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Fate of microphytobenthos nitrogen in subtropical subtidal sediments: A ^{15}N pulse-chase study

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Abstract

Microphytobenthos (MPB) are an important nitrogen (N) sink in coastal systems, but little is known about the fate of this N after it has been assimilated. We used an in situ ^{15}N pulse-chase experiment in subtidal sands to follow the assimilation, trophic transfer, transformation, and flux pathways of MPB-N over 33 d. Throughout the study MPB dominated ^{15}N uptake, on average representing only 18.1% of the biomass but 63.9% of the ^{15}N within 0–2 cm sediment. Following assimilation, ^{15}N was rapidly transferred to deeper sediment, with 32.1% below 2 cm and 16.5% below 5 cm after 60 h. In contrast to MPB, bacteria represented 39.5% of sediment biomass but accounted for only up to 27.3% of assimilated ^{15}N. Foraminifera accumulated and stored ^{15}N more than bacteria; their contribution to the ^{15}N remaining in 0–2 cm sediment at the end of the study was more than double their biomass contribution. Thirty-three days after the ^{15}N was assimilated by MPB 27% remained in the sediment, 16.5% had been effluxed as NO_3^-, 20.8% had been effluxed as NH_4^+, 20.7% had been effluxed as N_2 and 15.1% was missing. Most (12.6%) of ^{15}N label that was missing at the end of the study was probably lost as dissolved organic N (DON) fluxes. Of the ^{15}N remaining in 0–2 cm sediment, 80.4% was in MPB, 2.7% in bacteria, 1% in foraminifera and the remaining 15.9% was uncharacterized. Overall there was little benthic trophic transfer with most of the MPB-assimilated N remineralized over 33 d.

Microphytobenthos (MPB) make an important contribution to the primary production of shallow coastal systems (Cahoon 1999). During biomass synthesis MPB fix carbon (C) and assimilate nitrogen (N) from both sediment pore waters and the water column e.g., (Sundbäck and Graneli 1988; Ferguson et al. 2004; McGlathery et al. 2007). A number of studies have looked at the fate of C fixed by MPB e.g., (Middelburg et al. 2000; Oakes et al. 2012; Oakes and Eyre 2014), but little is known about the fate of assimilated N. The most comprehensive assessment of the fate of MPB-derived C in subtidal sediments showed that respiration was the major loss pathway for MPB-C (63%) (Oakes et al. 2012), whereas little MPB-C was lost via DOC fluxes (~3%) or resuspension (< 3%). After 33 d 31% of the MPB-C was still retained in the sediments. Of this, 7% was in MPB, 7% in bacteria, 2% in fauna and 84% was uncharacterized (Oakes et al. 2012).

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MPB-N in subtidal sediments will have some of the same flux and assimilation pathways as MPB-C, including loss via resuspension, and retention in bacteria and fauna, but the proportions are likely to be different due to the different reactivity of N, and the different N requirements of the organisms involved. In addition, MPB-N will have some unique flux pathways such as ammonification and loss as NH_4^+ effluxes across the sediment-water interface, ammonium oxidation (nitrification) and loss as NO_3^- effluxes, and denitrification and anammox and loss as N_2 effluxes. However, few studies have looked at the fate of MPB-N, and these have only considered limited pathways. For example, a whole estuary labeling study that measured benthic fluxes of $^{15}NH_4^+$ in intertidal sediments showed preferential mineralization of MPB over bulk sediment N and estimated that MPB could account for 50–100% of the N mineralized, with about 10% of the assimilated label returned to the water column over the 22 d study period (Tobias et al. 2003a,b). In the same study ^{15}N was also traced into a bacterial biomarker, suggesting a link between bacteria and MPB and the benthic NH_4^+ flux. In a short (4 d) laboratory-based pulse-chase stable isotope experiment with subtidal sediment, MPB-N was

rapidly transferred to consumers with a greater transfer of N, relative to C, to meiofauna than macrofauna (Evrard et al. 2010). A longer term (14 d) in situ study of intertidal sediments (mudflats and creek walls) traced MPB-N into saltmarsh infauna (Galván et al. 2008).

There have been no comprehensive assessments of the fate of MPB-N that include assimilation, trophic transfer and flux pathways. This study used a combination of ^{15}N-labeling, compound-specific isotope analysis of biomarkers, and isotope analysis of NH_4^+, NO_3^-, and N_2 to investigate the fate of MPB-N in subtropical photic subtidal sandy sediments. Specifically, we aimed to determine rates of N transfer within sediment compartments (assimilation and trophic transfer) and loss to the water column as fluxes across the sediment-water interface. We hypothesized that NH_4^+, NO_3^- and N_2 fluxes would be a major pathway for loss of MPB-N, due to the high loss of respired MPB-C as dissolved inorganic C (Oakes et al. 2012). The experiment was done in situ and over an extended time period (33 d) to allow natural loss processes to occur. Given the paucity of information on the transformation and fate of N fixed by MPB this is a valuable step towards understanding the role of MPB in coastal N cycling.

Methods

Study site

The subtidal study site (~ 1.5 m below average sea level) in the subtropical Brunswick River estuary, Australia, has been used in a number of previous studies (Webb and Eyre 2004; Oakes et al. 2010b; Oakes et al. 2012). During this study the site was net autotrophic (production : respiration ~ 1.2 over a diel cycle with gross CO_2 productivity of ~ 105 mmol C m^{-2} d^{-1}) (Oakes et al. 2012). The MPB assemblage was dominated by pennate diatoms and no cyanobacteria were observed under light microscopy (×100) (Oakes et al. 2012). Sediment to 10 cm depth had an organic carbon (OC) content of 18.6 mol C m^{-2} with higher organic matter content in surface sediments (0.18% C) than in deeper sediments (0.16% C at 2–5 cm; 0.15% C at 5–10 cm). Sediment at all depths was comprised primarily of fine (125–250 μm; 72–79%) and medium (250–500 μm; 17–24%) quartz sand grains, but they were not permeable (Oakes et al. 2012). MPB made the greatest contribution to OC in surface sediments (9.0% at 0–2 cm depth), contributing only 6.9% and 6.6% of the OC in sediment at depths of 2–5 cm, and 5–10 cm, respectively. The contribution of bacteria to OC was greater in deeper sediments (7.4% and 9.3% of OC at depths of 2–5 cm and 5–10 cm, respectively) than in the surface sediments (3.7% of OC). Foraminifera *Elphidium craticulatum* (referred to as *Cellanthus craticulatus* by Oakes et al. 2012), *Ammonia beccarii*, and *Elphidium advenum* contributed about 4% of the sediment OC across all depths. The remaining OC was uncharacterized.

^{15}N-labeling

The experimental plots, and the cores collected from these plots, were also used for a ^{13}C-labeling experiment (Oakes et al. 2010b; Oakes et al. 2012). Three physically similar areas of sediment without macrophytes or large animal burrows were chosen for experimental plots. Plots were defined by 30 cm tall hexagonal perspex benthic chambers that were placed on the sediment surface, enclosing a water volume of 360 L and a sediment area of 1.2 m^2. The NO_3^- pool in the enclosed water column of the chambers was labeled by adding 50 μmol ^{15}N L^{-1} via injection with KNO_3 (99% ^{15}N). This resulted in 93.4% of the NO_3^- (total concentration = 53.5 μmol N L^{-1}), and 86.3% of the dissolved inorganic nitrogen (DIN) (total concentration = 57.9 μmol N L^{-1}), in the water column being in the form of $^{15}NO_3^-$. A laminar flow of water across the sediment surface within the chambers was maintained with pumps, and the chambers were left in situ over a 24 h diel cycle, allowing uptake of the $^{15}NO_3^-$ by MPB. The chambers were then removed.

Sample collection

Two sediment cores (9 cm diameter × 20 cm deep, with 30 cm overlying water) from each labeled experimental plot and two control cores from an area 5–8 m away from any labeled sediment (for background isotope values) were collected immediately after chamber removal and after a further 1, 3, 10, 20, and 30 d. Control and labeled cores from each collection period were placed in separate tanks of unlabeled site water in the laboratory at in situ temperature (± 1°C) and average daily light levels (± 5%, ~400 μmol photons m^{-2} s^{-1} at sediment surface, 12 h). To maintain the natural in situ diel cycle the cores were pre-incubated uncapped (12 h dark, then 12 h light) and then sealed and incubated (12 h dark then 12 h light). Water samples were taken from one of each pair of collected cores (i.e., from 3 labeled and 1 control core) at the start and the end of the dark period, and these cores were then sacrificed. The remaining cores were treated similarly, with the same water volume removed during the dark incubation period, but not retained. These cores had water samples collected at the beginning and end of the light incubation and were then sacrificed. During pre-incubation and incubation the water column within each core was stirred, without causing resuspension, using magnetic bars ~10 cm above the sediment surface. Pre-incubation allowed the sediment microhabitats to re-establish following disturbance from coring. However, the combination of in situ chamber incubation and pre-incubation post-initial labeling prevented monitoring of ^{15}N losses over the first 48 h, and sediment transfers over the first 60 h. As such, this study is focused on longer-term transfer and loss processes.

Immediately following pre-incubation, 2 L of water from each tank was collected, filtered (pre-combusted GF/F) and retained for determination of background δ^{15}N values of NH_4^+ and NO_3^-. At the beginning and end of the light and

dark incubation periods, dissolved oxygen concentrations (± 0.01 mg L^{-1}) and temperature (± 0.01°C) were measured (Hach HQ40d, luminescent DO probe) through ports in the lid of the cores, and water samples were collected. As water was sampled, it was replaced with gravity-fed site water maintained within collapsible reservoirs under the same conditions as the cores. Triplicate samples for N_2:Ar analysis were collected directly from cores by allowing this replacement water to gently push sample water via tubing into 7 mL gastight glass-stoppered glass vials. The vials were filled to overflowing, killed with 20 μL of saturated $HgCl_2$ and stored submerged at ambient temperature. Approximately 60 mL of sample water was then withdrawn from each core into a plastic syringe. Samples to determine NH_4^+, NO_3^-, and DON concentrations were syringe filtered (0.45 μm cellulose acetate) into duplicate 10 mL polyethylene vials, leaving a headspace, and refrigerated for storage. To determine nitrogen stable isotope ratios ($\delta^{15}N$) of N_2, sampled water was filtered (0.45 μm cellulose acetate) into a 20 mL glass vial containing 500 μL of 2M NaOH. These samples were sealed without headspace using a lid containing a teflon-coated septum, then had a 4 mL helium headspace injected and were stored at ambient temperature until analysis. When cores were sacrificed at the end of the dark or light incubation periods, the water overlying sediment within each core (~ 1.9 L) was gently siphoned off, leaving ~1 cm depth of water in the core to avoid collection of loose surface sediment. This water was filtered (precombusted GF/F) for analysis of $\delta^{15}N$ in NH_4^+ and NO_3^-. Sediment within sacrificed cores was extruded and sectioned (0–2 cm, 2–5 cm, and 5–10 cm depths). Sediment for chlorophyll *a* analysis was scraped from the sediment surface (1 mL, ~2 mm depth) using a spatula and stored in a 10 mL centrifuge tube containing 5 mL of 90% acetone. Samples were stored in the dark at −20°C until analysis.

A known portion of each sediment sample was freeze-dried for $\delta^{15}N$ analysis of sediment organic nitrogen (ON) and the extraction and $\delta^{15}N$ analysis of D- and L-alanine (to determine ^{15}N uptake into bacteria and MPB). The remainder was stored frozen (−20°C) for separation of fauna, however, only foraminifera were found. Foraminifera were picked, by hand, from sediment samples to obtain sufficient material for isotope analysis.

Sample analysis

NH_4^+ and NO_3^- (including NO_2^-) for isotope analysis was collected on acidified GF-D (Whatman) filter papers via the acid-trap diffusion protocols described by Holmes et al. (1998) and Sigman et al. (1997). $^{15}NH_4^+$ and $^{15}NO_3^-$ trapped on the filter papers was determined by isotope ratio mass spectrometry (IRMS, Thermo Delta V Plus IRMS) after combustion on an elemental analyzer (Thermo Flash EA 1112). All EA-IRMS samples were standardized against the ultrapure calibration standard $(NH_4)_2SO_4$ (52%). %CV of eight replicate control samples = 0.2% for $^{15}NO_3^-$ and 0.4% for $^{15}NH_4^+$.

N_2 concentrations were determined from N_2:Ar measured using membrane inlet mass spectrometry with O_2 removal, as described by Eyre et al. (2002). Concentrations of DON, NH_4^+ and NO_3^- were determined using a four channel Flow Injection Analyzer (Lachat QuickChem 8000). Analytical methods, errors, and detection limits for these methods are detailed in Eyre and Ferguson (2005). The $^{15}N_2$ concentrations of the He headspace in N_2 samples were determined via gas chromatography-isotope ratio mass spectrometry, using a Thermo Trace Ultra gas chromatograph (GC) coupled with a Delta V Plus IRMS via a Thermo Conflo III interface. The IRMS was calibrated against pure tank N_2 standards. Values are reported with respect to N_2 in air. Aliquots of atmospheric air were analyzed prior to samples each day to test for accuracy and precision. %CV of thirty-seven replicate $^{15}N_2$ samples = 3.1%.

Transfer of ^{15}N into bacteria and MPB was determined through compound-specific stable isotope analysis of D-alanine and L-alanine following extraction and purification of total hydrolysable amino acids from lyophilized sediments. Only the 0–2 cm interval, and only one replicate per time was analysed for D-alanine and L-alanine due to the logistical constraints of this laborious analysis, and no peaks were obtained for day 33. Extraction and analysis was done as described by Veuger et al. (2005), except that a larger sample of sediment was required (~ 10 g). The sediment was initially washed with 5 mL of milli-Q and 1 mL of 12M HCl, and amino acids were eluted from glass columns containing Dowex 50WX8 resin using 12 mL of 2N NH_4OH. L-Norleucine was added to the samples after hydrolysis as an internal standard to check recovery of amino acids. Extracts were analyzed via gas chromatography-isotope ratio mass spectrometry using a Thermo Trace Ultra GC coupled with a Thermo Delta V Plus IRMS via a Thermo Conflo III interface. The column used was a 50 m length of Agilent CP Chirasil-L-Val (0.25 mm i.e., film thickness 0.12 μm), comprised of two 25 m columns connected together. Helium was the carrier gas, with a flow rate of 1 mL min^{-1}. Samples were manually injected in splitless mode. The GC oven was held at 60°C for 2 min, then ramped to 100°C at 40°C min^{-1}, then to 116°C at 1°C min^{-1}, then ramped at 20°C min^{-1} to 190°C and held at that temperature for 5 min. %CV of eight replicate control samples was 0.3% for D-alanine and L-alanine.

Only the particulate organic nitrogen (ON) in the sediment was analysed for ^{15}N by using KCl extraction to first remove N adsorbed to sediment particles. This was done by combining 4 g of dried homogenized sediment with 10 mL of 2M KCl, before shaking for 1 h and centrifuging. The supernatant was removed and discarded, and the sediment then rinsed three times with milli-Q. The sediment samples were then re-dried (60°C to constant weight). Foraminifera samples were also dried prior to analysis (60°C to constant

weight). Sediment and foraminifera samples were weighed into tin cups, then analyzed for $\delta^{15}N$ and %N using a Thermo Finnigan Flash EA 112 interfaced via a Thermo Conflo III with a Thermo Delta V Plus IRMS. Helium dilution of the carrier stream was turned off for foraminifera samples to reduce the required mass. %CV of seven replicate control samples was 0.2% for ^{15}N-ON. Chl *a* samples were sonicated (15 min), centrifuged (2500 rpm, 5 min) and the supernatant was analyzed using a spectrophotometer. The supernatant was then acidified with 1M HCl and reanalyzed to correct for the presence of phaeophytin.

Calculations

The total ON mass (biomass) of sediment and foraminifera was calculated as the product of %N determined during isotope analysis and total dry mass per unit area. For foraminifera, counts of individuals within sediment subsamples were scaled up to provide an estimate of the total number of individuals per m^2 at each depth. Total mass was determined by multiplying this estimate by the average mass of an individual, based on the mass of a known number of individuals. The total incorporation of ^{15}N into sediment ON and foraminifera was calculated as the product of excess ^{15}N (fraction ^{15}N in labeled sample—fraction ^{15}N in control) and the calculated ON mass.

The excess ^{15}N content of bacteria and MPB in 0-2 cm sediments was estimated based on excess ^{15}N within the amino acids D-alanine and L-alanine. The assumptions and uncertainty associated with this method are discussed in detail by Veuger et al. (2005) and Veuger et al. (2007*b*). The peak area of the L-Norleucine standard was used to calculate the concentration of D-alanine and L-alanine in sediment based on their peak areas. These concentrations were multiplied by the excess ^{15}N fraction for each peak (fraction ^{15}N in labeled sample—fraction ^{15}N in control) to determine the excess ^{15}N content of D- and L-alanine. Excess ^{15}N in bacterial D-alanine was calculated as follows:

$$\text{Excess}^{15}\text{N in total D}-\text{alanine} - 0.006 \times \text{excess}^{15}\text{N in total L}-\text{alanine}$$

where 0.006 is the assumed racemization background and corrects for conversion of L-alanine to D-alanine during acid hydrolysis (Veuger et al. 2007b). Although values as high as 0.02 are possible (Veuger et al. 2007b), ^{15}N D/L-alanine ratios as low as 0.004 were obtained, suggesting that the racemization rate was lower for the sediments used in the current study (Fig. 1). We therefore applied the lowest racemization value reported for liquid-phase hydrolysis-induced racemization of amino acids (0.006; Kaiser and Benner 2005).

Total excess ^{15}N within bacteria was determined by multiplying the excess ^{15}N content of bacterial D-alanine by 400, as described by Veuger et al. (2005), based on the D-alanine content of cultured bacteria (Veuger et al. 2005) and assuming a bacterial N content of ~12% (Madigan et al. 2000). The contribution of bacteria to ^{15}N uptake was calculated as follows:

$$\left(^{15}\text{N D/L}-\text{alanine} - 0.006\right)/(0.05 - 0.006) \times 100$$

Where ^{15}N D/L-alanine is the ratio of ^{15}N in total D-alanine to ^{15}N in total L-alanine, 0.006 corrects for racemization, and 0.05 is the assumed ratio of D-alanine to L-alanine in bacteria. This is at the lower end of the range reported by Veuger et al. (2007b) (0.05–0.1) and assumes a negligible contribution of Gram positive bacteria and/or cyanobacteria to the benthic community. The lack of cyanobacteria is consistent with our microscope work. The maximum ratio of 0.1 is based on 30% Gram-positive and 70% Gram-negative bacteria. Although Gram-positive bacteria can represent up to 30% of bacteria in anaerobic subsurface sediments (Moriarty and Hayward 1982), ^{15}N was mainly in the surface sediments until the end of the experiment, and given that the sediments are quite sandy, the anaerobic sediment community would most likely not have much access to ^{15}N. A value at the higher end of this range (0.1) would effectively halve the estimated contribution of bacteria to ^{15}N uptake. Uptake of ^{15}N into MPB was determined assuming that bacteria and MPB were the only components of the microbial community at the study site. The contribution of MPB to total ^{15}N uptake was therefore assumed to be 100% minus the bacterial contribution to ^{15}N uptake (i.e., MPB ^{15}N = sediment ON ^{15}N − bacterial ^{15}N). The total ^{15}N uptake by MPB was then determined based on the calculated ^{15}N uptake into bacteria and the proportion of total ^{15}N uptake into the microbial community that this represented.

Bacterial ON mass within deeper sediments, for which amino acids were not extracted, was estimated using C biomass data reported by Oakes et al. (2012) as part of the same labeling study. The molar C : N ratio was calculated for bacteria in 0–2 cm sediments and this was then used to estimate bacterial ON mass in deeper sediments based on the bacterial C biomass reported by Oakes et al. (2012) for these sediment depths. The N biomass of MPB was similarly determined based on C biomass data in Oakes et al. (2012), assuming a Redfield C : N ratio of 6.625.

The total excess ^{15}N in the water column in the form of $^{15}NH_4^+$, $^{15}NO_3^-$, and $^{15}N_2$ was calculated for the beginning and end of the dark incubation period and for the end of the light period as the product of excess ^{15}N in NH_4^+, NO_3^- and N_2 (fraction ^{15}N in labeled sample—fraction ^{15}N in the equivalent control), core volume, and the concentration of NH_4^+, NO_3^-, and N_2. The total flux of excess ^{15}N in NH_4^+, NO_3^-, and N_2 during dark or light incubation was then calculated as follows:

$$\text{Excess}^{15}\text{N flux} = \left(\text{Excess}^{15}\text{N}_{\text{start}} - \text{Excess}^{15}\text{N}_{\text{end}}\right)/\text{SA}/t$$

where excess $^{15}N_{start}$ and excess $^{15}N_{end}$ represent excess ^{15}N within NH_4^+, NO_3^-, or N_2 at the beginning and end of the

Table 1. Mean biomass of sediment compartments at depths of 0–2 cm, 2–5 cm, and 5–10 cm, based on control samples (standard errors in brackets). %N indicates the percentage N within each sediment depth that is accounted for by each compartment, with the exception of %N for sediment N, which indicates the percentage of total N (0–10 cm) detected within each sediment depth. Note that units are per area, not volume.

	0–2 cm		2–5 cm		5–10 cm	
Compartment	Biomass (mmol N m^{-2})	%N	Biomass (mmol N m^{-2})	%N	Biomass (mmol N m^{-2})	%N
Sediment*	335.8(25.0)	20.2	557.0(46.0)	33.5	771.5(60.1)	46.4
MPB	60.7(0.1)	18.1	58.2(5.2)	10.4	85.9(9.7)	11.1
Bacteria	132.3(8.3)	39.4	333.3(82.8)	59.9	640.2(95.9)	83.0
E. craticulatum	0.6(0.3)	0.2	0.1(<0.1)	<0.1	0.3(0.1)	0.2
A. beccarii	2.4(0.5)	0.72	3.6(0.6)	1.1	8.9(3.3)	2.7
E. advenum	1.3(0.3)	0.4	1.5(0.4)	0.5	0.5 (0.1)	0.2
Uncharacterized	138.5(26.9)	41.2	160.1(46.3)	28.8	35.4(114.7)	4.6

*%N for sediment N indicates the percentage of 0–10 cm N within each depth of sediment.

dark or light incubation period, SA is the sediment surface area within a core, and t represents hours of dark or light incubation. Net fluxes (excess ^{15}N m^{-2} d^{-1}) of excess $^{15}NH_4^+$, $^{15}NO_3^-$, and $^{15}N_2$ were calculated as follows:

$$\text{Net flux} = (\text{dark flux/dark hours}) + (\text{light flux/light hours}) \times 24 \text{ h}$$

We interpolated between measured net flux values between each of the measured time points and estimated the total quantity of ^{15}N lost via fluxes of NH_4^+, NO_3^-, and N_2 from the end of labeling up until each sampling period by determining the area under the curve.

Data analysis

Because excess ^{15}N values for sediment compartments and fluxes were determined for both dark and light periods analyses of variance (ANOVAs) were used to determine if the dark and light data should be treated separately. Three-way ANOVA with factors of depth, day, and light exposure (dark or light) were used to test for an effect of light exposure alone or in combination with other factors (interaction with depth and/or day) on excess ^{15}N within sediment ON and foraminifera. Two-way ANOVAs with factors of day and light exposure were similarly used to test for an effect of light exposure alone or in combination with day on excess ^{15}N in fluxes of NH_4^+, NO_3^-, and N_2. Where Levene's test was significant (variances were heterogeneous), data was transformed (log $(x + 1)$) before analysis. It was not possible to statistically test for an effect of light exposure on ^{15}N within bacteria and MPB, as only one replicate was analysed per sampling time.

Results

ANOVAs showed that there was no significant effect of light exposure during laboratory incubation on excess ^{15}N in sediment ON, foraminifera, or fluxes of NH_4^+, NO_3^- and N_2. Light and dark excess ^{15}N values were therefore combined for subsequent calculations. All days refer to days after labeling. The first samples were collected 3 d after label application and the last samples were at 33 d after label application.

Sediment Chl *a* and Nitrogen

Sediment chl *a* at 0–2 cm depth averaged 5.7 ± 3.1 mg m^{-2} (mean ± SD) across all cores collected over the study period. Sediment to 10 cm depth had an ON content of 1.7 mol N m^{-2} (Table 1) and a molar C:N ratio of ~ 7–10. There was higher organic matter content in surface sediments (0.03% N) than in deeper sediments (0.02% N at 2–5 cm; 0.01% N at 5–10 cm) (Table 1). MPB made the greatest contribution to ON in surface sediments (18.1%, at 0–2 cm depth), contributing less but similar amounts of the ON in sediment at depths of 2–5 cm and 5–10 cm (10.4% and 11.1%, respectively). The contribution of bacteria to ON was greater in deeper sediments (59.9% and 83.0% of ON at depths of 2–5 cm and 5–10 cm, respectively) than in surface sediments (39.4% of ON). Although Foraminifera made only a small contribution to ON, that contribution was highest at 5–10 cm (3.0%), and was dominated by the *Ammonia beccarii*. The remaining ON was uncharacterized, making the greatest contribution to total sediment ON (41.2%) at 0–2 cm.

Uptake of ^{15}N and transfer among sediment compartments

Sediment ON 0–10 cm deep contained 553 ± 139 μmol ^{15}N m^{-2} by the time the first sediment was collected (60 h after label addition; day 3). A comparison (three-way ANOVA) of δ ^{15}N, %N, fraction excess ^{15}N vs. control, and total excess ^{15}N (fraction * total mass N) in bulk (data not shown) and KCl-extracted sediment showed indirectly that there was no measureable residual $^{15}NO_3^-$ label in the porewater. When the in situ chamber was removed 24 h after label addition 665 ± 182 μmol ^{15}N m^{-2} was in 0–10 cm sediments, when corrected for measured loss of ^{15}N as NH_4^+, NO_3^-, and N_2 during the 36 h of incubation before the sediment sample collection. This equated to an incorporation of

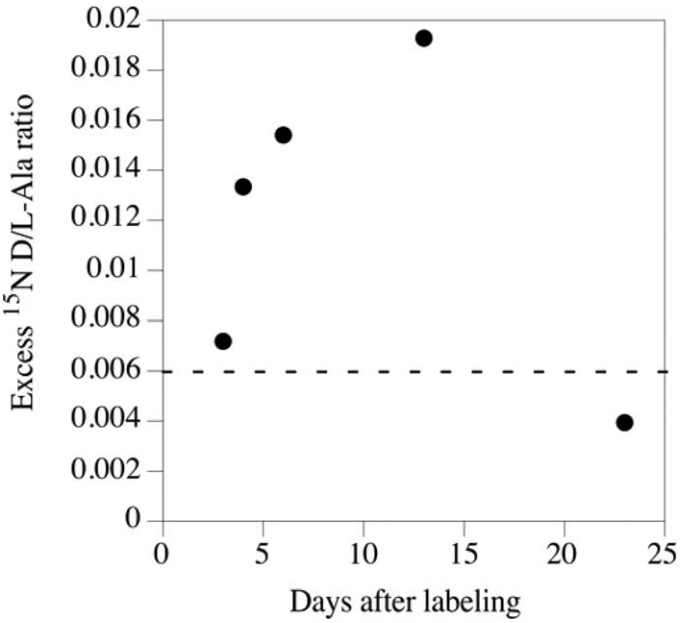

Fig. 1. Excess ^{15}N D/L-Ala ratios (not corrected for racemization). The racemization background is shown by the dashed line.

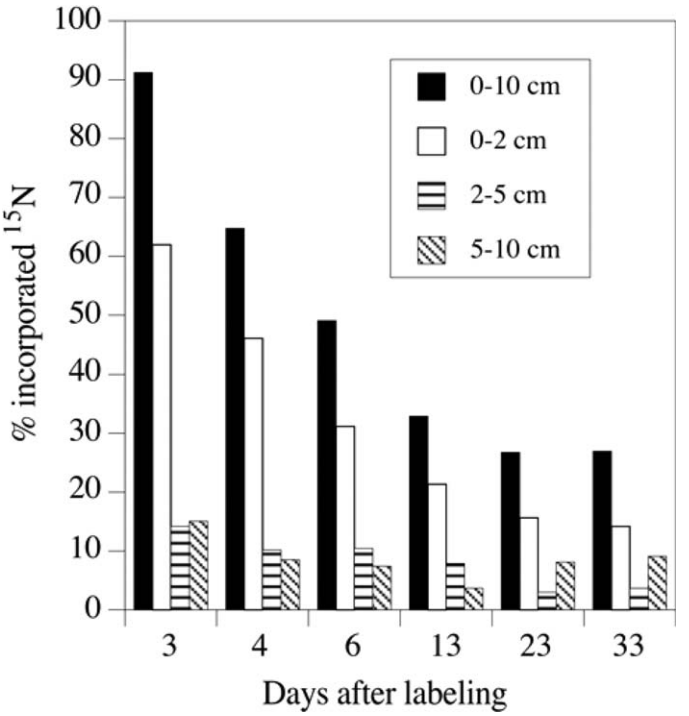

Fig. 2. Excess ^{15}N incorporation in total sediment organic nitrogen at sediment depths of 0–10 cm, 0–2 cm, 2–5 cm, and 5–10 cm throughout the study period as a percentage of the ^{15}N initially incorporated into sediment organic nitrogen (mean ± standard error).

28 μmol ^{15}N m^{-2} h^{-1}, with $\sim$ 0.04% of sediment ON replaced with ^{15}N.

Fixed ^{15}N had already been transported to deeper sediments (2–5 cm and 5–10 cm deep) by the first sampling time (Fig. 2). Within 60 h (day 3) of label addition 32.1% of the ^{15}N initially incorporated into sediment organic nitrogen was below 2 cm and 16.5% was between 5 cm and 10 cm. This gives transport rates of 3.0 μmol ^{15}N m^{-2} h^{-1} to below 2 cm and 1.5 μmol ^{15}N m^{-2} h^{-1} below 5 cm. Over the 33 d there was always significantly more excess ^{15}N measured in sediment ON at 0–2 cm than at 2–5 cm, and 5–10 cm (three-way ANOVA: $F_{2,\ 108} = 11.655$, $p < 0.001$; Fig. 2). Tukey tests showed that, at all times, there was similar excess ^{15}N in 2–5 cm and 5–10 cm sediments.

The sediment compartments considered (MPB, bacteria, and the three species of foraminifera) accounted for approximately 27.5% of the ^{15}N within sediment ON at 0–2 cm across all sampling times, but ranged from 50.8% on day 3 to 12.0% on day 23 (note: there is no MPB and bacteria data below 2 cm or for day 33) (Fig. 3). Most of the ^{15}N for these three compartments was in the MPB across all sampling times (average 73.6%). The contribution of MPB to the ^{15}N in sediment ON (63.9%) far outweighed the contribution of MPB biomass to sediment ON (18.1%; Table 1). Throughout the study, there was a marked decrease in the ^{15}N content of MPB in the 0-2 cm sediment. By day 23 post label addition, only 12.7% of the ^{15}N initially fixed into sediment ON remained in the MPB (Fig. 3); a rapid decrease from the first sampling of 49.5%. The contribution of bacteria to the ^{15}N in ON at 0-2 cm averaged 8.6%, and reached a maximum of 27.3% at day 6, which approaches the contribution of bacterial biomass to ON (39.4%; Table 1). The ^{15}N content of bacteria in 0–2 cm sediments increased up to day 6 post-labeling, then rapidly decreased (Fig. 3).

There was evidence of label uptake into all three foraminifera species by the time the first sediment sample was taken (Fig. 4). The greatest incorporation of ^{15}N was by *Ammonia beccarii*, which dominated the foraminifera. The greatest uptake of ^{15}N by *A. beccarii* was in sediment at 0–2 cm, but this represented only 0.24% of the total ^{15}N initially fixed into sediments. Uptake of ^{15}N by *Elphidium craticulatum* and *Elphidium advenum* accounted for less than 0.1% of the ^{15}N initially incorporated into sediment ON. Foraminifera in the deeper layers (2–5 cm and 5–10 cm) assimilated little (< 0.01%) of the total ^{15}N initially fixed into sediment ON (Fig. 4). The contribution of foraminifera to the ^{15}N that remained in 0–2 cm sediment at the end of the study (day 33) (2.67%) was more than double their biomass contribution (1.29%; Table 1), and 70% of this ^{15}N was in *Elphidium advenum*, equating to five times its biomass contribution.

Loss of ^{15}N from sediments

The decline in ^{15}N content of sediments at all depths throughout the study period was substantial (Fig. 2), with most of the decrease occurring at 0–2 cm. Most of this ^{15}N was lost from the sediments to the water column as NH_4^+, NO_3^- and N_2 fluxes (Figs. 5, 6). Initially, ^{15}N loss to the water

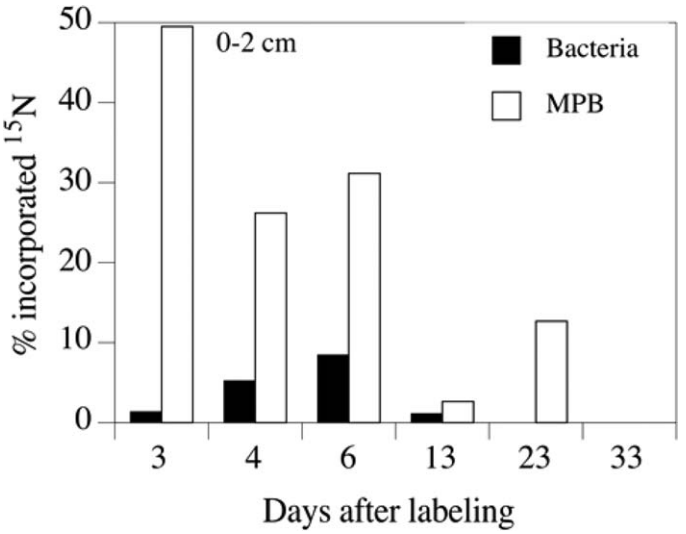

Fig. 3. Excess ^{15}N incorporation in microphytobenthos and bacteria at sediment depths of 0–2 cm throughout the study period as a percentage of the ^{15}N initially incorporated into sediment organic nitrogen ($n = 1$). There is no data for microphytobenthos and bacteria on day 33, or for 2–5 cm and 5–10 cm. ^{15}N incorporation into bacteria was based on a D/L-alanine ratio of 0.05. However, it is possible that this ratio may be as high as 0.1, which would effectively halve the estimated contribution of bacteria to ^{15}N uptake.

column was dominated by NH_4^+, accounting for 7.4% of the initially incorporated label loss by day 3, compared to only 1.33% loss via NO_3^- and N_2 combined. However, by day 33 very similar amounts had been lost via all three fluxes ($NH_4^+ = 20.8\%$; $NO_3^- = 16.5\%$; $N_2 = 20.7\%$) with an overall loss of 58% by the measured fluxes. At the end of the study (day 33) only 27.0% of the initially incorporated ^{15}N label remained. Of the remaining ^{15}N, 52.6% was in sediment at 0–2 cm, 13.7% was at 2–5 cm, and 33.7% was at 5–10 cm (Fig. 2). By day 33 15.1% of the initially incorporated ^{15}N label could not be accounted for by the fluxes and assimilation compartments considered (Fig. 6). Although not statistically significant, the mean loss of ^{15}N via sediment-water fluxes was generally greater in the dark for NH_4^+ and NO_3^-, and greater in the light for N_2.

Discussion

MPB act as an important N sink in coastal systems by assimilating dissolved inorganic and organic N directly from the water column and sediment pore waters, e.g., (Sundbäck and Graneli 1988; Ferguson et al. 2004; McGlathery et al. 2007). However, little is known about the fate of this N once it has been assimilated by MPB. We used an in situ ^{15}N pulse-chase experiment in subtidal sediments to follow the assimilation, transfer, transformation, and flux pathways of MPB-N. Our in situ experimental approach, in contrast to a laboratory study, allowed the fate of MBP-N to be studied under natural

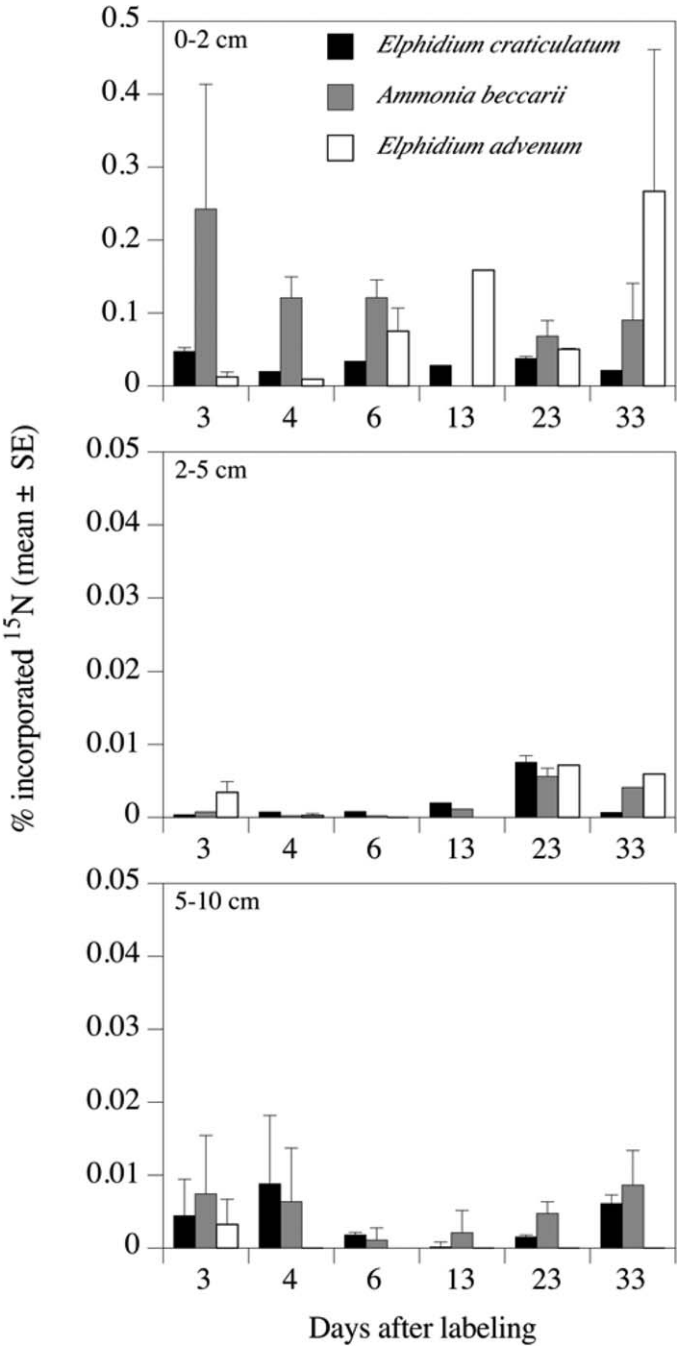

Fig. 4. Excess ^{15}N incorporation in foraminifera (*Elphidium craticulatum*, *Ammonia beccarii*, and *Elphidium advenum*) at sediment depths of 0–2 cm, 2–5 cm, and 5–10 cm throughout the study period as a percentage of the ^{15}N initially incorporated into sediment organic nitrogen (mean ± standard error). Note some bars and error bars are too small to be seen. Also note the order of magnitude scale change on the bottom two panels.

conditions of light, physical mixing and resuspension, bioturbation, and grazing. Thirty-three days after the ^{15}N pulse was assimilated by MPB 27% remained in the sediment, 16.5% had been effluxed as NO_3^-, 20.8% had been effluxed as NH_4^+,

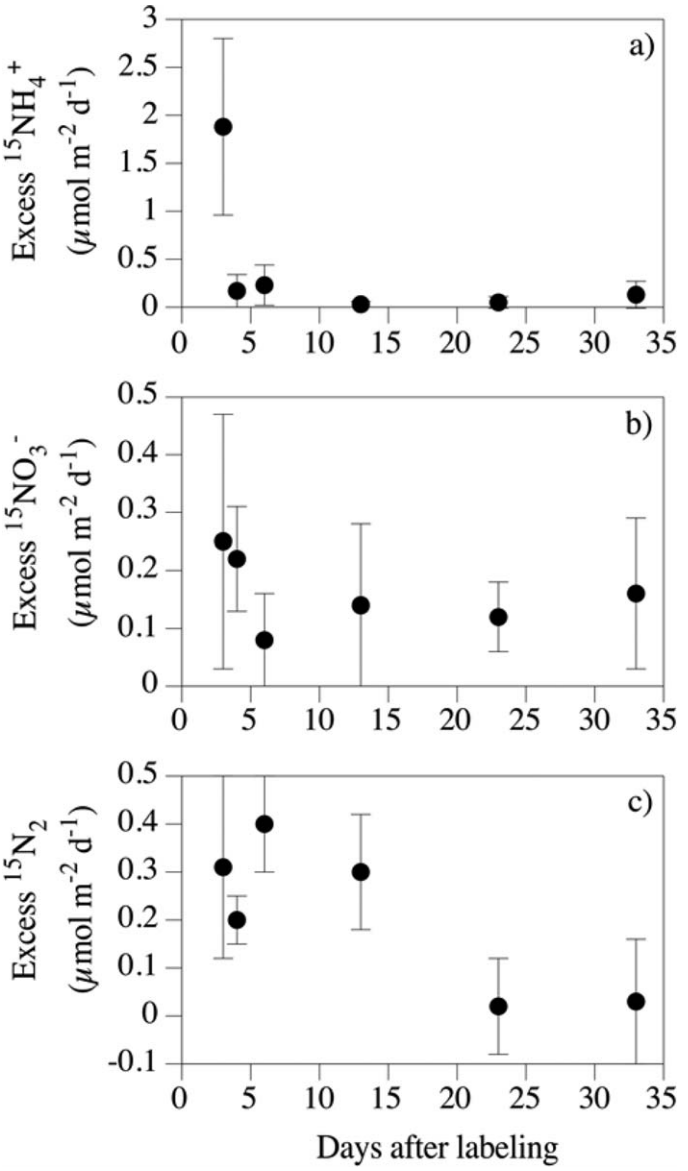

Fig. 5. Excess ^{15}N lost as net fluxes of **(a)** NH_4^+, **(b)** NO_3^-, and **(c)** N_2 across the sediment-water interface at each sample time (mean ± standard error).

20.7% had been effluxed as N_2 and 15.1% was missing (Fig. 7). Of the ^{15}N remaining in 0–2 cm sediment on day 23 80.4% was in MPB, 2.7% was in bacteria, 1% was in foraminifera and the remaining 15.9% was uncharacterized (Fig. 7). The % ^{15}N remaining in 0–2 cm sediment that was in foraminifera increased to 2.67% by day 33.

^{15}N incorporation and transfer within sediments

The quantity of ^{15}N incorporated into sediment ON by the end of the 24 h in situ chamber incubation equated to an assimilation rate of 0.66 mmol ^{15}N m^{-2}. This was for a CO_2 productivity rate of ~8.75 mmol C m^{-2} h^{-1} and 86.3% ^{15}N-labeling of the DIN pool (7.9 μmol N L^{-1}). A laboratory ^{15}N labeling study of MPB on physically similar subtidal quartz sediments found an assimilation rate of 0.91 mmol ^{15}N m^{-2} (average of light and dark) for an O_2 productivity rate of 1.68 mmol m^{-2} h^{-1} and 95% labeling of the DIN pool (< 5.0 μmol N L^{-1}) (Evrard et al. 2008). Despite a higher ^{15}N assimilation rate for a lower rate of benthic productivity, which probably reflects differences between in situ and laboratory studies or differential utilization of porewater vs. water-column N, both studies had a similar C : N ratio of incorporation. For example, using the C incorporation data of Oakes et al. (2012) and accounting for differences in the labeling of the DIC (23%) and DIN (86.3%) pools, this study had a ratio of incorporation of 10.2 compared to 12.6 in Evrard et al. (2008). The C:N ratio of incorporation was higher than required for microbial growth and is consistent with the production of carbon-rich extracellular organic material such as carbohydrate (Evrard et al. 2008), which accounted for 30% of the C assimilated by MPB over 60 h (Oakes et al. 2010b). The C:N ratio of incorporation for MPB was 4.8, which is similar to Redfield. In contrast, bacteria had a low C : N ratio of incorporation of 3.0 reflecting preferential uptake of ^{15}N. N-limitation of the microbial decomposition of high C : N organic matter, such as extracellular organic material, results in the uptake and accumulation of N by bacteria (Tupas and Koike 1991; Van Duyl et al. 1993; Lomstein et al. 1998) and may be more important in benthic tropical systems than benthic temperate systems (Eyre and Ferguson 2005). This extracellular organic material was probably also the source of much of the uncharacterized material in the sediment, and with a ^{13}C: ^{15}N ratio of incorporation of 63.2 is within the range of previously reported values (Cook et al. 2009).

The high percentage of incorporated ^{15}N (40%) that was found within MPB at the first sampling period suggests that MPB in the current study dominated the initial uptake of ^{15}N from the added KNO_3. In comparison, Veuger et al. (2007a) reported that the bacterial community dominated direct uptake of ^{15}N added to muddy subsurface sediments (porewaters) as NH_4^+. The marked difference in ^{15}N uptake pathways between the two studies may reflect differences in processing of the ^{15}N-labeled NH_4^+ compared to NO_3^-, or the method of addition (subsurface vs surface, see Hardison et al. (2011). MPB are concentrated in surface sediments and in the current study are therefore likely to have had greater access to ^{15}N, which was added to the overlying water. Differences in sediment characteristics such as grain size, organic matter content, and/or the biomass and composition of the microbial community may also have affected ^{15}N uptake. For example, the D/L-alanine ratio of sediment at the site of the current study was 0.031 ± 0.004 compared to 0.071 ± 0.005 at the site of the study by Veuger et al. (2007a) indicating either a lower relative abundance of bacteria and/or a dominance of Gram negative bacteria at the site of the current study.

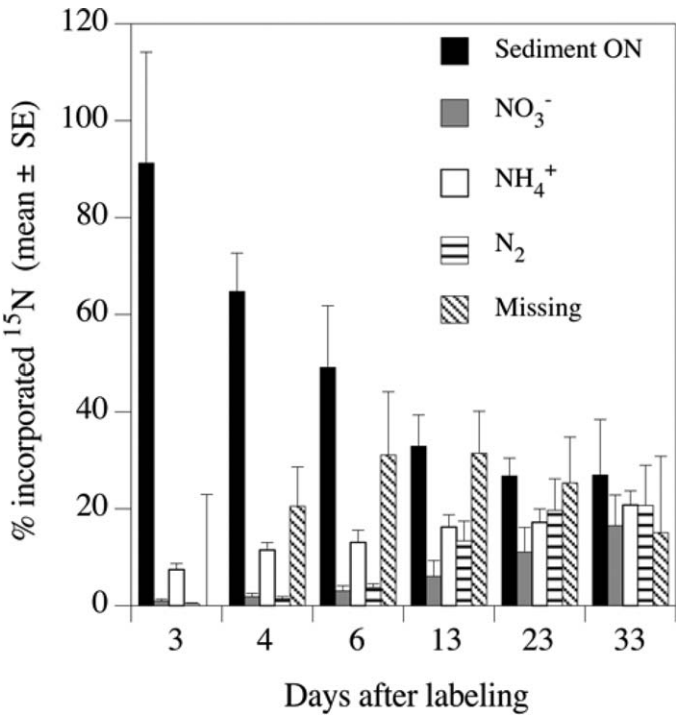

Fig. 6. Nitrogen budget showing excess ^{15}N within sediment organic nitrogen at 0–10 cm at each sampling time, and the cumulative excess ^{15}N lost via net fluxes of NH_4^+, NO_3^-, and N_2, and cumulative missing N, from the end of ^{15}N-labeling until each sampling time, as a percentage of the ^{15}N initially incorporated into sediment organic nitrogen (mean ± standard error).

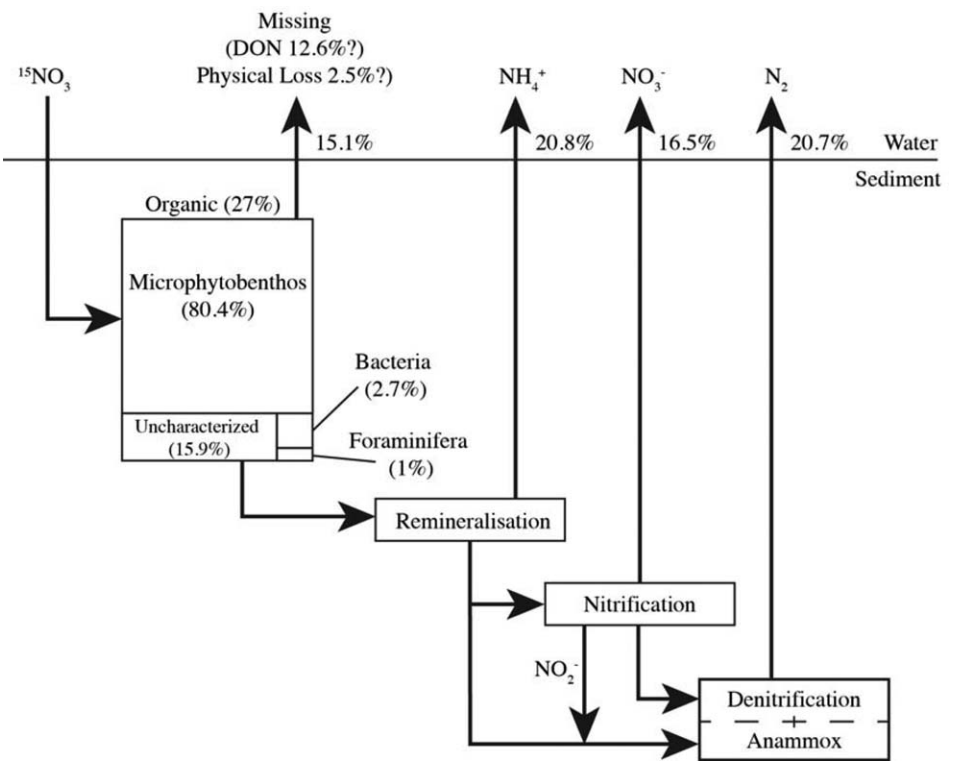

Fig. 7. Conceptual model of the net transfer, transformation and fate of microphytobenthos nitrogen 33 d after assimilation. Note: assimilation in the organic pool (microphytobenthos, bacteria, fauna and uncharacterized) is for 0–2 cm sediment 23 d after assimilation.

There are no other in situ MPB labeling studies with which to compare that have measured the downward transfer of ^{15}N within sediments. However, the downward transfer of N can be compared to the downward transfer of C (Oakes et al. 2012). The ratio of excess ^{13}C: ^{15}N that was in sediment organic matter 60 h (3 d) after label application increased with depth (9.16 : 1 at 0–2 cm; 10.19 : 1 at 2–5 cm; 13.75 : 1 at 5–10 cm). This shows either preferential transfer of C relative to N or lower transfer of N relative to C. N may be preferentially lost via NO_3^-, NH_4^+, and N_2 fluxes. Over the 33 d study period, there was sustained preferential loss of N relative to C from the sediment at 2–5 cm and 5–10 cm, as shown by the increasing sediment excess ^{13}C: ^{15}N ratios. In contrast, there was a decrease in excess ^{13}C: ^{15}N ratios at 0–2 cm from day 3 to 23, which may reflect the capture and assimilation of mineralized N from deeper sediments by MPB at the surface. Consistent with this are the decreasing ^{13}C: ^{15}N MPB ratios. Similar to carbon, the downward transfer of N may be due to MPB and/or heterotroph migration (i.e., migration by bacteria and/or meiobenthos) (Oakes et al. 2012). The low assimilation of ^{15}N by foraminifera in the deeper layers (2–5 cm and 5–10 cm) suggests that foraminifera probably only played a minor role in the downward transport of N. The considerable ^{15}N content of the 2–5 cm and 5–10 cm sediments probably mostly reflects the active downward migration of MPB within sands (Underwood 2002; Saburova and Polikarpov 2003) or coarser grain size. Greater light availability at low latitudes may enhance the ability of MPB in subtropical sands to persist at greater sediment depths (Oakes et al. 2012). MPB may also move to deeper, aphotic layers of sandy sediments to access nutrients (Saburova and Polikarpov 2003), although the surface enrichment with $^{15}NO_3^-$ would not have encouraged downward movement. Regardless of the mechanism involved, the rapid downward transport of N observed in this study has implications for the ultimate fate of N fixed by MPB. Labile ON will be respired, but the depth at which this occurs, and the pathways leading to its respiration, or ultimate long-term burial, will depend on mixing regimes.

There was excess ^{15}N in MPB, bacteria and foraminifera throughout the study period, despite their expected high turnover rates, reflecting recycling of MPB-N over the study period. The excess ^{15}N content of MPB declined over the study duration, as ^{15}N was transferred to other compartments and lost from the sediment. In contrast, the ^{15}N content of bacteria increased until at least 6 d after addition. This suggests that, rather than direct uptake of NO_3^-, bacteria source their N via remineralisation of MPB-derived organic matter and/or MPB exudates, resulting in a lag in ^{15}N uptake compared to MPB. The excess ^{15}N content of bacteria declined later in the study, as the excess ^{15}N content of MPB, and sediment organic matter in general, decreased. The decline in ^{15}N-labeled substrates for denitrifying bacteria, specifically, are also reflected in the similarity of patterns for excess ^{15}N in bacteria and excess ^{15}N loss via N_2 fluxes. The contribution of bacteria to ^{15}N uptake by the microbial community (bacteria and MPB) was relatively low throughout the current study, reaching a maximum of 30.2% at 13 d after label addition. This compares to 50–100% bacterial contribution to uptake of ^{15}N from NH_4^+ added to subsurface (porewater) intertidal muds (Veuger et al. 2007a) and 23–58% contribution to uptake of ^{15}N from NO_3^- added to the surface of subtidal sands (Evrard et al. 2008) and suggests that the bacterial community may have had less of a requirement for the added N in the current study than in these previous studies. However, these three studies used very different methods making comparisons difficult, and the differences may simply be related to different sediment types and light availability.

^{15}N was assimilated, stored and recycled differently in the three species of foraminifera. This suggests that there may be partitioning of N resource use among these dominant species, arising either from species having different access to the ^{15}N label, due to occupying different sediment depths within the 0–2 cm layer that was analyzed, or utilizing different food sources (Oakes et al. 2010a). Initially, *Ammonia beccarii* were responsible for the greatest uptake of ^{15}N among the foraminifera, but their ^{15}N content decreased over time, matching the pattern of ^{15}N content observed for MPB and surface sediments. Previous research suggests that *A. beccarii* selectively feeds on pennate diatoms, but does not consume bacteria (Murray 2014). It is likely that *A. beccarii* in the current study fed upon MPB and therefore rapidly accessed the assimilated ^{15}N. In contrast to *A. beccarii*, the ^{15}N content of *Elphidium advenum* increased over the study duration, and the ^{15}N content of *E. craticulatum* remained low. Foraminifera can utilize a variety of food sources, including MPB, bacteria, detritus, small animals, and dissolved organic material (Murray 2006). *E. advenum* and *E. craticulatum* potentially have a lower reliance on MPB as a source of N, relying more on bacteria and/or detritus (uncharacterized ON), which acquired ^{15}N later in the study.

The fraction of initially fixed ^{15}N that was in the foraminifera at 0–10 cm at day 3 (0.3%) was less than their biomass N contribution (1.29%). In contrast, the contribution of foraminifera to the ^{15}N that remained in the sediment 0–2 cm at the end of the study (day 33) (2.67%) was more than double their biomass N contribution (1.29%); 70% of this ^{15}N was in *Elphidium advenum*, representing five times its biomass N contribution. There was also a much larger amount of ^{15}N accumulated in foraminifera than fauna in a previous labeling study in muds in the same estuary (0.4%) (Veuger et al. 2007a); there were no foraminifera at that site. As such, the foraminifera appear to accumulate and store ^{15}N. One mechanism may be the uptake and accumulation of NO_3^-, which occurs in a wide range of different foraminiferal groups (Piña-Ochoa et al. 2010), possibly including species within

the same Order as *Elphidium advenum* (Rotaliida) (Nomaki et al. 2014).

Loss of ^{15}N from sediments

Sediment-water fluxes of NH_4^+, NO_3^-, and N_2 were the major pathway (58%) for loss of MPB-N from the sands studied (Figs. 5-7). Although no statistical difference was detected between dark and light loss of ^{15}N via fluxes of NH_4^+, NO_3^-, and N_2, this probably resulted from the high error and variability associated with the excess ^{15}N flux measurements, combined with the relatively low replication. It is likely that the lower mean excess ^{15}N loss via NO_3^- and NH_4^+ in the light reflects capture of these nutrients by MPB at the sediment surface, which would contribute to N retention within the sediment. This would, however, be partially offset by the increased loss of ^{15}N via N_2 fluxes in the light, possibly reflecting enhanced coupled nitrification-denitrification in the light (Risgaard-Petersen et al. 1994).

Most (85%) of the MPB-N that was lost from the sediment over the first three days was removed via NH_4^+ fluxes, most likely due to rapid mineralization of the freshly labeled MPB at the surface. However, over time nitrification (loss via NO_3^- fluxes) and coupled nitrification-denitrification and anammox (loss via N_2 fluxes) likely became more important. This may reflect the location of ^{15}N-labeled MPB in the sediment column. Later in the study, buried MPB would be mineralized, and the ^{15}N-labeled NH_4^+ produced would move upwards to the sub-oxic zone where coupled nitrification-denitrification can occur. MPB are typically thought to reduce nitrification and denitrification due to competition for available N between MPB on the one hand and nitrifiers and heterotrophic bacteria on the other (Risgaard-Petersen 2003). However, this study suggests that over longer time scales (33 d) MPB may enhance the loss of N via coupled nitrification-denitrification by assimilating inorganic N from the water column, which is later recycled.

This pathway may be of particular importance where there is little direct denitrification of water-column NO_3^- due to low concentrations. For example, a ^{15}N labeling study of benthic biofilms in a river found little loss via N_2 fluxes over 24 d ($< 0.1\%$) due to the high water-column NO_3^- concentrations (110 μmol N L^{-1}) (O'Brien et al. 2012). Similarly, $< 0.02\%$ of the incorporated ^{15}N was lost via denitrification over 31 d in a freshwater marsh with very high NO_3^- concentrations (450 μmol N L^{-1}) (Gribsholt et al. 2005). In contrast, water-column NO_3^- concentrations were low in this study (3.5 μmol N L^{-1}), and there was a large loss of ^{15}N as N_2 (20.7%) via coupled nitrification-denitrification over the 33 d. This control of water-column NO_3^- concentrations on the source of NO_3^- for denitrification has been seen previously in this system (Eyre and Ferguson 2005) and many other systems (Seitzinger et al. 2006). By the end of the study (33 d) all three sediment-water flux pathways were of similar importance for the loss of MPB-N.

At the end of the study 15.1% of the initially incorporated ^{15}N label could not be accounted for by the assimilation compartments and fluxes considered. The missing ^{15}N could have been removed via resuspension, grazing, burial below 10 cm, or as dissolved organic N (DON) fluxes to the water column. A carbon mass balance for the same experimental study found that only about 3% of the ^{13}C could not be accounted for at the end of the study, most likely lost to resuspension, grazing and burial below 10 cm (Oakes et al. 2012). These three processes would account for a similar loss of ^{15}N (2.5%) using the average ^{13}C:^{15}N sediment ratio over this study period. As such, the remaining missing 12.6% of the initially incorporated ^{15}N label was most likely lost as DON. Consistent with a loss of DON was the small loss of DOC from the same experimental study (Oakes et al. 2012), although the $DO^{13}C$:$DO^{15}N$ ratio would have to be low (0.6 : 1.0) to account for all (12.6%) the missing ^{15}N. Similarly low benthic DOC:DON flux ratios have been measured in coastal sediments (Apostolaki et al. 2010; Eyre et al. 2011), and probably represent the preferential release of high-molecular-weight, low C : N compounds during early diagenesis (Burdige and Zheng 1998; Eyre and Ferguson 2005). MPB would provide a source of fresh organic matter, with high MPB biomass associated with high benthic DON fluxes (Cook et al. 2004; Ferguson and Eyre 2010), and some of the DON may also have been released during grazing of MPB or viral lysis (Eyre and Ferguson 2002; Brussaard 2004).

Implications—attenuation and retention of N by MPB

Assimilation of dissolved inorganic and organic N directly from the water column and sediment pore waters by MPB is an important N sink in coastal systems (Sundbäck and Graneli 1988; Rizzo 1990; Ferguson et al. 2004; McGlathery et al. 2007). The assimilated N is typically considered to be retained in the MPB biomass, unless passed up the trophic food chain (Cabrita and Brotas 2000; Sundbäck et al. 2000; Sundbäck and Miles 2000). For example in the Indian River-Rehoboth Bay it was suggested that nutrients assimilated by MPB from the water column in late winter and spring are retained until summer when they are released (Cerco and Seitzinger 1997). Similarly, its been suggested that N assimilated by BMA may be retained during time of favourable phytoplankton blooms (Hardison et al. 2011). However, this study has shown that there is little benthic trophic transfer, with most of the MPB-assimilated N remineralized over 33 d and effluxed as NH_4^+ (20.8%), NO_3^- (16.5%), and DON (12.6%), which is then available for pelagic foodwebs. The remaining MPB-assimilated N is remineralized and lost as N_2 (20.7%), with only 27.0% of the initially incorporated ^{15}N label remaining at the end of the study (day 33).

Long-term ^{15}N labeling studies of freshwater and marine marshes have shown the highest losses during the first month (De Laune et al. 1983; White and Howes 1994; Gribsholt et al. 2009), although our losses were much higher. For

example, after one month 76% of the added ^{15}N remained in a salt marsh system (White and Howes 1994), 66% remained in a freshwater constructed wetland (Erler et al. 2010) and 50% remained in a tidal freshwater system (Gribsholt et al. 2009). All these studies with higher retention showed rapid transfer of ^{15}N into plants, which enhances the short-term retention. In contrast, in our study most of the ^{15}N was in MPB, which were rapidly remineralized over the 33 d with a large loss via N fluxes across the sediment-water interface. However, despite the low retention compared to sediment-plant systems the uptake of N by MPB and associated transfer to deeper sediment is still a mechanism that would enhance the burial of N.

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