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Protozoan Grazing and Bacterial Production in Stratified Lake Vechten Estimated with Fluorescently Labeled Bacteria and by Thymidine Incorporation

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In stratified Lake Vechten, The Netherlands, protozoan grazing was estimated on the basis of uptake of fluorescently labeled bacteria and compared with bacterial production estimated on the basis of thymidine incorporation. By using a grazer-free mixed bacterial population from the lake in continuous culture, an empirical relationship between cell production and thymidine incorporation was established. Thymidine incorporation into total cold-trichloroacetic-acid-insoluble macromolecules yielded a relatively constant empirical conversion factor of ca. 10^{18} (range, 0.38×10^{18} to 1.42×10^{18}) bacteria mol of thymidine^{-1} at specific growth rates (μ) ranging from 0.007 to 0.116 h^{-1}. Although thymidine incorporation has been assumed to measure DNA synthesis, thymidine incorporation appeared to underestimate the independently measured bacterial DNA synthesis by at least 1.5- to 13-fold, even if all incorporated label was assumed to be in DNA. However, incorporation into DNA was found to be insignificant as measured by conventional acid-base hydrolysis. Methodological problems of the thymidine technique are discussed. Like the cultures, Lake Vechten bacteria showed considerable thymidine incorporation into total macromolecules, but no significant incorporation into DNA was found by acid-base hydrolysis. This applied not only to the low-oxygen hypo- and metalimnion but also to the aerobic epilimnion. Thus, the established empirical conversion factor for thymidine incorporation into total macromolecules was used to estimate bacterial production. Maximum production rates (141×10^6 bacteria liter^{-1} h^{-1}; μ, 0.012 h^{-1}) were found in the metalimnion and were 1 order of magnitude higher than in the epi- and hypolimnion. In all three strata, the estimated bacterial production was roughly balanced by the estimated protozoan grazing. Heterotrophic nanoflagellates were the major consumers of the bacterial production and showed maximum numbers (up to 40×10^6 heterotrophic nanoflagellates liter^{-1}) in the microaerobic metalimnion.

Protozoa, especially heterotrophic nanoflagellates (HNAN; diameter, 2 to 20 μm), are recognized as major consumers of bacteria in aquatic ecosystems (3). Since most studies were performed in marine systems, relatively little is known about these protozoa in freshwater. In Lake Vechten, The Netherlands, maximum HNAN abundances of 40×10^6 HNAN liter^{-1} were found in the microaerobic metalimnion in 1985 (5). Long-term grazing experiments with selectively filtered lake water suggested that HNAN have the potential to harvest all daily bacterial production. Production was estimated on the basis of thymidine incorporation into total cold-trichloroacetic-acid (TCA)-insoluble macromolecules (11). However, in situ incubations with radioactive thymidine were not possible, and the samples had to be incubated after transport to the laboratory. Moreover, an empirical incorporation-to-production conversion factor was not established, and therefore a literature-derived value of 2×10^{18} bacteria mol of thymidine^{-1} (21) was used. Recently, McDonough et al. (19) reported that the thymidine approach can lead to underestimates of bacterial production, especially in meta- and hypolimnetic samples with low oxygen concentrations. Such samples showed high thymidine incorporation into TCA-insoluble macromolecules, but only 33% (range, 12 to 57%) was found in DNA by acid-base hydrolysis. Thus, more research was needed on thymidine incorporation and bacterial production in Lake Vechten.

Although the magnitude of protozoan bacterivory may be estimated on the basis of direct cell counts in long-term incubations (1, 5), short-term methods for in situ estimates are preferable, and recently Sherr et al. (29) introduced the use of fluorescently labeled bacteria (FLB) as tracers for in situ grazing experiments. In two-stage continuous cultures, protozoan ingestion rates appeared to be measured accurately by uptake of FLB (7) when 2% glutaraldehyde was used to fix HNAN. However, glutaraldehyde fixation has been found to cause egestion of FLB and severe underestimation of grazing, which was prevented by using van der Veer fixative, consisting of 2% acrolein, 2% glutaraldehyde, and 1% tannic acid (final concentrations after 1:1 dilution with fixative) (30).

In the present study, thymidine incorporation into total cold-TCA-insoluble macromolecules, as well as hot-NaOH and hot-TCA precipitates, was investigated in stratified Lake Vechten and in cultures. Methodological problems of the thymidine technique are discussed. From cultures an empirical conversion factor was obtained to estimate in situ bacterial production on the basis of thymidine incorporation into total macromolecules. The effect of glutaraldehyde and van der Veer fixative on FLB uptake was examined. On the basis of FLB uptake, in situ HNAN and ciliate grazing on bacteria was estimated and compared with the estimated bacterial production in the epi-, meta-, and hypolimnion of Lake Vechten.

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MATERIALS AND METHODS

Sampling. Lake Vechten is ca. 10 m deep, monomictic, and stratified from May until October, showing hypolimnetic oxygen depletion (5, 33). Oxygen and temperature profiles were measured with oxygen indicator model 2607 (probe 2112; Orbisphere, Geneva, Switzerland). Over the deepest area between 9 and 10 a.m., samples were taken with a Friedinger sampler (capacity, 5 liters; length, 0.60 m) for cell counts. For FLB uptake and thymidine incorporation measurements, a peristaltic pump was used to prevent aeration of low-oxygen samples (19). The incubation tubes were flushed with sample water, filled, and closed under water (in a bucket) with a screw cap containing a rubber septum. Through the septum, FLB, thymidine, and fixative were added with a syringe, while a second empty syringe received the excess water. The incubations started as soon as possible after sampling and were performed in dark (aluminum foil-wrapped) tubes at in situ temperatures in buckets with water from the depths at which the samples were taken.

Counting. Samples were fixed immediately with 1% (final concentration) glutaraldehyde, and protozoa (HNAN and ciliates) were stained with primulin and counted directly on polycarbonate membrane filters (Nuclepore Corp., Pleasanton, Calif.) by epifluorescence microscopy (6). For bacteria, the DAPI technique (24) was used, and chroococcoid cyanobacteria were counted by the autofluorescence of phycocyanin (5). For biovolume estimates, 100 organisms per sample were measured by eyepiece micrometer (standard deviation [SD] of duplicates, $\leq$20%), and volumes were calculated on the basis of lengths and widths, assuming a spherical or cylindrical shape.

Thymidine incorporation. It appeared necessary to sample low-oxygen meta- and hypolimnion water with a peristaltic pump and to perform thymidine incubations immediately after sampling. Storage for 1 h at the in situ temperature or on ice caused 50% reduced thymidine incorporation. A similar reduction was found when low-oxygen samples were aerated by using the Friedinger sampler.

In a field sample, thymidine incorporation was linear with time for 5 h with 5 nM thymidine. Higher concentrations of up to 30 nM did not increase incorporation. Thus, the thymidine concentration did not limit the incorporation rate (21). For cultures, 20 nM and occasionally also 200 nM was used, because yeast extracts in the medium may contain some (unlabeled) thymidine.

Two Formalin-killed blanks and five (or three) replicates were incubated for 30 min with [*methyl*-^{3}H]thymidine (3.3 TBq mmol^{-1}; Amersham Ltd., Amersham, United Kingdom). A thymidine concentration of 20 nM was used for 15-ml culture samples, and 5 nM was used for 40-ml field samples. The samples were fixed with 2% (final concentration) formaldehyde. Each replicate was split into three portions which were (i) extracted with ice-cold 5% TCA for 5 min, (ii) hydrolyzed at 60°C with 1 (or 0.5) N NaOH for 1 h, or (iii) hydrolyzed at 100°C with 20% TCA for 30 min as described by Riemann and Søndergaard (26) to obtain (i) total (cold-TCA-insoluble) macromolecules, (ii) DNA plus proteins, and (iii) proteins, respectively. This conventional acid-base hydrolysis may have serious shortcomings (27, 28) but facilitates comparison with most previous studies, which often reported a high fraction of label in DNA. The precipitated macromolecular fractions were collected on 0.2-μm-pore-size cellulose nitrate filters (Schleicher & Schuell, Inc., Keene, N.H.), which were dissolved in 1 ml of ethyl acetate. Then 10 ml of Instagel II (Packard Instrument Co., Inc., Rockville, Md.) was added, and radioactivity was assayed in a Packard Tricarb 4530 liquid scintillation counter. Counting efficiency was determined by automatic external standardization. The fraction of label incorporated into DNA was calculated on the basis of the difference between the hot-NaOH precipitate (ii) and the hot-TCA precipitate (iii) divided by the cold-TCA precipitate (i).

Conversion factor. On the basis of simultaneous measurements of production, determined from direct cell counts, and thymidine incorporation, an empirical conversion factor for calculation of bacterial production on the basis of thymidine incorporation can be obtained (15, 17, 25). Empirical conversion factors were determined with a grazer-free (filtered with a 1-μm-pore-size Nuclepore filter), mixed bacterial population from Lake Vechten. The absence of grazers was checked microscopically. The bacteria were grown on 0.001% (wt/vol) Knop solution (18) supplemented with 50 mg of yeast extract liter^{-1} (final concentration; Oxoid Ltd., London, United Kingdom) at 15°C in the dark in aerated two-stage continuous cultures (7) and a batch culture.

Exponentially growing bacteria from the latter were diluted 10-fold with sterile growth medium, and cell numbers (N) and incorporation rates (I) were monitored for 24 h. If bacterial growth and thymidine incorporation are closely coupled, linear regressions of ln N and ln I versus time must have equal slopes. The slope of either curve is then the specific growth rate (μ). Furthermore, the computed y intercept (b) of the incorporation regression, which is an estimate of the initial incorporation rate, can be used to calculate the conversion factor, C (8). According to Kirchman et al. (15), $C = [\mu \cdot N(0)]/e^b$, where $N(0)$ is the initial bacterial abundance.

In continuous cultures at steady state, bacterial production in stage 1 is $\mu \cdot N$, where μ equals the dilution rate, D, and production can simply be divided by the thymidine incorporation rate to obtain C. Thus, C was determined at specific growth rates of 0.007, 0.029, and 0.116 h^{-1}. In addition, bacterial DNA contents were determined fluorometrically by using Hoechst 33258 (22), and the frequency of dividing cells (FDC) of DAPI-stained bacteria was estimated as described by Hagström et al. (12). At least 1,000 cells and 100 fields per sample were counted.

FLB uptake. Bacteria from stage 2 of the continuous culture were concentrated by centrifugation (15 min; 22,000 × g; MSE High Speed 18; Measuring and Scientific Equipment Ltd.) and stained with 200 mg of 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (Sigma Chemical Co., St. Louis, Mo.) liter^{-1} (final concentration) for 2 h at 60°C as described by Sherr et al. (29). However, instead of sonication, a few minutes of vibration with a test tube mixer (Genie; The Vortex Manufacturing Co., Cleveland, Ohio) was used to disperse any remaining bacterial clumps. Thus, a suspension of 5.65×10^8 FLB ml^{-1} was obtained, and 2-ml portions were stored in a freezer. Before use, a portion was thawed, diluted to 15 ml with distilled water, and redispersed. On the lake, a 0.5-ml suspension containing 38×10^6 FLB was added per 14.5-ml tube with each field sample, resulting in a final concentration of 2.7×10^6 FLB ml^{-1}, which was 8 to 24% of the total bacterial abundance. Seven tubes per sampling depth were incubated, and for 30 min a tube was fixed every 5 min by injecting 1.5 ml of ice-cold 10% glutaraldehyde, resulting in a 1% final concentration, and put on ice. On the same day in the laboratory, the protozoa were collected on 1-μm-pore-size Nuclepore filters, stained with primulin, mounted on slides with immersion oil, and stored at −30°C (6). The increase in the average number of FLB

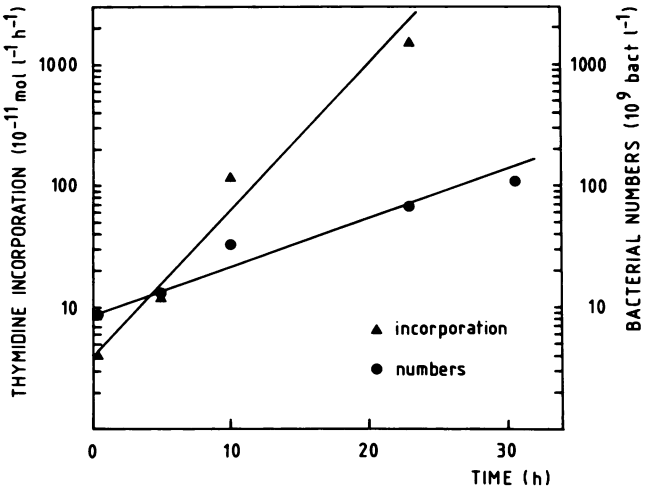

FIG. 1. Decoupling of bacterial growth and thymidine incorporation into cold-TCA-insoluble macromolecules in an exponentially growing batch culture diluted 10-fold.

cell^{-1} with time was determined by epifluorescence microscopic examination of 100 cells per time point sample, and protozoan ingestion rates were calculated. In the first experiment, the effect of fixation on FLB uptake was compared by using 1 and 2% glutaraldehyde and van der Veer fixative (30), which is toxic and explosive.

RESULTS

In the 10-fold-diluted exponentially growing batch culture, the bacterial numbers increased 8-fold in 23 h (Fig. 1), yielding a μ of 0.09 h^{-1}, and a conversion factor of 17 $\times$ 10^{18} bacteria mol of thymidine^{-1} was calculated. However, the thymidine incorporation rate (into cold-TCA-insoluble macromolecules) increased much faster (370-fold), indicating a μ of 0.26 h^{-1}. Thus, thymidine incorporation and growth (cell division) seemed to be decoupled. The 46-fold increase in thymidine incorporation per bacterium could not be explained by a major change in biovolume, and we supposed that unbalanced growth or a rapid increase in DNA contents might have occurred. Therefore, we switched to continuous cultures to determine thymidine incorporation rates into both total macromolecules and DNA during balanced growth at different rates. In addition, biovolumes, DNA contents, and FDC were determined.

In the continuous culture, both the biovolume and the DNA content increased with the growth rate, but the changes were relatively small (Table 1). A 17-fold higher growth rate resulted in a 1.5-fold bigger biovolume and a 3-fold higher DNA content. Also, the FDC increased with the growth rate, but the relationship appeared not to be linear. The differences in FDC were small at specific growth rates below 0.03 h^{-1}. Thus, at low growth rates, FDC is not a very sensitive measure of growth rate. Thymidine incorporation into DNA was found to be insignificant (Student t test; $P > 0.1$) and below 12% of the total incorporation (Table 2), except after addition of 200 nM thymidine at μ = 0.116 h^{-1}, when 63% of the label was found in the DNA fraction, and a conversion factor of 1.58 $\times$ 10^{18} bacteria mol^{-1} was calculated for incorporation into DNA. However, the total incorporations at 20 and 200 nM were similar, resulting in similar conversion factors for total incorporation (Table 1). Incorporation into total cold-TCA-insoluble macromolecules was proportional to the growth rate and yielded a relatively constant empirical conversion factor of around 10^{18} bacteria mol of thymidine^{-1}, which was used for field samples. To study unbalanced growth, the measurements were also performed within 1 generation time after the dilution rate had been shifted up. However, no great changes in conversion factor, DNA content, or biovolume were found.

On 29 September 1986, thymidine incorporation into total macromolecules in Lake Vechten showed a distinct peak at a 7-m depth (Fig. 2), just below the 6-m-deep oxycline in the lower metalimnion. With the conversion factor of 10^{18} bacteria mol^{-1}, a maximum production of 55 $\times$ 10^6 bacteria liter^{-1} h^{-1} and a specific growth rate of 0.009 h^{-1} were estimated. The same stratum showed maximum HNAN abundances of up to 40 $\times$ 10^6 HNAN liter^{-1} during the summer of 1986 (Fig. 3). In 1987, the metalimnion was ca. 2 m higher than in the previous years and therefore was sampled not at 7 m but at 5 m, where the oxygen concentration was below 0.5 mg liter^{-1} between 17 August and 21 September. On 28 September, it had increased to 7 mg liter^{-1}. Again, maximum HNAN numbers up to 11 $\times$ 10^6 HNAN liter^{-1} were found in the microaerobic metalimnion, whereas in the aerobic epilimnion (3-m depth) and the anaerobic hypolimnion (9-m depth) between 0.5 $\times$ 10^6 and 2.1 $\times$ 10^6 HNAN liter^{-1} were found (Fig. 4). Also, chroococcoid cyanobacteria (*Synechococcus* sp.; 1 to 2 μm in diameter) showed maximum numbers of up to 3.2 $\times$ 10^9 cells liter^{-1} in the metalimnion, in contrast to heterotrophic

TABLE 1. Empirical conversion factor for thymidine incorporation into total cold-TCA-insoluble macromolecules, DNA content, FDC, and biovolume of the bacteria in a continuous culture

μ (h^{-1}) or time[a]	Mean ± SD (no. of replicates)			
	Conversion factor (10^{18} bacteria mol^{-1})	DNA content (fg bacterium^{-1})	FDC (%)	Biovolume (μm^3 bacterium^{-1})
0.007	0.38 ± 0.11 (5)	4.86 ± 0.67 (3)	3.0 (1)	0.15 ± 0.00 (2)
20 h after shift up	1.06 ± 0.12 (5)	5.45 ± 0.00 (2)	3.2 ± 0.1 (2)	0.17 ± 0.01 (2)
0.029	1.42 ± 0.09 (5)	7.27 ± 1.14 (3)	3.6 ± 0.0 (2)	0.18 ± 0.00 (2)
	0.46 ± 0.03 (5)[b]			
6 h after shift up	ND[c]	10.10 ± 0.10 (3)	4.4 ± 0.0 (2)	0.19 ± 0.00 (2)
0.116	1.19 ± 0.14 (5)	14.20 ± 2.50 (3)	12.2 ± 0.6 (2)	0.23 ± 0.00 (2)
	1.00 ± 0.06 (5)[b]			

[a] Measurements were also performed within 1 generation time after shifting up of the dilution rate, when the new rate was assumed in the conversion factor calculation.
[b] Thymidine was added at 200 nM instead of 20 nM.
[c] ND, Not determined.

TABLE 2. Macromolecular distribution of ^3H following [*methyl*-^3H]thymidine incorporation by bacteria in a continuous culture[a]

μ (h^{-1}) or time	Mean ± SD (n = 5) [^3H]thymidine incorporation (10^8 dpm $liter^{-1}$ h^{-1}) in:			% of label in DNA
	Cold-TCA precipitate	Hot-NaOH precipitate	Hot-TCA precipitate	
0.007	3.41 ± 0.95	2.35 ± 0.72	2.11 ± 0.96	7
20 h after shift up	5.28 ± 0.62	3.68 ± 0.49	3.04 ± 0.47	12
0.029	4.12 ± 0.25	2.69 ± 0.13	2.56 ± 0.22	3
	13.50 ± 1.00[b]	7.52 ± 0.57	12.40 ± 0.60	−36
0.116	14.30 ± 1.70	9.15 ± 0.61	9.64 ± 0.48	−3
	17.10 ± 1.00[b]	14.70 ± 1.30	3.92 ± 0.23	63

[a] It was assumed that the cold-TCA, hot-NaOH, and hot-TCA precipitates represent total macromolecules, DNA plus proteins, and proteins, respectively.
[b] Thymidine was added at 200 nM instead of 20 nM.

bacteria, which showed similar numbers between 5.3×10^9 and 14.8×10^9 bacteria $liter^{-1}$ at all three depths.

The first FLB uptake experiment with epilimnion samples yielded nonsignificantly different ingestion rates of 5.2 ± 0.5 and 5.4 ± 0.7 bacteria $HNAN^{-1}$ h^{-1} after fixation with 1 and 2% glutaraldehyde (Fig. 5), whereas no FLB uptake was found with van der Veer fixative. Therefore, 1% glutaraldehyde was used in the following experiments. The average FLB biovolume was 0.13 μm^3 FLB^{-1}, similar to the 0.14 μm^3 $bacterium^{-1}$ found on 21 September 1987 for metalimnetic bacteria, which showed volumes between 0.10 and 0.27 μm^3 $bacterium^{-1}$ in 1985 (5). On 17 August 1987, the FLB uptake of metalimnion samples was measured in triplicate and showed little variation among the replicates (Fig. 6). Therefore, on 24 August and 7 and 21 September, single measurements were performed with epi-, meta-, and hypolimnion samples.

For epi- and hypolimnion samples, the ingestion rates were calculated on the basis of linear regressions of the number of FLB per HNAN against time. In metalimnion samples, however, the number of FLB $HNAN^{-1}$ did not show a linear increase with time at a constant rate, but the rate of increase decreased with time and became negative after ca. 20 min (Fig. 6), when the number of FLB $HNAN^{-1}$ decreased and apparently digestion and egestion exceeded ingestion. Because the ingestion rate appeared to decrease during incubation, we assumed that the ingestion rate was depressed by a hypothetical stress factor which increased linearly with time. The initial ingestion rate at $t = 0$ was then estimated as the intercept of a linear regression line of the change in FLB $HNAN^{-1}$ against time, because the differences in FLB $HNAN^{-1}$ between successive 5-min intervals decreased linearly with time. This stress model yielded ingestion rates from 9 to 17 bacteria $HNAN^{-1}$ h^{-1} for the metalimnion samples (Table 3), while simple linear regressions underestimated the initial ingestion rate and yielded values of 4.5 to 6.1 bacteria $HNAN^{-1}$ h^{-1}. For epi- and metalimnion samples, ingestion rates of 2 to 8 bacteria

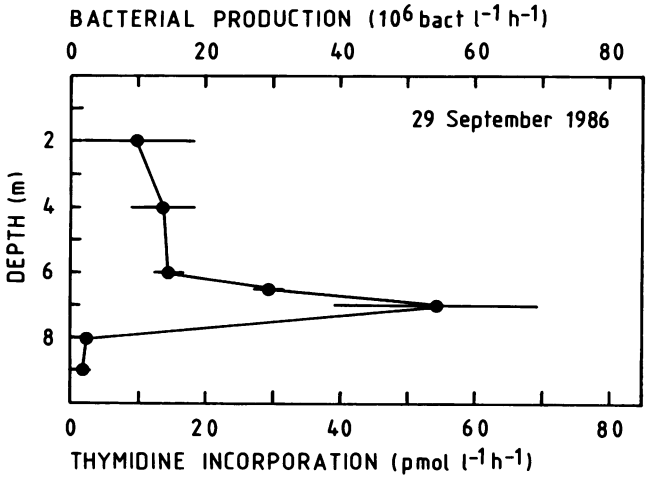

FIG. 2. Vertical distribution of bacterial production estimated on the basis of total thymidine incorporation (± SD; n = 3) in Lake Vechten.

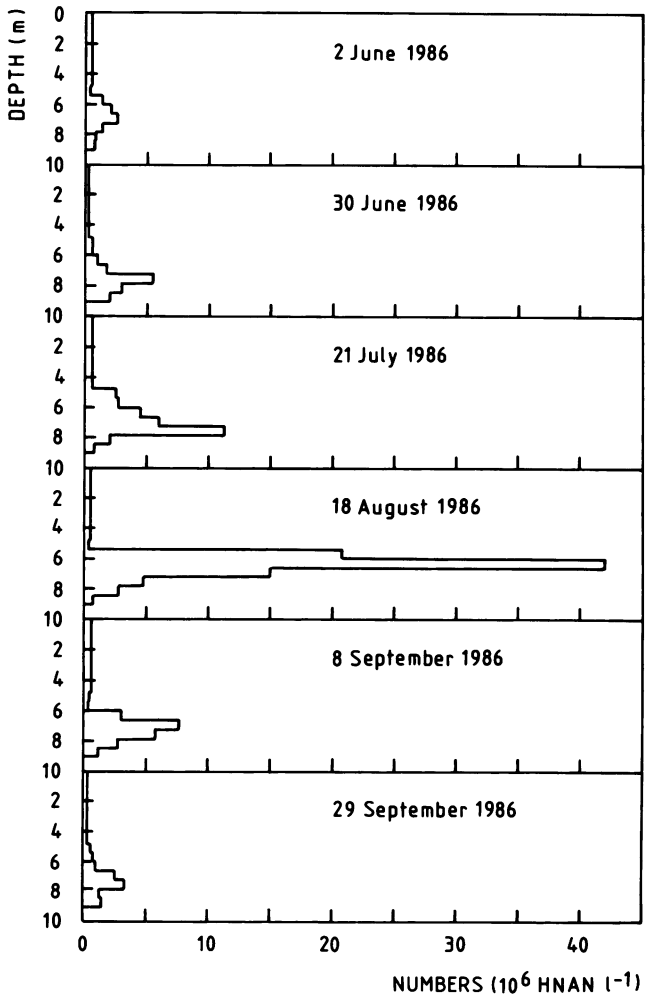

FIG. 3. Vertical distribution of HNAN in Lake Vechten during the summer of 1986.

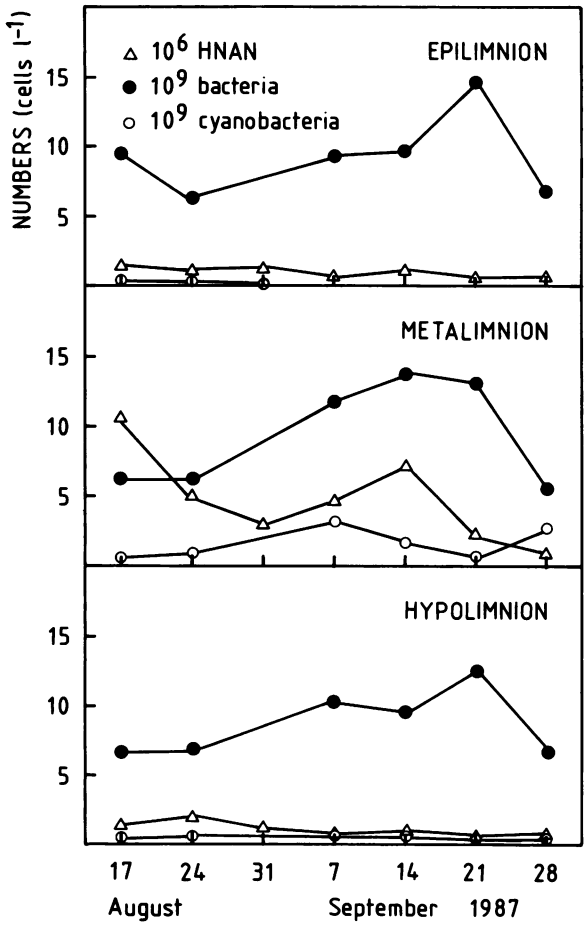

FIG. 4. Cell numbers of HNAN, bacteria, and chroococcoid cyanobacteria in the epi-, meta- and hypolimnion of Lake Vechten from August to September of 1987. Samples were taken at 3-, 5-, and 9-m depths.

$HNAN^{-1}$ h^{-1} were obtained. A similar ingestion rate was found for metalimnetic ciliates on 24 August (Table 3). However, since their numbers were 20-fold lower than those of the HNAN, their role in total protozoan bacterivory was negligible. In September, no FLB uptake by ciliates was found.

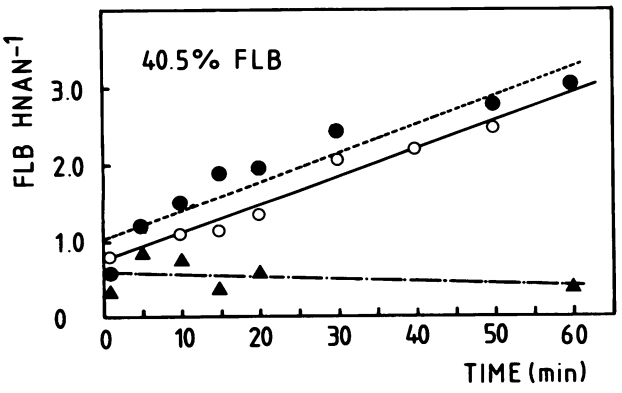

FIG. 5. FLB uptake by HNAN fixed with 1% (○) or 2% (●) glutaraldehyde or van der Veer fixative (▲).

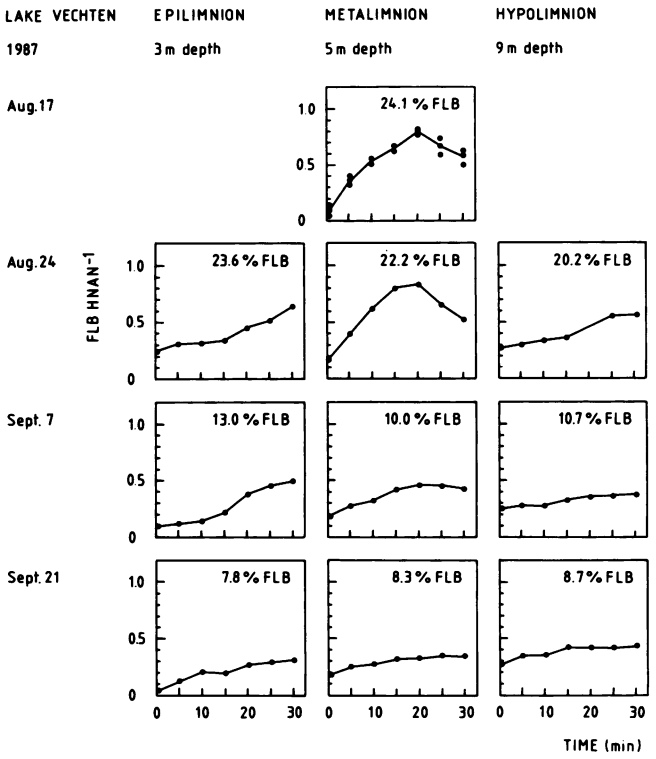

FIG. 6. FLB uptake by HNAN in stratified Lake Vechten from August to September of 1987.

On the basis of ingestion rates (Table 3) and HNAN numbers (Fig. 4), total protozoan grazing on Lake Vechten bacteria was estimated (Fig. 7). For dates when FLB uptake was not measured, the value of the nearest date or the average of the two nearest dates was taken. Thymidine incorporation was measured on all of the dates. Like the mixed bacterial population in the continuous culture, Lake Vechten bacteria generally showed no significant (Student t test; $P > 0.05$) thymidine incorporation into DNA at any sampling depth, although epilimnion samples showed a maximum of 17% incorporation into DNA on two dates (Table 4), as measured by acid-base hydrolysis. We used 1 N NaOH (26) to hydrolyze the so-called RNA fraction. Since 0.5 N NaOH has also been used (11, 27), we compared the two concentrations on 7 September 1987 with metalimnion samples. Both yielded no significant label in DNA. Thus, incorporation into total macromolecules had to be used to estimate production with the conversion factor established in the continuous culture. This purely empirical conversion factor is based not on assumptions about DNA synthesis but on direct comparison of thymidine incorporation into cold-TCA-insoluble macromolecules and cell production. The estimated bacterial production was of the same magnitude as the estimated protozoan grazing in the epi- and hypolimnion as well as in the metalimnion, but the latter showed maximum rates which were 1 order of magnitude higher than those in the other strata.

DISCUSSION

Decoupling of thymidine incorporation and growth. The thymidine technique is based on the assumption that thymidine incorporation measures bacterial DNA synthesis and is directly proportional to bacterial production (10, 11). Previ-

TABLE 3. Ingestion rates based on FLB uptake of HNAN and ciliates in the epi-, meta-, and hypolimnion of Lake Vechten from August to September 1987

Date	Mean ± SD ingestion rate[a]			
	Bacteria HNAN^{-1} h^{-1} in:			Bacteria ciliate^{-1} h^{-1} in metalimnion
	Epilimnion	Metallimnion	Hypolimnion	
17 August	ND	15.07 ± 4.38	ND	ND
24 August	2.65 ± 0.25	17.24 ± 5.46	2.27 ± 0.53	17.59 ± 13.00
7 September	5.82 ± 0.47	11.90 ± 4.32	2.27 ± 0.16	0
21 September	8.04 ± 0.66	9.18 ± 4.27	4.37 ± 0.46	0

[a] Samples were taken at 3-, 5-, and 9-m depths. ND, Not determined.

ous incubation studies showed a close correspondence between increases in thymidine incorporation rates and cell numbers in marine as well as freshwater systems (4, 15, 17, 25). However, in our batch culture, bacterial growth and thymidine incorporation seemed not to be coupled (Fig. 1). A similar decoupling was reported for oligotrophic oceanic bacteria by Ducklow and Hill (8). They concluded that the thymidine method is a useful tool but may involve some enticing mysteries associated with the biochemistry and physiology of thymidine incorporation and bacterial growth. They considered several possibilities which might explain the observed decoupling and suggested that oligotrophic bacteria may have the possibility to expand their DNA pools rapidly. However, the bacteria in our continuous cultures showed a relatively constant DNA content at different growth rates (Table 1). Alternatively, increasing cell size, isotope dilution, or incorporation into macromolecules other than DNA might cause apparent decoupling of thymidine incorporation and growth. The first possibility is unlikely, since the biovolume did not increase much with the growth rate (Table 1).

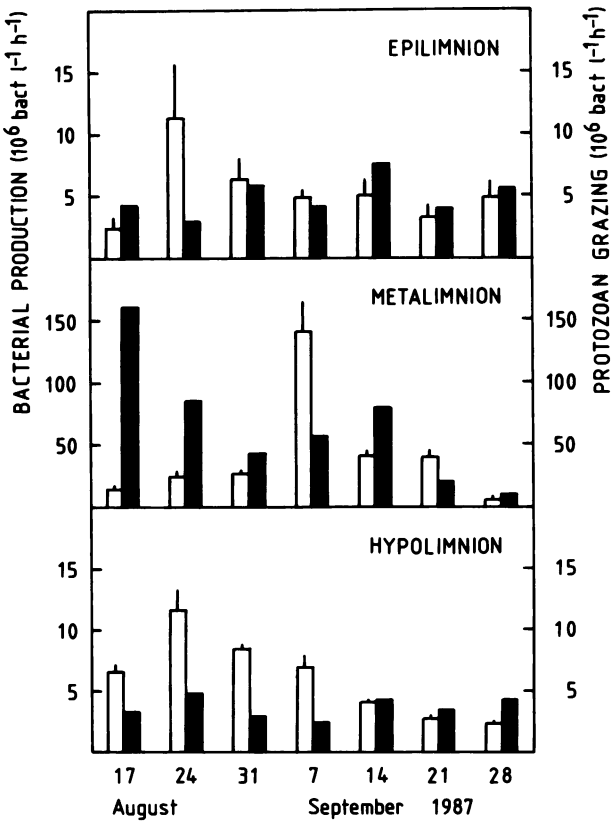

FIG. 7. Estimated bacterial production (open bars; error bars indicate 1 SD; $n = 5$) and protozoan grazing (filled bars) in stratified Lake Vechten for August to September of 1987. Samples were taken at 3-, 5-, and 9-m depths.

Isotope dilution, however, may have occurred in our batch culture, which showed a 46-fold increase in thymidine incorporation per bacterium in 23 h and a high initial conversion factor of 17×10^{18} bacteria mol^{-1}. Kirchman and Hoch (16) suggested that the most likely cause for high initial conversion factors and decoupling is extra- and intracellular isotope dilution. In our batch culture, exponentially growing bacteria were diluted 10-fold with fresh medium which may have contained unlabeled thymidine. This thymidine may have been assimilated by the growing bacteria, resulting in a decreasing concentration of unlabeled thymidine, decreasing isotope dilution, and increasing incorporation of labeled thymidine per bacterium. If such an artificial and decreasing isotope dilution caused the observed decoupling of thymidine incorporation and growth, the calculated high conversion factor is not ecologically relevant. Smits and Riemann (31) also found high conversion factors (average, 11.8×10^{18} bacteria mol^{-1}) in diluted batch cultures of freshwater bacteria at generation times below 20 h but not at longer generation times, at which an average of 2.15×10^{18} bacteria mol^{-1} was found. They hypothesized that in fast-growing bacteria, thymidine transport across the cell wall limits thymidine incorporation, resulting in intracellular isotope dilution and a high conversion factor. Such a high conversion factor was not found in our continuous culture at the shortest generation time of 6 h (Table 1).

Incorporation into macromolecules other than DNA also may have caused decoupling, because in the batch cultures of Ducklow and Hill (8), as well as in our batch cultures, only incorporation into total macromolecules was measured. In our continuous culture, usually no significant incorporation into DNA was found (Table 2), although in a previous study (7) the same hydrolysis procedure yielded 39 to 45% incorporation into DNA. Also, at all of the depths sampled in Lake Vechten, thymidine seemed not to be significantly incorporated into DNA (Table 4). Low (0 to 50%) incorporation into DNA has been reported, especially for deeper waters with low oxygen concentrations, whereas in aerobic surface samples often most (80%) of the label appeared in DNA (17, 19, 27). Sometimes also in surface samples very low (0%) incorporation into DNA has been found (13, 16). Robarts et al. (27) suggested that unbalanced growth may promote nonspecific macromolecule labeling, whereas balanced growth may be indicated by a high fraction of the label in DNA. This is not supported by our continuously cultured

TABLE 4. Macromolecular distribution of ^3H following [*methyl*-^3H]thymidine incorporation by bacteria in Lake Vechten[a]

Date (1987) and stratum	Mean ± SD (n = 5) [^3H]thymidine incorporation (10^5 dpm liter^{-1} h^{-1}) in:			% of label in DNA
	Cold-TCA precipitate	Hot-NaOH precipitate	Hot-TCA precipitate	
24 August				
Epilimnion	22.4 ± 9.20	8.66 ± 2.52	5.03 ± 2.54	16
Metalimnion	47.4 ± 6.70	27.5 ± 2.80	30.1 ± 3.40	−5
Hypolimnion	25.6 ± 3.20	6.11 ± 0.61	7.21 ± 0.49	−4
31 August				
Epilimnion	12.4 ± 3.40	12.9 ± 3.50	15.4 ± 10.0	−20
Metalimnion	54.8 ± 4.70	24.3 ± 1.50	23.9 ± 10.0	1
Hypolimnion	16.7 ± 0.50	6.76 ± 0.84	7.01 ± 2.26	−2
7 September				
Epilimnion	9.47 ± 1.21	6.96 ± 0.79	5.34 ± 0.89	17
Metalimnion	279 ± 46.0	233 ± 50	225 ± 27	3
Hypolimnion	13.9 ± 1.80	6.51 ± 0.83	7.65 ± 1.25	−8

[a] Samples were taken at 3-, 5-, and 9-m depths. It was assumed that the cold-TCA, hot-NaOH, and hot-TCA precipitates represent total macromolecules, DNA plus proteins, and proteins, respectively.

bacteria, which showed no significant DNA labeling during balanced growth at rates of 0.007 to 0.116 h^{-1}.

Conversion factors. On the basis of theoretical considerations, a conversion factor of about 0.2×10^{18} to 1.3×10^{18} bacteria mol^{-1} has been proposed for thymidine incorporation into DNA (10). Fuhrman and Azam (11) found empirical values of around 2×10^{18} bacteria mol^{-1} based on incorporation into DNA determined by acid-base hydrolysis. Although it is often recognized that thymidine incorporation into DNA should be measured (27), this is rather time consuming and usually only incorporation into total macromolecules has been determined. On the basis of incorporation into total macromolecules, empirical conversion factors of 1×10^{18} to 2×10^{18} bacteria mol^{-1} are generally found (17, 25, 31).

The empirical conversion factors found in the continuous culture for incorporation into macromolecules other than DNA (Table 1) are surprisingly close to the proposed values for incorporation into DNA (10). Since values between 0.38×10^{18} and 1.42×10^{18} bacteria mol^{-1} were found at growth rates ranging from 0.007 to 0.116 h^{-1}, a value of 10^{18} may be used to estimate the true growth rate within a factor of 2 or 3. Also, since the bacterial DNA content varied by a factor of 3, no more accuracy could be expected if all bacterial DNA synthesis could be measured by thymidine incorporation into purified DNA. However, measurement of only incorporation into DNA by conventional acid-base hydrolysis may seriously underestimate the real production of growing populations which seem to incorporate little or no tritiated thymidine into DNA, like Lake Vechten bacteria at all sampling depths. In meta- and hypolimnetic samples from Lake Oglethorpe, McDonough et al. (19) also found high thymidine incorporation into macromolecules but only a low fraction in DNA. In those samples, not incorporation into DNA but incorporation into total macromolecules yielded the most realistic production estimates compared with leucine incorporation and FDC.

Using conventional acid-base hydrolysis, we and sometimes others (13, 16, 27) found no significant incorporation of [methyl-^3H]thymidine into DNA. Still, we found reasonable (10, 25, 31) empirical conversion factors for incorporation into total macromolecules. A possible explanation may be that the measured incorporation into total cold-TCA-insoluble macromolecules really represents incorporation into DNA but that acid-base hydrolysis produces serious artifacts. The hot NaOH and hot TCA precipitates were assumed to represent DNA plus proteins and proteins, respectively, and the difference would yield the fraction of label in DNA (11). Servais et al. (28) found that this procedure is not specific enough. They showed that about 50% of labeled DNA was not hydrolyzed by hot-TCA extraction for 30 min, while 30% of a labeled protein was hydrolyzed. This suggests that the so-called protein fraction contains labeled DNA. This hot-TCA precipitate contained 61 ± 22% (± SD; n = 6) of the label in the continuous culture (Table 2) and 57 ± 31% (n = 9) in the field samples (Table 4). Alternatively, if acid-base hydrolysis yields reliable fractionation of macromolecules, incorporation into macromolecules other than DNA would be proportional to cell production (Table 1). However, it is not immediately clear how thymidine incorporation into other macromolecules can yield a conversion factor of 10^{18} bacteria mol^{-1}, because isotope dilution may be expected to cause a much higher conversion factor.

The bacterial DNA contents of 5 to 14 fg $cell^{-1}$ measured in the continuous culture equal the values measured for environmental bacteria by Jeffrey and Paul (14). On the basis of the measured DNA content (Table 1) and assuming 50% A · T base pair composition and an average molecular mass for a base pair of 624 g mol^{-1} (14) for our continuous culture, theoretical conversion factors of 0.09×10^{18} to 0.26×10^{18} bacteria mol^{-1} can be calculated. These values are 1.5- to 13-fold lower than the empirical conversion factors obtained, with the greatest difference at the highest growth rate. Thus, even if the measured thymidine incorporation into total cold-TCA-insoluble macromolecules would represent DNA synthesis only, the actual DNA synthesis would be significantly underestimated. Also, Jeffrey and Paul (14) reported that thymidine incorporation consistently underestimated DNA synthesis by a factor of 6 to 8. This indicates significant intracellular isotope dilution, which cannot be accounted for by standard isotope dilution assays and which may be expected if thymidine transport across the cell wall would limit incorporation into DNA (31). It may be hypothesized that not primarily thymidine incorporation into DNA but energy-dependent (21) thymidine uptake across the cell wall is more or less proportional to the growth rate. Therefore, the question of whether DNAs or other macromolecules are labeled may be of secondary importance. Thus, more research is needed on the biochemical and physiological bases of the thymidine method. Although the underlying mecha-

nism is not clear, the relationship between thymidine incorporation and cell production has been established empirically (17, 25, 31; Table 1) and can still be used to estimate bacterial production.

Production and grazing. Using the empirical conversion factor of 10^{18} bacteria mol^{-1} based on thymidine incorporation, we estimated bacterial production rates in Lake Vechten that varied from 2.4×10^6 to 141×10^6 bacteria $liter^{-1}$ h^{-1}. The estimated population doubling times were between 2.9 and 131 days (μ, 0.012 to 0.00022 h^{-1}). It is questionable whether these extremely low values represent growth or just biochemical turnover. The maximum production rates, which occurred in the metalimnion, were similar to values estimated for the metalimnion of Lake Oglethorpe (19).

In 1987, the maximum HNAN numbers (Fig. 4), and presumably also the grazing pressure on bacteria, were ca. threefold lower than in 1986 (Fig. 3) and 1985 (5). As in the previous years, maximum bacterial activity and HNAN numbers were found in the microaerobic metalimnion, which may be related to a metalimnetic primary production maximum (32). While the maximum rates in the metalimnion were 1 order of magnitude higher than those in the epi- and hypolimnion, in all three strata bacterial production appeared to be roughly balanced by HNAN grazing (Fig. 7). This seemed not to be true for the metalimnion on 17 August, when estimated grazing greatly exceeded estimated production. However, lags between production and grazing are possible, and HNAN may also consume food sources other than bacteria, such as chroococcoid cyanobacteria.

The protozoan grazing rates estimated on the basis of FLB uptake were probably not underestimated because of glutaraldehyde fixation. This method yielded accurate grazing estimates in two-stage continuous cultures (7), showing ingestion rates (ca. 9 bacteria $HNAN^{-1}$ h^{-1}) similar to those found in glutaraldehyde-fixed field samples (Table 3). Moreover, no FLB uptake was observed with van der Veer fixative (Fig. 5), in contrast to the findings of Sieracki et al. (30). The reason for this discrepancy is not clear, but addition of ice-cold glutaraldehyde and immediate cooling of the samples on ice may be important (R. W. Sanders, personal communication).

The estimated in situ ingestion rates of 2 to 17 bacteria $HNAN^{-1}$ h^{-1} (Table 3) are at the low end of the range reported in the literature. Similar low rates of 0.4 to 25 bacteria $HNAN^{-1}$ h^{-1} were estimated on the basis of direct cell counts in seawater (2) and with bacterium-sized fluorescent microspheres (20, 23). Much higher rates of up to 254 bacteria $HNAN^{-1}$ h^{-1} were calculated on the basis of direct counts in batch cultures (9). Even though the individual ingestion rates are relatively low, HNAN are the main consumers of bacterial production in stratified Lake Vechten, where maximum rates of bacterial production and protozoan grazing occur in the microaerobic metalimnion.

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