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The effects of phosphate on the biomineralization of the green alga, *Halimeda incrassata* (Ellis) Lam.

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ABSTRACT

Field surveys indicated that individuals of *Halimeda incrassata* (Ellis) Lamouroux, a rhizophytic alga, were significantly more mineralized when collected from phosphate-limited carbonate sediments of the Florida Keys than those collected from siliciclastic sediments at Tarpon Springs on the west coast of Florida. Results from field experiments in Tarpon Springs, which compared growth of *H. incrassata* in enriched conditions to unmanipulated controls, indicated that biomineralization of new growth was significantly lower when phosphate was added to plots. After 18 days in a tank culture experiment, newly produced segments of *H. incrassata* grown in 20 μM phosphate showed a significant decrease in mineralization compared to the new growth in control tanks. Phosphate enrichment also led to a non-significant increase in segment mortality and a significant decrease in amount of new growth. Results from the survey and experimental studies suggest that biomineralization of *H. incrassata* is negatively impacted by elevated phosphate levels.

1. Introduction

Green seaweeds of the order Bryopsidales are abundant in tropical oligotrophic waters presumably due to their ability to absorb nutrients from sediment pore waters through means of an extensive rhizoid system (Williams, 1984). These rhizophytic algae fasten themselves to loose, sandy substrates, consolidating and adding organic matter to sediment. In some cases these sedimentary changes have been linked to the facilitation of seagrass bed development (Williams, 1990). Several genera in this order (including *Udotea*, *Penicillus*, *Halimeda*, and *Rhipocephalus*) calcify, which has been shown to inhibit herbivory by some animals (Hay et al., 1994) and add significantly to the production of new $CaCO_3$ sediments (Neumann and Land, 1975).

Calcification, the formation of calcium carbonate as either aragonite or calcite crystals (Barsanti and Gualtieri, 2006), is common in many marine organisms including corals, mollusks, seaweeds, and plankton. Whereas some organisms spend metabolic energy on ion transport to orderly calcify (Palmer, 1992), calcareous Bryopsidalean algae can be viewed as adventitious calcifiers, in that the anatomy and physiology of these algae allow calcification to occur outside of the thallus, as a by-product of metabolic processes (Borowitzka and Larkum, 1976). Accordingly, the calcification process in these algae may be more susceptible than other calcareous organisms to changes in environmental conditions, such as those imposed by eutrophication, climate change, and ocean acidification.

The genus *Halimeda* contains species found world-wide in tropical waters and the alga may serve as a model organism for the study of macroalgal calcification. Close examination of *Halimeda* thalli reveals tightly compacted utricles that form invaginations separated from the seawater environment (Wilbur et al., 1969) referred to as the intercellular spaces (ICS). When the plant uptakes CO_2 for photosynthesis, the pH in the ICS rises markedly causing a shift in the CO_2 equilibrium from HCO_3^- to CO_3^{2-}, which leads to the precipitation of $CaCO_3$ onto Ca^{2+} binding polysaccharides attached to the cell wall (Böhm, 1972a; Bilan and Usov, 2001). Since these algae do not regulate calcification of their thalli, environmental factors, such as nutrient levels within the ICS, may be more important in controlling the calcification process. Accordingly, ions other than calcium have been reported in the calcium carbonate matrix (Böhm, 1972b).

Phosphate is known to act as a "crystal poison", preventing the precipitation of $CaCO_3$ (Simkiss, 1964; Lin and Singer, 2006). Likewise phosphate levels may vary considerably among areas where *Halimeda* exists given that species have been reported not only in tropical carbonate systems but also in subtropical siliciclastic areas (Dawes and Mathieson, 2008; Littler et al., 2008). Primary production in carbonate systems are generally phosphate limited presumably due to the adsorption of phosphate onto carbonate (Short, 1987) while siliciclastic environments are generally thought to experience less oligotrophic conditions and are generally nitrogen limited (Lapointe et al., 1992; Erftemeijer and Middelburg, 1993). Thus it is of interest to determine if amount of calcification of *Halimeda* is linked to ambient phosphate level.

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The goal of this study is to examine the effects of elevated phosphate concentrations on the calcification of *H. incrassata* (Ellis) Lamouroux, a calcareous member of the Bryopsidales common to the west coast of Florida (Dawes, 1974), as well as to the Florida Keys (Croley and Dawes, 1970; Dawes and Mathieson, 2008). However, since the dry-ash method we chose to evaluate calcification does not discriminate between Ca^{2+} and other bound ions, we will refer to our analysis as biomineralization (mostly $CaCO_3$ + other minerals in this matrix) from here on out. Specifically we ask: Is there a difference in the percent biomineralization of *H. incrassata* in the nitrogen limited waters north of Tampa Bay (Fourqurean and Cai, 2001) compared to those in phosphate limiting waters in the Florida Keys (Fourqurean and Zieman, 2002)? Does the addition of phosphate to the water column surrounding these seaweeds cause a change in the biomineralization of new growth? In a controlled laboratory setting, does phosphate enrichment lead to changes in growth and biomineralization of *H. incrassata*?

2. Materials and methods

2.1. Comparison of Halimeda calcification in field sites

A field survey of *H. incrassata* was carried out to determine the percent biomineralization at two sites in Florida. One shallow water site was Sunset Beach, at Tarpon Springs, Florida (28°08′43″ N, 82°47′27″ W). Both rhizophytic algae communities and seagrass beds dominated by *Thalassia testudinum* Koenig et Sims, *Syringodium filiforme* Kützing and *Halodule wrightii* (Ascheron) Ascheron are present. The dominant macroalgae are fleshy members of the Bryopsidales, including *Caulerpa prolifera* (Forsskål) Lamouroux, *C. cupressoides* (West) C. Agardh, and *C. ashmeadii* Harvey plus calcareous taxa (*H. incrassata*, *H. monile* (Ellis et Solander) Lamouroux, *Penicillus capitatus*, and *P. lamourouxii* Decaisne). Salinity was 28.8 ppt at time of collection and water temperature was 21.6 °C. Sediments at Sunset Beach are dominated by siliciclastic deposits and seagrass growth at the nearby Anclote Key is thought to be nitrogen limited (Fourqurean and Cai, 2001). Moreover, high phosphate availability and nitrogen-limited macroalgal growth was recently reported in other areas of Southwest Florida (Lapointe and Bedford, 2007).

The other site for surveys was located on Summerland Key in the Florida Keys at the Mote Marine Tropical Research Laboratory (24°39′27″ N, 81°27′19″ W). This site is similar to Sunset Beach in species composition, with additional species of *Caulerpa*, *Halimeda*, and *Penicillus*. The waters around this site are known to be phosphate limiting (Fourqurean and Zieman, 2002) and sediments are characterized as carbonates. The water temperature at the time of collection was 27 °C with a salinity of 36 ppt.

H. incrassata was collected from both field sites in November 2006. Sample sizes for Sunset Beach and Summerland Key were 28 and 25, respectively. Macroalgal thalli were returned to the laboratory, cut above the holdfast, and were dried for 24 h in an oven at 55 °C, and weighed (g). Dried thalli were then muffled at 500 °C for 3 h, allowed to cool in a dessicator, and the percent biomineralization determined (ash (g)/dry weight (g) × 100). The dry-ash method does not directly measure the $CaCO_3$ content of an alga, but rather the ratio of organic to inorganic (mineral) fractions. However, it can be used as a proxy for calcification in calcareous rhizophytic algae (see Ries (2006) and Davis and Fourqurean (2001) for example) because calcification occurs as a by-product of metabolism at a set ratio (Borowitzka and Larkum, 1976) in the absence of a calcification inhibitor.

2.2. Phosphate enrichment: field study

In February 2007 a field experiment was initiated to investigate the influence of enhanced phosphate levels on new growth of *H. incrassata*. Because this alga grows in chains of calcified segments, assessment of growth can be accomplished by marking intact segments and enumerating new unmarked components. Sixty clean *H. incrassata* plants were collected from Tarpon Springs, Florida, placed into buckets filled with seawater from the study site, and returned to the lab. These individuals were then placed in aquaria filled with seawater from the field site and dyed with Alizarin Red-S for 24 h (Multer, 1988).

The next day, algae were removed from aquaria and returned to the collection site. Algae were placed into twelve 0.25 m^2 quadrats and assembled into rings of five per quadrat. Quadrats were arranged in two parallel transects 10 m apart with 3 m between each quadrat. Any residing macroalgae were removed. Slow-release fertilizer pods (nylon netting with monohydrous NaH_2PO_4) were placed in the middle of each ring in every other quadrat, representing the phosphate-enriched treatment; other quadrats lacking fertilizer served as the control or ambient group. After 14 days, plants were collected from each quadrat and the thalli dried for 24 h in an oven at 55 °C, and weighed (g). The percent calcification of newly matured (non-dyed) segments was determined as described above for each quadrat of the enriched and control treatments.

2.3. Phosphate enrichment: Laboratory study

In order to observe plant responses in precisely manipulated PO_4^{3-} levels, we performed a study similar to the field fertilization experiment in a laboratory setting. Eighty clean, healthy *H. incrassata* individuals were collected from Sunset Beach on June 14, 2007 and were dyed with Alizarin Red-S in aquaria for 24 h as described above. Eight randomly selected individuals were placed in each of ten aquaria; 5 aquaria had an elevated phosphate level, and 5 aquaria served as controls. Individual algae were considered subsamples.

Experiments were conducted in 37 L (10 gal) aquaria each of which had been acid washed in 10% HCl and rinsed with distilled H_2O. Aquaria were filled with approximately 30 L of artificial seawater (Instant Ocean©) mixed to 34.5 ppt. Three replicates of seawater mixed with Instant Ocean© to 35.0 ppt were analyzed to determine nutrient levels before addition of nitrate and phosphate to aquaria. Phosphate levels were 0.15 μM + 0.025, while nitrate levels were 2.28 μM ± 0.05. Sufficient $NaNO_3$ was added to each aquarium to raise the nitrate levels to 1.5 μM, producing actual nitrate concentrations of approximately 3.78 μM in all aquaria. NaH_2PO_4 was also added to simulate PO_4^{3-} levels of 1.0 μM (1.15 μM) in 5 of the aquaria (representing the control treatment) and 20.0 μM (20.15 μM) in the other 5 to serve as the enriched treatment. Temperature and salinity were monitored daily and distilled water was added to maintain a salinity of 35.5 ppt.

Algae were placed into holders made of pieces of PVC (3.5 cm diameter) that were cut to a length of 3.8 cm and 5.2 cm of plastic mesh with openings of 3.5 cm rolled inside of the PVC holders that were then inserted into the aquaria. The holders allowed the thalli to remain upright in the absence of sand. After 18 days plants were harvested, the new growth removed from each plant, and percent calcification of new growth determined as described above. Any detached dead segments of *H. incrassata* were collected from the bottom of the aquarium, weighed, and divided by the total starting biomass (g) per aquarium (dead fragments and dyed living material) to determine percent lost due to segment mortality. Percent calcification of new growth was determined per plant using the ashing procedure and a mean calculated per aquarium. Biomass of new growth per aquarium (g) was divided by the starting biomass in each aquarium (dead fragments and dyed living material) to correct for variable starting sizes.

2.4. Statistical analysis

Data were analyzed using SigmaStat 3.5.0. One-way analysis of variance tests (ANOVA) were used to test for significant differences in % calcification of algae between survey sites and between treatments in experiments. Likewise, ANOVA was used to test for differences in growth rates and biomass of detached algal segments in enriched vs. control treatments in laboratory experiments. A difference was considered to be statistically significant with $P<0.05$.

3. Results

3.1. Comparison of Halimeda calcification in Florida sites

Percent mineralization (mean ± SD) of *H. incrassata* was 70.4 ± 2.8 and 79.7 ± 2.4 at Sunset Beach ($n = 28$) and Summerland Key ($n = 25$), respectively. The higher percent mineralization in plants from the phosphate limited site in the Florida Keys compared to ones from Tarpon Springs was highly significant ($F_{1,51} = 101.68$ *P*-value < 0.001).

3.2. Phosphate fertilization in the field

Sixty-six percent of quadrats in the fertilization experiment contained *H. incrassata* plants remaining at the end of the two week experiment. Plants were either dead or missing in other plots. The highest mean percent mineralization of newly formed segments of *H. incrassata* in a fertilized plot was lower than the lowest mean value from plants harvested from control plots (Fig. 1A). The average percent mineralization of new growth in fertilized plots ($\bar{x} = 69.3 \pm 3.0$, $n = 3$) was significantly lower than in control plots ($\bar{x} = 74.2 \pm 0.9$, $n = 5$) ($F_{1,6} = 10.597$ *P*-value = 0.017) (Fig. 1B).

3.3. Enrichment of phosphate in aquaria

In laboratory experiments, where phosphate levels were more closely controlled, results mirrored the responses noted in the field experiments (Fig. 2A, B). Mean percent mineralization of new growth of *H. incrassata* was significantly lower ($F_{1,8} = 6.389$ *P*-value = 0.035) in enriched ($\bar{x} = 64.7 \pm 5.3$, $n = 5$) vs. control ($\bar{x} = 72.2 \pm 3.9$, $n = 5$) phosphate treatments (Fig. 2B). Likewise, mean biomass of new growth per aquarium (as % dry wt. of initial biomass) over the 18d period was significantly lower ($F_{1,8} = 12.458$ *P*-value = 0.008) in phosphate enriched treatments (Fig. 3). Mean percent starting mass lost to segment shedding was higher in enriched treatments but this was not significantly different ($F_{1,8} = 3.341$ *P*-value = 0.101).

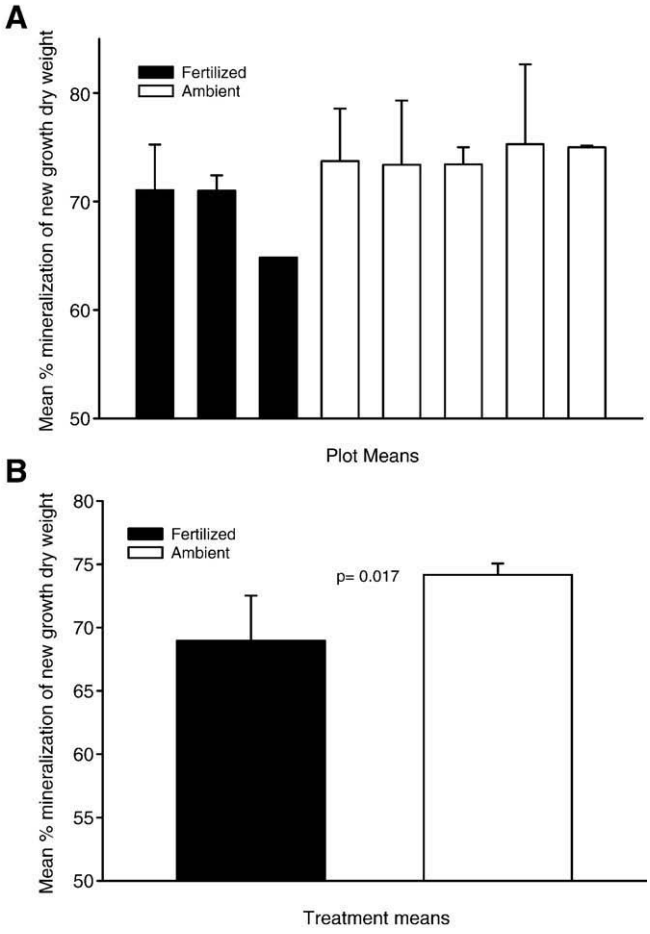

Fig. 1. Percent mineralization of new growth dry weight (g) in *Halimeda incrassata* plants in phosphate fertilized and control (ambient) plots per plot, where plants could be recovered (A) and per treatment (plants as subsamples) (B) in the field experiment. Values are mean ± SD. *P*-value for ANOVA is included.

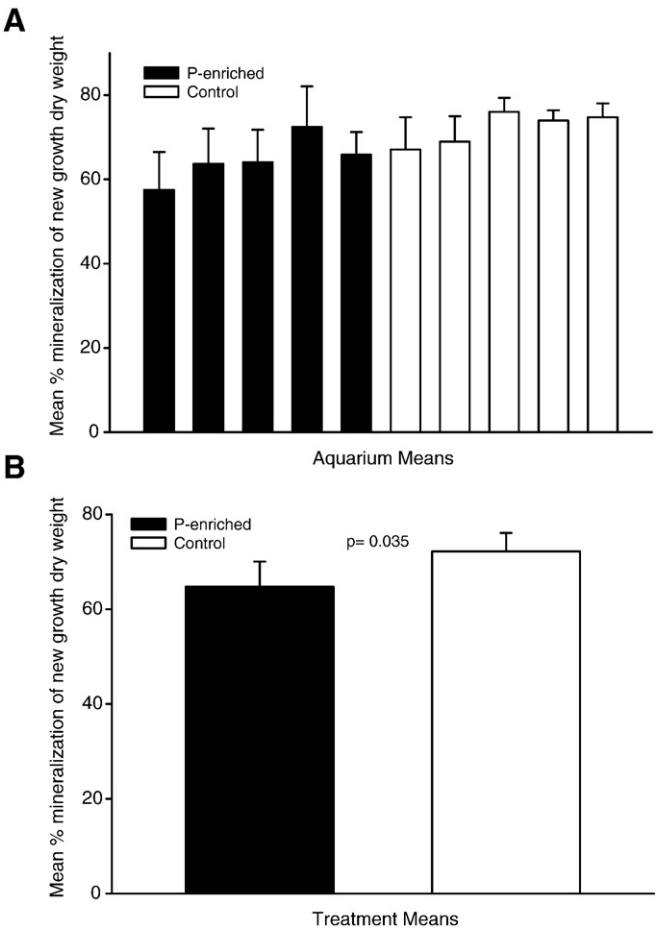

Fig. 2. Percent mineralization of new growth in g dry weight of *Halimeda incrassata* plants in phosphate enriched and control aquaria per aquarium (A) and per treatment (plants as subsamples) (B). Values are mean ± SD. *P*-value for ANOVA is included.

4. Discussion

In both field and laboratory experiments, a significant decrease in percent biomineralization of new growth of *H. incrassata* was recorded under enriched PO_4^{3-} conditions, supporting the hypothesis that elevated phosphate levels inhibit biomineralization. Likewise the results from the field survey indicate a higher percent biomineralization of above-ground biomass of *H. incrassata* collected from areas with documented low phosphate levels (Fourqurean and Zieman, 2002) compared to plants collected from siliciclastic sediments or in phosphate enriched experimental conditions (79 vs. 70–73%, respectively). While salinity is known to influence percent ash content of seaweeds (Lapointe et al., 1984), and might be a large factor causing the differences between field sites, phosphate also seems to be a likely contributor. Collectively these results suggest that increased phosphate levels may result in decreased biomineralization of this rhizophytic alga. Similarly, increased

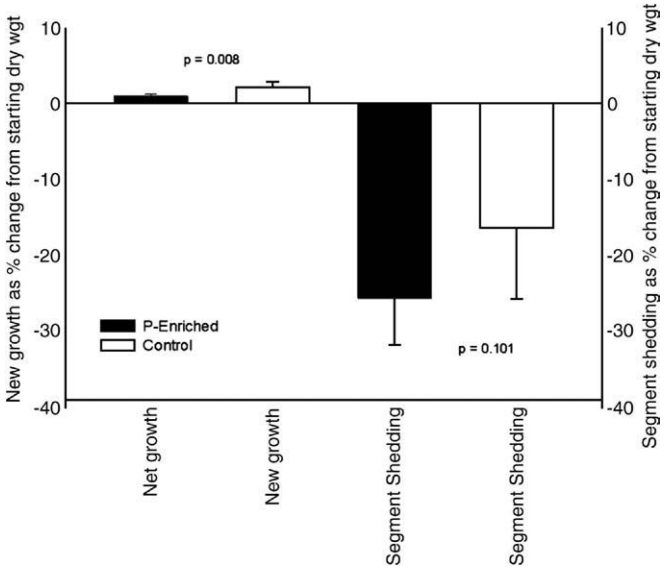

Fig. 3. Average biomass of *Halimeda incrassata* lost to segment shedding and gained via new growth as percent of starting biomass in fertilized and control aquaria over 18 days. Values are means per treatment ($n = 5$ for each) ± SD. *P*-values for ANOVA are included.

phosphate levels were found to decrease calcification of the related *P. dumetosus* (Delgado and Lapointe, 1994).

In lab experiments, not only was a decrease in biomineralization of new growth recorded for plants in aquaria with enriched phosphate levels, but on average more biomass was also lost in each tank to segment shedding than was added via new growth. This may suggest that the algae were stressed, potentially due to the artificial nutrient regime and/or limited water flow. Yet, biomineralization of algae in control conditions in the lab were at the level observed for field conditions so the reason remains unknown. However, despite stresses associated with the lab conditions, statistically significant differences in new growth and biomineralization were observed between treatments and the results resembled those from field experiments.

Reduced biomineralization of algae may have implications for higher trophic levels. It has been observed that, among algae, biomineralization serves a role in protecting against herbivory. For example, Hay et al. (1994) found that addition of $CaCO_3$ to food inhibited the urchin *Diadema antillarum* and the amphipod *Cymadusa filosa*. Further support for the idea that biomineralization evolved as an anti-grazing mechanism comes from the discovery that non-calcareous algae in the Bryopsidales contain greater concentrations of deterrent secondary metabolites than do their calcareous relatives (Paul, 1985). Accordingly, inhibition of the biomineralization process could pose a problem for calcareous organisms in areas of elevated phosphate levels if lower levels of biomineralization leave these algae more susceptible to herbivory.

Do high nutrient levels negatively influence the distribution of calcareous rhizophytic algae? Interestingly, rhizophytic algae that are common in algal beds in the Gulf of Mexico near Tampa Bay (Dawes, 1967), have not been reported as occurring within Tampa Bay itself, yet fleshy relatives, such as *C. prolifera*, occur abundantly (Stafford and Bell, 2006). Water column concentrations greater than 2 μM PO_4^{3-}, a level shown to inhibit coral calcification by up to 50% (Kinsey and Davies, 1979), have been recorded in Tampa Bay (Williamson et al., 2002). Such high levels of phosphate in Tampa Bay may explain, at least in part, the lack of calcified members of the Bryopsidales in Tampa Bay (Dawes, 1967), although other factors, such as salinity, may also be important in controlling the distribution of calcified rhizophytic algae (e.g. Biber and Irlandi, 2006). While our study provides initial assessment of a phosphate-biomineralization link, long term fertilization experiments on beds dominated by calcareous rhizophytic algae may help further address the impacts of eutrophication on this ecologically important group.

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