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Estimating Bacterioplankton Production by Measuring [^{3}H]thymidine Incorporation in a Eutrophic Swedish Lake

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Bacterioplankton abundance, [^{3}H]thymidine incorporation, $^{14}CO_2$ uptake in the dark, and fractionated primary production were measured on several occasions between June and August 1982 in eutrophic Lake Norrviken, Sweden. Bacterioplankton abundance and carbon biomass ranged from 0.5×10^9 to 2.4×10^9 cells liter^{-1} and 7 to 47 μg of C liter^{-1}, respectively. The average bacterial cell volume was 0.185 μm^3. [^{3}H]thymidine incorporation into cold-trichloroacetic acid-insoluble material ranged from 12×10^{-12} to 200×10^{-12} mol liter^{-1} h^{-1}. Bacterial carbon production rates were estimated to be 0.2 to 7.1 μg of C liter^{-1} h^{-1}. Bacterial production estimates from [^{3}H]thymidine incorporation and $^{14}CO_2$ uptake in the dark agreed when activity was high but diverged when activity was low and when blue-green algae (cyanobacteria) dominated the phytoplankton. Size fractionation indicated negligible uptake of [^{3}H]thymidine in the >3-μm fraction during a chrysophycean bloom in early June. We found that >50% of the ^{3}H activity was in the >3-μm fraction in late August; this phenomenon was most likely due to *Microcystis* spp., their associated bacteria, or both. Over 60% of the $^{14}CO_2$ uptake in the dark was attributed to algae on each sampling occasion. Algal exudate was an important carbon source for planktonic bacteria. Bacterial production was roughly 50% of primary production.

The assessment of bacterial activity in aquatic systems has attracted considerable attention since the work of Wright and Hobbie (75, 76). The approach of these workers—determination of the uptake kinetics of labeled organic compounds (heterotrophic potential), has shown that bacterial activity can exhibit diurnal and temporal variations that are often closely associated with fluctuations in primary production. However, it is practically impossible that any one compound is representative enough for assaying overall heterotrophic activity. For example, Overbeck (53) has found that glucose uptake is <5 to 10% of the total heterotrophic activity in Plussee and other German lakes. Methods are still needed which measure entire bacterial production in carbon units for a complete understanding of carbon metabolism in aquatic systems.

Several methods exist for the determination of bacterial production. The most widely used, determination of $^{14}CO_2$ dark uptake (60), is often criticized (54) but nonetheless can provide a first approximation (52). The data that Sorokin (66) obtained by this method have been criticized by Banse (7) for being one order of magnitude too high; Sieburth (63), on the other hand, considers the data realistic. Jordan and Likens (30) have found that determination of $^{14}CO_2$ dark uptake results in higher estimates, compared with those obtained by several other methods. Determination of radioactive-sulfate uptake has been used to measure bacterial production (14, 30, 31, 45, 55). This method is promising, but uncertainties still exist in determining C/S ratios (46), and bacterial and algal uptake rates must be separated, also a limitation of the $^{14}CO_2$ dark uptake method.

A number of new methods have recently been described. Sieburth et al. (64) have measured bacterial production as the ATP increase in in situ diffusion chambers. However, their a priori classification of the <3-μm fraction as heterotrophic is probably unwarranted: Larsson and Hagström (40) have found algal contamination in the <3-μm fraction. Hagström et al. (25) have measured bacterial production in the Baltic Sea as the frequency of dividing cells. This method requires only microscopy preparation but can give high coefficients of variation (48) and has only been calibrated for natural marine bacteria in chemostat cultures. This is inadequate since all cells in a chemostat grow at the same rate, and the distribution of growth within a bacterial community is critical to the calibration of the method.

Estimation of bacterial growth by determination of RNA (32–36) and DNA (22, 23) synthesis,

the latter being measured by determining [^{3}H]thymidine incorporation into trichloroacetic acid (TCA)-insoluble material, are valuable new methodological approaches, as well as the cause of controversy (22, 34). Thymidine is a specific precursor for DNA, and since DNA is only synthesized by growing cells, [^{3}H]thymidine incorporation into DNA would not occur in nonproliferating cells. Furthermore, the rate of DNA synthesis closely follows the actual growth rate, and bacterial DNA content varies as a function of growth rate by one order of magnitude less than RNA (41). Even the best determinations of biomass (ATP)-specific rates of RNA synthesis, based on [^{3}H]adenine incorporation, have therefore been interpreted on the basis of hypothetical cellular RNA concentrations (36). The measurement of simultaneous DNA and RNA synthesis (33) with [^{3}H]adenine has interesting applications (e.g., diurnal studies), but if the purpose is the estimation of bacterial carbon production, then the [^{3}H]thymidine method has theoretical and procedural advantages (22).

Fuhrman and Azam (22, 23) have applied the [^{3}H]thymidine method to coastal marine waters and by autoradiography have found that essentially all active bacteria take up [^{3}H]thymidine, with negligible algal uptake. These results, as well as the apparent absence of thymidine kinase from algae, fungi, and other eucaryotes, have led to the view that the incorporation of exogenous [^{3}H]thymidine into DNA via salvage pathways is predominantly bacterial (reviewed in reference 34). Since there are reports of [^{3}H]thymidine incorporation into eucaryotic DNA (34), an a priori assumption of specific bacterial uptake is perhaps not warranted, especially for eutrophic freshwaters.

Bacterial growth rate determinations with [^{3}H]thymidine have been reported for marine and estuarine waters (9, 16, 17, 19, 21–23) and sediments (47, 71). Riemann et al. (58) have recently tested the method in several freshwater systems and have presented field data from November and April. We present here [^{3}H]thymidine incorporation rates for a eutrophic lake during the summer of 1982. The potential for algal [^{3}H]thymidine incorporation was tested by size fractionation of the plankton and tests with algal cultures. In addition, we compared in situ [^{3}H]thymidine production estimates with estimates based on $^{14}CO_2$ dark uptake and bacterial uptake of algal extracellular products.

MATERIALS AND METHODS

Study site. Lake Norrviken (59°28′ N, 17°56′ E) is a eutrophic lake (maximum depth, 11 m; area, 2.7 km^2) located about 15 km north of Stockholm. Blue-green algae (cyanobacteria) start to proliferate by midsummer, and by September, the phytoplankton is usually >95% *Oscillatoria agardhii* Gom. The lake has been described previously (1, 3–5). Sampling dates and associated phytoplankton assemblages are given in Table 1. All sampling and incubation were performed over the deepest part of the lake (reference 4, station 5) between 10:00 a.m. and 2:00 p.m.

Laboratory experiments. To determine if appreciable algal uptake of [^{3}H]thymidine could occur, we added 20 ml of *O. agardhii* (96 μg of chlorophyll *a* liter^{-1}) and 20 ml of *Microcystis wesenbergii* Kom. (200 μg of chlorophyll *a* liter^{-1}) from healthy nonaxenic cultures (2) to replicate 125-ml flasks. The algae were washed through a Nitex net (25-μm pore size) before use. One replicate then received lake water (~5 × 10^6 bacteria ml^{-1}), and the other received sterilized culture medium Z8 (2). Water for the *Microcystis* replicate was taken from Lake Vallentunasjön, the source of the *Microcystis* culture. A third flask received lake water without the algae. Absorption blanks were fixed with Formalin (2% final concentration). We added 1 ml (1.35 nM) of [*methyl*-^{3}H]thymidine (46 Ci mmol^{-1}) and incubated the flasks for 2 h. Determination of [^{3}H]thymidine incorporation into cold-TCA-insoluble material was performed as described below. Experiments to determine the concentration of [^{3}H]thymidine needed for in situ experiments and the effects of Formalin fixation and dilution with unlabeled thymidine (10 mM) as means to stop the reaction were performed with water collected on the preceding day and stored in the dark at 4 to 8°C. Incubation was performed in the light (~100 μEinsteins m^{-2} s^{-1}) at room temperature (20 to 22°C).

Bacterioplankton abundance and biomass. Bacteria were counted and cell volumes were estimated by acridine orange staining and epifluorescent microscopy (26) with Nuclepore filters (0.2-μm [and occasionally 3-μm] pore size) stained with Sudan black (77). We took 50-ml samples from discrete depths with a Ruttner sampler and preserved them in the field with 40% Formalin (4% final concentration). We then added 2-ml portions of buffer (5 mM bicarbonate) and 4-ml

TABLE 1. Sampling dates, primary production, and associated phytoplankton assemblages

Date	Primary production (mg of C m^{-2} h^{-1})[a]	Dominant phytoplankton
2 June	142	*Rhodomonas minuta*, *Chrysochromulina* sp., *Oscillatoria agardhii*, *Scenedesmus* sp.
17 June		Detritus, Small chrysophyceae, *Oocystis* sp., *Anabaena* spp.
20 July	285	*Oocystis* sp., *Oscillatoria agardhii*, *Microcystis wesenbergii*
19 August	91	*Oscillatoria agardhii*, *Microcystis wesenbergii*, *Oocystis* sp.

[a] Integrated data from 0- to 3-m depth. Hourly midday rates are shown.

portions of 0.01% acridine orange to 2-ml subsamples. The filters were inspected at a magnification of ×1,250 under a Zeiss standard RA microscope equipped with a type 100 illuminator, a 50 W HBO high-pressure mercury lamp, K 490 and K 500 excitation filters, an RPI 510 reflector, and an LP 528 barrier filter. We counted 200 to 400 bacteria in at least 20 fields. Total bacterial biovolume was estimated by grouping each bacterium counted into one of four major size classes: rods of different length and diameter, cocci as 0.25-, 0.5-, and 1.0-μm-diameter spheres, diplococci, and oval forms. Mean cell volumes were calculated by dividing total biovolume by total count. Carbon biomass was computed from biovolumes with the assumption that carbon constituted 10% of the wet weight (67). Solution blanks were checked before each sample counting. All solutions were filter sterilized (0.2-μm pore size) if the blank was over 2% of the previous sample. Coefficients of variation (n = 3) averaged 9% (1.7 to 17.5) for total counts, 10.6% (6.2 to 14.7) for mean cell volume, and 15% (6.2 to 27.4) for total biovolume.

[^{3}H]thymidine incorporation. Bacterial production rates were estimated from the rate of [^{3}H]thymidine incorporation into cold-TCA-insoluble material (23). Duplicate samples were incubated in situ in 30- or 50-ml transparent Jena flasks with 5 nM [*methyl*-^{3}H]thymidine (40 to 50 Ci mmol^{-1}; Radiochemical Centre, Amersham, England) for 30 to 60 min. The reaction was stopped by the addition of Formalin to a final concentration of 2%. Absorption blanks prekilled with 2% Formalin were incubated at each depth.

Upon return to the laboratory, 3- to 10-ml subsamples were extracted in equal volumes of ice-cold 10% TCA for 10 to 30 min, filtered onto Nuclepore polycarbonate filters (0.2- and 3-μm pore sizes), rinsed three times with 2-ml portions of ice-cold 5% TCA, and put in scintillation vials. Radioactivity retained on the filters, representing material insoluble in cold TCA (DNA, RNA, and protein), was assayed on an Intertechnique SL-30 scintillation counter. Quenching was determined by the external-standard-channels-ratio method. The mean difference between duplicate thymidine incubations was 13%. Production was calculated from the following equation (equation 1):

micrograms of C liter^{-1} hour^{-1} = (nanomoles of [^{3}H]thymidine in cold-TCA-insoluble extract × conversion factor [cells nanomole^{-1}] × average cell carbon × 60)/(incubation time [minutes])

Conversion factor determination. A conversion factor which is the composite of several biochemical parameters (see Discussion) is needed to estimate carbon production from [^{3}H]thymidine incorporation. An alternative is to determine a relationship between increases in acridine orange direct counts (AODC) and changes in [^{3}H]thymidine incorporation rates of natural bacterial assemblages in the absence of bacteriovores (22, 23, 37). Growth is normally assumed to be linear in unenriched filtrate (e.g., 3-μm-pore-size filter) cultures (22, 23). Exponential growth can be studied by providing enrichment (11) or diluting the filtrate 1:10 with filter sterilized lake water to diminish nutrient competition (37).

Experiment 1. We followed the general procedure of Fuhrman and Azam (22, 23). A mixed epilimnion sample taken in July was prefiltered through a Nitex net (90-μm mesh size) to remove zooplankton (rotifers and ciliates were virtually absent [L. Bern, Institute of Limnology, Uppsala, Sweden, personal communication]). We incubated 2 liters in an acid-washed 5-liter flask with gentle mixing at a constant temperature (17 ± 1°C, ~50 μEinsteins m^{-2} s^{-1}). Samples were removed at 0, 2.5, 5, 10, and 18 h. Samples (5 ml) for AODC were placed in vials and preserved with Formalin (4% final concentration). Subsamples (30 ml) were incubated with 5 nM [*methyl*-^{3}H]thymidine for 60 min at the above constant temperature. Samples were processed as described above. Linear growth rate constants (Δ) were computed by the method of Christian et al. (11):

$$\Delta = (X_{t_2} - X_{t_1})/(X_{t_1})(t_2 - t_1) \quad (2)$$

where X is cell density or biovolume at specific times in hours (t_2, t_1). Increase in cell numbers between sequential time points was converted to production (cells liter^{-1} hour^{-1}) and compared with the concurrent [^{3}H]thymidine incorporation estimate (nanomoles liter^{-1} hour^{-1}) to obtain a conversion factor.

Experiment 2. A 5-liter water sample was filtered (10-μm pore size), and 50 mg of glucose was added to stimulate growth. A sample for AODC and biovolume determination was taken immediately after filtration. After 8 h, we assumed that growth was in the exponential phase (11) and removed the first subsamples (time zero). Subsequent samples were removed at 4, 7, and 10.5 h. For AODC, 18-ml portions were removed and placed in small bottles containing 2-ml portions of 40% Formalin. Incubation with [^{3}H]thymidine was performed as described for experiment 1. The growth rate, μ, was derived from semilog plots of bacterial abundance and thymidine incorporation rates, and a conversion factor (cells nanomoles^{-1}) was computed by the method of Kirchman et al. (37).

If the conversion factor is constant over the course of the experiment, as evidenced when the semilog plot of uptake rates versus time is a straight line, then uptake and production are related by the following equation of Kirchman et al. (37):

$$v(t) = C^{-1}\, dN(t)/dt \quad (3)$$

where $v(t)$ is uptake of thymidine, $dN(t)/dt$ is production of cells, and C is the conversion factor. One can define μ as follows:

$$dN(t)/dt = \mu\, N(t) \quad (4)$$

Equation 4 can then be substituted into equation 3 to give:

$$C = \frac{\mu\, N(t)}{v(t)} \quad (5)$$

At t = 0, equation 5 is equivalent to equation 4 presented by Kirchman et al. (37), and a conversion factor can be calculated from the following equation:

$$C = \frac{\mu\, N(0)}{v(0)} \quad (6)$$

where μ is the specific growth rate (hour^{-1}) determined from uptake rates and abundance regressions, $N(0)$ is the initial number of bacteria incorporating

TABLE 2. Thymidine incorporation by blue-green algae and bacteria[a]

Incorporation by:	dpm ml^{-1}
O. agardhii Gom.[b]	354
Bacteria[c]	5,531
O. agardhii Gom. + bacteria	5,890
Absorption blank	263
M. wesenbergii Kom.[b]	2,634
Bacteria[c]	1,704
M. wesenbergii Kom. + bacteria	1,894
Absorption blank	260

[a] Average of replicate determinations.
[b] Nonaxenic cultures.
[c] From lake water collected in early May. Blue-green algae were absent.

thymidine (assumed to be 100% if semilog plots are linear), and $v(0)$ is the y-intercept (initial measurement) of the uptake rate regression. If the semilog plots of abundance and uptake rates versus time are linear (see Fig. 4) and if the specific growth rates determined from both regressions are equal, then no further calculations are necessary. Calculations to account for complications due to increases in cell volume, changes in specific activity, and nondividing cells are discussed by Kirchman et al. (37). The conversion factor derived from equation 6 was used in equation 1 to compute bacterial production from in situ measurements.

$^{14}CO_2$ dark uptake. $^{14}CO_2$ dark uptake was estimated by incubating 125-ml samples in situ with 10-μCi portions of $NaH^{14}CO_3$ for 4 to 6 h. The samples were preserved with 0.3 mM $HgCl_2$, filtered through Nuclepore filters (3- and 0.2-μm pore sizes), rinsed with filter sterilized distilled water, and assayed by liquid scintillation. Dissolved inorganic carbon was determined from pH, conductivity, and alkalinity measurements. Bacterial heterotrophic production (0.2- to 3-μm fraction) was calculated with the assumption that 3 to 6% of carbon production is fixed as CO_2 (52, 60).

Primary production. Primary production was based on standard ^{14}C procedures (72). In situ incubation (1 μCi $flask^{-1}$) was for 3 to 4 h (10:00 a.m. to 2:00 p.m.). One dark bottle was included in each series. Bottles were stored in the dark until filtration. Subsamples (10 to 25 ml) were filtered through a sequence of Nuclepore filters (10-, 3-, and 0.2-μm pore sizes) in Millipore Swinnex holders. On several occasions, a 2-μm-pore-size filter was also inserted. A nonfractionated replicate was filtered directly through a 0.2-μm-pore-size filter. Filters were placed in scintillation vials and allowed to dry. We added 0.5-ml portions of TS-1 tissue solubilizer and allowed the mixtures to stand overnight. We then added 200-μl portions of isobutanol and 15-ml portions of Pugh IV scintillation solution (57) to the vials and mixed the contents. Quenching was determined by the external-standard-channels-ratio method. Loss of radioactivity due to sequential filtration was <10%.

Algal exudate was determined in the filtrate of the nonfractionated sample. Portions (3 to 5 ml) were transferred to scintillation vials, acidified to pH 2 with HCl, and bubbled with air for 15 min. Dioxan-based scintillation solution (15 ml) was then added, and radioactivity in the samples was determined by liquid scintillation counting. Total extracellular release (<0.2-μm fraction plus 0.2- to 3-μm fraction) was calculated only when the 0.2- to 3-μm fraction had negligible chlorophyll.

Chlorophyll. For chlorophyll determinations, water samples (150 to 300 ml) were filtered through GF/C filters. The filters were coated with 1% $MgCO_3$ and frozen in a desiccator until analysis. Chlorophyll was determined by the method of Strickland and Parsons (70) after homogenization and 24-h extraction in 90% aqueous acetone and measured spectrophotometrically. Extraction for fractionated samples was identical. Untreated lake water filtered onto GF/C filters was considered to have a chlorophyll content 100%, and the samples were measured fluorometrically with a Turner model 100 fluorometer.

RESULTS

Laboratory experiments. Incorporation of [^{3}H]thymidine by *O. agardhii* was negligible (Table 2). *M. wesenbergii*, on the other hand, seemed to incorporate [^{3}H]thymidine. It is more probable, however, that the associated bacteria in the mucilagenous sheath were responsible for most of the uptake. The *Microcystis* culture consisted, in fact, of amorphous 50- to 100-cell colonies containing various amounts of bacteria (G. Ahlgren, manuscript in preparation). Interestingly, bacterioplankton seemed inhibited in the presence of *M. wesenbergii*.

[^{3}H]thymidine incorporation rates are shown in Fig. 1 and 2. Uptake was linear for at least 1 hour at 5 nM [^{3}H]thymidine. Uptake was linear at all concentrations for the first 20 min (Fig. 2). The apparent near saturation at 5 nM suggests that at this concentration, external isotope dilution was minimal.

When the samples were filtered and extracted immediately after the reaction was stopped, the amounts of radioactivity in the cold-TCA-insoluble fractions were similar for Formalin-fixed

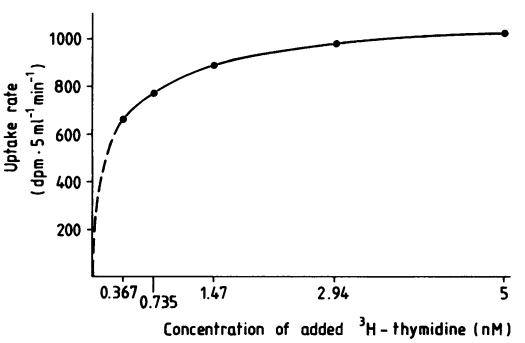

FIG. 1. [^{3}H]thymidine incorporation into cold-TCA-insoluble material, determined for water collected on 13 May. Uptake was linear for 1 h at all concentrations.

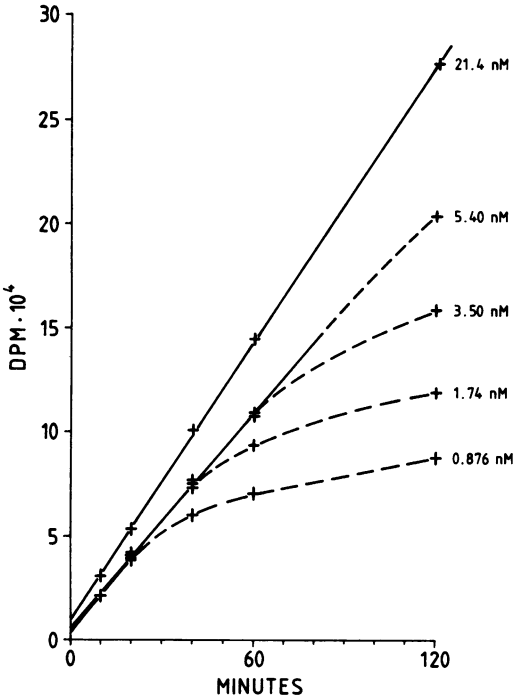

FIG. 2. [^{3}H]thymidine incorporation into cold-TCA-insoluble material, determined for water collected on 20 July.

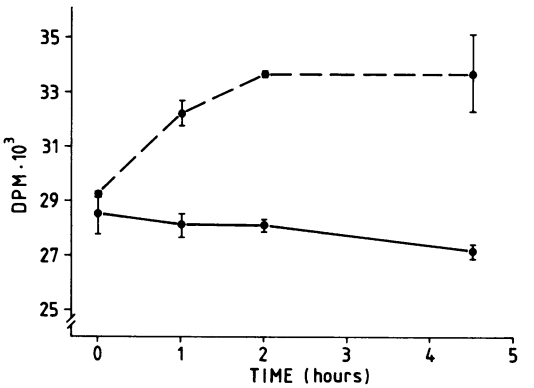

FIG. 3. Radioactivity in cold-TCA-insoluble material extracted after addition of Formalin or dilution with unlabeled thymidine. Formalin (——) or 10 mM unlabeled thymidine (– – –) was added after 1 h of incubation with 5 nM [^{3}H]thymidine. The times shown indicate intervals between stopping the reaction and extraction. Vertical bars indicate ranges of duplicate determinations.

samples and samples diluted with 10 mM unlabeled thymidine (Fig. 3, time zero). After 4.5 h at room temperature and before extraction, cold-TCA-insoluble radioactivity in the Formalin-fixed sample had decreased 5%. Radioactivity increased for 2 h after the addition of unlabeled thymidine. As a result, we used Formalin to stop the reaction of in situ incubations.

Conversion factor. Table 3 summarizes the results of experiment 1. With the exception of a decrease in abundance between 2.5 and 5 h, linear growth constants were similar throughout the experiment. Cell volume did not change significantly.

Experiment 2 is summarized in Table 4 and Fig. 4. Growth rate estimates determined from changes in bacterial abundance and incorporation rates were nearly identical. The amount of variation accounted for by both regression lines was 99%. These data and the linearity of the semilog plots of abundance and incorporation versus time indicate that nearly all bacteria were actively dividing. $N(0)$ was therefore not adjusted for nondividing cells in calculating the conversion factor from equation 6. Cell volume increased slightly (0.25 to 0.29 μm^3) during the exponential growth phase, which could explain the slightly lower growth rate determined from bacterial abundance. Bacterial volume doubled and abundance decreased in the first 8 h after addition of glucose (lag phase). The conversion factors derived from both experiments were similar. We used 2×10^9 cells nmol of [^{3}H]thymidine incorporated^{-1} in calculating in situ production from equation 1.

Bacterioplankton abundance and production. Bacterial abundance varied between 0.5×10^9 and 2.4×10^9 liter^{-1} and was usually greatest in

TABLE 3. Experiment 1 linear growth rate constants, bacterial production, [^{3}H]thymidine incorporation, and conversion factors

Time (h)	Cell density (h^{-1})	Bacterial production (10^7 cells liter^{-1} h^{-1})	[^{3}H]thymidine incorporation (10^{-2} nmol liter^{-1} h^{-1})	Conversion factor (10^9 cells nmol^{-1})
0–2.5	0.04	8.0	3.64	2.2
2.5–5	−0.1			
5–10	0.06	10.0	4.4	2.2
10–18	0.05	8.1	4.2	1.9

TABLE 4. Experiment 2 growth rates and conversion factors, as determined from changes in [^{3}H]thymidine incorporation rates and bacterial abundance

Determination from changes in:	Growth rate (h^{-1})	r^{2a}	Conversion factor (10^9 cells nmol^{-1})^b
Bacterial abundance	0.098	0.99	1.9
Incorporation rates	0.11	0.99	2.1

[a] r^2, Coefficient of determination.
[b] Calculated from equation 6.

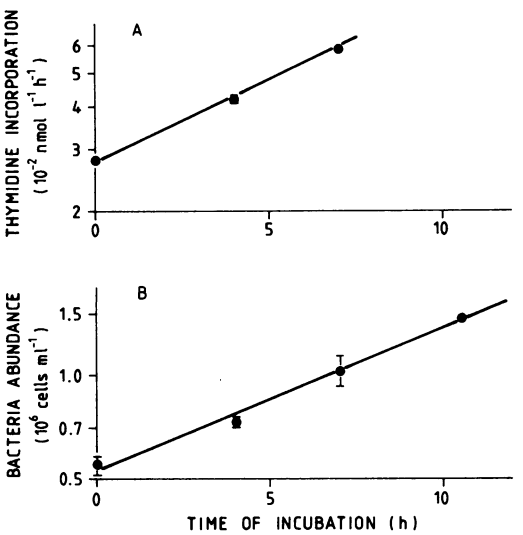

FIG. 4. Rates of [^{3}H]thymidine incorporation (A) and bacterial abundance (B) ($\log_e$ scale) in experiment 2. Vertical bars indicate ranges of triplicate determinations. When the ranges are smaller than the points, no vertical bars are shown.

the epilimnion (Table 5). Average cell volume varied between 0.08 and 0.28 μm^3. Between 30 and 70% of the cells were cocci. Less than 1% of the bacteria were bound to particles.

Thymidine incorporation was greater in the euphotic zone and varied 10-fold between sampling dates (Table 5). Specific activity (73) of the cells (10^{-12} nmol of thymidine $cell^{-1}$ h^{-1} and 10^{-5} nmol of thymidine μm^{-3} h^{-1}) was greatest at the surface and the 0.5-m depth. Activity was lowest on 17 June, both on a per-cell basis and a unit volume basis. [^{3}H]thymidine incorporation varied little in the hypolimnion.

The $^{14}CO_2$ dark uptake estimation of bacterial production was much greater than the [^{3}H]thymidine estimation for 17 June, when bacterial activity was very low (Table 6), but the [^{3}H]thymidine estimation fell within the $^{14}CO_2$ dark uptake estimation range when activity was high (20 July). [^{3}H]thymidine incorporation by the >3-μm fraction was usually less than 15% of the total activity on the 0.2-μm-pore-size filter (Table 7). AODC made on 3-μm-pore-size filters indicated that virtually all of the activity could be ascribed to free bacteria that were smaller than 3 μm and that remained on the filter (data not shown). Fractionation indicated that a high percentage of $^{14}CO_2$ dark uptake was algal (Table 7).

Primary production and extracellular release. Primary production and chlorophyll contents are shown in Tables 1 and 5, respectively. On 2 June, 50% of the primary production occurred in the 3- to 10-μm fraction. In July and August, 90% of the primary production occurred in the

TABLE 5. Data on bacterioplankton, [^{3}H]thymidine incorporation, temperature, and chlorophyll *a* (Chl *a*)

Date and depth	Abundance (10^9 liter^{-1})	Biomass (μg of C liter^{-1})	Avg vol (μm^3)	[^{3}H]thymidine incorporation			Temp (°C)	Chl *a* (μg liter^{-1})
				10^{-12} mol liter^{-1} h^{-1}	10^{-12} nmol $cell^{-1}$ h^{-1}	10^{-11} nmol μm^{-3} h^{-1}		
2 June								
Max (0.5 m)[a]	1.32	20.1	0.153	205	155.3	102		
Epi[b]	1.43	25.5	0.176	175.5	122.7	68.9	18.4	8.7
Hypo[c]	2.20	28.0	0.126	52.8	24.0	18.8	16.7	4.7
17 June								
Max (8 m)	0.43	7.5	0.177	12.1	28.4	16.1		
Epi	1.73	14.3	0.083	13.7	7.9	9.6	14.6	6.8
Hypo	0.48	7.3	0.154	12.1	25.2	16.7	14.2	2.7
20 July								
Max (0.5 m)	2.35	47.0	0.198	165	70.2	35.1		
Epi	2.08	44.0	0.212	138.3	66.4	31.4	21.8	35.8
Hypo	1.24	24.6	0.203	50.4	40.6	20.5	16.7	14.3
19 August								
Max (0 m)	0.994	24.4	0.245	60.5	60.9	24.8		
Epi	0.97	24.1	0.248	48.8	50.3	20.2	17.9	31.3
Hypo	0.82	22.7	0.279	25.7	31.3	11.3	16.4	18.0

[a] Values in parentheses indicate depths at which maximum (Max) [^{3}H]thymidine incorporation per cell was observed.

[b] Epi, Epilimnion (average, 0 to 2 m).

[c] Hypo, Hypolimnion (average, 4 to 8 m).

TABLE 6. Bacterioplankton production estimates for and specific growth rates in Lake Norrviken

Date and depth (m)	Bacterioplankton production (μg of C liter^{-1} h^{-1})			Exudate uptake as % of total bacterial production	Specific growth rate (day^{-1})
	Exudate uptake[a]	$^{14}CO_2$ dark uptake[b]	[^{3}H]thymidine incorporation[c]		
17 June					
0.5		2.2–4.4	0.27		0.55
1		1.7–3.4	0.21		0.24
2		1.5–3.0	0.24		0.44
20 July					
0	3.8	3.7–7.4	4.6	83	2.75
0.5	1.6	3.3–6.7	6.6	24	3.37
1	1.6	2.9–5.8	6.4	25	3.49
2	0.5	2.2–4.4	5.9	9	3.17
4		1.8–3.6	2.7		2.22
8		0.9–1.9	1.2		1.44
19 August					
0	1.3		3.0	45	2.92
0.5	0.6	0.3–0.6	1.8	32	1.69
1	0.25	0.3–0.6	2.2	11	2.38
2	2.2	0.5–1.0	2.7	80	2.78
4		0.4–0.8	2.2		2.56

[a] The 0.2- to 3-μm fraction used for primary production measurements equals heterotrophic uptake of algal extracellular products. Phytoplankton in this fraction was negligible (Table 8).

[b] Calculated under the assumption that CO_2 dark uptake was 3 to 6% of total carbon uptake (52, 60).

[c] Conversion factor of 2×10^9 cells produced per nmol of [^{3}H]thymidine incorporated × mean cell carbon (equation 1).

>10-μm fraction. No effective species separation resulted from size fractionation, however. Total extracellular carbon release was about 5% of the total carbon fixed. The lack of chlorophyll and ^{14}C radioactivity in the 2- to 3-μm fraction indicated adequate separation of algae and bacteria (Table 8). On 19 August, all labeled, released organic carbon was apparently taken up by the 0.2- to 3-μm (bacterial) fraction: the exudate had 0 dpm. This does not preclude, however, release of unlabeled algal extracellular products. Uptake of algal exudate by bacterioplankton amounted to 10 to 80% of the total heterotrophic production ([^{3}H]thymidine estimate) during midday (Table 6).

DISCUSSION

Because 5 nM [^{3}H]thymidine gave linear uptake for >1 h, the use of this concentration was justified. Hollibaugh et al. (28) have observed linearity for 5 to 70 min. Fuhrman and Azam (22) have observed linearity for up to 6 h in the Antarctic. We observed linear uptake for 2.5 h in Lake Norrviken, but longer in situ incubations were not attempted. A 20 nM addition increased the rate of incorporation by only 20%. A similar result has been reported by Fuhrman and Azam (22, 23). They have found a 5 nM addition suitable for a variety of marine environments.

TABLE 7. Size fractionation of $^{14}CO_2$ dark uptake and [^{3}H]thymidine activities[a]

Date and depth (m)	$^{14}CO_2$ dark uptake	[^{3}H]thymidine
2 June (0.5)		1.5
17 June		
0	60.1	
0.5	66.4	7.3
1	37.3	10.8
2	33.0	8.7
4	57.5	
20 July		
0	95.9	8.7
0.5	88.2	8.6
1	90.2	11.9
2	88.5	11.2
4	81.7	14.5
8	66.0	13.4
19 August		
0	61.2	50.5
0.5	82.3	56.0
1	83.0	56.2
2	78.6	56.6
4	73.6	59.1
8	62.1	28.8

[a] Each value indicates the activity retained on a 3-μm-pore-size filter as a percentage of the total activity on a 0.2-μm-pore-size filter.

TABLE 8. Size fractionation of phytoplankton[a]

Date and depth (m)	^{14}C activity (dpm) on filter with pore size:	
	3 μm	2 μm
20 July		
19 August		
0	36	0
0.25	35	0
0.5	19	0
1	47	0
2	24	3
3	2	1

[a] On 20 July, <1.5% of the total chlorophyll *a* content was in the <3-μm fraction. On 19 August, none of the total chlorophyll *a* content in each sample was in the <3-μm fraction.

Riemann et al. (58) have recommended a 10 nM addition for freshwater, but in some lakes, saturation is reached at 5 nM. The concentration needed should, of course, be tested for every system. Particularly poor results are obtained if the wrong [^{3}H]thymidine concentrations are chosen (34).

The assumption that [^{3}H]thymidine is assimilated predominantly or exclusively by bacteria has recently been questioned (34). Uptake of [^{3}H]thymidine by the >3-μm fraction was negligible during the chrysophycean bloom in early June. There was, however, substantial uptake in the >3-μm fraction in August. As with the cultures, the *Microcystis* colonies in situ had many bacteria embedded within the gelatinous matrix. Routine quantification was not possible, but some colonies contained over 500 bacteria. Obviously, autoradiography is needed to determine the partitioning of radioactivity within the >3-μm fraction. Aside from potential complications during blooms of blue-green algae which may require size fractionation, we conclude that algal uptake of [^{3}H]thymidine is not a substantial problem, in comparison with the problems caused by the non-specificity of other isotopes employed to measure bacterial production. For example, only 16% of the total $^{35}SO_4$ uptake and 58% of the total $^{14}CO_2$ dark uptake in Mirror Lake is bacterial (30). Karl (33, 34) claimed that there is no evidence that [^{3}H]thymidine is assimilated by the same subset of microorganisms. However, Karl and co-workers (35) used the same procedure, comparison with a tritiated-amino acid mixture via autoradiography, to claim that [^{3}H]adenine is assimilated by the majority of aquatic bacteria and unicellular algae. These workers also detected [^{3}H]adenine assimilation in marine bacterial isolates on nutrient agar, but in a water sample, the fraction of bacteria capable of forming a colony on agar medium is much smaller than the total number of active bacteria (27, 29, 44, 77). In freshwaters, the use of [^{3}H]adenine nearly always requires size fractionation if predominantly bacterial growth rates are desired. Our evidence indicates that this is not the case with [^{3}H]thymidine.

Bacterioplankton abundance and activity. Bacterial abundance in Lake Norrviken was rather low for a eutrophic lake. Although Hobbie and Wright (27) have stated that 1×10^6 to 2×10^6 ml^{-1} is typical for eutrophic lakes, other eutrophic lakes, such as Bysjön (13) and Møssø (59), commonly have over 10×10^6 ml^{-1}. Grazing could help keep the population low: Lake Norrviken has many zooplankters (*Diaphanosoma brachyurum, Chydorus sphaericus*, and *Daphnia cucullata*) considered to be effective bacterial feeders (24). Bacterioplankton from 0.01 to 0.3 μm^3 can be grazed (56), although apparently at lower rates than algae. The decline in bacterial abundance and the concomitant shift from green algae to blue-green algae between July and August (Tables 1 and 5) suggest that bacterioplankton is inhibited by actively growing blue-green algae. The decline cannot be entirely attributed to grazing (Bern, manuscript in preparation), although other mechanisms causing the decrease are certainly possible (55). Allelopathic relationships with blue-green algae are well documented (39, 42). The late summer dominance of blue-green algae is a yearly phenomenon in Lake Norrviken (1–5). Without exception, *O. agardhii* has dominated the phytoplankton community throughout the past decade. This must alter the microenvironment and create selective pressures within the bacterioplankton community.

The cell volumes observed in Lake Norrviken were within the ranges observed in other aquatic systems (13, 27, 40, 55). The mean bacterial cell volume, 0.186 μm^3, is comparable to the yearly average in eutrophic Lake Mendota—0.159 μm^3 (55). Although data based on AODC or scanning electron microscopy are not yet extensive, bacterial cell volumes seem to be larger in eutrophic lakes, a logical consequence of increased nutrient availability.

Bacterial production appeared to be tightly coupled to primary production in Lake Norrviken. Coveney (12) has also found that at times, over 50% of bacterial production is derived from algal extracellular products in eutrophic Lake Bysjön, and an intimate relationship between algae and bacteria has been revealed by studies of the Plussee (51–53). If one assumes a conversion efficiency of 60% (10), a rough calculation of integrated daily production (milligrams of C $meter^{-2}$ day^{-1}) from hourly rates [(bacterial rate × 24)/(algal rate × 10)] indicates that bacterial production was 40 to 70% of primary production. This is probably a slight overestimation,

since bacterial production was measured at midday, when rates in the euphotic zone may be highest (58). Net bacterial production in Lake Mendota is approximately 50% of gross primary production (55).

The low average bacterial cell size on 17 June and the low activity per cell (Table 5) suggest that many small inactive cells were present. Several studies indicate that small cocci are often inactive (18, 49, 69). One explanation is that cocci are rods which are in a state of dormancy or very low activity (49, 50). We have observed that small (0.25-μm diameter) cocci are very abundant when bacterial production is low, but more data are needed before general conclusions can be made. In contrast, some marine studies have shown that the smallest cells are more active on a per-volume basis (6, 20), but small cells can still be both active and nondividing. Nonetheless, a positive relationship between average cell size and growth rate could be expected (15, 40). A decrease in growth rate could occur when optimum cell size is exceeded, as observed for species in culture (68). Of course, the heterogeneity of natural bacterial populations might make detection of such a relationship confusing. We observed the highest production when cell size was average (0.15 μm^3).

Comparison of [^{3}H]thymidine and $^{14}CO_2$ dark uptake methods. The $^{14}CO_2$ dark uptake method is at best only a rough approximation of heterotrophic production. Although 3 to 6% of the carbon uptake is usually attributed to CO_2, variations of 0.4 to 30% have been reported, even for the same lake (52). The estimate from this method agreed best with the [^{3}H]thymidine estimate when bacterial and phytoplankton activities were both high (Tables 1 and 6). The poor agreement between the 17 June results could have resulted from several factors. Size fractionation could have been ineffective, thereby resulting in the overestimation of bacterial (0.2- to 3-μm) production by the $^{14}CO_2$ dark uptake method. Appreciable algal dark uptake and release with subsequent bacterial uptake could have occurred. Søndergaard and Schierup (65) have shown that dark extracellular release on a percentage basis can be very high. At any rate, CO_2 probably contributed much more than 3 to 6% of the substrate available for bacterial utilization. Søndergaard and Schierup (65) have also shown that during the spring diatom bloom in Lake Møssø, phytoplankton in the stationary phase excretes a higher percentage of high-molecular-weight products than algae in the logarithmic growth phase. The excreted high-molecular-weight molecules are probably less efficiently utilized by planktonic bacteria. This situation could have occurred in Lake Norrviken on 17 June, as the *Rhodomonas* bloom had crashed, detritus was common, and phytoplankton was very sparse (Table 1).

Concurrently, bacterial production could have been underestimated from [^{3}H]thymidine incorporation on 17 June if, in fact, a large percentage of small cells were inactive, since this would make the average biomass less than the true average biomass of cells undergoing division. Even so, this in itself would not explain the large difference between the $^{14}CO_2$ dark uptake estimates and the [^{3}H]thymidine estimates. Although several methods to determine numbers of active cells have been tried with some success (18, 29, 43, 44, 77), none has been shown to be free of interpretation problems or utilized concurrently with bacterioplankton production estimates and used to refine the latter. Fractionation of radioactivity into size fractions (0.2-, 0.4-, 0.8-μm, etc.) with Nuclepore filters and comparison of the results with AODC on similar-sized filters could, in theory, provide a measure of size-related production. We have, however, despite reports of success (27), found such data hard to interpret, since filters retain cells smaller than the stated pore size.

When blue-green algae dominated in August, the $^{14}CO_2$ dark uptake production estimate was much lower than the estimate based on [^{3}H]thymidine incorporation. A possible explanation can be extrapolated from the work of Overbeck (52), who has shown, using cultures of *Klebsiella* isolates from the Plussee, that the fraction of incorporated $^{14}CO_2$ increases with increasing amounts of added substrate (glucose). At the lowest levels of added substrate, practically no $^{14}CO_2$ is incorporated into the biomass. The lack of available algal exudate (a desired substrate) in Lake Norrviken on 19 August may, consequently, have influenced the uptake of $^{14}CO_2$.

Estimation of carbon production from [^{3}H]thymidine incorporation into cold-TCA-insoluble material is subject to several uncertainties (22, 23, 34), and thus the first conversion factors published by Fuhrman and Azam encompassed a sixfold range. By measuring the DNA content of the bacterioplankton, the percentage of [^{3}H]thymidine incorporated into DNA (80 to 95% of the cold-TCA-insoluble fraction), and the intracellular isotope dilution and assuming that bacterioplankton DNA is composed of 25% thymidylate residues, Fuhrman and Azam (23) later obtained a more reliable conversion factor. The conversion factor for the present study was very similar to the factor of 1.7×10^9 cells $nmol^{-1}$ estimated by Fuhrman and Azam (23) for a nearshore marine environment. The agreement is perhaps fortuitous, as Kirchman et al. (37) found different conversion factors for the environments they investigated. However, all of

their factors were greater than 1.9×10^9 cells $nmol^{-1}$. On the sole basis of published values for bacterial DNA content and assuming no isotope dilution, Moriarty and Pollard (47) estimated a conversion factor of 1.3×10^9 cells $nmol^{-1}$. The agreement between $^{14}CO_2$ dark uptake production estimates and [^{3}H]thymidine estimates for 20 July provides additional evidence that our conversion factor was acceptable, at least when the bacteria were doubling one to three times per day. We are less confident in applying our conversion factor to the data for 17 June, when the growth rate was much slower. We observed apparent linear growth when no enrichment was added to the filtrate, but that approach may not always be successful and, in general, cannot be applied to environments where growth is always slow, since incubation for more than 15 or 20 h can result in bottle effects (22). Since the purpose of filtration is to remove grazers, the use of a 3-μm-pore-size filter (11, 22) is not always essential. In July, we determined that prefiltration with 90-μm-pore-size filters removed essentially all of the zooplankton, with negligible loss of algae. This was important, since field data indicated that exudates were an important carbon source for the bacterioplankton. Although the dilution procedure employed by Kirchman et al. (37) to stimulate growth was less artificial, cell volumes and growth rates during our second conversion factor experiment (glucose addition) were well within the range of in situ measurements.

The constancy of a conversion factor in a specific environment has not yet been determined. At the very least, a new conversion factor may be needed for each major change in physical, chemical, and biological conditions in a temperate lake. Furthermore, only Fuhrman and Azam (23) have compared conversion factors derived from biochemical measurements with a factor derived from the change in biomass versus the change in incorporation rates. In that study, the factors agreed within 25%. Variation in the fraction of cold-TCA-insoluble material that is DNA and variation in internal isotope dilution from the de novo synthesis of deoxythymidine monophosphate are factors that have to be evaluated. Variation in the relationship between DNA synthesis via salvage and DNA synthesis via de novo pathways, in particular, could affect the temporal applicability of empirically derived conversion factors.

On the basis of the findings of Fuhrman and Azam (23), several studies have assumed that the amount of incorporated label in DNA is 80% of the total cold-TCA-insoluble label (16, 17, 19). Riemann et al. (58) have found, however, that the value is <20% in some Danish lakes and have recommended partial purification of the DNA fraction for freshwater studies. Karl (34) has also observed nonspecific macromolecular labeling with [^{3}H]thymidine, especially in a freshwater pond community. Despite the importance of conclusions based on various acid-base hydrolysis schemes (23, 34, 58) used to separate DNA, RNA, and protein, the applicability and efficiency of these schemes when applied to natural samples have not been adequately investigated. Riemann (personal communication) has subsequently determined that DNA comprises 60 to 90% of the cold-TCA-insoluble fraction in freshwater samples if the concentrations of extractants are increased. In addition, the purity of the DNA fraction was verified by the addition of DNase. We feel that it is premature to generalize on the extent of nonspecific macromolecular labeling.

Moriarty and Pollard (47) have suggested an approach to correct for both external and internal isotope dilutions which is based on the addition of nonradioactive thymidine to samples. Riemann et al. (58) have applied this method to several lakes and have found a linear plot of 1/disintegrations per minute incorporated into DNA in only one of four cases. Interpretation of

TABLE 9. [^{3}H]thymidine incorporation rates in various aquatic systems

Locality (reference)	Avg cell vol (μm^3)	Bacterial abundance (10^9 liter^{-1})	Thymidine incorporation		Specific growth rate (day^{-1})
			10^{-10} mol liter^{-1} day^{-1}	10^{-19} mol cell^{-1} day^{-1}	
Southern California Bight, cruise 14 (21)	~0.05	0.06–0.7	0.02–5	0.24–15.4	
Southern California Bight, cruise 15 (21)	~0.05	0.1–2	0.5–2	0.5–3	
California and British Columbia coastal waters (22)					<0.2–3
Hudson River Plume (17)			2–18	0.5–1.3	0.08
York River estuary, Virginia (16)	~0.06	1–8	10–100	2–10	0.2–1
Rhode Island River estuary[a]	0.06	1–50			0.05–>1
Lake Norrviken (this study)	0.185	0.5–2.4	3–49	1.2–37.4	0.2–7

[a] P. A. Rublee, S. M. Merkel, and M. A. Faust, Abstr. Annu. Meet. Am. Soc. Limnol. Oceanogr. 1982, p. 57.

TABLE 10. Aquatic bacterial production estimates

Locality	Method[a]	μg of C liter^{-1} h^{-1}	Reference
Marine environments			
Baltic Sea	FDC	0.09	25
Various oceanic sites	$^{14}CO_2$	0–2.92	66
Coastal waters, western U.S.	[^{3}H]thymidine	0.03–3	22
Kiel Fjord	DOC turnover	0.01–10	8
North Atlantic Ocean	Δ ATP	~5	64
York River estuary	[^{3}H]thymidine	0.3–3	16
Coastal waters, Georgia	FDC	0.6–17.6	48
Oligotrophic lakes			
Mirror, U.S.A.	$^{35}SO_4$	0.13	30
Humic lakes Sweden	$^{14}CO_2$	0.6–1.1	—[b]
Pääjarvi, Finland	$^{14}CO_2$	0.2–1	61
Various lakes, U.S.S.R.	$^{14}CO_2$	0.02–0.7	62
Eutrophic lakes			
Plussee, Germany	$^{14}CO_2$	0.5–3	53
Bysjön, Sweden	$^{14}CO_2$	2–5[c]	12
Møssø, Denmark	$^{14}CO_2$	4–10[d]	59
Various lakes, U.S.S.R.	$^{14}CO_2$	1.5–5.5	62
Mendota, Wisconsin	FDC, $^{35}SO_4$	0.3–8.3	55
Norrviken, Sweden	[^{3}H]thymidine	0.2–7.1	This study

[a] FDC, Frequency of dividing cells; $^{14}CO_2$, $^{14}CO_2$ dark uptake; DOC, dissolved organic carbon; Δ ATP, increase in ATP.

[b] —, J.-Å. Johansson, Dev. Hydrobiol., in press.

[c] Calculated under the assumption that 50% of the total bacteria were in the small particulate fraction (12).

[d] Estimated from integrated 14-day estimates.

multiphasic plots is difficult, if not impossible. The necessary assumption that the velocity of substrate utilization is independent of the concentration of the substrate added has been criticized in another context (74). Nevertheless, this approach deserves further testing since it is an alternative to the direct measurement of the specific activity of the nucleoside precursor pool. In conjunction with determinations of the DNA content of bacterioplankton and incorporation of [^{3}H]thymidine into a purified DNA fraction, measurement of isotope dilution by the method of Moriarty and Pollard (47) would yield a conversion factor which could be compared with that proposed by Kirchman et al. (37) and used in the present study.

Bacterioplankton production estimates for Lake Norrviken are compared with other [^{3}H]thymidine estimates in Table 9. Table 10 lists bacterial production estimates based on a variety of methods. Considering the assumptions inherent in each of the methods, the similarity in estimates for eutrophic lakes is encouraging. Even if the results of methods do not always correlate on a weekly basis, they are within the same order of magnitude (55).

We have shown that the determination of [^{3}H]thymidine incorporation into cold-TCA-insoluble material can, with proper precautions, be applied to freshwaters and, in conjunction with an empirically determined conversion factor, can produce realistic estimates of bacterial production. The repertoire of methods has increased significantly during the past few years. Further direct methodological comparisons will help illuminate the special advantages and disadvantages of each. Since each method measures a different aspect of bacterial growth, studies involving diurnal (38) and daily sampling are needed for further insights into the role of bacterioplankton in plankton dynamics and the overall carbon cycle.

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