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Estimates of Bacterial Growth from Changes in Uptake Rates and Biomass

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Rates of nucleic acid synthesis have been used to examine microbial growth in natural waters. These rates are calculated from the incorporation of [^{3}H]adenine and [^{3}H]thymidine for RNA and DNA syntheses, respectively. Several additional biochemical parameters must be measured or taken from the literature to estimate growth rates from the incorporation of the tritiated compounds. We propose a simple method of estimating a conversion factor which obviates measuring these biochemical parameters. The change in bacterial abundance and incorporation rates of [^{3}H]thymidine was measured in samples from three environments. The incorporation of exogenous [^{3}H]thymidine was closely coupled with growth and cell division as estimated from the increase in bacterial biomass. Analysis of the changes in incorporation rates and initial bacterial abundance yielded a conversion factor for calculating bacterial production rates from incorporation rates. Furthermore, the growth rate of only those bacteria incorporating the compound can be estimated. The data analysis and experimental design can be used to estimate the proportion of nondividing cells and to examine changes in cell volumes.

Estimates of growth rates and biomass are essential for determining the contribution of bacteria to total heterotrophic activity in natural waters. Although there are several accepted methods of measuring bacterial biomass (9, 15, 19, 30), there remains considerable controversy over methods of measuring bacterial growth rates and bacterial production (7, 8, 11–14, 21, 25). Estimation of rates of nucleic acid synthesis promises to be a powerful approach for assessing bacterial activity, and methods have been proposed that measure RNA and DNA syntheses from the incorporation of [^{3}H]adenine (11) and [^{3}H]thymidine (6, 28), respectively. However, there are unresolved problems concerning how bacterial growth and production can be estimated from these measurements.

Several parameters must be known to estimate growth rates from the incorporation of [^{3}H]adenine and [^{3}H]thymidine (6, 7, 11, 13). (i) The amount of radioactivity appearing in the nucleic acid fraction must be determined by either isolating the RNA or DNA or assuming that some portion of the radioactivity in trichloroacetic acid extracts is in the RNA or DNA fraction. (ii) The intracellular specific activity of ATP or dTTP must be known; ATP and dTTP are the immediate precursors for RNA and DNA, respectively, which would be ultimately derived from adenine and thymidine. (iii) The fraction of adenine or thymidine residues in RNA and DNA must be specified, as this fraction determines the relation of ATP and dTTP incorporation to RNA and DNA syntheses. This fraction has been assumed to be 25% (6, 7, 11, 12). These parameters allow the calculation of the rate of RNA or DNA synthesis. The amount of RNA or DNA per cell is needed to calculate the rate at which cells are produced per mole of nucleic acid synthesized. With this information it is possible to calculate bacterial production of those cells incorporating [^{3}H]adenine and [^{3}H]thymidine. To calculate a growth rate, bacterial production is divided by the number of bacteria incorporating the labeled compound. When bacterial abundance is determined by the direct-count method (9), the calculated growth rates are underestimated because of the presence of nondividing cells.

There have been several attempts to measure directly the parameters listed above, which are needed to interpret the incorporation of labeled nucleosides and bases (7, 13). Antibiotics, base analogs, and direct isolation of the nucleic acid fractions have been used to estimate the fraction of radioactivity in trichloroacetic acid extracts present as RNA or DNA. The importance of measuring specific activities of nucleic acid precursors is well recognized. Fuhrman and Azam

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(7) pointed out that intracellular specific activities should be measured in only those organisms incorporating the labeled compound, which may be difficult to accomplish. The specific activity of ATP has been measured directly for RNA synthesis (11). Isotope dilution was assessed indirectly for DNA synthesis by $^{32}PO_4$ incorporation (7) and by the addition of unlabeled thymidine (20). There is some information on the remaining parameters. Fuhrman and Azam (7) measured the amount of DNA per marine bacterium. Microautoradiography has been used to estimate the number of bacteria incorporating [^{3}H]thymidine (7).

The experiments of Karl et al. (13, 14) and Fuhrman and Azam (6, 7) provide valuable insight into the nucleic acid synthesis of bacteria in natural environments. However, unless these parameters are proven to be relatively constant, considerable work must be done to provide the necessary information for each estimate of bacterial production. This assumes that all of the parameters can be measured and applied to those cells incorporating the labeled compound.

We present here a simple experimental design for estimating the factor which converts rates of [^{3}H]thymidine incorporation to bacterial production. Furthermore, our analysis of the change in incorporation rates of any labeled compound yields an estimate of the growth rates of only those bacteria incorporating the labeled compound. This approach could be used to examine the coupling between incorporation of a compound (energy production or macromolecular synthesis) and bacterial growth (production of new cells or an increase in cell size).

MATERIALS AND METHODS

Change in uptake rates and bacterial abundance. The experimental procedure used to determine the change in uptake rates and bacterial abundance is a variation of the method described by Fuhrman and Azam (6). Bacterial abundance and the incorporation of [^{3}H]thymidine into the trichloroacetic acid-insoluble fraction was measured over time in water samples from three environments. These two parameters were measured in samples in which the particulate matter (including the bacteria) was diluted approximately 10-fold by adding 30 ml of unfiltered water to 270 ml of filter-sterilized water, which was prepared with a rinsed membrane filter (Millipore Corp.) with a pore diameter of 0.22 μm. The water samples were incubated in 300-ml dissolved oxygen bottles (Great Sippewisset Marsh, Mass.), 500-ml Pyrex flasks (Ice House Pond, Mass.), or acid-washed 500-ml polycarbonate flasks (New York Bight), all of which we designate here for convenience as the "growth bottles." The glass containers were combusted at 500°C for 4 h to remove possible contaminating organic compounds.

In the Great Sippewissett Marsh, unfiltered inocula were collected from the marsh at low tide and simultaneously from outside the marsh in Buzzards Bay (see reference 29 for description of the region). These inocula were added to filter-sterilized water collected within the marsh. In the New York Bight, inocula were collected from a station within the Hudson River Plume, which we will refer to as the "plume" sample, and from a station outside the plume, which we will refer to as the "shelf" sample. These inocula were added to filter-sterilized water from the plume (see references 2 and 17 for description of the area). Samples from Ice House Pond were taken from the middle of the pond (16).

Experiments on [^{3}H]thymidine incorporation were begun once the unfiltered water was added to the filtered water; this was time zero of the experiment. The growth bottles were then incubated in the dark with gentle mixing at ±2°C of the temperature of the water from which the samples were taken (±0.5°C for the New York Bight samples). Two 5.0-ml subsamples (three subsamples in Great Sippewissett) were taken at time zero from each growth bottle and [*methyl*-^{3}H]thymidine (>65 Ci mmol^{-1}; New England Nuclear) was added for a final concentration of 5 nM labeled compound. The subsamples were incubated under the same conditions as the growth bottles. After 30 or 60 min the reaction was stopped by adding unlabeled 10 mM thymidine and placing the subsamples in ice water. Cold trichloroacetic acid was added to a final concentration of 5% (wt/vol) once the subsamples had chilled. After 15 to 20 min the trichloroacetic acid-insoluble material was filtered onto Millipore filters with pore sizes of 0.45 μm (pressure differential, <150 mm of mercury) and rinsed twice with 3 ml of cold 5% trichloroacetic acid. The scintillation counting procedure was taken from Fuhrman and Azam (6). Subsequent [^{3}H]thymidine uptake rates were assayed on 5.0-ml subsamples taken from the growth bottle periodically throughout the experiment, and the protocol just described was followed.

Concurrently with assays on [^{3}H]thymidine incorporation, 10-ml subsamples were removed from the growth bottles and preserved with formaldehyde (final concentration, 2%) for subsequent direct counts of the bacterial abundance, according to the method of Hobbie et al. (9). The change in average cell volume was determined in one experiment by measuring at least 25 bacteria per sample with an ocular micrometer. The cells were classified as either spheres or cylinders.

In one experiment on Ice House Pond, [^{3}H]adenine (15 Ci mmol^{-1}; New England Nuclear) uptake rates were determined in subsamples from the growth bottle. The procedure was essentially the same as that used for [^{3}H]thymidine except the reaction was stopped only by placing the subsample in ice water. The subsample took less than 5 min to cool down before trichloroacetic acid was added. Subsamples which were cooled immediately after addition of [^{3}H]adenine showed no significant uptake, which indicated that rapid cooling was sufficient to stop uptake.

Formalin-killed controls were used to measure abiotic absorption of [^{3}H]thymidine and [^{3}H]adenine. The killed control values were subtracted from all data reported here.

Data analysis. The following model was used to analyze uptake rates and bacterial abundance which increase over time because of microbial growth. We first discuss the basic model, which is a simple procedure to calculate a conversion factor and specific growth rates. This conversion factor is the amalgama-

tion of those biochemical parameters that relate incorporation of a radiolabeled compound to bacterial growth. Subsequently, we explore the effect of nondividing cells and changes in cell biomass and in specific activity.

(i) Calculation of conversion factor and growth rate. We hypothesize that there is a linear relationship between uptake rate and bacterial production, which appears to be a reasonable first approximation. This implies that uptake and production are closely coupled, which can be expressed as:

$$v(t) = C^{-1}\, dN(t)/dt \quad (1)$$

where $v(t)$ is the uptake of the labeled compound, $dN(t)/dt$ is the production of cells, and C (units of cells per mole) is the conversion factor that allows uptake rates to be related to cell growth. Equation 1 holds if the parameters listed above, which are necessary to calculate bacterial production from incorporation rates, are constant. In particular, equation 1 holds even if the intracellular specific activities of the nucleic acid precursors are unknown but constant.

We hypothesized that growth is exponential, i.e.,

$$dN(t)/dt = \mu\, N(t) \quad (2)$$

The change in bacterial biomass will be discussed more thoroughly below. Equation 2 can be substituted into equation 1 to give $v(t) = C^{-1}\, \mu N(t)$. Since $N(t) = N(0)e^{\mu t}$, then

$$v(t) = C^{-1}\, \mu N(0)\, e^{\mu t} \quad (3)$$

$N(0)$ is the number of bacteria incorporating the compound at the beginning of the experiment. In this study, our data suggest that most bacteria incorporated the [^{3}H]thymidine and no correction was used.

Equation 3 offers a convenient way to analyze the changes in incorporation rates over time. A simple log transformation of equation 3 gives

$$\log_e v(t) = \mu t + \log_e [C^{-1}\, \mu N(0)] \quad (4)$$

The slope of a semilog plot of uptake rates versus time is the growth rate, μ, whereas the conversion factor C can be estimated from the y intercept (b). The equation to calculate C is

$$C = \frac{\mu\, N(0)}{\log_e{}^{-1} b} \quad (5)$$

It is worthwhile pointing out that C can be estimated even if μ is based on measurements other than $v(t)$. The conversion factor C could be calculated from a growth rate based on the change in bacterial biomass and one initial measurement of the incorporation rate, which is equivalent to $\log_e{}^{-1} b$. However, the basic assumption of the method, that uptake and production are closely coupled, then remains untested.

(ii) Increase in cell volume. The analysis is complicated somewhat by increases in the average cell volume. It is reasonable to hypothesize that the cell volume of naturally occurring bacteria increases exponentially, since the cell size of most species in pure culture increases exponentially with age (23). Therefore, let the change in average cell volume $B(t)$ (cubic micrometers per cell or micrograms of C per cell) over time be described as $B(t) = B(0)e^{st}$, where s is the specific growth rate (hour^{-1}) of the cell volume. With similar reasoning used to derive equation 6, we derive

$$\log_e v(t) = (\mu + s)t + \log_e [C^{-1}\, (\mu + s)N(0)B(0)] \quad (6)$$

Now the slope of $\log_e v(t)$ versus time is the growth rate of the total biomass ($\mu + s$), which is the result of increases in the biomass per cell and increases in the number of cells. Both μ and s can be determined independently of $v(t)$ by examining the changes in cell numbers and cell size. However, μ and s will be underestimated when based on direct counts of bacterial abundance because of the presence of nondividing or nongrowing cells. This is discussed in more detail below.

(iii) Changes in specific activity. A growth rate, μ or ($s + \mu$), can be calculated even if the specific activity of the precursors is unknown but constant. This implies that the conversion factor C is constant over the course of the experiment, which is supported when $\log_e v(t)$ versus time is a straight line. Bacterial production is not a linear function of uptake when the value of C varies during the course of the experiment.

The specific activity of nucleic acid precursors is probably the parameter that will change most rapidly since the specific activity depends on pools of low-molecular-weight compounds which would fluctuate more rapidly than macromolecules. Equation 1 can be modified to include a changing specific activity by separating the constant portions of C into C_a. By definition,

$$v_r(t) = C_a N(0)\, e^{\mu t} \quad (7)$$

where $v_r(t)$ is the true uptake rate corrected for dilution by unlabeled substrate. Note that

$$v_r(t) = \frac{S(t) + S^*(t)}{S^*(t)}\, v_a(t) \quad (8)$$

where $v_a(t)$ is the incorporation rate of labeled substrate. The symbols $S^*(t)$ and $S(t)$ are, respectively, the labeled and unlabeled substrate concentrations of the intracellular precursor pool. In nucleic acid synthesis $S(t)$ is the intracellular pool size of ATP or dTTP. After a few algebraic manipulations, equation 8 would appear similar to the presentation used by Moriarty and Pollard (20).

The effect of a changing specific activity on the change in [^{3}H]thymidine incorporation rates over time can be evaluated by substituting equation 8 into 7:

$$v_a(t) = \frac{S^*(t)}{S(t) + S^*(t)}\, C_a\, \mu N(0)\, e^{\mu t} \quad (9)$$

In general, a log transformation of equation 9 does not yield a straight line. The relationship between the slope of a $\log_e v_a(t)$-versus-time plot and the specific activity can be examined by looking at the derivative of the log transformation of equation 9:

$$\frac{d}{dt} \log_e [v_a(t)] = \frac{S^{*\prime}(t)S(t) - S'(t)S^*(t)}{S^*(t)[S(t) + S^*(t)]} + \mu \quad (10)$$

where $S'(t) = dS(t)/dt$ and $S'^*(t) = dS^*(t)/dt$. Equation 10 can be used to explain the possible effect of a

varying specific activity on incorporation rates.

(iv) Increase in bacterial abundance. Unicellular organisms that multiply by cell division undergo "exponential growth." A semilog plot of cell numbers versus time is a straight line and the slope is the specific growth rate. In natural environments, different populations of bacteria may be growing at different growth rates. A semilog plot of cell numbers versus time would be a straight line only if all growth rates were equal. In the simplest case we consider only two populations with different growth rates, one actively dividing and another "dormant" or not dividing during the experiment:

$$N(t) = N_a(0)e^{\mu t} + D \quad (11)$$

where $N_a(0)$ and D are the initial numbers of dividing and nondividing cells, respectively. A "dividing cell" is defined here as a cell, not necessarily "paired" (8), that will divide on the average by the measured doubling time. The $\log_e$ transformation of equation 11 will not give a straight line.

The presence of nondividing cells (D) "dilutes" the calculated growth rate of the relatively fast-growing cells. This bends the semilog plot of total bacterial abundance versus time to a concave curve as illustrated by Silvert (26). That report discussed the use of a particle counter to measure phytoplankton growth in the presence of detritus. We estimated values of μ and D with some of our data by minimizing the deviations of the calculated curve from the data with nonlinear regression techniques. Silvert (26) and Sheldon (24) discussed the problems with fitting data to equation 11.

(v) Calculating bacterial production. Bacterial production (in units of cells per liter per hour) was estimated from [^{3}H]thymidine incorporation in undiluted samples by using conversion factors calculated from equation 5: production = (nanomoles of [^{3}H]thymidine in trichloroacetic acid-insoluble extracts) × (conversion factor)/(incubation time). In addition, production was calculated from growth rates measured by the change in bacterial abundance and incorporation rates of [^{3}H]thymidine. These growth rates were multiplied by the bacterial abundance at the beginning of the experiment in the undiluted sample. This is justified by noting that production (dN/dt) is related to cell numbers $N(0)$ and the growth rate by equation 2.

(vi) Statistical analysis. The data were analyzed by standard linear regression techniques (27) except when the data were fit to equation 11. In these cases, values for μ, D, and $N_a(0)$ were determined by finding the least-squares curve with untransformed data. Replicate growth bottles were analyzed separately since the measurements of bacterial abundance and incorporation rates were taken independently. The rates calculated from slopes of the linear regression lines were compared with the F-test (27).

RESULTS

Growth rates from increases in incorporation rates and bacterial abundance. We measured the change in bacterial abundance and incorporation rates of [^{3}H]thymidine in samples from the Great Sippewissett Salt Marsh, Ice House Pond (a small freshwater pond near Woods Hole, Mass.), and the New York Bight. Incorporation rates of exogenous [^{3}H]thymidine increased exponentially over time and were coupled with growth as predicted by equation 4 in these three environments (Fig. 1a to 3a). Bacterial abundance also increased exponentially with time (Fig. 1b to 3b). The amount of variation explained by the linear regression line (r^2) was usually >90% in all three environments (Table 1). All points were used in our regression analyses except samples taken at 48 h in the New York Bight (data not shown).

The accumulation of [^{3}H]thymidine (nanomoles of labeled compound per liter or disintegrations per minute per liter) in cell extracts was linear during the determination of the incorporation rates (data not shown). This was tested at the beginning of experiments in the New York Bight and Ice House Pond but not in Great Sippewissett Marsh.

Our analysis shows that the changes in bacterial abundance and incorporation rates are estimates of the growth rate of the bacterial population. Specific growth rates of approximately 0.17 h^{-1} were measured by both the change in bacterial abundance and incorporation rates in Great Sippewissett (Table 2). These two methods gave similar estimates of 0.06 and 0.11 h^{-1} for two samples from the New York Bight (Table 2). Growth rates calculated from changes in bacterial abundance and in incorporation rates were not significantly different (Table 2) ($P > 0.05$; F-test).

In Ice House Pond on 11 July, in one sample, the growth rate calculated from the change in bacterial abundance was lower than that calculated from the change in [^{3}H]thymidine incorporation (Table 2). It appears that the difference was due to an increase in the average cell size from 0.23 (standard error = 0.08) to 0.52 (standard error = 0.08) μm^3 over the 20 h of the experiment. The specific increase (s) of the average cell size was 0.041 h^{-1}. As predicted by equation 6, the sum (0.095 h^{-1}) of the specific growth in cell numbers (μ = 0.054) and average cell size (s = 0.041) was close to the increase in rates of incorporation (0.088). The effect of an increase in total bacterial biomass is discussed below.

Effect of nondividing cells. Most of the variation (>90%) in bacterial abundance over time can be explained by simple exponential growth as predicted by equation 2 (Table 1). The points are close to a straight line on semilog plots of abundance versus time in samples from Great Sippewissett (Fig. 1b, sample A), the New York Bight (Fig. 2b), and Ice House Pond (Fig. 3b). These data indicate that nearly all of the bacteria were actively dividing in these samples. The agreement between growth rates calculated from

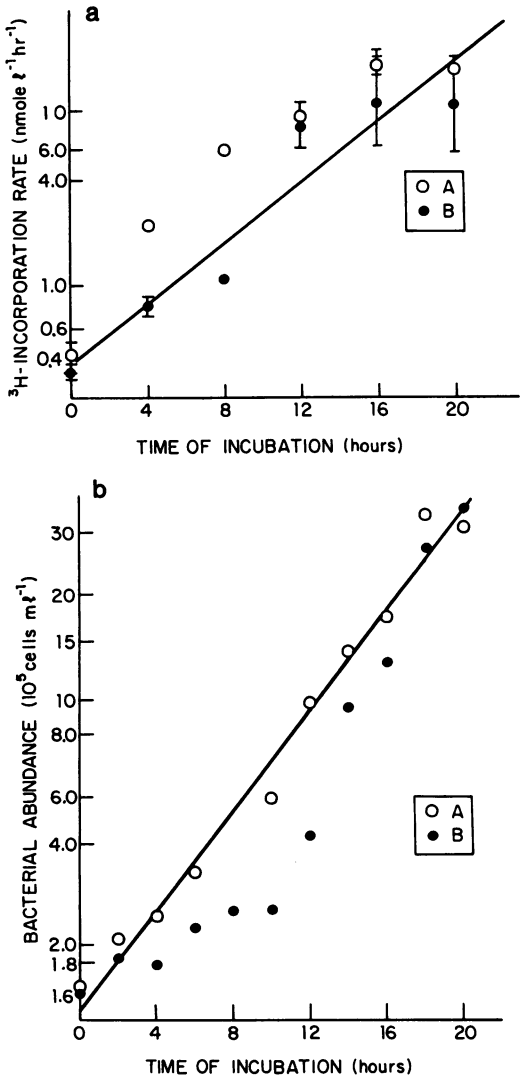

FIG. 1. Rates of [^{3}H]thymidine incorporation in samples from Great Sippewissett (a) and bacterial abundance in replicate samples A and B (b) ($\log_e$ scale). (a) Replicate growth bottles were prepared with an inoculum from the marsh. Each point is the mean ± standard deviation of three measurements on the incorporation rate for each growth bottle. Points without bars indicate that the standard deviation is smaller than the symbol. The line is from a linear regression based only on sample B for clarity. (b) The line is from a linear regression based only on sample A for clarity.

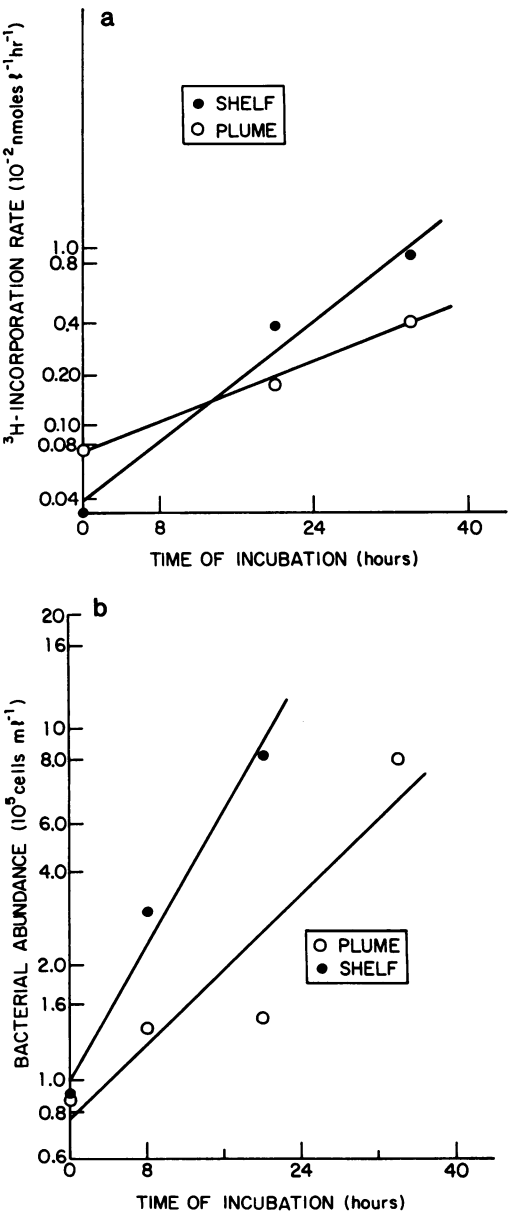

FIG. 2. Rates of [^{3}H]thymidine incorporation (a) and bacterial abundance (b) in samples from the New York Bight ($\log_e$ scale). Plume and shelf refer to the sources of the inoculum added to filtered plume water.

changes in bacterial abundance and in [^{3}H]thymidine incorporation rates supports this conclusion.

In one experiment there were clear deviations from linearity on the semilog plots of bacterial abundance versus time (Fig. 1b). This plot is concave for sample B from Great Sippewissett, which suggests the presence of nondividing cells (Fig. 1b). According to equation 11, approximately 62% of the cells were dividing in this sample. The usual interpretation of graphs such as Fig. 1b is that the population went through a lag phase or that growth was artificially enhanced by the manipulations of the sample. Both

TABLE 1. Summary of coefficients of determination (r^2) for semilog plots of bacterial abundance and incorporation rates of [^{3}H]thymidine versus time

Water sample[a]	Abundance	N[b]	Incorporation rate	N
Great Sippewissett Marsh				
A	0.98	10	0.86	6
B	0.88	10	0.91	6
Bay				
A	0.90	10	0.91	6
B	0.90	10	0.55	6
New York Bight				
Plume	0.86	3	0.92	3
Shelf	0.97	3	0.97	3
Ice House Pond				
11 July				
A	0.99	4	0.96	4
B	0.98	4	0.86	4
23 July				
A	0.98	4		
B	0.97	4		
C[c]	0.93	4		

[a] Samples A and B in Great Sippewissett and Ice House Pond were replicate growth bottles. Marsh, bay, shelf, and plume refer to the sources of the inoculum.

[b] N is the number of subsamples taken over the time course of the experiment.

[c] Unlike the other samples, this value is from undisturbed water samples.

explanations imply that cells divided slowly at first, but that growth rates increased during the incubation period. These alternative interpretations can be ruled out by comparing the semilog plots of abundance and incorporation rates versus time. In sample B from Great Sippewissett, the semilog plot of bacterial abundance is concave (Fig. 1b), whereas incorporation rates of [^{3}H]thymidine increased exponentially and the semilog plot is linear (Fig. 1a). This is not possible if growth rates changed during the incubation period. Our hypothesis seems to explain Fig. 1a and b; most of the bacteria grew at a constant rate, whereas a small number of cells grew slowly if at all during the incubation period.

Bacterial production. A "conversion factor" is needed to calculate bacterial production (cells per liter per hour) from [^{3}H]thymidine incorporation (nanomoles or disintegrations per minute per liter per hour). This factor is the product of several biochemical parameters which probably vary with the aquatic environment and bacterial community. Equation 5 was used to estimate this factor. Table 3 summarizes the conversion factors calculated from the regression analysis of data in Table 1 and Fig. 1 to 3. The conversion factors varied from sample to sample, but the order of magnitude was 10^9 cells per nmol of [^{3}H]thymidine incorporated into the trichloroacetic acid-insoluble fraction.

With these conversion factors bacterial production in the three environments was estimated from rates of [^{3}H]thymidine incorporation in unaltered water samples. Two more production

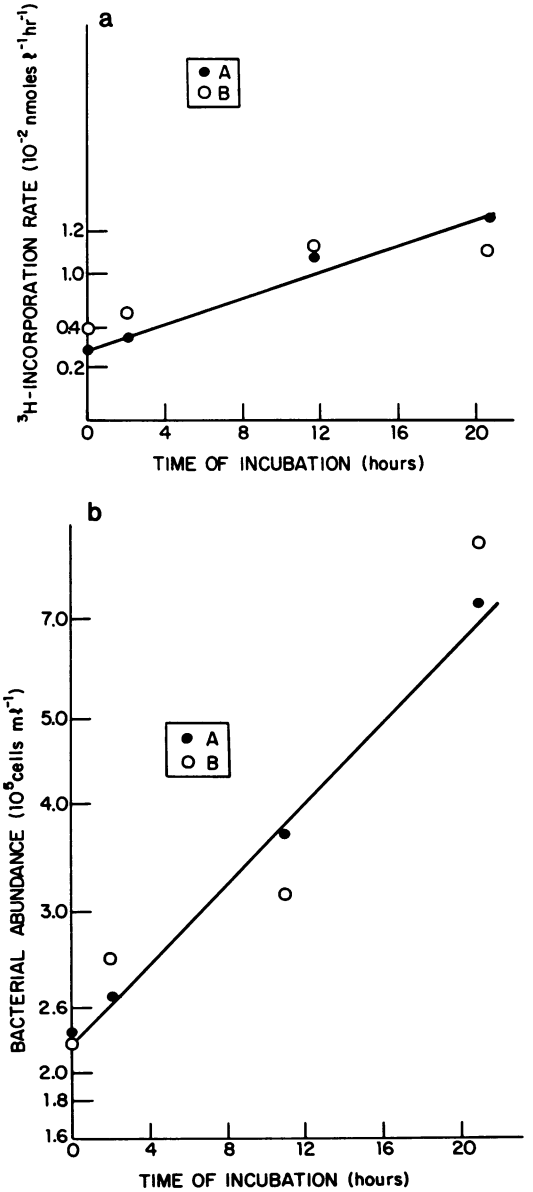

FIG. 3. Rates of [^{3}H]thymidine incorporation (a) and bacterial abundance (b) in replicate growth bottles from Ice House Pond on 11 July ($\log_e$ scale).

TABLE 2. Summary of growth rates measured by the change in [^{3}H]thymidine incorporation rates and bacterial abundance

Water sample	Temp (°C)	Growth rate (h^{-1})	
		Abundance	Incorporation rate
Great Sippewissett Marsh			
A	28	0.16	0.17
B		0.16	0.20
Bay			
A		0.17	0.18
B		0.16	0.13
New York Bight			
Plume	4	0.060	0.060
Shelf		0.11	0.12
Ice House Pond			
11 July			
A	25	0.054	0.088
B		0.054	0.057
23 July			
A	25	0.060	
B		0.075	
C[a]		0.022	
13 Aug.			
A[b]	25	0.037	0.51 (0.29)[c]
B[b]		0.041	0.35 (0.27)[c]
C[a]		0.011	0.051

[a] Unlike the other experiments, these growth rates were measured in unaltered water samples.

[b] See text for description of how these growth rates were calculated.

[c] Growth rates based on the change in [^{3}H]adenine incorporation are given in parentheses.

estimates were made based on growth rates calculated from the change in rates of [^{3}H]thymidine incorporation and bacterial abundance in our growth bottles. The growth rates were multiplied by the bacterial biomass [production = $\mu N(0)$] in the unaltered water sample.

Bacterial production estimated by these different approaches generally were within an order of magnitude of each other (Table 4). The production estimates based on the conversion factors applied to unaltered water samples were lower than estimates based on $\mu N(0)$ (where μ was measured in the growth bottle) in the New York Bight and in Great Sippewissett, but higher in Ice House Pond. There was no large difference between production estimates based on growth rates calculated from changes in [^{3}H]thymidine incorporation and bacterial abundance, which reflects the relatively small differences in these growth rate estimates.

Possible changes in specific activity. The slopes of $\log_e$ (rates of [^{3}H]thymidine incorporation) versus time were similar to slopes of $\log_e$ (bacterial abundance) versus time in Great Sippewissett Marsh, the New York Bight, and Ice House Pond (Table 2). This suggests that C was constant during the incubation period because incorporation of exogenous [^{3}H]thymidine was coupled with growth. In particular, the specific activity of the [^{3}H]thymidine in these experiments appeared to change slowly if at all relative to the growth rate. With one exception, equation 4 appears to describe all measurable changes in rates of [^{3}H]thymidine incorporation and accounted for most (90%) of the variation (Table 1). The single exception was an experiment in August with samples from Ice House Pond (described below).

There is some evidence of changes in specific activity in two experiments. First, the plot of $\log_e$ (incorporation rate) versus time is convex for sample A from Great Sippewissett Marsh (Fig. 1a). Equation 11 can be used to evaluate the magnitude of a possible change in specific activity. The increase in bacterial abundance, a conservative estimate of μ, was 0.16 h^{-1}, whereas the slope of $\log_e$ (rates of [^{3}H]thymidine incorporation) versus time was 0.40 h^{-1} based on samples taken at 0 and 4 h, which is the time period when incorporation rates increased most

TABLE 3. Summary of conversion factors for calculating bacterial production from [^{3}H]thymidine incorporation rates

Water sample	Factor (10^9 cells $nmol^{-1}$)
Great Sippewissett Marsh	
A	3.3
B	8.9
Bay	
A	3.7
B	1.9
New York Bight	
Plume	17
Shelf	68
Ice House Pond	
11 July	
A	5.9
B	3.0
13 Aug.	
A[a]	290
B[a]	62
C[b]	0.54

[a] These samples do not fit the model. See Fig. 5 and text for further details.

[b] This estimate of the conversion factor is based on measurements from an unaltered sample.

TABLE 4. Estimates of bacterial production (10^7 cells liter^{-1} h^{-1}) in unaltered water samples calculated with conversion factors and from specific growth rates estimated from the change in bacterial abundance and [^{3}H]thymidine incorporation

Water sample	Conversion factor[a]	Abundance[b]	Incorporation[b]
Great Sippewissett Marsh			
A	14	52	55
B	37	52	65
Bay			
A	20	58	62
B	10	55	45
New York Bight			
Plume	6.5	10	10
Shelf	5.8	14	16
Ice House Pond			
11 July, A	33	8.4	13
	15	2.3	8.4
25 July			
A		9.0	
B		11	

[a] Bacterial production was estimated by multiplying the rate of [^{3}H]thymidine incorporation in unaltered water samples by the conversion factor, which is given in Table 3.

[b] Production was calculated by multiplying the specific growth rate by the number of bacteria in the unaltered water samples. The growth rates (given in Table 2) were determined from the change in bacterial abundance and [^{3}H]thymidine.

rapidly. Thus, during the first 4 h of this experiment an increasing specific activity may have contributed 60% {[(0.40 − 0.16) × 100]/0.40} of the increase in rates of [^{3}H]thymidine incorporation. However, if all of the data points are used, an increasing specific activity could have contributed only 6% of the increase in incorporation rates. In this analysis we have ignored other possible changes in C and the increase in average cell size which we noticed but did not quantify.

Equations 4 and 6 did not describe all changes in incorporation rates in two samples from Ice House Pond in August. This is the second experiment in which a change in specific activity must be considered. Rates of [^{3}H]thymidine and [^{3}H]adenine incorporation did not increase exponentially for the first 4 h, contrary to equation 4 (Fig. 4). The rate of [^{3}H]thymidine incorporation decreased over the first 4 h and then increased exponentially. In contrast, the rate of [^{3}H]adenine incorporation increased rapidly from time zero to the second hour before following the exponential model (equation 4) from the fourth hour on to the end of the experiment. Both [^{3}H]thymidine and [^{3}H]adenine incorporation were coupled with growth after 4 h. The slopes range from 0.27 to 0.51 h^{-1} (Table 2). When semilog plots of [^{3}H]thymidine incorporation rates were compared with [^{3}H]adenine incorporation rates, there was a significant difference between these slopes in sample A but not in B, based on points after 2 h ($P < 0.05$; F-test).

Isotope dilution of [^{3}H]thymidine and [^{3}H]adenine by unlabeled compounds is one possible reason for the uncoupling of incorporation of the labeled compounds from growth. Rates of [^{3}H]thymidine incorporation per cell were much lower in the growth bottles than in the unaltered samples at 1 and 5 nM [^{3}H]thymidine, although the rates were similar at 10 and 20 nM (Fig. 5a). Rates of [^{3}H]adenine incorporation per cell were much lower over the entire concentration range examined (Fig. 5b). Similarily, [^{3}H]glucose rates of uptake per cell were lower in the growth bottles than in the unaltered samples (data not shown). However, it seems just as likely that the uptake kinetics (k_s and V_{max}) rather than the substrate concentrations changed during preparation of the growth bottle.

Change in average cell volume. Incorporation of exogenous [^{3}H]thymidine appears to be coupled with the production of new cells as hypothesized in equation 4 (Fig. 1 to 3a; Table 1). Cell volumes were measured in the August experiment on Ice House Pond to test whether incorporation rates also increased with the average cell size.

Incorporation of exogenous [^{3}H]thymidine and [^{3}H]adenine appears to be coupled to total production of bacterial biomass [$N(t)B(t)$] and not just the production of new cells. Biomass increased exponentially (Fig. 6a) at a rate of 0.21 h^{-1}. This rate, based on the interval of 6 to 14 h, was close to the increase of 0.27 to 0.51 h^{-1} in incorporation rates measured over the same time period. The rate of new cell production was 0.072 h^{-1} (Fig. 4c), which does not account for the increase in incorporation rates. The sum of the specific growth rates of the average cell volume (s = 0.15 h^{-1}) and cell numbers (μ = 0.072 h^{-1}) was the same as growth in total biomass (0.21 h^{-1}), as predicted by equation 6. Similar results were found in an 11 July sample from Ice House Pond as mentioned before.

The increase in biomass ($\mu + s$) was still smaller than the change in [^{3}H]thymidine and [^{3}H]adenine incorporation during this time period of the experiment. The concave plot of bacterial abundance versus time suggests that nondividing cells were present. When equation 11 was applied to the data, approximately 40 and 45% of the cells were dividing in replicate

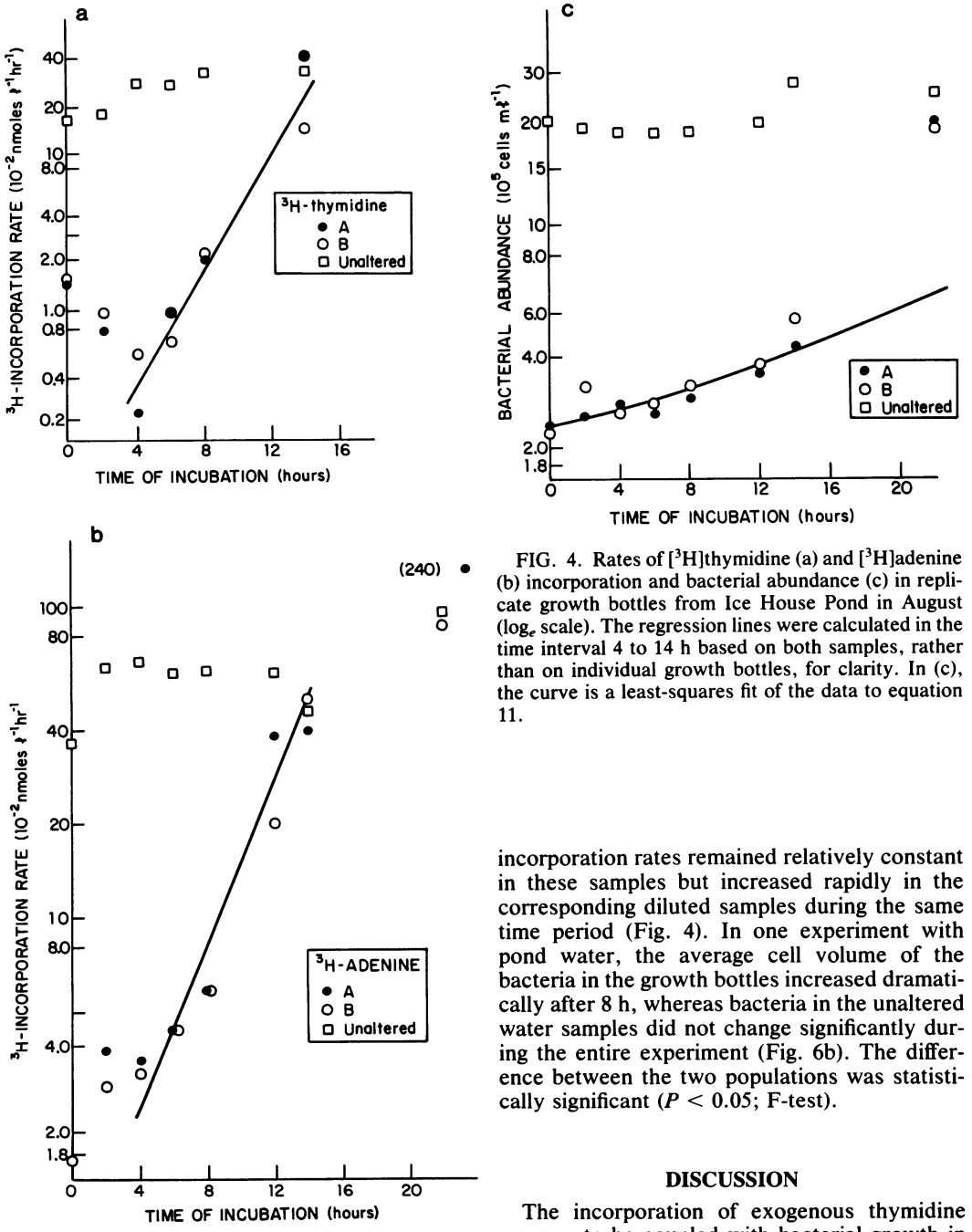

FIG. 4. Rates of [^{3}H]thymidine (a) and [^{3}H]adenine (b) incorporation and bacterial abundance (c) in replicate growth bottles from Ice House Pond in August ($\log_e$ scale). The regression lines were calculated in the time interval 4 to 14 h based on both samples, rather than on individual growth bottles, for clarity. In (c), the curve is a least-squares fit of the data to equation 11.

growth bottles A and B, respectively (Fig. 4c). Although μ was calculated with nondividing cells taken into account, s is still underestimated because of this factor.

There was no net bacterial growth in the unaltered samples. Bacterial abundance and incorporation rates remained relatively constant in these samples but increased rapidly in the corresponding diluted samples during the same time period (Fig. 4). In one experiment with pond water, the average cell volume of the bacteria in the growth bottles increased dramatically after 8 h, whereas bacteria in the unaltered water samples did not change significantly during the entire experiment (Fig. 6b). The difference between the two populations was statistically significant ($P < 0.05$; F-test).

DISCUSSION

The incorporation of exogenous thymidine appears to be coupled with bacterial growth in nearly all of the water samples we examined. This supports the correlations between thymidine incorporation and the increase in bacterial abundance observed by Fuhrman and Azam (6, 7). Our data suggest that thymidine incorporation is linked with cell growth (increase in cell volume) as well as cell division (increase in the number of cells). This coupling enables us to estimate the conversion factor necessary for

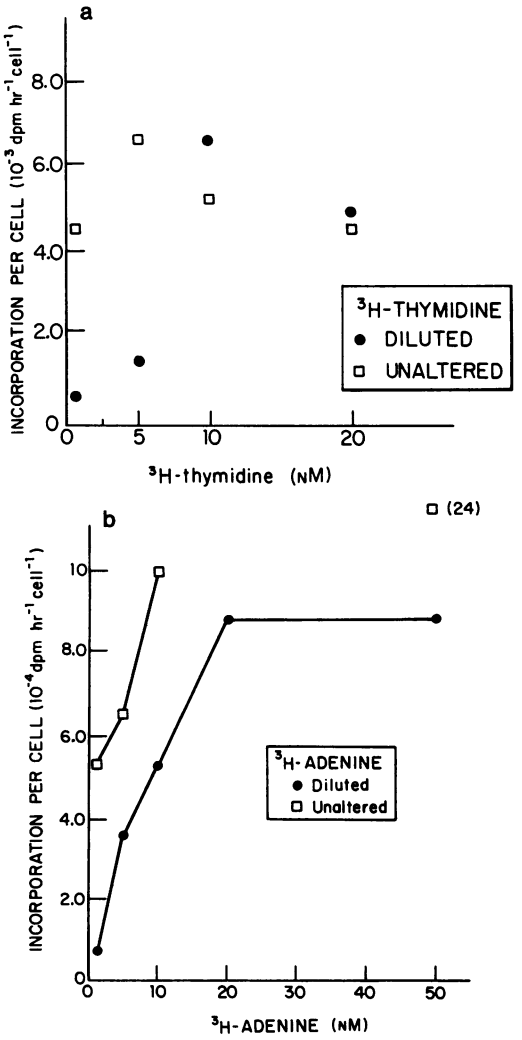

FIG. 5. Rates of [^{3}H]thymidine (a) and [^{3}H]adenine (b) incorporation per cell. The water in the "diluted" sample was prepared by the same method used to prepare the growth bottles in Fig. 4. This experiment was at time zero of the experiment in Fig. 4.

calculating bacterial production from rates of [^{3}H]thymidine incorporation. With the exception of two samples from the New York Bight, the conversion factor we measured was of the same order of magnitude as estimated by Fuhrman and Azam (6, 7), who used an entirely different approach. The values from the New York Bight may be high because 5 nM [^{3}H]thymidine did not saturate uptake (H. W. Ducklow and D. L. Kirchman, submitted for publication). Although the units used here were cells per nanomole, the factor can be expressed in micrograms of C per nanomole if the size of the bacteria is known. These units may be more

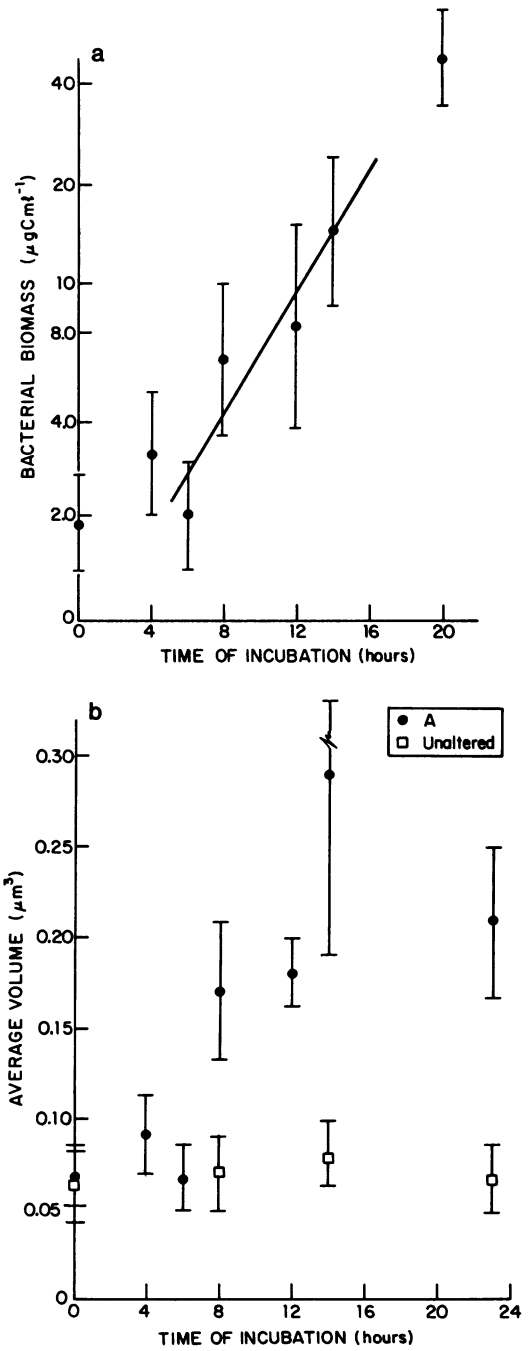

FIG. 6. Total bacterial biomass in growth bottle A (a) and bacterial cell volume in sample A (b) from Ice House Pond in August ($\log_e$ scale). (a) The total biomass was calculated from the number of bacteria (Fig. 4c) and the average cell volume (b). See text for description of regression line. Bacterial abundance and incorporation rates in growth bottle A and the unaltered sample are presented in Fig. 4. The mean and standard error of at least 20 measurements are given.

useful for evaluating the importance of bacterial production as well as for accounting for the variation in the conversion factor among water samples.

Conversion factors are used frequently in biological oceanography and limnology. Two familiar examples include estimating total living carbon by multiplying particulate ATP concentrations by 250 (10) and converting chlorophyll *a* measurements to phytoplankton carbon with a factor of 50 (3, 10). However, the conclusions based on this approach rest on the assumption that the factor is valid for the water mass being studied. As Fuhrman and Azam (6, 7) pointed out, the factor for converting [^{3}H]thymidine incorporation to bacterial production probably varies with the water body and bacterial community. Our approach allows one to calibrate this factor at least daily. At the expense of information about several biochemical parameters, we gain a simple approach for interpreting measurements of bacterial activity in natural environments.

Some water samples may not yield a conversion factor with our experimental design since incorporation of exogenous [^{3}H]thymidine may not always be coupled with growth. However, these exceptions are clearly identified. In one experiment [^{3}H]thymidine incorporation rates decreased before increasing exponentially. By examining the semilog plots of rates versus time, it was clear that this was an unusual case. The conversion factor also was much higher than the others (Table 3, Ice House Pond in August). Thus, it appears unlikely that conversion factors would be calculated erroneously with data to which equation 4 or 6 should not be applied. Furthermore, it is possible to assess the uncertainty of our approach by performing straightforward experiments to evaluate the effect of the sample manipulations on the bacterial populations. These experiments will be pointed out later.

It is difficult to determine the ecological significance of the growth rates calculated by our approach. The changes in bacterial abundance and incorporation rates were much smaller in unaltered water samples than in samples in which the particles were diluted 10-fold. This treatment appears to remove the limiting factors which usually maintain bacterial abundance in steady state. There are at least two explanations for these observations: (i) competition for limiting nutrients is lessened or eliminated when the bacterial density is diluted 10-fold, and (ii) predators cannot graze effectively when bacterial densities are decreased below a critical threshold (4, 5). These limiting factors would also determine the average cell size of natural bacteria, since nutrient limitation (22) and predation (4, 5) would select against large cells. The interpretation of the parameters (μ, s, and C) derived from these measurements is likely to differ in the two cases. Fuhrman and Azam (6) argued that bacteria increased in abundance in their experiments because bacteriovores were eliminated by filtering the water through filters with pore sizes of 3.0 μm. They did not mention any difference in changes in bacterial abundance between samples which were filtered to remove bacteriovores and samples in which a filtered inoculum was added to filter-sterilized water which diluted the particulate fraction 10-fold; we would not expect competition among cells for limiting nutrients to be lessened in 3.0-μm filtrates. We hypothesize that estimates of μ, s, and C measured in the altered water samples are valid if predation proves to be the dominant limiting factor of natural bacterial populations.

In spite of these difficulties, the data analysis and experimental design used here to examine the incorporation of thymidine, adenine, and other compounds merit further investigation. Although the factors limiting natural bacteria may remain unelucidated, the effect of sample manipulations on the bacterial community can be evaluated by comparing incorporation kinetics in altered and unaltered water samples even if the in situ concentrations or intracellular specific activities cannot be measured (20, 31) (Fig. 5). The approach we suggest here could be a powerful tool for examining the coupling of bacterial growth with the uptake of specific compounds used by bacteria for energy production or macromolecular synthesis (1, 12). Furthermore, this approach may be used to estimate the growth rate of bacteria utilizing specific compounds in aquatic systems.

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