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Evidence of grazer control on nitrogen fixation by eelgrass epiphytes in a temperate coastal bay

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ABSTRACT: In this study, we present data to support the hypothesis that removal of epiphytes by grazers is an important control of nitrogen fixation in temperate seagrass meadows during the summer. Previous work in West Falmouth Harbor, Massachusetts, USA, found highest rates of epiphytic nitrogen fixation in the part of the harbor (Snug Harbor) with the greatest nitrogen load and the lowest phosphate concentrations, a somewhat paradoxical result suggesting that biogeochemical controls are not the major factor regulating this nitrogen fixation. Here we report that the density of invertebrate grazers on epiphytic algae (predominantly *Bittiolum alternatum*) was least in Snug Harbor, where nitrogen fixation rates were greatest. Reciprocal transplant experiments showed that seagrass shoots transplanted into Snug Harbor from the part of the harbor (Outer Harbor) where external nitrogen loading was lower but grazer densities were 4-fold higher, had a more than 5-fold increase in epiphytic nitrogen fixation after a 12 d incubation period. Shoots transplanted from Snug Harbor to Outer Harbor showed a large, rapid reduction in epiphytic nitrogen fixation rates after only 6 d, likely due to consumption of epiphytes. Our results suggest that trophic control is a potentially important determinant of epiphytic nitrogen fixation rates in temperate seagrass meadows.

KEY WORDS: *Zostera marina* · Nitrogen fixation · Herbivory · Nutrient cycling

INTRODUCTION

Nitrogen fixation in seagrass meadows occurs both in the sediments and on the surface of leaves, and is attributed to both autotrophic and heterotrophic nitrogen fixers (O'Donohue et al. 1991, McGlathery et al. 1998, Cole & McGlathery 2012). Epiphytic fixation by cyanobacteria on seagrass leaves can occur at high rates and often accounts for a significant portion of total nitrogen fixation in these ecosystems (Capone & Taylor 1977, Capone et al. 1979, Howarth et al. 1988a,b). Autotrophic nitrogen fixation is often thought to be controlled primarily by physical and chemical factors such as light availability, nitrogen and phosphorus concentrations, and the water column N:P ratio (Capone & Taylor 1977, Howarth et al. 1988a,b, Welsh et al. 2000). Heterotrophic nitrogen fixation is controlled largely by carbon availability, although rates can be partially suppressed by high nitrogen concentrations (Goering & Parker 1972, Howarth et al. 1988a,b, Welsh et al. 1996, McGlathery 2008, Cole & McGlathery 2012).

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Grazing has been shown to constrain nitrogen fixation in some aquatic environments (Vitousek et al. 2002). For example, direct consumption of pelagic cyanobacteria by zooplankton can limit planktonic nitrogen fixation (Schaffner et al. 1994, Howarth et al. 1999, Marino et al. 2002, Chan et al. 2006). We hypothesize that grazing may also be an important control on epiphytic nitrogen fixation in seagrass meadows. The growth of epiphytes on seagrass leaves can be controlled by a number of factors (i.e. light availability, nutrient availability and grazing), and where present in significant numbers, grazers may be the most important factor regulating epiphyte abundance (e.g. Neckles et al. 1993, Moore & Wetzel 2000, Duffy & Harvilicz 2001, Whalen et al. 2013). Grazers decrease biomass, and can also influence the diversity of the epiphyte community by selective feeding (Duffy & Harvilicz 2001, Hughes et al. 2004, Jaschinski & Sommer 2008). It follows that by controlling epiphyte populations, grazers may also regulate nitrogen fixation, but this has not been explicitly studied in previous research.

As part of a multiple-year study on coupled biogeochemical cycles of carbon, nitrogen (N) and phosphorus (P) along a nutrient enrichment gradient in West Falmouth Harbor, Massachusetts, USA (Ganju et al. 2012, Hayn et al. 2013, Howarth et al. 2014), we made regular measurements of nitrogen fixation associated with epiphytic cyanobacteria on seagrass blades during the peak summer season. Somewhat surprisingly, the highest rates of fixation were found in the part of the ecosystem that has the highest nitrogen load and the lowest concentration of dissolved inorganic phosphorus, suggesting that these biogeochemical factors are not the dominant control on this process (Howarth et al. 2014, R. Marino unpubl. data). The goal of the study we report on here was to investigate whether the counter-intuitive patterns in the rates of epiphytic nitrogen fixation that we have observed can, at least in part, be explained by a difference in 'top down' (grazing) control, rather than well-recognized 'bottom up' factors such as water column N or P concentrations or the N:P ratio.

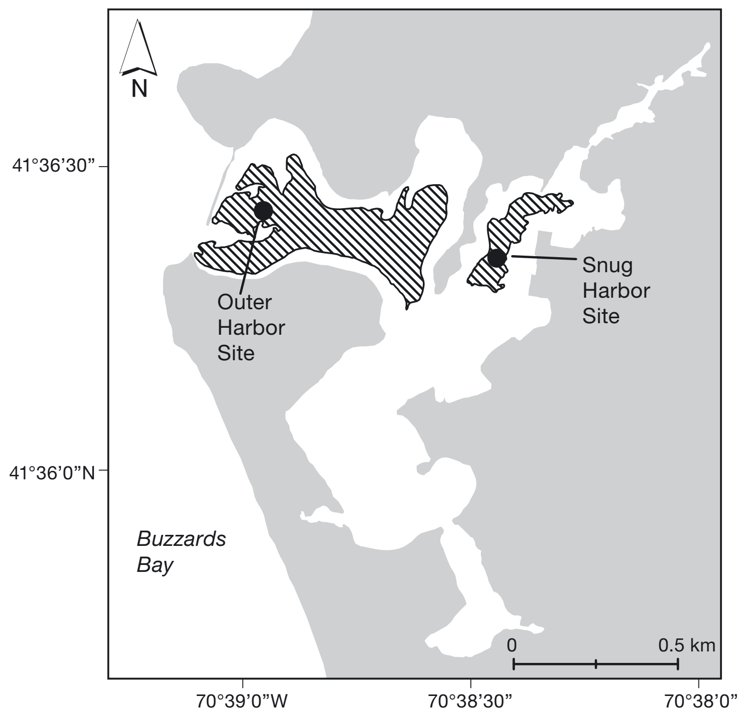

Fig. 1. West Falmouth Harbor System, Massachusetts, USA. The hatched area shows seagrass distribution in 2010 (Hayn 2012), and the dots show the approximate locations of the Snug Harbor and Outer Harbor sampling sites

MATERIALS AND METHODS

Site description

West Falmouth Harbor, MA, USA is a shallow lagoon that opens to Buzzards Bay through a single inlet on the western edge. This study was conducted in 2 discrete parts of the West Falmouth Harbor system: Snug Harbor (41.607° N, 70.638° W), located in the northeast part of the harbor, and Outer Harbor (41.607° N, 70.649° W), located just inside the inlet (Fig. 1). Mean external nitrogen load to the entire system was relatively high (4.2 mmol N m^{-2} d^{-1}), with two-thirds of this coming in via a plume of groundwater contaminated with nitrate from a municipal wastewater treatment facility (Hayn et al. 2013). This plume enters through Snug Harbor, and as a result the nitrogen load to that area was almost 4-fold higher (17 mmol N m^{-2} d^{-1}) than the whole-harbor average external N load (Howes et al. 2006, Hayn et al. 2013, Howarth et al. 2014).

Water column temperatures did not vary between basins, while oxygen concentrations and pH were lower in Snug Harbor than the Outer Harbor (Howarth et al. 2014). The mean (±SE) temperatures in Snug Harbor and Outer Harbor during the July–August period for 2008 and 2009 were 23.8 ± 0.8°C and 23.8 ± 0.9°C, respectively. Median values for daily minimum dissolved oxygen during July and August 2008 and 2009 in Snug Harbor ranged from 40 to 55% saturation, and from 60 to 75% saturation in the Outer Harbor; median pH values ranged from 7.6 to 8 in Outer Harbor and from 7.2 to 7.7 in Snug Harbor (Howarth et al. 2014).

In 2008, the mean (±SE) dissolved inorganic nitrogen (DIN) concentration in both systems for July and August was 0.5 ± 0.1 µM (R. Marino unpubl. data). In 2009, DIN concentrations were similar to 2008 in the Outer Harbor (0.4 ± 0.1 µM), while values were slightly higher in Snug Harbor (0.7 ± 0.4 µM). Mean dissolved inorganic phosphorus (DIP) values in 2008 at both sites were higher than in 2009 (Outer Harbor: 1.1 ± 0.1 vs. 0.3 ± 0.1 µM; Snug Harbor: 0.8 ± 0.1 vs 0.2 ± 0.0 µM) but did not vary significantly between basins within the same year. The relative abundance of water column inorganic nutrients in July and August did not vary between the 2 West Falmouth Harbor basins in 2008 (Outer Harbor molar DIN:DIP 0.5 and Snug Harbor DIN:DIP 0.6). DIN:DIP was higher in Snug Harbor (3.5) than in Outer Harbor (1.6) in 2009, but was still well below the Redfield ratio (16:1) and indicative of strong nitrogen limitation for microalgae (Howarth et al. 2014).

Experimental design and analyses

Differences in epiphyte nitrogen fixation between Snug Harbor and Outer Harbor were assessed using 2 reciprocal transplant experiments — one in early August of 2008 and one in late July of 2009. Intact replicate shoots collected from each harbor were attached to cotton line using zip ties and each end of the line was attached to a survey flag, creating one sampling unit. Sampling units were either placed back into eelgrass meadows in their original harbor (Control, denoted SH-SH or OH-OH in Figs. 2 & 3) or transplanted into eelgrass meadows in the alternate harbor (Transplant, denoted SH-OH or OH-SH), for a total of 4 experimental treatments. In 2008, plants were incubated *in situ* for 6 d, and in 2009, in order to better assess longer-term accumulation of epiphytes, half of the plants were removed after 6 d and half were incubated for 12 d. Sampling units were returned to the laboratory and epiphytic nitrogen fixation for each treatment was estimated on randomly selected shoots using the acetylene reduction technique (n = 5 in 2008, n = 7 in 2009). The top 15 cm of the second and third leaves (in order of age) were placed into 120 ml serum vials filled with 90 ml of 0.2 µm filtered seawater from the incubation site. Leaves 2 and 3 were chosen as representative of the whole shoots (i.e. both younger and older leaves), and a total length of 15 cm was used to keep leaf volume and headspace in the incubation bottles constant. Ten ml of acetylene-saturated, filtered seawater were added (Marino et al. 2002) and the samples were incubated at ambient site temperature and saturating light levels (approx. 650 µmol photons m^{-2} s^{-1}) for 4 h in a Conviron PGR15 growth chamber. We did not include acetylene-added dark incubation treatments for these experiments because ethylene production in dark incubations during routine N fixation measurements made as part of the larger, multiple-year study previously mentioned (see 'Introduction') rarely exceeded the blank level and was always orders of magnitude lower than in light incubations (R. Marino unpubl. data).

The incubation was ended by immediately immersing the bottles in a large bath of ice water (Marino et al. 2002); samples were held at 0°C until analysis. Bottles were well mixed and the headspace was sampled using a gas-tight syringe and analyzed for ethylene concentration using a Shimadzu GC-14A gas chromatograph equipped with a flame ionization detector and a 15.2 × 0.3 × 0.2 cm Teflon-coated column filled with Porapak N 80/100. Rates of ethylene produced from blades 2 and 3 were averaged to estimate a total shoot rate and were scaled to the area of the leaf (1-sided) incubated, and expressed as fixed N using the theoretical 3:2 molar conversion (Howarth et al. 1988a, Montoya et al. 1996). At the conclusion of the experiment in 2008 (but not in 2009), total biomass of epiphytes from each incubated leaf sample was quantified as described below. Differences in nitrogen fixation rates and epiphytic biomass were analyzed using a 1-way ANOVA with treatment (parent harbor–incubation harbor) as the fixed factor followed by post-hoc Tukey tests.

Epiphyte biomass was estimated from 10 whole shoots randomly chosen from the meadow area surrounding each experimental study site. Shoots were carefully collected stored in individual bags, and brought back to the laboratory to estimate total and organic epiphyte biomass. The epiphytes were scraped from each leaf of a replicate shoot using a razor blade; leaves 2 and 3 were pooled, and the remaining leaves were pooled. Epiphytes were rinsed on a glass fiber filter, dried, weighed, and then combusted at 500°C for 4 h and re-weighed. Empty tins and filters were used as a control to check for procedural error. Organic epiphyte biomass was calculated as ash-free dry weight, and both total and organic biomass were reported per shoot leaf area (measured length × width) and reported for leaves 2 and 3 (those used in the experiment) and for all leaves on the shoot pooled together. Differences in epiphyte biomass between Outer Harbor and Snug Harbor were determined using a 1-way ANOVA.

During the summers of 2008 and 2009, invertebrate grazers of seagrass epiphytes in both Outer Harbor and Snug Harbor were determined at 2 sites per harbor, adjacent to the locations of the reciprocal transplant experiment. Scuba divers collected duplicate samples (1–2 m apart) by carefully inserting polycarbonate core tubes (9.5 cm inner diameter × 30 cm length) to a depth of 20 cm below the sediment–water interface. Care was taken not to disrupt the seagrass canopy and associated mesograzers, which were included in the sample, and the core tube was immediately capped. Cores were chilled and transported to the laboratory for processing. Samples were passed through a sieve (1 mm) and fixed in a solution of buffered formalin (10%) and Rose Bengal (1%) prior to sorting and preservation in ethanol (70%). Invertebrates were separated from debris and plant material under a dissection microscope (10–70× magnification) with the aid of a mounted, digital camera, and identified to the lowest possible taxonomic level using standard keys (Pettibone 1963, Smith 1964, Gosner 1971, Bousfield 1973, Pollock 1998). A literature search was used to determine diet, and only species known to consume seagrass epiphytes were included in the analyses. The difference in grazer abundance was analyzed using a 1-way ANOVA on total number of grazers per site across years using harbor as the fixed factor.

RESULTS

While the magnitude of the rates of nitrogen fixation varied between years, the 2 reciprocal transplant experiments showed similar patterns with statistically significant differences between treatments (2008: $F = 4.6$, $p = 0.002$; 2009: $F = 10.8$, $p < 0.001$; Fig. 2). In the Outer Harbor, seagrass leaves had consistently lower rates of epiphytic nitrogen fixation per area of leaf than in Snug Harbor, and in both years, after only 6 d, plants from Snug Harbor that were transplanted into the Outer Harbor had similarly very low rates of nitrogen fixation (Fig. 2). In contrast, plants incubated in Snug Harbor had much higher rates of nitrogen fixation. In both years, when plants were transplanted from Outer Harbor to Snug Harbor, the rate of increase in nitrogen fixation was slower than the very rapid decrease in nitrogen fixation observed with the opposite transplant (SH to OH). In 2008, after 6 d, Outer Harbor plants transplanted into Snug Harbor had higher nitrogen fixation rates, albeit not significantly above the controls that remained in the Outer Harbor (Tukey's test, $p =$

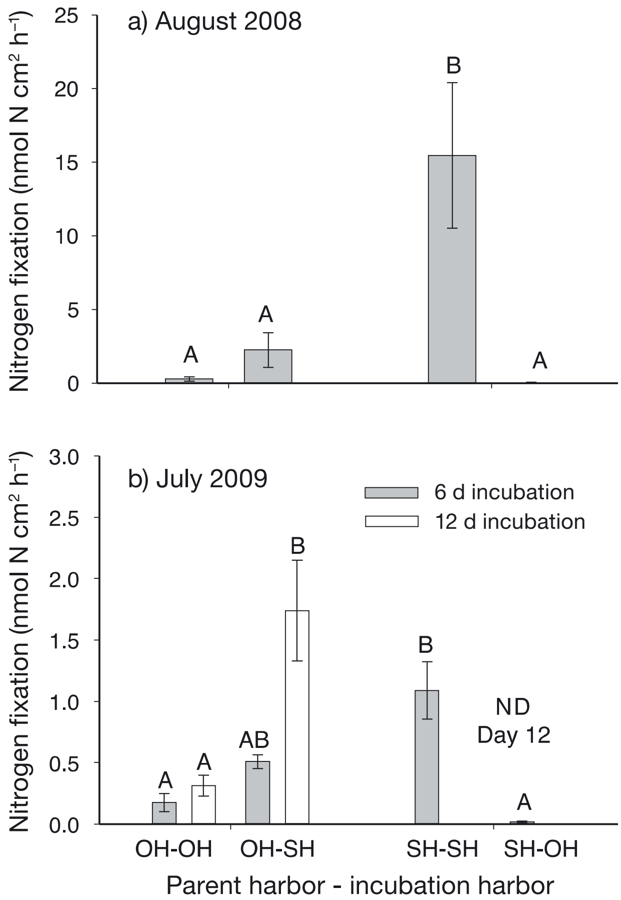

Fig. 2. Nitrogen fixation associated with epiphytes on seagrass blades in reciprocal transplant experiments after (a) 6 d in 2008 (n = 5) and (b) 6 and 12 d in 2009 (n = 7). Error bars are SE, and bars that share the same letter are not statistically different from one another. Note difference in *y*-axis scale between years. There is no data (ND) for Day 12 treatments using plants from Snug Harbor (SH), as they were too deteriorated to analyze. OH: Outer Harbor

0.95) and still significantly lower than the Snug Harbor controls (15.4 nmol N cm^{-2} h^{-1}) (Tukey's test, $p = 0.02$). Conversely, when plants were transplanted from Snug Harbor into Outer Harbor, epiphytic N fixation rates plummeted to well below those of the Snug Harbor controls after 6 d (Tukey's test, $p < 0.001$) and were similar to those of the Outer Harbor controls (Tukey's test, $p = 1.0$).

In 2009, nitrogen fixation rates were lower, but the pattern in experimental results was similar. After a 6 d incubation period, nitrogen fixation rates on plants transplanted from Outer Harbor to Snug Harbor had increased but were not significantly different from the Outer Harbor controls (Tukey's test, $p = 0.8$). However, after 12 d, the Outer Harbor to Snug Harbor transplants had epiphytic nitrogen fixation rates that were more than 5 times higher than the Outer

Harbor controls (Tukey's test, p = 0.0002), and similar to those for the Snug Harbor controls on Day 6 (Tukey's test, p = 0.2; Fig. 2). This is similar to the 2008 results, when plants were transplanted from Snug Harbor to the Outer Harbor, and epiphytic N fixation rates were reduced to those of Outer Harbor controls by Day 6 (Tukey's test, p < 0.001). After 12 d, both treatments with plants originating in Snug Harbor were too deteriorated to analyze; consequently, the Snug Harbor to Outer Harbor transplants and Snug Harbor control data are presented here and in Fig. 2 only for Day 6.

Epiphytic biomass on the seagrass blades analyzed for nitrogen fixation rates in the 2008 experiment mirrored the pattern in nitrogen fixation results and differed according to treatment (F = 4.8, p = 0.007). Shoots originating from and incubated in Outer Harbor tended to have low total epiphyte biomass, and the Outer Harbor shoots transplanted into Snug Harbor tended to have higher biomass, albeit the difference was not statistically significant (p = 0.5; Fig. 3). Conversely, control plants originating from and incubated in Snug Harbor had higher total epiphytic biomass, and transplants from Snug Harbor to the Outer Harbor had significantly reduced biomass (p = 0.006; Fig. 3).

Patterns of epiphytic biomass from the surrounding meadows at the Snug Harbor and Outer Harbor sites were similar to those from the experiment. Total seagrass epiphyte biomass was 3 times higher in Snug than in Outer Harbor when using only leaves 2 and 3 (F = 15.04, p = 0.003) and for whole shoots (F = 9.41, p = 0.01; Fig. 4). For these plants, we also estimated

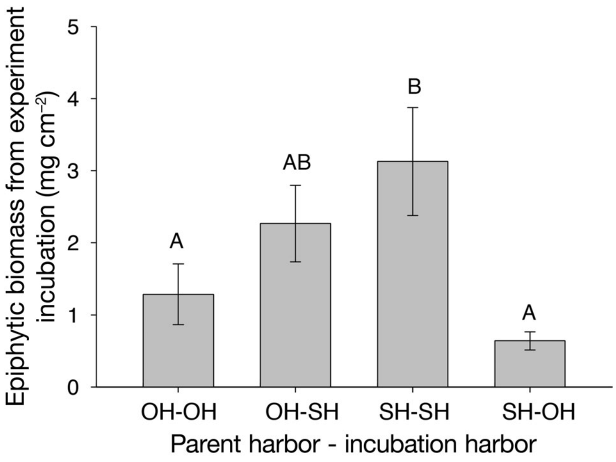

Fig. 3. Total epiphytic biomass from the 15 cm leaf segments incubated for nitrogen fixation analysis in the 2008 reciprocal transplant experiment (Fig. 2). Error bars are SE, and bars that share the same letter are not significantly different (p > 0.05) from one another

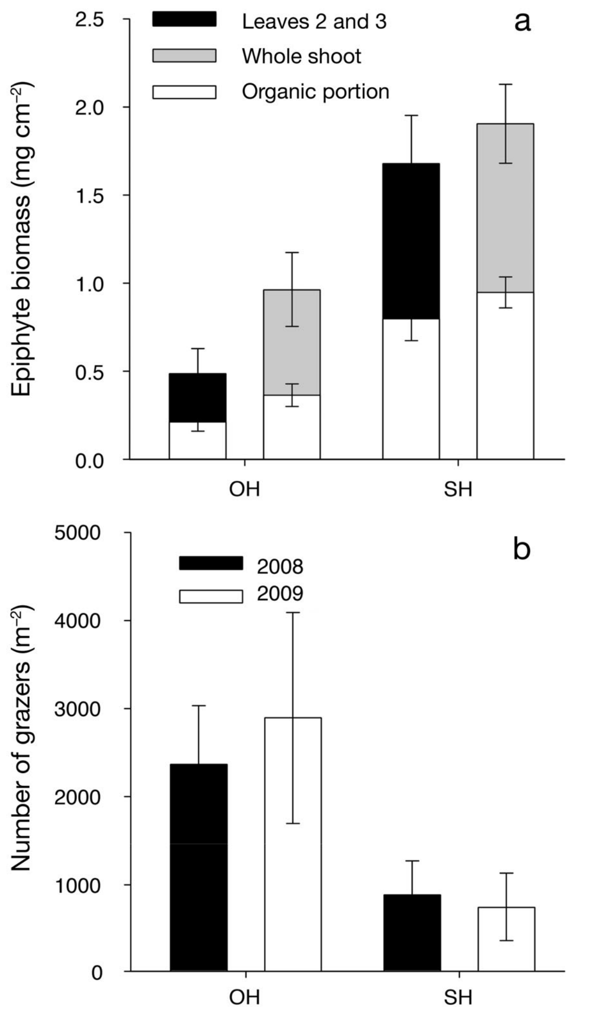

Fig. 4. (a) Epiphytic biomass in Outer Harbor (OH) and Snug Harbor (SH) in 2008. Total biomass is presented on a leaf area basis and is an average of 6 shoots. Error bars are SE. (b) Number of epiphyte grazers in the Outer Harbor (OH) and Snug Harbor (SH) in 2008 and in 2009. Abundance values represent the pooled sample at each site (2 sites per harbor). Error bars are SE

the portion of the epiphyte biomass that was organic. Patterns of organic epiphtye biomass were similar to total biomass, with 4 times more biomass for leaves 2 and 3 in Snug Harbor than in Outer Harbor (F = 18.54, p = 0.002), and nearly 3 times higher biomass on the whole shoots in Snug Harbor than in Outer Harbor (F = 28.6, p < 0.001). The whole shoots values are lower because they also incorporate the younger portions of the leaf that have not been colonized and were not used in the nitrogen fixation assay.

In both experimental years, invertebrate grazer density was higher in the Outer Harbor (Fig. 4). Invertebrate data were combined across years for statistical analysis to increase power, yielding inver-

Table 1. Number of epiphyte grazers sampled in 2008–2009 in Outer Harbor and Snug Harbor, West Falmouth Harbor, Massachusetts, USA. Densities are ind. m^{-2}

Phylum	Order	Species	Snug Harbor	Outer Harbor
Annelida	Aciculata	*Platynereis dumerilii*	18	0
Mollusca	Caenogastropoda	*Lacuna vincta*	0	335
		Bittiolum alternatum	652	1551
	Neogastropoda	*Costoanachis lafresnayi*	0	18
		Astyris lunata	35	141
		Costoanachis avara	88	212
	Patellogastropoda	*Acmaea testudinalis*	0	35
Arthropoda	Amphipoda	*Caprella penantis*	0	106
		Elasmopus levis	0	212
	Decapoda	*Hippolyte zostericola*	18	0
	Isopoda	*Erichsonella filiformis*	0	18
Total			811	2627

tebrate grazer densities that were 2 times higher in Outer Harbor (Fig. 4; $F = 8.9$, $p = 0.02$). Eleven species representing 8 invertebrate orders of epiphyte grazers were found. While 9 of the 11 species were found in Outer Harbor, only 5 were found in Snug Harbor. In both harbors, the dominant species was the gastropod *Bittiolum alternatum*, and densities of gastropod grazers in this group were nearly 2.5 times greater in Outer Harbor (Table 1). The epiphyte-grazing amphipods *Gammarus mucronatus* and *Ampithoe* sp., while observed in low numbers in both the Outer and Snug Harbors, were absent from these samples. While other amphipods were present, review of the literature suggests that they are scavengers (*Lysianopsis alba*; Pollock 1998), suspension feeders (*Corophium insidiosum*; Rasmussen 1973, Nair & Anger 1979, Moksnes et al. 2008), or grazers of larger macroalgae (*Microdeutopus gryllotalpa*; Jiménez et al. 1996, Jephson et al. 2008) and not important grazers on small epiphytes.

DISCUSSION

In West Falmouth Harbor, patterns of summertime epiphytic nitrogen fixation rates were consistent with the hypothesis of grazer control. Rates were higher in the area of the harbor (Snug Harbor) where grazer density was lower (Figs. 2 & 4). Further, when plants were transplanted into an environment with high grazer densities (Outer Harbor), epiphyte nitrogen fixation rates were quickly (within 6 d) reduced to local (parent harbor) values, presumably due to consumption of epiphytic nitrogen-fixer biomass (Figs. 2 & 3). With the reciprocal transplant into Snug Harbor, the slower increase in nitrogen fixation rates likely reflected the longer time needed for colonization and epiphyte growth once the seagrasses were placed in an environment with a reduced abundance of grazers.

The role of grazing as an important control on nitrogen fixation associated with seagrass epiphytes in our experiments is supported by the lack of evidence for alternative, bottom-up factors that have been shown to constrain nitrogen fixation. High inorganic nitrogen concentrations can limit nitrogen fixation by inhibiting the production of nitrogenase (Postgate 1982, Dixon 1984, Postgate & Kent 1984, Capone 1988); however, despite the high nitrogen load, particularly to Snug Harbor, DIN concentrations during the peak summer period of our study remained low in both Snug and Outer Harbor, presumably due to assimilation by primary producers (Hayn et al. 2013, Howarth et al. 2014). Water column DIN concentrations at both study sites were generally less than the 1 to 1.5 µM previously found to inhibit nitrogenase synthesis (Howarth et al. 1988b).

In Snug Harbor, high nitrogen loads lead to higher rates of gross primary productivity than in the Outer Harbor (Howarth et al. 2014) and also may be responsible for the patchy macroalgal blooms that occur during the mid-summer peak growing season (Yarrington et al. 2013) when all measurements in this study were taken. These conditions in turn contribute to the larger fluctuations in water column oxygen concentrations in Snug Harbor and explain contemporaneous observations of diurnal low oxygen and low pH (Howarth et al. 2014). The observed lower median oxygen concentrations and pH values during the peak summer months in Snug Harbor could result in the lower abundance of invertebrate grazers observed there relative to the Outer Harbor either directly, or indirectly by negatively impacting higher trophic level consumers that can constrain grazer populations. Data on consumer organism abundances were not available; however, a 2007 study in West Falmouth Harbor in the same areas as our experiments found that invertebrates tolerant of environmentally degraded, eutrophic conditions dominated the infaunal community in Snug Harbor (McLenaghan 2009). Other causative mechanisms such as high nutrient concentrations can affect total epiphyte loads on seagrasses (Williams & Ruckleshaus 1993). However, since there were little to no significant differences in standing stock concentrations of N, and since epiphyte biomass was reduced very quickly when plants were placed in Outer Harbor where grazers are abundant, it is likely that the relatively low abundance of grazers also contributed to the epiphyte proliferation in Snug Harbor (Fig. 4) and thus the higher measured rates of epiphyte nitrogen fixation in our Snug Harbor experimental treatments, either as the parent harbor or as transplants from the Outer Harbor (Fig. 2).

While grazing is known to impact nitrogen fixation in many different ecosystems (Vitousek et al. 2002) including in planktonic systems (Howarth et al. 1999, Marino et al. 2002, 2006, Chan et al. 2006), benthic algal/coral communities (Wilkinson & Sammarco 1983, Williams & Carpenter 1997), and stream communities (Arango et al. 2009), this is the first study providing evidence for the role of grazing in controlling nitrogen fixation associated with seagrass epiphytes. Interestingly, the densities of organisms capable of grazing on the epiphytes in West Falmouth Harbor during our study were similar or lower than those reported in other seagrass systems (Bologna & Heck 1999, Duffy et al. 2001) suggesting that the effects we demonstrate here could be of widespread importance. Conspecifics of the most common grazer in this system, *Bittiolum alternatum*, have been shown elsewhere to reduce epiphyte biomass by more than 50% and are particularly efficient at controlling diatom (van Montfrans et al. 1982), but not macroalgal (Whalen et al. 2013), growth. The specific effect of grazers on organisms capable of nitrogen fixation is unknown, but it appears that *B. alternatum* and other grazers in this study may be effective in their removal. Further, our data contribute to the growing understanding that grazing is an important but under-appreciated control in seagrass systems, impacting productivity (Nienhuis & Groenendijk 1986, Brearley & Walker 1995), reproduction (Reynolds et al. 2012, van Tussenbroek et al. 2012), biodiversity (Armitage & Fourqurean 2006), and nutrient cycling.

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