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Use of Metabolic Inhibitors to Characterize Ecological Interactions in an Estuarine Microbial Food Web

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ABSTRACT

Understanding microbial food web dynamics is complicated by the multitude of competitive or interdependent trophic interactions involved in material and energy flow. Metabolic inhibitors can be used to gain information on the relative importance of trophic pathways by uncoupling selected microbial components and examining the net effect on ecosystem structure and function. A eukaryotic growth inhibitor (cycloheximide), a prokaryotic growth inhibitor (antibiotic mixture), and an inhibitor of photosynthesis (DCMU) were used to examine the trophodynamics of microbial communities from the tidal creek in North Inlet, a salt marsh estuary near Georgetown, South Carolina. Natural microbial communities were collected in the spring, summer, and fall after colonization onto polyurethane foam substrates deployed in the tidal creek. Bacterial abundance and productivity, heterotrophic ciliate and flagellate abundance, and phototrophic productivity, biomass, and biovolume were measured at five time points after inhibitor additions. The trophic responses of the estuarine microbial food web to metabolic inhibitors varied with season. In the summer, a close interdependency among phototrophs, bacteria, and protozoa was indicated, and the important influence of microzooplanktonic nutrient recycling was evident (i.e., a positive feedback loop). In the fall, phototroph and bacteria interactions were competitive rather than interdependent, and grazer nutrient regeneration did not appear to be an important regulatory factor for bacterial or phototrophic activities. The results indicate a seasonal shift in microbial food web structure and function in North Inlet, from a summer community characterized by microbial loop dynamics to a more linear trophic system in the fall. This study stresses the important role of microbial loops in driving primary and secondary production in estuaries such as North Inlet that are tidally dominated by fluctuations in nutrient supply and a summer phytoplankton bloom.

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Introduction

Since the advent of the "microbial loop" concept [34], the complexity of planktonic trophodynamics in estuarine food webs has been increasingly recognized. Commonly demonstrated attributes of the microbial loop include high bacterial production, the predominance of small primary producers (e.g., pico- and nanoplankton) and microzooplanktonic grazers (e.g. flagellates, ciliates), and rapid nutrient cycling involving resource processing by multiple trophic components [2, 4, 6, 12, 13, 36, 39, 40]. There is general agreement on the components and trophic relationships within these microbial communities [19], but the consequences of microbial loop dynamics for energy transfer efficiency and ecosystem production are less clearly understood and likely vary greatly with estuarine type [5].

Stone and Berman [41] devised a mathematical model of the positive feedback loop in microbial communities, which emphasized the need to consider small spatial and temporal scales, based on the important influence of nutrient pulses. The timing and magnitude of nutrient inputs can affect plankton cell size, physiology, and community composition, and therefore drive phytoplankton bloom dynamics and community production. The model also suggested that the microbial loop does not approach steady state and that it will have different characteristics in different environments, based on nutrient flux patterns. Moloney et al. [29] also stressed the importance of microbial nitrogen regeneration in a plankton community subjected to nutrient pulses, and showed that recycling through the microbial loop provided significantly more of the nutrients needed to sustain phytoplankton and heterotrophic flagellates than did recycling by mesozooplankton. Thus, nutrient processing in microbial loop communities is not necessarily inefficient per se [17], and may even be considered a "beneficial" mechanism for supporting ecosystem production in environments such as estuaries marked by frequent fluctuations in nutrient supply [10].

Lewitus et al. [25] stressed the importance of microbial loop dynamics to material and energy flux in North Inlet (Georgetown, South Carolina), a high salinity salt marsh estuary characterized by the predominant influence of tidal exchange in driving nutrient flow. Freshwater input to North Inlet through surface- or groundwater is restricted (3% of tidal volume; [30]), and ca. 40% of the total water volume leaves the system on each ebb tide (semidiurnal) with an average hydrodynamic residence time of 15 h [22]. The frequent tidal flushing of this shallow estuary (2.2 m maximal tidal amplitude) results in a highly variable nutrient pool, regulated primarily by the magnitude of flux through the mouth, exchange between the water column and the intertidal sediments, and trophic interactions inside the estuary [30]. Lewitus et al. [25] characterized the annual phytoplankton bloom (peak in July–August) in North Inlet as a microbial loop, based on the predominance of phototrophic pico- and nanoflagellates, regenerated nitrogen sources (e.g., NH_4), and microzooplanktonic grazing control of phytoplankton population growth. More recent studies have also shown that bacteria and microzooplankton (ciliates, heterotrophic nanoflagellates) abundances during the summer in North Inlet were at the high end of those reported in the literature [26, 45]. Because the major proportion of annual planktonic primary production in North Inlet occurs during the summer [25, 44], knowledge of the trophodynamics of microbial communities during this period is important to understanding overall ecosystem function in this estuary.

In the present study, we further explored microbial food web dynamics at North Inlet through the use of artificial substrates (polyurethane foam) and the application of metabolic inhibitors (e.g., DCMU [3-(3,4-dichlorophenyl)-1,1 dimethylurea], an inhibitor of photosynthetic electron transfer; cycloheximide, an inhibitor of eukaryotic growth; and an antibiotic mix that inhibits prokaryotic growth). The use of artificial substrates provided some advantages over routine bottle sampling [7]. For example, they served as independent units to achieve a valid statistical design for resampling the same container over time. Polyurethane foam substrates minimize the effect of microbial assemblage patchiness and represent an accumulation of taxa over time as opposed to a single grab sample. Polyurethane foam substrates have been shown to permit colonization by swimming and sessile organisms, yield a high degree of species overlap among replicates, and contain colonized assemblages representative of natural microbial community composition and productivity [7, 11]. These substrates also collect planktonic and benthic microorganisms, including types associated with hard and soft substrates, and thus a composite community is collected [32].

We examined the interdependency of microbial components by comparing inhibitor responses of bacterial abundance and productivity, ciliate and heterotrophic flagellate abundance, and phototrophic productivity and biomass. We hypothesized that responses consistent with microbial loop dynamics would be most prevalent in the summer (at the peak of the phytoplankton bloom), least pronounced in the

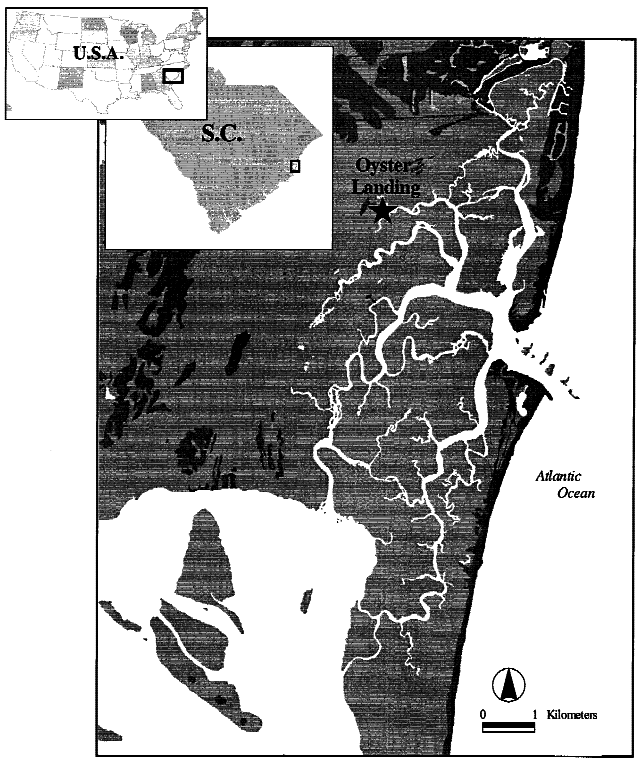

Fig. 1. Map of the study site, Oyster Landing at North Inlet, SC.

fall (postbloom), and intermediate in the spring (a period of transition from diatom- to flagellate-dominated phototrophic communities). Based on the hypothesis, we expected that, in the summer, bacterial activity would be regulated by positive feedback from microzooplanktonic regenerated nutrients, and therefore inhibition of grazing (by cycloheximide addition) would lead to decreased bacterial productivity and inhibition of phototrophic activity (DCMU) would have no effect on bacterial properties. In the fall, we expected responses consistent with the role of grazers in reducing bacterial biomass (e.g., cycloheximide addition should increase bacterial growth and productivity) and the predominant influence of "new" nutrients in regulating microbial population growth (e.g., inhibitor effects should reflect competition for limiting resources, such as stimulation of phototrophic activity by prokaryotic metabolic inhibitors and stimulation of bacterial activity by DCMU).

Methods

Artificial substrates (polyurethane foam cubes; 4 × 5 × 6 cm) were used to collect natural estuarine microbial communities from the Oyster Landing site of North Inlet, a NOAA National Estuarine Research Reserve and sanctuary site near Georgetown, SC (Fig. 1). The effects of metabolic inhibitors on the colonized community were then examined by incubating the collected substrates in aquaria and measuring parameters of microbial community structure and function. The experiment was conducted three times. Substrates were initially deployed on 14 May, 13 July and 15 October 1996, which represented periods during the upslope, peak, and downslope, respectively; of the annual phytoplankton bloom in North Inlet [25]. For example, at Oyster Landing in 1996, mean chlorophyll-*a* concentrations measured bi-hourly over two tidal cycles varied from 7.8 ± 3.3 in mid-May to 17.9 ± 9.7 in mid-July to 8.8 ± 4.3 and 7.9 ± 2.5 in early and late October, respectively (data from the NOAA North Inlet/Winyah Bay National Estuarine Research Reserve monitoring program).

Substrates (60 total) were attached to bricks with string and positioned just below the water surface at low tide. A 7-day colonization period was used in the present study, based on results from a preliminary experiment at the Oyster Landing site indicating that the number of microbial taxa collected on the substrates did not significantly increase with longer exposure (M. DeLorenzo, unpublished data). The composition of taxa retrieved was also similar to that collected by water column sampling; however, a greater proportion of the benthic microbial community was collected with the substrates than with water column sampling (A. Lewitus, personal communication). Substrates were collected at low tide by holding a reclosable plastic bag beneath the water surface, and sliding detached substrates into the bag. The bags were sealed, placed in a cooler, and transported immediately to the Baruch Marine Laboratory (adjacent to the study site).

In the laboratory, 5 substrates were placed in each of 12 glass aquaria containing 4 L of 0.45 μm filtered water from the sampling site. Temperature was adjusted to simulate the ambient condition at Oyster Landing (24°C in May, 26°C in July, and 17°C in October), and room lighting was set at a light:dark cycle simulating ambient conditions. Three replicate aquaria were used per treatment. The treatments included a control, a photosynthesis inhibitor (DCMU, 20 μM final concentration), a prokaryotic inhibitor (antibiotic mixture of 25 mg/L penicillin, 40 mg/L streptomycin, and 80 mg/L neomycin), and a eukaryotic inhibitor (cycloheximide, 50 mM final concentration). DCMU competes with quinone for the quinone-binding site in photosystem II, thus blocking photosynthetic electron transfer during noncyclic photophosphorylation [23]. The antibiotics inhibit murein biosynthesis in the prokaryotic cell wall [31]. Cycloheximide inhibits eukaryotic protein synthesis by blocking the peptidyl transferase of 80S eukaryotic ribosomes but not that of 70S prokaryotic ribosomes [23].

Because metabolic inhibitors do not affect all members of targeted communities equally, and can directly affect some components of nontargeted groups, quantitative interpretations of trophic responses are strictly limited [28, 33]. However, when attention is paid to time-course patterns, inhibitor effects can lend valuable qualitative information to interpreting trophic interactions as, for example, used here for comparing seasonal differences. The inhibitor concentrations used in this study were chosen based on previous demonstrations of effectiveness and specificity, including microscopic verification using North Inlet estuarine communities [24–26,42].

The aquaria were sampled before inhibitor addition ($T = 0$), and 8, 16, 24, 48, and 72 h after addition (except that a 16 h sampling time was not included in the May experiment). The samples were taken by randomly removing one substrate from each container at each time period and gently squeezing the contents into a sterile sample cup. Each substrate yielded approximately 60 mL. The samples were homogenized by gently stirring the contents, and aliquots were removed for microbial community analyses. Microbial community metrics included primary productivity (mg C assimilated/L/h), heterotrophic bacterial productivity (mol thymidine incorporated/L/h), chlorophyll *a* concentration (μg/L), phototrophic community composition (by abundance or biovolume), and bacterial, ciliate, or heterotrophic flagellate abundance (cells/mL). Salinity (ppt), temperature (°C), and dissolved oxygen (mg/L) were also measured at each sampling time.

Primary Productivity

A 5 mL sample was spiked with 1 μCi/ml $NaH^{14}CO_3$ (final concentration) and placed in an incubator set at *in situ* light and temperature regimes [27]. After 24 h, samples were filtered through 0.45 μm cellulose nitrate membrane filters, which were then rinsed with 0.2 μm filtered seawater. Filters were placed in scintillation vials and 1 mL of 10% HCl (v/v) was added. Samples were allowed to fume overnight in the dark. Scintillation fluid was added the next day, and after a 4-h stabilization period, radioactivity was measured as disintegrations per minute (DPM) using a Wallac liquid scintillation counter.

Heterotrophic Bacterial Productivity

Heterotrophic bacterial productivity was measured based on incorporation of tritiated thymidine [3, 15]. Samples (5 mL) were dosed with 20 nM/L (final concentration) [^{3}H] methyl-thymidine and incubated at *in situ* temperature in the dark for 30 min. The incubation was stopped by adding formalin, followed by 1 mL of unlabeled thymidine. Samples were immersed in icewater for 1 min. Five mL of ice-cold 10% trichloroacetic acid (TCA) was then added, and the samples were extracted on ice for 10 min. Samples were then filtered and rinsed 3 times with 1-mL aliquots of ice-cold 5% TCA, followed by five 1-mL aliquots of ice-cold 80% ethanol. Filters were then placed in scintillation vials, and 1 mL of ethyl acetate added. After 30 min, scintillation fluid was added and DPM determined.

Chlorophyll a

Chlorophyll *a* was used to estimate phototrophic biomass using a fluorometric method adapted from Glover and Morris [16]. Samples (3 mL) were filtered onto glass fiber filters (Whatman Type GF/F), the filters placed in vials with 1 mL of a saturated $MgCO_3$ solution, and the vials frozen at −20°C until extraction. For chlorophyll extraction, 9 mL of 100% acetone was added (90% acetone final concentration), and the vials were shaken, refrigerated overnight, shaken the next day, and refrigerated overnight again (a total extraction period of 48 h). On the following day, the samples were brought to room temperature in the dark, and fluorescence was measured using a Sequoia-Turner Model 450 fluorometer (correction for phaeophytin is not needed with this method).

Phototrophic Community Composition

Phototrophic community composition was determined by fixing 5-mL samples with 2% buffered formalin (final concentration). Samples were stored in the dark at 4°C until analysis. Phototrophic and heterotrophic flagellates were differentiated based on autofluorescence, using epifluorescence microscopy. Phototrophs were identified microscopically (500×) to genus, and the cell abundance, shapes, and dimensions recorded. Phototrophic biovolume (μm^3) was estimated assuming simple geometric shapes, following Wetzel and Likens [46].

Bacterial Abundance

Bacterial abundance (cells/mL) was determined by epifluorescent microscopic analysis of acridine orange-stained samples [20], modified from [18]. Chemical dispersant (sodium pyrophosphate, 0.25 mL) was added to the formalin-preserved sample (above), and the resulting mixture was sonicated briefly. The sample was then diluted with 0.2 μm filtered sea water, stained with the fluorochrome, acridine orange (100 mg/L final concentration), for 3 min, filtered onto a Poretics PCTE 0.2 μm black membrane filter, and mounted on a slide. Bacterial abundance was estimated under blue light excitation (470–490 nm) at 1,250×. Ten random grids were counted (ca. 400 cells total).

Ciliate and Heterotrophic Flagellate Abundance

Ciliate and heterotrophic flagellate abundances (cells/mL) were determined from the 24, 48, and 72 h formalin-preserved samples (above). The sample was diluted with 0.2 μm-filtered sea water, placed on a Sedgewick-Rafter slide (1 mL volume), and 10 random grids were counted at a total magnification of 500×. At least 200 organisms were counted in each sample. Ciliates and heterotrophic flagellates were sorted into size classes of > 20 μm and < 20 μm, but taxonomy was not determined.

Statistical Analysis

For each experimental endpoint (chlorophyll *a,* biovolume, etc.), analysis of variance (ANOVA) was used to test for significant difference among metabolic inhibitor treatments. Where data sets were nonparametric, a $\log_{10}(x+1)$ transformation was applied and ANOVA was repeated. Where ANOVA revealed a significant difference among treatments ($p<0.05$), Dunnett's procedure for multiple comparisons was used to determine which treatments differed significantly from the control [48].

Table 1. Microbial community measurements from the control substrates at time = 0 during each experiment (average of the three replicates ± standard deviation)

Variable	May	July	October
Chlorophyll *a* (mg/L)	0.81 ± 0.99	2.5 ± 0.3	1.3 ± 0.1
Phototrophic carbon assimilation (mgC/L/h)	226 ± 28	463 ± 54	689 ± 23
Phototrophic biovolume ($\mu m^3 \times 10^6$)	1.1 ± 0.11	2.51 ± 0.24	1.51 ± 0.11
Dissolved oxygen (mg/L)	3.53 ± 0.23	4.33 ± 0.12	3.90 ± 0.05
Bacterial abundance (cells/mL $\times 10^5$)	1.83 ± 0.13	2.52 ± 0.05	1.78 ± 0.06
Heterotrophic bacterial thymidine incorporation (nM/h)	0.11 ± 0.01	0.22 ± 0.01	0.27 ± 0.01
Ciliate abundance (<20 μm) (cells/mL)	364 ± 55	504 ± 60	420 ± 46
Flagellate abundance (<20 μm) (cells/mL)	368 ± 66	376 ± 46	198 ± 28
Ciliate abundance (>20 μm) (cells/mL)	220 ± 19	237 ± 12	279 ± 5
Flagellate abundance (>20 μm) (cells/mL)	265 ± 46	226 ± 17	241 ± 30

Results

Microbial Community at North Inlet

Seasonal trends in microbial community structure were observed. Chlorophyll *a* concentration, phototrophic biovolume, and bacterial abundance peaked in July (Table 1). Primary productivity and heterotrophic bacterial productivity rates were 2–3 times higher in July and October than in May. When normalized to phototrophic biomass (chlorophyll *a* or phototrophic biovolume), photosynthetic rates were 2–3 times higher in October than on the other dates, and bacterial productivity/cell was ca. 2 times higher in October. Ciliates and heterotrophic flagellates in the <20 μm size class were the most abundant protozoan groups in May, present in approximately equal densities. In July and October, small ciliates were the dominant protozoan group, being 25–53% more abundant than small flagellates. Large ciliate and flagellate abundance remained relatively constant regardless of sampling season (Table 1).

Phytoplankton community composition at North Inlet also changed with sampling period. In May and October, diatoms dominated total cell abundance (47.4% and 76%, respectively; Fig. 2A) and biovolume (>85%; Fig. 2B). Phototrophic flagellates peaked in relative abundance and biovolume in July, when their contribution to total phototrophic community biovolume was 30%, similar to that of diatoms (39%). The relative contribution of cyanobacteria also peaked in July, when they were twice as abundant as diatoms or phototrophic flagellates, and equivalent in biovolume to flagellates. In May, the most abundant diatom genera were *Nitzschia, Asterionella,* and *Navicula* (data not shown). The most abundant diatoms in July were *Melosira, Cymbella, Cylindrotheca,* and *Nitzschia.* In October, the most abundant diatoms were *Asterionella, Melosira, Coscinodiscus,* and *Amphiprora.*

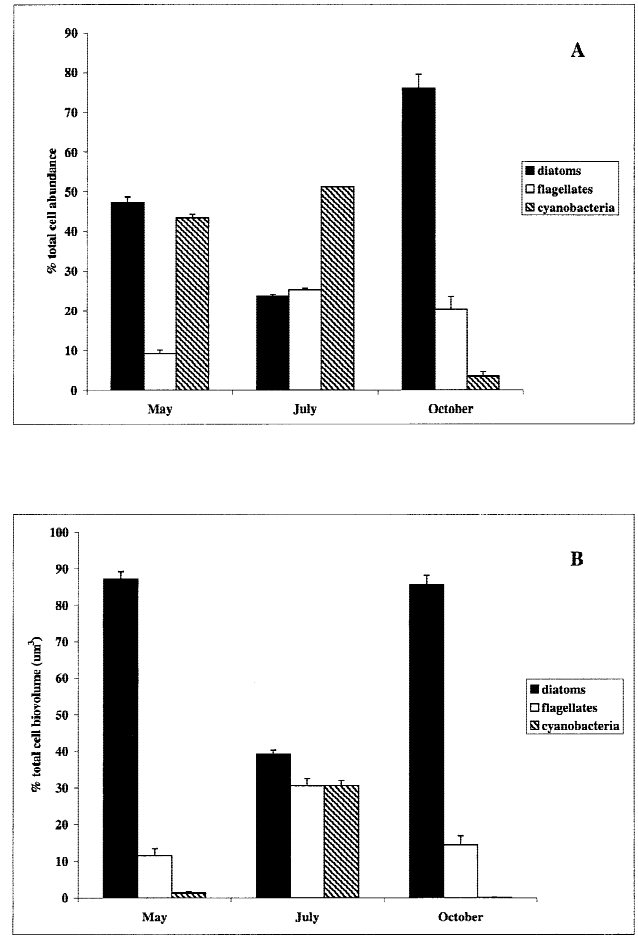

Fig. 2. Phototrophic community composition in terms of (A) cell abundance and (B) biovolume during May, July, and October. The proportion of diatoms, flagellates and cyanobacteria is expressed as percentage of the total.

Photosynthesis Inhibitor Treatment Effects

As expected, DCMU significantly reduced chlorophyll *a* concentration, phototrophic carbon assimilation, and pho-

Table 2. Summary of results using metabolic inhibitors[a]

Variable	Inhibitor	May exposure time (h)				July exposure time (h)					October exposure time (h)				
		8	24	48	72	8	16	24	48	72	8	16	24	48	72
Chlorophyll *a*	DCMU	−	−	−	−	0	−	−	−	−	−	−	−	−	−
	Antibiotics	+	−	0	0	0	0	0	0	−	−	0	0	+	+
	Cycloheximide	0	−	−	−	0	−	−	−	−	−	−	−	−	−
Phototrophic carbon assimilation	DCMU	0	−	−	0	0	0	−	−	−	−	−	−	−	−
	Antibiotics	0	0	0	0	0	0	−	0	0	0	0	0	+	+
	Cycloheximide	0	−	−	−	0	−	−	−	−	−	−	−	−	−
Phototrophic biovolume	DCMU	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Antibiotics	0	−	−	−	0	−	−	−	−	−	0	0	0	+
	Cycloheximide	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Dissolved oxygen	DCMU	0	−	−	−	0	−	−	−	−	−	0	−	−	−
	Antibiotics	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Cycloheximide	0	0	0	0	0	0	0	0	0	−	0	0	−	−
Bacterial abundance	DCMU	0	0	−	−	0	0	0	0	0	0	0	+	+	+
	Antibiotics	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Cycloheximide	0	−	−	0	0	0	0	0	0	0	+	+	+	+
Heterotrophic bacterial thymidine incorporation	DCMU	0	0	0	−	0	0	0	0	0	0	+	+	+	0
	Antibiotics	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Cycloheximide	0	0	0	−	0	0	0	−	−	+	+	+	+	0
Ciliate abundance (<20 μm)	DCMU		0	0	0			0	0	0			0	0	0
	Antibiotics	nd	0	0	0	nd	nd	0	0	0	nd	nd	−	−	−
	Cycloheximide		−	−	−			−	−	−			−	−	−
Flagellate abundance (<20 μm)	DCMU		0	0	0			0	0	0			0	0	0
	Antibiotics	nd	0	0	0	nd	nd	0	−	0	nd	nd	0	0	0
	Cycloheximide		−	−	−			−	−	−			−	−	−
Ciliate abundance (<20 μm)	DCMU		0	0	0			+	0	+			−	0	0
	Antibiotics	nd	0	0	0	nd	nd	0	0	0	nd	nd	0	0	0
	Cycloheximide		−	−	−			−	−	−			−	−	−
Flagellate abundance (<20 μm)	DCMU		0	+	0			0	0	0			0	0	+
	Antibiotics	nd	0	+	0	nd	nd	0	0	0	nd	nd	+	+	+
	Cycloheximide		−	−	−			−	−	−			−	−	0

[a] DCMU = photosynthesis inhibitor, Antibiotics = prokaryotic inhibitor, Cycloheximide = eukaryotic inhibitor). 0 = not statistically different from control, (+) = statistically greater than control, (−) = statistically less than control. (nd) = not determined.

totrophic biovolume over the course of each experiment (Table 2, Fig. 3). The DCMU inhibitory effect was observed at 8 h, except in July, when significant reductions in chlorophyll *a* and carbon uptake were not observed until the 16-h sampling time. DCMU noticeably decreased the abundance of most algal taxa relative to controls; however, the abundance of some diatoms (*Amphiprora, Coscinodiscus, Diploneis* in May; *Gomphonema* in July; *Cymbella, Diploneis, Gomphonema* in October) remained unaffected (data not shown).

Dissolved oxygen content was significantly reduced by DCMU addition in each experiment (Fig. 4). The degree of response ranged from a 2- to a 6-fold reduction compared to control values, depending on season. There was no recovery in dissolved oxygen content in the DCMU treatment after 72 h (data not shown).

Bacterial abundance in the DCMU treatment was signifi-

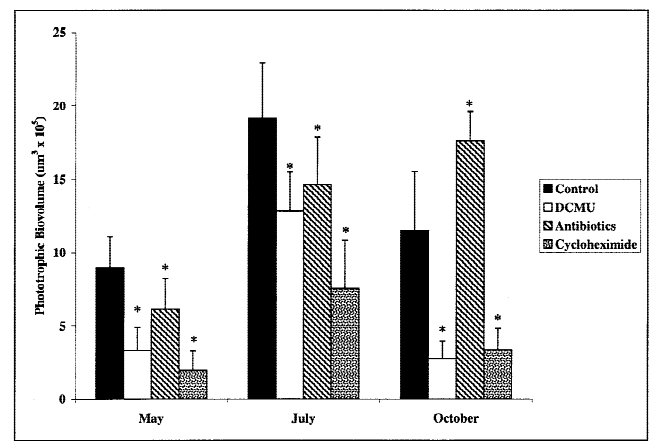

Fig. 3. Average phototrophic biovolume ($\mu m^3 \times 10^5$) measured in each treatment after 72 h during each experiment. Error bars indicate standard deviation. Asterisks indicate which treatments were significantly different from the control.

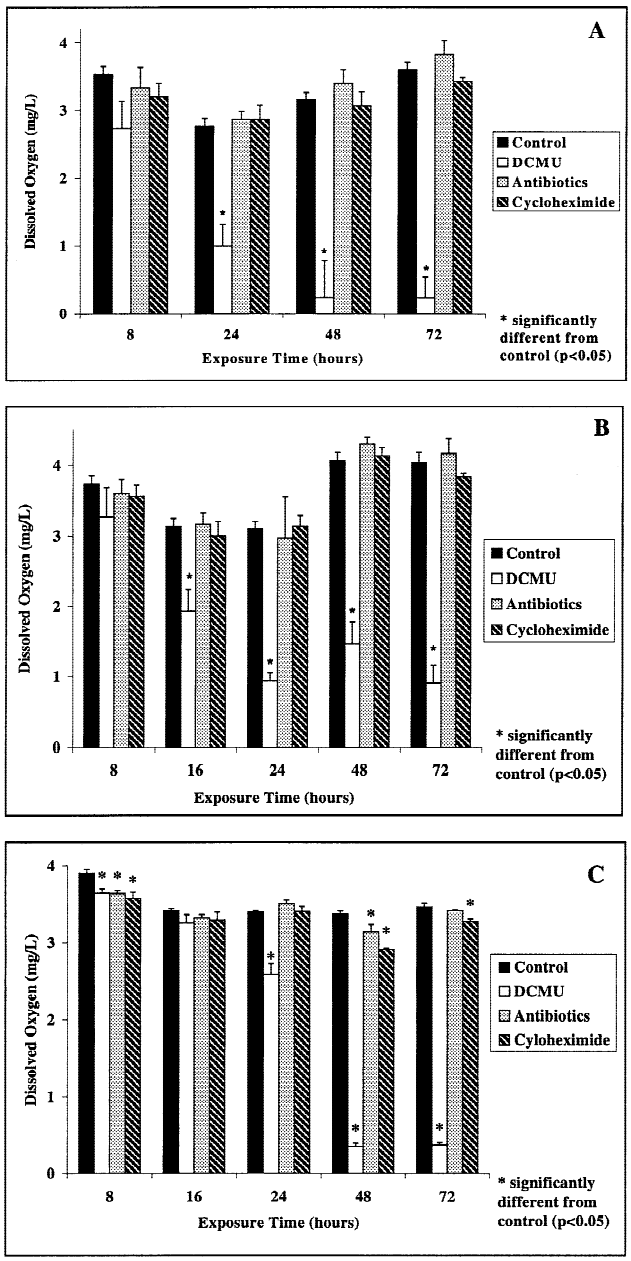

Fig. 4. Effects of metabolic inhibitors on dissolved oxygen content (mg/L) at each time point. (A) May experiment, (B) July experiment, (C) October experiment. Error bars indicate standard deviation. Asterisks indicate which treatments were significantly different from the control.

cantly less than controls after 48 and 72 h in the May experiment, was not significantly different from controls in the July experiment, and was significantly higher in the October experiment from 24 to 72 h (Table 2, Fig. 5). Heterotrophic bacterial productivity after DCMU addition followed a similar trend; significantly lower than controls after 72 h in the May experiment, no significant difference from controls in

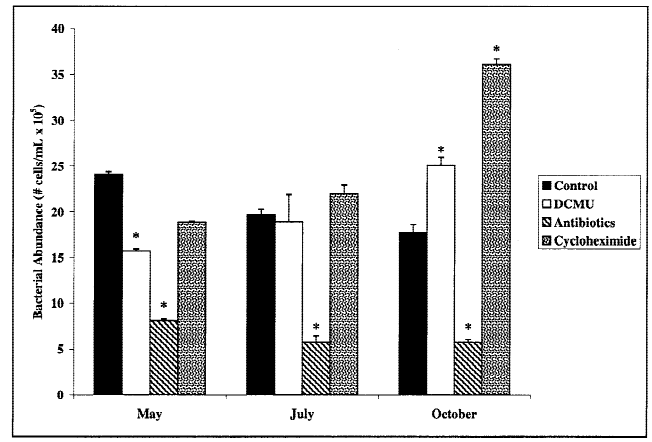

Fig. 5. Average bacterial abundance (cells/mL $\times 10^5$) measured in each treatment after 72 h during each experiment. Error bars indicate standard deviation. Asterisks indicate which treatments were significantly different from the control.

July, and significantly greater from 16 to 48 h in the October experiment (Table 2).

Heterotrophic ciliate and flagellate abundance was only measured at the 24, 48, and 72 h time points of each experiment (Table 2). There was no significant effect of DCMU on small (<20 μm) ciliate or flagellate abundance in any of the experiments, but the abundance of larger ciliates was significantly increased by DCMU addition in July (24, 72 h) and decreased in October (24 h). The abundance of larger flagellates (>20 μm) was also affected by DCMU, with higher numbers observed in May (48 h) and October (72 h).

Prokaryotic Inhibitor Treatment Effects

Following prokaryotic inhibitor addition, bacterial abundance and productivity were significantly less than in the controls in all samples (Table 2, Fig. 5). The inhibitory effects were observed at all sampling times and increased over incubation time. For example, after prokaryotic inhibitor addition, bacterial abundance was reduced by 40.4% to 79.0% at 8 h (data not shown), and 66.7% to 70.7% at 72 h (Fig. 5), while bacterial productivity was reduced by 67.0% to 68.6% at 8 h, and 67% to 82.8% at 72 h (data not shown).

Phototrophic responses to prokaryotic inhibitor addition varied with season (Table 2, Fig. 3). Although significantly higher chlorophyll *a* concentrations were observed in the prokaryotic inhibitor treatment at the 8 h sampling time in May, all other effects of this treatment on phototrophic properties in May and July were inhibitory. The most prevalent response to prokaryotic inhibitor addition during these months was a reduction in phototrophic biovolume from 16 h or 24 h on (Table 2, Fig. 3). In contrast, during October,

prokaryotic inhibitor addition either did not affect (16 to 48 h) or increased (72 h) phototrophic biovolume during this same incubation period. Also in October, stimulatory effects of prokaryotic inhibitor treatment on chlorophyll *a* and primary productivity were observed at the 48 and 72 h sampling times (Table 2).

The most apparent effect of prokaryotic inhibitor treatment on heterotrophic eukaryotes occurred in October, when the abundance of small (< 20 μm) ciliates was significantly lower, and that of large (> 20 μm) flagellates higher, than control values (Table 2). In May and July, the abundances of these groups were generally not affected by prokaryotic inhibitor addition, with the exception of a stimulatory effect on large flagellates after 48 h in May. The only significant effect of prokaryotic inhibitor treatment on the abundances of the other heterotrophic protozoan groups was observed at 48 h in July, when small flagellate abundance was lower in treated samples than in controls.

Eukaryotic Inhibitor Treatment Effects

The eukaryotic inhibitor cycloheximide significantly reduced chlorophyll *a* content, phototrophic carbon assimilation, and phototrophic biovolume compared to controls in all experiments (Table 2, Fig. 3). The reductions began 8–16 h after exposure and lasted for the 72-h experimental duration. In general, the eukaryotic inhibitor cycloheximide reduced the phototrophic variables to the same degree as the photosynthesis inhibitor (DCMU); however, cycloheximide reduced primary productivity to a greater degree than DCMU during the October experiment (approximately 6-fold after 72 h). Dissolved oxygen content was not significantly affected in the cycloheximide treatment in the May and July experiments, but was significantly reduced relative to control values during the October experiment (Table 2, Fig. 4).

Cycloheximide addition decreased bacterial abundance during May (24 and 48 h), did not affect abundance in July, and increased abundance during October (16–72 h) (Table 2, Fig. 5). Seasonally contrasting effects of this eukaryotic growth inhibitor were also observed on bacterial productivity. Following cycloheximide addition, a reduction in the rate of thymidine incorporation was observed in May (72 h) and July (48 and 72 h), but an increased rate was observed during October (8–48 h).

Cycloheximide significantly reduced the numbers of heterotrophic ciliates and flagellates of both size classes throughout all experiments (Table 2). Ciliate and flagellate abundance in the cycloheximide treatments was ca. 75% less than in the controls after 72 h (data not shown).

Discussion

The effects of metabolically inhibiting one component of a microbial community can cascade in several alternative directions, depending on which groups interact, their dependency on the interaction, and the nature of the interaction (e.g., prey/predator, competition for an exogenous resource, nutrient excretion/resource use). Despite this complexity, the net effects of metabolic inhibitors on the population growth and activity of microbial producers and consumers can yield valuable clues to understanding microbial food web function. Based on previous research from the Oyster Landing study site [25, 26], we hypothesized that metabolic inhibitor addition to microbial communities colonizing substrates would result in trophic responses consistent with "microbial loop" dynamics at the peak of the phytoplankton bloom (e.g., July) but not in the postbloom period (October). The responses in May were expected to be intermediary because this period is depicted as transitional, i.e., the upslope of the bloom when phototrophic nanoplankton (flagellates and small diatoms) are increasing in abundance. Consistent with the hypothesis, the results from inhibitor use indicated contrasting responses in July and October that suggest an interdependency (coupling) between bacteria and eukaryotes (phototrophic and heterotrophic) in July (and to some extent in May), and a competitive interaction between bacteria and phototrophs in October.

One of the key characteristics of the microbial loop is that microzooplanktonic grazing is an important regulatory factor in bacterial productivity and growth, and that this "top-down" control is manifested by negative (bacterivory) and positive (nutrient regeneration) influences on bacterial population growth. However, bacterial activity can also be tightly coupled to phototrophic exudation [43]. Therefore, the net effect of photosynthesis inhibitors (e.g., DCMU) or eukaryotic metabolism inhibitors (e.g., cycloheximide) on bacterial properties in microbial loop communities may vary as a function of the relative importance of grazing vs phototrophic production on bacterial population growth and productivity. DCMU or cycloheximide addition during May caused decreases in bacterial abundance and productivity, suggesting the dependency of bacteria on resources produced by phototrophs and/or microzooplankton. In July, the only response of bacteria to these inhibitors was a re-

duction in bacterial productivity after cycloheximide addition. These results suggest the relative importance of microzooplanktonic nutrient regeneration (positive feedback) in controlling bacterial growth and productivity during July. First, this is evident because cycloheximide (reduced substrate production by heterotrophs and phototrophs), but not DCMU (reduced production by phototrophs), caused a decrease in bacterial productivity. Secondly, cycloheximide did not cause an increase in bacterial abundance, suggesting that the reduction in bacterivory was offset by the reduced substrate availability (from nutrient regeneration) for growth.

In contrast to the observed patterns in May and July, DCMU and cycloheximide stimulated bacterial abundance and productivity in the October experiment. Therefore, during this latter period, bacterial dynamics apparently were not closely regulated by substrate production by phototrophs or microzooplankton, but rather competition for resources (with phototrophs) and bacterivory were most likely the important factors controlling bacterial growth and production. The competition between bacteria and phototrophs for nutrients during October, but not July, reflects the change in inorganic nitrogen availability over this period (e.g., NH_4 concentrations peak during the summer when N does not limit phytoplankton population growth, but decline and limit growth in the fall/winter) [25]. Because the DOC:DON ratio is characteristically high at this site (> 20; [26]), bacterial uptake of NH_4 may be expected [1, 8, 14, 21].

Consistent with the seasonal patterns in DCMU and cycloheximide responses, the effects of prokaryotic inhibitor treatment on phototrophic microbial properties also indicated a transition toward competitive interactions between bacteria and phototrophs in October. Whereas prokaryotic inhibitor addition led to reductions in chlorophyll *a,* primary productivity, and phototrophic biovolume in July, it led to increases in these properties in October. The July patterns may be explained as a shift in grazing from bacteria to phototrophs, or may simply reflect a reduction in cyanobacterial abundance.

In October, the abundance of small (< 20 μm) ciliates was decreased by prokaryotic inhibitor addition, and that of large (> 20 μm) heterotrophic flagellates increased, while the other two heterotrophic protozoan groups were unaffected by prokaryotic inhibitor treatment. These results reflect the role of protozoa as grazers, but not nutrient regenerators, at this time. The data suggest that small ciliates were the primary bacterivores and larger flagellates the predominant microbial algivores (i.e., these flagellates are responding to the increased abundance of phototrophic prey that resulted from the relief of bacterial competition for nutrients). However, it is interesting that DCMU did not decrease the abundance of the larger flagellates, which may indicate a capability for switching between bacterivory and algivory in this group. Other studies have demonstrated the predominant contribution of small ciliates to bacterivory in certain environments [9], including a Georgia salt marsh estuary [38]. In July, small flagellate abundance was reduced in the prokaryotic inhibitor treatment, while small ciliate abundance was not affected, suggesting that small flagellates may be the dominant bacterial consumer in the summer.

DCMU addition reduced dissolved oxygen concentration in all experiments, whereas cycloheximide decreased dissolved oxygen only during the October experiment. The decrease in dissolved oxygen was most likely a result of inhibited phototrophic activity. Cycloheximide may not have reduced dissolved oxygen in the summer because of the presence or stimulation of small, coccoid cyanobacteria (*Synechococcus*) that made up a large portion of the photosynthetic community during that time. These prokaryotes would be inhibited in the DCMU treatment, but not in the cycloheximide treatment [25, 42]. *Synechococcus* abundance was stimulated by the inhibition of heterotrophic ciliates and flagellates in the cycloheximide treatment (data not shown), suggesting that the cyanobacteria were actively grazed by these groups. In October, when diatoms became very abundant and relatively fewer *Synechococcus* were present, cycloheximide reduced dissolved oxygen concentration.

The trophic responses described in the present study are consistent with findings by Lewitus et al. [25] that demonstrated distinct seasonal differences in the factors controlling phytoplankton population growth at North Inlet estuary. The authors concluded that microzooplanktonic grazing was the dominant factor regulating growth in the summer, and nutrient supply limited phytoplankton growth in the winter. As in the present study, the authors found phototrophic growth to be depressed in treatments inhibiting bacteria during the summer. Thus, despite the abundance of NH_4^+ in the summer, nitrogen availability to phytoplankton was, to some extent, mediated by bacterial activity. Bacterivorous protozoa play a pivotal role in nutrient cycling by consuming nutrient-rich bacterial biomass, and by releasing phytoplankton from competition with bacteria for nutrients.

Our results concur with those of Wiebe et al. [47], who also found seasonal differences in microbial food web regulation in a mesotrophic, prealpine lake. In that study, bacterial or protozoan uptake of DOM produced from phytoplankton during winter and early spring was low, permitting

greater energy flow to zooplankton and benthic animals. During the spring bloom, however, more than 50% of primary production was channeled through the microbial loop to higher trophic levels. In late spring, summer, and fall, the microbial loop continued to dominate energy flux and organic carbon utilization. Thus, the nature of the trophic transfer, linear or feedback loop, may be dependent on temperature.

The results of Lewitus et al. [25] and the present study indicated the prevalence of microbial loop dynamics in North Inlet during the spring/summer, when high temperature, relatively high ammonia levels, and an abundance of small phytoplankton lead to energy fluxes in the form of a bacteria–nanophytoplankton–protozoa microbial food web. Because North Inlet is a tidally dominated estuary, nutrient inputs come in pulses, and therefore microbial regeneration of nutrients may provide an important resource in support of primary and secondary production [5, 41]. Moloney et al. [29] found that recycling through the microbial loop provided significantly more of the nutrients needed to sustain phytoplankton and heterotrophic flagellates than did mesozooplankton recycling in an estuarine ecosystem subjected to nutrient pulses. The rapid nutrient turnover rates of the microbial loop may increase the transfer efficiency of materials and energy to higher trophic levels in estuaries dominated by tidal exchange processes, rather than terrigenous exchange [35], and where nutrient supply is relatively low and continually fluctuating.

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