Denitrification in exposed intertidal mud-flats, measured with a new ^{15}N-ammonium spray technique

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ABSTRACT: Denitrification in exposed intertidal sediment was measured for the first time at locations in the Tagus Estuary, using a new ammonium spray method. With this method, $^{15}NH_4^+$ was sprayed on to the sediment surface, and nitrification-denitrification was subsequently estimated from accumulation of $^{29}N_2$ and $^{30}N_2$ in gas-tight glass chambers placed on the ^{15}N-labeled sediment. Coupled nitrification-denitrification ranged from 8.5 to 11.5 µmol N m^{-2} h^{-1} at night to below 2 µmol N m^{-2} h^{-1} during the day. Salt stress, light inhibition and depletion of NH_4^+ by benthic diatoms could contribute to the low daytime activity. The nighttime rates were confirmed by an independent estimate based on potential nitrification-denitrification and oxygen uptake. Coupled nitrification-denitrification activity at night during exposure was not significantly different from the activity measured when the sediment was inundated, but was 3 times lower than rates measured at a subtidal station in the estuary.

KEY WORDS: Intertidal mud flats · Exposed sediment · ^{15}N · Denitrification

INTRODUCTION

A unique feature of intertidal flats is the periodic exposure and inundation of the sediments. These create large temporal variation in physical and chemical conditions at the sediment surface. During exposure, drainage and evaporation can alter the water potential, temperature, and pore-water composition in the upper sediment strata (Anderson & Howell 1984, Howes & Goehringer 1994, Hollins & Ridd 1997) and increase the area of oxic/anoxic contact zones in cracks and burrows, thereby affecting nitrification and denitrification. The absence of a water column above the sediments also prevents loss of regenerated nutrients ions from the sediment, which therefore either will accumulate

(e.g. Gouleau et al. 1996, Rocha 1998) or be consumed in the benthic system. Accumulation of NH_4^+ and NO_3^- in the sediment could stimulate coupled nitrification-denitrification during exposure, while exclusion of the NO_3^- supply from overlying water could simultaneously have the opposite effect. Despite these potential effects of exposure, all measurements of denitrification in intertidal sediments have, to our knowledge, only been conducted during inundation (Joye & Paerl 1994, Jensen et al. 1996, Kristensen et al. 1997).

In this paper we introduce the ammonium spray method, a modified version of the ^{15}N isotope-pairing technique (Nielsen 1992), for measuring denitrification in air-exposed sediments, and present the first result from intertidal mud-flats in the Tagus Estuary (Portugal). The processes were measured *in situ*, and the diurnal variation in nitrification and denitrification activity was investigated. This study was conducted during a joint field campaign in June 1998, during

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which denitrification in inundated intertidal and permanently water-covered sediments was also measured (M. Bartoli et al. unpubl. data). Likewise, oxygen dynamics and photosynthesis on the intertidal flats were also studied (J. Serodio et al. unpubl. data). The data obtained in the present study will be discussed in relation to those results.

MATERIALS AND METHODS

Locations. The river Tagus runs through Spain and Portugal and flows into the Tagus Estuary (Fig. 1) at the city of Lisbon on the Atlantic coast of Portugal. The estuary covers an area of 320 km^2, and consists mainly of large mud-flats. With a tidal amplitude of 1 to 4 m, the intertidal area comprises 20% of the total area at neap tide and 40% at spring tide. Salinity varies from 5–30 psu at low tide to 20–33 psu at high tide. High concentrations of suspended matter (10 to 150 g m^{-3}) exclude benthic primary production during high tide (Brotas & Catarino 1995). The estuary receives discharge from Lisbon and its surroundings, and the most recent (1994/1995) estimate of annual average N-load to the estuary is 323 µmol N m^{-2} h^{-1}. From October to December 1995, the estimated loading rate was 509 µmol m^{-2} h^{-1} (Cabrita 1997), which compares well with an earlier fall estimate, 516 µmol m^{-2} h^{-1} (Seitzinger 1988). The daily riverine freshwater input (from 0.13×10^6 m^3 to 1.05×10^6 m^3) is low compared to the tidal exchange (from 395×10^6 m^3 to 806×10^6 m^3), and the residence time of the freshwater varies from 8 to 26 d. For a thorough description of the estuary, see Cabrita (1997).
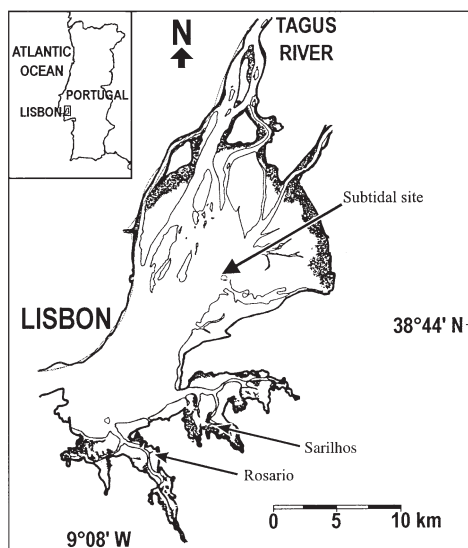

Fig. 1 Map of the Tagus Estuary with sampling sites Rosario and Sarilhos Pequenos

Two intertidal mud-flat stations (Sarilhos and Rosario) were selected in Tagus Estuary on the basis of accessibility. The Sarilhos station was characterized by a soft muddy sediment (porosity in top 5 cm from 0.50 to 0.55) which was densely populated with polychaetes (*Nereis diversicolor*, 800 to 1200 individuals m^{-2}), and remained water-saturated throughout the exposure period. It was impossible to walk on the mud-flat without sinking in knee deep, so wooden platforms were placed on the sediment as a working base, in order to facilitate accessibility and reduce the disturbance incurred by repeated visits to the site. The sediment at the Rosario station was much firmer, consisting of sand with a layer of mud on the top (porosity in top 5 cm from 0.50 to 0.90 and a *N. diversicolor* density of 500 to 2000 individuals m^{-2}). Both sediments were densely inhabited by benthic algae populations, consisting mainly of diatoms. Gross primary production (GPP) at Sarilhos, measured with O$_2$ microsensors during daytime exposure, ranged from 11 to 29 mmol O$_2$ m^{-2} h^{-1}. Oxygen penetration was approx. 1 mm, and showed but little diurnal variation during the exposure periods (J. Serodio et al. unpubl. data). At Rosario, GPP was 1 to 3 mmol O$_2$ m^{-2} h^{-1} and average oxygen penetration 0.9 mm.

Denitrification measurements. Denitrification measurements and supporting experiments were conducted *in situ* 5 times in June 1998, first 4 times during both day and night at the Sarilhos site, and 1 time at night at the Rosario station (Fig. 2). Each time, 3 flux chambers were used. Denitrification was estimated from $^{15}N_2$ ($^{29}N_2$, $^{30}N_2$) production, after adding $^{15}NH_4^+$ to the sediment. The tracer $^{15}NH_4^+$ was chosen since denitrification in an exposed sediment with high NO$_3^-$ turnover exclusively relies on the oxidation of NH$_4^+$ to NO$_3^-$. An area (30 × 40 cm) of the mud-flat was selected, and 1.5 to 2 ml 20 mM $^{15}NH_4^+$ solution was sprayed onto the chosen sediment-surface area with a water atomizer until a final minimum concentration of 100 to 200 µM $^{15}NH_4^+$ was attained at the surface. The intention was to ensure a large surplus of $^{15}NH_4^+$ in the nitrification zone throughout the incubation, so that the isotope would be well distributed in the oxic zone within 5 min (concentration at 1 mm depth >10 µM), as judged from model simulations of molecular diffusion (Crank 1975), assuming a diffusion coefficient of 19.6 cm^2 s^{-1} (Li & Gregory 1974). Gas-tight glass chambers (n = 3), with an inner diameter of 9 cm, were thereafter inserted in the ^{15}N-enriched sediment (Fig. 3), and closed with transparent lids. Atmospheric air, in the approximately 1 cm high headspace of the glass chambers, was replaced with a 20% O$_2$, 80% He gas mixture in order to reduce background N$_2$ and thereby increase the sensitivity of the assay. To minimize temperature and humidity differences between the inside
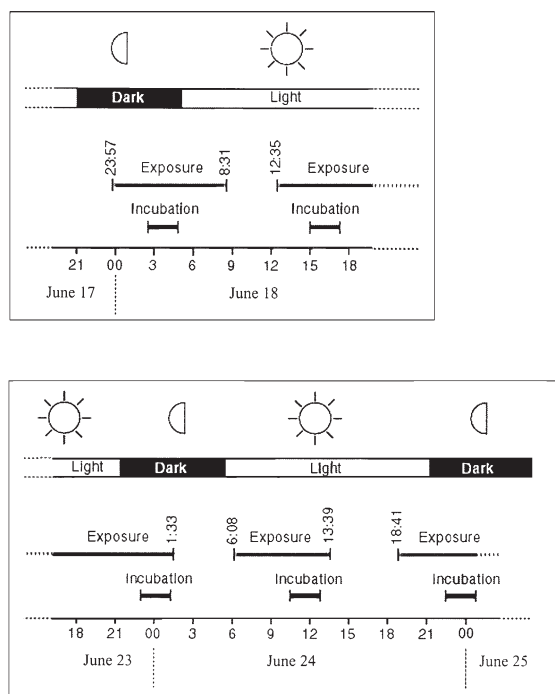
Fig. 2. Illustration of the sequence of light/dark and exposure/inundation at the sites on the days of investigation (June 17 and 18 and June 23 to 25, 1998). Period of denitrification measurements is indicated with black bars 'Incubation'

and outside of the chambers during the daytime measurements, we designed a gas circulation system with a 'cooling coil' and a water-vapor trap (Fig. 3). Because of this, sediment temperature inside the chambers never rose more than 2 degrees above ambient sediment temperature (23 to 27°C). Measurements at night were conducted without the gas-circulation system.

During the 2 to 3 h incubation period, gas samples for $^{15}N_2$ analysis were regularly collected from the headspace with a gas-tight glass syringe through a butyl rubber septum in the lids of the chambers (Fig. 3). The gas samples (2 ml) were transferred for storage in 5 ml glass vials (Labco, Exetainer, High Wycombe, UK), prefilled with He-purged milli-Q water that was replaced with the gas sample.

At each sampling time, 3 samples from the sediment for $^{15}N_2$ analysis were collected randomly within the isotope labeled area around the chambers. A plastic syringe (i.d. = 12 mm), from which the tip had been cut off, was used to sample the upper 2 cm of the sediment. The sediment material was gently mixed with 13 ml He-purged water in a small container. The resulting slurry was transferred to 5 ml glass vials (Labco, Exetainer, High Wycombe, UK) and preserved with 250 µl 7 M ZnCl$_2$. At the end of incubation, the glass chambers were removed and a sediment sample was taken from the area inside the chambers as described above.

The concentration of excess $^{15}N_2$ ($^{29}N_2$ and $^{30}N_2$) in the gas and slurry samples was determined on a mass spectrometer (Sira Series II, VG Isotech, Middlewich, UK) as described by Nielsen (1992). For each sample, the production of $^{28}N_2$ was calculated from the production of $^{29}N_2$ and $^{30}N_2$, which was measured directly on the mass spectrometer. The following equation was used where $p^{28}N_2$, $p^{29}N_2$, and $p^{30}N_2$ denote the production of the isotopic species:

$$p^{28}N_2 = \frac{{p^{29}N_2}^2}{4p^{30}N_2} \tag{1}$$

assuming uniform mixing of nitrogen-isotope species (Nielsen 1992). Denitrification rates were calculated by linear regression (see Fig 4).

Potential denitrification activity, relative to total O$_2$ consumption in surface sediment from the Sarilhos station, was evaluated from excess $^{29}N_2$ and $^{30}N_2$ production in slurry incubations with $^{15}NH_4^+$ and O$_2$ in known concentrations, as described by Ottosen et al. (1999).

Pore-water chemistry. A filter-extraction method was used to collect pore-water samples from the upper mm of the sediment for salinity and NH$_4^+$ determinations. Two dry GF/C glass-fiber filters (diam. = 4.5 cm) were placed on the sediment surface, and the upper filter was removed after 20 to 30 s, by which time both filters were saturated with pore water.

Salinity was determined by squeezing a few drops of the pore-water sample from the filter onto a refractometer (Type Meijo Techno Japan, salinometer S-1). Filters for NH$_4^+$ determination were rolled up, placed in polyethylene vials, and immediately frozen on dry ice for later centrifugation and NH$_4^+$ determination with the salicylate-hypoclorite method (Bower &
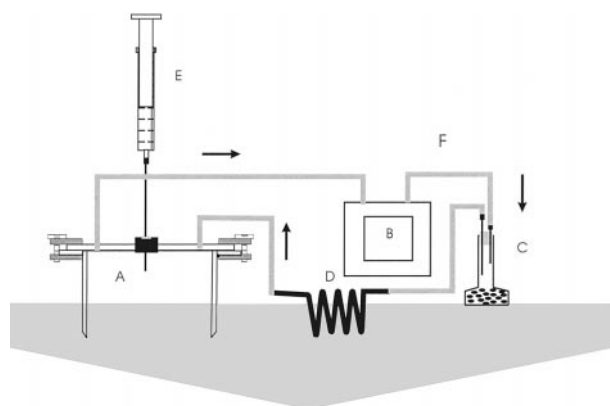

Fig. 3 Experimental set-up. A: glass chamber inserted in sediment; B: battery-driven electric air pump; C: vapor trap, container with absorbant material; D: air cooler, copper coil inserted in sediment; E: gas tight glass syringe for extracting gas samples from the chamber; F: gas-tight iso-versenic rubber tubes
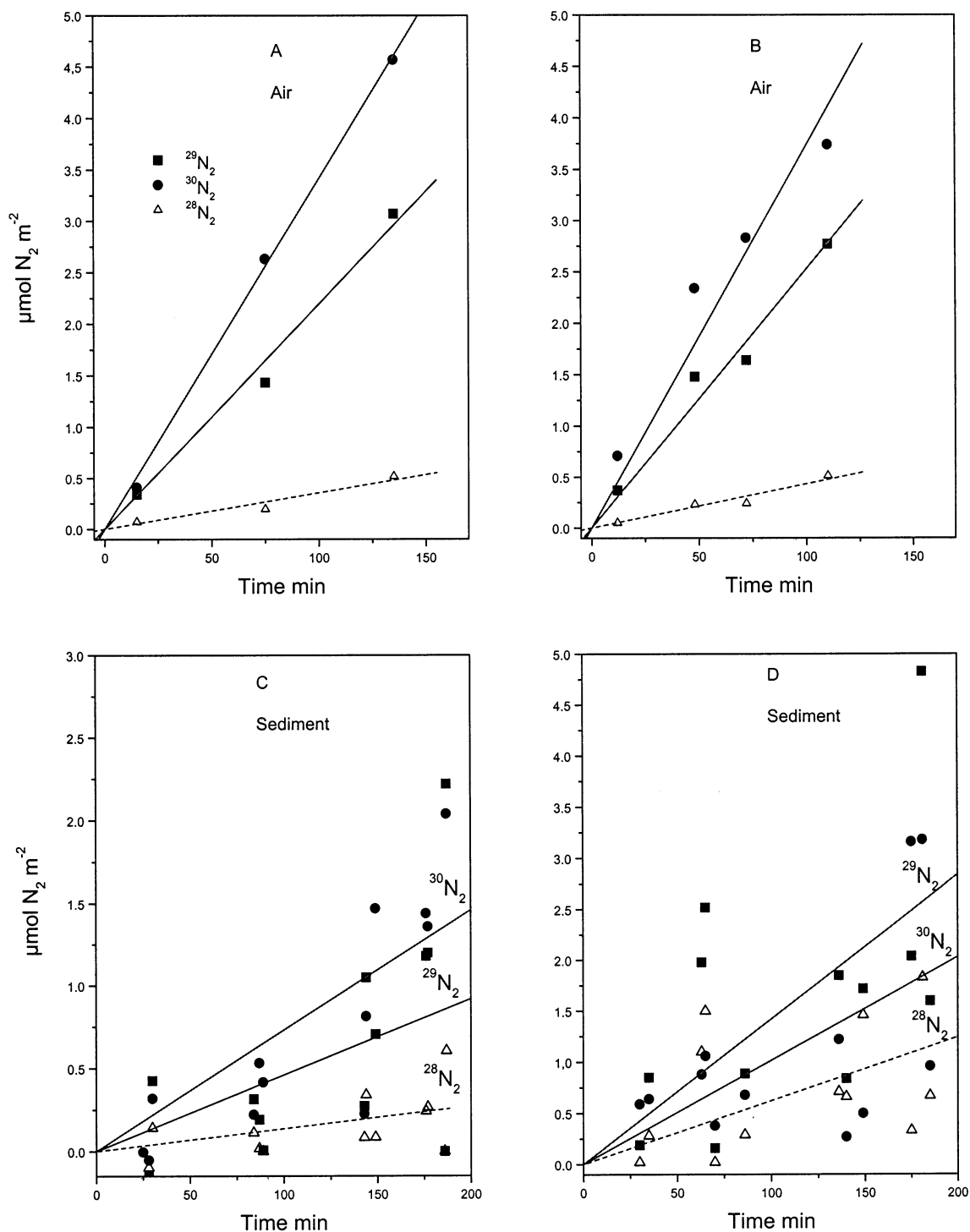
Fig. 4. Typical nighttime flux patterns of labeled N_2 after addition of $^{15}NH_4^+$. (A, B) Air from headspace of inserted chamber; (C, D) air within sediment. (A, C) Rosario; (B, D) Sarilhos

Holm-Hansen 1980). From the sediment porosity and the water content of the filter, it was calculated that a filter extracted pore water from approximately the top 0.8 to 0.9 mm of the sediment

After application of the $^{15}NH_4^+$ solution to the sediment surface and immediately after the denitrification measurements, pore-water samples for $^{15}NH_4^+$ determinations were taken from the sediment surface by

filters and frozen as described above. The content of $^{15}NH_4^+$ was determined by combined micro-diffusion hypobromite-oxidation (Risgaard-Petersen et al. 1995), whereby NH_4^+ is extracted and oxidized to N_2 for mass spectrometry.

Evaporation of pore water from the sediment surface during daytime incubation periods was measured as the weight loss over time of sediment slices in round trays (10 cm wide and 3 cm deep) embedded in the sediment, with the surface inside the tray aligned with that outside.

RESULTS

Throughout the dark incubations, $^{15}NH_4^+$ was present in the sediment with an average sediment surface concentration at the end of the incubation of 140 ± 40 µM. Rate measurements were therefore not complicated by exhaustion of the tracer. The concentration of $^{29}N_2$ and $^{30}N_2$ in the headspace of the incubation chambers increased linearly with time during all night measurements (Fig. 4A,B), suggesting stable fluxes of labeled N_2 across the sediment-air interface. The rates of $^{15}N_2$ accumulation in the sediment were less regular than the rate of N_2 accumulation in the headspace (Fig. 4 C,D). This was expected, as sediment samples represented small separate areas, whereas headspace samples represented repeated samplings integrating the flux from a larger area.

Denitrification during the nighttime was 11.5 ± 2.8 µmol m^{-2} h^{-1} (mean ± SE) at the Sarilhos station and 8.5 ± 1.3 µmol m^{-2} h^{-1} at the Rosario station (Fig. 5). For both sites, coupled nitrification-denitrification of added $^{15}NH_4^+$ accounted for approx. 65% of the activity. Denitrification activity during the day was below 2 µmol N m^{-2} h^{-1} (the statistically safe detection limit for the method). The rate of coupled nitrification-denitrification was equivalent to 0.88% ± 0.15% of total O_2 consumption in slurry incubations of surface sediment from Sarilhos. Average *in situ* oxygen uptake at the same station at night was 800 µmol O_2 m^{-2} h^{-1}, calculated from O_2 gradients at the sediment-air interface and direct flux measurements in air-filled gas-tight flux chambers (V. Brotas et al. unpubl. data). With this relative rate methodology, the estimated rate of the coupled nitrification-denitrification rate was thus 7 ± 1.2 µmol N m^{-2} h^{-1}, very close to the ^{15}N-based *in situ* results.

Evaporation from the sediment surface during the daytime (June 24) is shown in Fig. 6B. The exposure period began at about 13 h (13:00 h) that day, and evaporation measurements were initiated shortly after. Maximum water loss from the mud surface was in the early afternoon, being then about 1.1 mm h^{-1} (1100 ml m^{-2} h^{-1}) and gradually decreasing to 0.4 mm h^{-1} at 19 h (07:00 h). Integrated loss during exposure was 4.7 mm.

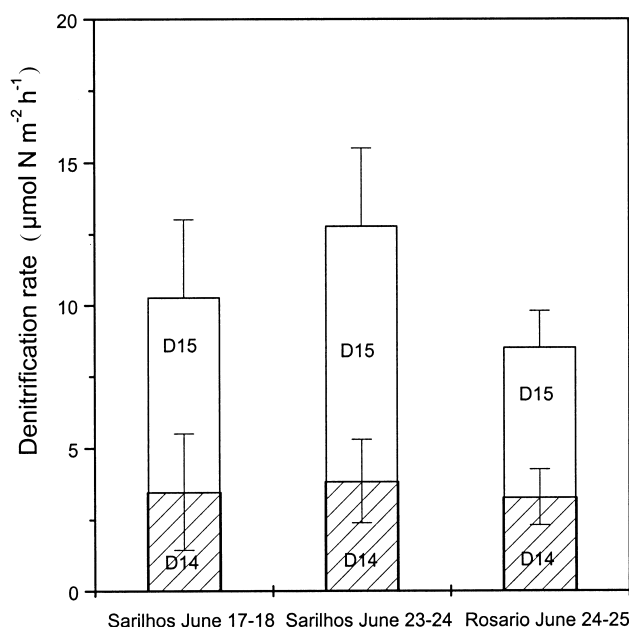Fig. 5. Denitrification rates (µmol N m^{-2} h^{-1}) in exposed sediment, Tagus Estuary June 1998. D14: coupled nitrification-denitrification of $^{14}NH_4^+$; D15: coupled nitrification-denitrification of $^{15}NH_4^+$

During the daytime exposure salinity tripled from ~25 psu up to ~75 psu at the driest spots on the sediment surface (Fig. 6A). During the night, salinity was constant at 20 to 25 psu. In contrast to surface salinity, there was no unambiguous development in NH_4^+. Average concentration in the top 0.8 mm was 32.9 ± 12.9 µM during daytime exposure. During the night, concentrations of NH_4^+ were substantially higher, ranging between 75 and 150 µM.

DISCUSSION

The high concentrations of NH_4^+ at the sediment surface during the nighttime suggests that nitrification was not NH_4^+-limited, and that the reaction rate would be largely unaffected by addition of further NH_4^+ (the tracer). The coupled nitrification-denitrification of the added $^{15}NH_4^+$ is assumed not to accelerate *in situ* rates; rather, the tracer $^{15}NH_4^+$ merely substitutes for naturally occurring NH_4^+. The total N_2 production during the experimental incubations therefore represented the best estimate of *in situ* coupled nitrification-denitrification. In the case of strong NH_4^+-limitation, addition of $^{15}NH_4^+$ would enhance nitrification and overestimate coupled rates. Denitrification of unlabeled nitrogen ($D_{14} = ^{29}N_2 + 2 \times ^{28}N_2$) alone would under such circumstances represent a better estimate of *in situ* activity.
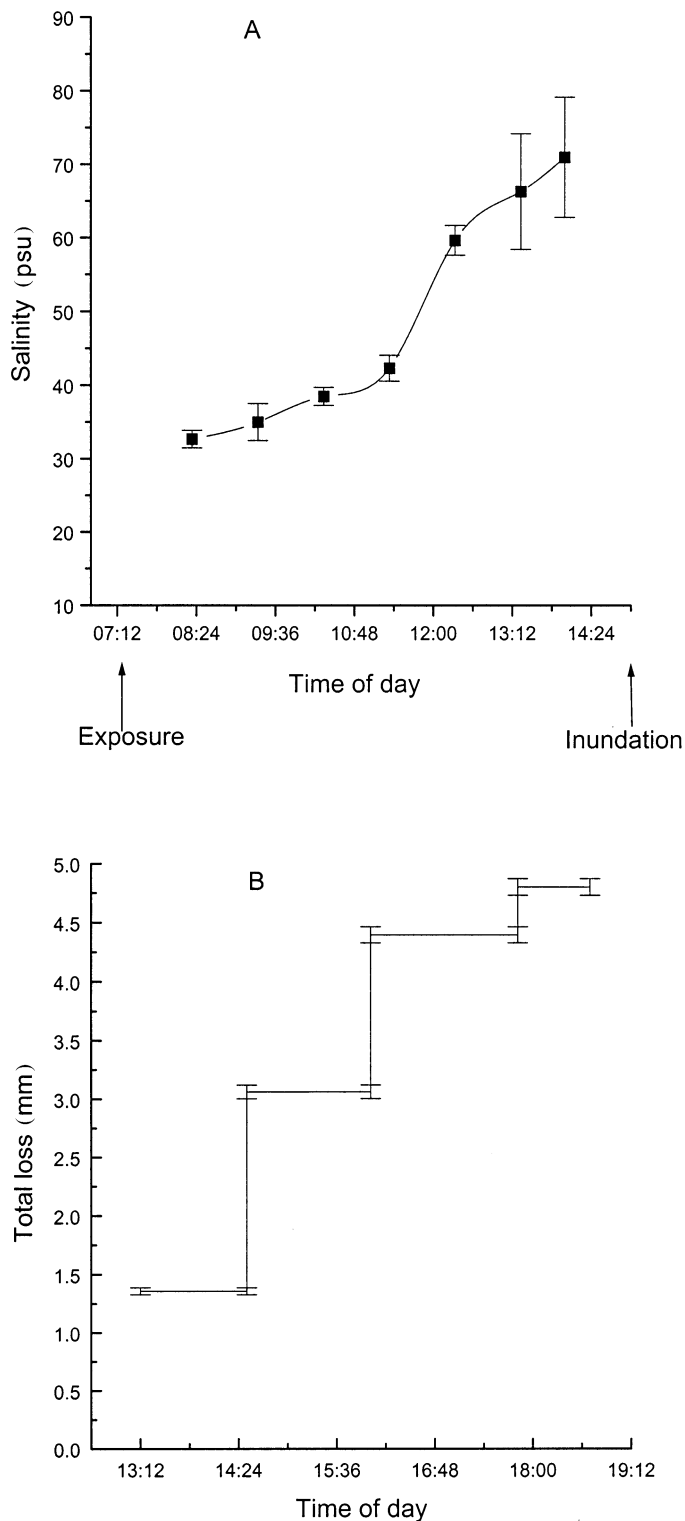
Fig. 6. (A) Salinity increase at sediment surface during exposure (June 18); (B) cumulative loss of water by evaporation from sediment surface during low tide (June 24). Note: water loss and salinity were not measured during the same period

To calculate the production of $^{28}N_2$, it is assumed that the mixing ratio of ^{14}N and ^{15}N in the denitrification zone is uniform. The data for Rosario indicated a homogeneous mixing of isotopes in the denitrification zone, and the rate estimate was therefore reliable. At Sarilhos, however, the more variable ratio of produced $^{29}N_2$ and $^{30}N_2$ in the small, discrete sediment samples shows that this assumption was not really fulfilled (Fig. 4). As a consequence, the production of $^{28}N_2$, and hence total denitrification, was underestimated (Boast et al. 1988, Nielsen 1992). In order to evaluate the error, we calculated $^{28}N_2$ accumulation for each discrete sample (as described in 'Materials and methods') and compared this to a rate estimate based on the average accumulation of $^{29}N_2$ and $^{30}N_2$ in the sediment samples.

The accumulation rate of $^{28}N_2$ in the sediment was calculated to be 0.373 μmol m^{-2} h^{-1} (using Eq. 1) when based on each discrete sediment sample, and 0.324 μmol m^{-2} h^{-1} (using Eq. 1) when based on the average production of $^{29}N_2$ and $^{30}N_2$ in all the sediment samples. Thus, estimating denitrification from the total integrated pools of $^{29}N_2$ and $^{30}N_2$ implies underestimation of $^{28}N_2$ production by <14%, and of total denitrification (sum of production of $^{28}N_2$ $^{29}N_2$ and $^{30}N_2$) with <3%. This calculation indicates that the applied ^{15}N enrichment was sufficiently high to ensure that most of the N_2 produced was recovered directly as $^{29}N_2$ or $^{30}N_2$, and the effect of heterogeneity therefore was minimal. With the presented methodology, we recommend that as much $^{15}NH_4^+$ as possible be added to minimize the effect of heterogeneity (Nielsen1992).

Theoretically we cannot rule out that heterogeneity, at a spatial scale much smaller than that analyzed here (0.7 cm^2 samples), might have played a role. A more detailed test of the isotope mixing in the sediment would have required many more parallel incubations with different levels of ^{15}N addition (Nielsen 1992). This might also have helped to explain the puzzling observation of generally higher $^{29}N_2/^{30}N_2$ ratios in sediment samples compared to gas-phase samples at the Sarilhos site. This finding could possibly reflect the presence of vertically separated zones of N_2 production, although more data is required to elucidate this.

The validity of the nighttime measurements of denitrification with the ammonium spray method (8.5 ± 1.3 μmol m^{-2} h^{-1} at Rosario) was also confirmed by the good correspondence with the independent estimate of denitrification based on oxygen uptake and relative rates of nitrification and denitrification in sediment slurries (7 ± 1.2 μmol N m^{-2} h^{-1}). For more crude surveys, the latter relative-rate methodology might in fact be an attractive alternative to the more direct ammonium spray method with in situ flux chambers.

The daytime measurements of denitrification were much more difficult to evaluate. No production of

labeled N_2 was detected, and at the end of the incubations none of the added $^{15}NH_4^+$ could be recovered from the sediment surface layer. Obviously the $^{15}NH_4^+$ had been assimilated by the abundant benthic microphytes which supported a net benthic production of 5 mmol O_2 m^{-2} h^{-1} (V. Brotas et al. ubpubl. data). It is tempting to conclude that none of the native $^{14}NH_4^+$ was available for nitrification-denitrification during the daytime (Nielsen & Sloth 1994). To further test this, much more $^{15}NH_4^+$ would have to be added to ensure penetration of the benthic microphyte mat, below which some nitrification-denitrification activity fed by NH_4^+ from deeper zones may be hidden, as demonstrated by Jensen et al. (1994). Curiously, the filter extraction consistently yielded considerable NH_4^+ concentrations (20 µM), even in the surface sediment, without any NH_4^+ addition, thus questioning the theory of N-limitation during the daytime. Moreover, we cannot exclude the possibility that the filters absorbed pore water from deeper anoxic layers through cracks and crevices, thus mixing pore water from the upper 0.8 mm with pore water from deeper layers. This could have obscured the true origin of the extracted pore water.

Mechanisms other than N-assimilation may have contributed to low daytime rates of nitrification-denitrification in exposed sediment. High salinity represses nitrification (e.g. Finstein & Bitsky 1972, Helder & De Vries 1983, Darrah et al. 1987), and in a parallel study G. Castaldelli et al. (unpubl. data) demonstrated 75% repression of potential nitrification in surface sediment at Sarilhos when salinity was elevated from 20 to 80 psu, as observed *in situ* during daytime exposure in the present study (Fig. 6). Light inhibition of marine nitrifiers is well known (e.g. Guerrero & Jones 1996) but it remains to be demonstrated whether light inhibition is important in a sediment such as that at Sarilhos, where light intensities strong enough to allow any photosynthesis are limited to the upper 0.5 mm of the sediment (Serodio et al. unpubl. data)

Coupled nitrification-denitrification, measured with a batch-version of the isotope-pairing technique when the sediments were inundated, was 7.3 ±1.4 µmol N m^{-2} h^{-1}. (M. Bartoli et al. unpubl. data). No light reached the sediment during inundation due to high turbidity of the overlying water. This activity was not significantly (p < 0.05) different from rates measured during dark exposure in the present study. The lack of any effect of inundation suggest that nitrification and denitrification was tightly coupled in the intertidal sediments studied, and that only a minor fraction of the NO_3^- produced by sedimentary nitrification diffused into the overlying water. Such a tight coupling has occasionally been demonstrated in freshwater sediments with concentrated nitrification activity at the oxic/anoxic inter-

face, most probably due to a limiting supply of NH_4^+ from the anoxic zone below (Jensen et al. 1994, Berg et al. 1998). Coupled nitrification-denitrification measured in sediments from a subtidal locality (water depth 6 m) in the Tagus Estuary was 27 ± 2.4 µmol m^{-2} h^{-1} (data not shown). This rate is about thrice the rates measured for the intertidal sediments. Probably conditions for nitrification were better at the subtidal locality, where the environment is more stable and protected and benthic primary production is absent.

The present study in the Tagus Estuary showed that the new ammonium spray method worked well to measure denitrification in exposed sediments during the nighttime, and probably, with more $^{15}NH_4^+$ tracer added, also during the daytime. Investigations of other types of intertidal sediments (e.g. with different levels of DIN and primary production) should be conducted before drawing general conclusions about the regulation of denitrification and the actual role of various intertidal areas as nitrogen sinks.

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