HANDBOOK OF METHODS IN

AQUATIC MICROBIAL ECOLOGY

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CHAPTER 55

Adenosine Triphosphate (ATP) and Total Adenine Nucleotide (TAN) Pool Turnover Rates as Measures of Energy Flux and Specific Growth Rate in Natural Populations of Microorganisms

David M. Karl

INTRODUCTION

Two fundamental goals in aquatic microbial ecology are to elucidate the pathways and to quantify the fluxes of carbon and energy through microbial communities in nature. In recent years, much attention has been focused on the measurement of microbial metabolism and productivity in natural aquatic habitats and a variety of experimental protocols is now available.[1] Among the most direct and meaningful assessments, however, is the measurement of microbial community specific growth rate (μ; or alternatively, generation time or doubling time) which can be compared to the extensive database derived from carefully controlled laboratory studies and to theoretical models relating specific growth rates to growth temperature or nutrient conditions.

Two general approaches have been used to estimate μ in natural populations of microorganisms: (1) direct measurements of the increase in cell numbers or biomass during timed bottle incubations, usually after removing the influence of grazer populations by size fractionation, chemical inhibition, or sample dilution and (2) direct and simultaneous measurements of standing stock (biomass) and production (rate of biomass increase) in order to calculate the mass-specific rate of biomass increase. A major limitation, however, with most experimentally derived estimates of specific growth rate, is the inability to ascertain heterogeneity in specific growth rate that is generally acknowledged or presumed to occur in natural populations or microorganisms.

In contrast to the availability of the above-referenced experimental methods providing either direct or derived estimates of carbon (or mass) flux, relatively few techniques exist for the measurement of microbiological energy flux. The generally assumed direct coupling between carbon and energy fluxes in nature require balanced cell growth and constant growth efficiencies. However, in nature, microbial cells and even entire populations of cells are known to exhibit unbalanced or transient growth dynamics. This not only invalidates the assumptions inherent in the application of certain productivity protocols, but also serves to uncouple the flux of energy from the production or turnover of cell biomass.

In a series of recent papers, Karl and colleagues[2-5] have described novel approaches for the independent estimation of energy flux and carbon flux by direct measurements of the

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turnover of the cellular adenosine triphosphate (ATP) and total adenine nucleotide (TAN) pools, respectively. In addition, a unique data analysis method has been devised for determining both the average turnover rate and the standard deviation thereof as a direct assessment of the heterogeneity of ATP or TAN pool turnover rates within microbial communities in nature.[6]

THEORETICAL PRINCIPLES

ATP Pool Turnover

The central role of ATP in the stoichiometric coupling of energy-yielding and energy-requiring metabolic reactions has been known since the pioneering research of Lipmann.[7] The steady-state intracellular concentration of ATP (referred to as the "ATP pool") in viable microorganisms (prokaryotes and eukaryotes) appears to be well-regulated at a value of 2 to 6 nmol ATP mg^{-1} dry weight (~1 to 3 m*M*) regardless of growth rate, culture condition, or mode of nutrition.[8] Previous studies of ATP pools in microorganisms and in environmental samples have emphasized the futility of extrapolating these static measurements to estimates of metabolic energy flux. Consequently, it is the turnover rate of the ATP pool, rather than the steady-state concentration of that pool, which varies in proportion to cellular metabolic energy requirements. It follows then, that direct measurements of ATP pool turnover rates, when coupled with independent estimates of ATP pool size, should provide useful information on biological energy flux in cells, populations, or natural microbial assemblages.

Cellular ATP pool turnover results from the hydrolysis of one or both of the anhydride-bound PO_4 groups (the β-P and γ-P or "high-energy" phosphate bonds) via orthophosphate or pyrophosphate cleavage, followed by subsequent regeneration of ATP by either substrate-level, oxidative, or photophosphorylation processes (Figure 1A and B). Because ATP pool turnover processes define a cycle, this essential set of metabolic processes neither results in net removal of adenine nucleotide molecules from the intracellular pool nor requires coupled biosynthesis thereof. This will become important in the analytical procedures employed to distinguish between the turnover cycles of ATP and TAN pools.

In theory, ATP pool turnover rates could be measured under steady-state metabolic conditions (i.e., $d[ATP]/dt = 0$) by estimating either the rate of ATP formation or rate of ATP utilization, if either process could be temporarily suspended without affecting the other. Such ATP pool transitions have been observed in microorganisms following the rapid removal of oxygen (for aerobic heterotrophs) or light (for phototrophs), but it is uncertain whether the results derived from these harsh experimental perturbations yield reliable estimates of ATP pool dynamics. Recently, Karl and Bossard[3] devised a novel method for ATP pool turnover in cell cultures or natural populations of microorganisms. The procedure relies upon the use of $^{32}PO_4$ (or $^{33}PO_4$) as a tracer for P-flux through the acid anhydride-bound P (β-P and γ-P; Figure 1A and B) groups of cellular ATP. The uptake of $^{32}PO_4$ also results in the labeling of the α-P group of ATP, if the population in question is actively growing (Figure 1C). Consequently, in order to uniquely assess the labeling of ATP derived from energy flux, one must be able to separate β-P and γ-P labeling from α-P.[3] On the other hand, α-P labeling defines TAN pool turnover and therefore, $^{32}PO_4$ labeling can be used to estimate both ATP pool and TAN pool turnover rates.

TAN Pool Turnover

The uptake, or salvage, of exogenously added nucleic acid precursors (e.g., adenine, thymidine, uridine) in preference to *de novo* synthesis is a well-documented characteristic

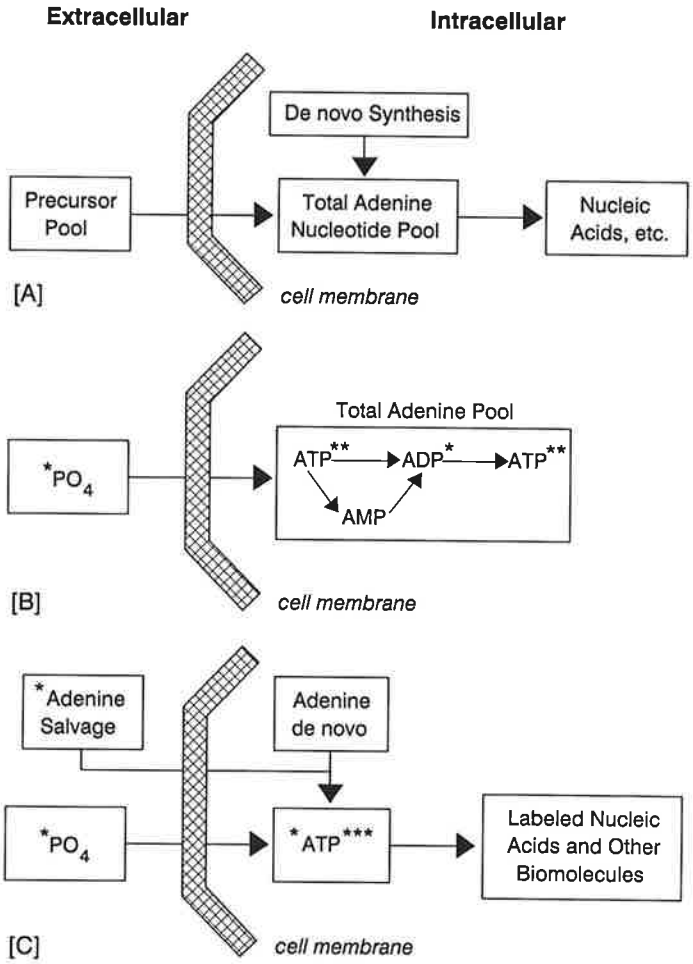


Figure 1. Schematic models for $^{32}PO_4$ ($^{33}PO_4$) labeling of the α-, β-, and γ-P positions of ATP and ^{3}H-adenine assimilation during microbial metabolism and biosynthesis. (A) Generalized model for the uptake of exogenously added precursor, incorporation into intermediate nucleotide pool, and into cellular macromolecules. The turnover rate of the adenine nucleotide pool is controlled by net removal as required for cellular biosynthesis. (B) Specific model for ATP pool turnover (energy flux) in absence of biosynthesis. Under these conditions, there is no net removal of nucleotide molecules and, therefore, no incorporation of radiolabeled phosphate (*P) into the α-P (i.e., AMP) position of ATP. Because of rapid interconversion between ATP, ADP, and AMP, the kinetic constants for labeling the β-P and γ-P positions of ATP are indistinguishable. Since there is no net removal of adenine for biosynthesis, ^{3}H-adenine would not be expected to label the ATP pool under these restrictive conditions. (C) During net macromolecular biosynthesis, $^{32}PO_4$ also labels the α-P of the ATP pool (although with a kinetic constant uncoupled from the labeling of β-P and γ-P positions of ATP) and ^{3}H-adenine is incorporated into cellular ATP pool. Net removal of adenine nucleotides for biosynthesis is balanced by the sum of salvage and *de novo* synthesis processes. In theory, the kinetic constant for labeling ATP by ^{3}H-adenine is identical to the $^{32}PO_4$ labeling constant of the α-P of ATP. (Adapted from Karl and Bossard.[3])

of aquatic microbial communities (References 9 and 10; see Figure 1C). This phenomenon comprises the theoretical basis for the use of [^{3}H]adenine, [^{3}H]thymidine, and [^{3}H]uridine in ecological studies of nucleic acid synthesis. An important aspect of the incorporation of nucleic acid precursors is the observation that the total flux of precursor into the nucleotide triphosphate pools (i.e., ATP, TTP, UTP, etc.), which is the result of the combined effects of salvage and *de novo* synthesis, is in equilibrium with the removal of the triphosphate

precursors required for cellular biosynthesis. Furthermore, it is well-documented that the TAN pool (i.e., steady-state intracellular concentration of [ATP + ADP + AMP]) in microorganisms does not vary with changes in the specific growth rate.[8,11] Because cellular biosynthesis is directly related to growth rate, a positive correlation is expected to exist between cellular adenine nucleotide flux and μ. It follows then that the turnover rate of the TAN pool (and most likely, of all ribonucleotide and deoxyribonucleotide pools in general) must vary in direct proportion to the rates of nucleic acid synthesis and hence, net growth.

The TAN pool turnover time is defined as the average residence time of a molecule in the intracellular pool before it is removed for macromolecular biosynthesis (i.e., the steady-state pool concentration divided by the steady-state rate of synthesis or removal). If a radioactive precursor such as [^{3}H]adenine is added to a growing culture or natural population of microorganisms, the TAN pool turnover time can be calculated by monitoring the change in the specific activity (SA) of the ATP pool with incubation time. TAN pool turnover rate can also be measured by monitoring the labeling kinetics of the α-P position of ATP following the addition of $^{32}PO_4$ (or $^{33}PO_4$) to a sample[3] (also see the section on ATP pool turnover). Exactly one turnover cycle has been completed when the ATP pool has achieved an SA that is equal to 50% of the value at isotopic equilibrium. Chapman and Atkinson[11] have estimated TAN pool turnover rates as a function of growth rate for *Escherichia coli* and *Salmonella typhimurium*. They conclude that the TAN pool ''turns over'' (i.e., is completely utilized for biosynthesis and is replenished through salvage and *de novo* synthesis) 30 to 50 times per generation regardless of generation time. This prediction has been tested using a diverse variety of marine microorganisms.[5] The results indicate that the TAN pool turnover time is positively correlated with generation time and averages 2.2% of the generation time (i.e., TAN pool turns over 45 times per generation).

MATERIALS REQUIRED

Equipment

- Water sampling gear (Niskin bottles, or equivalent)
- Polycarbonate incubation bottles (0.1 to 4 L, depending upon design of labeling experiments)
- On-deck temperature- and light-controlled incubator or *in situ* array
- 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), an adjustable vacuum pump with gauge (compressed N_2 or air can be used as an alternative to vacuum filtration), filters (24/25 mm diameter Whatman GF/F, Millipore HA/GS, or equivalent), filter forceps
- Block heater (Sybron Thermolyne Type #16500 Dri-bath, or equivalent) for ATP extraction, capable of heating extraction menstruum in test tubes to 100 $\pm$ 1°C (1 atm) and maintaining boiling temperatures throughout the extraction period
- Adjustable automatic pipettes (10 to 100 μl, 100 to 1000 μl, and 1 to 5 ml capacities)
- Storage freezer (-20°C)
- 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, Turner Designs, LKB Instruments, Biospherical Instruments Corporation, 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.

- Thin-layer chromatography (TLC) development tank (with glass cover)
- High-intensity ultraviolet (UV) lamp
- Liquid scintillation counter
- Gyrotatory shaker table
- Microcentrifuge
- Vacuum evaporator (Savant Speed-vac concentrator or equivalent)

Supplies

- TLC plates, 20 × 20 cm, polyethyleneimine (PEI) impregnated cellulose (MN #300 PEI, Brinkmann Instrument Company, Westbury, NY)
- ATP extraction and assay glassware: test tubes for ATP extraction (approximately 15 mm diameter × 125 to 160 mm length); ATP assay vials (18 mm diameter × 40 mm height), Note: size requirements may vary depending upon equipment/instruments used
- Disposable glass culture tubes (12 × 75 mm) and glass liquid scintillation counting (LSC) vials
- $^{32}PO_4$ ($^{33}PO_4$) or [2,^{3}H]-adenine (DuPont #NET-350, or equivalent) radioisotopic tracer
- LSC cocktail
- Microcentrifuge tubes, 1.5 ml capacity

Solutions and Reagents

- Firefly lantern extract (Sigma Chemical Company, cat. #FLE-50)
- ATPase, porcine cerebral cortex (EC 3.6.1.3; Sigma Chemical Company, cat. #A-7510) prepared in distilled water to a concentration of 1 unit ml^{-1} and stored frozen until needed
- Sodium arsenate buffer (100 m*M*, pH 7.4)
- Tris(hydroxy)aminomethane-HCl buffer (20 m*M*, pH 7.75)
- ATP (5×10^{-4} *M*) and AMP (3 m*M*) sodium salt solutions in Tris buffer
- Magnesium sulfate solution (40 m*M*)
- Potassium phosphate buffer (60 m*M*, pH 7.4) and potassium phosphate solution (0.85 *M*, pH 3.4)
- Magnesium chloride (0.7 *M* and 0.35 *M*), sodium chloride (5 *M*) and potassium chloride (1 *M*) solutions
- Activated charcoal (Sigma Chemical Company, cat. #C-4386)
- Phosphoric acid (concentrated)
- Formic acid (0.2 *M*)

PROCEDURES

Brief Summary

A freshly collected seawater sample is incubated under defined temperature and light conditions (e.g., *in situ*, simulated *in situ*, dark) in the presence of an appropriate radiolabeled precursor. After predetermined incubation periods, subsamples are removed and particulate ATP pools are extracted and purified. The isolated radiolabeled ATP is either directly counted (e.g., ^{3}H-adenine based determinations of TAN pool turnover rate) or subjected to enzymatic hydrolysis designed to selectively hydrolyze the γ-P position of ATP (e.g., $^{32}PO_4$ or $^{33}PO_4$ based determinations of ATP and TAN pool turnover rates). From time-course information on the change in ATP and TAN pool specific radioactivity, the turnover rates can be quantified and the results extrapolated to estimates of energy flux and specific growth rate.

Sample Collection, Incubation, and ATP Pool Extraction

1. Collect water sample for analysis using clean, noncontaminating PVC bottles, or equivalent. Try to minimize the time between sample collection and the beginning of the experiment if results are to be extrapolated to *in situ* rate processes. Keep water samples in ambient conditions (especially with regard to light and temperature regimes) during intermediate storage of samples in order to avoid carbon and energy flux transitions or perturbations caused by change in the environmental conditions. Remove subsamples of each water type for complementary chemical and microbiological measurements (e.g., total biomass, primary and secondary production, bacterial cell numbers, dissolved nutrients and gases, particulate matter composition, etc.)

2. Subsamples are placed into acid-cleaned, polycarbonate incubation bottles. The required volume is dependent upon microbial biomass. Typically for surface ocean samples, between 100 to 250 ml are used for each determination. Because time-course information on labeling kinetics is required, multiple (between 4 to 8) bottles are required for each water sample. Alternatively, one large polycarbonate bottle (2 to 4 L) which can be conveniently subsampled with minimum disruption to the microbial communities (light or temperature shock, turbulence, etc.) can also be employed. Depending upon the design of the experiment, light vs. dark bottles or size-fractionated water samples may also be prepared.

3. The timed incubations begin with the addition of either $^{32}PO_4$ (or $^{33}PO_4$) or ^{3}H-adenine, or both, to each sample to a final activity of between 0.1 to 0.5 μCi ml^{-1} ($^{32}PO_4$) or 0.05 to 0.2 μCi ml^{-1} (^{3}H-adenine) depending upon the desired sensitivity and expected ambient pool concentrations of nonlabeled substrates. The use of $^{32}PO_4$ enables one to measure both ATP and TAN pool turnover rates; ^{3}H-adenine labeling can only be used for growth rate estimation by TAN pool turnover rate measurements (also see the section on assay limitations and future applications).

4. At predetermined times, subsamples are filtered through 25 mm diameter, microfine glass fiber filters (Whatman GF/F type), and the filters containing the concentrated particulate matter are immersed into boiling phosphate (60 m*M*, pH 7.4) or Tris (20 m*M*, pH 7.7) buffer for ATP extraction. Alternatively, cellular ATP can be extracted by acidification of the entire water with H_3PO_4 (1 *M* final concentration). For additional details on ATP extraction techniques refer to Chapter 41.

ATP Purification

1. The aqueous ATP extracts are concentrated 10- to 20-fold by evaporation *in vacuo*.[12] The acid extracts are concentrated by the charcoal adsorption technique.[13] ATP concentration in an aliquot of the concentrated extract is determined using firefly bioluminescence (see Chapter 41).

2. Radiolabeled ATP is isolated by one-dimensional TLC using plastic backed (20 × 20 cm) sheets coated with PEI impregnated cellulose. Prior to use, the commercially available plates are soaked in a bath of 10% NaCl, air dried at room temperature, and subjected to an ascending development with distilled water. These pretreated TLC plates are dried and stored at 4°C until needed. Because ^{3}H-adenine and $^{32}PO_4$ ($^{33}PO_4$) label different suites of intracellular molecules, slightly different TLC procedures are required for ATP purification.

^{3}H-ATP Isolation

1. In a clean 12 × 75 mm disposable glass test tube, mix 10 μl of concentrated ATP extract and 10 μl of a nonradioactive solution of ATP (5×10^{-4} *M*) and spot 10 μl of this

mixture onto a predetermined location approximately 1 cm from the bottom edge of a PEI plate (i.e., "origin"). Approximately 8 to 10 lanes can be prepared per plate.

2. The plate is air dried, then washed twice (5 min each) by consecutive immersion into two 1-L distilled water baths. This procedure separates unused precursor (^{3}H-adenine) from labeled adenine nucleotides.[12]

3. The plate is then air dried (alternatively, drying can be accelerated using an electric hair dryer), and developed in one dimension with potassium phosphate (0.85 *M*, pH 3.4) in a closed glass TLC tank.[14]

4. When the solvent front has ascended to within 2 cm of the top edge, the plate is removed from the tank and air dried. Total separation time is approximately 2 to 3 h.

5. ATP (and ^{3}H-ATP) is visualized by irradiating the PEI plate with short-wavelength UV light and the spots are localized by circling lightly with a sharpened lead pencil.

6. The spots are cut out with a pair of scissors, placed into an LSC vial containing 1 ml of $MgCl_2$ (0.7 *M*) and the ATP is eluted by shaking the samples for 1 h. This elution step is necessary for ^{3}H-adenine labeled samples due to self-absorption on solid supports.

7. Liquid scintillation cocktail is added directly to the vials in preparation for ^{3}H counting.

8. The counts per min^{-1} are converted to disintegrations per min^{-1} (dpm) using standard techniques for quench correction. ATP pool SA (nCi $pmol^{-1}$) is calculated from the dpm in the isolated ATP spot (nCi) and ATP (pmol) in the concentrated extract after appropriate volume corrections have been applied. These data are used directly for the calculation of TAN pool turnover, as described in the section on data analysis.

^{32}P (or ^{33}P)-ATP Isolation

Because $^{32}PO_4$ labels many small molecular weight intracellular compounds including a variety of different nucleotides, the chromatographic purification procedure requires a two-step, ascending one-dimensional separation on PEI impregnated cellulose plates.

1. After sample application, distilled water washing and drying (as in the previous ^{3}H-ATP isolation section), the TLC plate is developed in formic acid (0.2 *M*) to the top of the plate. Under these conditions, nucleotide triphosphates (including ATP) remain at the origin while most other ^{32}P (^{33}P)-labeled molecules move with the solvent front.

2. The plate is removed and immediately washed in a distilled water bath to remove the formic acid and air dried before final development, in the same direction, with potassium phosphate (0.85 *M*, pH 3.4) in a closed TLC tank.

3. When the solvent front is within 2 cm of the top edge, the plate is removed, washed in two successive distilled water baths and air dried. The final wash is required to remove residual nonradioactive PO_4 (the developing solvent) which, if present, will inhibit the subsequent ATPase reaction (see section on selective ATP hydrolysis by ATPase). Total separation time is approximately 4 to 5 h.

4. The ATP spots are localized by UV light, circled with a pencil line and excised, as described previously.

5. The spots are eluted into 1 ml of $MgCl_2$ (0.35 *M*) by shaking for 1 h at room temperature.

6. A 10 μl aliquot is diluted into 1 ml of Tris buffer (pH 7.4, 0.02 *M*) and assayed for total ATP by the firefly bioluminescence reaction (see Chapter 41). This value is compared to a measurement of the total ATP applied to the plate to determine the overall recovery of ATP during the purification procedure, which is generally >90%.[3]

7. The remainder of the purified ATP (or a portion thereof) is transferred to a clean vial in preparation for selective enzymatic hydrolysis by ATPase, described in the following section.

Selective ATP Hydrolysis by ATPase

1. Stock solutions of ATPase are prepared in distilled water to a concentration of 1 unit ml^{-1} and stored frozen (−20°C) until needed. Under these conditions the ATPase reagent is stable for at least 1 month without appreciable loss of activity. For short-term storage (<2 to 3 d) the stock ATPase reagent should be stored at 4°C, rather than −20°C, in order to maximize stability.

2. To perform the ATPase hydrolysis reaction, 750 μl of the "TLC-purified" ATP solution (in 0.35 *M* $MgCl_2$) is placed into a 12 × 75 mm culture tube containing 250 μl distilled water, 10 μl of a 1 *M* KCl solution, 20 μl of a 5 *M* NaCl solution, and 25 μl of a 3 m*M* AMP solution, followed by the addition of 50 μl of the stock ATPase reagent. The AMP is added to repress the ADP dismutase reaction (2ADP → AMP + ATP) which is also catalyzed by ATPase. The contents of the tube are mixed thoroughly and a 10 μl subsample is immediately removed, diluted in 1 ml of 0.02 *M* Tris (pH 7.4) buffer to quench the reaction and assayed for determination of "time-zero" ATP concentration using the firefly bioluminescence reaction (Chapter 41).

3. The ATP assay procedure is repeated after approximately 10 to 15 min with one or two representative samples in order to establish the extent of ATP hydrolysis. Once the initial rate of hydrolysis is known one can estimate the time required for complete hydrolysis of ATP to ADP, which in our experience, ranges from 20 to 40 min. Since all samples contain identical concentrations of carrier ATP (from the TLC procedure) and equivalent amounts of enzyme, the rates of hydrolysis are expected to be similar. However, complete ATP hydrolysis should be confirmed for each individual sample before proceeding with the separation and purification of products.

4. Following ATP hydrolysis, a 250 μl aliquot of the sample is removed for measurement of "total radioactivity" (i.e., α-^{32}P + β-^{32}P + γ-^{32}P). A second 500 μl aliquot is added to a small plastic microfuge tube containing 500 μl of an activated charcoal slurry (25 mg charcoal per ml of 0.1 *M* H_3PO_4), mixed thoroughly and centrifuged for 10 min at 13,000 × g. A portion of the supernatant (usually 750 μl) is removed for LSC for determination of "$^{32}PO_4$ radioactivity" (i.e., γ-^{32}P). The phosphoric acid-charcoal treatment removes >99.5% of the ADP from solution and catalyzes a <1% nonspecific binding of $^{32}PO_4$.[3] Since γ-^{32}P- and β-^{32}P-specific activities are equal over all time periods (experimental evidence for this assumption is presented by Karl and Bossard[3] and Bossard and Karl[2]), the α-^{32}P activity can be calculated by difference (i.e., α-^{32}P = [total-^{32}P] − [2 × γ-^{32}P]). The γ- and β-P data are used for ATP pool turnover rate estimation and the α-P data are used for TAN pool turnover rate estimation (see following section).

Data Analysis and Derived Estimates of Energy Flux and Growth Rate

As predicted by radiotracer theory (Figure 2), the change in the ATP or TAN pool-specific radioactivity (SA; nCi $pmol^{-1}$) 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:

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

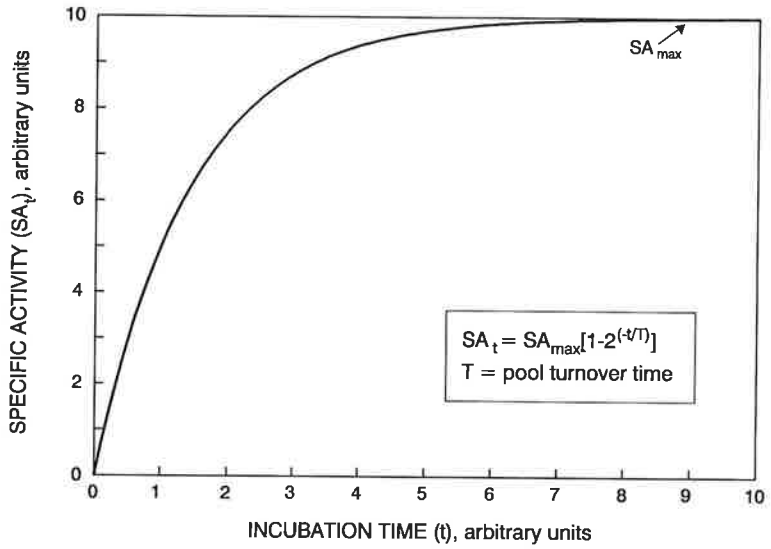


Figure 2. Change in ATP or TAN pool specific radioactivity (SA) vs. incubation time (t) following the addition of a suitable radiotracer. The general equation for this relationship is: $SA_t = SA_{max}[1-2^{-t/T}]$, which can be used to estimate SA_{max} and T from data derived from incubation experiments. When SA_t is expressed as a decimal equivalent of SA_{max} (e.g., $SA_{max} = 100$), the general relationship can also be expressed as: $SA_t = 1-(2^{-N})$ where N is the number of turnover cycles.

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:

$$T = t/N \tag{2}$$

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 (Figure 2).

Karl et al.[5] have compared two independent statistical curve-fitting methods for the analysis of the experimental kinetic data: (1) a computer-assisted nonlinear least squares regression program (PROC NLIN method, DUD; Statistics for the Applied Sciences [SAS] Institute Inc.) to obtain a best fit of the experimental data to the model equation (Equation 1), and (2) the Mangelsdorf[15] linear transformation method in which the ATP or TAN pool SA is determined at regular intervals of incubation time (Δt) and then analyzed by a linear regression analysis of a plot of SA at time (t) as the independent variable vs. the value at time ($t + \Delta t$) as the dependent variable. For the samples tested, both methods resulted in similar estimates of SA_{max} and turnover time (T) of the TAN pool,[5] but for reasons discussed by Karl et al.,[5] the authors recommended the use of the PROC-NLIN method of data analysis.

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. If we assign a value of -11 (± 1) kcal mol^{-1} to the free energy of ATP hydrolysis for either the β-P and γ-P positions under *in vivo* conditions,[16] then the total microbiological energy flux (EF = expressed in units of kcal L^{-1} of sample h^{-1}) can be calculated as:

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

where [ATP] is equal to the total particulate ATP pool in (mol L^{-1} of sample) T_{ATP} is ATP pool turnover time (h). This value for total available free energy can be directly compared 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[11] and empirical observations[5] 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 h) of the substrate responsive population is:

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

and specific growth rate (μ, expressed in units of h^{-1}) 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 these data, described in detail by Laws et al.,[6] 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.

COMMENTS: ASSAY LIMITATIONS AND FUTURE APPLICATIONS

Any technique which requires the uptake and assimilation of a radioactive precursor, as for the methods described in this chapter, are limited in that they can only detect the ''substrate responsive'' portion of the total microbial community. For $^{32}PO_4$ ($^{33}PO_4$), however, this is likely to be a larger subset (if not all viable cells) than for the uptake of more complex or exotic precursors. Since both algae and bacteria are able to assimilate PO_4, the methods are designed to measure total microbial energy flux and specific growth rate. If one desires information on the magnitude of the photophosphorylation energy flux, a light vs. dark incubation experiment could be performed.

With regard to ^{3}H-adenine assimilation, we are also confident that both bacteria and unicellular algae are substrate responsive even at nanomolar concentrations.[17-19] Consequently, TAN pool turnover rate determination by measuring the labeling kinetics of either the α-P position of the ATP pool or ^{3}H-ATP (from ^{3}H-adenine incubations) should provide complementary, redundant information on the specific growth rates of microorganisms in nature.[4] We should, however, emphasize the fact that the presence of nonresponsive microorganisms or metazoans will not adversely influence the growth rate estimated for the substrate responsive subpopulations.[5]

The growth rate derived from our analysis of TAN pool turnover rate is considered to represent a mass-weighted mean estimate for the growing population. As mentioned previously, a method now exists to determine the standard deviation of the average ATP pool

turnover rate.[6] With this additional information, it is possible to assess population heterogeneity. If there is a large variance in community growth rates, then the use of pre- or postincubation size fractionation procedures in order to obtain phytoplankton- and bacterial-enriched fractions for the measurement of size-fractionated TAN pool turnover rates may be used to help ascertain the source of this population variability. Future applications using flow cytometry and cell-sorting capabilities might also provide an opportunity to obtain population-specific estimates of growth using the TAN turnover method.

The theoretical basis of the method described in this chapter for total microbial community specific growth rate estimation could, in principle, be applied for the uptake of other radiolabeled molecules to provide complementary information on specific subcomponents of the total population. For example, the use of [^{3}H]thymidine, to the extent that it predominantly labels only heterotrophic bacteria,[20] is one such example. As expected from known metabolic models and experimental data, exponential labeling of the cellular TTP pool is similar to that described herein for ATP.[21] An exciting possibility is that the measurement of the thymidine nucleotide pool turnover in mixed assemblages of microorganisms may provide a direct measure of heterotrophic bacterial specific growth rate. Of course, the field application of this analysis would first require a laboratory calibration of the quantitative relationship between thymidine nucleotide pool turnover and generation time; however, from the data presented by Werner,[21] it appears that the quantitative relationship between TN pool turnover rate and generation time may be similar to that described herein for the TAN pool.

Another potential variation of the TAN pool turnover method might be to assess photoautotrophic (or chemolithoautotrophic) population growth rates using $^{14}CO_2$ labeling techniques. In theory, $^{14}CO_2$ should be incorporated into the ATP pool of microorganisms which use CO_2 as their principal carbon source (i.e., autotrophs). This method could be used to assess the specific growth rates of phytoplankton populations in mixed microbial assemblages without independent estimates of biomass or production. When used in conjunction with [^{3}H]adenine (bacteria plus algae), $^{32}PO_4$ (bacteria plus algae), and [^{3}H]thymidine (heterotrophic bacteria) the general approach of nucleotide pool turnover could eventually become a powerful method for assessing the growth rates of all microbial populations in nature.

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