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Sequential bioavailability of sedimentary organic matter to heterotrophic bacteria

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Summary

Aquatic sediments harbour diverse microbial communities that mediate organic matter degradation and influence biogeochemical cycles. The pool of bioavailable carbon continuously changes as a result of abiotic processes and microbial activity. It remains unclear how microbial communities respond to heterogeneous organic matrices and how this ultimately affects heterotrophic respiration. To explore the relationships between the degradation of mixed carbon substrates and microbial activity, we incubated batches of organic-rich sediments in a novel bioreactor (IsoCaRB) that permitted continuous observations of CO_2 production rates, as well as sequential sampling of isotopic signatures ($\delta^{13}C$, $\Delta^{14}C$), microbial community structure and diversity, and extracellular enzyme activity. Our results indicated that lower molecular weight (MW), labile, phytoplankton-derived compounds were degraded first, followed by petroleum-derived exogenous pollutants, and finally by higher MW polymeric plant material. This shift in utilization coincided with a community succession and increased extracellular enzyme activities. Thus, sequential utilization of different carbon pools induced changes at both the community and cellular level, shifting community composition, enzyme activity, respiration rates, and residual organic matter reactivity. Our results provide novel insight into the accessibility of sedimentary organic matter and demonstrate how bioavailability of natural organic substrates may affect the function and composition of heterotrophic bacterial populations.

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Introduction

Aquatic sediments cover almost ~75% of the Earth's surface and are one of the largest reservoirs of organic matter (Berner, 1982; Hedges and Keil, 1995; Cole *et al.*, 2007; Breithaupt *et al.*, 2012). Heterotrophic microorganisms living in these carbon-rich sediments are directly involved in the transformation and mineralization of organic matter and contribute to a significant fraction of respiration in these ecosystems (Jørgensen, 1983; Boudreau, 1992; Howarth, 1993; Del Giorgio and Williams, 2005). A wide array of bacteria are involved in mineralizing organic matter to carbon dioxide (CO_2), including groups that produce extracellular enzymes to catalyze the degradation of complex macromolecular compounds into smaller, more soluble substrates (Arnosti, 2011). The activity of heterotrophic microorganisms can significantly affect the type and quantity of carbon that is stored in sediments (Koho *et al.*, 2013), leading to profound impacts on both local and global biogeochemical cycles (Burdige, 2007). Therefore, to accurately predict carbon fluxes in aquatic systems we must understand how heterotrophic communities select and access carbon substrates in sediments.

Organic matter is comprised of a heterogeneous mixture of compounds that vary in chemical structure, origin, and age (Zonneveld *et al.*, 2010). These compounds can include recently photosynthesized phytoplankton biomass, terrestrial material introduced via erosion and/or rivers, as well as biomolecules generated by microorganisms (Ogawa *et al.*, 2001; Hagy *et al.*, 2005; Jiao *et al.*, 2010; Blair and Aller, 2012). Only a minor fraction of the total organic matter pool is available or accessible for utilization by heterotrophic microorganisms (Middelburg, 1989). The accessibility of the organic matter is thought to be influenced by its intrinsic chemical and physical properties including molecular size and sample history (fresh vs. aged organic material), interactions with mineral surfaces, as well as its origin (terrestrial vs. marine) (Hedges *et al.*, 1988; Keil *et al.*, 1994; Lee, 1994; Wakeham *et al.*, 1997; Lalonde *et al.*, 2012; Keil and Mayer, 2014). As a result, the pool of bioavailable carbon continuously changes and

may eventually be exhausted. It is unclear how microbial communities respond to this varying supply of carbon and how this ultimately affects heterotrophic respiration. Even small changes in the availability of organic substrates may trigger a shift in community composition and/or change the abundance of specific extracellular enzymes (Pinhassi *et al.*, 1999; Jones *et al.*, 2009; Dinasquet *et al.*, 2013; Sarmento *et al.*, 2016).

Understanding the relationship between the changing nature of carbon substrates and microbial respiration is a central question in carbon biogeochemistry, but it is complex and challenging to address. Microbial communities are taxonomically and metabolically diverse and derive organic carbon from varied pools that are heterogeneously distributed in space and time. Thus, it is difficult to distinguish between organic matter pools that support the growth of heterotrophic bacteria and those that do not. Natural abundance stable (^{13}C) and radiocarbon (^{14}C) analyses have become powerful tools for probing the sources and ages of natural organic matter consumed by microbes (Petsch *et al.*, 2001; Wakeham *et al.*, 2006; Pearson *et al.*, 2008; Ahad and Pakdel, 2013; Mailloux *et al.*, 2013; Mahmoudi *et al.*, 2013a,b). Heterotrophic microorganisms carry the same ^{14}C signatures and nearly the same ^{13}C signatures as their carbon sources; therefore, the ^{13}C and ^{14}C signatures of both microbial cellular components (e.g. membrane lipids) and respired CO_2 can be used to infer microbial utilization of isotopically-distinct carbon sources (Hayes, 2001). ^{14}C measurements of phospholipid fatty acids (PLFA) extracted from living microorganisms in agricultural soils demonstrate preferential utilization of carbon compounds derived from younger pools, i.e. plant material and/or root exudates (Rethemeyer *et al.*, 2004; 2005). In coastal ecosystems, ^{13}C measurements have been used to address the origin of carbon supporting bacterial populations, sometimes providing contrasting results and highlighting the complexity and variability of microbial carbon utilization. For example, ^{13}C measurements of PLFA extracted from three different salt marshes found that plant-derived organic matter was a minimal carbon source in one marsh while it was the primary carbon source at another (Boschker *et al.*, 1999). This is consistent with other studies that show microbial carbon sources are influenced by a number of factors including the sedimentation rate, the plant species and the total organic carbon content of the sediment (Bouillon and Boschker, 2006).

Unraveling the complexities of organic carbon metabolism and substrate availability is difficult because of the high levels of diversity of both natural organic matter and microbial communities. Here, we tracked the dynamics of carbon utilization by microbes present in a native sediment community after sediment processing and storage; the resulting community thus was composed almost exclusively of endospore-forming taxa. Using this approach, we reduced microbial diversity yet maintained fairly high levels of substrate diversity such that the microorganisms were exposed to a diverse mixture of carbon compounds. Although the selected microbial community may not resemble a natural assemblage, our goal was to understand the bioavailabity of sedimentary organic matter and assess the response of microbes to a heterogeneous supply of carbon substrates. While this approach limits our ability to extrapolate the observed rates to *in situ* environmental conditions, it allows us to examine key relationships between community dynamics and substrate utilization that may be occurring in the natural environment.

In the present study, we use a novel system called Isotopic Carbon Respirometer-Bioreactor (IsoCaRB) (Beaupré *et al.*, 2016) to investigate accessibility of sedimentary organic matter and microbial carbon utilization patterns. The IsoCaRB system allows us to continuously monitor and collect respiratory CO_2 quantitatively for natural-abundance isotopic analyses. These time-resolved isotopic measurements provide information on the age and source of natural organic matter being utilized, as well as its rate of remineralization. In addition to collecting successive fractions of microbially-respired CO_2 for natural abundance isotopic analysis ($\Delta^{14}C$ and $\delta^{13}C$), concurrent barcoded 16S rRNA Illumina sequencing and quantitative PCR (qPCR) were carried out on selected time points to assess the response of the microbial population. Lastly, extracellular enzyme activity was measured at these same time points to assess relative rates of polysaccharide (e.g. plant-derived cellulose) and protein utilization. The results indicate that organic matter degradation is indeed sequential and that substrate succession is a function of the inherent structure of the molecular class. Simultaneously, changes in microbial community composition appear to track the changing nature of the bioavailable substrate.

Results

Microbial CO_2 production and carbon utilization

The IsoCaRB system revealed three distinct but reproducible peaks in CO_2 production (Fig. 1). Both experiments commenced with a peak on day ~1.1, followed by a smaller peak on day 3.1, a final peak on day 5.25, and a gradual decrease to near baseline values by days 7 to 8. In addition to similar patterns of CO_2 concentration between experiments, the rates of CO_2 production were relatively consistent and ranged from ~ 1 to 4 μmol C hr^{-1}, in agreement with other studies (Hansen and Blackburn, 1991; Morris and Bradley, 1999). For the time-steps corresponding to the three major peaks, the maximum CO_2 yields over the intervals were respectively 0.8, 0.4 and 0.8 μg C l^{-1} min^{-1} in

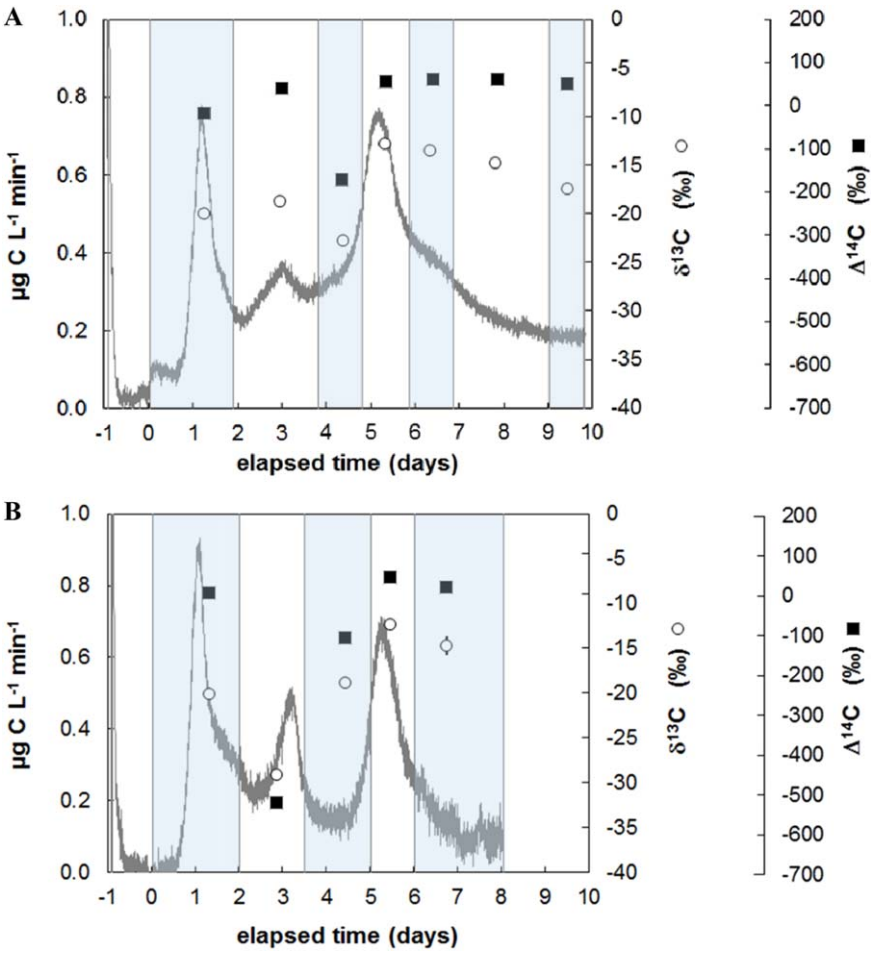

Fig. 1. Microbial CO_2 production rates (gray line), $\delta^{13}C$ (circles) and $\Delta^{14}C$ (squares) signatures of respired CO_2 observed during incubation of Salt Pond sediments for experiment #1 (A) and experiment #2 (B).
The width of each box spans the time interval during which each CO_2 fraction was collected for isotopic analysis, with each corresponding data point plotted at the time at which half the carbon in the fraction was collected. ~15g of sediment was incubated in the IsoCaRB system for each experiment and a 'three peak' respiration pattern was observed for both. During experiment #2, the sediment slurry was subsampled on a daily basis for enzyme analyses and DNA sequencing. [Color figure can be viewed at wileyonlinelibrary.com]

experiment #1, and 0.9, 0.5 and 0.7 μg C l^{-1} min^{-1} in experiment #2. The highest CO_2 production rate (peak 1) corresponds to the onset of respiration. During this interval, the increase (~2–3 fold) in CO_2 production and subsequent sharp decline all occurred within ~20–24 h. Interestingly, the lowest CO_2 production rates were observed midway, at days ~2–4, rather than towards the end of the incubation as one might expect.

In order to determine the source and age of the organic matter being degraded, successive CO_2 fractions were collected for natural abundance isotopic analysis ($\delta^{13}C$ and $\Delta^{14}C$) (Table 1). The duration for each fraction ranged from

Table 1. Masses and isotopic values of microbially-respired CO_2 collected during incubation of Salt Pond sediment.

	Fraction #	Collection times (days)	Collection Duration (hr)	Mass CO_2 (μg C)	$\delta^{13}C$ (‰)	$\Delta^{14}C$ (‰)
Experiment #1	1	0 −1.9	45.0	1315 ± 6	−19.9 ± 0.2	−15.1 ± 2.1
	2	1.9–3.8	46.2	1538 ± 7	−18.7 ± 0.2	38.9 ± 2.4
	3	3.8–4.8	23.7	1302 ± 6	−22.8 ± 0.2	−172.0 ± 1.9
	4	4.8–5.9	25.4	1665 ± 7	−12.7 ± 0.2	55.0 ± 2.2
	5	5.9–6.9	23.7	1170 ± 5	−13.4 ± 0.2	62.1 ± 2.4
	6	6.9–9.0	51.9	1587 ± 7	−14.8 ± 0.2	61.1 ± 2.6
	7	9.0–9.8	19.2	627 ± 3	−17.3 ± 0.2	51.8 ± 2.4
Experiment #2	1	0–2.0	47.3	1146 ± 5	−20.1 ± 0.2	1.5 ± 2.1
	2	2.0–3.5	36.2	1064 ± 5	−29.1 ± 0.4	−525.5 ± 2.2
	3	3.5–5.0	36.2	950 ± 4	−18.8 ± 0.6	−110.4 ± 2.4
	4	5.0–6.0	23.9	944 ± 4	−12.4 ± 0.5	41.5 ± 2.6
	5	6.0–8.0	49.1	751 ± 3	−14.7 ± 1.1	15.0 ± 2.7

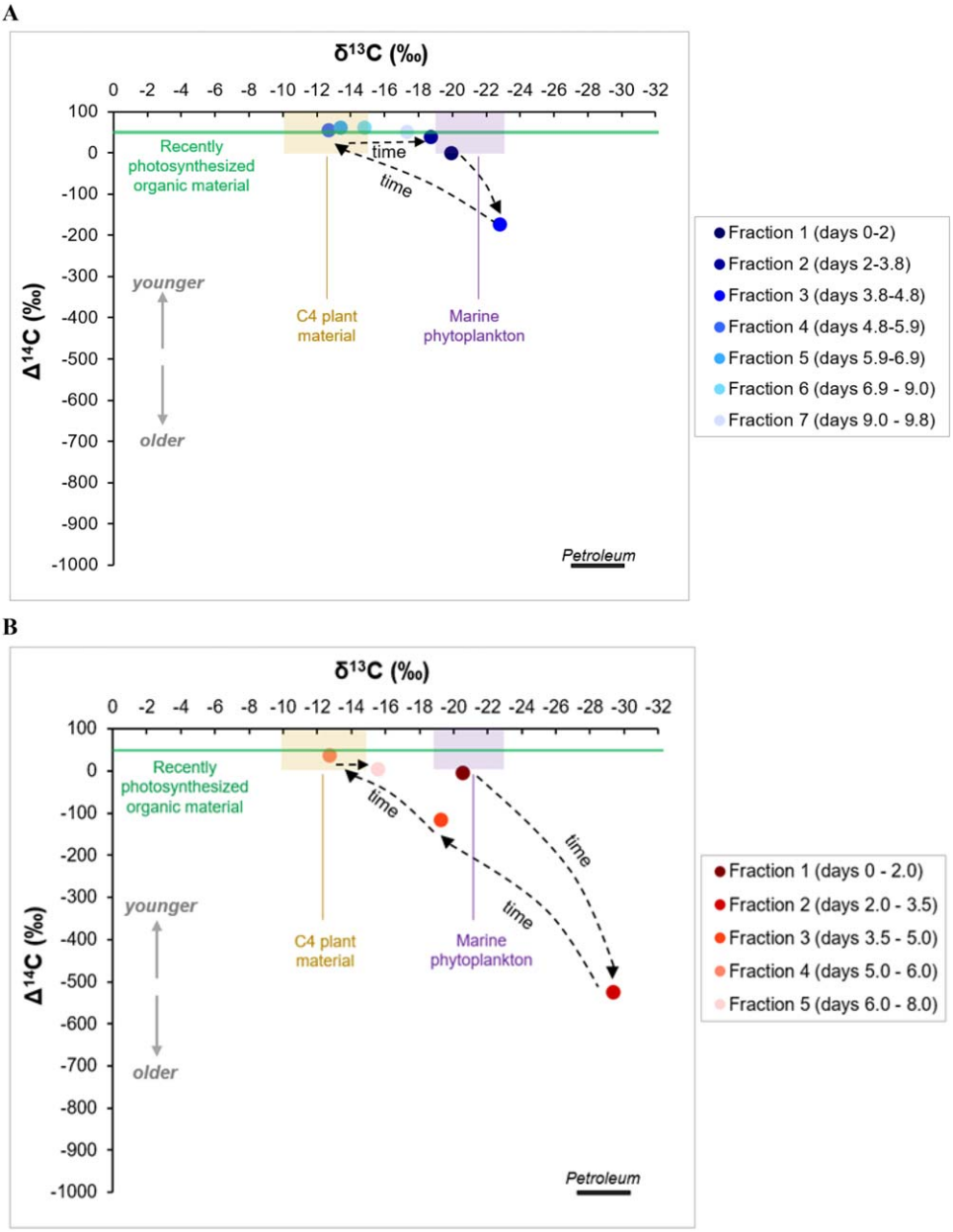

Fig. 2. Plot of stable carbon ($\delta^{13}C$) versus radiocarbon ($\Delta^{14}C$) signatures of respired CO_2 collected from incubation of Salt Pond sediments during (A) experiment #1 and (B) experiment #2. Also shown are $\delta^{13}C$ values for potential microbial carbon sources in Salt Pond sediment; C4 derived plant material (*Spartina* sp., $\delta^{13}C = -10$ to $-15‰$), marine phytoplankton ($\delta^{13}C = -19$ to $-23‰$) and petroleum hydrocarbons ($\delta^{13}C = -27$ to $-30‰$). Organic compounds that have been recently photosynthesized will have $\Delta^{14}C$ signature consistent with current atmosphere ($\Delta^{14}C = \sim +50‰$). In contrast, carbon compounds that are millions of years old (i.e. petroleum hydrocarbons) will have no ^{14}C due to radioactive decay ($\Delta^{14}C = -1000‰$). Range of $\Delta^{14}C$ values shown for C4 plant material and marine phytoplankton are estimated using depth of sediment sample and sedimentation rate of Salt Pond (Donnelly *et al.*, 2015).

~24 to 48 h to ensure that each fraction contained ~ 1 mg of carbon. A total of 9.30 mg and 4.86 mg of respired carbon was collected during experiments #1 and #2, respectively. The $\delta^{13}C$ signatures of CO_2 fractions ranged from -22.8 to -12.7 ‰ in experiment #1 and -29.4 to -12.7 ‰ in experiment #2 (Table 1; Fig. 1). In general, the respired CO_2 became more ^{13}C-enriched over time. The $\delta^{13}C$ signatures observed during days 0 to 5 (fractions 1–3 of exp. #1; fractions 1 and 3 of exp. #2) ranged from ~ -22 to $-19‰$, pointing to microbial degradation of marine-derived organic matter such as phytoplankton ($\delta^{13}C = -23$ to -19 ‰; Hoefs, 2004). The higher $\delta^{13}C$ values observed during days 5 to 9 (fraction 4–7 for exp. #1; fraction 4–5 for exp. #2) indicate a shift toward microbial degradation of predominantly C_4 plant material (i.e. *Spartina*-derived OM; $\delta^{13}C = -15$ to $-10‰$; Peterson *et al.*, 1985) (Fig. 2).

The $\Delta^{14}C$ signatures ranged from -172 to $+62‰$ for experiment #1 and -526 to $+42‰$ for experiment #2 (Table 1, Fig. 1). The majority of CO_2 respired during both incubations was modern in age (i.e. carbon derived from recently photosynthesized material, $\Delta^{14}C = +50‰$; Czimczik and Welker, 2010). However, the $\Delta^{14}C$ values higher than recently photosynthesized material (exp. #1, fraction #5 and 6; $\Delta^{14}C = \sim +62‰$) point to utilization of 'bomb carbon' originating from atmospheric testing of nuclear

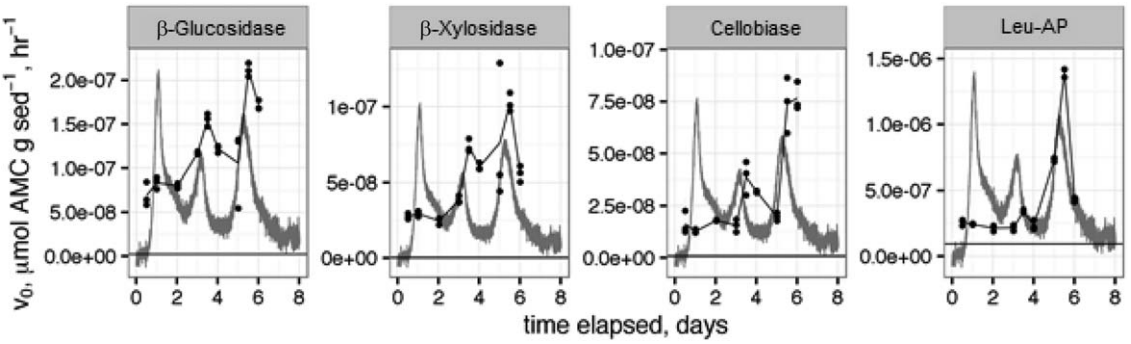

Fig. 3. Potential extracellular enzyme activities (black circles) of β-glucosidase, β-xylosidase, cellobiase, leucyl aminopeptidase and associated CO_2 concentrations (gray curve) during incubation of Salt Pond sediments (experiment #2). Enzyme activities (note different scales) were measured at 9 time points. Extracellular enzyme activities were expressed as micromole fluorophore per liter slurry per hour.

weapons during the 1950s and 1960s (Levin *et al*., 1985; Turnbull *et al*., 2007). Thus, the progression of lower to higher $\Delta^{14}C$ values in experiment #1 is indicative of utilization of progressively older compounds synthesized within the past 50 years. The exception to this trend is fraction #3 from exp. #1, which is significantly depleted in both $\Delta^{14}C$ (–172‰) and $\delta\ ^{13}C$ (–22.8‰) relative to all other fractions. Likewise, fraction 2 from exp. #2 was also highly depleted ($\Delta^{14}C = -526‰$; $\delta^{13}C = -29.1‰$) relative to all other fractions. This negative isotopic shift observed in both experiments may point to transient utilization of petroleum-derived carbon compounds having a $\Delta^{14}C$ signature of $-1000‰$ and $\delta^{13}C$ values near $-30‰$ (Reddy *et al*., 2002). Based on the geographic location of Salt Pond, it is likely that compounds such as petroleum hydrocarbons may regularly enter the pond from run-off of nearby roads (Supporting Information Fig. S9).

Enzyme activity

Potential activities of four extracellular enzymes were measured at 9 time points during exp. #2 (Fig. 3). Observed activities of all four enzymes increased at day 5 and peaked at day 5.5, corresponding to the third major CO_2 peak. The highest observed activity across all time points was associated with protein degradation, specifically leucine aminopeptidase; however, it remained low and relatively constant during days 0 to 4. A sudden rise in leucine aminopeptidase activity was observed at days 4–5. In contrast, there was much more variation in the activities of enzymes associated with polysaccharide degradation. Cellobiase and β-glucosidase are involved in the degradation of plant-derived cellulose through the hydrolysis of cellobiose into glucose. β-glucosidase activity was much higher relative to the two glucosidases (cellobiase and β-xylosidase), increasing slightly at day 3 and more dramatically at days 2 and 5. Cellobiase had the lowest activity across all time points and the activity of this enzyme slightly increased at day 3 and more dramatically at day 5. In general, the activity of β-xylosidase, which cleaves xylose from hemicellulose, continually increased during days 0 to 4 (with the exception of day 3.5). All four enzyme activities declined following day 5.5; this was particularly true for the activity of leucine aminopeptidase and β-xylosidase, which rapidly declined by day 6.

Microbial community composition and diversity

Paired-end sequencing of the 16S rRNA gene V4 region generated 2.6 x 10^6 filtered, high-quality sequences with an average length of 255 bp. These clustered into 285 OTUs (97% similarity). OTU rarefaction curves approached a saturation plateau (Supporting Information Fig. S6) and the Good's coverage, which is an estimator of sampling completeness (Good, 1953), ranged from 96 to 98% (Supporting Information Table S2).

Principal component analysis of weighted Unifrac distances showed that samples clustered into two separate groups corresponding to days 1–4 and days 5–8 (Fig. 4A). The first sample (day 0.5; prior to the appearance of any CO_2 peaks) did not cluster with either of these two groups and may include DNA from inactive cells since samples were not treated to eliminate extracellular DNA. NMDS analysis based on the Bray-Curtis distance matrix also showed similar results, further evidence that the major taxonomic shift in the system occurred between days 4 and 5 (Supporting Information Fig. S4). The NMDS stress value was 0.003 indicating good ordination. Accordingly, ADONIS analysis confirmed that length of incubation affected the variation in the microbial community ($R^2 = 0.36$, $p = 0.002$, strata = elapsedtime). Tight clustering of the two replicate experiments was also observed in both NMDS and PCoA analysis, further suggesting that the

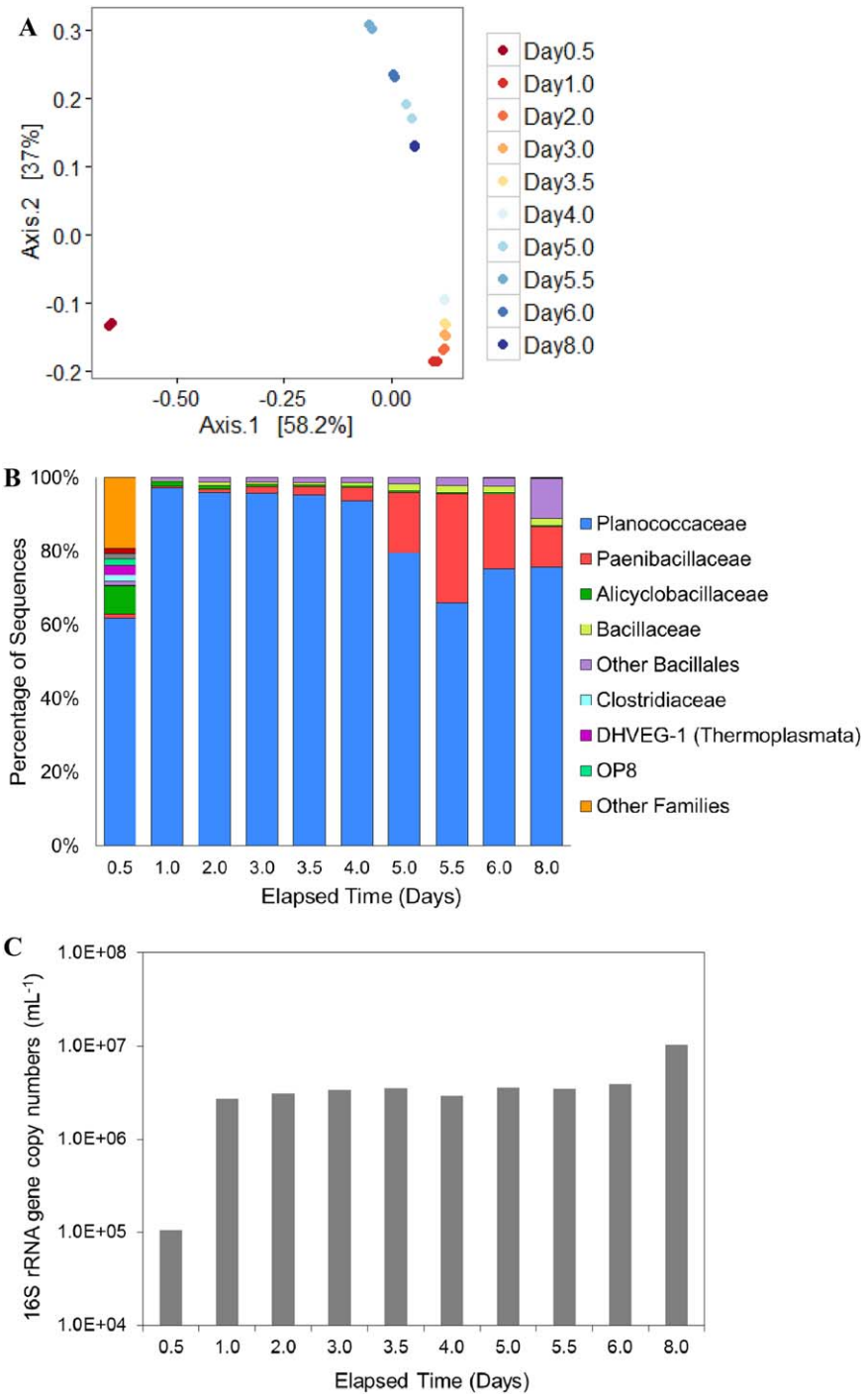

Fig. 4. Weighted UniFrac, taxonomic composition and bacterial 16S rRNA copy numbers of time points collected during incubation of Salt Pond sediments (experiment #2).
(A) Pairwise, weighted UniFrac distances visualized on principle coordinates plot (PCoA) with the percent of variation explained by each axis noted in parentheses. (B) Mean relative abundance of dominant families using 16S rRNA gene sequences observed during Salt Pond incubation. (C) Bacterial 16S rRNA copy numbers (per mL) as estimated by quantitative PCR (qPCR).

differences between time points reflect real shifts in community structure.

The taxonomic composition of the microbial community at day 0.5 included 20 different phyla, with the majority of sequences belonging to the phylum *Firmicutes* (almost 80% of total sequences). *Firmicutes* are capable of forming endospores that can survive desiccation and other extreme conditions (Onyenwoke *et al.*, 2004; Pedraza-Reyes *et al.*, 2012); thus it was not surprising that the community which remained following sediment preparation was primarily comprised of this group. *Firmicutes* was the predominant phyla detected during days 1 to 8. At the class and order level, *Bacilli* and *Bacillales*, respectively, accounted for over 99% of sequences at days 1 to 8 (Supporting Information Fig. S5). However, there were clear differences in the relative abundance of families within

Bacillales over time (Fig. 3B). Specifically, *Planococcaceae* accounted for 95% of sequences (on average) during Days 1 to 4; however it decreased to ~75% of sequences during days 5 to 8. *Paenibacillaceae* showed the opposite dynamic and accounted for less than 2% of sequences during Days 1 to 4, then increasing to 20% of sequences (on average). The relative abundance of *Paenibacillaceae* peaked at day 5.5 where it comprised of 30% of total sequences, directly coinciding with the third major CO_2 peak. At the OTU level, there were 17 dominant OTUs (i.e. > 1% of total reads at any given time point) whose abundances significantly changed during the incubation (G-test, $p < 0.05$) (Supporting Information Fig. S7). In particular, *Planococcaceae* OTU 1084045 was most abundant and contributed to 55% of total sequences at day 0.5, increasing to ~80% during days 1 to 4. Its abundance decreased to 54% at day 5.5 but slowly increased again during Days 6 to 8. *Paenibacillaceae* OTU 1107716 was the second most abundant OTU and displayed an opposite trend such that it comprised of 10 to 21% of total sequences during days 5 to 6 but was rarely detected during days 1 to 4.

Similar to the taxonomic composition, the diversity of the microbial community changed over time according to all metrics: divergence-based (Faith's phylogenetic diversity), OTU-based (observed species), or the Shannon and Simpson's indices (Supporting Information Fig. S8; Table S2). The highest diversity levels were observed at day 0.5 (prior to any respiration activity). Microbial diversity rapidly decreased with the onset of respiration during the first 48 h of incubation; diversity was lowest during days 2 to 4. Following this decline, diversity increased at day 4 and appeared to reach a steady plateau during days 6 to 8 as CO_2 concentrations declined to near baseline values.

Concentrations of 16S rRNA gene copies were used to determine if the respiration pattern corresponded to increases in bacterial cell numbers. Concentrations of 16S rRNA gene copies increased rapidly from 1×10^5 to $\sim 10^6$ copies ml^{-1} within the first 24 h of incubation and the onset of heterotrophic respiration, (Fig. 4C). However, 16S rRNA copy numbers did not vary dramatically following this initial increase and ranged from 2.9 to 3.9 x 10^6 for the remainder of the incubation with the exception of a slight increase at day 8 to 1.0×10^7 copies ml^{-1}.

Discussion

Aquatic sediments contain a diverse mixture of organic substrates that are thought to vary in their availability for microbial uptake and utilization (Middelburg, 1989; Canfield, 1994; Rothman and Forney, 2007). Based on intrinsic chemical properties of the compounds, macromolecular compounds such as polymeric plant material are thought to be more recalcitrant and less bioavailable compared with soluble, low molecular weight compounds such as oligosaccharides. Quantitative and isotopic measurements of algal and terrestrial biomarkers (such as fatty acids) in aquatic sediments have found that terrestrially derived plant material is often better preserved, which suggests that phytoplankton-derived organic matter is selectively utilized by microorganisms at higher rates (Canuel and Martens, 1996; Hedges *et al.*, 1997; Dauwe and Middelburg, 1998; Camacho-Ibar *et al.*, 2003; Burdige, 2005; Dai and Sun, 2007). However, empirically testing the selective utilization of organic matter by microorganisms is difficult due to the inherent complexity and high diversity of sedimentary organic matter and microbial communities. Natural abundance isotopic analysis of cellular biomarkers (e.g. lipids or DNA) is capable of identifying microbial carbon sources in complex environmental samples (e.g. Coffin *et al.*, 1990; Pearson *et al.*, 2008; Brady *et al.*, 2009; Mills *et al.*, 2010; Whaley-Martin *et al.*, 2016); however, it only provides a snapshot and is unable to resolve the potential selectivity that may occur over the short time span of hours to days. Other experiments performed over these shorter intervals used isotopically-labelled substrates (Benner *et al.*, 1984; Sun and Wakeham, 1998; Gontikaki *et al.*, 2013; van Nugteren *et al.*, 2009; Steen *et al.*, 2016). However, these studies added compounds of known accessibility at elevated concentrations and may not reflect their utilization at natural concentrations, or the accessibility of other compounds found in the sediment. In contrast, we investigated the accessibility of sedimentary organic matter and its potential utilization by continuously monitoring respiration rates and isotopic compositions of CO_2 released by a microbial community present in the sediment. Quantitative recovery of the resulting CO_2 for carbon isotopic analysis allows us to link the dynamics of carbon utilization to changes in microbial community composition and extracellular enzyme activity. Our results show sequential, selective initiation and uptake of different carbon pools as observed by a suite of isotopic, microbial, molecular techniques.

The coherence between the respiratory pattern and the isotopic composition of the recovered CO_2 in both replicate experiments suggests that each major CO_2 peak corresponds to the degradation of a specific carbon pool that varies in both origin and molecular class. Based on the geographic setting of Salt Pond, sedimentary organic matter in this system is likely dominated by a mixture of marine phytoplankton-derived compounds, C_4-derived plant material, and anthropogenic inputs of petroleum hydrocarbons. Given these diverse sources of sedimentary carbon available for microbial utilization, we attempted to constrain their respective contributions to microbial respiration for each collected fraction. Our model presumed three major carbon sources:

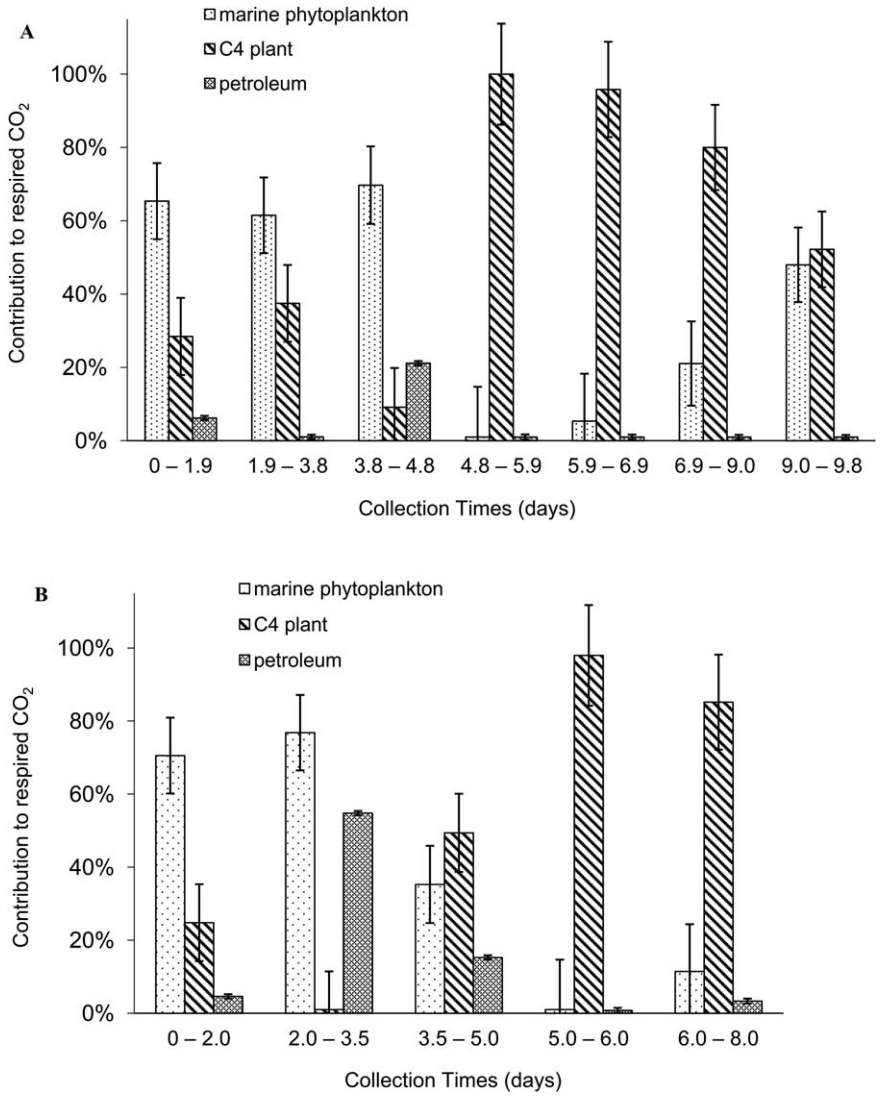

Fig. 5. Estimated contributions of potential carbon sources (C_4 plants, marine phytoplankton, petroleum hydrocarbons) to respired CO_2 during (A) experiment #1 and (B) experiment #2. Relative contributions were estimated using three-end mass balance model.

$$\delta^{13}C_{CO2} = f_{C4\ plant}\delta^{13}C_{C4\ plant} + f_{marine}\delta^{13}C_{marine} + f_{petroleum}\delta^{13}C_{petroleum} \quad (1)$$

$$\Delta^{14}C_{CO2} = f_{C4\ plant}\Delta^{14}C_{C4\ plant} + f_{marine}\Delta^{14}C_{marine} + f_{petroleum}\Delta^{14}C_{petroleum} \quad (2)$$

$$1 = f_{C4\ plant} + f_{marine} + f_{petroleum} \quad (3)$$

where $\delta^{13}C_{CO2}$ and $\Delta^{14}C_{CO2}$ correspond to the isotopic signature of the respired CO_2. For Eq. (1), the $\delta^{13}C$ end-members of marine phytoplankton and petroleum hydrocarbons were set to commonly accepted values of $-22 \pm 0.5‰$ and $-30 \pm 0.5‰$, respectively (Coleman and Fry, 1991). Since Salt Pond is surrounded by salt marshes that are dominated by C_4 grasses (such as *Spartina* sp.), the plant end member was set to the accepted value for *Spartina* sp., $-13 \pm 0.5‰$ (Hoefs, 2004); we considered the potential input of C_3 plant material to be negligible relative to the local dominance of *Spartina* sp. For Eq. (2), the $\Delta^{14}C$ value of petroleum is $-1000 \pm 0‰$, while recently photosynthesized carbon derived from phytoplankton ($\Delta^{14}C_{marine}$) and plant material ($\Delta^{14}C_{Spartina}$) were both set to $+50 \pm 5‰$. Percentages and uncertainties were estimated as means and standard deviations of solutions to 3 simultaneous mass-balance equations that were solved 10,000 times, in which normally-distributed pseudo-random noise was added to each isotope ratio measurement ($\Delta^{14}C \pm 5‰$; $\delta^{13}C \pm 0.5‰$) and isotopic signature. Using this approach, we predicted the fractional utilization of all three sources, including which pools were preferentially respired at different times of the incubation (Fig. 5; Supporting Information Table S3). It is important to note that all three carbon pools are present and likely reactive during the course of the incubation but that their relative contribution to microbial respiration varies dramatically such that a sequential utilization of each pool can be observed.

Specifically, marine phytoplankton was a dominant carbon source during the first two days of incubation, where it accounted for 65–71% of all respired carbon. Subsequently, there was an increase in the degradation of petroleum-derived carbon at day 3. This is particularly evident in experiment 2 where 55% of the respired carbon is estimated to come from petroleum sources. During the second half of the incubation, there was a clear shift to degradation of C_4 plant material and almost all respired carbon was derived from this pool at days 5 to 6. C_4 plant material then remained a predominant carbon source for the remainder of the incubations. Together with the respiration pattern, these results indicate that CO_2 peak 1 (Fig. 1) corresponds to the degradation of primarily phytoplankton-derived marine compounds, while peak 3 is almost exclusively linked to degradation of C_4 plant compounds. In contrast, peak 2 appears to reflect respiration of all three sources, with a substantial fraction being derived from degradation of petroleum hydrocarbons. Interestingly, the peak CO_2 production rates were consistently similar regardless of the substrates' origins, indicating that different substrates (i.e. phytoplankton products, C_4 plant material) were consumed at similar rates.

Patterns in extracellular enzyme activities have been used to infer the molecular composition of organic matter available to microorganisms (Boetius and Lochte, 1996; Boschker and Cappenberg, 1998). Plant materials are primarily made up of cellulose, hemicellulose (polysaccharides), and lignin (phenolic polymers), and are fairly low in proteins (Bacic *et al.*, 1988). In contrast, algae and cyanobacteria have a lower polysaccharide composition and a much higher protein content (Cowie and Hedges, 1984). The first CO_2 peak during days 0 to 2 appears to be a result of oxidation of small (less than 600 Da; Benz and Bauer, 1988; Weiss *et al.*, 1991), labile compounds that can be taken up directly into cells and/or simple sugars that are easily degradable using only glucosidases (Fig. 3). As this labile pool of primarily phytoplankton-derived material is rapidly utilized, there is an increase in the activity of all three glycosidases. Although temporally corresponding to the degradation of petroleum hydrocarbons at day 3, the mass-balance calculation shows that marine planktonic material continues to be consumed during this interval. Cells may be metabolizing high MW compounds ($>$ 600 Da) from marine sources, using a broader suite of extracellular enzymes, while also assimilating low MW petroleum-derived compounds. The final CO_2 peak that appears during days 5 to 6 appears to be driven by the oxidation of proteins and more highly resistant polysaccharides derived exclusively from C_4 plant material, as indicated both by the isotopic signatures (Figs 2 and 5) and changes in enzyme activity (Fig. 3). The enzyme activities measured in this study support the overall interpretations of the isotopic data and confirm that organic matter degradation is sequential: bacteria rapidly utilize the pool of labile, low MW ($<$ 600 Da) phytoplankton-derived carbon before utilizing the pool of complex, recalcitrant plant material.

Similar direct observations of preferential utilization of phytoplankton-derived carbon by bacteria have been found in other aquatic systems (Kritzberg *et al.*, 2004; Guillemette *et al.*, 2013; 2015) with respect to degradation of dissolved organic carbon (DOC) by bacterioplankton in the water column. This pattern of accessibility also holds true for sedimentary organic matter under our experimental conditions, namely that phytoplankton-derived carbon pools are more accessible to microbes than pools derived from plant material. That is, although our measured rates may not be representative of *in situ* conditions due to the effects of sediment pretreatment and the enriched, rather than original, microbial community, we expect the observed pattern of sequential utilization to hold true. Phytoplankton exudation and cell lysis frequently release low MW compounds such as amino acids, carboxylic acids and carbohydrates that are readily metabolized by bacteria and considered to be energetically rich (Fogg, 1983; Tranvik and Jørgensen, 1995; Weiss and Simon 1999; Rosenstock and Simon, 2001). Interestingly, although we observe sequential degradation of low MW vs. high MW organic matter, degradation of both pools occurs in a relatively short period of time (days) which indicates that microbial populations rapidly respond to changes in organic matter availability. Rapid uptake of low MW substrates during days 0 to 2 may have provided the microbial community with the energy to produce the extracellular enzymes needed to degrade the more recalcitrant compounds. This is often proposed as a mechanism for '*priming*', which is the increased degradation of recalcitrant organic matter resulting from an increase in overall microbial activity due to the energy and nutrients gained from the degradation of labile organic compounds (Kuzyakov *et al.*, 2000; Guenet *et al.*, 2010). Although our study did not directly investigate the priming effect – i.e. we did not add exogenous 'priming' substrates – our results suggest that degradation of labile compounds will precede and possibly enable the synthesis of extracellular enzymes required for degrading complex, polymeric compounds.

A major question in microbial ecology is the extent to which organic matter accessibility is taxon-specific, or if all bacteria can augment their metabolic capabilities to consume carbon from multiple pools. Taxon-specific availability implies that different populations specialize in the metabolism of specific carbon pools (Martinez *et al.*, 1996; Cottrell and Kirchman 2000; Riemann and Azam, 2002; Elifantz *et al.*, 2005). This model implies that changes in carbon degradation would result from observable shifts at the community level, i.e. a succession of specific bacterial populations (Geller, 1986; Crump *et al.*,

2003; Teeling *et al*., 2012). In the alternative hypothesis, organic matter degradation is a result of changes in cellular level processes and may not be accompanied by systematic or predicable population shifts. Such metabolic versatility (or plasticity) by generalists may allow them to access multiple carbon pools as needed, thereby making the microbial community less vulnerable to short-term changes in organic matter availability (Button *et al*., 2004; Mou *et al*., 2007; 2008).

Our results provide novel insight into how the differences in bioavailability of natural organic substrates may affect the function and composition of a heterotrophic bacterial population. Interestingly, the data indicate that *both* the taxon- and cellular-level accessibility models apply under our experimental conditions. During the first half of our incubation (days 1 to 4), we observe a relatively uniform community, dominated by a single OTU (*Planococcaceae* OTU 1084045). CO_2 respiration rates and isotopic results during this time show microbial utilization of multiple carbon substrates, including labile, low MW compounds (primarily from phytoplankton, and plant material) as well as petroleum hydrocarbons. Taken together, these results point to a sequence of cellular accessibility and highlight the metabolic versatility of this family (*Planococcaceae*). As the pool of available organic matter becomes more recalcitrant, however, there is selection for bacterial taxa able to produce the enzymatic machinery for processing complex biopolymers. At day 5, there is an increase in the relative abundance of *Paeinbacilliaceae*, which coincides with degradation of plant-derived material. Many genera within this family are known for their capacity to produce extracellular enzymes, and some can grow on xylan or cellulose as the sole carbon source (Pason *et al*., 2006; Waeonukul *et al*., 2009; Ghio *et al*., 2012). Although species within the family *Planococcaceae* have also been shown to produce extracellular enzymes, the concurrent shift in utilized carbon sources and emergence of *Paeinbacilliaceae* points to a correlation between taxon succession and the bioavailability of high MW polymeric material. This is consistent with Datta *et al*., (2016), who incubated a natural seawater community with chitin-coated beads and observed the emergence of a population that was devoid of genes to degrade chitin, but was able to consume the byproducts of chitin metabolizers. This implies that once a substrate is hydrolyzed to a smaller size, it may be consumed by a wider range of microbes, including those that cannot produce their own extracellular enzymes (Cotta, 1992). Thus it is difficult to say with certainty if *Paeinbacilliaceae* is a specialized population that produces high levels of extracellular enzymes and subsequently consumes the resulting hydrolyzed C_4 plant material, or whether it simply consumes byproducts or metabolites from the degradation of this material by *Planococcaceae*. Additional work to assess gene expression may confirm which populations are actively producing extracellular enzymes and further elucidate the dynamics of these two groups.

Understanding the interplay between the changing nature of organic matter and the response of heterotrophic bacteria is a major challenge and a key component of the carbon cycle. The present study provides insight into this process using well-defined experimental conditions that show sequential utilization of different carbon pools, which in turn induce observable changes at both the community and cellular level, including shifts in community composition, enzymatic activity, and respiration rates. Additional analyses such as metabolomics could further test the importance of liberating low vs. high MW substrates and the ensuing effect on metabolic pathways. Moreover, molecular-level analysis of the residual organic matter may also shed light on how the pool of available carbon changes both chemically and structurally due to microbial degradation. Ultimately, the methods and results of this study should motivate future investigations into how changing environmental conditions (e.g. temperature, oxic vs. anoxic) will affect the quantity and quality of organic matter that is degraded by microorganisms.

Experimental procedures

Sample collection

Sediment was collected from Salt Pond (Falmouth, Massachusetts; 41°32′44.14″N x 70°37′39.10″W; water depth 3 m) in September 2010 using a grab sampler from an approximate sediment depth of 20 cm. Salt Pond is a brackish coastal pond that is surrounded by approximately 0.02 km^2 of salt marsh containing C_4 grasses (such as *Spartina* sp.). The pond experiences sulfidic bottom waters at least 4 months of the year (Simmons *et al*., 2004), leading to high preservation of organic carbon (>5% by weight; Wakeham and Canuel, 1990). Salt Pond receives marine inputs via permanent tidal exchange with Vineyard Sound through a small inlet. Salt Pond also is surrounded by roadways which leads to inputs of anthropogenic pollutants via run-off (Donnelly *et al*., 2015).

Sample preparation

In order ensure that recovered CO_2 was derived only from microbial respiration, inorganic carbon (dissolved and particulate) was removed by titrating the sediments in dilute slurry to pH 2–2.5 with 10% hydrochloric acid (HCl) in an ice bath (Beaupré *et al*., 2016). This approach implies exposure to $[H^+]$ concentrations no higher than 0.003–0.01 M, while the cold temperatures minimize reactions between the acid and sedimentary organic matter. Subsequently, sediments were freeze dried and stored at −20°C until incubation. Consequently, the fraction of the native sediment microbial community incubated in the IsoCarRB system consisted only of groups that could withstand these treatments, i.e. primarily spore-forming taxa. The impact of the acidification treatment on subsequent organic matter reactivity was found to be

minimal using Ramped Pyrolysis/Oxidation (Ramped PyrOx). Details of the Ramped PyrOx tests are discussed in Supporting Information.

Incubation using the IsoCaRB system

The standard operating procedure for the IsoCaRB system, including sterilization and assembly, sample preparation, and CO_2 collection and purification is described in Beaupré *et al.* (2016) (Supporting Information, Fig. S2). Briefly, approximately 15 g (dry weight) of decarbonated sediments are incubated at room temperature (~22°C) in a custom Pyrex vessel containing 2 L of M9 growth medium (pH = 6; Sambrook and Russell, 2001) modified to be free of exogenous carbon (containing 2.6 g $Na_2HPO_4{\cdot}7H_2O$, 11 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.02 g $CaCl_2{\cdot}2H_2O$, and 10 mg $FeSO_4{\cdot}7H_2O$ per liter). The sediment slurry is stirred (90 rpm) under aerobic conditions to provide an unlimited supply of electron acceptors. The system is first purged of residual atmospheric CO_2 by sparging with 100 ml min^{-1} of CO_2-free helium for 24 h. Gas flow is then changed to 100 ml min^{-1} of 20% O_2 in helium. Respiratory CO_2 is carried to an online infrared CO_2 analyzer (Sable Systems CA-10) where concentrations are quantified in real time and continuously logged to a desktop PC using a custom LabVIEW program (National Instruments). This CO_2 is continuously collected as successive fractions in custom molecular sieve traps based on the evolving profile (i.e. capturing a peak in CO_2 production) and/or the integrated mass of carbon (~1mg C/fraction). CO_2 is recovered from the traps by baking (600°C for 75 min) under vacuum within ~24 h of collection, then cryogenically purified, quantified, and stored in flame-sealed in Pyrex tubes. Each experiment is allowed to proceed until CO_2 concentrations resume near baseline values.

Two replicate incubations (experiments '#1' and '#2') were performed. During experiment #2, approximately 45 mL of sediment slurry was subsampled every 12 h for molecular and enzymatic analyses (described below) and stored at −80°C. Accordingly, the final volume of medium in experiment #2 was approximately half of that of experiment #1.

Microbial CO_2 production

Gaseous CO_2 concentration measurements were corrected for baseline drifts (Beaupré *et al.*, 2016) and then rescaled to agree with the higher-precision manometric yields obtained from the trapped CO_2. These normalized CO_2 concentrations were then corrected for the confounding effects of mixing in the culture vessel headspace and decreasing media volume in order to constrain the rate of CO_2 generation per unit volume of growth medium (μg C l^{-1} min^{-1}), which serves as a proxy for the microbial CO_2 production rate (see Supporting Information for details).

Isotopic analysis

CO_2 fractions were sent to the National Ocean Sciences Accelerator Mass Spectrometry (NOSAMS) Facility at the Woods Hole Oceanographic Institution. A small aliquot of each sample was split for ^{13}C measurement, with the remainder reduced to graphite on an iron catalyst (Vogel *et al.*, 1987) for ^{14}C measurement by accelerator mass spectrometry (AMS). All isotopic data are corrected for background contamination associated with the IsoCaRB system as described in Beaupré *et al.*, (2016).

Stables isotope values (^{13}C) are reported in standard δ notation vs. the VPDB standard. Radiocarbon values (^{14}C) are reported in standard $\Delta^{14}C$ notation, wherein $\Delta^{14}C$ is the relative deviation from the $^{14}C/^{12}C$ ratio of the atmosphere in 1950 (Stuiver and Polach, 1977).

Extracellular enzyme assays

Potential activities of three extracellular glucosidases (β-glucosidase, β-xylosidase, cellobiase) and one peptidase (leucyl aminopeptidase) were assayed using fluorogenic substrate analogs (Hoppe, 1993), according to a modified version of the technique described in Steen and Arnosti (2013) (Supporting Information Table S1). Substrates were dissolved in 100% dimethylsulfoxide to a final concentration of 10mM and stored in the dark at −20°C until use. For each time point, 250 μL of sediment slurry was added in triplicate to a cuvette containing 40 μl of substrate and 710 μl of modified M9 medium (pH 6), followed by incubation at room temperature (~22°C). Fluorescence measurements were taken immediately after the addition of the sediment slurry and then after approximately 2, 4, 8, 10, 24 and 25 h using a Promega Glomax Jr. fluorometer. Fluorescence values were calibrated with 4-methylbellifoerone and 7-amino-4-methylcourmarin as appropriate.

Genomic DNA extraction and quantitative PCR

Genomic DNA was extracted in duplicate from experiment #2 (Supporting Information Fig. S3) using the PowerSoil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to manufacturer's protocol. DNA quality was assessed using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Total bacterial cell density was obtained by real time or quantitative PCR (qPCR) amplification of the 16S rRNA gene using the primer pair 1312-1332F and 1390-1396R (Corless *et al.*, 2000). Each qPCR mixture contained the following: 300 nM forward primer, 900 nM reverse primer, 125 nM Probe (5′ 6-FAM/ZEN/3′ IBFQ; Integrated DNA Technologies, Inc., Coralville, IA), 1X PerfeCTa qPCR ToughMix (Quanta Biosciences, Gaithersburg, MD, USA) and 2.5 μl of template DNA. Bacterial 16S copy numbers were amplified and quantified with the LightCycler 480 II real-time PCR system (Roche, Indianapolis, IN, USA). The results were analysed using the Roche LightCycler Software Version 1.5. The standard curve was made using three sample dilution series with eight replicates each. All qPCR analyses were carried out at Research and Testing Laboratory (Lubbock, TX, USA).

Amplicon sequencing of bacterial and archaeal SSU genes and sequence analysis

The V4 hypervariable region of the 16S rRNA gene in both bacteria and archaea (primer pair 515F and 806R; Caporaso *et al.*, 2012) was amplified and sequenced in duplicate for each time point (Supporting Information Fig. S3). Amplicons were

sequenced on an Illumina MiSeq (San Diego, CA, USA) platform at Molecular Research LP (Lubbock, TX, USA). Sequence data was processed and analysed using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline (version 1.9.1; Caporaso *et al*., 2010). Quality filtering and processing of paired-end reads was performed following Mahmoudi *et al*. (2015). Sequences were clustered into operational taxonomic units (OTUs) at 97% identity and taxonomy was assigned via the Ribosomal Database Project (RDP) classifier (Wang *et al*., 2007) against the Greengenes database. Sequences were then rarefied to the same depth (~62,000 sequences) to remove the effect of sequencing depth on analysis (Santos *et al*., 2014). Finally, evenness and alpha-diversity metrics were calculated using QIIME (Caporaso *et al*., 2010) and in R (v3.1) using the vegan (Oksanen *et al*., 2013) and Phyloseq (McMurdie and Holmes, 2013) packages (R Core Team, 2013). The 16S rRNA gene sequencing data for all the samples analysed in this study have been deposited at the NCBI Sequence Read Archive under accession number SUB2418808.

Statistical analyses

Differences between samples were calculated using weighted UniFrac (Lozupone and Knight, 2005) and Bray-Curtis similarity. Principal coordinate analysis (PCoA) was performed on weighted UniFrac distances, and non-metric multidimensional scaling (nMDS) was performed on Bray-Curtis dissimilarity matrices. For nMDS, a stress function was used to assess the goodness-of-fit of the ordination. Stress values range from 0 to 1; values below 0.2 suggest that the ordination accurately represents the observed dissimilarity between samples. The Adonis implementation of Permanova (non-parametric permutational multivariate analysis of variance; Anderson, 2001) was used to estimate the proportion of variation in microbial communities attributed to sampling time. Finally, the G-test of independence was used to identify OTUs whose abundance changed between time points, using 1000 Monte Carlo randomizations; the *p*-values were corrected for the number of comparisons (Bonferroni correction).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Thermogram for non-acidified (A) and acidified (B) homogenized sediment cored from 40–50 cm depth at Wilkinson Basin, 2014. Temperatures were ramped at 20°C min^{-1} under a 35 ml min^{-1} stream of ~3% O_2 in helium.
Fig. S2. Simple schematic of the IsoCaRB system during oxygenic incubation (adapted from Beaupré *et al.*, 2016). Major components include a gas panel (not shown), the culture vessel, a gas purification system, the CO_2 analyzer, and molecular sieve traps. The culture vessel gas supply, vent, and liquid sampling tube can be isolated via quarter turn plug valves Molecular sieve traps are rapidly and securely connected to the IsoCaRB system via 1/8 Ultra Unions.
Fig. S3. Microbial CO_2 production rates (gray curve) and sampling times (arrows) for 16S rRNA sequencing, qPCR and extracellular enzyme assays during experiment #2 using sediments collected from Salt Pond, MA.
Fig. S4. Community analysis using Nonmetric Multidimensional Scaling (NMDS) of Bray Curtis distance matrix showing dissimilarity among time points collected during incubation of Salt Pond sediments (experiment #2); stress = 0.005. Stress values range from 0 to 1; values below 0.2 suggest that the ordination accurately represents the observed dissimilarity between samples.
Fig. S5. Mean taxonomic distributions of microbial communities observed during incubation of Salt Pond sediments (experiment #2); depicted for the ranks of Class (A) and Order (B).
Fig. S6. Rarefaction curves of OTUs clustered at 97% sequence identity across all time points.
Fig. S7. Log scale heat map of abundant 16*S* rRNA operational taxonomic units (OTUs) observed during incubation of Salt Pond sediments (experiment #2). All OTUs that were >1% at any time point are shown.
Fig. S8. Estimates of alpha-diversity metrics observed during incubation of Salt Pond sediments (experiment #2).
Fig. S9. Map of Salt Pond (Falmouth, Massachusetts) indicating the location of nearby roadways that flood during storm events and heavy rainfall, leading to run-off into the pond.
Table S1. Extracelluar enzymes and substrate analogs used in this study and the potential biogeochemical function of the measured enzymes (Sinsabaugh *et al.*, 2008)
Table S2. Estimates of coverage and alpha-diversity metrics for time points collected during incubation of Salt Pond sediment (experiment #2).
Table S3. Estimated contribution of potential carbon sources (C_4 plants, marine phytoplankton, petroleum hydrocarbons) to respired CO_2.