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Nutrient Limitation of the Macroalga *Enteromorpha intestinalis* Collected along a Resource Gradient in a Highly Eutrophic Estuary

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ABSTRACT: We conducted a laboratory experiment to quantify nutrient (nitrogen and phosphorus) limitation of macroalgae collected along a gradient in water column nutrient availability in Upper Newport Bay estuary, a relatively nutrient-rich system in southern California, United States. We collected *Enteromorpha intestinalis* and water for use in the experiment from five sites ranging from the lower end of the estuary to the head. Initial algal tissue N and P concentrations and molar N:P ratios—as well as water column NO_3 and total Kjeldahl nitrogen (TKN)—increased along a spatial gradient from the lower end toward the head. Water column soluble reactive phosphorus (SRP) varied among sites as well but did not follow a pattern of increasing from the seaward end toward the head. Algae from each site were assigned to one of four experimental treatments: control (C), nitrogen enrichment (+N), phosphorus enrichment (+P), and nitrogen and phosphorus enrichment (+N+P). Each week for 3 wk we replaced the water in each unit with the appropriate treatment water to mimic a poorly flushed estuary. After 3 wk, the degree of nutrient limitation of *E. intestinalis* varied spatially with distance from the head of the estuary. Growth of *E. intestinalis* collected from several sites increased with N enrichment alone and increased further when P was added in combination with N. This indicated that N was limiting and that when N was sufficient, P became limiting. Sites from which *E. intestinalis* exhibited nutrient limitation spanned the range of background water column NO_3 (12.9 ± 0.4 to 55.2 ± 2.1 μM) and SRP (0.8 ± 0.0 to 2.9 ± 0.2 μM) concentrations. Algae that were N limited had initial tissue N levels ranging from 1.18 ± 0.03 to 2.81 ± 0.08% dry weight and molar N:P ratios ranging from 16.75 ± 0.39 to 26.40 ± 1.98.

Introduction

With the development of macroalgal blooms in estuaries throughout the world (McComb et al. 1981; Sfriso et al. 1987; Schramm and Nienhuis 1996; Hernández et al. 1997), it is critical to understand the factors regulating growth and biomass accumulation. Macroalgal blooms often occur following increased nutrient loading from watersheds (Valiela et al. 1992; Duarte 1995; Nixon 1995; Flindt et al. 1999; Schramm 1999) and are problematic as excessive production of macroalgae may reduce the habitat quality of an estuary by depleting oxygen levels (Sfriso et al. 1987; Valiela et al. 1992), leading to fish and invertebrate mortality (Raffaelli et al. 1991) and ultimately to changes in community structure (Raffaelli et al. 1989; Norkko and Bonsdorff 1996; Valiela et al. 1997; Bolam et al. 2000).

Macroalgae clearly respond to changes in availability of both nitrogen (N) and phosphorus (P) (Harlin and Thorne-Miller 1981; Valiela et al. 1992; Fong et al. 1993; Peckol et al. 1994). Growth is often stimulated when nutrient supply is increased, indicating that nutrients were limiting (Delgado and Lapointe 1994; Larned 1998; Lotze and Schramm 2000), as is common in marine and estuarine systems (Ryther and Dunstan 1971; Hanisak 1983; Howarth 1988; Holmboe et al. 1999; Smith et al. 1999).

Study of nutrient limitation of phytoplankton (Howarth 1988; Rudek et al. 1991; Fisher et al. 1992; Holmboe et al. 1999; Tomasky et al. 1999) indicates that the degree and nature of nutrient limitation may vary spatially within an estuary (Flemer et al. 1998). Water column N and P levels are generally higher near the head of a system and decrease toward the mouth (Rizzo and Christian 1996; Hernández et al. 1997; Nedwell et al. 2002), which may lead to increased nutrient limitation down-estuary. Due to differential rates of nutrient processing (Ryther and Dunstan 1971), the relative abundance of N and P available to primary pro-

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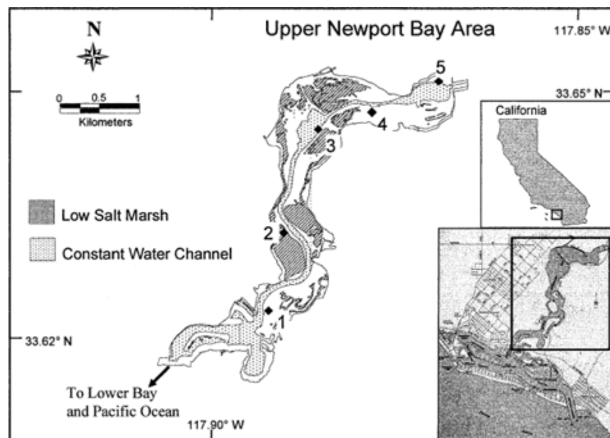

Fig. 1. Map of Upper Newport Bay estuary, California, United States, with five sites from which *Enteromorpha intestinalis* and water were collected for determination of nutrient limitation.

ducers may vary along a spatial gradient, generally causing nutrient limitation to shift from P to N as distance from the head of the estuary increases (Doering et al. 1995; Pitkänen and Tamminen 1995).

In southern California, blooms of *Enteromorpha intestinalis* are common (Peters et al. 1985; Rudnicki 1986; Kamer et al. 2001), and there is a need to further understand site-specific factors that limit their growth. Several southern California estuaries exhibit gradients in water column nutrient availability along an axis from the head to the mouth (Page et al. 1995; Fong and Zedler 2000) including Upper Newport Bay estuary (UNB; California Department of Fish and Game 1989; Boyle 2002), a highly eutrophic southern California system. Relative availability of water column N and P can differ between head and down-estuary areas (Boyle 2002). The objective of this study was to determine whether N or P limits growth of macroalgae collected along an estuarine nutrient gradient in UNB. We hypothesized that *E. intestinalis* from down-estuary sites would be nutrient limited and that the occurrence of nutrient limitation may decrease with increasing proximity to the head of UNB. We tested for possible limitation by both N and P to determine if the limiting nutrient changed with increasing distance from the head.

Materials and Methods

We conducted a laboratory experiment to test whether N or P limited growth of *E. intestinalis* from five sites in UNB. Sites ranged from the lower end of the estuary (Site 1) to the head (Site 5), where San Diego Creek, the primary surface water nutrient source (U.S. Environmental Protection Agency [EPA] 1998), enters UNB (Fig. 1). At each site, we collected algae from exposed intertidal mudflat and water from mid-water depth (~1 m) on June 11, 2001, on a flood tide. Materials were transported back to the laboratory within 5 h. In the laboratory, *E. intestinalis* from each site was cleaned of debris and other organisms and placed in shallow pans filled with aerated water from the corresponding site. Pans were kept outdoors overnight in a temperature controlled water bath (20 ± 2°C). The collected water was placed in the dark in a 6°C cold room until added to experimental units.

On June 12, 2001, a portion of the water from each site was divided into 4 aliquots to create the experimental treatments (control [C], nitrogen enrichment [+N], phosphorus enrichment [+P], and nitrogen and phosphorus enrichment [+N+P]), resulting in a three-factor experimental design: site × N enrichment × P enrichment. Control treatments were ambient water from each site with no additions. Nutrient amendment treatments were enriched with NO_3 and PO_4 to increase N and P concentrations by 400 and 40 μM, respectively, over background levels. Enrichment concentrations were based on known levels of NO_3 in UNB (Boyle 2002; Kamer unpublished data). P was added in a 10:1 N:P ratio based on the work of Boyle (2002) showing a year-long water column N:P grand mean of 10.4:1. Due to collection of water during a flood tide, water column nutrient concentrations (Table 1) were considerably lower than values measured at low tide in other studies of UNB (Boyle 2002; Kamer unpublished data) or other southern California estuaries (Page et al.

TABLE 1. Mean (±SE) background water column nutrient concentrations at the time of collection from each of the 5 sites in UNB. n = 3. Superscripts a–e denote means that are significantly different from each other ($p < 0.05$, Fisher's LSD following significant 1-factor ANOVA). Values below detection limits (3.57 μM for N and 1.61 μM for P) were set to half the detection limit.

Site	Salinity (psu)	Water Column Nutrients (μM)			
		NO_3	NH_4	SRP	TKN
1	37	12.9 (0.4)[a]	6.2 (1.2)[b]	0.8 (0.0)[a]	92.9 (8.4)[a]
2	35	28.3 (1.2)[b]	25.7 (1.2)[d]	2.9 (0.2)[b]	95.2 (38.3)[a]
3	33	36.2 (0.6)[c]	12.6 (1.4)[c]	1.1 (0.3)[a]	100 (14.9)[a]
4	31	55.2 (2.1)[e]	17.4 (2.7)[c]	2.5 (0.3)[b]	238.1 (41.7)[b]
5	33	42.4 (0.6)[d]	1.79 (0.0)[a]	0.8 (0.0)[a]	302.4 (56.9)[b]

1995; Fong and Zedler 2000). Over the course of a year, Boyle (2002) measured higher mean water column nutrient concentrations near the head of the estuary, proximal to this study's Site 5 (158–800 μM NO_3; 4.3–16.7 μM total P) relative to down-estuary areas near Site 1 (5–90 μM NO_3; 1.8–11.5 μM total P), and Kamer (unpublished data) also found a gradient in water column NO_3 in UNB of 414 μM at Site 5, 101 μM at Site 3, and 49 μM at Site 1 2 mo before the current study.

E. intestinalis from each site was placed in nylon mesh bags and spun in a salad spinner for 1 minute to remove excess water. Algae were wet weighed and 8.0 ± 0.1 g sub-samples were added to glass experimental units (1.5 l total volume), each containing 800 ml of the appropriate solution. Five additional sub-samples of algae were processed for initial tissue N and P analyses. Experimental units were placed in a randomized array outdoors in a temperature controlled water bath (20 ± 2°C) and covered with window screening to reduce incident light (2,200–2,500 μmoles m^{-2} s at midday) by ~30% to simulate coastal levels (1,405–1,956 μmoles m^{-2} s^{-2}, Arnold and Murray 1980). Replication was five-fold with the exception of the site 3 Control and site 3 +N treatments, which only had three replicates each due to not having collected enough algae from that site. There were a total of 96 units. Salinity was monitored every 2 d with a hand-held refractometer and deionized water was added to compensate for evaporation. Salinity was maintained within 2 psu of initial levels measured for each site at the time of collection.

The experiment ran for 3 wk with weekly exchange of treatment water to simulate low turnover characteristic of poorly flushed estuaries in southern California (Zedler 1982, 1996; Largier et al. 1997; Fong and Zedler 2000). Weekly exchange is appropriate based on hydrodynamic models of UNB showing that 55% of a dissolved, conservative tracer remained in the bay for 3 d following a storm flow of 100 cfs (Resource Management Associates, Inc. 2003), which is considerably higher than normal base flow conditions (Public Facilities and Resources Department 2001). Units were re-randomized in the water bath each week.

At the end of the experiment, algae were removed from each unit and wet weighed after being spun in nylon mesh bags in a salad spinner for 1 min. Growth was determined as the percent change from initial wet biomass. Each sample was rinsed briefly in freshwater to remove external salts, dried in a forced air oven at 60°C to a constant weight, and ground with mortar and pestle for subsequent tissue N and P analysis. N and P content of algae are reported as concentration (% dry weight).

Water samples were filtered (Whatman GF/C) prior to being frozen for subsequent analysis. Water column NO_3 was reduced to NO_2 via cadmium reduction; NO_2 was measured spectrophotometrically after diazotation (Switala 1999; Wendt 1999). Water column NH_4 was heated with solutions of salicylate and hypochlorite and determined spectrophotometrically (Switala 1999; Wendt 1999). Water column total Kjeldahl nitrogen (TKN, which is all forms of N except NO_3 and NO_2) was determined by the wet oxidation of nitrogen using sulfuric acid and digestion catalyst. The procedure converts organic nitrogen to NH_4, which is subsequently determined (Carlson 1978). Water column soluble reactive phosphorus (SRP) was determined spectrophotometrically following reaction with ammonium molybdate and antimony potassium under acidic conditions (American Public Health Association 1998). These automated methods have detection limits of 3.57 μM for all forms of N and 1.61 μM for P. Values of samples below detection limits were set to half the detection limit to allow statistical analysis of data.

Algal tissue N was determined using an induction furnace and a thermal conductivity detector (Dumas 1981). Algal tissue P was determined by atomic absorption spectrometry (AAS) and inductively coupled plasma atomic emission spectrometry (ICP-AES) following a nitric acid-hydrogen peroxide microwave digestion (Meyer and Keliher 1992). Algal tissue N:P ratios were calculated on a molar basis.

All data were tested for normality and homogeneity of variance; no transformations were needed. Differences in initial water nutrient concentrations (NO_3, NH_4, TKN, SRP) were analyzed by 1-factor AVOVA (site). Multiple pairwise comparison procedures (Fisher's Least Significant Difference test [LSD]) were employed following significant ANOVA ($p < 0.05$) to determine differences among individual sites. Among treatment differences in growth, tissue nutrient concentrations, and tissue N:P ratios were analyzed using three-factor ANOVA (site × N enrichment × P enrichment). Significant interactions did not occur unless otherwise noted. Following a significant ANOVA, multiple comparisons were used to determine differences among individual treatments within factors (Fisher's LSD).

Results

Nutrient concentrations of water collected June 11, 2001, varied with site (ANOVA, $p < 0.001$ for NO_3, NH_4, and SRP, $p = 0.005$ for TKN). NO_3 was lower in water from the more seaward sites (Sites 1 and 2) compared to the sites further up-estuary, and the highest NO_3 levels were found in water from site 4 (Table 1). NH_4 and SRP were variable

TABLE 2. Mean (±SE) initial *Enteromorpha intestinalis* tissue nitrogen and phosphorus concentrations and molar N:P ratios from each of the 5 sites in UNB. n = 5. superscripts a–d denote means that are significantly different from each other ($p < 0.05$, Fisher's LSD following significant 1-factor ANOVA).

Site	Tissue Nutrient Content (as % dry weight) N	P	Molar N:P
1	1.18 (0.03)[a]	0.156 (0.002)[a]	16.75 (0.39)[a]
2	1.47 (0.06)[b]	0.164 (0.007)[a]	20.09 (1.37)[b]
3	2.21 (0.03)[c]	0.230 (0.003)[b]	21.26 (0.26)[b]
4	2.81 (0.13)[d]	0.238 (0.009)[b]	26.40 (1.98)[c]
5	2.62 (0.08)[d]	0.320 (0.009)[c]	18.16 (0.40)[a,b]

among sites and did not increase with proximity to San Diego Creek. TKN was similar in water from Sites 1–3 and higher from Sites 4 and 5 (Table 1). Tissue N and P content of *E. intestinalis* increased along a spatial gradient from the lower end of the estuary toward the head (Table 2). Tissue N:P molar ratios ranged from 16.75 to 26.40 (Table 2) and increased from Site 1 to Site 4.

After 3 wk, growth of *E. intestinalis* was significantly affected by site (ANOVA $p = 0.0001$). With the exception of Site 5, growth increased with proximity of collection sites to the head of the Bay (Fig. 2; Sites 1–4). *E. intestinalis* growth was affected by N enrichment (ANOVA $p = 0.0001$) but not by P enrichment (ANOVA $p = 0.6268$). Compared to controls, growth of algae from Sites 1, 2, and 4 increased in the N enrichment alone treatments (Fisher's LSD $p < 0.05$ for C versus +N at each site), indicating that N was limiting. The effect of N enrichment on growth was strongest for algae from the down estuary sites. Growth of algae from Site 1 enriched with N was 14.8 ± 2.3% greater than growth of controls; algae from Site 2 enriched with N grew 16.3 ± 2.0% more than controls. These values were not different (Fisher's LSD $p = 0.657$), but algae from Site 2 enriched with N grew more relative to controls than algae from site 4 enriched with N did (8.3 ± 2.4%; Fisher's LSD $p = 0.027$).

The increase in biomass of *E. intestinalis* from Site 3 when N was added compared to controls was statistically insignificant (Fisher's LSD $p = 0.054$) as variability in the control treatment was relatively high and replication of the control and +N treatments was three-fold. There was no significant difference in growth of algae from site 5 between control and +N treatments (Fisher's LSD $p = 1.000$), which, along with the response of algae from Site 3, resulted in an interaction between site and N enrichment (ANOVA $p = 0.0024$).

For *E. intestinalis* collected from Sites 1, 2 and 4, greater growth when P was added in combination with N (Fig. 2; Fisher's LSD $p < 0.005$ for +N versus +N+P at each site) resulted in an interaction between N and P enrichment (ANOVA $p = 0.0039$). This indicated that P became limiting when N limitation was relieved. Growth of *E. intestinalis* from Site 5 was less when P was added, resulting in an interaction between site and P enrichment (ANOVA $p = 0.0001$).

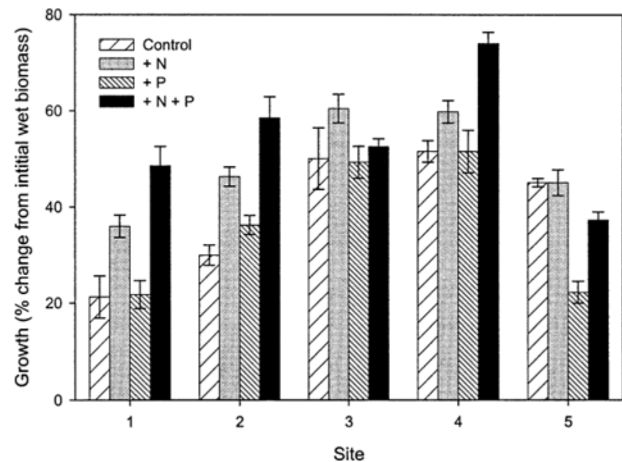

Fig. 2. *Enteromorpha intestinalis* biomass (as % change from initial) grown with ambient (Control), nitrogen enrichment (+N), phosphorus enrichment (+P), or nitrogen and phosphorus enrichment (+N+P) solutions. Bars represent ±1 SE.

There was a gradient in *E. intestinalis* final tissue N concentration across sites (ANOVA $p = 0.0001$) in all treatments (Fig. 3). To some degree these differences are a reflection of the gradient in initial tissue N concentrations (Table 2) with the exception of algal tissue N from Site 4. When N was added, final tissue N levels of algae from Sites 1 and 2 were 39.7 ± 2.8% and 21.0 ± 2.3% (mean ± SE for +N and +N+P treatments, n = 10) higher than initial levels, respectively. Tissue N of algae from site 4 was 22.6 ± 1.1% lower, and for algae from Sites 3 and 5, final tissue N was within 6% of initial levels.

Tissue N was significantly affected by N enrichment (ANOVA $p = 0.0001$) but not by P enrichment (ANOVA $p = 0.2878$). For all sites, tissue N concentration was greatest when N was added regardless of P addition. There was an interaction between site and N enrichment (ANOVA $p = 0.002$) probably due to a slightly higher concentration of tissue N in the +N versus +N+P treatments from Site 4.

E. intestinalis final tissue P concentration was significantly affected by site and P enrichment (ANOVA $p = 0.0001$ for both factors) but not by N enrichment (ANOVA $p = 0.4439$). For all sites, tissue P concentration was greatest when P was added regardless of N addition (Fig. 3). Tissue P concentrations in every experimental treatment were

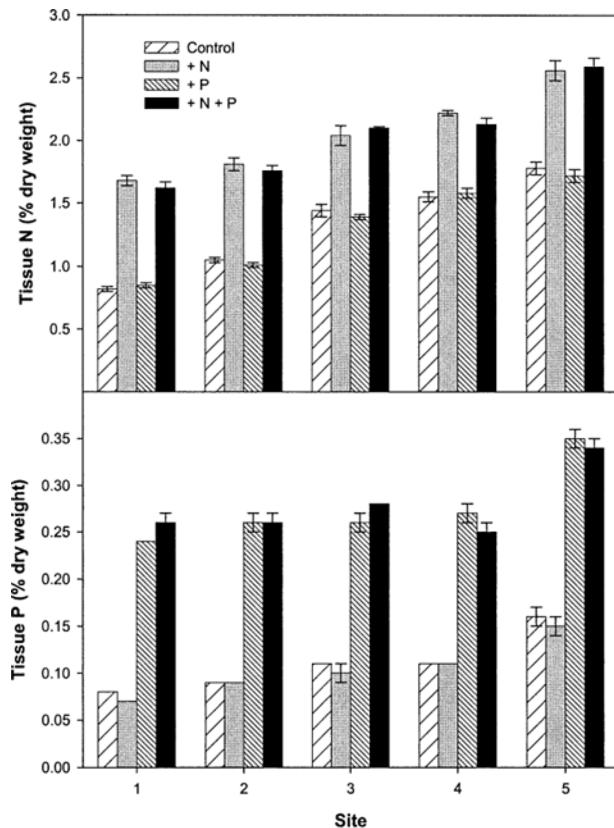

Fig. 3. *Enteromorpha intestinalis* tissue nitrogen and phosphorus concentration as % dry wt after 3 wk in ambient (Control), nitrogen enrichment (+N), phosphorus enrichment (+P), or nitrogen and phosphorus enrichment (+N+P) solutions. Bars represent ±1 SE.

greatest from Site 5. This was likely due to high initial tissue P concentration (Table 2) combined with only moderate growth relative to the algae from the other sites, and caused an interaction between site and P enrichment (ANOVA $p = 0.0038$). In the treatments not enriched with P, tissue P of algae increased among algae collected along the nutrient gradient in UNB, possibly reflecting the initial algal tissue P gradient (Table 2) similar to tissue N. In treatments where P was added, tissue P was similar among algae collected from different sites within UNB with the exception of algae from Site 5.

E. intestinalis tissue N:P ratios were significantly affected by N enrichment, P enrichment, and site (ANOVA $p < 0.0001$ for each factor) and there was significant interaction between N and P enrichment (ANOVA $p < 0.0001$). Lowest N:P ratios occurred in treatments receiving P enrichment alone (Table 3) followed by +N+P treatments. The highest ratios were in the +N only treatments. There was significant three-way interaction (ANOVA $p < 0.0001$) due to different patterns in tissue N:P ratios among sites within each experimental treatment.

TABLE 3. Mean (±SE) final molar N:P ratios of *Enteromorpha intestinalis* tissue from all experimental treatments.

	Treatment			
Site	Control	+N	+P	+N+P
1	22.70 (0.45)	52.01 (1.80)	7.77 (0.23)	13.98 (0.19)
2	26.44 (0.29)	44.58 (1.27)	8.58 (0.21)	15.11 (0.27)
3	29.89 (0.16)	43.94 (1.16)	11.76 (0.24)	16.85 (0.21)
4	32.35 (0.58)	45.62 (0.99)	12.99 (0.29)	18.71 (0.30)
5	25.27 (0.40)	38.94 (0.83)	10.93 (0.16)	17.09 (0.34)

Discussion

Nutrient limitation of *E. intestinalis* from UNB varied spatially throughout the bay. The degree of nitrogen limitation increased with distance from the head of the estuary. Algae from down-estuary sites, where background water column NO_3 concentrations were lowest, were more N-limited than *E. intestinalis* from Site 4, which had the highest background water column NO_3 concentration. Water from Site 4 had one of the highest background water column TKN levels, yet algae were still N limited in spite of this potential source of N (Probyn and Chapman 1982; Hanisak 1983; Navarro-Angulo and Robledo 1999; Lotze and Schramm 2000; Tyler et al. 2001). There was no indication of N limitation of algae from Site 5, but growth of algae from this site was relatively moderate. Site 5 was closest to the mouth of San Diego Creek, which is known to contain selenium, heavy metals, organic pesticides, and PCBs (EPA 2002). These compounds may have inhibited algal growth.

Once N limitation was relieved, P limitation occurred in algae from several sites, regardless of water column or tissue P availability. Similarly, Delgado and Lapointe (1994) also observed P limitation of two species of tropical, fleshy macroalgae once N limitation was relieved. Lapointe (1987) found increases in *Gracilaria tikvahiae* growth with either N or P enrichment and synergistic interaction between the two nutrients, and Schaffelke and Klumpp (1998) used tissue nutrient concentrations and estimated nutrient requirements to infer that both N and P limited *Sargassum baccularia* growth. In several instances, it has been found that phytoplankton abundance was most limited by N and that P became limiting when N supply increased (McComb et al. 1981; Taylor et al. 1995).

Initial tissue N concentration was not always an effective predictor of nutrient limitation over the experimental period. N limitation occurred in *E. intestinalis* with initial tissue N concentrations that varied by more than two-fold at the beginning of the experiment. N limitation may have been ex-

pected in algae from Sites 1 and 2, which had initial tissue N concentrations < 2% dry weight, the suggested critical concentration below which macroalgal maximal growth rates are limited by internal N concentration (Birch et al. 1981; Hanisak 1983; O'Brien and Wheeler 1987; Pedersen and Borum 1996). N limitation of algae from Site 4 occurred as well. Algae from this site had initial tissue N concentrations of ~2.8% dry weight, above which further increases in N supply should not stimulate increased growth. Algae became limited as growth reduced internal stores over the 3-wk experiment. In some treatments, tissue N concentrations decreased over the course of the experiment, indicating that algae were using internal stores of N as well as water column supplies to sustain growth.

Neither initial nor final tissue molar N:P ratios of *E. intestinalis* were consistently good indicators of nutrient limitation. Earlier work suggests that macroalgal tissue molar N:P ratios < 16 indicate N limitation, ratios between 16 and 24 indicate that N and P are present in sufficient supply relative to each other, and ratios > 24 indicate P limitation (Björnsäter and Wheeler 1990; Wheeler and Björnsäter 1992). In our experiment, algae from Site 4 with the highest initial N:P ratio of ~26 displayed N limitation when P limitation may have been predicted based on tissue nutrient content. After 11 to 12 d, Larned (1998) found N limitation of *Ulva fasciata* with an initial tissue N:P ratio of 48.3:1. Our end of experiment tissue N:P ratios of *E. intestinalis* from control treatments were also generally in the range (>24) of P limitation (Björnsäter and Wheeler 1990), yet the algae from Sites 1, 2, and 4 were N limited. These data in combination suggest that algal tissue N:P ratios indicative of nutrient limitation may vary over regional or local scales. They may be site- and species-specific, indicating a need for direct experimental determination of nutrient limitation rather than relying on tissue N:P ratios alone.

Several of our results were more in agreement with earlier work (Björnsäter and Wheeler 1990). N:P ratios of *E. intestinalis* in +N only treatments (>38) and +P only treatments (<13) were consistent with the Björnsäter and Wheeler paradigm as algae from Sites 1, 2, and 4 became P-limited when N was supplied and remained N-limited when P was supplied.

The results of our experiment demonstrate spatial variability in one species of macroalgae in one season. Future investigation of nutrient limitation of macroalgae in southern California should include other dominant macroalgal species such as *Ulva,* and seasonal manipulations should be performed to determine the temporal extent of nutrient limitation. Incorporation of sediments into experimental units should be considered in order to better approximate field conditions and obtain more realistic insight into nutrient limitation of macroalgae, or nutrient limitation experiments could be done in situ. Additional efforts should also be made to determine how prevalent patterns of nutrient limitation are across estuaries in the southern California region. Increased understanding of factors that control seasonal algal blooms is vital to efforts to prevent them.

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