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## Microphytobenthic extracellular polymeric substances (EPS) in intertidal sediments fuel both generalist and specialist EPS-degrading bacteria

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### Abstract

Microphytobenthic biofilms contain high concentrations of carbohydrate-rich extracellular polymeric substances (EPS) that are important in sediment carbon cycling. Field measurements at two locations in the Colne Estuary, U.K., showed that a significant curvilinear relationship explained 50% of the variability in chlorophyll *a* and EPS content. Estimates of EPS production, based on field data and published rates of production by diatoms, revealed that EPS turnover of 52% to 369% over the tidal cycle was required to account for field standing stocks. We investigated EPS degradation in sediment slurries using purified <sup>13</sup>C-EPS produced by the diatom *Nitzschia tubicola*. Although EPS constituted only 5% of the sediment dissolved organic carbon (DOC) pool, 100% of the added EPS was utilized within 30 h, before decreases in other sediment-carbohydrate fractions and DOC concentrations. A general <sup>13</sup>C enrichment of phospholipid fatty acids (PLFAs), representative of Gram-positive and Gram-negative bacteria, occurred within 6 h, with the PLFAs *a*15:0, *i*15:0, and 18:1 $\omega$ 7c being highly enriched. The diatom PLFA 20:5 $\omega$ 3 had relatively low but significant <sup>13</sup>C enrichment. Stable isotope probing of 16S ribosomal ribonucleic acid (RNA-SIP) at 30 h revealed <sup>13</sup>C-enriched sequences from the diatom genus *Navicula*; further evidence that diatoms assimilated the EPS, or EPS-breakdown products, from other diatom taxa. RNA-SIP also demonstrated a diverse range of highly <sup>13</sup>C-enriched bacterial taxa, including a distinct subset (Alphaproteobacteria and Gammaproteobacteria) found only in the heavily labeled microbial assemblages. Thus, cycling of diatom EPS is rapid, and involves a wide range of microbial taxa, including some apparent specialists.

Marine sediments contain substantial quantities of both particulate and dissolved carbohydrates, which are important constituents of the total pool of organic carbon existing in the world's oceans (Burdige et al. 2000; Verdugo 2012). Substantial differences in the biological availability of different components of the sediment carbohydrate pool are observed across a range of marine sediment habitats (e.g., sublittoral, littoral, sandy, muddy; Böer et al. 2009; Mayor et al. 2012; McKew et al. 2013). In littoral environments dominated by fine sediment, a significant proportion of the biologically reactive dissolved organic carbon (DOC) originates from the primary production of diatom-rich microphytobenthic biofilms (Underwood and Kromkamp 1999). Microphytobenthos (MPB) produce copious amounts of carbohydrate-rich extracellular polymeric substances (EPS), which contribute to the characteristic physical properties of biofilms (Underwood and Paterson 2003) and are a major source of carbon and energy in benthic food webs (Middelburg et al. 2000; Cook et al. 2007).

Marine benthic diatoms produce a range of different EPS, mainly heteropolymers rich in glucose and galactose (Bellinger et al. 2005, 2009). Production and secretion of EPS is moderated by a number of environmental factors, e.g., light, nutrient stress (Underwood and Paterson 2003), rhythms of vertical migration (Smith and Underwood 1998; Hanlon et al. 2006), and by interactions between microbial taxa (Grossart et al. 2005; Bruckner et al. 2011). The

production of large quantities of biologically labile material with a high C:N ratio provides an available carbon source for bacteria in sediments, which can lead to increased competition for available nitrogen between autotrophs and heterotrophs, altering rates of various biogeochemical processes (Cook et al. 2007).

Varying levels of micro- and meso-spatial patchiness and temporal variability are significant factors in determining general patterns of MPB biomass and production at larger, habitat scales (Underwood and Kromkamp 1999; Spilmont et al. 2011; Weerman et al. 2011). Using two large data sets from the Colne Estuary, we aimed to determine whether there is an underlying broad-scale relationship between MPB biomass (chlorophyll *a* [Chl *a*]) and EPS content in estuarine intertidal sediment biofilms present over seasonal timescales, and estimate the required in situ turnover of colloidal EPS (cEPS) on the basis of field standing stocks and published rates of EPS production.

The large molecular size of EPS precludes direct uptake into bacterial cells, and heterotrophic bacteria rely on a suite of extracellular enzymes to hydrolyze complex polysaccharides into smaller oligosaccharides and monosaccharides (Thornton et al. 2010; Arnosti 2011). Studies with sediment slurries (Goto et al. 2001; Haynes et al. 2007; Hofmann et al. 2009) and field measurements (Bhaskar and Bhosle 2008) have shown that there can be an initial rapid utilization of lower molecular weight carbohydrates, followed by subsequent reductions in the concentrations of various EPS fractions; with decreases associated with increased extracellular enzyme activity.

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In situ and experimental studies using  $^{14}\text{C}$  have found very rapid (0.5–4 h) incorporation of dissolved inorganic carbon into EPS (Smith and Underwood 1998; Goto et al. 2001).  $^{13}\text{C}$ -bicarbonate stable isotope probing (SIP) has demonstrated differential labeling of constituent sugars in diatom carbohydrate fractions from MPB (Bellinger et al. 2009; Oakes et al. 2010); with subsequent incorporation of  $^{13}\text{C}$ -labeled carbon into the phospholipid fatty acids (PLFAs) of various bacterial groups (Middelburg et al. 2000; Bellinger et al. 2009; Gihring et al. 2009). Though bacterial utilization may result in significant mineralization of diatom EPS to dissolved inorganic carbon (Oakes et al. 2010, 2012), a proportion of this carbon is incorporated into bacterial biomass, e.g., peptidoglycans, where it can contribute to the longer term retention of organic carbon within sediments (Veuger et al. 2006).

Given that diatoms excrete a variety of structurally diverse EPS, it is likely that a wide range of bacteria are involved in EPS degradation. Bacteroidetes, Alphaproteobacteria, and Gammaproteobacteria play a part in the degradation of DOC and EPS in estuaries (Haynes et al. 2007; Webster et al. 2010), and have been shown to form associations with marine diatoms (Grossart et al. 2005). Shifts in natural bacterial communities are caused by the addition of EPS (Haynes et al. 2007; Li et al. 2011), which have been tracked by adding  $^{13}\text{C}$ -labeled algal dissolved organic matter followed by deoxyribonucleic acid (DNA)–SIP or ribonucleic acid (RNA)–SIP (Chipman et al. 2010). There is some evidence that benthic diatoms utilize dissolved carbohydrates present in the environment surrounding their cells (Smith and Underwood 2000; de Brouwer and Stal 2002). However, it is not clear whether diatoms utilize complex EPS directly or benefit from increased bacterial activity within biofilms (i.e., depolymerization of EPS to smaller molecules available to the diatoms or through the production of growth factors; Croft et al. 2005; Bruckner et al. 2011).

Previous field and experimental SIP studies have relied on either a general labeling of the system with either  $^{13}\text{C}$ -labeled dissolved inorganic carbon or DOC, or addition of uncharacterized EPS extracted from sediments. Because labeled carbon will also be incorporated into other organic compounds and be excreted or liberated by cell death or grazing, it is not possible to be certain that EPS is the vehicle for such carbon transfer. One solution is to use a model organism to produce the compounds that are to be examined (Franco et al. 2008). The major advantage of this approach is that specific labeled compounds can be characterized and added to sediments in controlled amounts. In this study we set out to measure, using a defined diatom EPS source, the rates of EPS utilization by sediment microbial communities; and determine (using PLFA-SIP and RNA-SIP) which microbial taxa utilized this specific diatom EPS carbon. Bellinger et al. (2009) proposed (based on in situ  $^{13}\text{C}$  labeling of PLFAs) that diatom exudates (lower molecular weight organic material and EPS) would be preferentially utilized by a limited number of (mainly Gram-negative) bacterial taxa. By using a defined diatom EPS, we aimed to test this prediction and determine any specificity or preference by particular

bacterial taxa for diatom EPS. Because EPS are ubiquitous (Verdugo 2012), understanding the pathways of microbial EPS utilization has broad significance for carbon flow in aquatic systems.

## Methods

*Field sampling*—Sediment Chl *a* and EPS content of intertidal biofilms was determined at two locations (Pye-fleet mudflat, 51°48'12"N, 0°59'32"E; and Alresford Creek, 51°50'18"N, 0°59'22"E) in the Colne Estuary, U.K., a well-described mesotidal (tidal range 3.5–4.5 m) estuary on the east coast of England (Thornton et al. 2002; Hanlon et al. 2006). Alresford Creek mudflat was sampled from March to November 2003 and in June 2004, and the Pyefleet mudflat was sampled between January and November 2007. At each location, replicate ( $n = 10$  for Alresford,  $n = 6$  for Pyefleet) minicores (0.95 cm<sup>2</sup> area, top 2 mm of sediment, approximately 0.25 g wet weight) were taken randomly within individual square 1 m<sup>2</sup> quadrats. Sets of six quadrats were randomly placed across the midshore mudflat at > 10 m apart on the midshore over three consecutive days during each period of sampling. An additional spatial factor of upper mudflat and midshore mudflat stations (> 100 m apart) was used at Alresford Creek. Sediments were frozen in dry ice in the field, stored at  $-20^{\circ}\text{C}$ , then lyophilized, and Chl *a* and cEPS content were determined using the methods of Hanlon et al. (2006; see following section).

*Diatom isolation, identification, culturing, and harvesting of EPS*—Our approach was to purify  $^{13}\text{C}$ -labeled EPS produced by a benthic estuarine diatom isolated from mudflats in the Colne Estuary, U.K. This defined  $^{13}\text{C}$ -labeled EPS was added to sediment slurries and the pathway of carbon flow was followed using PLFA- and RNA-SIP. The methods of Smith and Underwood (1998) were used to obtain axenic cultures of benthic diatom taxa from Pyefleet channel at the mouth of the Colne Estuary. Cultures were grown in ESAW artificial seawater media (Berges et al. 2001) enriched with *f/2* vitamins and trace metals (Guillard and Ryther 1962). A small *Nitzschia* species producing abundant EPS was selected for further study. The species was identified by light microscopy of living cells and acid-cleaned frustules, DNA extraction (McKew et al. 2011), and polymerase chain reaction (PCR) amplification of the variable region of the 18S rRNA gene with primers Euk 1A (5'-CTG GTT GAT CCT GCC AG-3'; Sogin and Gunderson 1987) and 516r (5'-ACC AGA CTT GCC CTC C-3'; Amann et al. 1990). The PCR product was cleaned using a MinElute PCR Purification Kit (Qiagen), and sequenced using an Applied Biosystems Prism 3100 (Applied Biosystems), as outlined in Gramain et al. (2011). The partial 18S rRNA gene sequence (GenBank accession number JX992718) had 100% identity to that from several species of diatom including *Nitzschia*. On the basis of phylogeny and morphology, the isolate was identified as *Nitzschia tubicola*.

Cultures of *N. tubicola* were grown in 16 conical flasks of 1 liter volume containing 800 mL of ESAW salts (Berges et

al. 2001) with f/2 nutrients (Guillard and Ryther 1962), at 17°C and with a light regime of 10:14 dark:light (70  $\mu\text{mol photons m}^{-2} \text{ h}^{-1}$ ). One set (eight flasks) contained 5 mmol L<sup>-1</sup> of <sup>13</sup>C-labeled sodium bicarbonate (99.8% <sup>13</sup>C label, CK Gas Products) as an inorganic carbon source, and another set (eight flasks) contained 5 mmol L<sup>-1</sup> unlabeled sodium bicarbonate (Sigma). *N. tubicola* cultures were harvested in late logarithmic phase (after 8 d), when colloidal carbohydrate concentrations were  $5.46 \pm 0.25 \mu\text{g glucose equivalents (glu. equiv.) mL}^{-1}$  (standard error [SE],  $n = 16$ ). Cultures were centrifuged at  $3000 \times g$  for 15 min and the supernatant containing colloidal carbohydrate (Underwood et al. 1995) transferred to a fresh centrifuge tube, frozen at -20°C, and freeze-dried. These colloidal carbohydrate samples were desalted to a final salinity < 1 by dialysis using 8 kDa membrane tubing against ultrapure water for 18 h, with stirring and three changes of the water. Dialysis with an 8 kDa cut-off membrane retains polysaccharides larger than  $\sim 40$  monosaccharide units. The desalted cEPS (concentration of  $2.10 \pm 0.58 \mu\text{g glu. equiv. mL}^{-1}$ , representing  $\sim 40\%$  of the colloidal carbohydrate present in the culture media) was frozen, freeze-dried, and stored at -20°C. Freeze-dried cEPS were redissolved in 6 mL of ultrapure water (a final concentration of 0.9 mg [glu. equiv.] mL<sup>-1</sup> for both the <sup>13</sup>C-labeled EPS and the <sup>12</sup>C unlabeled EPS), and 5 mL of each was used in the experiments and the final 1 mL used for sugar and protein analysis. The monosaccharide composition of this extract was analyzed by gas chromatography–mass spectrometry (GC-MS). Protein analysis was carried out in all samples using a BioRad® Lowry standard kit with a bovine serum albumin standard curve.

There were no significant differences in the cell-normalized production of cEPS or in the relative proportions of different monosaccharides produced by *N. tubicola* grown with either the <sup>13</sup>C-bicarbonate or <sup>12</sup>C-bicarbonate in the medium (Table 1). Further fractionation of a subsample of the purified cEPS using a 100 kDa filter (Amicon) found that  $\sim 60\%$  of the extracted cEPS was made up of polymers larger than 100 kDa (Table 1). The most abundant monosaccharides were glucose, rhamnose, galactose, and mannose, collectively contributing 77% of the monosaccharides (Table 1). Protein concentrations in the both cEPS extracts were below the detection limit (1  $\mu\text{g mL}^{-1}$ ) of the Bradford assay, and so proteins were a minor component of the extracts.

**Sampling and experimental design for slurry experiments**—Sediment samples (500 g wet weight of the top 2 mm of sediment) for use in slurries were taken from mudflats adjacent to the Pyefleet channel on 17 February 2009. In the laboratory the sediment was gently mixed, passed through a 2 mm sieve to remove large organisms, portions of sediment (24 g wet weight) were decanted into 20 separate sterile 250 mL conical flasks with 120 mL of ESAW (salts' solution), and thoroughly homogenized. Four treatments were established: (1) addition (1 mL of 0.9 mg mL<sup>-1</sup> EPS stock solution to each replicate) of <sup>13</sup>C-labeled cEPS termed “<sup>13</sup>C addition”; (2) addition (1 mL of 0.9 mg mL<sup>-1</sup> EPS stock) of unlabeled cEPS—“<sup>12</sup>C

addition”; (3) no additional cEPS—“control”; and (4) inhibited using sodium azide (5 mmol L<sup>-1</sup> final concentration)—“killed controls.” Five replicates were established for each treatment.

Constituent and fraction	<sup>13</sup> C-labeled extract	<sup>12</sup> C unlabeled extract
CHO size		
>100 kDa (%)	59.86±1.76	59.24±2.71
8–100 kDa (%)	40.13±1.76	40.76±2.71
CHO conc. (mg glu. equiv. mL <sup>-1</sup> )	0.33	0.27
DOC (mg C mL <sup>-1</sup> )	0.15	0.12
Protein	not detected	not detected
% relative abundance		
Rhamnose	22.15	20.06
Fucose	11.33	13.00
Ribose	0.02	0.05
Arabinose	1.15	1.01
Xylose	10.00	8.71
Mannose	14.99	13.85
Galactose	18.73	19.42
Glucose	21.64	23.90

addition”; (3) no additional cEPS—“control”; and (4) inhibited using sodium azide (5 mmol L<sup>-1</sup> final concentration)—“killed controls.” Five replicates were established for each treatment.

Sediment slurries were incubated in the dark at 15°C on an orbital shaker (110 revolutions min<sup>-1</sup>) for 72 h. After ensuring that the slurries were well mixed, subsamples were taken from flasks at 0, 6, 18, 30, 48, and 72 h. At each time point: 2 mL of slurry was transferred to 50 mL centrifuge tubes for determination of maximum potential  $\beta$ -glucosidase activity and analyzed using 4-methylumbelliferone (MUF)- $\beta$ -glucopyranoside (McKew et al. 2011); 2 mL was removed to immediately measure the reducing-sugar concentration (Hanlon et al. 2006; McKew et al. 2011); 2 mL was taken for total organic carbon analysis (Shimadzu TOC total organic carbon analyzer with a Shimadzu SSM 5000A Solid Sample Module and autosampler; McKew et al. 2011); 4 mL was used to measure two water-soluble carbohydrate fractions: colloidal carbohydrate, which is all the dissolved carbohydrates, and a subfraction termed cEPS, material retained by an 8 kDa dialysis membrane. In addition to carbohydrate analysis, the DOC concentrations within each of these fractions were determined. The sediment pellet remaining after the extraction of the water-soluble fraction was then used to sequentially obtain hot-water-soluble and hot-bicarbonate-soluble carbohydrate fractions (Hanlon et al. 2006; McKew et al. 2011). After 0, 6, 18, and 30 h incubation, samples of each slurry were stored at -80°C for subsequent PLFA extraction (10 mL) and RNA extraction (5 mL). After the final sample point (72 h), 20 mL of slurry was remaining in each flask.

**PLFA extraction, GC-MS analysis, and GC-isotope ratio mass spectrometry analysis**—Frozen replicate slurry extracts

(10 mL) were freeze-dried, pooled, and homogenized. The freeze-dried sediment was weighed (~ 5 g) into a 20 mL glass vial, and subjected to a total lipid extraction using the method of Bligh and Dyer (1959). Organic layers from repeat extractions for each sample were pooled and solvent evaporated under N<sub>2</sub> and then stored under N<sub>2</sub> in glass vials at -20°C until fractionation.

Fractionation of the PLFAs from the total lipid extract employed the method of Dickson et al. (2009). A glass column (15 cm × 0.8 cm diameter) of oven-activated silica gel (0.5 g of 600 nm particle size; Sigma) was conditioned with chloroform (4 mL). The total lipid extract was then resuspended in 1 mL of chloroform and applied to the column in two volumes of 500 μL. The neutral lipid fraction was eluted with 5 mL of chloroform, the glycolipid fraction with 20 mL of acetone, and the phospholipid fraction with 5 mL of methanol. The solvent was evaporated from this final fraction under N<sub>2</sub> and stored at -20°C.

Fatty-acid methyl esters (FAMES) were prepared at the NERC Life Sciences Mass Spectrometry Facility, Bristol, using base-catalyzed trans-esterification. The phospholipid fractions were dissolved in a mixture of sodium diethyl ether (0.5 mL) and methyl acetate (20 μL). Twenty microliters of sodium methoxide (0.5 mol L<sup>-1</sup>) dissolved in methanol ( $\delta^{13}\text{C}$  value = -46.45‰ ± 0.03‰) was added, and after brief mixing left at room temperature for 5 min. The reaction was stopped by the addition of acetic acid (2 μL) and the solvent was evaporated under a gentle flow of N<sub>2</sub>. Hexane (1 mL) was added and the sample centrifuged (2500 g, 1 min), after which the solution of FAMES was decanted into a fresh vial from which the solvent was evaporated, leaving the residue to be dissolved in hexane for analysis by GC-MS and gas chromatography combustion isotope ratio mass spectrometry (GC-IRMS).

The position of the double bond in unsaturated FAMES was identified by further derivitization with dimethyl disulfide (DMDS; Evershed 1992). FAMES were first dissolved in 100 μL hexane; 100 μL DMDS was then added followed by 2 drops of iodine solution (6% w:v in diethyl ether). This solution was vortex mixed for 30 s, and allowed to stand at room temperature for 15 h, after which 0.5 mL of hexane was added followed by 0.5 mL of sodium thiosulfate (5% v:v), and vortex mixed in order to remove the iodine. More sodium thiosulfate was added until the solution became colorless, indicating that all the iodine had been reduced to iodide. The organic layer containing the DMDS adducts was then transferred to a clean vial and the hexane extraction of the remaining aqueous solution was repeated. All organic washings were combined and dried over anhydrous sodium sulfate, decanted into a glass vial, and solvent evaporated under N<sub>2</sub>, then stored at -20°C. When required, DMDS adducts were dissolved in hexane prior to analysis.

GC-MS was performed using a ThermoQuest Trace GC-MS system (ThermoQuest) with a split or splitless injector (used in splitless mode) and MS in electron ionization mode set to scan the range *m/z* 50 to 650, detector voltage 350 V. A Factor Four VF23-ms fused silica column of size 60 m × 0.32 mm with 0.15 μm film thickness (Varian Chrompack)

was used. The oven profile had an initial temperature of 50°C followed by two temperature ramps, first to 120°C at 15°C min<sup>-1</sup>, and then up to 250°C at 3°C min<sup>-1</sup>, with a final hold time of 15 min. Samples were injected manually (1 μL from a volume of 6 μL in hexane). Data were acquired and processed using the XCalibur (Thermo-Fisher Scientific) data acquisition and processing software. Identification was based on mass spectra in conjunction with relative retention times of known standards, including both individual FAMES (Sigma Aldrich) and mixtures (bacterial fatty acid methyl esters and Supelco<sup>®</sup> 37 component FAME standards, Sigma Aldrich), and comparison of mass spectra to the National Institute of Standards and Technology database (version NIST02).

$\delta^{13}\text{C}$  values of individual FAMES (1 μL) were determined by GC-IRMS, using a Delta V plus with a Trace Ultra GC fitted with a Programmed Temperature Vaporizing injector operating on splitless mode. A Factor Four VF23-ms 60 m × 0.32 mm with 0.15 μm film thickness column (Varian Chrompack) was used. The oven profile had an initial temperature of 50°C with a hold time of 2 min, followed by two temperature ramps, first up to 100°C at 10°C min<sup>-1</sup>, and then up to 250°C at 4°C min<sup>-1</sup>, with a hold time of 15 min. Injections were made in duplicate and mean values taken, and the instrument precision was ± 0.3‰. Isotopic ratios were corrected to take into account the exogenous carbon in the added methyl group after Rieley (1994). All  $\delta^{13}\text{C}$  values are reported relative to the Vienna PeeDee Belemnite standard. Each value is a mean of duplicate determinations with results displayed as  $\delta^{13}\text{C}$  (‰).

Taxonomic attributions based on PLFA profiles were first checked against previous studies in similar habitats (Boschker and Middelburg 2002; MacGregor et al. 2006; Bellinger et al. 2009), and then from the wider literature. Gram-positive bacteria were represented by *a14:00*, *a15:0*, *i15:0*, *15:1*, *i16:0*, *i17:0*, and *16:1ω9* (Tunlid and White 1992; Zelles 1997). Gram-negative bacteria were represented by *16:1ω5*, *16:1ω7c*, *18:1ω7c*, and *cyl8* (Tunlid and White 1992; Zelles 1997). Unspecific bacterial markers were *17:0*, *17:1ω8*, and *17:1ω7* (Zelles 1997). General markers present in both algae and bacteria were assigned as *14:0*, *15:0*, *16:0*, *18:0*, *19:1ω7*, *19:1ω8* (Tunlid and White 1992). The PLFAs *20:0*, *20:1*, *19:3*, *20:4*, *22:1*, and *22:6* were all representative of eukaryotes (Dijkman and Kromkamp 2006). The PLFA biomarker *20:5ω3* is widely recognized as being diatom specific within estuarine sediments (Middelburg et al. 2000; Boschker and Middelburg 2002). Although *20:5ω3* has the highest abundance in the diatoms, it is also present in other algal groups, though absent from the Chlorophyta (Dijkman and Kromkamp 2006). Given that diatoms dominate (in terms of abundance and biomass) within estuarine sediment biofilms, *20:5ω3* has been considered a marker for diatoms in this study.

*Preparation and analysis of carbohydrate extracts*—The neutral monosaccharide composition of the <sup>13</sup>C-labeled and unlabeled cEPS extracts was determined on a freeze-dried 1 mL sample of the concentrated salt-free cEPS solution. Samples were prepared using the protocols of

Bellinger et al. (2005). The freeze-dried samples were hydrolyzed with 200  $\mu\text{L}$  of 2 mol  $\text{L}^{-1}$  trifluoroacetic acid at 100°C for 3 h, dried under a  $\text{N}_2$  stream, saponified with 1 mol  $\text{L}^{-1}$  ammonium hydroxide (Sigma), and reduced to the corresponding alditols using sodium borohydratedimethyl sulfoxide (Sigma). The alditols were then acetylated, extracted with 500  $\mu\text{L}$  dichloromethane, then dried and stored at  $-20^\circ\text{C}$ . The dried extracts of alditol acetates were redissolved in 100  $\mu\text{L}$  dichloromethane, and 3  $\mu\text{L}$  aliquots were injected into a Thermo Trace Dual Stage Quadrupole GC-MS system. Separation was carried out through an Rtx-2330 column (Restek) with helium as the carrier gas (1 mL  $\text{min}^{-1}$ ), with a temperature gradient of 60°C for 10 min, increasing by 8°C  $\text{min}^{-1}$  to 240°C and then by 1°C  $\text{min}^{-1}$  to 250°C for 10 min. Monosaccharides were identified according to their retention time and mass spectra, and quantified using rhamnose, fucose, ribose, arabinose, xylose, mannose, galactose, glucose, and inositol standards (Sigma) that underwent the same hydrolyzation and acetylation procedure. Inositol was added as an internal standard to all samples to check for the extent of loss during the preparation process. Reproducibility of standards was within 1%. Monosaccharide data are presented as percent relative abundance of the total analyzed fraction.

**RNA extraction**—RNA was extracted as outlined by McKew et al. (2007). Extracted RNA from slurry subsamples was quantified using a Nanodrop (Thermo Scientific), and 100  $\mu\text{L}$  ( $\sim 300$  ng of RNA) was added to a mixture of 7.83 mL cesium trifluoroacetate (GE Healthcare; density  $2.0 \pm 0.1$  g  $\text{mL}^{-1}$ ), 75  $\mu\text{L}$  formamide (Sigma), and 3.55 mL of diethylpyrocarbonate-treated ultrapure water. The mixture was then placed in 11.5 mL ultracentrifuge tubes (Kendro Laboratory Products) that were crimp sealed and checked to ensure no air bubbles were present, then centrifuged ( $129,743 \times g$  for 48 h) in a Sorvall Discovery 90SE Ultraspeed centrifuge with 41.14 rotor.

After centrifugation the contents of the tubes were fractionated into 0.5 mL aliquots by piercing the bottom of the centrifuge tube with a needle (19G, 25 mm  $\times$  1 mm; Becton Dickinson), then injecting sterile ultrapure water into the top using a peristaltic pump at a rate of 10  $\mu\text{L}$   $\text{sec}^{-1}$ , and collecting fractions from the bottom. Refractive indices were measured for each fraction with a refractometer (Bellingham Stanley). In order to recover RNA from the fractions, 100  $\mu\text{L}$  was added to 1 mL of ice-cold isopropanol (Fisher Scientific), kept at  $-20^\circ\text{C}$  for 15 min, and then centrifuged at  $16,000 \times g$  at room temperature. The supernatant was removed and the pellet was washed with 70% v:v ethanol and centrifuged at  $16,000 \times g$  at room temperature. Pellets were air-dried and resuspended in 10  $\mu\text{L}$  of diethylpyrocarbonate-treated ultrapure water.

**Amplification, cloning, sequencing, and identification of microbial taxa from RNA extracts**—Reverse transcription PCR was performed on the RNA extracts from the RNA-SIP fractions using BIOScript™ reverse transcriptase (Bioline) and protocols recommended by the manufacturer, with 0.1 mmol  $\text{L}^{-1}$  reverse primer (5'-ATT ACC GCG

GCT GCT GG-3'; Muyzer et al. 1993). Two microliters of reverse transcription products were used in PCR reactions to amplify the variable V3 region of the bacterial 16S rRNA gene (*Escherichia coli* positions 341–534; Muyzer et al. 1993). PCR products were separated by electrophoresis on a 2% agarose gel in Tris(hydroxymethyl)amino-methane-acetate-ethylenediaminetetraacetic acid buffer. Denaturing gradient gel electrophoresis (DGGE) was performed as previously described (McKew et al. 2007) and silver stained using the modified protocol of Acuña Alvarez et al. (2009). Bands of interest were excised and placed in 20  $\mu\text{L}$  sterile water at 4°C for 5 d to elute DNA from the gel, and 5  $\mu\text{L}$  of eluent served as template in a PCR amplification. PCRs were performed as above (except that the GC clamp on the forward primer was not used). Triplicate PCRs were then pooled and purified with a QIAquick gel extraction kit according to the manufacturer's instructions (Qiagen) and used in subsequent cloning reactions.

Cloning was carried out with a "TA Cloning Kit" version W (Invitrogen) following the manufacturer's instructions. The cloned partial 16S rRNA gene was amplified from positive clones by PCR (as above, but using the primers: M13 [5'-GAC GTT GTA AAA CGA CGG CCA G-3'] and RM13 [5'-CAC AGG AAA CAG CTA TGA CCA TG-3']). PCR products were then cleaned using a PCR purification kit (Qiagen) following the manufacturer's instructions and sent for sequencing (GATC Biotech) using reverse T7 primer (5'-TAA TAC GAC TCA CTA TAG G-3'). Manually checked and edited sequences were subjected to a Basic Local Alignment Search Tool (BLAST) search (Altschul et al. 1990).

**Statistical analysis**—Analysis of variance with Tukey's Honestly Significant Difference multiple comparison tests and was performed using XLSTAT Version 2010 (Addinsoft). Analysis of patchiness patterns were carried out using a General Linear Model in Minitab version 13.1 (Minitab). Electrophoresis gels were analyzed with Quantity One 1-dimensional gel analysis software (BioRad) and then presence and absence data were analyzed using Primer-E (Plymouth) to determine Jaccard similarity indices, which were then used in construction of multidimensional scaling plots. Significant enrichment with  $^{13}\text{C}$  in individual PLFAs was determined by calculating statistical confidence intervals ( $p = 0.001$ ) based on the distribution of natural  $\delta^{13}\text{C}$  values in the no-addition controls and in the addition treatments at time 0 ( $n = 6$ ).

## Results

**Relationship between cEPS and Chl a, and predicted turnover of EPS**—A combined data set (Fig. 1A), based on 440 individual sediment cores taken over seasonal time-scales during 2003–2005 and during 2007 at two intertidal mudflat locations in the Colne Estuary, was used to determine the relationship between MPB biomass (Chl *a*) and EPS content in estuarine intertidal sediment biofilms. Alresford Creek supported significantly higher microalgal biomass during 2003–2005 compared to Pyefleet in 2007

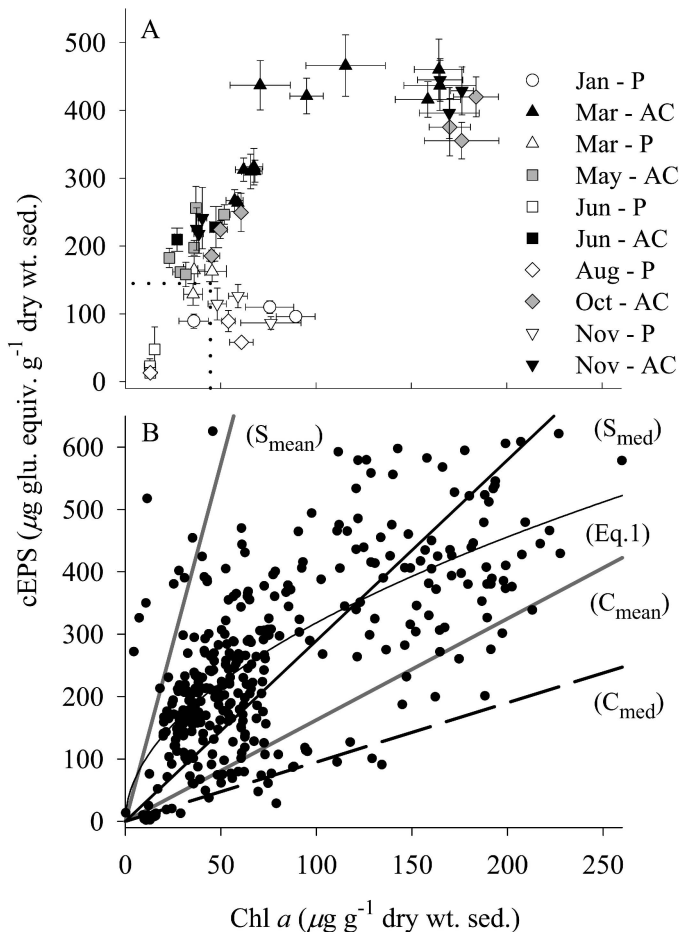


Fig. 1. (A) Sediment chlorophyll *a* (Chl *a*) and colloidal EPS (cEPS;  $\mu\text{g glu. equiv. g}^{-1}$  dry weight) content (top 2 mm) in intertidal sediment biofilms from two locations (Pyefleet [P]; Alresford Creek [AC]) in the Colne Estuary, U.K., during seven different months during 2003–2005 (AC) and 2007 (P). Mean values of spatially separate patches  $\pm$  standard error,  $n = 6$  or 10. Dotted lines indicate the values for the sediments used in the slurry experiment. (B) Best fit relationship (Eq. 1) between sediment Chl *a* and cEPS content (all data,  $R^2 = 50\%$ ,  $n = 437$ ), and estimates of new cEPS production over a single 6 h tidal emersion period, based on published rates of cEPS production ( $\text{ng cEPS } \mu\text{g Chl } a^{-1} \text{ h}^{-1}$ ) using mean ( $S_{\text{mean}}$ ,  $C_{\text{mean}}$ ) and median ( $S_{\text{med}}$ ,  $C_{\text{med}}$ ) values from in situ sediment (S) and diatom culture (C) studies.

( $F_{1,439} = 39.42$ ,  $p \leq 0.001$ ), particularly on the upper intertidal mudflat locations, though the range of Chl *a* content values from the Alresford midshore stations overlapped with values determined at Pyefleet. Highest Chl *a* and cEPS content at each site occurred in January, March, October–November, with marked reduction in biomass in the summer months (Fig. 1A). Sediment samples used in subsequent SIP experiments fell within the observed range of Chl *a* and cEPS (Fig. 1A).

Microspatial variability (differences in individual microcores within 1 m<sup>2</sup>) was high, representing 36% and 39% of the total variance in the Chl *a* and EPS data, respectively. Significant differences in both Chl *a* and cEPS content at the spatial scale  $> 100$  m ( $F_{1,437} = 508.4$ ,  $p \leq 0.001$ ;  $F_{1,439}$

$= 382.9$ ,  $p \leq 0.001$  for Chl *a* and cEPS, respectively), and at inter-month temporal scales (Chl *a*:  $F_{8,439} = 25.5$ ,  $p \leq 0.001$ ; EPS content:  $F_{8,437} = 22.65$ ,  $p \leq 0.001$ ), accounted for 43% and 18% of the Chl *a* variability, and 43% and 23% of the EPS content variability, respectively. Variability of Chl *a* and cEPS content at the  $> 10$  m scale was not significant within the estuary.

Standing stock of cEPS was significantly positively correlated with sediment Chl *a* (Fig. 1B) across all sites, times, and spatial scales ( $r_s = 0.66, 0.71, 0.39$ ,  $p \leq 0.001$  for the combined data set and for the separate Alresford and Pyefleet data sets, respectively). A power function derived from nonlinear regression was the best-fit description to explain the relationship between sediment (sed.) Chl *a* and cEPS content:

$$\text{cEPS } (\mu\text{g glu. equiv. g}^{-1} \text{ dry weight [dry wt.] sed.}) = 29.2 \times (\text{Chl } a \text{ content } [\mu\text{g g}^{-1}])^{0.519}, \quad (1)$$

$$R^2 = 50\%, p \leq 0.001, \text{ df} = 437$$

Estimates of new cEPS production by microphytobenthic assemblages over a single 6 h tidal emersion period were made using published mean and median rates of Chl *a*-normalized cEPS production by benthic diatoms in both culture and in natural intertidal sediments (Underwood and Paterson 2003; Hanlon et al. 2006; Fig. 1B). Rates derived from changes in sediment concentrations over daily exposure periods indicated the potential for a clear excess of new cEPS production compared to measured standing stocks. The average in situ production rate ( $1904 \text{ ng cEPS [glu. equiv.] } \mu\text{g Chl } a^{-1} \text{ h}^{-1}$ ) resulted in an estimate of new cEPS production representing up to 369% of the cEPS standing stock (Line  $S_{\text{mean}}$ , Fig. 1B), while median in situ rates ( $483 \text{ ng cEPS } \mu\text{g Chl } a^{-1} \text{ h}^{-1}$ ) would generate on average 93% of the in situ cEPS content (Line  $S_{\text{med}}$ , Fig. 1B) over a 6 h period. At Chl *a* concentrations  $> 125 \mu\text{g g}^{-1}$  dry wt. sed., cEPS production over a 6 h exposure period at the median rate would exceed the predicted standing stock. Rate of cEPS production derived from diatom culture experiments were lower (mean rate =  $270.9 \text{ ng cEPS [glu. equiv.] } \mu\text{g Chl } a^{-1} \text{ h}^{-1}$ ; Line  $C_{\text{mean}}$ , Fig. 1B), equivalent to approximately 52% of the predicted in situ cEPS concentration using Eq. 1. Median culture-based production rates would contribute 30% of the standing stock of EPS over a 6 h emersion period ( $C_{\text{med}}$ , Fig. 1B). These estimates indicate that there need to be significant loss processes reducing cEPS content within MPB biofilms over a 24 h period to balance the production of new cEPS in situ over tidal emersion periods. Determining the rate and organisms responsible for these degradation processes were the focus of the subsequent SIP experiments.

*Changes in concentration of cEPS, low-molecular-weight, and colloidal carbohydrates in sediment slurries*—Compared to control slurries (Table 2), the cEPS additions significantly increased the initial concentrations of colloidal carbohydrate (Tukey test,  $p \leq 0.05$ ), cEPS carbohydrate

Table 2. Concentrations (conc.) of colloidal carbohydrate (CHO), colloidal EPS carbohydrate (cEPS > 8 kDa molecular size), and dissolved organic carbon (DOC) in colloidal and EPS fractions, and  $\beta$ -glucosidase activity and reducing-sugar monosaccharide concentration in initial conditions in the control and treatment slurries after dilution and addition of cEPS. The percentage change (and whether significant) caused by cEPS additions are given. Mean values  $\pm$  standard error ( $n = 5$ ).

Variable	Sediment	Control	$^{13}\text{C}$ addition	% change	$^{12}\text{C}$ addition	% change
Colloidal carbohydrate ( $\mu\text{g}$ glu. equiv. $\text{g}^{-1}$ wet wt. sed.)	—	54.35 $\pm$ 4.0	89.85 $\pm$ 3.44	65.3*	92.75 $\pm$ 10.40	70.6*
cEPS >8 kDa ( $\mu\text{g}$ glu. equiv. $\text{g}^{-1}$ wet wt. sed.)	91.55 $\pm$ 7.55	29.71 $\pm$ 1.85	47.75 $\pm$ 2.15	60.7*	48.27 $\pm$ 4.91	62.5*
DOC colloidal fraction ( $\mu\text{g}$ C $\text{g}^{-1}$ wet wt. sed.)	616.1 $\pm$ 76.0	395.1 $\pm$ 72.0	402.23 $\pm$ 83.02	1.8 ns	395.2 $\pm$ 51.30	0
DOC EPS >8 kDa ( $\mu\text{g}$ C $\text{g}^{-1}$ wet wt. sed.)	336.7 $\pm$ 41.6	24.49 $\pm$ 7.85	49.98 $\pm$ 13.49	104*	41.22 $\pm$ 13.07	68.3*
$\beta$ -glucosidase activity (nmol MUF $\text{g}^{-1}$ wet wt. sed. $\text{h}^{-1}$ )	14.35 $\pm$ 1.20	16.60 $\pm$ 2.74	12.30 $\pm$ 2.28	-25.9 ns	13.83 $\pm$ 1.07	-20.0 ns
Reducing-sugar conc. ( $\mu\text{g}$ glu. equiv. $\text{g}^{-1}$ wet wt. sed.)	23.87 $\pm$ 2.10	27.94 $\pm$ 0.86	20.30 $\pm$ 2.61	-27.3 ns	23.38 $\pm$ 2.93	-19.5 ns

\* Significant change ( $p < 0.05$ ); ns, not significant.

(Tukey test,  $p \leq 0.005$ ), and EPS-organic carbon by between 60–70% at time 0. These starting concentrations were comfortably within the natural range of cEPS concentrations found in sediments (Fig. 1A), and so reflect a realistic situation. Changes in other variables were not significant. The addition of cEPS did not significantly increase the overall concentration of DOC in the slurries, and the added polysaccharide represented only 5% of the total carbohydrate load present in the sediments.

The slurries were EPS rich, with cEPS carbohydrate representing around 40–50% of the colloidal carbohydrate concentration in all treatments (Fig. 2). Approximately 37  $\mu\text{g}$  glu. equiv. wet weight (wet wt.)  $\text{sed.}^{-1}$  was added to each flask in the cEPS inoculum. After mixing with the sediment slurry and then undergoing an extraction procedure, the measured starting concentration of cEPS was equal in both addition treatments and  $\sim 18$  to 18.5  $\mu\text{g}$  glu. equiv. wet wt.  $\text{sed.}^{-1}$  higher than in the no-addition control (Fig. 2A). cEPS carbohydrate concentrations showed substantial changes over time, with significant net cEPS degradation in the  $^{13}\text{C}$  ( $F_{5,24} = 15.85$ ,  $p \leq 0.001$ ) and unlabeled  $^{12}\text{C}$  ( $F_{5,24} = 17.10$ ,  $p \leq 0.001$ ) addition treatments (Fig. 2A). The decreases in cEPS carbohydrate concentrations in the  $^{13}\text{C}$  addition and unlabeled addition occurred between 18 and 30 h (Tukey test,  $^{13}\text{C}$  additions,  $p \leq 0.05$ ; unlabeled additions,  $p \leq 0.001$ ), with total cEPS concentrations decreasing by almost 50% over this period ( $^{13}\text{C}$  addition treatments: 43.55–26.36  $\mu\text{g}$  glu. equiv.  $\text{g}^{-1}$  wet wt.  $\text{sed.}$ , unlabeled additions 41.52–17.25  $\mu\text{g}$  glu. equiv.  $\text{g}^{-1}$  wet wt.  $\text{sed.}$ ), an amount accounting for 100% of the added cEPS. Between 30 and 72 h there was little further change in cEPS concentration ( $^{13}\text{C}$  additions,  $p = 0.718$ ; unlabeled additions,  $p = 1.00$ ). Overall, the no-addition controls showed a significant decrease over time in cEPS concentration of 26% between time 0 and 72 h (Tukey test,  $p \leq 0.001$ ). Concentrations of lower molecular weight carbohydrate (non-EPS components) in the colloidal fraction showed significant increases for the first 30 h in both of the addition treatments (Tukey test,  $p \leq 0.001$ ) and in the controls (Tukey test,  $p \leq 0.001$ ), before declining

significantly by 72 h (Tukey test, addition treatments,  $p \leq 0.001$ ; controls,  $p \leq 0.001$ ; Fig. 2B).

Concentrations of colloidal carbohydrates (Fig. 2C) in both the EPS-supplemented treatments remained relatively constant, and higher than the controls for the first 30 h, after which there were significant decreases ( $^{13}\text{C}$  addition,  $F_{5,24} = 9.01$ ,  $p \leq 0.0001$ ; unlabeled addition,  $F_{5,24} = 3.07$ ,  $p \leq 0.05$ ), so that by 48 h, colloidal carbohydrate concentrations in all three treatments were similar. Overall, there was a significant decrease in colloidal carbohydrate concentration in the  $^{13}\text{C}$ -addition treatment (of 46%) between 0 and 72 h (Tukey test,  $p \leq 0.005$ ) and in the unlabeled addition (of 42%) between 0 and 72 h (Tukey test,  $p \leq 0.05$ ). The no-addition controls showed a significant increase in colloidal carbohydrate concentrations between 18 and 30 h and then a subsequent decrease between 30 and 72 h (Tukey test,  $p \leq 0.05$ ) of around 36%. At 18 h the concentration of colloidal carbohydrate in the additions was almost double that of the control (Tukey test,  $p \leq 0.005$ ).

In contrast to the changes in sediment carbohydrate concentrations, there were no significant differences in DOC concentrations (Fig. 3A) in the colloidal fractions of both treatment and control slurries, and DOC concentrations did not significantly change over the first 30 h of the experiment. There was an overall significant decline in DOC of  $\sim 40\%$  between 30 and 72 h (Tukey test,  $p \leq 0.01$ ; Fig. 3A). The organic carbon content of the cEPS fractions (Fig. 3B) was significantly enhanced at the beginning of the “addition” treatments (Tukey test,  $p \leq 0.001$ ), and showed significant declines (Tukey test,  $p \leq 0.001$ ), reflecting the changes seen in the carbohydrate concentrations in this fraction. Carbohydrate contributed approximately half of the organic carbon present in the cEPS fractions, but cEPS carbon only represented a small percentage (0.21%) of the total organic carbon (particulate and dissolved) present in the sediment slurries.

*$\beta$ -glucosidase activity and reducing-sugar production—* Compared with the killed controls, the maximum potential

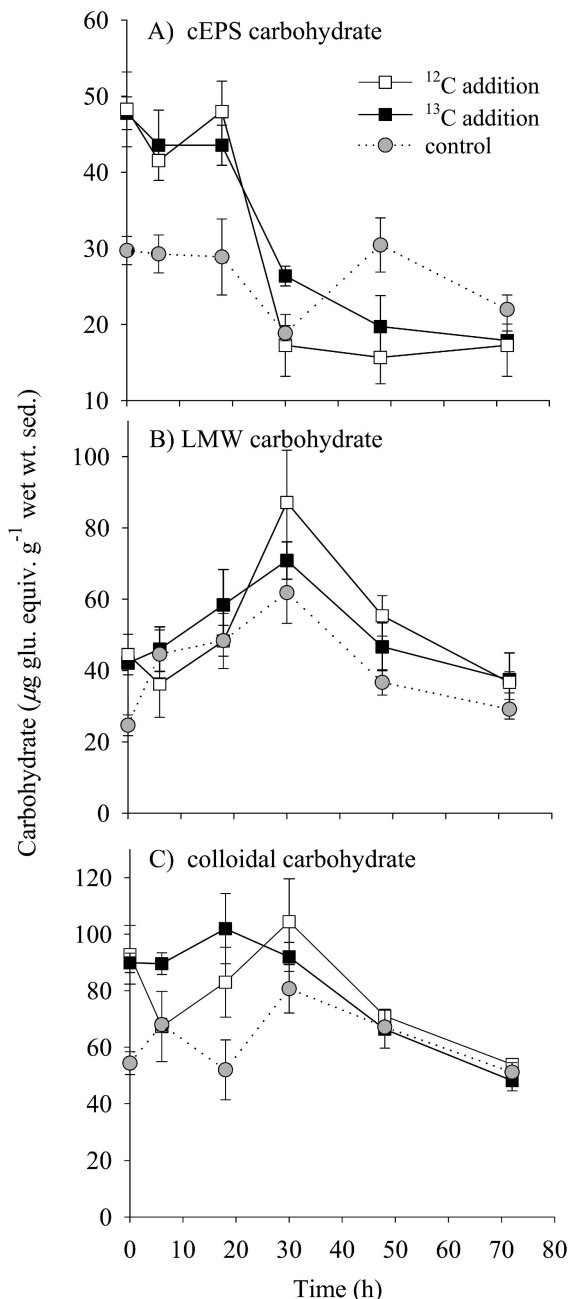


Fig. 2. Concentrations of three carbohydrate fractions: (A) cEPS (cEPS > 8 kDa), (B) low-molecular-weight (LMW) carbohydrate (< 8 kDa), and (C) colloidal carbohydrate, in estuarine sediment slurries (24 g sediment from the top 2 mm; 120 mL artificial seawater [ASW]) over 72 h. Treatments were: addition of <sup>13</sup>C-labeled and unlabeled (<sup>12</sup>C) EPS harvested from cultures of the diatom *Nitzschia tubicola*, and control slurries with no additional EPS. Values are means ( $n = 5$ )  $\pm$  standard error (SE), expressed as  $\mu\text{g}$  glucose equivalents  $\text{g}^{-1}$  wet weight of original sediment.

$\beta$ -glucosidase activity (Fig. 4A) showed a significant increase over time in all three treatments (<sup>13</sup>C addition  $F_{5,24} = 2.843$ ,  $p \leq 0.05$ ), unlabeled addition ( $F_{5,24} = 3.86$ ,  $p \leq 0.01$ ), control ( $F_{5,24} = 2.90$ ,  $p \leq 0.05$ ). All treatments showed a similar pattern with no significant difference in

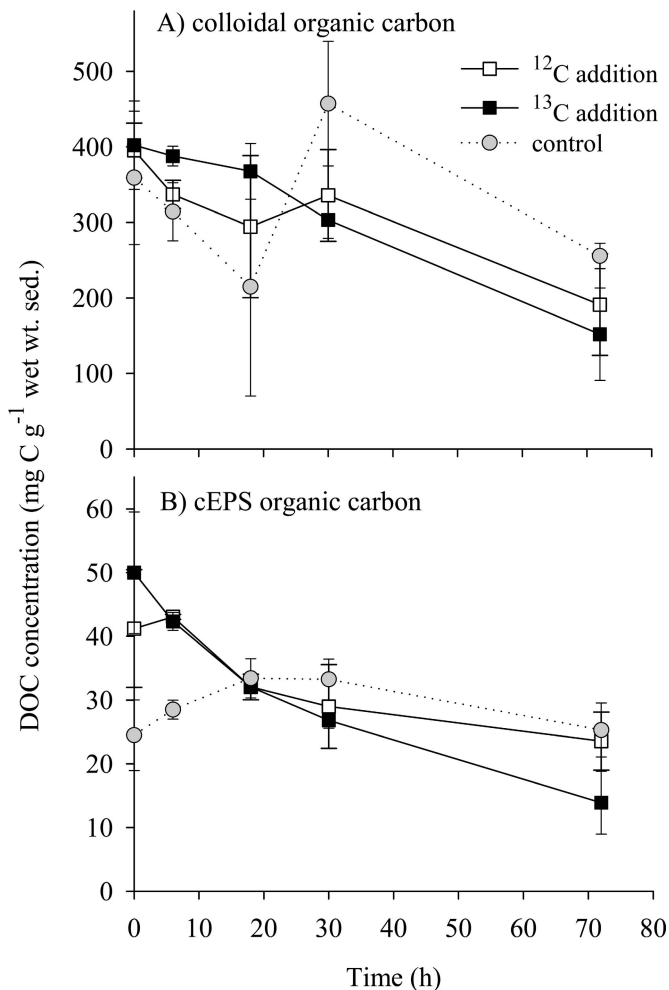


Fig. 3. Concentrations of dissolved organic carbon (DOC) in (A) colloidal fractions and (B) cEPS fractions (cEPS > 8 kDa;  $\text{mg C g}^{-1}$  wet weight sediment<sup>-1</sup>) in estuarine sediment slurries (24 g sediment, from the top 2 mm; 120 mL ASW) over 72 h. Treatments were addition of <sup>13</sup>C-labeled and unlabeled (<sup>12</sup>C) EPS harvested from cultures of the diatom *N. tubicola*, and control slurries with no additional EPS (gray circles). Values are the mean ( $n = 5$ )  $\pm$  SE, expressed as  $\text{mg C g}^{-1}$  wet weight of original sediment.

maximum potential rate between treatments. There were significant increases between 0 and 48 h of around 20% (Tukey test,  $p \leq 0.05$ ) and a further 20% increase in the unlabeled addition between 48 and 72 h (Tukey test,  $p \leq 0.005$ ). Pooling all the time point data from both the <sup>13</sup>C and <sup>12</sup>C addition treatments revealed significant negative correlations between the maximum potential  $\beta$ -glucosidase activity and cEPS carbohydrate concentration ( $r = -0.63$ ,  $p \leq 0.05$ ), and between  $\beta$ -glucosidase activity and colloidal DOC concentrations ( $r = -0.56$ ,  $p \leq 0.05$ ), whereas there were no such significant correlations in the controls.

The concentration of available reducing-sugar moieties, an indicator of the abundance of individual polysaccharide molecules, showed a general decrease (Fig. 4B) of around 50% in all treatments between 0 and 72 h (<sup>13</sup>C additions:  $F_{5,24} = 5.24$ ,  $p \leq 0.005$ ; unlabeled additions:  $F_{5,24} = 4.172$ ,



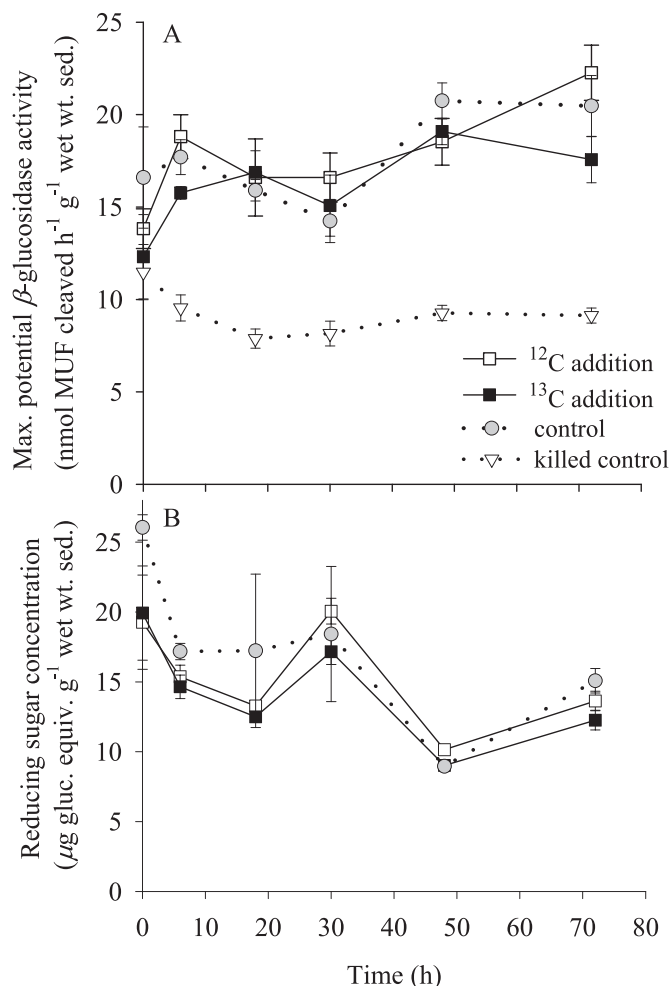


Fig. 4. Temporal changes in (A) maximum (max.) potential  $\beta$ -glucosidase activity, and (B) concentration of reducing sugars ( $\mu\text{g}$  glucose equivalents  $\text{g}^{-1}$  wet weight of original sediment) in estuarine sediment slurries (24 g sediment, the top 2 mm 120 mL ASW) incubated at  $15^\circ\text{C}$  for 72 h with additions of  $^{13}\text{C}$ -labeled EPS, additions of unlabeled EPS, no-addition controls, and killed sediment controls. Values are the mean ( $n = 5$ )  $\pm$  SE.

$p \leq 0.01$ ; controls:  $F_{5,24} = 47.63$ ,  $p \leq 0.001$ ). There was no significant difference between treatments ( $p > 0.05$ ).

**PLFA profiles and  $^{13}\text{C}$  enrichment of PLFAs**—In order to identify changes in the microbial community and utilization of the  $^{13}\text{C}$ -labeled EPS, PLFA profiles and the degree of  $^{13}\text{C}$  enrichment in individual PLFAs were determined from pooled samples at selected time points (Table 3). In total, 32 PLFAs representative of a wide range of taxonomic groups were identified. Of these, 16 PLFAs were detected with consistent patterns of relative concentration and occurrence across the three treatments. There were no clear treatment effects in the overall sediment PLFA profile (relative abundance) over the first 30 h of the experiment. The PLFA with the highest relative concentration was 16:0 (a general bacterial and eukaryotic biomarker), accounting for  $30.8\% \pm 5.0\%$  (based on a mean concentration of the total identified PLFAs at time 0

in  $^{13}\text{C}$  additions, unlabeled additions, and control), with a range of other bacterial markers contributing  $\sim 45\%$  of the total PLFA pool (Table 3), with the balance made up of other bacterial and eukaryote PLFAs.

The  $\delta^{13}\text{C}$  values for PLFAs from all no-addition and  $^{12}\text{C}$ -addition time points, as well as the time-zero values for the  $^{13}\text{C}$  addition, were very similar ( $-16.9\% \pm 0.27\% \text{ SE}$ ,  $n = 70$ ). In contrast, after 6 h incubation in the presence of  $^{13}\text{C}$ -labeled EPS, there was wide variation in the degree of  $^{13}\text{C}$  enrichment in different PLFAs (from a  $\delta^{13}\text{C}$  of  $-4.6\%$  for the diatom marker 20:5 $\omega$ 3 to  $+404.7\%$  for the Gram-positive bacteria marker *a*14:0; Table 3), implying widespread but differential significant assimilation by various microbes. Bacterial assimilation of  $^{13}\text{C}$ -EPS was high, but there was no distinction in  $\delta^{13}\text{C}$  between PLFAs from Gram-positive (range 62‰ to 404.7‰) and Gram-negative (range 66‰ to 472.6‰) bacteria after 6 h incubation. Some PLFAs (e.g., 16:1 $\omega$ 7, 17:1 $\omega$ 7, and 19:1 $\omega$ 7) were below the limit of detection by GC-IRMS at time 0, but were detected in later time point samples.

Levels of enrichment remained similar between 6 and 30 h for many PLFAs. Further increases in  $\delta^{13}\text{C}$  values occurred between 6 and 30 h in the Gram-negative-specific PLFA 18:1 $\omega$ 7c (Table 3), whereas the unspecific PLFA 19:1 $\omega$ 7 exhibited a substantial decline. In the diatom marker lipid 20:5 $\omega$ 3 (which contributed 3.4% of the PLFA pool after 30 h), there was a low but significant ( $p \leq 0.001$ ) level of  $^{13}\text{C}$  enrichment after 6 h and further  $^{13}\text{C}$  enrichment by 30 h (Table 3).

**Utilization of  $^{13}\text{C}$ -labeled diatom EPS by specific microbial taxa**—Because a significant decrease in the concentrations of cEPS carbohydrate and cEPS-DOC was observed between 18 and 30 h (Figs. 2A, 3B), RNA-SIP was performed on the 30 h samples. Community rRNA varies naturally in mol% G + C content, and so will separate along a cesium trifluoroacetate density gradient. To differentiate this natural variation in buoyant density from that caused by uptake of  $^{13}\text{C}$  EPS and incorporation of  $^{13}\text{C}$  into cellular macromolecules, a comparison was made with the rRNA from the  $^{12}\text{C}$  EPS gradient. Fractions from both gradients were collected (Fig. 5), their density measured, and after reverse transcription of 16S rRNA, were subjected to PCR amplification, which yielded amplicons from the  $^{13}\text{C}$ -labeled gradient fractions L2 to L9 (with densities of 1.85–1.73  $\text{g mL}^{-1}$ ) and from the unlabeled gradient fractions U5 to U10 (with densities of 1.79–1.73  $\text{g mL}^{-1}$ ; Fig. 5). These buoyant densities are consistent with those reported by Manefield et al. (2002) for distinguishing  $^{13}\text{C}$ -labeled and unlabeled RNA. Amplicons were analyzed by DGGE (Fig. 6) and multi-dimensional scaling analysis based on presence or absence assessment of bands (data not shown), revealing that the bacterial assemblage in the densest  $^{13}\text{C}$ -labeled rRNA samples (L2, L3, and L4) were all distinct, with a low similarity ( $< 20\%$ ) to each other, whereas the less dense  $^{13}\text{C}$ -labeled fractions (e.g., L5, L6, and L7) clustered more closely with the unlabeled fractions (U6, U7, U8, and U9). The many common bands in the DGGE of amplicons from different labeled fractions suggest that the majority

Table 3. Relative abundance (RA; % of total PLFA determined) and  $\delta^{13}\text{C}$  isotopic values (‰) for selected PLFAs extracted from sediment slurries with three different treatments: slurries with no addition of cEPS (control), addition of unlabeled cEPS ( $^{12}\text{C}$  addition), and with addition of  $^{13}\text{C}$ -labeled cEPS ( $^{13}\text{C}$  addition) at various time points between 0 and 30 h. \*\*\* = PLFA with significant ( $p \leq 0.001$ )  $\delta^{13}\text{C}$  enrichment in the  $^{13}\text{C}$  treatments.

PLFA	Group	Control		$^{12}\text{C}$ addition			$^{13}\text{C}$ addition		
		Time (h)		Time (h)			Time (h)		
		0	18	0	18	30	0	6	30
<i>a14:0</i> ***	Gram-positive bacteria	1.6	0.5	1.0	0.8	0.8	1.2	1.0	1.1
RA		—	—	—	—	—16.9	—19.9	404.7	—
$\delta^{13}\text{C}$									
<i>14:0</i> ***	general	7.7	4.5	3.9	5.7	4.6	5.7	6.0	6.4
RA		—16.5	—16.1	—15.6	—16.6	—15.1	—15.6	110.0	134.0
$\delta^{13}\text{C}$									
<i>a15:0</i> ***	Gram-positive bacteria	3.6	1.4	2.0	1.4	2.6	2.2	2.2	1.5
RA		—18.8	—19.0	—17.3	—15.8	—15.9	—18.2	376.9	350.7
$\delta^{13}\text{C}$									
<i>i15:0</i> ***	Gram-positive bacteria	2.9	1.6	2.1	1.8	2.2	2.4	2.9	2.0
RA		—19.2	—18.6	—18.2	—17.5	—17.0	—18.1	298.9	293.1
$\delta^{13}\text{C}$									
<i>15:0</i> ***	general	7.8	5.5	4.3	5.3	5.7	5.0	7.5	5.9
RA		—15.1	—14.2	—14.5	—15.1	—14.9	—15.0	45.6	21
$\delta^{13}\text{C}$									
<i>16:0</i> ***	general	40.8	28.4	26.8	24.8	47.8	24.8	35.2	27.2
RA		—18.4	—	—16.4	—17.0	—16.2	—15.7	97.6	110.1
$\delta^{13}\text{C}$									
<i>16:1<math>\omega</math>7</i> ***	Gram-negative bacteria	11.1	15.8	23.3	23.5	11.5	20.0	7.1	16.9
RA		—	—17.8	—17.0	—17.1	—16.3	—	66.0	—
$\delta^{13}\text{C}$									
<i>i17:0/16:1<math>\omega</math>9</i> ***	Gram-positive bacteria	—	2.8	—	—	1.97	—	12.7	1.4
RA		—18.3	—18.4	—	—	—19.0	—12.0	77.1	62.0
$\delta^{13}\text{C}$									
<i>17:0</i> ***	bacteria	1.8	2.9	3.0	3.5	2.3	4.2	1.8	2.8
RA		—15.9	—13.8	—18.5	—18.3	—13.7	—14.6	76.1	59.4
$\delta^{13}\text{C}$									
<i>17:1<math>\omega</math>8</i> ***	bacteria	1.5	2.4	3.2	3.3	1.9	3.4	2.0	3.1
RA		—15.3	—13.1	—14.6	—14.9	—13.6	—13.5	19.0	—2.0
$\delta^{13}\text{C}$									
<i>17:1<math>\omega</math>7</i> ***	bacteria	0.4	1.2	1.8	1.7	0.8	1.6	0.7	1.5
RA		—	—	—	—16.6	—20.9	—	32.8	—
$\delta^{13}\text{C}$									
<i>18:0</i> ***	general	5.0	4.3	0.4	3.3	3.3	3.7	3.5	5.5
RA		—20.7	—18.7	—17.7	—18.5	—20.0	—25.9	51.6	31.8
$\delta^{13}\text{C}$									
<i>18:1<math>\omega</math>9c</i>	fungi	4.1	3.2	0.2	3.2	5.1	2.7	—	4.6
RA		—	—16.3	—19.5	—21.7	—	—	—	—
$\delta^{13}\text{C}$									
<i>18:1<math>\omega</math>7c</i> ***	Gram-negative bacteria	—	7.0	13.3	9.2	—	10.3	5.4	4.9
RA		—18.2	—18.4	—18.8	—19.4	—16.8	—16.9	396.5	472.6
$\delta^{13}\text{C}$									
<i>19:1<math>\omega</math>7</i>	general	0.64	0.82	1.05	0.74	0.55	1.00	—	1.57
RA		—	—	—	—	—	—	339.2	72.6
$\delta^{13}\text{C}$									
<i>20:5<math>\omega</math>3</i> ***	diatom	0.4	4.4	6.9	4.4	1.0	4.6	0.8	3.4
RA		—	—15.9	—15.8	—15.5	—15.9	—15.8	—4.6	2.8
$\delta^{13}\text{C}$									

of bacteria are generalists, consuming EPS (hence their presence in the L2 and L3) as well as other unlabeled organic carbon (hence their presence elsewhere). Sequencing focused on DGGE bands unique to fractions L2 and L3 (densities of 1.849 and 1.836, respectively), which contain RNA from those organisms that have been most

active in specifically consuming  $^{13}\text{C}$  EPS and converting it into cellular material.

PCR products were obtained from five of 13 bands (bands A–E, Fig. 6) excised from these lanes in the DGGE gel, from which 21 clones were sequenced (Table 4). Bands A and B contained sequences that derived from the two

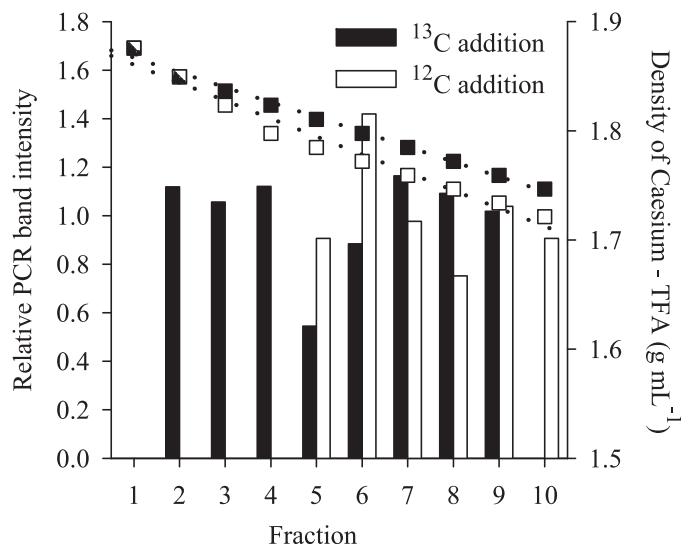


Fig. 5. Relative band intensity of PCR amplicons of cDNA from each of the fractions, (bars left-hand y-axis) and the fraction density (squares,  $\text{g mL}^{-1}$ , right-hand y-axis) based on a cesium trifluoroacetate (TFA) gradient for the sequential fractions 1 to 10 recovered by ultracentrifugation of RNA extracts from sediment slurries (24 g surface sediment, 120 mL ASW) after addition of  $^{13}\text{C}$ -labeled *N. tubicola* EPS (shaded bars) or additions of unlabeled EPS (open bars) and 30 h incubation. No PCR products were obtained past fraction 10.

related Alphaproteobacteria genera, *Azospirillum* and *Skermanella*. Band C contained sequences from two distinct genera of Gammaproteobacteria, *Pseudomonas* and *Pantoea*; while the faint band E was derived from the Gammaproteobacteria genus, *Acinetobacter*. Band D must have consisted of several overlapping bands, and yielded sequences with similarity to: Chromatiales (two sequences), *Pseudomonas stutzeri* (one sequence), *Lactococcus* (one sequence), and interestingly, 16S rRNA chloroplast sequences from the diatom genus *Navicula* (three sequences).

## Discussion

EPS are a major constituent of microphytobenthic assemblages, contributing to the structural integrity of biofilms, and provide a carbohydrate-rich carbon source supporting heterotrophic activity (Underwood and Paterson 2003; Cook et al. 2007; McKew et al. 2013). Our aims were to describe the broad-scale relationship between MPB biomass and colloidal EPS in estuarine mudflats, estimate the rates of turnover of EPS, and to determine which microorganisms are involved in the heterotrophic utilization of diatom-derived EPS carbon.

*Patterns of cEPS distribution and turnover of EPS carbon in sediments*—We found a significant curvilinear relationship between sediment Chl *a* and sediment cEPS concentrations, explaining 50% of the variability across an interannual and multisite data set. This pattern is different from previously published relationships (Underwood and Smith 1998; Bellinger et al. 2009; Thornton 2009) that have reported linear relationships between colloidal carbohydrate

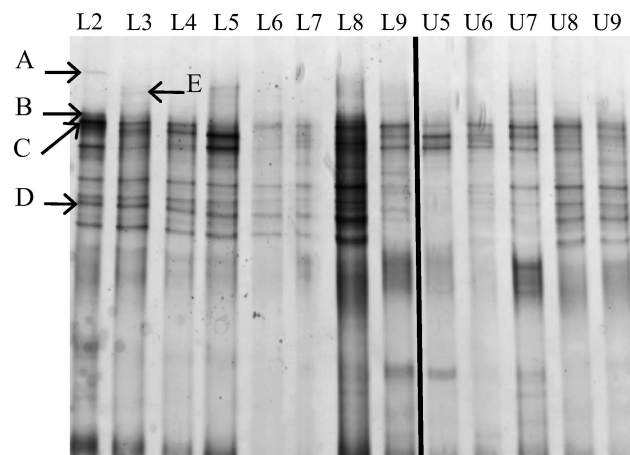


Fig. 6. DGGE gel of bacterial 16S rRNA PCR amplicons derived from reverse-transcribed RNA fractions separated by ultracentrifugation. RNA was obtained from sediment slurries incubated for 30 h with either  $^{13}\text{C}$ -labeled EPS (L) or unlabeled EPS (U) harvested from the diatom *N. tubicola*. Codes in the top row relate to specific density fractions of the gradient shown in Fig. 5. DGGE bands A–E, uniquely present in the most labeled fractions, were excised from the gel, PCR amplified, cloned, and sequenced to identify bacterial taxa.

(i.e., all water-extractable polymeric and non-polymeric carbohydrate) and Chl *a*. However, this difference is expected, as both the concentrations and relative proportions of EPS vary as a fraction of the total extracellular carbohydrate pool produced under changing environmental conditions by MPB. In dense biofilms (concentrations exceeding  $\sim 150\text{--}200 \text{ mg Chl } a \text{ m}^{-2}$ ,  $\approx 80 \mu\text{g Chl } a \text{ g}^{-1}$ ), photosynthesis and growth can become temporarily nutrient limited (Blanchard et al. 2006), which increases the production of non-polymeric carbohydrate material during photosynthesis, and reduces the relative production of EPS (Yallop et al. 2000; Perkins et al. 2001; Underwood et al. 2004). This results in a linear relationship with concentrations of dissolved carbohydrates (termed “colloidal,” Hanlon et al. 2006; or “dissolved,” Thornton 2009), at lower Chl *a* concentrations (Underwood and Smith 1998; Thornton 2009), but a relative reduction in the concentrations of large, complex EPS and a curvilinear response at higher Chl *a* concentrations (see fig. 2B in Bellinger et al. 2009).

The underlying cEPS to Chl *a* relationship (Eq. 1) encompasses the high levels of spatial patchiness and temporal variability that are recognized features of MPB (Spilmont et al. 2011; Weerman et al. 2011). A high degree of microspatial patchiness (at spatial scales within  $1 \text{ m}^2$ ) can effectively mask the presence of any significant differences in biomass at scales of  $< 10 \text{ m}$  (Spilmont et al. 2011). However,  $> 60\%$  of the variability in our data set was attributed to differences at large spatial scales ( $> 100 \text{ m}$ ), and between months, reflecting the broad-scale seasonal and landscape-scale patterns in the distribution of MPB biomass found in many systems (Underwood and Paterson 1993; Underwood and Kromkamp 1999; Thornton et al. 2002). Though Alresford Creek supported higher levels of MPB biomass, at both sites their lowest relative

Table 4. Genetic similarity of sequences of clones from bands A–E in fractions L2 and L3 (Fig. 6) to nearest cultured relatives derived from National Center for Biotechnology Information BLAST searches. PCR products were obtained from bands extracted from the DGGE gel (Fig. 6).

Clone	Closest relatives	Group	% similarity	Accession No.
A1	<i>Azospirillum, Skermanella</i>	Alphaproteobacteria	100	JX992719
A4	<i>Azospirillum, Skermanella</i>	Alphaproteobacteria	100	JX992720
A5	<i>Azospirillum, Skermanella</i>	Alphaproteobacteria	99	JX992721
B2	<i>Azospirillum, Skermanella</i>	Alphaproteobacteria	100	JX992722
B4	<i>Azospirillum, Skermanella</i>	Alphaproteobacteria	99	JX992723
B5	<i>Azospirillum, Skermanella</i>	Alphaproteobacteria	99	JX992724
B7	<i>Azospirillum, Skermanella</i>	Alphaproteobacteria	99	JX992725
C1	<i>Pantoea</i>	Gammaproteobacteria	99	JX992726
C2	<i>Pseudomonas</i>	Gammaproteobacteria	100	JX992727
C3	<i>Pseudomonas</i>	Gammaproteobacteria	100	JX992728
C4	<i>Pantoea</i>	Gammaproteobacteria	100	JX992729
C6	<i>Pantoea</i>	Gammaproteobacteria	100	JX992730
C7	<i>Pantoea</i>	Gammaproteobacteria	98	JX992731
D2	<i>Navicula</i>	Diatom chloroplast	100	JX992732
D3	<i>Navicula</i>	Diatom chloroplast	100	JX992733
D4	<i>Lactococcus</i>	Firmicutes	100	JX992734
D5	<i>Pseudomonas stutzeri</i>	Gammaproteobacteria	100	JX992735
D6	<i>Navicula</i>	Diatom chloroplast	99	JX992736
D7	Chromatiales	Gammaproteobacteria	95*	JX992737
D8	Chromatiales	Gammaproteobacteria	95*	JX992738
E3	<i>Acinetobacter</i> sp.	Gammaproteobacteria	100	JX992739

\* Sequences are 99% similar to many uncultivated bacteria from marine sediments.

biomass values were measured between May and August. This is when temperature stress and significant grazing pressure are known to decrease MPB activity, resulting in summer declines in standing stocks (Blanchard et al. 2006; Bellinger et al. 2009; Weerman et al. 2011).

Using Eq. 1, and published Chl *a*-normalized rates of EPS production by diatoms in culture and from in situ studies of diatom-dominated biofilms (Underwood and Paterson 2003), we estimated that the amount of new EPS production over a 6 h exposure period could contribute considerably to (or even exceed) the standing stock of EPS present in biofilms. Field measurements over tidal emersion periods have found significant accumulation of DOC, carbohydrate, and EPS (Smith and Underwood 1998; Staats et al. 2000; Hanlon et al. 2006), though concentrations return to initial levels after periods of tidal cover and darkness (Hanlon et al. 2006). To offset the continuous production of EPS by biofilm autotrophs (even if only the more conservative median production rates  $S_{med}$ ,  $C_{med}$  are considered), there needs to be substantial EPS loss processes occurring over a 24 h period, which will prevent the accumulation of EPS. Dissolution and physical wash-away of surface biofilm material into the water column during tidal cover can account for between 25% and 65% of the required reductions (Blanchard et al. 2006; Hanlon et al. 2006; Thornton 2009), whereas microbial activity will lead to the continuous degradation of EPS (Hanlon et al. 2006; Bellinger et al. 2009; Oakes et al. 2010).

*Conditions in the slurry experiments*—Previous studies have used  $^{13}\text{C}$ - or  $^{14}\text{C}$ -bicarbonate enrichment approaches to label diatom exudates (Goto et al. 2001; Bellinger et al. 2009; Oakes et al. 2010), and follow the transfer of that label to bacteria and eukaryotes (Middelburg et al. 2000;

Cook et al. 2007; Bellinger et al. 2009). Because our specific aim was to follow EPS degradation, we used a characterized  $^{13}\text{C}$ -enriched EPS produced by *N. tubicola* in our slurry experiments. Species in the genus *Nitzschia* are very common benthic taxa, with species characterized by small lineolate growth form, such as *N. tubicola*, recorded widely (but often not fully identified) from estuarine biofilms (Thornton et al. 2002; Forster et al. 2006). The EPS isolated from *N. tubicola* (a range of heteropolysaccharides > 8 kDa, with 60% of the material > 100 kDa), was very similar to EPS from cultures of other benthic marine diatoms (Underwood and Paterson 2003; Bellinger et al. 2005) and to EPS isolated from biofilm-dominated intertidal mudflats (de Brouwer and Stal 2001; Hanlon et al. 2006). We are therefore confident this provided a realistic purified EPS to use in the slurry experiments.

Addition of diatom EPS resulted in significant increases in EPS and colloidal carbohydrate concentrations, but final concentrations were within the natural range present in intertidal mudflats (Hanlon et al. 2006) or used in previous EPS enrichment studies (Haynes et al. 2007; Hofmann et al. 2009). Although EPS concentrations were increased, we did not significantly increase sediment DOC (which had a concentration an order of magnitude higher). Therefore, the concentration and composition of the added carbon source was relevant for the studied environment; and the increased EPS content in the enrichments would result in no major perturbation of the sediment organic carbon loading beyond what may be found under natural conditions. This is important because we wished to avoid substantial enrichments of total organic carbon that can result in significant shifts in sediment properties and the microbial community (Mayor et al. 2012).

*Degradation of cEPS by heterotrophic activity*—Major decreases in cEPS from *N. tubicola* occurred in both enriched treatments between 18–30 h (Fig. 2), similar timescales to those seen in previous cEPS slurry experiments (Haynes et al. 2007; Hofmann et al. 2009) and field measurements (Hanlon et al. 2006). Similar decreases in EPS concentrations (but of lower magnitude), were seen in the no-addition controls, indicating that the degradation rates of the added diatom cEPS was identical to those of EPS from natural diatom assemblages. Despite the substantial pool of DOC present in the slurries, it was specifically the cEPS fractions that showed the most rapid decreases. This suggests a high level of preferential utilization of this material by the microbiota present in the slurries.

The added diatom EPS contained a substantial proportion of glucose and galactose (~ 40% relative abundance). These monosaccharides may act as weak links, or access points, for bacterial degradation, as opposed to those polymers rich in fucose or rhamnose (Giroldo et al. 2003). Similar preferential turnover of glucose within EPS fractions has been reported (Bellinger et al. 2009; Oakes et al. 2010). Though monosaccharide composition is not necessarily a good proxy for polysaccharide structure (Arnosti 2011), it is clear that the large polysaccharides (> 100 kDa) present in diatom EPS can be rapidly, and preferentially, degraded in sediments (Goto et al. 2001; Hanlon et al. 2006; Oakes et al. 2010).

Associated with the increased EPS concentrations at the beginning of the experiment were significant declines in extracellular  $\beta$ -glucosidase activity, an enzyme that has been used as a proxy for microbial hydrolysis of carbohydrates in aquatic systems (Thornton et al. 2010; McKew et al. 2011). Extracellular hydrolysis of the polysaccharide chains is necessary as EPS are too large to be taken up across the bacteria cell membrane (Arnosti 2011).  $\beta$ -glucosidase activity in the killed controls reflected the original activity in the sediments used to make the slurry, and this activity slowly declined over time as the existing pool of enzymes became inactive. Extracellular enzyme activity is upregulated in the absence of easily accessible substrates, and the control treatment (no additional cEPS) showed elevated  $\beta$ -glucosidase activity at the beginning of the experiment. Where additional EPS had been added, there was a slight increase over the killed controls, but then activity increased over time in both addition treatments. Other studies have also identified increased rates of  $\beta$ -glucosidase activity in intertidal sediments in response to enrichment with organic matter (Goto et al. 2001; Köster et al. 2005). While simple MUF-linked substrates may not be ideal analogues for studying the degradation of complex natural polysaccharides (Arnosti 2011), it is clear that during the first 6 h of the experiment, the bacterial community in the enrichment treatments was utilizing carried-over labile material, before beginning to utilize the added high-molecular-weight EPS, via the production of extracellular  $\beta$ -glucosidases or similar enzymes (Thornton et al. 2010). Enzyme activity increased in all treatments later in the experiment, indicative of extracellular degradation of more refractory carbohydrates

(Haynes et al. 2007; McKew et al. 2013). The increases in non-polymeric (lower molecular weight) carbohydrates in the slurries during the first 30 h (Fig. 2B) can be explained by the accumulation of less labile small products of EPS degradation (Haynes et al. 2007; Hofmann et al. 2009), as well as additional bacterial DOC production (Eichinger et al. 2009). Reductions in the lower molecular weight carbohydrate pool only occurred after cEPS concentrations had declined, indicating a preference by the microbial assemblages for the original added diatom EPS over subsequent degradation products.

These rapid loss rates for diatom EPS (50% of total concentration in slurries over 30 h) demonstrates the potential for very close coupling between production and loss, and for high rates of EPS turnover in biofilms (Middelburg et al. 2000; Hanlon et al. 2006; Cook et al. 2007). Though EPS are large molecules, the preferential use of this material would indicate that at least some members of the sediment microbial community are preadapted to utilize it as a carbon source. Such a high turnover is necessary to explain the pattern of EPS and Chl *a* distributions in intertidal MPB (Fig. 1).

*Response of the heterotrophic microbial community to cEPS addition*—We conducted slurry experiments because our aim was to determine the relative rates of utilization under standard conditions over a time course and identify preferential uptake of a specific EPS carbon source into microbial assemblages, rather than determine actual rates, which is better done in situ (Middelburg et al. 2000; Bellinger et al. 2009; Oakes et al. 2010). Short-term slurry experiments have been used successfully to determine microbial responses to EPS additions (Haynes et al. 2007). The absence of major changes in PLFA profiles between treatments suggest that we did not induce significant community-level changes over the first 36 h of our experiments.

Incorporation of  $^{13}\text{C}$  from diatom EPS into bacteria-specific PLFAs occurred by 6 h, similar to the timescale (4 h) of in situ PLFA-labeling studies in estuarine intertidal sediments (Middelburg et al. 2000; Bellinger et al. 2009). This, together with the EPS-degradation data, provides clear evidence that bacteria inhabiting diatom-rich sediments (Thornton et al. 2002; Hanlon et al. 2006) were preadapted and primed to utilize diatom EPS as a source of carbon and energy. The PLFA profile obtained from the slurries was very similar to that found in intertidal sediments from estuarine systems (Middelburg et al. 2000; Boschker and Middelburg 2002; MacGregor et al. 2006), suggesting no major shifts in the bacterial community composition in the slurries. The fact that all PLFAs in the  $^{13}\text{C}$ -addition experiments became enriched in  $^{13}\text{C}$  suggests that a wide range of bacteria can utilize diatom EPS. However, differences in levels of  $^{13}\text{C}$  enrichment across the range of PLFAs identified (some of the most enriched PLFAs represented only a small proportion of the total PLFA extracted) can be partially explained if diatom EPS was being used preferentially by a subset of specialist microorganisms (with a taxonomically constrained PLFA composition) in the sediment slurries. Taxonomic attribution of

PLFA profiles is not reliable below broad taxonomic levels, but we did find very high  $^{13}\text{C}$  labeling in some PLFAs present at low relative concentrations indicative of Gram-positive and Gram-negative bacteria (Table 3), and lower levels of enrichment in general markers. Bellinger et al. (2009) proposed, on the basis of PLFA enrichment patterns, that Gram-negative bacteria may be initial degraders of EPS followed by the Gram-positive bacteria. In our slurries, high enrichment in both PLFA *a15:0*, *i15:0* (specific for Gram-positive bacteria), and PLFA *18:1 $\omega$ 7c* (specific for Gram-negative bacteria) were very similar after 6 h, suggesting that was not the case.

The presence of several common bands in the DGGE profiles over a wide range of  $^{13}\text{C}$ -labeled fractions supports the PLFA data, indicating that many bacteria can consume diatom-derived EPS as well as a range of other carbon sources. Such a generalist lifestyle is typical of many coastal bacteria (Mou et al. 2008). However, the absence of bands from, and the presence of bands unique to, the densest fractions (L2, L3, Fig. 6), demonstrates that EPS consumption is by no means universal and that there are specialist EPS-degrading bacteria in the intertidal sediment community. These findings are consistent with previous studies that showed only small changes in overall bacterial community composition in response to added EPS (Hanlon et al. 2006; Haynes et al. 2007; Bellinger et al. 2009). Given that 30 h elapsed prior to RNA extraction from the sediment slurries, there will have been a progressive dilution of label within the sediment carbon pool, as EPS is broken down and newly produced  $^{13}\text{C}$ -labeled organic products (both intracellular and extracellular; Eichinger et al. 2009) would become available for subsequent uptake by other microbiota. However, though the major decreases in EPS concentrations occurred between 18 and 30 h, we did see significant enrichment of PLFAs within 6 h of addition of  $^{13}\text{C}$ -EPS, indicating degradation of and incorporation of EPS-carbon into PLFAs had been initiated over that much shorter timescale.

The bands identified as unique to the RNA-SIP heavy fractions came mainly from the bacterial classes Alphaproteobacteria and Gammaproteobacteria that possess a wide range of metabolic functions and are common in coastal sediments and waters (McKew et al. 2011). Representatives of both classes feed on organic carbon derived from diatom blooms, expressing polysaccharide-degradation genes (Teeling et al. 2012), and Alphaproteobacteria have been linked to  $\beta$ -glucosidase activity in diatom blooms (Rieman et al. 2000). However, the specific genera identified by us as uniquely degrading benthic diatom EPS differ from those typically abundant in coastal sediments. This does not mean that common bacteria do not utilize EPS carbon—our results focus on taxa that have taken up the greatest amount of  $^{13}\text{C}$  label. The higher taxonomic resolution of RNA-SIP allows for the identification of these specialists, while the PLFA profiles show that a general labeling of the microbial community also occurred.

Some of the DGGE bands present in the heavily  $^{13}\text{C}$ -labeled fractions are from taxa known to have interactions with phototrophs; for example, members of the genus *Azospirillum* (A and B, Table 4) have been shown to

interact with plants (Bashan and Holguin 1997) and to have a positive effect on the growth of microalgae (Perez-Garcia et al. 2010). The Gammaproteobacterial genera *Pseudomonas* and *Acinetobacter* were found in the heavy fractions in this experiment. Haynes et al. (2007) also found large increases in the relative abundance of members of the Gammaproteobacteria, particularly *Acinetobacter* and also *Pseudomonas*, in response to cEPS additions. Sequence comparison showed that the clones identified as *Acinetobacter* by Haynes et al. (2007) were similar, but not identical, to the clones identified in this study (data not shown). Members of the genus *Acinetobacter* possess genes for  $\beta$ -glucosidase production (Tajima et al. 2001) and some members are also capable of producing a wide range of glycosidases (Kim et al. 2008). They have also been linked to degradation of macromolecules in complex lichen communities (Grube et al. 2009). Importantly, representatives of both genera were recently shown to degrade polysaccharides from, as well as enhance growth of, the alga *Tetraselmis indica* (Arora et al. 2012). In band C (Fig. 6), which was the most dense unique band in the heaviest fraction, the bacterial genus *Pantoea* was identified in some of the clones (Table 4). *Pantoea* is a member of the Enterobacteriaceae, but is found in a wide range of habitats. Members of the genus have been linked to EPS degradation in cooling tower biofilms (Ceyhan and Ozdemir 2008), and a marine isolate of the genus has been found to produce chitinase and glucosidases capable of degradation of complex macromolecules (Gohel et al. 2006). Given that Bacteroidetes have been found associated with diatoms and estuarine biofilms (Sapp et al. 2007; Teeling et al. 2012), it is notable that we did not identify them in the most heavily  $^{13}\text{C}$ -labeled fractions. This does not preclude their playing a role in EPS degradation because only those bands that were not also found in lighter fractions were sequenced.

#### *Heterotrophic utilization of cEPS by diatoms in the dark—*

The diatom-specific PLFA 20:5 $\omega$ 3 became increasingly enriched over 6 and 30 h, although the degree of enrichment was much lower than for other PLFAs. Moreover, diatom chloroplast 16S rRNA sequences with a high similarity to 16S rRNA genes from the chloroplasts of *Navicula* spp. were detected in the band D of the densest fraction from the RNA-SIP experiment (Fig. 6; Table 4). This labeling of diatom RNA (after 30 h in darkness) matches the significant increases in the level of  $^{13}\text{C}$  enrichment in the diatom-specific PLFA 20:5 $\omega$ 3, and strongly suggests a heterotrophic pathway of carbon acquisition from diatom EPS into living diatoms. Cross contamination of  $^{13}\text{C}$ -labeled RNA or PLFA from the original *Nitzschia* culture can be discounted. PLFAs exhibit low aqueous solubility, the EPS extraction procedure was completely water based and subject to a number of purification steps, and the  $\delta^{13}\text{C}$  values for this diatom-specific PLFA at time 0 was in the range of all the others. It is very unlikely that nucleic acid would have been isolated in the extraction because the nucleic acid content of diatom EPS is low (Bhaskar and Bhosle 2008), and we measured an extremely low ribose content in the EPS (< 0.05% of the

monosaccharides determined, Table 1). There may have been some slight carryover of  $^{13}\text{C}$ -labeled bicarbonate, (despite the washing and dialysis steps carried out), but the slurries were incubated in darkness, preventing photosynthesis. Anapleurotic uptake of  $\text{CO}_2$  can occur but it is negligible (Smith and Underwood 1998). Finally, the heavy chloroplast sequences found were from *Navicula*, a genus phylogenetically distinct from *Nitzschia* (Rimet et al. 2011). *Navicula* species would have been present in the slurries, as they are one of the most common benthic diatoms in the Colne Estuary system (Thornton et al. 2002), and can survive extended periods of darkness and anaerobic conditions (McKew et al. 2013).

Diatoms could be taking up mono- or disaccharides released from  $^{13}\text{C}$ -EPS by bacterial extracellular enzymes or even polysaccharides from the originally added EPS. Heterotrophic uptake is known in benthic diatoms, though work has concentrated on small molecules, e.g., acetate, glucose, and amino acids (Admiraal et al. 1987; Liu et al. 2009). It is not known if diatoms secrete extracellular enzymes to degrade EPS (some diatoms are reported to be able to degrade macroalgal polysaccharides; Armstrong et al. 2000). Decreases in extracellular carbohydrates in the dark in axenic diatom cultures have been reported (Smith and Underwood 2000; Staats et al. 2000), and a number of species of apochlorotic diatoms, including species in the genus *Nitzschia*, can utilize algal polysaccharides in axenic culture (Armstrong et al. 2000). Our experimental design does not allow us to determine whether diatoms present in the slurries were directly utilizing the  $^{13}\text{C}$ -labeled *N. tubicola* EPS or other labeled by-products of microbial degradation of this EPS. However, labeled diatom PLFA were present after 6 h, so if carbon transfer was via a bacterial intermediary, this suggests a tightly coupled and rapid process. A potential indirect pathway could be the production by associated (mutualistic) bacteria of some required compound using carbon derived from the  $^{13}\text{C}$  EPS (e.g., amino acids, essential vitamins), subsequently made available to the algae (Croft et al. 2005). There could be selective pressures to have evolved such a strategy. Diatoms in estuarine sediments can spend long periods buried under the sediment in the dark and may switch to heterotrophy to survive. Diatoms have previously been shown to utilize complex carbohydrates when conditions become unfavorable for autotrophic growth (de Brouwer and Stal 2002). The data presented here provide strong evidence that benthic diatoms can access diatom EPS-carbon produced by another diatom species and incorporate that into their lipid and RNA. Further work is needed to determine the mechanisms and cost–benefits of such a strategy.

*Microbial EPS cycling in intertidal sediments*—Extracellular polymeric substances are important components of the carbon cycle in surficial sediments dominated by MPB. Standing stocks of EPS are a product of both production and consumption rates, yet are closely correlated to MPB biomass. Despite MPB excreting a range of extracellular carbon compounds (Underwood and Paterson 2003; McKew et al. 2013), colloidal EPS molecules are rapidly and preferentially degraded by heterotrophic bacteria

under aerobic conditions. While the general sediment bacterial community utilizes diatom EPS (with no temporal distinction, contrary to the predictions of Bellinger et al. 2009), we have provided evidence supporting earlier work that some taxa might be considered EPS specialists (e.g., *Acinetobacter*; Hanlon et al. 2006; Haynes et al. 2007). The physical structured nature of biofilms increases the likelihood that these sediment systems will support many potential species–species interactions (Croft et al. 2005; Chipman et al. 2010; Teeling et al. 2012), whereas studies of freshwater-lake biofilms have shown diatom EPS production to be positively influenced by the presence of particular bacteria (Bruckner et al. 2011). We have recently shown that different, more insoluble, EPS fractions produced by estuarine MPB are preferentially degraded in anaerobic compared to aerobic conditions (McKew et al. 2013). Given the ubiquity of EPS in aquatic systems (Verdugo 2012), it is likely that similar interactions and processes are occurring in many different habitats.

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