Vol. 13: 161–175, 1997 | AQUATIC MICROBIAL ECOLOGY Aquat Microb Ecol | Published August 21

Cascading trophic effects on aquatic nitrification: experimental evidence and potential implications

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ABSTRACT: Experiments, using natural plankton collected from a eutrophic site in Saginaw Bay, Lake Huron (USA) and from a hypereutrophic wetland of southern Lake Erie (USA), were conducted to test the hypothesis that bacterivory can control aquatic nitrification rates. The dynamics of nitrogen and protists in these experiments revealed a consistent pattern: an increase in concentrations of nitrates due to oxidation of NH_4^+ always followed the collapse of bacterivorous nanoplankton populations. This collapse was, in turn, caused by predation pressure of larger ciliates and metazooplankton. Experiments, using enrichment batch cultures maintained at near-ambient concentrations of NH_4^+, indicated that bacterivorous protists can inhibit nitrification directly by reducing bacterial numbers and indirectly by promoting bacterial aggregation. The latter experiments also suggest that feeding strategies of microbial grazers, e.g. suspension-feeding *Spumella* sp. versus surface-feeding *Bodo saltans*, may determine their grazing impacts on nitrifiers. Finally, ingestion rates of fluorescently labeled nitrifying bacteria (FLNB) by the natural planktonic assemblage from Saginaw Bay demonstrated that nanoflagellates were able to efficiently prey on low concentrations of FLNB. Our study suggests that previously neglected trophic factors may be of potential importance for mediating nitrification rates in the pelagic environment.

KEY WORDS: Nitrification · Protozoa · Zooplankton · Bacterivory · Predation

INTRODUCTION

Nitrification, a fundamental process in aquatic environments, is mediated by 2 groups of obligatory autolithotrophic bacteria that use NH_4^+ and NO_2^- as their sole energy sources and CO_2 as their main carbon source (Winogradsky 1890). The ammonium-oxidizers and nitrite-oxidizers are Proteobacteria (Woese et al. 1984a, b) that are most commonly represented in the freshwater environment by the genera *Nitrosomonas* and *Nitrobacter*, respectively (Hall 1986). Most nitrifiers grow optimally in cultures at nitrogenous substrate concentrations ranging from 2 to 10 mM (Bock et al. 1986). Combined with the high energy cost of carbon fixation in nitrifiers via the Calvin cycle (Wood 1986) and photooxidation of their cytochrome *c* by visible blue and UV light (Bock et al. 1986), these observations may suggest that the growth and metabolisms of nitrifying bacteria should be very slow at the µM to nM concentrations of inorganic N characteristic of most marine and freshwater systems.

Ambient populations of nitrifiers appear, however, to be well adapted to a wide range of environmental conditions. Studies of nitrification in marine pelagic environments, reviewed by Kaplan (1983) and Ward (1986), indicate that natural populations of nitrifiers can oxidize their substrates at rates several orders of magnitude higher than those predicted from pure cultures. Furthermore, nitrification was detected under 24 h light cycle incubations in the photic zone (Ward 1986), suggesting that light inhibition may be not so

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severe in the surface waters as previously believed (Yoshioka & Sajo 1984). Nitrification occurs in such extreme environments as hot springs (Bock et al. 1989) and Antarctic ice (Arrigo et al. 1995). Although autolithotrophic nitrification is strictly aerobic, nitrifiers remain active at very low oxygen tensions (Lipschultz et al. 1990).

The biotic factors that have been studied with respect to nitrifying bacteria are mostly those affecting substrate availability and abiotic growth conditions. Nitrifiers can efficiently compete with phytoplankton for NH_4^+ due to their high substrate affinities (Kaplan 1983, Ward 1986). Similar competition between aquatic nitrifiers and heterotrophic bacteria should occur because heterotrophs often compete with phytoplankton for dissolved inorganic nutrients (Bratbak & Thingstad 1985, Elser et al. 1995). At the same time, nitrifying activity in the pelagic zone positively correlates with the amount of labile organic matter (Hall 1986, Lipschultz et al. 1990), probably implying a dependency on NH_4^+ regeneration. At the sediment-water interface, microalgae compete with nitrifiers for NH_4^+ (Rysgaard et al. 1995) and change their growth conditions by increasing both pH (Henriksen & Kemp 1988) and O_2 penetration (Risgaard-Petersen et al. 1994), whereas benthic macrofauna can accelerate nitrification by aerating sediments, regenerating NH_4^+, and producing PON (particulate organic nitrogen)-rich fecal pellets (Gardner et al. 1987, Henriksen et al. 1993).

Although nitrifying bacteria are susceptible to grazing (Fenchel & Blackburn 1979, Meyer-Reil 1983, Henriksen & Kemp 1988), direct evidence for a trophic control of aquatic nitrification is lacking. In both the marine and freshwater environments, planktonic protists (i.e. unicellular eukaryotic organisms capable of phagotrophy) play the pivotal role in consuming heterotrophic bacterial production (Azam et al. 1983, Sanders et al. 1989, Sherr et al. 1989) and recycling NH_4^+ in the pelagic zone (Caron & Goldman 1990, Selmer et al. 1993, Haga et al. 1995). In turn, the trophic cascade involving bacterivorous protists can profoundly affect bacterial community structure (Jürgens et al. 1994) as well as bacterial production (Rivkin et al. 1996) and community N regeneration (Miller et al. 1995, Suzuki et al. 1996).

Our major goals were to test the hypothesis that bacterivory by planktonic protists can control nitrification rates and to determine whether this process can be mediated by changes in invertebrate community trophic structure. Specifically, we conducted experiments to address the following questions that arose as the study progressed:

(1) Are nitrification rates related to the compositional and density dynamics of the planktonic protist community and does the presence of metazooplankton affect this relationship?

(2) What is the 'unrestricted' (i.e. under no substrate competition/no predation pressure) effect of bacterivory on nitrification?

(3) Do differences in feeding strategies of bacterivores and grazing resistance in nitrifying bacteria affect nitrification rates?

(4) What is the potential for the natural planktonic assemblage and its major components to graze on nitrifying bacteria?

MATERIAL AND METHODS

Sampling sites. The natural plankton used in this study were collected from Saginaw Bay, Michigan, USA, and Old Woman Creek, Ohio, USA. Saginaw Bay is a large (about 82 km long and 42 km wide) bay extending off the western edge of Lake Huron. Its shallow (3.5 m mean depth) inner part receives large inflows of enriched waters from the Saginaw River and is considered to be eutrophic. Details of the bay ecosystem are provided by Nalepa & Fahnenstiel (1995). Old Woman Creek, located in the southwestern shore of Lake Erie east of Sandusky Bay, is a shallow (ca 0.5 m mean depth) hypereutrophic coastal wetland that receives high inputs of allochthonous nutrients and organic matter from the surrounding agriculture (Kepner & Pratt 1996 and references therein). The concentration of chlorophyll *a* at the time of sample collection was 7.5 and 50.5 µg l^{-1} in Saginaw Bay and Old Woman Creek, respectively (J. F. Cavaletto, GLERL, pers. comm.).

Experimental design. ***Expt 1:*** To address Question 1 of this study we examined nitrification and protist dynamics in the presence and absence of net zooplankton in a bottle experiment. A flow diagram (Fig. 1) outlines the following experimental manipulations. Water was collected from Old Woman Creek (15°C) and inner Saginaw Bay (13.5°C) in October 1994 by submerging 20 l polycarbonate carboys under the surface. The carboys were kept in insulated coolers in the laboratory for 2 d, allowing water temperature to gradually reach 20°C. Subsequently, 3.5 l aliquots of the water were placed in four 4 l acid-washed and distilled water-rinsed polycarbonate bottles and incubated in an indoor Percival incubator at 20°C in the dark.

By conducting the experiments in the dark we eliminated such strong factors as light inhibition and algal N uptake. This approach allowed us to measure nitrification rates by simply tracing the concentrations of NH_4^+ and nitrates without using specific inhibitors and isotope dilution techniques that are based on the numer-

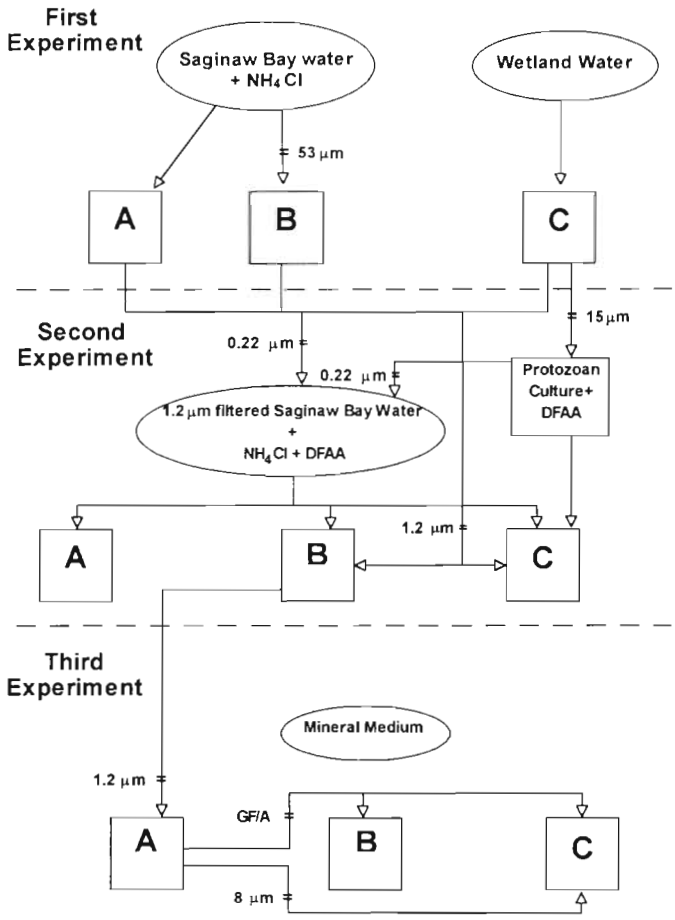

Fig. 1. Flow diagram outlining the experimental manipulations. Letters (A, B, C) refer to experimental treatments in Expts 1, 2, and 3 shown in Figs. 2, 3 and 4, respectively, and in Table 2

ous hard-to-test assumptions (see Ward 1986). All experiments were conducted at the same temperature to equalize its effects and ensure the fast growth of nitrifier populations. This temperature change was well within the range typical for the shallow coastal habitats of the experimental organisms. Our observations indicated no immediate change in the abundance or composition of planktonic organisms that could be associated with a temperature-induced stress.

Before incubation, Saginaw Bay water (Fig. 1A, B) was enriched with 4 µM NH_4Cl to give a total NH_4^+ concentration of 6 µM. One of the bottles was filled with water pre-filtered through a 53 µm mesh size Nitex net to remove zooplankton (Fig. 1B). Duplicate bottles with Old Woman Creek water were incubated without nutrient additions (Fig. 1C) because NH_4^+ concentrations were already high (ca 30 µM). The concentrations of NH_4^+, nitrates ($NO_2^- + NO_3^-$), and protists in the bottles were simultaneously monitored over time as described below. Zooplankton were collected from the carboys at the beginning and from the bottles at the end of experiment by passing 3.5 l aliquots of water through a 53 µm mesh size screen. Zooplankton from the bottle with filtered Saginaw Bay water were collected only at the end. The experiment was terminated when the concentration of NH_4^+ dropped below detection (ca 0.2 µM).

Expt 2: To examine the effect of bacterivory on nitrification in the absence of predation pressure on bacterivores and substrate competition (Question 2) we conducted another bottle experiment. A mixed culture of bacterivorous protists was established by filtering the Old Woman Creek water through a 15 µm mesh size Nitex net to remove metazoans and larger protists and then enriching the resulting filtrate with a mixture of amino acids (AA-S-18, Sigma) to provide an organic substrate for bacteria. The following manipulations were done to equalize conditions in experimental treatments and promote the growth of nitrifiers. Water from the first experiment was filtered through a 0.22 µm pore size membrane filter, mixed with 0.22 µm filtrate from the culture, diluted 1:1 with freshly collected and 1.2 µm filtered Saginaw Bay water, and put into 3 clean 1 l polycarbonate bottles. One bottle was used as a control (Fig. 1A). Aliquots of 1.2 µm filtrate from the first experiment were added into the 2 remaining bottles (Fig. 1B, C), and the cultured bacterivorous flagellates and ciliates in the exponential phase were inoculated into 1 of these 2 bottles (Fig. 1C). Finally, the concentrations of NH_4^+ and amino acids were adjusted to 10 µM and about 4 µM, respectively, in all 3 bottles. Amino acids were added to prevent possible competition between heterotrophic and nitrifying bacteria for NH_4^+, assuming that heterotrophic bacteria would preferentially obtain N from amino acids (Kirchman et al. 1989). The bottles were incubated at 20°C in the dark. The concentrations of NH_4^+, nitrates, and protists were simultaneously monitored over time. As in the first experiment, the bottles were monitored until NH_4^+ fell below detection in any of the bottles.

Expt 3: The effects of grazing resistance of bacteria and feeding strategies of bacterivores on nitrification rates (Question 3) were examined in an enrichment batch culture of nitrifying bacteria. Synthetic freshwater (Lehman 1980; 20 mg $CaCl_2$ $2H_2O$, 10 mg $MgSO_4$, 20 mg $NaHCO_3$ per liter of deionized water) enriched with NH_4Cl to 20 µM (final concentration) was used as a culture medium. The pH, adjusted with 0.5 N NaOH, was 7.3 after sterilization. To enrich nitrifiers, water from the treatment without added grazers in Expt 2 (Fig. 1B) was filtered through a 1.2 µm pore size filter and transferred (dilution 1:500) into a glass Erlenmeyer flask containing 500 ml of medium and

equipped with inoculation and sampling ports, and a sterile air intake. The flask was incubated at 20°C in the dark. The concentration of dissolved oxygen (DO) monitored with a DO meter remained relatively constant between 0.4 and 0.5 mM. Regular transfers into a fresh medium were done with a dilution factor of 1:20. After phagotrophic flagellates were detected, nitrogen concentrations and microbial numbers in the culture were monitored (Fig. 1A). Subsequently, 1 portion of the culture was filtered through a GF/A fiber glass filter (pore size ca 1.7 µm) to remove grazers and bacterial aggregates and a second portion was filtered through a 8 µm pore size membrane filter to remove bacterial aggregates but leave grazers. The 5 ml aliquots of resulting GF/A filtrate were added to 2 flasks containing 100 ml of fresh medium (Fig. 1B, C) and 1 of the flasks was additionally inoculated with 5 ml of the 8 µm filtrate (Fig. 1C).

We measured grazing rates of flagellates that developed in the enrichment culture using fluorescently labeled bacteria (FLB; Sherr & Sherr 1993) prepared from GF/A filtrates of the same culture. To make ingestion rate measurements, a 60 ml sample of culture was taken on the 10th day after inoculation (Fig. 1A), spiked with pre-sonicated FLB at ca 15% to the total bacterial concentration, and incubated in a 75 ml Erlenmeyer flask at 20°C in the dark. Sub-samples were taken at 15 min intervals and preserved with sequential additions of acid Lugol's iodine (1% final concentration), a few drops of 3% sodium thiosulfate, and buffered formaldehyde (1% final concentration). Numbers of FLB in grazer cells were determined microscopically.

Expt 4: To examine the potential ability of the natural planktonic assemblage to graze on nitrifying bacteria (Question 4) we conducted a field grazing experiment in August 1995. Water was collected from the subsurface layer in the inner Saginaw Bay as described above. Net zooplankton were collected by slowly hauling a 30.5 cm diameter, 53 µm mesh size net from 1 m to the surface. One third of the resultant 250 ml zooplankton sample was placed into a 1 l clear polycarbonate bottle filled with 750 ml of 53 µm filtered lake water. Pre-sonicated FLB prepared from the enrichment culture (Expt 3) were dispensed into a 1 l clean polycarbonate bottle. We added FLB at 4% of the total bacterial population to examine the potential ability of the natural planktonic assemblage to consume nitrifying bacteria at approximately natural proportions relative to total bacterioplankton in the pelagic zone. Given that the average size of ambient bacterial cells from Saginaw Bay was 0.42 µm ESD (equivalent spherical diameter) at the time of experiment, the added FLB constituted ca 19% of the ambient bacterial C. The bottle was incubated in an outdoor incubator at ambient temperature (21°C) and about 50% of incident radiation. Before taking each sample, the water was gently but thoroughly mixed by inverting the bottle. The rest of the FLB experiment was conducted as described above, except that an additional sample was taken at 10 min after FLB were added. Zooplankton were screened from the bottle on a 53 µm mesh size screen at the end of the experiment. In addition to measuring species-specific grazing rates we used a slightly modified community approach based on the disappearance of FLB from the water over time (Landry et al. 1995).

Sample processing. ***Nutrient analyses:*** The concentrations of NH_4^+ and amino acids (primary amines) were measured via high performance liquid chromatography (Gardner & St John 1991). The total concentration of nitrates (NO_2^- + NO_3^-) was measured via a Technicon AutoAnalyzer using the standard column wet chemical procedure of nitrate reduction by a copper-cadmium reductor column (Armstrong et al. 1967).

Bacteria: In the enrichment culture (Expt 3), free-suspended bacterial cells collected onto a 0.22 µm pore size black polycarbonate filter were counted under a Leitz Laborlux fluorescent microscope (magnification ×1000) using acridine orange (Hobbie et al. 1977). Total bacteria were counted similarly in samples sonicated for 5 min to break up bacterial aggregates. Bacterial cell sizes were measured via scanning electron microscopy (SEM; Nation 1983, Bratbak 1993) under an Amray 1820I scanning electron microscope (magnification ×11 500) at an acceleration voltage of 10 kV. In the case of the field grazing experiment (Expt 4), ambient bacteria were counted using acriflavine (Bergstrom et al. 1986), and bacterial dimensions were measured using fluorescent spheres (Bratbak 1993). Allometric relationships (Norland 1993) were used to convert bacterial biovolume to carbon. Carbon estimates were then averaged. Specific growth rates of cultured bacteria (r, d^{-1}) were estimated assuming the exponential growth $r = \ln(N_t/N_0)/t$, where N_0 and N_t were abundance of organisms at the beginning and at the end of incubation period t. Their specific metabolic rates and the efficiency of autolithotrophic growth were estimated assuming the ratio of NH_4^+-oxidizers to NO_2^--oxidizers of 1:1, and gross growth efficiencies of 10% (Wood 1986).

Protists and metazoa: Heterotrophic flagellates, preserved with cacodylate buffered glutaraldehyde (1% final concentration), were counted (magnification ×1000) following the DAPI-staining (Porter & Feig 1980). For enrichment experiments and grazing experiments, the entire filter area was scanned (magnification ×500). Ciliates, preserved with acid Lugol's iodine (1% final concentration), were counted in settling

chambers under a Wild phase-contrast inverted microscope (magnification ×125 to ×500). In addition, protists were qualitatively examined *in vivo* and those from the bacterial culture were also examined under SEM. Protistan biomass was estimated from cell linear dimensions by approximating geometric solids and assuming carbon content of 21 fg C μm^{-3} (Putt & Stoecker 1989). Metazooplankton were counted and identified after narcotization with carbonated water and preservation with sucrose-formalin.

RESULTS

Expt 1: nitrification and protist dynamics in the presence and absence of net zooplankton

In Saginaw Bay water (Fig. 2A, B), numbers of the predominant ciliates *Strobilidium* (*Rhimostrombidium*) *humile*, *Urotricha ristoi*, *U. pelagica*, and *Pelagostrombidium* sp. gradually declined as the experiment progressed. The abundance of nanoflagellates, largely the chrysophyte *Chromulina* sp (ESD 4.3 µm), increased, peaking at the end of the second week of the experiment. In 53 µm filtered Saginaw Bay water (Fig. 2B) the increase in abundance of nanoflagellates coincided with the appearance of the large (ESD 55.5 µm) choreotrichous ciliate *Strobilidium* (*Rhimostrombidium*) *lacustris*. This ciliate species reached its maximum by the 20th day. The microflagellates *Paraphysomonas* sp. (ESD 18.2 µm) and *Collodictyon triciliatum* (ESD 12.0 µm) also appeared in Saginaw Bay water at that time. After Day 20, the protistan populations declined in both bottles. This trend was more pronounced in unfiltered water, where decreases in protistan numbers began earlier and resulted in their virtual disappearance by the end of the experiment. In Old Woman Creek water (Fig. 2C), the large (ESD 69.5 µm) ciliate *Hypotrichidium conicum*, which dominated protistan biomass in the initial sample, was replaced by rapidly growing populations of nano-sized flagellates (largely cryptophytes and euglenids, ESD 5.0 to 7.2 µm) and the scuticociliates *Cyclidium* sp. (ESD 12.5 µm) and *Cinetochilium margaritaceum* (ESD 17.8 µm). Nanoplankton peaked at the 5th day, coinciding with the occurrence of the large ciliates *Dileptus* sp. (ESD 40.0 µm) and *Euplotes affinis* (ESD 45.2 µm) and the euglenid flagellate *Peronema* sp. (ESD 10.4 µm). The protistan populations declined as fast as they developed, and had almost disappeared by the 12th day. In contrast, zooplankton abundances increased by the end of experiment in both Saginaw Bay and Old Woman Creek treatments (Table 1).

In unfiltered Saginaw Bay water, the concentration of NH_4^+ gradually increased until the 20th day, when it

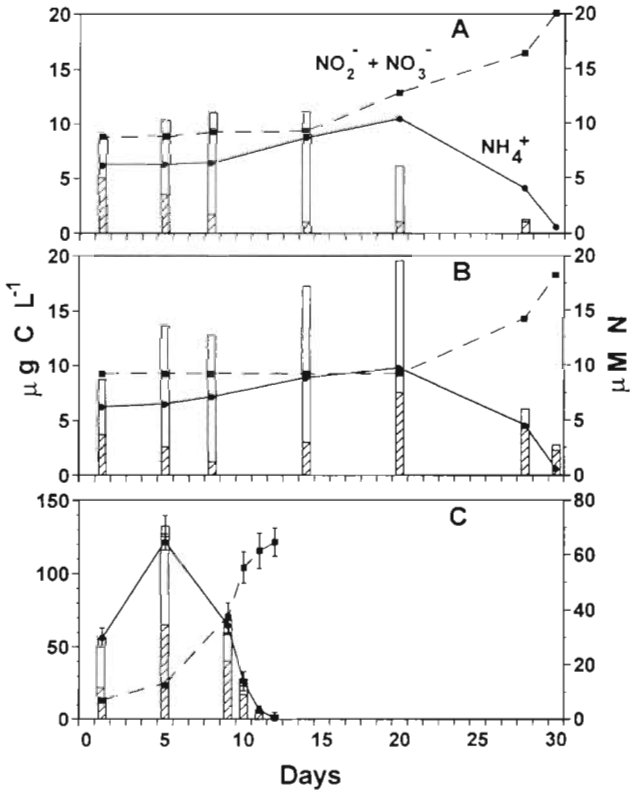

Fig. 2. Dynamics of NH_4^+ (solid line with dots), nitrates (NO_2^- + NO_3^-, dashed line with squares), and biomass of protists (open bars: flagellates; striped bars: ciliates) in Expt 1: (A) in the bottle with unfiltered Saginaw Bay water, (B) in the bottle with 53 µm filtered Saginaw Bay water, and (C) in duplicate bottles with unfiltered Old Woman Creek water (±SE)

Table 1 Numbers (ind. l^{-1} ± SE) of net zooplankton at the beginning (T_0) and the end (T_{inc}) of Expt 1. Incubation time: Saginaw Bay water, 30 d; Old Woman Creek, 14 d

Taxon	T_0	T_{inc}
Saginaw Bay unfiltered water		
Bosmina longirostris	12	44
Cyclops sp. (adults + copepodites)	5	12
Copepod nauplii	28	59
Keratella cohlearis	34	67
Polyarthra sp.	125	210
Saginaw Bay 53 µm filtered water		
Copepod nauplii	–	24
Keratella cohlearis	–	75
Polyarthra sp.	–	1185
Old Woman Creek unfiltered water (duplicate treatments)		
Asplanchna priodonta	5.5 ± 1.2	5.0 ± 2.4
Acanthocyclops viridis (adults + copepodites)	4.5 ± 0.18	5.5 ± 0.21
Bosmina sp.	12.1 ± 1.0	17.8 ± 0.88
Chydorus sp.	<0.2	–
Copepod nauplii	86.0 ± 6.4	120.5 ± 5.5
Diaptomus sp. (adults + copepodites)	22.3 ± 0.44	27.6 ± 0.5

began to decrease falling into nanomolar concentrations by the 30th day (Fig. 2A). The concentration of nitrates did not show any appreciable changes until the 15th day when it began to increase, reaching a rate of 1.5 µM d^{-1} at the end of experiment. The dynamics of nitrogen in filtered Saginaw Bay water was basically the same as in unfiltered water, except that there was a 5 d lag in the increase in nitrates (Fig. 2B). The final concentration of these compounds was slightly higher in unfiltered water (20 vs 18 µM). In contrast to these slow changes, the dynamics of nitrogen in the Old Woman Creek water was quite dramatic. There was a rapid increase in the concentration of NH_4^+ from 30 to almost 70 µM within a few days, which was followed by even sharper decrease in NH_4^+ and corresponding accumulation of nitrates at a rate of up to 20 µM d^{-1} (Fig. 2C). Despite this striking difference, all the treatments revealed a similar pattern: NH_4^+ concentrations generally followed dynamics of protists, while accumulation of nitrates followed the collapse in their populations.

Expt 2: isolated effect of bacterivory on nitrification

In the control treatment (not enriched with bacteria from the first experiment; Fig. 3A), the concentration of NH_4^+ increased steadily during the experiment, while the concentration of nitrates changed little, except for a slight increase at the end of experiment. At the same time, a 2-fold difference in accumulation of these compounds (65 vs 33% of the initial level) was observed between treatments without (Fig. 3B) and with (Fig. 3C) the addition of an inoculum from a mixed culture of nanoplankton-sized bacterivorous protists mostly

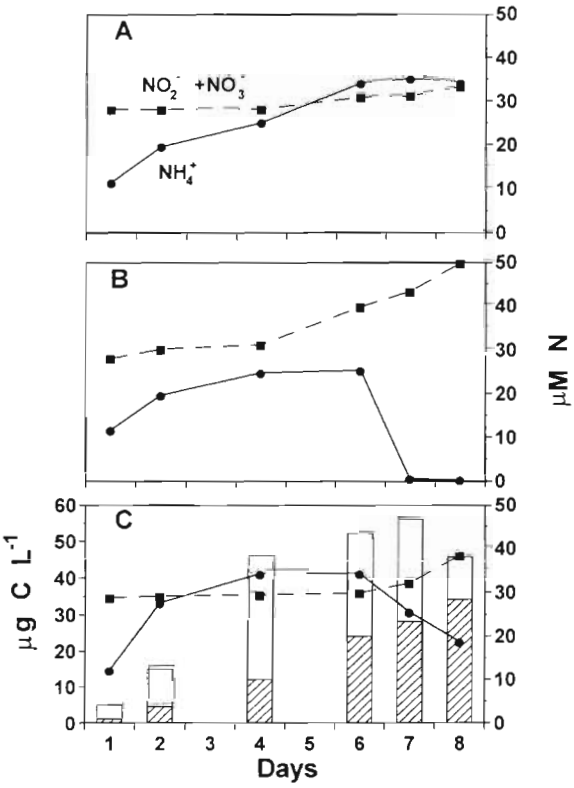

Fig. 3. Dynamics of NH_4^+, nitrates, and protists in Expt 2. (A) Control, (B) addition of 1.2 µm filtrate from Expt 1, and (C) the same plus an inoculum of protist culture. Legend as in Fig. 2

represented by *Cyclidium* sp., *C. margaritaceum*, *Bodo saltans*, *Spumella* sp., and *Goniomonas* sp. The carnivorous ciliate *Dileptus* sp. developed in the treatment with the inoculum by the end of incubation.

Table 2. Dynamics of nitrification rates and selected microbial parameters in an enrichment batch culture of nitrifying bacteria (Expt 3). Letters A, B, and C denote experimental treatments (for explanation see Fig. 1 and the text). C_p: increase in bacterial biomass over time; C_f: fixed carbon (assuming gross growth efficiency of 10%); N_o: oxidized nitrogenous substrate. The range of the published specific rates and ratios for ammonium- (upper row) and nitrite-oxidizing (lower row) bacteria are given after Kaplan (1983), Glover (1985), Bock et al. (1986) and Ward (1986). The range of *in situ* nitrification rates in the pelagic zone of freshwater lakes is given after Hall (1986)

Treatment period (d)	A					B	C	Published data
Nitrification rate (µM d^{-1})	2.12	1.15	0.52	0.42	0.38	18.66	8.9	0–78.5
Specific nitrification rate (fmol N $cell^{-1}$ h^{-1})	3.16	1.36	0.52	0.39	0.33	70.0	48.2	0.9–83 0.24–42
Bacterial specific growth rate (d^{-1})	0.17	0.06	0.04	0.02	0.015	2.53	1.30	0.03–2.67 0.01–1.66
Carbon yield from nitrification (C_p:N_o mmol:mol)	4.7	4.5	7.1	5.9	6.2	1.6	1.85	4–7 1.4–3.1
Utilization efficiency (N_o:C_f mol:mol)	21.3	22.2	14.1	16.9	16.1	62.5	54.5	5–42 25–132

Expt 3: grazing resistance of bacteria and feeding strategies of bacterivores

From 85 to 90% of the enrichment culture consisted of uniform rod-shaped bacteria (ESD 0.47 to 0.51 µm). The rates of NH_4^+ uptake, and corresponding accumulation of nitrates in the culture (which was regularly transferred into a fresh medium), were relatively constant at ca 20 µM d^{-1} (the initial concentration of NH_4^+ was always set at 20 µM) for several weeks until they fell to ca 2 µM d^{-1} as the flagellates *Spumella* sp. (ESD 2.0 µm) and *Bodo saltans* (ESD 3.9 µm) appeared in the culture (Table 2A). At the end of the 20 d incubation the concentration of NH_4^+ decreased from the initial 20 µM to 3.4 µM, while nitrates increased from ca 1 to 19.2 µM (Fig. 4A). Shortly after the occurrence of flagellates, bacteria began to form large (18 to >70 µm ESD) gelatinous colonies. The ratio of flagellate to bacterial C and the proportion of aggregated bacteria both increased over time (Fig. 4A), whereas nitrification rates gradually decreased down to 0.38 µM N d^{-1} (Table 2A). The specific N-oxidation metabolism of bacteria, estimated assuming that NH_4^+-oxidizers and NO_2^--oxidizers were numerically equal, decreased from 3.16 to 0.33 fmol N $cell^{-1}$ h^{-1}, and their net specific growth rates decreased from 0.168 d^{-1} to 0.015 d^{-1}. The 2 latter variables were tightly correlated ($r = 0.99$, $p < 0.001$ and $r = 0.98$, $p < 0.01$, respectively) with nitrification rates. The efficiency of autolithotrophic growth expressed as the ratios of N oxidized to C produced and C fixed did not change appreciably, even though the average bacterial cell volume increased by about 11% by the end of incubation.

The growth of the initially predominant *Spumella* sp. slowed down as bacterial clumping progressed, whereas the abundance and growth rates of *Bodo saltans* increased. When the grazers and bacterial clumps were removed by differential filtration, and the resulting filtrates were transferred into a fresh medium, nitrification immediately increased to the initial rate of 18.66 µM N d^{-1} (Table 2B). The corresponding net specific growth rate of bacteria of 2.53 d^{-1} and their specific N-oxidation rate of 70 fmol N $cell^{-1}$ h^{-1} were much higher compared to the previous incubation, whereas the parameters indicating growth efficiency were lower. Intentional inoculation of flagellates into the new culture (Table 2C), however, lowered the total and cell-specific rates of nitrification by 52% and 30%, respectively, and bacterial net specific growth rate by ca 50%. The autolithotrophic growth-efficiency parameters remained at about the level observed in the ungrazed treatment. About 18% of the total bacterial population in the new culture occurred in aggregates when flagellates were present versus less than 2% when they were absent (Fig. 4B, C).

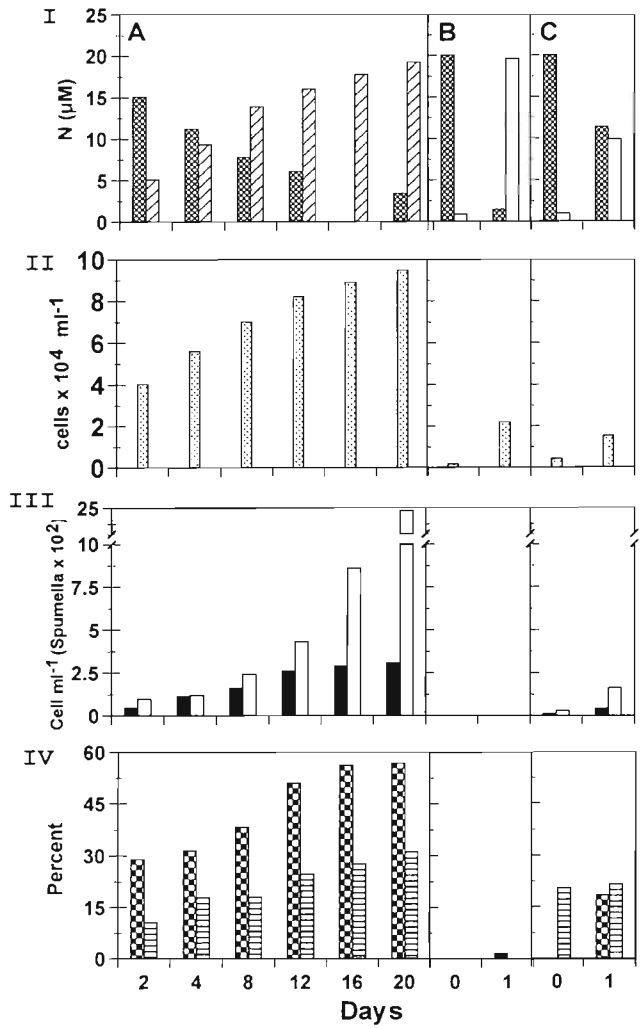

Fig. 4. Dynamics of: (I) dissolved inorganic nitrogen (NH_4^+: shaded bars; nitrates: striped bars); (II) bacteria (shaded bars); (III) flagellates (*Spumella* sp.: solid bars; *Bodo saltans*: empty bars); and (IV) the ratios of bacterial aggregates to the total bacterial abundance (shaded bars) and flagellate to bacterial biomass (striped bars) in the enrichment culture (Expt 3). Letters (A, B, C) refer to experimental treatments described in the text

A FLB experiment conducted on the 10th day yielded grazing rates of 5 ± 0.6 and 1.2 ± 0.4 bacteria $flagellate^{-1}$ h^{-1} for *Spumella* sp. and *Bodo saltans*, respectively. Given respective specific growth rates of 0.118 and 0.140 d^{-1} for the 2 flagellates, their gross growth efficiencies (GGE) were ca 30 and 50%, respectively. Based on their abundance and specific growth rates (1.69 d^{-1} *B. saltans* and 1.29 d^{-1} *Spumella* sp.) and the above GGE, the combined daily carbon requirement of the flagellates added back to the fresh medium was 300 pg C ml^{-1} d^{-1} or 9.94×10^3 cells ml^{-1} d^{-1} that was approximately equal to the difference in total bacterial cell yield between the grazer (Fig. 4B) and control treatments (Fig. 4C).

Expt 4: grazing on nitrifying bacteria by natural plankton

The abundance (ind. l^{-1}) of plankton in Saginaw Bay sample was as follows: heterotrophic flagellates 1.25×10^6, ciliates 1.13×10^4, rotifers 2.15×10^2, copepod nauplii 65, cyclopoid copepods (adults and copepodites) 10.5, calanoid copepods (adults and copepodites) 3.2, cladocera 17.5, *Dreissena* larvae 62. The ambient concentration of bacteria in the bay water was 4.6×10^6 cells ml^{-1}. Based on changes in FLB concentration over time in the experimental vessel, the total community grazing rate on FLB was 5.4×10^3 FLB ml^{-1} h^{-1} or nearly 3% of the added amount. Because Lugol's color was not adequately removed with thiosulfate in some chlorophyll-bearing protists, and because several species were not found in sufficient numbers, it was only possible to examine their FLB uptake qualitatively (Table 3). However, we were able to estimate specific ingestion rates for flagellates and the choreotrichous ciliate *Strobilidium humile* (ESD 20.1 µm) which formed nearly 70% of the total ciliate abundance. The numbers of ingested FLB in protists increased linearly over time ($r = 0.94$, $p < 0.05$). For flagellates these numbers ranged from 0.8 cells flagellate^{-1} h^{-1} in the *Ochromonas nana* (ESD 2.3 µm) to 20.2 cells flagellate^{-1} h^{-1} in *Paraphysomonas* sp. (ESD 12.9 µm), averaging 2.78 cells flagellate^{-1} h^{-1}. *S. humile* ingested 89.4 cells ciliate^{-1} h^{-1}. Based on the abundance of these protists their contributions to the total grazing loss of FLB were 64 and 12% for flagellates and *S. humile*, respectively.

Table 3. Qualitative data on ingestion of fluorescently labeled nitrifying bacteria (FLNB) by planktonic protists and metazooplankton from Saginaw Bay (Expt 4)

Species	Ingested FLNB
Ciliates	
Askenasia volvox	No
Cyclidium sp.	Yes
Disematostoma sp.	No
Pelagohalteria viridis	Yes
Pelagostrombidium sp.	Yes
P. fallax	No
Prorodon sp.	No
Pseudobalanion planktonicum	No
Strobilidium sp.	Yes
S. lacustris	Yes
S. humile	Yes
Urostyla sp.	No
Urotricha furcata	No
U. pelagica	No
U. ristoi	Yes
Vaginicola sp.	No
Vorticella anabaenae	Yes
Flagellates	
Bodo sp.	Yes
Chromulina sp.	Yes
Codosiga sp.	Yes
Cryptomonas sp.	Yes
C. erosa	No
Chrysochromulina parva	No
Ceratium herudinella	No
Dynobrion bavaricum	Yes
Gymnodinium helveticum	No
Ochromonas nana	Yes
Peridinium sp.	No
Rhodomonas minuta	No
Peranema sp.	Yes
Paraphysomonas sp.	Yes
Zooplankton	
Bosmina longirostris	Yes
copepod nauplia	Yes
Cyclops sp.	No
Diaptomus sp.	No
Dreissena larvae	Yes
Filinia sp.	Yes
Keratella cohlearis	Yes
Polyarthra sp.	No

DISCUSSION

Direct effect: grazing mortality in nitrifiers

The results of this study point out the potential ability of planktonic protists to control nitrifier populations via grazing. In Expt 4, a significant proportion of FLB was removed by the natural assemblage of flagellates and nanociliates despite the low concentration of labeled prey. This fact may suggest that protists would efficiently prey on nitrifying bacteria even in the environments where these bacteria form only a small proportion of the total bacterial assemblage. Since the FLB used in our experiments were large compared to ambient bacteria from Saginaw Bay, they may have been selectively grazed (Chrzanowski & Šimek 1990, Gonzalez et al. 1990). Because of the necessity to accommodate intracytoplasmic membranes, nitrifier cells tend to be larger then cells of most other planktonic bacteria. Hence, the above experimental situation may have been not too far from reality. This aspect of the protist-nitrifier interactions remains to be clarified in further studies.

The results of Expt 2 imply that bacterial grazing was the major factor causing N-oxidation rates to be lower in the treatment with added protists. A 2-fold decrease in nitrification rate and the corresponding decrease in the bacterial cell yield observed in the grazer treatment in Expt 3 provide further evidence for grazing mortality of nitrifiers being a major cause of lower nitrification rates in the presence of protists. Also, the bacterial growth rates found here in the absence of grazers were close to those reported for natural assemblages of heterotrophic bacteria (Coveney & Wetzel 1995). This observation implies that

protistan bacterivory may be partially responsible for the 'low growth rates' of ambient populations of nitrifiers that have been previously observed (Hall 1986 and references therein). Thus, our findings are consistent with the idea that nitrification rates may be directly affected by protistan bacterivory.

Trophic interactions involving nitrifiers previously studied in soils and activated sludge provide useful data for comparison with our data, even though in many aspects the above 2 environments are different from the aquatic environments. Predominantly filter-feeding protists were able to remove a large proportion of free-suspended nitrifying bacteria in the biofilm reactor, but the 2-fold increase in nitrification rates was observed only after rotifers grazing upon attached bacteria were inhibited (Lee & Welander 1994). In contrast, no significant change in nitrification rates was observed in a soil chemostat culture despite a 70 to 80% reduction in numbers of free-suspended nitrifiers caused by selective protistan grazing. This result was attributed to the stimulating effect of bacterivory on the nitrifier cell-specific activities (Verhagen & Laanbroek 1992). Although stiff competition between nitrifiers and heterotrophic bacteria for NH_4^+ under high C:N ratios in the latter study may have caused this difference, it remains unclear whether the only grazer present in the soil culture, the flagellate *Adriomonas peritocrescens*, was able to efficiently handle attached nitrifier cells. These results should draw our attention to the potential effects of bacterial behavior and invertebrate grazing strategies on nitrification.

Indirect effects: grazing resistance and feeding modes

Our findings also suggest that protists could indirectly reduce nitrification rates via forcing bacteria to grow in aggregates. The tendency of nitrifiers to form large colonies surrounded by gelatinous slime material first noted by Winogradsky (1890) has been frequently observed in enrichment cultures and activated sludge (Prosser 1986). This slime material was thought to protect nitrifiers from unfavorable conditions, specifically from high pH (Cox et al. 1980). The proportional increase in bacterial aggregates with the increasing ratio of protistan to bacterial carbon (Table 2A) and the occurrence of the bacterial aggregates after inoculation of the flagellates both strongly suggest that the bacterial clumping was related to bacterivorous activities. Aggregation in response to grazing has been repeatedly observed in heterotrophic bacteria in activated sludge (Curds 1982) and cultures (Patterson 1990) as well as in experiments using natural bacterioplankton (e.g. Van Wambeke & Bianchi 1985, Caron et al. 1988). Bacterial growth in aggregates was incorporated as a part of the concept of grazing resistant bacteria (Jürgens & Güde 1994), where it was largely associated with protists. The extracellular enzyme activities observed in bacterivorous protists (Šimek et al. 1994) are likely to have an allelochemic effect on bacterial behavior, but the exact mechanism triggering bacterial clumping remains to be studied.

Since nitrifiers tend to use aggregation as a refuge from grazing, the overall impact of bacterivory may depend upon the ability of grazers to adapt to these changes in bacterial behavior. The occurrence of *Spumella* sp. at the initial stage of incubation and the following development of *Bodo saltans* are consistent with the idea that these species are adapted to feed upon free-suspended and attached bacteria, respectively (Caron 1987, Sibbald & Albright 1988). Our grazing experiment using dispersed FLB likely yielded a conservative estimate of *B. saltans* ingestion rates, taking into account its feeding patterns. On the other hand, this mechanism may explain the higher growth rates of *B. saltans* despite its larger cell volume as compared to *Spumella* sp. The proportional increase in aggregates observed in Expt 3 corresponded to decreasing bacterial nitrification activity. Although this decrease may have been related to the physiological changes in the aging culture (Glover 1985), a decrease in the active cell surface due to the growth in aggregates could also cause this result. Aggregated *Nitrobacter* cells had lower activity due to the development of the slime layer that was only found in large older aggregates but not in young small colonies (Prosser 1986). The latter result could explain why bacterial cell-specific nitrification activity in Expt 3 became much higher in the fresh medium, even in the presence of grazers, after the old large aggregates had been removed. The growth in aggregates as a general pattern of bacterial behavior usually occurs when the concentration of substrate is not a limiting factor (Jürgens & Güde 1994).

We did not attempt to accurately determine abundance of nitrifying bacteria in this pilot study because the conventional most probable number and immunofluorescence techniques used for their enumeration often yield numbers that show little correlation with the observed ambient nitrifying activities (Hall 1986). The polymerase chain reaction assay recently adapted for detecting nitrifiers in the natural bacterial assemblages (Derange & Bardin 1995, Voytek & Ward 1995) appears to overcome this problem but is not yet refined. Thus, as mentioned above, we assumed that NH_4^+-oxidizers and NO_2^--oxidizers were numerically equal in our enrichment culture, even though in fact their physiological requirements may differ as well as their ambient distribution (Ward 1986).

In addition, a part of the culture may have consisted of heterotrophic bacteria since soluble organic compounds exuded by nitrifiers may support the growth of a small heterotrophic bacterial population in a pure inorganic medium (Rittman et al. 1994). These bacteria could develop as the culture ages and organic compounds accumulate (treatment A). Also, we could expect bacterial carbon yield per N oxidized to increase with the increasing proportion of bacteria growing on substrate other then NH_4^+ or NO_2^-. However, this variable remained relatively constant (Table 2A). The tight correlation between net specific growth rates of bacteria and nitrification rates in this treatment also implies the absence of significant changes in bacterial composition over time. Because of the above factors, our calculated rates are conservative. For example, if we assume that 50% of the enrichment culture consisted of bacteria other than nitrifiers, the specific nitrification rate observed in treatment B would increase to a value that is almost twice as high as the maximum published specific nitrification rates (Table 2). Further, the N:C ratios estimated for the enrichment culture are well within the range reported in the literature. However, if we use the above assumption of 50% values of the culture due to heterotrophs, these ratios in treatments B and C would increase to unrealistically high values for a mixed culture of NH_4^+-oxidizers and NO_2^--oxidizers. Combined with the morphological homogeneity of bacterial cells, frequent culture transfers, and the simultaneous accumulation of nitrates with NH_4^+ uptake, this observation indicates that nitrifying bacteria may have constituted a large fraction of the culture.

Most importantly, heterotrophic bacteria growing in a mineral medium are almost certainly C-limited, and, therefore, they are unlikely to compete with nitrifiers for inorganic nitrogen. Hence, independent of their proportion in the enrichment culture (Expt 3), heterotrophs were not responsible for the observed changes in nitrification rates. In contrast, addition of bacterivores always lowered these rates in this and other experiments. Interestingly, the maximum values of cell-specific N-oxidation activity and carbon yield per unit of N oxidized were similar to those reported for the nitrifiers grown in pure cultures at millimolar substrate concentrations (Table 2). This result may suggest that substrate affinity of nitrifying bacteria in our experiments remained close to that of the original bacteria.

Cascading top-down effects: importance of taxonomic composition

The results of Expt 1 suggest that trophic cascades involving bacterivorous (mostly nano-sized) protists, larger protists, and metazooplankton can affect nitrification rates. The occurrence of the specialized bacterivorous scuticociliates may partially explain the rapid dynamics in the water from Old Woman Creek as their specific ingestion rates may be 50 times higher than those of nanoflagellates (e.g. Šimek et al. 1994). High numbers of these ciliates found in the wetland water are typical for hypereutrophic situations (Beaver & Crisman 1989, Christoffersen et al. 1990). In contrast, nanoflagellates were the major bacterial grazers in Saginaw Bay water as was observed in Expt 4. Similarly, nanoflagellates played the leading part in consuming bacteria in Lake Michigan (Carrick et al. 1991) as well as in other waters of low or moderate productivity (Gaedke & Straile 1994, Sommaruga & Psenner 1995).

Bacterivory has been reported in rotifers (Sanders et al. 1989, Ooms-Wilms et al. 1995), cladocera (Vaqué et al. 1992, Burns & Schallenberg 1996), and in copepod nauplii (Roff et al. 1995). Some metazoa from Saginaw Bay contained ingested FLB in Expt 4 (Table 3). However, none of the zooplankton species in this study should be considered to be highly efficient bacterial grazers based on construction of their food gathering structures (Vanderploeg 1994). On the other hand, the same or similar zooplankton species prey upon protists (Hartmann et al. 1993, Jack & Gilbert 1993, Sanders et al. 1994). The grazing control of nanoheterotrophs by planktonic microciliates is also well documented (e.g. Bernard & Rassoulzadegan 1990, Verity 1991).

The direct and indirect grazing impacts of protists on nitrification and trophic interactions with metazoa discussed above suggest that the consistent patterns observed in the dynamics of protists and nitrification rates in Expt 1 are unlikely the result of simple coincidence. A plausible scenario for the observed dynamics is as follows: (1) die-offs of phytoplankton at the beginning of the dark incubation resulted in disappearance of most algivorous ciliates and produced large quantities of labile organic material, fostering development of heterotrophic bacteria; (2) heterotrophic nanoplankton (HNANO) relieved from ciliate grazing pressure thrived at the plentiful bacterial substrate; (3) depletion of this substrate and increases in omnivorous mesozooplankton in unfiltered water, or larger, potentially carnivorous ciliates, flagellates, and rotifers in filtered water, removed HNANO; (4) these predators were not efficient in filtering bacteria-sized particles and allowed the nitrifiers to proliferate.

If the above scenario is true, the cascading top-down effect of zooplankton on nitrifying bacteria would resemble those previously described to mediate the structure and activity of heterotrophic bacterioplankton (Jürgens et al. 1994, Rivkin et al. 1996). This similarity should not be unexpected as nitrifiers are a sub-

set of the total bacterial assemblage. It is important to note, however, that the consequences of top-down impacts on nitrifying and heterotrophic bacteria may be different because of the difference in their physiology and functions in the ecosystem. Protist grazing upon heterotrophic bacteria, as portrayed in numerous studies, crops bacterial production and recycles nutrients for algal use, whereas grazing upon nitrifiers, as follows from our experiments, may modify nitrification rates and thus affect the biogeochemical cycle of nitrogen. In an ambient multistep food web, the composition of crustacean zooplankton may also be important: predominance of copepods will increase predation pressure on larger ciliates (Wiackowski et al. 1994, Burns & Schallenberg 1996), while cladocera will have a strong impact on nanoplankton (Massana et al. 1994, Pace & Vaqué 1994, Gasol et al. 1995). The survival rates and, therefore, potential trophic impacts of protists under predation pressure will also depend on their swimming abilities and growth rates (Gilbert 1994, Lavrentyev et al. 1995). Occasionally, trophic interactions between similarly sized protists may be also important (e.g. Hansen 1991).

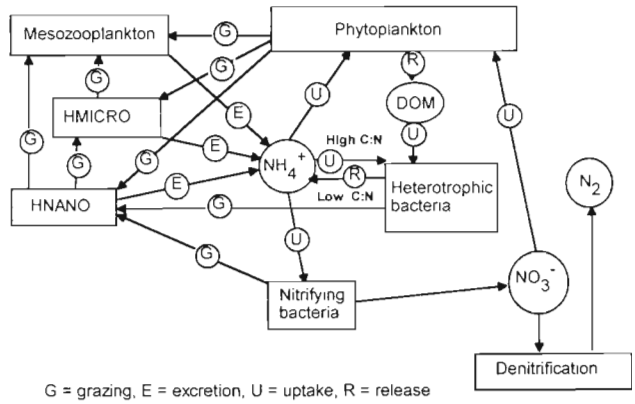

Fig. 5. Simplified conceptual representation of potential interactions between nitrifying bacteria and the microbial food web in the pelagic zone. Denitrification may occur under anoxic conditions in the deeper layers (Seitzinger 1988). Not shown on the figure: zooplankton and ciliate grazing activities also contribute to the labile organic pool (Jumars et al. 1989, Van Wambeke 1995)

Potential implications

Although results from laboratory experiments may not always accurately reflect natural-system processes, we think that our results may be relevant to the pelagic environment because: (1) both the substrate concentrations used and nitrification rates observed in this study were generally comparable (same order of magnitude) to the ambient concentrations and rates reported from limnetic environments (Hall 1986); (2) in 2 of the 4 experiments conducted in this study we used the natural assemblage of aquatic organisms rather than cultured organisms; (3) the patterns in trophic control of nitrification were consistent under the different experimental conditions. The major implication of our study is that previously neglected trophic factors may be a potentially important mechanism for mediating aquatic nitrification rates. Given that nitrification presents a significant sink for oxygen (Pakulski et al. 1995, Balls et al. 1996), provides a substrate for denitrification (Seitzinger 1988), and affects other biogeochemical cycles (Vandenabeele et al. 1995), 'top-down' impacts on nitrifying bacteria may potentially have major feedback effects on the ecosystem level. Obtaining direct evidence from natural aquatic systems remains a subject for further research.

A simplified concept (Fig. 5) illustrates direct and indirect trophic interactions that might involve nitrifying bacteria and thus affect the N cycle in the pelagic zone. Under this scenario, phagotrophic nanoflagellates (or bacterivorous ciliates in hypereutrophic situations) control nitrification directly by reducing bacterial numbers and indirectly by prompting bacterial aggregation. In turn, larger omnivorous ciliates and flagellates diminish grazing control of nitrifiers via predation on bacterivores, while cascading effects of metazooplankton will depend on their composition (i.e. filter-feeders vs raptorial feeders). Nitrifiers growing in aggregates or associated with particles might be also vulnerable to zooplankton filter-feeders as shown for heterotrophic bacteria (Lawrence et al. 1993).

We expect trophic factors to be important in environments characterized by favorable, or at least tolerable, abiotic conditions for nitrification, e.g. transitional zones of estuaries (Feliatra & Bianchi 1993, Pakulski et al. 1995), reduced-oxygen regions (Ward 1986, Lipschultz et al. 1990), and the primary nitrite maximum (Olson 1981, Ward et al. 1990). In more N-limited situations, such as oligotrophic areas of the open ocean, the cascading top-down effects on nitrifiers may essentially depend upon the extent of their competition for NH_4^+ with phytoplankton and heterotrophic bacteria (when the C:N ratio is high). In other words, we cannot exclude the possibility that under certain circumstances a stimulating effect of bacterivory as NH_4^+ supply for nitrification will exceed its inhibitory effect.

Although this study is concerned with water-column organisms, the pivotal role of protists in consuming heterotrophic bacterial production at the sediment-water interface and in sediments (e.g. Bott & Kaplan 1990, Bak et al. 1995) may suggest that similar cascading effects on nitrification are possible in benthic environments. In recent experiments using a flow-through

system filled with Saginaw Bay sediments, we have found a 3-fold decrease in the protistan to bacterial C ratio in the overlying water caused by the filter-feeding bivalve *Dreissena polymorpha* to correspond to a 4-fold increase in nitrification rates (authors' unpubl. data).

Autolithotrophy and the microbial food web: a missing link

The increasing understanding of microbial interactions based on the numerous studies in different aquatic environments has led to gradual transformation of the original 'microbial loop' concept (Pomeroy 1974, Azam et al. 1983) into the microbial food web (Sherr et al. 1988), which is now spanning from submicron viruses to metazoan invertebrates (Legendre & Rassoulzadegan 1995). However, chemosynthetic bacteria are not mentioned in any of the above concepts. At the same time, multiple evidence exists for their direct involvement in trophic interactions with different planktonic organisms. Both purple and colorless sulphur bacteria have been repeatedly shown to be grazed by protists (Massana & Pedros-Alio 1994, Bernard & Fenchel 1995, Guhl et al. 1996) and metazoan zooplankton (Sorokin 1970, Gophen et al. 1974, Caumette et al. 1983). Microplankton-sized amoeba preyed upon hydrogen- and methane-oxidizing bacteria (Sorokin et al. 1994), whereas acidophilic iron-oxidizers were grazed by *Bodo* sp. and *Cinetochilium* sp. (Johnson 1995). Distribution of most autolithotrophs is limited to specific habitats, e.g. the boundary of anoxic/hypoxic zones (Miracle et al. 1992, Sorokin et al. 1995), where their maximums usually coincide with maximum concentrations of protists (Finlay 1983, Zubkov et al. 1992, Fenchel et al. 1995). While trophic interactions between the established components of the microbial food web unanimously play the pivotal role in transferring energy in the pelagic zone, we may hypothesize that, in environments supporting large concentrations of chemosynthetic bacteria, they also can provide the microbial food web with significant quantities of newly-synthesized POM (particulate organic matter). More importantly, combined with a widespread symbiosis between protists and chemosynthetic bacteria (Stumm & Vogel 1989 and references therein), the almost ubiquitous distribution of protists suggests their inherent involvement in many important biogeochemical processes. As far as we know, no attempt has been made to estimate effects of bacterivory on chemosynthesis rates in the aquatic environment. This concept presents an obvious challenge for both microbial food web and biogeochemical studies.

Acknowledgements. We thank Thomas Johengen for help with NO_3^- analyses, Irina Lavrentyeva for making microscope preparations, Joann Cavaletto for sharing unpublished data on chlorophyll *a*, David Patterson for suggestions on the protist taxonomy, and the Electron Microscopy Laboratory of the Eastern Michigan University for letting us use SEM. The manuscript benefited from criticisms and discussion provided by Harvey Bootsma, Marie Bundy, James Cotner, Diane Stoecker, Bess Ward, and 3 anonymous reviewers. This study was supported by the Great Lakes Environmental Research Laboratory and by Fulbright grant 18965 to P.L. This is GLERL contribution 971.

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Responsible Subject Editor: Farooq Azam, La Jolla, California, USA

Manuscript received: September 15, 1996
Revised version accepted: March 6, 1997