Short-term fate of intertidal microphytobenthos carbon under enhanced nutrient availability: A $^{13}$C pulse-chase experiment

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ABSTRACT:

Shallow coastal waters in many regions are subject to nutrient over-enrichment. Microphytobenthos (MPB) can account for much of the carbon (C) fixation in these environments, depending on the depth of the water column, but the effect of enhanced nutrient availability on the processing and fate of MPB-derived C is relatively unknown. In this study, MPB were labeled (stable isotope enrichment) in situ using $^{13}$C-sodium bicarbonate. The processing and fate of the newly-fixed MPB-C was then traced using ex situ incubations over 3.5 d under different concentrations of nutrients ($\text{NH}_4^+$ and $\text{PO}_4^{3-}$: ambient, 2× ambient, 5× ambient, and 10× ambient). After 3.5 d, sediments incubated with increased nutrient concentrations (amended treatments) had increased loss of $^{13}$C from sediment organic matter as a portion of initial uptake (95% remaining in ambient vs 79-93% for amended treatments) and less $^{13}$C in MPB (52% ambient, 26-49% amended), most likely reflecting increased turnover of MPB-derived C supporting increased production of extracellular enzymes and storage products. Loss of MPB-derived C to the water column via dissolved organic C was minimal regardless of treatment (0.4-0.6%). Loss due to respiration was more substantial, with effluxes of dissolved inorganic C increasing with additional nutrient availability (4% ambient, 6.6-19.8% amended). These shifts resulted in a decreased turnover time for algal C (419 d ambient, 134-199 d amended). This suggests that nutrient enrichment of estuaries may ultimately lead to decreased retention of carbon within MPB-dominated sediments.
1.0 Introduction

Intertidal sediments are important sites for the processing of carbon (C) within estuaries, producing, remineralizing, and transforming considerable amounts of organic material prior to its export to the coastal shelf (Bauer et al. 2013). Algal production is a key source of C within the coastal zone, and is primarily derived from microphytobenthos (MPB) in shallow photic sediments (Hardison et al. 2013; Middelburg et al. 2000). In addition to algal cells being a labile carbon source, MPB exude large amounts of carbohydrates as extracellular polymeric substances (EPS) (Goto et al. 1999) that allow for vertical migration and enhance sediment stability (Stal 2010). A better understanding of the carbon pathways utilized during processing of algal cells and exudates within sediments is important for determining the quality and quantity of carbon exported from estuarine waters to continental shelves.

Application of stable isotope tracers cause isotopic enrichments that usually serve to make negligible the variability associated with fractionation effects in natural abundance stable isotope techniques. As such, stable isotope tracers have been useful for elucidating pathways for the processing and loss of MPB-derived C within estuarine sediments. Loss pathways for MPB-derived C include resuspension (Oakes and Eyre 2014), fluxes of dissolved inorganic C (DIC) due to mineralization and respiration (Evrard et al. 2012; Oakes et al. 2012), fluxes of dissolved organic C (DOC) comprised of microbial exudates and products from cell lysis (Oakes et al. 2010a), and direct production of CO₂ (Oakes and Eyre 2014). Stable isotope tracer studies have also enabled quantification of the trophic transfer (Middelburg et al. 2000; Miyatake et al. 2014; Nordström et al. 2014; Oakes et al. 2010a) and

When $^{13}$C is combined with analysis of phospholipid-linked fatty acids (PLFAs), it becomes possible to trace C transfer into individual microbial groups that account for the living biomass within sediment organic C (OC) (Drenovsky et al. 2004; Hardison et al. 2011; Oakes et al. 2012; Oakes and Eyre 2014; Spivak 2015). This allows for the quantification of microbial transfers of newly produced algal C between MPB and bacteria and the relative contributions of MPB and bacteria to microbial biomass in sediment OC. This technique has shown that EPS produced by MPB is readily utilized as a C source for heterotrophic bacteria (Oakes et al. 2010; van Oevelen et al. 2006). Pathways for processing of MPB-derived C have been reasonably well described, but the response of these pathways to local environmental changes remains a significant knowledge gap.

A major source of environmental change in coastal systems is nutrient over-enrichment (Cloern et al. 2001), which may affect the assimilation and flux pathways of MPB-derived carbon through 1) increased microbial biomass resulting from relaxation of nutrient limitation, 2) increased algal production that drives elevated heterotrophic processes as bacteria utilize newly produced C, and 3) increased loss of C as DIC via respiratory pathways as heterotrophic processes dominate. MPB are able to use both porewater and water column nutrients, and although MPB biomass can increase with elevated nutrient availability (Armitage and Fong 2004; Cook et al. 2007), this is not always the case, with multiple studies finding no corresponding increase in MPB biomass (Alsterberg et al. 2012; Piehler et al. 2010; Spivak and Ossolinski 2016). Processing and mineralization of C are significantly affected by changes in the relationship between MPB and bacteria (Evrad et al. 2012). Both EPS
production and bacterial utilization of newly produced EPS may decrease with increasing nutrient availability (Cook et al. 2007). Increased autochthonous production driven by nutrient enrichment can lead to increased heterotrophy, as newly produced organic matter is mineralized (Fry et al. 2015), resulting in increased DIC production. Increased remineralization of newly produced MPB-C will result in greater loss of DIC from intertidal sediment via bacterial respiration (Hardison et al. 2011).

In this \(^{13}\)C pulse-chase study we aimed to quantify the processing pathways of MPB-derived C within subtropical intertidal sediments and to determine how this is affected by increased nutrient loading. The in situ MPB community was used to provide a pulse of labeled MPB-C of similar quantity and quality to normal production. Even application of separate label applications for each plot prior to incubation served to isolate the subsequent effect of increased nutrient availability on the processing of MPB-derived C. Pathways considered included transfer through sediment compartments (MPB, bacteria, uncharacterized and sediment OC), and loss via fluxes of DOC and DIC. We expected increased nutrient availability to stimulate MPB production of EPS after initial labeling, resulting in decreased turnover times for MPB-C as well as a shift towards dominance of heterotrophic processes as bacteria utilize this additional labile C. We further hypothesized that enhanced heterotrophy would increase loss of newly fixed algal-derived C via respiration as DI\(^{13}\)C. Incorporation of \(^{13}\)C into biomarkers should reflect the shift towards heterotrophy, with quicker shifts towards increased bacterial utilization of newly produced algal C corresponding with increased nutrient load. Both DIC and DOC should be significant loss pathways for newly produced algal C as labile OM is readily processed by heterotrophs.

2.0 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). The 6900 km² catchment has an 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 dry period. The site experiences semidiurnal tides with a range of ~2 m. Samples were collected in summer January 2015 with average site water temperature of 25.6 ± 2.3°C.

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 C content of 17.5 ± 0.02 mol C m⁻². Sediment molar C:N was lowest at 2-5 cm, but comparable across all other depths (top scrape (TS) 14.4 ± 1.6, 0-2 cm 17.2 ± 1.7, 2-5 cm 10.9 ± 0.5, 5-10 cm 16.2 ± 2.2).

2.2 Overview

We labeled MPB with ^13C via in situ application of ^13C-labeled sodium bicarbonate to exposed intertidal sediments. Unincorporated ^13C was flushed from the sediment during the next tidal inundation of the site. Sediment cores were collected and incubated in the laboratory over 3.5 d under four nutrient enrichment scenarios (ambient, minimal, moderate, and elevated) using pulsed nutrient additions. Incubation of cores ex situ allowed for explicit control of nutrient additions and examination of the short-term processing and fate (loss to overlying water) of MPB carbon. Sediments remained inundated during incubations with minimal water exchange, as might be expected during neap tide at this site. Inundation also
served to minimize C loss via physical resuspension and export while we were examining sediment processing.

### 2.3 $^{13}$C-labeling

Bare sediment within a 2 m$^2$ experimental plot was $^{13}$C-labeled when sediments were first exposed during the ebbing tide in the middle of the day by using motorized sprayers to evenly apply 99% NaH$^{13}$CO$_3$ onto individual 400 cm$^2$ subplots, closely following the method outlined in Oakes and Eyre (2014). Label applications were prepared using NaCl-amended Milli-Q matching site salinity (34.6), and 20 ml aliquots (1.7 mmol $^{13}$C) were applied to each individual subplot, resulting in application of 42.5 mmol $^{13}$C m$^{-2}$. The use of individual aliquots of label ensured even $^{13}$C application across the sediment surface. Assimilation of label by the sediment community occurred over ~4 hours with average light exposure of 1376 µE m$^{-2}$ s$^{-1}$, before tidal inundation removed the majority of unincorporated $^{13}$C. Removal was confirmed by loss of 99.0% of the applied $^{13}$C from treatment applications within initial cores sampled in the field.

### 2.4 Collection of sediment cores

Prior to label application, 3 cores (9 cm diameter, 20 cm depth) were collected from unlabeled sediment surrounding the treatment plots and immediately extruded and sectioned (0-0.2 cm (top scrape, TS), 0.2-2 cm, 2-5 cm, and 5-10 cm) to provide control natural abundance sediment OC $\delta^{13}$C values for sediment depths within the study site. Eleven hours after label application, at low tide, 35 sediment cores were similarly collected from the labeled plot using Plexiglas core liners. Immediately, three cores were extruded and sectioned as above to determine initial $^{13}$C uptake and grain size distribution for all sediment depths, and chlorophyll-$\alpha$ (Chl-$\alpha$) concentrations in 0-1 cm sediments. All samples were placed within
plastic zip-lock bags, transported to the laboratory on ice, and stored frozen in the dark (-20°C). Plexiglas plates were used to seal the bottom of the core liners, and cores for incubation were then transported to the laboratory within 2 hours of sampling. Site water (400 L) was collected and transported to the laboratory for use in incubations.

2.5 Nutrient amendment

Pulsed applications of nutrients for each treatment amendment were used to mimic a range of nutrient concentrations without exceeding sediment capacity for uptake. The treatment tanks were set up at ambient concentration (site water, DIN of 2.5 ± 0.04 µmol N L⁻¹, measured on incoming tide), and with N (NH₄⁺) and P (H₃PO₄) amendment 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 NH₄⁺ and 5 µmol L⁻¹ PO₄³⁻; Eyre (1997; 2000). To allow thorough mixing, the initial pulse of nutrients was added to both incubation tanks and bags holding replacement water for sampling, one hour prior to cores being transferred into the incubation tanks. An additional pulse of NH₄⁺ was applied to incubation tanks at 1.5 d (after sample collection) to mimic the nutrient availability that occurs with regular inundation of tidal sediments. Silica (Si) was also added to all incubation tanks at 2.5 d (after sample collection) to ensure that isolation of the benthic diatom-dominated sediment from regular water turnover did not result in secondary limitation of Si. There was no significant accumulation of NH₄⁺ within treatment tank water, as nutrients were readily processed (Supplemental Fig. 1).

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 85
L treatment tanks (Ambient, Minimal, Moderate, Elevated; eight cores per treatment). Water in the treatment tanks and cores was continuously recirculated, held at in situ temperature (25 ± 1°C) by a chiller on each tank, and aerated. Cores were stirred via a rotating magnet at the center of each treatment tank, which interacted with the magnetic stir bars. Stirring occurred at a rate below the threshold for sediment resuspension (Ferguson et al. 2003). Three sodium halide lamps suspended above the treatment tanks provided 824 ± 40 µE m\(^{-2}\) s\(^{-1}\) to the sediment/water interface within the cores on a 12 h light/12 h dark cycle which approximated the average light level measured at the sediment surface during inundation (941.4 ± 139 µE m\(^{-2}\) s\(^{-1}\)). Cores were allowed to acclimate in tanks for 6 h prior to the start of incubation. 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). Dissolved oxygen (± 0.01 mg L\(^{-1}\)) and pH (± 0.002 pH) were measured optically and electrically (Hach HQ40d multi-parameter meter) via a sampling port in the lid. Initial samples were taken 30 min after closure of the lids, dark samples were taken after ~12 hours incubation with no light, and light samples were taken after 3 hours of illumination following the end of the dark period. During sampling, 50 ml of water was syringe-filtered (precombusted GF/F) into precombusted 40 ml glass vials with Teflon coated septa, killed with HgCl\(_2\) (20 µL saturated solution), and refrigerated prior to analysis for concentration and \(\delta^{13}\)C of DIC and DOC. Sample water was simultaneously replaced by water held in replacement bags as sampling occurred at each time point. No samples were collected for analysis of gaseous CO\(_2\) fluxes from exposed sediments, as this was previously determined to be a negligible pathway for loss of MPB-C at this site (Oakes and Eyre 2014). At the end of sampling for the light period, cores were extruded, sectioned and sampled for Chl-α in the
same manner as control cores and stored frozen (-20°C). Eight cores (two cores per treatment) were sampled in this manner for water column fluxes, PLFAs, and sediment OC after 1.5 d, 2.5 d and 3.5 d of incubation. Additionally, 8 cores (two cores per treatment) were sampled for only PLFAs and sediment OC at 0.5 d of incubation.

2.7 Sample analysis

Chl-α was measured by colorimetry (Lorenzen 1967) for each core (0-1 cm depth). MPB-C biomass was calculated assuming a C:Chl-α ratio of 40, within the range reported for algae in Australian subtropical estuaries (30-60 Ferguson et al. 2004; Oakes et al. 2012). Biomass measurements utilizing Chl-α were used to compare biomass across controls and treatments and were not utilized in calculations for uptake of $^{13}$C into MPB or bacteria using PLFAs. Bacterial C biomass for controls was estimated based on MPB-C biomass derived from Chl-α and the ratio of MPB to bacterial biomass obtained from PLFA analysis of the control cores (n=3).

Sediment samples were lyophilized, loaded into silver capsules, acidified (10% HCl), dried (60°C to constant weight), and analyzed for %C and $\delta^{13}$C using a Thermo Flash Elemental Analyzer coupled to a Delta V IRMS via a Thermo ConFlo IV. Samples were run alongside glucose standards that are calibrated against international standards (NBS 19 and IAEA ch6). Precision for $\delta^{13}$C was 0.1‰ with decreasing precision for enrichments above 100‰.

PLFAs specific to bacteria (i + a 15:0) were used as biomarkers for this group. However, although visual analysis confirmed the presence of a large number of pennate diatoms at the study site and diatom-specific PLFAs (e.g. 20:5(n-3)) were detected,
chromatographic peaks for these PLFAs were sometimes indistinct. The 16:1(n-7) PLFA, which represents 27.4% of total diatom PLFAs (Volkman et al. 1989), was therefore used as a biomarker for diatoms, following correction for contributions from gram-negative bacteria and cyanobacteria as described below and in Oakes et al. (2016), as it was consistently present across all samples. Extraction of PLFAs used 40 g of freeze-dried sediment and a modified Bligh and Dyer technique. Sediment was spiked with an internal standard (500 µL of 1 mg ml$^{-1}$ tridecanoic acid, C$_{13}$), immersed in 30-40 ml of a 3:6:1 mixture of dichloromethane (DCM), methanol, and Milli-Q water, sonicated (15 min), and centrifuged (15 min, 9 g). The supernatant was removed into a separating funnel and the pellet was re-suspended in 30-40 ml of the DCM:MeOH:Milli-Q mixture, sonicated, and centrifuged twice more to ensure complete removal of biomarkers. DCM (30 ml) and water (30 ml) were added to the supernatant, gently mixed, and phases were allowed to separate prior to removal of the bottom layer into a round bottom flask. The top layer was then rinsed with 15 ml of DCM, gently shaken, and phases allowed to separate prior to addition to the round bottom flask. This extract was then concentrated under vacuum and separated using silica solid phase extraction columns (Grace; 500 mg, 6.0 ml) by elution with 5 ml each of chloroform, acetone, and methanol. The fraction containing methanol was retained, reduced to dryness under N$_2$, methylated (3 ml 10:1:1 MeOH:HCl:CHCl$_3$, 80°C, 2 h), quenched using first 3 ml and then 2 ml of 4:1 hexane:DCM, evaporated to ~ 200 µl under N$_2$, transferred to a GC vial for analysis, and stored frozen (-20°C). PLFA concentrations and $\delta^{13}$C values were measured using a non-polar 60 m HP5-MS column in a Trace GC coupled to a Delta V IRMS with a Thermo Conflo III interface following the protocol outlined in Oakes et al. (2010a).
DIC and DOC concentrations and δ\(^{13}\)C values were measured via continuous-flow wet oxidation isotope-ratio mass spectrometry using an Aurora 1030W total organic C analyzer coupled to a Thermo Delta V isotope ratio mass spectrometer (IRMS) (Oakes et al. 2010b). Sodium bicarbonate (DIC) and glucose (DOC) of known isotopic composition dissolved in He-purged Milli-Q were used to correct for drift and verify both concentration and δ\(^{13}\)C of samples. Reproducibility was ± 0.2 mg L\(^{-1}\) and ± 0.1 ‰ for DIC and ± 0.2 mg L\(^{-1}\) and ± 0.4 ‰ for DOC.

2.8 Calculations

Incorporation of \(^{13}\)C into sediment OC, bacteria, and MPB (mmol \(^{13}\)C m\(^{-2}\)) was calculated as the product of excess \(^{13}\)C (fraction \(^{13}\)C in sample – fraction \(^{13}\)C in control) and the mass of OC within each pool. For sediment, OC was the product of %C and dry mass per unit area.

Excess \(^{13}\)C for PLFAs was determined only for 0-2 cm, 2-5 cm, and 5-10 cm depths, as there was inadequate sample mass for the 0-0.2 cm top scrape. Due to limitations of time and cost, PLFA samples were taken from only one of the two cores incubated for each treatment at each sampling period. PLFA excess \(^{13}\)C for both bacteria and diatoms was the product of excess \(^{13}\)C contained in the PLFA (fraction \(^{13}\)C in PLFA in sample – fraction \(^{13}\)C in PLFA in control) and the concentrations of C within respective biomarkers. Concentrations of PLFA C were calculated from their peak areas relative to the internal C\(_{13}\) standard spike. Biomass of diatoms and bacteria were calculated using the method described by Oakes et al. (2016).

Briefly, bacterial biomass was calculated as:

1. \(\text{Biomass}_{\text{bacteria}} = \frac{\text{Biomass}_{i+a15:0}}{a \times b}\)
where \( a \) represents the average concentration of PLFA (0.056 g C PLFA per g C biomass; Brinch-Iversen and King 1990) in bacteria and \( b \) represents the average fraction of PLFA accounted for by \( i+a15:0 \) within bacteria-dominated marine sediments (0.16, Osaka Bay, Japan; Rajendran et al. 1994; Rajendran et al. 1993). Biomass estimates for bacteria calculated using the minimum and maximum fraction values (16-19% for \( i+a15:0 \); Rajendron et al. 1993) resulted in a 16% difference.

For diatoms, a mixing model was used to correct the concentration and \( \delta^{13} \)C value of 16:1(n-7) for the any contribution from non-diatom sources. Due to the scarcity of cyanobacteria observed using light microscopy (1000×), low sediment D-/L- alanine ratios measured previously at this site (as low as 0.0062, Riekenberg et al. 2017), and lack of the characteristic 18:2(n-6) peak (Bellinger et al. 2009) cyanobacteria were assumed to make a negligible contribution to the 16:1(n-7) peak. A two-source mixing model was applied to correct the concentration and \( \delta^{13} \)C value of the 16:1(n-7) peak for the contribution of gram-negative bacteria, based on a typical ratio of 18:1(n-7) to 16:1(n-7) for gram-negative bacteria of 0.7 (Edlund et al. 1985) as previously applied in Oakes et al. (2016). Biomass for diatoms was calculated using the formula:

\[
\text{Biomass}_{\text{Diatom}} = \frac{\text{Biomass}_{\text{corrected16:1(n-7)}}}{(c \times d)}
\]

where \( c \) is the average fraction of diatom PLFAs accounted for by corrected 16:1(n-7) (0.67; Volkman et al. 1989) and \( d \) is the average PLFA concentration in diatoms (0.035 g PLFA C per g C biomass; Middelburg et al. 2000). Biomass estimates for diatoms calculated using maximum and minimum fraction values for 16:1(n-7) (18-33%; Volkman et al. 1989) were within 50% of estimates based on the average value.
Fluxes across the sediment-water interface were calculated as a function of incubation time, core water volume and sediment surface area. Dark flux rates were calculated using concentration data from the dark incubation period and light flux rates from the light incubation period. The following parameters were calculated from dark and light rates:

3. Respiration (R) = Dark DO flux h⁻¹
4. Net primary production (NPP) = Light DO flux h⁻¹
5. Gross primary production (GPP) = NPP + R
6. Production/respiration (P/R) = GPP x daylight hours (12 h) / R x 24 h (Eyre et al. 2011)

Total $^{13}$C in DIC and DOC ($\mu$mol $^{13}$C) in the overlying water in the sediment core was calculated for initial, the end of the dark period, and the end of the light period as the product of excess $^{13}$C (excess $^{13}$C in labeled sample versus relevant natural abundance control), core volume, and concentration. Total excess flux of $^{13}$C as DIC or DOC ($\mu$mol $^{13}$C m⁻² h⁻¹) was then calculated as:

7. Excess $^{13}$C flux = (Excess $^{13}$C start – Excess $^{13}$C end) / SA / t

where excess $^{13}$C start and excess $^{13}$C end represent excess $^{13}$C of DIC or DOC at the start and end of dark and light incubation periods, SA is sediment surface area, and t is incubation period length (h). Net fluxes of excess $^{13}$C (excess $^{13}$C m⁻² h⁻¹) for DIC and DOC were calculated as:

8. Net flux = ((dark flux * dark hours) + (light flux * light hours))/ 24 hours

Total $^{13}$C lost via flux to the water column from initial labeling to each sampling period was interpolated from measured net flux values by calculating the area underneath the curve for
each treatment. To prevent the potential development of limitations during incubations, $O_2$ concentrations were not allowed to drop below 60% saturation in the dark and light incubations were shortened (~3 h) to ensure that production was not limited due to available resources within limited core volume.

### 2.9 Data Analysis

MPB-C biomass was determined using chl-a data for cores within all treatments for 0.5 d, 1.5 d, 2.5 d and 3.5 d. We therefore used a two-way analysis of variance (ANOVA) to determine whether MPB-C biomass was affected by treatment and/or time. P/R ratios were determined for 1.5 d, 2.5 d and 3.5 d to determine whether significant differences occurred between treatments within each time period ($\alpha = 0.05$). Levene’s tests indicated that variances were homogeneous in all cases and there were no significant interactions between variables in either analysis. For significant effects of treatment or time, post hoc Tukey tests were used to identify significant differences between groups.

Total uptake for $^{13}$C into both MPB and bacteria, and relative $^{13}$C uptake into MPB were determined for only a single core across all treatments from 0.5 d, 1.5 d, 2.5 d and 3.5 d. To increase replication for statistical analysis, and therefore increase the power to detect a significant difference, we therefore grouped data across times into two levels: before 1.5 d (including 0.5 d and 1.5 d) and after 1.5 d (including 2.5 d and 3.5 d). There was no pooling of data across treatments. A two-way ANOVA was then used to determine whether significant differences occurred among treatments within each pooled time period ($\alpha = 0.05$). No significant interactions were observed for total uptake into MPB or bacteria, but there was a significant interaction observed for relative $^{13}$C uptake into MPB. For significant effects of
interaction, treatment, time, post hoc Tukey tests were used to identify significant differences between groups.

The $^{13}$C remaining in microbial biomass and sediment OC were fitted with an exponential decay function for each treatment across 3.5 d. Loss rate constants for these exponential relationships were compared across treatments and are reported as positive numbers following mathematical conventions associated with loss rates.

3.0 Results

3.1 Uptake of nutrient additions

Uptake of the added nutrients into the sediment was rapid and substantial, as indicated by decreases in dissolved inorganic nitrogen (NH$_4^+$ + NO$_3^-$) concentrations in the overlying core water to $<1.2 \pm 0.1$ µM L$^{-1}$ by 0.5 d. Across the incubation periods, elevated DIN concentrations in overlying water were occasionally observed (Supplemental Fig. 1), but corresponded with times when the cores were sealed for light and dark incubations, indicating that DIN production was a result of in-core processing rather than nutrient amendments.

3.2 Sediment characteristics

Control sediment OC content was greater in the 2-5 cm depth (187.5 ± 27.7 µmol C g$^{-1}$) than at all other sediment depths (112.3 ± 11.4 µmol C g$^{-1}$ in the TS, 149.8 ± 31.6 µmol C g$^{-1}$ at 0-2 cm, and 120.1 ± 16.5 µmol C g$^{-1}$ at 5-10 cm). Natural abundance $\delta^{13}$C values were most enriched in surface sediments ($-18.7\%o$ in TS) and became progressively depleted within deeper sediments to $-22.1\%o$ at 5-10 cm (Table 1). In the 0-2 cm depth of the control sediment,
MPB-C biomass was 321.9 ± 42.0 mmol C m$^{-2}$ and bacterial biomass was 500.4 ± 65.3 mmol C m$^{-2}$ (Table 1).

3.3 Initial $^{13}$C uptake

Uptake of $^{13}$C into sediment OC occurred rapidly and was observed in the first cores collected (11 h after labeling, after tidal flushing, and with cores sectioned in the field). At this time, prior to laboratory incubation and nutrient amendment, 1549 ± 140 µmol $^{13}$C m$^{-2}$ had been incorporated into sediment OC. Sediment OC was $^{13}$C-enriched across all sediment depths at this time (Table 1), but 78% of the initially incorporated $^{13}$C was in the uppermost 2 cm of sediment (compared to 12.8% 2-5 cm, 9.4% 5-10 cm). Prior to incubation, $^{13}$C uptake into microbial biomass at 0-2 cm was dominated by MPB (92.7 ± 1.6%), despite their lower biomass (200.2 ± 26.5 mmol C m$^{-2}$) compared to bacteria (311.3 ± 56.4 mmol C m$^{-2}$) within the labeled cores. Conversely, bacteria dominated $^{13}$C uptake in 2-5 cm sediment (66.8 ± 17.2% of the $^{13}$C within microbial biomass). Although sediment OC at 5-10 cm was $^{13}$C-enriched, minimal uptake was detected in microbial biomarkers.

3.4 Effect of nutrient additions on P/R

Average MPB biomass remained similar across treatments over the 3.5 d incubation (two-way ANOVA: treatment $F_{3,31}=0.04$, $p=0.99$; time $F_{3,31}=0.1$, $p=0.94$, Supplemental Figure 2). However, there were changes in P/R ratio that varied among treatments. Examination of the effects of treatment and time on P/R showed no significant differences (two-way ANOVA: treatment $F_{3,23}=3.0$, $p=0.08$; time $F_{2,23}=2.7$, $p=0.11$), although the post hoc Tukey comparison between ambient and elevated treatments was nearly significant.
(p=0.0506). For the ambient, minimal and moderate treatments, P/R ratios were dominated by autotrophy and changed little over the first 2.5 d (1.5 \pm 0.8, 1.2 \pm 0.4, and 1.3 \pm 0.1, respectively, Fig. 1b) as any increases in production were offset by increased respiration (Fig. 1a). By 3.5 d the minimal treatment had shifted into heterotrophy (0.6 \pm 0.1) as a result of increased respiration and decreased production, whereas P/R ratios for the ambient and moderate treatments remained essentially unchanged (1.3 \pm 0.2, 1.3 \pm 0.4). P/R in the elevated treatment was initially high compared to all other treatments (2.2 \pm 0.2 at 0.5 d) indicating strong dominance of autotrophic production (Fig. 1a & B). However, P/R in the elevated treatment generally decreased to 1.1 \pm 0.4 after 3.5 d (Fig. 1), indicating a strong shift away from autotrophy and towards dominance of heterotrophic processes as respiration increased and production decreased (Fig. 1a).

3.5 Incorporation of $^{13}$C into sediment organic carbon

3.5.1 Uptake of $^{13}$C into 0-2 cm sediment

At 0.5 d, the $^{13}$C incorporated into sediment OC was predominantly contained in the 0-2 cm depth across all treatments (~65%-90%, Fig. 2) and was statistically similar across treatments (one-way ANOVA: $F_{3,7}= 4.2, p=0.1$). By 3.5 d, $^{13}$C retention was lower within sediment from nutrient amended treatments compared to the ambient treatment. Whereas the $^{13}$C contained in the 0-2 cm depth in the ambient treatment was similar across 3.5 d (78.9 \pm 8.8\% 1.5 d, 77.0 \pm 16.4\% 2.5 d, 81.6 \pm 4.4\% 3.5 d), the $^{13}$C content decreased in the minimal, moderate and elevated treatments to 70.3 \pm 8.3\%, 73.6 \pm 16.4\%, and 68.8 \pm 7.6\%, respectively (Fig. 2).
3.5.2 Downward transport below 2 cm

Downward transport of newly labeled material to 2-5 cm depth was low across all treatments, but was higher for the elevated treatment at both 0.5 and 2.5 d. At 0.5 d there was less downward transport in minimal and moderate treatments compared to the ambient and elevated treatments. By 2.5 d downward transport was similar for ambient, minimal and moderate treatments (10%, 9%, 10%, respectively; Fig. 2), but was considerably higher in the elevated treatment (28.4%). By 3.5 d, $^{13}$C incorporation into 2-5 cm sediment OC was similarly low for ambient, minimal and moderate treatments ($8.0 \pm 2.1\%$, $11.1 \pm 0.1\%$, and $8.7 \pm 2.1\%$, respectively), but lower in the elevated treatment ($4.8 \pm 2.1\%$). At 0.5 d, downward transport into the 5-10 cm layer was a relatively small portion of initial $^{13}$C, but was higher in ambient and minimal treatments ($8.7 \pm 2.4\%$ and $11.6 \pm 1.5\%$) when compared to moderate and elevated treatments ($2.3 \pm 1.9\%$ and $6.8 \pm 0.1\%$, Fig. 2). Downward transport below 5 cm was similar (5-11%) for all treatments at 2.5 d and 3.5 d.

3.6 $^{13}$C distribution amongst sediment compartments

3.6.1 Microbial biomass

The total $^{13}$C content of MPB (mmol $^{13}$C m$^{-2}$; Fig. 4a) decreased significantly from before 1.5 d to after 1.5 d for all treatments (two-way ANOVA: $F_{1,8}=12.2$, $p=0.008$), but there was no significant difference among treatments (two-way ANOVA: $F_{3,8}=2.7$, $p=0.12$). The total $^{13}$C content of bacteria (mmol $^{13}$C m$^{-2}$; Fig. 4a) did not change significantly with time, and was not significantly affected by treatment. The majority of the $^{13}$C assimilated into the cores was present in the 0-2 cm depth ($0-2$ cm 2.5 cm $9 \pm 0.8\%$; and $5-10$ cm $5.2 \pm 0.5\%$ Supplemental Fig. 3a, b & c). $^{13}$C incorporation was largely dominated by bacteria across all treatments in sediments below 2 cm, with few exceptions. Increased bacterial contribution
occurred more quickly and was more pronounced in nutrient amended treatments at both 2-5 cm and 5-10 cm (Supplemental Figs. 4 & 5).

Total uptake of excess $^{13}$C (Fig. 4a), while informative about the amount of label contained within each core at each time period, is not as useful for comparison between microbial groups due to variations in the total amount of $^{13}$C assimilated between cores. It is important to consider the relative contribution to $^{13}$C uptake (Fig. 4b) of both microbial groups as each data point was sampled from separate cores that assimilated similar, but different, initial concentrations of newly fixed $^{13}$C. Significant MPB contribution (%) decreased for after 1.5 d (two-way ANOVA: $F_{1,8} = 83.1, p<0.0001$) but showed no difference between treatments ($F_{3,8} = 8.2, p=0.008$), although interaction between the variables was significant ($F_{3,8} = 8.2, p=0.008$). Tukey tests found that MPB contributed less to microbial uptake of $^{13}$C in the elevated treatment than in the ambient treatment ($p=0.01$) as well as the moderate treatment being lower than the minimal treatment ($p=0.014$). MPB dominated the relative incorporation of $^{13}$C into microbial biomass at 0-2 cm in all treatments initially (0.5d; 90% ambient, 90% minimal, 92% moderate, and 92% elevated; Fig. 4b) and throughout the 3.5 d incubation (81-90% ambient, 82-91% minimal, 74-92% moderate, and 65-92% elevated; Fig. 4b). The relative bacterial contribution to microbial $^{13}$C incorporation increased across all treatments as the incubations progressed, but increases in the moderate and elevated treatments at 2.5 and 3.5 d (Fig. 4b) corresponded with decreased $^{13}$C incorporation into MPB (Fig. 4a).

3.6.2 Uncharacterized

A portion of the $^{13}$C contained within sediment OC was uncharacterized, i.e., not contained within the viable microbial biomass measured using PLFA biomarkers. Initially (0.5 d) the uncharacterized pool accounted for less sediment $^{13}$C within the nutrient-amended
treatments (1-3\%) than within the ambient treatment (12\%; Fig. 3), indicating that there was more \(^{13}\)C contained in viable microbial biomass under increased nutrient availability after 12 h of incubation. By 3.5 d increased contribution to the uncharacterized pool in the moderate and elevated treatments (29\% ambient, 32\% minimal, 41\% moderate and 45\% elevated; Fig. 3) corresponded with decreased \(^{13}\)C contained in MPB (52\% ambient, 49\% minimal, 42\% moderate and 26\% elevated). In contrast, changes in the \(^{13}\)C in the uncharacterized pool did not relate to \(^{13}\)C contained in bacteria, as the bacterial contribution to \(^{13}\)C remained relatively unchanged (17\% ambient, 14\% minimal, 15\% moderate and 15\% elevated) and was similar among treatments at 3.5 d.

3.7 Loss of \(^{13}\)C from sediment OC

Rates of \(^{13}\)C loss from sediment OC to the water column were highest in the moderate and elevated treatments (total lost at 3.5 d: ambient 5\%, minimal, 7\%, moderate 11\% and elevated 20\%; Fig. 5 & 6). Reflecting this, loss rate constants for the \(^{13}\)C remaining in sediment OC after accounting for losses of DI\(^{13}\)C and DO\(^{13}\)C across 3.5 d were equivalent for ambient and minimal treatments (0.018 ± 0.024, \(R^2 = 0.95\) and 0.021 ± 0.001, \(R^2 = 0.99\), respectively; Fig. 6), but were higher for both moderate and elevated treatments (0.0383 ± 0.009, \(R^2 = 0.86\), 0.0566 ± 0.003, \(R^2 = 0.99\), respectively; Fig. 6).

Across all treatments, most of the \(^{13}\)C loss from sediment during the incubation occurred via DIC fluxes (Fig. 5). Cumulative \(^{13}\)C export to the water column via DIC fluxes was considerably larger than via DOC fluxes for all treatments (9× ambient, 11× minimal, 10× moderate and 17× elevated). Initial DI\(^{13}\)C loss (0.5 d) was higher in the elevated treatment than in the ambient, minimal, and moderate treatments (5.3 ± 3.4\%, versus 0\%, 1.1 ± 0.3\% and 1.4 ± 1.4\%, respectively; Fig. 5). After 3.5 d, cumulative losses of DI\(^{13}\)C were higher in
moderate and elevated treatments (12.4 ± 11.6% moderate, 19.8 ± 10.8% elevated; Fig. 5) than in ambient (4.0 ± 3.2%) and minimal treatments (6.6 ± 2.0%; Fig. 5 & 7).

DOC export was a less important pathway for $^{13}$C loss than DIC across all treatments. $^{13}$C loss via DOC export was comparable and low across all treatments with similar maximum export at 3.5 d (0.5 ± 0.2% ambient, 0.5 ± 0.2% minimal, 0.4 ± 0.2% moderate, and 0.6 ± 0.5% elevated; Fig. 5).

4.0 Discussion

This study examined the effects of enhanced nutrient loading on the processing pathways for MPB-derived C in intertidal estuarine sediments. Enhanced nutrient availability 1) increased loss of MPB-derived C from sediment via DIC efflux (Fig. 5 & 6), 2) shifted benthic metabolism to be less autotrophic (Fig. 1), and 3) decreased retention of C within MPB (Fig. 3 & 4). These multiple lines of evidence indicate that intertidal sediments in areas experiencing increased nutrient loading are likely to process C differently, resulting in reduced potential for C retention within the sediment.

4.1 Loss pathways for $^{13}$C

Increased nutrient additions caused additional loss of $^{13}$C from sediment OC, largely driven by DIC fluxes to the water column (Fig. 5 & 6). Complete loss of newly produced C from sediment OC, as estimated from exponential decay functions, occurred more quickly in nutrient amended treatments than in ambient (15% increase minimal, 210% increase moderate and 310% increase elevated, Fig. 6). Increased loss rates indicated reduced turnover time for newly produced MPB-derived C under increased nutrient load (419 d ambient versus 199 d moderate and 134 d elevated). It should be noted that the loss rate constant for the minimal
treatment (0.021 ± 0.001, $R^2 = 0.99$, 366 d) was comparable to that for the ambient treatment (0.018 ± 0.024, $R^2 = 0.95$, 419 d), indicating that a small nutrient addition may not cause significant decreases in C turnover time. Increased loss rates imply that C retention and burial in MPB-dominated photic sediments are greatest when nutrients are limiting and that increased nutrient availability alters the processing of MPB-C within the sediment. Increased nitrogen availability appears to have decreased the retention of C within MPB biomass (Fig. 3). Increased turnover of the newly fixed MPB-derived C from the sediment likely occurred as the net result of exudation of material and breakdown of cells. This increased turnover may have caused the increased efflux of MPB-derived C as exudates and cell components were increasingly available to support respiration.

Across all treatments, DIC was the main loss pathway for MPB-C, DOC was a minor pathway and loss via CO$_2$ was considered negligible (Oakes and Eyre 2014) (Fig. 5 & 6). Loss of $^{13}$C via the DIC pathway appears to be stimulated by nutrient additions, resulting in increased export occurring earlier within incubations as a result of increased bacterial remineralization (Fig. 2 & 5). Increased DI$^{13}$C export represents the portion of DI$^{13}$C produced via respiration in excess of that which is re-captured and utilized by MPB to drive production. Given the close proximity of bacteria and MPB in the sediment, there is the potential for considerable utilization of the DI$^{13}$C arising from bacterial remineralization to support algal production. Relatively low fluxes of DI$^{13}$C to the water column in the ambient treatment across 2.5 d likely indicate more complete utilization and recycling of DI$^{13}$C to support algal production (Fig. 5). Export of DI$^{13}$C was considerably higher in both the moderate and elevated treatments, indicating production of DI$^{13}$C during bacterial remineralization in excess of utilization of DI$^{13}$C by MPB. Decreased recycling of DI$^{13}$C from
remineralization in elevated treatments could develop due to 1) decreased DIC demand as algal production decreased after initial stimulation or 2) increased production of unlabeled DIC through remineralization of previously refractory organic material providing an alternative unlabeled source to support algal production.

Cumulative losses of DO$^{13}$C were low for all treatments across 3.5 d (<1.5 % of total $^{13}$C, Fig.5) and did not appear to change significantly with increased nutrient availability. Previous studies have also found that DOC fluxes are a relatively minor contributor to loss of MPB-derived carbon (Oakes et al. 2012; Oakes and Eyre 2014), as observed in the current study, but DOC may be a significant export pathway in other settings. Produced DOC may be labile and respired to DIC prior to loss from the sediment, but this pathway was not greatly altered in this study due to increased nutrient availability.

**4.2 Shifts in benthic metabolism**

Each nutrient amendment produced a different shift in benthic metabolism within the core incubations (Fig. 1) with no clear dose-effect relationship between increased nutrient availability and P/R observed among nutrient-amended treatments. Heterogeneity in both bacterial and MPB biomass are routinely observed within intertidal sediment and can lead to substantial variability between the production and respiration observed between cores (Eyre et al. 2005; Glud 2008). Despite a background of variability between cores, both minimal and elevated treatments display a decrease in autotrophy. The minimal treatment shifted into heterotrophy (P/R<1) and the elevated treatment stimulated initial algal production sufficient to cause a subsequent spike in respiration. Increased respiration by 3.5 d was partially offset by maintained production that kept P/R above 1. In contrast, the moderate treatment maintained a steady P/R across 3.5 d, although substantial error bars indicate considerable
variability between the cores within the treatment. Differences in the response among nutrient-amended treatments appear to result from increased initial production that was supported in both the elevated and moderate treatments, but that decreased by 3.5 d in the minimal treatment. MPB-dominated sediment is expected to be net autotrophic, with positive GPP (Tang and Kristensen 2007) that may be further stimulated by nutrient inputs (Underwood and Kromkamp 1999). Increased algal production of labile organic matter subsequently stimulates heterotrophic respiration, increasing oxygen consumption and lowering P/R (Glud 2008; McGlathery et al. 2007). Quick increases in MPB productivity followed by increased respiration have been observed in response to pulses of organic matter in both oligotrophic and estuarine sediments (Eyre and Ferguson 2005; Glud et al. 2008). Rapid increases in respiration rates, as reflected in the oxygen fluxes for the elevated treatment (Fig. 1a), are often associated with an increased supply of labile C and can occur at rates higher than expected for in situ temperature. This has been observed in subtropical sediments (Eyre and Ferguson 2005) as well as polar and temperate systems (Banta et al. 1995; Rysgaard et al. 1998). Although the sediments in this study were not oligotrophic, the extent of the shift towards heterotrophy is still likely controlled by the amount and relative quality (C/N ratio) of the organic matter available for processing (Cook et al. 2009; Eyre et al. 2008). The similarity in initial P/R ratios between ambient and minimal treatments indicate that a small nutrient addition did not stimulate large increases in algal production, but rather a small increase in production that was offset by increased respiration in the minimal treatment (Fig. 1). The moderate treatment had a distinctly different reaction to increased nutrient availability, with stable P/R as both production and respiration were maintained across 3.5 d. The elevated treatment had increased algal production at 1.5 d, with the highest production rate observed in
this study, and this was followed by a considerable increase in respiration by 3.5 d (increased dark uptake of O\textsubscript{2}; Fig. 1). Decreased autotrophy by 3.5 d was a result of both elevated respiration driven by increased bacterial decomposition of labile material and declining MPB production. It is important to note that the elevated treatment did not shift to a P/R less than 1, but did display a considerable increase in respiration. The rapid increase in respiration in the elevated treatment suggests that the newly produced organic matter was readily bioavailable and quickly processed by bacteria as a result of increased nutrient availability.

4.3 Retention of carbon within microphytobenthos biomass

Within surface sediments, MPB biomass did not increase with increased nutrient load, despite apparent increases in productivity (Supplemental Fig. 2). Although MPB biomass did not change, by 3.5 d the \textsuperscript{13}C retained within MPB biomass in the nutrient-amended treatments appears to have decreased (Fig. 4a) indicating increased turnover of newly fixed C out of MPB biomass. This aligns with many previous reports that increased productivity does not necessarily correspond with increased algal biomass (Alsterberg et al. 2012; Ferguson and Eyre 2013; Ferguson et al. 2007; Hillebrand and Kahlert 2002; Pielhler et al. 2010; Spivak and Ossolinski 2016). Lack of change in MPB biomass, despite increased productivity, may occur as a result of grazing or secondary nutrient limitation (Hillebrand and Kahlert 2002), but these explanations are unlikely for the current study. Grazing is likely to have occurred at only a low level. There was very little fauna, including grazers, within sediment at the study site and, although any grazers such as copepods that were within the site water would have been included in the incubations, larger, mobile grazers were excluded. Secondary nutrient limitation of P or Si was avoided through additions of both elements at 0 d for P and 2.5 d for Si during incubation. It is more likely that the microbial community responded to pulses of
increased nutrients through increased production of extracellular compounds (MPB: carbohydrates; bacteria: enzymes) rather than increasing their biomass (Thornton et al. 2010). This may be a strategy to optimally utilize intermittently available nutrient resources, given that increased cell numbers (biomass) within a biofilm community may otherwise increase competition among cells (Decho 2000; Drescher et al. 2014). Allocation of additional N towards increased production of extracellular enzymes or storage molecules rather than new biomass may therefore benefit the community. Strong competition between MPB and bacteria for available N resulted in a minimal contribution from denitrification as a pathway for N loss likely as a result of limited availability of NO$_3^-$ for denitrifying bacteria (unpubl. data).

**4.4 $^{13}$C distribution within the sediment**

**4.4.1 Microbial biomass**

Decreased autotrophy is somewhat reflected in the relative partitioning of $^{13}$C from newly produced algal C between MPB and bacteria within the individual treatments (Fig. 1 & Fig. 4b). Initially, uptake of $^{13}$C was strongly dominated by MPB amongst treatments, with minimal incorporation by bacteria. As incubations progressed, a shift towards increased relative contribution by bacteria was apparent in all treatments, but was more substantial in the elevated treatment (3.5 d; 19% ambient, 18% minimal, 26% moderate, and 35% elevated, Fig. 4b). This quicker shift towards bacterial dominance of $^{13}$C incorporation corresponded with the largest decrease in P/R ratios observed in this study, as increased respiration and decreased production caused the elevated treatment to become less autotrophic (Fig. 1). These corresponding factors are likely a result of a tight coupling and intense recycling between algal production and bacterial processing of newly produced MPB-derived C. EPS can be a large export pathway for newly fixed C from algal cells (up to 70.3% Goto et al. 1999) and
can provide a labile C source for heterotrophic or denitrifying bacteria. The $^{13}$C incorporated into bacteria represents the balance of respiration and uptake and is expected to become increasingly muddled by $^{13}$C being processed through other pathways (denitrification) as incubations progress. Therefore, this study only considered the transfer of MPB-C into bacteria at the 0.5 d sampling. However, given the low initial transfer of $^{13}$C to bacteria in all treatments over 24 h following labeling (0.5 d; 0.8% h$^{-1}$ ambient, 0.8% h$^{-1}$ minimal, 0.7% h$^{-1}$ moderate, 0.7% h$^{-1}$ elevated; Fig. 3) it appears that either production or utilization of EPS containing newly fixed C was relatively low in the current study, regardless of nutrient addition. This transfer was the net result of EPS production and bacterial remineralization and would have become increasingly muddled as $^{13}$C-containing detrital material accumulated as incubations progressed. Low EPS production at 0.5 d may indicate that N is not limiting for MPB in these sediments, as exuded EPS does not appear to be copious, as would be expected under severe N limitation (van Den Meersche et al. 2004). Similarly low rates of C transfer from MPB to bacteria were previously reported for the site (0.83% h$^{-1}$, Oakes and Eyre 2014) and are towards the lower end of the range of EPS production rates for benthic diatoms (0.05 to 73% h$^{-1}$; Underwood and Paterson 2003). At 0.5 d nutrient availability appears to have had little effect on the initial transfer rates from MPB to bacteria, but appears to have decreased the turnover of MPB-C out of the microbial community, as contributions of $^{13}$C to the uncharacterized pool were lower in the nutrient-amended treatments (Fig. 3). By 3.5 d, increased nutrient availability appears to stimulate the transfer of $^{13}$C from microbial biomass in the uncharacterized pool, but had no effect on $^{13}$C in bacteria as the bacterial pool was equal across all treatments (15-18%, Fig. 3 & 7).
A portion of the $^{13}$C incorporated into sediment OC was uncharacterized (i.e., not within microbial biomass). By 3.5 d, the portion of initially incorporated $^{13}$C that was within the uncharacterized pool varied substantially among the treatments (29-46%, Fig. 7). This uncharacterized C is likely to represent a mixture of both labile and refractory OC (Veuger et al. 2012), including metabolic byproducts, senescent cells undergoing breakdown, EPS, extracellular enzymes, carbohydrates, and a variety of complex, molecularly uncharacterized organic matter (Hedges et al. 2000). Collectively, these molecules form a pool of labeled intra- and extra-cellular material remaining in sediment OC derived from both MPB and bacteria that is not characterized as microbial biomass when using PLFAs to estimate microbial biomass (e.g., $^{13}$C contained in storage products or enzymes that was not incorporated into phospholipids). Given that MPB can direct up to 70% of their newly fixed C to EPS (Goto et al. 1999), carbohydrates are likely to form a considerable portion of the uncharacterized $^{13}$C.

A study using a similar $^{13}$C-labeling approach reported that 15-30% of MPB-derived carbon was transferred to intra- and extracellular carbohydrates within 30 d after an initial transfer rate of ~0.4% into bacteria (2 d; Oakes et al. 2010a). In light of the higher transfer rates for $^{13}$C into bacteria observed in this study (0.7 to 0.9% h$^{-1}$), there is potential for a considerable portion of the uncharacterized pool to be accounted for by EPS.

When quantified, the uncharacterized C pool typically has a high C:N ratio (10 to 60; Cook et al. 2009; Eyre et al. 2016a), indicating that nitrogen availability may have a role in regulating its content and accumulation. Given that nitrogen limitation has been observed to suppress processing pathways of otherwise labile OM in soils (Jian et al. 2016; Schimel and Bennett 2004), a similar mechanism may be possible in estuarine sediments. This mechanism
may include a priming effect due to either increased production of extracellular enzymes or
due to increased energy from labile C compounds allowing for the increased breakdown of
sediment OM (Bianchi 2011). Increased extracellular enzyme production would result in more
complete utilization of sediment OM through promotion of hydrolysis (Arnosti 2011; Huettel
et al. 2014), a potentially limiting step during the breakdown of organic material. This would
result in more complete utilization of $^{13}$C by microbial biomass and a smaller pool of
uncharacterized C within sediment OC, as was observed in the nutrient-amended treatments at
0.5 d (Fig. 3). This is further supported by the increased turnover of MPB-C from microbial
biomass into the uncharacterized pool observed within the nutrient amended treatments (2.5 d,
Fig. 3) indicating $^{13}$C that was previously incorporated into MPB was processed into the
uncharacterized pool more quickly with increased nutrient availability. After 2.5 d, the $^{13}$C
content of the uncharacterized pool was substantially larger for the elevated treatment (Fig. 3
& 7) and looks to have been largely sourced from MPB $^{13}$C, given that bacterial contribution
to sediment OM remained stable. Composition of the uncharacterized pool will be study-
specific depending on the different biomarker techniques utilized to estimate microbial
biomass incorporating different pools of material. The metabolic pathways and ecological
strategies regulating the portion of $^{13}$C entering the uncharacterized pool warrant further
investigation.

4.5 Downward transport

Increased nutrient availability reduced the downward transport of fixed $^{13}$C,
particularly within 2-5 cm, mainly as a result of increased export of MPB-C to the water
column. In the ambient treatment, downward transport to 2-5 cm (10.0%) and 5-10 cm (9.2%)
across 60 h was comparable to that reported by Oakes and Eyre (2014) for the same site (8.3%
2-5 cm, 14.9% 5-10 cm, 60 h). Oakes and Eyre (2014) suggested that resuspension resulting
from a flood event limited the downward transport of $^{13}$C, but a comparable and lower rate of
downward transport at 60 h (12.1% 2-5 cm, 9% 5-10 cm, ambient treatment) was observed in
the current study in the absence of marked freshwater inflow. Downward transport is not a
large pathway for loss of $^{13}$C within this system as transport to sediment below 2 cm was
minimal, and appeared further reduced in the elevated treatment (Supplemental Fig. 3b & c).
Decreased downward transport of MPB-derived C under increased nutrient load may reflect 1)
decreased transport to depth as diatoms reduce migration downward to find nutrients
(Saburova and Polikarpov 2003) or 2) relaxation of the tight recycling and retention of newly
fixed C between MPB and bacteria within surface sediments allowing for increased export of
labile C to the water column (Cook et al. 2007). Decreased downward transport in this study
likely reflects a combination of reduced algal transport of $^{13}$C to depth and increased loss of
$^{13}$C from surface sediments to the water column.

### 4.6 Implications

This study has provided valuable insight into the processing of MPB-derived C under
increased nutrient availability using multiple lines of evidence (budgeting $^{13}$C within sediment
compartments and sediment-water effluxes, partitioning of C pools via biomarkers, and
changes in P/R) and is among the first to have addressed this problem. However, some caveats
on interpretation are important to note, as follows: 1) *Ex situ* incubation of sediment cores
may not be directly comparable to processes occurring *in situ* and may overestimate C
retention, as there is reduced potential for loss via sediment resuspension due to tidal
movement, water currents, and grazing. 2) Removal of grazers may also increase MPB
production and their release of exudates (Fouilland et al. 2014), which could enhance $^{13}$C
transfer to bacteria. However, given the lack of apparent grazers at the site of the current study, and the low observed $^{13}$C transfer rate to bacteria (0.7-0.9% h$^{-1}$ Fig. 4b) that was comparable to previously measured in situ rates in Oakes and Eyre (2014), grazers appear to have had little potential impact on sediment processing in this study.

The findings show that increased nutrient availability reduced C retention, but the main export pathway for algal carbon remained the same (primarily loss via DIC). Coastal environments are recognized as important sites for carbon storage. Although the focus has primarily been on vegetated environments (Duarte et al. 2005), which store the most carbon, unvegetated sediments also have capacity for longer-term retention (e.g. 30% after 30 d Oakes and Eyre 2014; 31% after 30 d Oakes et al. 2012). Based on N burial rates (and corresponding unpublished C burial rates) some coastal systems can have higher C burial rates in subtidal and intertidal macrophyte-free MPB sediments than in macrophyte-dominated sediments (Eyre et al. 2016b; Maher and Eyre 2011) although this was shown in only one of the three estuaries studied. Increased nutrient loading into coastal settings has been implicated in historical decreases of long-term carbon storage through a shift from macrophyte dominated systems (seagrass and mangrove) towards MPB dominated systems (Macreadie et al. 2012) within coastal environments. Carbon storage potential within MPB dominated sediments remains a significant knowledge gap within the carbon budgets of estuaries. At 30 d, estimates of retention of C identified for ambient and minimal treatments were considerable in the current study (58% and 54%), however, increased nutrient loading reduced this retention considerably (32% moderate, 18% elevated). Given that nutrient inputs have increased globally and bare photic sediment accounts for a large surface area within estuaries, these two factors could have resulted in substantial release of currently stored carbon and demonstrate
the capacity for further substantial reduction of C storage potential globally if elevated

nutrient inputs continue within estuarine systems.

Although MPB-dominated sediments probably have less decadal-scale long-term storage of C than macrophyte-dominated sediments, this study clearly demonstrates that the existing storage potential is further degraded by increased nutrient loading within MPB-dominated sediments. These sediments may lock away less C per area, but are fairly ubiquitous within photic coastal and oceanic sediment and may contribute significantly to carbon storage within coastal systems due to this increased area. The observed increases in mobility of newly fixed algal carbon from intertidal sediments (Fig. 5) as a result of elevated anthropogenic nutrient loading will directly translate to increased carbon export to coastal oceans and reduced carbon storage potential within shallow photic estuarine sediments.

References


**Author Contribution**

PR planned the experimental design and field work, performed the field work, isolation of biomarkers and laboratory analysis, and wrote the manuscript. JO planned the experimental...
design and field work, contributed to the data interpretation and assisted with statistical analysis and writing of the manuscript. BE planned the experimental design and field work, contributed to the data interpretation and assisted with the writing of the manuscript. The group of co-authors has approved the submission of this manuscript.

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Figures and Tables

Table 1: $\delta^{13}$C values (‰) and carbon biomass (µmol C m$^{-2}$) for control (natural abundance, n=3) and initially labeled cores (n=3, 0 d). Microphytobenthos and bacterial biomass are only provided for control cores.
Figure 1: Oxygen fluxes and ratio of production to respiration (P/R) for all treatments across 24 h calculated from oxygen fluxes for individual cores. Values are mean ± SE.
Figure 2: Carbon budget for excess $^{13}$C within sediment OC at top scrape (TS), 0.2 to 2 cm, 2 to 5 cm, 5-10 cm, and the cumulative excess $^{13}$C exported to the water column via the combined efflux of DIC and DOC for each treatment at each sampling time. All values are as a percentage of the $^{13}$C initially incorporated into sediment OC (0-10 cm). Some error bars are too small to be seen (mean ± SE).
**Figure 3:** Excess $^{13}$C incorporation into microphytobenthos, bacteria, and uncharacterized OC as a percentage of $^{13}$C contained in sediment OC in 0-10 cm. There are no error bars as PLFAs were analyzed for only one replicate sample from each time period.
Figure 4: $^{13}$C within MPB and bacterial biomass in sediment at 0-2 cm depth as A) total excess $^{13}$C (mmol $^{13}$C m$^{-2}$) and B) a percentage of the total $^{13}$C in microbial biomass at 0-2 cm at each time period. There are no error bars as PLFAs were analyzed for only one replicate sample from each time period.
Figure 5: Effluxes of $^{13}$C from the sediment as dissolved organic carbon (DOC) and dissolved inorganic carbon (DIC) as a percentage of the total $^{13}$C contained in sediment at 0-10 cm depth at each sampling time (mean ± SE).
Figure 6: The percentage of $^{13}$C remaining in sediment OC (0-10 cm depth) after accounting for losses of DI$^{13}$C and DO$^{13}$C to the water column (mean ± SE). Lines are exponential decay functions for each treatment across the 3.5 d of incubation (Loss rate constant, $R^2$ of function).
Figure 7: Distribution of $^{13}$C at 3.5 d of incubation of inundated sediment including loss pathways for DIC and DOC. The $^{13}$C contained in sediment organic carbon (sediment OC) is further partitioned into microphytobenthos, bacteria, and uncharacterized organic carbon as a percentage of the $^{13}$C in sediment organic carbon at 0-10 cm 3.5 d after labeling (Figure layout from Eyre et al., 2016).