Limnol. Oceanogr., 54(1), 2009, 13–22

Long-term ^{15}N-nitrogen retention in tidal freshwater marsh sediment: Elucidating the microbial contribution

Britta Gribsholt,[1,2] *Bart Veuger, Anton Tramper, Jack J. Middelburg, and Henricus T. S. Boschker*
Netherlands Institute of Ecology, Center for Estuarine and Marine Ecology, P.O. Box 140, 4400AC Yerseke, The Netherlands

Abstract

The long-term retention of nitrogen in sediment of a tidal freshwater marsh in the Scheldt estuary (Belgium) was investigated by an in situ ^{15}N-labeling experiment. Sediment of an unvegetated creek bank and sediment vegetated by common reed (*Phragmites australis*) were labeled with ^{15}N-enriched NH_4^+ after which ^{15}N was traced into pore-water dissolved NH_4^+, NO_3^-, and N_2; plant roots and leaves; and bulk sediment over a 1-yr period. Label retention in the sediment organic matter was further characterized by analyzing KCl-extracted sediment and hydrolyzable amino acids (including the bacterial biomarker D-alanine). Within weeks all added $^{15}NH_4^+$ was transformed and/or assimilated by the biota. Between 42% and 48% of the added label was recovered in plants, root, and sediment (collectively) after 3–6 months, whereas 24% remained after 1 yr. Transfer to plants and roots was rapid and although retention in leaves was transient, the ^{15}N assimilated by roots was retained for the entire 1-yr period. Root assimilation could account for 12–23% of the ^{15}N retained in vegetated plots. The majority of ^{15}N was retained in the organic matter pool within the sediment (represented by ^{15}N in the KCl extracted sediment and total hydrolyzable amino acids), primarily through efficient recycling of the ^{15}N within the microbial community. ^{15}N incorporation into D-alanine confirmed that bacteria were the major group of microorganisms responsible for the strong retention of ^{15}N. This study shows the strong potential of reed roots and especially bacteria to retain nitrogen in tidal freshwater sediment over longer periods of time.

Tidal freshwater marshes are diverse habitats that differ widely in terms of plant community composition, sediment type, marsh elevation, and nutrient availability. They often occur where estuaries are most enriched in particles and nutrients and are hotspots of biogeochemical transformations (Bowden et al. 1991; Struyf et al. 2006). Tidal freshwater marshes provide a range of ecosystem services, including acting as water quality filters, and are often regarded as seasonal or long-term sinks for nutrients (Bowden 1987; Fisher and Acreman 2004; Neubauer et al. 2005). Despite their ecological significance, tidal freshwater marshes have not been studied nearly as well as their brackish and salt marsh counterparts.

Traditionally, tidal freshwater marsh studies have focused on nutrient exchange between marshes and tidal waters. Bowden et al. (1991) and Neubauer et al. (2005) were the first to provide comprehensive mass balances for the nitrogen cycle in freshwater tidal marshes. Both studies of North American systems revealed that exchanges of dissolved inorganic nitrogen (DIN; ammonium and nitrate) between the marsh and tidal water are relatively small compared to internal marsh nitrogen recycling. Using deliberate ^{15}N tracer additions to intact ecosystems, Gribsholt et al. (2005, 2006, 2007) studied the fate of watershed-derived ammonium in a tidal freshwater marsh fringing the Scheldt estuary (Belgium). These marsh-scale experiments conducted during the early and late growing seasons confirmed the limited exchange between marshes and tidal waters because the majority of the ammonium entering the marsh was exported again by subsequent tides. Moreover, they revealed that tidal freshwater marshes function primarily as nutrient transformers, because nitrification represented the largest sink of added ^{15}N (Gribsholt et al. 2005, 2006). About 4% of the added ^{15}N-NH_4^+ was retained by the marsh ecosystem during the 2-week study periods. Despite their large biomass, uptake by macrophytes was of limited importance, and most retention was associated with the sediment and plant litter and attributed to assimilation by the diverse microbial community associated with the surfaces of these components (Gribsholt et al. 2007). Identification of the various microbial groups involved was, however, not achieved; thus, the relative importance of different groups of microbes (i.e., benthic algae, fungi, and bacteria) for nitrogen retention remains unresolved. Moreover, ^{15}N enrichment levels in the sediment were low, precluding detailed assessment of nitrogen cycling within marsh sediments. Finally, these whole-ecosystem ^{15}N-labeling studies of tidal freshwater marshes lasted only 2 weeks and thus did not provide information on the processes governing the longer-term (i.e., months to years) retention of N.

White and Howes (1994) pioneered the use of in situ ^{15}N labeling to trace the long-term retention of nitrogen in salt marsh sediments. They observed that about 40% of the

[1] Corresponding author (britta.gribsholt@biology.au.dk).
[2] Present address: Department of Biological Sciences, Center for Geomicrobiology, University of Aarhus, Building 1540, Ny Munkegade, DK-8000 Aarhus C, Denmark.

Acknowledgments

We thank two anonymous reviewers for constructive feedback and the staff at the Netherlands Institute of Ecology, Center for Estuarine and Marine Ecology (NIOO-CEME) analytical laboratory for their assistance. This research was supported by the Netherlands Organization for Scientific Research and is part of the Dutch-Flemish cooperation on Coastal Research (VLANEZO). This is NIOO publication 4347.

added tracer could be recovered after 7 yr and that label initially incorporated in aboveground and belowground biomass of *Spartina alterniflora* was gradually transferred to the sediment organic matter pool. This long-term ^{15}N retention was attributed to accumulation of refractory belowground plant material and microbial incorporation, but the relative contributions of the two could not be assessed. Recent advances in analytical techniques now allow us to combine ^{15}N labeling with the analysis of ^{15}N incorporation into hydrolyzable amino acids (Veuger et al. 2005). ^{15}N incorporation into total hydrolyzable amino acids (THAAs) provides a measure for ^{15}N incorporation in total microbial biomass, and analysis of ^{15}N incorporation into bacteria-specific amino acids makes it possible to trace ^{15}N into bacteria (Tobias et al. 2003*b*; Veuger et al. 2007*a*,*b*). The objective of this study was to determine the long-term (months) retention and processing of nitrogen in sediments of a tidal freshwater marsh in the Scheldt estuary (Belgium). To accomplish this we injected ^{15}N-labeled NH_4^+ into the sediment and traced the ^{15}N into different compartments of the ecosystem (bulk sediment, KCl-extracted sediment, THAAs, pore-water dissolved NH_4^+ and NO_3^-, plant leaves, and plant roots) over a 1-yr period. Analysis of the ^{15}N incorporation into the bacteria-specific amino acid D-alanine (D-Ala; Veuger et al. 2005) enabled us to verify the importance of bacteria in the long-term nitrogen retention in the marsh sediment.

Methods

Study site and experimental design—The study was conducted in the Tielrode marsh (51°06′N, 4°10′E) fringing the Scheldt and Durme Rivers, Belgium (Fig. 1a). The vegetation of this ~100,000-m^2 marsh is typical for freshwater marshes, with large, monospecific stands of the common reed *Phragmites australis* interspersed by smaller patches of willows (2–6-m-high specimens of *Salix* sp.) and ruderal vegetation (*Impatiens glandulifera*, *Epilobium hirsutum*, and *Urtica dioica*). The vegetation cover is very dense, with *P. australis* growing to exceptional heights of up to 4 m, as is commonly observed in the ~4.5-km^2 Scheldt marshes. A semidiurnal tide (up to 5.3 m) floods the marsh with low-oxygen, nutrient-rich Scheldt water with DIN concentrations of >400 μmol L^{-1} (Gribsholt et al. 2005; Soetaert et al. 2006). River NH_4^+ generally accounts for 25–30% of total DIN. However, water flooding the study site varies greatly in ammonium concentration (<20 to >100 μmol L^{-1}) both within and among tides, because N undergoes considerable processing during the long distance traveled by water over marsh creeks and surfaces before arriving at the study site (Gribsholt et al. 2005). Groundwater input is negligible because the marsh is higher than the neighboring embanked polder. A detailed description of the study area can be found in Gribsholt et al. (2005).

Four experimental plots (1 m^2) were established in the northern part of the marsh in April 2002 by placing 30-cm-wide boards on the sediment surface (Fig. 1b). Boards were nailed together and held down by large metal clamps inserted approximately 0.5 m into the sediment. All plots were situated in close proximity (Fig. 1b) to the marsh area utilized for two (May 2002 and September 2003) large-scale whole-ecosystem ^{15}N-labeling studies (Gribsholt et al. 2005, 2006, 2007). Two plots (hereafter referred to as R1

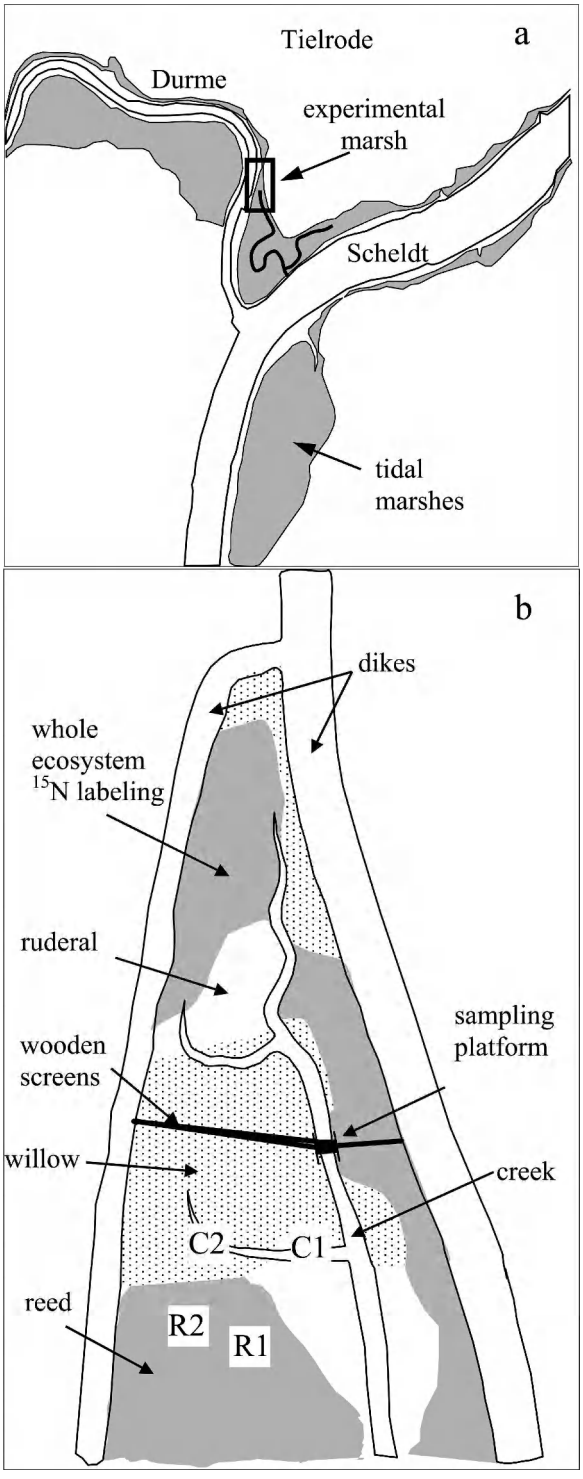

Fig. 1. (a) Tielrode tidal freshwater marsh fringing the Scheldt and Durme Rivers, with location of experimental marsh marked, and (b) location of experimental plots (C1, C2, R1, and R2) and vegetation distribution. Plots were situated south of the enclosed whole-ecosystem ^{15}N-labeling area (*see* text for details).

and R2) were established within monospecific stands of reed, and two plots (hereafter referred to as C1 and C2) were established on the unvegetated creek bank. Plots were left undisturbed for 2 months before the experiment was initiated on 05 June 2002, when the ^{15}N label was applied to each experimental plot by line injection (0–10-cm depth) of 1.0 mL tracer solution in 100 evenly spaced points (10 × 10) 8 cm apart, ensuring a homogenous distribution of the label. The label solution consisted of 12.0 g of ammonium chloride (NH_4Cl; 99 atom% ^{15}N) dissolved in 1 liter MilliQ H_2O, and a total of 1.2 g ^{15}N was injected per plot, equivalent to 34.7 mmol ^{15}N m^{-2} surface area. Each injection point was marked by a small wooden stick. Great care was taken to prevent damage to the vegetation and sediment surface within plots as much as possible during both labeling and subsequent sampling.

Field sampling—Samples were collected at days 1, 2, 7, 14, 28, 58, 91, 176, and 380 after $^{15}NH_4^+$ injection. In addition, samples were collected just before tracer addition (day 0) to establish natural abundance levels of ^{15}N. On each sampling occasion ~30 mL pore water was collected by inserting a standard Eijkelkamp Rhizon soil moisture sampler, consisting of 10-cm porous polymer tubing with a mean pore size of 0.1 μm, into the sediment at three a priori randomly selected label injection points. Potential sampling points were limited a priori to only 32 (evenly spaced) of the 100 label injection points to minimize edge and sampling effects on label distribution. For dissolved $^{15}N_2$ analysis, ~5 mL pore water was collected into a He-evacuated Venoject Vacutainer (Terumo, Belgium) attached directly to one Rhizon sampler. For concentration and isotopic analysis of NH_4^+, NO_3^-, and NO_2^-, 5–10 mL pore water was then collected from all three samplers by applying vacuum with 60-mL syringes, pooled into one sample, and stored frozen.

Following pore water extraction, one reed plant (stem + leaves) growing within a 4-cm radius of each pore-water sampling point was collected in the R plots by cutting at the sediment surface, and samples were pooled. From the otherwise unvegetated C plots, watercress (*Rorippa* sp.) was sampled when present. Subsequently, a metal corer (7.5-cm inner diameter) was inserted around each pore-water sampling point (i.e., 3 cores per plot) and a ~35-cm-deep core was extruded. Sediment cores were immediately sliced into 10-cm depth intervals down to 30-cm depth, triplicate sediment horizons were pooled into one well-mixed sample, and a 50–100-g subsample (avoiding live and dead roots) was collected and brought to the laboratory for further handling. A subsample of roots was collected from the remaining sediment by handpicking, except when root biomass was determined (*see* below). To prevent collapse of the sediment, water table disturbance, and air entry, we immediately filled each core hole with sediment collected from an area adjacent to each experimental plot.

Sample handling and analysis—Nitrogen isotopic composition of pore-water NH_4^+ and of $NO_3^- + NO_2^-$ (hereafter collectively referred to as nitrate [NO_3^-]), was determined in two steps using a modification of the ammonium diffusion procedure described in Gribsholt et al. (2005), followed by isotope ratio mass spectrometry (IRMS). Because of very high isotopic ratios in the pore water and the detection limits of the IRMS, it was necessary to dilute samples with unlabeled DIN before isotopic analysis. For determination of $^{15}NH_4^+$, 0.25–5 mL of sample was diluted in a 10- or 15-μmol L^{-1} NH_4^+ standard solution to a final volume of 30 mL containing 0.3–0.5 μmol N. For subsequent determination of $^{15}NO_3^-$, the samples were spiked with nitrate (0–0.5 μmol N depending on in situ [NO_3^-]). Concentrations of NH_4^+, NO_3^-, and NO_2^- were determined on separate subsamples using automated colorimetric techniques. Isotopic composition and concentration of dissolved dinitrogen ($^{29}N_2$, $^{30}N_2$) were determined in Vacutainer headspace gas (after vigorous shaking) by elemental analyzer (EA)-IRMS equipped with a Haysep Q column as described in Gribsholt et al. (2005).

Bulk sediment, plant, and root samples were analyzed for isotopic composition and total N on a Carlo Erba EA following Nieuwenhuize et al. (1994) and a Fisons EA coupled online to a Finnigan Delta S IRMS. Collected plant and root material had initially been rinsed, dried to constant weight (70°C), and shredded (Retsch cutting mill), and sediment samples had been freeze-dried, before a well-mixed subsample was ground to a fine, homogenous powder for analysis.

Surface sediment (0–10 cm) from one reed (R1) and one creek bank plot (C1) was analyzed for ^{15}N incorporation into THAAs including the bacterial biomarker D-Ala, according to Veuger et al. (2005). ^{15}N in THAAs represents ^{15}N incorporated in proteinaceous material. Furthermore, sediment-bound inorganic nitrogen was extracted by standard KCl technique (2 mmol L^{-1} KCl, 1 h). Following centrifugation and removal of the supernatant, the remaining sediment pellet was rinsed in MilliQ water as described in Gribsholt et al. (2007) and subsequently analyzed for ^{15}N as described above (all plots; 0–10 cm only). The ^{15}N in this KCl-extracted sediment represents ^{15}N incorporated in organic matter and will hereafter be referred to as Sed_{org}.

Standing reed biomass (aboveground) was determined at the onset of the experiment by harvesting all vegetation in 3 randomly chosen 30 × 30-cm quadrants in close proximity to the experimental plots. Root biomass was determined at days 0, 58, 91, 176, and 380 by washing all sediment remaining after subsampling (*see* above) on a 1-mm mesh. Samples were sorted into dead and live roots based on appearance and dried to constant weight (70°C). A subsample was used for root ^{15}N determination (*see* above). Although watercress was sometimes present on the otherwise unvegetated creek banks, its biomass was not determined. Instead, literature values were applied for budget calculations. Sediment porosity was calculated from water loss of a known sediment volume after freeze-drying.

Calculations—^{15}N is presented directly as the atomic percentage of ^{15}N in the sample (atom% ^{15}N) or as δ^E, quantified according to

$$\delta^E = \left(\frac{\delta^{15}N_s + 1000}{(\delta^{15}N_b + 1000) - 1} \right) \times 1000 \qquad (1)$$

where b is background and s sample. This enrichment notation δ^E can be compared directly with the previously used $\Delta\delta$ ($\delta^{15}N_s - \delta^{15}N_b$) notation but has the advantage that it is exact and thus valid at high levels of enrichments as observed here. ^{15}N retention in sediment, plant, and roots was calculated per surface area as the excess amount of ^{15}N above natural abundance. Total ^{15}N excess in leaves and roots was calculated from the product of the N content of the leaves per biomass unit, biomass (m^{-2}), and the ^{15}N excess atomic percentage in tissue.

Excess ^{15}N in D-Ala is presented relative to that in L-alanine (L-Ala) as excess ^{15}N D : L-Ala ratio (excess ^{15}N in D-Ala divided by excess ^{15}N in L-Ala). The racemization background (D : L-Ala ratio resulting from hydrolysis-induced racemization of L-Ala; Kaiser and Benner 2005) is indicated graphically. Excess ^{15}N D : L-Ala ratios were used to estimate the percentage bacterial contribution to total ^{15}N incorporation as described in Veuger et al. (2007*b*). Because the D : L-Ala ratio of natural bacterial communities ranges between 0.05 and ~0.1 (Veuger et al. 2007*b*), bacterial D : L-Ala ratios of 0.05 and 0.1 were used to derive a maximum and a minimum estimate of the bacterial contribution, respectively.

The isotopic composition of DIN following dilution with unlabeled N was calculated according to

$$\text{atom\%}_{pw} = \frac{\text{atom\%}_{mix} - \text{atom\%}_{st} * \left(\frac{N_{st}}{N_{mix}}\right)}{\left(\frac{N_{pw}}{N_{mix}}\right)} \quad (2)$$

where atom% is percentage of ^{15}N and N the total amount of N, and the subscript *pw* denotes the pore-water sample, *mix* the mixture of pore water diluted in NH_4^+ standard solution, and *st* the standard solution.

Results

^{15}N in pore-water DIN—Initial pore-water ammonium concentrations were similar in the two reed plots (17 ± 0.3 μmol L^{-1}), and 2 and 3.5 times higher in C1 (34 μmol L^{-1}) and C2 (65 μmol L^{-1}), respectively. Label injection increased the concentrations dramatically (5–12 times), and most of the ammonium was $^{15}NH_4^+$ on day 1 (Fig. 2). The degree of label, however, rapidly decreased by ammonium regeneration in both habitats, but the decrease was slightly faster in reed (atom% $^{15}NH_4^+$ = 143 $d^{-0.94}$; R^2 = 0.96) than in creek bank sediments (atom% $^{15}NH_4^+$ = 121 $d^{-0.79}$; R^2 = 0.96; Fig. 2). At day 28, the atom% ^{15}N of the ammonium pool was <10%, but the ammonium pool was still ^{15}N enriched after 1 yr. Initial nitrate concentrations were low (3–10 μmol L^{-1}) except in C1 (120 μmol L^{-1}). In spite of differences in nitrate concentrations, the ^{15}N enrichment of nitrate in both C plots increased to 100 atom% ^{15}N after 2 d (Fig. 2). A more heterogeneous response was observed in the R plots, where the maximum atom% ^{15}N of nitrate was 19% and 98% in R1 and R2, respectively. Moreover, ^{15}N enrichment of the nitrate pool decreased rapidly, but background levels were not reached during the experimental period. The isotopic enrichment of the dissolved dinitrogen pool varied greatly among plots. In both R plots, ^{15}N labeled N_2 was detected at all sampling occasions during the first 2 weeks, with maximum δ^E values of 21‰ and 49‰ at day 1 in R1 and R2, respectively. A much higher δ^E value of 687‰ was observed in C1 (day 2), where significant enrichment was observed until day 28. In C2, however, significant but low (δ^E = 8‰) enrichment of the N_2 pool was detected only on day 28.

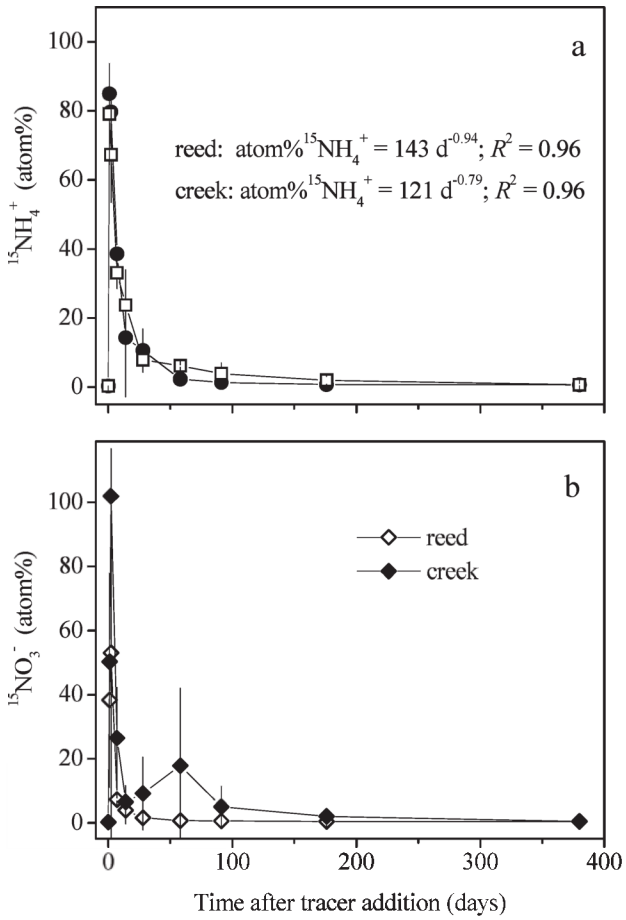

Fig. 2. Isotopic composition (atom%) of interstitial (a) ammonium and (b) nitrate (mean ± SD; n = 2). Equations in (a) are power functions describing the average decrease in ^{15}N labeling to the ammonium pool in reed and creek bank plots in relation to time (d = days after tracer addition).

^{15}N in bulk sediment and (microbial) biomass—Transfer of ^{15}N from the dissolved pool to the sediment occurred rapidly, with maximum δ^E values of ~375‰ after 2 d in both R and C plots (Fig. 3). Initially, almost all ^{15}N recovered in the sediment was present in the 0–10-cm depth horizon (Fig. 3a,b), but increasingly more (up to 51%) was found in the 10–20-cm depth horizon over time, especially in the C plots. Generally, <2% of the ^{15}N in the upper 30 cm of the sediment was recovered in the 20–30-cm horizon, except for the C plots at day 380 (1 yr), in which 14–18% of the recovered label was present in the 0–30-cm horizon. The average amounts of ^{15}N retained by the sediment 3–12 months after label injection were 9.3 ± 5.0 and 13.0 ± 7.8 mmol m^{-2} (0–30-cm depth) in R and C

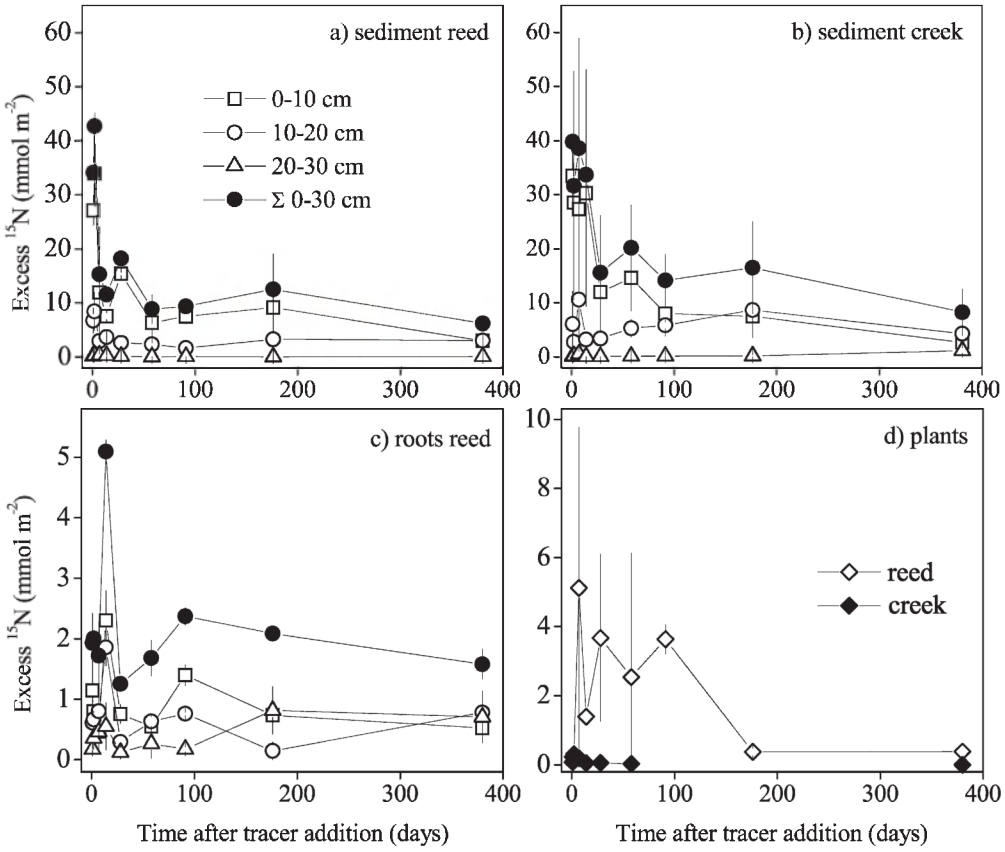

Fig. 3. Excess ^{15}N per surface area unit (mmol m^{-2}) in (a) reed and (b) creek bulk sediment, (c) reed roots, and (d) plants. Sediment and root data are presented for each 10-cm depth interval separately (open symbols), and collectively (closed symbols, 0–30-cm depth). Note that watercress was absent from creek banks on days 91 (September) and 176 (November). (Mean ± SE; n = 2.)

plots, respectively. After 1 yr, 17% and 19% of the injected label (34.7 mmol ^{15}N m^{-2}) had been retained in the sediment of the two reed plots. Retention was more variable between the two creek plots, with 12% and 36% of the injected label recovered in C1 and C2 after 1 yr, respectively.

Sed_{org} was also significantly enriched in ^{15}N, and maximum δ^E values of up to 145‰ were generally observed on day 2. Excess ^{15}N values generally peaked within the first few days, but development over time showed considerable variability between replicates (Fig. 4). In R1, excess ^{15}N in Sed_{org} increased sevenfold from 2.6 to 19.1 mmol ^{15}N m^{-2} between 2 and 6 months after label injection, and subsequently decreased to only 1.4 mmol ^{15}N m^{-2} after 1 yr. In R2, however, values remained low (2.1–3.3 mmol ^{15}N m^{-2}; 2–12 months) but increased slightly with time. Following the initial peak, excess ^{15}N Sed_{org} in C1 was relatively constant (3.1–3.4 mmol ^{15}N m^{-2}) over time until 6 months after injection, but then decreased 50% after 1 yr. In C2, maximum enrichment was observed after 2 months (8.8 mmol ^{15}N m^{-2}), after which it remained relatively constant (6.3 ± 0.5 mmol ^{15}N m^{-2}). Peak values generally (except in C2) coincided with peak values in ^{15}N excess in the bulk sediment (Sed_{tot}), and excess ^{15}N values for Sed_{org} and Sed_{tot} converged over time. The percentage of excess ^{15}N in organic matter increased from 27.5% ± 10.6% and 18.0% ± 16.3% in R and C, respectively, to >50% after 1 month and 74.0% ± 24.0% and 115.5% ± 16.3% after 1 yr in R and C, respectively. Estimated excess ^{15}N in Sed_{org} exceeded that in Sed_{tot} on two occasions (day 176 in R1 and day 380 in C2). This is likely an artifact stemming from insufficient mixing of the sediment sample prior to subsampling for KCl extraction (*see* Methods).

Trends for enrichment (δ^{E15}N, data not shown) and excess ^{15}N in THAAs over time were very similar to those for Sed_{org} (Fig. 4a,c), and there was good agreement between these independent estimates of ^{15}N in proteinaceous material and total organic material respectively (excess ^{15}N-THAAs = 0.41 × excess ^{15}N-Sed_{org} + 0.73; R^2 = 0.76). Between months 3 and 12, excess ^{15}N in THAAs made up 46–64% of total excess ^{15}N in the sediment. The composition of the ^{15}N-labeled THAA pool remained relatively constant over time, except for a gradual increase (from 15% to 26%; R^2 = 0.92) in the fraction of ^{15}N in THAAs present in glycine (Gly) in the C plot. In the R plot only a slight (from 18% to 20%) and more erratic (R^2 = 0.25) increase in ^{15}N-Gly : THAAs ratio was observed over the entire experimental period (data not shown).

^{15}N in bacteria (D-Ala)—Trends for excess ^{15}N in D-Ala (a proxy for bacterial ^{15}N incorporation) over time were

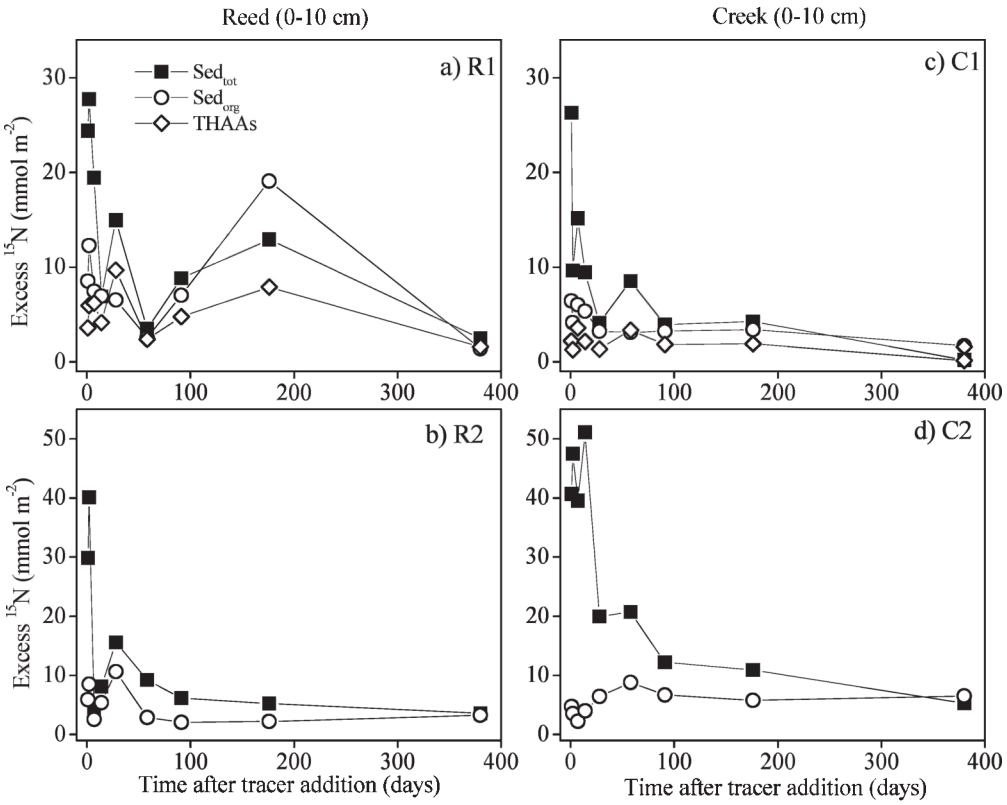

Fig. 4. Excess ^{15}N in bulk sediment (Sed$_{tot}$), microbial pool associated with the sediment (Sed$_{org}$; *see* text for details) and in the total hydrolyzable amino acids pool (THAAs) in the 0–10-cm depth horizon in the four plots. Incorporation into THAAs was analyzed only in R1 and C1. Note the different scales for the *y*-axes on the top and bottom plots.

very similar to those for THAAs, including ~3 times higher values observed in R1 compared to C1 in the period between days 91 and 380. Excess ^{15}N D : L-Ala ratios (Fig. 5)—the ratio between label recovered in D-Ala and in L-Ala, which is a measure of bacterial vs. eukaryote incorporation (Veuger et al. 2005)—were similar for both plots, with somewhat higher values for C1 (0.06, vs. 0.05 for R1), and remained rather stable over time, except for a dip after 2–4 weeks in R1. The excess ^{15}N D : L-Ala ratio could not be determined properly in C1 after 1 yr because the ^{15}N enrichment level was too low. For the period 3–12 months after label injection, the minimum and maximum bacterial contributions to excess ^{15}N in THAAs were estimated from the excess ^{15}N D : L-Ala ratios (Veuger et al. 2007*b*) and ranged from 41% to 103% and 49% to 124% in R and C, respectively (Fig. 5).

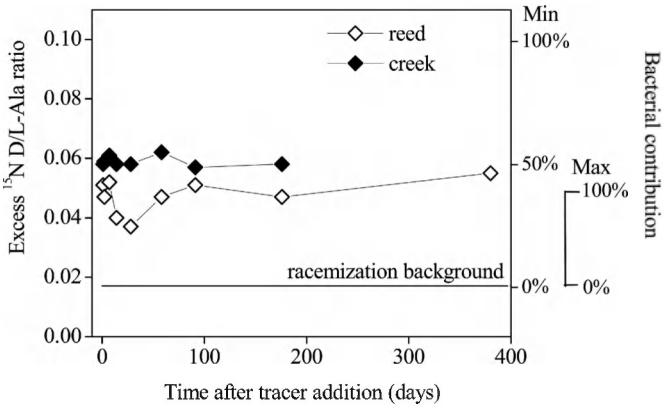

Fig. 5. Excess ^{15}N D : L-alanine ratios (excess ^{15}N in D-alanine divided by excess ^{15}N in L-alanine) in reed and creek plots. Dotted line indicates hydrolysis-induced racemization background. Values on second *y*-axis indicate minimum and maximum estimates of bacterial contribution to total microbial ^{15}N incorporation.

^{15}N in leaves and roots—Transfer of ^{15}N to the vegetation occurred almost as rapidly as to the bulk sediment. Reed roots were highly ^{15}N-labeled, with δ^{E} values up to 1250‰. In each 10-cm depth horizon, the reed root ^{15}N recovery showed an erratic temporal pattern (Fig. 3c). However, although the degree of labeling decreased with depth, root biomass increased, which resulted in relatively similar absolute amounts of ^{15}N assimilated in all depth horizons (0–30 cm). Following an initial peak 2 weeks after label injection (5.1 mmol m^{-2}), excess ^{15}N in roots was relatively constant over time, accounting for 4–7% of the injected label and 14–32% of excess ^{15}N in the bulk sediment (Sed$_{tot}$). Initially, ^{15}N was rapidly translocated to the leaves, as evident from an increase in excess ^{15}N in leaves on day 7 (R; Fig. 3d), and reflected in a high excess ^{15}N leaves : roots ratio (2.6–3.0 after 14 and 28 d). After 1 month, however, the ratio decreased linearly with time, to 0.2 on days 176 and 380.

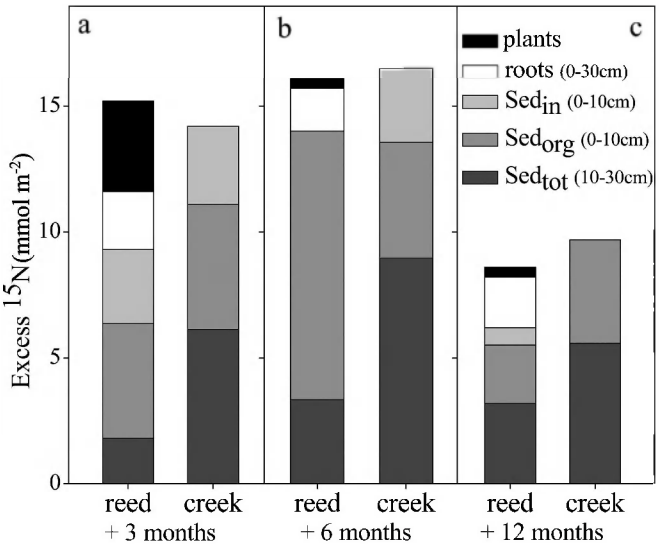

Fig. 6. Average excess ^{15}N (mmol m^{-2}) in plants (black), roots (0–30 cm; white), and sediment (0–30 cm; gray colors collectively) in reed and creek bank plots (a) 3 months, (b) 6 months, and (c) 12 months (days 91, 176, and 380, respectively) after label injection. Sediment ^{15}N incorporation is shown as Sed$_{tot}$ in the deeper 10–30-cm depth horizon (dark gray), which was not further analyzed, and as incorporated in organic matter (Sed$_{org}$; mid-gray) and sediment-bound inorganic nitrogen (Sed$_{in}$; light gray) in the 0–10-cm surface sediment.

Watercress growing on the otherwise unvegetated creek banks was highly labeled immediately following tracer injection. The highest enrichment (δ^E = 4370‰ on day 2) was more than twice the maximum observed in reed (δ^E = 1310‰, day 7). However, because the watercress biomass was low, the absolute amount of ^{15}N assimilated was negligible.

The total amount of ^{15}N retained by the plant, root, and sediment compartments (0–30-cm depth) collectively decreased slightly over the 3–12 month period after label injection and was relatively similar among the creek and reed biota (Fig. 6). Collectively, 42–48% of the injected label was recovered after 3–6 months (all plots), whereas 24% remained after 1 yr.

Discussion

Methodological aspects—Application of stable isotopes as deliberate tracers in field studies has gained increasing use in recent years. This technique has provided new insight into N cycling and retention in a broad range of freshwater (Kling 1994; Peterson et al. 1997; Webster et al. 2003), estuarine (Holmes et al. 2000; Hughes et al. 2000; Tobias et al. 2003*a*), and marine ecosystems (White and Howes 1994; Evrard et al. 2005; Barron et al. 2006). The advantage of this approach is that it allows for the examination of N flow through multiple pools simultaneously, while maintaining natural hydrological and biogeochemical processes. To enforce the latter, the heavy isotope is usually added only in trace amounts (<5–10% of ambient concentrations), so that it does not accelerate ambient rates but merely substitutes for ambient ^{14}N, and most field studies have therefore been restricted to relatively short periods of time (days to weeks), and only very few (White and Howes 1994) have examined seasonal and/or more long-term (months to years) N transformation processes and retention.

In order to trace the fate of ^{15}N though the different sediment pools over a relatively long period of time (months), we deliberately injected high concentrations of $^{15}NH_4^+$ directly into the marsh sediment. Injected concentrations were much higher than ambient NH_4^+ concentrations, which dramatically increased both concentrations and ^{15}N enrichment of the total NH_4^+ pool. Therefore, measured transformation rates for $^{15}NH_4^+$ and $^{15}NO_3^-$ do not reflect ambient rates. However, DIN concentrations quickly dropped to ambient concentrations and isotopic enrichments to <10 atom%. More importantly, ^{15}N enrichments of the total sediment, Sed$_{org}$, and THAA pool were always well below 1 atom%, and consequently this experiment can still be considered a tracer study.

Ammonium is usually the dominant form of biologically available nitrogen in reduced, waterlogged marsh sediments (Bowden 1987). The ^{15}N label was therefore administered as $^{15}NH_4^+$ and injected directly into the sediment (i.e., not sprayed on top of the sediment or added to the overlying water) to best mimic the release of dissolved nitrogen during degradation of organic matter within the sediment. We recognize that a truly homogeneous distribution of the label after line injection may take 1–2 months to establish in the rather impermeable marsh sediment. Consequently, some initial excess ^{15}N estimates extrapolated to a square-meter basis were higher than the added 34 mmol m^{-2} (Figs. 3, 4). In our budget calculations we assume an even distribution of the label for the 3–12-month period.

Turnover and losses of ^{15}N—In spite of the experimental limitations described above, tracing of the ^{15}N in dissolved pools did deliver some useful insight. The ^{15}N added as ammonium was rapidly recovered in the nitrate pool, indicating active nitrification. Rates of ammonium regeneration based on isotope dilution (Laws 1984) during the first 2 d were 12 ± 4 and 37 ± 10 μmol L^{-1} d^{-1} for R and C plots, respectively. Nitrification rates based on ^{15}N transfer from ammonium to nitrate during the first 2 d were 6 ± 4 and 26 ± 21 μmol L^{-1} d^{-1} for R and C plots, respectively. These initial ammonification and nitrification rates should be interpreted with caution, because pore-water ammonium concentrations were elevated because of label additions.

Pore-water pools of ^{15}N-NH_4^+ and ^{15}N-NO_3^- were diluted by regeneration of ammonium and nitrate over the entire period. Regeneration of ^{15}N from labeled organic nitrogen complicates the use of simple isotope dilution models (Laws 1984), and derived rates of ammonification and nitrification after day 2 are consequently not accurate. However, they do indicate active recycling of ^{15}N, and ammonification and nitrification rates were well correlated (r^2 = 0.94), suggesting strong coupling of ammonification and nitrification. Moreover, detection of ^{15}N in the dissolved dinitrogen pool suggests that denitrification in turn is coupled to nitrification. Reed (*P. australis*) has a

well-developed aerenchymatous root–rhizome system specially adapted to the reducing conditions in waterlogged sediment. By releasing oxygen from its roots, it creates a mosaic of oxic–anoxic interfaces in the otherwise anaerobic sediment, stimulating nitrification–denitrification (Bodelier et al. 1996; Eriksson and Weisner 1999; Gribsholt and Kristensen 2002).

Although detection of ^{15}N in the dissolved dinitrogen pools (data not shown) provides unequivocal evidence for nitrogen losses caused by coupled nitrification–denitrification, it does not allow us to quantify ^{15}N losses caused by dinitrogen gas emissions. Moreover, other ^{15}N losses, such as lateral exchange of dissolved inorganic and organic nitrogen to creeks, may also have contributed and have not been quantified. Bioturbation and roots and rhizome growth may have caused lateral transfer of ^{15}N tracer outside the experimental plot or vertical transport to larger depth, as evidenced by the recovery of 14–18% of the tracer in the deepest horizon sampled (20–30 cm).

Label transformation rates and losses were relatively high during the first 2 months, and then ^{15}N levels stabilized (Figs. 2–4). Between 42% and 48% of the label remained after 3–6 months, whereas about 24% was recovered after 1 yr. This pattern of rapid initial losses followed by stabilization of tracer level is consistent with observations made in marine (De Laune et al. 1983; White and Howes 1994) and freshwater marshes (De Laune et al. 1986), although our ^{15}N retention efficiencies are at the lower end of the range reported in the literature (typically 70% to 93% after 2–4 months; White and Howes 1994). White and Howes (1994) studied the retention of ^{15}N in the sediments of a New England salt marsh and recovered 62% after 1 yr and 40% after 7 yr. Although direct comparison of our retention efficiencies with literature studies is complicated by difference in climate, plant species, experimental setups (field vs. laboratory), and marsh sediment organic matter contents composition, the relatively low retention might be because of the high nitrogen loadings of tidal freshwater marshes in the Scheldt estuary (Soetaert et al. 2006).

Nitrogen retention by P. australis—In addition to the benthic microbial community (*see* below), the vegetation in R plots represents a strong (temporary) sink for N from pore-water NH_4^+. Transfer of the ^{15}N from the dissolved pool to the vegetation was very rapid, and although the amount of label decreased considerably (by ~90%) in the aboveground parts after 6 months, there was very little net loss from the root compartment over the 1-yr experimental period. Considering the life history of the plant and the time of sampling, the dynamics of the ^{15}N content in the plant (aboveground biomass) is not surprising. The experiment was initiated in early June, when plants were actively growing and building up biomass, with high N demand; thus, a rapid uptake of readily available N would be expected. Plant production is often limited by the availability of mineralizable nitrogen (Bowden 1987), and the rapid incorporation of ^{15}N into plant biomass suggests that plant uptake competes with nitrification–denitrification for remineralized NH_4^+ early in the growing season, reducing the availability of N for denitrification. Competition between plants and nitrifiers for uptake of limited quantities of N is known in a variety of other systems, including salt marshes, seagrass meadows, and hardwood forests (Zak et al. 1990; Welsh et al. 2000; Hamersley and Howes 2005). However, because the Scheldt marshes are flooded twice daily with very nutrient-rich (>400 μmol L^{-1} DIN) estuarine water, the system is generally not considered to be N-limited, and denitrification is more important later in the growing season (Gribsholt et al. 2006).

After 3 months (September), plants were in a flowering and/or early senescent state, beginning to translocate nutrients to the roots and rhizomes. By late November (day 176), plants had withered, and only leafless stems remained. The stems did, however, retain a green, live core, but the ^{15}N content was reduced by 90% compared to September (3 months), and the average plant total nitrogen content (%N) decreased linearly ($R^2 = 0.95$) from 1.75% to 0.50% from June to November. Nitrogen translocation to roots and rhizomes (storage organs) could account for the increase in root ^{15}N observed in late summer (1–3 months); however, this increase could also be caused by direct root uptake from the sediment. Although transfer of nitrogen from dying annual tissue to storage in perennial tissue (rhizomes) would seem a rational strategy, low translocation rates have previously been reported for *P. australis* in natural wetlands (Bowden 1987; Meuleman et al. 2002), and perennial belowground tissue does not necessarily cease to take up nitrogen coincidently with tissue death aboveground. It is likely that translocation and post-senescence uptake by roots and rhizomes operate simultaneously. The general loss of ^{15}N from plants (November) is likely because of N loss through seed dispersal (Meuleman et al. 2002) and litter fall with subsequent export from the system. Regrettably, isotopic compositions of these components were not determined in this study. The lack of increase in ^{15}N on the last sampling occasion (after 1 yr) suggests limited re-translocation of N stored in rhizomes.

Microbial nitrogen retention—The sediment compartment was by far the largest pool for long-term (3–12 months after injection) ^{15}N retention, even in the vegetated reed plots. Rather than merely looking at the ^{15}N retained by the sediment as one entity (bulk sediment; black box approach) we were able to distinguish between the ^{15}N that was merely sorbed to sediment particles (sediment-bound) and that which had been actively assimilated by microbes. Furthermore, using the novel technique of tracing ^{15}N into D-Ala, we were able to demonstrate that bacteria are by far the most important microbes responsible for N retention and recycling in these marshes.

In this study we included two independent measures of ^{15}N incorporation into organic matter, namely ^{15}N in the KCl-extracted sediment (Sed_{org}; representing total organic matter) and ^{15}N in THAAs (representing proteinaceous material). In general, trends for these two pools correlated nicely, with a slope of 0.41, indicating that proteins account for ~41% of total organic nitrogen. This contribution is very similar to the THAA content of bacterial biomass (50–

60%; Cowie and Hedges 1992). During the initial few weeks there was a large additional pool of ^{15}N present in the sediment, as reflected by the difference between ^{15}N in the bulk sediment and that in the organic matter (Sed_{org}). This pool must primarily have consisted of added $^{15}NH_4^+$ loosely bound to the sediment and/or dissolved in the pore water. During the first weeks of the experiment this pool was rapidly depleted, and the $^{15}NH_4^+$ and $^{15}NO_3^-$ available for incorporation into microbial biomass were completely exhausted after 1–2 months. Consequently, retention of ^{15}N in the organic matter after 1–2 months was no longer biased by potential continued incorporation of "new" ^{15}N from $^{15}NH_4^+$.

The remarkably strong retention of ^{15}N in (proteinaceous) organic matter up to 1 yr is likely because of retention in living microbial biomass though efficient recycling of the ^{15}N, i.e., reincorporation of ^{15}N released during degradation of dead, labeled microbial biomass (detritus). High turnover (hours to days) of microbial biomass in these (organic-rich) sediments (Alongi 1994; Buesing and Gessner 2006) and efficient degradation exclude survival of the microorganisms that initially assimilated ^{15}N or accumulation of labeled detritus as important ^{15}N retention mechanisms. Furthermore, the observed increase in excess ^{15}N Gly : THAA ratios over time can be explained only by active reworking of the original ^{15}N-labeled material. Excess ^{15}N D : L-Ala ratios remained at values typical for a living bacterial community. In case of substantial accumulation of detritus, this ratio is expected to increase, because D-Ala is abundant in relatively refractory biomass components such as peptidoglycan, whereas L-Ala is predominantly present in relatively labile proteinaceous biomass (Tremblay and Benner 2006).

Our data on excess ^{15}N in the bacterial biomarker D-Ala relative to its commonly present stereoisomer L-Ala (Fig. 5) provides further information on the bacterial contribution to total microbial ^{15}N incorporation and retention. These excess ^{15}N D : L-Ala ratios remained in the range for natural bacterial communities and showed little change over time. The temporary dip in excess ^{15}N D : L-Ala ratio in C1 after 2–3 weeks may reflect a temporary contribution by benthic microalgae (BMA) to total ^{15}N incorporation. However, excess ^{15}N D : L-Ala ratios indicate that ^{15}N incorporation and retention was generally dominated by bacteria, which is also reflected in the estimated theoretical bacterial contributions of 50–100% (Fig. 5). The actual bacterial contribution was likely near 100% and ^{15}N sequestration by BMA negligible, given that this study deals with the upper 10-cm layer of the sediment, with active BMA typically restricted to the upper few millimeters of the sediment. Moreover, the presence of BMA in the experimental marsh was very low (except in early spring) because of shading by surrounding vegetation (Gribsholt et al. 2007). Fungi are another microbial group that may potentially be involved in the uptake and recycling of ^{15}N. They have long been considered unimportant in water-saturated, partially anoxic sediments, but recent work has shown that mycorrhizal fungi are sometimes abundant in wetlands, where they may play an important role in various processes, including nitrogen retention (Gutknecht et al. 2006; Balasooriya et al. 2007). Although fungi associated with plant litter (aboveground dead stems and leaves) do play an important role in N sequestering (Findlay et al. 2002; Gribsholt et al. 2007), the importance of fungi in tidal freshwater marsh sediments remains unclear. Although we cannot entirely exclude a contribution by fungi in the present study, we are confident that bacteria were the major microbial group involved in incorporation and recycling of the ^{15}N.

Our data add to the accumulating evidence that nitrogen cycling in tidal freshwater marshes is relatively closed (Neubauer et al. 2005), i.e., rates of internal recycling are high (Bowden et al. 1991) and exchanges of nitrogen between marsh and tidal waters are relatively small (Gribsholt et al. 2005, 2006). Internal recycling involves regeneration of ammonium from organic nitrogen. The ^{15}N ammonium added to marsh pore water was partly nitrified (and subsequently denitrified and thus lost from the system), but the majority was retained by plant uptake and in particular bacterial incorporation (Fig. 6). The ^{15}N incorporated by the bacteria was retained over a full year, although it was continuously recycled. This implies that bacterial incorporation and regeneration are tightly linked, because retention of nitrogen would otherwise have been much lower. This efficient retention of nitrogen by bacterial recycling occurs despite that these marshes receive tidal waters highly enriched in DIN (Soetaert et al. 2006). The combination of nitrogen uptake from tidal waters (Gribsholt et al. 2005, 2006, 2007), efficient retention by bacteria, and marsh accretion by sediment deposition (Temmerman et al. 2004) constitutes a long-term nitrogen sink. Whether this long-term sink by microbial nitrogen retention is more important than denitrification losses cannot be assessed with the data at hand and would also require construction of nitrogen mass balance models that resolve both total and tracer nitrogen flows.

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Edited by: H. Maurice Valett

Received: 19 February 2008
Accepted: 27 September 2008
Amended: 01 October 2008