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

Philip M. Riekenberg[1,a*], Joanne M. Oakes[1], Bradley D. Eyre[1]

[1]Centre for Coastal Biogeochemistry, Southern Cross University, PO Box 157, Lismore, NSW, 2480, Australia

[a]Present address: NIOZ Royal Netherlands Institute for Sea Research, Department of Marine Microbiology and Biogeochemistry, PO Box 59, 1790AB Den Burg

[*] Corresponding author: phrieken@gmail.com, (0031) 222 369 409

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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 (NH_4^+ and 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 rare isotope tracers can render fractionation effects and variability that affect natural abundance stable isotope techniques negligible and has 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 CO_2 (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 flux of newly produced C from sediments (Andersson et al. 2008; Oakes et al. 2012; Van Nugteren et al. 2009).

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; Hardison et al. 2011). 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 or an increase in production of extracellular enzymes 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; Pascal et al. 2013; 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 (Evrard 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 short-term effects of increased nutrient concentrations on the processing pathways for MPB-derived C within subtropical intertidal sediments. 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 production of MPB-C 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 km^2 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, 0.13± 0.02 %OC; 0-2 cm 17.2 ± 1.7, 0.18± 0.07 %OC;~~,~~ 2-5 cm 10.9 ± 0.5, 0.23± 0.06 %OC;~~,~~ 5-10 cm 16.2 ± 2.2, 0.14± 0.03 %OC).

2.2 Overview

We labeled MPB with ^{13}C via in situ application of ^{13}C-labeled sodium bicarbonate to exposed intertidal sediments. Unincorporated ^{13}C 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 δ^{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-*a* (Chl-*a*) 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 (NH_4^+) and P (H_3PO_4) amendment to unfiltered 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 (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 NH_4^+ 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 NH_4^+ within treatment tank water, as nutrients were readily processed (Supplemental Fig. 1). Processing of newly fixed MPB-C occurred in the 13 h prior to incubation with nutrient amendments, but was likely similar across cores as they were kept in identical conditions prior to incubation before random allocation to treatments. Although

MPB-C was not freshly fixed at 13 h and likely more refractory as a result, the available C was still relatively labile and readily processed across all treatments.

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^{o}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 to allow for the re-establishment of microclimates and anaerobic zonation that was potentially disturbed by coring. 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 (~15 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. Light incubations were of shorter duration to prevent supersaturation of dissolved oxygen which would have

compromised additional analyses required for a complementary study. 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. These sampling time periods were chosen to capture the active dynamics of ^{13}C processing that were expected to occur over the first few days of the study, based on previous work by Oakes and Eyre (2014). 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 consistently present across all samples and was used as a biomarker for diatoms, following correction for contributions from gram-negative bacteria, cyanobacteria, and sulfate reducing bacteria, determined using 18:1n7 as described in the calculations section below and in Oakes et al. (2016). 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

239 supernatant, gently mixed, and phases were allowed to separate prior to removal of the bottom
240 layer into a round bottom flask. The top layer was then rinsed with 15 ml of DCM, gently
241 shaken, and phases allowed to separate prior to addition to the round bottom flask. This
242 extract was then concentrated under vacuum and separated using silica solid phase extraction
243 columns (Grace; 500 mg, 6.0 ml) by elution with 5 ml each of chloroform, acetone, and
244 methanol. The fraction containing methanol was retained, reduced to dryness under N_2,
245 methylated (3 ml 10:1:1 MeOH:HCl:$CHCl_3$, 80˚C, 2 h), quenched using first 3 ml and then 2
246 ml of 4:1 hexane:DCM, evaporated to ~ 200 µl under N_2, transferred to a GC vial for analysis,
247 and stored frozen (-20˚C). PLFA concentrations and $\delta^{13}C$ values were measured using a non-
248 polar 60 m HP5-MS column in a Trace GC coupled to a Delta V IRMS with a Thermo Conflo
249 III interface following the protocol outlined in Oakes et al. (2010a).

250 DIC and DOC concentrations and $\delta^{13}C$ values were measured via continuous-flow wet
251 oxidation isotope-ratio mass spectrometry using an Aurora 1030W total organic C analyzer
252 coupled to a Thermo Delta V isotope ratio mass spectrometer (IRMS) (Oakes et al. 2010b).
253 Sodium bicarbonate (DIC) and glucose (DOC) of known isotopic composition dissolved in
254 He-purged Milli-Q were used to correct for drift and verify both concentration and $\delta^{13}C$ of
255 samples. Reproducibility was ± 0.2 mg L^{-1} and ± 0.1 ‰ for DIC and ± 0.2 mg L^{-1} and ± 0.4 ‰
256 for DOC.

257 **2.8 Calculations**

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

unit area. Percentages calculated from these pools are presented as portions of the sum of total ^{13}C contained within the sediment and the interpolated fluxes of DIC and DOC that were estimated to have occurred from 0 d until each sampling time.

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. $\text{Biomass}_{\text{bacteria}} = \text{Biomass}_{\text{i+a15:0}} / (a \times b)$

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:

2. $\text{Biomass}_{\text{Diatom}} = \text{Biomass}_{\text{corrected16:1(n-7)}} / (c \times d)$

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 of 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. Microbial biomass is the sum of calculated diatom and bacterial biomass. Uncharacterized ^{13}C was calculated as:

3. $^{13}C_{\text{uncharacterized}} = {}^{13}C_{\text{sediment organic}} - {}^{13}C_{\text{microbial biomass}}$

where $^{13}C_{\text{sediment organic}}$ represents total ^{13}C in sediment organic carbon and $^{13}C_{\text{microbial biomass}}$ represents the ^{13}C contained in microbial biomass within the same core.

Fluxes across the sediment-water interface were calculated from two measured concentrations, at the start and finish of each dark and light period (e.g., Oakes and Eyre 2014), 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.~~4. Respiration (R) = Dark DO flux h^{-1}

~~4.~~5. Net primary production (NPP) = Light DO flux h^{-1}

~~5.~~6. Gross primary production (GPP) = NPP + R

7. Production/respiration (P/R) = GPP x daylight hours (12 h) / R x 24 h (Eyre et al. 2011)

To prevent the potential development of resource limitations during incubation, 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 allowed to become supersaturated.

Total ^{13}C in DIC and DOC (µmol ^{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 (µmol ^{13}C m^{-2} h^{-1}) was then calculated as:

~~6.~~8. 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:

~~7.~~9. 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. Because all ^{13}C was contained within the cores, values for ^{13}C budgets add to 100%. Starting values were estimated by looking at how much ^{13}C remained in the sediment and how much was lost to the water column (initial ^{13}C= ^{13}C remaining + ^{13}C lost).

2.9 Data Analysis

^{13}C remaining in sediment and 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 either ^{13}C remaining in sediment or MPB-C biomass were 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 data for ^{13}C remaining in sediment OC were further examined by fitting an exponential decay function for each treatment across 3.5 d using the Exp2PMod1 function in OriginPro 2017 and ^{13}C turnover estimates were then determined by solving for y = 0.05 % remaining ^{13}C (a value close to 0) and x = 30 d for each treatment. Exponential decay functions were compared across treatments by dividing by 100 to convert percentages to proportions and transforming with log(n+1) to convert into linear functions with 95% confidence bands (Supplemental Fig. 2). Loss rate constants are reported as positive numbers following mathematical conventions associated with loss rates (Fig. 6).

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 ($NH_4^+ + NO_x$) 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‰ in TS) and became progressively depleted within deeper sediments to -22.1‰ 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 3). 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 ± 0.8, 1.2 ± 0.4, and 1.3 ± 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 ± 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 ± 0.2, 1.3 ± 0.4). P/R in the elevated treatment was initially high compared to all other treatments (2.2 ± 0.2 at 0.5 d) indicating strong dominance of autotrophic production (Fig. 1a & B). However, P/R in the elevated treatment decreased to 1.1 ± 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

411 ^{13}C contained in the 0-2 cm depth in the ambient treatment was similar across 3.5 d (78.9 ±
412 8.8% 1.5 d, 77.0 ± 16.4% 2.5 d, 81.6 ± 4.4% 3.5 d), the ^{13}C content decreased in the minimal,
413 moderate and elevated treatments to 70.3 ± 8.3%, 73.6 ± 16.4%, and 68.8 ± 7.6%, respectively
414 (Fig. 2).

415 **3.5.2 Downward transport below 2 cm**

416 Downward transport of newly labeled material to 2-5 cm depth was low across all
417 treatments, but was higher for the elevated treatment at both 0.5 and 2.5 d. At 0.5 d there was
418 less downward transport in minimal and moderate treatments compared to the ambient and
419 elevated treatments. By 2.5 d downward transport was similar for ambient, minimal and
420 moderate treatments (10%, 9%, 10%, respectively; Fig. 2), but was considerably higher in the
421 elevated treatment (28.4%). By 3.5 d, ^{13}C incorporation into 2-5 cm sediment OC was
422 similarly low for ambient, minimal and moderate treatments (8.0 ± 2.1%, 11.1 ± 0.1%, and 8.7
423 ± 2.1%, respectively), but lower in the elevated treatment (4.8 ± 2.1%). At 0.5 d, downward
424 transport into the 5-10 cm layer was a relatively small portion of initial ^{13}C, but was higher in
425 ambient and minimal treatments (8.7 ± 2.4% and 11.6 ± 1.5%) when compared to moderate
426 and elevated treatments (2.3 ± 1.9% and 6.8 ± 0.1%, Fig. 2). Downward transport below 5 cm
427 was similar (5-11%) for all treatments at 2.5 d and 3.5 d.

428 **3.6 ^{13}C distribution amongst sediment compartments**

429 **3.6.1 Microbial biomass**

430 The total ^{13}C content of MPB (mmol ^{13}C m^{-2}; Fig. 4a) decreased significantly from
431 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
432 was no significant difference among treatments (two-way ANOVA: $F_{3,8}$=2.7, p=0.12). The
433 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 ± 0.8%; and 5-10 cm 5.2 ± 0.5% Supplemental Fig. 4a, 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. 5 & 6).

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

The total ^{13}C remaining in sediment (Fig. 6) varied significantly among treatments (two-way ANOVA: $F_{3, 31}= 5.7$, $p=0.008$) and across sampling times ($F_{3, 31}= 3.9$, $p=0.03$). Throughout the study, there was generally less ^{13}C remaining within the elevated treatment than in than either the ambient ($p=0.008$) or minimal treatments ($p=0.02$), and there was significantly less ^{13}C remaining within the sediment at 3.5 d than at 0.5 d ($p=0.02$). 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

480 minimal treatments (0.018 ± 0.024, R^2 = 0.95 and 0.021 ± 0.001, R^2 = 0.99, respectively; Fig.
481 6), but were higher for both moderate and elevated treatments (0.0383 ± 0.009, R^2 = 0.86,
482 0.0566 ± 0.003, R^2 = 0.99, respectively; Fig. 6). Since the intercept is known, i.e., the initial
483 value equals 100% at time 0, linear models where only the slopes were estimable, were fitted
484 to further analyze the differences between slopes. Assuming an exponential decay, the
485 percentage remaining ^{13}C (Y) was $\log_{10}$ transformed and the value 2 was subsequently
486 subtracted ($Z=\log_{10}(Y) -2$), which implies that the intercept of Z versus time equals 0. The
487 model with different slopes for each treatment fitted significantly better than the model with a
488 single slope (F-test, $F_{3,28}=9.84$, $P <0.001$, Supplemental Fig. 2). The analysis was performed
489 in R.

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

498 DOC export was a less important pathway for ^{13}C loss than DIC across all treatments.
499 ^{13}C loss via DOC export was comparable and low across all treatments with similar maximum
500 export at 3.5 d (0.5 ± 0.2% ambient, 0.5 ± 0.2% minimal, 0.4 ± 0.2% moderate, and 0.6 ±
501 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

525 the net result of exudation of material and breakdown of cells. This increased turnover may
526 have caused the increased efflux of MPB-derived C as exudates and cell components were
527 increasingly available to support respiration. The focus of this study was short-term fate of
528 MPB-C, but our findings also show potential implications for longer- term retention. Our
529 calculated retention times may be under or over-estimated due to their reliance on short-term
530 data. However, the relative differences between treatments (decreased retention with increased
531 nutrient amendment) are clear. The rationale for utilizing exponential functions in this study
532 follows previous findings in Oakes et al. (2014) that ^{13}C export from subtidal sediments at this
533 site were well-described by an exponential decay function across a longer time period (31 d).
534 Additionally, the 30 d estimates provided within this study (18-58%) fall across a range
535 similar to that of other previous labeling experiments (30-50%; Hardison et al. 2011; Oakes
536 and Eyre 2014; Oakes et al. 2012), leading the authors to conclude that the use of exponential
537 functions to describe this relationship was valid in this study.

538 Across all treatments, DIC was the main loss pathway for MPB-C, DOC was a minor
539 pathway and loss via CO_2 was considered negligible (Oakes and Eyre 2014) (Fig. 5 & 6). Loss
540 of ^{13}C via the DIC pathway appears to be stimulated by nutrient additions, resulting in
541 increased export occurring earlier within incubations as a result of increased bacterial
542 remineralization (Fig. 2 & 5). Increased $DI^{13}C$ export represents the portion of $DI^{13}C$
543 produced via respiration in excess of that which is re-captured and utilized by MPB to drive
544 production. Given the close proximity of bacteria and MPB in the sediment, there is the
545 potential for considerable utilization of the $DI^{13}C$ arising from bacterial remineralization to
546 support algal production. Relatively low fluxes of $DI^{13}C$ to the water column in the ambient
547 treatment across 2.5 d likely indicate more complete utilization and recycling of $DI^{13}C$ to

548 support algal production (Fig. 5). Export of $DI^{13}C$ was considerably higher in both the
549 moderate and elevated treatments, indicating production of $DI^{13}C$ during bacterial
550 remineralization in excess of utilization of $DI^{13}C$ by MPB. Decreased recycling of $DI^{13}C$ from
551 remineralization in elevated treatments could develop due to 1) decreased DIC demand as
552 algal production decreased after initial stimulation or 2) increased production of unlabeled
553 DIC through remineralization of previously refractory organic material providing an
554 alternative unlabeled source to support algal production.

555 Cumulative losses of $DO^{13}C$ were low for all treatments across 3.5 d (<1.5 % of total
556 ^{13}C, Fig.5) and did not appear to change significantly with increased nutrient availability.
557 Previous studies have also found that DOC fluxes are a relatively minor contributor to loss of
558 MPB-derived carbon (Oakes et al. 2012; Oakes and Eyre 2014), as observed in the current
559 study, but DOC may be a significant export pathway in other settings. Produced DOC may be
560 labile and respired to DIC prior to loss from the sediment, but this pathway was not greatly
561 altered in this study due to increased nutrient availability.

562 **4.2 Shifts in benthic metabolism**

563 Each nutrient amendment produced a different shift in benthic metabolism within the
564 core incubations (Fig. 1) with no clear dose-effect relationship between increased nutrient
565 availability and P/R observed among nutrient-amended treatments. Heterogeneity in both
566 bacterial and MPB biomass are routinely observed within intertidal sediment and can lead to
567 substantial variability between the production and respiration observed between cores (Eyre et
568 al. 2005; Glud 2008). Despite a background of variability between cores, both minimal and
569 elevated treatments display a decrease in autotrophy. The minimal treatment shifted into
570 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_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. 3). Although MPB biomass did not change, by 3.5 d the ^{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; Piehler 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

617 included in the incubations, larger, mobile grazers were excluded. Secondary nutrient
618 limitation of P or Si was avoided through additions of both elements at 0 d for P and 2.5 d for
619 Si during incubation. It is more likely that the microbial community responded to pulses of
620 increased nutrients through increased production of extracellular compounds (MPB:
621 carbohydrates; bacteria: enzymes) rather than increasing their biomass (Thornton et al. 2010).
622 This may be a strategy to optimally utilize intermittently available nutrient resources, given
623 that increased cell numbers (biomass) within a biofilm community may otherwise increase
624 competition among cells (Decho 2000; Drescher et al. 2014). Allocation of additional N
625 towards increased production of extracellular enzymes or storage molecules rather than new
626 biomass may therefore benefit the community. Strong competition between MPB and bacteria
627 for available N resulted in a minimal contribution from denitrification as a pathway for N loss
628 likely as a result of limited availability of NO_3^- for denitrifying bacteria (unpubl. data).

629 **4.4 ^{13}C distribution within the sediment**

630 **4.4.1 Microbial biomass**

631 Decreased autotrophy is somewhat reflected in the relative partitioning of ^{13}C from
632 newly produced algal C between MPB and bacteria within the individual treatments (Fig. 1 &
633 Fig. 4b). Initially, uptake of ^{13}C was strongly dominated by MPB amongst treatments, with
634 minimal incorporation by bacteria. As incubations progressed, a shift towards increased
635 relative contribution by bacteria was apparent in all treatments, but was more substantial in the
636 elevated treatment (3.5 d; 19% ambient, 18% minimal, 26% moderate, and 35% elevated, Fig.
637 4b). This quicker shift towards bacterial dominance of ^{13}C incorporation corresponded with
638 the largest decrease in P/R ratios observed in this study, as increased respiration and decreased
639 production caused the elevated treatment to become less autotrophic (Fig. 1). These

640 corresponding factors are likely a result of a tight coupling and intense recycling between
641 algal production and bacterial processing of newly produced MPB-derived C. EPS can be a
642 large export pathway for newly fixed C from algal cells (up to 70.3% Goto et al. 1999) and
643 can provide a labile C source for heterotrophic or denitrifying bacteria. The ^{13}C incorporated
644 into bacteria represents the balance of respiration and uptake and is expected to become
645 increasingly muddled by ^{13}C being processed through other pathways (denitrification) as
646 incubations progress. Therefore, this study only considered the transfer of MPB-C into
647 bacteria at the 0.5 d sampling. However, given the low initial transfer of ^{13}C to bacteria in all
648 treatments over 24 h following labeling (0.5 d; 0.8% h^{-1} ambient, 0.8% h^{-1} minimal, 0.7% h^{-1}
649 moderate, 0.7% h^{-1} elevated; Fig. 3) it appears that either production or utilization of EPS
650 containing newly fixed C was relatively low in the current study, regardless of nutrient
651 addition. This transfer was the net result of EPS production and bacterial remineralization and
652 would have become increasingly muddled as ^{13}C-containing detrital material accumulated as
653 incubations progressed. Low EPS production at 0.5 d may indicate that N is not limiting for
654 MPB in these sediments, as exuded EPS does not appear to be copious, as would be expected
655 under severe N limitation (van Den Meersche et al. 2004). Similarly low rates of C transfer
656 from MPB to bacteria were previously reported for the site (0.83% h^{-1}, Oakes and Eyre 2014)
657 and are towards the lower end of the range of EPS production rates for benthic diatoms (0.05
658 to 73% h^{-1}; Underwood and Paterson 2003). At 0.5 d nutrient availability appears to have had
659 little effect on the initial transfer rates from MPB to bacteria, but appears to have decreased
660 the turnover of MPB-C out of the microbial community, as contributions of ^{13}C to the
661 uncharacterized pool were lower in the nutrient-amended treatments (Fig. 3). By 3.5 d,
662 increased nutrient availability appears to stimulate the transfer of ^{13}C from microbial biomass

663 in the uncharacterized pool, but had no effect on ^{13}C in bacteria as the bacterial pool was equal
664 across all treatments (15-18%, Fig. 3 & 7).

665 **4.4.2 Uncharacterized**

666 A portion of the ^{13}C incorporated into sediment OC was uncharacterized (i.e., not
667 within microbial biomass). By 3.5 d, the portion of initially incorporated ^{13}C that was within
668 the uncharacterized pool varied substantially among the treatments (29-46%, Fig. 7). This
669 uncharacterized C is likely to represent a mixture of both labile and refractory OC (Veuger et
670 al. 2012), including metabolic byproducts, senescent cells undergoing breakdown, EPS,
671 extracellular enzymes, carbohydrates, and a variety of complex, molecularly uncharacterized
672 organic matter (Hedges et al. 2000). Collectively, these molecules form a pool of labeled intra
673 and extra-cellular material remaining in sediment OC derived from both MPB and bacteria
674 that is not characterized as microbial biomass when using PLFAs to estimate microbial
675 biomass (e.g., ^{13}C contained in storage products or enzymes that was not incorporated into
676 phospholipids). Given that MPB can direct up to 70% of their newly fixed C to EPS (Goto et
677 al. 1999), carbohydrates are likely to form a considerable portion of the uncharacterized ^{13}C.
678 A study using a similar ^{13}C-labeling approach reported that 15-30% of MPB-derived carbon
679 was transferred to intra- and extracellular carbohydrates within 30 d after an initial transfer
680 rate of ~0.4% into bacteria (2 d; Oakes et al. 2010a). In light of the higher transfer rates for
681 ^{13}C into bacteria observed in this study (0.7 to 0.9% h^{-1}), there is potential for a considerable
682 portion of the uncharacterized pool to be accounted for by EPS.

683 When quantified, the uncharacterized C pool typically has a high C:N ratio (10 to 60;
684 Cook et al. 2009; Eyre et al. 2016a), indicating that nitrogen availability may have a role in
685 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. 4b & 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

732 movement, water currents, and grazing. 2) Removal of grazers may also increase MPB
733 production and their release of exudates (Fouilland et al. 2014), which could enhance ^{13}C
734 transfer to bacteria. However, given the lack of apparent grazers at the site of the current
735 study, and the low observed ^{13}C transfer rate to bacteria (0.7-0.9% h^{-1} Fig. 4b) that was
736 comparable to previously measured in situ rates in Oakes and Eyre (2014), grazers appear to
737 have had little potential impact on sediment processing in this study.

738 The findings show that increased nutrient availability reduced C retention, but the
739 main export pathway for algal carbon remained the same (primarily loss via DIC). Coastal
740 environments are recognized as important sites for carbon storage. Although the focus has
741 primarily been on vegetated environments (Duarte et al. 2005), which store the most carbon,
742 unvegetated sediments also have capacity for longer-term retention (e.g. ~50% after 21 d
743 Hardison et al. 2011, 30% after 30 d Oakes and Eyre 2014; 31% after 30 d Oakes et al. 2012).
744 Based on N burial rates (and corresponding unpublished C burial rates) some coastal systems
745 can have higher C burial rates in subtidal and intertidal macrophyte-free MPB sediments than
746 in macrophyte-dominated sediments (Eyre et al. 2016b; Maher and Eyre 2011) although this
747 was shown in only one of the three estuaries studied. Increased nutrient loading into coastal
748 settings has been implicated in historical decreases of long-term carbon storage through a shift
749 from macrophyte dominated systems (seagrass and mangrove) towards MPB dominated
750 systems (Macreadie et al. 2012) within coastal environments. Carbon storage potential within
751 MPB dominated sediments remains a significant knowledge gap within the carbon budgets of
752 estuaries. The primary focus of this study was short-term fate of MPB-C, but the significant
753 decrease in retention observed with nutrient amendments imply that short-term processes may
754 have implications for longer term retention. It is interesting to consider how these short-term

changes may affect the longer-term retention (30 d) reported by previous studies (e.g., Oakes & Eyre 2014), with the caveat that the substantial extrapolation required could introduce considerable error to estimates of retention. 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.

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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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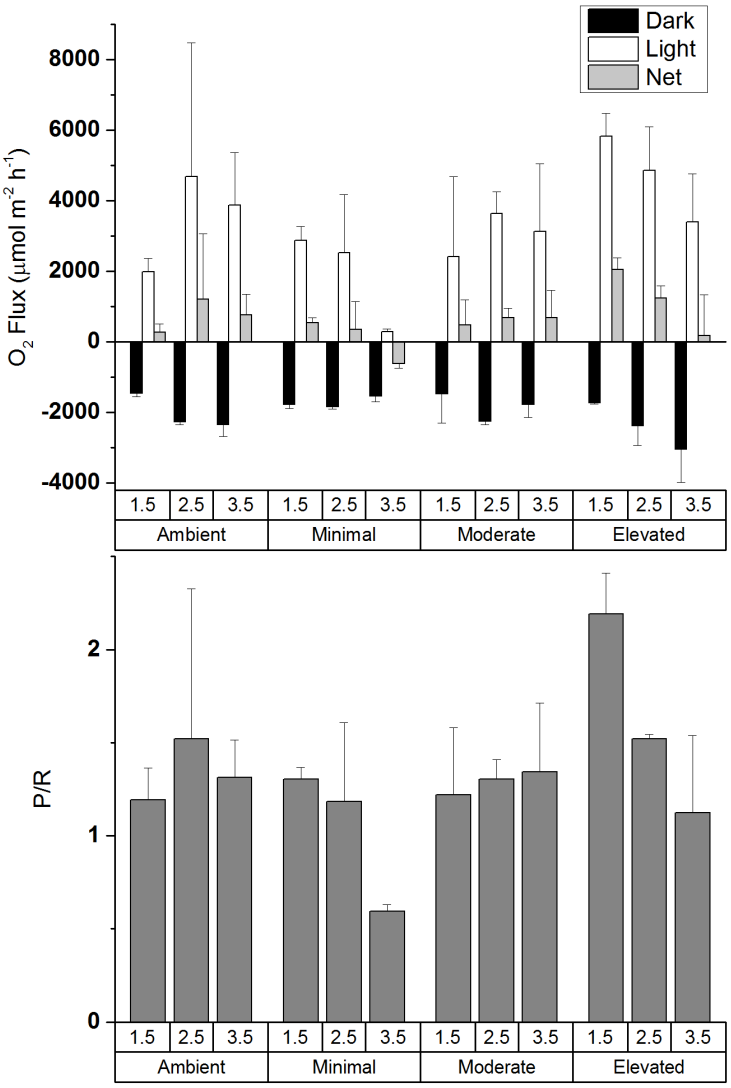
978

Figures and Tables

		Top Scrape				0 to 2 cm				2 to 5 cm				5 to 10 cm			
		Biomass	SE	$\delta^{13}C$	SE	Biomass	SE	$\delta^{13}C$	SE	Biomass	SE	$\delta^{13}C$	SE	Biomass	SE	$\delta^{13}C$	SE
Control cores	Sediment organic carbon	318.0	32.3	-18.7	0.3	3818.0	804.2	-20.7	0.3	7963.3	1174.9	-22.0	0.4	8498.2	1165.2	-22.1	0.4
	Microphytobenthos biomass					321.9	42.0			226.2	33.1			227.3	37.3		
	Bacterial biomass					500.4	65.3			286.0	68.8			244.1	66.1		
Initial cores	Sediment organic carbon	376.4	4.5	121.4	23.7	3693.6	382.4	-7.5	2.1	5056.8	117.8	-19.4	1.0	8397.0	492.5	-21.4	0.7

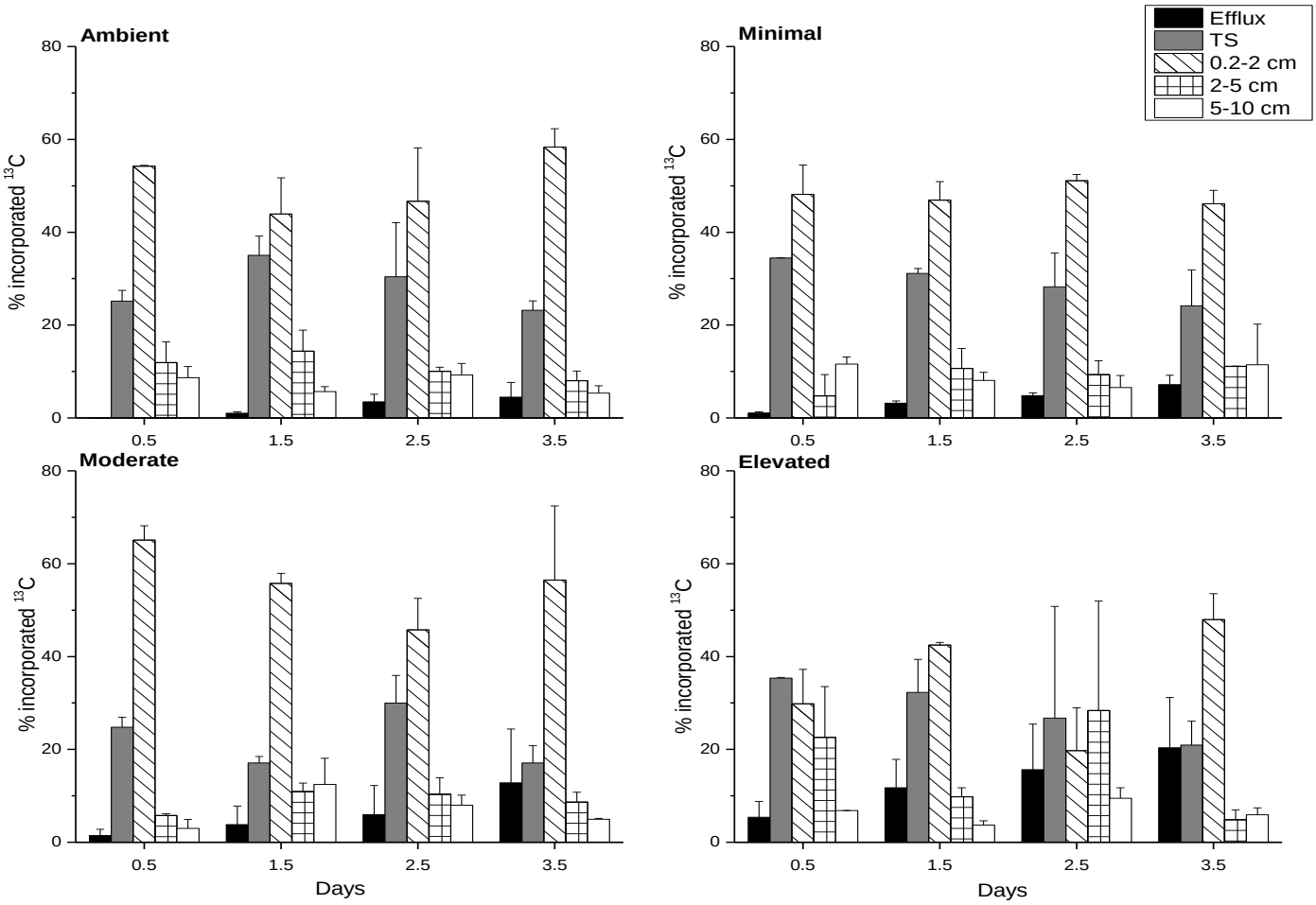
Table 1: $\delta^{13}C$ values (‰) and carbon biomass (µmol C m^{-2}) for control (natural abundance, n=3) and initially labeled cores (n=3, 0 d). Microphytobenthos and bacterial biomass are only provided for control cores.



984

985 **Figure 1:** Oxygen fluxes and ratio of production to respiration (P/R) for all treatments across

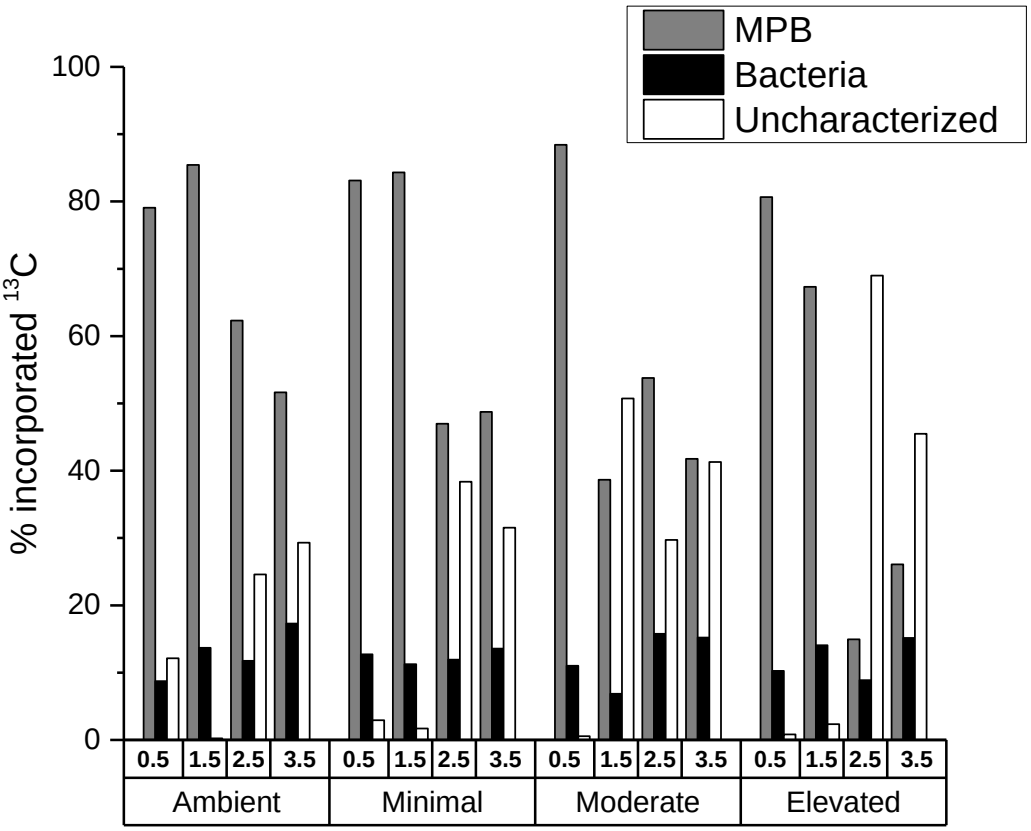
986 24 h calculated from oxygen fluxes for individual cores. Values are mean ± SE.



987

988 **Figure 2:** Carbon budget for excess ^{13}C within sediment OC at top scrape (TS), 0.2 to 2 cm, 2
989 to 5 cm, 5-10 cm, and the cumulative excess ^{13}C exported to the water column via the
990 combined efflux of DIC and DOC for each treatment at each sampling time. All values are as
991 a percentage of the ^{13}C initially incorporated into sediment OC (0-10 cm). Some error bars are
992 too small to be seen (mean ± SE).

993



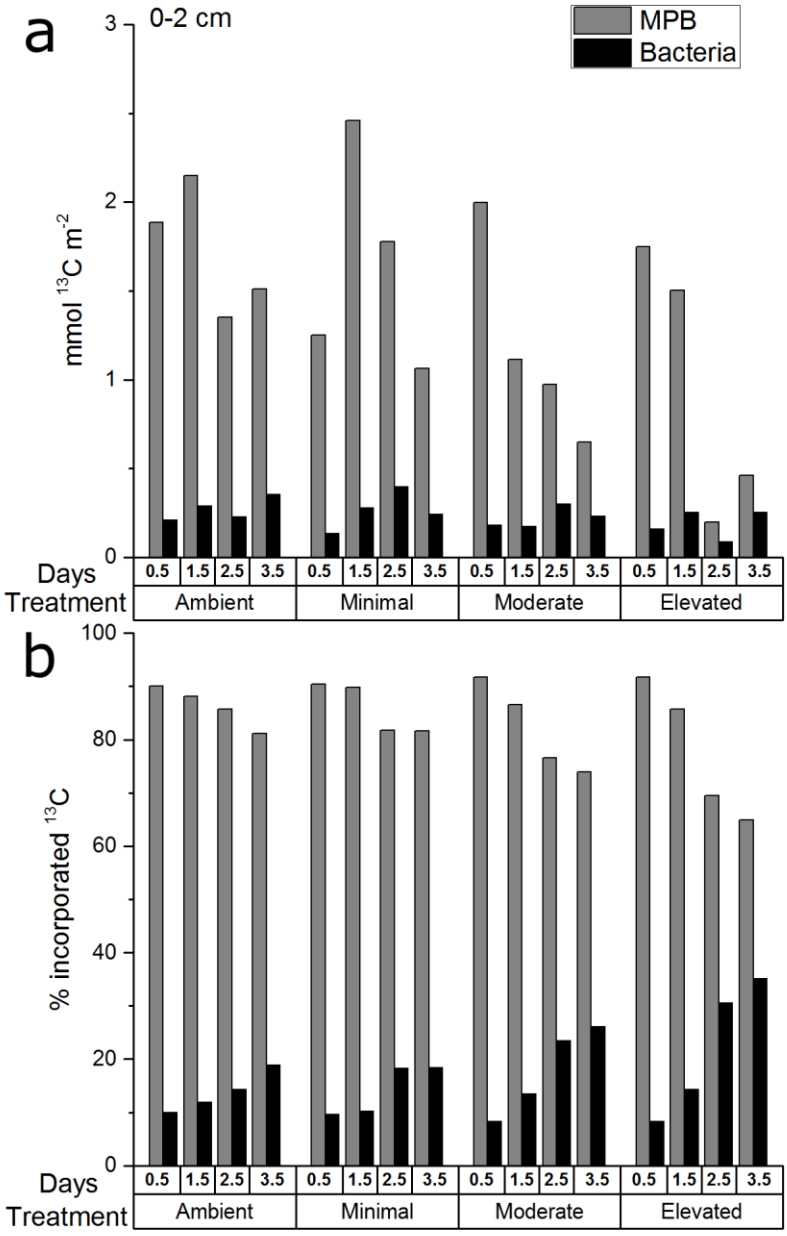
994

995 **Figure 3:** Excess ^{13}C incorporation into microphytobenthos, bacteria, and uncharacterized OC

996 as a percentage of ^{13}C contained in sediment OC in 0-10 cm. There are no error bars as PLFAs

997 were analyzed for only one replicate sample from each time period.

998



999

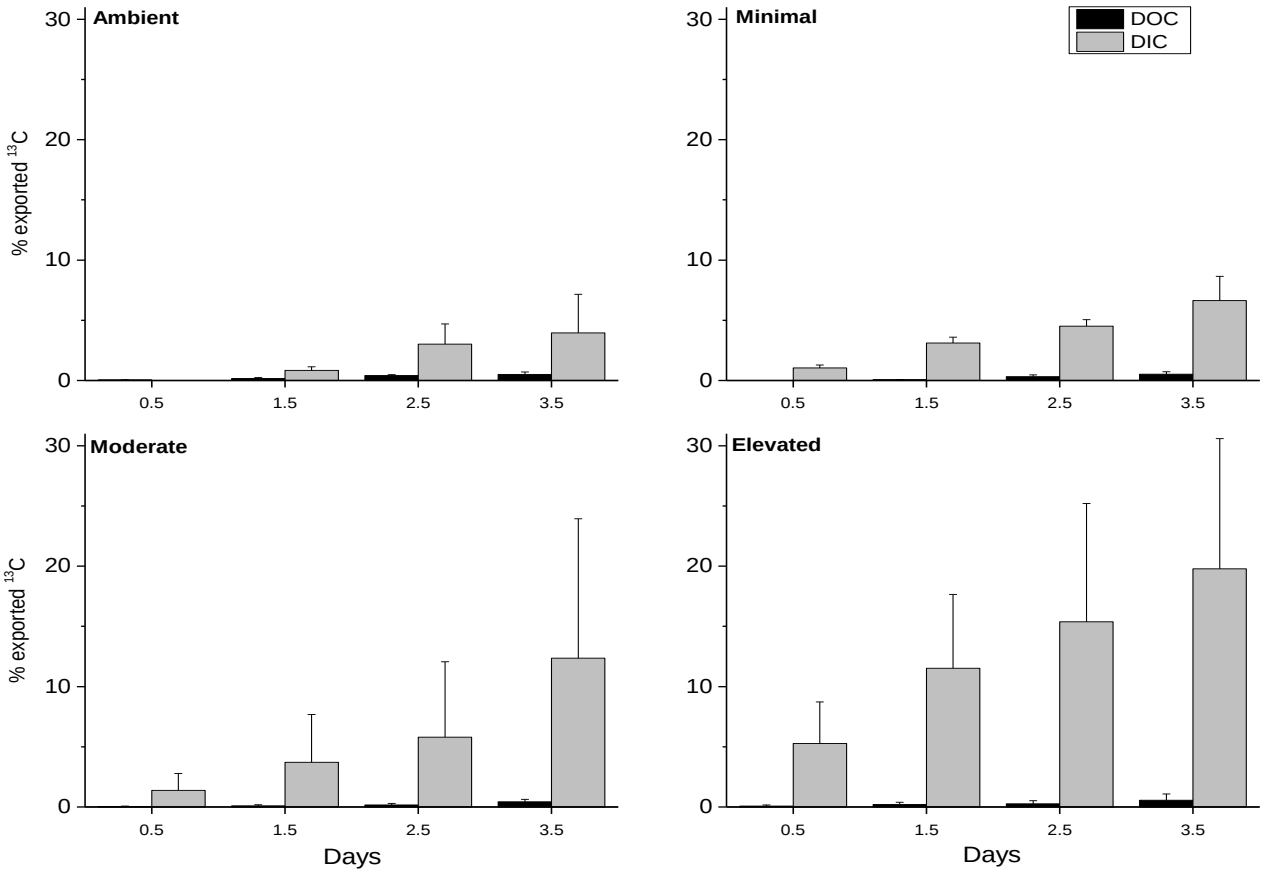
1000 **Figure 4:** ^{13}C within MPB and bacterial biomass in sediment at 0-2 cm depth as A) total

1001 excess ^{13}C (mmol ^{13}C m^{-2}) and B) a percentage of the total ^{13}C in microbial biomass at 0-2 cm

1002 at each time period. There are no error bars as PLFAs were analyzed for only one replicate

1003 sample from each time period.

1004

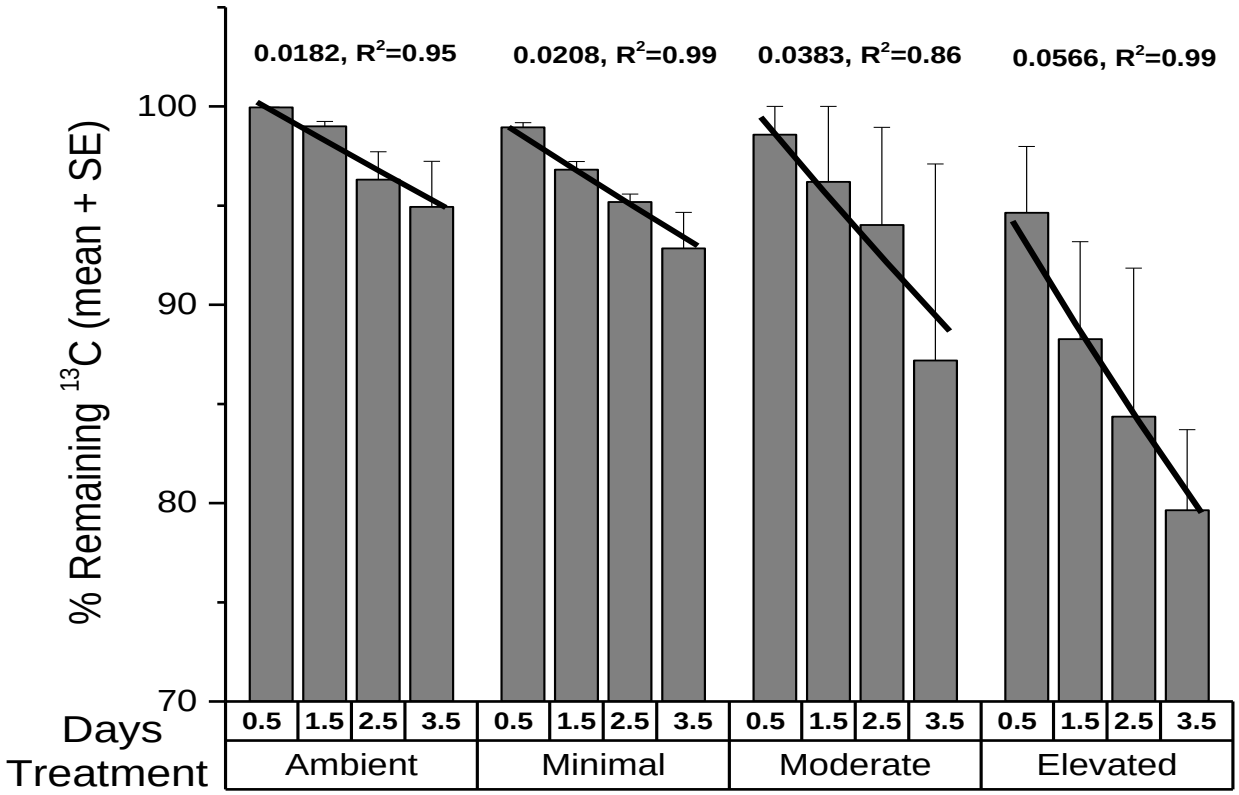


1005

1006 **Figure 5**: Effluxes of ^{13}C from the sediment as dissolved organic carbon (DOC) and dissolved

1007 inorganic carbon (DIC) as a percentage of the total ^{13}C contained in sediment at 0-10 cm

1008 depth at each sampling time (mean ± SE).



1009

1010 **Figure 6**: The percentage of ^{13}C remaining in sediment OC (0-10 cm depth; mean ± SE) from

1011 the calculated budget for total ^{13}C. Lines are exponential decay functions for each treatment

1012 across the 3.5 d of incubation (Loss rate constant, R^2 of function).

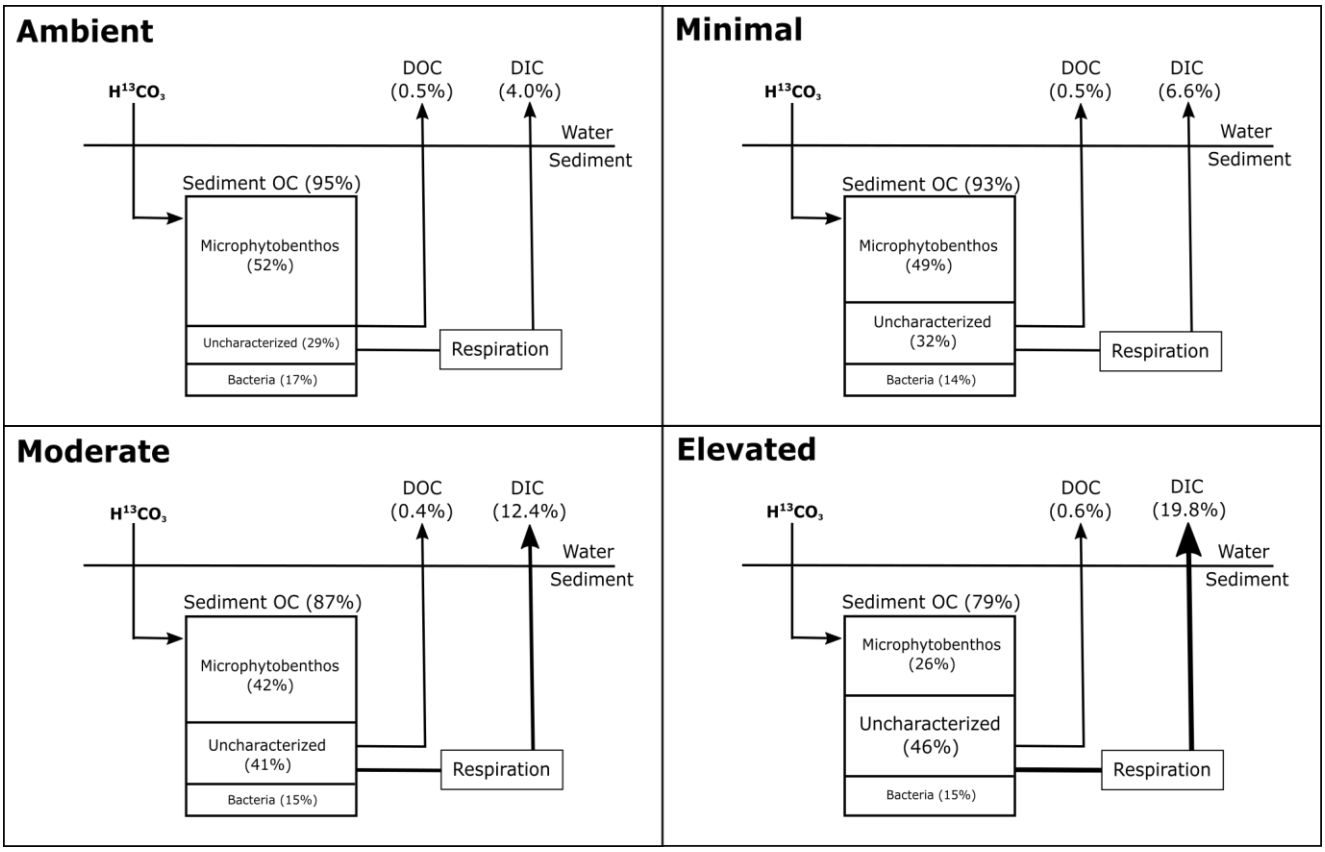
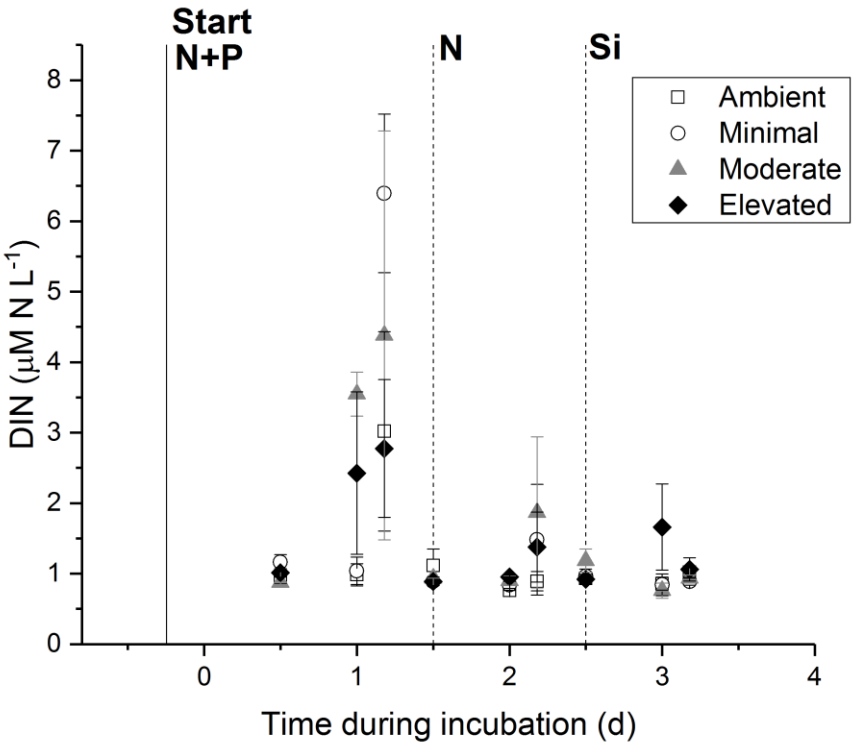
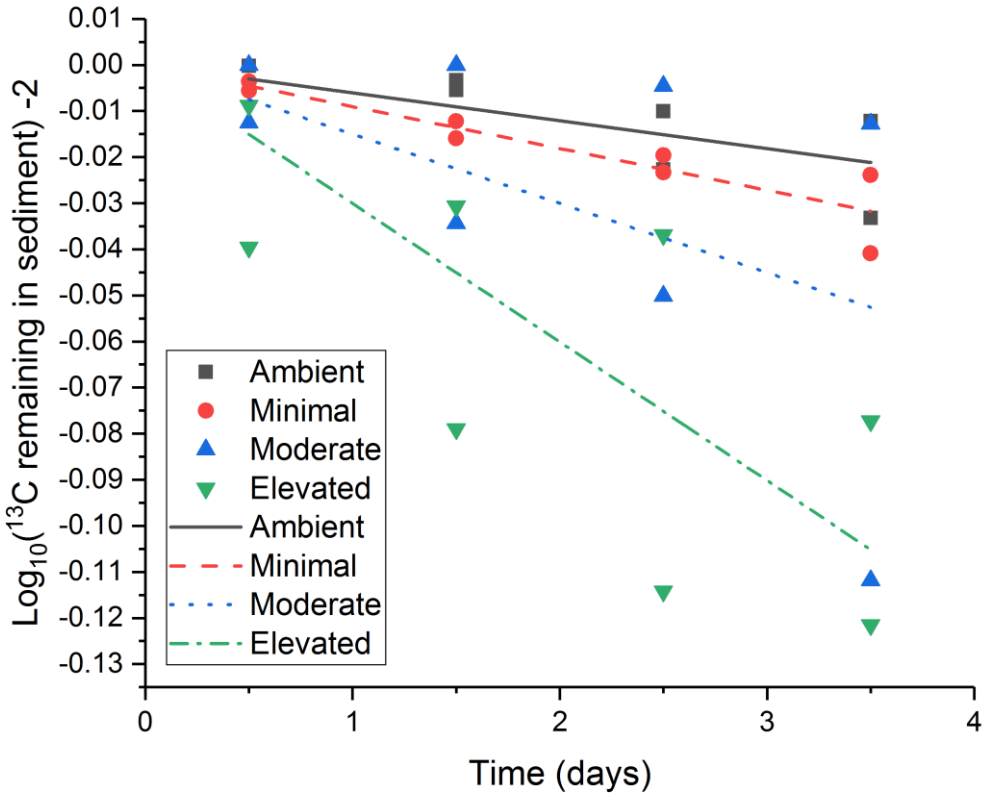


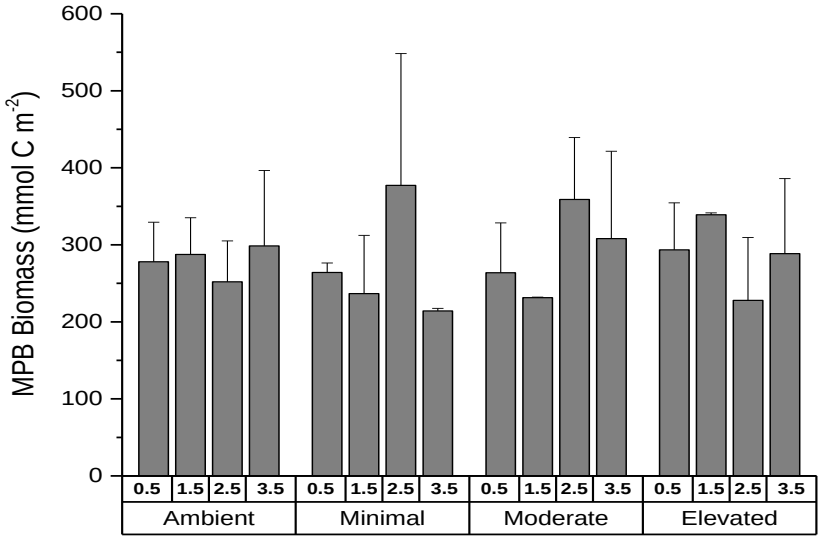
Figure 7: Distribution of ^{13}C at 3.5 d of incubation of inundated sediment including loss pathways for DIC and DOC. The ^{13}C contained in sediment organic carbon (sediment OC) is further partitioned into microphytobenthos, bacteria, and uncharacterized organic carbon as a percentage of the ^{13}C in sediment organic carbon at 0-10 cm 3.5 d after labeling (Figure layout from Eyre et al., 2016).



Supplemental Figure 1: Dissolved inorganic nitrogen in the overlying core waters during sampling for light and dark incubations. The y-axis intercept represents the initial application of $H^{13}CO_3$ to the sediment. The solid line is when cores were placed into treatment tanks prior to the start of incubation. Dashed lines represent additional treatment pulses ($N=NH_4^+$, $P=PO_4^{3-}$, $Si=SiO_3$) that occurred during incubation.



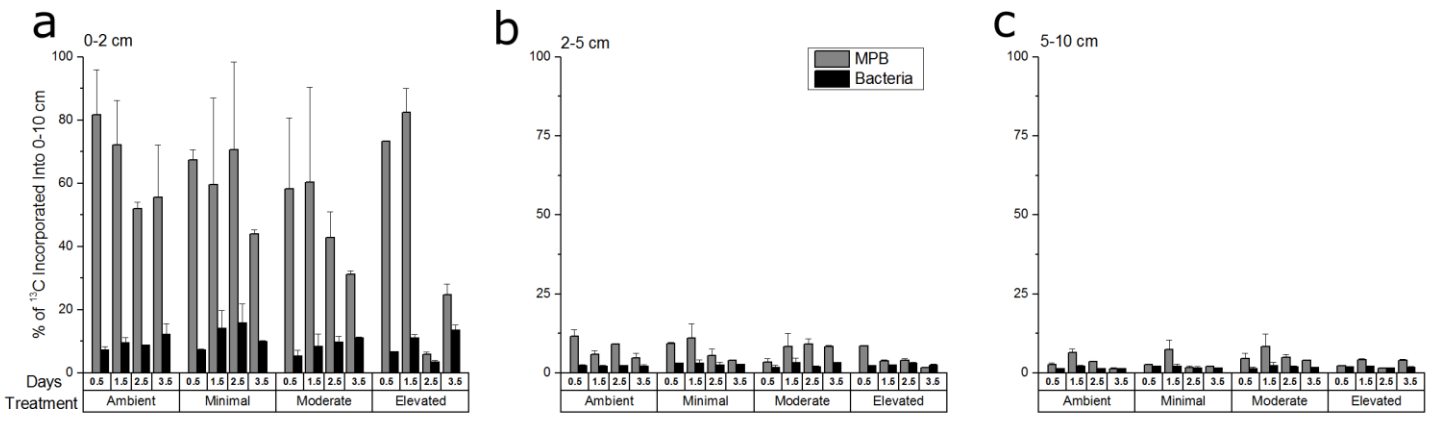
Supplemental Figure 2: Slope comparison between treatments for log_{10} transformed ^{13}C remaining in sediment. The model with different slopes for each treatment fitted significantly better than the model with a single slope (F-test, $F_{3,28}$=9.84, P <0.001).



1034

1035 **Supplemental Figure 3:** MPB carbon biomass in 0 to 2 cm sediments calculated from

1036 chlorophyll *a* concentrations, assuming a C:Chl-*a* ratio of 40 (mean ± SE).

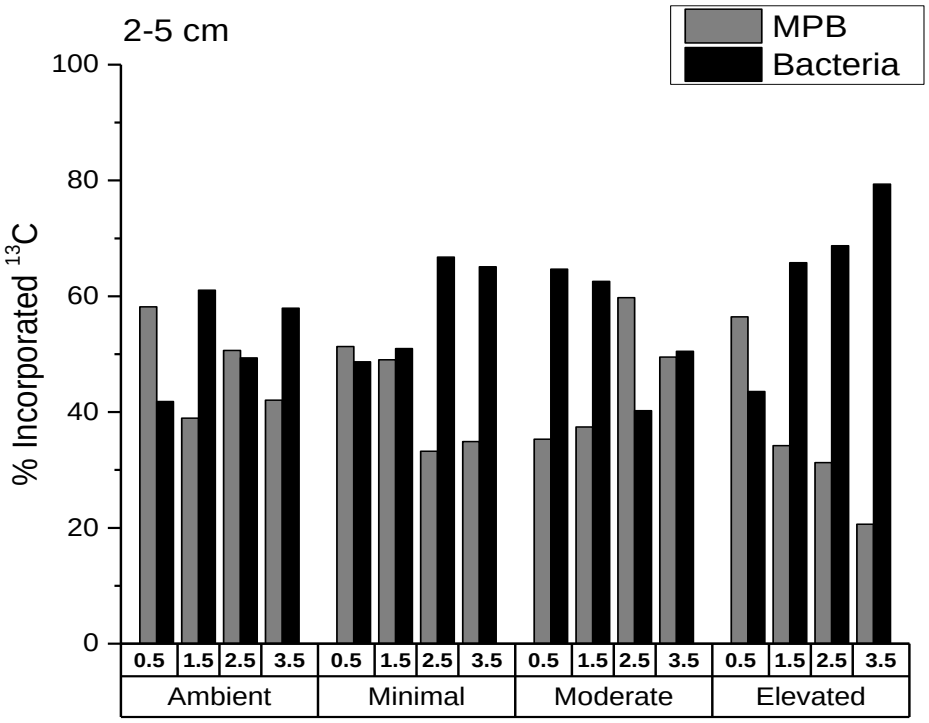


1037

1038 **Supplemental Figure 4:** Excess ^{13}C incorporation into MPB and bacterial biomass at depths

1039 a) 0-2 cm, b) 2-5 cm and c) 5-10 cm as a portion of the total ^{13}C in 0-10 cm sediment OC at

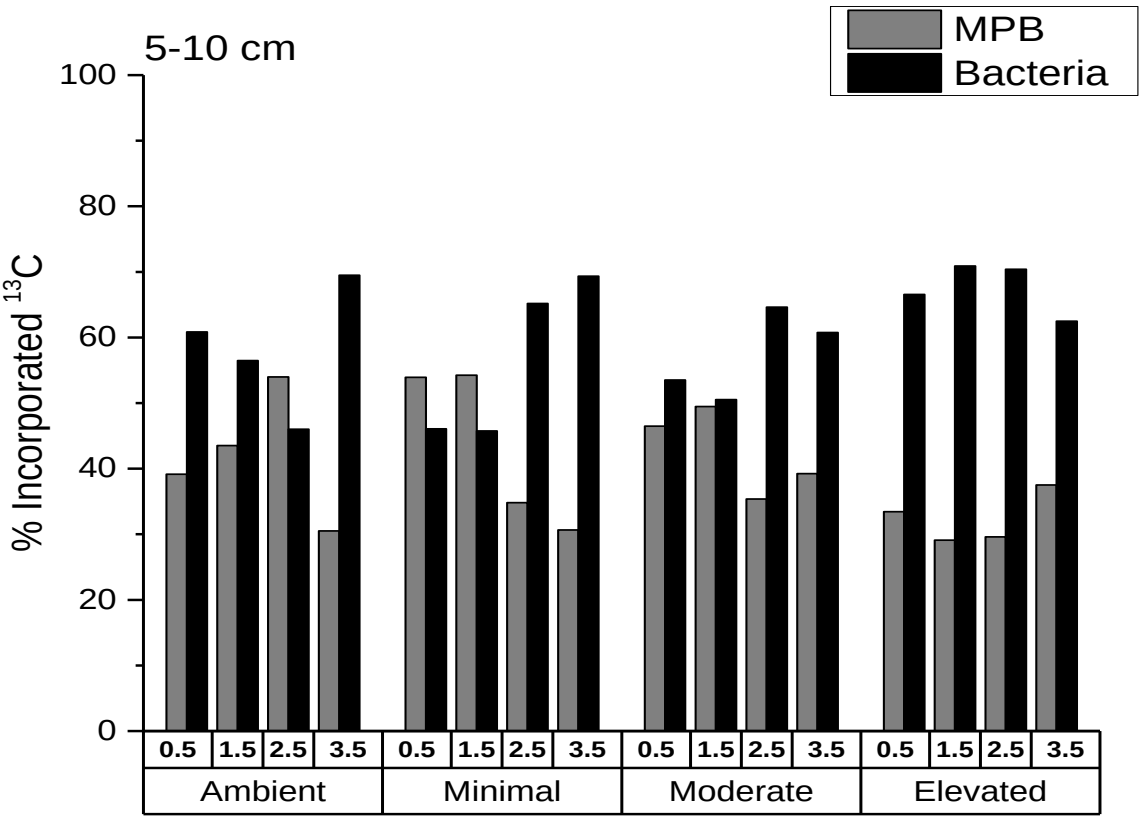
1040 each time.



1041

1042 **Supplemental Figure 5:** Excess ^{13}C incorporation into MPB and bacterial biomass at a depth
1043 of 2-5 cm as a percentage of the total ^{13}C in microbial biomass at 2-5 cm at each time period.
1044 There are no error bars as PLFAs were analyzed for only one replicate sample from each time
1045 period.

1046



1047

1048 **Supplemental Figure 6:** Excess ^{13}C incorporation into MPB and bacterial biomass at a depth
1049 of 5 -10 cm as a percentage of the total ^{13}C in microbial biomass at 5-10 cm at each time
1050 period. There are no error bars as PLFAs were analyzed for only one replicate sample from
1051 each time period.

1052

We thank the two anonymous reviewers for their feedback. The suggested changes have helped to improve this manuscript.

The structure of the response is:
1) Reviewer comment number
1. Reviewer comment,
2. Author response
3. Changes to manuscript.

Highlighted text reflects portions of the text added as a result of comment.

Reviewer 1 main comments
1)
1. The authors applied a ^{13}C label to exposed surface sediments at low tide and then waited 11 h to collect cores (lines 141-143, 144-145). Approximately 2 h after collection, the cores were placed in the experimental tanks and exposed to the nutrient treatments (lines 150, 154-161). Carbon exchange, recycling, and loss between MPB and bacteria occurs quickly – over just a few hours. My concern is that at least 13 h passed between the ^{13}C label addition and the application of the nutrient treatments. It seems that carbon exchange between MPB and bacteria that occurred prior to nutrient additions would confound the effects of the nutrient treatments.

2. *Prior to incubation, all cores were maintained in conditions that were as close to identical as possible (both in situ and during transport) to maintain as much homogeneity as possible between the cores. As a result of this care, the carbon exchange between MPB and bacteria are expected to have been similar between cores prior to exposure to the nutrient additions as they were both handled in an identical manner and subsequently randomly allocated (LN 175) to treatment group prior to being placed in the incubation tanks.*

As a result of this, carbon processing that occurred after the treatment application should be similar across cores within the treatments, regardless of the microbial processing that occurred in the 13 h prior to nutrient amendment. Any differences among treatments are therefore solely due to the application of different nutrient concentrations. The reviewer is correct that there was processing of newly fixed MPB-C that occurred prior to nutrient additions, but this manuscript focuses on the processing that occurred on the remaining MPB-C that was still labile and present (as evidenced by the ^{13}C found within both bacteria and MPB) within the sediment during our incubations.

3. *We have added a clarifying statement about the the effect of the 13 h prior to nutrient amendment. LN 168 now reads: "*Processing of newly fixed MPB-C occurred in the 13 h prior to incubation with nutrient amendments, but was likely similar across cores as they were kept in identical conditions prior to incubation before random allocation to treatments. Although MPB-C was not freshly fixed at 13 h and likely more refractory as a result, the available C was still relatively labile and readily processed across all treatments."

*We have also clarified the focus of the study by more clearly stating the focus in the concluding paragraph of the introduction. LN 85 now reads: "*In this ^{13}C pulse-chase study we aimed to quantify the short-term effects of increased nutrient concentrations on the processing pathways for MPB-derived C within subtropical intertidal sediments"

2)
1. In addition, it is not clear from S1 that the nutrient treatments were effective.

2. *As per text on ln 166-167, figure S1 was included to demonstrate that nutrient additions were incorporated into the biomass and were not sufficient to overload the capacity for nutrient utilization and leave a large concentration of DIN in the water column. We added nutrients in pulses that were processed and removed from the water column at both 0 d and 1.5 d. The sampling at 1.5 d was capped for incubation prior to nutrient addition and therefore was not expected to contain the nutrient addition that occurred at 1.5 d. Nutrient treatments were effective, as evidenced by the increased export of ^{13}C from the sediment within the elevated treatment (LN 471), and the changes in distribution of labeled material as processing pathways were affected (Fig. 7).*

3. *No changes were made in response to this comment.*

3)

1. Further, it seems from the methods that the cores in the same nutrient treatment tank all shared the same water column (lines 176-179). This would affect the independence of processes across cores and could confound the results.

2. *The reviewer is correct that the water column was shared by all cores within each treatment tank. It was not logistically possible to separate paired cores into individual treatment tanks for each sampling and adding treatments to sealed cores would have exacerbated any 'bottle effect' given the relatively small volumes of water contained in each core.*

Sharing a water column could have been a problem if ^{13}C produced within one core found its way into another through transport of enriched DIC or DOC produced from MPB-C. However, this was not a problem as the volume in each treatment tank was sufficient to dilute any label coming from these sources. We measured $\delta^{13}C$ of water column DIC and DOC; initial values for each light incubation (collected immediately after capping of cores) reflect the treatment tank water. No considerable enrichment in $\delta^{13}C$ values for initial DIC or DOC was observed for any of the sampling times across the 3.5 d incubations, confirming that there was no ^{13}C transfer among cores within each treatment tank.

3. *No changes were made in response to this comment.*

4)

1. For the incubations, cores were sealed for 30 mins before initial samples were collected. Final samples were collected 3 h (light), 12 h (dark), or up to 16 h later (lines 186, 188-192). It would be good to clarify the exact duration of light and dark incubations and why the times differed between conditions.

2. *The 3 h duration of the light incubation was intended to prevent dissolved oxygen from becoming supersaturated as a requirement for N_2/Ar analysis that was being run simultaneously during this experiment as part of another study focused on N.*

3. *We have clarified that the incubation period was ~15 h (LN 188). LN 192 now reads:* "Light incubations were of shorter duration to prevent supersaturation of dissolved oxygen which would have compromised additional analyses required for a complementary study."

5)

1. The authors should also clarify whether rates were calculated based on concentration changes between two time points. Generally, at least 3 time points are needed to calculate fluxes and capture nonlinear dynamics.

2. *Several incubation studies have been performed by this working group across the last 15 years, and while they began with 3 to 5 point incubations (Ferguson et al. 2003, AME 33: 137-154, Veuger et al. 2007, L&O 52(5): 1930-1942) for flux measurements, the dynamics observed were largely linear. More recent studies have used two point dark and light incubations due to the considerable reduction of cost and sampling effort (e.g., Oakes & Eyre 2014, Biogeosciences 11: 1927-1940) with minimal loss of information and accuracy.*

3. *We have clarified on LN 300:* "Fluxes across the sediment-water interface were calculated from two measured concentrations, at the start and finish of each dark and light period (e.g., Oakes and Eyre 2014), as a function of incubation time, core water volume and sediment surface area."

6)

1. Moreover, long incubations, particularly under dark conditions, likely created 'bottle' effects that could affect metabolic rates. The authors should discuss potential artifacts of the sampling approach – particularly the potential impact of low DO on sediment respiration rates.

2. *DO did not fall below 4.85 mg/L (DO% 58.5%) during the dark incubations, minimizing potential long term effects or 'bottle' effects. DO consistently increased rapidly when the lights were turned on, resulting in the need to shorten the light period in order to sample below supersaturation as required for N_2/Ar analysis for another study occurring simultaneously with this one. The quick recovery of production indicates that any potential bottle effect during the longer incubation was negligible and did not result in considerable lag between respiration and production periods.*

*3. We have clarified that DO was not allowed either to drop too low or go above saturation. LN 310 now reads: "*To prevent the potential development of resource limitations during incubation, 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 allowed to become supersaturated."

7)

1. Additional rationale for the time course of measuring fluxes 6 h after adding nutrients (and 19 h after adding ^{13}C), and then again 1.5 d, 2.5 d, and 3.5 d would also be useful.

2. *This project focused on initial processing of C, with multiple incubations over a relatively short period (3.5d after label addition), due to the observation in a comparable previous study at the same site that most ^{13}C transformation occurs within this ~4d (Oakes & Eyre 2014). Sampling was only feasible every ~24 hours, with light/dark periods aligned with in situ conditions, and each light/dark incubation taking ~15h. Acclimation time was allowed for any microclimates or disturbed anaerobic zonation to re-establish after disturbance with coring as this has the potential to influence P/R dynamics.*

3. *We have added a clarifying statement about the spacing of our sampling times. LN 204 now reads*: "These sampling time periods were chosen to capture the active dynamics of ^{13}C processing that were expected to occur over the first few days of the study, based on previous work by Oakes and Eyre (2014)."

*We have added a clarifying statement about the rationale behind the 6 h acclimation period. LN 184 now reads: "*Cores were allowed to acclimate in tanks for 6 h prior to the start of incubation to allow for the re-establishment of microclimates and anaerobic zonation that was potentially disturbed by coring."

8)

1. The authors need to provide more rationale for using 16:1n7 as a marker for diatoms. While this compound is an important component of diatom lipids, it is also produced by other groups of algae as

well as by iron reducing bacteria and sulfate reducing bacteria. These communities are likely active in shallow coastal sediments and could be in close proximity to MPB if the sediments are anoxic. The absence of polyunsaturated C20 and C22 PLFAs seems to suggest that diatoms and other microalgae were at low abundances. I am not convinced that the current approach adequately isolates contributions from diatoms vs. non-diatoms.

2. *We have used a previously published method (Oakes et al. 2016, L&O 61:2296-2308) for calculation of diatom associated 16:1n7. This calculation provides diatom associated 16:1n7 after subtraction of the estimated contribution of 16:1n7 from other sources, calculated from 18:1n7 using a two source mixing model described on LN 280. 18:1n7 is a monosaturated PLFA that is associated with cyanobacteria, Gram negative bacteria, and sulfate reducing bacteria. By removing the amount of 16:1n7 estimated from 18:1n7, we removed the contribution of these bacterial groups from the total pool of 16:1n7. We did not investigate PLFAs associated with Fe-reducing bacteria as it was outside of the scope of this study. Furthermore, we are confident that pennate diatoms had substantial biomass at the site, and the contribution from other algal species was minimal based on visual analysis of the sediments (via microscopy) that confirmed strong dominance of pennate diatoms (LN 225). On this basis, we conclude that the remaining portion of 16:1n7 is predominately derived from diatoms.*

3. *We have clarified that 18:1n7 was used to account for the contribution of Gram negative, cyanobacteria, and SRB to 16:1n7 in the initial description of PLFA analysis. LN 227 now reads "*The 16:1(n-7) PLFA, which represents 27.4% of total diatom PLFAs (Volkman et al. 1989), was consistently present across all samples and was used as a biomarker for diatoms, following correction for contributions from gram-negative bacteria, cyanobacteria, and sulfate reducing bacteria, determined using 18:1n7 as described in the calculations section below and in Oakes et al. (2016)."

9)

1. The carbon mass balance calculations should be clarified. For instance, the authors report the %C loss from the sediments, but it is unclear how this was calculated. Did this reflect the % change between the initial core collections and each sampling time point (0.5, 1.5, 2.5, and 3.5d)? Or was this calculated by subtracting out ^{13}C losses via DIC and DOC (lines 490-491)? If the former, then that should automatically account for losses by respiration and exudation.

2. *Neither method that the reviewer suggests reflects the accounting presented in this study*.

Figure 6 shows ^{13}C remaining in the sediment, not ^{13}C loss. ^{13}C remaining in the sediment was calculated as the sum of the exported DI^{13}C and DO^{13}C and sediment derived ^{13}C (bulk OC) divided by the sediment derived ^{13}C and multiplied by 100 for each core at the end of each dark/light incubation. We then went on to use exponential decay functions to explore how the export of ^{13}C from the sediment differed between nutrient amended treatments and ambient treatments.

*We feel that we have adequately described how the turnover of ^{13}C was calculated from our response to Reviewer 1 comment 10, briefly repeated here: "*The data for ^{13}C remaining in sediment OC were further examined by fitting an exponential decay function for each treatment across 3.5 d using the Exp2PMod1 function in OriginPro 2017 and ^{13}C turnover estimates were then determined by solving for y = 0.05 % remaining ^{13}C (a value close to 0) and x = 30 d for each treatment*."*

To further clarify, the %'s presented in the budget for Figure 7 reflect the portion of the ^{13}C within each pool (MPB, Bacteria, uncharacterized) at each sampling time, with the interpolated fluxes of DIC and DOC included.

3. *We have clarified our method of accounting for ^{13}C on LN 261*. "Percentages calculated from these pools are presented as portions of the sum of total ^{13}C contained within the sediment and the interpolated fluxes of DIC and DOC that were estimated to have occurred from 0 d until each sampling time."

10)

1. Similarly it is unclear how the exponential decay functions were calculated.

2. *Exponential decay functions were fitted using the function Exp2pmod1 in OriginPro 2017. ^{13}C turnover estimates were provided by solving for y = 0 % remaining ^{13}C and x = 30 d.*

3. *LN 350 now reads*: "The data for ^{13}C remaining in sediment OC were further examined by fitting an exponential decay function for each treatment across 3.5 d using the Exp2PMod1 function in OriginPro 2017 and ^{13}C turnover estimates were then determined by solving for y = 0.05 % remaining ^{13}C (a value close to 0) and x = 30 d for each treatment."

11)

1. The error bars in Figures 5 and 6 are large, particularly for the moderate and elevated treatments; this variability makes the substantial differences across treatments (lines 475-478) somewhat unexpected.

2. *Some of the error bars in Figs. 5 & 6 are large, as is expected for calculated fluxes across the multiple cores contained in each treatment. We have further investigated the ^{13}C remaining in the sediment (Fig. 6) with a two-way ANOVA and found the elevated treatment to be significantly lower than both the ambient and minimal treatments. We have now included this analysis in our results.*

To further investigate the differences between the loss rate constants presented in Fig. 6 resulting from the export of DIC and DOC shown in Fig. 5, we now present an analysis of the transformed data for ^{13}C remaining in the sediment. This compared a model using a single slope for all of the data (i.e. including all treatments) with a model utilizing separate slopes for each treatment. We have added supplemental figure 2 that presents the slopes being compared as well as the underlying data being modeled.

3. *LN 471 now describes the two-way ANOVA run for ^{13}C remaining in sediments. LN 471 reads*: "The total ^{13}C remaining in sediment (Fig. 6) varied significantly among treatments (two-way ANOVA: $F_{3, 31}$= 5.7, p=0.008) and across sampling times ($F_{3, 31}$= 3.9, p=0.03). Throughout the study, there was generally less ^{13}C remaining within the elevated treatment than in than either the ambient (p=0.008) or minimal treatments (p=0.02), and there was significantly less ^{13}C remaining within the sediment at 3.5 d than at 0.5 d (p=0.02)."

LN 482 now describes how the comparison between slopes were analyzed: "Since the intercept is known, i.e., the initial value equals 100% at time 0, linear models where only the slopes were estimable, were fitted to further analyze the differences between slopes. Assuming an exponential decay, the percentage remaining ^{13}C (Y) was $\log_{10}$ transformed and the value 2 was subsequently subtracted ($Z=\log_{10}(Y) -2$), which implies that the intercept of Z versus time equals 0. The model with different slopes for each treatment fitted significantly better than the model with a single slope (F-test, $F_{3,28}$=9.84, P <0.001, Supplemental Fig. 2). The analysis was performed in R."

Supplemental Figure 2 has been added to LN 1030 along with a figure caption that reads:
"Supplemental Figure 2: Slope comparison between treatments for $\log_{10}$ transformed ^{13}C remaining in sediment. The model with different slopes for each treatment fitted significantly better than the model with a single slope (F-test, $F_{3,28}$=9.84, P <0.001)."

12)

1. The discussion should be more concise and focused. It would have been helpful if the authors considered processes driving variability across the treatments and compared their findings to other studies examining MPB-bacterial responses to surface water nutrient additions – particularly other stable isotope labeling experiments. Along these lines, I was somewhat surprised that nutrient additions would not stimulate MPB production and thereby promote C retention in the sediments.

2. *We have examined the discussion and chosen to eliminate some included detail (previously* LN 571-574 & LN 587-597) *about patterns within the P/R data, as they were covered in too much detail in the text.*

The reviewer's description of N stimulating MPB production describes only one side of the interaction that occurs within the intertidal biofilm. Opposite of MPB production, bacteria are working to actively hydrolyze both the EPS and biomass produced by MPB. Bacteria rely heavily on MPB-derived material for N under N-limitation. It may be possible that with increased N availability, bacteria are able to offset increased MPB production through increased hydrolysis and bacterial production. Figs. 3 and 4b support this, with increased incorporation of ^{13}C into both bacteria and the uncharacterized pool (processed C) within the elevated treatments. It appears that the net balance between production and consumption of C within mudflat biofilms regulates whether there is net uptake or net loss of C within the sediment (Cook et al. 2007, Hardison et al. 2013, Spivak et al. 2016).

Labeling applications in coastal sediments with limited fauna and/or macroalgal influence that also include nutrient amendments are sparse in the literature. We have now included a comparison of retention of ^{13}C within the sediment to Hardison et al. (2011). Given that there was very little fauna at our study site, and certainly no macrofauna within our incubated cores, we consider it inappropriate to directly compare to other studies such as Pascal et al. 2013 (6 year enrichment study for both C + N) or Spivak et al. 2016 (seasonal comparison using labeled detritus to work out MPB contribution) due to commentary within both studies about grazing pressure potentially masking bottom up effects of fertilization on MPB due to the faunal abundance found within the study site.

3. *We have deleted a substantial amount of text from the discussion of P/R (previously* LN 571-574 and LN 587-597).

LN 742 now includes a direct comparison to Hardison et al. 2011 for retention of ^{13}C in sediment during a water column nutrient amended study.

Specific comments

Introduction:

13)

1. The authors acknowledge that other studies have examined the effects of nutrient loading on MPB (lines 73-77), but should discuss the subset of studies that used isotope tracer techniques in more depth, as these are directly relevant to the current manuscript.

2. *The subset of studies that have used both biomarkers and labeled SI additions have not usually gone on to subsequently partition out the DIC and DOC portions of the carbon budget and therefore do not fully discuss these processing pathways. To fully address both portions of the budget that this study examines (sediment and DIC/DOC), we have broadened our focus and included a combination of references for both tracer studies and non-tracer studies that investigated nutrients and MPB on LN 80-84*: "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)." *The reason for this diverse focus is that most tracer studies utilizing biomarkers focus solely on partitioning of the budget directly involved with those biomarkers and do not present accounting of the DIC and DOC pathways as a portion of the budget within the framework of the study.*

*Additionally, we have added Pascal et al. (2013) as another relevant stable isotope study in addition to Cook et al. (2007) which was already included (a tracer study examining the effects of nutrient enrichment) and discussed on LN 78: "*Both EPS production and bacterial utilization of newly produced EPS may decrease with increasing nutrient availability".

3. We have added the reference to Pascal et al. (2013) to LN 76.

14)
1. Line 113 and elsewhere: instead of reporting carbon concentration per surface area, please report % organic carbon.

2. *We have now provided sed %OC in the study site description in addition to our previous units. Elsewhere in the manuscript we have retained units as per surface area as it is convention for work of this nature (e.g. Oakes et al. 2014, Hardison et al. 2011, and Middelburg et al. 2000) and allows for comparison across studies as well as conversion into %OC if anyone should wish to do so.*

3. *%OC is now included on LN 113 in addition to the previously provided units for concentration per surface area.*

15)
1. Lines 155-160. What were the target concentrations of each of the treatments and was the site water filtered before the treatments were applied?

2. LN 155 reads: "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 (NH_4^+) and P (H_3PO_4) amendment to unfiltered 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 (1997; 2000)."

The target concentrations for pulsed nutrient amendments were for 8, 25, and 40 µmol L^{-1} NH_4^+ and 10, 25, and 50 µmol L^{-1} PO_4^{3-} respectively for minimal, moderate and elevated treatment pulses. Site water was unfiltered.

3. *We have now specified that the water was not filtered (LN 155). No changes were made to further specify the target concentrations of each treatment, as these can clearly be derived from the text on LN 155 (2x, 5x, and 10x average water column concentrations of 4umol/L NH_4 and 5umol/L PO_4).*

Calculations:
16)
1. The biomass calculations use several conversion factors to scale from lipid concentrations to bacterial and diatom biomass. It is unclear how well constrained the conversion factors are and the amount of uncertainty they introduce into the calculations. For instance, does the concentration of lipid per unit biomass change with algal growth or nutrient condition? It is unclear why the lipid concentrations need to be scaled to biomass – why not compare the ratio of bacterial to microalgal lipids?

2. *This comment addresses concerns about methodology that are not unique to this study, but which are common to methods routinely applied in studies using biomarkers. Conversion factors are frequently applied to FAMEs (fatty acid methyl esters) to estimate and compare biomasses of different microbial groups (bacteria, MPB).*

When examining uptake of ^{13}C, we need to account differences in biomass to adequately account for the total ^{13}C present in different pools. It is possible to have a large enrichment of ^{13}C contained in a small pool of biomass that may contain less ^{13}C than a minimally enriched pool of biomass that is considerably larger. Without accounting for the relative biomass that is contained within each pool, it would appear the more enriched biomarker is more involved in the processing of ^{13}C, but upon full accounting of both biomass and enrichment, it becomes abundantly clear that the larger, less enriched pool has a more significant impact.

As such, thorough investigation of "the concentration of lipid per unit biomass change with algal growth or nutrient condition" falls outside of the scope of this study.

3. *We have referred to previous work that utilize these conversion estimates on LN 227: "*The 16:1(n-7) PLFA, which represents 27.4% of total diatom PLFAs (Volkman et al. 1989), was consistently present across all samples and was used as a biomarker for diatoms, following correction for contributions from gram-negative bacteria, cyanobacteria, and sulfate reducing bacteria, determined using 18:1n7 as described in the calculations section below and in Oakes et al. (2016).*"*

17)
1. Equations 3-4 are descriptions more so than equations.

2. *The reviewer is technically correct, but these serve to efficiently communicate how the fluxes for both respiration and net primary productivity were calculated in order to further calculate gross primary productivity.*

3. *We consider the current presentation to be both clear and concise; no changes have been made.*

18)
1. Line 330: ANOVA is not the most appropriate test because the cores within a treatment were not independent of one another as they shared a water column.

2. As stated in the previous response to Reviewer 1 comment 3: "Sharing a water column could have been a problem if ^{13}C produced within one core found its way into another through transport of enriched DIC or DOC produced from MPB-C. However, this was not a problem as the volume in each treatment tank was sufficient to dilute any label coming from these sources. We measured $\delta^{13}C$ of water column DIC and DOC; initial values for each light incubation (collected immediately after capping of cores) reflect the treatment tank water. No considerable enrichment in $\delta^{13}C$ values for initial DIC or DOC was observed for any of the sampling times across the 3.5 d incubations, confirming that there was no ^{13}C transfer among cores within each treatment tank."

3. The reviewer does not provide an alternative analysis for us to investigate, so we have considered their case for a lack of independence and provided refuting evidence. No changes were made as a result of this comment.

19)
1. Line 341: What is the ecological rationale for grouping time points 0.5 and 1.5 vs. 2.5 and 3.5?

2. *As stated in the text on LN 341: "*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).*", the rationale is largely statistically driven. The division provides an equal group weighting between the examined groups. There is some geochemical rationale in doing this as the additions of tracers are often observed to change the most in the earliest part of the experiment, and taper off as the carbon becomes more recalcitrant and dispersed within the system, but primarily, this split was designed to allow for sufficient replication within the groups and, therefore, robust statistical analysis.*

3. *No changes were made as a result of this comment.*

20)

1. Lines 415-426. How did downward transport occur in these cores? MPB are generally restricted to the top 2 cm and it does not seem that there was pore water flow during the lab incubations. Was there mixing by animal communities? Is it possible that contamination occurred during core collection in the field?

2. *Saburova et al. 2003 examined diatom migration in detail and found 4 cm migration in sediment with clay sublayers, and up to 8 cm migration in sediments with a coarse sand sublayer, so the reviewer's statement that MPB migration is limited to 2 cm is incorrect. As our site sediments were quite sandy, and the stir bars suspended above the sediments were sufficient to stimulate moderate porewater flow in the upper layer, we consider it plausible that downward transport occurred via MPB transport across depth during the incubations. Saburova et al. 2003 is referenced in the downward transport section of the discussion (LN 717) supporting MPB migration as an effective pathway for downward transport in this study.*

Contamination is possible during collection, but would have been minimal and fairly uniform across the cores since the core liners were placed into the sediment in an identical manner. Additionally, our initial cores displayed minimal downward presence of ^{13}C, which also supports that contamination was minimal during collection as they were collected in exactly the same manner as sample cores.

3. *No changes were made as a result of this comment.*

21)

1. Line 457: Was the uncharacterized fraction defined as PLFAs that were not i,a-15 or 16:1n7? If so, additional rationale is needed to justify this approach.

2. *The ^{13}C content of the uncharacterized fraction was calculated by subtracting the ^{13}C in contained microbial biomass (diatom and bacteria calculated from PLFAs) from the ^{13}C contained in the sediment organic carbon. This is now described in the methods section.*

3. *LN 295 now reads:* "Microbial biomass is the sum of calculated diatom and bacterial biomass. Uncharacterized ^{13}C was calculated as:

3. $^{13}C_{\text{uncharacterized}} = {}^{13}C_{\text{sediment organic}} - {}^{13}C_{\text{microbial biomass}}$

where $^{13}C_{\text{sediment organic}}$ represents total ^{13}C in sediment organic carbon and $^{13}C_{\text{microbial biomass}}$ represents the ^{13}C contained in microbial biomass within the same core."

22)

1. Graphs: It would be helpful if the error bars were positive and negative

2. *We attempted to add both positive and negative errors bars, but with the complexity of the experimental design that is presented, positive and negative error bars achieve little other than cluttering the bar graphs being presented. This is especially true for figures 1A and 2 where both positive and negative fluxes are presented and with several pools being displayed in the same figure.*

3. *No changes were made as a result of this comment.*

We thank the two anonymous reviewers for their feedback. The suggested changes have helped to improve this manuscript.

The structure of the response is:
1) Reviewer comment number
1. Reviewer comment,
2. Author response
3. Changes to manuscript.

Highlighted text reflects portions of the text added as a result of comment.

Reviewer 2 main comments

1)
1. The manuscript by Riekenberg et al. describes data from ^{13}C-incubation experiments whereby microphyobenthos was labeled with ^{13}C in situ, and then incubated under controlled conditions over a period of 3.5 days; either with background nutrient levels or with higher than ambient N &/or P concentrations. While generally a well performed study, I am surprised by the short duration of the experiments (3.5 days). When fitting exponential decay functions on the resulting data (Figure 6), I feel this is somewhat thin ice – the data should be spread more in time for a convincing exponential fit.

2. *The reviewer is correct, the short time frame and relatively few data points means that the rate of loss may not be entirely representative of longer time loss at the site. However, the aim of this part of the study was to determine the relative differences in loss rates between the nutrient amended treatments and the ambient treatment. The difference between treatments is clear (now supported with a two-way ANOVA on LN 471) and similar whether an exponential or linear fit is required. The use of an exponential relationship was supported given that previous studies using a similar method of ^{13}C application have found robust exponential relationships when describing the loss of ^{13}C from sediment over a longer period, including data over the first few days following labeling (Oakes et al. 2014 Fig. 4; Veuger et al. 2012). Oakes et al. 2014 is a labeling study that occurred in the intertidal range within this same site that found exponential loss to be adequate to model ^{13}C loss from this site.*

In response to the 'thin ice' comment, we have more thoroughly investigated the differences in sediment retention of ^{13}C between treatments using a two-way ANOVA. This analysis indicated significant differences between the means for the elevated and both the ambient and minimal treatments. We subsequently explored the relationships between treatments and found the difference observed between the elevated and ambient treatments to be robust as demonstrated by the analysis now provided (LN 471) that examines the differences between the slopes of loss found for each treatment (Supplemental Figure 2). It is also interesting to note the apparent dose-effect relationship within the data set, as the moderate treatment appears to fall in between that of the minimal and elevated treatment.

Regarding the short duration of this study, we have addressed this in a previous comment from Reviewer 1 comment 7 briefly repeated here: "This project focused on initial processing of C, with multiple incubations over a relatively short period (3.5d after label addition), due to the observation in a comparable previous study at the same site that most ^{13}C transformation occurs within this ~4d (Oakes & Eyre 2014)."

3. *As a result of this comment, we have now performed a two-way ANOVA on the % of ^{13}C retained in the sediment provided on LN 471 and we have also included an analysis comparing the differences between slopes for our loss rates. LN 471 now reads: "*The total ^{13}C remaining in sediment (Fig. 6) varied significantly among treatments (two-way ANOVA: $F_{3, 31}$= 5.7, p=0.008) and across sampling times ($F_{3,}$

$_{31}$= 3.9, p=0.03). Throughout the study, there was generally less ^{13}C remaining within the elevated treatment than in than either the ambient (p=0.008) or minimal treatments (p=0.02), and there was significantly less ^{13}C remaining within the sediment at 3.5 d than at 0.5 d (p=0.02).*"*

Our response to Reviewer 2's second comment also further addresses the relevance of the turnover times calculated from this study and how they compare to previous labeling experiments performed in similar environments.

*We have also added a clarifying statement about the duration of the study in response to Reviewer 1 comment 7 repeated here: LN 204 now reads: "*These sampling time periods were chosen to capture the active dynamics of ^{13}C processing that were expected to occur over the first few days of the study, based on previous work by Oakes and Eyre (2014)."

2)

1. Abstract, line 30 and in Discussion: clearly define in the manuscript how you define and calculate the turnover time, to avoid any ambiguity. I find these turnover times surprisingly high (i.e., long), and in line with other comments, wonder whether the short incubation period did not lead to a bias in this estimate – with 3 time points very early on it seems not ideal to fit an exponential fit to these data.

2. *We disagree with the reviewer that turnover times are high. Our estimates of retention at 30 d (18-58%, LN 757) fall around the range found for other studies in unvegetated sediments (30-50%) as stated on LN 740:*

*"*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. ~50% after 21 d Hardison et al. 2011, 30% after 30 d Oakes and Eyre 2014; 31% after 30 d Oakes et al. 2012)."

The relatively short incubation times were ideal to explore the short-term fate of MPB-C which was the primary focus of this study. We then tried to use that data to see what implications these dynamics may have for longer-term retention. This is clearly not ideal, as it requires extrapolation from a small data set, but the relative differences between treatments are robust. Future studies examining nutrient effects on ^{13}C retention should examine this relationship across a longer time period if possible.

3. We have now clearly defined how we have calculated turnover time with the inclusion of our clarifying statement on LN 350: "The data for ^{13}C remaining in sediment OC were further examined by fitting an exponential decay function for each treatment across 3.5 d using the Exp2pMod1 function in OriginPro 2017 and ^{13}C turnover estimates were then determined by solving for y = 0.05% remaining ^{13}C (a value close to 0) and x = 30 d for each treatment."

We have included a statement supporting our utilizing exponential functions on LN 527: "The focus of this study was short-term fate, but our findings also show potential implications for longer- term retention. Our calculated retention times may be under or over-estimated due to their reliance on short-term data. However, the relative differences between treatments (decreased retention with increased nutrient amendment) are clear. The rationale for utilizing exponential functions in this study follows previous findings in Oakes et al. (2014) that ^{13}C export from subtidal sediments at this site were well-described by an exponential decay function across a longer time period (31 d). Additionally, the 30 d estimates provided within this study (18-58%) fall across a range similar to that of other previous labeling experiments (30-50%; Hardison et al. 2011; Oakes and Eyre 2014; Oakes et al. 2012), leading the authors to conclude that the use of exponential functions to describe this relationship was valid in this study."

3)

1. Also, it is not unambiguously clear what your t=0 is (after the 6 hour 'acclimation period' ? See next comment). On page 9, line 184, the authors mention that the cores were allowed to 'acclimate for 6 hrs prior to the start of the incubation'. I'm not sure what this means, it's not as if no microbial activity would take place during this period, hence for me it would seem to be an integral part of the incubation period. Why not simply define t=0 as the moment the cores were no longer exposed to 13C-DIC labeling ? Are these 6 hours part of the incubation times mentioned throughout the ms ? If not, this may bias the estimates of turnover times.

2. *The purpose of the acclimation period was to allow for re-establishment of any disturbed sediment redox zonation that occurred during coring (see changes due to reviewer 1 comment 7). Therefore, we omitted the 6 h period prior to measuring in an effort to obtain more robust measurements for P/R and water column fluxes for DOC and DIC. As this study was focused primarily on the development of differences between the treatments kept under the same conditions, we do not agree with the reviewer that this 6 h period is integral to the results presented.*

The described shift used with the exponential functions from the current 0 to 19 h before (when the ^{13}C was at its maximum) describes the mathematical parameter of an x transform shift by 0.8 d. Calculation of the resulting change results in materially the same estimates for turnover time (shifted longer by 0.8 d). There is no apparent bias within the dataset resulting from this as the functions used to model loss rates were not forced to 100% at the starting point (x=0).

3. *This comment further supports the previous changes that resulted from Reviewer 1, comment 7, copied here: We have added a clarifying statement about the rationale behind the 6 h acclimation period. LN 184 now reads: "*Cores were allowed to acclimate in tanks for 6 h prior to the start of incubation to allow for the re-establishment of microclimates and anaerobic zonation that was potentially disturbed by coring."

4)

1. In the abstract (line 26-27), the authors mention that treatments with higher nutrient levels showed higher loss of ^{13}C label, "supporting increased production of extracellular enzymes and storage products". I have two reservations here: First, this pattern would equally be consistent with a scenario in which the heterotrophic bacterial community was N and/or P-limited ? Eg Keuskamp et al. Sci Total Environ. 2015 doi: 10.1016/j.scitotenv.2014.11.092. I would suggest to add this as a possible mechanism in the introduction on page 4 (section starting at line 67).

2. *We agree with the reviewer that relaxation of nutrient limitation may have increased the extracellular products being released. We previously postulated that relaxation of nutrient limitation had potential to affect microbial biomass on LN 67.*

3. *We have added this possibility to our previous statement in LN 67*: "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 or an increase in production of extracellular enzymes 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."

5)

1. Secondly, this conclusion contradicts the statements in the introduction that "EPS production and bacterial utilization of newly produced EPS may decrease with increasing nutrient availability' (page 5, first lines). It is indeed generally assumed that extracellular release is a higher fraction of total primary production under nutrient-limiting conditions. On page 5 line 92-93 you write that you expected that

increased nutrient availability would stimulate EPS production – I don't see why you would assume this, it is the opposite of what the literature suggests?

2. *The reviewer is correct that the fraction of primary productivity that EPS represents is higher in nutrient limited settings as MPB produce EPS as a way to manage excess C. We agree that the nutrient addition should stimulate overall MPB-C production and not just that of EPS and have changed the wording to reflect that.*

3. *LN 92 now reads:* "We expected increased nutrient availability to stimulate production of MPB-C 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"

6)

1. I feel the quantitative handling of the data is not always transparent or easy to follow. For the overall budgets in Figure 7, it is not clear to me how these were closed: you have concentrations and d^{13}C data on all these compartments, so you can calculate them individually – but they add up to 100% each time; you could add confidence to these numbers by verifying which % of the initial ^{13}C-labeled biomass you can account for.

2. *We closed the budgets in Figure 7 by accounting for the total ^{13}C contained in the bulk sediment organic C and including the calculated fluxes for DIC and DOC that were interpolated from measurements across the 3.5 d for both DIC and DOC.*

3. *To make it clear how the budgets were constructed and closed, we have included a brief statement in the methods section about how the budget was calculated. LN 326 now reads:* "Because all ^{13}C was contained within the cores, values for ^{13}C budgets add to 100%. Starting values were estimated by looking at how much ^{13}C remained in the sediment and how much was lost to the water column (initial ^{13}C= ^{13}C remaining + ^{13}C lost)."

7)

1. Figure 6: why are these first 'accounted for by loss of ^{13}C in DIC & DOC" ? My first impression would be that you should simply look at the amount of ^{13}C remaining in the sediment, without this 'correction'? Please explain the rationale behind this in the text.

2. *Since these core incubations are a contained system, the estimate of original ^{13}C present adds to 100%, with the measured fluxes of DIC and DOC needing to be incorporated in order to correctly portion the amount of ^{13}C still contained in the sediment versus the amount exported to the water column across the incubation period.*

3. *We have further clarified how the ^{13}C budgets were constructed as a result of Reviewer 2 comment 6, briefly repeated here: To make it clear how the budgets were constructed and closed, we are including a brief statement in the methods section about how the budget was calculated. LN 326 now reads:* "Because all ^{13}C was contained within the cores, values for ^{13}C budgets add to 100%. Starting values were estimated by looking at how much ^{13}C remained in the sediment and how much was lost to the water column (initial ^{13}C= ^{13}C remaining + ^{13}C lost)."

8)

1. Towards the end of the discussion (line 757), the authors mention estimates of C retention at 30 days. This is odd, as the experiment ran over only 3.5 days and I would not consider extrapolations to 30 days very reliable (see also first comments).

2. *We address this with quoted text in reviewer 2's comment 2, but will expand further here. On LN 740, we detail findings of other studies that determined ^{13}C retention across 30 days. Further in that paragraph (LN 757) we provide our estimates of retention at 30 d taken from our exponential functions. Our ambient and minimal estimates fall close to the range of these previous studies. This finding allows us to feel reasonably confident that we are not too far out of the ballpark working with our exponential fits in the data set. Being able to gauge our results against the findings of other studies that extended over this period (e.g. Oakes & Eyre 2014) was the goal behind extrapolating our data to 30 d. We realize that extrapolation is not terrifically reliable when based on a data set formed over a short time period, but extrapolation in this case allows us to compare our short-term loss rates to other longer-term studies in an effort to gauge whether the rates we have found agree and are in a reasonable range.*

3. *A clarifying statement about the 30 d comparisons is now provided, pointing out that substantial extrapolation is required in order for us to compare to other studies looking at longer term retention of MPB-C. LN 752 now reads:* "The primary focus of this study was short-term fate of MPB-C, but the significant decrease in retention observed with nutrient amendments imply that short-term processes may have implications for longer term retention. It is interesting to consider how these short-term changes may affect the longer-term retention (30 d) reported by previous studies (e.g., Oakes & Eyre 2014), with the caveat that the substantial extrapolation required could introduce considerable error to estimates of retention."

Minor corrections

9)

1. Abstract, line 15: what is meant with 'over-enrichment' ? I assume 'enrichment' suffices.

2. *Enrichment does suffice, corrected as suggested.*

10)

1. Line 148: chlorophyll a (not alpha)

2. *Fixed throughout manuscript.*

11)

1. Line 46-47: re-write this sentence, structure is odd.

3. *LN 46 now reads:* "Application of rare isotope tracers can render fractionation effects and variability that affect natural abundance stable isotope techniques negligible and has been useful for elucidating pathways for the processing and loss of MPB-derived C within estuarine sediments."