique "B" at concentrations which exceed the chelating ability of the magnesium disodium ethylenediamine tetraacetate (Mg-EDTA) incorporated into the extractant.

5.3.2 Strong electrolytes interefere with the ATP bioluminescence reaction if their concentration in the extracts is sufficiently high as to result in a final concentration in the bioluminescence reaction mixture of greater than 0.0005 M. This can be minimized by standard addition technique.

5.3.3 Soluble humic substances such as fulvic acid and lignosulfonic acid salts, which may be extracted from sediment samples, interfere with the ATP bioluminescence reaction if their concentration in the bioluminescence reaction mixture exceeds 2 mg/litre.

5.4 Other energy-mediating compounds, such as adenosine diphosphate, GTP, cytidine-5-triphosphate, and inosine-5-triphosphate, also react with luciferase to produce light, but do not constitute a significant source of error because their relative bioluminescence with high purity firefly agent is negligible, and they are usually present only in small amounts to relative ATP.

5.5 High-viscosity samples may not mix adequately with the reagents upon injection into the bioluminescence cell of the instrument. If this occurs, reaction rate may be reduced (reaction will not go to completion and the reaction rate will be decreased with improper mixing) or the results may not be reproducible.

5.6 Extracted ATP may be lost in the presence of particulate matter due to sorption or chemical interaction.

5.6.1 A variable and substantial loss of ATP can result from the extraction of ATP with glycine buffer in the presence of undissociated mineral constituents such as calcium carbonate, ferric hydroxide and aluminum hydroxide, which may be present in sediment samples.

5.6.2 A slow progressive loss of ATP can also result from the storage of extracts containing cell debris and insoluble sedimental material.

6. Apparatus

6.1 An appropriate luminescence photometer is recommended[3]. The stability of the instrument should be checked before each use with a standard light source available from the

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3. DuPont Model 760 Luminescence Biometer (No. 760000) or SAI Model 3000 ATP Photometer (No. 01-03-02), or equivalent, complete with sample injector, reagent dispenser and reaction vials.

manufacturer. It is advisable to maintain a record of the instrument response to permit detection of any instability or changes in response levels.

6.2 Vacuum filtration equipment, suitable for the simultaneous removal of particulates and chloroform from the extracts of technique "A"[4].

6.3 Vacuum Filtration Equipment, suitable for the concentration of microorganisms in aqueous samples[5].

6.4 Heating Block capable of accepting glass scintillation vials with a snug fit and maintaining a constant uniform temperature of 110 to 115°C[6].

6.5 Scintillation Vials, 20 mL with linerless polyethylene screw caps[7].

6.6 Homogenizer[8].

6.7 Centrifuge, capable of a relative centrigal force (RCF) of 1500 to 2000 xg using 12 mL centrifuge tubes[9].

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4 DuPont Vacuum Filter Stand (No. 760308) and Filter Assemblies (No. 760312) or equivalent.

5 Fisher Filtrator (No. 9-788) and Millipore Microanalysis Filter Holder (No. XX10-025-00) and Reeve Angel (No. 984H), 2.4 cm diameter glass fiber filter circles with 0.3 to 0.5 μM pore size or equivalent.

6 Pierce Reacti-Therm Heating module (No. 18800) and Pierce Reacti-Block K (No. 18810) milled with five holes 1-1/8 inch (2.86 cm) deep and suitable diameter for a snug fit with standardized glass scintillation vials or equivalent.

7 John Scientific (No. 66022-217) or equivalent.

8 Willems Polytron with model PT10 generator or equivalent.

9 IEC model CL or equivalent.

6.8 Sediment Sampler, capable of delivering 0.6 cubic centimetres of wet sediment with a coefficient of variability of ±2% with respect to dry weight of sediment. A disposable one cubic centimetre tuberculin syringe with the tip cut off at the zero cubic centimetre mark on the barrel and sharpened with a cork borer sharpener or equivalent.

6.9 Automatic Pipettes and Disposable Tips.

6.10 ATP -- Free Glassware. Rinse chemically clean glassware three times with 2 N HCl, rinse three times with 0.01 M Glycine Buffer (7.9) and rinse three times with low response water (7.1) and dry in the oven for 30 minutes at 150°C.

7. Reagents

7.1 Low Response Water (LRW) -- sterile deionized, ATP-free water, which gives a bioluminescence response equivalent to 0.03 µg/l ATP or less is prepared by treatment in a suitable system involving carbon treatment with deionization and filtration[10].

7.2 Hydrochloric Acid (HCl) Solution, 2M -- dilute 168 mL of concentrated hydrochloric acid (sp. gr. 1.19) up to 1000 mL with LRW (7.1) water and transfer to a polyethylene wash bottle.

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10 Millipore 3-Housing Milli-Q2 Reagent Grade Water System (No. AD29 000 70) or equivalent.

7.3 Sodium Hydroxide (NaOH) Solution, 2.5 M — dissolve 10 g of sodium hydroxide pellets in LRW and dilute to 100 mL. Transfer to a polyethylene botle and store refrigerated (4°C).

7.4 Glycine Buffer, 0.1 M — dissolve 1.50 g of glycine in approximately 190 mL of LRW and titrate to pH 10.0 with approximately 5.2 mL of 2.5 M NaOH (7.3) using a pH meter. Dilute to 200 mL and sterilize by filtering through a 0.45 M membrane filter. Transfer to a pyrex storage bottle and store refrigerated (4°C).

7.5 Magnesium Disodium Ethylenediamine Tetraacetate (Mg-EDTA) solution, 0.1 M -- dissolve 3.58 g of magnesium disodium ethylenediamine tetraacetate in approximately 90 mL of LRW and titrate to pH 10.0 with approximately 0.45 mL of 2.5 M NaOH using a pH meter. Dilute to 100 mL and sterilize by filtering through a 0.45 μm membrane filter. Transfer to a pyrex storage bottle and store refrigerated (4°C).

7.6 Trisodium Phosphate (Na_3PO_4) Solution, 0.2 M -- dissolve 7.60 g of dodecahydrated trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$) in approximately 90 mL of LRW and dilute up to 100 mL. Sterilize by filtering through a 0.45 μM membrane filter. Transfer to a pyrex storage bottle and store refrigerated (4°C).

7.7 Extraction Technique "A" Extraction Solution -- dilute 25 mL of 0.2 M Na_3PO_4 solution (8.6) to 500 mL with LRW. Store refrigerated (4°C) in a pyrex storage bottle.

7.8 Extraction technique "B" extraction solution — dilute 50 mL of 0.1 M glycine buffer (7.4) and 25 mL of 0.1 m Mg-EDTA solution (7.5) to 500 mL with LRW. Store refrigerated (4°C) in a pyrex storage bottle.

7.9 Glycine Buffer, 0.01 M — dilute 50 mL of 0.1 M glycine buffer to 500 mL with LRW and store refrigerated (4°C) in a pyrex bottle.

7.10 Adenosine-5'-triphosphate (ATP) Stock Solution, 1.0 mg/mL — Weight 119.3 mg of crystalline trihydated adenosine-5'-triphosphate disodium salt ($Na_2ATP \cdot 3H_2O$) into a 100 mL volumetric flask. Dissolve and dilute to 100 mL with 0.01 M glycine buffer (7.9). Transfer to a pyrex storage bottle and store refrigerated (4°C).

7.11 Luciferase/Luciferin Reaction Mixture — a number of crude and purified preparations are commercially available[11]. To obtain a sensitivity comparable with the crude reagent preparations, a much larger volume of sample extract must be used. This results in varying and inaccurate results possibly due to the introduction of higher concentrations of bioluminescence quenchers and interfering materials and possibly due to a higher response of the crude reagent to other related ribonucleotides. The reagent should be prepared in accordance with the supplier's instructions. Note the following when preparing this material:

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11 Purified preparations such as the DuPont Reagent Kit (No. 760145-901) or equivalent are recommended because they are 50 to 100 fold more sensitive than crude preparations.

7.11.1 Clean glassware must be used.

7.11.2 The luciferase/luciferin reaction mixture must be mixed gently without shaking.

7.11.3 Bacteria may live and multiply in the LRW and extraction solutions; this can introduce an ATP interference. The quality of the LRW and extraction solutions should be periodically tested.

8. Sampling

8.1 The method of sampling and the choice of sampling sites will depend upon the objectives of the project. The extraction procedures should be performed in triplicate on each sample, as soon as possible, and preferably immediately after collection. The natural environmental conditions of the sample, such as temperature, lighting and aeration, should be maintained until extraction. Once extracted, the sample ATP is stable for weeks at 4°C, using extraction technique "B" or at 20 to -25°C using extraction technique "A".

9. Pretreatment of Samples

9.1 Aqueous Samples.

9.1.1 Where necessary, prefilter samples through a 200 μm plastic screen for the removal of materials which interfere with the precision and accuracy of the determination. This step is particularly applicable

to natural water samples which may contain large zooplankton and macrophyte fragments.

9.1.2 Stir the sample to maintain uniformity of composition during subsampling.

9.1.3 For samples known to contain free (extracellular) ATP or less than 10^7 cells per millilitre, filter a suitable aliquot of 5 to 100 mL, depending upon the nature of the sample, through a 0.3 to 0.5 µm glass fiber filter using a vacuum of 250 mm of mercury. Ensure that the time of filtration does not exceed 3 minutes and that the filter pad is not allowed to dry during the process. Break the vacuum the instant the level of water disapperas below the surface of the filter pad and quickly remove the filter into the boiling or homogenizing extractant.

9.1.4 Perform replicate (triplicate) extractions on each sample directly or after concentration using extraction technique "A" or "B".

9.2 Sediment Samples

9.2.1 Remove excess water from sediment samples at the time of collection.

9.2.2 Manually mix wet surface sediment samples and loosely pack a portion of the mixed sample into a 100 mL beaker to a depth of 5 cm.

9.2.3 For sediment cores, carefully transfer, top side up, core segments, 5 cm in length, to beakers, with an inside diameter slightly larger than the diameter of the extruded core.

9.2.4 Perform replicate (triplicate) extractions using extraction technique "A".

9.2.5 Determine in triplicate the dry weight of 0.6 cubic centimetre aliquots of wet sediment after drying overnight at 105°C in preweighed 20 mL beakers which have been preheated at least one hour at 150°C and cooled to room temperature in a desiccator. Record weights to the nearest tenth of a milligram after cooling to room temperature in a desiccator. Calculate the dry weight of the sediment by subtracting the initial weight of the beaker from the final weight of the beaker and dry sediment.

10. Extraction Procedures

10.1 Extraction Technique "A" -- homogenizing chloroform and trisodium phosphate solution.

10.1.1 Transfer 10 mL of Extraction Technique "A" extraction solution (7.7) and 3 mL of glass-distilled chloroform (Caledon Laboratories or equivalent) to a 30 mL beaker and immerse the homogenizer probe into the solution such that the tip of the probe rests a couple of millimeters from the bottom of the beaker.

10.1.2 Just prior to the introduction of the sample, turn the homogenizer on full speed.

10.1.3 Inject 0.10 to 1.00 mL of aqueous sample of 0.6 cubic centimetres of wet sediment sample, into the homogenizing extractant. Incase of filter paper completely immerse the filter pad upon which the aqueous microorganisms are concentrated during homogenization and extraction.

10.1.4 Homogenize the sample at full speed for 2 minutes.

10.1.5 Turn off the homogenizer and let the extracts of aqueous samples stand a few minutes to facilitate the setting of debris and chloroform.

10.1.6 For sediment extracts transfer the extract to a centrifuge at 1500 to 2000 xg for 10 minutes.

10.1.7 Filter a carefully decanted portion of sample extract (10.1.5) or a few millilitres of centrifuged sediment extract (10.1.6) applying a vacuum of at least 720 mm of mercury. Maintain the vacuum for an additional minute after the filtrate has been collected to remove dissolved chloroform[12].

10.1.8 If the clarified extract is not analyzed immediately it may be stored at room temperature in suitable size stoppered or capped tube for up to two weeks.

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12 CAUTION: Dissolved chloroform interferes with the ATP bioluminescence if the final concentration in the bioluminescence reaction mixture is greater than 0.01%

10.1.9 Dilute clarified sediment extract with nine volumes of extraction technique "B" extraction solution prior to analysis (7.8).

10.1.10 Likewise, if crude firefly reagent is used for analysis, the clarified extract of an aqueous sample must also be diluted with nine volumes of extraction technique "B" extraction solution (7.8) prior to analysis.

10.2 Extraction Technique "B" -- boiling glycine buffer.

10.2.1 Transfer 5 mL of Extraction Technique "B" extraction solution (7.8) to a scintillation vial and loosely secure the cap.

10.2.2 Prior to the introduction of the sample, preheat the extractant for 5 minutes, in a heating block, maintained at 110 to 115°C.

10.2.3 Inject 0.10 to 0.20 mL of aqueous sample into the preheated extraction solution, or completely immerse the filter pad upon which the sample microorganisms are concentrated.

10.2.4 Quickly replace the cap loosely on the vial and agitate the heating block to facilitate mixing.

10.2.5 Heat for 5 minutes, maintaining the block temperature between 110 and 115°C and periodically agitate the heating lock during the extraction.

10.2.6 Remove the vial from the heating block and cool the extract to room temperature with the aid of an ice bath.

10.2.7 Transfer the extract to a centrifuge tube and centrifuge at 1500 to 2000 X g for 5 to 10 minutes.

10.2.8 Carefully decant the supernate into a suitable size stoppered or capped tube.

10.2.9 If the analysis is not performed the same day the extract may be stored refrigerated for up to two weeks.

11. Calibration

11.1 Prepare standard solutions containing concentrations of ATP at the lower end, upper end and middle of the range expected in the extracts or diluted extracts of the samples.

11.2 Prepare the standards by serial dilution of the stock 1.0 mg/mL ATP solution (7.10) with the appropriate extraction solution (7.7 or 7.8), depending upon the final matrix of the sample extracts.

11.3 Following the instruction manual of the luminescence photometer, determine the average instrument response for each standard ATP solution using a minimum of three replicate measurements. The volume of solution that is used for the measurement will depend upon the volume and quality of firefly reagent employed. Using purified reagent, this volume

is one-tenth that of the reagent and, using crude reagent, it is eight-tenths that of the regent.

11.4 Using triplicate measurements, determine the average instrument response to the same volume of reagent blank, consisting of the appropriate extraction solution (7.7 or 7.8) and subtract this value from that obtained for each standard ATP solution.

11.5 Plot the blank-corrected bioluminescence response versus the ATP concentration on linear graph paper if purified reagent was used or on log-log graph paper if crude reagent was used.

12. ATP Measurement

12.1 Determine the average instrument response for the final sample extract using triplicate measurements with the same volume of solution as employed for the calibration standards.

12.2 Using triplicate measurements, determine the average instrument response to the same volume of appropriately treated extraction solution and substract this value from that obtained for the final sample extract.

12.3 Determine the concentration of ATP in the final extract from the calibration curve (11.5). If purified reagent was used for the analysis, calibration curves are usually linear and the concentration of ATP can be calculated by dividing the

blank corrected bioluminescence response obtained for the final extract by the value obtained during calibration for the average bioluminescence response per unit concentration of standard ATP.

12.4 The concentration of ATP in the original sample prior to concentration and/or extraction and/or dilution is calculated using the following equations:

12.4.1 µg ATP/l of aqueous sample = µg ATP/l of final extract

$$\times \frac{\text{(ml of final extract)}}{\text{(ml of orginal sample)}}$$

12.4.2 µg ATP/g of dry sediment

= µg ATP/mL of final extract $\times \frac{\text{(ml of } Na_3PO_4 \text{ extract}}{\text{(g of dry sediment)}}$

$\times \frac{\text{(ml of diluted extrac}}{\text{(ml of extract dilute}}$

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