BACTERIAL PRODUCTION, PROTOZOAN GRAZING, AND MINERALIZATION IN STRATIFIED LAKE VECHTEN

Cover: Heterotrophic nanoflagellates (Monosiga sp.) from Lake Vechten under the epifluorescence microscope. The primulin-stained cells with a diameter of about 6 micrometer, show yellow-white fluorescence and have one flagellum surrounded by a collar. The red cells are autofluorescent chroococcoid cyanobacteria with a diameter of 1 to 2 micrometer.

Omslag: Heterotrofe nanoflagellaten (Monosiga sp.) uit de plas Vechten onder het epifluorescentie microscoop. De met primuline gekleurde cellen, met een diameter van ongeveer 6 micrometer, fluoresceren geel-wit en bezitten een kraag rond de flagel. De rode cellen zijn autofluorescerende cyanobacteriën met een diameter van 1 tot 2 micrometer.

STELLINGEN

1. Het belang van bacteriën in een oecosysteem hangt niet af van hun aantal maar van hun activiteit.

2. De relatie tussen thymidine-inbouw en bacteriële groei berust waarschijnlijk niet op de DNA synthese, in tegenstelling tot de heersende opvatting (D. J. W. Moriarty. 1986. Measurement of bacterial growth rates in aquatic systems from rates of nucleic acid synthesis. Adv. Microbial Ecol. **9**:245-292).

3. Bacteriële afbraak moet niet worden verward met mineralisatie (F. B. van Es. 1984. Decomposition of organic matter in the Wadden Sea. Netherlands Institute for Sea Research - Publication Series **10**:133-144).

4. Het terugdringen van protozoën leidt mogelijk tot helderder water (B. Riemann. 1985. Potential importance of fish predation and zooplankton grazing on natural populations of freshwater bacteria. Appl. Environ. Microbiol. **50**:187-193).

5. Statistiek maakt niet recht wat krom is (Prediker 1:15).

6. Aan gesaneerde gifgrond zit vaak nog een luchtje (R. Buisman en A. van Pelt. Grondreiniging moet beter. Natuur en Milieu, November 1987 :12-16).

7. Ruimtelijke ordening omvat het plannen van zaken die nooit zullen gebeuren, en het legaliseren van ontwikkelingen die al begonnen zijn.

8. Gezien de Belgische verdeeldheid, getuigt de keuze van Brussel als centrum van de Europese eenheid van een grenzeloos optimisme.

9. De redenering dat een hogere maximum snelheid leidt tot een lagere gemiddelde snelheid en minder milieuvervuiling, is even dwaas als het grote aantal 100-km-borden langs sommige weggedeelten.

10. Een goede stelling is een groot probleem.

Stellingen bij het proefschrift "Bacterial production, protozoan grazing, and mineralization in stratified Lake Vechten" van Jaap Bloem.

Wageningen, 20 oktober 1989.

Jaap Bloem

BACTERIAL PRODUCTION, PROTOZOAN GRAZING, AND MINERALIZATION IN STRATIFIED LAKE VECHTEN

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. H. C. van der Plas,
in het openbaar te verdedigen
op vrijdag 20 oktober 1989
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

Promotor: dr. A. J. B. Zehnder, hoogleraar in de microbiologie.
Co-promotor: dr. Th. E. Cappenberg, werkgroepleider, Limnologisch Instituut.

Cast your bread upon the waters,
for you will find it after many days.
(Ecclesiastes 11. 1)

CONTENTS

PREFACE

The research reported in this thesis was performed between August 1984 and June 1988, at the Limnological Institute of the Royal Netherlands Academy of Arts and Sciences in Nieuwersluis, The Netherlands. I am indebted to many people who contributed to this study. The subject was proposed by Thomas Cappenberg, who pleasantly stimulated and supervised the project, together with Alex Zehnder (Department of Microbiology, Wageningen Agricultural University). Excellent technical support was given by Marie-José Bär-Gilissen, who also drew most figures. Important contributions were provided by the students: Marion van Drunen, who conscientiously fixed and counted innumerable flagellates; Mathieu Starink, who enthusiastically evaluated methods for measuring protozoan grazing, and reached control of fluorescently labelled bacteria; and Frank Ellenbroek, who patiently worked on disentanglement of the thymidine incorporation method, and reached impressive activity in the radioisotope-laboratory.

Measurements with radioisotopes were kindly supported by Guus Postema, Willem de Kloet, and Klaas Siewertsen, in the laboratory as well as on the lake. Fast and safe transport to and from the lake was punctually performed by Heb Roon. Advice and support with chemical and physical analyses was provided by Harry Korthals, Kees Steenbergen, Ten Dekkers, Henk Verdouw, Anja Sinke, and Frans de Bles, and also by Wim Roelofsen and Mark van Loosdrecht (both of the Wageningen Agricultural University). Onno van Tongeren energetically created and solved statistical problems, and critically helped to improve manuscripts, together with the reviewers acknowledged in the papers. Thanks are also due to Ed Mariën for very accurate photography, to Cecilia Kroon for typing some large tables, and to all other colleagues of the Limnological Institute for (incidental) support and a pleasant working atmosphere.

I thank Claudia Albert and Thomas Berman (Kinneret Limnological Laboratory, Israel) for the fruitful cooperation, and Tom Fenchel and Ilse Jensen (University of Aarhus, Denmark), who kindly introduced me in protozoology. I enjoyed the hospitality in the U.S.A., and the interesting discussions with a.o. Bob Sanders and Karen Porter (University of Georgia, Athens), David Kirchman (University of Delaware, Lewes), Michael Pace (Institute of Ecosystem Studies, Millbrook, New York), John Hobbie (Marine Biological Laboratory, Woods Hole. Massachusetts), and Joel Goldman (Woods Hole Oceanographic Institution).

Finally, I thank my colleagues of the Institute for Soil Fertility (Haren, The Netherlands) for giving me the opportunity to finish this thesis.

INTRODUCTION

Stratified Lake Vechten

Lake Vechten (52°04'N and 5°5'E) is situated in the central part of The Netherlands, near the city of Utrecht. The lake was formed in 1941 by excavation of sand used for the construction of a nearby highway. Since the foundation of the Limnological Institute in 1957, Lake Vechten has been a major research site of the Vijverhof Laboratory (Gulati and Parma, 1982).

The lake is small with a surface area of 4.7 ha, and is slightly eutrophic, about 10 m deep and monomictic, i.e. stratified in summer (Steenbergen and Verdouw, 1982). In years with a severe winter and a long period of ice cover, stratification occurs also in winter (i.e. dimictic). In early spring, the lake is well mixed and has a relatively low temperature of about 5°C and a high oxygen concentration of about 11 mg $O_2.l^{-1}$ in the whole water column. From late April onwards, the water is warmed up starting at the surface. Since the density of water is greatest at 4°C, the warming up results in a lower density of the upper water layer, which prevents mixing with deeper colder layers. Thus, in May a stable thermal stratification is established, which lasts to the end of October or the beginning of November when the upper stratum cools down, becomes heavier and is mixed again with the deeper strata.

During summer stratification, three strata can be distinguished. The layer between the surface and a depth of about 5 m is the epilimnion, with a water temperature of about 20°C and a high oxygen concentration (Fig. 1, chapter 3). In the metalimnion, roughly between 5 and 8 m depth, both temperature and oxygen concentration show a steep decline (thermocline and oxycline). Due to the steep density gradient of the thermocline, this layer is very stable. The anaerobic bottom layer with a temperature of 8 to 10°C is the hypolimnion.

Microbial activity in the metalimnion

During 1969 and 1970, Cappenberg (1972) studied the distribution of specific bacterial groups in Lake Vechten by counting viable bacteria grown on selective media. Because on any culture medium only a small fraction of the natural bacteria can be grown, viable counts yield much lower numbers than direct microscopical counts. However, viable counts are considered as a relative measure of active bacteria. Due to the dispersion of bottom detritus (dead organic particles) and nutrients in spring, increasing numbers of

heterotrophic bacteria were found in the bottom layers of the lake, depleting the oxygen in the hypolimnion by their metabolic activities. In summer, the largest numbers of viable heterotrophic bacteria were found in the metalimnion, which was attributed to the gradients of oxygen tension and available organic matter.

In the years 1977 to 1980 during summer stratification, Blaauboer et al. (1982) and Steenbergen and Korthals (1982) found the highest chlorophyll concentrations and primary production rates in the metalimnion of Lake Vechten. In this stratum the phytoplankton was dominated by Synechococcus sp. (Cyanobacteria) and Mallomonas caudata (Chrysophyceae). Maxima of phytoplankton biomass and primary production near the thermocline can be explained by an equilibrium between downward light penetration and upward nutrient flux at this site (Sorokin, 1981; Lovell and Konopka, 1985). Moreover, sedimenting plankton and detritus may accumulate at the thermocline, due to reduced sinking velocities in the pronounced density gradient (Cappenberg, 1972; Herndl and Malačič, 1987). Because accumulation of detritus as well as high primary production may promote the growth of heterotrophic bacteria, a high bacterial production may be expected in the metalimnion. However, the depth distribution of bacterial abundance and production in stratified lakes with an anaerobic hypolimnion has not been examined thoroughly (McDonough et al., 1986).

The microbial loop

Realistic estimates of bacterial numbers and production can now be obtained by recently developed techniques (Van Es and Meyer-Reil, 1982). Bacteria can be stained with fluorescent dyes and counted directly by epifluorescence microscopy (Hobbie et al., 1977). Thymidine incorporation and frequency of dividing cells (FDC) are proportional to growth rate, and are considered to be the most promising indicators of in-situ bacterial production rates (Azam et al., 1983). The frequency of dividing cells (Hagström et al., 1979) is tedious to determine and difficult to calibrate. Tritium-labelled thymidine incorporation (Fuhrman and Azam, 1980; 1982) is relatively rapid, sensitive, specific for heterotrophic bacteria, and most widely used. Thymidine is a precursor of DNA synthesis, which is incorporated into DNA by thymidine-kinase. This enzyme has not been found in other organisms than heterotrophic bacteria (Moriarty, 1986). A major problem of the thymidine technique is the determination of the conversion factor needed to convert thymidine incorporation to bacterial production.

Heterotrophic aquatic bacteria utilizing dissolved organic matter, which mainly originates from phytoplankton, were found to

sustain high growth rates. Although population doubling times (generation times) of less than 1 day have often been reported (Van Es and Meyer-Reil, 1982; Moriarty, 1986; Pace, 1988), the variation in bacterial numbers is relatively small, suggesting a sink of bacteria. Azam et al. (1983) hypothesized that actively growing marine bacteria (size 0.2-2 µm) are kept below a density of 5 to 10 x 10^9 bact.l^{-1} primarily by heterotrophic nanoflagellates (HNAN, size 2-20 µm), which reach abundances of around 10^6 HNAN.l^{-1}. HNAN are in turn consumed by microzooplankton, such as ciliates, of the same size range as the larger phytoplankton (20-200 µm). Thus, energy released as dissolved organic matter by phytoplankton may be returned to the main food chain via a microbial loop of bacteria-heterotrophic nanoflagellates-microzooplankton. In the conventional concept of aquatic ecosystems, only carbon and energy flow via the main food chain of phytoplankton-zooplankton-fish was recognized. Previously, the role of smaller organisms was largely ignored because of technical limitations.

Protozoan grazing and mineralization

There are mechanical limitations to the size difference between predator and prey. A 15-m-long whale feeding on krill smaller than 10 cm is an extreme example. Compared with a bacterium smaller than 1 µm, however, a 150-µm-long ciliate is a protozoan whale (Fenchel, 1986). In contrast to bigger zooplankton, HNAN are very efficient bacterivores because of their small size and high maximum growth rates, which are close to those of their prey. Evidence for protozoan control of bacterial numbers in-situ was presented by Fenchel (1982), who observed predator-prey oscillations between marine bacteria and HNAN in Limfjorden (Denmark), with a lag of 4 days between bacterial and flagellate peaks. It was calculated that HNAN are capable of filtering 10 to 70% of the water column per day.

Johannes (1965) reported that the mineralization of dissolved inorganic phosphate from organic detritus proceeds faster and more completely in the presence of bacteria and protozoa than in the presence of bacteria alone. He suggested that the grazing protozoa keep the bacteria in a prolonged state of physiological youth, resulting in an increased rate of organic matter decomposition. Similarly, Sherr et al. (1982) observed that the decomposition of phytoplankton cells (Peridinium cinctum, Dinophyceae) was accelerated when HNAN were present. Although the mechanisms of this stimulation were not exactly identified, their results suggested that protozoan grazing increased the availability of mineral nutrients, especially phosphorus, to bacteria which

degrade mineral-poor detritus. Thus, HNAN can play a dual role in aquatic ecosystems: (1) they can be a link in the food chain between small bacteria and algae on the one hand and bigger (micro)zooplankton on the other, and (2) they can accelerate the decomposition and mineralization of organic matter.

Protozoa in freshwater

Whereas most studies on the role of HNAN have been performed in the sea, little is known about these protozoa in freshwater. Stratified lakes may be especially interesting because of the steep physical, chemical and biological gradients. Peak population densities of ciliates have been found at or just below the oxic-anoxic boundary (Finlay, 1981; Pace and Orcutt, 1981; Finlay and Fenchel, 1986a,b). The oxic epilimnion is avoided by the ciliates and their numbers decrease with depth in the anoxic hypolimnion. The ciliate Loxodes sp. shows peak abundances at extremely low oxygen tensions (less than 0.05 mg $O_2.l^{-1}$) or even in anoxic water. This behaviour may be a compromise between avoidance of high (toxic) oxygen concentrations and a periodic requirement for aerobic metabolism. Although Loxodes can switch to nitrate respiration under anaerobic conditions, the meta- and hypolimnetic ciliates are probably aerobes and cannot survive indefinite in anoxia (Bark and Goodfellow, 1985).

The formation of metalimnetic ciliate peaks has the obvious benefit that it brings cells into a zone where competition and predation by bigger metazoan zooplankton are probably unimportant, because the latter avoid these microaerobic and anaerobic zones (Finlay and Fenchel, 1986a,b). Moreover, in this zone bacteria and sedimenting particles tend to accumulate, and the algal primary and bacterial secondary production tend to be high. Thus, the metalimnion seems to be a layer with a high food availability and a relatively low predation pressure. Although predatory ciliates cannot be avoided by HNAN in the metalimnion, these benefits may also apply to HNAN. However, the vertical distribution of HNAN in stratified lakes has hardly been studied. Near the thermocline in Dalnee Lake (Kamchatka), Sorokin and Paveljeva (1972) reported a HNAN peak of 0.75×10^6 HNAN.l^{-1}. This number may have been an underestimate, however, because the flagellates were counted alive. Living HNAN are very fragile and hard to see without fluorescent staining.

Aim of the thesis

This study is aimed at the role of HNAN in grazing on bacteria and in mineralization of organic matter in stratified Lake Vechten. When the research was started in 1984, several methods had been published for fixing and counting of HNAN. However, effects of these manipulations on HNAN had hardly been checked, although it had often been recognized that nanoflagellates are very fragile. Therefore quantitative effects of several fixatives, and of filtration and centrifugation, on HNAN were investigated (chapter 2).

During summer stratification in 1985, the vertical distribution and temporal variation in numbers of chroococcoid cyanobacteria, bacteria, HNAN, and ciliates in Lake Vechten were investigated to obtain indications for predator-prey relationships (chapter 3). In addition, bacterial activity was estimated from thymidine incorporation, and potential grazing on bacteria by HNAN was estimated from experiments with selectively filtered lake water incubated in the laboratory.

Batch incubations with selectively filtered Lake Vechten water (with bacteria alone and with bacteria and protozoa) were also used to study effects of protozoa on the breakdown of organic carbon, and the mineralization of inorganic phosphorus and nitrogen from dead chroococcoid cyanobacteria (Synechococcus sp.) (chapter 4). Also ingestion rates of HNAN and ciliates grazing on bacteria were estimated.

Estimates from dynamic batch cultures with rapidly changing conditions, however, are rather crude. Much more accurate estimates of grazing and mineralization can be obtained with continuous cultures in steady-state, which facilitate maintenance of stable low growth rates, small bacterial biovolumes and low bacterial concentrations, similar to values found in the lake. Therefore, HNAN grazing on bacteria was quantified in two-stage continuous cultures at various specific growth rates, and effects of protozoan grazing on carbon, phosphorus and nitrogen mineralization rates were examined (chapter 5). Moreover, gross growth efficiencies (yields) and carbon and phosphorus contents of bacteria and protozoa were estimated. Protozoan ingestion rates determined in continuous culture were compared with results of independent, recently published techniques for determination of in-situ protozoan grazing. Thus, the validity of ingestion rate measurements with procaryotic inhibitors, bacteria-sized fluorescent microspheres, and fluorescently labelled bacteria was evaluated.

Problems with the thymidine technique were also studied in continuous culture. At various specific growth rates, bacterial DNA synthesis was compared with thymidine incorporation, and an

empirical conversion factor was obtained to estimate in-situ bacterial production (chapter 6). During summer stratification in 1987, bacterial production rates estimated from thymidine incorporation were compared with protozoan grazing rates estimated with fluorescently labelled bacteria, in the epi-, meta- and hypolimnion of Lake Vechten.

Finally (chapter 7) the role of bacteria and protozoa in the microbial food web of Lake Vechten is discussed.

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Fixation, Counting, and Manipulation of Heterotrophic Nanoflagellates

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Quantitative effects of several fixatives on heterotrophic nanoflagellates (HNAN) and phototrophic nanoflagellates (PNAN) were investigated by hemacytometer and epifluorescence counting techniques. Counts of *Monas* sp. cultures before and after fixation with unbuffered 0.3% glutaraldehyde and 5% formaldehyde showed no loss of cells during fixation, and cell concentrations remained constant for several weeks after fixation. Buffering of fixatives with borax caused severe losses, up to 100% within 2 h. Field samples from Lake Vechten showed no decline of HNAN and total nanoflagellate concentrations for at least 1 week after fixation with 5% formaldehyde and with 1% glutaraldehyde. With 1% glutaraldehyde, the chlorophyll autofluorescence of PNAN was much brighter than with 5% formaldehyde, although it was lost after a few days and thus limited the storage time of samples. However, when primulin-stained slides were prepared soon after fixation and stored at −30°C, the loss of autofluorescence was prevented and PNAN and HNAN concentrations were stable for at least 16 weeks. Effects of filtration and centrifugation on HNAN were also studied. Filtration vacuum could not exceed 3 kPa since 10 kPa already caused losses of 15 to 20%. Similar losses were caused by centrifugation, even at low speed (500 × *g*).

Heterotrophic nanoflagellates (HNAN; size, 2 to 20 μm) are now being recognized as major consumers of bacterial production and as catalysts for mineralization in aquatic ecosystems (4, 9, 18, 21, 31). Quantitative field studies on HNAN and phototrophic nanoflagellates (PNAN) have been permitted by recently developed epifluorescence microscopic counting techniques (2, 5, 10, 20). Since nanoflagellates are very fragile (20, 23), loss of cells as a result of fixation and manipulation before counting must be prevented. Moreover, chlorophyll autofluorescence must be preserved for distinction between phototrophic and heterotrophic cells.

Although various fixatives are used (11, 26, 29), buffered (2, 20, 22) and unbuffered aldehydes are the most common in quantitative studies. The highest final concentration was used by Fenchel (7), who could keep samples fixed with 5% unbuffered formaldehyde for at least 1 month. However, formaldehyde is reported to cause loss of flagella, distortion of cell shape, and loss of cells in many flagellates (16, 27). Some authors recommend a buffered (8, 26) or unbuffered (28) mixture of 1% glutaraldehyde (killing-fixing agent) and 0.03 to 1% paraformaldehyde (preservative) as the best fixative for fragile flagellates. Haas (10) considered a low concentration to be an advantage and used 0.3% glutaraldehyde.

Despite the variability in fixation methods, hardly any quantitative control experiments have been published. Tsuji and Yanagita (28) used the percentage of unbroken cells after fixation as an index for evaluation of fixatives and found 100% unbroken cells in a mixture of 1% glutaraldehyde and 0.03 to 1% paraformaldehyde. However, the presence of perfectly preserved cells does not prove that no losses occur during fixation. Therefore, counts of living flagellates before fixation should be compared with counts at several times after fixation. We know of only one such study, which was performed with electronic particle counters and showed drastic losses of cells in both autotrophic and heterotrophic marine flagellates with all fixatives used (13).

This paper describes quantitative effects of several fixatives on HNAN and PNAN from laboratory cultures as well as freshwater field samples. In addition, effects of filtration and centrifugation on HNAN are discussed.

MATERIALS AND METHODS

Monas sp. (6 to 10 μm) and *Bodo* sp. (6 to 10 μm) were isolated from Lake Vechten, The Netherlands (25), by successive dilutions from enrichment cultures and cultured in 0.025% Knop solution (15) enriched with 100 mg of yeast extract · liter^{-1} (Oxoid). *Chlorella* sp. (3 μm) was isolated from the same lake, and *Haematococcus pluvialis* (20 μm) was isolated from a gutter at our institute. The last two species were cultured in mineral medium no. 11 as described by Allen (1). Field samples from Lake Vechten were obtained with a 5-liter Friedinger sampler from a depth of 4.8 to 6.0 m.

Fixatives at various final concentrations were prepared from stocks of 35 to 38% formaldehyde stabilized with 8 to 13% methanol, 50% glutaraldehyde (Baker grade) in distilled water, and paraformaldehyde powder. In some experiments, fixatives were buffered at pH 7 with 0.01 M phosphate buffer (Na_2HPO_4 plus KH_2PO_4) or at pH 8 with borax ($Na_2B_4O_7 \cdot 10H_2O$) (24) or hexamine (26).

The staining procedure for nanoflagellates was a modification of the method of Caron (2). The fluorochrome primulin (Janssen Chimica, Belgium) was dissolved in distilled water buffered at pH 4 with 0.1 M Trizma hydrochloride (Sigma Chemical Co., St. Louis, Mo.). Initially, a primulin concentration of 250 mg · liter^{-1} was used, which was lowered to 63 mg · liter^{-1} later (see Results). The solution was prepared fresh and was filtered through a 0.2-μm-pore-size membrane filter (Schleicher & Schüll) before each use. Fixed samples (5 to 10 ml) were filtered onto a 1.0-μm-pore-size Nuclepore polycarbonate filter (diameter, 25 mm), prestained with Dylon no. 8 ebony black dye, at a vacuum not exceeding 3 kPa. A 1.2-μm-pore-size

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TABLE 1. Hemacytometer (volume, 0.8 mm^3) counts of living HNAN from three *Monas* cultures

Sample	No. of *Monas* cells/0.8 mm^3[a] for culture:		
	I	II	III
1	141	165	102
2	138	162	85
3	143	174	107
4	140	171	106
5	128	155	115
6	140	175	87
7	133	166	110
8	151	170	109
9	142	164	87
10	142	164	89

[a] For cultures I through III, there were 1.75×10^8, 2.08×10^8, and 1.25×10^8 cells · $liter^{-1}$, respectively.

Schleicher & Schüll membrane filter was used as a backing under the Nuclepore filter. The filter was then rinsed twice with 2 ml of 0.2-μm-filtered Trizma buffer (pH 4.0) and flooded with 2 ml of primulin solution. The vacuum was removed, and the sample was allowed to stain, initially for 15 min and later for 5 min (see Results). The stain was then filtered through, and the filter was rinsed four times with 2 ml of Trizma buffer. The removed filter was mounted with immersion oil between a glass slide and a cover glass. Primulin staining on 0.2-μm Nuclepore filters sometimes resulted in long filtration times and heavy staining of the whole filter surface, making counts impossible. These problems, probably owing to clogging, did not occur with 1.0-μm filters.

The slides were examined at ×1,000 magnification by epifluorescence microscopy in a dark room with a Zeiss Universal II microscope equipped with a Neofluar 100× objective lens, an HBO 50 mercury lamp, and a filter housing (Zeiss 466249-9904) which allowed the rapid exchange of four filter sets. The following filter sets were used: for primulin, a BP 365 exciter filter, an FT 395 beam splitter, and an LP 397 barrier filter (Zeiss filter set 487701); for chlorophyll *a*, a BP 450-490 exciter filter, an FT 510 beam splitter, and an LP 520 barrier filter (Zeiss filter set 487709); and for phycobilins, a BP 546 exciter filter, an FT 580 beam splitter, and an LP 590 barrier filter (Zeiss filter set 487715) (19, 30). For each water sample, at least 100 fields were counted on each of two filters. Usually, between 250 and 500 cells were counted per filter.

Nanoflagellates were centrifuged with an MSE High Speed 18 centrifuge (Measuring & Scientific Equipment Ltd.) equipped with rotor no. 69181 (8 by 50 ml; angle, 30°) at 5°C, and with a Fixette table centrifuge equipped with a swing-out rotor at room temperature.

RESULTS

Counting of living HNAN. To study quantitative effects of fixation, HNAN concentrations before and after fixation should be compared. Therefore, a technique was needed to count living HNAN. This is possible with an electronic particle counter if the medium in which the cells are suspended has a fairly high conductivity, like seawater (12). For freshwater samples, this implies the addition of an electrolyte to a final concentration of at least 0.6%. In two experiments, we added 0.6% Ringer solution (containing, in grams per liter, NaCl, 5.44; KCl, 0.254; $CaCl_2$, 0.109; $NaHCO_3$, 0.121; and citric acid, 0.082, final concentrations) to cultures of *Monas* sp. and found a loss of ca. 20% within 2 h, which increased up to ca. 70% within 20 h. Therefore, freshwater HNAN did not withstand the addition of low electrolyte concentrations and consequently could not be counted with an electronic particle counter.

However, it appeared to be possible to accurately count living, actively swimming HNAN from cultures with a hemacytometer (Table 1). The 95% confidence limits did not exceed 10% of the mean (means ± 95% confidence limits for three cultures were 140 ± 4.4, 167 ± 4.3, and 100 ± 8.2 cells per 0.8 mm^3) if the cell concentrations were in a suitable range (resulting in 5 to 10 cells per field) and counting was performed fast. Each of 10 replicate samples was counted at ×400 magnification within 5 min. If the samples were allowed to stand in the hemacytometer for longer than 10 min, the counts were drastically reduced because the cells migrated to the edges of the cover glass, probably owing to oxygen depletion.

Fixation of cultures. Hemacytometer counts of living HNAN were not possible with field samples because relatively high cell concentrations are required. Therefore, fixation experiments were started with cultures of *Monas* sp. The live cells were counted by hemacytometer before fixation. After fixation, counts were performed by hemacytometer as well as epifluorescence microscopy. In the first experiment, however, the fluorescence counts appeared to be up to 45% lower than the hemacytometer counts. One cause of the differences was the distribution of HNAN over the effective filter area. Near the edges, the HNAN densities per field were as low as 10% of the densities in the central filter area. These low densities may be explained by penetration of the sample under the edges of the filter tower. When the edges were excluded from the calculation, ca. 30% higher HNAN concentrations were obtained. Counting was therefore not started as soon as HNAN were observed but at a distance of 20 fields from the edges.

Another cause of differences was the rinsing procedure with Trizma buffer during staining. When the rinse fluid was sucked down until the meniscus just reached the filter surface (5), lower HNAN concentrations were obtained than when the meniscus was kept 5 mm above the filter. With a *Monas* culture, the first method yielded $(0.53 \pm 0.12) \times 10^8$ cells · $liter^{-1}$ (mean ± standard deviation, $n = 2$). The second method yielded $(0.80 \pm 0.08) \times 10^8$ cells · $liter^{-1}$ ($n = 5$), which agreed well with hemacytometer counts of $(0.90 \pm 0.11) \times 10^8$ cells · $liter^{-1}$ ($n = 4$). Thus hemacytometer and fluorescence counts yielded identical results if the meniscus was not allowed to reach the filter surface during rinsing and if the edges of the filter area were not taken into account.

In the first fixation experiment, three fixatives were tested: 0.3% glutaraldehyde, a borax-buffered mixture of 1% glutaraldehyde and 1% paraformaldehyde (pH 8), and 5% formaldehyde (final concentrations). Hemacytometer counts showed no loss of cells during 4 weeks after fixation with 0.3% glutaraldehyde and 5% formaldehyde. In the buffered mixture, however, 40% of the cells were lost after 2 weeks. Because of the disagreement between hemacytometer and fluorescence counts in the first experiment, it was repeated, with good agreement between the two counting methods. The results were similar to those of the first experiment (Table 2). With 0.3% glutaraldehyde and 5% formaldehyde, the heterotrophic *Monas* cells were well preserved for several weeks, whereas cells in the borax-buffered mixture showed considerable losses.

For distinguishing between heterotrophic and photo-

TABLE 2. *Monas* concentrations before and after fixation[a]

Fixative[a]	Counting method[b]	*Monas* concn (10^8 cells/liter): Before fixation[c]	After fixation[d] 2 h	1 day	1 wk	2 wk	4 wk	% Loss after 4 wk
A	F		1.15 ± 0.04	1.15 ± 0.00	1.18 ± 0.06	1.19 ± 0.01	1.17 ± 0.01	6
	H	1.25 ± 0.14	1.16 ± 0.04	1.17 ± 0.02	1.19 ± 0.07	1.15 ± 0.04	1.29 ± 0.06	0
B	F		1.11 ± 0.01	1.04 ± 0.08	0.98 ± 0.02	0.97 ± 0.00	0.84 ± 0.02	33
	H	1.25 ± 0.14	1.11 ± 0.05	1.05 ± 0.01	1.05 ± 0.08	1.04 ± 0.06	0.93 ± 0.05	26
C	F		1.20 ± 0.01	1.17 ± 0.01	1.16 ± 0.01	1.18 ± 0.03	1.00 ± 0.06	20
	H	1.25 ± 0.14	1.23 ± 0.04	1.17 ± 0.09	1.24 ± 0.01	1.18 ± 0.04	1.11 ± 0.02	11

[a] Fixatives: A, 0.3% glutaraldehyde; B, 1% glutaraldehyde plus 1% paraformaldehyde, buffered by borax (pH 8.2); C, 5% formaldehyde.
[b] Abbreviations: F, fluorescence; H, hemacytometer.
[c] Values are given as mean ± standard deviation of 10 determinations.
[d] Values are given as mean ± standard deviation of two determinations.

trophic nanoplankton cells in field samples, a good preservation of chlorophyll autofluorescence is also required. This was studied in a third experiment, with a mixture of heterotrophic *Monas* and phototrophic *Chlorella* cells, both from cultures. Because of the previous results, 0.3% glutaraldehyde and 5% formaldehyde were tested again. In addition, phosphate-buffered 0.3% glutaraldehyde (pH 7) and borax-buffered 5% formaldehyde (pH 8) were used. Hemacytometer as well as fluorescence counts showed no considerable loss of *Monas* cells after 4 weeks with three of the four fixatives (Table 3). With borax-buffered formaldehyde, however, all cells were lost within 2 h. The hemacytometer counts showed no loss of *Chlorella* cells during 4 weeks with all fixatives used. The fluorescence counts of *Chlorella* were lower than the hemacytometer counts, probably owing to dead cells, which lack chlorophyll autofluorescence. The chlorophyll autofluorescence was preserved for a maximum of 1 week with 0.3% glutaraldehyde. The phosphate buffer did not improve the results. The autofluorescence was completely lost after 4 weeks. In 5% formaldehyde, the autofluorescence of *Chlorella* cells was preserved for several weeks, although it was hard to see with borax buffer.

Fixation, staining, and storage of field samples. Cultures showed the best preservation of cells and autofluorescence with 5% formaldehyde. With 0.3% glutaraldehyde, cell outlines tended to fade after a few weeks and chlorophyll autofluorescence was lost more rapidly. To obtain a better preservation, the glutaraldehyde concentration was raised to 1% in experiments with field samples, which were counted by epifluorescence microscopy after fixation with 1% glutaraldehyde and 5% formaldehyde (Table 4). HNAN and PNAN were distinguished by chlorophyll autofluorescence. However, some of the cells showed no bright autofluorescence and were classified as doubtful cases. Their autofluorescence was not improved when the fixatives were buffered at pH 8 with hexamine. HNAN and total nanoflagellate concentrations were constant for 1 week with both

TABLE 3. Concentrations of heterotrophic (*Monas*) and phototrophic (*Chlorella*) nanoplankton cells before and after fixation

Fixative[a]	Species	Counting method[b]	Concn (10^8 cells/liter)[c]: Before fixation	After fixation 2 h	1 day	1 wk	2 wk	4 wk	% Loss after 4 wk
A	*Monas*	F		1.15 ± 0.08	1.23 ± 0.00	1.22 ± 0.01	1.21 ± 0.08	1.02 ± 0.02	15
		H	1.20 ± 0.26	1.17 ± 0.06	1.20 ± 0.00	1.21 ± 0.07	1.21 ± 0.10	1.19 ± 0.09	1
	Chlorella	F		0.89 ± 0.01	0.82 ± 0.00	0.82 ± 0.10	0.55 ± 0.08	0.00 ± 0.00	100
		H	1.18 ± 0.09	1.08 ± 0.01	1.05 ± 0.03	1.08 ± 0.07	1.06 ± 0.04	1.08 ± 0.07	9
Ab	*Monas*	F		1.21 ± 0.02	1.23 ± 0.03	1.20 ± 0.00	1.23 ± 0.01	1.24 ± 0.04	0
		H	1.20 ± 0.26	1.16 ± 0.03	1.20 ± 0.06	1.24 ± 0.03	1.20 ± 0.02	1.19 ± 0.04	1
	Chlorella	F		0.88 ± 0.01	0.78 ± 0.01	0.71 ± 0.01	0.47 ± 0.11	0.00 ± 0.00	100
		H	1.18 ± 0.09	1.05 ± 0.06	1.10 ± 0.08	1.10 ± 0.03	1.06 ± 0.04	1.08 ± 0.04	9
C	*Monas*	F		1.27 ± 0.03	1.23 ± 0.01	1.19 ± 0.04	1.29 ± 0.02	1.21 ± 0.01	10
		H	1.35 ±0.16	1.38 ± 0.01	1.38 ± 0.04	1.38 ± 0.10	1.36 ± 0.10	1.34 ± 0.01	1
	Chlorella	F		1.18 ± 0.10	1.10 ± 0.01	1.03 ± 0.08	1.12 ± 0.01	0.98 ± 0.00	27
		H	1.35 ± 0.18	1.33 ± 0.03	1.27 ± 0.04	1.24 ± 0.06	1.15 ± 0.03	1.18 ± 0.04	13
Cb	*Monas*	F		0.23	0.06	0.07 ± 0.02	0.06 ± 0.01	0.00	100
		H	1.35 ± 0.16	0.00	0.00	0.00	0.00	0.00	100
	Chlorella	F		1.15	0.69	0.89 ± 0.04	0.90 ± 0.04	0.99 ± 0.10	27
		H	1.35 ± 0.18	1.36 ± 0.02	1.29 ± 0.08	1.33 ± 0.13	1.37 ± 0.00	1.18 ± 0.04	13

[a] Fixatives: A, 0.3% glutaraldehyde; Ab, 0.3% glutaraldehyde plus phosphate buffer (pH 7.0); C, 5% formaldehyde; Cb, 5% formaldehyde plus borax buffer (pH 8.2).
[b] Abbreviations: F, fluorescence; H, hemacytometer.
[c] Mean ± standard deviation of two determinations.

TABLE 4. Fluorescence counts of nanoplankton from Lake Vechten (sampling depth: 4.8 to 6.0 m, 7 May 1985) at several times after fixation

Fixative	Cell type	10^6 Cells/liter at time after fixation[a]:		
		1 h	1 day	1 wk
1% Glutaraldehyde	HNAN	2.89 ± 0.01	2.65 ± 0.05	2.73
	PNAN	3.51 ± 0.05	0.97 ± 0.11	0.09
	?NAN[b]	1.15 ± 0.10	4.43 ± 0.23	5.55
5% Formaldehyde	HNAN	2.41 ± 0.32	2.47 ± 0.17	2.49
	PNAN	0.63 ± 0.35	0.37 ± 0.05	0.00
	?NAN[b]	4.24 ± 0.04	4.93 ± 0.27	5.49

[a] Values are given as mean ± standard deviation of two determinations.
[b] ?NAN, Doubtful cases (autofluorescence not clear).

fixatives. However, chlorophyll autofluorescence was lost rapidly, resulting in low PNAN counts and a high fraction of doubtful cases. The brightest autofluorescence of PNAN was observed with 1% glutaraldehyde, although it was lost after 1 day.

The loss of autofluorescence was studied with a cultured phototrophic flagellate, *H. pluvialis*. This species showed bright autofluorescence in water 5 days after fixation with either 1% glutaraldehyde or 5% formaldehyde. Trizma buffer (pH 4.0) did not affect autofluorescence. However, autofluorescence strongly decreased when primulin was added, dissolved either in Trizma buffer or in distilled water. Since primulin seemed to cause loss of autofluorescence, the staining procedure was modified. Reduction of staining time from 15 to 5 min and of primulin concentration from 250 to 63 mg · liter^{-1} strongly reduced the fraction of cells showing doubtful autofluorescence in field samples (Table 5). The ratio of doubtful cases to PNAN was reduced from 0.57 to 0.08, whereas HNAN and total nanoflagellate concentrations were not affected. Thus bright autofluorescence of PNAN was achieved up to 2 days after fixation with 1% glutaraldehyde. After 6 days, the chlorophyll autofluorescence had been lost, although the autofluorescence of phycobilins was still intact. The fixed samples were stored at 5°C in a refrigerator.

Because the chlorophyll autofluorescence of PNAN was preserved for only a few days, the effect of storage in a freezer was studied. Field samples were fixed with 1% glutaraldehyde immediately after being taken, and the staining procedure was performed within 1 day. On the day of sampling (t = 0), 3 slides were counted and 25 slides were stored in a freezer at −30°C. At regular time intervals, three of the slides were taken out of the freezer and nanoflagellates were counted. HNAN and PNAN counts were constant for at least 16 weeks, and no decrease of autofluorescence was observed (Table 6).

Manipulation of HNAN. Regarding the fragility of HNAN and the required filtration in the staining procedure, the effect of filtration vacuum (i.e., the pressure differential over the filter) on HNAN was investigated. Exponentially growing cultures of *Monas* and *Bodo* spp. were fixed with 0.3% glutaraldehyde and counted 10 times by hemacytometer to obtain concentrations without filtration (vacuum, 0.00 kPa). HNAN in field samples could not be counted by hemacytometer because of their lower concentrations. After fixation with 5% formaldehyde, they were gravity filtered on 1-μm Nuclepore filters without vacuum applied (0.00 kPa) and counted in duplicate by epifluorescence microscopy. Further epifluorescence counts were performed of *Monas* sp., *Bodo* sp., and the field samples after filtration on 0.2-μm Nuclepore filters at 3, 10, and 30 kPa, respectively. At a vacuum exceeding 3 kPa, the cultures as well as the field samples showed considerable losses, between 15 and 36%, whereas no significant losses were observed at 3 kPa (Table 7). Therefore, a vacuum of 3 kPa was used as an upper limit for filtration of HNAN.

In addition, the effects of centrifugation on *Monas* and *Bodo* spp. were studied. Samples (15 ml) from exponentially growing cultures were centrifuged alive for 30 min in an MSE centrifuge at forces ranging from 500 × g (2,000 rpm) to 38,000 × g (18,000 rpm). After centrifugation, pellet and supernatant were mixed and the HNAN were fixed with 5% formaldehyde. Fixed samples were counted by hemacytometer before and after centrifugation. Even low-speed centrifugation (500 × g) caused significant losses (18 to 34%) of HNAN (Table 8). High-speed centrifugation (38,000 × g) destroyed 55% of the *Monas* cells and 98% of the *Bodo* cells.

TABLE 5. Effect of staining time and primulin concentration on nanoplankton counts (Lake Vechten, 21 May 1985)

Time after fixation (days)	Primulin concn (mg/liter)	Staining time (min)	10^6 cells/liter		
			HNAN	PNAN	?NAN[a]
1	250	15	7.35	2.62	1.49
1	250	10	6.53	2.95	0.87
1	250	5	7.11	3.24	0.28
2	250	5	6.70	2.93	0.91
2	125	5	6.83	3.70	0.66
2	63	5	7.00	3.84	0.30

[a] ?NAN, Doubtful cases.

TABLE 6. Counts of HNAN and PNAN (Lake Vechten, 12 November 1985) after up to 16 weeks storage in a freezer at −30°C

Storage time	Counts (10^6 cells/liter) of:	
	HNAN	PNAN
0	2.93 ± 0.24	0.96 ± 0.07
1 day	3.09 ± 0.04	0.98 ± 0.06
5 days	2.96 ± 0.12	1.04 ± 0.02
1 wk	3.00 ± 0.18	1.06 ± 0.06
2 wk	2.97 ± 0.12	1.00 ± 0.08
4 wk	2.93 ± 0.08	1.07 ± 0.07
8 wk	2.89 ± 0.07	0.97 ± 0.05
16 wk	2.89 ± 0.13	1.01 ± 0.06

TABLE 7. Effect of filtration vacuum on counts of *Monas* and *Bodo* cultures and HNAN from Lake Vechten

Filtration vacuum (kPa)	Counts (10^8 cells/liter) (% loss) of[a]:		Counts (10^6 cells/liter) (% loss) of HNAN
	Monas sp.	*Bodo* sp.	
0	2.19 ± 0.26[b] (0)	2.28 ± 0.26[b] (0)	3.06 ± 0.25 (0)
3	2.14 ± 0.10 (2)	2.15 ± 0.14 (6)	2.97 ± 0.16 (3)
10	1.79 ± 0.08 (18)	1.82 ± 0.07 (20)	2.60 ± 0.14 (15)
30	1.41 ± 0.04 (36)	1.64 ± 0.04 (28)	2.39 ± 0.30 (22)

[a] Values are given as mean ± standard deviation of two determinations.
[b] Values are given as mean ± standard deviation of 10 determinations.

For experimental purposes, it is desirable to increase HNAN concentrations and to separate HNAN from their growth medium. Therefore, living HNAN were concentrated by filtration, centrifugation, and settling. A 100-ml volume of a *Monas* culture was filtered down to a volume of 10 ml to obtain a 10-fold-higher cell concentration. However, on 1-μm Nuclepore filters, only a twofold concentration was achieved from $(1.39 \pm 0.05) \times 10^8$ to $(3.16 \pm 0.16) \times 10^8$ cells · liter^{-1} (mean ± 95% confidence limits, as counted by hemacytometer). At a vacuum not exceeding 3 kPa, the filtration took 2 to 3 h. Filtration was considerably faster on a 5-μm Nuclepore filter, but this did not raise the cell concentrations at all. Some of the cells passed the 5-μm filters and were observed in the filtrate. Microscopic observations of both 1- and 5-μm filters showed many HNAN sedimented on the filters. These cells could not be resuspended by gentle syringing. To prevent sedimentation of cells and clogging of the filter during filtration, the fluid above the filters was continuously stirred. Despite stirring, it was impossible to filter 100 ml of a *Bodo* culture because 1-μm as well as 5-μm filters became clogged. We were therefore not able to concentrate HNAN by filtration.

Centrifugation for 30 min at 1,000 × *g* in the swing-out rotor of the Fixette centrifuge resulted in the concentration of a *Monas* culture from $(2.37 \pm 0.36) \times 10^8$ to $(8.08 \pm 0.73) \times 10^8$ cells · liter^{-1} (mean ± 95% confidence limits). Since the pellet and the supernatant contained 96 and 4% of the cells, respectively, a good separation was achieved. However, 50% of the cells were lost during centrifugation. Losses were limited to 20% when a *Bodo* culture was centrifuged for 30 min at 500 × *g* in the angle rotor of the MSE centrifuge. Because the pellet contained only 31% of the cells, no good separation was achieved, although the cell concentration was raised from $(2.31 \pm 0.31) \times 10^8$ to $(9.03 \pm 1.38) \times 10^8$ cells · liter^{-1}. In conclusion, HNAN could be concentrated fourfold by low-speed centrifugation, but a good separation was not obtained without severe losses.

No losses occurred when 300-ml volumes of *Bodo* and *Monas* cultures were incubated in 35-cm high-settling cylinders at room temperature in the dark. After 24 h, both species showed a threefold concentration in the bottom 30 ml, but this bottom volume contained only 29% of the *Monas* cells and 55% of the *Bodo* cells and therefore yielded a poor separation.

TABLE 8. Effect of centrifugation on *Monas* and *Bodo* cultures

Centrifugal force (× *g*)	Counts (10^8 cells/liter) (% loss) of *Monas* sp.[a]	Counts (10^7 cells/liter) (% loss) of *Bodo* sp.[a]
0	5.25 ± 0.13 (0)	7.43 ± 0.86 (0)
500	3.45 ± 0.23 (34)	6.06 ± 0.30 (18)
1,000	3.75 ± 0.19 (29)	4.86 ± 1.05 (35)
5,000	4.10 ± 0.24 (22)	2.31 ± 0.21 (69)
10,000	2.82 ± 0.16 (46)	0.74 ± 0.20 (90)
38,000	2.34 ± 0.11 (55)	0.14 ± 0.07 (98)

[a] Values are given as mean ± 95% confidence limits.

DISCUSSION

Fragility of HNAN. HNAN appeared to be very vulnerable to common manipulations such as centrifugation and filtration. Although, as far as we know, no control experiments have been published, different authors used different upper limits for the filtration vacuum. Sherr and Sherr (20) used a vacuum of not more than 0.7 kPa, well below our upper limit of 3 kPa which yielded no losses of HNAN in our experiments (Table 7). However, higher values (7 kPa [10], 13 kPa [2, 3, 31], and 16 kPa [5]) have also been used. At 10 kPa, losses of 15 to 20% were found with cultures as well as field samples. Considerable losses, of at least 20%, were also caused by centrifugation, even at low speed (500 × *g*) (Table 8). Since the *Bodo* sp. showed substantially higher losses than did the *Monas* sp., centrifugation of field samples may cause shifts in species composition. Determination of species composition in field samples requires concentrated samples of live HNAN which can be observed by light microscopy and fixed by electron microscopy. For this purpose, Fenchel (7) centrifuged 0.5-liter water samples until a ca. 1-ml particle concentrate remained. For comparison, we concentrated 15 ml to 1 ml at 500 to 1,000 × *g* and found losses between 20 and 50%. At 1,000 × *g*, *Monas* sp. showed a loss of 29% in the angle rotor (Table 8), whereas 50% of the cells were lost in the swing-out rotor. The higher loss in the latter rotor can be explained by higher hydrostatic pressures which are generated in a swing-out rotor. Despite the risks of centrifugation, it still seems to be the best method available for concentrating HNAN.

Fixation of HNAN. Sherr and Sherr (20) fixed nanoplankton with 2% borax-buffered formaldehyde. The stored samples did not lose counts after 2 weeks, but the total number of heterotrophs and autotrophs did decline, by 46 and 18%, respectively, after 4 weeks. Borax-buffered fixatives also caused losses in our experiments. Within 2 weeks of fixation by a borax-buffered mixture of 1% glutaraldehyde and 1% paraformaldehyde, up to 40% of the *Monas* cells were lost (Table 2). With 5% borax-buffered formaldehyde, and thus a higher borax concentration, all *Monas* cells were completely destroyed within 2 h of fixation, whereas unbuffered 5% formaldehyde did not cause losses (Table 3). Borax tends to produce an internal swelling of crustacea, which makes them turgid (24) and may therefore also cause collapse of nanoflagellates. Cell recovery and chlorophyll autofluorescence were neither decreased nor improved by buffering with hexamine and phosphate buffer. Therefore, buffers were omitted.

Counts before and after fixation with unbuffered 0.3% glutaraldehyde and 5% formaldehyde showed no losses of

Monas cells during fixation, whereas cell concentrations in the stored samples remained constant for several weeks after fixation (Tables 2 and 3). Field samples showed no decline in HNAN and total nanoflagellate concentrations for at least 1 week after fixation with 1% glutaraldehyde and 5% formaldehyde (Table 4). Losses during fixation could not be checked, because live counts before fixation could not be made with field samples. However, instantaneous losses during fixation are not probable, because cell concentrations of *Oxyrrhis marina* and other flagellates decreased only slightly (up to 11%) during and soon after fixation, whereas after 24 h, up to 75% of the cells had disappeared (13). The decrease continued over time.

Klein Breteler (13) and Sorokin (23) concluded that naked protozooplankton cells are hardly preserved, if at all, in common fixatives. Quantitative controls with particle counters showed drastic losses of both heterotrophic and autotrophic flagellates after fixation with low concentrations of acetic acid, trichloroacetic acid, sublimate, benzoic acid-5-hydroxy-sulfo, and Lugol iodine solution (13). According to Taylor (26), Lugol iodine produces a high residue of unidentifiable nanoplankton cells and cannot compare with 2 to 5% glutaraldehyde in its capacity to fix flagella and cell contents. However, Pomroy (17) obtained comparable counts of marine microprotozoans from samples preserved with Lugol iodine and glutaraldehyde. Cultures of fragile *Pavlova lutheri* and *Prorocentrum triestinum* gave 96% of unbroken cells after fixation with 1% glutaraldehyde, whereas reduction of the glutaraldehyde concentration resulted in a considerable decrease in the percentage of unbroken cells (28). This indicates that fixatives at concentrations that are too low may kill cells without fixing them. In our experiments, 0.3 to 1% glutaraldehyde yielded a good fixation.

According to Porter et al. (18), formaldehyde is highly disruptive to soft flagellates and ciliates. The cell shape may become distorted, and flagella may be thrown off in many flagellates (27). Only very weak formaldehyde solutions, with a final concentration below 3%, should be used (26). The concentration of the stock solution of formaldehyde added should not exceed 4%, because the addition of concentrated stock solution to a sample exposes many cells to highly concentrated preservative before mixing is effective, and these cells will be destroyed or become unrecognizable (26). However, as suggested by Fenchel (7), we added concentrated (38%) formaldehyde to our samples; this resulted in a final concentration of 5%, and we found no loss of HNAN cells or flagella. Only cells with visible flagella were counted as HNAN. HNAN and total nanoflagellates in field samples showed similar concentrations after fixation with 5% formaldehyde and 1% glutaraldehyde (Table 4). Although ruminal protozoa may be less vulnerable than planktonic protozoa, the total counts of ruminal protozoa were not affected when the formaldehyde concentration was raised from 4 to 10 or even 18.5% (6). Therefore, no detrimental effects of formaldehyde were observed in our experiments.

Fixation of PNAN. In field samples, the estimate of HNAN concentrations depends upon an accurate estimation of PNAN concentrations (2, 5). Since PNAN cannot always be recognized by shape, a good preservation of chlorophyll autofluorescence is required. Although HNAN could be preserved for several weeks, the autofluorescence of PNAN was lost more rapidly and thus limited the storage time of samples. In field samples, 5% formaldehyde strongly decreased the autofluorescence of PNAN, whereas 1% glutaraldehyde yielded a much brighter autofluorescence and thus higher PNAN concentrations (Table 4, 1 h after fixation). Therefore, 1% glutaraldehyde was used for fixation of field samples. Unfortunately, the autofluorescence was lost after 2 days of storage at 5°C in a refrigerator. A drop in the fluorescence of chlorophyll pigments at 2 days after fixation for samples preserved with 1% formaldehyde was also noted by Davis and Sieburth (5). They used 1% glutaraldehyde when samples had to be counted more than 2 days after fixation. Algae fixed with 5% glutaraldehyde showed sufficient retention of chlorophyll pigments to allow counting after 9 months of sample storage at 9°C (30). The retention time of chlorophyll autofluorescence after fixation depends upon the type of organism. Cultured *Chlorella* sp. and *H. pluvialis* cells, as well as green algae and cyanobacteria in field samples, showed a much longer retention of chlorophyll autofluorescence than did PNAN in field samples from Lake Vechten. The loss of autofluorescence in PNAN was prevented when primulin-stained slides were prepared within 1 day of fixation and then stored in a freezer at −30°C. PNAN and HNAN concentrations were stable for at least 16 weeks (Table 6), and no decrease in the fluorescence intensity was observed. Haas (10) observed significant fading of fluorescence in proflavine-stained slides after 1 to 2 weeks of storage at −15°C. However, Landry et al. (14) observed an excellent condition of nanoplankton cells after 4 months of storage at −15°C: Quantitative data were provided by Tsuji and Yanagita (28), who noted a loss of only 10% of *Pavlova lutheri* cells with red chlorophyll autofluorescence after 2 months of storage at −20°C. Prolonged storage for nearly 6 months did not cause a further decrease in the fluorescent cell number. Their technique was more complicated than ours and involved mounting of filters with glycerol jelly and storage in a desiccator.

Concluding, HNAN were well preserved with 5% formaldehyde as well as 0.3 to 1% glutaraldehyde. With 1% glutaraldehyde, the chlorophyll autofluorescence of PNAN could be preserved for a few days, whereas 5% formaldehyde strongly decreased the autofluorescence. Prepared primulin-stained slides could be stored at −30°C for at least 16 weeks without loss of counts and autofluorescence.

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Bacterial activity and protozoan grazing potential in a stratified lake

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Abstract

During summer stratification in Lake Vechten, heterotrophic nanoflagellates (HNAN) showed peak densities at very low oxygen concentrations in the lower metalimnion at a depth of 6–7 m. In this stratum, high HNAN numbers ($>30 \times 10^6$ HNAN liter^{-1}) coincided with high bacterial activity. Assuming a conversion factor of 2×10^{18} bacteria mol^{-1} thymidine, we estimated a maximal bacterial production of about 4×10^9 bacteria liter^{-1} d^{-1} from thymidine incorporation. In the upper metalimnion (5–6-m depth) we found a low bacterial activity (estimated production 1×10^8 bacteria liter^{-1} d^{-1}) and low HNAN numbers (3×10^6 HNAN liter^{-1}). HNAN seem to be a link in the food web between bacteria and cyanobacteria on the one hand and ciliates on the other. Grazing experiments with selectively filtered lake water indicated ingestion rates of about 7 bacteria HNAN^{-1} h^{-1}. We calculated that the metalimnetic HNAN are able to consume the entire bacterial production even at high bacterial growth rates.

Increasing evidence suggests that an important part of the carbon and energy flow in aquatic ecosystems is channeled through the microbial loop in planktonic food webs (Azam et al. 1983; Porter et al. 1985). Dissolved organic matter, mainly from phytoplankton, is used as a carbon and energy source by heterotrophic bacteria, which can sustain high growth rates. Although population doubling times of <1 d have been reported (Van Es and Meyer-Reil 1982), the temporal variation in bacterial abundance is relatively small, suggesting a sink of bacteria. Azam et al. (1983) hypothesized that the bacterial production is grazed, primarily by heterotrophic nanoflagellates (HNAN, size 2–20 μm), which in turn are consumed by microzooplankton (size 20–200 μm) such as ciliates. Thus carbon and energy released as dissolved organic matter by phytoplankton may be returned to the main food chain via a microbial loop of bacteria-HNAN-microzooplankton. The efficiency of this process depends on the yield (gross growth efficiency) of the grazers.

Fenchel (1982*b*) observed a coupling between numbers of bacteria and HNAN in Limfjorden (Denmark) that suggested a predator–prey relationship. He calculated that, per day, between 10 and 70% of the water column was cleared of bacteria by HNAN. Grazing experiments with seawater samples indicated that HNAN control bacterial densities in situ (Andersen and Fenchel 1985). Most studies on the role of HNAN have been performed in marine systems; little is known about these Protozoa in freshwater systems. Stratified lakes may be especially interesting, because peak densities of Protozoa (ciliates) have been observed near the oxycline in the metalimnion (Bark and Goodfellow 1985; Finlay and Fenchel 1986). Maximal bacterial activities have also been found in the metalimnion (Cappenberg 1972; Lovell and Konopka 1985).

The aim of our study is to investigate the role of HNAN in grazing on bacteria in stratified Lake Vechten, The Netherlands. We studied the vertical distribution and temporal variation in numbers of bacteria, chroococcoid cyanobacteria, HNAN, and ciliates. Concurrently, bacterial production was estimated by [^{3}H-methyl]thymidine incorporation. In addition, grazing experiments with selectively filtered lake water were performed to provide a crude estimate

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of ingestion rates and yield of HNAN grazing on bacteria.

Materials and methods

Samples—Lake Vechten is a small (4.7 ha), monomictic, mesotrophic, deeply dug pit (max depth, 11.9 m; avg depth, 6 m). The lake is isolated, protected from wind, stratified from May until October, and shows hypolimnetic oxygen depletion (Steenbergen and Verdouw 1982). In the eastern depression over the deepest area, water samples were collected with a Friedinger sampler (capacity, 5 liters; length, 0.60 m). All samples were taken between 0900 and 1000 hours. Before each sampling, temperature and oxygen profiles were measured in situ with an Orbisphere oxygen indicator model 2607 (probe 2112).

Field samples were fixed immediately with 1% glutaraldehyde. HNAN were counted directly on 1-μm pore-size Nuclepore filters by epifluorescence microscopy at 1,000× magnification after primulin staining with a BP 365, FT 395, LP 397 filter set (Bloem et al. 1986). We counted ciliates with the same method on 5-μm Nuclepore filters at 400× magnification. Bacteria and chroococcoid cyanobacteria were counted at 1,000× magnification on 0.2-μm Nuclepore filters. For bacteria we used the DAPI technique (Porter and Feig 1980) with a final stain concentration of 1 μg ml^{-1}. Cyanobacteria were counted by autofluorescence of phycocyanin with a BP 546, FT 580, LP 590 filter set. No filtration exceeded a vacuum of 3 kPa (Bloem et al. 1986). For biovolume estimations, we measured 100 individuals per sample by eyepiece micrometer (SD of duplicates <20%) and calculated volumes from length and width, assuming a spherical or cylindrical shape. To study the qualitative composition of the HNAN population, we concentrated two 200-ml samples by centrifugation (1 h, max 1,000 × *g*) to about 2 ml (Fenchel 1982*b*). We observed 100 individuals alive per sample by phase contrast microscopy and identified them with several guides (Pascher 1914; Jahn 1949; Lackey 1959; Kudo 1966; Lee et al. 1985).

Total chlorophyll concentrations were determined spectrophotometrically after hot (80°C) extraction with 90% acetone (Steenbergen and Korthals 1982).

Bacterial production was estimated by measuring thymidine incorporation into cold trichloroacetic acid (TCA)-insoluble material (Fuhrman and Azam 1982). Water samples were stored on ice and transported to the lab, where incubations started ~1 h after sampling. On two dates the effect of 1 h of storage on ice was compared with 1 h of storage at in situ temperature; no significant differences (<13%) were found. Two Formalin-killed blanks and five replicates of 5 ml containing 5 nM [^{3}H-methyl] thymidine (3.3 TBq mmol^{-1}, Amersham) were incubated in acid-cleaned, 30-ml screwcap bottles in the dark at in situ temperature for 1.5–2 h. After incubation samples were fixed with 2% formaldehyde (final concn) and an equal volume of 10% ice-cold TCA was added. After 45 min of extraction on ice, the cold TCA-insoluble material was collected by filtration on 25-mm-diameter, 0.2-μm pore-size cellulose nitrate filters (Schleicher and Schuell). The filters were then rinsed three times with 5 ml of ice-cold 5% TCA and placed in scintillation vials. To hydrolyze the DNA, we added 0.5 ml of 0.5 N H_3PO_4 and heated the vials at 100°C for 1 h. After cooling, 1 ml of ethylacetate was added to dissolve the filters. Then 10 ml of Insta-Gel II (Packard) was added and radioactivity assayed in a Packard Tricarb (model 4530) liquid scintillation counter. Counting efficiency was determined by automatic external standardization.

From the amount of thymidine incorporated we calculated bacterial production, assuming a conversion factor of 2×10^{18} cells mol^{-1} of thymidine (Fuhrman and Azam 1982; Bell et al. 1983; Lovell and Konopka 1985; Moriarty 1986). We tried to determine an empirical conversion factor for Lake Vechten bacteria by comparison of thymidine incorporation and increases in cell numbers in 1-μm-filtered, 10-fold diluted lake water (Kirchman et al. 1982; Lovell and Konopka 1985). Although metalimnion samples showed a considerable initial thymidine incorporation, filtered and diluted samples showed no significant increase of bacteria within 24 h, even after enrichment. This finding was the same for

aerobic, microaerophilic (N_2 + 5% air), and anaerobic incubations. Thus a conversion factor was not obtained. A time-course experiment with upper metalimnion samples showed linear thymidine incorporation during 5 h, at a concentration of 5 nM. When higher thymidine concentrations, up to 30 nM, were added, thymidine incorporation did not increase. Thus isotope dilution by external pools did not occur, and thymidine concentration was not limiting incorporation rate (Moriarty 1986).

Grazing experiments—To estimate HNAN ingestion rates, we performed grazing experiments with upper metalimnion samples. In neither field samples nor exponentially growing HNAN cultures were bacteria-sized fluorescent particles (McManus and Fuhrman 1986) ingested during short (<1 h) incubations. After selective filtration (Wright and Coffin 1984), no significant growth and grazing were observed within 24 h. Therefore, following Andersen and Fenchel (1985), long-term (1–2 weeks) experiments were done, the first in June and the second in August 1985. Two samples of 500 ml each were filtered gently (≤3 kPa) through Nuclepore filters with pore sizes of 10 and 1 μm. The 10-μm filtrate contained both bacteria and HNAN. The 1-μm filtrate contained bacteria only and served as a grazer-free control. HNAN were not found in the 1-μm filtrates at the start or during the course of the experiments. Ciliates—potential HNAN grazers—were absent from the cultures. Both filtrates were enriched, in June with 1 mg of yeast extract per liter and in August with 10 mg of glucose and 10 mg of yeast extract per liter, to promote bacterial growth. The filtrates were incubated at 15°C in the dark in sterilized 500-ml culture vessels, which were stirred and aerated with sterile air. Two to three times a day, subsamples (10 ml) were taken and fixed with 5% formaldehyde (final concn) for counts of bacteria and HNAN. By following the disappearance of bacteria and the appearance of HNAN in the cultures, we monitored HNAN grazing and growth.

Growth and grazing parameters were calculated over a 12-h interval, in which exponential HNAN growth coincided with a maximal decline of bacteria. For an interval of T (h), the specific growth rate μ (h^{-1}) of the HNAN was calculated as:

$$\mu = (\ln y_T - \ln y_0)/T$$

where y_0 and y_T are the HNAN numbers at the beginning and end of the interval (Sherr et al. 1983). The population doubling time T_d (h) was calculated as

$$T_d = \ln 2/\mu.$$

During exponential growth the HNAN numbers at the beginning and end of interval T ($t = 0$ to $t = T$) are related by $y_T = y_0\exp(\mu T)$. Integration of the protozoan numbers over interval T, i.e.

$$y_0 \int_0^T \exp(\mu t)\, dt = y_0/\mu[\exp(\mu T) - 1],$$

yields the total protozoan × time units, denoted P (HNAN h) (Fenchel 1986). If Δx bacteria disappear during T, $\Delta x/P$ is the ingestion per individual per unit of time (bacteria $HNAN^{-1}\ h^{-1}$). The clearance F (nl $HNAN^{-1}\ h^{-1}$) was calculated as

$$F = I/\bar{x}$$

where $\bar{x}$ is the average bacterial density in time interval T. During exponential bacterial decline, $\bar{x}$ is given by

$$(x_T - x_0)/(\ln x_T - \ln x_0)$$

(Heinbokel 1978). The clearance was also expressed as a volume-specific clearance [$=F$/body volume (body vol h^{-1})], which is useful to compare the clearance of Protozoa of different sizes. The gross growth efficiency or yield Y (HNAN cells $bacterium^{-1}$) of the HNAN was calculated as μ/I (Fenchel 1986). If the biovolumes are measured, the yield can also be expressed as the HNAN volume grown per bacterial volume consumed (% vol).

Results

Vertical distribution—During a period of frequent sampling between August and November 1985, depth profiles of temperature and oxygen concentrations showed very stable summer stratification until mid-October. The metalimnion was between 5 and 7 m deep, and in this layer the oxygen concentration dropped to <0.1 mg $liter^{-1}$ (Fig. 1). The oxycline moved downward from

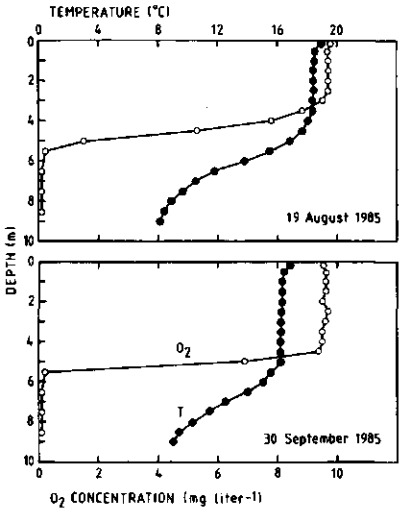

Fig. 1. Typical depth profiles of oxygen concentration and temperature in Lake Vechten during summer stratification.

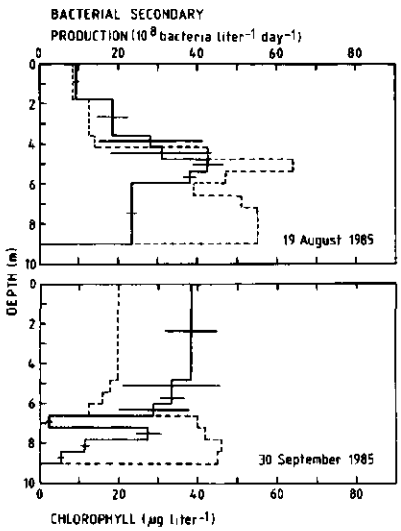

Fig. 2. Depth profiles of bacterial production (——) (mean ± SD, n = 3) estimated by thymidine incorporation, assuming a conversion factor of 2 × 10^{18} bacteria mol^{-1} thymidine, and of total chlorophyll concentration (– – –) in Lake Vechten.

mid-October until mid-November when the lake was completely mixed.

At the start of the sampling period on 19 August, depth profiles of HNAN, bacterial numbers and thymidine incorporation, chroococcoid cyanobacteria (*Synechococcus* sp.), and chlorophyll concentrations were studied. These profiles were repeated on 30 September. Maximal chlorophyll concentrations of 40–60 µg $liter^{-1}$ were observed in the meta- and hypolimnion (Fig. 2). In these layers, the highest bacterial production (4 × 10^9 cells liter d^{-1}) was also found on 19 August; on 30 September it was found in the epilimnion. Bacterial numbers showed no stratification on either date, with values between 5 and 11 × 10^9 bacteria $liter^{-1}$ over the entire water column (Fig. 3). In contrast to the bacteria, cyanobacteria showed high numbers (up to 3 × 10^9 cells $liter^{-1}$) only below a depth of 6 m.

HNAN were distinguished from phototrophic nanoflagellates (PNAN), which showed red chlorophyll autofluorescence. PNAN showed maximal numbers in the epilimnion, and their abundance decreased with depth on both dates. On 19 August, up to 13 × 10^6 PNAN $liter^{-1}$ were found in the epilimnion, whereas in the meta- and hypolimnion, 0.7 and 0.2 × 10^6 PNAN $liter^{-1}$ were counted. On 30 September, ciliates were also counted and showed a distinct peak in the metalimnion with 23 × 10^4 ciliates $liter^{-1}$ at 6.0–6.6-m depth; in the epi- and hypolimnion the densities were < 3 × 10^4 ciliates $liter^{-1}$.

Temporal variation—Because of pronounced differences in HNAN numbers (Fig. 3), the upper (4.8–6.0 m) and lower (6.0–

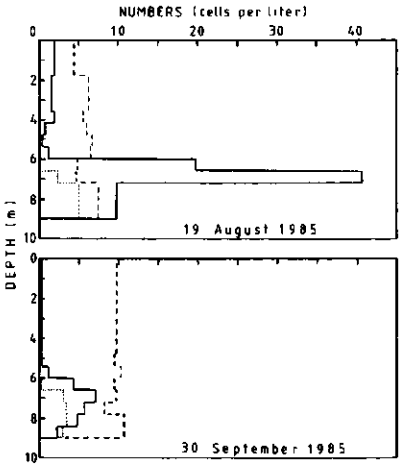

Fig. 3. Depth profiles of numbers of bacteria (×10^9, dashed lines), chroococcoid cyanobacteria (×10^9, dotted lines), and HNAN (×10^6, solid lines) in Lake Vechten.

7.2 m) layers of the metalimnion were chosen for frequent sampling to study temporal variations and to obtain indications of predator–prey relationships.

During a 6-week period, thymidine incorporation was measured twice a week. In early September the two layers showed large differences in estimated bacterial production (Fig. 4A). A very low production of ~1 × 10^8 bacteria liter^{-1} d^{-1}, corresponding with a population doubling time (T_d) of 50 d, was found in the upper metalimnion. The lower metalimnion showed a much higher production of ~40 × 10^8 bacteria liter^{-1} d^{-1} (T_d = 1 d). Coincident with maximal thymidine incorporation rates (Fig. 4A), the average biovolume per bacterium reached its maximum (0.27 μm^3 bacterium^{-1}) in the lower metalimnion in mid-September (Fig 4B) when ~40% of the cells were >1 μm. Most of the bacteria, however, were small cocci and rods with sizes <1 μm. Few bacteria seemed to be attached to particles.

Maximal chlorophyll concentrations (~80 μg liter^{-1}; Fig. 4C) also coincided with maximal bacterial activity (Fig. 4A) in the lower metalimnion. Cyanobacterial numbers in the lower metalimnion (max 3.1 × 10^9 cells liter^{-1}) were >10-fold higher than in the upper metalimnion (max 0.17 × 10^9 cells liter^{-1}; Fig. 5). In both layers the bacterial numbers were similar (4–10 × 10^9 bacteria liter^{-1}) despite the much higher bacterial production in the lower metalimnion (Fig. 4A). In the upper metalimnion the HNAN numbers were <3 × 10^6 HNAN liter^{-1}, but were >30 × 10^6 in the lower metalimnion. Thus high HNAN numbers coincided with high bacterial activity and high numbers of cyanobacteria (size, 1–2 μm), suggesting predator–prey relationships. HNAN showed a steep decline in September (Fig. 5), which could not be explained by depletion of food because bacterial production as well as numbers of bacteria and cyanobacteria were still high at the onset of the decline. The decline of HNAN was followed, however, by a peak of ciliates (max 28 × 10^4 cells liter^{-1}), suggesting consumption of HNAN by ciliates. Three types of ciliates were observed, two *Coleps* spp. with cell lengths of 25 and 40 μm and a smaller spherical ciliate with a diameter of 15–20 μm. The most

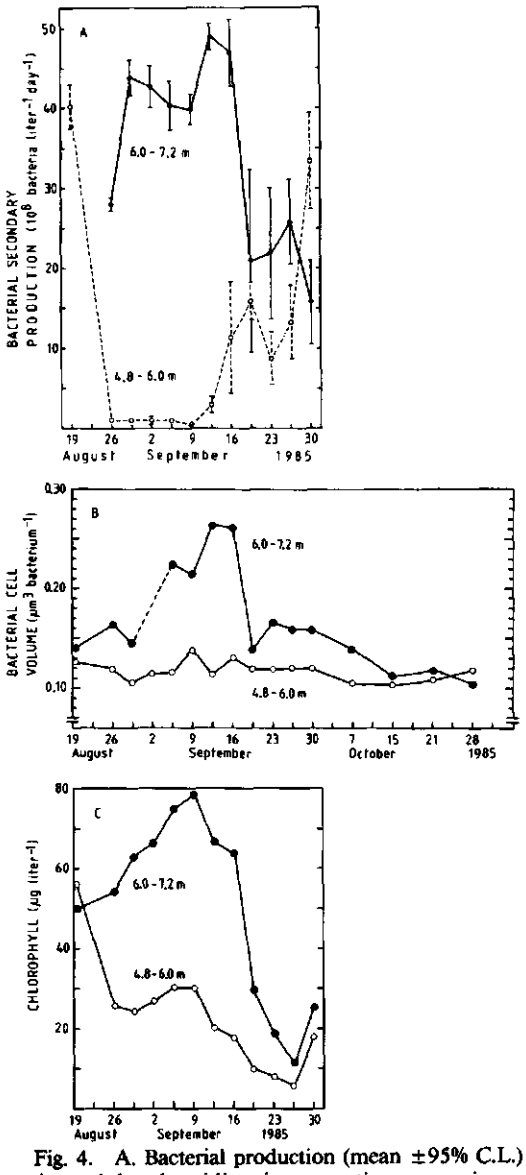

Fig. 4. A. Bacterial production (mean ±95% C.L.) estimated by thymidine incorporation, assuming a conversion factor of 2 × 10^{18} bacteria mol^{-1} thymidine. B. Average biovolume per bacterium. C. Total chlorophyll concentrations in the upper (4.8–6.0-m depth) and lower (6.0–7.2-m depth) metalimnion of Lake Vechten.

abundant was the smaller *Coleps*, which reached a density of 16 × 10^4 cells liter^{-1} on 12 September, when the bigger *Coleps* and the small ciliate reached 7 and 5 × 10^4 cells liter^{-1}.

The HNAN population was strongly dominated by *Monosiga* sp. (relative abun-

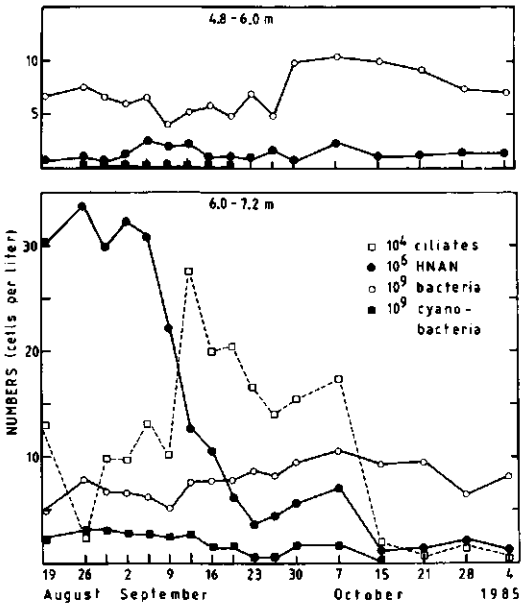

Fig. 5. Numbers of ciliates, HNAN, bacteria, and chroococcoid cyanobacteria in the upper (4.8–6.0-m depth) and lower (6.0–7.2-m depth) metalimnion of Lake Vechten.

dance up to 90%; Table 1), with a cell diameter of ~6 μm. Other important species were *Monas* and *Bodo* spp., with relative abundances of up to 30%. Compared to HNAN, PNAN showed low numbers of up to 2.2 × 10^6 liter^{-1} in the lower metalimnion. In October, however, when the HNAN numbers were low, the PNAN contributed 50% to the total nanoflagellate density. Their contribution reached 75% in the upper metalimnion, with a maximum of 7 × 10^6 PNAN liter^{-1}.

Grazing experiments—Grazing of bacteria by HNAN was estimated in two experiments with selectively filtered lake water. The filtrates were enriched to promote bacterial growth. Hardly any bacterial growth occurred in the grazer-free control (1-μm filtrate) in the first experiment (June 1985) after enrichment with 1 mg of yeast extract per liter (Fig. 6A). Therefore, in the second experiment (August 1985) the filtrates were enriched with the higher concentrations of 10 mg of glucose and 10 mg of yeast extract per liter, which did result in exponential growth of bacteria (Fig. 6B). After 100 h of incubation, bacteria reached the stationary phase and densities remained stable for at least 10 d in the grazer-free controls, indicating that grazing was the only cause of mortality. In the presence of grazers (10-μm filtrates), however, the developed bacterial population was rapidly grazed down by an exponentially growing HNAN population (Fig. 6). In the first experiment, the HNAN density increased from 56.5 to 221 × 10^6 liter^{-1}, while bacteria decreased from 2.41 to 1.83 × 10^{10} liter^{-1} between t = 190 and 202 h.

From this time interval we calculated the growth and grazing parameters in Table 2. The parameters for the second experiment were derived from t = 128–140 h, when HNAN increased from 47.4 to 108 × 10^6 liter^{-1} and bacteria decreased from 2.00 to 1.37 × 10^{10} liter^{-1}. Because no bacterial growth occurred in the controls during these intervals, we assumed that bacteria in the 10-μm filtrates also did not grow when they were grazed down by the HNAN. Further we assumed that grazing was the only cause of bacterial decline. From the bacterial de-

Table 1. Qualitative composition of the metalimnetic HNAN population in Lake Vechten, August–October 1985.

	Relative abundance (%) on								
		2	5	9	12	16	19	23	26	
Genus	29 Aug	Sep								28 Oct
Monosiga	90	93	85	96	91	89	79	67	59	4
Monas	0	0	0	0	0	1	0	0	0	27
Bodo	5	5	8	2	6	6	3	16	10	27
Pleuromonas	2	1	2	0	1	0	4	5	15	0
Rhabdospira	3	1	3	2	1	0	11	4	3	0
Misc. forms	0	0	2	0	1	4	3	8	14	44

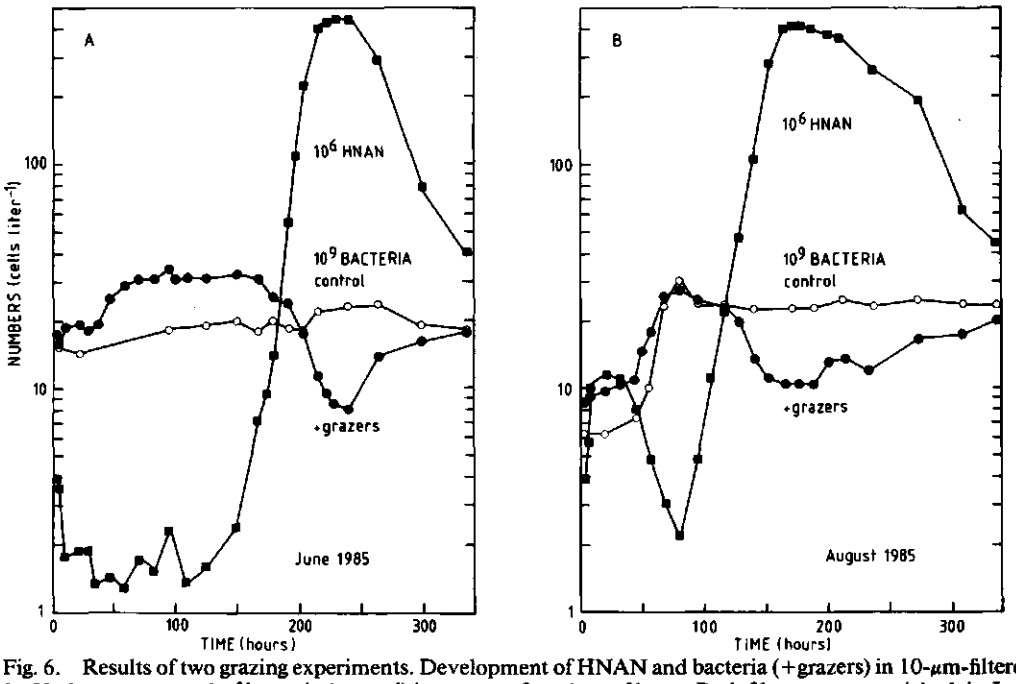

Fig. 6. Results of two grazing experiments. Development of HNAN and bacteria (+grazers) in 10-μm-filtered Lake Vechten water, and of bacteria (control) in a grazer-free, 1-μm filtrate. Both filtrates were enriched: in June with 1 mg liter^{-1} of yeast extract; in August with 10 mg liter^{-1} of yeast extract and 10 mg liter^{-1} of glucose.

cline and the integrated protozoan activity (HNAN h) in the intervals, we calculated ingestion rate.

One HNAN ingested ~7 bacteria h^{-1} and cleared ~0.4 nl of water of bacteria h^{-1} (Table 2). In the second experiment, the yield was 9.6×10^{-3} HNAN cells bacterium^{-1}, which means that an average HNAN cell consumed 104 bacteria before it divided (reciprocal of yield). Since the average biovolume per bacterium was 0.9 μm^3, $104 \times 0.9 = 93.6$ μm^3 was consumed to synthesize 1 HNAN cell with an average biovolume of 25 μm^3. Thus the volumetric yield or gross growth efficiency was 25/93.6 = 27%. A much higher yield of 79% was calculated from the first experiment. In both experiments, the developed HNAN population was strongly dominated by a small (3–4-μm diam) *Monas* sp. which showed high specific growth rates up to 0.11 h^{-1}.

During the first grazing experiment the average biovolume per bacterium varied. It increased from 0.5 μm^3 in the beginning to 0.9 μm^3 at the bacterial peak and did not increase further in the control. In the presence of grazers, however, it had doubled to 1.7 μm^3 after the bacterial decline. At that time (t = 238 h), 25% of the bacteria were filaments longer than 8 μm. Because these filaments were thought to be unsuitable for consumption by HNAN, they were not taken into account in Fig. 6 and in biovolume calculations. Apparently, the larger bacteria escaped from predation. This phenomenon was also observed by Güde (1986). Such a

Table 2. Growth and grazing parameters of HNAN in the grazing experiments of June and August 1985.

Grazing exp.	Biovolume (μm^3)		Specific growth rate μ (h^{-1})	Pop. doubling time T_d (h)	Ingestion rate (bact. HNAN^{-1} h^{-1})	Clearance (nl HNAN^{-1} h^{-1})	Specific clearance (body-vol h^{-1})	Yield	
	Bact.	HNAN						(cells bact.$^{-1}$)	(% vol)
Jun	0.9	25	0.114	6.1	4.00	0.20	8×10^3	28.5×10^{-3}	79
Aug	0.9	25	0.068	10.2	7.13	0.43	17×10^3	9.61×10^{-3}	27

shift in bacterial community structure did not occur in the second experiment, in which the biovolume stayed between 0.8 and 1.0 μm^3 bacterium^{-1} and few filaments were observed. The bigger bacterial biovolume during 1-μm filtration at the start of the second experiment caused a higher filtration loss of 36% (vs. 11% in the first experiment).

Discussion

High microbial activity and a HNAN peak in the metalimnion—During summer stratification in Lake Vechten, chlorophyll concentrations and primary production rates are maximal in the metalimnion (Blaauboer et al. 1982; Steenbergen and Korthals 1982). Metalimnetic phytoplankton maxima can be explained by an equilibrium between downward light penetration and upward nutrient flux (Sorokin 1981). Moreover, sedimenting plankton and detritus can accumulate at the thermocline (Cappenberg 1972). High primary production as well as detrital accumulation may promote bacterial production in the metalimnion.

In the lower metalimnion of Lake Vechten, high bacterial production rates were estimated in early September (Fig. 4A). Bacterial production had pronounced maxima in the metalimnion of Lake Oglethorpe (McDonough et al. 1986) and Little Crooked Lake (Lovell and Konopka 1985). In these lakes the bacterial numbers were also maximal in the metalimnion, in contrast with Lake Mendota where no stratification of bacterial numbers was found (Pedrós-Alió and Brock 1982). Lake Vechten showed no stratification of bacterial numbers (Fig. 3), and higher bacterial activity did not coincide with higher densities (Figs. 2, 3). In early September the estimated bacterial production in the lower metalimnion was 40-fold higher than in the upper metalimnion (Fig. 4A), but bacterial numbers were similar in both layers (Fig. 5). This finding indicates that the high bacterial production is completely consumed by grazers, probably Protozoa.

In Lake Vechten, HNAN were clearly stratified and showed a distinct peak in the lower metalimnion (Fig. 3). Sorokin and Paveljeva (1972) also observed a HNAN peak near the thermocline in Dalnee Lake, but the maximal densities of 0.75×10^6 HNAN liter^{-1} were much lower than the 40×10^6 HNAN liter^{-1} we found in Lake Vechten. During summer stratification, the maximal HNAN numbers in Lake Vechten are relatively high. Although similar densities occur in estuaries (Sieburth and Davis 1982). HNAN abundances are often 10-fold lower in marine systems (Azam et al. 1983; Porter et al. 1985).

Ciliates also reached maximal numbers in the metalimnion of Lake Vechten. Whereas little is known about HNAN peaks, metalimnetic ciliate peaks have been observed and studied more extensively in several lakes. Like the HNAN peak in Lake Vechten (Figs. 1, 3), peak densities of ciliates normally occur at or just below the oxic–anoxic boundary (Finlay and Fenchel 1986). Bigger zooplankton avoid this zone, and Protozoa may benefit from low predation and high food availability in the metalimnion. Although the ciliate *Loxodes* can switch to nitrate respiration under anaerobic conditions, the meta- and hypolimnetic ciliates are probably aerobes and cannot survive indefinite anoxia (Bark and Goodfellow 1985). The HNAN peak suggests that the HNAN have a microaerophilic metabolism and behavior similar to that of the ciliates. In spite of the low oxygen concentrations, the oxygen flux in the metalimnion of Lake Vechten is more than enough to mineralize the whole carbon input in the 5.0–7.2-m stratum (Steenbergen and Verdouw 1984). Although sulfate reduction and nitrate respiration are negligible in this layer, the electron acceptor consumption is completely dominated by oxygen. This result indicates that the active microbial populations in the microaerophilic metalimnion are aerobic rather than anaerobic.

The metalimnetic HNAN peak in Lake Vechten was strongly dominated by a choanoflagellate of the genus *Monosiga* (Table 1). Choanoflagellates are extremely common in seawater and may comprise the most numerous phagotrophic organisms on earth (Fenchel 1986). Other abundant genera in Lake Vechten include *Monas* and *Bodo,* which are also important in marine

systems (Davis et al. 1985). Like the bacteria, HNAN in Lake Vechten appeared to be unattached. In the grazing experiments, maximally 15% of the HNAN were retained by 10-μm filters, whereas Fenchel (1982*a*) lost 60% by 20-μm filtration and concluded that many pelagic flagellates were attached to large particles. Andersen and Sørensen (1986) observed that distinct maxima of episymbiotic (attached to diatoms) and free-swimming *Monosiga* spp. occurred in different periods.

Microbial activity and trophic links in the metalimnion—Maximal bacterial activity occurred in the lower metalimnion in early September 1985, when production was estimated at 40×10^8 bacteria liter^{-1} d^{-1}. This production is similar to the 60×10^8 estimated for metalimnetic bacteria in Lake Oglethorpe (McDonough et al. 1986). These estimates both are based on thymidine incorporation into cold TCA-insoluble macromolecules, assuming a conversion factor of 2×10^{18} cells mol^{-1} of thymidine. This conversion factor is considered to be in general agreement with information currently available (Moriarty 1986) and has been confirmed for marine as well as freshwater aerobic systems (Bell et al 1983; Lovell and Konopka 1985) and also for mixed cultures of anaerobic bacteria (Pollard and Moriarty 1984). Higher conversion factors, however, have also been published (Kirchman et al. 1982; Scavia et al. 1986) and may be expected if, for example, part of the growing population is unable to incorporate thymidine (McDonough et al. 1986).

An empirical conversion factor for the microaerophilic metalimnion of Lake Vechten was not obtained because filtered diluted samples showed no bacterial growth within 24 h. It seems contradictory that lower metalimnion samples showed considerable initial thymidine incorporation, but no increase of bacteria in 24-h incubations. This situation was not due to grazing, since grazers were removed by 1-μm filtration and their absence was checked microscopically. Samples for measuring initial thymidine incorporation were incubated for only a few hours, however, and were not aerobically filtered and diluted. Thus more manipulation or longer incubation seemed to prevent significant bacterial growth in the 24-h incubations.

Although thymidine incorporation was high in our lower metalimnion samples, changes in oxygen concentration and flux during sampling and incubation may have depressed incorporation rates. The many problems involved in measuring bacterial production in a stratified lake with low oxygen concentrations were studied by McDonough et al. (1986). In epilimnetic samples they found most of the incorporated thymidine (70%) in DNA. This fraction was much lower in meta- and hypolimnion samples that showed a high incorporation into other macromolecules, however, suggesting that many bacteria were able to transport thymidine into the cell but lacked thymidine kinase to incorporate thymidine into DNA. In these samples, incorporation into total macromolecules yielded the best production estimates. Comparison with leucine incorporation and frequency of dividing cells indicated that thymidine incorporation may underestimate bacterial production, especially in the meta- and hypolimnion, and that a higher conversion factor may yield more realistic estimates. Given the uncertainties in conversion factors, especially for low-oxygen waters, our thymidine-based estimates must be regarded cautiously.

Further uncertainties arise when the estimated cell production is converted to carbon production, using a conversion factor to convert biovolume to biomass. Although higher values have been reported (Bratbak 1985), we used 1.21×10^{-13} g C μm^{-3} (Watson et al. 1977; Nagata 1986). A biovolume of 0.27 μm^3 cell^{-1} (Fig. 4B), found for active bacteria in the lower metalimnion of Lake Vechten, yields a bacterial carbon content of 33 fg C cell^{-1}, which is in the range (14–71 fg C cell^{-1}) reported by Lovell and Konopka (1985), Bjørnsen (1986), and Lee and Fuhrman (1987). From a carbon content of 33 fg C cell^{-1} and a production of $\sim 40 \times 10^8$ cells liter^{-1} d^{-1} (Fig. 4A), bacterial production in the lower metalimnion of Lake Vechten can be estimated at 130 μg C liter^{-1} d^{-1}, assuming conservative conversion factors.

High bacterial activity in the lower metalimnion in mid-September coincided with

a high chlorophyll concentration (Fig. 4A, C), suggesting a link between phytoplankton and bacteria. Evidence that bacteria in Lake Vechten can grow on dissolved organic compounds excreted by phytoplankton was presented by Blaauboer et al. (1982). They observed a metalimnetic phytoplankton maximum which excreted up to 55% of the photosynthetically fixed carbon at rates up to 10 μg C liter^{-1} h^{-1}. This would be sufficient to support our estimated bacterial production of 130 μg C liter^{-1} d^{-1}, if a yield of 50% is assumed (Azam et al. 1983). If the yield or the phytoplankton extracellular release are lower, other carbon sources are needed, such as decaying algae and sedimentation. The latter, estimated at 7 mg C liter^{-1} d^{-1} at 7.2-m depth (Steenbergen and Verdouw 1984), greatly exceeds the bacterial carbon requirements, but it is not known how much of this carbon can be used.

In the lower metalimnion of Lake Vechten, a high bacterial activity and high numbers of cyanobacteria coincided with high HNAN numbers, and an apparent coupling between HNAN and ciliates was observed. It indicates that HNAN are a link in the microbial food web between small bacteria and cyanobacteria on the one hand and bigger ciliates on the other. In Limfjorden, similar trophic couplings were observed between bacteria, HNAN, and ciliates (Andersen and Sørensen 1986). Ciliates in turn are consumed by bigger zooplankton such as copepods and cladocerans (Sorokin and Paveljeva 1972; Porter et al. 1979). In the lower metalimnion of lake Vechten, however, hardly any bigger zooplankton are present due to low oxygen concentrations. We focused on consumption of bacteria by HNAN, which are considered to be the dominant grazers of pelagic bacteria (Fenchel 1986). The dominating *Monosiga* sp. is specialized to feed on the smallest procaryotes. Bacteria may also be consumed, however, by small ciliates (Sherr and Sherr 1987) and mixotrophic flagellates (Porter et al. 1985). The latter would be counted as autofluorescent PNAN, which showed relatively low numbers in the metalimnion. Besides bacteria, the grazers may also consume small algae and cyanobacteria (Campbell and Carpenter 1986). We observed *Synechococcus* sp. inside HNAN, but ingestion rates were not quantified. Thus the microbial food web of Lake Vechten is still poorly known.

Grazing by HNAN—During the grazing experiments the composition of the microbial populations may have changed. The bacterial biovolume per cell increased in the first experiment but not in the second. The developed HNAN population was almost a monoculture of small (25 μm^3) *Monas* cells, whereas the field population of a month later (Table 1) was dominated by bigger *Monosiga* cells (~110 μm^3) that may have higher ingestion rates. Therefore the calculated grazing parameters (Table 2) may differ from in situ rates. Attempts to isolate *Monosiga* in microaerophilic cultures failed. More recent in situ grazing measurements with short incubations (30 min) of Lake Vechten water and fluorescently labeled bacteria, however, yielded similar ingestion rates of 2–17 bacteria HNAN^{-1} h^{-1} in 1987 (Bloem et al. in prep.).

In our experiments bacteria in grazer-free controls showed a long stationary phase (Fig. 6), probably caused by nutrient depletion. The same limitation probably stopped exponential growth in the grazer flasks. Alternatively, grazing by HNAN might have balanced bacterial growth and prevented further increase. Significantly grazed bacteria would probably not reach the same maximal densities, however, as those in the controls (Andersen and Fenchel 1985), especially in the second experiment after enrichment. Moreover, it is unlikely that a balance between grazing and growth would maintain stable bacterial numbers for 100 h (Fig. 6A). It should also be noted that bacterial increase stopped at the minimum of HNAN abundance. In the calculation of ingestion and clearance rates we therefore assumed that the bacteria were not growing.

In Andersen and Fenchel's (1985) experiments HNAN growth and grazing started before the bacterial peak, and grazing parameters were calculated at this peak. It was assumed that the bacteria had maintained their initial growth rates and that the increase in bacterial numbers was stopped only by grazers. If growth rates decreased near the bacterial peak, however, this approach

may lead to overestimated ingestion rates. Andersen and Fenchel calculated a clearance of 15–20 nl HNAN^{-1} h^{-1} and ingestion rates between 45 and 73 bacteria HNAN^{-1} h^{-1}, which is much higher than our estimates. Our ingestion and clearance rates of 4–7 bacteria HNAN^{-1} h^{-1} and 0.2–0.4 nl HNAN^{-1} h^{-1} (Table 2) are at the low end of the range reported in the literature and similar to values reported by Andersen and Sørensen (1986) and McManus and Fuhrman (1986). With *Monas* sp. (30 μm^3) similar to ours, ingestion rates of 4–75 bacteria HNAN^{-1} h^{-1} and clearances of <0.2–1 nl HNAN^{-1} h^{-1} were found (Sherr et al. 1983; Cynar and Sieburth 1986).

The relatively low ingestion rate of 7 bacteria HNAN^{-1} h^{-1} (Table 2) in our second grazing experiment was probably not an underestimate because the yield of the HNAN was only 27% (vol), which is not high. High growth yields will be calculated if consumption is underestimated. Such underestimation might occur if HNAN grazing restores and promotes bacterial growth; we assumed no growth in the time interval used for calculations. From the first experiment we calculated an ingestion rate of 4 bacteria HNAN^{-1} h^{-1}, which may be an underestimate since the yield (79%) was very high. For HNAN, yields between 10 and 50% have been reported (Fenchel 1982*a*; Caron et al. 1985).

The results of the grazing experiments, combined with the field observations, facilitate a rough estimate of potential grazing on bacteria by the HNAN population in the metalimnion of Lake Vechten. In the lower metalimnion ~30 × 10^6 HNAN liter^{-1} were found in August–September (Fig. 5). In August, the grazing experiment showed an ingestion rate of 7 bacteria HNAN^{-1} h^{-1} (Table 2). Thus the total consumption can be estimated at 5 × 10^9 bacteria liter^{-1} d^{-1}, which is 60–100% of the bacterial standing stock (5–8 × 10^9 bacteria liter^{-1}; Fig. 5). This consumption equals the maximal bacterial production, estimated at 4 × 10^9 bacteria liter^{-1} d^{-1} (Fig. 4A). Thus HNAN have the potential to consume the whole bacterial production, even at high bacterial growth rates and low protozoan ingestion rates. In the aerobic waters of Lake Oglethorpe, bacterial production seemed to be slightly greater than protozoan grazing (Sanders and Porter 1986).

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Nutrient cycling through phytoplankton, bacteria and protozoa, in selectively filtered Lake Vechten water

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Abstract. The breakdown of organic carbon of dead *Synechococcus* cell walls, added to selectively filtered Lake Vechten water, was not accelerated by protozoa. During 4 weeks of incubation at 15°C no significant decrease of total organic carbon was observed. However, heterotrophic nanoflagellates (HNAN) and ciliates strongly increased the remineralization of N and especially P, from both cell walls and cell extract. Bacterioplankton growth did not result in net P mineralization but in P uptake. P was remineralized only in the presence of protozoan grazers. Both HNAN and ciliates grazed on bacteria, with ingestion rates estimated at 27–96 bact $HNAN^{-1}$ h^{-1} and 129 bact $ciliate^{-1}$ h^{-1} respectively. Grazers increased N mineralization too, although N was also mineralized in the absence of protozoa. The phytoplankton cell walls yielded less P but more N remineralization than the cell extract. Thus, protozoa can strongly accelerate cycling of specific nutrients through plankton. Nuclepore filters were found to cause artificial DOC release during selective filtration.

Introduction

In aquatic ecosystems protozoa, especially heterotrophic nanoflagellates (HNAN, size 2–20 μm), are recognized as major consumers of bacteria (Azam *et al.*, 1983). Consumption of bacteria by protozoa may accelerate the decomposition and mineralization of organic matter. This can be direct, via consumption of living bacteria and small phytoplankton cells. A part of the consumed biomass is then respired and remineralized (Caron *et al.*, 1985; Goldman *et al.*, 1985; Güde, 1985), and nutrients for new phytoplankton growth are supplied (Berman *et al.*, 1987). The acceleration may also be indirect, because grazing may promote bacterial growth, and consequently the decomposition of detritus (Johannes, 1965; Fenchel and Harrison, 1976). Whereas increased mineralization of dead macrophytes and living phytoplankton by protozoa has been demonstrated, not much is known about their role in the mineralization of dead phytoplankton. Dead dinoflagellates (*Peridinium cinctum*) in Lake Kinneret water showed an accelerated decomposition in the presence of HNAN (Sherr *et al.*, 1982). The HNAN enhanced the bacterial breakdown of the polysaccharide cell walls, but not of the cell protoplasm. Sherr *et al.* (1982) therefore suggested that microprotozoa may selectively facilitate the breakdown of detritus with a high structural carbohydrate and a low mineral content.

During summer stratification in Lake Vechten, high organic carbon sedimentation rates (Steenbergen and Verdouw, 1984), and maximum chlorophyll

concentrations and primary production rates (Blaauboer *et al.*, 1982; Steenbergen and Korthals, 1982) are found in the metalimnion. In this layer thymidine incorporation indicated a high bacterial production, which coincided with maximum HNAN concentrations of more than 30×10^6 HNAN l^{-1} (Bloem and Bär-Gilissen, 1989). Grazing experiments suggested that the HNAN are able to consume the whole metalimnetic bacterial production. Therefore we hypothesized that in the metalimnion of Lake Vechten protozoa accelerate the mineralization of dead phytoplankton cells.

The aim of this study was to investigate the role of protozoa in the decomposition of organic carbon, and the remineralization of inorganic phosphorus and nitrogen from dead phytoplankton cells. Since the phytoplankton in the metalimnion of Lake Vechten is dominated by small chroococcoid *Synechococcus*-like cyanobacteria (Steenbergen and Korthals, 1982), the decomposition of autoclaved *Synechococcus* cells was studied. The mineralization of cell walls, which have a low phosphate content (Drews and Weckesser, 1982), and cell protoplasm was studied separately in selectively filtered Lake Vechten water, both in the absence and presence of protozoa. In addition, ingestion rates of protozoa grazing on bacteria were estimated.

Materials and methods

Synechococcus preparation

As described by Korthals and Steenbergen (1985), *Synechococcus* sp. isolated from Lake Vechten was grown in the presence of bacteria under light limitation at 15°C in semi-continuous culture ($\mu = 0.005$ h^{-1}). Samples from the culture were centrifuged (20 min, 27 000 *g*) to collect *Synechococcus* cells. The cells were washed three times by centrifugation in distilled water to remove nutrients from the culture medium. The washing did not cause obvious cell damage. Then the samples were autoclaved (20 min, 120°C, 200 kPa) to break all the cells. The broken cell suspension was filtered through 0.2 μm membrane filters (Schleicher and Schüll BA 83) to prepare cell extract (protoplasm). Cell walls were collected from broken cell suspensions by centrifugation (20 min, 27 000 *g*) and washed three times to remove dissolved nutrients.

Chemical analyses

The total carbon content of freeze dried samples was determined with a Carlo Erba Elemental Analyzer model 1106 (Carlo Erba, Milano, Italy). Carbonate carbon was determined after ignition (2 h, 550°C) in the residue. By subtracting carbonate carbon from total carbon, the TOC (Total Organic Carbon) concentration was derived. After filtration through 0.45 μm membrane filters (Millipore type HA), dissolved nutrients were analyzed. DOC (Dissolved Organic Carbon) was measured with a Skalar Continuous Flow Analyzer (Skalar Analytical, Breda, The Netherlands), which oxidized DOC by UV radiation to CO_2. The CO_2 was measured colorimetrically at 550 nm with phenolphthalein indicator (Schreurs, 1978). Also PO_4-P, analyzed as molybdate reactive

phosphorus (Murphy and Riley, 1962), was measured with a Skalar auto-analyzer. NH_3-N determinations were based on indophenol formation with sodium salicylate (Verdouw *et al.*, 1978). NO_3-N was determined by cadmium–copper reduction to nitrite (Wood *et al.*, 1967).

Counting

Samples were fixed with 5% formaldehyde (final concentration). Field samples at the start of the experiments were fixed by 1% glutaraldehyde for distinction between heterotrophic and phototrophic nanoflagellates. These unbuffered fixatives were found to cause no loss of HNAN cells (Bloem *et al.*, 1986), and were assumed to preserve ciliates as well. HNAN and ciliates were stained with primulin and counted directly on 1 μm Nuclepore filters by epifluorescence microscopy. With the same method unstained autofluorescent *Synechococcus* cells were counted. For bacteria the DAPI technique was used (Porter and Feig, 1980), with a final stain concentration of 1 μg ml^{-1}. For biovolume estimations 100 individuals were measured by eyepiece micrometer, and volumes were calculated from length and width assuming a spherical or cylindrical shape.

Experiments

Two experiments were performed, the first in October 1986, to study the breakdown of *Synechococcus* cell extract, and the second in November using cell walls. Water samples were taken from Lake Vechten, The Netherlands (52°4'N, 5°5'E), a small (4.7 ha) mesotrophic 12 m deep stratifying lake. From the metalimnion at 6.0–7.2 m depth samples were collected with a Friedinger sampler, and sieved through a 125 μm plankton gauze. At a vacuum not exceeding 3 kPa (Bloem *et al.*, 1986), two 1000 ml vols were filtered through Nuclepore polycarbonate filters (25 mm dia.) with pore sizes of 1 and 10 μm respectively. The 10 μm filtrate contained both bacteria and protozoa. The 1 μm filtrate contained bacteria only and served as a grazer free control. To prevent protozoan growth in the control, 50 mg l^{-1} cycloheximide (BDH Chemicals Ltd, Poole, UK) was added (Fuhrman and McManus, 1984). The filtrates were incubated at 15°C in the dark in sterilized 1000 ml culture vessels, which were stirred and aerated with sterile air.

Both filtrates were enriched, in experiment 1 with *Synechococcus* extract, at a final concentration of 10 times the field concentration, corresponding to an extract of 30×10^9 cells added l^{-1}. In experiment 2 cell walls were added at 100 times the field concentration, corresponding to 300×10^9 cells l^{-1}. These concentrations were expected to yield DOC and TOC concentrations well above the detection limits of our analyses. By measuring DOC and POC (Particulate Organic Carbon = TOC − DOC) concentrations, the decomposition of cell extract and cell walls, respectively, would be followed. Remineralization of inorganic P and N was followed by measuring PO_4-P, NH_3-N and NO_3-N concentrations. By comparing the 10 μm filtrates, containing both bacteria and protozoa, with the 1 μm filtrates, containing bacteria only, the effect of protozoa on decomposition and mineralization was investigated. By following

the disappearance of bacteria and the appearance of protozoa in the filtrates, protozoan grazing and growth was monitored.

Growth and grazing parameters were calculated from a 12 or 24 h time interval, in which exponential HNAN growth coincided with a maximal decline of bacteria. For a time interval of T (h), the specific growth rate μ (h^{-1}) of the HNAN was calculated as

$$\mu = (\ln y(T) - \ln y(0))/T$$

where $y(0)$ and $y(T)$ are the HNAN concentrations at the beginning and end of the interval (Sherr *et al.*, 1983). During exponential growth the HNAN concentrations at the beginning and end of the time interval T ($t = 0 - T$) are related by

$$y(T) = y(0).\exp(\mu T)$$

The integrated protozoan number over interval T, denoted P (HNAN.h), is then given by

$$y(0) \int_0^T \exp(\mu t)dt = y(0)/\mu \ (\exp(\mu T) - 1)$$

(Fenchel, 1986). If Δx bacteria disappear during T, $\Delta x/P$ is the ingestion rate I (bact $HNAN^{-1}$ h^{-1}). The clearance (nl $HNAN^{-1}$ h^{-1}) was calculated as $I/\bar{x}$, where $\bar{x}$ is the average bacterial concentration in the time interval T. During exponential bacterial decline, $\bar{x}$ is given by $(x(T) - x(0))/(\ln x(T) - \ln x(0))$ (Heinbokel, 1978).

Results

Experiment 1

After addition of the *Synechococcus* cell extract ($t = 0$ h), exponential growth of bacteria occurred in both filtrates (Figure 1A). The stationary phase was reached after 72 h, and the concentrations remained stable for ~10 days in the grazer-free control. However, in the presence of grazers, the developed bacterial population was grazed down rapidly by an exponentially growing HNAN population. The HNAN (average biovolume 30 μm^3 $HNAN^{-1}$) increased from 10.2 to 59.0 $\times$ 10^6 HNAN l^{-1} ($\mu = 0.15$ h^{-1}), while the bacteria (average biovolume 0.16 μm^3 $bact^{-1}$) decreased from 27.1 to 18.4 $\times$ 10^9 bact l^{-1}, between $t = 72$ and 84 h. From this time interval an ingestion rate of 27 bact $HNAN^{-1}$ h^{-1} and a clearance of 1.20 nl $HNAN^{-1}$ h^{-1} were calculated.

DOC concentrations (Figure 1B) were much higher in the control (82 mg l^{-1}) than in the grazer flask (31 mg l^{-1}). This can largely be explained by the 50 mg l^{-1} cycloheximide, containing 35 mg C l^{-1}, which was added to inhibit eukaryotic growth in the control. Since the DOC decline rate was similar in both flasks, the grazers did not accelerate the disappearance of DOC.

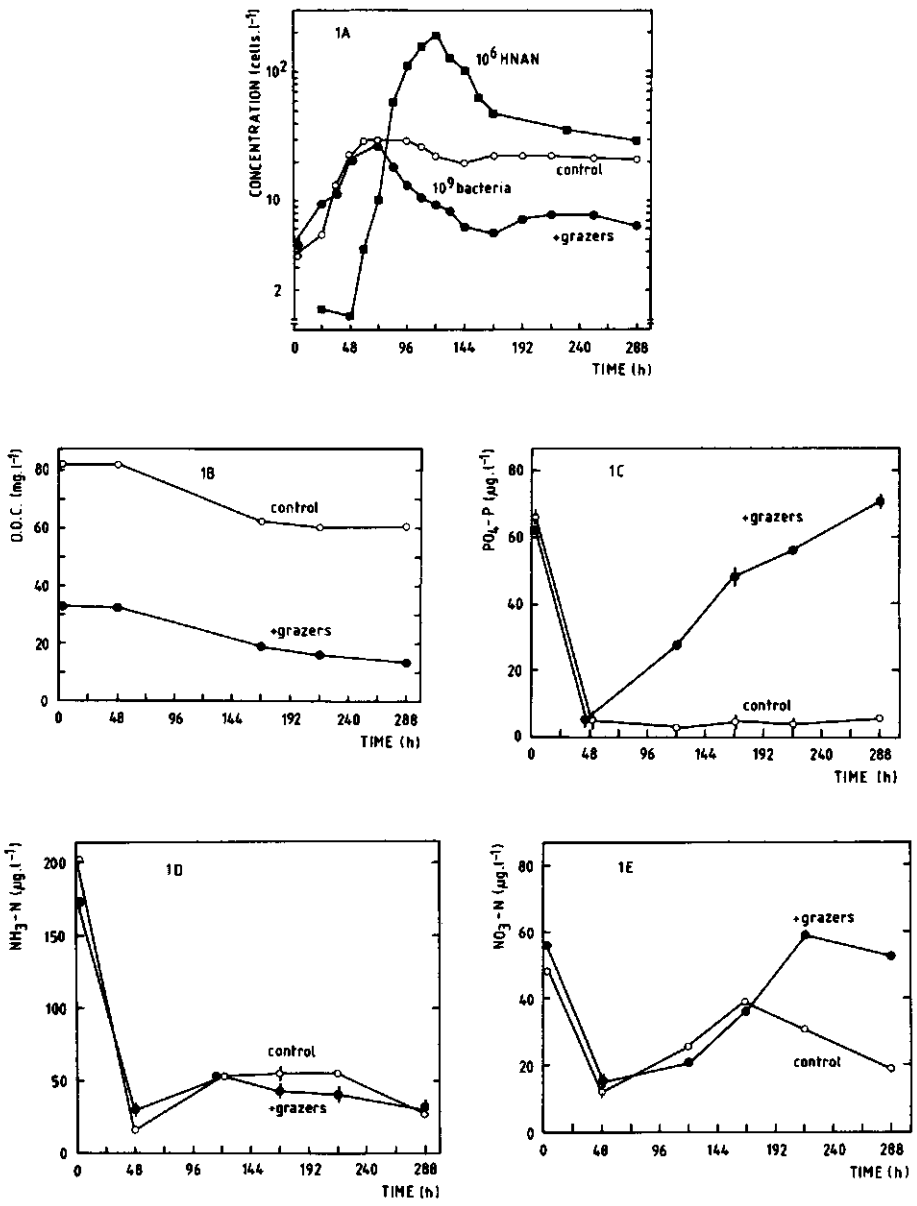

Fig. 1. Concentrations of bacteria (○, ●) and heterotrophic nanoflagellates (■, HNAN) (**A**); DOC (**B**); PO_4-P (**C**); NH_3-N (**D**); and NO_3-N (**E**), in 1 µm (control, open symbols) and 10 µm (+ grazers, filled symbols) filtered Lake Vechten water, after enrichment with *Synechococcus* cell extract (bars indicate SD, $n = 2$).

After addition of the *Synechococcus* extract (t = 0 h) both flasks contained 60 μg PO_4-P l^{-1}. During exponential growth in the first 48 h (Figure 1A) the PO_4-P concentrations dropped below 6 μg l^{-1} (Figure 1C). The concentration remained low in the control, whereas in the grazer flask the PO_4-P concentration increased again to 70 μg l^{-1} after 288 h. Thus, bacterial growth did not result in P mineralization but in P uptake, and the P immobilized by bacteria was remineralized only in the presence of HNAN grazers. In the grazer flask the final PO_4-P concentration was 10 times higher than in the control. From the similar initial and final concentrations (~60 μg PO_4-P l^{-1}) it should not be concluded that 100% of the added dissolved inorganic P was remineralized. Part of the remineralized P may have originated from dissolved organic P added with the extract, which was not included in the analyses.

Also NH_3-N and NO_3-N concentrations were relatively high at the start of the experiment, and decreased during bacterial growth (Figure 1D and E). In both flasks no clear increase in NH_3-N was observed. Although the final NO_3-N concentration in the grazer flask (53 μg l^{-1}) was 2.5 times higher than in the control (19 μg l^{-1}), the effect of grazers was less pronounced than with PO_4-P (Figure 1C).

Experiment 2

After the addition of *Synechococcus* cell walls to filtered lake water, again exponential bacterial growth occurred in the first days (Figure 2A). In contrast with the previous experiment, aggregates of big cells were observed and the bacteria showed a wide variation in size and shape. Because of the complicated population structure, biovolumes were not estimated. In the control the stationary phase of the bacteria was maintained for 4 weeks, and no decline was observed in the absence of grazers. In the grazer flask a HNAN population developed first, coinciding with a decrease of bacteria (t = 96 h). A second decrease of bacteria was observed when a population of ciliates (size 15–40 μm) developed (t = 384 h), after the HNAN peak. Because the ciliate peak occurred after the decline of HNAN and coincided with a decline of bacteria, the bacteria and not the HNAN must have been the major prey of the ciliates.

Between t = 384 and 408 h, the ciliates increased from 2.35 to 5.68 × 10^6 cil l^{-1} (μ = 0.037 h^{-1}), while the bacteria decreased from 34.1 to 22.4 × 10^9 bact l^{-1}. From this interval an ingestion rate of 129 bact cil^{-1} h^{-1} and a clearance of 4.65 nl cil^{-1} h^{-1} were estimated. The HNAN showed an ingestion rate of 96 bact $HNAN^{-1}$ h^{-1} and a clearance of 2.33 nl $HNAN^{-1}$ h^{-1}. These values were estimated between t = 96 and 108 h, when the HNAN increased from 3.8 to 11.9 × 10^6 HNAN l^{-1} (μ = 0.095 h^{-1}) and the bacteria decreased from 45.6 to 37.4 × 10^9 bact l^{-1}.

Because of the added cycloheximide, higher DOC and TOC concentrations were expected in the control. Indeed, TOC in the control was ~35 mg l^{-1} higher than in the grazer flask (Figure 2B). In both flasks no significant TOC decrease occurred in 4 weeks, and this was not affected by the protozoan grazers. DOC concentrations were much higher than expected, especially in the control, where

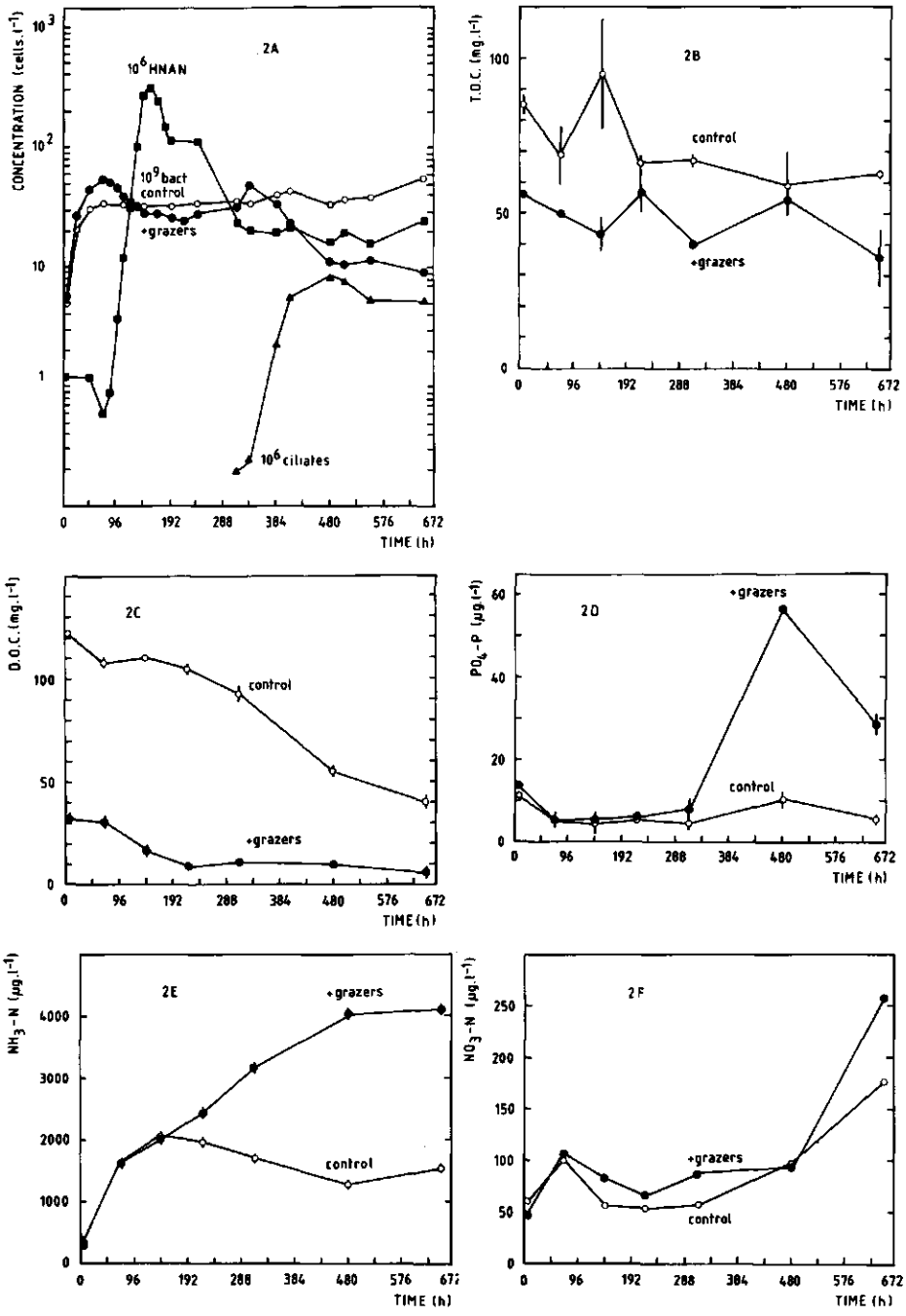

Fig. 2. Concentrations of bacteria (○, ●), heterotrophic nanoflagellates (■, HNAN), and ciliates (▲) (**A**); TOC (**B**); DOC (**C**); PO_4-P (**D**); NH_3-N (**E**); and NO_3-N (**F**), in 1 µm (control, open symbols) and 10 µm (+ grazers, filled symbols) filtered Lake Vechten water, after enrichment with *Synechococcus* cell walls (bars indicate SD, $n = 2$).

DOC exceeded measured TOC (Figure 2C). The DOC decline was not accelerated by grazers.

As in experiment 1, bacterial growth after enrichment resulted in PO_4-P uptake rather than mineralization (Figure 2D). PO_4-P concentrations remained low in the control. Only in the presence of grazers was P remineralized. P remineralization did not coincide with HNAN but with ciliate grazing, in contrast with experiment 1. Whereas in experiment 1 NH_3-N was taken up rather than remineralized, in experiment 2 NH_3-N was mineralized from the beginning in both flasks (Figure 2E). In the presence of grazers the final NH_3-N concentration was 2.7 times higher than in the control. Such an obvious stimulation by grazers was not found for NO_3-N, which was also mineralized in both flasks (Figure 2F).

Nuclepore filtration and enrichment

In both experiments lake water was filtered through 1 and 10 μm Nuclepore filters, to remove grazers of bacteria and protozoa, respectively. Subsequently, the filtrates were enriched with *Synechococcus* extract or cell walls. Already before the enrichment, however, DOC concentrations were higher than expected. Whereas lake water contained only 4 mg DOC l^{-1}, the filtrates contained up to 84 mg l^{-1}, indicating that DOC was released by the Nuclepore filters. This was confirmed by filtering 50 ml distilled water four times through each of two 1 μm Nuclepore filters for 1 h without vacuum applied. Such a filtration raised the DOC from 0 to 117 mg l^{-1}. The addition of the *Synechococcus* extract increased the DOC only with a few mg l^{-1}. Thus the DOC in the filtrates originated mainly from the Nuclepore filters. In contrast to DOC, inorganic P and N were not released by the filters but originated from the algal extract. The extract strongly increased PO_4-P concentrations from 6 to 60 μg l^{-1} and NH_3-N from 70 to 200 μg l^{-1}, whereas NO_3 was not affected. Addition of cell walls did not increase these nutrients much, but increased the measured TOC from below the detection limit to 50 mg l^{-1}.

Discussion

Grazing of bacteria by protozoa

In grazer-free controls bacterial concentrations were stable for up to 4 weeks (Figures 1A and 2A), indicating that no lysis occurred and that grazing was the major cause of bacterial mortality. In the presence of protozoa the bacteria were grazed down rapidly. Ingestion rates of HNAN were estimated at 27 and 96 bact $HNAN^{-1}$ h^{-1} in experiments 1 and 2 respectively. This is much higher than the ingestion rates of ~7 bact $HNAN^{-1}$ h^{-1} found in earlier experiments with Lake Vechten water (Bloem and Bär-Gilissen, 1989), but well in the range reported in the literature. With similar sized HNAN (30 μm^3) from Lake Kinneret, Sherr *et al.* (1983) found ingestion rates between 10 and 75 bact $HNAN^{-1}$ h^{-1}. The statistics of the grazing estimates are relatively poor because the decrease of bacteria is relatively small during the period of balanced exponential growth of

HNAN (Fenchel, 1986). Nevertheless, these crude estimates confirm the potential importance of HNAN as grazers of bacteria in the metalimnion of Lake Vechten, as discussed by Bloem and Bär-Gilissen (1989).

Not only HNAN but also ciliates grazed on bacteria (Figure 2A). The development of ciliates in experiment 2 may be explained by the presence of big and/or aggregated bacteria, which did not occur in experiment 1. Probably, the small HNAN (<10 μm) grazed on small bacteria and the bigger ciliates (15–40 μm) on big bacteria. This is supported by the PO_4-P mineralization, which coincided with the ciliate and not the HNAN peak (Figure 2A and D). This indicates that the total bacterial biomass consumed was much higher at the ciliate peak. Since at both peaks the total numbers of consumed bacteria (~20×10^9 cells) were similar, the biomass per consumed bacterium seemed to be much bigger at the ciliate peak.

Decomposition of organic carbon

The grazing and decomposition processes in our filtrates may have differed from in-situ processes, because the organic carbon concentrations were increased 10- to 100-fold to overcome the detection limits of our analyses. Moreover, the algal cell material added was probably denaturized by autoclaving. However, we wanted to add a sterile substrate, and a coarse method was needed to break the *Synechococcus* cells. Because of the low concentrations and complexity of natural systems, artificial manipulations are often inevitable to investigate ecological principles.

It appeared that in size fractionation studies Nuclepore filters should be pre-rinsed thoroughly to prevent artificial DOC release. Because most of the DOC originated from the Nuclepore filters, no conclusions can be drawn about the effect of HNAN on the decomposition of *Synechococcus* extract in experiment 1. In experiment 2 the DOC exceeded the measured TOC (Figure 2B and C), and the DOC decline was not reflected in a TOC decline. This indicates that the DOC contamination from the Nuclepore filters was not included in the TOC measurements. In contrast to the DOC samples, the samples for TOC measurement were freeze-dried, and apparently most of the DOC evaporated during this treatment.

The measured TOC appeared to represent the *Synechococcus* cell walls, because the addition of the cell wall suspension increased the measured TOC in the filtrates from below the detection limit to ~50 mg l^{-1}. Since no significant TOC decrease occurred in 4 weeks at 15°C (Figure 2B), the *Synechococcus* cell walls appeared to be very resistant to decomposition. At 26°C POC from marine *Synechococcus* sp. showed a considerable decomposition, of 50% in 2 weeks (Biddanda, 1988). Whereas some algae were destroyed in short periods others, such as *Microcystis* sp., withstood microbial digestion for more than 4 weeks (Fallon and Brock, 1979). These differences may be related to the relative biodegradabilities of specific components of the algal cell walls. The cell wall is a major determinant of algal resistance to degradation (Gunnison and Alexander, 1975; Cappenberg *et al.*, 1982). The decomposition of *Synechococcus* cell walls

was not increased by protozoa (Figure 2B), in contrast to the decomposition of dead macrophytes (Johannes, 1965; Fenchel and Harrison, 1976) and *Peridinium* cell walls (Sherr *et al.*, 1982).

The increased decomposition of POC from *Peridinium* cell walls was caused indirectly by protozoan grazing, which stimulated bacterial activity (Sherr *et al.*, 1982). Protozoa can also accelerate POC decomposition directly, by consumption of phytoplankton cells. Caron *et al.* (1985) observed that direct consumption of living diatoms by HNAN of nearly the same size (7–12 μm) was the primary mechanism for remineralization of POC. Approximately 65% of the POC initially present was lost in 8 days. The carbon mineralization of living *Chlorella* cells was increased 3-fold by HNAN which grazed on algae and bacteria (Güde, 1985). Apparently the dead *Synechococcus* cell wall fragments in our experiment were not consumed and mineralized directly by protozoa (Figure 2B). This may have been caused by rapid dispersion of the cell walls into particles too small for protozoan ingestion. Many wall fragments were visible during microscopical counting of microorganisms, immediately after the enrichment. However, these visible fragments disappeared within 1 week, whereas in 4 weeks no significant TOC decrease occurred. Similarly, Fallon and Brock (1979) observed that recognizable algal cellular structure disappeared much more rapidly than POC.

Remineralization of N and P

Although lake water enriched with *Synechococcus* cell walls showed no significant decrease of TOC (Figure 2B), considerable amounts of N (Figure 2E) and P (Figure 2D) were remineralized. N and P are often mineralized faster than organic C (Fallon and Brock, 1979). Whereas 10 times more cell walls than cell extract were added, remineralization in experiments 1 and 2 yielded similar amounts of PO_4-P (~60 μg l^{-1}, Figures 1C and 2D). Thus much more PO_4-P was remineralized from *Synechococcus* cell extract than from cell walls, which have a low P content (Drews and Weckesser, 1982). On the other hand, cell walls yielded much more NH_3-N.

The remineralization of N and especially P from dead *Synechococcus* was greatly increased by the presence of protozoan grazers (Figures 1C and E, and 2D and E). Bacterial growth after enrichment with DOC and POC did not result in remineralization but in uptake of PO_4-P (Figures 1A and C, and 2A and D). Because bacteria usually have a higher P content than their substrate (Thingstad, 1987), and can store P in great excess of their demands, the PO_4-P uptake will generally exceed the release (Güde, 1985). Thus PO_4-P is generally immobilized and not mineralized by bacteria. According to Johannes (1965) P is remineralized mainly by protozoan ingestion of bacteria and subsequent excretion of dissolved P. This direct nutrient regeneration by protozoa can continue through the stationary phase (Goldman *et al.*, 1985; Andersen *et al.*, 1986). Also in experiment 1, PO_4-P mineralization continued after protozoan growth (Figure 1A and C). In contrast to Johannes (1965), Barsdate *et al.* (1974)

and Fenchel and Harrison (1976) argued that protozoa indirectly increased P mineralization from dead macrophytes, by accelerating bacterial activity and P cycling. In the presence of grazers they observed an increased P turnover in bacterial biomass, as short as 1 h. An increased P recycling, however, only indicates a rapid exchange between intra- and extracellular pools, and not necessarily a net P mineralization by bacteria.

The absence of net P mineralization during bacterial growth in our experiments suggests that direct consumption of bacteria by protozoa was the major mechanism of P remineralization. The protozoa consumed about 20×10^9 bact l^{-1} (Figures 1A and 2A). Assuming a P content of 2.6×10^{-9} μg P bacterium^{-1} (Barsdate *et al.*, 1974), the consumption by protozoa can roughly be estimated at 50 μg P l^{-1}, which is of the same magnitude as the observed mineralization of up to 60 μg P l^{-1} (Figures 1C and 2D). Direct mineralization by protozoa in our experiments cannot be proved because the exact bacterial P content is not known. However, in late continuous culture experiments with a mixed population of Lake Vechten bacteria we measured $3.55 \pm 0.47 \times 10^{-9}$ μg P bacterium^{-1} (mean ± 95% confidence limits), which is close to the value assumed here. In systems with living micro-algae, bacteria and protozoa, direct consumption of algae by protozoa caused the bulk of P remineralization, whereas bacteria did not remineralize P, but competed with algae for PO_4-P (Güde, 1985; Andersen *et al.*, 1986).

Although often bacteria seem to be a P sink, this is not always true. If the substrate for bacteria contains elements such as P and N in excess of the amount incorporated in bacterial biomass, these nutrients would have to be excreted and the bacteria would function as remineralizers (Thingstad, 1987). Remineralization by bacteria occurred after enrichment of Lake Vechten water with *Synechococcus* cell walls. Both in the absence and presence of grazers, NH_3-N concentrations increased strongly during bacterial growth in the first days of incubation (Figure 2A and E). However, in the presence of protozoa the final NH_3-N concentration was 2.7 times higher. An increased N mineralization by protozoa was also observed by Goldman *et al.* (1985). Güde (1985) found 3.7 times higher N and 45 times higher P mineralization rates in the presence of HNAN grazers. Also in our experiments protozoa increased P mineralization more than N mineralization.

In conclusion, protozoa can strongly accelerate cycling of specific nutrients through plankton in aquatic ecosystems. Although bacteria can mineralize nutrients from dead phytoplankton, often nutrients are trapped in bacterial biomass, and remineralized only by protozoan grazing. Living microphytoplankton may also be consumed and mineralized directly by protozoa. Nutrients remineralized by protozoa can enhance new phytoplankton growth.

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Protozoan Grazing, Bacterial Activity, and Mineralization in Two-Stage Continuous Cultures

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In two-stage continuous cultures, at bacterial concentrations, biovolumes, and growth rates similar to values found in Lake Vechten, ingestion rates of heterotrophic nanoflagellates (HNAN) increased from 2.3 bacteria · $HNAN^{-1}$ · h^{-1} at a growth rate of 0.15 day^{-1} to 9.2 bacteria · $HNAN^{-1}$ · h^{-1} at a growth rate of 0.65 day^{-1}. On a yeast extract medium with a C/N/P ratio of 100:15:1.2 (Redfield ratio), a mixed bacterial population showed a yield of 18% (C/C) and a specific carbon content of 211 fg of C · μm^{-3}. The HNAN carbon content and yield were estimated at 127 fg of C · μm^{-3} and 47% (C/C). Although P was not growth limiting, HNAN accelerated the mineralization of PO_4-P from dissolved organic matter by 600%. The major mechanism of P remineralization appeared to be direct consumption of bacteria by HNAN. N mineralization was performed mainly (70%) by bacteria but was increased 30% by HNAN. HNAN did not enhance the decomposition of the relatively mineral-rich dissolved organic matter. An accelerated decomposition of organic carbon by protozoa may be restricted to mineral-poor substrates and may be explained mainly by protozoan nutrient regeneration. Growth and grazing in the cultures were compared with methods for in situ estimates. Thymidine incorporation by actively growing bacteria yielded an empirical conversion factor of 1.1×10^{18} bacteria per mol of thymidine incorporated into DNA. However, nongrowing bacteria also showed considerable incorporation. Protozoan grazing was found to be accurately measured by uptake of fluorescently labeled bacteria, whereas artificial fluorescent microspheres were not ingested, and selective prokaryotic inhibitors blocked not only bacterial growth but also protozoan grazing.

In the metalimnion of Lake Vechten, thymidine incorporation indicated high bacterial growth rates of up to 0.7 day^{-1}, which coincided with maximum heterotrophic nanoflagellate (HNAN; size, 2 to 20 μm) concentrations of more than 30×10^6 HNAN $liter^{-1}$ (J. Bloem and M.-J. B. Bär-Gilissen, Limnol. Oceanogr., in press). Grazing experiments in batch cultures with selectively filtered Lake Vechten water suggested that HNAN have the potential to crop the high bacterial production even at relatively low ingestion rates of about 7 bacteria · $HNAN^{-1}$ · h^{-1}. Consumption of bacteria by protozoa can accelerate the mineralization of organic matter either directly by consumption of bacteria and small phytoplankton (1, 19) or indirectly by stimulation of bacterial growth (16, 20).

In batch cultures of Lake Vechten water enriched with autoclaved cyanobacteria, N and especially P mineralization rates were strongly increased by protozoan grazing (Bloem et al., J. Plankton Res., in press). We estimated ingestion rates of between 4 and 96 bacteria $HNAN^{-1}$ · h^{-1} from decreasing bacterial numbers during exponential HNAN growth in batch cultures. However, because the bacterial decrease is relatively small during the period of balanced HNAN growth, the statistics of estimates from dynamic batch cultures are relatively poor (15). Much more accurate estimates of grazing and mineralization can be obtained with continuous cultures in steady state (11, 19), which facilitate maintenance of stable low growth rates, small bacterial biovolumes, and low bacterial concentrations, similar to values found in the lake.

In addition to estimates from direct counts of cell numbers in cultures, several methods to estimate in situ protozoan grazing, such as measurement of protozoan uptake of fluorescently labeled bacteria (FLB) (42) or artificial fluorescent microspheres (26, 32), which can be added as tracers to field samples, have been published recently. Grazing can also be estimated from the disappearance of bacteria after addition of selective procaryotic inhibitors which block bacterial growth (4).

The aim of this study was to quantify HNAN grazing on bacteria in two-stage continuous cultures at various growth rates and to examine the effect of protozoan grazing on carbon, nitrogen, and phosphorus mineralization rates. Moreover, gross growth efficiencies (yields) as well as C and P contents of bacteria and protozoa were estimated. Protozoan grazing in the cultures was compared with results from procaryotic inhibitor experiments and with uptake of FLB and fluorescent microspheres in subsamples of the cultures. Bacterial production was compared with thymidine incorporation into different macromolecular fractions to obtain an empirical conversion factor needed for in situ estimates (18).

MATERIALS AND METHODS

Chemical analyses. Total organic carbon was determined with a model 915A TOC analyzer (Beckman Instruments, Inc., Fullerton, Calif.). After filtration through acid-rinsed 0.45-μm-pore-size membrane filters (Millipore HA; Millipore Corp., Bedford, Mass.), dissolved nutrients were determined. Dissolved organic carbon (DOC) was measured with a continuous-flow autoanalyzer (Skalar Analytical, Breda, The Netherlands), which oxidized DOC by UV radiation to CO_2. The CO_2 was measured colorimetrically at 550 nm with phenolphthalein indicator (39). Particulate organic carbon (POC) was calculated as TOC minus DOC. With a similar autoanalyzer, PO_4-P was determined as soluble molybdate-reactive phosphorus (28). After destruction with a persulfate-sulfuric acid mixture (12), total phos-

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phorus and total dissolved phosphorus were determined as molybdate-reactive phosphorus. Dissolved organic phosphorus was calculated as total dissolved phosphorus minus molybdate-reactive phosphorus, and particulate phosphorus was calculated as total phosphorus minus total dissolved phosphorus. Dissolved Kjeldahl N (NH_3-N plus dissolved organic nitrogen) was converted to NH_3-N as described by Scheiner (38). NH_3-N determinations were based on indophenol formation with sodium salicylate (48). NO_3-N was determined by cadmium-copper reduction to NO_2-N (49) and measured according to the method of Freier (17).

Counting. Subsamples (10 ml) were fixed with 1% glutaraldehyde (final concentration), and HNAN were stained with primulin and counted directly on membrane filters (Nuclepore Corp., Pleasanton, Calif.) by epifluorescence microscopy (5). For bacteria, the 4,6-diamidino-2-phenylindole technique was used (33). For biovolume estimates, immediately after fixation 100 organisms per sample were measured by eyepiece micrometer (standard deviation [SD] of duplicates, <20%), and volumes were calculated from length and width, assuming a spherical or cylindrical shape.

Cultures. The principles of two-stage continuous cultures to study grazing of bacteria by protozoa (ciliates) were described by Curds and Cockburn (11), and the culture vessels were described by Steenbergen (45). From a 10-liter reservoir, sterile culture medium was pumped at a constant rate by a two-channel peristaltic pump (Minipuls 2; Gilson, Villiers le Bel, France) to a sterilized coupled system of two 1,000-ml culture vessels and a waste vessel. The cultures were kept at 15°C in the dark, thoroughly stirred, and aerated. Sterile air entered the first culture vessel and left via the waste vessel to promote the flow of culture fluid through the system. Bacteria were grown in the first stage and fed to the second stage containing HNAN, which strongly reduced the bacterial concentrations. In steady state, the specific growth rate μ (per hour) of the organisms equals the dilution rate D (per hour) (flow rate divided by vessel volume), assuming complete mixing and negligible death rates. Negligible mortality was indicated by Lake Vechten bacteria in grazer-free batch cultures, which showed stable numbers for more than 1 week (Bloem and Bär-Gilissen, in press; Bloem et al., in press). The protozoan ingestion rate I (bacteria per HNAN per hour) is then given by $D\ (b_1 - b_2)/n$, where b_1 and b_2 are the bacterial concentrations in the first and second stages and n is the number of HNAN per liter (11). The clearance F (nanoliters per HNAN per hour) is calculated as I/b_2. The protozoan yield Y_{HNAN} (percent) is given by $[n/(b_1 - b_2)] \times 100$, where b and n are expressed as biovolume or biomass. The bacterial yield Y_{bact} (percent) is calculated as $[b_1/(C_m - C_1]) \times 100$, where C_m and C_1 are the DOC concentrations in the medium reservoir and the first stage, respectively, and b_1 is expressed as biomass (in milligrams of C per liter). Net nutrient mineralization rates R (in micrograms per liter per hour) are calculated as $D \times C_{diff}$, where C_{diff} is the dissolved nutrient concentration difference between inlet and outlet of a vessel (19).

Thymidine incorporation. Two Formalin-killed blanks and five replicates of 15 ml containing 20 nM [*methyl*-^{3}H]thymidine (3.3 TBq · mmol^{-1}; Amersham Ltd., Amersham, United Kingdom) were incubated at 15°C in the dark for 20 min. The samples were then fixed with 2% formaldehyde (final concentration). Each replicate was split into three 5-ml portions, which were (i) extracted with ice-cold 5% trichloroacetic acid (TCA), (ii) hydrolyzed at 60°C with 1 N NaOH, or (iii) hydrolyzed at 100°C with 20% TCA, as described by Riemann and Sondergaard (35), to obtain (i) total (cold-TCA-insoluble) macromolecules, (ii) DNA plus proteins, or (iii) proteins. The precipitated macromolecular fractions were collected on 0.2-μm-pore-size cellulose nitrate filters (Schleicher & Schuell, 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 (model 4530) liquid scintillation counter. Counting efficiency was determined by automatic external standardization. By subtraction of the macromolecular fractions, incorporation into DNA, RNA, and proteins was calculated. Incorporation into DNA is a measure of DNA synthesis and thus of bacterial production (18). Since bacterial production in the first stage of the continuous cultures is known ($b_1 \times \mu$), production divided by incorporation yields an empirical conversion factor to calculate bacterial production from thymidine incorporation.

Ingestion rate measurements. Protozoan ingestion rates were measured with subsamples of the HNAN-containing culture vessel 4 (experiment 2). From the grazer-free second stage (vessel 2), bacteria were concentrated by centrifugation for 15 min at 22,000 × *g* and stained with 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (Sigma Chemical Co., St. Louis, Mo.) at 200 mg · liter^{-1} (final concentration) for 2 h at 60°C as described by Sherr et al. (42). 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 bacterial clumps. The FLB thus obtained were added to 20-ml subsamples of the HNAN culture at a final FLB concentration of 10 to 20% of the total bacterial abundance. Growth rates of *Monas* batch cultures were not affected by 30% FLB. At 5-min intervals for 40 min, 1-ml subsamples were fixed with 4 ml of cold (5°C) glutaraldehyde at a final concentration of 2%. The HNAN were collected on 1-μm-pore-size Nuclepore filters, mounted on slides with immersion oil, and stored at −30°C (5). By epifluorescence microscopic examination of 100 HNAN per time point sample, the increase in average number of FLB per HNAN with time was determined, and protozoan ingestion rates were calculated by linear regression. Similar uptake experiments were performed with bacterium-sized (about 0.4 μm in diameter) fluorescent paint particles (grade JST 300, color orange yellow 322; Radiant Color Co.) (26) and with fluorescent microspheres (0.61 μm in diameter; PolySciences Fluoresbrite carboxylated microspheres). Selective-inhibitor experiments were performed by using a combination of penicillin and streptomycin (Sigma) at final concentrations of 50 mg · liter^{-1} (4).

RESULTS

Two experiments were performed. In experiment 1, only grazing, not mineralization, was studied. The culture medium consisted of 0.001% (wt/vol) Knop solution (24) enriched with yeast extract (Oxoid Ltd., London, United Kingdom) at a final concentration of 10 mg · liter^{-1}, close to DOC concentrations of 5 mg · liter^{-1} found in Lake Vechten. In experiment 2, 50 mg of yeast extract liter^{-1} was used because higher DOC concentrations were required to measure C mineralization. This medium was applied to approach dissolved complex natural substrates. The molar C/N/P ratio (100:15:1.2) of the medium is similar to the Redfield ratio (47). In experiment 1, the first stage was inoculated with an unidentified strain of heterotrophic bacteria isolated from Lake Vechten. However, this pure culture did not incorporate thymidine. Because thymidine in-

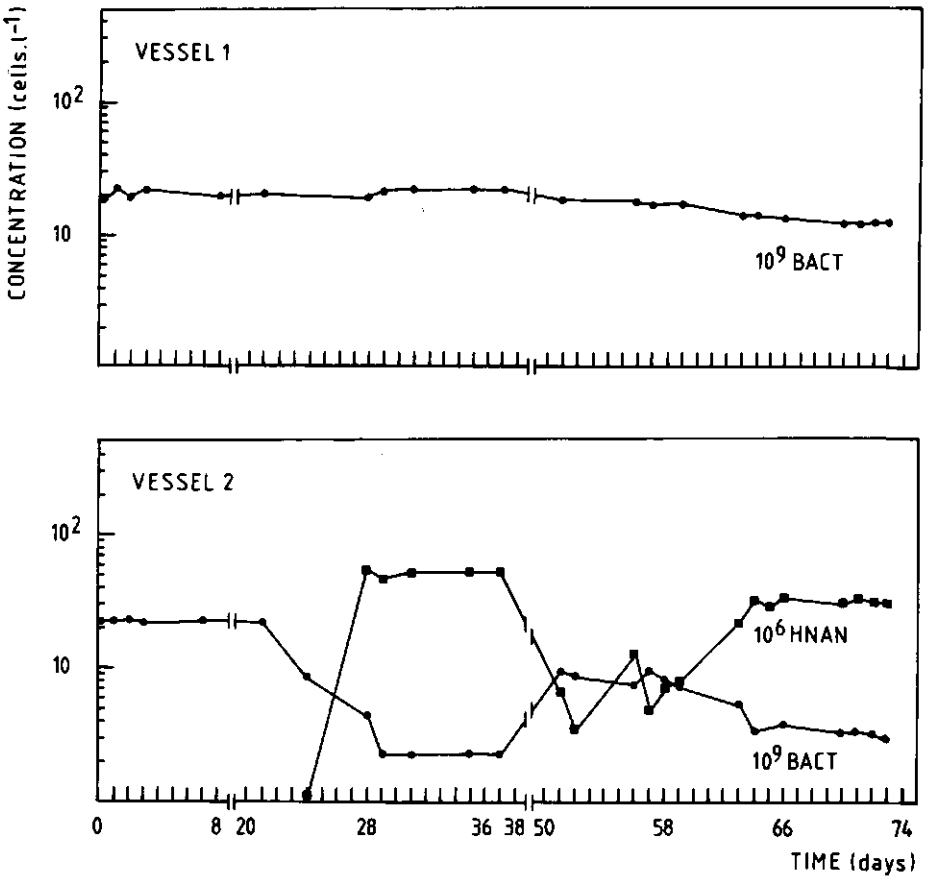

FIG. 1. Bacterial (●) and HNAN (■) concentrations in the two-stage continuous culture at dilution rates of 0.15 (days 0 to 37) and 0.31 (days 38 to 73) day^{-1}.

corporation was intended to monitor bacterial production in the cultures, in experiment 2 a grazer-free mixed bacterial population from Lake Vechten that was found to incorporate thymidine was used. The grazer-free bacteria were obtained by sieving lake water through 1-μm-pore-size Nuclepore filters. Ten bottles, each containing 50 ml of filtrate enriched with yeast extract (10 mg · liter^{-1}, final concentration), were incubated during 2 weeks. A bottle in which no protozoa were observed by epifluorescence microscopy was used as a grazer-free bacterial inoculum. *Monas* sp. (about 6 μm in size), a HNAN isolated from Lake Vechten, was inoculated as a grazer in the second stage.

In experiment 1, a steady state of bacterial numbers was reached within 1 week at a dilution rate of 0.15 day^{-1}. During week 1 the bacterial numbers were similar in both vessels, which indicated that no net growth or mortality occurred in the second stage (Fig. 1). HNAN were inoculated in vessel 2 on day 24 and reached a steady state (50 × 10^6 HNAN · liter^{-1}) on day 29. The HNAN reduced the bacterial concentration from 20 × 10^9 to 2.7 × 10^9 bacteria · liter^{-1}. This situation was maintained for 1 week to determine the steady-state concentrations and biovolumes of bacteria and HNAN, which were used to calculate the ingestion rate, clearance, and yield at a specific growth rate of 0.15 day^{-1}. Then the dilution rate was doubled to establish a growth rate of 0.31 day^{-1}. After some oscillations, on day 66 a new steady state was reached at 32 × 10^6 HNAN · liter^{-1} and 3.4 × 10^9 bacteria · liter^{-1} in vessel 2 and 12 × 10^9 bacteria · liter^{-1} in vessel 1. At the end of the experiment some wall growth became apparent. Wall growth may lead to reduced bacterial numbers in the fluid and to underestimated ingestion rates.

In experiment 2, two parallel two-stage systems were used instead of a single two-stage system. Bacteria from vessel 1 (first stage) were fed to vessel 2 (second stage), which contained no protozoa and served as a grazer-free control. In the parallel system, bacteria from vessel 3 (first stage) were fed to vessel 4 (second stage), which contained the HNAN. After 4 weeks at a dilution rate of 0.65 day^{-1}, the mixed bacterial population reached a steady state at 83 × 10^9 bacteria · liter^{-1} (Fig. 2). In the absence of grazers during days 31 to 35 (phase I), all vessels showed the same bacterial numbers. To prevent wall growth, after day 35 the culture systems were broken down, cleaned, sterilized, and restarted at the same dilution rate. After 3 weeks the bacteria showed the same stable numbers as in phase I. HNAN were inoculated in vessel 4 on day 26 and reduced the bacteria from 83 × 10^9 to 2.3 × 10^9 bacteria · liter^{-1}. A steady state of 240 × 10^6 HNAN · liter^{-1} was reached after day 33 (phase II). Wall growth was not observed. The fivefold-higher yeast extract concentration in experiment 2 resulted in higher bacterial numbers in the first stage, whereas in the grazed second stage the HNAN rather than the bacteria were increased (Fig. 1 and 2).

From the experiments 1 and 2, results were obtained at three growth rates (Table 1). The ingestion rate increased from 2.3 bacteria · HNAN^{-1} · h^{-1} at a growth rate of 0.15 day^{-1} to 9.2 bacteria · HNAN^{-1} · h^{-1} at 0.65 day^{-1}. The

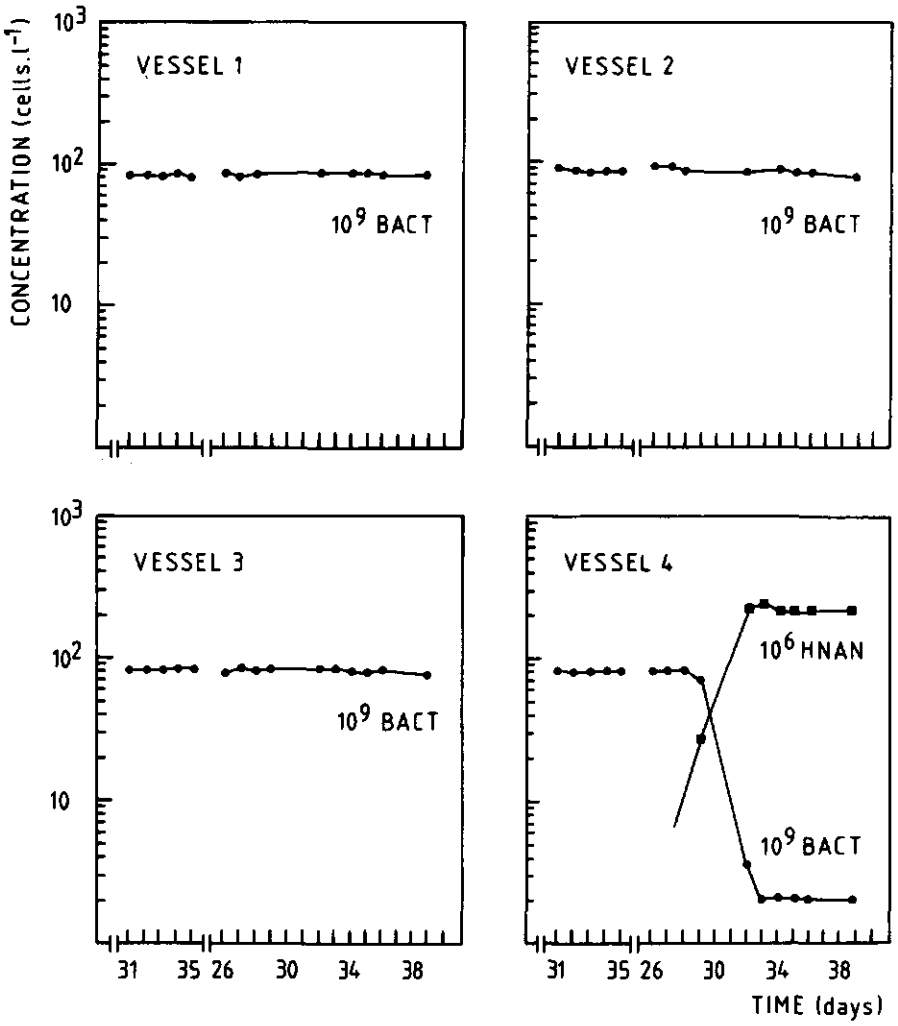

FIG. 2. Bacterial (●) and HNAN (■) concentrations in the two parallel two-stage continuous cultures during two runs at a dilution rate of 0.65 day^{-1}. Vessel 4 was fed by vessel 3, and vessel 2 was fed by vessel 1.

yields calculated from the HNAN biovolume grown per bacterial biovolume consumed (percent by volume) were very high. An overestimated yield may be caused by overestimation of the HNAN biovolume or underestimation of the bacterial biovolume. Since fixed cells were used, the effect of fixation was checked by comparing biovolumes before and after fixation. Living cells were measured by phase contrast, and fixed cells were measured by both phase contrast and epifluorescence microscopy. The bacterial biovolumes had not changed within a few hours after fixation with 1% glutaraldehyde or 5% formaldehyde, although after 1 week a 50% reduction was found. However, the HNAN showed a 50% reduction within a few hours.

In experiment 2, at a dilution rate of 0.027 h^{-1} during 5-day steady-state periods, C, N, and P concentrations were determined every other day. Figure 3 shows the average concentrations per vessel. Microbial C and P contents, growth yields, and mineralization rates were calculated for each sample separately rather than from the averages and then averaged over time. In the absence of grazers (phase I),

TABLE 1. Results of two-stage continuous culture experiments at three growth rates[a]

Expt	Bacteria					HNAN (in stage 2)					
	Specific growth rate (day^{-1})	Concn (10^9 bacteria · liter^{-1})		Biovolume (μm^3 · bacterium^{-1})		Concn (10^6 HNAN · liter^{-1})	Biovolume (μm^3 · HNAN^{-1})	Ingestion rate (bacteria · HNAN^{-1} · h^{-1})	Clearance (nl · HNAN^{-1} · h^{-1})	Yield (10^{-3} HNAN cells · bacterium^{-1})	Yield (%, vol/vol)
		Stage 1	Stage 2	Stage 1	Stage 2						
1	0.15 ± 0.01	20.82 ± 0.55	2.34 ± 0.05	0.15	0.17	50.4 ± 2.11	48	2.29 ± 0.23	0.98 ± 0.12	2.73 ± 0.12	92.0 ± 4.0
	0.31 ± 0.01	12.21 ± 0.46	3.41 ± 0.36	0.17	0.14	32.5 ± 1.54	77	3.53 ± 0.16	1.05 ± 0.13	3.69 ± 0.17	161.0 ± 7.0
2	0.65 ± 0.01	82.79 ± 1.42	2.27 ± 0.08	0.14	0.13	239.7 ± 6.92	40	9.16 ± 0.06	4.04 ± 0.16	2.98 ± 0.03	83.0 ± 0.9

[a] Mean ± SD, $n = 4$.

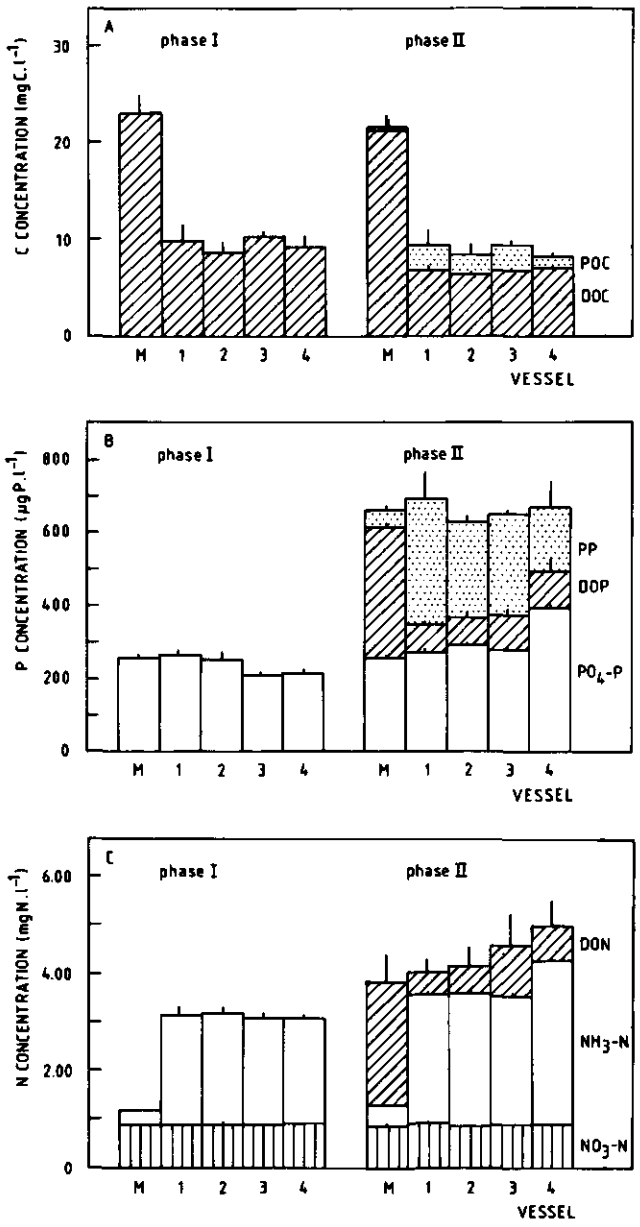

FIG. 3. Carbon (A), phosphorus (B), and nitrogen (C) concentrations in the medium (M) and culture vessels (1 through 4) without (phase I) and with (phase II) HNAN grazers in vessel 4. Error bars indicate 1 SD; n = 3. POC, Particulate organic carbon; DOC, dissolved organic carbon; PP, particulate phosphorus; DOP, dissolved organic phosphorus; DON, dissolved organic nitrogen.

the concentrations in vessels 1 and 2 equaled those in vessels 3 and 4. Therefore, vessel 2 was used as a grazer-free control of vessel 4 in the presence of HNAN (phase II).

In the first stages (vessels 1 and 3), the bacteria reduced DOC from 21.1 ± 1.04 (SD) to 6.66 ± 0.29 mg · liter^{-1} (n = 6) (Fig. 3A). The consumed DOC (14.4 ± 0.99 mg · liter^{-1}) was converted partly to POC (2.63 ± 1.17 mg · liter^{-1}). Thus, a bacterial yield of 18.2 ± 8.26% (C/C) was calculated, assuming that all POC in the first stages was bacterial biomass. From the concentrations of POC (2.41 ± 0.89 mg · liter^{-1} ± 95% confidence limits; n = 9) and bacteria (82.3 ± 2.28 × 10^9 bacteria · liter^{-1}) in grazer-free vessels 1, 2, and 3 (Fig. 2 and 3A), a bacterial C content of 29.5 ± 1.11 fg of C · bacterium^{-1} was calculated (Table 2). Since the biovolume was 0.14 µm^3 · bacterium^{-1} (Table 1), the biovolume-to-biomass conversion factor can be estimated at 211 ± 79 fg of C · µm^{-3}. In vessel 4, the HNAN reduced the number of bacteria to 2.35 × 10^9 ± 0.11 × 10^9 (SD) bacteria · liter^{-1} (n = 3). Assuming the above C content, the bacteria contributed 0.069 ± 0.003 mg · liter^{-1} to the total POC concentration of 1.23 ± 0.55 mg · liter^{-1}. If the remaining POC (1.16 ± 0.55 mg · liter^{-1}) consisted of HNAN (232.9 × 10^6 ± 7.42 × 10^6 HNAN · liter^{-1}), the protozoan C content can roughly be estimated at 5.02 ± 2.42 pg of C · HNAN^{-1} (Table 2). From the HNAN biomass (1.16 ± 0.55 mg of C · liter^{-1}) and the bacterial biomass consumed (2.66 ± 0.68 mg of C · liter^{-1}, calculated from the differences in bacterial POC between vessels 3 and 4), the yield of the HNAN can roughly be estimated at 47 ± 28% (C/C). A similar HNAN yield of 62 ± 24% (P/P) was calculated by using the estimated bacterial and protozoan P contents (Table 2). P contents were estimated at 3.55 fg of P · bacterium^{-1} and 716 fg of P · HNAN^{-1}, with calculations similar to those used for C.

TABLE 2. Carbon and phosphorus content of fixed bacteria (0.14 µm^3 · cell^{-1}) and HNAN (40 µm^3 · cell^{-1}) in the continuous cultures of experiment 2

Determination	Bacteria (n = 9)			HNAN (n = 3)[a]	
	Mean	SD	95% Confidence limit	Mean	SD
C					
fg · cell^{-1}	29.5	14.4	11.1	5,020	2,420
fg · µm^{-3}	211	103	79	127	61
P					
fg · cell^{-1}	3.55	0.61	0.47	716	281
fg · µm^{-3}	25.4	4.4	3.4	18.1	7.1

[a] 95% confidence limits were not determined.

In the first stages (vessels 1 and 3), most of the dissolved organic phosphorus from the medium was converted to particulate phosphorus, whereas the PO_4-P concentrations were not increased (Fig. 3B). Thus, dissolved organic phosphorus was incorporated into bacterial biomass rather than mineralized. In the second stages, no changes occurred without HNAN (vessel 2), whereas in the presence of HNAN (vessel 4) PO_4-P increased and particulate phosphorus decreased. Thus, significant P mineralization occurred only in the presence of grazers. Most of the dissolved organic nitrogen from the medium disappeared in the first stages (vessels 1 and 3, Fig. 3C), whereas NH_3-N was greatly increased. In the second stages no changes occurred without HNAN (vessel 2), but in the presence of HNAN (vessel 4) NH_3-N increased further. NO_3-N concentrations were the same in all vessels.

C was mineralized mainly by bacteria in the first stages at rates of 310 µg of C · liter^{-1} · h^{-1} (vessels 1 and 3, Fig. 4A). Some C mineralization (about 30 µg of C · liter^{-1} · h^{-1}) occurred in the second stages (vessels 2 and 4), but this rate was not significantly increased by HNAN (vessel 4, phase II). The grazers strongly increased the PO_4-P mineralization rates (Fig. 4B), from 0.51 ± 0.13 to 3.09 ± 0.11 µg of P · liter^{-1} · h^{-1}(n = 3). Also, NH_3-N mineralization (Fig. 4C) in the second stages was strongly increased in the presence of HNAN, from 2.22 ± 2.98 to 20.3 ± 1.90 µg of N · liter^{-1} · h^{-1}. However, much higher rates of 61.4 ± 2.25 µg of N · liter^{-1} · h^{-1} were observed in the grazer-free first stages.

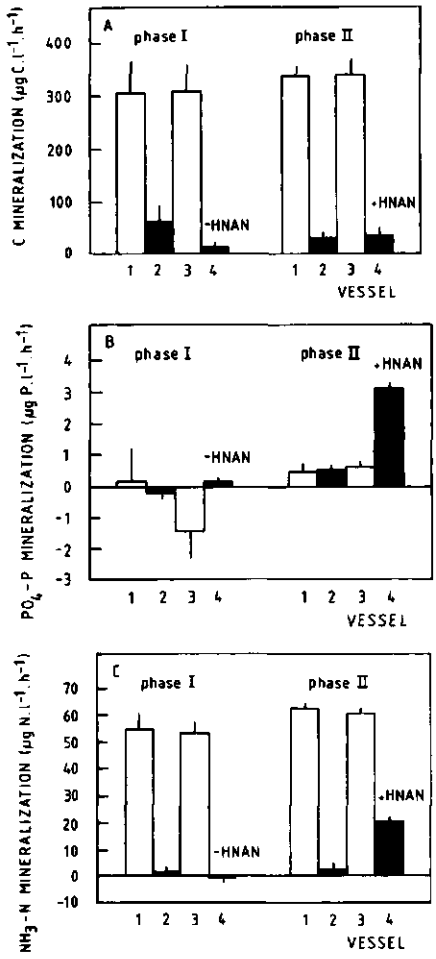

FIG. 4. Carbon (A), phosphorus (B), and nitrogen (C) mineralization rates in the culture vessels (1 through 4) without (phase I) and with (phase II) HNAN grazers in vessel 4. Error bars indicate 1 SD; $n = 3$.

In subsamples of vessel 4, FLB uptake yielded an ingestion rate of 8.67 ± 0.78 bacteria · $HNAN^{-1}$ · h^{-1} (Fig. 5), which is not significantly different from the calculated rate of 9.16 bacteria · $HNAN^{-1}$ · h^{-1} (Table 1). Subsamples treated with the procaryotic inhibitors did not show any bacterial decrease in 24 h, whereas in untreated subsamples the bacteria strongly decreased during the first 5 h of incubation, and linear regression yielded an ingestion rate of

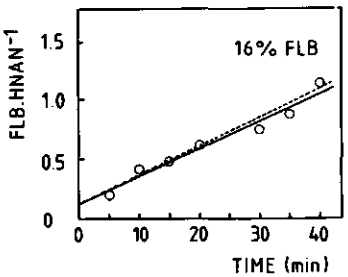

FIG. 5. Uptake of FLB by HNAN in a subsample of vessel 4. The measured uptake (——) is not significantly different from the calculated uptake (----) and yields an ingestion rate of 8.67 ± 0.78 bacteria · $HNAN^{-1}$ · h^{-1}.

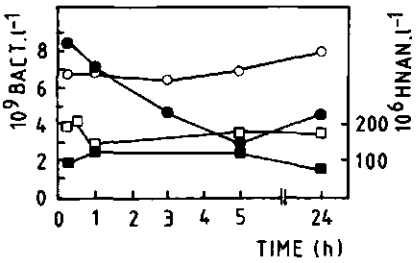

FIG. 6. HNAN (□, ■) and bacterial (○, ●) concentrations in subsamples of vessel 4 with (□, ○) and without (■, ●) penicillin and streptomycin. The bacterial decrease indicates an ingestion rate of 9.66 ± 0.67 bacteria · $HNAN^{-1}$ · h^{-1}.

9.66 ± 0.67 bacteria · $HNAN^{-1}$ · h^{-1} (Fig. 6). Apparently, the procaryotic inhibitors blocked protozoan grazing completely. This was confirmed by FLB uptake experiments. In the presence of inhibitors no FLB uptake occurred, whereas in the absence of antibiotics an ingestion rate of 8.39 ± 0.97 bacteria · $HNAN^{-1}$ · h^{-1} was found (Fig. 7).

From the thymidine incorporation by the actively growing bacteria in the first stages of the continuous cultures (Table 3, vessels 1 and 3) and the calculated bacterial production, an empirical conversion factor of 0.47×10^{18} bacteria · mol of $thymidine^{-1}$ was calculated for total incorporation into cold-TCA-insoluble macromolecules. Since 39 to 45% of the label appeared in DNA, for incorporation into DNA a conversion factor of 1.1×10^{18} bacteria · mol of $thymidine^{-1}$ was derived. In the grazed vessel 4, incorporation into DNA was only 6%, and 60% of the label appeared in the RNA fraction, which is twice as high as the percentage in the ungrazed vessels. In all vessels, about 30% of the label was found in proteins. Nongrowing bacteria in vessel 2 showed a relatively high thymidine incorporation into DNA (about 60% of that in vessel 1), whereas subsamples from vessel 2 showed stable bacterial numbers during 24-h incubations. Thus, thymidine incorporation and bacterial growth (cell division) seemed not to be closely coupled.

DISCUSSION

Grazing. The growth rates, biovolumes, and cell concentrations in the two-stage continuous cultures (Table 1) were similar to natural values. In Lake Vechten, we estimated bacterial growth rates of 0.01 to 0.6 day^{-1}, bacterial biovolumes of 0.10 to 0.27 μm^3 · $bacterium^{-1}$, densities of 2×10^9 to 20×10^9 bacteria · $liter^{-1}$, and up to 40×10^6 HNAN $liter^{-1}$ (Bloem and Bär-Gilissen, in press). To measure

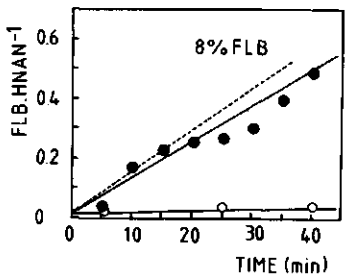

FIG. 7. Uptake of FLB by HNAN in subsamples of vessel 4 with (○) and without (●) penicillin and streptomycin, showing ingestion rates of 0 and 8.39 ± 0.97 bacteria · $HNAN^{-1}$ · h^{-1}. ----, Calculated uptake.

TABLE 3. Thymidine incorporation rates in the four culture vessels of experiment 2

Vessel	Thymidine incorporation (10^{-9} mol · l^{-1} · h^{-1}) in:				Specific thymidine incorporation (10^{-20} mol · bacterium^{-1} · h^{-1}) in:			
	Cold-TCA-insoluble macromolecules	DNA	RNA	Proteins	Cold-TCA-insoluble macromolecules	DNA	RNA	Proteins
1	4.57	2.07	1.19	1.31	5.65	2.56	1.47	1.62
2	3.77	1.26	1.11	1.40	5.39	1.80	1.50	2.00
3	4.89	1.90	1.27	1.72	6.33	2.46	1.65	2.22
4	0.37	0.02	0.23	0.13	12.30	0.72	7.46	4.14

mineralization, in experiment 2 a fivefold-higher substrate concentration was used than in experiment 1. This resulted in about fivefold-higher HNAN concentrations, whereas the bacterial concentration remained constant at about 2×10^9 bacteria · liter^{-1}. This seems to be the minimum level that can support HNAN growth (14) and is similar to minimum Lake Vechten concentrations.

Due to the steady state in the continuous cultures, the variability in the calculated ingestion rates was low (SD, <10%; Table 1). This does not necessarily mean that the rates are accurate. They might be underestimated if the grazed bacteria were growing, whereas no growth was assumed in the second stages. However, protozoan grazing may stimulate bacterial growth (16), and therefore thymidine incorporation was measured (Table 3). Since the nongrowing bacteria in the ungrazed vessel 2 showed an unexpectedly high incorporation into DNA, no firm conclusions can be drawn about growth of the grazed bacteria. The effect of possible growth on the calculated ingestion rates was negligible, however, because the bacterial numbers in vessel 4 were low compared with numbers in the inflow from vessel 3 and because the same ingestion rates were found in FLB uptake experiments. The good agreement between the different methods supports the validity of the FLB method for in situ estimates. In contrast to Sieracki et al. (44), we found no underestimation of ingestion rates caused by FLB egestion after glutaraldehyde fixation or by FLB digestion. Unlike FLB, fluorescent microspheres were ingested neither by the *Monas* culture nor in field samples. Microsphere ingestion and fixation effects may depend on the HNAN species and on the type (surface chemistry) and size of the particles used.

The ingestion rates of 2 to 9 bacteria · HNAN^{-1} · h^{-1} (Table 1) are at the low end of the range reported in the literature. Values of 10 to 250 bacteria · HNAN^{-1} · h^{-1} were found in batch cultures (13, 41). The low values in the continuous cultures may have been caused, at least in part, by the relatively low growth rates and low bacterial concentrations.

To estimate C and energy fluxes in natural ecosystems, the microbial C content is needed. For bacteria, Norland et al. (29) established a size-dependent conversion factor of 50 to 100 fg of C · μm^{-3}. With natural-sized Lake Vechten bacteria, we obtained a somewhat higher factor of 211 fg of C · μm^{-3}, close to the values reported by Bratbak and Dundas (9) and Scavia and Laird (37). Higher values of 380 to 560 fg of C · μm^{-3} have also been reported (7, 23) but were considered to be physiologically unreasonable (21). Volume-to-carbon conversion factors are strongly influenced by the method used to measure biovolumes and by fixatives which may change biovolumes (9). In our experiment, soon after fixation the bacterial biovolumes had not changed, but the HNAN had shrunk by about 50%. Similar shrinkage of fixed *Monas* sp. was also reported by Børsheim and Bratbak (6). They proposed conversion factors of 100 (for living cells) and 220 (for fixed cells) fg of C · μm^{-3}, in general agreement with the results of Fenchel (13) and our crude estimate of 127 fg of C · μm^{-3} for fixed HNAN (Table 2).

The uncertainties in HNAN biovolume estimates may have caused part of the very high biovolume-based yields (Table 1), although an excessive apparent yield indicates swelling instead of shrinkage of HNAN. However, fixed *Oxyrrhis* cells showed an initial swelling followed by shrinkage (22). Moreover, the bacteria showed a 70% higher specific carbon content than did the HNAN (Table 2), and thus the biovolume-based yield will be 70% higher than the preferable carbon-based yield. In experiment 2, *Monas* sp. showed a yield of 83% (vol/vol) and 47% (C/C). Similarly, Bratbak (8) found apparent HNAN yields of 70% (vol/vol) and 24% (C/C). Carbon-based yields of 34 and 43% were reported for *Ochromonas* sp. and *Pleuromonas jaculans* (13). For *Monas* sp., Sherr et al. (41) determined dry-weight-based yields of 25 to 45%. For bacteria we calculated a yield of 18% (C/C), similar to the values of 11 to 27% (C/C) found for marine bacterioplankton growing on complex natural substrates in continuous cultures (3). However, bacterial yields below 10% and over 50% have been reported and may vary widely with growth conditions (25, 31).

Mineralization and decomposition. The presence of protozoan grazers greatly increased the remineralization of inorganic P from dissolved organic matter (Fig. 4). Whether such a stimulation by protozoa is caused directly because of consumption of bacteria and excretion of surplus nutrients or indirectly via stimulation of bacterial activity has been subject to controversy (2, 16, 20). In systems with living microalgae, bacteria, and protozoa, direct consumption of algae by protozoa caused the bulk of P remineralization, whereas bacteria did not remineralize but competed with algae for PO_4-P (1, 19). The mineralization of dissolved organic matter leached from algal cells and detritus is probably mediated by bacteria. However, the active bacteria in the continuous cultures showed only slight net P mineralization (Fig. 4). Considerable remineralization occurred in vessel 4, where the HNAN consumed 2.19×10^9 bacteria · liter^{-1} · h^{-1}, containing 3.6 fg of P · bacterium^{-1} (Table 2). Thus, the protozoa consumed 7.8 μg of P · liter^{-1} · h^{-1} as bacterial biomass. Since 60% of the consumed P was incorporated in protozoan biomass, the surplus of 3.0 μg of P · liter^{-1} · h^{-1} was excreted and can account for all P mineralization in vessel 4. Thus, direct consumption of bacteria by HNAN appeared to be the major mechanism of P remineralization.

The relative importance of protozoa and bacteria as nutrient regenerators in the detrital food web depends largely on the C/N/P ratio of the substrate (10). If the substrate contains N and P in excess of the amount incorporated in bacterial biomass, the bacteria will function as remineralizers (47).

This was obviously the case with N in our cultures (Fig. 4). From N-limited batch cultures, Caron et al. (10) concluded that the role of protozoa as remineralizers of a growth-limiting nutrient is maximal when the carbon/nutrient ratio of the substrate is high, i.e., under severe nutrient limitation. However, in our cultures protozoa were the major P remineralizers, whereas the substrate C/P ratio of 83 was not high and P was not limiting. P and N concentrations were relatively high in the medium (Fig. 3), which had a C/N/P composition similar to the Redfield ratio. With such a medium, bacteria should be C limited and are expected to remineralize P according to the mathematical chemostat model of Thingstad (47). This model predicts bacterial P remineralization, assuming that the bacterial biomass composition fits the Redfield ratio, with a C/P ratio of about 100. However, a much lower ratio of 21 was calculated (from Table 3) for the bacteria in the continuous cultures. Thus, the high bacterial P content, which may be common (19, 47), prevented bacterial P remineralization, as already suggested by Thingstad (47). The HNAN showed a C/P ratio of 18, similar to that of their prey, and therefore protozoan consumption of bacteria resulted in a greatly increased P remineralization. Thus, even if P is not growth limiting, HNAN appear to be important remineralizers of inorganic P and may therefore have a significant effect on the primary production and eutrophication of lakes.

Protozoa can also enhance the decomposition rates of organic detritus and pollutants, as observed in mineral-poor substrates such as eelgrass, barley hay (16), and glucose (30). The substrate in our cultures was not mineral poor, and the rate of organic carbon decomposition was not significantly accelerated by HNAN (Fig. 4). Sherr et al. (40) found that the decomposition of dinoflagellate cell protoplasm was not accelerated either, whereas the bacterial breakdown of mineral-poor cell walls was significantly enhanced. They concluded that protozoa may selectively facilitate the breakdown of detritus with a high structural carbohydrate and a low mineral content.

Several mechanisms have been suggested by which HNAN may stimulate bacterial activity and organic carbon decomposition (43). Protozoa can accelerate nutrient regeneration (Fig. 4) and thus the activity of P- and N-limited bacteria. In grazer-free cultures, Sherr et al. (40) stimulated cell wall decomposition by adding inorganic P. A similar stimulation was obtained by periodic removal of bacteria by filtration to simulate cropping by protozoa, which may maintain bacteria in an active metabolic state. However, this cropping effect may have been influenced by cell breakage and nutrient release during the filtration. In our continuous cultures, cropping of bacteria by HNAN did not stimulate C mineralization (Fig. 4). The observed mineralization of 34 μg of C · $liter^{-1}$ · h^{-1} can largely be explained by consumption of bacteria. The HNAN consumed 65 μg of bacterial C · $liter^{-1}$ · h^{-1}, with a yield of 47% (C/C). Thus, the HNAN could mineralize up to 34 μg of C · $liter^{-1}$ · h^{-1}, assuming a negligible excretion of organic carbon. Taylor et al. (46) found that bacterivorous ciliates contributed quantitatively and qualitatively to the dissolved organic matter pool and suggested that this may stimulate bacterial activity and growth. The DOC pool was not increased significantly by HNAN grazing in vessel 4 (Fig. 3), although qualitative changes cannot be excluded. Moreover, excreted DOC could have been taken up rapidly by the bacteria.

The specific thymidine incorporation into cold-TCA-insoluble macromolecules indicated that the activity per bacterium was at most twofold higher in the presence of grazers (Table 3). However, incorporation by nongrowing bacteria in vessel 2 was also high, which suggests that thymidine incorporation does not always indicate growth, although the empirical conversion factor derived for actively growing bacteria in the first stages was similar to the generally accepted value of 1×10^{18} to 2×10^{18} bacteria · mol of $thymidine^{-1}$ (27, 34). Robarts et al. (36) speculated that the RNA fraction obtained by the acid-base hydrolysis may represent labeled lipids and other macromolecules. This hypothesis is supported by the twofold-higher incorporation into "RNA" (60%) in subsamples of vessel 4, which probably contained consumed bacterial biomass incorporated by protozoa.

Even if the twofold-higher specific thymidine incorporation into total macromolecules indicated a twofold-higher specific bacterial growth rate, the overall effect on DOC degradation would be negligible because the bacterial numbers were reduced 36-fold by HNAN (Fig. 2). Such a drastic reduction of bacteria is not an artifact of two-stage continuous cultures with separated production and grazing compartments. It occurred also when HNAN were growing in the first stage. In single continuous cultures, HNAN reduced bacterial numbers 200-fold and also stimulated the degradation of glucose, which was attributed to protozoan P remineralization (30). Since the relatively mineral-rich substrate in our cultures was not decomposed faster in the presence of HNAN, an accelerated decomposition of organic carbon by protozoa may be restricted to mineral-poor substrates and may be explained mainly by protozoan nutrient regeneration.

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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

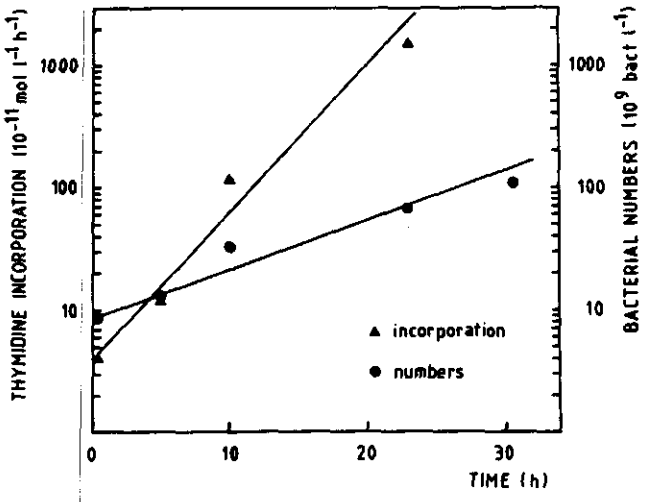

FIG. 1. Decoupling of bacterial growth and thymidine incorporation into cold-TCA-insoluble macromolecules in an exponentially growing batch culture diluted 10-fold.

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 17×10^{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 1.58×10^{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 55×10^6 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 40×10^6 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 11×10^6 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 0.5×10^6 and 2.1×10^6 HNAN liter^{-1} were found (Fig. 4). Also, chroococcoid cyanobacteria (*Synechococcus* sp.; 1 to 2 μm in diameter) showed maximum numbers of up to 3.2×10^9 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

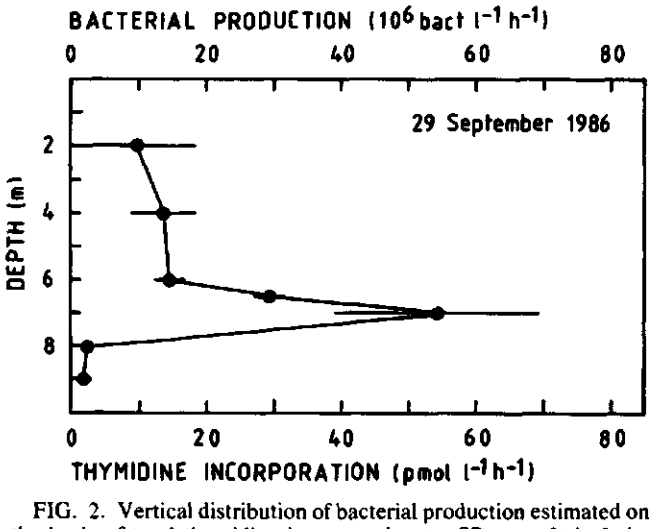

FIG. 2. Vertical distribution of bacterial production estimated on the basis of total thymidine incorporation (± SD; n = 3) in Lake Vechten.

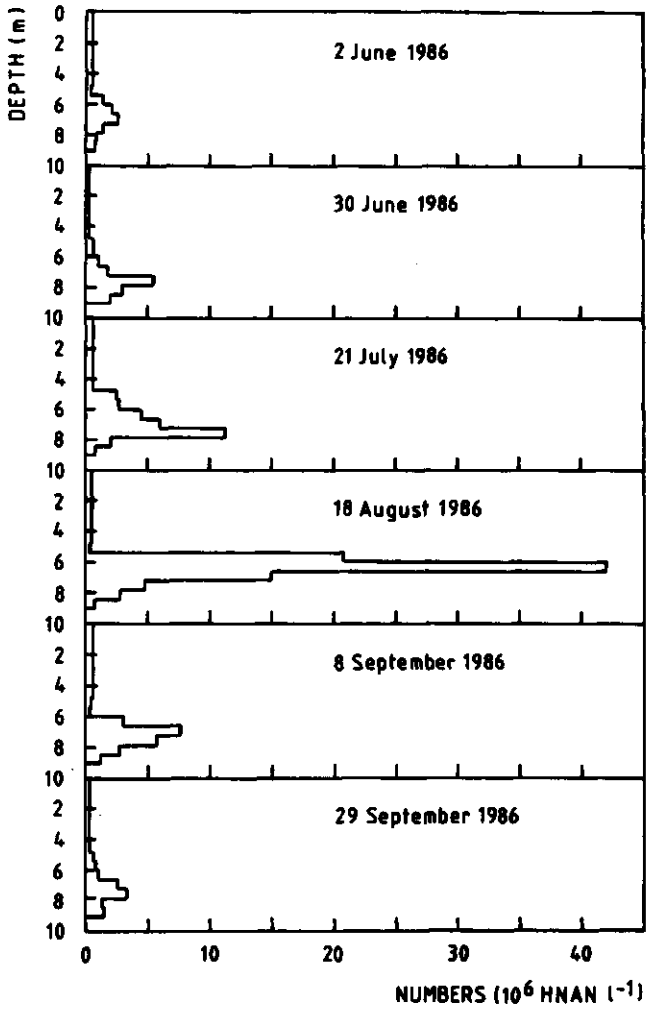

FIG. 3. Vertical distribution of HNAN in Lake Vechten during the summer of 1986.

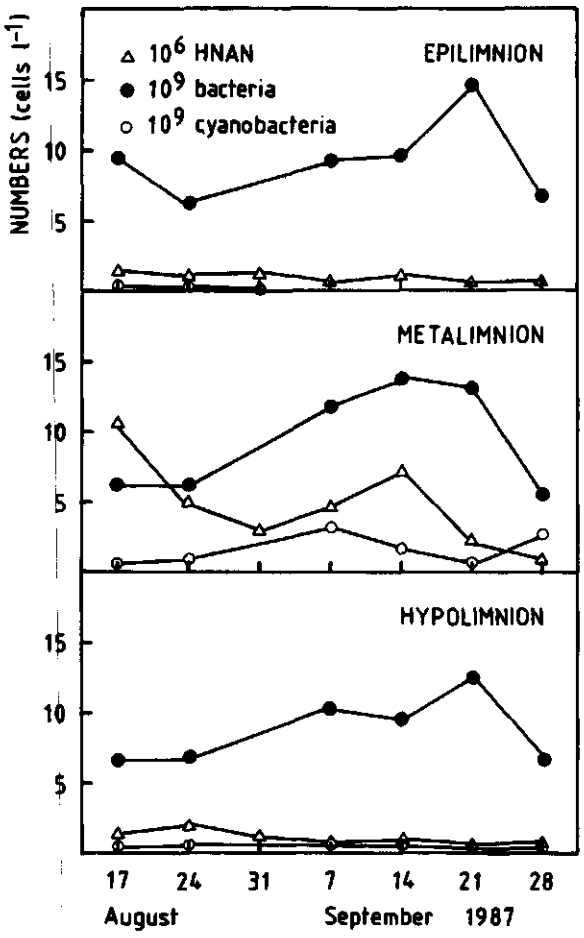

FIG. 4. Cell numbers of HNAN, bacteria, and chroococcoid cyanobacteria in the epi-, meta- and hypolimnion of Lake Vechten from August to September of 1987. Samples were taken at 3-, 5-, and 9-m depths.

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.

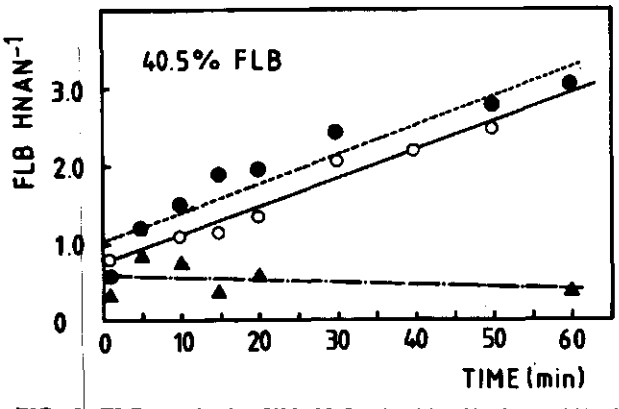

FIG. 5. FLB uptake by HNAN fixed with 1% (○) or 2% (●) glutaraldehyde or van der Veer fixative (▲).

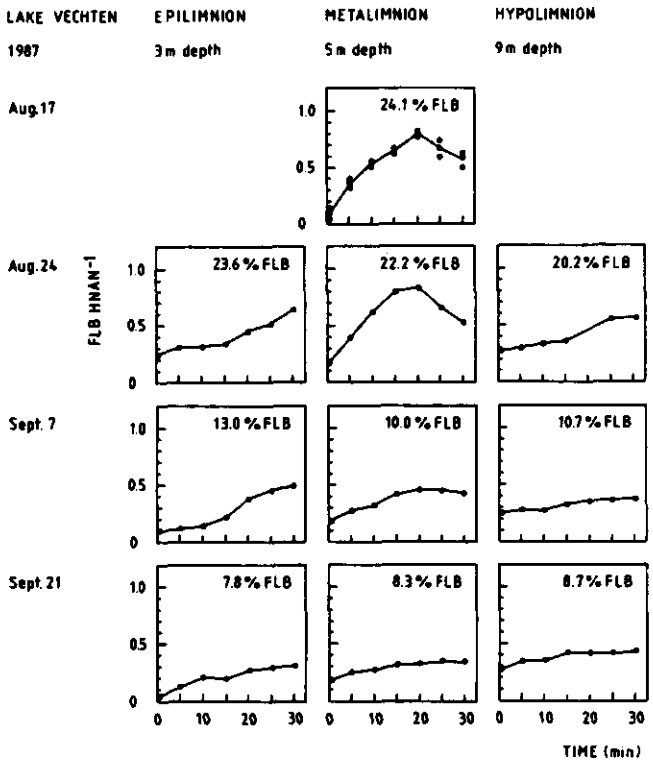

FIG. 6. FLB uptake by HNAN in stratified Lake Vechten from August to September of 1987.

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).

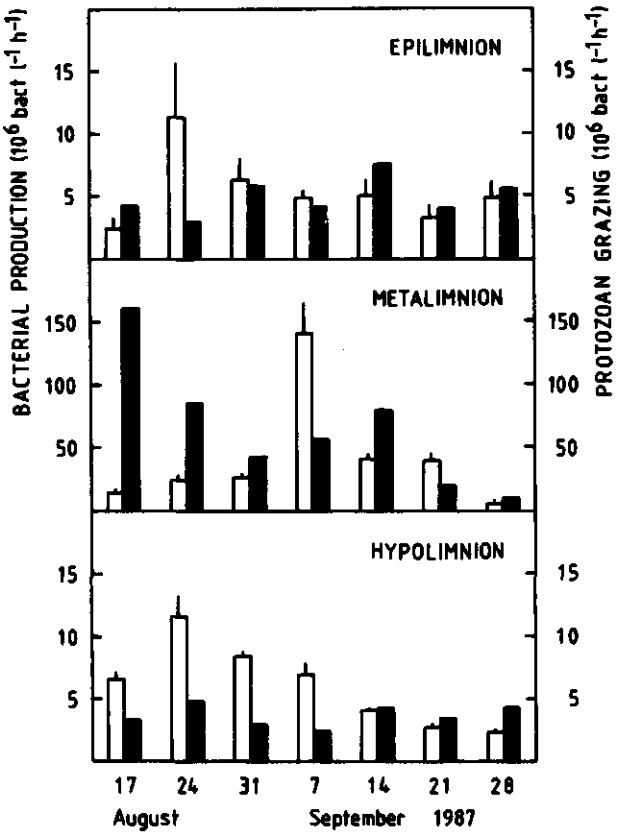

FIG. 7. Estimated bacterial production (open bars; error bars indicate 1 SD; $n = 5$) and protozoan grazing (filled bars) in stratified Lake Vechten for August to September of 1987. Samples were taken at 3-, 5-, and 9-m depths.

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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DISCUSSION

Regulation of bacterial numbers

In Lake Vechten the spatial and temporal variation in bacterial numbers (4 to 15 x 10^9 bact.l^{-1}) was small compared with other organisms such as chroococcoid cyanobacteria, HNAN, and ciliates, which varied one to two orders of magnitude (chapters 3 and 6). Also the estimated bacterial production rates varied two orders of magnitude, and were approximately balanced by HNAN grazing rates. A balance between production and grazing does not prove that bacterial densities are regulated by HNAN, as hypothesized by Azam et al. (1983), who suggested that marine bacteria are kept below a density of 5 to 10 x 10^9 bact.l^{-1} by HNAN grazing. However, an alternative possibility is that another density-dependent limiting factor, e.g. a nutrient, keeps the bacteria at a maximum density i.e. the carrying capacity of the system. Then, grazing losses would be compensated for by production which keeps the bacterial numbers at the carrying capacity.

Kuno (1987) reviewed predator-prey interactions in various systems and concluded that populations in nature are never in a state of delicate dynamic balance with their predators, and are not subject to consistent predatory regulation. Kuno did not find natural populations which have been proved to be regulated by their natural enemies. The regulatory factor is almost invariably the intraspecies competition for restricted food or other related resources. The importance of predators as key factors in population fluctuations is usually minor, even among insect populations, and it seems to be virtually negligible in most populations of birds and mammals. However, the role of predators as mortality factors is fairly important in general, with a tendency to become more significant in lower organisms.

Theoretically it is surely possible for predators to achieve strict regulation of prey populations at low density levels. This requires: a high searching efficiency of the predators; similar reproductive capacity of predator and prey; a stable environment; and a habitat which provides refuges for the prey. The state of regulation is, therefore, quite fragile, being liable to shift to a state of non-regulatory coexistence of both populations if anyone of the above conditions is violated (Kuno, 1987). In contrast to most higher animals, bacterivorous HNAN appear to be able to meet the above conditions: HNAN are efficient and fast growing grazers; aquatic ecosystems may at least temporarily provide a relatively stable environment, e.g. during summer stratification in the metalimnion; and bacterial densities below the threshold which just supports protozoan growth can be regarded

as a refuge for bacteria.

In our two-stage continuous cultures (chapter 5), HNAN clearly controlled bacterial numbers, which were kept below 4 x 10^9 bact.l^{-1} in the grazed vessel, whereas ungrazed vessels contained 20 to 80 x 10^9 bact.l^{-1} depending on the nutrient (yeast extract) concentration in the growth medium. The regulation of bacterial numbers in natural waters, however, is a much more complicated problem. Theoretically, grazer-controlled bacteria will show increasing numbers after removal of protozoa by selective filtration through 1 µm pore-size Nuclepore filters. Such increases have been reported for marine bacteria (Wright and Coffin, 1984; Andersen and Fenchel, 1985). However, a problem not mentioned in the literature before is that Nuclepore filtration can enrich natural water samples with dissolved organic carbon (chapter 4), which may promote bacterial growth. Moreover, growth lags may occur. In Lake Vechten water containing 5 to 15 x 10^9 bact.l^{-1} the numbers of bacteria increased significantly after selective filtration and enrichment with yeast- or Synechococcus extract, resulting in peak densities of 30 x 10^9 bact.l^{-1} (chapters 3 and 4). This indicates that either organic or inorganic nutrients, or protozoan grazing limited bacterial numbers.

Wright (1988) presented a model for short-term control of bacterioplankton by substrate and grazing, which indicates that bacterial density is determined primarily by the ratio of substrate input to grazing rate, and that bacterial production is balanced by a combination of losses due to grazing, death and maintenance, and occurs at a rate determined by the rate of substrate input and growth yield. The model predicts in substrate-controlled systems: high bacterial densities, low bacterial growth rates, low grazer densities, low grazing rates, and low ambient substrate concentrations. On the other hand, grazer-controlled systems show: moderate bacterial densities, high bacterial growth rates, moderate to high grazer densities, high grazing rates, and relatively high ambient substrate concentrations. Thus, the high bacterial growth rates, high HNAN densities, and high grazing rates in the metalimnion of Lake Vechten (chapters 3 and 6) are indicative of a grazer-controlled system.

Probably the metalimnetic peaks of phytoplankton biomass, primary production, and extracellular excretion (Blaauboer et al., 1982; Steenbergen and Korthals, 1982), as well as detritus accumulation, result in a relatively high supply of nutrients for bacterial growth in the metalimnion. If the metalimnetic bacteria would be nutrient-limited, the relatively high availability of nutrients in the metalimnion should cause a peak in bacterial numbers. However, the bacterial numbers are not increased, whereas the major consumers of bacteria, i.e. the HNAN, show peak

densities in the metalimnion. Thus, HNAN seem to prevent an increase in bacterial numbers when nutrient supply and bacterial production are high. It is not likely that Lake Vechten bacteria are always controlled by HNAN grazing. The system may switch to nutrient limitation when the nutrient supply is low, and when HNAN numbers are low, e.g. due to predation by (micro)zooplankton.

The microbial food web

In the epi-, meta-, and hypolimnion of Lake Vechten, bacterial production rates were approximately balanced by HNAN grazing rates (chapters 3 and 6). Recently, such a balance has also been reported for the epilimnion of Lake Biwa (Nagata, 1988). However, in most marine and freshwater systems bacterial production was found to be considerably higher than grazing, as reviewed by Pace (1988). Grazing by metazoan zooplankton and other mortality factors appeared to be of minor importance compared with protozoan grazing. The apparent lack of balance between grazing and production was attributed to inaccurate methods for measuring bacterial mortality and growth.

Estimates of bacterial production from 70 fresh- and saltwater ecosystems were examined by Cole et al. (1988). For all planktonic systems analysed, bacterial production ranged from 0.017 to 6.25 $\mu g\ C.l^{-1}.h^{-1}$, and averaged 20% (median 16.5%) of planktonic primary production. In the photic zones of lakes and oceans, bacterial production showed a significant linear relationship with planktonic primary production, suggesting: either (1) that both bacteria and phytoplankton grow in response to common factors (nutrient load, temperature); or (2) that phytoplankton or material produced by phytoplankton are important substrates for bacterial growth. The second possibility is the most likely for stratified Lake Vechten, where the highest bacterial production rates (chapters 3 and 6) and the highest rates of primary production and extracellular release by phytoplankton (Blaauboer et al., 1982; Steenbergen and Korthals, 1982) have been found in the metalimnion.

Metalimnetic maxima of bacterial production near or below the depth of maximum primary production and phytoplankton biomass have also been reported for Crooked Lake, Little Crooked Lake (Lovell and Konopka, 1985), and Lake Arlington (Chrzanowski and Hubbard, 1988). In these lakes adequate light penetration to the metalimnion occurs, because the relatively nutrient-poor epilimnion is not too turbid and fairly shallow. In the much larger and deeper Lake Constance (Güde et al., 1985), Lake Michigan (Scavia and Laird, 1987), and Lake Biwa (Nagata, 1987), maximum primary and bacterial secondary production have been found

in the epilimnion.

In the metalimnion of Lake Vechten, the greatest activity of the microbial populations occurs in August and September. Assuming instantaneous rates near the maximum values observed, a simplified carbon flow diagram of the microbial food web in the metalimnion can be constructed (Fig. 1). The main components of the food web are phytoplankton (mainly chroococcoid cyanobacteria), heterotrophic bacteria, HNAN, and ciliates. The primary production of phytoplankton reaches maximum rates of about 20 µg $C.l^{-1}h^{-1}$ (Blaauboer et al., 1982; Steenbergen and Korthals, 1982), of which approximately 8 µg $C.l^{-1}.h^{-1}$ can be excreted as dissolved organic carbon. At 7.2 m depth, the input of particulate organic carbon by sedimentation can be estimated to be 290 µg $C.l^{-1}.h^{-1}$ (Steenbergen and Verdouw, 1984). Primary production as well as sedimentation provide substrates for bacterial secondary production. We found bacterial production rates of about 140 x 10^6 $bact.l^{-1}.h^{-1}$ (chapters 3 and 6), and a carbon content of 30 fg $C.bacterium^{-1}$ (chapter 5). Thus, the bacterial production can be estimated to be 4.2 µg $C.l^{-1}.h^{-1}$. Assuming a bacterial growth yield of 20% (chapter 5), the bacterial respiration (= carbon mineralization) is estimated to be 16.7 µg $C.l^{-1}.h^{-1}$, and the bacterial carbon consumption 21 µg $C.l^{-1}.h^{-1}$. The HNAN consumed up to 160 x 10^6 $bact.l^{-1}.h^{-1}$, i.e. 4.7 µg $C.l^{-1}.h^{-1}$. Assuming a protozoan growth yield of 40% and a negligible excretion (chapter 5), the HNAN production can be estimated to be 1.9 µg $C.l^{-1}.h^{-1}$, and the respiration 2.9 µg $C.l^{-1}.h^{-1}$. The maximum consumption by HNAN seemed to be slightly greater than the estimated maximum bacterial production. It should be remembered, however, that the production estimates are rather crude due to the 3-fold variation in conversion factors for thymidine incorporation (chapter 6).

In the metalimnion of Lake Vechten, the bacterial secondary production appears to be approximately 20% of the phytoplankton primary production (Fig. 1), which is similar to values found in other aquatic ecosystems (Cole et al., 1988; Schwaerter et al., 1988). An important part of the bacterial carbon requirements can be supplied by phytoplankton extracellular release of dissolved organic carbon. Most of these substrates are easily decomposable and can support bacterial growth with a gross growth efficiency of 50% (Findlay et al., 1986; Schwaerter et al., 1988). Thus, at maximum yield and excretion rates phytoplankton extracellular release may temporarily support all bacterial production. However, extracellular release and growth yield are not always maximum and depend on growth conditions. The fraction of labile photosynthate released is probably increased under stress due to nutrient limitation (Azam et al., 1983) or inappropriate light conditions,

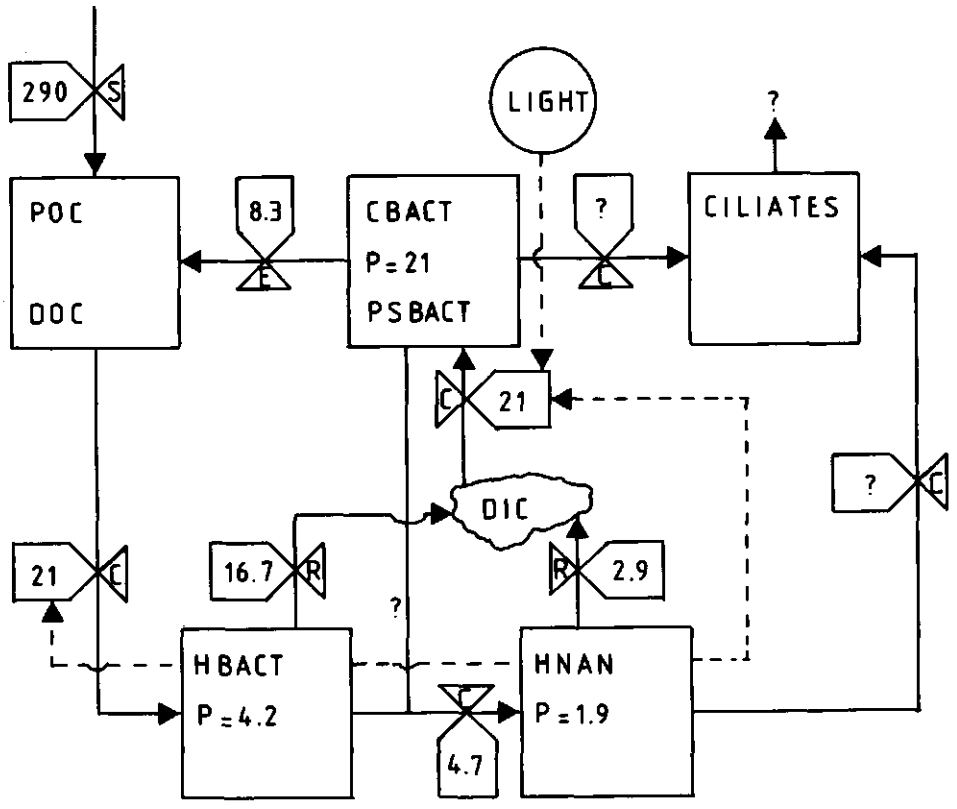

FIG. 1. Simplified carbon flow diagram of the microbial food web in the metalimnion of Lake Vechten. Estimated maximum rates of sedimentation (S), excretion (E), consumption (C), production (P), and respiration (R) are given in μg $C.l^{-1}.h^{-1}$. DIC = dissolved inorganic carbon, DOC = dissolved organic carbon, POC = particulate organic carbon, CBACT = cyanobacteria, PSBACT = photosynthetic sulfur bacteria, HBACT = heterotrophic bacteria (with a yield of 20%), and HNAN = heterotrophic nanoflagellates (with a yield of 40%).

and varies between 0 and 55% of photosynthetic carbon fixation in Lake Vechten (Blaauboer et al., 1982). Depending on substrate quality, also bacterial growth yield may vary widely. Values between 10 and 40% have been reported for natural bacteria in eutrophic lakes (Schwaerter et al., 1988). In addition to dissolved organic matter excreted by phytoplankton, decaying plankton cells, methane diffusing from deeper strata and sedimenting organic particles may serve as substrates for bacterial growth (Cappenberg and Verdouw, 1982).

About 50% of the input of sedimenting particulate organic carbon at 7.2 m depth (290 $\mu g\ C.l^{-1}.h^{-1}$) was found to disappear between 7.2 and 9.6 m depth (Steenbergen and Verdouw, 1984). Thus, averaged over this 2.4 m depth interval, sedimentation may contribute up to 6 $\mu g\ C.l^{-1}.h^{-1}$ (0.5 x 290/24) to the total bacterial carbon consumption (= decomposition), and may support a bacterial production of about 1.2 $\mu g\ C.l^{-1}.h^{-1}$, assuming a yield of 20%.

Most of the metalimnetic carbon mineralization appears to be performed by bacteria. Their respiration is estimated to be about 17 $\mu g\ C.l^{-1}.h^{-1}$, while HNAN contribute about 3 $\mu g\ C.l^{-1}.h^{-1}$ (Fig. 1). In contrast to carbon, phosphorus appears to be mineralized mainly by protozoan grazers, which regenerate nutrients immobilized in bacterial biomass (chapters 4 and 5). Because of their relatively high phosphorus content, bacteria generally act as consumers of inorganic phosphorus. Recently, net bacterial phosphorus consumption was found to be four times higher than that of phosphorus-limited phytoplankton in a eutrophic Norwegian lake (Vadstein et al., 1988). Thus, if inorganic phosphorus is limiting the growth rate of phytoplankton or bacteria, protozoan grazing and nutrient regeneration can accelerate phytoplankton and bacterial production, as well as carbon mineralization rates.

The simplified carbon flow diagram of the microbial food web in the metalimnion of Lake Vechten (Fig. 1) is based on maximum instantaneous rates which have been observed usually in the morning between 0800 and 1100 hours, during August and September when optimum stratification and high microbial activity are found. However, day-night rhythms in microbial activity may occur and have not been investigated. Also seasonal cycles remain to be studied. To complete the carbon flow diagram, grazing of HNAN and ciliates on chroococcoid cyanobacteria, and of ciliates on HNAN, have to be quantified. Further, it is not clear whether the abundant metalimnetic protozoa (HNAN and ciliates) are linked to the "conventional" food chain via grazing by bigger zooplankton, such as rotifers and cladocerans.

The role of protozoa in the mineralization in sediments, and in nutrient (especially phosphorus) cycling in shallow turbulent lakes of different trophic states, may be important and remains to

be examined. Since the role of bacteria in the functioning of ecosystems is not determined primarily by their numbers but by their metabolic activity, more research is needed on the biochemical and physiological basis of the empirical relationship between thymidine incorporation and growth rate, to improve estimates of in-situ bacterial production.

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SUMMARY

The role of heterotrophic nanoflagellates (HNAN, size 2-20 µm) in grazing on bacteria and mineralization of organic matter in stratified Lake Vechten was studied.

Quantitative effects of manipulation and fixation on HNAN were checked. Considerable losses were caused by centrifugation, even at low speed, and by filtration if the vacuum exceeded 3 kPa. HNAN were well preserved for several weeks with unbuffered 5% formaldehyde as well as with 0.3 to 1% glutaraldehyde (final concentrations). However, the chlorophyll autofluorescence of phototrophic nanoflagellates was strongly decreased by formaldehyde, whereas it could be preserved for a few days with 1% glutaraldehyde. Loss of autofluorescence and of cells was prevented for at least one year if prepared primulin-stained slides were stored in a freezer.

During summer stratification in 1985, HNAN showed peak densities in the microaerobic lower metalimnion at 6-7 m depth. In this stratum, high HNAN numbers (more than 30 x 10^6 HNAN.l^{-1}) coincided with high bacterial production, as estimated by thymidine incorporation. Despite the high production, bacterial numbers (about 5 x 10^9 bacteria.l^{-1}) did not peak in the metalimnion but were similar throughout the whole water column, indicating that the produced bacteria were consumed by HNAN. A steep decline of HNAN was followed by a peak of ciliates. Thus, HNAN seemed to be a link in the food web between bacteria and ciliates. Batch incubations with selectively filtered lake water, with bacteria alone and with bacteria plus protozoa, indicated ingestion rates of about 7 bacteria.HNAN^{-1}.h^{-1}, and suggested that the HNAN are able to balance the estimated bacterial population doubling times of about 1 day.

The breakdown of organic carbon (C) of dead cyanobacterial cell walls added to selectively filtered lake water was not accelerated by protozoa. However, HNAN and ciliates strongly increased the mineralization of nitrogen (N) and especially phosphorus (P), both from Synechococcus cell walls and cell extract. Bacterial growth did not result in net P mineralization, but in phosphate uptake. P was remineralized only in the presence of protozoan grazers. Grazers increased N mineralization too, although N was also mineralized by bacteria in the absence of grazers. Nuclepore filters were found to release dissolved organic carbon during selective filtration.

In two-stage continuous cultures, ingestion rates of HNAN increased from 2.3 to 9.2 bacteria.HNAN^{-1}.h^{-1} at specific growth rates from 0.15 to 0.65 day^{-1}. On a yeast extract medium with a molar C/N/P ratio of 100/15/1.2 (Redfield ratio), a mixed bacterial population had a gross growth efficiency of 18% (C/C)

and a specific carbon content of 211 fg C.μm^{-3}, at a biovolume of 0.14 μm^3.$cell^{-1}$. The HNAN carbon content, biovolume and yield were estimated to be 127 fg C.μm^{-3}, 40 μm^3.$cell^{-1}$ and 47% (C/C). Although P was not growth limiting, HNAN accelerated the mineralization of P from dissolved organic matter 6-fold. The major mechanism of P remineralization appeared to be direct consumption of bacteria by HNAN. N mineralization was performed mainly (70%) by bacteria, but was increased 30% by HNAN. HNAN did not enhance the decomposition of the relatively mineral-rich dissolved organic matter. An accelerated decomposition of organic carbon by protozoa may be restricted to mineral-poor substrates, and may be explained mainly by protozoan nutrient regeneration. With subsamples from the continuous cultures, protozoan grazing was found to be accurately measured by uptake of fluorescently labelled bacteria.

In continuous culture an empirical relationship between bacterial cell production and thymidine incorporation was established. Tritium-labelled thymidine incorporation into total cold-TCA-insoluble macromolecules yielded a relatively constant empirical conversion factor of about 1 (range 0.38-1.42) x 10^{18} bacteria produced per mol of thymidine incorporated, at specific growth rates ranging from 0.007 to 0.116 h^{-1}. Although thymidine incorporation has been assumed to be a measure of DNA synthesis, thymidine incorporation appeared to underestimate the independently measured DNA synthesis at least by 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. These observations suggest that the empirical relationship between thymidine incorporation and bacterial growth rate is not based primarily on DNA synthesis.

In 1987, thymidine incorporation into total macromolecules yielded maximum bacterial production rates of 141 x 10^6 bacteria.l^{-1}.h^{-1} (4.2 μg C.l^{-1}.h^{-1}) in the metalimnion, which was one order of magnitude higher than in the epi- and hypolimnion. In all three strata, the estimated bacterial production was roughly balanced by the in-situ protozoan grazing determined with fluorescently labelled bacteria. HNAN showed ingestion rates of 2 to 17 bacteria.$HNAN^{-1}$.h^{-1}, and were the main consumers of bacteria.

The moderate bacterial densities, high bacterial growth rates, high HNAN densities and high grazing rates in the metalimnion are indicative of protozoan control of bacterial numbers. The bacterial secondary production in the metalimnion is estimated to be about 20% of the phytoplankton primary production, and may be largely supported by extracellular release of labile photosynthate and decaying plankton.

SAMENVATTING

In de kringlopen van koolstof (C), fosfor (P) en stikstof (N) in het water spelen bacteriën een belangrijke rol. Ze breken dood organisch materiaal af, dat grotendeels afkomstig is van algenproductie. Een deel van dit organische materiaal wordt omgezet in bacteriebiomassa, de rest wordt verademd tot CO_2 en uitgescheiden als minerale stikstof en fosfaat (= mineralisatie). Deze mineralen dienen op hun beurt weer als voedingsstoffen voor nieuwe algengroei.

Bacteriën blijken in natuurlijke wateren snel te kunnen groeien, met verdubbelingstijden van ongeveer 1 dag. Desondanks blijven hun aantallen betrekkelijk constant. Dit wijst erop dat de bacteriën ongeveer even snel verdwijnen als ze geproduceerd worden. Op grond van onderzoek in zeewater werd verondersteld dat de geproduceerde bacteriën worden weggegraasd door protozoën, met name door heterotrofe nanoflagellaten. Dit zijn eencellige diertjes met afmetingen van 2 tot 20 micrometer (1 µm = 1/1000 millimeter), die zich voeden en voortbewegen door middel van zweepharen (flagellen). Begrazing van bacteriën door protozoën zou kunnen leiden tot een versnelde mineralisatie van organisch materiaal, omdat grazers ook minerale stikstof en fosfaat uitscheiden en bovendien de activiteit van bacteriën zouden kunnen stimuleren. Het meeste onderzoek naar protozoën werd verricht in zeewater, en er is nog weinig bekend over hun rol in zoete wateren.

Dit proefschrift beschrijft de resultaten van onderzoek naar de rol van heterotrofe nanoflagellaten bij de begrazing van bacteriën en bij de mineralisatie van organisch materiaal in de gestratificeerde plas Vechten. Deze 10-meter diepe plas vertoont in de zomer een sterke gelaagdheid in de watertemperatuur (thermische stratificatie).

Tijdens de zomerstratificatie in 1985 vertoonden heterotrofe nanoflagellaten maximale dichtheden op 6 tot 7 meter diepte onderin het zuurstofarme metalimnion, de spronglaag waar de temperatuur snel afneemt. In deze laag vielen hoge aantallen flagellaten (meer dan 30 miljoen per liter) samen met een hoge bacterieproductie, terwijl de aantallen bacteriën (rond 5 miljard per liter) niet verhoogd waren, maar gelijk waren over de hele waterkolom. Dit wijst erop dat de geproduceerde bacteriën werden weggegraasd door flagellaten. Een sterke afname van flagellaten werd gevolgd door een piek van ciliaten; dit zijn grotere protozoën die zich voeden en voortbewegen met behulp van vele trilharen (ciliën). Flagellaten lijken dus een schakel te vormen in het voedselweb tussen bacteriën en ciliaten. Waarnemingen aan geincubeerd, selectief gefilterd Vechtenwater, met alleen bacteriën en met bacteriën en protozoën samen, leverden

graassnelheden op van ongeveer 7 bacteriën.flagellaat^{-1}.uur^{-1}. Deze graassnelheid is voldoende om bij de gevonden dichtheden van 30 miljoen flagellaten.liter^{-1} het aantal bacteriën constant te houden, ondanks de snelle bacteriegroei met een geschatte verdubbelingstijd van ongeveer 1 dag.

De afbraak van organische koolstof van dode cyanobacterie-(blauwalgen)celwanden die waren toegevoegd aan selectief gefilterd Vechtenwater, werd niet versneld door protozoën. Flagellaten en ciliaten veroorzaakten echter een sterke toename van de mineralisatie van stikstof en in het bijzonder van fosfor, uit zowel Synechococcus celwanden als celextract. Bacteriegroei ging niet gepaard met netto fosformineralisatie, maar met opname van fosfaat. Fosfaat kwam alleen vrij in de aanwezigheid van protozoën. Grazers verhoogden ook de stikstofmineralisatie, hoewel anorganische stikstof eveneens werd vrijgemaakt door bacteriën in afwezigheid van grazers.

In tweetraps continuculturen namen de graassnelheden van flagellaten toe van 2,3 tot 9,2 bacteriën.flagellaat^{-1}.uur^{-1}, bij specifieke groeisnelheden van 0.15 tot 0.65 dag^{-1} (verdubbelingstijden van 4,6 tot 1 dag). Op een gistextract voedingsmedium met een molaire C/N/P verhouding van 100/15/1,2 (de Redfield ratio), vertoonde een gemengde bacteriepopulatie een bruto groei-efficiëntie (= productie/consumptie) van 18% (gebaseerd op koolstof) en een specifiek koolstofgehalte van 211 femtogram C.μm^{-3} (1 femtogram = 10^{-15} gram), bij een biovolume van 0,14 μm^3 per cel. Het koolstofgehalte, biovolume en de groei-efficiëntie van flagellaten werden geschat op 127 femtogram C.μm^{-3}, 40 μm^3.cel^{-1} en 47% (C/C). Hoewel fosfor niet groeisnelheidsbeperkend was, bleken de bacteriën veel fosfor in te bouwen en weinig te mineraliseren. De flagellaten echter, versnelden de mineralisatie van fosfaat met 600%. Het belangrijkste mechanisme van fosformineralisatie was de consumptie van bacteriën door flagellaten. Hierdoor werd fosfaat vrijgemaakt dat was vastgelegd in bacteriebiomassa. In tegenstelling tot de fosformineralisatie werd de stikstofmineralisatie voornamelijk (voor 70%) uitgevoerd door bacteriën. De stikstofmineralisatie werd met 30% verhoogd door flagellaten. Deze versnelden niet de afbraak van het opgeloste organische materiaal, dat betrekkelijk veel mineralen bevatte. Een versnelde afbraak van organische koolstof door protozoën treedt waarschijnlijk alleen op bij mineraalarm materiaal, en wordt dan voornamelijk veroorzaakt door het vrijkomen van bacteriegroeibeperkende mineralen ten gevolge van begrazing door protozoën. Graassnelheden van protozoën bleken nauwkeurig te kunnen worden bepaald door middel van de opname van fluorescerend gemerkte bacteriën.

In continucultuur werd een verband vastgesteld tussen de bacterieproductie en de inbouw van radioactief gemerkte thymidine

in bacteriële macromoleculen. Er werd een betrekkelijk constante empirische conversiefactor gevonden van ongeveer 1 (0,38 tot 1,42) x 10^{18} geproduceerde bacteriën per mol ingebouwde thymidine. De veel gebruikte thymidinemethode berust op de aanname dat thymidine voornamelijk wordt ingebouwd in bacteriëel DNA. Door middel van thymidine-inbouw zou dan de DNA-synthese worden gemeten, wat een maat zou zijn voor de celproductie omdat het DNA-gehalte per cel betrekkelijk constant is. In de continucultuur bleek echter dat met thymidine-inbouw de werkelijke DNA-synthese 1,5 tot 13 maal werd onderschat, indien werd aangenomen dat alle ingebouwde thymidine in DNA zat. Met de gangbare zuur-base hydrolysemethode werd echter geen inbouw gevonden in DNA, maar wel in andere macromoleculen. Deze waarnemingen wijzen erop dat het proefondervindelijke verband tussen thymidine-inbouw en bacterieproductie niet direct berust op de DNA synthese.

In 1987 werden in de plas Vechten, met metingen van thymidine-inbouw in bacteriële macromoleculen, maximale productiesnelheden gevonden van 141 x 10^{6} bacteriën.liter^{-1}.uur^{-1} (4,2 microgram C.liter^{-1}.uur^{-1}), met in het metalimnion ongeveer 10 maal hogere waarden dan in het epilimnion (warme bovenlaag) en het hypolimnion (koude onderlaag). In alle drie waterlagen werd de geschatte bacterieproductie ruwweg gecompenseerd door begrazing van protozoën, die werd bepaald met fluorescerend gemerkte bacteriën. De flagellaten vertoonden graassnelheden van 2 tot 17 bacteriën.flagellaat^{-1}.uur^{-1}, en waren de belangrijkste bacterie-eters.

De matige bacteriedichtheden, hoge bacteriële groeisnelheden, hoge flagellatendichtheden en hoge graassnelheden in het metalimnion wijzen erop dat de bacterie-aantallen door protozoën onder controle worden gehouden. De bacteriële secundaire productie in het metalimnion wordt geschat op ongeveer 20% van de primaire productie van het fytoplankton, en kan grotendeels in stand worden gehouden door uitgescheiden fotosyntheseproducten en afgestorven plankton.

CURRICULUM VITAE

Jaap Bloem was born on 9 February 1958, in Rotstergaast, Friesland, The Netherlands. In 1975 and 1976, as a hobby he studied zooplankton grazing on algae in Lake Tjeukemeer, supported by Mr. Th. H. Frank and Dr. J. Vijverberg of the Tjeukemeer Laboratory of the Limnological Institute in Oosterzee, and funded by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.). From August 1976, he studied Biology at the Groningen State University, and obtained his M.Sc. degree on 25 August 1983. As an M.Sc. student he studied three subjects: Ecological Microbiology with Prof. Dr. H. Veldkamp, Dr. F. B. van Es, and Dr. L. A. Bouwman; Plant Ecology with Dr. D. M. Pegtel and Dr. A. P. Grootjans; and Limnology with Dr. J. R. Moed (Tjeukemeer Laboratory, Limnological Institute) and Prof. Dr. C. van den Hoek.

Between 1 August 1984 and 1 June 1988 he was employed at the Vijverhof Laboratory of the Limnological Institute (Royal Netherlands Academy of Arts and Sciences) in Nieuwersluis. In the working group Mineralization of Organic Matter (leader Dr. Th. E. Cappenberg), he worked on the role of protozoa in grazing on bacteria and mineralization of organic matter in stratified Lake Vechten. This Ph.D. study was directed by Prof. Dr. A. J. B. Zehnder, Department of Microbiology, Wageningen Agricultural University. Since 1 June 1988 he has been working as a microbiologist at the Institute for Soil Fertility in Haren.

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Beware of anything beyond these. Of making many books there is no end, and much study is a weariness of the flesh.
(Ecclesiastes 12. 12)