HANDBOOK OF METHODS IN

AQUATIC MICROBIAL ECOLOGY

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CHAPTER 41

Total Microbial Biomass Estimation Derived from the Measurement of Particulate Adenosine-5′-Triphosphate

David M. Karl

INTRODUCTION

Microbial biomass, defined here as the total amount of living cellular material and generally expressed as g C m^{-3} for water samples or g C m^{-2} for sediments, is a fundamental state variable in quantitative microbial ecology. In trophic studies of aquatic environments it is necessary to know the total standing stock or biomass of microorganisms (e.g., all organisms less than or equal to approximately 200 μm) present in a given habitat or size-fractionated subsample (i.e., protozoan, algal, or bacterial cell-enriched fraction). The biomass of viable microorganisms, when used in conjunction with estimates of metabolic activity, productivity, or rates of cell division (see Notes and Comments) can be used to place limitations on material and energy fluxes through aquatic microbial food webs.

Conventional laboratory-derived methods including measurement of dry weight or carbon to estimate biomass or cell enumeration by direct microscopy are generally unreliable for estimating microbial biomass in natural aquatic environments. Causes for the relative ineffectiveness of these methods include the presence of a relatively large and variable proportion of nonliving particulate matter and the difficulty in distinguishing viable from nonviable cells. Furthermore, the heterogeneous distribution of cell sizes and morphologies and variable cell number-to-biovolume-to-biomass extrapolation factors complicate methods involving direct microscopy to determine total microbial biomass.

ATP has several unique characteristics which make it a reliable indicator of microbial biomass in aquatic environments. It is ubiquitous in all living cells, has a relatively short half-life following cell death and autolysis, and is present at a fairly constant intracellular concentration regardless of nutritional mode (e.g., photoautotroph, chemoheterotroph, chemolithoautotroph, phagotroph) and growth rate. Furthermore, particulate ATP (P-ATP) can be rapidly and efficiently extracted from cells and stabilized in solution using boiling buffers, cold mineral or organic acids, or a variety of organic solvents. The preferred method of ATP quantification is by the firefly bioluminescence reaction; however, a variety of analytical techniques is available for either discrete sample or continuous flow analyses. Data on the P-ATP content of a water or sediment sample can be extrapolated to total microbial biomass using C:ATP relationships derived from either laboratory or field studies.

The advantages of using P-ATP to measure total microbial biomass are (1) the relative ease of P-ATP preconcentration (if necessary), extraction, and analysis including the simplicity of the required equipment, (2) the low detection limit (less than 10^{-12} mol) and

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acceptable level of precision for field replicates (typically 1 to 10%, depending upon ATP concentration and operator), (3) the high degree of objectivity compared with methods requiring operator recognition of "live" cells and estimation of cell dimensions, and (4) the potential for near "real time" analyses and continuous flow applications. Furthermore, the P-ATP biomass assay is founded on fundamental first principles of cell physiology; both the obligate association of P-ATP with viable organisms and the constancy of intracellular ATP pool concentrations are well established. Compared to most other biomass assays, there is also an extensive laboratory and field database for comparisons, conclusions, and ecological interpretations.

The P-ATP method has been used, and abused, but for the most part has withstood the critical test of time since the first field application to oceanic waters off Southern California.[1] Often, the P-ATP biomass method is criticized for its failure to differentiate among individual groups of microorganisms (except by size or specific density partitioning). Although this clearly is a limitation of the technique, one should hasten to add that the unique value of P-ATP measurements is for the determination of total microbial biomass. Other limitations include the presence of potentially interfering substances or adsorption losses in certain sample materials (especially marine sediments) which require careful internal standardization, variations in cellular C:ATP ratio as a result of nutrient starvation and possibly other forms of environmental stress, and the absence of commercially available certified reference standards for estimating the accuracy of field determinations.

MATERIALS REQUIRED

Equipment

- Sampling gear (water column): Niskin bottles (or equivalent), kevlar line or equivalent, subsampling bottles, 202-μm Nitex mesh
- Sampling gear (sediment column): corer device, core tubes, syringe samplers, glove box (optional)
- Filtration gear: PVC or stainless steel manifold (3 or 6 place), equipped with glass filter bases with stainless steel screens for 25-mm diameter filters and large volume funnels (100- to 200-ml capacity)
- Vacuum pump with gauge (compressed N_2 or air can be used as an alternative to vacuum filtration)
- Block heater (Sybron Thermolyne Type #16500 Dri-bath, or equivalent): capable of heating extraction menstruum in test tubes to 100 ± 1°C (1 atm) and maintaining boiling temperatures throughout the extraction period
- Storage freezer (−20°C)
- pH meter and reference buffers
- Magnetic stirrer and stir bars
- Light-detection instrument: Any one of a number of commercially available general instruments, such as fluorometers, spectrophotometers, or liquid scintillation counters, are suitable for measuring light emission. However, in order to obtain efficient and reliable data, with maximum sensitivity, a specially designed ATP photometer is required. Instruments specifically designed for ATP analyses are marketed by several corporations including, but not limited to, E. I. DuPont, LKB Instruments, Biospherical Instruments Corp., Turner Designs, and United Technologies. Whatever light-measuring device is selected, it is imperative to have either a strip chart recorder, integrator, or suitable computer-assisted data station to quantify light emission.

Supplies

- Filters (24/25 mm diameter Whatman GF/F, Millipore HA/GS, or equivalent)
- Filter forceps
- Extraction and assay glassware: test tubes for ATP extraction (approximately 15 mm diameter × 125- to 160-mm length); assay vials (18-mm diameter × 40-mm height). Size requirements may vary depending upon equipment/instruments used.
- Adjustable automatic pipettes (0.1- to 1-ml and 1- to 5-ml capacities)

Solutions and Reagents

(Unless otherwise indicated, catalog numbers or information refers to items sold by Sigma Chemical Company.)

- Firefly lantern extract (FLE-50)
- Potassium arsenate buffer, 100 m*M*, pH 7.4
- Tris(hydroxy)aminomethane-HCl buffer, 20 m*M*, pH 7.75
- ATP, ADP, and AMP, sodium salt, 2 μ*M* solutions in Tris buffer, in 1- to 5-ml aliquots, and stored frozen
- Magnesium sulfate solution, 40 m*M*
- Potassium phosphate buffer, 60 m*M*, pH 7.4
- D-Luciferin, optional (L-9504)
- H_3PO_4 (1 to 1.5 *M*)
- NaOH (1 and 0.1 *M*)

PROCEDURES

Sampling, Subsampling, Extraction, and Sample Storage

Water Column

Typically, oceanic samples are collected at predetermined depths throughout the water column using standard, commercially available polyvinylchloride (PVC) bottles (e.g., General Oceanics Niskin or Go-Flo bottles, or equivalent), mounted on a CTD-rosette sampler. In addition to obtaining complementary information on physical and chemical characteristics of the water column, this sampling protocol allows interactive, directed sampling at specific regions of interest (e.g., particle, fluorescence or oxygen maxima or minima, density discontinuities, etc.). Alternatively, water can be obtained using submersible pumps, manually operated evacuated bottles, syringe samplers, or any other effective means. Selected habitats, such as the sea-surface microlayer or high-temperature hydrothermal vents require the use of specialized samplers.

1. Prior to use, the samplers are cleaned with dilute HCl (0.5 to 1 *M*), or ethyl alcohol (95%) and rinsed thoroughly with distilled water; sterilization of the samplers is neither required nor practical for most field studies.

2. To measure microbial ATP, it is necessary to remove metazoans and other nonmicrobial ATP prior to sample extraction. This is done most conveniently by passing the water sample through a 202-μm Nitex mesh as part of the subsampling procedures (e.g., during subsampling from Niskin bottles). It is conceivable that, in certain aquatic environments, this procedure may also remove large detrital particles to which microorganisms are attached. In these extreme cases, metazoans can be hand sorted from the respective water samples prior to analyses.

3. Following collection and subsampling, the particulate matter is concentrated and extracted as soon as possible. Although numerous methods have been described for the extraction of ATP from microorganisms,[2] the most commonly used method for aqueous samples involves P-ATP concentration by vacuum or pressure filtration and extraction into boiling TRIS (0.02 *M*, pH 7.4 to 7.7) or boiling phosphate (60 m*M*, pH 7.4) buffers. The latter is recommended for samples suspected of containing alkaline phosphatase.[3] After concentration of microbes onto a filter, the filter should be immersed into the boiling buffer as quickly as possible after the final portion of liquid passes through the filter. If left on the filtration manifold for an extended period of time, measured in seconds, the cells dessicate causing a loss of viability. It is imperative that the extraction buffer is at its boiling point at the time of filter insertion. At temperatures below approximately 90°C, inefficient extraction occurs.
4. After the filter is placed into the boiling buffer, the sample is heated for an additional 5 min during which time the test tubes are partially covered to minimize evaporation and resultant volume changes.
5. Following extraction, the samples are removed from the heating block or temperature-controlled bath, cooled, then stored frozen (−20°C) until assayed. At this point the sample extracts are extremely stable with ATP losses of less than 1% $year^{-1}$ in properly buffered solutions.

Sediment Column

Many aquatic sediments are well stratified and characterized by steep depth gradients in microbial biomass. Consequently, it is imperative that the sampling and subsampling methods used to collect sediment for microbial ATP analysis preserve the unique depth distribution. For intertidal or shallow subtidal habitats, sediment cores can be collected manually by inserting PVC or acrylic tubes (10- to 15-cm diameter, 30- to 50-cm length) into the sediment and placing stoppers on both ends during retrieval. Deeper samples require the use of a spade box corer (or equivalent device) which is designed to minimize both sediment disruption during sampling and winnowing during sample recovery. Unfortunately, most gravity corers, which are easier to operate than box corers, create an unacceptable bow wave prior to penetration into the sediment. This results in a disruption of the microbial biomass gradients that are of greatest interest to the microbial ecologist. As for water column samples, the greatest variability is expected to occur at the level of sample (i.e., sediment core) replication. This variability is also of greatest relevance to the microbial ecologist.

Once collected, the core samples should be processed as quickly as possible and, as discussed above, with care taken to minimize changes in environmental conditions. For most anoxic sediments, this requires the use of a N_2-filled glove box to prevent sample oxidation and subsequent transitions in intracellular ATP pools or potential loss of obligate anaerobe viability.

A variety of procedures has been described for core subsampling, including techniques for the collection of the water-sediment interface where microbial biomass and activity are expected to be the highest,[4] and for the preservation of millimeter-scale habitat variability.[5] If required, the sediment samples can be screened to remove ATP-containing metazoa, macroalgae, or higher plant rhizomes prior to analysis. The replicated subsamples from the different depth strata are then mixed thoroughly using a spatula in preparation for final subsampling for ATP extraction.

1. Triplicate subsamples (1 to 2 cm^3) of each sediment fraction are collected using a 3-cc plastic syringe barrel (luer lock end removed). Additional replicates are also taken for the determination of wet volume-to-dry weight conversion and for other bulk chemical parameters (e.g., total carbon).

2. The plugs of sediment are immediately discharged into test tubes containing 10 ml of cold H_3PO_4 (0.5 *M*, 4°C), capped, and thoroughly mixed. Additional subsamples should be prepared with a known amount of ATP added as an internal standard in order to assess and correct for ATP losses by the combined effects of adsorption, hydrolysis, and various potential sources of chemical interference. Representative sediment porewaters should also be collected (by centrifugation or pressure filtration through a 0.2-μm filter) to assess the potential interference due to dissolved ATP (D-ATP). If the sediment samples contain $CaCO_3$, occasional venting to release accumulated CO_2 gas may be required.
3. After an extraction period of 15 to 20 min at 4°C, the extracted nucleotides are separated from the solid phase by centrifugation or vacuum filtration.
4. The pH of the acid extracts is adjusted to 7.4 by titration with NaOH (1.0 and 0.1 *M*). At this point, the ATP is stable in samples stored at −20°C. If the ATP concentration in the acid extract is >100 n*M*, the sample can be diluted with 60 m*M* PO_4 buffer as an alternative to base titration.
5. If the ATP concentration is ≤1 n*M*, the acid extract must be concentrated by either the activated charcoal procedure[6] or brucite coprecipitation[7] prior to analysis. At this point, the ATP is relatively stable and the samples may either be stored at 4°C for up to 2 to 3 weeks or processed immediately.

Detection of ATP by Firefly Bioluminescence

Although ATP (and other adenine and non-adenine nucleotides) can be measured using any one of a variety of analytical detection systems, the firefly bioluminescence assay and high-performance liquid chromatography (HPLC) are most commonly used in ecological studies. A major advantage of HPLC is the ability to separate complex nucleotide mixtures (i.e., cell extracts) into individual components (ATP, ADP, AMP, etc.) that can be quantified during a single sample run. This additional information on the concentrations of non-ATP nucleotides can provide useful data on the metabolic states and *in situ* growth rates of microbial communities in nature.[2,8] The more commonly used bioluminescence assay, however, has a much lower ATP detection limit, is straightforward and inexpensive to perform, has a high level of precision, and requires less-specialized instrumentation. Furthermore, non-ATP nucleotides (e.g., ADP, AMP, GTP, etc.) can also be measured by the firefly bioluminescence reaction following stoichiometric generation of ATP from other nucleotide triphosphates via specific transphosphorylation reactions.[9]

Several reviews have been published concerning the specificity, kinetics, and mechanism of the firefly bioluminescence reaction. The postulated steps are[10]

$$LH_2 \text{ (luciferin)} + ATP \xrightarrow{Mg^{2+}\text{–luciferase}} E\text{-}LH_2\text{-}AMP + PP_i \quad (1)$$

$$E\text{-}LH_2\text{-}AMP + O_2 \xrightarrow{\text{neutral pH}} \text{oxyluciferin} + E + CO_2 + \text{light} \quad (2)$$

When all necessary reactants are present in excess, the *in vitro* light emission is directly proportional to the concentration of ATP in solution. Reaction kinetics and specificity are dependent upon the purity of the enzyme preparation; sensitivity is controlled by luciferin concentration.[11] The measurement of the initial rise of the luminescence curve (0 to 2 s), the peak height of luminescence (0 to 5 s), or a predetermined integrated portion (e.g., 15 to 75 s) of the light emission decay curve can be used to relate ATP concentrations in reference standards to those in the unknown sample extracts. The major advantages of the integrated mode are increased sensitivity, ease and reliability of mixing, and nonreliance on

the peak-height response, which is difficult or impossible to measure using certain instruments. However, a major disadvantage of the integrated mode is the nonspecificity of light emission with certain crude enzyme preparations.[12] Reliability of peak-height analyses depends upon a very rapid and complete mixing of all reactants. This is best accomplished using an automatic injection system, which ensures consistent mixing velocities for all samples. The peak-height mode of analysis offers the advantages of speed of assay and minimum interference from other enzymes or substrates (e.g., non-ATP nucleotides) that may affect the rate of the luciferase-catalyzed reaction.

1. Firefly lantern extract (catalog FLE-50 or FLE-250, Sigma Chemical Co., St. Louis, MO) is stored with dessicant at −20°C. To activate lyophilized FLE, a 50-mg vial is hydrated in 5 ml of distilled water. This enzyme preparation is allowed to "age" at room temperature for a minimum of 4 to 6 h to a maximum of 24 h, depending upon the desired sensitivity. During the aging process, endogenous ATP initially present in the crude extract is consumed, thereby decreasing the background light emission. Due to variations among individual enzyme preparations, it is imperative that only a single batch of enzyme be used for the analysis of a given set of samples and ATP standards.
2. Next, the hydrated, aged FLE is further diluted using equal volumes (generally 10 ml each for a single 50-mg vial of FLE) of $MgSO_4$ (0.04 *M*) and $KHAsO_4$ buffer (0.1 *M*, pH 7.4) and incubated at room temperature for 1 h.
3. If desired, the FLE preparation can be diluted to greater final volume depending upon the required sensitivity of the assay. However, if a single vial of FLE-50 is diluted to a working volume of >50 ml, the bioluminescence reaction can become limiting for luciferin which must be added back in order to maintain first-order reaction kinetics with respect to ATP.[11]
4. Immediately prior to use, the insoluble residue is removed by vacuum filtration (Whatman GF/F filter) or centrifugation (1500 × g, 5 min).

ATP Standards

For each enzyme preparation, an ATP standard curve is prepared and analyzed with the sample extracts.

1. A stock ATP solution containing 1 to 2 μ*M* ATP is prepared in TRIS buffer (0.02 *M*, pH 7.4 to 7.7) and stored at −20°C in 1 ml-aliquots until needed. Under these conditions, ATP hydrolysis is <1% $year^{-1}$. The exact concentration of the stock solution is determined by absorption spectrophotometry at 259 nm, using the relationship

$$A = Cle \tag{3}$$

where C = ATP *(M);* 1 = absorption pathlength (cm); and e = 15.4×10^3 (ATP molar extinction coefficient at pH 7.4).

2. A working ATP standard solution is prepared on the day of the assay by diluting the stock ATP preparation with the appropriate extraction menstruum (TRIS or phosphate buffer depending upon sample type, and preferably the same batch as used for sample extraction).
3. Between seven and eight ATP standards (including a buffer blank) covering the expected range of the sample concentrations (approximately 1 to 100 n*M* ATP) are prepared. If ≥100 samples are analyzed or if the analysis time exceeds approximately 1 h, it is desirable to measure a set of standards at the beginning and at the end of the analysis to monitor temporal changes in the response of the FLE preparation. Otherwise, a single standard curve measured midway through the experiment is sufficient. Generally, there is no significant hydrolysis of the diluted ATP standard solutions during a typical 3- to 4-h working period.

4. Following a single use, all thawed ATP stock standards and serial dilutions thereof should be discarded.

Data Reduction and Extrapolations of ATP to Biomass

The relationship between ATP and light emission is linear provided substrate (O_2 and luciferin) limitation does not occur. Peak heights or integrated areas are regressed on ATP standard concentrations. From a model-1 linear regression analysis of these data, the ATP concentrations in sample extracts can be calculated. By correcting for the proportion of the sample actually assayed and the volume of medium originally extracted, the ATP L^{-1} of the original water sample can be determined.

The C:ATP ratio in microorganisms varies considerably, although somewhat predictably, among taxa and even for a given species when grown under different nutritional constraints (data summarized by Karl[2]). Among the most conspicuous differences in C:ATP ratios are those observed between unicellular microorganisms (i.e., bacteria and microalgae: C:ATP $\approx$ 200 to 350, by weight) and micrometazoa (C:ATP = 50 to 150), and the large increases in the C:ATP ratio when microorganisms are starved for phosphorus.[2,8] However, under most conditions found in nature, the C:ATP ratio of the microbial community is about 250:1. Although originally developed to estimate total microbial "biomass," ATP concentrations are, in theory, more closely coupled to "protoplasm" biomass and, more specifically, total biovolume. Because of the obligate role of ATP in cellular bioenergetics, intracellular ATP levels are carefully maintained at a concentration of approximately 1 to 2 m*M*.[13] Unfortunately it is difficult to use biovolume estimates directly, however accurate, in most studies of microbial ecology. Consequently, we rely upon empirically determined C:ATP ratios to extrapolate P-ATP determinations to estimates of total microbial biomass. In so doing, we decrease both the level of precision and the accuracy of the initial ATP determination. Furthermore, in habitats where copious amounts of capsular materials, extracellular secretions, or slimes occur,[14] ATP-based values of total biomass probably provide only minimum estimates.

NOTES AND COMMENTS

Sampling

The investigator must be aware of three basic areas where variability can be introduced into field measurements: replication at the level of sampling (i.e., multiple water bottles at a given depth), replication at the level of subsampling (multiple samples from a single collection), and analytical replication (i.e., multiple analyses of a single sample extract). Because of the heterogeneous distribution of microbial communities in nature and problems inherent to the collection of particulate matter from aquatic environments, variance between sampling bottles is generally the largest. Ideally, the entire contents of multiple water samples collected at each depth should be analyzed for P-ATP.

Exposure of viable microorganisms to environmental conditions that are substantially different from the collection site should be avoided to minimize short-term transitions in intracellular ATP pools. However, this becomes nearly impossible during the collection of many samples, for example, abyssal water samples from the equatorial ocean. Decreases in pressure and increases in temperature, even during the time required for the samples to reach the ocean's surface, are almost certain to alter microbial ATP concentrations and perhaps even cell viability. However, at the present time these effects have not been systematically evaluated. In the future, a technique which provides for the *in situ* extraction of ATP from

microorganisms needs to be developed and compared to conventional sampling procedures in order to provide quantitative constraints on the potential changes in P-ATP during routine sample recovery.

Extraction

The concentration step, which is necessary for most low biomass environments (i.e., <1 g C m^{-3}), must be performed with extreme care and predetermined judgment regarding total sample volume. It has been shown that the recovery of microbial ATP from certain high particulate load samples is volume dependent.[15] Initially, the observed P-ATP losses were thought to be the result of cell lysis during prolonged filtration. However, it is now known to be caused by a filtration-induced metabolic stress that results in the hydrolysis of ATP to ADP and AMP.[16] Consequently, if the total adenine nucleotide pool (the sum of ATP + ADP + AMP) rather than ATP is measured, filtration volume becomes less critical.

In certain eutrophic habitats where total microbial biomass exceeds 1 g C m^{-3}, water samples can be extracted directly, thereby eliminating the preextraction concentration step.[17] It is imperative, however, that the sample volume injected does not exceed 5% (by volume) of the extractant volumes; otherwise temperature changes affect the efficiency of ATP extraction. If desired, multiple injections can be made allowing time for the extraction menstruum to return to its boiling temperature. Furthermore, if direct injection is employed for P-ATP extraction it is necessary to measure, and correct for, D-ATP (operationally defined as passing through a 0.2-μm filter) which is also present in most marine[18,19] and freshwater[20] ecosystems.

Sensitivity, Precision, and Accuracy

The sensitivity of the ATP assay is determined by the instrumentation used to detect light emission and by the purity of the luciferase preparation. Using the crude FLE-50 luciferase preparation prepared as described in this chapter and a commercially available ATP photometer (Biospherical Instruments Corp.), the lower limit of ATP detection (i.e., twice the background light emission) is about 0.2 n*M* ATP. For greater sensitivity, exogenous luciferin is added to the enzyme preparations enabling the detection of 1 p*M* ATP.[11] The precision of the peak height assay procedure as routinely performed in our laboratory is ± 1 to 2% of the mean ($n = 8$) throughout the entire range of ATP standards. Accuracy is estimated by analyzing diluted ATP standards and treating them as unknown samples. At the present time, there are no commercially available certified reference materials available for independent determination of accuracy.

Analytical Interferences and Use of Internal Standards

In addition to the potential problems discussed in the previous sections, several sources of analytical interference are possible. These include (1) the presence of inorganic and organic ions in the sample extracts, resulting in loss of ATP in solution (i.e., through chelation) or in decreased luciferase activity, (2) the presence of humic acid-like substances in the sample extracts that impart a yellow color to the solution, thereby resulting in attenuation of the emitted light, (3) turbidity of the final extracts resulting in light scattering and absorption, (4) the presence of a high concentration of inorganic particulate material in the final extracts resulting in loss of ATP through adsorption, and (5) the presence of contaminating enzymes, in either the sample extracts or the luciferase preparation, that compete with luciferase for the ATP in solution (e.g., ATPase or adenylate kinase) or that result in the production of ATP through transphosphorylase reactions (e.g., nucleoside diphosphate kinase or pyruvate kinase).

Most of the above sources of error are detected, and corrected for, through the use of an ATP internal standard, as discussed by Strehler.[21] The internal standard may be added in the form of an ATP salt solution, as live or lyophilized bacterial cells, or as radiolabeled ATP. To minimize the effects of ionic interference, it is imperative that the standard ATP solutions be prepared in an ionic medium identical to that of the samples. Peak-height measurements significantly decrease the analytical interference due to the presence of non-adenine nucleotide triphosphates and therefore, are strongly recommended. If available, a strip-chart recorder or oscilloscope should be interfaced with the photometer in order to detect deviations from the standard ATP-dependent light-emission reaction kinetics.

REFERENCES

1. Holm-Hansen, O. and Booth, C. R., The measurement of adenosine triphosphate in the ocean and its ecological significance, *Limnol. Oceanogr.*, 11, 510, 1966.
2. Karl, D. M., Cellular nucleotide measurements and applications in microbial ecology, *Microbiol. Rev.*, 44, 739, 1980.
3. Karl, D. M. and Craven, D. B., Effects of alkaline phosphatase activity on nucleotide measurements in aquatic microbial communities, *Appl. Environ. Microbiol.*, 40, 549, 1980.
4. Novitsky, J. A., Heterotrophic activity throughout a vertical profile of seawater and sediment in Halifax Harbor, Canada, *Appl. Environ. Microbiol.*, 45, 1761, 1983.
5. Craven, D. B., Jahnke, R. A., and Carlucci, A. F., Fine-scale vertical distributions of microbial biomass and activity in California Borderland sediments, *Mar. Biol.*, 83, 129, 1986.
6. Hodson, R. E., Holm-Hansen, O., and Azam, F., Improved methodology for ATP determination in marine environments, *Mar. Biol.*, 34, 143, 1976.
7. Karl, D. M. and Tien, G., MAGIC: a sensitive and precise method for measuring dissolved phosphorus in aquatic environments, *Limnol. Oceanogr.*, 37, 105, 1992.
8. Karl, D. M., Determination of *in situ* microbial biomass, viability, metabolism, and growth, in *Bacteria in Nature*, Vol. 2, Poindexter, J. S. and Leadbetter, E. R., Eds., Plenum Press, New York, 1986, 85.
9. Karl, D. M., A rapid sensitive method for the measurement of guanine ribonucleotides in bacterial and environmental extracts, *Anal. Biochem.*, 89, 581, 1978.
10. DeLuca, M. and McElroy, W. D., Purification and properties of firefly luciferase, in *Methods in Enzymology*, Volume 57, DeLuca, M. A., Ed., Academic Press, New York, 1978, 3.
11. Karl, D. M. and Holm-Hansen, O., Effects of luciferin concentration on the quantitative assay of ATP using crude luciferase preparations, *Anal. Biochem.*, 75, 100, 1976.
12. Karl, D. M., Occurrence and ecological significance of GTP in the ocean and in microbial cells, *Appl. Environ. Microbiol.*, 36, 349, 1978.
13. Chapman, A. G. and Atkinson, D. E., Adenine nucleotide concentrations and turnover rates. Their correlation with biological activity in bacteria and yeast, *Adv. Microb. Physiol.*, 15, 253, 1977.
14. Costerton, J. W., Irvin, R. T., and Cheng, K. J., The bacterial glycocalyx in nature and disease, *Annu. Rev. Microbiol.*, 35, 299, 1991.
15. Sutcliffe, W. H., Jr., Orr, E. A., and Holm-Hansen, O., Difficulties with ATP measurements in inshore waters, *Limnol. Oceanogr.*, 21, 145, 1976.
16. Karl, D. M. and Holm-Hansen, O., Methodology and measurement of adenylate energy charge ratios in environmental samples, *Mar. Biol.*, 48, 185, 1978.
17. Jones, J. G. and Simon, B. M., Increased sensitivity in the measurement of ATP in freshwater samples with a comment on the adverse effect of membrane filtration, *Freshwater Biol.*, 7, 253, 1977.
18. Azam, F. and Hodson, R. E., Dissolved ATP in the sea and its utilization by marine bacteria, *Nature*, 267, 696, 1977.
19. Nawrocki, M. P. and Karl, D. M., Dissolved ATP turnover in the Bransfield Strait, Antarctica during a spring bloom, *Mar. Ecol. Prog. Ser.*, 57, 35, 1989.

20. Maki, J. S., Sierszen, M. E., and Remsen, C. C., Measurements of dissolved adenosine triphosphate in Lake Michigan, *Can. J. Fish. Aquat. Sci.*, 40, 542, 1983.
21. Strehler, B. L., Bioluminescence assay: principles and practice, *Methods Biochem. Anal.*, 16, 99, 1968.