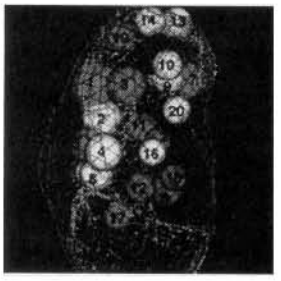
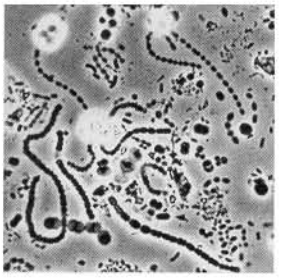
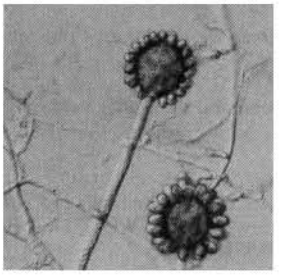
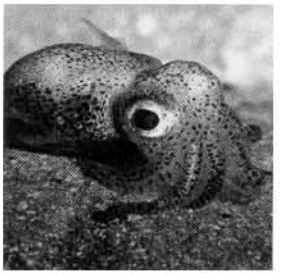
Methods for General and Molecular Microbiology

3rd Edition

C. A. Reddy, *Editor in Chief*

T. J. Beveridge, J. A. Breznak, G. A. Marzluf, T. M. Schmidt, and L. R. Snyder, *Editors*

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37

Microbial Nucleotide Fingerprints in Nature

DAVID M. KARL

37.1. INTRODUCTION 869
.2. SAMPLING AND SUBSAMPLING THE ENVIRONMENT 871
.3. BIOMASS DETERMINATION BY ATP 871
.4. MATERIALS REQUIRED 871
37.4.1. Equipment 871
.2. Supplies 872
.3. Solutions and Reagents 872
37.5. PROCEDURES 872
37.5.1. Sampling, Subsampling, Extraction, and Sample Storage 872
37.5.1.1. Water Column 872
.2. Sediment Column 872
37.5.2. Detection of ATP by Firefly Bioluminescence 873
.3. ATP Standards 874
.4. Data Reduction and Extrapolations of ATP to Biomass 874
37.6. NOTES AND COMMENTS 874
37.6.1. Sampling and Subsampling 874
.2. Extraction 874
.3. Sensitivity, Precision, and Accuracy 875
.4. Analytical Interferences and Use of Internal Standards 875
37.7. ENHANCING THE MICROBIAL NUCLEOTIDE FINGERPRINT 875
.8. CONCLUSION 876
.9. REFERENCES 876

37.1. INTRODUCTION

Microorganisms are the key to Earth's habitability. They harvest light energy, produce organic matter, and facilitate the turnover of key bioelements like nitrogen (N), phosphorus (P), and sulfur (S). During the process of microbial cell metabolism and growth, inorganic nutrients are assimilated into new biomass that fuels a complex series of interactions between various groups of microorganisms and among microbes and multicellular organisms. In summary, microbes make things happen.

Microbial communities in nature are diverse and complex. With the advent of modern techniques in molecular biology, one can now identify and track even the "virtual" microbes—those species that have successfully evaded pure culture isolation. It is also possible to interrogate ecosystems for the presence of specific genes and gene products, and even determine in situ expression of target genes. The present level of analytical sophistication and the continued improvement of techniques are impressive and hold great promise to yield new, fundamental information regarding the phylogenetic and physiological structure of microbial assemblages in nature. By comparison with the development of novel autecological approaches, progress in the development and refinement of methods that target the entire microbial community, specifically estimation of total living biomass, metabolic activity, growth, and reproduction, has been less dramatic.

For many ecological studies, accurate estimates of total microbial biomass, metabolic activity, and productivity are essential; they are the key master variables for a complete ecological assessment. Despite the recognized importance of total microbial community assessments, however, few methods currently exist, and those that have been developed have unique limitations that derive from the fact that the assay targets are generally diverse, partially uncharacterized, and in variable states of metabolism and growth. A forward-looking review article by Madsen (40), "Epistemology of environmental microbiology," presents an up-to-date progress report of the constraints imposed by the complexity of natural ecosystems and by limited methodology.

In pure culture studies, the measurement of biomass is fairly easily satisfied. However, as stated by Koch (36) in his authoritative review on growth measurements, "the methods for measuring biomass seem obvious and straightforward, but in fact they are complicated if accuracy is sought." He goes on to review the methods that are most often applied to laboratory studies of pure culture isolates, namely wet and dry weight, light scattering and turbidimetry, and various enumeration techniques. These last include light and electron microscopy and flow cytometry for direct counts, as well as spread plates, most probable number, and other culture methods for viable cell counts. If the average dimensions (or average mass or carbon content) of the cells in culture are known, or measured, then the direct or viable colony count can be extrapolated to biomass. However, even with pure culture isolates, cell size, mass, and elemental composition may vary depending on composition of the growth medium, culture conditions (temperature, light, pressure), and growth rate. These laboratory-based methods for direct and indirect estimation of biomass

are not easily adapted to the complex mixed cultures of microorganisms that one generally finds in nature.

The direct measurement of carbon, nucleic acids, protein, or other similar cell-derived compounds is generally unacceptable due to the relatively long residence times in the environment after cell death and lysis. For example, in most open-ocean habitats there is more particulate carbon (PC) in nonliving than in living materials, and the proportion varies considerably with water depth (approximately 50% of total carbon is living near the surface, decreasing to about 5% at 1,000 m) (29). There is even more nonliving particulate DNA in most natural ecosystems than there is inside all of the cells that are present. Nevertheless, measurements of total PC or DNA can be used to set upper bounds on total biomass and, as such, they have been used extensively in ecological studies.

An effective biochemical or molecular biomarker of microbial biomass should satisfy the following criteria (22): (i) the biomarker selected must be present in all living cells or in all organisms of a specific population; (ii) it must be readily metabolized, hydrolyzed, or otherwise decomposed following cell death; (iii) it must exist as a uniform and constant percentage of total biomass regardless of environmental or physiological conditions; (iv) a convenient method must exist to extract and purify (if necessary) the compound from environmental samples; and (v) a sensitive, quantitative assay procedure must be available. Microbial biomass in many natural ecosystems is often low, so assay sensitivity is an important criterion.

Only a few common features are shared by all of the diverse members of natural microbial assemblages that might serve as the basis for an environmental biomass assay system, and for this reason, progress in these important areas of microbial ecology has been slow. Two promising candidate classes of molecular biomarkers for ecological investigation are membrane lipids and cellular nucleotides. Other compounds have been used in environmental studies to detect the biomass of a portion of the total microbial assemblage. These include chlorophyll *a* for the sum of all photosynthetically active bacteria and eukarya, lipopolysaccharide for gram-negative bacteria, and muramic acid for gram-positive plus gram-negative bacteria (29).

Determination of total lipid phosphate (LP) as a quantitative measure of microbial biomass was originally described by White et al. (51) in their application to marine sediments. The general method was subsequently expanded to include (i) LP production rate, using radioactive phosphate (^{32}P or ^{33}P) as a precursor, as a measure of total microbial production (50); (ii) the pattern of signature ester-linked phospholipids fatty acids (PLFA) and hydroxyl fatty acids in the lipopolysaccharide of gram-negative bacteria as measures of microbial community structure (46, 52); and (iii) changes in the concentrations of poly-β-hydroxyalkanoic acid (PHA) in bacteria or triglyceride in microeukaryotes as measures of nutritional-physiological status (13). These diagnostic lipid-based profiles, or environmental microbial lipid fingerprints, have proven invaluable in a variety of ecological studies (11). Major limitations, according to White et al. (52), are the nontraditional units that result from these analyses (e.g., picomoles of PLFA per sample, rather than total cell mass or carbon), the relative insensitivity (detection limit of approximately 10^{10} bacteria per sample for biomass by LP analysis; for oligotrophic ocean seawaters that would equate to ~10 liters), and the requirement for specialized analytical equipment (gas chromatograph–mass spectrometer [GC-MS]) for the lipid profile analyses. Furthermore, it now appears that certain ubiquitous marine microorganisms, e.g., *Synechococcus* and *Prochlorococcus*, may have reduced cell quotas of membrane phospholipids (3, 9), so they would not be accurately represented in the environmental microbial biomass assessment.

The second and, perhaps, more broadly applicable class of cellular biomarkers is nucleotides and their derivatives. All living cells studied to date have an identical suite of more than 100 different nucleotides that are essential for viability, energy transduction, metabolic regulation, and biosynthesis. Although it is impossible to be absolutely certain on this point, it is highly unlikely that any microorganisms will be discovered with alternative pathways; the universal role of nucleotides and their derivatives appears to be well established (21).

The central role of the adenine nucleotides, and especially ATP, as intermediate carriers of chemical energy linking catabolism and biosynthesis has been known since the publication of Lipmann's classic paper (39). Nucleotides have at least three other crucial cellular functions: (i) synthesis of DNA and RNA; (ii) activation and transfer of precursors for cellular biosynthesis; and (iii) control and regulation of cellular metabolism. By measuring the intracellular concentrations of key nucleotides and ratios thereof, the intracellular pool turnover rates, and nucleic acid (DNA, RNA) synthesis rates, an environmental nucleotide fingerprint can be compiled that provides valuable information concerning in situ biomass and metabolic activity of the total microbial community.

Over 40 years ago, methods were developed for quantitative extraction of ATP from cells and for its detection in cell extracts. Initially, the ATP detection method was promoted as a life detection system for possible use on the Martian spacecraft *Viking* in 1974 (38), but a few years later the method was expanded into a total microbial biomass assay by Holm-Hansen and Booth (15). The steady-state intracellular concentration of ATP (referred to as the "ATP pool") in metabolically active microorganisms (prokaryotes and eukaryotes) appears to be well regulated at a value of 2 to 6 nmol of ATP mg^{-1} dry weight (1 to 3 mM) regardless of growth rate, culture condition, or mode of nutrition (21). This served as the theoretical basis for its use in total microbial biomass estimation.

Subsequent investigation led to the development of the adenylate energy charge (EC_A) concept and its application to microbial assemblages in nature (53). The EC_A is equal to one-half of the number of anhydride-bound phosphate groups per adenine moiety, or EC_A = ([ATP] + [ADP])/([ATP] + [ADP] + [AMP]), and is equivalent to a linear measure of the total amount of chemical potential energy momentarily stored in the adenine nucleotide pool (1). In vitro rate responses of several "key" enzymes in cellular metabolism to variations in EC_A have provided the background data for this control hypothesis. The well-established correlation between cellular EC_A and metabolic or growth potentials of individual organisms, in theory, provides a framework for estimating metabolic potentials of naturally occurring microbial populations (6).

In addition to ATP, the measurement of non-adenine NTPs may provide relevant ecological information. Many important cellular reactions, notably those associated with microbial biosynthesis and growth, are coupled to energy derived from NTPs other than ATP (20). For example, guanosine-5′-triphosphate (GTP) and uridine 5′-triphosphate (UTP) are both required for the activation and interconversion of carbohydrate precursors for polysaccharide

biosynthesis; cytidine 5′-triphosphate (CTP), GTP, and UTP are required for RNA transcription; and the deoxyribose derivatives of CTP, GTP, ATP, and thymidine 5′-triphosphate are required for DNA replication. In addition, GTP is an obligate requirement for the initiation, the aminoacyl-tRNA binding, and the translocation processes of protein synthesis. Unlike the ATP pool, which is maintained at a relatively constant level independent of growth rate, the intracellular concentrations of non-adenine NTPs fluctuate in direct proportion to their requirements for biosynthesis (12, 20, 47). Consequently, quantitative measurements of GTP and the estimation of total GTP:ATP ratios of microbial communities in nature constitute a key component of the environmental nucleotide fingerprint.

Some previous studies of ATP in microorganisms and in environmental samples have emphasized the futility of extrapolating these static pool measurements to estimates of metabolic energy flux. It is the turnover rate of the ATP pool, rather than the steady-state concentration, that varies in proportion to cellular metabolic energy requirements. It follows, then, that direct measurements of ATP pool turnover, when coupled with independent estimates of ATP pool size, should provide useful information on biological energy flux in cells, populations, or natural microbial assemblages (26). Finally, the measurement of rates of RNA and DNA synthesis, using the uptake and incorporation of specific radiolabeled precursors (e.g., ^{3}H-adenine, ^{3}H-thymidine, ^{32}P/^{33}P-phosphate) provides invaluable information to the environmental nucleotide fingerprint (24, 27, 43). The correlations between nucleic acid synthesis, protein synthesis, and cell growth are so universally accepted that they lend themselves well to the study of complex microbial assemblages in nature.

This chapter will focus on the most basic and most widely used aspect of the environmental microbial nucleotide fingerprint, namely, the measurement of cellular ATP as a biomass indicator. The stepwise methodology that is presented will focus on aquatic habitats. However, ATP measurements have also been used for the analysis of soils and in wastewater treatment and clinical applications (21). The ATP extraction and analysis protocols will vary considerably with sample type and expected microbial biomass. Other features, including the EC_A ratio, GTP:ATP ratio, total energy flux determination by cellular ATP turnover, and measurement of rates of RNA and DNA synthesis, will be mentioned, but the detailed analytical procedures published elsewhere (16, 23–25, 31, 32) will not be repeated here.

37.2. SAMPLING AND SUBSAMPLING THE ENVIRONMENT

Sampling is one of the most important aspects of quantitative microbial ecology, but sampling design, including frequency of sampling, replication, and other relevant issues, is sometimes overlooked. All naturally occurring microbial communities are variable in space and in time, and the number of samples typically collected in any single study is usually small and, therefore inadequate for a complete description of the habitat under investigation. To the extent possible, multiple samples should be collected, covering as many microenvironments of the bulk habitat as possible to obtain a true representation of the ecosystem under investigation. For relatively homogeneous aquatic environments this is straightforward, but for sediments and soils it is not. The nature of the sampler itself is also important. For example, when is a sterile sampler absolutely necessary, and when can that requirement be relaxed?

The number of samples that are necessary to accurately describe microbial processes in a given ecosystem depends to a certain extent on the structure of the habitat. Because most natural habitats are actually mosaics of many microhabitats with a heterogeneous distribution of microorganisms, variance between multiple samples generally exceeds variance between subsamples of a single collection. Therefore, replication for microbial biomass and activity estimation is most meaningful when performed at the highest level (35). Unfortunately, this is rarely achieved. Automation in sample collection and, especially, in sample analysis—including remote sensing—should greatly improve the current situation with respect to the overall accuracy of ecosystem measurement.

37.3. BIOMASS DETERMINATION BY ATP

ATP has several unique characteristics that 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) or 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 (21). The preferred method of ATP quantification is the firefly bioluminescence reaction, but a variety of analytical techniques are available for either discrete sample or continuous flow analyses. Data on the P-ATP content of a water, sediment, or soil sample can be extrapolated to total microbial biomass using C:ATP relationships derived from either laboratory or field studies. Other advantages include the low detection limit (less than 10^{-12} mol; roughly equivalent to $\sim 10^{3}$ *Escherichia coli* cells) and acceptable level of precision for field replicates (typically 1 to 10%, depending on ATP concentration and operator), the high degree of objectivity compared with methods requiring operator recognition of "live" cells and estimation of cell dimensions, and the potential for near "real time" analyses and continuous flow applications. Finally, compared with most other biomass assays, there is also an extensive laboratory and field database for comparisons, conclusions, and ecological interpretations.

37.4. MATERIALS REQUIRED

37.4.1. 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: polyvinyl chloride (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 many commercially available general instruments, such as fluorometers, spectrophotometers, or liquid scintillation counters, is suitable for measuring light emission. However, 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 manufacturers. 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.

37.4.2. 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 by 125– to 160–mm length); assay vials (18–mm diameter by 40–mm height). Size requirements may vary depending on equipment/instruments used.
- Adjustable automatic pipettes (0.1– to 1–ml and 1– to 5–ml capacities)

37.4.3. Solutions and Reagents

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

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

37.5. PROCEDURES

37.5.1. Sampling, Subsampling, Extraction, and Sample Storage

37.5.1.1. Water Column

Typically, oceanic or lake samples are collected at predetermined depths throughout the water column using standard commercially available 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 water samples 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 before analysis.

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 (21), 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 mM, pH 7.4) buffers. The latter is recommended for samples suspected of containing alkaline phosphatase, as would be expected in phosphorus-depleted habitats (28). After concentration of microbes onto a filter, the filter is 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, measured in seconds, the cells desiccate, causing a loss of cellular ATP. It is imperative that the extraction buffer be at boiling point at the time of filter insertion. At temperatures below approximately 90°C, inefficient extraction occurs due to enzyme-catalyzed ATP hydrolysis.

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% per year in properly buffered solutions.

37.5.1.2. 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 prior to 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 have 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 (45), and for the preservation of millimeter-scale habitat variability (8). 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 with 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–ml 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 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-pore-size 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 nM, the sample can be diluted with 60 mM PO_4 buffer as an alternative to base titration.

5. If the ATP concentration is ≤1 nM, the acid extract must be concentrated by either the activated charcoal procedure (14) or brucite coprecipitation (4, 34) 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.

37.5.2. Detection of ATP by Firefly Bioluminescence

Although ATP (and other adenine and nonadenine 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 (21, 22). 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, as discussed later in this chapter.

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

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

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

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 depend on the purity of the enzyme preparation; sensitivity is controlled by luciferin concentration (30). The measurement of either 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 with certain instruments. However, a major disadvantage of the integrated mode is the nonspecificity of light emission with certain crude enzyme preparations (20). Reliability of peak-height analyses depends on 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. Firely lantern extract (catalog FLE-50 or FLE-250; Sigma Chemical Co., St. Louis, MO) is stored with desiccant 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 on the desired sensitivity. During the aging process, endogenous ATP initially present in the crude extract is consumed, thereby decreasing the background light emission. Because of 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 the mixture is incubated at room temperature for 1 h.

3. If desired, the FLE preparation can be diluted to a greater final volume depending on 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 to maintain first-order reaction kinetics with respect to ATP (30).

4. Immediately before use, the insoluble residue is removed by vacuum filtration (Whatman GF/F filter) or centrifugation (1500 × *g*, 5 min).

37.5.3. 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 μM 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% per year. 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); l = absorption pathlength (cm); and $e = 15.4 \times 10^3$ (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 on 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 nM 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. In general, no significant hydrolysis of the diluted ATP standard solutions occurs 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.

37.5.4. Data Reduction and Extrapolations of ATP to Biomass

The relationship between ATP concentration 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 I 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 per liter of the original water or sediment 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 [21]). Among the most conspicuous differences in C:ATP ratios are those observed between unicellular microorganisms (i.e., bacteria and microalgae: C:ATP ≈ 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 (21, 22). 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 mM (6). Unfortunately, it is difficult to use biovolume estimates directly, however accurate, in most studies of microbial ecology. Consequently, one must rely on empirically determined C:ATP ratios to extrapolate P-ATP determinations to estimates of total microbial biomass. In so doing, both the level of precision and the accuracy of the initial ATP determination are decreased. Furthermore, in habitats where copious amounts of capsular materials, extracellular secretions, or slimes occur (7), ATP-based values of total biomass probably provide only minimum estimates.

37.6. NOTES AND COMMENTS

37.6.1. Sampling and Subsampling

Exposure of metabolically active 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 that provides for the in situ extraction of ATP from microorganisms needs to be developed and compared with conventional sampling procedures in order to provide quantitative constraints on the potential changes in P-ATP during routine sample recovery.

37.6.2. Extraction

The concentration step, which is necessary for most low-biomass environments (i.e., <1 g of C m^{-3}), must be performed with extreme care; sample size is especially important. It has been shown that the recovery of microbial ATP from certain high-particulate-load samples is volume dependent (49). 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 (31). 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 of 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 may affect the efficiency of ATP extraction. If desired, multiple injections can be made, allowing time between single injections 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, dissolved ATP (operationally defined as passing through a 0.2–μm-pore-size filter), which is also present in most marine (2, 44) and freshwater (41) ecosystems.

37.6.3. 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 luciferase and luciferin concentration in the enzyme preparation. Using the crude FLE-50 luciferase mixture prepared as described in this chapter and commercially available ATP photometers, the lower limit of ATP detection (i.e., twice the background light emission) is about 0.2 nM ATP. For greater sensitivity, exogenous luciferin is added to the enzyme preparations, enabling the detection of 1 pM ATP (30). The precision of the peak height assay procedure as routinely performed 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, no commercially available certified reference materials are available for independent determination of accuracy.

37.6.4. 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 (i) 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; (ii) 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; (iii) turbidity of the final extracts, resulting in light scattering and absorption; (iv) the presence of a high concentration of inorganic particulate material in the final extracts, resulting in loss of ATP through adsorption; and (v) 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 preceding sources of error are detected, and corrected for, through the use of an ATP internal standard, as discussed by Strehler (48). 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 with that of the samples. Peak-height measurements significantly decrease the analytical interference due to the presence of nonadenine nucleotide triphosphates and therefore are strongly recommended. A review of analytical issues concerned with ATP extraction efficiency from soils has recently appeared (42).

37.7. ENHANCING THE MICROBIAL NUCLEOTIDE FINGERPRINT

The basic method of ATP measurement as an indicator of total microbial biomass has given rise to the more comprehensive protocol of environmental nucleotide fingerprinting (21). The development of specific and sensitive techniques for quantitative measurements of selected non-ATP intracellular nucleotides has enabled researchers to estimate the rates of protein and nucleic acid biosyntheses, nucleotide metabolism, metabolic activity, and growth. Furthermore, Karl and Bossard (26) developed a method to estimate the turnover rates of ATP to quantify energy flux in natural populations of marine microorganisms. This expanded approach of nucleotide fingerprinting is strongly recommended for field investigations to obtain corroborative data relating to the in situ physiological and growth states of microbial assemblages in nature.

To calculate the mean cellular EC_A ratio, measurements of ADP and AMP must be made in addition to ATP determination, as described above. ADP and AMP are both quantitatively coextracted with ATP, so the methods described above for sample collection and initial processing are identical to those for ATP determination. After the extraction of cellular nucleotides, ADP and AMP are quantitatively converted to ATP by stepwise enzymatic reactions involving pyruvate kinase and adenylate kinase plus pyruvate kinase, respectively. ATP is measured again in each sample, and ADP and AMP concentrations are calculated by difference (16).

For GTP concentration measurements, the ATP and UTP present in the nucleotide extracts are first destroyed by specific enzymatic reactions involving the addition of hexokinase, glucose-6–phosphate dehydrogenase, and UDP-glucose pyrophosphorylase. The remaining NTPs, mainly GTP and CTP, are separated kinetically in the light emission reaction involving firefly luciferase (18–20).

For the determination of ATP and total adenine nucleotide (TAN) pool turnover rates as estimates of energy flux and specific growth rate of microbial populations in nature, an environmental sample is incubated with radioactive phosphate (^{32}P- or ^{33}P-PO_4), as a tracer for P-flux through the acid anhydride-bound P groups of ATP (β-P and γ-P) and into the α-P position as adenine nucleotide molecules are removed for net growth (27). With increasing incubation time, the three phosphate moieties of the "community" ATP pool (or isolated fraction thereof) become labeled, albeit at different rates. Experimental work has demonstrated that the kinetic constants of γ-P and β-P labeling are indistinguishable, both in laboratory cultures and in field samples, because of the rapid intracellular interconversions of ADP and ATP. Because both anhydride bonds have identical free energies of hydrolysis, this simplifies the measurement of total metabolic energy flux (5). By comparison, the α-P labeling lags behind the γ-P and β-P moieties because the α-P position of ATP becomes labeled only as a result of the net removal of adenine nucleotides for biosynthesis, the sum of salvage and de novo synthesis processes. The exact turnover time of the ATP or the TAN pool is determined by an analysis of the time rate of change in the specific activity of either the γ-P, β-P, or α-P positions of ATP. Labeling of the individual positions is determined by selective hydrolysis of ATP to ADP + P_i. Since the γ-P radioactivity is equal to the β-P radioactivity, α-P can be calculated by difference: ($\alpha\text{-P}^* = [\text{total ATP}^*] - [2 \times \gamma\text{-P}^*]$).

As predicted by radiotracer theory, the change in the ATP or TAN pool-specific radioactivity (SA) (nanocuries per picomole) following the addition of an appropriate radioactive precursor is an exponential function of incubation time (t). The initial SA (at t = 0) is zero, and the relative SA (at isotopic equilibrium with precursor pool) is 1.000. Under these conditions, the decimal equivalent of SA at any time (SA_t) can be described by the equation

$$SA_t = 1 - (2^{-N}) \quad (1)$$

where N is equal to the number of turnover cycles (N is dimensionless) observed during the incubation period. ATP or TAN pool turnover time (T) can then be calculated from the expression

$$T = t/N \quad (2)$$

This relationship is applicable for all incubation periods (t) less than or equal to $5N$, at which time the pools are expected to be near isotopic equilibrium and, in theory, would not change until the exogenous radioactive precursor pool is exhausted. For oligotrophic surface seawaters, a 3- to 4-h incubation period is sufficient to reach isotopic equilibrium (5).

Once the turnover time (T) of the ATP or TAN pool has been determined, it is straightforward to extrapolate these data to the more meaningful ecological parameters of energy flux and specific growth rate. Assuming a value of -11 (± 1) kcal mol^{-1} for the free energy of ATP hydrolysis for either the β-P and γ-P positions under in vivo conditions (54), then the total microbiological energy flux (EF, expressed in units of kilocalories per liter of sample per hour) can be calculated as

$$\text{EF (kcal liter}^{-1}\text{ h}^{-1}) = -22([\text{ATP}]/T_{\text{ATP}}) \quad (3)$$

where [ATP] is equal to the total particulate ATP pool in moles per liter^{-1} of sample and T_{ATP} is ATP pool turnover time (h). This value for total available free energy can be compared directly to the solar energy flux, to the energy stored by photosynthesis, or to respiration. In this way, carbon and energy fluxes can be directly compared.

Extrapolation of the measured TAN turnover time (T_{TAN}) to specific growth rate (μ) is based on the theoretical predictions (6) and empirical observations (33) that the T_{TAN} is equivalent to a value that is 2 to 3% of the generation time (i.e., TAN pool turns over, on average, 40 times per generation). Consequently, the doubling time (T_d, expressed in hours) of the substrate-responsive population is

$$T_d = T_{\text{TAN}} \times 40 \quad (4)$$

and specific growth rate (μ, expressed in units per hour) is

$$\mu = 1/T_d \times \ln(2) \quad (5)$$

These kinetic model formulations will provide "ideal" results when all of the radiotracer-responsive microorganisms in a given habitat are growing at identical rates. A novel, statistical treatment of field data, described in detail by Laws et al. (37), provides a method for determining the coefficient of variation among the individual estimates of T_{ATP} and T_{TAN}. Consequently, one can quantify the variability among individual subcomponents of the microbial community. Although applied here for the turnover of ATP and TAN pools, this mathematical formulation can be used to assess heterogeneity in the turnover rate of any intracellular pool for which a suitable radioactive or stable isotopic precursor exists.

37.8. CONCLUSION

No single approach to the study of microorganisms in their natural environments is universally accepted, or acceptable (22). Early in the development of microbiology as a scientific discipline, specific groups of microorganisms were isolated from nature and studied in the laboratory. This led to major advances in microbiology, especially physiology, genetics, and metabolism. Now, armed with new information about the phylogenetic and metabolic diversity of microorganisms in nature and the probable presence of novel, as yet uncultivatable microbes, there is a renewed interest in microbial ecology and in whole-community assays. Despite recent and significant progress, the field of microbial ecology is still "methods-limited" with regard to the most fundamental properties of natural microbial assemblages, namely, biomass and metabolic activity estimation of the total population. One of the most significant challenges for the future is to link microbial phylogeny to biogeochemistry and ecology to gain a better understanding of material and energy flow in the biosphere.

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