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Growth, nutrient storage, and release of dissolved organic nitrogen by *Enteromorpha intestinalis* in response to pulses of nitrogen and phosphorus

Peggy Fong*, Jon J. Fong, Caitlin R. Fong

Department of Organismic Biology, Ecology, and Evolution, University of California Los Angeles, 621 Charles E. Young Drive South, Los Angeles, CA 90095-1606, USA

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Abstract

Enteromorpha intestinalis was subject to nitrogen (N) and phosphorus (P) enrichment supplied in four weekly pulses in a 10:1 molar ratio with six concentrations ranging from no addition to 1000 μM NO_3 + 100 μM PO_4. The alga reduced inorganic N to very low concentrations (<3.5 μM) across all nutrient additions while a larger proportion of the added P remained in the water at the end of the experiment (up to 20 μM in highest addition). Growth of *E. intestinalis* increased in proportion to enrichment across the lower four of the six treatments; range of growth was −6 to 60% change from initial wet wt. N concentration in the tissue decreased at the four lower loading rates due to dilution by growth, but increased greatly (from an initial of 2.7–5% dry wt.) in the highest loading rate partially due to lack of growth in this treatment. In contrast, the mass of N in the tissue increased linearly (slope = 0.012, r^2 = 0.779, P = 0.0001) over all treatments, showing that uptake and storage were not always coupled to growth. Patterns of tissue P concentration and accumulation were similar to N. Significant amounts of dissolved organic N accumulated in the water, especially in the highest N pulses. Net retention of N in algal tissue ranged from 73 to 98% while retention of P ranged from 79 to 88%; the % lowest retention occurred when nutrient pulses were either very high or low. We hypothesize that this opportunistic, bloom-forming alga is adapted to pulses of nutrients, such that, when pulses are of very high concentration, *E. intestinalis* can delay growth in favor of saving energy to maximize nutrient uptake and storage.

Keywords: Nitrogen; Phosphorus; Estuary; Southern California; Algal blooms; Nutrient cycling

* Corresponding author. Tel.: +1-310-825-5444; fax: +1-310-206-3987.
E-mail address: pfong@biology.ucla.edu (P. Fong).

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1. Introduction

Nutrient enrichment of coastal marine ecosystems has become an increasing problem world-wide (for reviews see Nixon et al., 1996; Corredor et al., 1999). Increased loading of nutrients, especially nitrogen (N) and phosphorus (P), often cause macroalgal blooms in shallow estuaries (e.g. Valiela et al., 1997) and these blooms may dominate estuarine nutrient cycles. Massive accumulations of macroalgae take up available inorganic nutrients from the water as well as intercept benthic fluxes, and sequester and store large pools of nutrients in their tissues during bloom development (Peckol et al., 1994; Fong and Zedler, 2000). The presence of macroalgal mats may increase rates of sedimentary N cycling, supporting mats during times when external N supplies are low and acting to retain large amounts of N within the estuary (Trimmer et al., 2000). Macroalgae can be both a source (Tyler et al., 2001) and a sink (Cohen and Fong, unpublished data) of water column dissolved organic N (DON). Large accumulations of macroalgae can function as a DON source to the water during active growth (Tyler et al., 2001) and decomposition (Boyle, Kamer and Fong, unpublished data). Therefore, it is important to study both uptake and release of nutrients to understand the net importance of macroalgae in estuarine nutrient cycles, and especially their role in retention of nutrients within the estuary.

Macroalgal blooms are often comprised of opportunistic species in the genera *Enteromorpha*, *Ulva*, *Cladophora*, or *Gracilaria* (e.g. Delgado and Lapointe, 1994; Borum and Sand-Jensen, 1996) with life-history characteristics that enhance their response to increased nutrient supplies, including rapid nutrient uptake at high nutrient concentrations with resultant rapid growth (Rosenburg and Ramus, 1984; Fugita, 1985; Duke et al., 1989; Duarte, 1995). These characteristics result in high nutrient requirements in order to sustain maximum growth (Pedersen and Borum, 1997). However, when nutrient supply exceeds the demand for growth, as can occur when nutrients are supplied in high concentration pulses, they have the ability to store nutrients in their tissues to sustain growth during periods of low nutrient supply (Fugita, 1985; Duke et al., 1989; Fong et al., 1994a; Aisha et al., 1995). Thus, these opportunistic species can proliferate when nutrient inputs are episodic, as long as the duration between nutrient pulses does not exceed the storage capacity of the algal tissue (McGlathery et al., 1996). In addition, these same characteristics may allow them to dominate the algal community when nutrient loading is enhanced by anthropogenic sources (Fong et al., 1993, 1994b).

Nutrient dynamics of southern California estuaries and coastal lagoons are understudied (Williams and Zedler, 1992; Bricker et al., 1999); however, from the few studies that exist, it is clear that these estuaries are subject to anthropogenic nutrient sources that enhance an already pulsed natural supply (Peters et al., 1986; Fong and Zedler, 2000). In southern California estuaries, *Enteromorpha* spp. form expansive blooms (Peters et al., 1986; Fong and Zedler, 2000; Kamer et al., 2001). Although largely undocumented, many believe that these macroalgal blooms are responsible for the historic loss of eelgrass throughout southern California; at present, only a few estuaries support remnant seagrass populations (Stewart, 1991). In addition, blooms of *Enteromorpha intestinalis* outcompete phytoplankton under conditions of moderate to high nutrient concentrations (Fong et al., 1993, 1994b). Thus, bloom species currently dominate primary producers in southern Californian estuaries (Kamer et al., 2001), probably replacing a more diverse community. Growth, nutrient

accumulation, and release of DON was measured for *E. intestinalis* in response to pulses of N and P enrichment within the range found in eutrophic southern California estuarine systems.

2. Materials and methods

To test the effects of N and P enrichment on growth and net accumulation of N and P in *E. intestinalis*, we conducted a single factor experiment adding nutrients weekly to model episodic pulses associated with rainfall. Six levels of enrichment (as NO_3 and PO_4) were used in a 10:1 molar ratio (μM N:μM P in treatments = 0:0, 200:20, 400:40, 600:60, 800:80, 1000:100). Values were within the range of N concentrations found in southern California estuaries during the rainy season(Irvine Ranch Water District, 1997; Boyle, Kamer and Fong, unpublished data). We used a 10:1 molar ratio as this approximated the mean (10.3, S.E. = 0.59) found in one local system in a year-long study (Boyle, unpublished data). Replication was five-fold for a total of 30 experimental units.

E. intestinalis was collected from Upper Newport Bay, Orange County, CA, on 21 March 1998, and was batch cultured in shallow pans in a greenhouse for 14 days to equalize internal nutrients (Fong et al., 1994a). Pans were filled with aerated low nutrient seawater (<3.57 μM NO_3, <1.61 μM PO_4) and covered with screening to reduce light by 30% to values typical of coastal areas.

On 4 April 1998, we started the experiment. Low nutrient seawater was divided into two individual 20 l batches; one was kept as an ambient stock solution and the other enriched to make a 1000 μM NO_3:100 μM PO_4 stock solution. Experimental nutrient treatments were made by combining appropriate volumes of stock solutions. Each experimental unit (clear plastic jars) was filled with 300 ml of the appropriate nutrient solution. Algae were placed in mesh bags, spun in a salad spinner for 1 min to remove excess water, and weighed. Replicate 5 g subsamples were placed in each unit; $n = 5$ for each treatment. Units were placed outdoors in a randomized array and covered with nylon window screens as during culture. Water temperature was ambient, fluctuating daily between approximately 18–22 °C (to 24 °C on 2 days), reflecting diurnal variations in poorly flushed, shallow estuaries in southern California in spring (Fong, 1986). The six treatment solutions were mixed from stock solutions weekly and water in each experimental unit was exchanged to simulate pulses of nutrients associated with storms. Salinity was monitored daily with a hand held refractometer and distilled water was added to compensate for evaporation. Salinity was maintained at 34 ± 2 ppt, which is typical for southern California estuaries between rainfall events (Fong, 1986). The experiment was run for 4 weeks.

Wet biomass was measured at the end of the experiment on 3 May 1998. Algae were removed from experimental units, placed in individually labeled nylon mesh bags, spun in a salad spinner for 1 min to remove excess water, and weighed. Growth was calculated as percent increase from initial wet biomass. After weighing, algae were rinsed briefly in distilled water to remove external salts, dried to a constant weight at 60 °C, re-weighed, ground with a mortar and pestle, and analyzed for tissue N and P. For total N, samples were ignited in a quartz combustion tube containing helium and oxygen in an induction furnace at approximately 900 °C. An aliquot of the combustion gases was passed through

a copper catalyst to convert nitrous oxides to N_2, scrubbed of moisture and carbon dioxide, and the nitrogen content was determined by thermal conductivity (Sweeney, 1989). For total P, we utilized a nitric acid/hydrogen peroxide microwave digestion (Johnson and Ulrich, 1959) and the analyte concentration was determined by inductively coupled plasma-atomic emission spectrometry (ICP-AES) with a vacuum spectrometer (Franson, 1985).

We reported tissue nutrients as a percentage of dry weight (concentration). We also calculated the total mass of N or P contained within all algal tissue in each experimental unit at the end of the experiment (% dry wt. N or P/100* mg total dry wt. of algae = N or P mass of algae as mg N or P per experimental unit). We used this measure of tissue nutrients in order to be able to compare N content of algae with different amounts of growth over the course of the experiment. Growth may dilute concentration, so measuring total mass in the tissue shows overall accumulation.

Water samples were collected from each experimental unit after the final week of the experiment. Our other studies using similar methods demonstrated that nutrient concentrations in the water were similar at the end of every weekly sampling over the course of several weeks (Kamer, Fong, and Kennison, unpublished data). Water samples were filtered through Whatman GF/C glass fiber filters and frozen until analyzed for NO_3, NH_4, DON (calculated as TKN–NH_4) and total P (TP). Total Kjeldahl Nitrogen (TKN) is measured by wet oxidation of N using sulfuric acid and digestion catalyst and subsequent determination of NH_4. The procedure does not include oxidized forms. NH_4 and NO_3 ($NO_3 + NO_2$) are measured by the diffusion-conductivity method as described by Carlson (1978). TP in seawater used the same methods as for tissue.

Among treatment differences in algal growth rates were analyzed with one factor ANOVA. Histograms of data and plots of residuals versus fitted Y values were examined to ensure data complied with ANOVA assumptions. No transformations were necessary. A significant ANOVA was followed by a Fisher's protected least significant difference test (PLSD) to compare means among treatments. Regression analysis was used to relate tissue N and P content (% dry wt.), N and P mass (mg per experimental unit), tissue N:P ratio, and final water column DON and TP with nutrient treatments. The relationship between tissue N and P was also quantified by simple linear regression. At the end of the experiment, water column NO_3 and NH_4 values were below the detection limit (lower limit of detection is 3.57 μM for all N analyses, 1.61 μM for P) in all experimental units, regardless of treatment and therefore no statistical analyses were possible. We calculated the net removal of N and P from the last pulsed nutrient addition by subtracting the total amount of N and P in the water at the end of the final week from the total amount in the weekly pulse (dissolved organic nutrients + added inorganic forms). This removal rate was normalized to biomass (g dry wt.) of algae. Regression analysis was used to relate net removal of N and P with nutrient treatments.

3. Results

There was a significant effect of nutrient treatment on growth of *E. intestinalis* (Fig. 1; ANOVA $P = 0.0001$). Growth exceeded 30% in 4 weeks in all treatments but the 1000 μM N:100 μM P addition (subsequently abbreviated 1000:100); growth in this treatment was

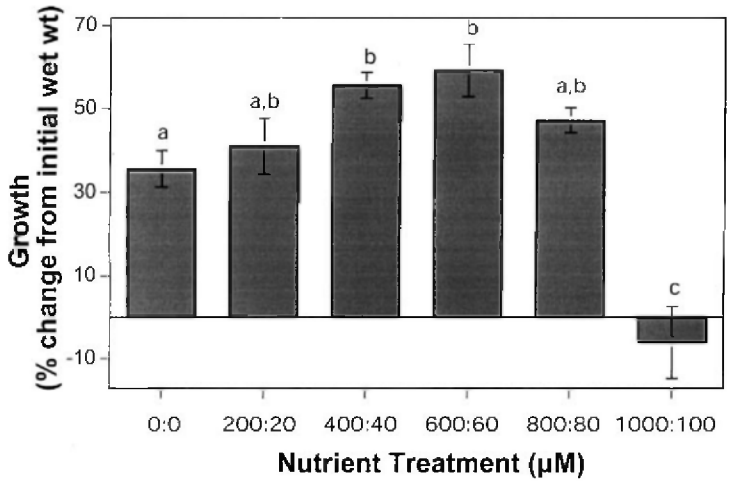

Fig. 1. Growth of *Enteromorpha intestinalis* after 4 weeks in nutrient enrichment treatments. Initial wet weight was 5 g and growth ranged from −2.5 to 21 mg wet wt. g wet wt.$^{-1}$ per day. Bars are ±1 S.E., $n = 5$. Letters represent the results of the post-hoc multiple comparisons. Means that are not significantly different share a letter.

lower than all other treatments (PLSD $P = 0.0001$ for all comparisons). Growth increased with nutrient treatment from the 0:0 through the 600:60 treatment, although mean values among treatments were not always significantly different.

Tissue N concentration increased with nutrient treatment (Fig. 2a); the best fit regression was an exponential equation. Initial tissue N concentration was 2.68% dry wt. (S.E. = 0.18), which is relatively high for algae in southern California estuaries (Kamer et al., 2001). Final mean values were lower than initial in all nutrient treatments from 0:0 through 600:60, probably due to dilution by growth. In the 800:80 treatment, tissue N concentration was extremely variable, but the mean approximated initial values. In the 1000:100 treatment, mean tissue N doubled from initial values due to continued uptake coupled with a lack of growth. In contrast to tissue N concentration, the total mass of N in algal tissue in each experimental unit increased linearly with nutrient treatment (Fig. 2b). Mean mass of nitrogen was about 10 mg in the 0:0 treatment and increased to over 22 in the 1000:100 treatment.

Tissue P showed the same overall pattern as tissue N (Fig. 3). When measured as concentration (% dry wt.), the relationship between nutrient treatment and tissue P was best described with an exponential equation. When calculated as total mass of P in algal tissue, there was a linear increase with nutrient treatment. However, there were two differences between accumulation of N and P during the course of the experiment. First, fit of both equations is better for P than N, demonstrating that accumulation of P in the tissue of this alga is less variable than N. Second, mean tissue P concentration only decreased below initial levels in 0:0 and 200:20 treatments; in all higher nutrient treatments tissue P concentration either remained the same or increased during the experiment, suggesting that P was more abundant than N compared to physiological requirements for growth.

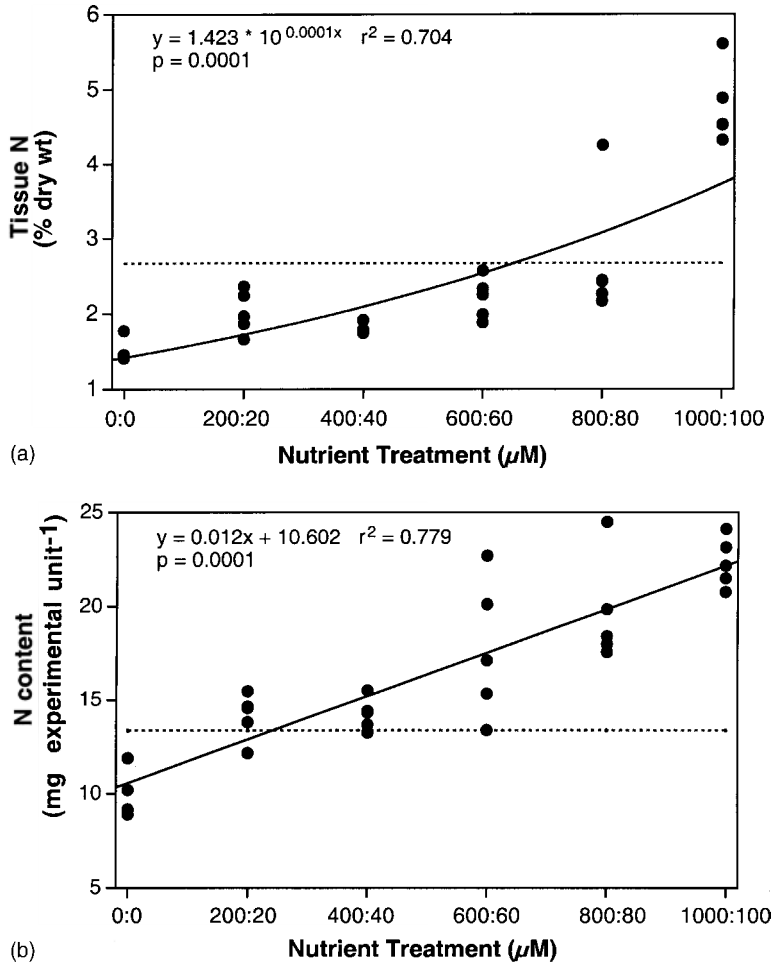

Fig. 2. Relationship between nutrient treatment and (a) tissue N and (b) accumulation of N in *Enteromorpha intestinalis*. Dotted horizontal lines indicate initial concentration or mass of N.

There was a linear relationship between the concentration of N and P in algal the tissue (Fig. 4a). N and P accumulated in a predictable proportion across all experimental treatments. Despite a constant N:P addition ratio of 10:1, N:P ratio in tissue decreased from an initial 26.2:1 (S.E. = 0.9) to a mean of 18.7:1 (S.E. = 0.6) across all treatments. In addition, there was a linear decrease in N:P ratio with increasing concentration (Fig. 4b), further supporting that P may have accumulated in tissue beyond requirements at the highest concentrations.

Both forms of inorganic N in the water (NO_3 and NH_4) were below detection limit in all 30 experimental units at the end of the experiment, demonstrating that all added NO_3 was taken up. DON in the water increased across all treatments (Fig. 5a), although the

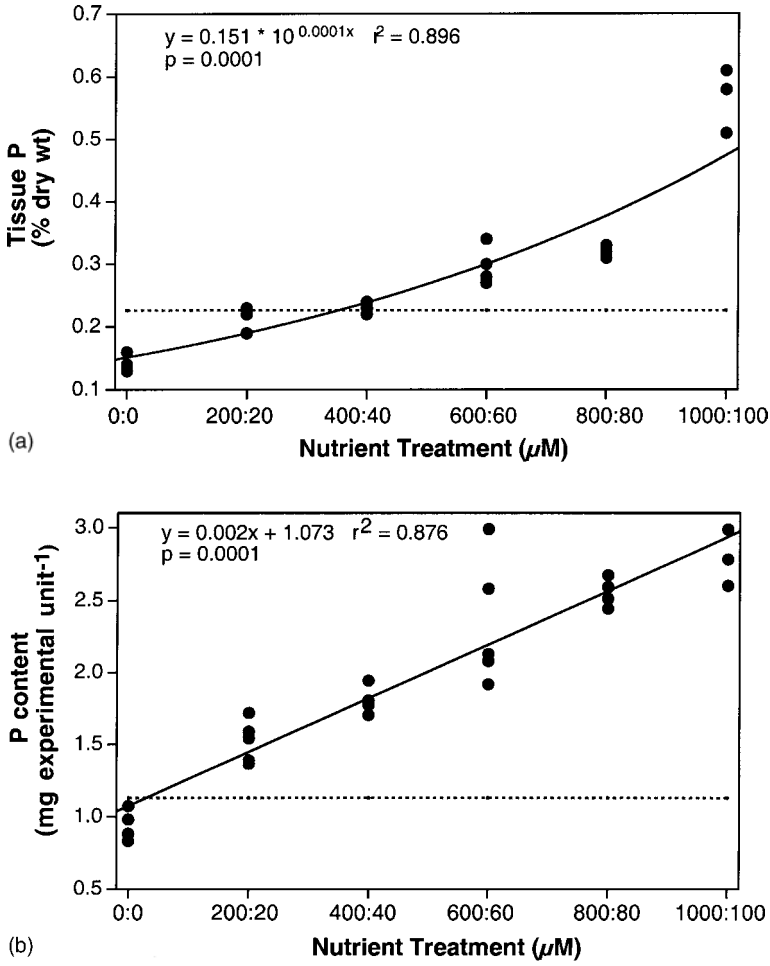

Fig. 3. Relationship between nutrient treatment and (a) tissue P and (b) accumulation of P in *Enteromorpha intestinalis*. Dotted horizontal lines indicate initial concentration or mass of P.

relationship is highly variable. This demonstrates that uptake of inorganic N was followed by the release of DON, especially at high N addition rates. TP also increased with nutrient treatment (Fig. 5b) in all treatments where it was measurable, though data were below detection limit in 13 of 30 experimental units. Most below detection measures were in addition rates of 400:40 and lower.

A second order polynomial produced the best fit between the N content of the nutrient pulses and net retention of N in tissue (Fig. 6a). Retention rates of N ranged from 73 to 98% with the lowest percent retention at both the highest and lowest enrichment treatments (Table 1). Where retention within the algal tissue was lower, it was a result of accumulation of DON in the water, as added NO_3 was all removed after 1 week. When retention was normalized on a biomass basis, retention increased with the magnitude

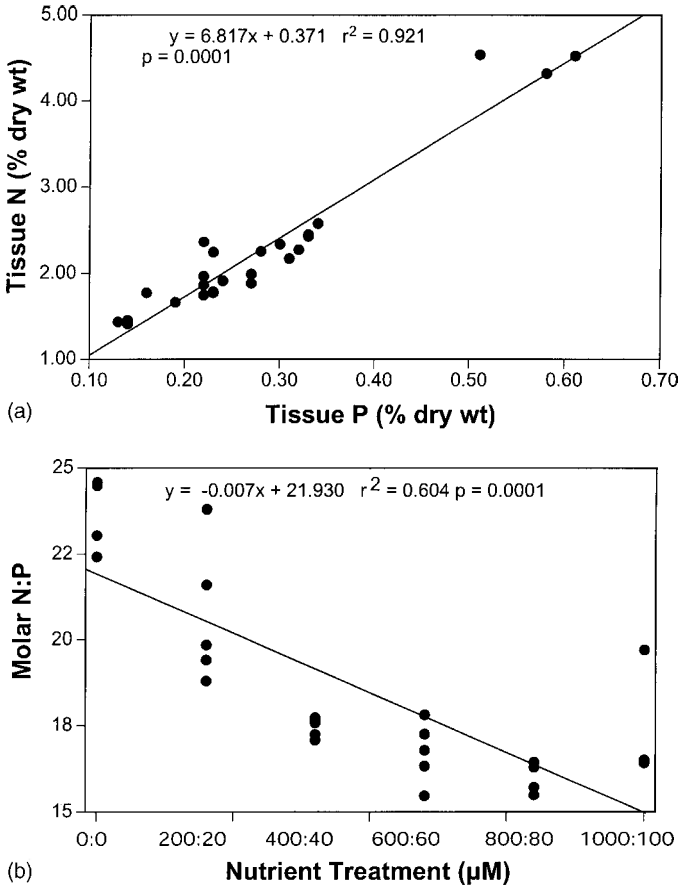

Fig. 4. Relationship between the concentration of N and P in tissue of *Enteromorpha intestinalis* in each experimental treatment.

of the N pulse, especially as pulses became very high; the line of best fit was a second order polynomial (Fig. 6b). The differences between these two regressions can be explained by the lack of growth but continued uptake at the highest nutrient addition rates.

Table 1
Percent retention of N and P supplied at weekly intervals in the tissue of *Enteromorpha intestinalis*

Treatment (µmol, NO_3:PO_4)	N retention (% of initial N supply)	P retention (% of initial P supply)
60:6	72.6 (9.8)	78.6 (n = 2)
120:12	98.4 (1.7)	87.5 (n = 1)
180:18	89.2 (6.2)	84.2 (3.7)
240:24	97.3 (1.9)	88.2 (2.1)
300:30	80.9 (5.4)	78.9 (2.6)

Data are means (standard error). $N = 5$ except where sample sizes are indicated in parentheses.

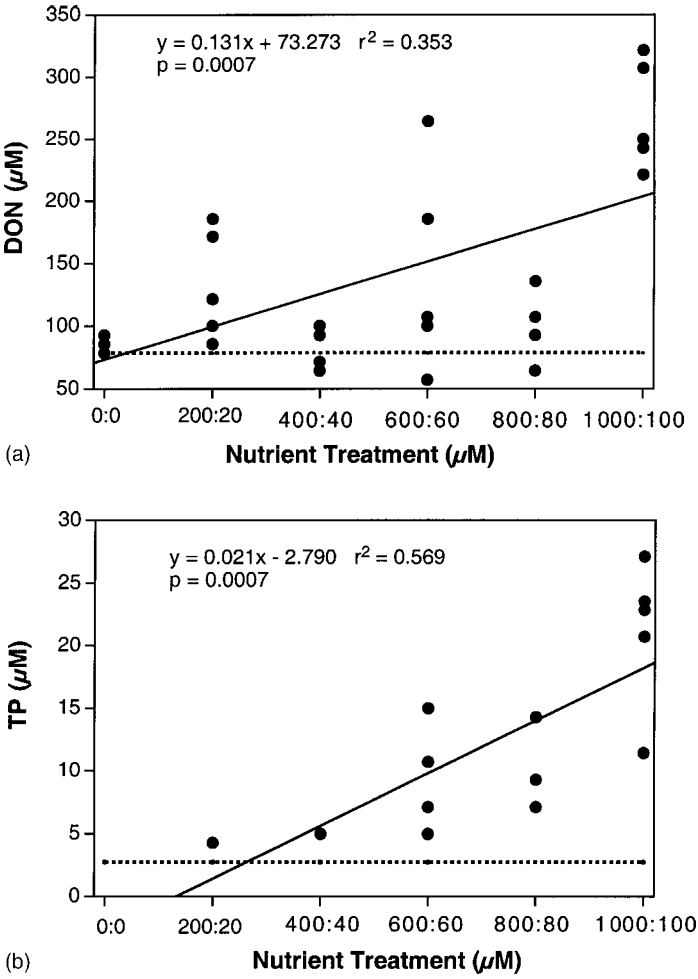

Fig. 5. Relationship between nutrient treatment and (a) DON and (b) TP in the water at the end of the experiment. Dotted horizontal lines indicate initial concentration of DON or P.

Retention of P followed a similar pattern as N, although the mechanism may have been different. The best fit relationship between P content of the nutrient pulses and net retention of P in the algal tissue was a second order polynomial as for N (Fig. 6c). Also, net retention was least at the lowest and highest nutrient pulse (Table 1). However, net retention rates were less variable across enrichment treatments for P than N (range = 79–88%). As we only measured total P in the water, it is unclear whether the lowest P retention rates were related to uptake of inorganic P and release of dissolved organic P, or simply that uptake was not as complete for P compared to N. In contrast to N, net removal of P normalized to biomass followed an exponential relationship due to the much greater relative retention at the highest P addition rate (Fig. 6d).

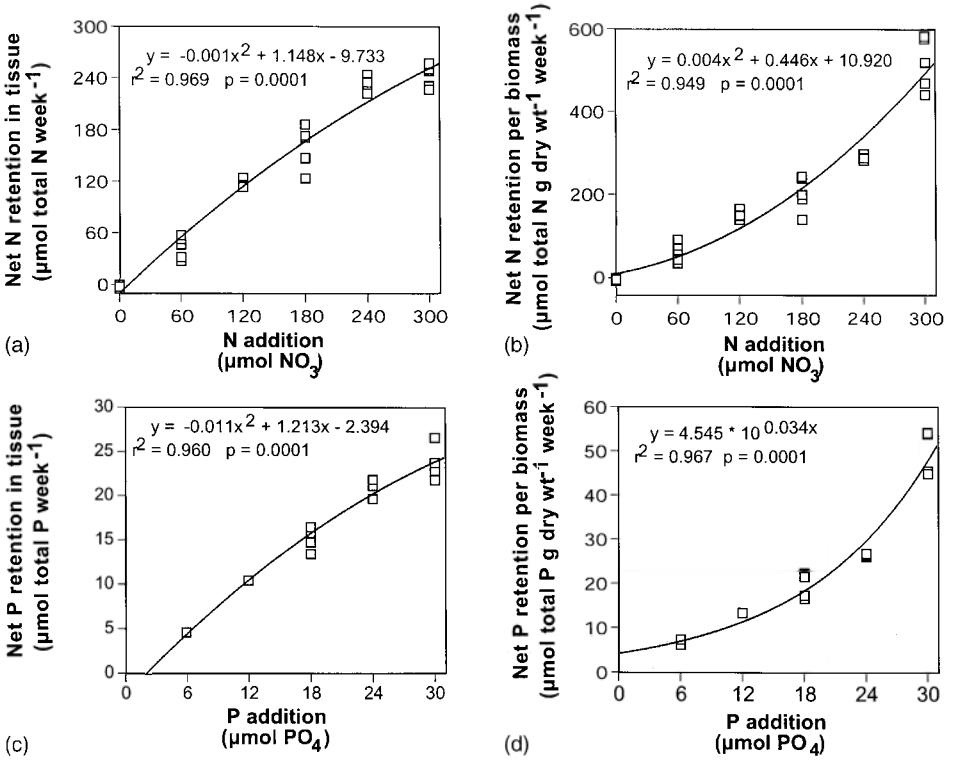

Fig. 6. Retention of N and P in algal tissue in relation to the magnitude of the weekly nutrient pulse. Retention was expressed as the total µmol of (a) N or (c) P retained in the tissue as well as normalized to biomass (b) and (d).

4. Discussion

Our study demonstrated that opportunistic macroalgae play an important role in estuarine nutrient cycling, functioning to retain pulsed supplies of N and P within estuaries, especially in eutrophic systems with Mediterranean climates. Although many studies have quantified the rapid uptake ability of opportunistic green macroalgae over the short term (e.g. Rosenburg and Ramus, 1984; Fugita, 1985; Duke et al., 1989; Duarte, 1995), and one has documented release of significant amounts of DON during active growth (Tyler et al., 2001), the longer term role of macroalgae as both sinks and sources of nutrients has not been investigated. We found that over 4 weeks, macroalgae were able to take up significant amounts of dissolved inorganic nitrogen (DIN) supplied in high concentration pulses typical of southern California estuaries and sequester it in their tissue. In addition, the largest percentage of the N supply was retained within tissues under conditions of moderate to high concentration pulses. In shallow eutrophic systems, the primary estuarine function of nutrient retention (Day et al., 1989) may be performed almost exclusively by macroalgae. Other primary producers such as seagrasses and longer-lived macroalgae that

traditionally function in nutrient retention are often absent in eutrophic estuaries such as those of southern California (Zedler, 1982; Stewart, 1991). Therefore, without blooms of opportunistic macroalgae, nutrients may be transported directly into the coastal ocean with the tides (Valiella et al., 1997), eliminating an important estuarine function.

Another important step in the N-cycle of estuaries that may be facilitated by macroalgae is the transformation of DIN to DON in the water column. DON is often an important component of the total dissolved N pool in estuaries (e.g. Fong and Zedler, 2000; Tyler et al., 2001). We found that concurrently with uptake of DIN, *E. intestinalis* released DON to the water column, and that, while variable, release was related to supply of DIN. In one field study, Tyler et al. (2001) found that healthy blooms of *Ulva lactuca*, another opportunistic green macroalga, can be a source of DON to the water during active growth. Uptake of DIN and transformation to DON contributes to the processing of N within the estuary. In addition, increasing supply of DON to the water may support a microheterotrophic food web, further processing pulses of N within estuaries and aiding in retention of N within the estuary.

No growth occurred in the 1000 μM N + 100 μM P treatment throughout the 4 weeks of the experiment. One possible explanation for this lack of growth was that the nutrient concentration in the pulses was so high that NO_3 or PO_4 reached toxic levels. We think this is unlikely as there is a rich literature on the toxicity of NH_4 to aquatic organisms (e.g. Peckol and Rivers, 1995; Underwood and Provot, 2000), but we found no evidence in the literature of NO_3 or PO_4 toxicity. An alternative explanation is that these opportunistic algae have evolved mechanisms to take advantage of extremely high concentration nutrient pulses by prioritizing allocation of available energy and carbon skeletons to nutrient uptake and assimilation of N. Turpin et al. (1988) found that addition of NO_3 resulted in increased rates of respiration and use of fixed carbon to synthesize amino acids. We hypothesize that at lower nutrient concentrations, algae were able to take up all available nutrients, and still have energy and fixed carbon left over for growth. However, at the highest nutrient addition rate it is possible that all resources were required for nutrient uptake due to greater supply, and none was left for growth.

In conclusion, although there are many ecosystem-level negative impacts of macroalgal blooms (e.g. Raffaelli et al., 1989; Valiela et al., 1997; Bolam et al., 2000), blooms of *E. intestinalis* play an important role in N retention and cycling in eutrophic estuaries of southern California. This role includes efficient uptake and storage of N in response to high concentration N pulses typical of human-dominated Mediterranean regions as well as release of DON in proportion to DIN supply, further contributing to N processing within estuaries. One mechanism that may aid in this function is the ability of this alga to allocate most of its energy toward nutrient uptake when pulses are large, delaying growth in favor of maximizing uptake and storage. These characteristics, coupled with the ability to accumulate large masses in the field (Kamer et al., 2001) suggest that *E. intestinalis* plays a significant role in nutrient cycling in shallow lagoons and estuaries.

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