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Response of microphytobenthic biomass to experimental nutrient enrichment and grazer exclusion at different land-derived nitrogen loads

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ABSTRACT: Effects of eutrophication on the relative importance of nutrients and macroherbivores as controls of microphytobenthic standing crop were examined in estuaries with different nitrogen loading rates: Sage Lot Pond (14 kg ha^{-1} yr^{-1}), Green Pond (178 kg ha^{-1} yr^{-1}), and Childs River (601 kg ha^{-1} yr^{-1}). We selected 5 sites with similar salinity ranges on shallow-water, sandy substrates per estuary. In year-round experiments, we fertilized sediments with nitrogen + phosphorus to examine nutrient limitation. We conducted exclusion experiments to determine the significance of macroherbivores as controls of microphytobenthic biomass and examined possible interactions between nutrients and grazing in cages fertilized with nitrogen + phosphorus. Cages fertilized with nitrogen only were also included to determine if nitrogen availability was limiting. Nitrogen + phosphorus addition increased sediment chlorophyll *a* (chl *a*) content (herein used as a proxy for biomass) by a similar magnitude across estuaries. Grazer exclusion also increased chl *a*, but to a different extent across estuaries: the magnitude of the response increased with increasing nitrogen loading rates. We found no interactions between nutrients and grazing. Strong chl *a* increases in response to nitrogen only addition indicated N limitation in Sage Lot Pond and Green Pond. In the highly eutrophic Childs River estuary we found virtually no response to nitrogen-only additions, suggesting the possibility of phosphorus limitation in this estuary.

KEY WORDS: Microphytobenthos · Biomass · Nutrients · Grazers · Eutrophication · Nitrogen load

INTRODUCTION

Microphytobenthos consists of unicellular algae (primarily diatoms, euglenoids, chlorophytes, dinoflagellates) and cyanobacteria that live on the bottom of aquatic environments (MacIntyre et al. 1996). In marine systems it occurs from the upper intertidal to continental shelf environments (Round 1971, Admiraal et al. 1982, Cahoon & Laws 1993, Cahoon et al. 1993), and is commonly dominated by pennate diatoms (Round 1971). Primary production by benthic microphytes can reach 50% or more of total primary production (Underwood & Kromkamp 1999) and surpasses that of phytoplankton in many shallow systems (Grøntved 1960, Hargrave 1969, Cahoon 1999, Underwood & Kromkamp 1999).

In recent decades there has been a drastic increase in anthropogenic nutrient input to coastal water bodies as a result of human population growth along the land–sea margin, frequently leading to eutrophication (GESAMP 1990, National Academy of Sciences 1994, Valiela 1995). In temperate coastal systems, eutrophication is driven by increasing nitrogen loads, as the availability of nitrogen tends to be limiting to primary production in these areas (Howarth 1988, Valiela 1995). Shifts in dominant flora (Duarte 1995, Valiela et al. 1997) and fauna (Heip et al. 1995) have resulted. For instance, along the NE coast of the United States and parts of Europe, algal blooms have caused overgrowth and shading of native seagrasses resulting in a widespread decline in seagrass meadows (Duarte 1995, Short & Burdick 1996, Valiela et al. 1997). The reliance

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of many commercial species on these habitats as feeding and nursery grounds has led to negative impacts on local fisheries (Curley et al. 1971, Valiela et al. 1992).

While the effects of eutrophication on phytoplankton and macrophytes have been subject to many studies in recent years, the effects on microphytobenthos have received less attention. Therefore, the effects of eutrophication on microphytobenthic biomass are still poorly understood. While growth of microphytobenthos is not considered nutrient-limited in fine sediments because of high organic matter degradation and remineralization (Admiraal et al. 1982, Underwood & Kromkamp 1999), it can be nutrient-limited on sandy substrates (Granéli & Sundbäck 1985, Sundbäck & Snoeijs 1991, but see Hillebrand & Kahlert 2002). Therefore, direct effects of eutrophication on biomass seem more likely in sandy sediments. In addition to nutrients, light availability (van Raalte et al. 1976, Kühl & Jørgensen 1994) and herbivores such as ciliates, nematodes, ostracods, copepods, amphipods, bivalves or gastropods can regulate microphytobenthic biomass (Fenchel & Kofoed 1976, Pace et al. 1979, Connor et al. 1982, Admiraal et al. 1983, Montagna et al. 1995, Hillebrand et al. 2000, Middelburg et al. 2000).

We were interested in the potential effects of estuarine eutrophication on the relative significance of nutrients and grazing as controls of microphytobenthic biomass. Because of their location at the land–sea margin, estuaries are particularly subject to increasing land-derived nitrogen loads, and hence eutrophication (Nixon et al. 1986). Changes in the nutrient regime may alter the relative balance between nutrients and grazers as controls of primary producers in these systems (Hauxwell et al. 1998). In this study we examine the relative importance of nutrients and macroherbivores as controls of microphytobenthic biomass in 3 estuaries subject to different nitrogen loads. We focus on macrofaunal consumers rather then meiofauna, as most previous studies have suggested that meiofauna consume at most 10% of the microphyte standing crop (Admiraal et al. 1983, McCormick 1991, Jönsson et al. 1993, Moens et al. 2002) and are quantitatively less-important consumers of microphytobenthic biomass than macrofauna (Middelburg et al. 2000).

We address the following questions: (1) Is microphytobenthos nutrient-limited? (2) If so, is nitrogen the limiting nutrient? (3) What is the relative significance of 'top-down' (grazers) versus 'bottom-up' (nutrients) controls on microphytobenthic standing crop? (4) Does the relative importance of top-down versus bottom-up effects vary between estuaries subject to different nitrogen loads?

MATERIALS AND METHODS

Study area. This study was carried out in the Waquoit Bay estuarine system (Fig. 1) of Cape Cod, Massachusetts, USA, from April 2001 to January 2002. Land-derived nitrogen, primarily in the form of nitrate and dissolved organic nitrogen, is transported to the Waquoit Bay estuarine system chiefly via groundwater flow (Valiela et al. 1997). Nitrogen loading rates to estuaries around Waquoit Bay range over almost 2 orders of magnitude (14 to 601 kg N ha^{-1} yr^{-1}), making them well suited for the study of anthropogenic eutrophication in estuarine ecosystems.

In a parallel study, we examined a potential correlation between microphytobenthic biomass and nitrogen loading rate across 6 estuaries in monthly samplings at 5 sites per estuary. The results were inconclusive as evidenced by a weak correlation and peripherally significant regression slope (R^2 = 0.57, p = 0.08) (Lever 2002). In the present study, we investigated the relationship between microphytobenthic standing crop and nitrogen loading rates by experimentally manipulating variables known to control microphytobenthic biomass, such as porewater nutrient concentrations and grazing by macroherbivores. We compared differences in the significance of nutrient availability and grazing in the context of estuarine nitrogen loading rates. As study sites we chose 3 estuaries with low, medium, and high loading rates: Sage Lot Pond (14 kg N ha^{-1} yr^{-1}), Green Pond (178 kg N ha^{-1} yr^{-1}) and Childs River (601 kg N ha^{-1} yr^{-1}).

Since our research focused on the significance of grazers and nutrients, we attempted to minimize dif-

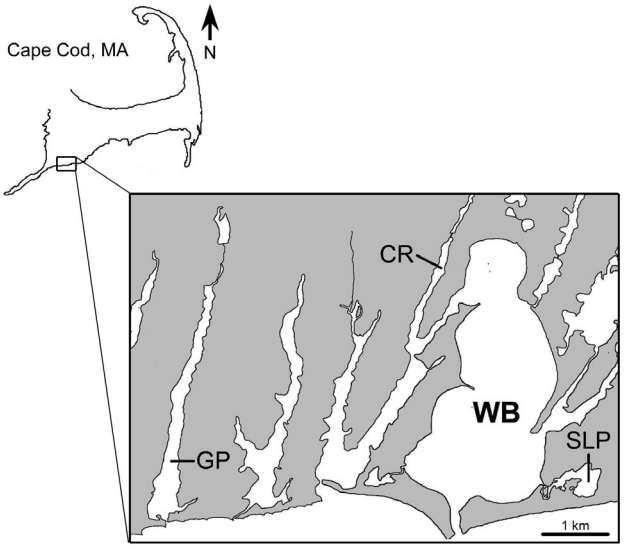

Fig. 1. Waquoit Bay and adjacent estuaries. WB: Waquoit Bay, SLP: Sage Lot Pond, GP: Green Pond, CR: Childs River

ferences in other environmental variables that could contribute to differences in microphytobenthic biomass between sites and estuaries. In each of the 3 estuaries we chose 5 sampling stations with similar salinities (23.5 to 31.0) and depths (0.02 to 0.34 m below mean low tide level). Stations were located on bare strands with similar current regimes, as indicated by primarily (77 to 93% dry mass) medium (Ø = 0.25 to 0.5 mm) to coarse (Ø = 0.5 to 1 mm) sands and similar porosities (16 to 23%). All 3 estuaries were microtidal, with mean tidal amplitudes of ~0.3 m. Microphytobenthic communities were typically dominated by diatoms; 2 exceptions occurred in Sage Lot Pond: from June to August 2001 filamentous cyanobacteria became dominant and formed a mat in all 3 cages at 1 station; in December 2001 filamentous and coccoid cyanobacteria became dominant coincident with a period of extreme neap tides during which this site was exposed for most of the tidal cycle.

Grazer exclusion and fertilization experiment. To determine the significance of consumption by macroherbivores, we built cages for grazer exclusion from transparent plastic containers (23 × 23 × 10 cm). The bottoms were removed, so the container could be pushed into the sediment. To ensure water-exchange and minimize sedimentation, we cut windows into the lids and sides of the containers. To exclude macroherbivores, each window was provided with a 4 mm polypropylene square-mesh. We chose this large mesh size because (1) the predominant macroherbivores were mud snails (*Ilyanassa obsoleta*) and grass shrimp (*Palaemonetes pugio*, *P. vulgaris*), none of which could typically enter through the mesh; (2) it allowed for better flushing than a 1 mm mesh size, that would exclude the smallest macrofauna; and (3) it minimized shading. Cages were pushed into the sediment to a depth of ~5 cm to prevent macroherbivores from entering by burrowing. Wooden dowels, each ~40 cm long, were attached to the corners of each cage, and driven into the sediment to anchor the cages. Cages were visited frequently (up to once a week) to remove algae and fouling communities. All macrograzers were removed from the cages at the onset of the experiment, as well as after each sampling or cleaning.

We fertilized sediments using diffusers made of perforated 1.5 ml microcentrifuge tubes, filled with time-release fertilizer pellets (Osmocote, Scotts Company). The bottom of each diffuser was tied to a thin wooden dowel (~0.4 cm thick, 30 cm long) and vertically pushed into the sediment with the lid pointing up, until the lid was ~1 cm below the sediment surface. The purpose of the dowels was to firmly hold the diffusers in place and prevent removal by physical or biological disturbance.

There were 2 nutrient addition treatments, NP and N, each using a different time-release fertilizer. Fertilizer for the NP treatment contained nitrogen (59.4% of mass as NO_3^-, 40.6% as NH_4^+) and phosphorus (as orthophosphate, PO_4) at an atomic ratio of ~6.9 N:1 P, applied at a dose of 60 g N and 8.7 g P m^{-2}. The NP treatment was used to test for general nutrient limitation. The fertilizer used in the N treatment contained only nitrogen. The N treatment served to specifically test for N limitation. Nitrogen in N treatments was in the form of urea and added at a dose of 196 g N m^{-2}. For optimal comparability, a fertilizer containing the same N species as the NP treatment would have been superior, however no such fertilizer was produced by the manufacturer. Nevertheless, since urea [$NO(NH_2)_2$] is hydrolyzed to ammonium within hours or a few days in sediments (Lomstein et al. 1989, Therkildsen et al. 1996), urea addition was analogous to enrichment with ammonium.

In the NP treatments, 9 diffusers were inserted at equal distances from each other in a 3 × 3 grid, whereas in N treatments 16 diffusers were inserted at equal distances from another in a 4 × 4 grid. The number of diffusers, and hence N dose per area, differed between NP and N treatments because of different release times of the 2 fertilizers. NP treatments were refertilized every 3 to 4 mo, N treatments only once after 7 mo. The first fertilization was carried out after the initial sampling on April 2 to 6, 2001.

At each sampling station there were 6 experimental units, each subjected to a different treatment: (1) control (C); (2) control cage (CX)—a cage without mesh, diffusers filled with autochthonous sediment rather than fertilizer; (3) N and P addition (NP); (4) exclusion cage (X); (5) exclusion cage with N and P addition (NPX); (6) exclusion cage with N addition (NX). We chose the NX treatment over N enrichment without a cage because of the possibility of grazers keeping up with enhanced growth rates. Higher consumption rates by grazers could potentially prevent biomass increases due to enrichment and lead to erroneous conclusions regarding N limitation. Following the same rationale, we included an NPX treatment in addition to NP and X treatments. The NPX treatment also allowed for examination of possible interactions between enrichment and grazer exclusion. Each experimental unit consisted of a 23 × 23 cm quadrat of sediment (equivalent to the area of a cage).

Sampling. To measure the response of microphytes to treatments, we measured sediment chlorophyll *a* contents in the experimental units. We collected sediment samples with small cores made of cut-off syringes (Ø = 0.95 cm, depth = 2.5 cm). We collected and analyzed sediment cores to a depth of 2.5 cm, even though the photic zone was likely to be less than 1 cm. Previ-

ous measurements had revealed significant amounts of chl *a* to a depth of 2.5 cm, even though sands were coherent and subject to little mechanical turnover. To account for spatial patchiness of microphytobenthic biomass we took 6 randomly spaced replicate cores at each experimental unit. Replicates were pooled and stored in 250 ml polyethylene bottles, that had previously been wrapped in aluminum foil to prevent photodegradation of chl *a*.

To determine whether experimental fertilizations were successful, we collected porewater samples for measurements of nitrate, ammonium and phosphate concentrations. Porewater was sampled using 'sippers' constructed of 10 ml syringes. Needles (with their sheaths) were inserted onto syringes and sealed at the base with glue (Marine Goop, Eclectic Products). Parallel rows of small holes were drilled at 1 mm intervals starting at the tip of the sheath over a 1 cm stretch down from the tip. To sample porewater, the tip of the sheath was pushed 1 cm into the sediment. Water was sucked into the syringe through the holes, which were small enough to prevent intrusion of sand grains. Each filled syringe contained a pooled sample of porewater from the upper 1 cm of sediment. To account for spatial heterogeneity in porewater nutrient concentrations, we collected 6 replicates at each experimental unit. Porewater samples were pooled and stored in 125 ml polyethylene bottles.

We sampled sites on average every 5 to 6 wk, and collected porewater before coring sediment for chl *a* sampling because of the more disruptive nature of the latter. While in the field, we kept sediment cores and porewater samples on ice. On arrival at the laboratory, we filtered porewater samples through 47 mm glass-fiber filters of 0.7 µm nominal pore size to remove fine sediment and organic matter. We froze all samples at –20°C.

Analyses. Porewater concentrations of phosphate (Strickland & Parsons 1972), ammonium (Holmes et al. 1999), and nitrate (QuikChem method for LACHAT autoanalyzer) were measured. Sediment chl *a* was extracted via a modification of the protocol of Riaux-Gobin & Klein (1993): to each 250 ml bottle containing sample, acetone was added to a final concentration of ~90%. Bottles were stored at 4°C for 24 h. To suspend and break cells, samples were repeatedly vortexed for 10 s followed by sonication for 30 s, the first time immediately after acetone addition, the second time after ~20 h. Directly before analysis, extracts were transferred to 50 ml Falcon tubes and centrifuged for 2 min at 3000 rpm. Chl *a* concentrations of supernatants were measured spectrophotometrically following the acidification method of Lorenzen (1967).

RESULTS

In the following sections we will first examine the effectiveness of the fertilizer treatments. Secondly, we will compare chl *a* values of different treatments and estuaries over the course of the study. We will conclude by examining potential relationships between mean chl *a* responses to treatments (treatment minus control) and regional nitrogen loading rates.

Effectiveness of treatments

NP treatments increased phosphate concentrations on average 2.8 to 6.8 times relative to other treatments (Table 1). Throughout the study, phosphate concentrations were higher in NP than other treatments (Fig. 2, top panels), with the exception of 1 sampling date in Childs River. The presence of cages did not influence porewater phosphate concentrations (Table 1).

Compared to unfertilized treatments, porewater ammonium concentrations increased on average by 3.8 to 7.0 and 20 to 43 times in the NP and N treatments, respectively (Table 1). Throughout the sampling period ammonium concentrations remained higher in NP and N treatments than in unfertilized sediments (Fig. 2, middle panels). Cages did not affect ammonium concentrations.

Table 1. Sediment porewater mean concentrations (µM) of orthophosphate (PO_4), ammonium (NH_4^+), and nitrate (NO_3^-) for controls (C), control cages (CX), exclusion cages (X), NP-enriched open plots (NP), NP-enriched cages (NPX), and N-enriched cages (NX) in Sage Lot Pond (SLP), Green Pond (GP), and Childs River (CR). Values are means ±1 SE. SE were calculated from means of all sampling dates after treatments were started

	PO_4			NH_4^+			NO_3^-		
	SLP	GP	CR	SLP	GP	CR	SLP	GP	CR
C	0.6 ± 0.1	0.5 ± 0.2	0.4 ± 0.2	10 ± 2	7 ± 2	5 ± 2	0.5 ± 0.0	2.7 ± 1.0	2.1 ± 0.7
CX	0.6 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	7 ± 1	6 ± 1	4 ± 2	0.5 ± 0.0	2.7 ± 1.2	2.1 ± 0.6
X	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	7 ± 1	8 ± 1	4 ± 1	0.8 ± 0.2	2.6 ± 1.0	2.2 ± 0.6
NP	2.7 ± 0.6	1.6 ± 0.5	1.4 ± 0.7	76 ± 47	35 ± 8	19 ± 7	7.8 ± 4.2	8.9 ± 4.3	8.9 ± 3.0
NPX	2.1 ± 0.5	1.9 ± 0.8	1.8 ± 0.8	59 ± 37	42 ± 12	25 ± 9	7.9 ± 5.0	9.7 ± 4.6	8.1 ± 2.8
NX	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	204 ± 75	160 ± 55	173 ± 89	0.6 ± 0.1	4.1 ± 1.2	2.3 ± 0.6

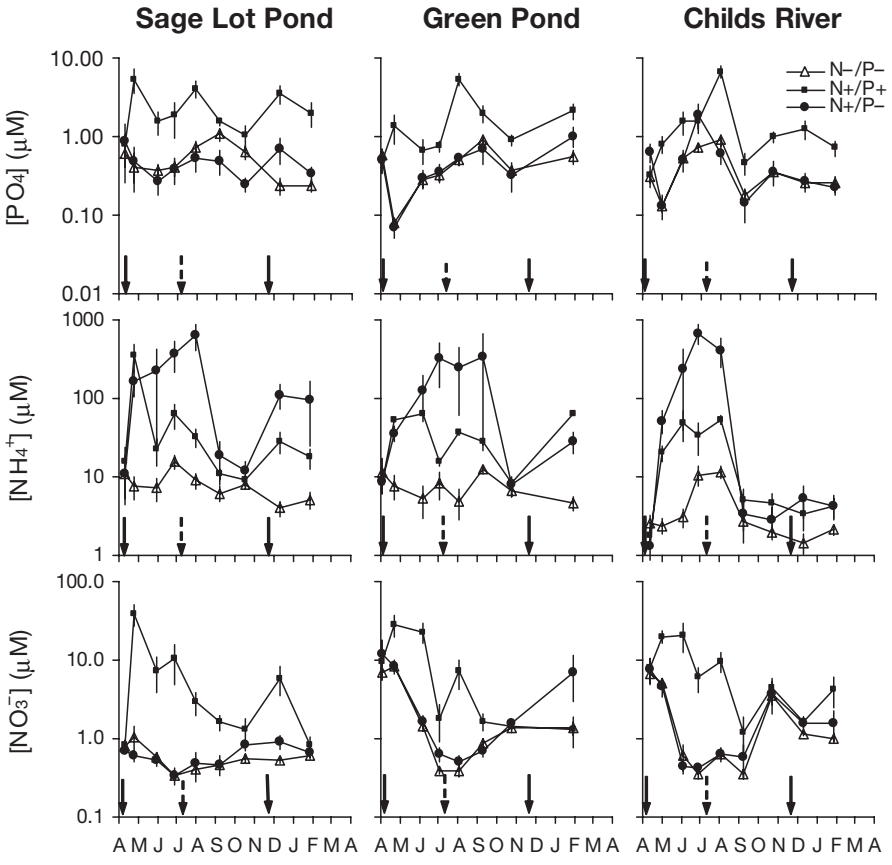

Fig. 2. Porewater concentrations of orthophosphate (PO_4), ammonium (NH_4^+), and nitrate (NO_3^-) throughout study period. N–/P–: unfertilized, comprises controls (C), control cages (CX) and exclusion cages (X); N+/P+: N + P added, comprises open plots (NP) and cages (NPX); N+/P–: N added, comprises only cages (NX). Arrows mark dates of fertilization, whereby solid arrows indicate dates when fertilizer was added to both NP and N enriched plots and dashed arrows dates when fertilizer was added only to NP enriched sediments. Data points are means ± SE. SEs obtained from the 5 sampling sites per estuary

Table 2. Mean concentrations of chlorophyll *a* in controls (C), control cages (CX), cages (X), NP enriched open plots (NP), NP enriched cages (NPX), and N enriched cages (NX) throughout study period. Horizontal bars indicate results of a Tukey's post-hoc test, whereby means underscored by same bar are not significantly different and means not underscored by same bar are significantly different ($p < 0.05$). Values are means ± 1 SE. SLP: Sage Lot Pond; Green Pond; CR: Childs River

Estuary	Chlorophyll *a* (mg m^{-2})					
SLP	CX	C	X	NP	NPX	NX
	61.0 ± 6.4	65.8 ± 5.5	74.0 ± 7.6	74.1 ± 6.6	86.5 ± 8.4	92.3 ± 12.8
GP	C	CX	NP	X	NPX	NX
	56.2 ± 6.8	61.6 ± 6.8	72.5 ± 8.1	73.8 ± 6.8	86.6 ± 12.9	97.8 ± 13.4
CR	CX	C	NP	X	NX	NPX
	98.3 ± 6.1	109.8 ± 6.1	122.6 ± 7.9	140.2 ± 6.3	144.6 ± 11.9	151.2 ± 6.7

Nitrate concentrations were elevated by NP treatments, but not by N treatments (Table 1). NP treatments had, on average, 3.4 to 15.8 times higher nitrate concentrations than unfertilized treatments. Compared to controls, the high mean nitrate concentration of NX treatments in Green Pond is due to a single high value in January 2002 (Fig. 2, bottom panels). Throughout the sampling period, nitrate concentrations were consistently higher in NP treatments than in all other treatments with the exception of October 2001 and January 2002 in Green Pond (Fig. 2). Cage presence did not affect nitrate concentrations.

We found no relationship between background porewater nutrient concentrations and nitrogen loading rates.

We tested whether treatments had a significant effect on chl *a* with a 2-factor (treatment × site) analysis of variance (ANOVA) randomized blocks design. Treatment and site had highly significant effects ($p < 0.001$) on chl *a* in all 3 estuaries.

We used a Tukey's post-hoc comparisons test to test for differences between individual chlorophyll means (Table 2). Patterns were similar across estuaries: (1) controls (C) and control cages (CX), (2) NP-enriched sediments (NP) and cages (X), and (3) NP and N enriched cages did not differ significantly. NP and N enriched cages had significantly higher chl *a* than controls or control cages, and only differed significantly from NP enriched plots and cages in Green Pond. NP enriched plots and cages had significantly higher means than controls or control cages in some but not all cases.

The ANOVA and post-hoc comparisons tests have limited resolution, as only annual means can be compared while seasonal variability is ignored. In the next subsection we will examine treatment responses closer by looking at the time courses of chlorophyll.

Time course of chlorophyll *a* treatments

To simplify the presentation of data we combined controls and control cages to 1 category — 'controls' (C) (Figs. 3 to 5). We compared the chl *a* of treatments and controls (Fig. 3) and examined the relative effects of cages and nutrients on microphytobenthic biomass (Fig. 4). We used the number of runs as a test of randomness (Bradley 1968) to assess if the time courses of any 2 treatments/controls differed. Since we had not started treatments until after the first sampling (early April 2001), we did not include chl *a* means from the first sampling in the tests.

Annual chl *a* peaks of controls differed in timing between estuaries. In Green Pond the peak was in April, in Childs River in June, and in Sage Lot Pond in July. Annual lows in chl *a* uniformly occured in mid-winter.

Throughout the year chl *a* was significantly higher in NP enriched sediments (Fig. 3, top panels). Cages had significantly higher chl *a* in Green Pond and Childs River, but not in Sage Lot Pond (Fig. 3, 2nd row of panels). The response to grazer exclusion approximated that to NP enrichment in Sage Lot and Green Pond. In Childs River the biomass responded much more to grazer exclusion than NP enrichment. Chl *a* was significantly higher in NP- and N-enriched cages than in controls (Fig. 3, 3rd and 4th row of panels, respectively).

There was a significant increase in chl *a* in NP fertilized cages compared to NP fertilized plots (Fig. 4, top panels). The magnitude of the difference increased with increasing nitrogen load. NP enriched cages had significantly higher biomass than unfertilized cages (Fig. 4, middle panels). The magnitude of the response was fairly constant across estuaries. N enrichment significantly elevated chl *a* in Green Pond, but not in Sage Lot Pond or Childs River (Fig. 3, bottom panels). While the mean response to N exceeded that to NP enrichment in Sage Lot Pond and Green Pond, there was virtually no response to N addition in Childs River.

There were no significant interactions between cages and NP enrichment (Fig. 5). Adding chl *a* responses to NP fertilization (ΔNP) and grazer exclusion (ΔX) to monthly chl *a* measurements in controls (C) resulted in a close fit to chl *a* values in NP enriched cages (Fig. 5).

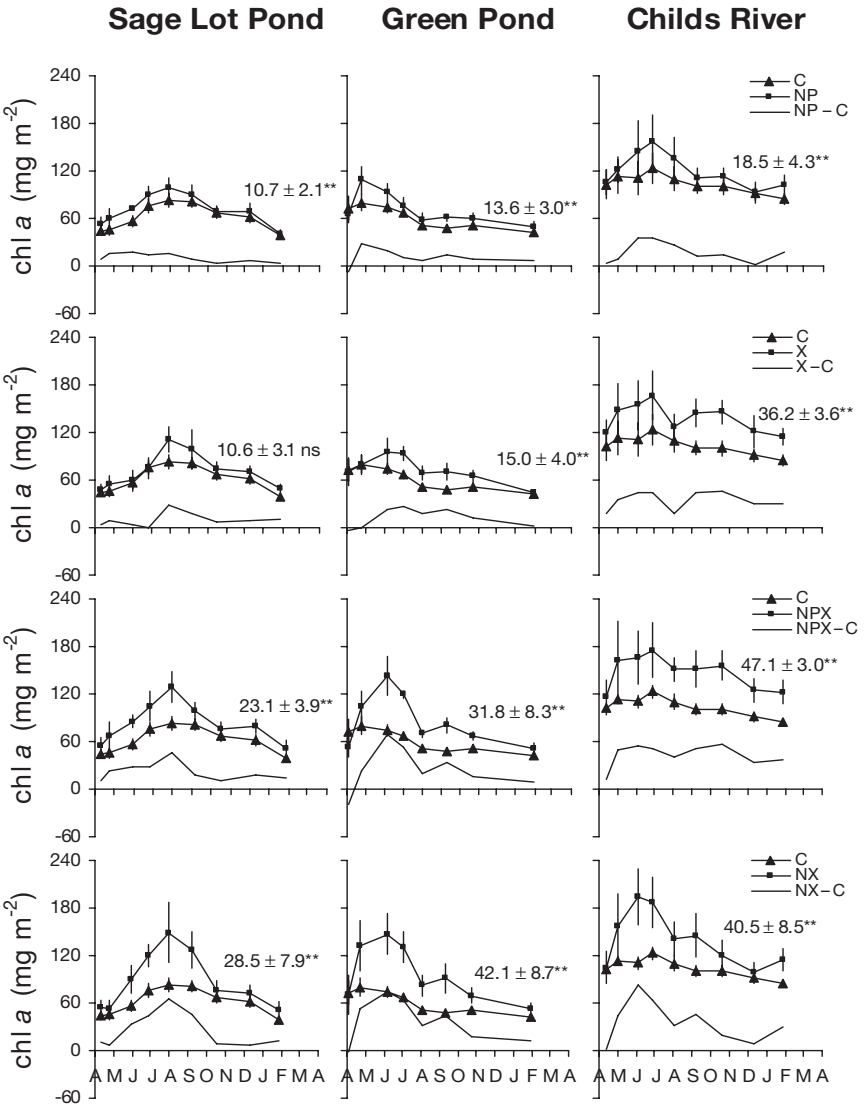

Fig. 3. Time courses of chlorophyll *a* responses to treatments. Controls and control cages combined into 1 category (C). Each graph shows treatment, control and treatment response (treatment minus control). Top panels: NP-enriched plots (NP); 2nd row of panels: cages (X); 3rd row of panels: cages fertilized with NP (NPX); 4th row of panels: cages fertilized with N (NX). Data are means ± SE. SEs were obtained from 5 sites per estuary. Included in each graph are mean difference ± SE between treatments and controls over entire study, and results of a runs test (ns: $p > 0.05$; **: $p < 0.01$)

Mean chlorophyll *a* responses across nitrogen loads

The responses to NP enrichment, cages and NP-enriched cages increased the higher the regional nitro-

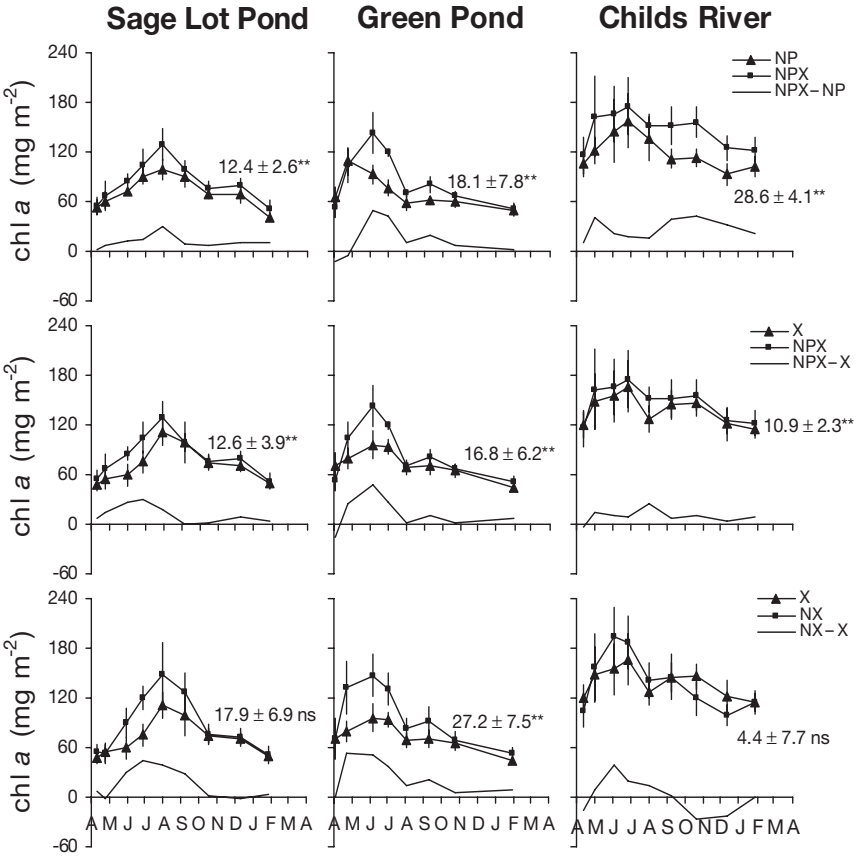

Fig. 4. Comparison of chlorophyll *a* in NP enriched sediments outside (NP) and inside (NPX) cages (top panels), unfertilized cages (X) and NP enriched cages (NPX) (middle panels), and unfertilized cages (X) and N enriched cages (NX) (bottom panels). Data are mean ± SE of 5 stations per estuary. Included in each graph are time courses of difference between the 2 treatments compared, values of mean difference ± SE, and results of a runs test (ns: $p > 0.05$; **: $p < 0.01$)

gen loading rates (Fig. 6). All 3 linear regressions performed were only peripherally significant at $p = 0.05$. Responses to N enrichment in cages did not increase linearly: while Sage Lot Pond and Green Pond had strong increases in chl *a* relative to unfertilized cages, there was practically no increase attributable to N addition in Childs River.

We investigated possible relationships between the mean chl *a* in controls and the magnitude of treatment responses and found none.

DISCUSSION

Nutrient availability was limiting to microphytobenthic biomass in all 3 estuaries as indicated by a significant response to NP enrichment (Fig. 3, top panels). Similarly, consumption by macrograzers represented a significant control of biomass in the estuaries studied (Fig. 3, 2nd row of panels). A combination of NP enrichment with grazer exclusion resulted in significantly greater biomass than in individual enrichment or grazer exclusion treatments across estuaries (Fig. 4, top panels). The biomass increment resulting from a combination of NP enrichment and grazer exclusion approximately equalled the sum of biomass increments for the individual treatments (Fig. 5). Hence, there were no interactions between the effects of NP enrichment and grazer exclusion or, in other words: (1) nutrient enrichment did not reduce herbivore effects by compensating biomass removal with enhanced growth rates, and (2) herbivores did not respond to enhanced microphyte growth by grazing fertilized plots more heavily. Biomass responses to NP enrichment, grazer exclusion, and the combination of NP enrichment and grazer exclusion became stronger with increasing nitrogen loading rate, while there was no such trend for N enrichment with grazer exclusion.

Importance of bottom-up effects

In Sage Lot Pond and Green Pond, nitrogen was the limiting nutrient (Fig. 6). Cages with N only addition showed greater increases in chl *a* than cages fertilized with NP because of higher doses of N. In Childs River there was a greater response to NP than to N only enrichment in cages (Figs. 3 & 4), despite a vastly higher nitrogen dose in cages enriched with N only (Fig. 2, Table 1). Compared to unfertilized cages, N enrichment in cages increased chlorophyll *a* only for a brief period in early summer (Fig. 4, bottom panels). There are several scenarios that may account for the absence of a growth response to N only enrichment in Childs River: (1) phosphorus became limiting when only nitrogen was added; sediments may be barely N limited and require but little N addition to shift to P limitation. (2) Phosphorus and nitrogen were co-limiting; addition of 1 nutrient alone resulted in growth limitation by the availability of the other nutrient, and hence no significant growth response by microphytes. (3) Phosphorus, and not nitrogen, was the limiting nutrient for most of the year; seasonal phosphate limitation of macroalgae in the lower reaches of Childs River was documented previously (Peckol et al. 1994). Phosphate adsorbs to parti-

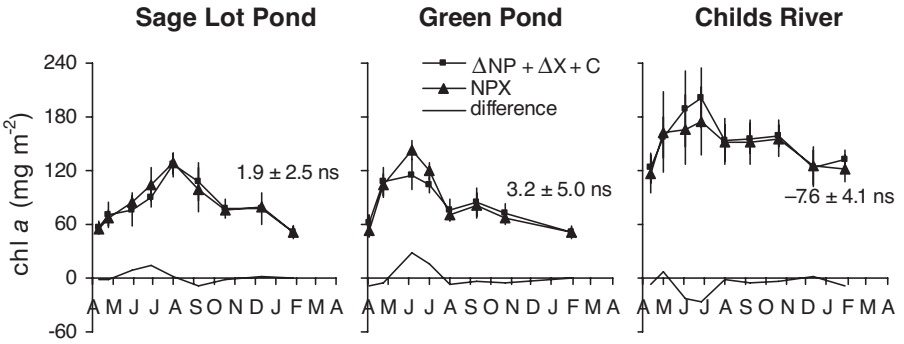

Fig. 5. Examination of treatment interactions (treatment abbreviations as in Fig. 2). Chlorophyll *a* responses to single-factor manipulations (ΔNP: NP–C, ΔX: X–C) are added to chl *a* in controls (C) and compared to treatments that are combinations of both factors (NPX). All data points represent mean response ± SE at 5 sampling stations. Included are also values of mean difference ± SE over entire study period and results of a runs test (ns: $p > 0.05$)

cles under aerobic conditions (Weiskel & Howes 1992) and does not travel through the oxic aquifers of the primarily groundwater-fed Waquoit Bay estuaries. Despite high phosphorus input to the aquifers, e.g. from septic tanks, only a minor portion is transported through the aquifers into the estuaries. In contrast, nitrate is highly soluble in groundwater. While most ammonium is probably also adsorbed to particles (Caezan et al. 1987, Kipp 1987), large amounts of nitrate, derived from breakdown of sewage and nitrification, enter the more eutrophic estuaries (Valiela et al. 1992). Nitrate contributes by far the most dissolved inorganic nitrogen to these systems, and a large portion of the total nitrogen load (McClelland & Valiela 1997). Phosphate limitation in Childs River occurr naturally or as an artifact of enrichment with N only, is the most likely explanation for the lesser (or absent) response of microphytobenthic biomass to N than to NP addition.

Trends towards phosphate limitation have been found in other eutrophic estuaries (D'Elia et al. 1986, Peckol et al. 1994, Wasmund et al. 2001). The measured Redfield ratios in our study were not consistently higher in Childs River than in the other estuaries (Lever 2002, masters thesis). Phosphate concentrations in sediment porewater may not reflect the total phosphate present. However, because of adsorption under oxic conditions as in sandy, surface sediments; and it remains unknown what proportion of adsorbed phosphate, if any, is available to benthic microphytes. Besides phosphate, organic phosphorus can be utilized as a P source by some aquatic phototrophs (Cotner & Wetzel 1992, Björkman & Karl 1994), yet its significance to the benthic microphytes studied herein remains unknown.

Sediment chl *a* content of control plots was not correlated with ambient porewater nutrient concentrations (Lever 2002 and present study). This may be explained by differences in nutrient supply rates and turnover times. Besides, porewater nutrient concentrations did not vary drastically within or between estuaries (Table 1). Perhaps concentration differences were too small to prevail over other environmental factors such as grazing, light, or flow regime. Moreover, water-column nutrients may contribute significantly to microphytobenthic nutrient uptake (Tobias et al. 2003) and lessen the importance of porewater nutrients in estuaries with elevated water-column nutrient concentrations, such as Childs River (Valiela et al. 1992).

At ambient conditions we found an only peripherally significant correlation ($p = 0.08$, $R^2 = 0.57$) between microphytobenthic biomass and estuarine nitrogen loading rate (Lever 2002 and present study). The eutrophic estuaries Green Pond and Childs River receive most of their freshwater, and hence nitrate, in the middle and upper reaches, at salinity ranges lower than those we investigated in this study (Valiela et al. 2000). Perhaps this explains why nitrate concentrations of the sediment porewater at our sites were not correlated with nitrogen loading rate (Lever 2002 and pre-

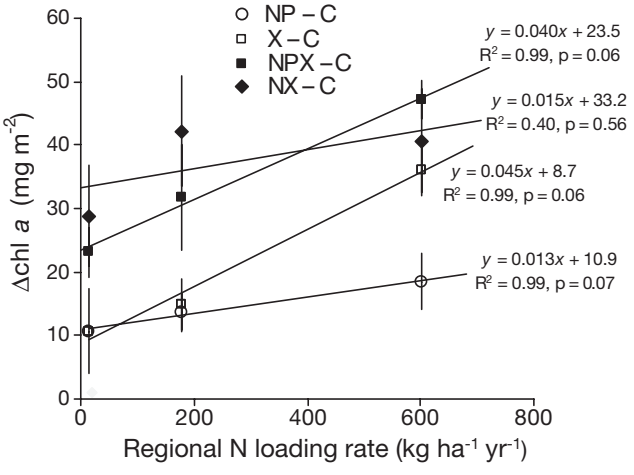

Fig. 6. Mean chlorophyll *a* response to treatment plotted against nitrogen loading rate of the respective estuary. NP: NP enriched; X: cage; NPX: NP enriched cage; NX: N enriched cage. Each data point represents mean difference between treatment and control (= average of control and control cage) of all but initial sampling dates. Error bars were calculated from mean chl *a* values from each of 5 sampling stations per estuary throughout study period

sent study). Sampling sites located in the middle and upper reaches, directly at the main sources, might be more suited to examine the effect of increased anthropogenic nitrogen loads on microphytobenthic biomass. Unlike phytoplankton, which derives nutrients primarily from the well-mixed water column and increases in biomass throughout the euthrophic estuaries, microphytobenthic biomass may respond to increased nutrient inputs more locally, e.g. at sediment–water interfaces in areas with high rates of groundwater discharge and hence nutrient supply. Variations in periphyton biomass in rivers have been linked to variations in groundwater flow and nutrient delivery (Pepin & Hauer 2002). Problems can arise when comparing estuaries with different salinity ranges, as salinity may affect microphytobenthic biomass (Underwood & Provot 2000). Sage Lot Pond, the only estuary around Waquoit Bay without human development in its recharge area, has high salinities throughout and is not suitable for this type of comparison.

To our knowledge, there have been no prior studies on nutrient limitation of sand-inhabiting microphytobenthos across estuaries with different degrees of eutrophication. However, our finding of nutrient limitation across a wide range of nitrogen loading rates is consistent with previous studies on macroalgae (Peckol et al. 1994, Valiela et al. 1997), phytoplankton (Sheridan et al. 1995, Tomasky & Valiela 1995) and periphyton (Hillebrand 2002). While the magnitude of the growth response to enrichment appears correlated with initial biomass in periphyton (Hillebrand 2002), our results suggest a weak correlation with nitrogen loading rate instead (Fig. 6). Controls in Green Pond had lower average biomass than in Sage Lot Pond (Table 2), but mean annual chl *a* response to NP enrichment was greater than in Sage Lot Pond (Fig. 3).

Microphytobenthos inhabiting fine sediments is generally not nutrient-limited, (Admiraal et al. 1982, Underwood & Kromkamp 1999), while periphyton typically is (Hillebrand 2002). Microphytobenthos inhabiting coarser sediments, such as sand, is nutrient-limited in some cases (Granéli & Sundbäck 1985, Sundbäck & Snoeijs 1991, but see Hillebrand & Kahlert 2002). Arguably, conditions in sandy sediments are intermediate to those in fine sediments and the habitats occupied by periphyton, e.g. cobblestones, rocks or macrophytes. Sandy sediments are porous and permeable. Organic matter can accumulate at the surface, but is also buried via bioturbation and physical processes. Remineralization of organic matter leads to release of nutrients that can be utilized by microphytes. High permeability of sandy beds allows advective transport of water column organic matter (Rusch & Huettel 1995) and nutrients into porewater (Huettel et al. 1998, 2003). While advection may be a significant source of nutrients in nutrient-poor sands, it may, however, also lead to an overall loss of nutrients in sands with high organic matter remineralization rates, or in high-energy areas. Groundwater flow across sandy sediment–water interfaces can contribute further to the nutrient budget. In studies with nitrate-rich groundwater, more than half of the nitrate was removed during passage through the sediment–water interface (Valiela et al. 1992). Although denitrification accounts for part of this loss, the importance of microphytes cannot be excluded.

Fine sediments tend to accumulate more organic matter than sandy sediments. Because of less turbulence and lower flow velocity of overlying water, sedimentation is higher and deposited organic matter less subject to resuspension and export. Higher organic matter content results in greater organic matter remineralization and nutrient regeneration. Porewater concentrations of ammonium and phosphate are generally higher than in sandy sediments and much higher than in the overlying water. Even though fine sediments are less permeable, leading to less groundwater and advective delivery of nutrients, microphytes on fine sediments are exposed to higher nutrient supplies, which are transported primarily via diffusion (Huettel et al. 2003). Because of non-limiting nutrient supplies and less resuspension, microphyte biomass on fine sediments tends to be higher than on sandy sediments (Underwood & Kromkamp 1999).

Because of the nature of their habitat, periphyton assemblages are likely to obtain nutrients primarily from the overlying water column. Groundwater nutrient delivery can play an important role locally in river beds (Ford & Naiman 1989, Pepin & Hauer 2002). The high-energy conditions typical of the periphyton habitat result in less accumulation of organic matter and hence nutrient regeneration than in sediments, even though regeneration plays an important role in the total nutrient budget (Wetzel 1996). Since water column nutrient concentrations are commonly low, even in highly eutrophic systems, and groundwater flow is extremely patchy — only affecting periphyton in certain locations — it is not surprising that periphyton is prone to nutrient limitation across habitats.

Even though sand-inhabiting microphytes can exploit groundwater and advective nutrient delivery to a greater extent than microphytes on fine sediments, they appear more prone to nutrient limitation because of the lower organic matter remineralizaton within their environment. Nutrient regeneration from diagenetic processes appears to be a key variable determining nutrient limitation in benthic microphytes from soft to hard substrata.

Importance of top-down effects

In addition to nutrients, macroherbivores played an important role in controlling microphytobenthic biomass (Fig. 3, 2nd row of panels, & Fig. 6). While the increase in biomass arising from grazer exclusion approximately equals that arising from NP enrichment in Sage Lot Pond and Green Pond, it is about twice as high in Childs River (Fig. 6). At the NP fertilizer dose and mesh size applied in our study, top-down effects are more important than bottom-up effects in regulating microphytobenthic biomass in Childs River, and equally important in Sage Lot Pond or Green Pond.

We found the response to herbivore exclusion to increase with nitrogen loading rate (Fig. 6). We are not aware of other studies that have examined the significance of top-down effects on sand-inhabiting microphytobenthic biomass across different nitrogen loading rates. Numerous studies have, however, examined responses of periphyton biomass to top-down controls across systems of different background productivity (Hillebrand 2002). The significance of top-down effects depended on initial periphyton biomass, rather than trophic status.

Our results also contrast with those of a previous study on macroalgae in Waquoit Bay (Hauxwell et al. 1998), in which the significance of top-down effects in controlling macroalgal biomass declined with decreasing nitrogen loading rate. Hauswell et al. (1998) suggested 2 possible explanations: first, higher water column nutrient concentrations in eutrophic estuaries may stimulate macroalgal growth rates sufficiently to surpass grazing rates; second, lower grazer densities in eutrophic estuaries may result in reduced impact of grazers on macroalgal biomass. Lower grazer densities could be the result of episodic anoxic conditions in bottom waters of eutrophic estuaries (D'Avanzo & Kremer 1994). Even without heightened mortality of grazers, greater macroalgal biomass may not result in more food. Only the upper 6 to 10 cm of macroalgal canopies may be accessible to grazers, since conditions below are frequently anaerobic (Waquoit Bay Land Margin Ecosystem Research project unpubl. data). Macroalgae in eutrophic systems may, moreover, escape grazing due to higher biomass remaining from the previous growing season (May 1977, Geertz-Hansen et al. 1993). Greater biomass at the onset of the growth season may allow production rates sufficient to exceed consumption rates.

While macroalgae expand into the water column, where given favorable conditions they can form canopies >75 cm thick (Hersh 1995), the photic zone of sand-inhabiting microphytobenthos, and hence its growth zone, is confined to the upper few millimeters of sediment (Kühl & Jørgensen 1994, Paterson et al. 1997). Thus, macroalgae have a much greater potential to increase in biomass. They may also partially escape grazing by growth into the water column, where they are less available to epibenthic macrograzers such as snails or bottom-dwelling amphipods and shrimp. Microphytobenthos occurs within close proximity to these herbivores. Its biomass may be more accessible and easier to control, even when growth rates are high, as might be expected under conditions of high nutrient availability.

We counted densities of the dominant macroherbivore, the gastropod *Ilyanassa obsoleta*, and found no correlation with nitrogen loading rate (Lever 2002). Higher consumption rates per specimen may have resulted in higher individual biomass rather than higher snail densities. The growth rates of mud snail species (Hydrobiidae) have been shown to correlate with microphytobenthic standing crop (Fenchel & Kofoed 1976, Levinton & Bianchi 1981). Past studies have shown densities of the grass shrimp *Palaemonetes pugio* to increase with increasing nitrogen loading rate (Millman et al. 2002). Increases in shrimp populations could account for the greater importance of top-down effects in high N load estuaries. However, when we counted populations of the dominant grass shrimp species (*Palaemonetes pugio* and *P. vulgaris* combined), we found these were absent or at low average densities (0 to 5 m^{-2}; median = 0 m^{-2}) at our sampling stations. Mud snails were present at all stations at much greater average densities (24.1 to 1190 m^{-2}, median = 259 m^{-2}), and were thus probably more important consumers of the microphytobenthic biomass.

Hypoxic or anoxic events are unlikely to have played a role in regulating grazer densities. Over the summers of 2000 and 2001 we took diel oxygen measurements at all sites, and not once were dissolved concentrations in the hypoxic or anoxic range. Hypoxic and anoxic events have been detected in macroalgal canopies in Waquoit Bay estuaries (Waquoit Bay Land Margin Ecosystem Research project unpubl. data) and at greater, more stratified depths (D'Avanzo & Kremer 1994). The sites selected for the present study were located at shallow depths and within close proximity to shore (~0.5 to 10 m at mean low tide). Moderate but constant wave action along the shore prevented vertical stratification and enhanced atmospheric mixing.

CONCLUSIONS

We found microphytobenthos on sandy substrates to be nutrient-limited. Nutrient limitation occurred independent of state of eutrophication and resulted in ele-

vated microphytobenthic biomass on plots fertilized with N and P throughout the study period. In a pristine and moderately eutrophic estuary, N was the limiting nutrient, whereas in the most eutrophic estuary P availability was limiting, or co-limiting with N, for most of the year. The importance of nutrients (N and P) and macrograzers as controls of microphytobenthic standing crop increased with increasing nitrogen loading rates. While nutrients (bottom-up effects) were about equally important as macrograzers (top-down effects) in the pristine and moderately eutrophic estuaries, macrograzers were more important than nutrients in the highly eutrophic estuary. We currently have no compelling explanation for the observed results. Further work is required to explain why (e.g.) top-down effects may become more important with increasing nitrogen loads, or whether phosphorus was limiting or co-limiting with nitrogen in the most eutrophied estuary.

To our knowledge, this study is the first to experimentally investigate shifts in the significance of top-down versus bottom-up effects as controls of sediment-inhabiting, microphytobenthic biomass in the context of anthropogenic eutrophication. The estuaries investigated cover a span of nitrogen loading rates that includes the range of approximately ~75% of the world's estuaries (Valiela 1995). With estuarine mean chl *a* measurements of 57 to 109 mg m^{-2} (range 21 to 248 mg m^{-2}) at ambient conditions, our values lie well within the published range for estuarine microphytobenthos (Underwood & Kromkamp 1999). Given similar conditions (i.e. bare sandy substrates in shallow water at high salinities), the results of this study are likely to be relevant to estuarine systems elsewhere.

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