Vol. 32: 169–179, 1986 | MARINE ECOLOGY – PROGRESS SERIES Mar. Ecol. Prog. Ser. | Published September 18

Trophic interactions between heterotrophic Protozoa and bacterioplankton in estuarine water analyzed with selective metabolic inhibitors

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ABSTRACT: We estimated *in situ* grazing rates of heterotrophic nanoplankton (HNAN) on bacterioplankton in a salt marsh estuary using a selective metabolic inhibitor technique. Seasonal experiments with prokaryotic inhibitors showed a significant, positive relation between bacterial abundance and HNAN grazing rates. On average, only 40 to 45 % of bacterioplankton production in the estuary appeared to be consumed by phagogrophic Protozoa <20 µm in size. Eukaryotic inhibitor experiments suggested that protozoan grazing can have a positive feedback effect on bacterioplankton production, and that nitrogen regeneration by Protozoa may be of particular importance to bacterial growth. Although eukaryotic inhibitors did not prove to be routinely useful to estimate protozoan grazing rates on bacterioplankton, they may contribute to understanding qualitative control mechanisms in the microbial food web.

INTRODUCTION

Heterotrophic bacteria process a significant fraction (up to 65 %) of total phytoplankton production in marine ecosystems (Fuhrman & Azam 1982, Linley et al. 1983, Azam & Fuhrman 1985). In addition, heterotrophic bacteria produce nutritionally valuable biomass via the consumption of dissolved and particulate non-living organic matter which would otherwise be lost to marine food webs (Pomeroy 1980). A major question in marine microbial ecology at present is how, and to what extent, does bacterial production contribute to biomass production at the higher levels of marine food chains?

Apochlorotic Protozoa in the size range of nanoplankton (2 to 20 µm) have been identified as the major grazers of bacteria in the sea (Fenchel 1982b, 1984, Caron et al. 1982, Sieburth 1984, Sherr et al. 1984). This group of heterotrophic nanoplankton (HNAN) is numerically dominated by colorless microflagellates (Fenchel 1982b, 1984). However, aloricate ciliates with effective cell diameters of <20 µm can compose a significant fraction of the biomass of the HNAN and may compete with microflagellates for bacteria (Rivier et al. 1985, Sherr et al. 1986).

There is only limited information on the *in situ* grazing rate of populations of heterotrophic microprotozoa on bacteria in marine waters. Various direct methods have been used to estimate protozoan bacterivory (Børsheim 1984, Fuhrman & McManus 1984, Landry et al. 1984, Wright & Coffin 1984, Lessard & Swift 1985, McManus & Fuhrman 1986). We report here experimental studies designed to investigate the magnitude and nature of the trophic link between heterotrophic microprotozoa and suspended bacteria in the water column of a salt marsh estuary using selective metabolic inhibitors. Our initial experiments with eukaryotic inhibitors suggested that the method produced useful results (Newell et al. 1983). Furthermore, in comparison to other techniques, the selective inhibitor approach involves minimal manipulation of water samples, does not require use of radioisotopes, and also measures grazing on natural bacterial populations.

MATERIALS AND METHODS

Site description. Water was collected from the Duplin River Estuary adjacent to Sapelo Island, Georgia, USA, a pristine, 12.5 km long tidal embayment

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with an 1100 ha marsh drainage dominated by *Spartina alterniflora,* and no major freshwater input (Pomeroy & Wiegert 1981). Sampling was confined to the distinct tidal prism at the headwaters of the Duplin, which makes a twice daily tidal excursion onto the surrounding salt marshes (Imberger et al. 1983). The Duplin River is a net heterotrophic system, with intense microbial activity in the headwaters because of the intimate association with the marsh (Hopkinson & Hoffman 1984, Sherr et al. 1986, Fallon et al. in press).

Experimental methods. Various concentrations of 4 prokaryotic and 4 eukaryotic antibiotics (Table 1) were tested for specificity and effectiveness using monospecific cultures of Protozoa, natural populations of bacteria, and natural HNAN assemblages. All the inhibitory compounds except thiram were purchased from Sigma Chemical Co.; thiram was obtained from Aldrich Chemical Co.

Clonal cultures of an unidentified colorless monad (3 to 5 µm diameter) and a ciliate, *Uronema* sp., were obtained using initially unenriched or bacterial-nutrient enriched Duplin River water, serially diluted and then plated out as microdrops in silicon oil using the technique of Soldo & Brickson (1980). These cultures were maintained by periodic transfer of an aliquot into 20 ‰ artificial seawater (ASW) (Lyman & Fleming 1940) containing 5 % Cerophyll extract, or bacterial suspensions of either *Escherichia coli* (CGSC Strain #4842) or a *Pseudomonas* sp. originally isolated from the Duplin.

Natural assemblages of bacterioplankton and HNAN were obtained from depth-integrated samples of estuarine water collected with a hand pump. The water was pre-screened through 17 µm mesh Nitex netting by reverse-flow filtration (to separate HNAN from larger zooplankton; we have found that 17 µm mesh is superior to 20 µm mesh in separating nanoplankton from microplankton). For bacterial assemblages, subsamples of the water were filtered through 0.6 or 0.8 µm Nuclepore filters (to separate bacteria from HNAN). To ensure sufficient substrate for bacterial growth, the filtrates were then either diluted 1:1 with filter-sterilized estuarine water or were amended with nutrient broth to a final concentration of 1 mg l^{-1}.

To assay for effects of the antibiotics on bacterial ingestion rates and growth kinetics of bacterivorous Protozoa in the Duplin River, laboratory-cultured Protozoa or the natural assemblages of bacterioplankton or HNAN were incubated with the inhibitors and the changes in bacterial or protozoan numbers with time were compared to those of uninhibited controls. To ensure that the HNAN were not food limited by addition of bacterial growth inhibitors, natural estuarine bacteria were concentrated from 0.8 µm filtered Duplin water by centrifugation and added to the experimental treatments to yield a final concentration of about 10^7 bacterial cells ml^{-1}. The abundance data were used to calculate instantaneous bacterial grazing rates or protozoan growth rates (µ).

Stock solutions and subsequent dilutions of each antibiotic or mixture of antibiotics (except cycloheximide) were made using ASW, and 50 or 100 µl aliquots pipetted into 200 or 400 ml of water containing the organisms to be tested. Because of its limited solu-

Table 1. Qualitative results of assays evaluating the effectiveness and specificity of pro- and eukaryotic antibiotics on bacterioplankton and HNAN

Antibiotics tested	Range of concentrations (mg l^{-1})	Effect on growth kinetics			
		Natural bacterioplankton	Test flagellate	Test ciliate	Natural HNAN assemblage
Prokaryotic					
Chloramphenicol	100	C	C	NA	NA
Cephapirin	1–100	x	NA	NA	NA
Penicillin	1–100	P, C	P, C, x[a]	x	NA
Vancomycin	100–200	P	x	x	x
Vancomycin + Penicillin	200+1	C	x	x	x
Eukaryotic					
Thiram	1–100	P	C	C	C
Cycloheximide	100–200	x	C	P	NA
Demicolcine	0.1–1.0	NA	x	x	x
Colchicine	25–200	x	x	P	NA
Cycloheximide + Colchicine	200+100	x	C	C	C

x: no effect; NA: not assayed; P: partial inhibition; C: complete inhibition; P, C: partial or complete inhibition depending on concentration used

[a] 1 mg l^{-1}

bility, appropriate amounts of cycloheximide were weighed out separately and added directly to each replicate sample. Each treatment was run in duplicate with triplicate subsamples collected at various time intervals during the incubations, which lasted between 1 and 24 h. All incubations were carried out in 750 ml WhirlPak bags at 25 °C in the dark. Soaking the bags overnight in 10 % HCl improved the treatment replication.

Bacterial counts and cell biovolume measurements were made using epifluorescence microscopy and acridine orange staining as described by Newell & Fallon (1982). Biovolumes were converted to carbon equivalents using the recent estimate reported by Bratbak & Dundas (1984) of 2.2×10^{-10} mg C μm^{-3}. Short term assays of [^{3}H]-thymidine incorporation into bacterial DNA (Fuhrman & Azam 1980, 1982, Newell & Fallon 1982) provided a relative index of bacterial growth responses to added antibiotics. During some of the incubation experiments we analysed the frequency of dividing bacterial cells (FDC) (Hagström et al. 1979, Newell & Christian 1981) as another relative index of bacterial growth. Determination of HNAN population abundance and biovolume were made using the double-staining epifluorescence microscopy procedure (Sherr & Sherr 1983, Sherr et al. 1986).

Three methods were used to assess the rates of grazing of natural assemblages of HNAN on suspended bacteria at various times during 1984–1985. Methods 1 and 2 involved the use of pro- and eukaryotic inhibitors, respectively. If the bacterioplankton assemblage is simultaneously producing biomass and being cropped by the HNAN, then terminating bacterial production or protozoan grazing should with time alter the bacterial standing stock. In both cases we compared the relative rates of change in the bacterioplankton biomass between control and inhibited treatments during incubations of replicate 17 µm screened water samples. Water containing both pro- and eukaryotic antibiotics was used as a control for inhibitor-induced bacterial cell lysis in Method 1. The rate of decrease of suspended bacteria in this control (due to only cell lysis) was then subtracted from the rate of decrease in the samples containing only prokaryotic inhibitors to obtain an estimate of bacterial loss due to HNAN grazing. To initially determine if the rates of change in bacterial numbers in controls and inhibitor treatments were significant, regression analyses of bacterial biomass versus time, generally using 6 to 8 time points, were performed. The data nearly always fit a linear model, as we and others have found in previous experiments (Newell et al. 1983, Fuhrman & McManus 1984, Wright & Coffin 1984). The resultant regression coefficients were subjected to the T' and GT2 analyses for unplanned comparisons of multiple slopes (Sokal & Rohlf 1981). Grazing rate estimates were made only if the slopes were different at the 95 % level.

As an independent means of assessing protozoan bacterivory, Method 3 consisted of determining HNAN net biomass productivity in the control treatments, and then estimating the amount of bacterial carbon ingested by assuming that all of the HNAN production occurred at the expense of bacterioplankton and that the average gross growth efficiency of the HNAN assemblage was 33 % (Sherr & Sherr 1984, Sherr et al. 1984). HNAN production was obtained by monitoring with time the cumulative increase in total HNAN biovolume ml^{-1} and multiplying by the average carbon:protozoan biovolume conversion factor of 8×10^{-11} mg C μm^{-3} (Sherr & Sherr 1984).

In 4 experiments NH_4^+ was added to control and eukaryotic inhibited samples. Reagent grade NH_4Cl was sublimated to remove trace organics and added to inhibited samples throughout a 24 h incubation at concentrations theoretically required (based on the Redfield C:N ratio of 7:1) to support at least one doubling of the bacterial assemblage during the incubation period. The NH_4^+ concentrations added varied according to initial (zero time) bacterial carbon standing stock estimates. Changes in NH_4^+ concentrations during the incubations were determined by the indophenol method of Koroleff (1976). In one experiment in which we monitored the ammonium pool in control and inhibited treatments over time, α-D glucose was added to separate replicate bags containing control or eukaryotic inhibited water. Sterile, 40 µl aliquots of a glucose solution were added 4 times during the 24 h experiment at the rate of 7.5 µg glucose per 200 ml of water sample per time interval in order to simulate the average daily turnover rate of glucose in the Duplin River estuary, estimated by Hanson & Snyder (1980).

RESULTS

Effectiveness and specificity of prokaryotic antibiotic mixture

Of the 4 prokaryotic antibiotics tested, only a mixture of 200 mg l^{-1} vancomycin and 1 mg l^{-1} benzyl penicillin was effective at inhibiting bacterial growth without also affecting protozoan growth (Table 1). Typically, inhibition was essentially complete within 1 h of addition of the prokaryotic antibiotics to natural assemblages of bacterioplankton in early log phase of growth (Fig. 1). There was a slight decrease in bacterial numbers with time after the inhibitor mixture was added. This bacteriocidal effect was apparent in all of our grazing rate experiments. Bacterial cell loss

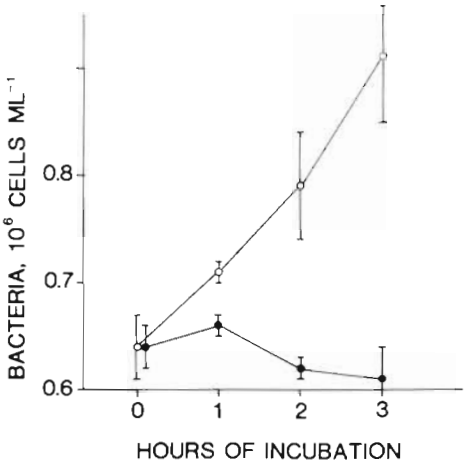

Fig. 1. Inhibitory effects of prokaryotic antibiotic mixture (vancomycin, 200 mg l^{-1} + penicillin, 1 mg l^{-1}) on reproduction of a natural assemblage of estuarine bacterioplankton in early log phase of growth. Inhibitors were added at 0 h. (○) uninhibited controls; (●) inhibited samples. Line bars represent ± 1 SD

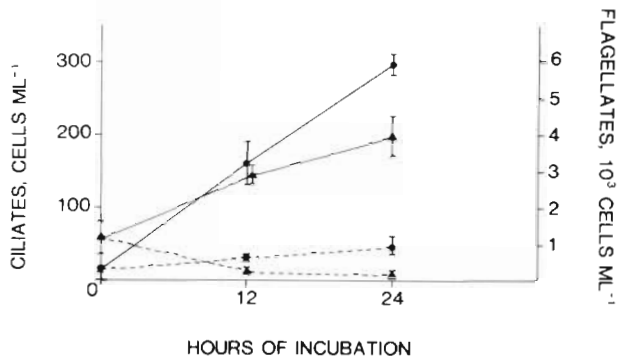

Fig. 2. Typical population growth response of a natural assemblage of colorless microflagellates (▲) and nanoplanktonic ciliates (●) to the addition of the eukaryotic inhibitor mixture (cycloheximide, 200 mg l^{-1} + colchicine, 100 mg l^{-1}). Solid lines: uninhibited controls; broken lines: inhibited samples; line bars: ± 1 SD

ascribed to lysis was on average 21 % of the decrease in cell numbers which occurred as a result of grazing.

We found no significant effects (Student's t-test) on population growth rates of either the cultured flagellate or *Uronema* sp. when the vancomycin-penicillin mixture was added to actively growing protozoan cultures containing 10^6 to 10^8 cells ml^{-1} of *Escherichia coli* or the *Pseudomonas* sp. isolate. Natural assemblages of HNAN also showed no difference in growth rate with or without the prokaryotic antibiotics.

Effectiveness and specificity of eukaryotic antibiotic mixture

Of the eukaryotic antibiotics evaluated, only a cycloheximide-colchicine mixture at 200 and 100 mg l^{-1}, respectively, was both an effective inhibitor of protozoan reproduction and feeding and had no direct effect on bacterial growth. The final cell yield of *Uronema* sp. was only 1 % that of the control when this antibiotic mixture was used. The inhibition of ciliate growth was less complete when either antibiotic was used alone. Growth of the test flagellate was 100 % inhibited by the antibiotic mixture as well as by cycloheximide alone. Colchicine by itself did not inhibit reproduction of the flagellate.

A typical growth response of a natural species assemblage of heterotrophic ciliates and flagellates after addition of the eukaryotic inhibitors is shown in Fig. 2. The inhibitory effect was consistent with that obtained using the monospecific protozoan cultures. The population growth of heterotrophic Protozoa was similarly inhibited in every incubation experiment we conducted with Duplin River water, suggesting that the combined effect of cycloheximide and colchicine was of a sufficiently broad spectrum to inhibit the entire species assemblage of bacterivorous Protozoa.

Elimination of the population growth of heterotrophic Protozoa does not necessarily imply that the Protozoa have ceased feeding. We tested the efficacy of the cycloheximide-colchicine mixture in terminating feeding activity of a natural assemblage of heterotrophic flagellates fed 10^6 cells ml^{-1} of the *Pseudomonas* sp. Flagellate cell-specific bacterial ingestion rates fell to 0 between 60 and 90 min after addition of the inhibitors. In a similar experiment with the *Uronema* sp., bacterial ingestion ceased between 90 and 120 min after addition of inhibitors. Since our grazing rate experiments were typically run for 24 h, the time to complete cessation of grazing by heterotrophic flagellates and ciliates was less than 6 % of the total incubation time and can be considered essentially an instantaneous effect.

At the concentrations of cycloheximide and colchicine which produced essentially complete inhibition of protozoan growth and feeding, no significant effect was found on either population growth rates or on cell-specific incorporation of [^{3}H]-thymidine into DNA during log-phase growth of natural bacterioplankton in Duplin River water.

Use of prokaryotic and eukaryotic inhibitors to estimate HNAN grazing rates on Duplin River bacterioplankton

Below we present the results of our initial set of experiments conducted with natural estuarine water and both sets of inhibitors during the winter and summer-fall of 1985.

Table 2. *In situ* temperatures, abundance of bacterioplankton, and abundance and biomass production of HNAN obtained during grazing rate experiments conducted during winter and summer-fall of 1985

Date	Temperature °C	Bacterial abundance[a] mg C m^{-3}	HNAN abundance[b], mg C m^{-3} Flagellates	Ciliates	Total	HNAN net biomass production mg C m^{-3} h^{-1}
Winter						
24 Jan 85	5	58	11.1	1.7	12.8	0.8
6 Feb 85	14	36	15.1	0.9	16.0	0.6
18 Feb 85	11	74	21.4	1.2	22.6	0.6
28 Feb 85	21	121	4.9	3.7	8.6	0.4
$\bar{X}$[c]	12.8 (6.6)	72 (36)	13.1 (6.9)	1.9 (1.3)	15.0 (5.9)	0.6 (0.2)
Summer-fall						
7 Jun 85	31	93	1.1	2.1	3.2	1.9
1 Jul 85	31	105	8.7	1.2	9.9	0.4
16 Sep 85	26	60	4.4	2.3	6.7	1.5
23 Sep 85	28	42	3.7	0.9	4.6	0.4
$\bar{X}$[c]	29 (2.4)	75 (29)	4.5 (3.2)	1.6 (0.7)	6.1 (2.9)	1.1 (0.8)
Difference[d]	$P<0.002$	ns	$P<0.005$	ns	$P<0.02$	$P<0.1$

[a] Based on a conversion factor of 2.2×10^{-10} mg C μm^{-3} of cell volume (Bratbak & Dundas 1984). Average biovolume $cell^{-1}$ ranged from 0.06 to 0.08 μm^3

[b] Based on a conversion factor of 8×10^{-11} mg C μm^{-3} of cell volume (Sherr & Sherr 1984)

[c] Mean value, 1 SD in parentheses

[d] Student's t-test of difference between winter and summer-fall mean values; ns no significant difference between means

Background data: measured bacterial and protozoan parameters

In spite of the 16 C° higher average *in situ* water temperature during the summer-fall period, there were no statistically significant differences between the 2 sampling periods in bacterioplankton biomass abundance (Table 2), which corresponded to bacterial numbers of 3.3×10^6 to 11×10^6 cells ml^{-1}. All protozoan parameters except for nanoplanktonic ciliate biomass were significantly different between the 2 sampling periods (Table 2). Total protozoan biomass was 2-fold higher during the winter, with aloricate ciliates representing an average of from 13 % of total protozoan biomass in winter to an average of 26 % during the summer. Numbers of heterotrophic Protozoa at the beginning of the experimental incubations averaged $1.3\pm0.3\times10^3$ flagellates ml^{-1} and 11 ± 5 ciliates ml^{-1} in summer, and $2.2\pm0.7\times10^3$ flagellates ml^{-1} and 12 ± 8 ciliates ml^{-1} in winter. During the summer period of this study, the average net biomass productivity of the HNAN assemblage was proceeding twice as rapidly as during the winter. The rates of biomass production of the Protozoa during the experimental incubations were used to derive independent, upper estimates of potential rates of bacterivory (Table 3, Method 3).

Grazing rates estimated by prokaryotic inhibition

Although there was considerable variation between experiments, the mean grazing rate estimates (Method 1, Table 3) were not different for the 2 sampling periods in spite of the 2-fold seasonal difference in protozoan biomass. Linear regression analysis of initial bacterial concentrations versus the HNAN grazing rates estimated by use of prokaryotic inhibitors revealed a highly significant ($r = 0.97$, $p<0.001$) positive correlation coefficient (Fig. 3), indicating that, although the bacterial standing stocks were not high enough at any time to elicit a saturation feeding response by the HNAN, they were above minimum concentrations below which grazing cannot occur because of morphological and physiological constraints (Fenchel 1984). The rates of bacterial cell decrease corresponded to an average consumption per protozoan of 20 to 80 bacteria h^{-1} and an average clearance rate of 2 to 10 nl $protozoan^{-1}$ h^{-1}. These rates are within the range of values found by other investigators for both monospecific cultures of flagellates and populations of heterotrophic flagellates in other marine environments (Table 4).

Comparison of Method 1 grazing rates with previous estimates of average winter and summer *in situ* bacterial net productivity in the upper Duplin, based on

Table 3. Results of 3 methods of estimating grazing rates of natural HNAN assemblages on Duplin River bacterioplankton in 8 incubation experiments during 1985, compared to net bacterial growth in control treatments and to average rates of bacterioplankton production previously measured during winter and summer in the upper Duplin River

Date	HNAN grazing rates Method 1: pro. inh.	Method 2: eu. inh.	Method 3: HNAN prod.	Net bacterial growth in controls	Seasonal average bacterial production[a]
Winter					
24 Jan 85	0.5	0.9	2.4	2.4	
6 Feb 85	0.3	0.5	1.7	1.0	
18 Feb 85	1.0	0	1.9	0	
28 Feb 85	2.4	0	1.1	4.4	
$\bar{X}$[b]	1.1 (0.9)		1.8 (0.5)	2.0 (2.0)	2.4 (1.3)
Summer					
7 Jun 85	1.9	2.3	5.6	1.0	
1 Jul 85	2.0	2.5	1.3	1.0	
16 Sep 85	0.7	0	4.4	1.1	
23 Sep 85	0.3	0	1.2	2.1	
$\bar{X}$[b]	1.2 (0.8)		3.1 (2.2)	1.3 (0.5)	3.0 (2.0)

[a] Calculated from [^{3}H]-thymidine uptake measurements during 16 d periods in Feb 1984 and Aug–Sep 1983 (Sherr et al. 1986)
[b] Mean value, 1 SD in parentheses

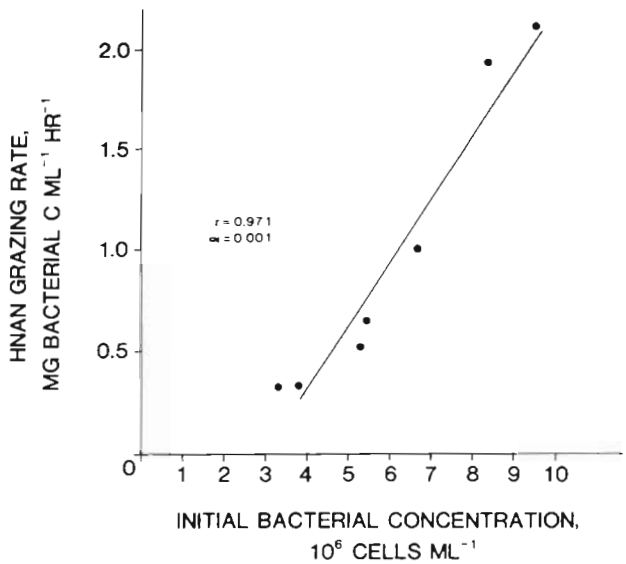

Fig. 3. Plot of Method 1; estimated grazing rates of heterotrophic nanoplankton (HNAN) on bacterioplankton (in units of mg bacterial carbon consumed ml^{-1} h^{-1}) *vs.* initial (i.e. *in situ*) bacterial cell concentration at beginning of incubation. r: correlation coefficient; α = significance level of r

uptake of [^{3}H]-thymidine (Table 3; see Sherr et al. 1986), indicated that the HNAN were only cropping about 45 % of the net bacterial production in the winter and about 40 % during the summer-fall season. In 7 out of 8 experiments, we observed a net linear increase of bacterial biomass in the uninhibited controls (Table 3), which independently suggested that bacterial production was greater than the HNAN grazing rate in the headwaters of the Duplin River.

In contrast, to the grazing rates obtained with the prokaryotic inhibitors, the grazing rate values obtained with Method 3 (HNAN biomass production, see 'Materials and Methods') were higher, averaging 75 % of average bacterial production (Table 3) in winter, and 103 % in summer. Many species within the HNAN assemblage are not obligate bacterivores, but can also ingest other prey including chroococcoid cyanobacteria and a variety of eukaryotic cells (Johnson et al. 1982, Sherr & Sherr 1984, Goldman & Caron 1985). Thus, if the HNAN assemblage was producing biomass at the expense of other food in addition to heterotrophic bacteria, Method 3 would result in an overestimation of bacterial cropping by the HNAN.

Grazing rates estimated by eukaryotic inhibitors

In only 4 out of 8 experiments did bacterial concentrations in the eukaryotic inhibited treatments increase significantly above uninhibited controls (Method 2, Table 3). In these experiments, the calculated HNAN grazing rates agreed fairly closely with those estimated by prokaryotic inhibition (Method 1). In the other 4 experiments, rates of bacterial increase were either not significantly different from the controls, or actually less than the controls. We also found that the FDC values of bacterial assemblages in these eukaryotic inhibitor treatments were generally much lower than the FDC values of the control assemblages (example shown in Fig. 4).

Table 4. Comparison of rates of protozoan bacterivory for monospecific flagellate cultures and for natural HNAN assemblages estimated using various methods

Method	Protozoan	Concentration of bacteria ml^{-1}	Bacteria consumed proto-zoan^{-1} h^{-1}	Clearance rate nl proto-zoan^{-1} h^{-1}	Population grazing rate 10^4 bacteria ml^{-1}	Source
Disappearance of cultured bacteria	Monospecific flagellate cultures	10^{-6}–10^{-8}	27–254	1.4–79	–	Fenchel 1982a
Disappearance of cultured bacteria	*Monas* sp.	10^{7}–10^{9}	10–75	0.2–0.95	–	Sherr et al. 1983
FDDC method	Monospecific flagellate cultures from Rhode Island estuary, shelf & Sargasso Sea	10^{5}–10^{7}	0–300	17–336	2.5–60	Davis & Sieburth 1984
Differential filtration	Natural assemblage, Massachusetts coast	10^{6}–10^{7}	–	–	4.2–29	Wright & Coffin 1984
Dilution method	Natural assemblage, Hawaiian coast	10^{6}	17–38	2.5–29	1.5–3.4	Landry et al. 1984
Uptake of microbeads	Natural assemblage, Chesapeake Bay	–	2–25	0.6–1.9	–	McManus & Fuhrman 1986
Selective inhibition	Natural assemblage, New York coast	10^{6}–10^{7}	–	–	4.6–63	Fuhrman & McManus 1984
Selective inhibition	Natural assemblage, Georgia coast	10^{6}–10^{7}	20–80	2–10	2.2–18	This study

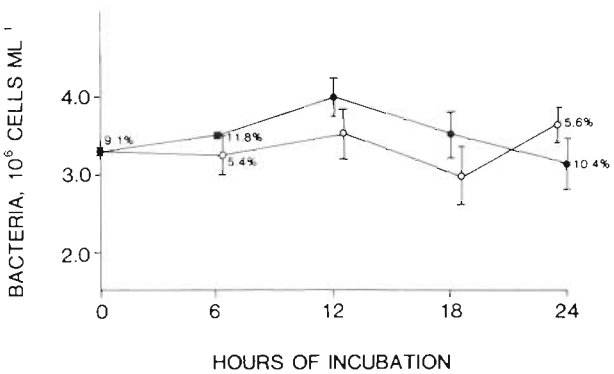

Fig. 4. Results of an eukaryotic inhibitor experiment conducted on 18 Feb 1985. Data are bacterial cell abundances of uninhibited controls (●) and eukaryotic inhibited samples (○) at various times during a 24 h incubation. Percentages are frequency of dividing bacterial cells (FDC values) estimated at 0, 6, and 24 h after addition of the eukaryotic inhibitor mixture. Line bars: ± 1 SD

Experiments on potential nitrogen limitation

Table 5 shows the results of 4 experiments where we found an increase in the observed rates of growth of suspended bacteria when NH_4^+ was added to eukaryotic inhibited samples (see 'Materials and Methods'). In 2 of these experiments (28 Feb and 23 Sep 1985), bacterial growth in the inhibited treatment was significantly lower than the controls; addition of NH_4^+ returned bacterial growth rates to control levels (Table 5). In the other 2 experiments, NH_4^+ addition further enhanced bacterial growth observed in the eukaryotic inhibitor treatments.

Variations in the natural ammonium concentration were followed in an experiment conducted on 15 Nov 1984 (Fig. 5); by the end of 24 h, the NH_4^+ concentrations in the eukaryotic inhibited samples had decreased by almost an order of magnitude below the controls. Addition of glucose to control or inhibited samples resulted in a more rapid decline of NH_4^+, to near zero concentration, compared to that observed in the treatment with only eukaryotic inhibitor (Fig. 5).

Table 5. Stimulatory effect of NH_4^+ addition to eukaryotic inhibited samples on the observed rates of bacterioplankton growth[a]. Data are mean observed rates of change of bacterial standing stocks during incubation in mg C m^{-3} h^{-1}; superscript numbers indicate which row means are significantly different from each other (1-way ANOVA + SNK testing, $P<0.05$)

Experiment	Controls	Eukaryotic inhibitors	Eukaryotic inhibitors + NH_4^+
28 Feb 85	7.7^{2}	$4.8^{1,3}$	6.8^{2}
28 Jun 85	$0.7^{2,3}$	$2.0^{1,3}$	$4.1^{1,2}$
16 Sep 85	3.5^{3}	5.1^{3}	$10.2^{1,2}$
23 Sep 85	5.0^{2}	$2.5^{1,3}$	4.7^{2}

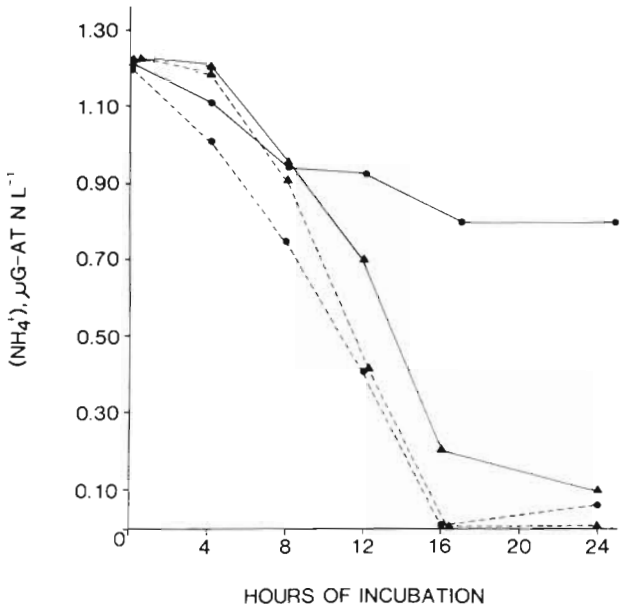

Fig. 5. Temporal changes in the NH_4^+ concentrations of uninhibited 17 µm screened water samples (●) compared to those of eukaryotic inhibited samples (▲) with (broken lines) and without (solid lines) addition of glucose

DISCUSSION

In using a metabolic blocking agent (or combination of agents) to address ecological quesitons such as those being asked in the present study, 3 basic criteria must be met. (1) The agent(s) must act only on the target component of the observed system, i.e. they must not produce any direct stimulatory or inhibitory effect on any of the other interacting components, the subsequent response (if any) of the latter groups developing only as a consequence of the removal of the target component from the system. (2) The agent(s) must be sufficiently broad spectrum to include all of the species populations within the target component. (3) The agent(s) must perform rapidly to inhibit the particular activity of the target component relative to the experimentally induced response of the other components. By testing a number of antibiotics, we arrived at separate mixtures of prokaryotic and eukaryotic inhibitors which met these criteria.

Prokaryotic inhibitors

There is no accepted standard method by which to judge the accuracy of the grazing rates estimated from the prokaryotic inhibitor experiments. In terms of average rates of bacterial consumption and volume cleared per hour by individual HNAN cells, and of the total grazing rate on bacteria by the HNAN population, our results are well within the range of values for these parameters derived from other studies (Table 4). However, due to the diversity of habitats sampled combined with the bias inherent in each method used, there is a 10 to 100-fold variation in the ranges of these values. The large differences in clearance rates can be partly explained by the wide range in bacterial abundance, 10^5 to 10^8 cells ml^{-1}, in the various studies. The very low clearance rates reported by McManus & Fuhrman (1986) may result from discrimination against microbeads by some species of phagotrophic Protozoa (Pace & Bailiff unpubl.).

A second test of the accuracy of experimentally-derived grazing rates is comparison with estimates of bacterial growth. If grazing by the HNAN is responsible for most of bacterial mortality in marine waters, then estimates of HNAN bacterivory should, on average, be equivalent to estimates of bacterial productivity in the same system. Equivalency between rates of protozoan bacterivory and of bacterioplankton growth has been reported in Massachusetts (Wright & Coffin 1984) and in New York (Fuhrman & McManus 1984) coastal waters.

Our data, however, suggested that the HNAN may be consuming less than one-half of the instantaneous bacterial production in the headwaters of the Duplin River. In a previous study (Sherr et al. 1984), we calculated that, based on population size and biomass production, HNAN in Georgia estuaries and offshore waters grazed 30 to 50 % of daily bacterioplankton production. The fact that in nearly all of the present experiments there was net bacterial growth in the control treatments (Table 3) is further evidence that the HNAN were not cropping 100 % of bacterial production. Since bacterial biomass does not accumulate in the water column of the Duplin River (Sherr et al. 1986, Fallon et al. in press), the excess bacterial production must be lost to other sinks. Lessard & Swift (1985) have shown that microplanktonic (>20 µm) Protozoa can ingest bacterioplankton. Other potentially bacterivorous organisms in the estuarine-marsh system are benthic filter feeders. The water mass at the upper station of the Duplin makes a twice daily tidal excursion onto the surrounding *Spartina* marsh, and 2 studies have reported a net important of estuarine bacterioplankton into similar marshes (Erkenbrecher & Stevenson 1975, Rublee et al. 1983). Also, Wright et al. (1982) demonstrated that the salt marsh mussel *Geukensia demissa* is capable of efficient clearance of natural bacterioplankton from the water column.

The lack of equivalency between HNAN grazing and bacterioplankton production found in this study is similar to the conclusion of Landry et al. (1984) that in the shallow waters of Kaneohe Bay, Hawaii, maintenance of relatively stable bacterial populations could

not be attributed solely to grazing by small heterotrophic flagellates, but was likely also due to grazing by larger zooplankton and by benthic organisms.

Eukaryotic inhibitors

Even though the HNAN may not be grazing all of bacterial production, the results of the eukaryotic inhibitor experiments indicated that phagotrophic Protozoa may indirectly influence bacterial growth. The absence of a relative increase in bacterial biomass in half of the eukaryotic-inhibited treatments (Table 3) was apparently due to an experimentally induced decrease in growth rate, as suggested by the lower FDC values of bacterial populations in those treatments compared to the controls (Fig. 4). Since the eukaryotic antibiotics undoubtably effect the physiology of phytoplankton cells as well as phagotrophic Protozoa, we cannot dismiss the possibility that disruption of algal metabolism affected subsequent bacterial production, for example by reducing the amount of utilizable DOM released from the phytoplankton cells. However, in the eutrophic Duplin River system, bacterioplankton dynamics may be more strongly coupled to organic carbon sources originating in the surrounding salt marshes and to fecal pellet production in the water column than to phytoplankton exudates (Christian et al. 1981). The other possibility for the induced decrease in growth rate is that the decoupling of the bacterioplankton from HNAN grazing disrupted a feedback interaction between the 2 assemblages.

Several authors have stressed the role of the HNAN as the major regenerators of microbially utilized inorganic forms of nitrogen and phosphorous in marine pelagic systems (Johannes 1965, Glibert 1982, Harrison 1983, Goldman & Caron 1985). The organic substrates in the Duplin River which originate in the surrounding salt marshes have high C:N ratios, characteristic of vascular plant detritus (Hanson & Snyder 1980), and bacterial production in the estuary may be nitrogen limited (Haines & Hanson 1979). Kirchman & Wheeler (unpubl. data) have found that bacterioplankton in the Duplin estuary preferentially utilize ammonium as a nitrogen source and are likely to be active competitors with phytoplankton for dissolved inorganic nitrogen.

On the basis of these reports, we tested the hypothesis that eukaryotic inhibition of protozoan metabolism may indirectly limit bacterial growth by a decreased availability of regenerated nitrogen. When supplemental ammonium was added to eukaryotic inhibitor treatments, we found significantly enhanced bacterial growth compared to inhibitor treatments without ammonium addition. When the natural concentration of ammonium was followed with time in another inhibitor experiment with and without added glucose, the ammonium concentration in the control without eukaryotic inhibitor or glucose remained close to the initial level, while the ammonium concentrations in the other treatments dropped to near zero. These results support the idea that phagotrophic Protozoa are important in nutrient recycling (Taylor 1982, Goldman & Caron 1985), and are a powerful argument in favor of predator feedback control on bacterioplankton production operable under conditions of nitrogen limitation. They also suggest that addition of mineral-poor organic substrates can stress the system, at least on the short term, since apparently the Protozoa in the control with added glucose were not able to regenerate ammonium as rapidly as it was taken up.

There are other possible feedback effects between bacteria and their protozoan grazers which we did not test. For example, Sieburth & Davis (1982) concluded that in the Sargasso and Caribbean Seas the HNAN have a 'nurturing' effect on their bacterial prey by excreting dissolved organic compounds which supplement photosynthate released primarily during the daylight hours. Subsequently, Taylor et al. (1985) reported direct experimental evidence for a significantly greater release of low molecular weight compounds in the presence of bacterivorous ciliates and natural microzooplankton, compared to protozoan-free controls, and for rapid bacterial utilization of the compounds released in the presence of grazers.

In summary, the rates of HNAN grazing on bacterioplankton in the Duplin River estuary determined by the use of prokaryotic inhibitors were similar to rates of protozoan bacterivory reported for other coastal waters, although on average the HNAN cropped less than half of estimated bacterial production. To adequately evaluate the accuracy of the method, comparative studies should be conducted in which grazing rates are determined simultaneously on the same water sample using other methods as well as the inhibition technique. Addition of eukaryotic inhibitors is not a reliable method for determining grazing rates, apparently because the inhibitors curtail indirect, positive effects of eukaryotic cells on bacterial growth. On the other hand, our data showed that eukaryotic inhibitors can be a useful tool to detect and evaluate the nature of feedback relations between microbial predators and their prey. Conceptual models of bacterioplankton dynamics in the sea (e.g. Azam et al. 1983, Pace et al. 1984, Wright 1984) have, in general, considered only organic substrate availability and physical removal of cells by grazers as regulators of bacterial growth. The results obtained in this study suggest that feedback control effects should also be explicitly included in any future modelling effort.

Acknowledgements. We thank Dr. Jed Fuhrman and anonymous reviewers for their comments on the manuscript, as well as Lea Kneib for preparation of tables and Cindy Holcombe for preparation of figures. This work was supported by NSF grant OCE-82-19866, by NIH grant BRSG 507 RR07025-21, and by grants from the Sapelo Island Research Foundation. Contribution No. 561 of the University of Georgia Marine Institute.

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This article was presented by Professor T. Fenchel; it was accepted for printing on June 24, 1986