APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1988, p. 2018–2026
0099-2240/88/082018-09$02.00/0

Vol. 54, No. 8

Experimental Evaluation of Conversion Factors for the [^{3}H]Thymidine Incorporation Assay of Bacterial Secondary Productivity†

MICHAEL F. COVENEY‡* AND ROBERT G. WETZEL§

Michigan State University, W. K. Kellogg Biological Station, Hickory Corners, Michigan 49060

Received 4 February 1988/Accepted 5 May 1988

The relationship between bacterial growth and incorporation of [*methyl*-^{3}H]thymidine in oligotrophic lake water cultures was investigated. Prescreening, dilution, and addition of organic and inorganic nutrients were treatments used to prevent bacterivory and stimulate bacterial growth. Growth in unmanipulated samples was estimated through separate measurements of grazing losses. Both bacterial number and biovolume growth responses were measured, and incorporation of [^{3}H]thymidine in both total macromolecules and nucleic acids was assayed. The treatments had significant effects on conversion factors used to relate thymidine incorporation to bacterial growth. Cell number-based factors ranged from 1.1×10^{18} to 38×10^{18} cells mol of total thymidine incorporation^{-1} and varied with treatment up to 10-fold for the same initial bacterial assemblage. In contrast, cell biovolume-based conversion factors were similar for two treatment groups across a 16-fold range of [^{3}H]thymidine incorporation rates: 5.54×10^{17} μm^3 mol of total thymidine incorporation^{-1} and 15.2×10^{17} μm^3 mol of nucleic acid incorporation^{-1}. Much of the variation in cell number-based conversion factors was related to changes in apparent mean cell volume of produced bacteria. Phosphorus addition stimulated [^{3}H]thymidine incorporation more than it increased bacterial growth, which resulted in low conversion factors.

Bacterial productivity is commonly assayed indirectly by uptake rates of specific labeled compounds. Measurements have primarily involved the uptake of $^{14}CO_2$ (29), $^{35}SO_4^{2-}$ (20), labeled nucleosides and nucleic acid bases (see, e.g., references 4, 26, and 51), and labeled protein precursors (22, 43). Both CO_2 and SO_4^{2-} uptake measurements in the dark suffer from serious interference by contaminating algae and require size fractionation to isolate the bacterial uptake component. Incorporation of [^{3}H]adenine into nucleic acids is similarly nonspecific. Incorporation of [*methyl*-^{3}H]thymidine ([^{3}H]TdR) into DNA is specific for heterotrophic bacteria with the assay protocols usually followed (3, 10, 30, 34). This specificity and the relative ease of the assay have resulted in the widespread use of [^{3}H]TdR incorporation to measure the synthesis of bacterial DNA, bacterial cells, and cell biomass carbon.

A serious shortcoming in the application of the [^{3}H]TdR incorporation assay is uncertainty regarding the conversion factor to relate measured [^{3}H]TdR incorporation to estimated production of bacterial cells (45). Two approaches are followed to establish this factor. In the first approach various subfactors describing the [^{3}H]TdR uptake and incorporation process are determined and combined (D. J. W. Moriarty, Ergeb. Limnol., in press). These include extra- and intracellular dilution of [^{3}H]TdR, [^{3}H]TdR moles percent in bacterial DNA, and DNA content per bacterial cell (1, 10, 11). In the second, empirical approach a conversion factor is obtained by relating [^{3}H]TdR incorporation directly to measured cell production in a specific sample (see, e.g., references 21 and 32). With either method, bacterial cell volume and carbon content must be considered if productivity is to be expressed in biomass carbon units.

Kirchman et al. (21) presented a detailed methodology for derivation of empirical conversion factors from exponential growth curves. In its basic form, their calculation procedure requires that all cells be active and that both cell number and [^{3}H]TdR incorporation increase with similar exponential trajectories. These conditions are not always met in practice, and an alternate conversion factor calculation used here is simply the ratio of cell production to integrated [^{3}H]TdR incorporation during an incubation period (11, 32).

Use of an empirical conversion factor eliminates the need to quantify each step in [^{3}H]TdR incorporation, and an empirical factor is the only way to compensate for bacteria that show little or no incorporation of exogenous [^{3}H]TdR because of uptake or biosynthetic limitations (14, 30, 37). However, determination of an empirical factor requires that bacterial growth or removal processes be perturbed to eliminate or reduce grazing losses in natural communities. These pretreatments include (i) screening (pore size, 1 to 3 μm) to remove bacterivores (11, 31), (ii) dilution of the original sample into filtered sample water to reduce density-dependent grazing and relieve nutrient competition (7, 21, 32), and (iii) organic nutrient addition (2, 27).

Clearly an empirical conversion factor is useful only inasmuch as it is constant or predictable in each assay situation. In fact, conversion factors determined for various systems and by different techniques vary over 10-fold (32, 39; Moriarty, in press). This variation has not yet been explained, and factors have been difficult to determine for oligotrophic systems (7). To better understand the causes of variability in conversion factors for the [^{3}H]TdR assay, we examined experimentally the effects of several pretreatments on the subsequent relationship between [^{3}H]TdR incorporation and measures of bacterial growth in oligotro-

* Corresponding author.

† Contribution no. 631 from the W. K. Kellogg Biological Station, Michigan State University.

‡ Present address: Water Resources Department, St. Johns River Water Management District, P.O. Box 1429, Palatka, FL 32078-1429.

§ Present address: Department of Biology and Great Lakes Research Division, The University of Michigan, Ann Arbor, MI 48109-1048.

TABLE 1. Summary of experimental treatments and grouping of treatments for regression analyses

Group	Treatment code	Description
C (whole water)	C	Control, whole water, no amendments
	C/5	Whole water plus 5× dilution
	CO	Whole water plus organic substrates
F (prescreened)	F	Prescreened
	F/5	Prescreened plus 5× dilution
	FO	Prescreened plus organic substrates
P (P_i addition)	CP	Whole water plus P_i
	FP	Prescreened plus P_i
	FPO	Prescreened plus P_i and organic substrates
	FPOT	Prescreened plus P_i, organic substrates, and trace metals

phic lake water. Conversion factors were determined in parallel samples without pretreatment by separate estimation of loss of bacterial cells to grazers. We demonstrate that growth increments in bacterial biovolume, rather than cell number, correlate closely with [^{3}H]TdR incorporation and suggest that this fact contributes to the wide variation in reported conversion factors.

MATERIALS AND METHODS

Experimental design. Experiments were conducted on three occasions during August and September 1986 with integrated epilimnetic (depth, 0 to 4 m) water samples from Lawrence Lake, an oligotrophic, hard-water lake in Michigan (50). Water was returned to the laboratory in a 15-liter carboy and dispensed into flasks. Duplicate control flasks received no further manipulation. Treatments varied somewhat for the three experiments and included prescreening (pore size, 0.8 μm; Nuclepore Corp.) to remove bacterivores; fivefold dilution in filtered (pore size, 0.2 μm) lake water; and additions of an organic substrate mixture, P_i, or a trace-metal mixture (Table 1). Most treatment combinations were done in duplicate. Two additional flasks contained whole lake water plus penicillin G (100 mg liter^{-1}; Sigma Chemical Co.) to inhibit bacterial cell division. Experimental manipulations were completed and incubations were initiated within 2.4 to 3.8 h of lake sampling. Flasks were incubated for ca. 36 h at in situ temperatures in the dark and were subsampled every 6 h for determination of [^{3}H]TdR incorporation rates. Unless specified otherwise, only initial and final samples were removed for enumeration and sizing of bacterial cells.

Nutrient additions were made immediately when flasks were filled and again after 12 and 24 h. The mixed-organic addition consisted of five low-molecular substrates, all of which were utilized by oligotrophic bacterial strains in one study (19). Glutamic acid, glycine, serine, glycolic acid, and glucose were each added at 200 μg liter^{-1}. P_i (K_2HPO_4) was added at 5 μg of P_i liter^{-1} (161 nM). A trace-metal mixture was added at 15% of the concentrations used by Stanier et al. (46), except for Fe^{3+}, which was at 4% and was added as EDTA ferric sodium salt.

Bacterial cell numbers and biovolume. Samples were fixed with filtered (pore size, 0.8 μm) 25% glutaraldehyde to 5% and stored refrigerated. Cells were stained with acridine orange and counted on black 0.2-μm-pore-size Nuclepore filters (16). The best preparation quality was obtained when samples were filtered and then stained on the filters and when the filters were placed for 30 s on a dry cellulosic membrane filter to remove water in the pores before they were mounted in immersion oil. Cells in 15 random fields were counted in four size/morphotype categories for biovolume calculation. We determined the mean cell volume in each category by photographing 10 random fields (Kodak Tri-X, 400 ASA) and projecting negatives on a Science Accessories Corp. GP-7 digitizer work area to measure cell dimensions. The total magnification was about $\times 10^4$. Approximately 400 cells were counted and an additional 350 cells were measured for each preparation. Because changes in cell number during incubation usually were modest, three to five replicate preparations were counted from each preserved sample. The sizing procedure was checked by diameter measurements of fluorescent microsphere standards (diameter, 0.51 μm; Polysciences Inc.). Microspheres were photographed, and a series of negatives were chosen in which microsphere exposures were judged to bracket the usual exposure of bacterial cells. These negatives were projected, and sphere diameters were measured as above.

[^{3}H]TdR incorporation. We used a [^{3}H]TdR incorporation assay slightly modified from those of Fuhrman and Azam (11) and Riemann et al. (33). High-specific-activity [*methyl*-^{3}H]TdR (ca. 80 Ci/mmol) was lyophilized immediately before use and taken up in reagent-grade water. Flask subsamples received 10 nM [^{3}H]TdR and were incubated under experimental conditions for 60 min. Incubation was terminated with Formalin (to 1%). Samples were extracted on ice in 5% trichloroacetic acid for 10 min and filtered (pore size, 0.2 μm). Filters were rinsed four times with ice-cold 5% trichloroacetic acid and once with 50% ethanol. The filter rims were excised before radioassay, which greatly reduced blank values.

We assayed ^{3}H activity in both the total cold trichloroacetic acid-insoluble material and a hot-acid extract. In the extraction procedure, carrier protein and DNA (50 μg of bovine serum albumin, 50 μg of DNA) plus 6.0 ml of 0.5 M $HClO_4$ were added to each sample filter in a glass vial. After extraction for 45 min at 70°C, the vials were chilled on ice. The extract was decanted and centrifuged (20 min at 12,000 × *g* and 4°C), and ^{3}H in a sample was assayed by liquid scintillation counting. The extraction procedure measured ^{3}H in DNA and RNA (15) and provided a ^{3}H incorporation value free from the major contaminant, protein. We refer to this value as nucleic acid incorporation, which should represent incorporation primarily into DNA, because ^{3}H labeling of RNA is minimal during short incubations (26, 31, 35). To assay ^{3}H activity which remained on the filter for calculation of total incorporation, 1.0 ml of 1 M HCl was added to the vial and the material was hydrolyzed at 90°C for 5 h. Addition of 2.0 ml of ethyl acetate solubilized the filter for liquid scintillation counting.

Conversion factor calculations. For flasks which received prescreened water (pore size, 0.8 μm) (Table 1, group F and most of group P) grazing losses were assumed to be negligible, and measured bacterial production was used as gross production. For other flasks (Table 1, group C and treatment CP) the observed net bacterial production was corrected for grazing losses by the use of loss rates derived from penicillin treatments. Cell division was assumed to be prevented by penicillin, while grazing continued unabated. A negative specific growth rate (or specific grazing rate), g was calculated from initial (N) and final (N_t) cell densities as $(\ln N_t - \ln N)/T$, where T is the incubation time. The number of cells

removed by grazers in flasks without prescreening was calculated by integration over time of the product of the specific grazing rate, g, and instantaneous cell density [N_o exp(kt)], i.e.,

$$\int_0^T gN_0 \exp(kt)dt, \text{ or}$$

$$gN_0k^{-1}[\exp(kT) - 1], \quad (1)$$

where N_o is the initial cell density in the flask, k is the net observed specific growth rate in the flask, and g and T are as above. These calculations were done for each morphotype/size category of bacterial cell and summed across categories to give the total number and total biomass of cells removed by grazers. Grazing losses were added to the net observed change in the flasks without prescreening to give the gross bacterial production.

Integrated [^{3}H]TdR incorporation for the 36-h incubation period was calculated by rectangular integration over time of values measured at 6-h intervals. For each experimental flask we obtained two measures of gross bacterial production (increments in cell number and in cell biovolume) and two measures of integrated [^{3}H]TdR incorporation (total cold trichloroacetic acid insoluble and nucleic acid) over the incubation period. Four alternate conversion factors were calculated as the ratio of each gross bacterial production measure to each integrated [^{3}H]TdR incorporation value.

Conversion factors were also evaluated by using data pooled across treatments and experiments by regression of each gross production measure on each integrated [^{3}H]TdR incorporation value (7, 11, 25). Measurement precision for gross bacterial production (cell number or biovolume) varied greatly for these datum points because of variable specific growth rates. We accounted for these differences in regression models by weighting each production value by the inverse of its variance (44), which was estimated from the mean coefficient of variation (14%) for 80 sets of replicate cell counts and propagation of error formulae (17). For biovolume production only the variance due to cell counts was considered, since it was the predominant component.

RESULTS

Measurements of the diameter of standard 0.51-μm fluorescent microspheres revealed a tendency to underestimate fluorescent-particle dimensions. When microspheres were photographed at an image density equivalent to that of most bacterial cells and measured, linear dimensions were underestimated by 5.9%. A multiplicative correction factor of 1.20 was therefore applied to cell biovolumes under an assumption of independent measurement errors. Additional measurements of microspheres photographed at twice and one-half this exposure resulted in 1 and 19% underestimation of diameters, respectively, but the images exceeded the range of exposures observed with cells. Both this variation in apparent image size with exposure and the necessary correction factor were similar to results with 0.21- to 0.70-μm-diameter microspheres and color photography (24).

Time courses for [^{3}H]TdR incorporation rates were qualitatively similar in all experiments. Treatments other than P_i addition showed constant or slowly increasing rates over 36 h (Fig. 1). In sharp contrast, P_i addition resulted in greatly increased [^{3}H]TdR incorporation, which reached a maximum at ca. 24 h and subsequently declined. In each experiment prescreening lowered [^{3}H]TdR incorporation much

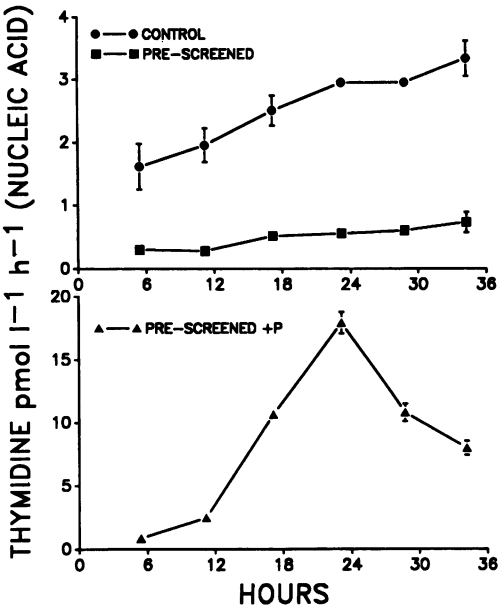

FIG. 1. Time course of [^{3}H]TdR incorporation rate into nucleic acid for three treatments in one experiment. The treatments are explained in Table 1. Symbols are mean values for duplicate treatment flasks, and error bars show ± 1 standard error if larger than symbol. Note the different scales on the y axes.

below that in control flasks (Fig. 1). This depression was not caused simply by loss of bacterial cells, because densities after prescreening were about 80% of whole-water values (range for cell number, 77 to 88%; range for cell biovolume, 70 to 81%).

Conversion factors relating bacterial production to [^{3}H]TdR incorporation for each of the three experiments are shown in Fig. 2 and 3. Only two of the four possible factors are given: cell production per mole of total [^{3}H]TdR incorporation, which is the form comparable to published factors, and biovolume production per mole of nucleic acid incorporation. Qualitative results were similar for the other two factors. Different treatments resulted in up to 10-fold variation in conversion factors for the same initial bacterial assemblage (Fig. 2 and 3). One-way analysis of variance after logarithmic transformation to equalize variance showed significant treatment effects on both types of conversion factor in the first two experiments and on the biovolume-based factor in the third (Fig. 2 and 3). For three experiments, cell number-based conversion factors varied from 1.1 × 10^{18} to 38 × 10^{18} cells mol of total [^{3}H]TdR incorporation^{-1}. This range is similar to that of published values from a wide variety of habitats (39; Moriarty, in press).

With cell number-based factors (Fig. 2) there was a clear tendency for prescreening (F, FO, and F/5 [defined in Table 1]) to result in high conversion factors, whereas whole-water treatment (C, CO, and C/5) factors were intermediate. P_i addition (CP, FP, FPO, and FPOT) consistently resulted in the lowest number-based conversion factors, whether with whole or prescreened water and with or without other amendments (Fig. 2). Biovolume-based factors showed less consistent segregation of the prescreened and whole-water treatment groups, but P_i addition again resulted in the lowest conversion factors (Fig. 3). In most cases organic addition did not result in [^{3}H]TdR conversion factors which differed significantly from those in treatments without added organic

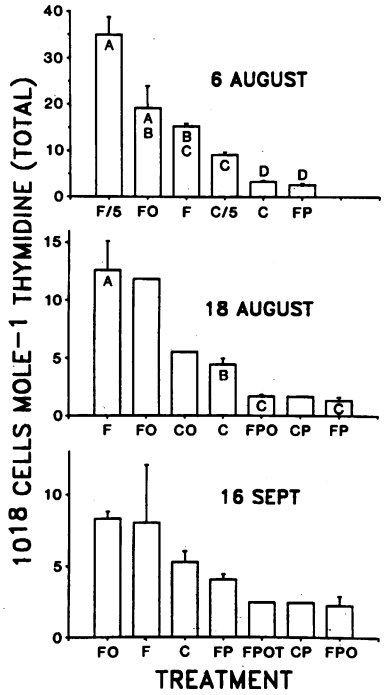

FIG. 2. Conversion factors relating increases in bacterial cell number to total [3H]TdR incorporation for three experiments with lake water batch cultures. Treatments are ordered by conversion factor, and treatment codes are defined in Table 1. Error bars (±1 standard error) identify treatments done in duplicate; other treatments were not replicated. For replicated values, codes in bars identify means which were not significantly different by one-way analysis of variance after logarithmic transformation. No treatment effects were significant for transformed means on 16 September.

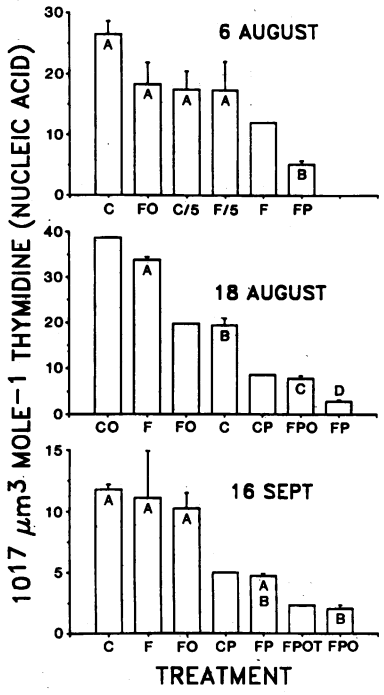

FIG. 3. Conversion factors relating increase in bacterial biovolume to [3H]TdR incorporation into nucleic acids for three experiments with lake water batch cultures. Treatments are ordered by conversion factor, and treatment codes are defined in Table 1. Error bars (±1 standard error) identify treatments done in duplicate; other treatments were not replicated. For replicated values, codes in bars identify means which were not significantly different by one-way analysis of variance after logarithmic transformation.

substrates. This similarity was true in every instance for cell number-based factors and in four of seven cases for biovolume-based factors. Dilution treatments were used only on 6 August; factors differed significantly from undiluted treatments for cell number but not for biovolume (Fig. 2 and 3).

Data were pooled for all experiments to establish mean conversion factors for the three groups of treatments distinguished above (C, F, and P [Table 1]) by using weighted regression of production measures on integrated [3H]TdR incorporation values (Fig. 4 and 5). If the conversion factor is a constant, these regressions should have zero y intercepts. In fact, all regressions for cell number production on both total and nucleic acid [3H]TdR incorporation had significantly nonzero y intercepts (all $P < 0.05$; most $P < 0.001$) whether regressions were calculated for treatment groups individually or for all points combined.

In contrast to cell production, weighted regressions of bacterial biovolume production on both measures of integrated [3H]TdR incorporation showed nonsignificant or only marginally significant y intercepts (Fig. 5; Table 2). For these cases, slopes were also fitted with regressions forced through the origin (Table 2). Slopes for forced regressions for two treatment groups (C and F) did not differ significantly, and these groups were pooled for composite slope estimates (Fig. 5; Table 2). The slopes provide biovolume-based conversion factors: 5.54×10^{17} μm^3 mol of total [3H]TdR incorporation^{-1} and 15.2×10^{17} μm^3 mol of nucleic acid incorporation^{-1}. A direct relationship between biovolume production and total [3H]TdR incorporation might be expected if incorporation into macromolecules other than DNA were large. However, the regression of biovolume production on nucleic acid incorporation was equally good (Table 2).

DISCUSSION

The failure of regressions of bacterial cell production on [3H]TdR incorporation (Fig. 4) to show zero y intercepts, both for individual treatment groups and for pooled data, is important. Under these conditions the regression slope alone does not provide a conversion factor. This fact has not always been recognized, and composite conversion factor estimates have been derived from the slope of a linear

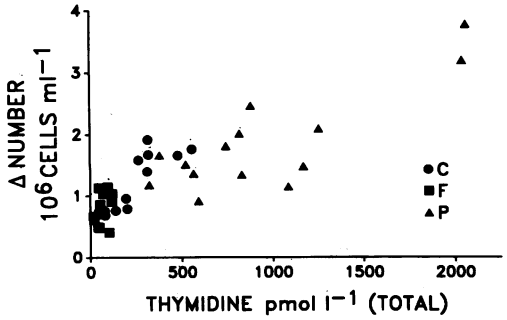

FIG. 4. Increase in bacterial cell number related to integrated total [3H]TdR incorporation for 38 lake water batch cultures. Symbols designate treatment groups, which are defined in Table 1.

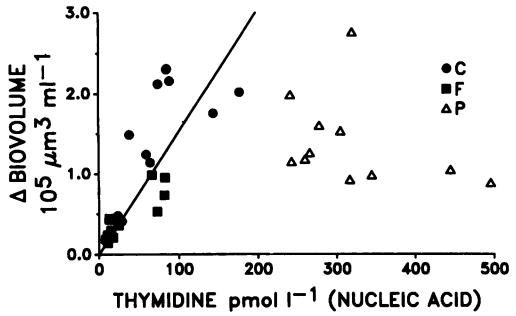

FIG. 5. Increase in bacterial biovolume related to integrated [^{3}H]TdR incorporation into nucleic acids for 38 lake water batch cultures. Symbols designate treatment groups, which are defined in Table 1. Three points in group P are off scale. The line is a weighted linear regression for pooled treatment groups C and F and is forced through the origin.

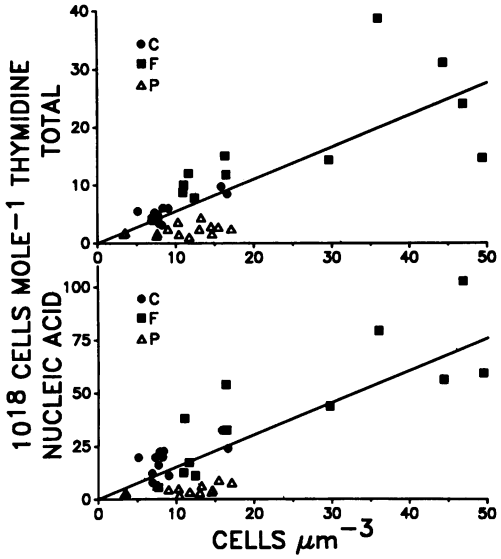

FIG. 6. Bacterial cell number-based conversion factors related to the apparent mean number of cells per cubic micrometer of biovolume produced (see Discussion). Conversion factors are ratios of cell production to total macromolecular [^{3}H]TdR incorporation (top) and to incorporation in nucleic acids (bottom). Symbols designate treatment groups, which are defined in Table 1. Lines are shown with slopes equal to biovolume-based conversion factors for pooled treatment groups C and F (Table 2) to illustrate equation 2.

regression with a nonzero intercept (7). One solution (11, 25) is to force the regression through the origin if the original y intercept is small. This treatment would not be appropriate for cell number-based regressions in the present data set, in which y intercepts are highly significant (Fig. 4).

Intuitively, bacterial cell number and biovolume production cannot both be constant multiples of [^{3}H]TdR incorporation unless the mean volume of produced cells is constant. To visualize the relationship between number-based and biovolume-based factors, we express biovolume production, V, as a linear function of [^{3}H]TdR incorporation, I, with a conversion factor C and a y intercept of zero: $V = CI$. This relationship is appropriate for pooled treatment groups C and F in our data set (Fig. 5; Table 2). Both sides of the equation are divided by cell number production, N, and the equation is solved for the cell number-based conversion factor, N/I,

$$N/I = C(N/V) \quad (2)$$

Thus, if biovolume production is directly related to [^{3}H]TdR incorporation, then the cell number-based conversion factor, N/I, is a linear function of the ratio of cell production to biovolume production, N/V (i.e., the inverse of the apparent mean volume of produced cells). The y intercept is zero, and the slope is the biovolume-based conversion factor C. The apparent mean volume of produced cells has a concrete interpretation if both growth and division of cells occur. If cells change in size without division, the interpretation is more abstract. Regardless, it is a useful and necessary convention to relate cell number-based and biovolume-based conversion factors.

Pooled data for the three experiments were recast in the form of equation 2 (Fig. 6). Clearly, the apparent mean cell volume of produced cells was a key variable in our experiments and explained the wide range in cell number-based conversion factors. This was a consequence of close association between [^{3}H]TdR incorporation and change in bacterial biovolume rather than cell number (Fig. 5) and was true whether total or nucleic acid [^{3}H]TdR incorporation was measured (Fig. 6).

Prescreening resulted in a low apparent mean cell volume of produced cells and correspondingly high number-based conversion factors (Fig. 6). The effects of treatments on bacterial growth in these experiments will be discussed elsewhere (M. F. Coveney and R. G. Wetzel, manuscript in preparation), but production of small cells can be an adap-

TABLE 2. Slope and y intercept values for weighted linear regressions of bacterial biovolume production on integrated [^{3}H]TdR incorporation

Independent variable	Group[a]	Fitted model[b]		Slope ± SE for forced model (10^{17} μm^3 mol^{-1})
		Slope (10^{17} μm^3 mol^{-1})	Intercept (10^5 μm^3 ml^{-1})	
Total incorporation	C	4.72***	0.076^{ns}	5.34 ± 0.32
	F	2.86*	0.103*	5.77 ± 0.76
	P	1.85*	−0.108^{ns}	1.73 ± 0.26
	C+F	4.33***	0.070*	5.54 ± 0.40
Nucleic acid incorporation	C	15.2*	0.053^{ns}	16.7 ± 1.5
	F	9.12*	0.072*	14.3 ± 1.3
	P	3.64*	0.043^{ns}	3.76 ± 0.55
	C+F	13.9***	0.024^{ns}	15.2 ± 1.01

[a] Treatment groups are identified in Table 1.
[b] Significance levels: ns, not significant; *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$. Significance levels are based on F tests.

tive response to low-nutrient conditions (36). Prescreening and confinement of seawater can improve nutrient conditions for bacterial populations (9). However, separation of components of the plankton by prescreening might also elicit nutrient stress, because particles larger than bacteria can be sources of both organic and inorganic nutrients. Phosphorus limitation was apparent in our experiments (see below), and addition of P_i after prescreening increased [^{3}H]TdR incorporation (Fig. 1) and prevented a reduction in cell size (Fig. 6). For the pooled C and F treatment groups, cell number-based conversion factors varied approximately 10-fold, and much of this variation was due to prescreening treatments. The similar range found in published number-based factors (39; Moriarty, in press) may be related to differences in cell size, either inherent among sites or artifactual through qualitative changes in bacterial assemblages after sample pretreatment.

The relationship between bacterial [^{3}H]TdR incorporation and biovolume production was not explicitly examined in earlier work. Other biovolume-based conversion factors are not available for comparison, nor can we evaluate the degree to which the apparent mean volume of produced cells covaries with published cell number-based factors (Fig. 6). Because the DNA content per bacterial cell is taken to be constant, albeit poorly known, conversion factors have been based universally on cell number (11, 26). The rate constant for [^{3}H]TdR incorporation more closely approximated that of bacterial biovolume production than of cell production in one case (21). In a similar treatment of bacterial growth and [^{3}H]TdR incorporation kinetics, Ducklow and Hill (7) concluded that changes in cell size did not explain the major discrepancies in rate constants which they observed. Riemann et al. (32) rejected cell size as an important variable in [^{3}H]TdR conversion factors. However, in neither of these cases was the relationship between incremental cell biovolume production and [^{3}H]TdR incorporation systematically examined. Comparative studies with a variety of natural bacterial assemblages is necessary to test the generality of our observations.

It is important to note that the apparent mean cell volume of produced cells could differ greatly from both initial and final mean cell volume in our experiments. Initial mean cell volume for prescreened and whole-water treatments ranged from 0.042 to 0.052 μm^3 (19 to 24 cells μm^{-3} [Fig. 6]), whereas the mean volume of produced cells varied from 0.020 to 0.301 μm^3 (3.3 to 50 cells μm^{-3} [Fig. 6]). Whether a cursory examination of the initial and final mean cell volume of assemblages (32) reveals the extent of these differences depends on the overall specific growth rate and incubation time. For three flasks which showed low growth rates in our experiments, the mean cell volume of produced cells averaged 51% of initial values, whereas mean cell volumes of final assemblages averaged 85% of initial values.

The fact that [^{3}H]TdR incorporation was more directly related to production of bacterial biovolume than to production of cells implies a positive relationship between cell size and DNA content. Both mean cell size and DNA content per cell increase with growth rate in bacterial cultures (18). The DNA content increases initially because an increasing portion of cells are undergoing replication. Eventually, multiple replication forks appear as the generation time becomes shorter than the DNA replication time (18). However, this change in DNA content is not a change in the haploid genome size; the amount of DNA synthesis which results in a replicated genome remains constant. The pertinent question is whether genome size varies with cell size over the greater-than-20-fold range in cell volumes found in our samples and in aquatic systems in general. Our results are consistent with this relationship, but there is presently no evidence.

Gross bacterial-cell or biovolume production in whole-water treatments (C, Table 1) was estimated by calculation of grazing losses from specific loss rates assayed in penicillin treatments (equation 1). Antibiotics are widely used to derive specific grazing rates in this fashion (39). Necessary assumptions in our approach to the calculation of grazing losses were that removal of cells by bacterivores could be described as a first-order process, that the antibiotic prevented bacterial division without inducing mortality, and that the number and grazing activity of bacterivores were not affected by antibiotic treatment. These assumptions can be supported, as follows.

Our specific loss rates were based on initial and final cell densities, rather than time courses, so that kinetics were not directly assessed. First-order relationships are commonly used to describe the grazing process (39, 40). Expression of bacterivory as water clearance rates for grazers (8) implies a first-order removal of prey. Both zero- and first-order kinetics have been used to describe bacterial growth (6). At the low net growth rates which we observed in whole-water samples, the difference was negligible and a first-order relationship (equation 1) was appropriate. Penicillin has been used successfully to inhibit bacterial division in grazing studies (12, 38 [for aerobic incubations]). Penicillin can cause bacterial lysis (5), but this was not found with marine assemblages (12). Effects of penicillin on ciliate bacterivory ranged from moderate inhibition to moderate stimulation (38); effects on rotifer and cladoceran bacterivores would not be expected.

Separate experiments done to evaluate penicillin effects on bacterial growth in samples from Lawrence Lake showed a potential for both under- and overestimation of grazing losses (M. F. Coveney, unpublished data). Cocci increased in number despite penicillin treatment, which would result in underestimation of grazing rates. Numbers of rods did not change significantly, although the mean length decreased somewhat, which would lead to an overestimate of biovolume loss. Grazing losses expressed as cell biovolume were probably more accurate than cell number estimates, since division of rods was inhibited by penicillin and rods constituted most (80 to 85%) of the biovolume (50 to 55% of cell numbers). On two occasions we compared specific loss rates for rods, based on penicillin treatments, with independent estimates obtained in dilution assays (23). Penicillin values were 71 and 78% of dilution assay estimates, which supports our contention that cell biovolume losses were correctly estimated.

The grazing correction amounted to 12 to 80% of gross biovolume production in whole-water samples, and this proportion was not correlated with biovolume-based conversion factors after angular transformation ($r = 0.12$ for total [^{3}H]TdR incorporation; $r = 0.13$ for nucleic acid incorporation). Thus, although the use of antibiotics to estimate grazing losses can be criticized, we find no evidence for serious bias in the resulting [^{3}H]TdR conversion factors. It is increasingly clear that undesirable side effects, low efficacy, or effects on nontarget organisms are common problems with the use of inhibitors in natural communities (38, 47, 48). Alternate techniques to estimate grazing losses, for example, through disappearance of fluorescently labeled bacteria (42), could be used in the approach developed here to measure [^{3}H]TdR conversion factors in enclosed but otherwise unmanipulated aquatic bacterial assemblages.

Addition of P_i to lake water cultures consistently stimulated [^{3}H]TdR incorporation out of proportion to stimulation in bacterial cell or biovolume production, which resulted in low [^{3}H]TdR conversion factors (Fig. 2 and 3). Kinetics of [^{3}H]TdR incorporation after P_i addition were consistent with a synchronization of cell cycles by P_i addition; i.e., [^{3}H]TdR incorporation in nucleic acid increased and then decreased as cells passed through DNA replication simultaneously (Fig. 1). A synchronization effect of periodic P_i addition has been shown in P-limited bacterial cultures (13). A single pulse of P_i initiated cell division after a period of P_i uptake in P-limited cultures of a marine *Vibrio* species (28). Epilimnetic phytoplankton was shown to be frequently and severely phosphorus limited in Lawrence Lake during summer stratification (49). Time courses of [^{3}H]TdR incorporation after P_i addition (Fig. 1) suggest that bacterioplankton was also phosphorus limited and responded to P_i addition in a synchronous fashion.

Even partially synchronized bacterial division could bias empirical [^{3}H]TdR conversion factors. For calculation techniques based on kinetics of [^{3}H]TdR incorporation (7, 21), phased DNA replication would result in [^{3}H]TdR time courses that are intractable or apparently uncoupled from cell production. With the simpler calculation model which we used (32), chance termination of an incubation immediately following phased replication could result in low apparent production of cells. Biovolume-based factors should be less affected, since the cell size increases before replication commences (18). We examined this possible artifact of cell cycle timing by comparing bacterial production with integrated [^{3}H]TdR incorporation during sequential 12-h segments after P_i addition to the flasks in the experiment shown in Fig. 1. Production remained well correlated with [^{3}H]TdR incorporation in nucleic acids over the shorter period (cell number, $r = 0.90$; biovolume, $r = 0.83$; $P < 0.05$). Synchronization of cell division by P_i addition may have occurred, but this was not the cause of lower [^{3}H]TdR conversion factors in P_i-treated flasks. A potential causal factor was decreased isotopic dilution of [^{3}H]TdR (below), if abrupt improvement in P status increased the utilization of exogenous [^{3}H]TdR compared with de novo synthesis of dTMP. This explanation is speculative at present, however. Until these differences are understood, bacterial productivity assays after experimental manipulation should be verified by direct methods.

We conclude that biovolume-based [^{3}H]TdR conversion factors derived from pooled whole-water and prescreened treatment groups (Table 2, C + F) are the most appropriate. A subset of these points was based on experiments with control flasks which were not manipulated other than by enclosure. In addition, biovolume conversion factors were consistent for whole-water and prescreened treatments, despite >16-fold differences in [^{3}H]TdR incorporation. This robust behavior suggests that the factors are valid. Use of the factor relating biovolume production to [^{3}H]TdR incorporation into nucleic acid (Table 2, 15.2×10^{17} μm^3 mol^{-1}) with in situ measurements of summer epilimnetic [^{3}H]TdR incorporation in Lawrence Lake resulted in bacterial-specific growth rates of 0.06 to 0.13 h^{-1}. These growth rates are similar to values obtained in other oligotrophic to mesotrophic systems (39; Table 5 in reference 6).

We pooled data across three experiments for regression analyses (Fig. 4 and 5; Table 2). The time spanned by our data set was too limited for us to identify seasonal variation which would simply increase residual variance in the regression calculations. One-way analysis of variance on biovolume factors (Fig. 3) after log transformation showed a significant difference among experiments for pooled means of groups C and F. Lovell and Konopka (25) found a constant cell number-based empirical factor over two summer stratification periods. Scavia and Laird (39) described distinct seasonal variation in empirical factors over 10 months. Interaction between mean cell size and a constant biovolume-based conversion factor could produce a seasonal change in cell number-based factors.

Dilution of added [^{3}H]TdR, by either exogenous or endogenous sources, increases empirical [^{3}H]TdR conversion factors (1, 11, 30). We did not assay [^{3}H]TdR dilution in our samples, but note that 10 nM addition was sufficient to saturate incorporation on two occasions (Coveney, unpublished data). A saturation response does not mean that dilution is negligible, but it indicates conditions under which variation in dilution is minimized (1). Variable isotopic dilution could have contributed to residual variation in our biovolume-based empirical factors. It has been suggested that [^{3}H]TdR conversion factors might increase with bacterial growth rate through greater isotopic dilution (7). We could not examine the relationship between measured growth rate and conversion factors in our experiments. Both variables were derived from endpoint cell counts and biovolumes, and errors were potentially large and highly correlated.

We assayed ^{3}H incorporated into both total macromolecules and nucleic acid. From 16 to 85% of integrated [^{3}H]TdR incorporation resided in nucleic acid for individual treatments. The composite biovolume-based conversion factors obtained through regression analysis (Table 2) were equivalent to 32 to 46% nucleic acid (ratio of each factor for total incorporation to equivalent factor for nucleic acid incorporation). This range is lower than values commonly reported (41) and lower than most values determined in situ in Lawrence Lake. There is no evident reason why the percent nucleic acid incorporation would be lower in experiments, but this fact underscores the importance of DNA purification in assay procedure.

Servais et al. (41) questioned the efficiency of acid extraction to isolate DNA in the [^{3}H]TdR assay and suggested an enzymatic procedure. We used a modified perchloric acid extraction method which was suggested for microbial cells and was found to give almost complete recovery of nucleic acids (15). The procedure involved milder hydrolysis conditions than those tested by Servais et al. (41) and may have avoided the protein hydrolysis which they observed. There is presently disagreement regarding the macromolecular distribution of ^{3}H in [^{3}H]TdR incorporation assay samples. The label is not expected to appear in RNA after a short incubation (26, 31). Robarts et al. (35) showed that apparent RNA labeling after base and acid extraction resided in other macromolecules, probably lipids. This contrasts with high RNA labeling reported when an enzymatic procedure was used (41). These discrepancies can be resolved only through specific biochemical separations.

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation (grant BSR 8517039) and the Department of Energy (grants DE-FG02-87ER60515 and COO-1599-312).

N. L. Consolatti assisted with bacterial enumeration and [^{3}H]TdR assays, and D. L. Kirchman commented on the manuscript.

LITERATURE CITED

1. **Bell, R. T.** 1986. Further verification of the isotope dilution approach for estimating the degree of participation of [^{3}H]

thymidine in DNA synthesis in studies of aquatic bacterial production. Appl. Environ. Microbiol. **52**:1212–1214.
2. **Bell, R. T., G. M. Ahlgren, and I. Ahlgren.** 1983. Estimating bacterioplankton production by measuring [^{3}H]thymidine incorporation in a eutrophic Swedish lake. Appl. Environ. Microbiol. **45**:1709–1721.
3. **Bern, L.** 1985. Autoradiographic studies of [*methyl*-^{3}H]thymidine incorporation in a cyanobacterium (*Microcystis wesenbergii*)-bacterium association and in selected algae and bacteria. Appl. Environ. Microbiol. **49**:232–233.
4. **Brock, T. D.** 1967. Bacterial growth rate in the sea: direct analysis by thymidine autoradiography. Science **155**:81–83.
5. **Brock, T. D.** 1970. Biology of microorganisms, p. 238. Prentice-Hall, Inc., Englewood Cliffs, N.J.
6. **Ducklow, H. W., and S. M. Hill.** 1985. The growth of heterotrophic bacteria in the surface waters of warm core rings. Limnol. Oceanogr. **30**:239–259.
7. **Ducklow, H. W., and S. M. Hill.** 1985. Tritiated thymidine incorporation and the growth of heterotrophic bacteria in warm core rings. Limnol. Oceanogr. **30**:260–272.
8. **Fenchel, T.** 1982. Ecology of heterotrophic microflagellates. II. Bioenergetics and growth. Mar. Ecol. Prog. Ser. **8**:225–231.
9. **Ferguson, R. L., E. N. Buckley, and A. V. Palumbo.** 1984. Response of marine bacterioplankton to differential filtration and confinement. Appl. Environ. Microbiol. **47**:49–55.
10. **Findlay, S. E. G., J. L. Meyer, and R. T. Edwards.** 1984. Measuring bacterial production via rate of incorporation of [^{3}H]thymidine into DNA. J. Microbiol. Methods **2**:57–72.
11. **Fuhrman, J. A., and F. Azam.** 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. (Berlin) **66**:109–120.
12. **Fuhrman, J. A., and G. B. McManus.** 1984. Do bacteria-sized marine eukaryotes consume significant bacterial production? Science **224**:1257–1260.
13. **Goodwin, B. C.** 1969. Synchronization of *Escherichia coli* in a chemostat by periodic phosphate feeding. Eur. J. Biochem. **10**: 511–514.
14. **Güde, H.** 1984. Test for validity of different radioisotope activity measurements by microbial pure and mixed cultures. Ergeb. Limnol. **19**:257–266.
15. **Herbert, D., P. J. Phipps, and R. E. Strange.** 1971. Chemical analysis of microbial cells, p. 210–344. *In* J. R. Norris and D. W. Ribbons (ed.), Methods in microbiology, vol. 5B. Academic Press, Inc., New York.
16. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225–1228.
17. **Horrocks, D. L.** 1974. Applications of liquid scintillation counting. Academic Press, Inc., New York.
18. **Ingraham, J. L., O. Maaloe, and F. C. Neidhardt.** 1983. Growth of the bacterial cell. Sinauer Associates, Inc., Sunderland, Mass.
19. **Ishida, Y., and H. Kadota.** 1981. Growth patterns and substrate requirements of naturally occurring obligate oligotrophs. Microb. Ecol. **7**:123–130.
20. **Jordan, M. J., and G. E. Likens.** 1980. Measurement of planktonic bacterial production in an oligotrophic lake. Limnol. Oceanogr. **25**:719–732.
21. **Kirchman, D., H. Ducklow, and R. Mitchell.** 1982. Estimates of bacterial growth from changes in uptake rates and biomass. Appl. Environ. Microbiol. **44**:1296–1307.
22. **Kirchman, D., E. K'nees, and R. Hodson.** 1985. Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. Appl. Environ. Microbiol. **49**:599–607.
23. **Landry, M. R., L. W. Haas, and V. L. Fagerness.** 1984. Dynamics of microbial plankton communities: experiments in Kaneohe Bay, Hawaii. Mar. Ecol. Prog. Ser. **16**:127–133.
24. **Lee, S., and J. A. Fuhrman.** 1987. Relationships between biovolume and biomass of naturally derived marine bacterioplankton. Appl. Environ. Microbiol. **53**:1298–1303.
25. **Lovell, C. R., and A. Konopka.** 1985. Seasonal bacterial production in a dimictic lake as measured by increases in cell numbers and thymidine incorporation. Appl. Environ. Microbiol. **49**:492–500.
26. **Moriarty, D. J. W.** 1986. Measurement of bacterial growth rates in aquatic systems using rates of nucleic acid synthesis. Adv. Microb. Ecol. **9**:245–292.
27. **Murray, R. E., and R. E. Hodson.** 1985. Annual cycle of bacterial secondary production in five aquatic habitats of the Okefenokee Swamp ecosystem. Appl. Environ. Microbiol. **49**: 650–655.
28. **Nissen, H., M. Heldal, and S. Norland.** 1987. Growth, elemental composition and formation of polyphosphate bodies in *Vibrio natriegens* cultures shifted from phosphate-limited to phosphate-pulsed media. Can. J. Microbiol. **33**:583–588.
29. **Overbeck, J.** 1979. Dark CO_2 uptake—biochemical background and its relevance to in situ bacterial production. Ergeb. Limnol. **12**:38–47.
30. **Pollard, P. C., and D. J. W. Moriarty.** 1984. Validity of the tritiated thymidine method for estimating bacterial growth rates: measurement of isotope dilution during DNA synthesis. Appl. Environ. Microbiol. **48**:1076–1083.
31. **Riemann, B.** 1984. Determining growth rates of natural assemblages of freshwater bacteria by means of ^{3}H-thymidine incorporation into DNA: comments on methodology. Ergeb. Limnol. **19**:67–80.
32. **Riemann, B., P. K. Bjørnsen, S. Newell, and R. Fallon.** 1987. Calculation of cell production of coastal marine bacteria based on measured incorporation of [^{3}H] thymidine. Limnol. Oceanogr. **32**:471–476.
33. **Riemann, B., J. Fuhrman, and F. Azam.** 1982. Bacterial secondary production in freshwater measured by ^{3}H-thymidine incorporation method. Microb. Ecol. **8**:101–114.
34. **Rivkin, R. B.** 1986. Incorporation of tritiated thymidine by eucaryotic microalgae. J. Phycol. **22**:193–198.
35. **Robarts, R. D., R. J. Wicks, and L. M. Sephton.** 1986. Spatial and temporal variations in bacterial macromolecule labeling with [*methyl*-^{3}H]thymidine in a hypertrophic lake. Appl. Environ. Microbiol. **52**:1368–1373.
36. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51**:365–379.
37. **Saito, H., H. Tomioka, and S. Ohkido.** 1985. Further studies on thymidine kinase: distribution pattern of the enzyme in bacteria. J. Gen. Microbiol. **131**:3091–3098.
38. **Sanders, R. W., and K. G. Porter.** 1986. Use of metabolic inhibitors to estimate protozooplankton grazing and bacterial production in a monomictic eutrophic lake with an anaerobic hypolimnion. Appl. Environ. Microbiol. **52**:101–107.
39. **Scavia, D., and G. A. Laird.** 1987. Bacterioplankton in Lake Michigan: dynamics, controls, and significance to carbon flux. Limnol. Oceanogr. **32**:1017–1033.
40. **Servais, P., G. Billen, and J. V. Rego.** 1985. Rate of bacterial mortality in aquatic environments. Appl. Environ. Microbiol. **49**:1448–1454.
41. **Servais, P., J. Martinez, G. Billen, and J. Vives-Rego.** 1987. Determining [^{3}H]thymidine incorporation into bacterioplankton DNA: improvement of the method by DNase treatment. Appl. Environ. Microbiol. **53**:1977–1979.
42. **Sherr, B. F., E. B. Sherr, and R. D. Fallon.** 1987. Use of monodispersed, fluorescently labeled bacteria to estimate in situ protozoan bactivory. Appl. Environ. Microbiol. **53**:958–965.
43. **Simon, M.** 1987. Biomass and production of small and large free-living and attached bacteria in Lake Constance. Limnol. Oceanogr. **32**:591–607.
44. **Snedecor, G. W., and W. G. Cochran.** 1980. Statistical methods, 7th ed. Iowa State University Press, Ames.
45. **Staley, J. T., and A. Konopka.** 1985. Measurement of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. **39**:321–346.
46. **Stanier, R. Y., R. Kunisawa, M. Mandel, and G. Cohen-Bazire.** 1971. Purification and properties of unicellular blue-green algae (order *Chroococcales*). Bacteriol. Rev. **35**:171–205.

47. **Taylor, G. T., and M. L. Pace.** 1987. Validity of eucaryote inhibitors for assessing production and grazing mortality of marine bacterioplankton. Appl. Environ. Microbiol. **53**:119–128.
48. **Tremaine, S. C., and A. L. Mills.** 1987. Inadequacy of the eucaryote inhibitor cycloheximide in studies of protozoan grazing on bacteria at the freshwater-sediment interface. Appl. Environ. Microbiol. **53**:1969–1972.
49. **Wetzel, R. G.** 1981. Longterm dissolved and particulate alkaline phosphatase activity in a hardwater lake in relation to lake stability and phosphorus enrichments. Verh. Int. Verein. Limnol. **21**:369–381.
50. **Wetzel, R. G.** 1983. Limnology, 2nd ed. Saunders College Publishing, New York.
51. **Winn, C. D., and D. M. Karl.** 1984. Laboratory calibrations of the [^{3}H]adenine technique for measuring rates of RNA and DNA synthesis in marine microorganisms. Appl. Environ. Microbiol. **47**:835–842.