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Ongoing biodegradation of *Deepwater Horizon* oil in beach sands: Insights from tracing petroleum carbon into microbial biomass

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ABSTRACT

Heavily weathered petroleum residues from the *Deepwater Horizon* (*DwH*) disaster continue to be found on beaches along the Gulf of Mexico as oiled-sand patties. Here, we demonstrate the ongoing biodegradation of weathered Macondo Well (MW) oil residues by tracing oil-derived carbon into active microbial biomass using natural abundance radiocarbon (^{14}C). Oiled-sand patties and non-oiled sand were collected from previously studied beaches in Mississippi, Alabama, and Florida. Phospholipid fatty acid (PLFA) analyses illustrated that microbial communities present in oiled-sand patties were distinct from non-oiled sand. Depleted ^{14}C measurements of PLFA revealed that microbes on oiled-sand patties were assimilating MW oil residues five years post-spill. In contrast, microbes in non-oiled sand assimilated recently photosynthesized carbon. These results demonstrate ongoing biodegradation of weathered oil in sand patties and the utility of ^{14}C PLFA analysis to track the biodegradation of MW oil residues long after other indicators of biodegradation are no longer detectable.

1. Introduction

The *Deepwater Horizon* (*DwH*) disaster was one of the largest accidental marine oil spills in history. An estimated 779 million L of fluid, consisting of 5.3×10^{11} g of oil and 1.7×10^{11} g of gas, from the damaged Macondo well (MW) were released into the Gulf of Mexico beginning on April 20, 2010 and continued flowing for 87 days (Reddy et al., 2012). The spilled oil and gas were altered, removed, and transported through various physical, biological, and mechanical processes (Ramseur, 2010). An estimated 10% of the released petroleum fluid formed a surface slick (Reddy et al., 2012; Ryerson et al., 2011; McNutt et al., 2012) that oiled approximately 965 km of sandy beaches along the northern Gulf of Mexico coastline (Nixon et al., 2016). While large aggregates of oil on coastlines were manually and mechanically removed during initial cleanup efforts, oiled-sand patties (~1–5 cm in diameter) are still being washed ashore today, likely derived from submerged oiled mats offshore (Hayworth et al., 2015). Due to their continued deposition, oiled-sand patties represent the most accessible samples of MW oil residues remaining.

For this pool of remaining oil washing ashore on sandy beaches, the most likely natural process of removal is microbial degradation. Generally, microbes degrade lower molecular weight saturated and smaller aromatic compounds first, while heavier, more aromatic compounds are degraded at a much slower rate (Wang et al., 1998). Within oiled-sand patties, many of the lower molecular weight saturated and aromatic hydrocarbons were lost rather quickly (< 1 year post-spill) (Aeppli et al., 2012). Additionally, loss of some normal, branched, and cyclic alkanes (> C_{22}) in oiled-sand patties was attributed to microbial degradation (Gros et al., 2014). Therefore, oiled-sand patties were heavily weathered by one to two years post-spill (Wenger et al., 2002), with some MW oil residues (including sand patties) becoming dominated by oxygenated hydrocarbon degradation products that are not present in fresh MW oil (Aeppli et al., 2012; White et al., 2016). Such newly formed oxygenated hydrocarbons have been hypothesized to be recalcitrant, but this hypothesis has not been tested in situ (Aeppli et al., 2012). Because lower molecular weight saturated and aromatic hydrocarbons are amenable to traditional analytical approaches, these compounds are conventionally used to gauge degradation. Many of these conventional indicators used to gauge biodegradation, such as the ratio of octadecane (C_{18}) to phytane, have been removed from heavily

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weathered MW oil in sand patties, therefore ongoing degradation is difficult to verify using these molecular-ratio based methods.

Measurements of carbon (C) isotopes in microbial lipids have been used as an alternative approach to using molecular ratios for gauging microbial degradation. A commonly used class of microbial lipids in C source elucidation is phospholipid fatty acids (PLFA) (Mahmoudi et al., 2013a; Wakeham et al., 2006; Slater et al., 2005; Slater et al., 2006; Mahmoudi et al., 2013b; Ahad et al., 2010; Ahad and Pakdel, 2013; Boschker et al., 1999; Boschker et al., 2005). Because PLFA readily degrade after cell death (White et al., 1979; Harvey et al., 1986), they provide a snapshot of the viable microbial community and their C sources at the time of sampling. C source elucidation has primarily been accomplished by measuring the stable carbon isotopic (^{13}C) composition of microbial membranes (Boschker et al., 1999; Londry et al., 2004; Petsch et al., 2003).

Natural abundance radiocarbon (^{14}C) measurements of microbial lipids provide powerful resolution for elucidating microbial C sources in oiled systems. Petroleum C ($\Delta^{14}C = -1000‰$) (White et al., 2005) is easily distinguishable from modern C produced by photosynthesis over the past 20 years ($\Delta^{14}C = 40–100‰$) (Graven et al., 2012). Since petroleum and modern organic matter are separated by a large dynamic range in $\Delta^{14}C$ (> 1000‰) and the analytical uncertainty (~20‰) represents only a small percentage of that signal, radiocarbon is a powerful tool for constraining estimates of C sources to microbes. ^{14}C of microbial lipids ($^{14}C_{PLFA}$) has been previously used in a number of studies related to oil spills (Table S1) (Mahmoudi et al., 2013a; Wakeham et al., 2006; Slater et al., 2005; Slater et al., 2006; Mahmoudi et al., 2013b; Ahad et al., 2010; Ahad and Pakdel, 2013; Petsch et al., 2001; Cowie et al., 2010; Mills et al., 2013; Morrill et al., 2014). In particular, $^{14}C_{PLFA}$ was used in oil-spill contaminated salt marsh sediments and rocky intertidal zones to demonstrate that microbes may initially assimilate oil (Slater et al., 2006), but eventually preferentially consume natural organic matter (Mahmoudi et al., 2013a; Wakeham et al., 2006; Slater et al., 2005). Specific to the *DwH* disaster, ^{14}C was used to show that oxygenated hydrocarbons in weathered MW oil were formed post-spill from ^{14}C-free precursors (i.e., physical or biological degradation of petroleum, and not natural organic matter) (Aeppli et al., 2012). Additionally, natural abundance ^{14}C was used to trace residues deposited on the Gulf of Mexico seafloor (Chanton et al., 2015) and assimilation of oil-derived C into the marine food web (Chanton et al., 2012).

Here we provide evidence of active microbial degradation of highly weathered oil using the ^{14}C signature of microbial lipids. The primary goal of this study was to assess if microbes actively degrade weathered MW oil residues on affected beaches. Oiled-sand patties and non-oiled sand were collected from three Gulf of Mexico beaches in July 2015 that have been previously studied (Hayworth et al., 2015; Aeppli et al., 2012; White et al., 2016), and compound-class ^{14}C analysis of microbial lipids demonstrated that microbes continue to assimilate weathered MW oil.

2. Materials and methods

2.1. Sample location

Oiled-sand patties and visibly non-oiled sand samples were collected in July 2015 from Ship Island (SI), MS; Fort Morgan (FM) AL; and Fort Pickens (FP), FL (Fig. 1) following previously described procedures (Aeppli et al., 2012). Samples were obtained from both intertidal and supratidal locations. Intertidal (IT) regions were defined as the area between low tide and high tide while supratidal (ST) regions were the areas between the high tide and dune line (Operational Science Advisory Team (OSAT), 2011). Samples were placed in pre-combusted glass containers, transported on ice for one to three days, and then stored at −20 °C until further analysis.

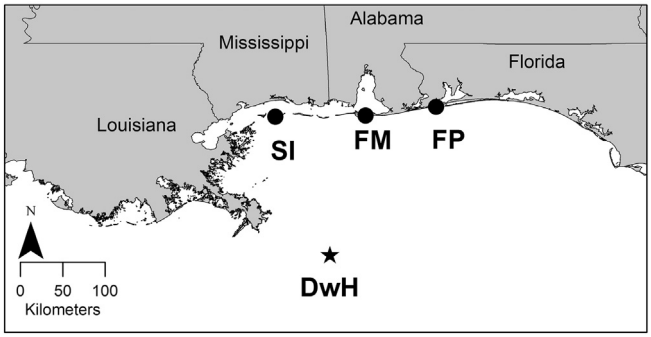

Fig. 1. Oiled-sand patty sample locations in relation to *DwH* drilling platform (SI = Ship Island, Mississippi; FM = Fort Morgan, Alabama; FP = Fort Pickens, Florida). Sample locations have been sampled in previous research (Hayworth et al., 2015; Aeppli et al., 2012; White et al., 2016).

2.2. Oil characterization and fingerprinting

To determine the extent of weathering and the source of the oil residues in the sand patties, samples were solvent-extracted with dichloromethane/methanol (9:1 v:v) and analyzed using gas chromatography coupled to a mass spectrometer (GC–MS), comprehensive two-dimensional gas chromatography coupled to a flame ionization detector (GC × GC-FID), as well as thin layer chromatography coupled to a flame ionization detector (TLC-FID) according to previously described methods (Aeppli et al., 2012; Aeppli et al., 2014). See the Supporting information (SI) for further details on oil characterization and fingerprinting.

2.3. PLFA extraction for ^{14}C analysis

PLFA were extracted using a modified Bligh and Dyer method (Bligh and Dyer, 1959). Oiled-sand patties and non-oiled sand (10–80 g) were sonicated for 2 min in a 1:2:0.8 dichloromethane/methanol/phosphate ($DCM/MeOH/PO_4$) buffer mixture (Fig. S1) and left to sit overnight at room temperature. Extracts were then filtered (VWR Glass Microfibre Filter, 691–1.5 μm particle retention) into separatory funnels (1 L) and augmented with DCM and H_2O to form a 1:1:0.9 $DCM/MeOH/PO_4$ buffer mixture to produce organic (lower) and aqueous (upper) phases. The organic phase, or total lipid extract (TLE), was collected and evaporated to ~1 mL under ultrahigh purity (UHP) N_2. The TLE was then charged onto fully activated silica gel (baked at 400 °C for 4 h, Alfa Aesar, 215–400 mesh) in glass columns (25 mm i.d. and ~25 cm silica gel height) and separated into fractions of increasing polarity by elution with DCM, acetone, and MeOH. Care was taken to use sufficiently large silica gel columns to ensure that the columns were not overloaded. The MeOH fraction contained the PLFA, which were then converted to fatty acid methyl esters (FAME) via mild alkaline methanolysis. As this is base-catalyzed transesterification, free fatty acids were not methylated (Chowdhury and Dick, 2012), which is critical for separating the viable organisms from those that are non-living. The free fatty acids were not analyzed as part of this project.

As our goal was to measure the bulk ^{14}C of the PLFA-derived FAME, further separation from co-occurring non-FAME peaks (presumably oxygenated petroleum hydrocarbon degradation products) using secondary silica gel chromatography was utilized. To this end, samples were loaded onto fully activated silica gel and eluted with 4:1 hexane/DCM, DCM, and MeOH in disposable glass pipettes. The FAME were eluted in the DCM fraction. An additional separation on the secondary silica gel system was completed for selected samples (denoted with a plus (+) sign) to remove remaining petroleum degradation products contained within the FAME fraction that could be monitored using gas chromatography (Fig. S2).

FAME were identified and quantified using an Agilent 7890B/

5977A GC–MS with a DB-5MS column (30 m, 0.25 mm i.d., 0.25 μm film thickness) with UHP He as the carrier gas. The temperature program began at 50 °C, ramped 20 °C min^{-1} to 130, then ramped 4 °C min^{-1} and held isothermal at 160 °C (5 min), ramped up 8 °C min^{-1} to 300 (held for 5 min) and MS was operated in full scan mode. FAMEs were identified by comparing retention times with commercially available standards (*Supelco 37 Component FAME Mix*). For compounds not present in the commercial standard, FAME were tentatively identified using relative retention times and mass fragmentation patterns. Some FAME that exhibited mass fragmentation patterns typical of FAME but were not easily identified were classed as "other FAME". Concentrations of PLFA-derived FAME were normalized to the mass of sand extracted (PLFA pmol/g sand) and relative abundance of PLFA in a given sample (mole percentage). Cell abundances were calculated using a conversion factor of 2×10^4 cell $pmol^{-1}$ PLFA (Green and Scow, 2000). Calculated PLFA concentrations varied by < 5% between replicate injections. PLFA nomenclature convention is described in SI text.

2.4. Radiocarbon measurements

^{14}C was measured for whole oiled sand-patty samples and PLFA extracted from both oiled sand-patties and non-oiled sand. Oiled-sand patties were treated with 5% HCl to remove carbonates and placed into pre-combusted quartz tubes. PLFA extracts were dried under UHP N_2 in pre-combusted quartz tubes. Cupric oxide and silver wire were added to the quartz tubes before being evacuated on a vacuum line and flame sealed. After tubes were combusted at 900 °C for 4 h, the evolved CO_2 was purified, quantified manometrically, and sealed into Pyrex tubes then submitted for ^{14}C analysis at the Center for Applied Isotope Studies at the University of Georgia. ^{14}C values were normalized to $\delta^{13}C$ values of −25‰ to correct for isotope fractionation and presented as $\Delta^{14}C$ (‰) relative to the standard NBS Oxalic Acid I (NIST-SRM-4990) (Stuvier and Polach, 1977). To correct the isotopic value of ΣPLFA for the fossil derived methanol C atom added during the conversion of PLFA to FAME (e.g. transesterification), an isotopic mass balance was used (SI Text). Uncertainty of $\Delta^{14}C$ was assumed to ± 10‰ for bulk oiled-sand patties and ± 20‰ for PLFA. Combustion blanks contained < 3 μg C, while method blanks, extracts without any sample that had been through the entire process, were an additional 1 μg C, which was at least 25 × smaller than samples sizes for ^{14}C analysis that ranged from 100 to 1000 μg C.

2.5. Accounting for oil-derived C using isotope mass balance

To account for the potential influence of GC-amenable non-PFLA oil degradation products in ΣPLFA fractions, an isotopic mass balance approach was used. The following isotope mass balance equations were used to constrain the isotope value of PLFA:

$$f_{PLFA} + f_{oil} = 1 \quad (1)$$

$$\Delta^{14}C_{measured} = \Delta^{14}C_{\Sigma PLFA} * f_{PLFA} + \Delta^{14}C_{oil} * f_{oil} \quad (2)$$

To calculate the isotope value of PLFA ($\Delta^{14}C_{\Sigma PLFA}$), the isotope value of the sample analyzed ($\Delta^{14}C_{measured}$) and the relative mass contributions of oil (f_{oil}) and PLFA (f_{PLFA}) to the sample must be known. The mass contribution from oil (f_{oil}) to PLFA fractions was calculated using the ratio of non-PLFA peak area to total peak area (PLFA + non-PLFA peak area), as measured by GC–MS (using total ion current). $\Delta^{14}C_{measured}$ was the reported AMS value and $\Delta^{14}C_{oil}$ is assumed to be −1000‰.

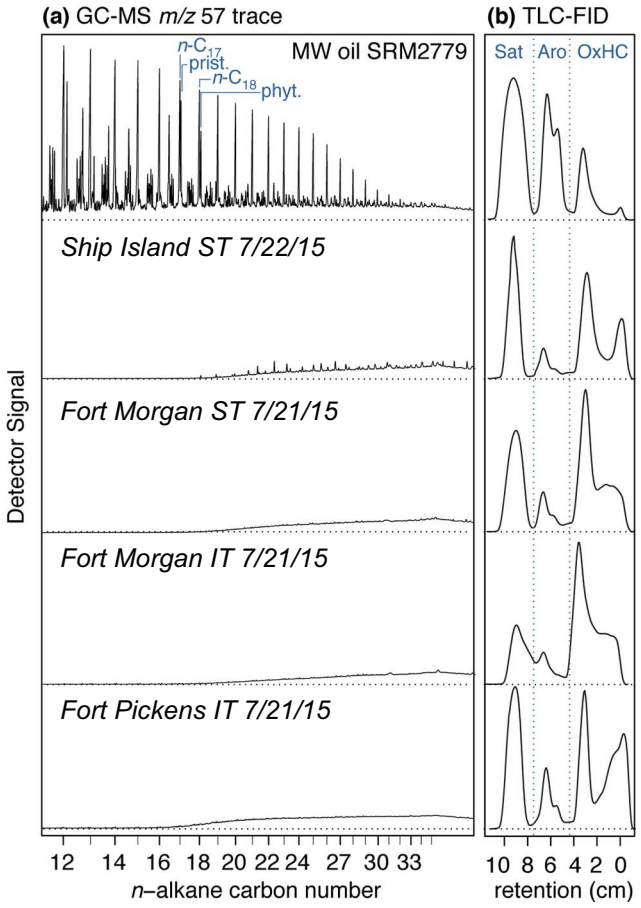

Fig. 2. Analysis of sand patties collected five years after the *DwH* and the spilled MW oil (*NIST SRM 2779: Gulf of Mexico Crude Oil*): (a) The extracted ion current profile of *m/z* 57 from GC–MS and (b) TLC-FID chromatograms. Various weathering processes have completely removed *n*-alkanes (based on the *m/z* 57 traces), decreased the abundance of saturated and aromatic hydrocarbons and increased the abundance of oxygenated hydrocarbons (TLC-FID traces) in the five years post-spill (SI = Ship Island, Mississippi; FM = Fort Morgan, Alabama; FP = Fort Pickens, Florida; ST = Supratidal, IT = Intertidal). For reference, the GC retention time has been replaced with the elution order for normal alkanes. The dotted lines in the chromatograms are the solvent blank.

3. Results

3.1. Oil fingerprinting and weathering state of oiled samples

To confirm that the oil residues from oiled-sand patties in this study were derived from the MW oil, the hopanoid biomarkers were measured as they have shown to be the most resistant to weathering compared to other biomarkers (Aeppli et al., 2014). Overall, the ratio of hopanoid biomarkers in the oiled sand patties in this study were consistent with MW oil as well as with sand patty samples previously fingerprinted as MW oil, they were also significantly different from other reference crude oils form the Gulf of Mexico (Table S3, Table S4). While one of the nine investigated hopanoid biomarkers ratios (4HH(R)/4HH (S)) ratio was a non-match to MW oil, it has been shown previously that this particular ratio can be affected by oil biodegradation (Aeppli et al., 2014). Overall, the hopanoid biomarkers—along with the visual appearance and sampling location—of the investigated oiled sand patty samples are consistent with MW as the source oil.

As expected and in line with previous studies (Aeppli et al., 2014), the oiled sand patty samples were found to contain heavily weathered oil. GC–MS analysis (Fig. 2) and GC × GC-FID analysis (Fig. S4) of the oil residues show that most *n*-alkanes and isoprenoids were completely degraded, and an unresolved complex mixture (UCM) of highly branched and cyclic alkanes as well as hopanoids and (dia)steranes

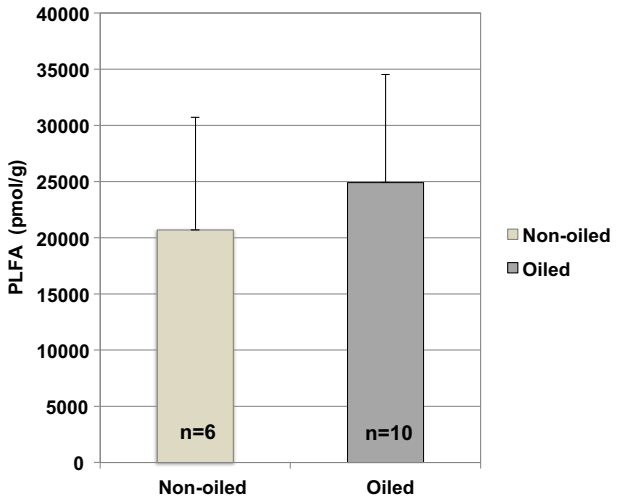

Fig. 3. PLFA concentrations in non-oiled and oiled sand showing the average concentration (bar) and the standard deviation (error bar).

remained. In addition, TLC-FID analysis showed that most of the solvent extractable material in the oiled-sand patties was oxygenated hydrocarbons (53–69%) (Fig. 2). In contrast, MW oil is dominated by saturated (50%) and aromatic (34%) hydrocarbons, and the fraction of more polar compounds is < 20% (Table S5). Oxygenated hydrocarbons have been observed post-*DwH* spill and have been suggested to be oil degradation products caused by photo-oxidation and/or biodegradation (Aeppli et al., 2012; Hall et al., 2013).

3.2. Microbial lipid (PLFA) concentrations and distributions

The concentrations of phospholipid fatty acids (pmol PLFA g^{-1} sand) in oiled sand-patties were similar to the non-oiled sand in all samples collected (Fig. 3). Oiled-sand patties contained $2.5 \times 10^4 \pm 9.6 \times 10^3$ pmol PLFA g^{-1} sand ($n = 10$), while non-oiled sand contained $2.1 \times 10^4 \pm 1.0 \times 10^4$ pmol PLFA g^{-1} sand ($n = 6$). The samples analyzed originated from both supratidal and intertidal environments. When the oiled samples were separated into supratidal ($n = 8$) and intertidal samples ($n = 2$), the difference between the average concentration of PLFA in the two environments was within the sample variability ($2.6 \times 10^4 \pm 1.0 \times 10^4$ pmol PLFA g^{-1} sand versus $2.3 \times 10^4 \pm 9.4 \times 10^3$ pmol PLFA g^{-1} sand). Conversely, when the non-oiled samples were separated into supratidal ($n = 1$) and intertidal ($n = 5$) samples, the abundance of PLFA was different in the two environments (4.7×10^3 PLFA g^{-1} sand versus $2.4 \times 10^4 \pm 7.0 \times 10^3$ pmol PLFA g^{-1} sand, respectively). The PLFA content was not influenced by the implementation of an additional silica gel clean-up (see Materials and methods).

Often, PLFA concentrations are converted to cell abundances (Green and Scow, 2000). Using this approach, the cellular abundance ranged from $3.0–7.9 \times 10^8$ cells g^{-1} sand in oiled-sand and $0.93–7.0 \times 10^8$ cells g^{-1} sand in non-oiled sand, which are similar magnitudes to previous work studying microbial communities using a petroleum C source (Petsch et al., 2001). These calculated microbial abundances, as measured via PLFA, indicated that the oiled-sand was sustaining a similarly abundant microbial population as the non-oiled sand.

To facilitate comparison between samples, we organized the observed PLFA into groups based on their chemical structure (saturated, monounsaturated, polyunsaturated, branched, cyclic, and other PLFA). The most striking difference between the oiled and non-oiled PLFA is the increased abundance of monounsaturated PLFA in non-oiled samples (Fig. 4). The microbial lipids extracted from non-oiled samples were composed of approximately half monounsaturated PLFA, while the PLFA extracted from the oiled sand patties contained a much smaller proportion of monounsaturated PLFA (~14 mol%). Additionally the non-oiled sand contained a slightly higher proportion of polyunsaturated PLFA (~8 mol%) than the oiled sand patties (~3 mol %). Most oiled samples (80%) were far more abundant in "other PLFA" than all non-oiled samples. These "other PLFA" compounds were (*a*) mostly medium molecular weight lipids (weighted average = 15 carbons) that had GC–MS mass fragments characteristic of PLFA (*m/z* 74, 87) (McLafferty, 1959; Bobbie and White, 1980) but poor baseline resolution made it difficult to identify them, (*b*) multiply branched PLFA with either 2-methyl (*m/z* 88, 101) or 3-methyl branching (*m/z* 74, 101) (Hedrick et al., 2008), and (*c*) PLFA compounds that were both branched and unsaturated. The proportion of "other PLFA" was as high as 33 mol% of the total measured PLFA in the oiled sand patty samples. Therefore, based on the PLFA distributions, the microbial communities inhabiting the oiled sand-patties and the non-oiled sand were distinct.

3.3. Radiocarbon of sand patties and microbial lipids

To assess if microbes were consuming oil-derived carbon or more modern C sources, we measured the natural abundance radiocarbon (or ^{14}C) content of both oiled-sand patties and microbial lipids (Fig. 5). The bulk total organic carbon (TOC) of oiled-sand patties had radiocarbon values that ranged from −996 to −966‰ ($n = 6$), consistent with previous ^{14}C measurements of oiled-sand patties (Aeppli et al., 2012). Microbial lipid (PLFA) extracts of oiled-sand patties comprised < 1% of TOC, and were all highly depleted in radiocarbon ($\Delta^{14}C_{measured} = -990$ to −921‰, Table S2). Because GC–MS chromatograms of the PLFA extracts contained a number of peaks that were not PLFA compounds and likely originated from weathered oil, we used a mass-balance approach to account for the ^{14}C values of the non-PFLA fraction of these samples. As a conservative estimation, this calculation, outlined in the Methods section, assumed that all non-PLFA peaks were oil-derived, as non-oiled samples did not contain non-PLFA peaks. Based on this isotopic mass balance method, f_{oil} was between 1 and 81% in the PLFA extracts from oil sand patties. The samples that underwent additional silica gel chromatography purification steps (denoted by a "+" in Fig. 5) contained a smaller proportion of petroleum carbon. However, after adjusting the $\Delta^{14}C$ values of all oiled sand-patty PLFA samples for the presence of oil, we obtained consistent highly depleted $\Delta^{14}C_{PLFA}$ values of −953 to −852‰ (Table S2). In sharp contrast, microbial lipids from non-oiled sand contained modern carbon ($\Delta^{14}C_{\Sigma PLFA}$ = 34‰ and 36‰), which is consistent with the radiocarbon content of the present day atmospheric CO_2 (~20‰) assuming the uncertainty on these $\Delta^{14}C_{\Sigma PLFA}$ measurements is ± 20‰ (Levin et al., 2013). Therefore, natural abundance radiocarbon measurements of phospholipid fatty acids show that microbes in oiled-sand patties were primarily assimilating depleted C (e.g. oil), while microbes in non-oiled sand were assimilating modern carbon (e.g. recently photosynthesized).

4. Discussion

4.1. Microbial communities differ between oiled and non-oiled sand

The different distribution of PLFA compounds in oiled and non-oiled sand samples suggests they are composed of different microbial communities. Therefore, if the microbial lipids are any indication of the diversity of the microbial community present, these data support there being different microbial communities inhabiting the oiled and non-oiled sand. It should be noted that when PLFA distributions in this study were compared to other studies that employed ^{14}C of PLFA to assess microbial degradation of petroleum or fossil C sources (Table S1, Fig. S5), PLFA distributions of microbes degrading weathered shale under a laboratory setting were most similar (Petsch et al., 2005).

Using the microbial lipid data, we followed an approach of a previous study to determine if there was active oil degradation occurring.

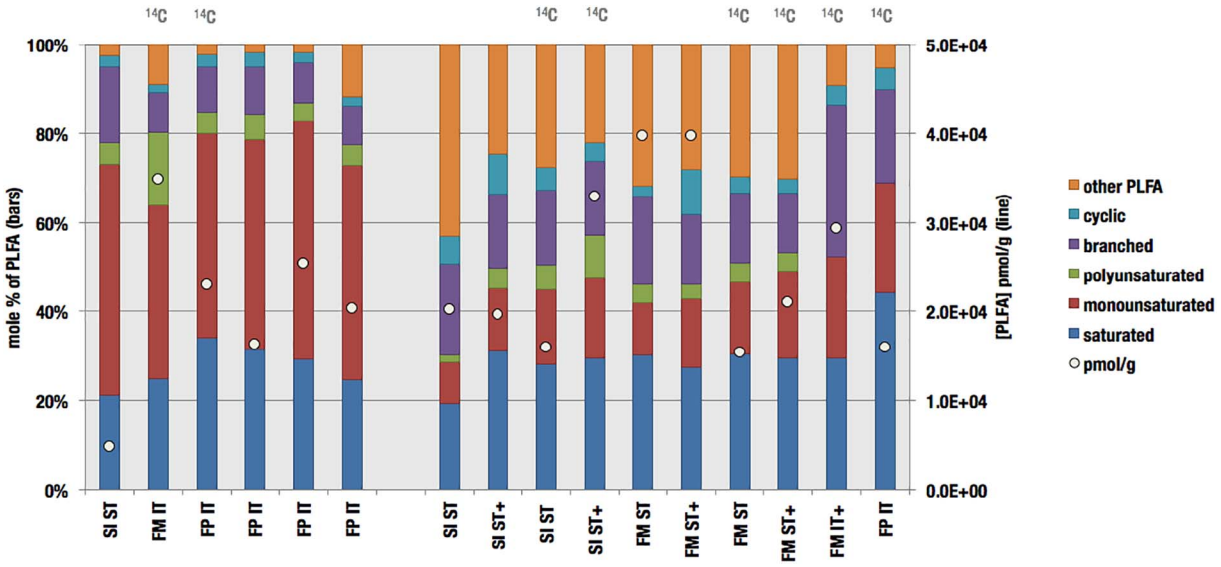

Fig. 4. Mole % of PLFA structures between non-oiled (left) and oiled (right) sand collected in intertidal (IT) relative to supratidal (ST) settings. Differences in PLFA structures were used to make inferences regarding differences in microbial community composition. The line and points on the right y-axis represent the measured PLFA concentration in pmol/g. Samples measured for radiocarbon are denoted with a ^{14}C marker on top of the stacked bar. (SI = Ship Island, MS, FM = Fort Morgan, AL, FP = Fort Pickens, FL).

This approach defines a hydrocarbon degrading activity index (HDAI) based on the observed PLFA distributions of known oil degrading bacteria, with higher HDAI values indicating oil-degrading microbes (Aries et al., 2001). Indeed, microbial lipids from oiled-sand patties in this study had a higher HDAI value (2.5) than microbial lipids from non-oiled sand (0.4), indicating that oiled-sand patties contain microbes associated with oil degradation (Table S6). Therefore, based on the measured PLFA distributions, our results indicate that there were different types of microbial communities inhabiting the oil and non-oiled sand, and that the microbial communities inhabiting the oiled sand were actively consuming the oil.

4.2. Microbes in sand patties are consuming petroleum carbon

Based on the depleted ^{14}C content of the microbial lipids, we conclude that petroleum carbon is the primary source of carbon to the microbes living on all investigated oiled sand patties. The predominant $\Delta^{14}C$ signal of the microbial lipids, or PLFA, extracted from oiled sand patties was extremely depleted (i.e. $\Delta^{14}C < -850‰$, Fig. 5). Additionally, the ^{14}C content of the microbial lipids was similarly depleted regardless of the sample collection location. The fact that intertidal microbial lipids were equally depleted in radiocarbon as the supratidal microbial lipids suggests that the proximity to fresh carbon sources and moisture in the intertidal zone did not impact the ^{14}C content of the microbial lipids. Collectively, these results demonstrate that carbon within the cellular membranes is consistent with the incorporation of petroleum carbon and that the microbes are primarily consuming carbon that originated from the oil in the sand patties.

While the microbial communities living on the oiled sand patties contained ^{14}C that was consistent with the incorporation of oil, the microbial lipids extracted from non-oiled sand samples were consistent with present-day atmospheric carbon. The $\Delta^{14}C$ value of atmospheric CO_2 is estimated to have been $+30 \pm 10‰$ in 2015 when these samples were collected (Graven et al., 2012). Due to sample size limitations for ^{14}C analysis, PLFA ^{14}C samples were only analyzed for non-oiled samples from the intertidal zone, and those microbial lipids samples contained carbon consistent with atmospheric C and within error of each other ($\Delta^{14}C = +34 \pm 20‰$ and $+36 \pm 20‰$). The modern carbon in microbial lipids in the non-oiled sand could be a combination of autotrophs directly fixing modern-day CO_2 and heterotrophs consuming recently fixed organic matter that has a modern ^{14}C signature. Based on the isotopic composition of the microbial lipids

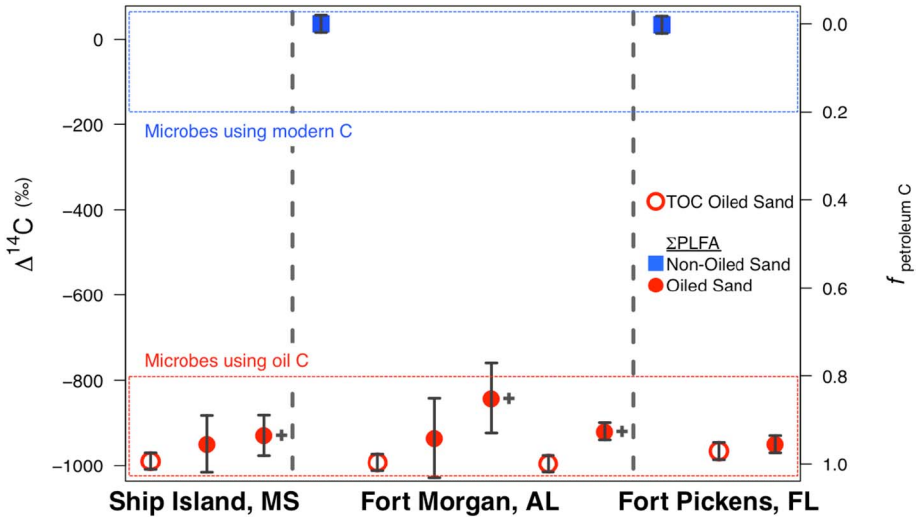

Fig. 5. $\Delta^{14}C$ and $f_{petroleum\ C}$ for bulk oiled-sand patties (unfilled red circles), ΣPLFA from oiled-sand (filled red circles), and ΣPLFA from non-oiled sand (filled blue squares). ΣPLFA $\Delta^{14}C$ indicates microbes present on oiled-sand patties are assimilating a petroleum source of C while microbes on non-oiled sand are assimilating a modern source of C. (+ notates samples that underwent an additional secondary silica gel step). $\Delta^{14}C$ and $f_{oil\ C}$ values of oiled sand PLFA (filled red circles) are corrected for the presence of oil or oil degradation products. Sample names (from left to right): Bulk SI ST, SI ST, SI ST +, FM IT, Bulk FM ST, FM ST, FM ST +, Bulk FM IT, FM IT +, FP IT, Bulk FP IT, FP IT. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

extracted from non-oiled sand, it is clear that the microbial communities living on oiled sand patties are using different carbon source(s) than the microbes living on the non-oiled sand.

Since the microbial lipids in the non-oiled sand contain a significantly more modern $\Delta^{14}C$ than the PLFA from oiled sand within our reported uncertainty, we explored the possibility that the depleted microbial lipid $\Delta^{14}C$ signal from the oil-sand patties was due to the presence of oil or oil degradation products in the PLFA extracts. As described in the methods, the amount of oil remaining in the ΣPLFA samples was estimated based on the amount of carbon in the samples as calculated by GC–MS. Regardless of the oil content, it is possible to account for the presence of that oil in the $\Delta^{14}C$ values. Even after the most conservative estimation of how much oil or oil degradation products could be in the isolated PLFA fraction of our samples (assuming all non-PLFA signal was oil derived), PLFA from the oiled sand is consistently highly depleted ($\Delta^{14}C_{\Sigma PLFA}$ ranged from -952 to -853‰). Even after adjusting the measured $\Delta^{14}C$ values for the presence of oil, the samples were still uniformly depleted in ^{14}C (Fig. 5), indicating that the microbes were primarily eating oil. If ΣPLFA from the oiled-sand patties actually contained modern carbon like the non-oiled sand patties and the depleted ^{14}C was simply from the oil contaminating the ΣPLFA, we calculated that these samples would have had to contain between 92 and > 99% oil to have the same ^{14}C value as the non-oiled sand patties, which is much larger than the f_{oil} we calculated for our samples (1–81%) based on the proportion of the total GC–MS signal represented by PLFA peaks. Additionally, based on the amount of PLFA in the samples we can reject the hypothesis that > 90% of the carbon in the sample was oil as there were significant amounts of PLFA typical of microbial communities in the samples (Fig. S2). For example, the sample that was calculated to contain the least amount of oil (FM intertidal, $f_{oil} = 1\%$) was still extremely depleted in ^{14}C ($\Delta^{14}C_{PLFA} = -920$‰), indicating that the microbes are in fact consuming oil derived carbon. Therefore, the depleted ^{14}C in the microbial lipids are due to the incorporation of oil-derived carbon by the microbes rather than contamination of the samples by oil.

This study presents the radiocarbon data of the ΣPLFA, which determined the ^{14}C content for all the viable microbial lipids in a sample. Previous studies that employed $^{14}C_{PLFA}$ (Table S1) did not find significant differences in the ^{14}C content of individual PLFA compounds from the same sample (or extract) (Mahmoudi et al., 2013a; Wakeham et al., 2006; Slater et al., 2005; Cowie et al., 2010). This method of using ΣPLFA ^{14}C rather than individual PLFA is more cost effective and can provide larger sample sizes than measuring the ^{14}C of individual PLFA. While isolating individual compounds via preparative-GC may have minimized the fraction of oil in the samples, samples isolated with a fraction collector would still contain unresolved complex mixture that co-elutes with the PLFA compounds (White et al., 2013).

4.3. Implications for microbial degradation of weathered oil

Continued microbial degradation of heavily weathered MW oil has implications for oil continuing to wash ashore. Our results clearly demonstrate that microbes contained oil derived carbon, most likely from the weathered oil that continues to be deposited on Gulf Coast beaches. These findings suggest microbial degradation of weathered oil occurred and may continue to occur, as oiled-sand patties are expected to wash ashore for several years (Hayworth et al., 2015). It should be noted that a limitation of this study is that it only provides a snapshot of in situ carbon usage of the microbes and not the rate of oil carbon being incorporated. The removal of oiled-sand patties through microbial degradation is surprising given that heavily weathered oil is often assumed to be recalcitrant (Wang et al., 1998). While the microbial community as a whole may be degrading the weathered MW oil, it is unclear if the microbes are consuming the oil directly or the oxygenated oil degradation byproducts. Therefore, a logical next step would be to use a combination of environmental manipulations (e.g. photochemistry) and incubations to determine if microbes are using oil directly or oil residue compounds. Lastly, quantifying degradation rates of remaining MW oil using incubations (Röling et al., 2002) will allow for better estimates of when affected beaches will return to pre-*DwH* conditions.

In conclusion, our ^{14}C analysis of microbial lipids demonstrates that microbes on Gulf of Mexico beaches are continuing to degrade weathered oil residues from the *DwH* spill after more than five years in the environment. Microbial degradation of weathered oil represents a loss term for MW oil that continues to wash ashore on Gulf Coast beaches. Our results, showing microbial degradation of MW oil, demonstrate that microbes are capable of continued degradation of weathered oil residues from the *DwH* spill.

Author contributions

The manuscript was written through equal contributions of all authors. All authors have given approval to the final version of the manuscript.

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Appendix A. Supplementary data

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