Supplementary Information for:

Nitrogen fixation associated with epiphytes on the seagrass *Zostera marina* in a temperate lagoon with moderate to high nitrogen loads

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Text S1: Calculations to scale nitrogen fixation to rates per shoot and per area of seagrass bed

A summary of parameters used in our scaling calculations is in Table S1. We based our scaling on factors determined from several additional acetylene reduction experiments, carried out using the same methodology as our routine assays:

- Incubation of samples from 18 different locations within the OH seagrass bed (July 2019) to assess spatial variability and how representative our annual sample site is of overall site fixation rates. Results from this experiment are presented in the main publication and in Figure S5.
- Incubation of the entire length of blades 2 and 4 (July 2013) and blade 4 (July 2019) to calculate the percentage of total blade fixation we are capturing by only assessing the top 15cm. Rates of fixation were far higher on the top 15 cm than on the lower blade area, particularly for older blades (Figure S6), with 84% of total blade N fixation occurring in the top 15cm of blade 2, and 68% in the top 15cm of blade 4.
- Incubation of the top 15cm of all blades present on each seagrass shoot (July 2006, July 2009, June 2010) to assess what fraction of total shoot fixation we capture with our measures of blades 2 and 4. On average, 36% of total plant fixation occurred on blades 2 + 4 if there were 5 blades present, and 75% if there were only 4 blades present.

Additionally, we used data on the morphometry of plants at our sites and the density from field counts to inform our calculations (Table S1). OH plants average 4.75 blades per plant, with most plants having either 4 or 5 blades, so for calculation purposes we assume 75% of them have 5 blades and 25% have 4 blades. SH (Snug Harbor) plants averaged 4.5 blades, so we assume 50% have 4 blades and 50% have 5 blades.

Our shoot densities collected in the field counted total shoot density, and we made the conservative estimate that there is no N fixation occurring on smaller shoots at the site. In 2018 and 2019 we conducted detailed surveys at the 15 sites in the OH seagrass bed where we counted and measured plants by size class. We found on average that 57% of shoots are in the medium-to-large size classes (mean leaf area per shoot of 97cm^2 with middle two quartiles from 62 to 120cm^2), of similar size to the OH plants sampled for N Fixation (mean leaf area per shoot of 103cm^2, with middle two quartiles from 88 to 120 cm^2). Since shoot density has remained constant over our measurements, we also assume that plant size distribution has been roughly constant over the duration of our study. Thus, we reduce our average shoot density to 57% of field values to provide a conservative estimate of field fixation rates across the entire seagrass bed. We do not have these data available in Snug Harbor, so we apply the same scaling factor. This is likely to under-estimate fixation in SH, since the plants are smaller on average (see Table 1 and Table S1) and more heavily epiphyte-laden in general (Discussion Section and Reynolds et al. 2015).

We used the following equations to calculate rates of N_2 fixation per square meter of seafloor (N_s in mmol N m^{-2} day^{-1}) from our annual rates reported in the manuscript for the top 15cm of blades 2 and 4 (R2 and R4, in nmol N cm^{-2} day^{-1}). The intermediate value N_{2+4} represents the sum of whole-blade fixation on blades 2 and 4. Where the values differed by basin, we used the appropriate values for that basin. Parameters are defined in Table S1.

$$Equation\ S1: \qquad N_{2+4} = \frac{R2 * LA2}{PF2} + \frac{R4 * LA4}{PF4}$$

$$Equation\ S2: \qquad N_S = \left[\frac{P5 *\ N_{2+4}}{WP5} + \frac{(1 - P5) *\ N_{2+4}}{WP4}\right] * (D * DSF) * \frac{1}{1E6}$$

Parameter	Value	Variable Name
Percent of fixation in top 15cm	84% - Blade 2 68% - Blade 4	PF2 PF4
Percent of fixation accounted for in blades 2 and 4	36% - 5 blades present 75% - 4 blades present	WP5 WP4
Total Shoot Density	OH: 450 SS/m2 SH: 330 SS/m2	D
Shoot Density Scaling Factor	57%	DSF
Average blades per plant	OH: 4.75 SH: 4.5	
Percent of plants at site with 5 blades	OH: 75% SH: 50%	P5
Mean leaf area in top 15cm of Blade 2	OH: 7.1 cm2 SH: 6.6 cm2	LA2
Mean leaf area in top 15cm of Blade 4	OH: 7.2 cm2 SH: 6.9 cm2	LA4

Table S1. Parameters used in calculation scaling from fixation rates measured in the top 15cm of blades 2 and 4 to rates per m2 of seafloor. When parameters differed between the two study sites, OH and SH are used to designate the site differences.

Text S2: Methods Details – Additional ARA methodological details

An ethylene standard curve was prepared in serum bottles set up identically to the sample bottles; 100 ml of 0.2 µ-filtered seawater or salinity-matched artificial seawater were dispensed in 120-ml serum bottles which were then sealed, and aliquots of an ethylene gas standard in N_2 were added to the headspace of each bottle with a 21G BD Needle. They were vigorously mixed for 30 seconds, then kept on ice with the samples until run on the GC. Since standards were prepared identically to the samples (same volume of liquid and headspace analyzed at the same temperature) with a known number of moles of ethylene added, a simple linear standard curve between GC area counts and nmol ethylene in the bottle is used to calculate ethylene in the samples. Blanks were prepared at the start and end of the incubation setup as well as regularly alongside the samples (since ethylene contamination in the source acetylene could drift slightly along the run), and the appropriate blank values were subtracted from the final concentrations.

The solubility of ethylene is very sensitive to temperature (Flett et al. 1976), so it is critical that the same temperature is used for sample bottles and standards. Keeping both samples and standards in an ice-water solution until and during headspace sampling for GC analysis ensures a constant temperature of 0^o C (Howarth et al. 1993).

To run samples on the Shimadzu GC-14a, samples (or standards) were vigorously shaken for 30 seconds to equilibrate gases between liquid and head space, then allowed to settle for 30 seconds in an ice-water solution before a 4mL sample was withdrawn from the head space in the serum bottles using a 10mL BD syringe. A pressure transducer (Honeywell BP357BN Model S with digital display and luer adapter to 21G BD PrecisionGlide needle) was inserted through the septa into the headspace of each vial and used to monitor pressure changes as the gas was withdrawn to detect possible leaks; when leaks were detected, samples were discarded. The sample was injected into the GC through a 1mL sample loop, and the resulting peak area was recorded. Several carryover tests were conducted to ensure that a 4mL sample thoroughly purged the sample loop.

Text S3: Methods Details – Calibration of ARA estimate to N_2 Fixation

Ten replicate samples were used for each of the three assays for the methods inter-comparison, run for 4 hours and at the same light level used for the routine ARA assay. We used a homogenized slurry of epiphytes for these comparisons. Epiphytes were carefully scraped from the top 15 cm of blade 4 of plants collected in the OH and put into a solution with 0.2 µm-filtered site water (31.5 ppt salinity) that had been equilibrated with the atmosphere for 2 days at temperature (23.7 ˚C) and very gently stirred continuously during the setup of each assay. Rates for all three assays in this calibration are reported per gram of epiphyte biomass.

For the ARA, we followed the method outlined in the manuscript, except adding 5 ml of epiphyte slurry to 85 ml of filtered seawater in a serum bottle (instead of a grass blade with 90 ml of seawater). At the end of the incubation, the contents of each incubation bottle were filtered onto pre-ashed and weighed GF/F to quantify epibiota biomass. We also ran ethylene production and time-zero blanks as described in the main manuscript on the filtered seawater used for the incubations and no ethylene production was found.

For the N_2:Ar measurements, we prepared 10 replicate samples by slowly adding 5 ml of the epiphyte slurry with a wide tip pipet to 6.5 ml of the temperature and atmosphere-equilibrated, filtered seawater that had been slowly siphoned into a 12 ml exetainer, being careful at every step not to entrain any air bubbles. The exetainer was capped carefully and tightly, so as not to introduce any bubbles, since there cannot be any air in the vials for this assay. An additional six replicate samples were prepared in the same way and immediately injected with 250 µl of $ZnCl_2$ solution; these time-zero "killed" samples were considered to be the most accurate blanks. Incubation water blanks with no epiphyte slurry and $ZnCl_2$ added were taken at the start and the finish of the setup as well. Samples were incubated for 4 hours, as with the ARA at a constant temperature of 23.7 ± 0.2 ˚C. At the end of the incubation, 250 µL of $ZnCl_2$ were injected into to each vial, and they were stored upright, underwater, and in the dark at a constant temperature of 23 ± 1˚C until analysis on the MIMS, within 2 weeks.

The MIMS system used includes a specially designed gas inlet system attached to a Balzers quadrapole mass spectrometer (Todd Kana, Bay Instruments http://www.hpl.umces.edu/dga/DGAhome.htm), with a furnace retrofitted to allow for measurement of mass 28, 29, and 30. Because we also are measuring $^{30}N_2$, and there can be some mass 30 formed at the filament by NO, we use a furnace to remove oxygen from the gases before they enter the mass spectrometer. This not only eliminates the issue of mass 30 formation but further increases the precision to better than 0.03% (see discussion in Kana and Weiss 2004; Eyre et al. 2004). A cryotrap is used before the furnace to remove water and CO_2. The sample is directly pumped from the storage vial past a capillary bore silicone tubing interface in a vacuum which then goes to the MS. This avoids any issues of having to degas the sample or make corrections for the water/gas ratio, as there is never any headspace. As with the slurry ARA, the contents of each exetainer were filtered after the assay was completed and analyzed for epiphyte biomass, and the change in N_2 was reported per gram of epiphyte biomass incubated.

For the ^{15}N-uptake assay, we prepared a spike solution using $^{15}N_2$ gas (98+%; 5 liters in a pressurized cylinder; Cambridge Isotope Labs lot # 1-21-65/AR0664758 (NLM-363-5)) as follows: A 0.6 liter Kynar (PVDF) gas bag was filled with 400 ml of 0.2 µm-filtered seawater, making sure to purge out any introduced air before 5 ml of the $^{15}N_2$ were added, and the solution was equilibrated with for 24 hours. To set up the assay, 5 ml of the epiphyte slurry was pipetted as described above into 20, 122-ml serum bottles containing 100 ml of the filtered seawater, the bottles were topped off and capped with no gas head space. Then 10 ml of the $^{15}N_2$-enriched water solution was injected into each of 10 bottles using a 10 ml syringe with a long needle, and a shorter vent needle simultaneously inserted to release pressure. A dilution measurement using 10 other replicate serum bottles prepared similarly and injected with 10 ml of a dye solution (instead of the $^{15}N_2$-enriched water) determined that the final volume of $^{15}N_2$ enrichment solution in the serum bottle incubations added in this way was 9.5 ml. Separate aliquots of the $^{15}N_2$ in the enrichment spike solution were transferred into exetainers as described above (N_2:Ar assay) and measured by membrane inlet mass spectrometry (MIMS) as $^{30}N_2$. The isotopic enrichment in the solution was 40.27‰, giving an effective spike concentration in the incubation vials of the $^{15}N_2$-uptake assay of 3.5‰ $^{15}N_2$. After incubation as described above for ARA and N_2:Ar measurements, the contents of each bottles were filtered onto a pre-ashed GF/F filter, dried at 60 ˚C, and run at the MBL Stable Isotope facility for $\delta^{15}N$. From the $\delta^{15}N$ enrichment of the samples (10 replicates) and comparison with un-enriched controls (10 replicates), we calculated the incorporation of ^{15}N into epibiota biomass over the duration of the incubation following the calculations in Montoya et al. (1996), assuming no fractionation during the process.

A bootstrap analysis (Efron and Tibshirani, 1993) was used to calculate the ratio of ethylene produced (ARA) to N_2 fixed for each of the assays that measures a change in N_2 because the data were not always normally distributed (see manuscript Figure 2). Each calculation randomly sampled the analytical observations 10x with replacement and calculated the mean ratio of the rates. This creates many (in our case, 1000) simulated datasets sampled from our experimental results, without making assumptions about data distribution. We then use these to calculate the average ratios between our methods, to get a mean and confidence interval that are independent from assumptions about data normality. Figures S2 and S3 show the resulting histograms.

Text S4. Details on PAR Measurements

The PAR values shown in Figures 7, S8, and S10 are based on continuously monitored short-waved solar radiation at the Woods Hole Oceanographic Institution (WHOI), 7.5 km from West Falmouth Harbor. These data are converted to mean daily PAR by regressing our more limited PAR data in the harbor against the WHOI data for several 24-hour integrated periods in 2012 (Supplemental figure S9; R^2= 0.96, P<0.0001).

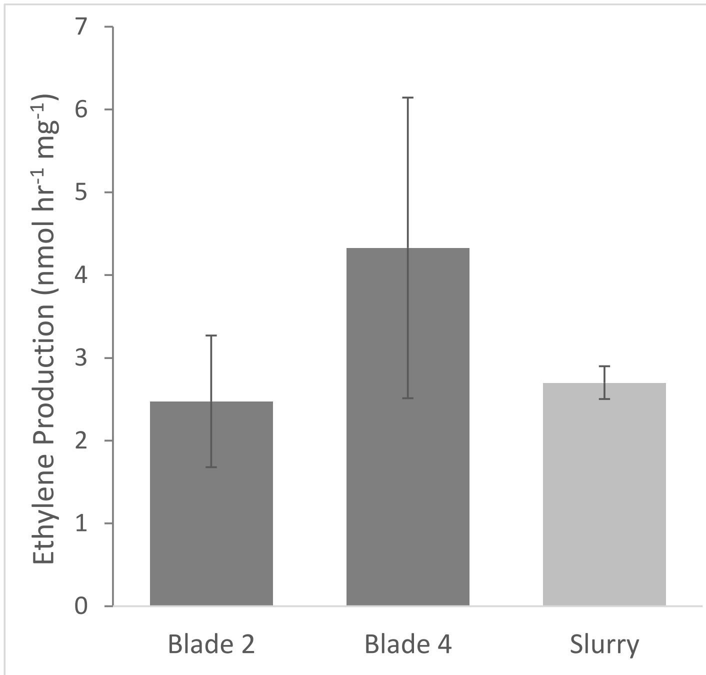

Fig. S1 Rates of ethylene production expressed per biomass of epiphytes for intact grass leaves (dark gray boxes, blades 2 and 4) and for slurried epiphytes taken from older leaves (light gray boxes, blade 4). Vertical lines represent the standard deviation of the reps run (n=10 for Blade 4 and slurry, n=4 for Blade 2). Acetylene reduction measured on the epiphyte slurry was quite comparable to that measured on intact leaf-epiphyte assemblages, when normalized to epiphyte biomass, indicating that fixation rates weren't suppressed by the removal of epibiota from the seagrass blade surface.

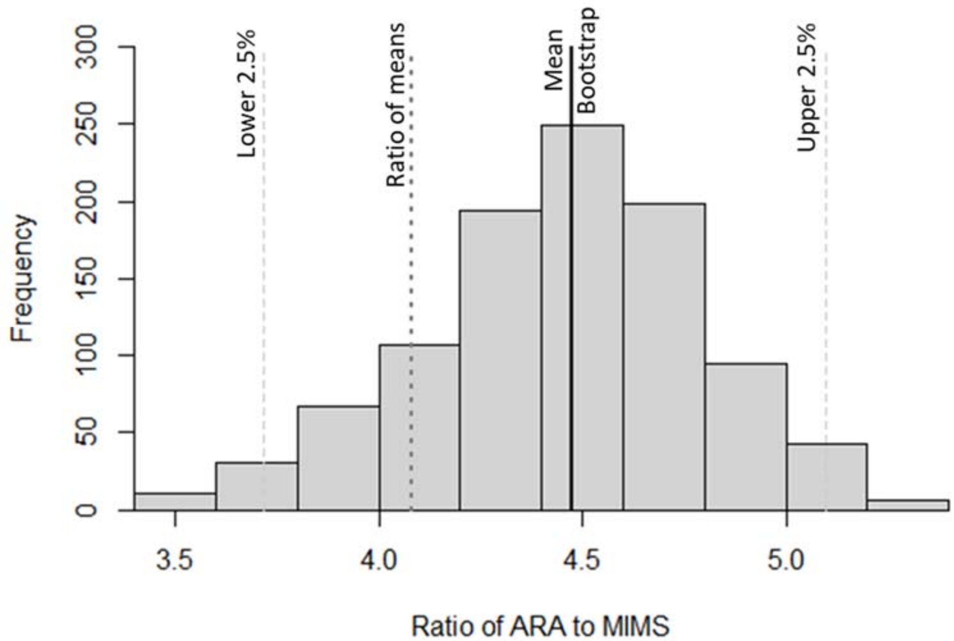

Fig. S2 Frequency distribution of the ratio of acetylene reduction (ARA) to nitrogen fixation measured by decreased in dissolved N_2 by MIMS (MIMS) based on a bootstrap analysis. Note that the mean of the bootstrap analysis is greater than that represented by the simple ratio of the means of the ten observations (dotted line). The MIMS data are not normally distributed, and so the mean of the bootstrap analysis provides a better calibration than does the ratio of the means.

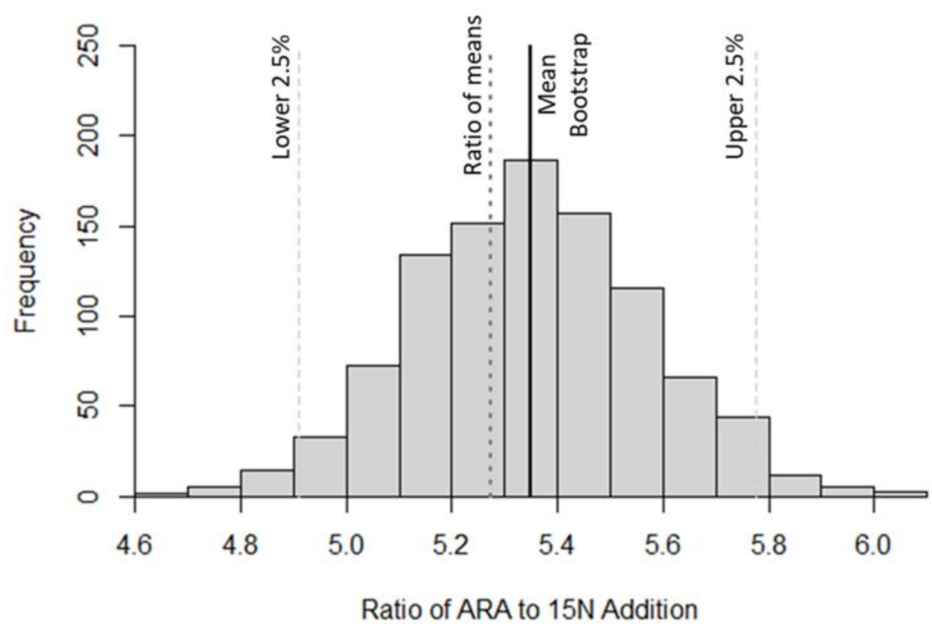

Fig. S3 Frequency distribution of the ratio of acetylene reduction (ARA) to nitrogen fixation measured by uptake of $^{15}N_2$ based on bootstrap analysis. Note that since both the ARA and 15N addition results were close to normally distributed, the ratio of the means and the mean from the bootstrap analysis are similar.

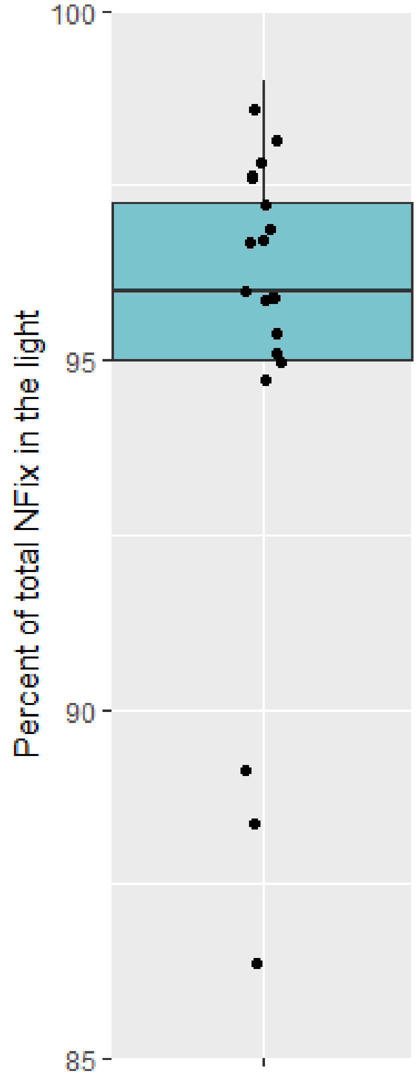

Fig. S4 Percent of nitrogen fixation over 24-hour periods that occurs in the light, combined for blades 2 and 4 and including Outer Harbor and Snug Harbor data. Each point represents a year at one of the sites. The median is shown by the horizontal line, the box represents the middle two quartiles of data, and black dots are individual observations.

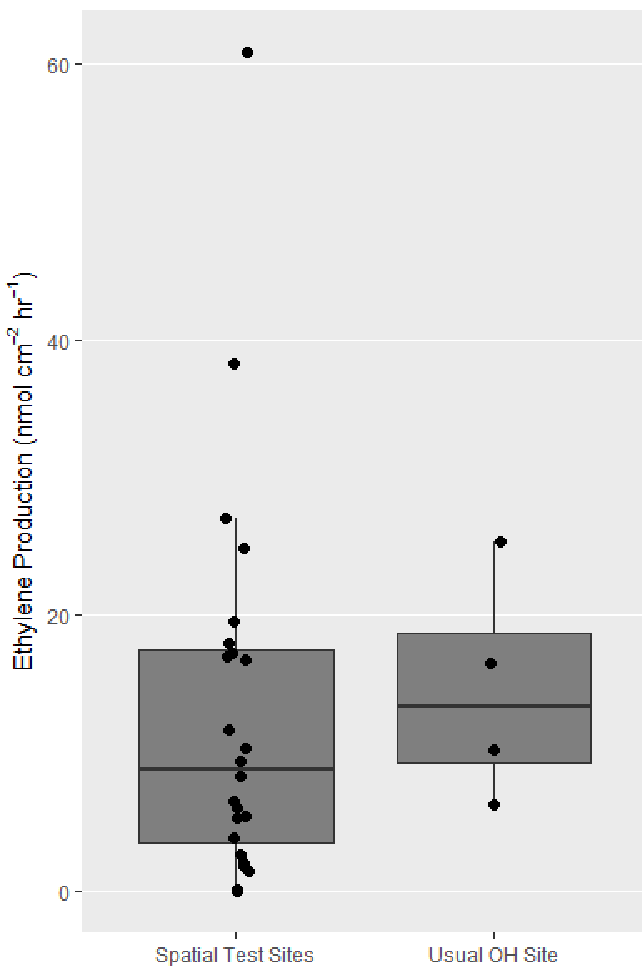

Fig. S5 Rates of ethylene production per surface area of leaf in the light across multiple sites in the Outer Harbor in 2019. The "Usual OH Site" represents 4 replicates taken from the long-term Outer Harbor sampling site, and "Spatial Test Sites" represents the 18 other sites within the seagrass bed taken simultaneously for comparison. Site locations are shown in Figure 1 in the manuscript.

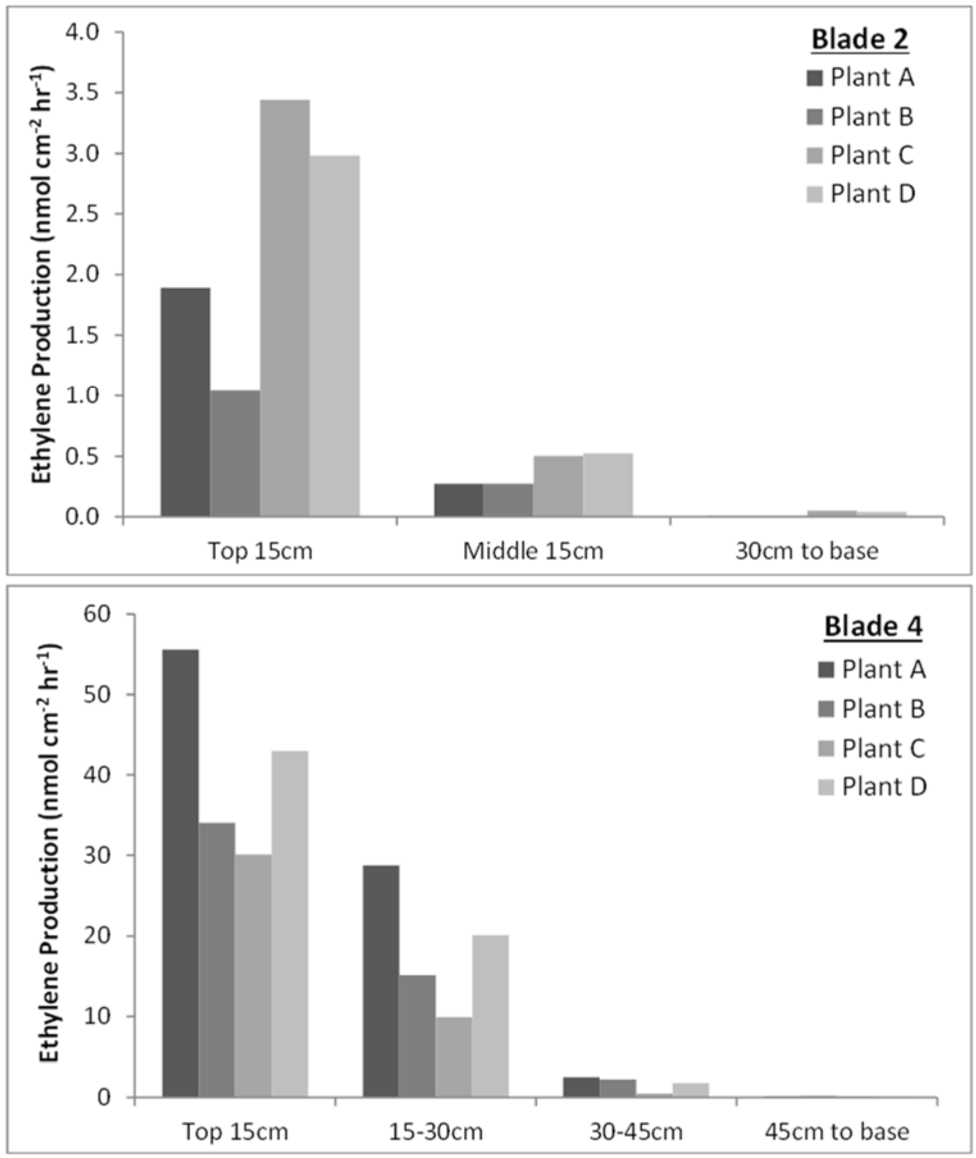

Fig. S6 Comparison of rates of ethylene production in the light for younger leaves (blade 2, top) and older leaves (blade 4, bottom) for the top 15 cm of the leaves and lower portions of leaves. Routine long-term measurements were just for top 15 cm. Data are shown for 4 individual plants in 2013. Data for 2019 are not shown.

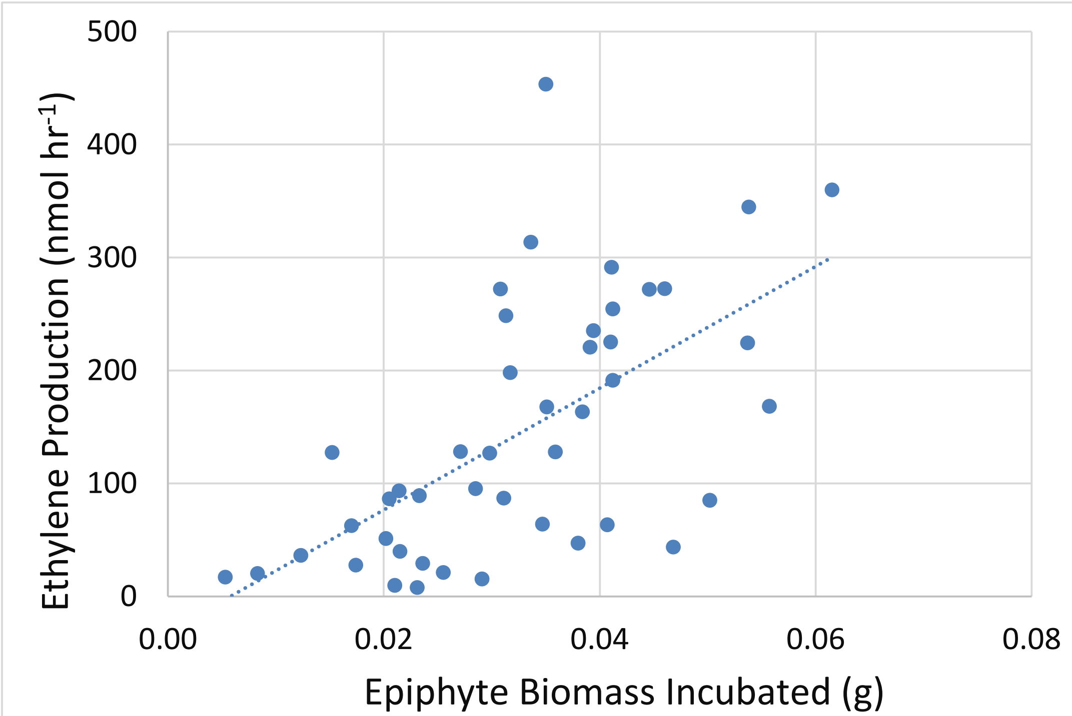

Fig S7 Rates of ethylene production as a function of the total dry biomass of epiphytes on portion of *Zostera* leaf incubated for ARA. Data are for 2016 to 2019 for the Outer Harbor site.

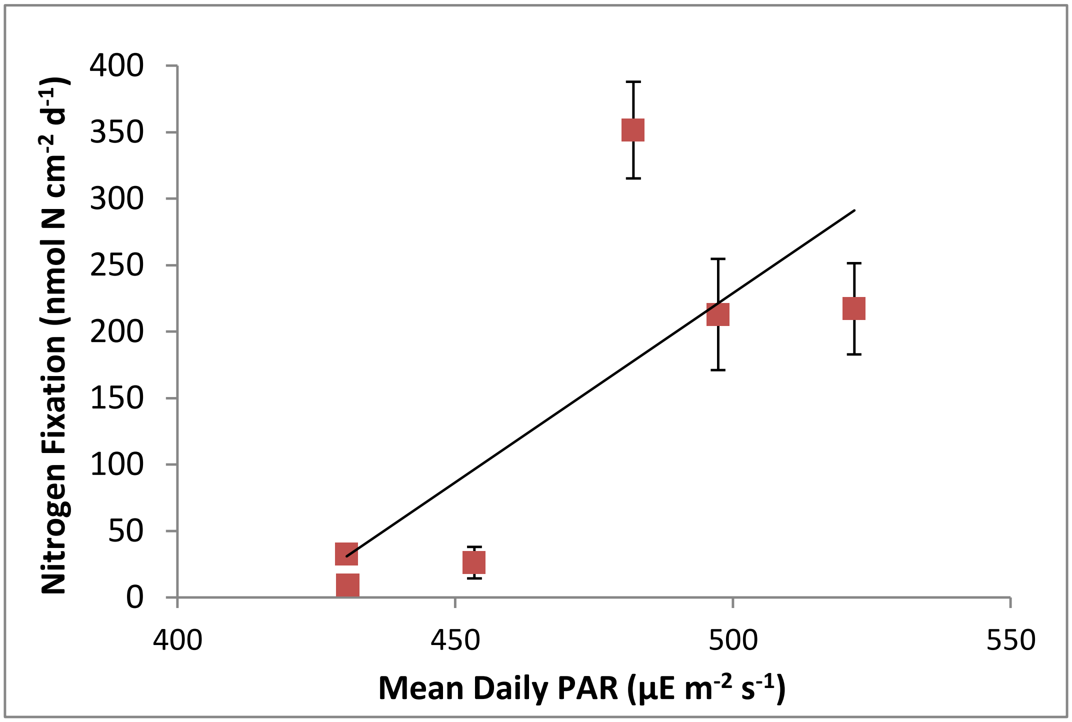

Fig. S8 Rates of nitrogen fixation per area of leaf in Snug Harbor for each year until the beds were lost from the site, as a function of the mean daily PAR from May 1 through the date nitrogen fixation rates were measured in each year.

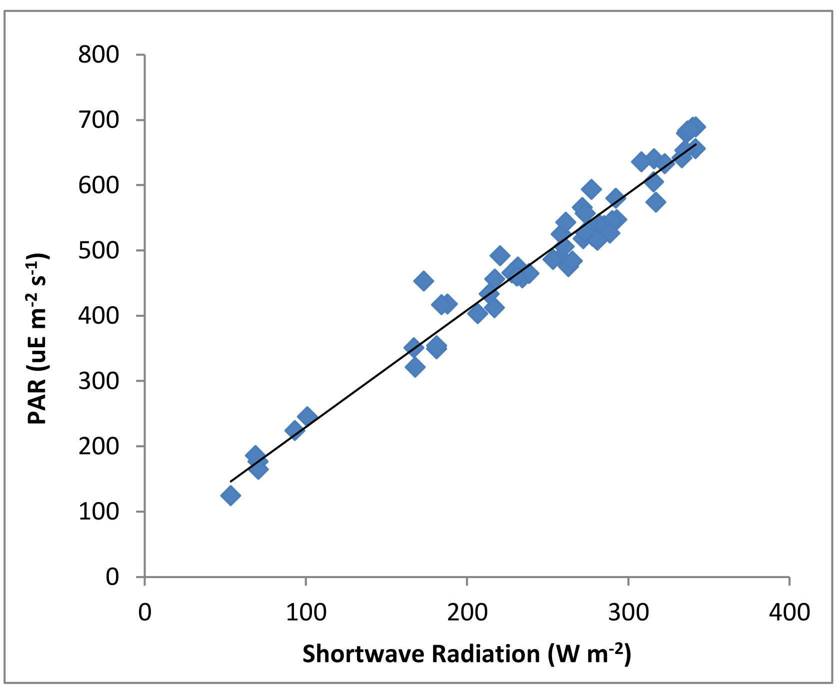

Fig. S9 Average PAR over 24 hours as measured on multiple days in Outer Harbor compared to shortwave radiation measured on the same days at the Woods Hole Oceanographic Institution (WHOI) in 2012.

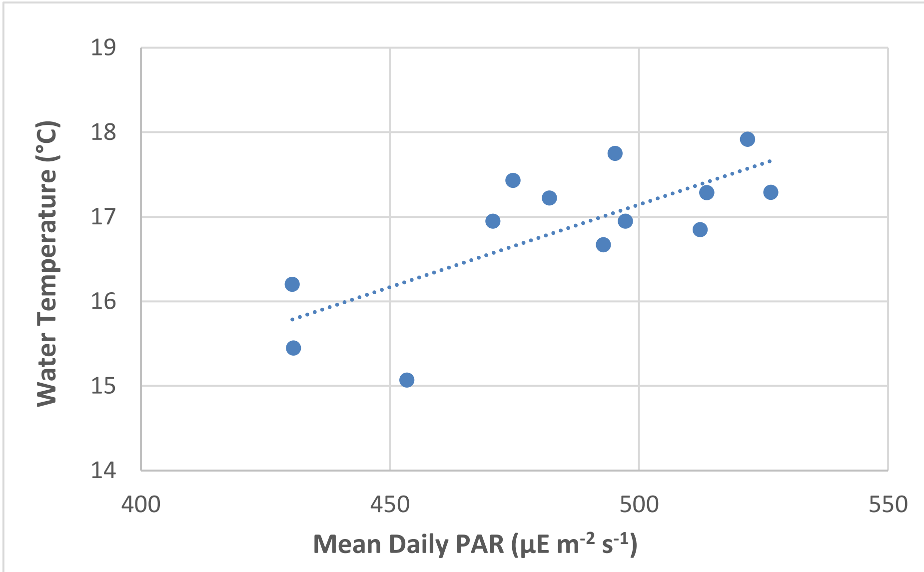

Fig. S10 Average water temperature in Woods Hole as a function mean PAR for the period from May 1 to the time nitrogen fixation was measured in multiple years. Woods Hole is near West Falmouth Harbor.

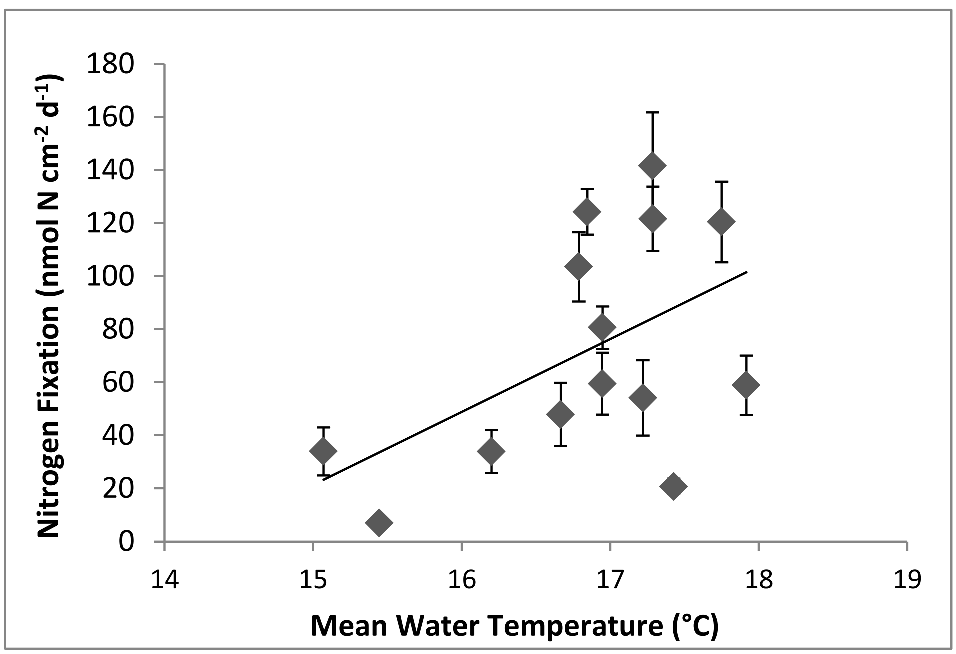

Fig. S11 Nitrogen fixation rates per area of leaf in Outer Harbor as a function of the mean water temperature in Woods Hole for the period from May 1st through the time the nitrogen fixation measurement was made.