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Temporal and spatial variation in nitrogen fixation activity in the eelgrass *Zostera marina* rhizosphere

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ABSTRACT: A perfusion technique for measuring nitrogen fixation in the eelgrass rhizosphere was developed and used to investigate the patterns and controls of nitrogen fixation in sediment cores containing live eelgrass *Zostera marina* plants collected from the Limfjord, Denmark. Detailed temporal and spatial patterns of acetylene reduction were consistent with the hypothesis that heterotrophic nitrogen fixation in the eelgrass rhizosphere is stimulated by organic root exudates derived from plant photosynthesis. Nitrogen fixation activity was approximately 3× higher in vegetated than unvegetated sediments, and showed strong seasonal patterns and differences in light-dark incubations that corresponded to variations in plant productivity. Rates in both the light and dark were lowest in the winter months, and increased steadily through the spring and summer to a peak activity in August that coincided with the maximum eelgrass aboveground biomass. Light-incubated cores had significantly higher rates (25 to 40%) than those incubated in the dark during the growth season, and this difference was greatest during the summer months when plant productivity was highest. Nitrogen fixation activity also showed strong (5-fold) spatial variation with depth in the eelgrass root zone, with highest rates corresponding to the largest root+rhizome biomass and correlating with seasonal changes in belowground biomass distribution. Additions of glucose or NH_4^+ showed that the nitrogen-fixing bacteria were limited by organic substrate and were not sensitive to NH_4^+ concentrations. Molybdate additions indicated that sulfate reducers were responsible for about 25% of the nitrogen fixation activity in the eelgrass rhizosphere. Overall, daily nitrogen fixation rates integrated to a depth of 14 cm in the sediment (1 to 6 mg N m^{-2} d^{-1}) were comparable to rates measured in other temperate seagrass meadows, but were lower than those determined for tropical seagrass beds.

KEY WORDS: Nitrogen fixation · Eelgrass · Rhizosphere · Sediment

INTRODUCTION

Nearshore coastal ecosystems are typically characterized by high primary production of phytoplankton and benthic macrophytes, which in temperate areas is usually limited by nitrogen availability (Nixon & Pilson 1983, Howarth 1988). The high productivity of these temperate systems is largely supported by external inputs and by the recycling of remineralized nitrogen, but in the absence of significant external loading, new inputs from nitrogen fixation are essential to offset losses via denitrification (Capone 1988). Shallow benthic areas are believed to be the main sites of nitrogen fixation in the oceans, especially seagrass, coral reef and salt marsh ecosystems, where fixation provides from <5 to 100% of the plant nitrogen demand (Capone 1988, Howarth et al. 1988). Heterotrophic bacteria located in the rhizosphere of both tropical and temperate seagrasses have particularly high nitrogen fixation rates, and these rates typically exceed those in nearby unvegetated sediments (Hemminga et al. 1991, O'Donohue et al. 1991a, Welsh et al. 1996a).

Submersed vascular plants strongly influence the microbially mediated N transformation processes within the sediments (ammonification, nitrification, denitrification, nitrate reduction, nitrogen fixation), both directly through uptake of NH_4^+ and NO_3^- and indirectly by altering redox conditions in the rhizosphere (Boon et al. 1986a, b, Christensen & Sørensen 1986, Caffrey & Kemp 1990, 1992). Seagrasses also transport

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organic compounds synthesized during photosynthesis to the roots (Oremland & Taylor 1977, Wetzel & Penhale 1979, Iizumi et al. 1980, Moriarty et al. 1986); some of this photosynthate is exuded to the sediment and is believed to provide an oxidizable carbon source that enhances heterotrophic bacterial activity (Moriarty & Pollard 1982, Moriarty et al. 1986). Moriarty et al. (1986) showed that between 6 and 28% of the carbon fixed by the leaves of the tropical seagrass *Halodule wrightii* was translocated to the rhizomes and roots, and that about 10% of the total fixed carbon was released within 6 h to the surrounding sediment where it stimulated bacterial production. Bacterial growth in the seagrass rhizosphere also generally follows a diurnal cycle, which suggests a tight coupling to seagrass metabolism, with highest activity during the early part of the day decreasing in the afternoon to a low constant nighttime rate (Moriarty & Pollard 1982).

Nitrogen fixation in seagrass sediments is often stimulated by the addition of a wide range of oxidizable organic carbon compounds (Capone & Budin 1982, Capone 1988, Welsh et al. 1996b), and it has been suggested that the activity of heterotrophic nitrogen-fixing bacteria is associated with the release of photosynthetically derived organic carbon in both tropical and temperate seagrass beds (Capone & Taylor 1980, Smith & Hayasaka 1982, O'Donohue et al. 1991a, Welsh et al. 1996a). High rates of sulfate reduction and the presence of a short-lived organic pool (5 to 10 h) in the rhizosphere of the tropical seagrass *Halodule* sp. also point to a subsurface input of organic carbon probably originating from root exudates (Blackburn et al. 1994). These sulfate-reducing bacteria may be responsible for some of the nitrogen fixation that occurs in the seagrass rhizosphere (Capone 1982, 1988, Welsh et al. 1996b).

The purpose of this study was to characterize in detail the temporal and spatial patterns of nitrogen fixation in the rhizosphere of the temperate eelgrass *Zostera marina* to evaluate the hypothesis that these patterns are linked to variations in seagrass productivity. We used a perfusion technique with intact vegetated cores that allowed us to measure higher resolution profiles of acetylene reduction activity in the seagrass rhizosphere than have been recorded previously, without disrupting the direct root-microbe interactions during the incubations. Large numbers of heterotrophic nitrogen-fixing bacteria have been shown to occur in the rhizosphere of *Z. marina* compared to unvegetated sediments (Shieh et al. 1989), and it is likely that these bacteria are dependent on root exudates as a source of labile organic carbon. Previous studies based on either quarterly or semi-annual measurements, and with less spatial detail in slurry or perfusion incubations, have shown that nitrogen fixation activity in the seagrass rhizosphere was highest during the plant's most active growth period for the subtropical seagrass *Zostera capricorni* (O'Donohue et al. 1991a) and the temperate species *Zostera noltii* (Welsh et al. 1996a) and *Z. marina* (Smith & Hayasaka 1982). We measured depth profiles of acetylene reduction activity with a 2 cm spatial resolution at nearly monthly intervals during a full annual cycle. Our study is also the first to relate directly variation in depth profiles of acetylene reduction activity during light and dark incubations to the biomass and distribution of eelgrass roots and rhizomes. To determine both the extent to which nitrogen fixation was enhanced by organic substrate or inhibited by NH_4^+ (Capone 1988), intact vegetated cores or sediment slurries were amended with additions of organic carbon or NH_4^+ in separate experiments. Finally, eelgrass sediment cores were perfused with molybdate, a specific inhibitor of sulfate reduction (Taylor & Oremland 1979, Oremland & Capone 1988), to determine the contribution of sulfate-reducing bacteria to nitrogen fixation activity.

METHODS

Site description. This study was conducted from January through December 1995 in the Limfjord (57°N, 9°E), a shallow bay in northern Jutland, Denmark. The site chosen for this work was the same as that used in an integrated study of the effect of benthic primary producers on the nitrogen balance in Danish shallow marine waters, and was colonized by patchy, dense seagrass beds (960 to 1500 shoots m^{-2}). A detailed characterization of the aboveground (leaves) and belowground (roots+rhizomes) biomass of *Zostera marina* at the study site was made during 2 intensive field campaigns, one in April at the onset of rapid growth and the second in August at the time of maximum eelgrass biomass (N. Risgaard-Petersen, S. Rysgaard, P. B. Christensen, J. Borum, K. J. McGlathery & L. P. Nielsen unpubl.). Belowground biomass was most dense in the upper 6 cm of the sediments, but fine roots reached to a depth of at least 14 cm. The sediments were comprised of fine sands with an organic content of <1%.

Perfusion technique. Rates of acetylene reduction were measured on intact sediment cores containing live eelgrass plants (9.3 cm diameter plexiglass core; sediment depth of 14 cm, water column depth of 20 cm). A perforated PVC bottom was attached to each core and the sediment pore water was slowly drained by vacuum into a reservoir, temporarily replacing the pore water with water overlying the sediment that was obtained at the time of coring (Fig. 1). A subsample of the anoxic pore water was placed into a laminated plastic bag (NEN/PE 80/100, Danisco Flexible Otto Nielsen a/s, Denmark) in which the headspace was

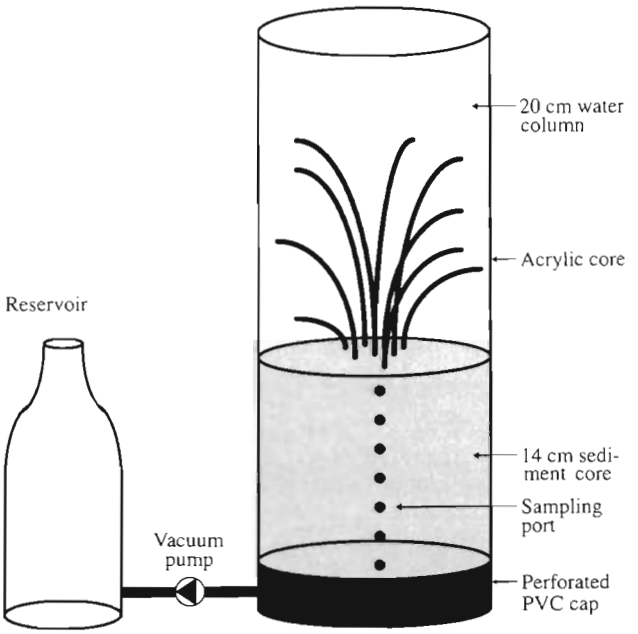

Fig. 1. Diagram of perfusion apparatus used for aceytlene reduction studies. Pore water was drained by vacuum into a reservoir through a perforated PVC cap attached to each core. The acetylene-amended pore water was drawn back into the intact sediment core by vacuum. Depth profiles of acetylene reduction activity were obtained by sampling with needle and syringe from silicone-filled holes located at 2 cm intervals in the clear acrylic core wall

filled with acetylene gas, and the bag was shaken to equilibrate the liquid and gas phases. The water sample was then added back to the reservoir to achieve a final acetylene concentration of 20% (v/v), and the amended pore water was drawn back into the intact sediment core by vacuum. At the end of a 6 to 8 h incubation, depth profiles of acetylene reduction rates in the rhizosphere were made by taking duplicate 1 ml sediment pore water samples with a syringe and needle through silicone-filled holes located at 2 cm intervals (1, 3, 5, 7, 9, 11, 13 cm depth) in the core wall. Twenty-four hour time course experiments confirmed that 6 to 8 h was the optimal incubation time in which a linear response could be measured accurately without the artifacts of a lag in response that can sometimes occur with too short an incubation time (0.5 to 2 h), or of substrate depletion from too long an incubation time (>12 h) (Capone 1982, Seitzinger & Garber 1987).

The perfusion process took <5 min per core and there was no visual indication of compaction of the core or channeling of the perfused pore water in the sandy sediments. Detailed spatial sampling of acetylene concentrations in the sediment core through multiple series of silicone holes in the core wall showed that the acetylene was uniformly distributed throughout the sediment core. This perfusion technique is particularly suitable for measuring nitrogen fixation rates in sandy sediments, but may be less effective for sediments with a high clay content. Bromide additions to the water column of the experimental cores showed that the sampling did not result in appreciable dilution of sediment pore water by drawing water down from the overlying water column (<5% dilution for samples taken from the upper 3 cm of sediment, and <1% in the deeper layers). Oxygen and nutrient gradients were disturbed by the perfusion process, but, unlike the continuous perfusion of sediment cores described by Capone & Carpenter (1982) where pore water is constantly flushed through the sediment core, the short perfusion time and subsequent 6 to 8 h incubation probably allowed microgradients to become reestablished around the roots because nutrient turnover time in the pore water is high (Capone 1982, Boon et al. 1986a, O'Donohue et al. 1991b). Pore water samples taken during the incubation period also smelled strongly of H_2S, indicating anoxic conditions in the rhizosphere. The advantages of this perfusion technique are that it maintains the direct root-microbe interactions during the incubation period that are disturbed using the slurry method, and also avoids the potential release of labile organic carbon compounds from damaged roots that may be a source of error in slurry incubations (O'Donohue et al. 1991b, Hines et al. 1994, Welsh et al. 1996a). The perfusion method also has a distinct advantage over the lacunal diffusion technique, because the latter relies on gas exchange through the plant lacunal system and may only saturate bacteria adjacent to the roots due to slow diffusion rates in the sediments (O'Donohue et al. 1991b, Welsh et al. 1996a). This may result in uneven and unsaturating concentrations of acetylene below the root zone (O'Donohue et al. 1991b, Moriarty & O'Donohue 1993) and an underestimation of nitrogen fixation rates.

Pore water samples were stored in 3 ml evacuated tubes (Venoject) containing 25 µl $ZnCl_2$ (50% w/w) to stop bacterial activity, and were analyzed for acetylene and ethylene concentrations within a week of sampling. Separate time course studies showed that samples could be preserved for this time period without measurable loss of ethylene from the tubes. Ethylene and acetylene concentrations were measured by flame ionization gas chromatography on a sample taken from the headspace after several minutes of shaking to equilibrate the liquid and gas phases. Sediment porosity was determined from weight differences before and after drying a known volume of sediment at 105°C for 24 h. Acetylene reduction rates at each depth were calculated using the appropriate Bunsen coefficient as

$$\Delta C \varphi / \Delta t \quad (1)$$

where ΔC is the measured change in pore water ethyl-

ene accounting for the ethylene present in the pore water at the start of the incubation, φ is the sediment porosity, and Δt is the incubation time. To determine the potential effect of the vertical diffusion of ethylene on our results, we recalculated the acetylene reduction rates using a modified version of the discrete approximation of Fick's second law given by Rysgaard & Berg (1996). These calculations gave the same ethylene production rates within 1%, indicating that the diffusive transport of ethylene during the incubation period was insignificant. Acetylene reduction rates were related to nitrogen fixation rates using the stoichiometric relationship of 1 mol dinitrogen fixed for every 3 mol acetylene reduced. Though this ratio is known to vary in marine sediments (Seitzinger & Garber 1987), we based our calculations on the calibration of acetylene reduction with $^{15}N_2$ fixation made by O'Donohue et al. (1991a) in *Zostera capricorni* sediments, which showed little departure from the theoretical 3:1 ratio. Results are reported as calculated nitrogen fixation rates on an area or volume basis.

Temporal patterns of nitrogen fixation activity. On each of the sampling dates during 1995, 8 replicate cores containing live eelgrass plants were collected and held overnight in a water bath in a temperature-controlled incubation room, and were processed the following day to determine nitrogen fixation activity. Acetylene was added to the sediment pore water using the perfusion technique, and then half the cores were incubated in the light and half in the dark (representing day and night, respectively) at *in situ* temperatures. The illuminated cores were submerged in an outdoor bath of seawater taken from the site. The water column above the cores was a similar depth to that in the field at low tide so that the incubation light intensities were as similar as possible to field light intensities. A comparison of depth profiles of acetylene reduction activity in vegetated and bare sediments was made in October

Carbon, ammonium and molybdate effects on nitrogen fixation. Twelve sediment cores containing intact eelgrass plants were incubated in laboratory experiments during June 1995 to determine the extent of carbon limitation of the heterotrophic nitrogen-fixing bacteria. Pore water was drained from the cores, and a source of labile dissolved organic carbon (1 mM glucose) was added with acetylene (20% v/v) to the reservoir of half the cores. The other 6 cores received acetylene additions only and served as controls. Half of the cores (3 glucose amended, 3 controls) were then incubated in the dark and half at the *in situ* light level. After a 6 h incubation, detailed pore water samples were taken as described above and analyzed for acetylene reduction activity.

The interaction of pore water NH_4^+ concentrations and acetylene reduction activity in the seagrass rhizosphere was determined both at *in situ* NH_4^+ levels and in NH_4^+ addition experiments. First, depth profiles of acetylene reduction activity were made in June 1995 in triplicate perfused cores containing intact eelgrass plants as described above, and acetylene reduction rates were correlated with pore water NH_4^+ concentrations from the same depth interval. For the NH_4^+ analysis, 1 ml of pore water from each 2 cm depth interval was removed through the silicone-filled holes in the core wall using a needle and syringe, and concentrations were determined immediately using the salicylate-hypochlorite method of Bower & Holm-Hansen (1980). Second, we tested the effect of NH_4^+ additions on acetylene reduction activity in slurry incubations of sediment collected from the depth interval of maximum activity in the eelgrass rhizosphere. We chose slurry incubations for this experiment so that we could isolate the effects of varying levels of NH_4^+ on acetylene reduction activity in the sediment from responses linked to variations in plant activity. The 2 to 6 cm depth intervals from 6 vegetated cores were pooled, and 6 cm^3 of sediment was added to 4 replicate 11 ml gas-tight vials with Butyl-rubber stoppers (Exetainer, Labco, High Whycombe, UK) for each of 5 treatments: no NH_4^+ addition (control), and 200, 400, 600 and 800 µM NH_4^+ additions. The vials were immediately filled with N_2-bubbled nutrient-free seawater amended with acetylene (20% v/v) and the appropriate NH_4^+ concentration, and were incubated for 6 h on a shaker table. At the end of the incubation, the vials were shaken vigorously to form a suspension and a 1 ml subsample was removed with a needle and syringe and immediately placed in a 3 ml Venoject containing 25 µl 50% (w/w) $ZnCl_2$ to stop bacterial activity. Ethylene concentrations were measured by gas chromatography as described above, and net reduction rates of acetylene were calculated and expressed on a g dry weight (gDW) basis.

Molybdate (20 mM) was added as Na_2MoO_4 using the perfusion technique to cores containing live eelgrass plants to determine the extent to which sulfate-reducing bacteria were responsible for the patterns of nitrogen fixation in these sediments. The cores were incubated in triplicate in the light for 6 h and acetylene reduction rates measured as described above.

Nitrogen fixation in excised roots and rhizomes. To determine the relative contribution by heterotrophic bacteria either attached to or embedded within the eelgrass roots and rhizomes to the total nitrogen fixation activity in the rhizosphere, separate measurements of acetylene reduction were made on roots and rhizomes picked out of vegetated sediment cores. Roots and rhizomes were incubated separately (n = 4) in 11 ml glass vials (Exetainer, Labco) to which 20% (v/v) acetylene-saturated, nutrient-free seawater was added. Incuba-

tions lasted for 6 h and samples were shaken continuously. One set of roots and rhizomes was separated from the sediments under anoxic conditions and incubated in anoxic seawater. The second set was exposed to oxygen during both processing and incubation. At the end of the incubation, a 1 ml subsample was drawn from the incubation vial and placed into a 3 ml Venoject containing 25 µl of 50% (w/w) $ZnCl_2$, and ethylene concentrations were measured on gas samples drawn from the headspace after shaking to equilibrate the water and gas phases. A set of root and rhizome incubations also were run under both oxic and anoxic conditions, to which nutrient-free seawater was added without acetylene. This was done to check if ethylene was produced by the excised roots and rhizomes as a damage response, independent of acetylene reduction.

RESULTS

Depth-integrated nitrogen fixation rates

The set of whole-core incubations with no acetylene added to the perfused water showed that ethylene was not produced from root damage during the perfusion and incubation procedures independent of acetylene reduction, and that the perfused pore water could be used as an appropriate blank for the entire depth profile. In addition, there was no measurable nitrogen fixation activity in 6 to 8 h incubations of excised roots and rhizomes, regardless of the treatment (oxic vs anoxic). There also was no ethylene produced from root damage in the control incubations of roots and rhizomes that were incubated without the addition of acetylene.

Heterotrophic nitrogen fixation was stimulated by the presence of eelgrass, and showed strong temporal variations in activity that corresponded to the general seasonal pattern of plant productivity (Pedersen & Borum 1993). Rates of nitrogen fixation integrated to a depth of 14 cm in the eelgrass rhizosphere ranged from about 4 to 20 µmol N m^{-2} h^{-1} (Fig. 2). There was a strong seasonal variation in nitrogen fixation activity (repeated-measures ANOVA: $F_{7,21} = 25.9$, $p < 0.0001$), where rates in both the light and dark were lowest in the winter months, and increased steadily through the spring and summer to a peak activity in August that coincided with the maximum eelgrass biomass (Risgaard-Petersen et al. unpubl.). Rates in the light-incubated cores declined during the fall and winter months as temperatures and eelgrass productivity also declined. There was a slight increase in dark nitrogen fixation activity during the fall.

Depth-integrated nitrogen fixation activity also showed significant variation between light and dark incubations throughout the year (Fig. 2; repeated-measures ANOVA: $F_{7,21} = 3.69$, $p = 0.009$), with cores incubated in the light having consistently higher rates than those incubated in the dark, except during the winter when bacterial and plant productivity were both negligible (Fig. 2). This difference was greatest when plant productivity was highest during the summer months, when light-incubated rates were 40 to 50% higher than dark-incubated rates (Fig. 2).

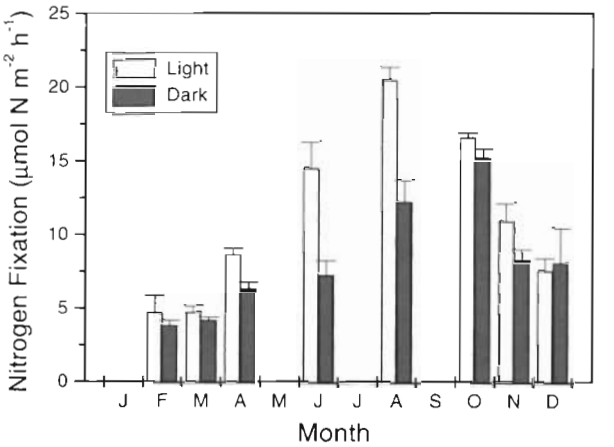

Fig. 2. Seasonal variation in depth-integrated nitrogen fixation activity (acetylene reduction) in sediments colonized by *Zostera marina*. Sediment cores were perfused with acetylene-saturated pore water and incubated at *in situ* temperature and light levels for 6 h. Values represent means (n = 3) +1 SE

Depth profiles of nitrogen fixation

Nitrogen fixation rates were typically 3× higher in vegetated than in unvegetated sediments, and activity in the eelgrass rhizosphere showed a marked variation with depth that was absent in the bare sediments (Fig. 3; repeated-measures ANOVA: $F_{6,18} = 2.92$, $p = 0.036$). This depth variation in nitrogen fixation was apparent throughout the year in the eelgrass rhizosphere in both the light (repeated-measures ANOVA: $F_{42,114} = 5.11$, $p < 0.0001$) and dark (repeated-measures ANOVA: $F_{42,126} = 4.60$, $p < 0.0001$), but was most prominent during the summer months (Fig. 4). Depth-specific rates of nitrogen fixation showed that activity was low throughout the rhizosphere in both the light (Fig. 4A) and dark (Fig. 4B) incubations during the winter and early spring, and then reached a subsurface maximum in activity in the spring and early summer that was 4× higher than the low rates measured in the top and bottom few cm of the sediment core. Nitrogen fixation rates were highest throughout the rhizosphere in summer and showed a strong (5-fold) spatial variation, with maximum activity in the surface layers of the sediment core (Fig. 4). During the fall, nitrogen fixation

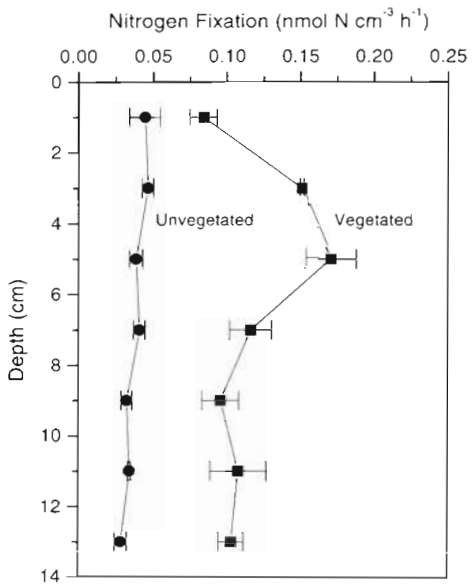

Fig. 3. Comparison of depth profiles of nitrogen fixation activity (acetylene reduction) in sediments colonized by *Zostera marina* and in nearby uncolonized sediments. Values represent means (n = 3) ±1 SE

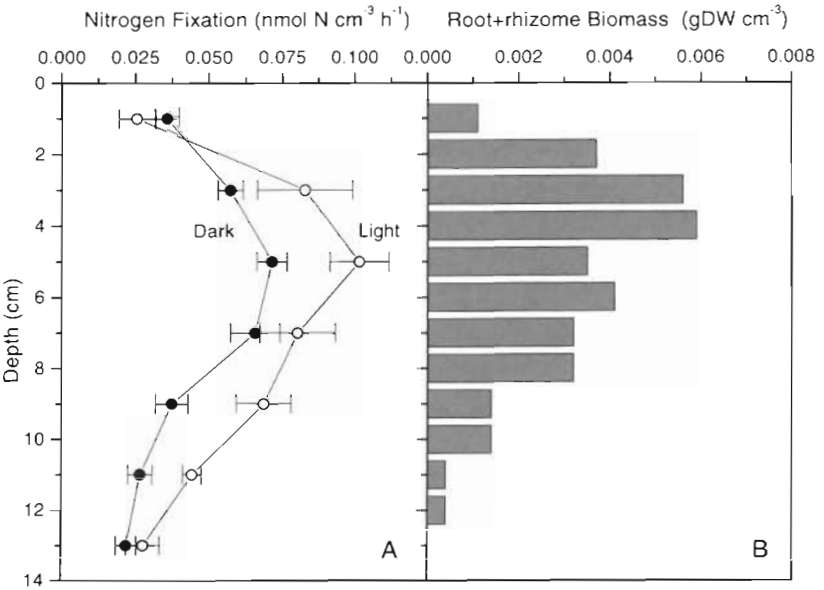

Fig. 5. Comparison of depth profiles of (A) nitrogen fixation (acetylene reduction) activity in *Zostera marina* vegetated cores incubated under light and dark conditions (means ± SE, n = 3) and (B) belowground root+rhizome biomass of *Z. marina* at the spring onset of rapid growth in April. Biomass was estimated from pooled samples (n = 3)

activity declined throughout the core, and again showed a slight subsurface peak in activity in both the light and dark (Fig. 4).

A comparison of depth profiles of both nitrogen fixation activity and belowground biomass of *Zostera marina* in April and August showed that the variation in nitrogen fixation activity with depth in the eelgrass root zone corresponded to root+rhizome biomass and was consistent with seasonal changes in belowgound biomass distribution (Figs. 5 & 6). In April, at the onset of rapid eelgrass growth, nitrogen fixation correlated with belowground root+rhizome biomass (r = 0.629, p = 0.016, n = 14) and the peak activity occurred at 4 to 6 cm depth, where the seagrass belowground biomass was also maximal (Fig. 5). In August, when eelgrass biomass was highest, the zone of maximum nitrogen fixation moved upward to the top 0 to 2 cm of sediment (Fig. 6A) and was correlated with a similar shift in root+rhizome biomass (Fig. 6B; r = 0.723, p = 0.004, n = 14). Depth-specific nitrogen fixation rates were about 2× higher in light-incubated cores than in cores incubated in the dark (Fig. 6A). Belowground biomass of *Z. marina* was roughly 3× higher at the depth interval where it reached its maximum in August than in April and corresponded to an increase in nitrogen fixation rates of the same magnitude (Fig. 5).

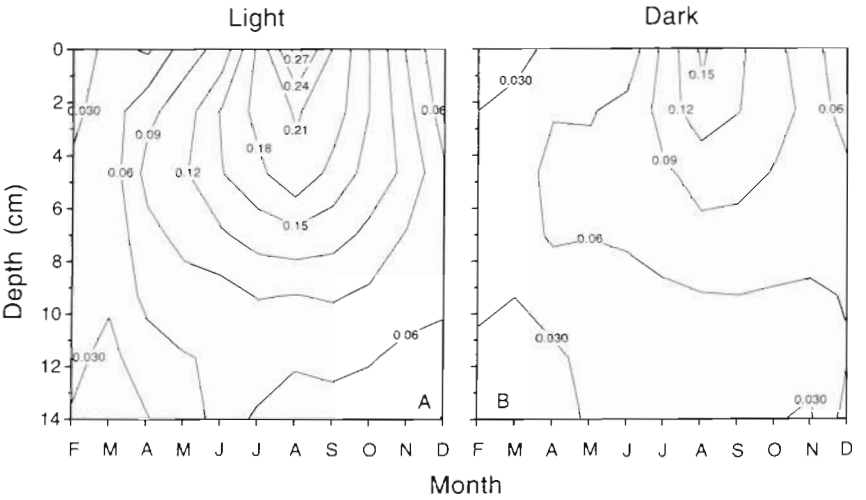

Fig. 4. Depth distribution and seasonal variation of nitrogen fixation activity (acetylene reduction) in perfused sediment cores vegetated by *Zostera marina* during 6 h (A) light and (B) dark incubations. Duplicate pore water samples were taken at 2 cm depth intervals on triplicate cores for each treatment for the 8 sampling dates. Units are nmol N cm^{-3} h^{-1}

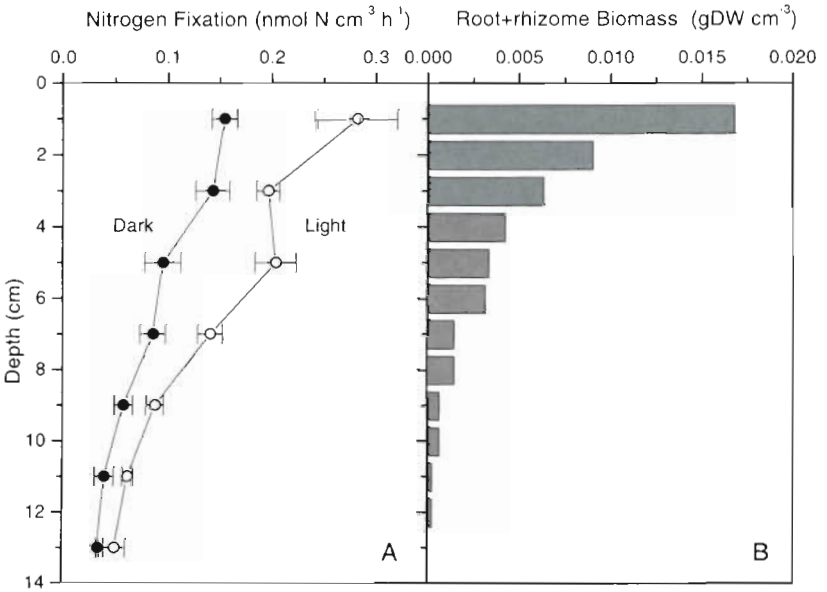

Fig. 6. Comparison of depth profiles of (A) nitrogen fixation (acetylene reduction) activity in *Zostera marina* vegetated cores incubated under light and dark conditions (means ± SE, n = 3) and (B) belowground root+rhizome biomass of *Z. marina* at the summer peak in seagrass biomass in August. Biomass was estimated from pooled samples (n = 6)

Factors controlling nitrogen fixation in the eelgrass rhizosphere

Depth-integrated nitrogen fixation rates were nearly 2-fold higher in carbon-amended cores that were incubated in both the light and dark compared to controls to which only acetylene was added [Fig. 7; *t*-test: $p < 0.06$ (light), $p < 0.003$ (dark)]. The depth profile also shows that both dark and light rates of nitrogen fixation were stimulated throughout the rhizosphere, and that the largest increase occurred in the upper 5 cm, where the root+rhizome biomass was highest (Fig. 8).

Pore water NH_4^+ was found to have no negative effect on nitrogen fixation acitivity, even at *in situ* concentrations up to 650 µM (Fig. 9). This was shown both in the absence of NH_4^+ inhibition in the NH_4^+ addition experiments (Table 1; ANOVA: $F_{4,14} = 0.61$, $p = 0.662$), and in the lack of a negative correlation between *in situ* NH_4^+ concentrations and acetylene reduction in perfused cores (Fig 9). There was actually a positive correlation between *in situ* NH_4^+ levels and acetylene reduction ($r = 0.349$, $p = 0.04$, $n = 36$).

Sulfate reducers appear to be responsible for at least part of the nitrogen fixation activity. Additions of molybdate with acetylene to the pore water using the perfusion technique decreased nitrogen fixation activity in the light by 25% (Fig. 7; *t*-test: $p = 0.244$). This response is also shown in the depth profile of nitrogen fixation in the eelgrass root zone (Fig. 10), where molybdate additions reduced nitrogen fixation activity throughout the depth profile in the light-incubated cores.

Table 1. Rates of anaerobic nitrogen fixation (acetylene reduction) of sediment from *Zostera marina* beds incubated for 6 h in slurries with different levels of NH_4^+ additions. Values are means (±1 SE)

NH_4^+ addition (µM)	Acetylene reduction (nmol C_2H_4 gDW^{-1} h^{-1})
Control	0.058 (0.003)
+ 200	0.061 (0.003)
+ 400	0.062 (0.003)
+ 600	0.055 (0.003)
+ 800	0.057 (0.001)

DISCUSSION

The temporal patterns of nitrogen fixation rates integrated to a depth of 14 cm in the *Zostera marina* vegetated sediment indicated a clear coupling between plant productivity and heterotrophic bacterial activity in the seagrass rhizosphere. This was evident both in the absolute magnitude of nitrogen fixation, which showed a variation that was consistent with the annual trends in plant biomass and productivity typical of temperate populations (Pedersen & Borum 1993), and in

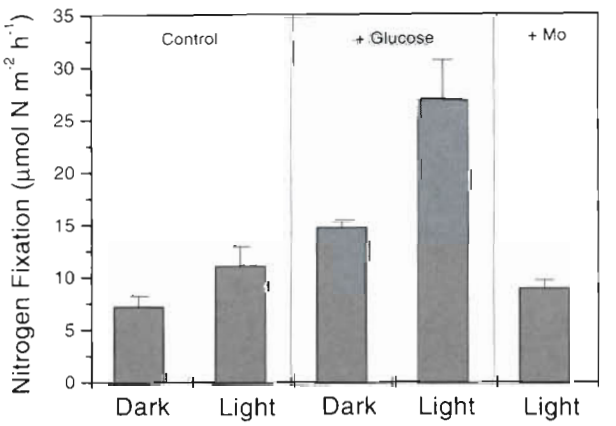

Fig 7. Depth-integrated rates (means + SE, n = 3) of nitrogen fixation (acetylene reduction) in sediment cores vegetated by *Zostera marina* and amended using the perfusion technique with glucose as a labile carbon source or molybdenum to inhibit the activity of sulfate-reducing bacteria

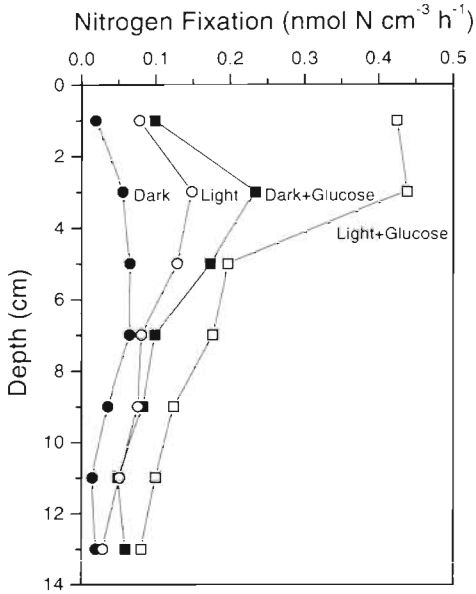

Fig. 8. Depth profiles of light- and dark-incubated intact sediment cores from a *Zostera marina* bed with or without the addition of glucose to the sediment pore water using the acetylene-reduction perfusion technique

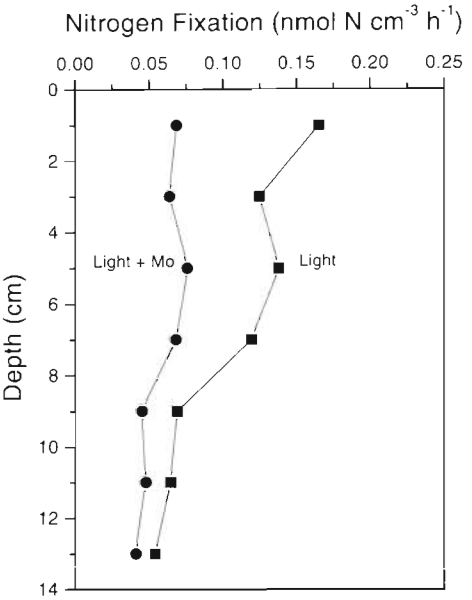

Fig 10. Depth profiles of light-incubated intact sediment cores from a *Zostera marina* bed using the acetylene-reduction perfusion technique with or without the addition of molybdate to the sediment pore water as an inhibitor of sulfate-reducing bacteria

the differences between light and dark incubations throughout the year (Fig. 2). Although temperature also clearly influences bacterial activities (Smith & Hayasaka 1982), it alone cannot account for the observed differences between light and dark incubations. On a seasonal basis, depth-integrated nitrogen fixation rates were lowest in the winter months, when eelgrass biomass was at a minimum, and then began to increase at the onset of rapid growth in April, reaching a maximum in August some 4-fold higher than winter rates that coincided with the high summer *Z. marina* biomass and productivity (Pedersen & Borum 1993, Risgaard-Petersen et al. unpubl.). Rates declined in the fall as colder temperatures and shorter daylength reduced eelgrass growth, but remained relatively high compared to the spring (Fig. 2), perhaps due to the greater eelgrass standing crop or to nitrogen fixation associated with decaying roots and rhizomes (Kenworthy et al. 1987). This seasonal pattern of nitrogen fixation in the eelgrass rhizosphere shows a more detailed correspondence between plant productivity (seagrasses and salt-marsh grasses) and nitrogen fixation than has been shown previously by studies based on quarterly or semi-annual measurements (Capone & Taylor 1980, Smith & Hayasaka 1982, Whiting et al. 1986, O'Donohue et al. 1991a, Welsh et al. 1996a).

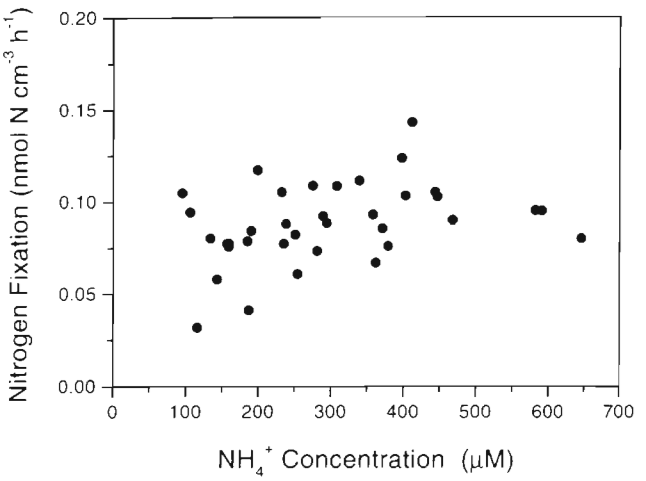

Fig. 9. Relationship between nitrogen fixation (acetylene reduction) and pore water NH_4^+ concentrations on samples taken at 2 cm depth intervals in triplicate sediment cores containing intact *Zostera marina* plants

Differences in the degree of light stimulation of nitrogen fixation activity throughout the year also clearly show the link between plant metabolism and heterotrophic bacterial activity in the eelgrass rhizosphere. This relationship has also been suggested from laboratory experiments that found direct light stimulation of nitrogen fixation in the root zone of the seagrass *Zostera carpricorni* (O'Donohue et al. 1991a) and the salt-marsh grass *Spartina alterniflora* (Whiting et al. 1986). In the eelgrass rhizosphere, there was no stimulation of the low nitrogen fixation rates by light during the cold winter months (December to February), presumably because both plant biomass and growth were

low (Montcreiff et al. 1992, Pedersen & Borum 1993) and light availability thus had an insignificant effect on plant metabolism. As the growing season progressed with higher temperatures and greater light availability, heterotrophic nitrogen fixation in the light was enhanced 25 to 40% relative to the dark rates, with the largest differences occurring when seagrass productivity was highest. Welsh et al. (1996a) also found that the difference between nitrogen fixation activity in the rhizosphere of the temperate seagrass *Zostera noltii* during 4 seasonal light and dark incubations was maximal in the summer and minimal in the winter. In the *Z. marina* rhizosphere, the degree of light stimulation decreased in the fall as lower temperatures and irradiance levels reduced seagrass productivity. The relatively high nitrogen fixation rates in the dark could be due to a larger subsurface carbon input in the fall from decaying roots and rhizomes (Kenworthy et al. 1987) and other organic matter not directly linked to plant metabolism.

Spatial trends in the depth profiles of nitrogen fixation throughout the year provide further evidence that is consistent with the positive influence of plant metabolism on heterotrophic bacterial activity in the rhizosphere. First, nitrogen fixation rates were 2 to 4× higher in the seagrass-vegetated sediments than in nearby unvegetated sediments, and showed a distinct subsurface peak in activity that corresponded to the depth of maximum seagrass belowground biomass (Fig. 3). Other studies also have shown high nitrogen fixation activity in sediments vegetated by tropical and temperate seagrasses compared to bare sediments, but have not related the subsurface peak to seagrass belowground biomass (Capone & Taylor 1980, O'Donohue et al. 1991a, Moriarty & O'Donohue 1993, Welsh et al. 1996b). Second, variations in spatial patterns of depth-specific nitrogen fixation rates during the year were consistent with variations in plant biomass and productivity (Pedersen & Borum 1993; Fig 4). In the winter months, nitrogen fixation was low and relatively homogenous throughout the sediment core in both the light and dark, a pattern that would be expected if plant metabolism had little effect on bacterial activities when productivity was low. The subsurface peaks in nitrogen fixation activity that later developed during the spring and summer corresponded directly to the variations in *Zostera marina* belowground biomass measured in April and August (Figs. 4, 5 & 6). Previous studies have shown some depth variation in nitrogen fixation rates in the rhizosphere of several tropical and temperate seagrass species, with highest rates typically in the upper 4 cm of the sediment (Capone 1982, O'Donohue et al. 1991a, Moriarty & O'Donohue 1993, Welsh et al. 1996a), but have not related these patterns directly to seagrass root+rhizome biomass. Welsh et al. (1996a) found an increase in nitrogen fixation activity in the upper few cm in the *Z. noltii* rhizosphere between the spring and summer in the Bassin d'Arcachon in southwest France, and suggested that this was due to increased activity of sulfate-reducing bacteria, which were the dominant component of the nitrogen-fixing microflora. Other studies have not shown variation in bacterial activity with depth in seagrass-vegetated sediments (Moriarty & O'Donohue 1993, Blackburn et al. 1994), possibly because the results were from slurry incubations, or because seagrass metabolism had little effect on sediment bacteria due to low shoot and root densities.

Plant metabolism may directly influence heterotrophic nitrogen fixation activity in the rhizosphere by releasing photosynthetically derived organic carbon from the roots (Moriarty & Pollard 1982, Moriarty et al. 1986) or by altering NH_4^+ and O_2 levels in the rhizosphere (Caffrey & Kemp 1990, 1992). Both the laboratory experiments with intact root-microbe associations and the high spatial and temporal resolution of the field results indicate that the most plausible explanation for the strong coupling of nitrogen fixation in the eelgrass rhizosphere to plant productivity is stimulation of heterotrophic bacteria by the release of organic exudates from seagrass roots. Heterotrophic bacteria in the seagrass rhizosphere depend to a large extent on the supply of a readily oxidizable organic carbon pool, of which root exudates are a large component (Moriarty & Pollard 1982, Moriarty et al. 1986). The laboratory experiments with glucose-amended sediment cores clearly showed that the nitrogen-fixing bacteria in the rhizosphere were carbon limited. There was a doubling of nitrogen fixation activity in both the light and dark incubations (Fig. 7), and this stimulation was most pronounced (3 to 5×) in the 1 to 3 cm depth strata where root biomass was highest (Fig. 8). Slurry incubations of other seagrass-vegetated sediments have sometimes (Capone 1982), but not always (Welsh et al. 1996b), shown carbon stimulation of heterotrophic nitrogen fixation. Unlike the perfusion method which uses whole cores with intact plants, the slurry technique may provide equivocal results in carbon addition experiments because seagrass roots may be damaged during preparation of the sediment slurries, and this may result in the release of internal pools of labile carbon which may artificially increase carbon availability during the incubations (Welsh et al. 1996b). The temporal (seasonal and light vs dark) and spatial (depth profiles) trends in nitrogen fixation activity in the *Zostera marina* rhizosphere discussed earlier also point to the stimulation of heterotrophic bacteria by a subsurface carbon input linked to plant productivity. Low nitrogen fixation rates and a lack of light stimulation during the winter probably were due to low

organic carbon inputs from root exudates when plant productivity was low. In the spring and summer, the large differences between light and dark rates and the direct correlation between shifts in root biomass and patterns of nitrogen fixation activity indicate a carbon source related to plant productivity, since root exudation would be expected to be greater during the active growth season and should be most pronounced in the depth interval where belowground biomass is highest.

The link between dissolved organic matter release from plant roots and bacterial activities also has been made to explain temporal dynamics of sulfate reduction in sediments vegetated by the temperate seagrass *Zostera noltii* (Welsh et al. 1996b), the tropical seagrass *Halodule* sp. (Blackburn et al. 1994), salt-marsh grasses (Nedwell & Aziz 1980, Hines et al. 1989), and mangroves (Nedwell et al. 1994). Pollard & Moriarty (1991) suggested that the spatial pattern of sulfate reduction in sediments colonized by several tropical seagrass species correlated with the belowground distribution of seagrass roots and rhizomes. Additions of molybdate to perfused cores in the *Z. marina*-vegetated sediments showed that sulfate reducers were responsible for 25% of the nitrogen fixation activity (Figs. 7 & 10). Other studies have found that sulfate-reducing bacteria were responsible for a higher proportion of the nitrogen fixation acitivity in sediment slurries (80% for *Z. noltii*, Welsh et al. 1996a; 95% for *Z. marina*, Capone 1982).

Ammonium control of nitrogen fixation has been demonstrated for a variety of photosynthetic and heterotrophic bacteria, where NH_4^+ additions repress the synthesis of the nitrogenase enzyme responsible for nitrogen fixation (Yoch & Whiting 1986, Capone 1988). Heterotrophic nitrogen fixation, however, tends to be high in organic-rich marine sediments with relatively high NH_4^+ concentrations (Capone 1988), even though studies on salt marsh sediments have shown that NH_4^+ can inhibit nitrogen fixation at concentrations as low as 100 to 200 µM (Carpenter et al. 1978, Teal et al. 1979, Yoch & Whiting 1986). We found no direct evidence that NH_4^+ concentrations regulated nitrogen fixation at our study site, and we believe it is unlikely that changes in NH_4^+ availability could account for the temporal and spatial variation in nitrogen fixation during the year. Ammonium concentrations were high (100 to 650 µM) in the eelgrass rhizosphere, and showed no inverse correlation with acetylene reduction, nor was there an effect of NH_4^+ additions (200 to 800 µM) on acetylene reduction rates (Fig. 9, Table 1). Repression of nitrogenase activity by NH_4^+ is often variable in sediments, both with and without vegetation, and may partly reflect differences in bacterial populations that vary in their susceptibility to NH_4^+ inhibition (Capone 1988, O'Neil & Capone 1989). It is possible, however, that we did not observe NH_4^+ repression of nitrogen fixation in either the intact cores or sediment slurries because *in situ* nitrogen fixation activities were already below maximum rates because of the presence of NH_4^+ (Capone 1988), and thus NH_4^+ additions would be unlikely to further decrease nitrogen fixation rates. This has been shown in studies on the seagrass *Zostera marina* and the salt-marsh grass *Spartina alterniflora*, where additions of MSX (L-methionine-D, L-sulfoximine), which is known to negate the repression of nitrogenase synthesis in the presence of NH_4^+, caused a marked increase in nitrogenase activity and indicated that *in situ* rates were submaximal (Yoch & Whiting 1986, Capone 1988). It is also possible that even at the high NH_4^+ concentrations measured in the seagrass rhizosphere, bacterial nitrogen fixation may be favored in localized microzones close to the seagrass roots. Seagrasses decrease NH_4^+ concentrations in the vicinity of the roots through direct uptake (Caffrey & Kemp 1990, 1992), and may therefore compete with bacteria for NH_4^+ (Welsh et al. 1996b). Non-nitrogen-fixing bacteria also place a demand on available nitrogen in the rhizosphere. Localized nitrogen limitation could therefore occur in microzones around the roots, but could be missed in the bulk measurements of NH_4^+ concentrations and acetylene reduction activity.

We also have no evidence that variations in O_2 conditions in the rhizosphere could account for the patterns of nitrogen fixation activity in these *Zostera marina* sediments. Nitrogen fixation in the seagrass rhizosphere (roots+rhizomes and sediments) is typically higher in anoxic or microaerophilic conditions than in oxic conditions (Capone & Budin 1982, Capone 1988, O'Donohue et al. 1991a) because the nitrogenase enzyme is highly O_2 sensitive (Capone 1988). Oxygen released from the roots would tend to have a negative effect, if any, on acetylene reduction rates in the areas of highest root biomass (Capone & Budin 1982, O'Donohue et al. 1991a). We did not see this negative effect in our study, which suggests that either O_2 excretion by the roots was low, or O_2 consumption near the roots was high presumably in part due to high sulfate reduction activity (Blaabjerg et al. 1998). The exudation of labile organic compounds from the seagrass roots may also counteract the effects of oxygen release by promoting areas of localized O_2 consumption around the roots that may be particularly conducive to heterotrophic nitrogen fixation. Nitrogen fixation activity of bacteria directly associated with the seagrass roots and rhizomes can sometimes be stimulated by fully oxic conditions (Smith & Hayasaka 1982, O'Donohue et al. 1991a), which could represent a link between seagrass metabolism and heterotrophic nitrogen fixation activity. However, the absence of measurable activity in excised roots and rhizomes in our study

Table 2. Comparison of nitrogen fixation estimates by acetylene reduction in the rhizosphere of temperate and tropical seagrass beds

Site	Species	Season/ Month	Nitrogen fixation (mg N m^{-2} d^{-1})	Source
Temperate				
Limfjord, Denmark	*Zostera marina*	Winter	1.2–2.7[a]	This study
Limfjord, Denmark	*Zostera marina*	Spring	1.5–2.6[a]	This study
Limfjord, Denmark	*Zostera marina*	Summer	4.2–6.0[a]	This study
Limfjord, Denmark	*Zostera marina*	Fall	3.1–5.5[a]	This study
Great South Bay, New York, USA	*Zostera marina*	Summer	3.9–6.5[b]	Capone (1982)
Vaucluse Shores, Virginia, USA	*Zostera marina*	Summer	5.2[b]	Capone (1982)
Bassin d'Arachon	*Zostera noltii*	Spring	0.2–0.4[c]	Welsh et al. (1996a)
Bassin d'Arachon	*Zostera noltii*	Summer	2.0–7.3[c]	Welsh et al. (1996a)
Bassin d'Arachon	*Zostera noltii*	Fall	1.8–4.4[c]	Welsh et al. (1996a)
Bassin d'Arachon	*Zostera noltii*	Winter	0.1–0.2[c]	Welsh et al. (1996a)
Tropical and subtropical				
Gulf of Carpentaria, Australia	*Syringodium isoetifolium* *Cymodocea serrulata*	Feb	16–47[d]	Moriarty & O'Donohue (1993)
Gulf of Carpentaria, Australia	*Thalassia hemprichii* *Cymodocea serrulata*	Feb	13–19[e]	Moriarty & O'Donohue (1993)
Gulf of Carpentaria, Australia	*Enhalus acoroides*	Nov	25[f]	Moriarty & O'Donohue (1993)
Moreton Bay, Australia	*Zostera capricorni*	Summer	25–40[g]	O'Donohue et al. (1991a)
Moreton Bay, Australia	*Zostera capricorni*	Winter	10[g]	O'Donohue et al. (1991a)
Barbados	*Thalassia testudinum*	Sep	27–140[h]	Patriquin & Knowles (1972)
Bimini Harbor, Bahamas	*Thalassia testudinum*	Jul	5.1–5.3[i]	Capone et al. (1979)
Florida, USA	*Thalassia testudinum*	Mar, Jan	0.03[j]	McRoy et al. (1973)
Biscayne Bay, Florida, USA	*Thalassia testudinum*	Aug, Oct	7.5–24.3[k]	Capone & Taylor (1980)
Bimini Harbor, Bahamas	*Thalassia testudinum*	Aug	5.3–12.1[k]	Capone & Taylor (1980)
Jamaica	*Halodule beaudetti*	Dec	14[l]	Blackburn et al. (1994)

[a]Acetylene added to pore water of intact cores containing plants by perfusion and incubated in light or dark. Daily rates integrated to depth of 14 cm calculated using average number of daylight and dark hours per month and dinitrogen conversion ratio of 3:1

[b]Slurry incubations of 12 cm deep sediment cores containing both sediments and roots. Daily rates calculated using dinitrogen conversion ratio of 2.6:1 and assuming a constant rate for 24 h

[c]Acetylene added to headspace of intact sediment cores containing plants. Nitrogen fixation calculated using 3:1 dinitrogen conversion ratio

[d]Range of nitrogen fixation values determined in mixed seagrass bed by both slurry incubations and pore water perfusion prior to incubation; rates integrated to a depth of 12 cm using using dinitrogen conversion ratio of 3:1. Average values for the techniques were 33 mg N m^{-2} d^{-1} (slurry) and 41 mg N m^{-2} d^{-1} (perfusion)

[e]Range of nitrogen fixation values determined in mixed seagrass bed by both slurry incubations and pore water perfusion prior to incubation; rates integrated to a depth of 7 cm using using dinitrogen conversion of 3:1. Average values for the techniques were 16 mg N m^{-2} d^{-1} (slurry) and 15 mg N m^{-2} d^{-1} (perfusion)

[f]Fixation determined by slurry incubation of sediments without roots, integrated to a depth of 12 cm using 3:1 dinitrogen conversion ratio

[g]Pore water of intact sediment cores containing plants perfused with acetylene-amended water prior to incubation. Calculation of fixation based on ^{15}N calibration yielding dinitrogen conversion ratio of 3:0.95

[h]Slurry incubations of sediments including roots and rhizomes. Annual fixation rates based on dinitrogen conversion ratio of 3:1 were converted to daily rates assuming 365 d yr^{-1} in tropics

[i]Slurry incubation of 20 cm sediment cores divided into 0–10 cm and 10–20 cm sections; fixation calculated using dinitrogen conversion ratio of 3:1

[j]Maximum rate measured on sediments containing roots and rhizomes in flasks to which acetylene-amended seawater was added

[k]Slurry incubations including roots, rhizomes and sediments integrated to a depth of 20 cm and converted to nitrogen fixation based on 3:1 dinitrogen conversion ratio. Hourly rates were converted to daily rates by multiplying by 24

[l]Acetylene-saturated seawater injected at 0.5 cm intervals into 7 cm sediment core; overlying water adjusted to 10% saturation. Samples taken from sediment slurries following incubation

indicates that this was probably not responsible for the patterns we observed.

Overall, daily nitrogen fixation rates integrated to a depth of 14 cm measured in this study (1 to 6 mg N m^{-2} d^{-1}) were lower than rates typically measured in tropical and subtropical seagrass beds (5 to 140 mg N m^{-2} d^{-1}; Table 2), but were comparable to rates measured in other eelgrass beds (3 to 7 mg N m^{-2} d^{-1}; Table 2) and temperate seagrass sediments (<1 to 7 mg N m^{-2} d^{-1}; Table 2). The annual rate of heterotrophic nitrogen fixation in the *Zostera marina* rhizosphere based on monthly light/dark rates and corrected for the average number of light and dark hours each month was 1.3 g N m^{-2} yr^{-1}. This value is very similar to that calculated for *Z. marina* in New York (0.84 g N m^{-2} yr^{-1}) and Virginia, USA (1.0 g N m^{-2} yr^{-1}) (Capone 1982, calculated by Howarth et al. 1988), and for *Z. noltii* in southwest France (0.4 to 1.1 g N m^{-2} yr^{-1}; Welsh et al. 1996a). Kenworthy et al. (1987) also reported a somewhat lower rate of 0.57 g N m^{-2} yr^{-1} only for nitrogen fixation associated with *Z. marina* detritus in a North Carolina (USA) seagrass meadow. This fixed N, once mineralized, may be taken up by the plant roots, diffuse to the water column, or be lost via denitrification (Caffrey & Kemp 1990, Hemminga et al. 1991, Pedersen & Borum 1993). If we use the annual N incorporation value of 34.5 g N m^{-2} yr^{-1} determined by Pedersen & Borum (1993) for a comparable *Z. marina* population in another location in Denmark, we can estimate the potential significance of the measured nitrogen fixation rates to *Z. marina*. Of the total annual N demand, 73% of which we assume was provided by external N sources (51% from sediment, 49% from water column) and 27% by internal N recycling (Pedersen & Borum 1993), our nitrogen fixation rates could potentially provide approximately 5% of the annual external N incorporation and 10% of that derived from sediment sources. Nitrogen fixation in these sediments accounted for less than 4% of the measured N inputs from April through August (Risgaard-Petersen et al. unpubl.). Depth-integrated rates of denitrification in the same eelgrass meadow measured during April and August were 30% (April) and 75% (August) lower than nitrogen fixation rates, and thus did not balance inputs from nitrogen fixation (Risgaard-Petersen et al. unpubl.).

Our data from intact sediment-plant associations clearly indicate a direct coupling of rhizosphere nitrogen fixation to *Zostera marina* productivity and support the hypothesis that heterotrophic nitrogen fixation is at least partly dependent on the exudation of photosynthetically derived organic carbon from seagrass roots. The detailed temporal and spatial patterns of nitrogen fixation reported here from perfusion incubations that maintain direct root-microbe associations indicate the importance of macrophytes as a source of labile carbon to heterotrophic bacteria, with NH_4^+ and O_2 concentrations in the sediment pore water having minor or secondary roles. What remains unclear is the exact nature of the association between the eelgrass and microbes that defines this close coupling of plant productivity and nitrogen fixation. Previous studies have suggested the possibility of a symbiotic association between marine macrophytes (seagrasses and salt-marsh grasses) and nitrogen-fixing heterotrophic bacteria in direct contact with the roots, involving a bidirectional exchange of materials (Capone & Budin 1982, Whiting et al. 1986). Capone (1988) provided some evidence of this from incubations of individual *Z. marina* plants in which the roots and rhizomes were exposed to $^{15}N_2$, showing that a significant amount of fixed N was incorporated into the root, rhizome and leaf tissue after 48 h. Further studies using isotopic tracers would help clarify whether this exchange is rapid through direct transfer of metabolites in both directions or whether it is more indirect, depending on the longer-term dynamics of mineralization and plant uptake.

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