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Denitrification in an intertidal seagrass meadow, a comparison of ^{15}N-isotope and acetylene-block techniques: dissimilatory nitrate reduction to ammonia as a source of N_2O?

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Abstract Tests of the isotope-pairing technique over a range (15–200 μM) of ^{15}N-nitrate additions to the water column of intact or defoliated cores of the seagrass *Zostera noltii* demonstrated that whilst overall rates of denitrification were highly dependent on the nitrate concentration in the water column, the calculated in situ rates, D_{14}, D_W and D_N, were not significantly affected. Rates of coupled nitrification–denitrification determined following additions of ^{15}N-ammonium to the sediment porewater were within the range of D_N values determined by isotope pairing. Thus, this technique appears to accurately measure denitrification rates in these seagrass-colonised sediments, probably due to the effective limitation of coupled nitrification–denitrification to the surficial sediments, which ensures the homogenous mixing within the denitrification zone of the added ^{15}N-nitrate tracer and the ^{14}N-nitrate generated by nitrification in the sediment. Denitrification rates of nitrate diffusing from the water column were compared using the isotope-pairing and acetylene-block techniques. Whilst rates were similar during dark incubations, during light incubations, rates of N_2O accumulation during acetylene blocking were consistently twofold greater than denitrification rates measured by isotope pairing. We propose that this excess N_2O could have been generated during dissimilatory nitrate reduction to ammonium by fermentative and/or sulphate-reducing bacteria.

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Introduction

Denitrification is a major sink in the nitrogen cycle, with fixed-N eliminated as gaseous products. In sediments, denitrification can be sustained by nitrate diffusing from the overlying water column or generated in the sediment by nitrification. In recent years, there has been increasing interest in denitrification processes in the coastal zone, due to the capacity of denitrification to reduce the impacts of anthropogenic nitrogen loads. Several methodologies have been employed to measure denitrification rates, including N-mass balance approaches (Knowles 1982; van Luijn et al. 1996), measurements of N_2 fluxes or N_2:argon ratios (Devol 1991; van Luijn et al. 1996; Kana et al. 1998), determination of nitrate gradients using ion-specific microelectrodes (De Beer and Sweets 1989; Revsbech 1994) or N_2O gradients following acetylene blocking (Christensen et al. 1989), ^{15}N isotopic methods (Koike and Hattori 1977; Nielsen 1992; Seitzinger et al. 1993) and acetylene blocking (Kemp et al. 1990; Seitzinger et al. 1993). However, amongst these methods, acetylene blocking, due to its sensitivity and ease of application, and the ^{15}N-isotope-pairing technique, which allows simultaneous estimation of denitrification of nitrate diffusing from the water column and coupled nitrification–denitrification, have been most extensively employed in recent years.

Acetylene inhibits the final step of denitrification, the reduction of N_2O to N_2 (Koike and Sørensen 1988), and

thus the accumulation of N_2O gives a measure of denitrification. However, acetylene is also a highly potent inhibitor of nitrification (Koike and Sørensen 1988; Knowles 1990) and therefore only measures denitrification based on pre-existing nitrate. The isotope-pairing technique relies on homogenous mixing within the denitrification zone of the added ^{15}N-nitrate diffusing from the water column with the nitrate produced in the sediment by nitrification, to allow accurate calculation of coupled nitrification–denitrification from the ratio of $^{29}N_2$ and $^{30}N_2$ (Nielsen 1992). In seagrass sediments such uniform mixing may not be achieved, as coupled nitrification–denitrification could occur deep in the rhizosphere associated with O_2 release by the plant roots, in areas remote from the diffusion zone of the added ^{15}N-nitrate. Thus, at least in some seagrass sediments, rates of denitrification could be underestimated by the isotope-pairing technique.

In the present study, we have calibrated the isotope-pairing technique over a range of ^{15}N-nitrate concentrations in intertidal seagrass sediments. Rates of denitrification dependent upon water column nitrate were compared with those determined by acetylene blocking. To assess the effects of plant photosynthesis (root oxygen release) and competition between plant nitrate assimilation and denitrification, on denitrification rates, these experiments were performed under both light and dark conditions in cores containing intact or defoliated plants. Finally, rates of ^{15}N-N_2 species production following ^{15}N-ammonium additions to the sediment porewater were included as a qualitative control for rates of coupled nitrification–denitrification measured by the isotope-pairing technique.

Materials and methods

Sampling site and sampling methods

The sampling station was situated in an intertidal *Zostera noltii* meadow of the Ile aux Oiseaux, Bassin d'Arcachon, France (44°40′N; 1°10′W). A full description of this station can be found in Welsh et al. (1996). Data, for the *Z. noltii* biomass, sediment temperature and water column inorganic nitrogen concentrations during the sampling period are listed in Table 1.

Table 1 Biomass of *Zostera noltii*, sediment temperature and water column concentrations of inorganic nitrogen species measured during the sampling period in October 1997. Data for the *Z. noltii* biomasses are means of five replicate samples and standard deviations are shown in *parentheses*

Parameter	
Sediment temperature	17°C
Z. noltii biomass (g dry wt m^{-2})	
Shoots	87.8 (12.8)
Roots and rhizomes	66.4 (24.7)
Total	154.2 (28.1)
Water column inorganic N concentrations (μM)	
Ammonium	7.9
Nitrate	1.7
Nitrite	0.7

Cores were hand collected in October 1997 at low tide using 30×8 cm i.d. (internal diameter) for isotope pairing or 20×4.4 cm i.d. plexiglass core tubes for acetylene-block and ^{15}N-ammonium-addition experiments. Cores of defoliated plants were collected by cutting the leaves from an area of the seagrass bed prior to core collection. At the laboratory, cores for isotope pairing were transferred to a custom-built incubation tank containing natural seawater from the Bassin, and a small magnetic stirrer was suspended within each core. The incubation tank contained a central electric motor to drive the individual magnets in the cores. Cores for ^{15}N-ammonium-addition experiments and acetylene-block determinations were stored in a 600 l mesocosm tank flushed (~200 l h^{-1}) with seawater from the Bassin. All cores were left to equilibrate for 12–18 h before experiments were initiated, and experiments were completed within 24 h.

Isotope-pairing determinations of denitrification

Rates of denitrification were determined using the isotope-pairing technique of Nielsen (1992). Prior to experiments the water in the cores was exchanged with that in the incubation tank to ensure homogeneity. To initiate determinations, the water level in the incubation tank was lowered below that of the cores and zero time samples were collected for determination of initial nitrate concentrations. Sufficient of a 30 mM stock (99 at.%) was added to give final water column concentrations of 15–200 μM ^{15}N-nitrate, and the cores closed with floating transparent lids. Cores were incubated under either natural ambient light or dark (double-wrapped in aluminium foil) conditions for 1 h.

Following incubation, activity was stopped by the addition of 7 M $ZnCl_2$ to a final concentration of 10 mM, and the cores were gently slurried in order to homogenise the dissolved N_2 pools in the water column and porewater. Subsamples of the slurry were transferred to 12-ml Exetainer vials, fixed with 100 μl 7 M $ZnCl_2$ and stored at 4°C until analysed within 2–3 weeks. Samples were analysed for ^{29}N and ^{30}N-N_2, and production rates of the isotope pairs calculated as previously described (Welsh et al. 2000). Rates of D_{15} (denitrification of added ^{15}N-nitrate), D_{14} (denitrification of ambient ^{14}N-nitrate), D_W (denitrification of nitrate diffusing from the water column) and D_N (denitrification of nitrate generated by nitrification in the sediment) were calculated according to the equations derived by Nielsen (1992).

Determination of coupled nitrification–denitrification using ^{15}N-ammonium

Sediment cores were adjusted to a length of approximately 5 cm and closed with rubber bungs; two injections of 100 μl of 50 mM 99 at.% $^{15}NH_4Cl$ were made at 90° to each other at each 1 cm depth interval of the sediment (equivalent to increasing the exchangeable ammonium concentration by circa 1 mM; Welsh et al. 1996). Cores were incubated under natural ambient light conditions at in situ temperature in the mesocosm tank. At indicated time points during the 3 h incubation, individual cores were sacrificed, activity was stopped, cores slurried, and samples collected and analysed for ^{29}N and ^{30}N-N_2 as described for the isotope-pairing experiments. Denitrification rates were calculated from the production with time of ^{29}N and ^{30}N-N_2 such that: $D = p(^{29}N_2) + 2p(^{30}N_2)/M$, where $p(^{29}N_2)$ and $p(^{30}N_2)$ are the production rates of the labelled N_2 species and M is the mole fraction of ^{15}N-ammonium, which was nominally 0.8 for all cores, based on previously measured sediment-exchangeable ammonium pools (Welsh et al. 1996).

Acetylene-block determinations of denitrification

The length of cores was adjusted to approximately 5 cm, two injections at 90° to each other of 0.5 ml acetylene-saturated seawater were made at each 1 cm depth interval of the sediment via silicon-sealed ports, and 10% of the water column replaced with acetylene-

saturated seawater via a Suba-sealed sampling port; excess water was allowed to escape via a hypodermic needle inserted through the sampling port. Nitrate additions to the water column were made by injection via the sampling port to give final concentrations of 0–200 μM. Cores were incubated for 6 h under natural ambient light or dark (double-wrapped in aluminium foil) conditions in the mesocosm tank. Following incubation, a 50 ml subsample of the water column was transferred to a partially evacuated 120 ml serum bottle containing 10 ml of 100 mM $ZnCl_2$. The remainder of the water was discarded, and the sediment transferred to a 360 ml glass bottle containing 80 ml of 100 mM $ZnCl_2$. Bottles were sealed with screw tops fitted with rubber-sealed injection ports and vigorously shaken to stop further activity. Following gas equilibration, aliquot volumes (3 cm^3) of the headspace gas were transferred to 3-ml draw volume Venoject tubes for storage. Samples were analysed for N_2O content by gas chromatography, with an electron capture detector, using authentic N_2O as standard.

Analytical techniques

Ammonium concentrations were measured by the indophenol-blue method (Koroleff 1970), nitrite was measured using a Technicon autoanalyser II following diazotation (APHA 1975), nitrate was determined by the same method, following reduction over cadmium columns.

Statistical analysis

Normality of data was assumed, homoscedasticity was confirmed using the Cochran test. Data were analysed by one-way ANOVA with an a posteriori comparison of the means performed using the *T*-method (Sokal and Rolf 1995).

Results

Denitrification rates were determined by isotope pairing following additions of ^{15}N-nitrate of 15–200 μM nitrate, or approximately 10–100 times the ambient water column nitrate concentration during the experimental period (Table 1). Overall denitrification rates based on the added ^{15}N-nitrate (D_{15}), in cores of intact plants (*Zostera noltii*) increased with increasing ^{15}N-nitrate concentration during both light and dark incubations with nitrate additions up to 100 μM. Above this threshold denitrification rates saturated at a little over 50 μmol N m^{-2} h^{-1} (Fig. 1). In contrast, in cores containing defoliated plants, although denitrification rates based on the labelled nitrate were similar following low nitrate additions they did not saturate within the range of 15–200 μM nitrate and attained maximal values of approximately 100 μmol N m^{-2} h^{-1} (Fig. 1). No differences in the denitrification rates were apparent between light and dark incubations (ANOVA; $P > 0.05$), for incubations of either intact or defoliated plants. However, whilst overall denitrification rates increased with increasing ^{15}N-nitrate concentration, calculated rates of total in situ denitrification (D_{14}), coupled nitrification–denitrification (D_N) and denitrification based on the ambient water column ^{14}N-nitrate (D_W), although low and variable at all tested ^{15}N-nitrate concentrations, were unaffected by the level of ^{15}N-nitrate added (Fig. 2). No significant differences were detected

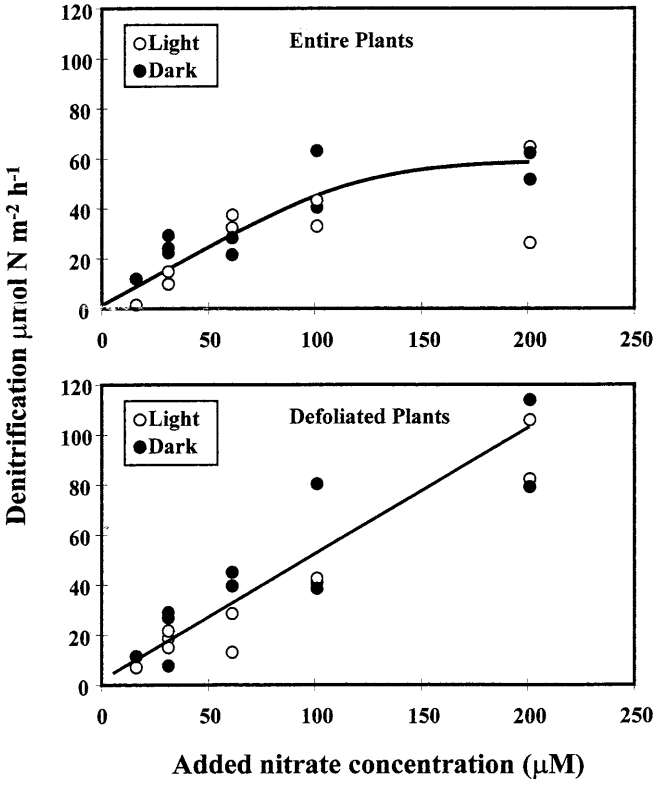

Fig. 1 Effect of added ^{15}N-nitrate concentration on denitrification rates, based on ^{15}N-nitrate (D_{15}) diffusing from the water column in cores containing intact or defoliated *Z. noltii* plants, during light (*open circles*) and dark (*filled circles*) incubations. Data points represent values determined for individual cores

between light and dark incubations (ANOVA; $P > 0.05$), and pooled rates for all incubations were 3.8 ± 2.2, 3.0 ± 2.1 and 0.8 ± 0.3 μmol N m^{-2} h^{-1} for D_{14}, D_N and D_W, respectively (Fig. 2). Similarly, in parallel incubations of defoliated cores no trends in the calculated rates of D_{14}, D_N and D_W were observed with increasing ^{15}N-nitrate concentration in the water column. Data from light and dark incubations were not significantly different (ANOVA; $P > 0.05$), with pooled light and dark denitrification rates being 5.6 ± 2.4, 4.4 ± 2.3 and 1.2 ± 0.5 μmol N m^{-2} h^{-1} for D_{14}, D_N and D_W, respectively (data not shown). Although, in incubations of defoliated plants, all the mean measured rates were somewhat higher than for cores containing intact plants, these differences were not significant (ANOVA; $P > 0.05$).

Rates of accumulation of labelled $^{29}N_2$ and $^{30}N_2$ in cores to which ^{15}N-ammonium was added to the interstitial water to a final concentration of 1 mM (nominal 80 at.% enrichment) are shown in Fig. 3. Accumulation rates of the labelled species were low, 0.55 and 2.4 μmol N_2 m^{-2} h^{-1} for $^{29}N_2$ and $^{30}N_2$, respectively, and are equivalent to a coupled denitrification rate of 1.3 μmol N m^{-2} h^{-1}, which falls well within the range of the values of D_N determined by the isotope-pairing technique (Fig. 2b).

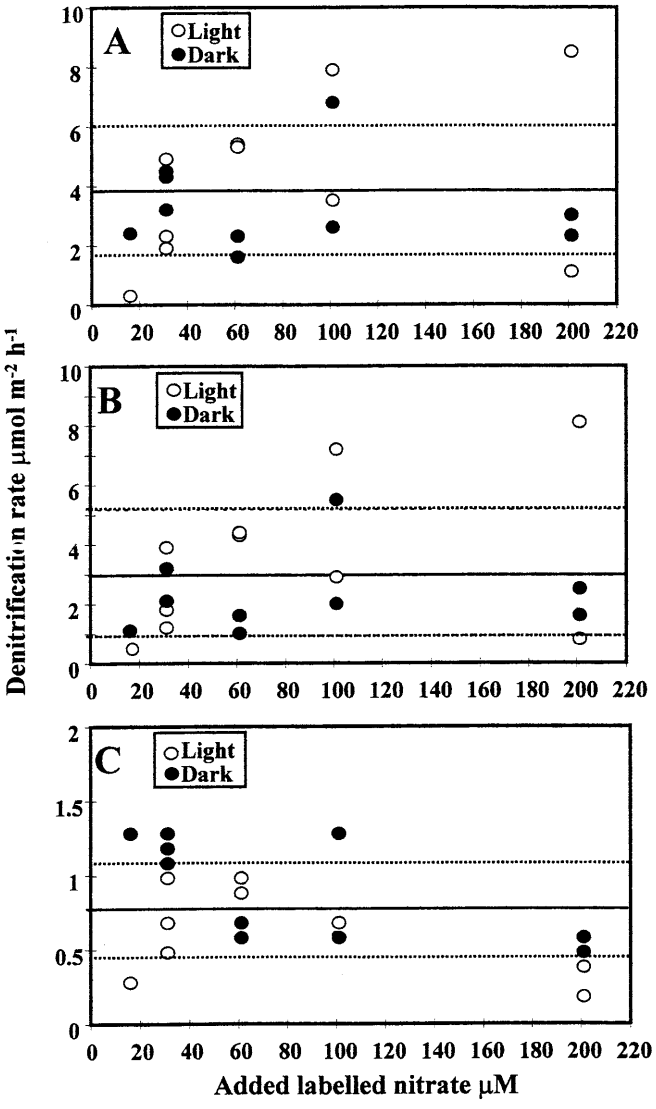

Fig. 2a–c Effect of added ^{15}N-nitrate concentration on calculated rates of: **a** total in situ denitrification (D_{14}), **b** denitrification coupled to nitrification activity in the sediments (D_N), and **c** denitrification of nitrate diffusing from the overlying water column (D_W). Data points represent values determined for individual cores under natural light (*open circles*) or dark (*filled circles*) conditions. *Solid line* represents the mean of all data points; *dashed lines* indicate one standard deviation

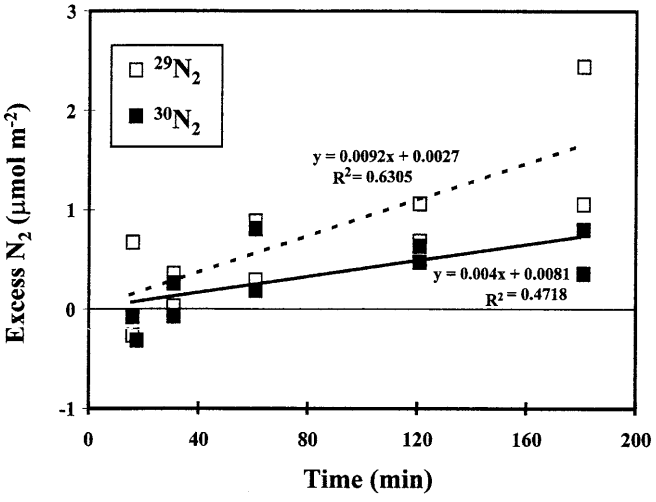

Fig. 3 Accumulation of labelled $^{29}N_2$ and $^{30}N_2$ during light incubations of cores to which ^{15}N-ammonium was added to the porewater to a nominal concentration of 1 mM (approx. 80 at.% enrichment of the exchangeable ammonium pool). *Data points* represent values determined for individual cores; *lines* represent linear regressions for $^{29}N_2$ (*dashed line*) and $^{30}N_2$ (*solid line*) productions, percentage atomic excess of $^{29}N_2$ and $^{30}N_2$ were determined by reference to air blanks

The influence of water column nitrate concentration on rates of denitrification of nitrate diffusing from the water column to the sediment was determined by the acetylene-block and isotope-pairing techniques (Fig. 4). During dark incubations rates of N_2O production following acetylene blocking were very similar to denitrification rates of water column nitrate ($D_{15}+D_W$) determined by isotope pairing at the equivalent nitrate concentration (Fig. 4). In contrast during light incubations, rates of denitrification determined by the acetylene-block technique were consistently twofold or more greater than those determined by isotope pairing at the equivalent nitrate concentration (Fig. 4), indicating that

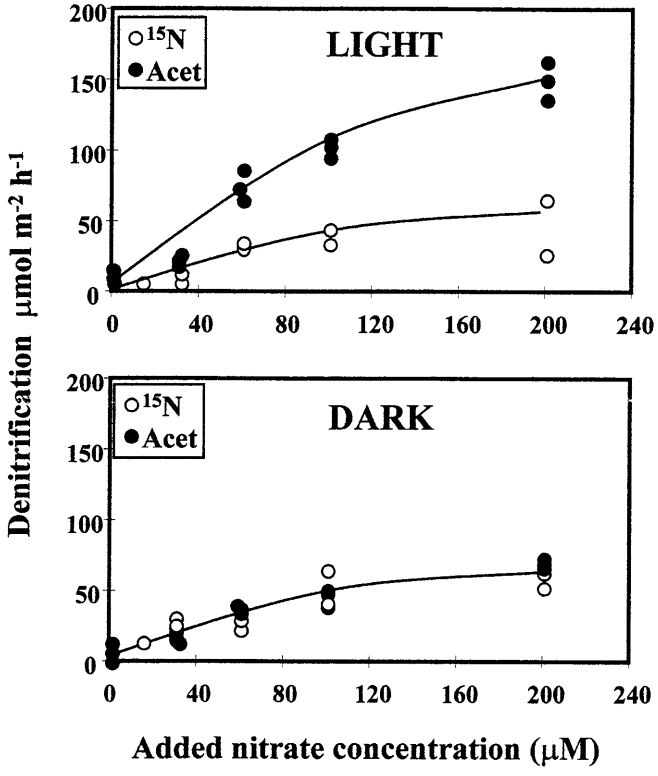

Fig. 4 Effect of water column nitrate concentration on rates of total denitrification of water column nitrate measured using the isotope-pairing ($D_{15}+D_W$; *open circles*) and acetylene-blocking (N_2O production; *filled circles*) techniques during light and dark incubations of *Z. noltii*–colonised sediments. For comparison all data are expressed as μmol N m^{-2} h^{-1}. Data points represent values for individual cores

a nitrate dependent source of N_2O other than denitrification was active.

Discussion

Coupled nitrification–denitrification

Rates of coupled nitrification–denitrification determined by isotope pairing were low and spatially variable, but showed no significant variations with either the light environment or between incubations with intact or defoliated plants. However, due to the principles on which the isotope-pairing technique is based, the measured rates potentially represent an underestimate of the true rates of coupled nitrification–denitrification in the rhizosphere, since, for the calculation of denitrification rates, it is assumed that there is a homogenous mixing in the denitrification zone of the ^{15}N-nitrate added to the water column and that generated by nitrification in the sediment (Nielsen 1992). In seagrass-colonised sediments such mixing may not be attained, as nitrification may occur in microzones, deep in the sediment associated with oxygen release from the plant roots (Caffrey and Kemp 1992; Risgaard-Petersen and Jensen 1997; Pedersen et al. 1998), which are remote from the diffusion zone of the added tracer (Nielsen 1992). The degree of oxygen release from roots, and thus the potential rate of coupled nitrification, varies considerably between aquatic plant species (Sand-Jensen et al. 1982). Although no studies have investigated oxygen release by roots of *Zostera noltii*, previous studies have reported large populations of aerobic sulphur-oxidising bacteria inhabiting their rhizospheres (Schaub and van Gemerden 1996), indicating that oxygen is available at least periodically. However, this oxygen does not appear to support nitrification, as populations of nitrifying bacteria monitored between August 1996 and November 1997 at the same sampling station were consistently below 500 and 200 cells cm^{-3} sediment for ammonium- and nitrite-oxidising bacteria, respectively (Riou 1998). These small populations of nitrifiers may reflect their comparatively low affinity for oxygen compared to heterotrophic and chemolithotrophic bacteria (Sharma and Ahlert 1977; Laanbroek and Gerards 1993; Laanbroek et al. 1994). Therefore, in the rhizosphere, nitrifying bacteria may be out-competed for limiting oxygen concentrations by both heterotrophs and chemolithotrophs. In addition to the probable low nitrification rates, in these sediments, dissimilatory nitrate reduction to ammonium (DNRA) is the dominant sink for nitrate rather than denitrification (Rysgaard et al. 1996). Thus, due to the combination of small populations of nitrifiers and the dominance of DNRA as a sink for nitrate, coupled nitrification–denitrification is unlikely to be a quantitatively significant process. This hypothesis is supported by our data from incubations in which 1 mM ^{15}N-ammonium was added to the interstitial water. This experiment, however, can only be considered as a qualitative control, as the true ^{15}N-enrichment of the ammonium pool in the nitrification zone(s) is unknown and would have changed during the incubation (Blackburn et al. 1994), particularly near to the root surfaces, where ammonium assimilation by the roots would result in a faster turnover of ammonium pools than in the bulk sediment. The fact that the production rates of the labelled N_2 species were low and the estimated rate of coupled nitrification–denitrification of 1.3 μmol N m^{-2} h^{-1} falls within the range determined by isotope pairing provides further evidence that little coupled nitrification–denitrification occurred deep within the rhizosphere. Overall, our data indicate that in the studied *Z. noltii* meadow denitrification was essentially limited to the surficial sediments, where it would be accurately determined by the isotope-pairing technique, since in this sediment layer the ^{15}N-nitrate diffusing from the water column would be homogenously mixed with that generated by nitrification in the sediment.

Denitrification of water column nitrate

Rates of denitrification of water column nitrate measured by isotope pairing were highly dependent upon the water column nitrate concentration during incubations of cores containing intact or defoliated plants. Such a relationship would be expected since the slope of the nitrate gradient to the denitrification zone in the sediment is a major determinant of rates (Christensen et al. 1990). In incubations of cores containing intact plants, denitrification of water column nitrate saturated at around 100 μM nitrate, but did not saturate up to 200 μM nitrate when the *Z. noltii* shoots had been removed, indicating that competition between the plants and denitrifying bacteria for nitrate influenced denitrification to some extent. Whilst total rates of denitrification of nitrate diffusing from the water column increased with increasing nitrate concentration in both intact and defoliated cores, the final concentration of the labelled nitrate used during incubations had no significant influence on the calculated rates of in situ denitrification of water column nitrate (D_W). These robust data sets indicate that the isotope-pairing technique accurately estimated rates of denitrification of water column nitrate in this seagrass bed.

Denitrification rates of water column nitrate measured by the isotope-pairing technique were compared to those determined by the acetylene-blocking technique over a range of added nitrate concentrations under both light and dark conditions. Acetylene inhibits the final step of denitrification involving the reduction of N_2O to N_2 by nitrous oxide reductase, and thus denitrification can be determined from the production rate of N_2O (Knowles 1990). However, acetylene, at much lower concentrations than used for denitrification assays, also inhibits the mono-oxygenase catalysed oxidation of ammonia to hydroxylamine, a key step in nitrification (Knowles 1990), and therefore

only measures denitrification of pre-formed nitrate and not coupled nitrification–denitrification. Thus, the acetylene-blocking technique essentially only measures denitrification of nitrate diffusing from the water column and can be directly compared with total rates of denitrification of water column nitrate ($D_{15}+D_W$) determined by isotope pairing. During dark incubations, rates of N_2O production and rates of denitrification of water column nitrate measured by isotope pairing were highly similar. In contrast, during light incubations of acetylene-blocked cores, N_2O accumulation rates were consistently twofold or more greater than denitrification rates of water column nitrate measured by isotope pairing over the entire range of nitrate concentrations tested. These data are at variance with those of previous studies using unvegatated sediments, which have generally concluded that acetylene blocking tends to underestimate denitrification rates due to incomplete inhibition of nitrous oxide reductase, particularly at low nitrate concentrations or in sulphidic environments (e.g. Christensen et al. 1989; Knowles 1990; Binnerup et al. 1992; Seitzinger et al. 1993). Our results indicated that, in acetylene-treated cores, there was a nitrate-dependent source of N_2O other than denitrification. Whilst many studies have shown that nitrification can also evolve N_2O (Poth and Focht 1985; Cofman Anderson et al. 1993), it is extremely unlikely that this was the source of the excess N_2O produced during our experiments. As discussed previously, acetylene effectively inhibits nitrification at much lower concentrations than those employed (Knowles 1990), the nitrifier populations in the rhizosphere were extremely small (Riou 1998), and N_2O production during nitrification would not be expected to show the observed dependence upon water column nitrate concentration.

Other than denitrification, the major nitrate-consuming process in marine sediments is DNRA (Koike and Sørensen 1988; Cole 1990). Unlike denitrification, DNRA can proceed by several pathways; for example, in at least some sulphate-reducing bacteria, DNRA is a true respiration process coupled to electron transport phosphorylation (Seitz and Cypionka 1986), whereas, in fermentative bacteria, nitrate acts as an electron sink, allowing reoxidation of reduced NADH and continued substrate level phosphorylation (Cole 1990). Populations of dissimilatory nitrate reducers in the rhizosphere of *Z. noltii* determined using a most probable number technique to range between 10^6–10^8 cells cm^{-3} (Welsh and Herbert, unpublished data), and, in a previous study at an adjacent sampling station, Rysgaard et al. (1996) reported that DNRA was by far the dominant pathway of nitrate reduction in these sediments. Similarly, Boon et al. (1986) reported that DNRA was the major sink for nitrate and that denitrification played a relatively minor role in a *Z. capricornia* meadow. Additionally, sulphate reduction rates and numbers of sulphate-reducing bacteria in the rhizosphere of *Z. noltii* in the Bassin d'Arcachon are extremely high (Isaksen and Finster 1995; Schaub and van Gemerden 1996; Welsh et al. 1996), as is commonly the case in seagrass meadows (Blackburn et al. 1994; Holmer and Nielsen 1997; Blaabjerg et al. 1998), and DNRA activity is known to be widespread within this group of microorganisms (Mitchell et al. 1986; Seitz and Cypionka 1986; Dalsgaard and Bak 1994). Indeed, kinetic data indicate that sulphate-reducing bacteria could effectively compete with denitrifiers for nitrate at the low concentrations found in sediment porewaters (Seitz and Cypionka 1986; Dalsgaard and Bak 1994). Moreover, in rhizosphere sediments, many bacterial activities, including sulphate reduction, are coupled to the photosynthetic activity of the plants, due to the root exudation of photosynthates (Blackburn et al. 1994; Holmer and Nielsen 1997; Welsh et al. 1997; Blaabjerg et al. 1998; Welsh 2000). Thus, a similar coupling between photosynthetically driven carbon exudation by the *Z. noltii* root system and DNRA by sulphate-reducing and/or fermentative bacteria could explain the excess production of N_2O during light incubations of acetylene-blocked cores.

Several studies have demonstrated that, in at least some fermentative bacteria, N_2O is produced during the dissimilatory reduction of nitrate or nitrite (Kaspar and Tjiede 1981; Bleakley and Tjiede 1982; Kaspar 1982; Scott Smith 1983; Allison and Macfarlane 1989). In *Escherichia coli*, N_2O evolution has been attributed to a non-specific activity of nitrate reductase, which does not appear to be coupled to growth (Scott Smith 1983), although in other bacteria N_2O evolution has been proposed to function as a detoxification mechanism to remove nitrite generated during DNRA (Kaspar 1982; Allison and Macfarlane 1989). Reported rates of N_2O production are extremely variable, ranging from 0.1% to 100% of the total nitrate or nitrite reduced (Kaspar and Tjiede 1981; Kaspar 1982; Bleakley and Tjiede 1982; Scott Smith 1983; Allison and Macfarlane 1989), dependent on the bacterial strain(s) and the growth conditions. However, due to the dominance of DNRA over denitrification in the studied sediments (Rysgaard et al. 1996), even low percentage yields of N_2O from DNRA could account for the observed excess N_2O production in acetylene-blocked cores. For example, Rysgaard et al. (1996) reported that in these *Z. noltii*–colonised sediments 95% of total nitrate diffusing from the water column was recovered as ammonium and only 5% as N_2. Assuming a similar relationship between DNRA and denitrification rates during our experiments, an N_2O production equivalent to only 1% of the DNRA activity would be required to produce an N_2O flux equivalent to that generated by denitrification during acetylene blocking. Thus, production of N_2O during DNRA could easily account for the excess production of N_2O we observed in acetylene-blocked cores. Few studies have investigated the influence of acetylene on rates of N_2O evolution during DNRA, although Kaspar and Tjiede (1981) reported inhibition of up to 35% by 5–50% acetylene, N_2O evolution was reported to be unaffected by acetylene in two strains of *Propionibacterium acne* (Allison and Macfarlane 1989).

Thus, it is plausible to suppose that the excess N_2O production may be an intrinsic feature of DNRA in the rhizosphere sediments, although in the absence of acetylene much of this N_2O may be consumed by denitrifying or other bacteria (Conrad 1996; Klüber and Conrad 1998). However, further studies are required to determine whether DNRA does represent a significant source of N_2O in these or other seagrass-colonised sediments and to elucidate the effect(s) of acetylene on this process.

Applicability of denitrification methods to seagrass-colonised sediments

Determination of denitrification rates in seagrass meadows presents a number of difficulties due to the inherent limitations of the denitrification assays themselves and specific features of the rhizosphere sediments. No single denitrification assay is universally applicable to these ecosystems. Acetylene blocking both fails to measure coupled nitrification–denitrification due to the inhibition of nitrification and may underestimate denitrification of pre-existing nitrate if inhibition of nitrous oxide reductase is incomplete. Additionally, our results indicate that this technique may overestimate denitrification rates based on pre-formed nitrate in seagrass-colonised sediments, where DNRA rather than denitrification is the dominant pathway of nitrate reduction, due to the evolution of N_2O during DNRA. These problems also limit the use of methods based on acetylene blocking and microsensor profiling of N_2O gradients, and similar techniques using nitrate-selective microsensors cannot be applied in marine environments due to interference from other ions (Revsbech 1994). Determinations of denitrification from N_2 fluxes or N_2:argon ratios are also of only limited value, as seagrass meadows are often zones of intense N-fixation (see Welsh 2000 for review), which can more than compensate for N-losses via denitrification (McGlathery et al. 1998; Welsh et al. 2000). Since, methods based on N_2 reflect the net balance between denitrification and N-fixation, the data would require correction for the N-fixation activity, the measurement of which also presents some difficulties (Welsh 2000).

Due to these problems stable-isotope methods would appear to offer the most promising avenue for the investigation of denitrification processes in seagrass ecosystems. Our data indicate that the isotope-pairing technique accurately estimates denitrification rates based on water column nitrate and, at least in the case of the studied seagrass meadow, also provides a good estimate of coupled nitrification–denitrification rates, as this process was essentially limited to the surficial sediments. However, the technique would not be suitable for seagrass meadows in which significant coupled nitrification–denitrification occurred in the deeper sediments associated with oxygen release from the plant roots (Nielsen 1992). In these cases, a combination of the isotope-pairing technique, to allow determination of surface-associated rates of D_N and D_W, and a method based on ^{15}N-ammonium additions to the porewater, to assess coupled nitrification–denitrification in the rhizosphere would be advisable. Although, unfortunately, rates based on ^{15}N-ammonium additions can only be considered as rough estimates. Firstly, the considerable additions of labelled ammonium required to attain a significant enrichment of the N_2 pool may stimulate nitrification and thereby coupled nitrification–denitrification rates, if these are ammonium limited. Secondly, uncertainty exists as to the degree of enrichment of the porewater ammonium pool, as conventional porewater extraction techniques damage the seagrass root system releasing internal ammonium pools, which substantially elevate the measured porewater concentrations (Hansen and Lomstein 1999), and the ammonium assimilation activity of the plant roots will cause changes in the ^{15}N:^{14}N-ammonium ratio during incubations and spatial heterogeneity in porewater concentrations within the rhizosphere sediments.

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