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A shift in the pool of retained microphytobenthos nitrogen under enhanced nutrient availability

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ABSTRACT

Sediment microbial communities are an important sink for both organic and inorganic nitrogen (N), with microphytobenthos (MPB) biomass making the largest contribution to short-term N-assimilation and retention. Coastal waters are increasingly subject to anthropogenic nutrient enrichment, but the effect of nutrient enrichment on microbial assimilation, processing, and fate of MPB-derived N (MPB-N) remains poorly characterised. In this study, an MPB-dominated microbial community was labeled in situ with a pulse of $^{15}NH_4^+$-N. Laboratory core incubations of this labeled sediment under increasing nutrient concentrations (NH_4^+ and PO_4^{3-}: ambient, 2 × ambient, 5 × ambient, and 10 × ambient) were used to investigate changes in the processing and flux pathways of the ^{15}N-labeled MPB-N across 10.5 d under nutrient enrichment. Short-term retention of MPB-N by MPB was stimulated by nutrient addition, with higher ^{15}N in MPB in the nutrient amended treatments (71–93%) than in the ambient treatment (38%) at 0.5 d After 10.5 d, the nutrient amended treatments had increased turnover of MPB-N out of MPB biomass into an uncharacterised pool of sediment ON (45–75%). Increased turnover of MPB-N likely resulted from decreased recycling of MPB-N between MPB and heterotrophic bacteria as inorganic nutrients were preferentially used as an N source and remineralisation of sediment ON decreased. Decreased breakdown of sediment ON reduced the efflux of MPB-N via DON in the amended (3.9–5.2%) versus the ambient treatment (10.9%). Exports of MPB-N to the water column were relatively small, accounting for a maximum of 14% of ^{15}N exported from the sediment, and were predominantly exported DON and N_2 (denitrification). Overall, there was considerable retention of MPB-N over 10.5 d, but increased nutrient loading shifted N from MPB biomass into other sediment ON.

1. Introduction

Estuaries have a considerable role in altering and processing terrestrial and riverine derived organic matter prior to export to the coastal oceans. Within shallow photic coastal settings, much of the organic matter processing is mediated within the sediments by the microbial community (Bauer et al., 2013). In benthic sediments, microphytobenthos (MPB) contribute significantly to primary production and biomass by fixing carbon (C) and utilizing nitrogen (N) from the water column and porewater (Cook et al., 2004a; Dalsgaard, 2003; McGlathery et al., 2007). Coupling between MPB production and bacterial remineralisation within these sediments can result in strong retention of N and recycling of C. Heterotrophic bacteria utilize the extracellular polymeric substances (EPS) produced by MPB, while MPB rely on inorganic sources or bacterially-excreted NH_4^+ from remineralisation of organic matter (Cook et al., 2007; Forehead et al., 2013). The fate and processing of MPB-derived C (MPB-C) within sediments has been well described, with studies quantifying the incorporation of MPB-C into sediment organic matter and its loss from the sediment via efflux of primarily dissolved inorganic carbon (DIC) with some dissolved organic carbon (DOC) (Middelburg and Nieuwenhuize, 2001; Oakes et al., 2012; Oakes and Eyre, 2014, 2016).

In contrast to MPB-C, the processing and fate of MPB-derived N (MPB-N) in coastal sediments is poorly understood. MPB-N is expected to be processed along the same pathways as MPB-C, but transfer of N will proceed at different rates according to the biological needs of the microbial community and stoichiometric limitation in comparison to C (Eyre et al., 2016). Two in situ studies have quantified MPB-N processing and loss of N via effluxes of DIN, DON and N_2 during tidal inundation and observed

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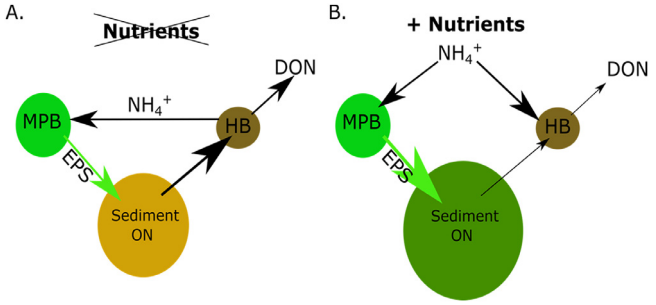

Fig. 1. Conceptual diagram of the hypothesised mechanism and change in relationship between microphytobenthos (MPB) and heterotrophic bacteria (HB) under A) nutrient limiting and B) nutrient replete settings. A) When nutrients are scarce there is strong recycling between bacterial remineralisation of MPB-N within sediment ON and competition for nutrients between MPB and HB. Increased remineralisation of MPB-N within sediment ON results in increased turnover of MPB-N between MPB, bacteria, and uncharacterised ON and increased export of MPB-N as DON from remineralisation. B) When inorganic nutrients are available, bacterial remineralisation decreases as inorganic nutrients are preferentially used over sediment ON resulting in decreased competition between MPB and HB for nutrients from remineralisation. Reduced HB remineralisation of ON results in decreased production of DON and the buildup of MPB-N contained in sediment ON.

different outcomes for the fate of the initially incorporated ^{15}N over ~30 d (Eyre et al., 2016; Oakes et al., 2020). Subtidal losses due to denitrification were considerably higher than intertidal losses as processing of MPB-N provided substrates for denitrifying bacteria (Eyre et al., 2016), while intense competition from MPB for N resulted in considerable retention of MPB-N within intertidal sediments (Oakes et al., 2020). In both studies, MPB dominated the uptake of ^{15}N initially, but only remained the dominant reservoir for MPB-N within sediment organic matter in subtidal sediments. In intertidal sediments, MPB and uncharacterised material (^{15}N contained in sediment ON that is not incorporated into microbial biomass) contained comparable amounts of MPB-N after 31 days, due to increased turnover of MPB-N into uncharacterised sediment organic matter. Increased turnover into the uncharacterised pool between these two studies may indicate quicker turnover of newly assimilated MPB-N into sediment ON in settings where MPB outcompete ammonia-oxidizing bacteria for N substrates (Risgaard-Petersen et al., 2004).

Coastal waters are increasingly subject to anthropogenic nutrient enrichment (Howarth and Marino, 2006) which alters biogeochemical pathways and can cause eutrophication within otherwise healthy estuaries (Howarth and Marino, 2006; Rabalais et al., 2009). Within intertidal sediments, increased availability of inorganic nutrients from anthropogenic sources is expected to alter the processing of MPB-N as the microbial community responds to an additional source of N that was previously in limited supply (Cook et al., 2007). Efflux of N as DIN or N_2 occurs as the net result of mineralisation and competition between MPB, heterotrophic bacteria, and ammonia-oxidizing bacteria for uptake of available NH_4^+. In N-limited settings, strong competition for nutrients arising from bacterial remineralisation of sediment ON results in strong retention of remineralised MPB-N within sediment organic matter (OM) compartments (MPB, bacteria, and uncharacterised) (Fig. 1A) and little N is available for export via DIN or the coupled nitrification-denitrification pathways (Cook et al., 2004b, 2007; McGlathery et al., 2007; Sundback et al., 2000). With additional nutrient inputs increased MPB productivity has been observed, coinciding with decreasing efflux of DIN and N_2 to the water column (Dalsgaard, 2003; Ferguson and Eyre, 2013; Ferguson et al., 2004). Few studies have examined how increased N availability in the water column alters the processing and fate of MPB-N within shallow photic coastal settings. A laboratory study partitioned the algal and bacterial contribution to uptake of ^{15}N within the sediment microbial community through the use a biomarker technique (D/L-Alanine) and found that the addition of nutrients (NH_4^+, $Si(OH)_4$, HPO_4^-) increased MPB incorporation of ^{15}N and decreased competition for inorganic nutrients between MPB and bacteria (Cook et al., 2007); Fig. 1B). However, Cook et al. (2007) did not quantify water column efflux of DIN, DON or N_2.

In this ^{15}N pulse-chase study we aimed to quantify changes in the processing and fate of MPB-N within subtropical intertidal sediments due to increased water column nutrient availability. Pathways considered included transfer of MPB-N through sediment ON compartments (MPB, bacteria and uncharacterised), and export via efflux of NH_4^+, DON, and N_2 to the overlying water column. We expected decreased remineralisation, re-capture, and recycling of processed MPB-N through the sediment compartments as introduced inorganic nutrients were preferentially utilised by the microbial community. Utilization of inorganic nutrients by the microbial community could result in either increased retention of MPB-N within the uncharacterised sediment organic matter pool due to decreased utilization of MPB-N by both MPB and bacteria, or increased MPB-N export as MPB-N is effluxed to the water column as DON without being re-captured and remineralised. The resulting decreased remineralisation of MPB-N within the microbial community should decrease the export of ^{15}N via efflux of NH_4^+, DON, and N_2 and result in increased sediment retention of MPB-N within N replete treatments (Fig. 1B).

2. Methods

2.1. Study site

The study site was a subtropical intertidal shoal ~2 km upstream of the mouth of the Richmond River estuary in New South Wales, Australia (28°52′30″S, 153°33′26″E) that was simultaneously used for a ^{13}C pulse-chase study (Riekenberg et al., 2018). The 6900 km^2 Richmond River catchment has a mean annual rainfall of 1300 mm (McKee et al., 2000) and an average flow rate of 2200 ML d^{-1} (daily gauged flow adjusted for catchment area, averaged over years for which data was available; 1970–2013). Although the Richmond River estuary has highly variable flushing, salinity, and nutrient concentrations associated with frequent episodic rainfall events and flooding (Eyre, 1997; McKee et al., 2000), this study was undertaken during a period of average rainfall (~200 mm in the month prior). The site experiences semidiurnal tides with a range of ~2 m. Samples were collected in January 2015 (summer) with average site water temperature of 25.6 ± 2.3 °C.

2.2. Experimental overview

An in situ application of ^{15}N (99% NH_4^+) was used to introduce a pulse of ^{15}N-labeled MPB-N into sediment. Unincorporated ^{15}N was flushed from the sediment during the next tidal inundation of the site. Sediment cores were then collected and incubated in the laboratory under four nutrient enrichment scenarios (ambient, minimal, moderate, and elevated) using pulsed nutrient additions. In situ label application and uptake into the microbial community followed with laboratory incubation of cores allowed for explicit control of nutrient additions and their effect on the fate and processing of the pulse of MPB-N over 10.5 d This time period was chosen based on previous work that has indicated that the majority of microbial processing of MPB derived C and N occurs within 10 to 15 d of initial uptake within benthic sediment under photic conditions (Eyre et al., 2016; Oakes et al., 2020). Sediments remained inundated during incubation to minimize loss of MPB-N through physical processes as we were primarily interested

in biological sediment processing and previous studies have shown that physical loss processes can have an important seasonal role in the processing of MPB-N (Nielsen et al., 2017).

2.3. ^{15}N labeling

An experimental plot (2 m^2) of bare sediment free of large animal burrows was labeled with ^{15}N (99% $^{15}NH_4^+$) when sediments were initially exposed during the ebbing tide in the middle of the day. To ensure even application, the plot was divided into 400 cm^2 subplots and a 20 ml aliquot of 40 µmol $^{15}NH_4^+$ solution was sprayed onto each subplot using motorized sprayers. This gave a label application rate of 2 mmol $^{15}NH_4^+$ m^{-2}. The labeled solution was prepared with NaCl amended Milli-Q to match site salinity (34.6). Assimilation of ^{15}N by the sediment community occurred during the 4 h prior to tidal inundation. Tidal inundation and flushing then removed unincorporated ^{15}N predominately through dilution and physical removal (^{15}N remaining in the sediment that was not taken up by the microbial community or adsorbed to sediment grains); as confirmed by measurement of 90.3% removal of ^{15}N from the initial application (in 0–10 cm) from non-KCl extracted sediment ON between treatment application and core removal.

2.4. Sample collection

Prior to application of ^{15}N, 3 cores (9 cm diameter, 20 cm depth) were collected immediately adjacent to the treatment plot and were immediately extruded and sectioned (0–2 cm, 2–5 cm, and 5–10 cm) to provide unlabeled control samples for sediment ON $\delta^{15}N$. At the next low tide (11 h after label application), 43 sediment cores were collected from the labeled plot using clear acrylic core liners (9 cm diameter, 47 cm height). Immediately, three cores were extruded and sectioned, as described above. The sediment samples were placed into plastic bags, transported to the laboratory on ice, stored frozen in the dark (−20 °C), and were later used to determine initial ^{15}N uptake, grain size distribution across sediment depths, and chlorophyll-α (Chl-a) concentration within 0–1 cm sediments. The 40 remaining core liners were sealed with acrylic bottom plates and transported to the laboratory for incubation within 2 h of sampling. Site water (400 L) was collected and transported to the laboratory for use during incubations.

2.5. Nutrient amendment

Sediment cores were incubated in the laboratory with a range of nutrient concentrations in the overlying water that were below the sediment capacity for uptake. Incubation tanks (85 L volume), each containing ten randomly assigned cores from the application plots, were established with nutrients at ambient concentration (site water: DIN of 2.5 ± 0.04 µmol N L^{-1}, TP 0.9 ± 0.09 µmol P L^{-1} measured on incoming tide), and with N (NH_4^+) and P (H_3PO_4) amendments to site water at 2 × (minimal treatment), 5 × (moderate treatment), and 10 × (elevated treatment) average water column concentrations near the study site (4 µmol L^{-1} NH_4^+ and 5 µmol L^{-1} PO_4^{3-}, Eyre (2000). Nutrient amendments were added to the incubation tanks and to bags of replacement water an hour prior to cores being transferred into treatment tanks for incubation. Two additional identical amendments of NH_4^+ were added to the incubation tanks at the respective treatment concentrations after sampling at 1.5 d and 3.5 d of incubation to maintain treatment applications of NH_4^+ (Supplementary Fig. 1). These additions were designed to maintain increased DIN availability to the microbial community without developing secondary limitation for P or Si that would limit uptake of DIN into the diatom-dominated MPB community. The lower than expected water column values for DIN observed throughout the experiment indicate that uptake of the NH_4^+ applications occurred quickly and NH_4^+ was readily removed from the water column by the benthic microbial community within 24 h of additions. Processing of NH_4^+ varied between treatments, likely as a result of the variability in the microbial community present in each core, but there was no significant accumulation of DIN within treatment tank waters. The equimolar application of nutrients was equivalent to 2.5 × the trigger concentration for increased trophic status under the Australian and New Zealand Environment Conservation Council guidelines (ANZECC, 2018). An addition of sodium metasilicate (Na_2SiO_3, 17 µmol Si L^{-1}) was also added to all incubation tanks at the end of the 2.5 d of incubation to prevent secondary limitation of Si.

2.6. Benthic flux incubations

In the laboratory, cores were fitted with magnetic stir bars positioned 10 cm above the sediment surface, filled with ~2 L of site water, and randomly allocated to one of the four treatment tanks (ambient, minimal, moderate, elevated; ten cores per treatment). Water within treatment tanks and cores was continuously recirculated, held at in situ temperature (25 ± 1 °C) using a temperature controller, and aerated. Cores were stirred at a rate below the threshold for sediment resuspension (Ferguson et al., 2003) via a rotating magnet at the center of each treatment tank, which interacted with the magnetic stir bars. Three sodium halide lamps suspended above the treatment tanks approximated the average light level measured at the sediment surface during inundation (941.4 ± 139 µE m^{-2} s^{-1}) by providing 824 ± 40 µE m^{-2} s^{-1} to the sediment/water interface within the cores on a 12 h light/12 h dark cycle. Cores were allowed to acclimate in the treatment tanks for 6 h prior to the start of incubation, allowing sediment microhabitats and any disturbed redox conditions to re-establish. Cores remained open to the tank water until 30 min before sampling when clear Plexiglas lids were fitted to each core liner to seal in overlying water within the core for the duration of the incubation (~16 h). On each sampling occasion, 8 cores (2 per treatment at 1.5, 2.5, 3.5 and 10.5 d) were sampled for dissolved oxygen (± 0.01 mg/L) and temperature (± 0.01 °C) using a Hach HQ40d multiparameter meter via a sampling port in the core lid.

For each sampling period, measurements were made at three time points (initial, dark end/light start, and light end) to allow dark, light, and net flux calculations. Initial samples were taken 30 min after closure of the lids, dark end/light start samples were taken after ~12 h incubation with no light, and light end samples were taken 3 h after illumination at the end of the dark sampling. At each initial, dark end/light start, and light end sampling duplicate samples for N_2:Ar analysis were collected by allowing water from suspended replacement water bags to gently displace sample water out of the core via tubing into 7 ml gastight glass-stoppered glass vials. These vials were allowed to overflow by 2–3 vol, killed with 20 µl of saturated $HgCl_2$ and stored submerged at ambient temperature. To determine NH_4^+, NO_3^-, and DON concentrations and $\delta^{15}N$ values, sample water was syringe-filtered (0.45 µm cellulose acetate) into 10 ml and 50 ml polyethylene vials, leaving a headspace, and stored frozen (−20 °C). To determine $\delta^{15}N$ values for N_2, sample water was filtered (0.45 µm cellulose acetate) into a 20 ml glass vial containing 500 µl of 2 M NaOH. These samples were sealed without headspace using a lid containing a teflon-coated septum and refrigerated for storage. After completion of the dark/light flux incubation, cores were sacrificed and sediment was extruded and sectioned (top scrape, 0.2–2 cm, 2–5 cm, and 5–10 cm depths). A subsample (1 cm^3) of sediment was taken for Chl-α analysis from the 0–1 cm depth prior to sectioning of the 0–2 cm layer using a spatula and placed in a 10 ml centrifuge vial containing 5 ml of 90% acetone. Sediment samples were placed in ziplock bags and stored in the dark at −20 °C until analysis.

2.7. Sample analysis

A portion of each sediment sample was freeze-dried and KCl-extracted (2 M KCl) for analysis of concentration and $\delta^{15}N$ of sediment ON. Both KCl-extracted and non-amended sediment samples were dried (60 °C) and weighed into tin capsules for analysis of $\delta^{15}N$ and %N using a Flash elemental analyzer coupled on-line to a Thermo Fisher Delta V Plus isotope ratio mass spectrometer (IRMS). Reproducibility of $\delta^{15}N$ values for samples with $\delta^{15}N$ enrichment <100‰ was ± 0.2‰. Precision decreased with enrichment beyond 100‰. Additional freeze-dried sediment was analyzed for D- and L- alanine concentration and $\delta^{15}N$ to determine the relative contribution to uptake of ^{15}N by both MPB and bacteria.

Concentrations for DON, NH_4^+, and NO_3^- were determined through colorimetric analysis on a four channel Flow Injection Analyser (FIA, Lachat QuickChem 8000) (Eyre et al., 2011; Eyre and Pont, 2003). DON was quantified via measurement of total nitrogen (TN) through flow injection analysis using persulfate digestion (Valderrama, 1981) and subsequent subtraction of DIN ($NH_4^+ + NO_3^- + NO_2^-$) concentrations (Lachat, 1994). Prior to analysis, vials for N_2 analysis had 4 ml of water displaced with a helium headspace and were held at ambient temperature. N_2 concentrations were analysed using N_2:Ar measured with a membrane inlet mass spectrometer with O_2 removal (Eyre et al., 2002). $\delta^{15}N_2$ values were determined using the He headspace in N_2 samples analyzed via gas chromatography isotope ratio mass spectrometry using a Thermo Trace Ultra Gas Chromatograph coupled to a Delta V Plus IRMS via a Thermo Conflo III interface. Detailed description of $\delta^{15}N$ determination for NH_4^+ and $NO_3^-$is included in Supplement 1 in Supplementary Materials.

The relative contribution of MPB and bacteria to ^{15}N uptake and transfer was determined through compound-specific analysis of D- and L- alanine following acid hydrolysis and extraction of total hydrolysable amino acids from 7 g of freeze-dried sediment following methods described in Riekenberg et al. (2017). Due to the laborious extraction process, concentrations and $\delta^{15}N$ values from single replicates from the 0.2–2, 2–5 and 5–10 cm depths per sampling time were determined via gas chromatography-combustion-isotope ratio mass spectrometry on a HP 6890 GC interfaced via a Thermo Conflo III interfaced with a Thermo Delta V Plus IRMS using the column and ramp schedule described in Eyre et al. (2016).

Detailed description of the calculations for sediment ON and microbial biomass for MPB and heterotrophic bacteria, calculations for excess fluxes for NH_4^+, DON and N_2, and the statistical analyses that support this analysis are included in Supplements 2–4 in Supplementary Materials.

3. Results

3.1. Sediment characteristics

Sediment at depths of 0–2 cm, 2–5 cm and 5–10 cm was dominated by fine sand (66%−73%) and sediment across 0–10 cm had an organic N content of 1.2 ± 0.1 mol N m^{-2}. Sediment molar C:N was lowest at 2–5 cm, but comparable across other depths (0–2 cm 17.2 ± 1.7, 2–5 cm 10.9 ± 0.5, 5–10 cm 16.2 ± 2.2). The MPB assemblage was dominated by pennate diatoms with few larger heterotrophs (>500 µm, foraminifera) and no cyanobacteria observed under light microscopy (1000 ×) as has been described previously for this site (Oakes and Eyre, 2014, Riekenberg et al. submitted); Riekenberg et al., 2017, 2018). Foraminifera were not considered in the current study, as they have previously been found to make a minimal contribution to uptake of MPB-N (~1%) in sediments in the adjacent Brunswick Estuary (Eyre et al., 2016).

Sediment Chl-α at 0.2–2 cm depth averaged 48.3 ± 2.9 mg m^{-2} (mean ± SE) across all cores collected in this study and was not affected by time or treatment (two-way ANOVA: $F_{3,20}$=0.8, $p = 0.5$). Sediment organic matter across the 0–10 cm depth had a molar C:N ratio of 18.1 ± 1.1 and contained 1.2 ± 0.1 mol N m^{-2}. %N of sediment was low and even across all sediment layers (0.02%). Within initial cores taken after labeling, but prior to incubation, MPB biomass had the greatest contribution to sediment ON (20%) in the 0–2 cm depth, with bacteria representing only 4% (Table 1). Due to uptake of ^{15}N being largely confined to ON in the uppermost sediment layer (0–2 cm) in the initial cores, deeper depths (2–5 cm and 5–10 cm) were not examined for uptake into MPB, bacteria, and uncharacterised sediment compartments. Within the ambient treatment across all samplings in this study (0.5, 1.5, 2.5, 3.5 and 10.5 d), MPB had the greatest contribution to ON in the uppermost layer (0–2 cm, 28.4%) and contributed less in deeper depths (2–5 cm, 10.7%; 5–10 cm, 6.4%, Table 1). Bacterial contribution to ON was less than MPB in the uppermost layer (0–2 cm, 17.4%) and decreased with depth (2–5 cm, 7.1%; 5–10 cm, 6.0%). Uncharacterised ON made the largest contribution to both initial (75%) and ambient (54–88%, Table 1) sediments within this study.

3.2. Uptake of ^{15}N

The pulse of ^{15}N was rapidly incorporated into sediment ON, with 193 ± 44.8 µmol ^{15}N m^{-2} detected in 0–2 cm of sediment 11 h after label application, when the first ^{15}N-labeled cores were collected after tidal flushing. At this time ^{15}N was largely confined to the top scrape (upper 2 mm of sediment) and 0–2 cm depths (84.8 mmol N m^{-2}, 43.8% and 85.4 mmol N m^{-2}, 44.1% of total incorporated ^{15}N, respectively, with a total N of 193.4 mmol N m^{-2}), but some downward transport of ^{15}N was evident in the 2–5 cm and 5–10 cm depths (10.2 mmol N m^{-2}, 5.3%; 13.1 mmol N m^{-2}, 6.8%, respectively). At 0–2 cm, ^{15}N uptake into the microbial community was dominated by MPB (MPB 53.8 mmol N m^{-2}, 83%; bacteria 11.1 mmol N m^{-2}, 17%, Table 1). Due to limited labeling uptake within the lower depths during the initial sampling, we did not run D/L-Ala-analysis for the microbial community in the 2–5 cm and 5–10 cm depths.

^{15}N was incorporated into deeper sediments (2–5 cm and 5–10 cm) within all treatments by the initial sampling time (Fig. 2). By 0.5 d, similar amounts of the assimilated MPB-N (8–16%) was in sediment below 2 cm (One-way ANOVA: $F_{3,7} = 5.3$, $p = 0.07$, ambient 12%; minimal 16%; moderate 8%; and elevated 10%). After 10.5 d there was significantly more ^{15}N contained in the 0–2 cm layer (TS + 0.2–2 cm) than the 2–5 cm or 5–10 cm layers across all treatments (two-way ANOVA: $F_{2,\ 108} = 1070$, $p<0.001$; Fig. 2; ambient 83.4%, minimal 81.9%, moderate 79.1%, and elevated 78.6%). Post-hoc Tukey tests indicated that incorporation into the 2–5 and 5–10 cm depths was similarly low for all treatments across sampling times ($p > 0.1$ in all comparisons).

Uptake of ^{15}N into microbial biomass (MPB and bacteria) accounted for 22–95% of the total ^{15}N incorporated into sediment ON across all treatments and sampling times (Fig. 3). Initially, ^{15}N retained (0.5 d) in MPB during the incubation was higher in the elevated treatment (one-way ANOVA: $F_{3,7} = 6.9$, $p = 0.046$, minimal, 71±21%; moderate, 75±12%; and elevated, 93±1%, mean±SE) than in the ambient treatment (38±4%) with Tukey tests indicating a significant difference between elevated and ambient treatments ($p = 0.04$: Fig. 3) with values for minimal and moderate treatments falling intermediate between them. This indicates that initial retention of MPB-N was stimulated by increased nutrient availability and this assimilated ^{15}N remained in MPB at 0.5 d. Within the microbial community after 0.5 d, distribution of ^{15}N continued to be dominated by MPB for all treatments (ambient 83±3%; minimal 79±4%; moderate 74±2%; elevated 75±5%, Fig. 4). Bacterial contri-

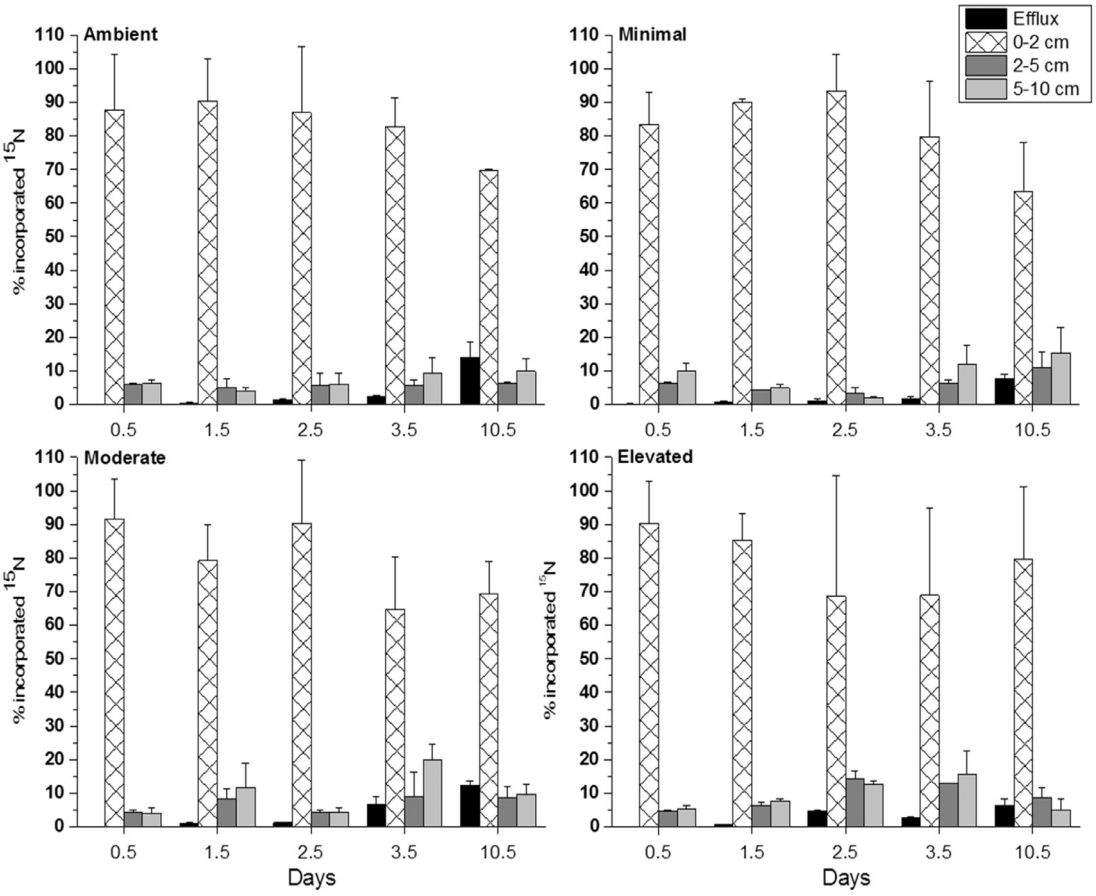

Fig. 2. Nitrogen budget for excess ^{15}N within sediment ON at 0–2 cm, 2–5 cm, and 5–10 cm, and cumulative excess ^{15}N exported to the water column combined via effluxes of NH_4^+, DON, and N_2 for each treatment at each sampling time. All values are as a percentage of the ^{15}N initially incorporated into sediment ON (0–10 cm). Some error bars are too small to be seen (mean + SE).

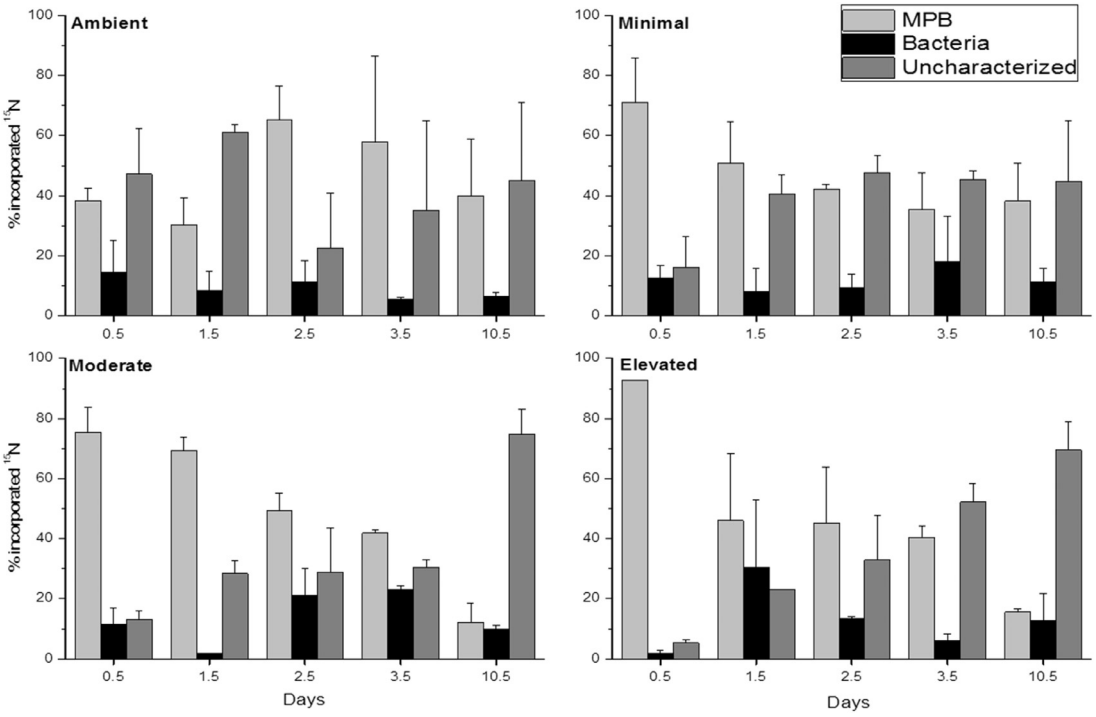

Fig. 3. Incorporation of ^{15}N into bacteria, MPB, and uncharacterised sediment compartments (mean + SE). The uncharacterised compartment is derived by subtracting ^{15}N contained in microbial biomass from bulk sediment organic ^{15}N.

Table 1
Mean biomass for sediment compartments for 0–2 cm for initially sampled cores after tidal flushing and for 0–2 cm, 2–5 cm, and 5–10 cm for all cores sampled in the ambient treatment (mean ± SE) across the 10.5 d sampling. %N is the percentage N within each sediment depth for individual sediment compartments (MPB, Bacteria, and Uncharacterised) except for sediment N, where it represents the portion of N relative to the total N in 0–10 cm of sediment.

	0–2 cm			2–5 cm			5–10 cm		
	mmol N m^{-2}	SE	%N	mmol N m^{-2}	SE	%N	mmol N m^{-2}	SE	%N
Initial									
Sediment	268.3	19.1	21.2	465.7	29.7	36.9	529.7	44.7	41.9
MPB	53.8	3.1	20.1						
Bacteria	11.1	0.0	4.1						
Uncharacterized	201.6	36.1	75.1						
Ambient									
Sediment	231.1	18.8	19.8	421.4	93.3	36.1	515.7	73.3	44.1
MPB	65.6	8.1	28.4	45.2	10.5	10.7	33.2	13.2	6.4
Bacteria	40.3	10.5	17.4	29.8	4.6	7.1	31.1	7.3	6.0
Uncharacterized	125.2	23.0	54.2	346.4	94.0	82.2	451.3	74.9	87.5

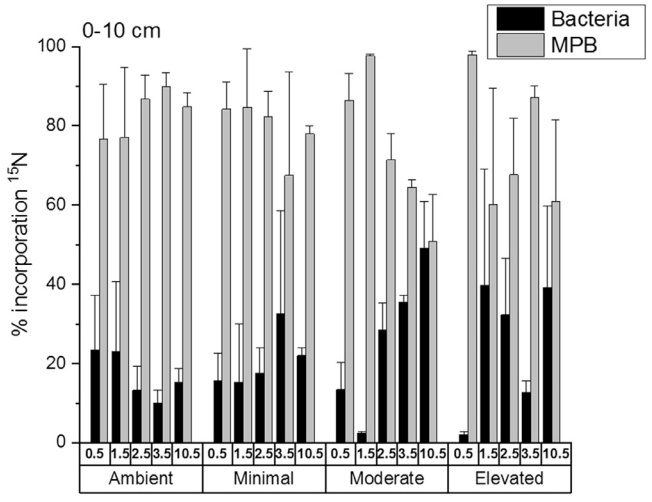

Fig. 4. Excess ^{15}N incorporation into MPB and bacterial biomass within 0–10 cm depth as a percentage of the total ^{15}N incorporated into the microbial community for each treatment at each time period (mean + SE).

bution to ^{15}N uptake was comparable or higher during the later sampling times (1.5 to 10.5 d) in the nutrient amended treatments (minimal, 15–23%; moderate, 2–49%; elevated, 2–40%, Fig. 4) compared to the ambient treatment (10–23%).

In the ambient treatment, distribution of ^{15}N between microbial biomass and uncharacterised sediment ON remained comparable across the 10.5 d incubation (one-way ANOVA: $F_{4,9} = 0.5$, $p = 0.8$; Fig. 3). In contrast, in the nutrient amended treatments ^{15}N incorporation was initially dominated by microbial biomass, primarily MPB, but shifted towards increased incorporation into the uncharacterised pool as the incubations progressed (two-way ANOVAs: minimal, $F_{1,\ 10} = 10$, $p = 0.01$; moderate, $F_{1,\ 10} = 10$, $p < 0.001$; elevated, $F_{1,\ 10} = 10$, $p < 0.001$; Fig. 3). In the nutrient amended treatments, the ^{15}N contributing to the increased uncharacterised pool appeared to be largely sourced from MPB, resulting in reduced MPB contributions by 10.5 d (minimal 38%; moderate 12%; elevated 16%; Fig. 3). Bacterial contributions to ^{15}N remained similar across the incubation period in nutrient amended treatments (two-way ANOVA: $F_{2,\ 15} = 0.1$, $p = 0.9$).

3.3. Loss of ^{15}N from sediments

There was relatively little efflux of ^{15}N from the sediment to the water column in this study, with loss pathways accounting for a maximum of 14% of the initially incorporated ^{15}N across treatments (Fig. 2). Across all treatments, most of this loss to the water column occurred in the form of DON and N_2 fluxes (Fig. 5), with only a minor contribution from DIN (NH_4^+, maximum of 0.3%). Within the ambient treatment, DON was the largest export pathway accounting for loss over 10.5 d of 10.5% of the initially incorporated ^{15}N (Figs. 5 & 6). Loss of ^{15}N from the sediments via DON effluxes was lower within the nutrient amended treatments (minimal, 4.8%; moderate, 5.2%; elevated, 5.2%) but was not statistically significant. Export of ^{15}N via N_2 was comparably low between ambient, minimal and elevated treatments (3.3%, 2.8%, and 2.3%, respectively), but was higher in the moderate treatment (7.1%, Figs. 4 & 6). Overall export via combined efflux pathways was low across all treatments, with the bulk of MPB-N remaining within the sediment by 10.5 d (ambient 86 ± 4%, minimal 90 ± 2%, moderate 88 ± 2%, and elevated 94 ± 3%, Fig. 6). The majority of ^{15}N remaining in the sediment was found in the 0–2 cm depth for all treatments (ambient 70%; minimal 63%; moderate 69%; and elevated 80%, Fig. 2 while ^{15}N incorporation remained lower for depths below 2 cm (ambient 16%; minimal 27%; moderate 18%; and elevated 14%).

4. Discussion

By combining a pulse-chase application of the rare isotope ^{15}N with the D/L-Ala-biomarker technique, we identified that increased nutrient availability as a pulsed addition of 5 to 50 µmol L^{-1} NH_4^+ across three treatments: 1) stimulated initial retention of MPB-N within MPB 2) increased microbial turnover of MPB-N into the uncharacterised sediment ON compartment as incubations progressed, and 3) decreased the amount of MPB-N lost via DON effluxes. By the end of the incubation (10.5 d) 86–94% of the ^{15}N incorporated remained in the sediment, with 3.9 - 10.9% effluxed as DON, 2.3 −7.1% effluxed as N_2, and less than 1% effluxed as NH_4^+ (Fig. 6). Of the ^{15}N contained in the sediment 12–40% was in MPB, 6–15% was in bacteria and the remaining 45–75% within uncharacterised sediment ON (Fig. 6). This study tracks the fate and processing of a pulse of MPB-N produced in situ under an increasing gradient of water column nutrient availability.

4.1. Initial incorporation and downward transfer of ^{15}N

Initial retention of ^{15}N into microbial biomass was dominated by MPB across all treatments as has been observed previously at this site (Riekenberg et al., 2017), and was stimulated by increased nutrient availability (ambient 53% versus 84–95% nutrient amended treatments, 0.5 d, Fig. 4). Retention of MPB-N may have resulted from decoupling of bacterial processing from MPB production, with heterotrophic bacteria preferentially utilizing inorganic nutrients in lieu of MPB-N in the nutrient amended treatments.

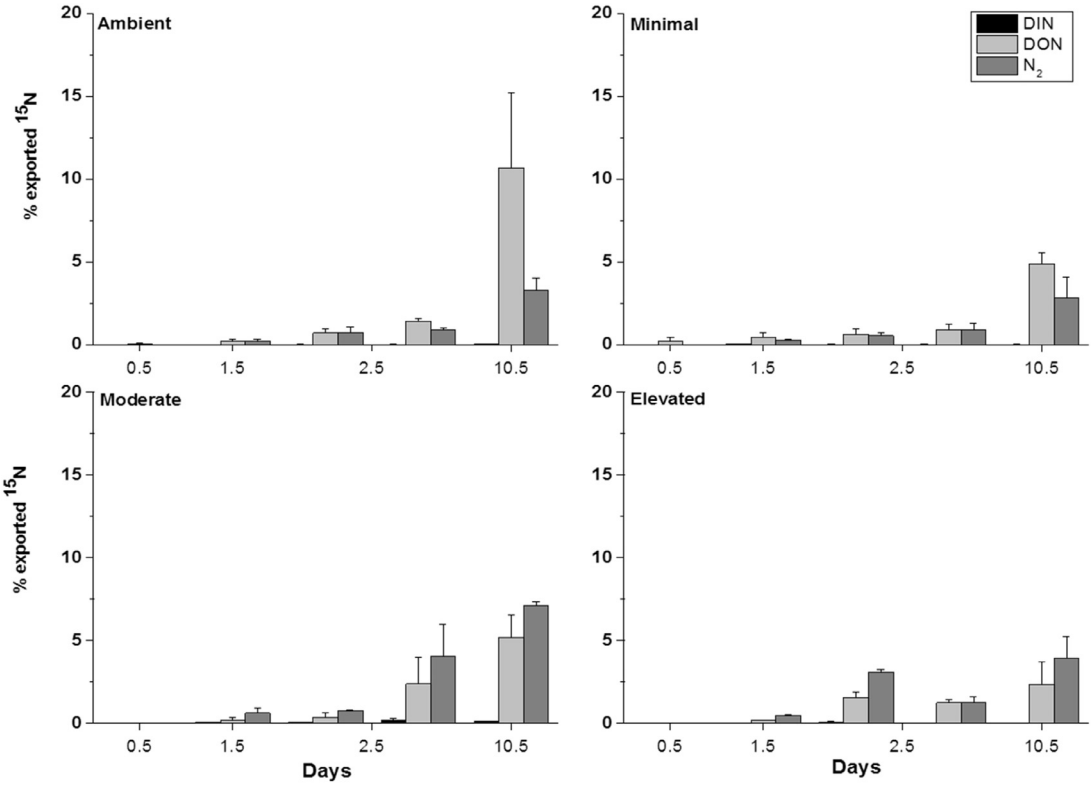

Fig. 5. Cumulative excess ^{15}N lost via efflux of DIN, DON, and N_2 at each sampling time. All values are as a percentage of the ^{15}N incorporated into sediment ON (0–10 cm). Some bars and error bars are too small to be seen (mean + SE).

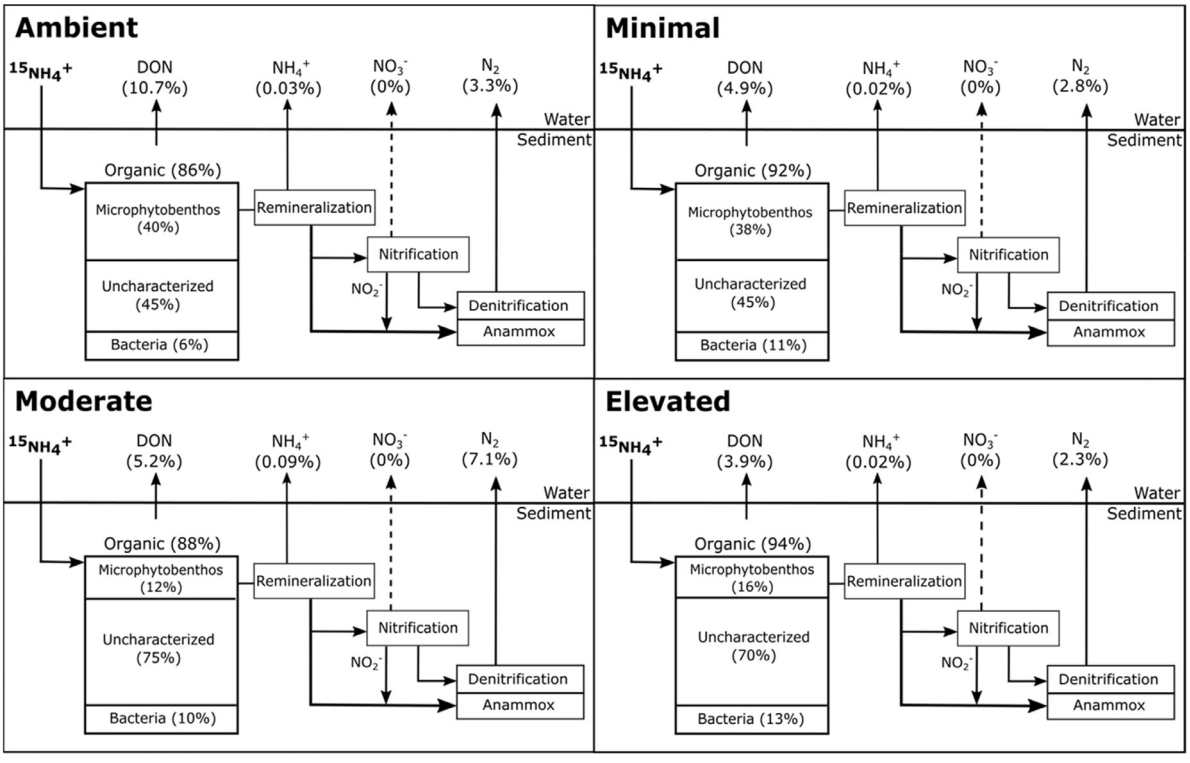

Fig. 6. Conceptual model comparing the processing and fate of microphytobenthos nitrogen after 10.5 d of incubation amongst treatments. Sediment organic nitrogen is partitioned into sediment compartments (microphytobenthos, bacteria, and uncharacterised) as a percentage of ^{15}N contained in 0–10 cm depth using biomarker analyses for 0–2 cm, 2–5 cm, and 5–10 cm depths. The dashed line for efflux of NO_3^- indicates that concentrations were consistently below the detection limit for $^{15}NO_3^-$ analysis (figure modified from Eyre et al., 2016.

Resource switching by heterotrophic bacteria is further supported by decreased initial MPB-N turnover into uncharacterised ON. Considerable turnover of MPB-N into uncharacterised ON occurred in the ambient treatment (47.3%, 0.5 d), but was significantly lower in the nutrient amended treatments (5–16%, $t = 3.8$, $p = 0.009$; Fig. 3). The uncharacterised pool represents ^{15}N within bulk sediment OM that was not accounted for by ^{15}N contained within microbial biomass. This pool is composed of ^{15}N-containing compounds such as EPS, enzymes, and OM derived from the remineralisation of MPB-N. Increased breakdown and initial transfer of MPB-N into the uncharacterised pool is consistent with bacterial utilization of MPB-N during remineralisation that resulted in increased initial turnover in low nutrient settings (Fig. 3). Increased coupling between MPB and bacteria in low nutrient settings (Cook et al., 2007; Oakes et al., 2012) has previously been attributed to increased reliance on the limited nutrients arising from bacterial remineralisation of sediment ON.

Ratios of microbial biomass ^{13}C:^{15}N were estimated based on excess ^{13}C incorporation determined in a complementary study (Riekenberg et al., 2018). ^{13}C:^{15}N ratios potentially over-estimate relative uptake of ^{15}N into the microbial community as phospholipid fatty acid analysis only accounts for ^{13}C contained in living biomass and D/L-Ala-accounts for ^{15}N in both living and dead biomass, particularly as incubations progress. Therefore, ^{13}C:^{15}N ratios for microbial biomass were only examined during the initial sampling (0.5 d). The ^{13}C:^{15}N ratio in the ambient treatment (16.7 ± 2.8) was considerably higher than in nutrient amended treatments (minimal 8.3 ± 3.8; moderate 5.2 ± 3.4, and elevated 7.5 ± 2; One-way ANOVA, $F_{3,7} = 5.4$, $p = 0.068$), and aligns well with previous estimates where preferential excretion of fixed C as EPS would occur due to N limitation within algal cells (e.g., ~20, Van den Meersche et al. (2004). A lower ^{13}C:^{15}N ratio within nutrient amended treatments indicates uptake occurring at a ratio closer to the Redfield ratio expected for algal uptake (6.7) as a result of increased N availability (Cook et al., 2007). Bacteria under N replete conditions utilize inorganic nutrients to support production of low C:N biomass, regardless of the C:N ratio of the substrate being processed to produce that biomass (Goldman and Dennett, 2000). Shifting retention of MPB-N between sediment compartments due to nutrient amendment indicates changed short-term processing of recently fixed organic matter and was observed despite considerable time having elapsed prior to incubation (18 h allowing for flushing of tracer, and core acclimation).

Over 10.5 d, the majority (81%) of ^{15}N incorporated into the sediment was recovered from the 0–2 cm depth, but deeper sediment layer incorporation occurred rapidly (within 0.5 d, Fig. 2), resulting in a transport rate of 2.7 – 4.4 μmol ^{15}N m^{-2} h^{-1}. Downward transport was substantially lower than previously observed at this site (e.g., 19.6 μmol ^{15}N m^{-2} h^{-1} over 1.5 d, (Oakes et al., 2020) likely driven by the draining and re-filling of cores to simulate intertidal conditions in that study. Downward transport for ^{15}N in this study is similar to that observed for MPB-N in subtropical subtidal sediments (e.g., ~2.7 μmol ^{15}N m^{-2} h^{-1} over 3 d with ambient DIN of ~8 μmolL^{-1}, Eyre et al. (2016), but represent a smaller portion of the total incorporated ^{15}N (8.3 – 16.4% compared to ~29%). Similar downward transport for MPB-N between the two studies likely reflect comparable downward migration by MPB within subtropical sands (Saburova and Polikarpov, 2003). Incorporation of ^{15}N into 2–10 cm depth increased slowly thereafter and resulted in higher downward transport in the nutrient amended treatments as the incubations progressed (average over 3.5–10.5 d; ambient 15.6 ± 2.0%; minimal 22.6 ± 2.8%; moderate 23.6 ± 2.7%; and elevated 21.2 ± 2.0%, Fig. 2). Downward migration of MPB can be enhanced by light stress (Underwood 2002), but given that light intensity was consistent across treatments, increased downward transfer of MPB-N within nutrient amended treatments more likely reflects increased downward migration for nutrients and cell division as water column nutrients quickly became limiting later in the incubations (Saburova and Polikarpov, 2003).

4.2. Transfer of MPB-N within sediments

Transfer and processing of MPB-N between sediment compartments within sediment ON decreased between the ambient and nutrient amended treatments due to decreased bacterial remineralization of MPB-N, reducing recycling and transfer of MPB-N between sediment compartments (Fig. 3). Across 10.5 d, the ambient treatments contribution of MPB to ^{15}N within microbial biomass increased (77–90%), bacterial contributions declined (23 to 10%, Fig. 4), and the uncharacterised pool of ^{15}N contributed considerably to the excess ^{15}N in sediment ON (23–61%, Fig. 3). These combined factors indicate MPB-N was efficiently recycled when nutrients are relatively scarce. Bacteria in the ambient treatment re-mineralised MPB-N within the uncharacterised pool (Fig. 1A), providing inorganic nutrients that were increasingly used by MPB as they competed for available nutrients under nutrient limitation (Cook et al., 2007).

In contrast, across 10.5 d in the nutrient amended treatments, tight recycling of nutrients from bacterial remineralisation decreased as bacteria preferentially utilised inorganic nutrients instead of degrading organic matter for N. This switch resulted in accumulation of ^{15}N within sediment ON as MPB-N was not remineralised and remained unavailable for recycling back into MPB. With increased nutrient loading, MPB contributions to ^{15}N within microbial biomass declined, bacterial contributions increased (Fig. 3), and resulted in increased ^{15}N contained in the uncharacterised pool (minimal, 41–47%; moderate 29–75%; elevated 23–70%; Fig. 3). Accumulation of ^{15}N in uncharacterised sediment ON reflects decreased recycling of MPB-N contained in OM (likely as EPS) and detritus (MPB and bacterial biomass) as bacteria preferentially utilised inorganic nutrients instead of MPB-N within the uncharacterised pool (Fig. 1B).

4.3. Effluxes of MPB-N

Export of ^{15}N from the sediments to the water column occurred via fluxes of DON, N_2, and NH_4^+ (Figs. 2 & 6). Fluxes of NH_4^+ from remineralisation (0–0.3%) contributed little to exported ^{15}N across all treatments, indicating strong retention of nutrients arising from MPB-N remineralisation. Retention of N from remineralization is expected as competition between MPB and heterotrophic bacteria for N has been previously observed under nutrient limiting settings (Agogue et al., 2014; Risgaard-Petersen et al., 2004). Limited export of ^{15}N as NH_4^+across all treatments suggests that the microbial community maintained capacity for further N uptake throughout the incubations. Cumulative flux of NH_4^+ in this study was considerably smaller than observed in subtropical subtidal sediments over 33 d (20.8%, Eyre et al., 2016) but similar to previous observations for this site across 31 d (0.2%, (Oakes et al., 2020). Similar NH_4^+ fluxes between studies at this site likely result from decreased bacterial remineralisation of sediment ON and increased retention of MPB-N in comparison to subtidal processing from increased competition between heterotrophic bacteria and MPB for available N.

Low fluxes of N_2 may be caused by competition for inorganic nutrients between MPB and bacteria. Intense competition for recycled inorganic nutrients between MPB and bacteria would have starved both ammonia oxidizing and subsequently denitrifying bacteria of substrates (McGlathery et al., 2007; Sundbäck and Miles, 2002) and reduced N_2 efflux from denitrification (2.3–7.1%, 10.5 d; Fig. 6). This finding is comparable with previously re-

ported denitrification rates for this site (2.6% by 31 d; (Oakes et al., 2020), and suggests that competition from MPB for NH_4^+ was similarly limiting during that period as ambient NH_4^+ was higher in (Oakes et al., 2020)), 2.1 ± 1.8 μmolL^{-1}) than in this study (0.9 ± 0.1 μmolL^{-1}). Limited availability of substrates for denitrification was maintained as intense competition between MPB and heterotrophic bacteria for NH_4^+ occurred, starving ammonia-oxidizers that produce NO_3^-. Substrate limitation helps to explain the low percentage for N_2 efflux observed in this study versus the higher rates found in subtropical subtidal sediments (20.7%, Eyre et al. (2016). Enhanced efflux of MPB-N as both N_2 and NH_4^+ in a subtidal setting suggests that increased remineralisation produced inorganic nutrients sufficient to overcome the N limitation from MPB. In the moderate treatment, denitrification, bacterial incorporation of MPB-N, and the flux of NH_4^+ were higher by 10.5 d, indicating that competition between MPB and heterotrophic bacteria did not limit substrate availability for denitrification. Both moderate and elevated treatments provide examples of decreased competition between MPB and heterotrophic bacteria that resulted in increased turnover of MPB-N into uncharacterised sediment OM. Between these treatments, only the moderate had increased denitrification indicating that changed processing between the sediment compartments may occur with or without considerably increased denitrification as a symptom.

Increased inorganic nutrient availability decreased bacterial remineralisation as inorganic nutrients were preferentially utilised instead of sediment ON (Fig. 1B). In the ambient treatment, efflux of MPB-N via DON accounted for 10% of the ^{15}N assimilated within the system and is evidence of increased remineralisation, hydrolysis of freshly deposited material (Ferguson et al., 2004; Glud et al., 2008), and export of MPB-N from the uncharacterised pool within sediment ON. With nutrient amendment, export of MPB-N via DON decreased to 3.9–5.2% (Fig. 6) despite enhanced MPB-N retention at 0.5 d and the equal or increased presence of ^{15}N within the uncharacterised pool within the nutrient amended treatments. Decreased export of MPB-N via DON likely occurred due to added DIN being preferentially used by heterotrophic bacteria instead of sediment ON via remineralisation.

4.4. Retention of MPB-N

MPB have a considerable role in regulating processing of nutrients within shallow unvegetated benthic settings through primary production and N uptake (Ferguson and Eyre, 2013; Ferguson et al., 2004; McGlathery et al., 2007; Nielsen et al., 2017; Sundbäck and Miles, 2002). Newly assimilated N is considered to be retained within MPB biomass, with increased MPB biomass resulting in reduced N efflux from sediments (Sundbäck et al., 2006). Excluding Eyre et al. (2016), Oakes et al. (2020), and Veuger et al. (2007), the chiral forms of alanine have not been used to partition sediment ON into MPB and bacterial compartments as well as processed MPB-N remaining in sediment ON. Partitioning the uncharacterised pool within sediment ON reveals that much of the initially assimilated MPB-N is transferred there, presumably via excretion of EPS by MPB and turnover of microbial cellular material. Under nutrient-limiting conditions, this material is quickly remineralised by bacteria, releasing $^{15}NH_4^+$ that is then recycled as MPB and bacteria compete for available nutrients (Fig. 1A). Under ambient conditions, this coupling resulted in efficient bacterial remineralisation and recycling of MPB-N resulting in turnover of ^{15}N among all the sediment compartments. Effluxes of DIN or N_2 were not stimulated by the altered processing of MPB-N in sediment ON, which differs greatly from our stated hypothesis. Sediment retention of MPB-N was somewhat reduced by increased efflux of DON through increased hydrolysis and bacterial remineralisation of freshly deposited MPB-N. With added nutrient amendments, hydrolysis and bacterial remineralisation of MPB-N slowed, resulting in reduced processing and accumulation of MPB-N within sediment ON and decreased ^{15}N export via DON efflux. These changes caused decreased retention of MPB-N in MPB and increased turnover into sediment ON under nutrient amendment. Retained MPB-N occurred largely outside of the microbial biomass, within the uncharacterised sediment ON pool (i.e., retained as EPS and other N-containing molecules). This is in stark contrast to previous findings that MPB-N is predominately strongly retained in MPB-biomass (Eyre et al., 2016; Nielsen et al., 2017) and reflects the considerable turnover of MPB-N within this system.

5. Conclusion

The microbial community is an important sink for organic and inorganic N within benthic coastal settings (Ferguson et al., 2004; McGlathery et al., 2007; Thornton et al., 2002). Uptake of N is typically viewed as being MPB dominated, with N retained in MPB biomass (Evrard et al., 2008; Hardison et al., 2011; Sundbäck et al., 2006; Veuger et al., 2007) unless grazing causes trophic transfer of MPB-N (Eyre and Ferguson, 2002, 2005). Addition of inorganic nutrients appears to initially stimulate N retention in MPB, but then causes reduced bacterial remineralisation and recycling of remineralised MPB-N as inorganic nutrients are preferentially utilised. This leads to decreased MPB-N retention within MPB biomass, greater accumulation of MPB-N within sediment ON as uncharacterised material and corresponds with decreased efflux of MPB-N as DON. Uncharacterised material is a mixture of EPS, enzymes and other N-bearing OM that is either associated with the microbial biofilm and is fairly resistant to breakdown and export or has accumulated due to the low physical transport processes associated with this study. Either way, turnover of MPB-N between the sediment compartments has decreased and is no longer strongly retained within MPB biomass. These changes resulted in increased accumulation of relatively labile MPB-N in the shallow surface sediments that may stimulate long-term support of increased bacterial respiration. Increased accumulation of newly assimilated OM in the sediment occurred with short-lived pulses of nutrients within shallow MPB-dominated sediments and occurred under nutrient replete settings regardless of whether denitrification of ^{15}N increased. This indicates that short-term pulses of nutrients into coastal ecosystems can result in considerable accumulation of newly assimilated OM, regardless of whether N_2 export is stimulated.

Declaration of Competing Interest

None.

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