Natural-Abundance Radiocarbon as a Tracer of Assimilation of Petroleum Carbon by Bacteria in Salt Marsh Sediments

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ABSTRACT

The natural abundance of radiocarbon (^{14}C) provides unique insight into the source and cycling of sedimentary organic matter. Radiocarbon analysis of bacterial phospholipid lipid fatty acids (PLFAs) in salt-marsh sediments of southeast Georgia (USA) – one heavily contaminated by petroleum residues – was used to assess the fate of petroleum-derived carbon in sediments and incorporation of fossil carbon into microbial biomass. PLFAs that are common components of eubacterial cell membranes (e.g., branched C_{15} and C_{17}, 10-methyl-C_{16}) were depleted in ^{14}C in the contaminated sediment (mean $\Delta^{14}C$ value of +25 ± 19 ‰ for bacterial PLFAs) relative to PLFAs in uncontaminated "control" sediment ($\Delta^{14}C$ = +101 ± 12 ‰). We suggest that the ^{14}C-depletion in bacterial PLFAs at the contaminated site results from microbial metabolism of petroleum and subsequent incorporation of petroleum-derived carbon into bacterial membrane lipids. A mass balance calculation indicates that 6-10% of the carbon in bacterial PLFAs at the oiled site could derive from petroleum residues. These results demonstrate that even weathered petroleum may contain components of sufficient lability to be a carbon source for biomass production by marsh sediment microorganisms. Furthermore, a small but significant fraction of fossil carbon is assimilated even in the presence of a much larger pool of presumably more-labile and faster-cycling carbon substrates.

1. INTRODUCTION

Isotopic signatures of heterotrophic organisms, including bacteria, reflect the isotopic composition of organic carbon sources being assimilated (Abraham *et al.*, 1998; Boschker and Middelburg, 2002; Londry *et al*., 2004), thus directly linking microbial communities with biogeochemical processes. Natural-abundance radiocarbon analyses add the dimension of "age" to the character of organic matter. The dynamic range of $\Delta^{14}C$ (−1000 to ~ +200 ‰) is much greater than for $\delta^{13}C$ (−32 to −12 ‰) since fossil materials contain no radiocarbon ($t_{1/2}$ = 5740 yr vs ages >10^6 yr) compared to modern organic carbon pools enriched in ^{14}C ($\Delta^{14}C > 0$) from atmospheric nuclear bomb testing. Compound-specific radiocarbon measurements are valuable since the history (age) of a given biomarker ties it to its provenance (Ingalls and Pearson, 2005). A bomb-derived ^{14}C signal in organic matter is an unambiguous indicator of recent photoautotrophy utilizing atmospheric CO_2, whereas ^{14}C-depletions in organic substances reflect incorporation of carbon originating from other reservoirs isolated from the atmosphere. Incorporation of old carbon into recently-biosynthesized biomass will be detectable in its lower $\Delta^{14}C$, thereby offering an opportunity to begin to apportion carbon sources on the basis of ^{14}C content and to ascertain the lability of "old" and presumably refractory carbon pools.

Fossil organic substances, including petroleum and kerogen, have traditionally been considered refractory towards biodegradation in aquatic environments. The diversity of molecular types in petroleum leads to a wide range in labilities, and a variety of microorganisms are capable of biodegrading components of petroleum. Studies using enriched and natural abundance stable and radiocarbon isotopes have shown hydrocarbon

degradation by aerobic bacteria and fungi (e.g., Leahy and Colwell, 1990; Bugna *et al*., 2004) as well as anaerobes utilizing nitrate, ferric-iron, and sulfate as electron acceptors (e.g., Aeckersberg *et al*., 1998; Coates *et al*., 1998; Heider *et al*., 1999; So and Young, 1999; Fukui *et al*., 2000; Lovley and Coates, 2000; Tor and Lovley, 2001; Widdel and Rabus, 2001; Rothermich *et al*. 2002). Anaerobic hydrocarbon degradation generally proceeds with lower free energy yields and at slower growth rates than for aerobes. As for kerogen, Petsch *et al.* (2001) used natural abundance ^{14}C to show that a microbial enrichment culture was able to grow on black shale organic matter and incorporate this fossil carbon into phospholipid fatty acids.

Although petroleum hydrocarbons are efficiently remineralized by bacteria, with up to >80% of tracer ^{14}C recovered as $^{14}CO_2$ (Coates *et al*., 1997; Rothermich *et al*., 2002; Hayes *et al*., 1999), the extent to which hydrocarbon-derived carbon is assimilated and goes toward biomass remains uncertain. In order to address this deficiency, we investigated the distribution of ^{14}C in organic matter fractions in a marsh sediment that is contaminated with petroleum hydrocarbons with the goal of determining the extent to which fossil carbon might be incorporated into bacterial biomass. Specifically, we hypothesized that incorporation of fossil C would lead to depletions in the ^{14}C content of bacterial membrane-derived phospholipid fatty acid (PLFAs) as determined by compound-specific radiocarbon analyses. Phospholipid fatty acids were chosen as target compounds because their origin in membrane-bound phospholipids would insure that they likely derive from viable bacteria inhabiting the sediment at the time of sampling, as phospholipids degrade quickly upon cellular death (Harvey *et al*., 1986). We show here that in a chronically petroleum-contaminated salt marsh, PLFAs, notably *iso*- and

anteiso-C_{15} and C_{17}, 10-methyl-C_{16}, and monounsaturated $C_{16:1}$ common in the salt marsh sediment bacterial community, are depleted in ^{14}C compared to other PLFAs and to an uncontaminated control sediment. This fossil carbon is being assimilated by salt marsh bacteria even in the presence of more abundant "natural" labile organic materials.

2. MATERIALS AND METHODS (may be set in small type)

2.1. Sediments. Sediment samples (Table 1) were collected in March, 1999, from two tidal marsh sites (LCP and Sapelo) in southeastern Georgia. Both sites are dominated by the marsh cordgrass, *Spartina alterniflora*. The LCP Chemicals Superfund Site in Brunswick, GA http://www.epa.gov/region4/waste/npl/nplga/lcpincga.htm) is severely contaminated with petroleum residues and polycyclic aromatic hydrocarbons, mercury, polychlorinated biphenyls, and toxaphene as it has been occupied by a petroleum refinery, a chlor-alkali plant, a paint manufacturing site, and a power plant over the last 70 years. Refinery operations ceased in 1935. "Control" sediments were collected at the southern end of Sapelo Island in the tidal marsh that has been designated a NOAA Estuarine Research Reserve.

Sediments at both sites are subjected to aerobic decomposition as oxygenated tidal waters flood the sites and oxygen is transported into subsurface sediments by porewater advection and by *Spartina* roots and rhizomes. However, oxygen is rapidly depleted in the top few millimeters of Georgia salt marsh sediments (Furukawa et al., 2004), and dissolved sulfate or solid phase Fe(III) minerals are the most abundant electron acceptors available to microorganisms (Kostka *et al*., 2002a,b; Koretsky *et al*., 2005). Thus, although tidal floods may briefly replenish porewater oxygen in the upper sediments, organic matter decomposition below-ground is dominated by sulfate reduction and Fe(III) reduction (Kostka *et al*., 2002a,b; Gribsholt *et al*., 2003; Furukawa *et al*., 2004).

Sediment samples were obtained from stands of tall *Spartina* in the upper marsh at both sites. A shovel was used to cut through the root and rhizome mass and ~1 kg (wet) of ~0-5 cm sediment from each site was placed in double zip-lok bags, transported back to the laboratory on ice in separate coolers, and then stored frozen and separately until further analysis. Both sediments were brown to black in color, and contained extensive root biomass. The LCP sediment smelled strongly of petroleum and there was a sheen of oil in the adjacent tidal creek.

2.2. Extraction and Purification of PLFAs. The analytical scheme for isolating PLFAs is shown in Fig. 1. Thawed, wet sediments, with most root matter removed, were extracted for 48 hr in a soxhlet extractor with chloroform-methanol (2:1) followed by partitioning between $CHCl_3$ and 5% NaCl solution in a separatory funnel. The aqueous phase was discarded and a solvent lipid-extract (SLE) in the $CHCl_3$ phase was dried over Na_2SO_4. Extracted sediments were dried and weighed; about 110 g dry weight of sediment had been extracted for each sample. SLEs were separated into operationally-defined neutral lipid (NL), glycolipid (GL), and phospholipid (PL) fractions by chromatography on activated silica gel (Merck silica gel G) using chloroform, acetone, and methanol as eluants, respectively (Guckert *et al*., 1985). NL and PL fractions were saponified with 0.5N KOH in toluene-methanol-water (1:1:0.5), and nonsaponified lipids and acids were obtained by sequential extraction of the saponification mixture at pH 14 and then pH 2. Acids from the PL fraction were methylated with BF_3-MeOH and were purified on a silica gel column, with the PL-FAMES eluting with 15% ethylacetate in hexane. An α-hydroxy acid composite was eluted with 20% ethylacetate in hexane and used to determine isotope compositions of lipids derived from *Spartina* leaf and root cutin and suberin. Hydrocarbons were isolated from the non-saponifiable fraction of the NL fraction via a silica gel column and were treated with urea to separate straight-chained (urea non-adductable) compounds from branched and cyclic (urea adductable) compounds (i.e., the unresolved complex mixture or UCM).

PL-FAMES and hydrocarbons were analysed by gas chromatography (GC) using a Carlo Erba 8160 Fractovap with on-column injection, J&W DB-1 column (60 m x 0.25 mm id x 0.25 μm film) and with hydrogen as carrier gas. PL-FAME identities were determined by gas-chromatography-mass spectrometry (GC-MS) using a Hewlett Packard 5890II GC coupled to a Finnigan INCOS 50 mass spectrometer and with a J&W DB-5 column (30 m x 0.25 mm id x 0.25 μm film thickness) and helium as carrier gas.

2.3. Preparative Capillary Gas Chromatography (PCGC). Collection of purified individual fatty acids was by PCGC as described by Eglinton *et al*. (1996) and Pearson *et al*. (2001). A HP 5980II GC equipped with an HP 7673 autoinjector, a Gerstel CIS-3 cooled injection system and a Gerstel preparative fraction collector (PFC) was fitted with RTX-1 megabore (60 m x 0.53 mm id x 0.5μm film) capillary column. The GC temperature program was 60°C (1 min), 20°C/min to 160°C, 4°C/min to 300°C and isothermal at 300°C for 20 min. An effluent splitter directed 1% of the column effluent to the FID and the remaining 99% was sent to the zero-dead-volume splitter of the PFC. The PFC was operated at 320°C and U-tube traps were held at -10°C using a recirculating chiller containing a water-polyethylene glycol mixture. Purity of fractions and the amounts of fractions collected were checked by GC. Individual or composited compounds or operational classes (see below) were transferred to glass ampules and flame sealed for isotope analysis.

2.4. Stable and Radiocarbon Analysis. All stable and radio-carbon isotope measurements were made at the National Ocean Sciences Accelerator Mass Spectrometry (NOSAMS) Facility at the Woods Hole Oceanographic Institution. Sediment samples were acidified [10% (v/v) HCl] and dry subsamples were transferred to pre-combusted Vycor tubes containing CuO and Ag powder. Sample extracts and individual compounds were transferred with solvent to a pre-

combusted Vycor tube. The solvent was evaporated and 100 mg CuO was added to the tube. After evacuation, samples were sealed and combusted to CO_2 at 850°C for 5 hours. After purification and quantification, a split of the CO_2 was analyzed for $\delta^{13}C$ on a VG Micromass Optima isotope ratio mass spectrometer. The remainder of the gas was reduced to filamentous carbon (commonly referred to as graphite) over either Fe or Co powder. Radiocarbon analyses of both large and small samples were performed using standard NOSAMS procedures (McNichol *et al*., 1994; von Reden *et al*., 1998; Pearson *et al*., 1998). Delta-^{14}C values and errors reported for all combusted samples have been corrected for a combustion blank. Recently photosynthesized material (*Spartina* root tissues) had a $\Delta^{14}C$ value of +98 ‰, whereas small samples of petroleum analyzed as part of this study had $\Delta^{14}C$ values of −999 to −979 ‰.

The contribution of the methyl carbon derived from methanol in FAMEs was removed by isotopic mass balance (Pearson, 2000): $\delta^{13}C_{FAME} = [(N+1)* \delta^{13}C_{measured} - \delta^{13}C_{MeOH}] / N$ and $\Delta^{14}C_{FAME} = [(N+1)*\Delta^{14}C_{measured} - \Delta^{14}C_{MeOH}] / N$, where N is the number of carbon atoms. We determined the $\delta^{13}C$ and $\Delta^{14}C$ values of carbon in the methylating reagent two ways. First, we directly measured the $\Delta^{14}C$ of the carbon in methanol of BF_3-MeOH and obtained a value of !998 ‰. Second, we measured the $\Delta^{14}C$ values of a palmitic acid standard (+111 ‰), methylpalmitate prepared using the same lot of BF_3-MeOH (+59 ‰) and methylpalmitate isolated by PCGC (+35 ‰). The $\delta^{13}C$ and $\Delta^{14}C$ values of the derivatizing carbon were calculated by mass balance to be −51.5 ‰ and −777 ‰, respectively. We believe isotope corrections obtained after PCGC are more reflective of the entire derivatization and isolation procedure through which all compounds undergo and we therefore use the derived value for our subsequent calculations. If we were to use the value obtained directly for the methanol, the final corrected $\delta^{13}C$ and $\Delta^{14}C$ values of individual compounds do not differ greatly and do not affect our overall conclusions. Errors for $\delta^{13}C$'s are ±0.1 ‰, and cumulative errors for $\Delta^{14}C$ are given in Tables 2 and 3.

2.5. Elemental Analysis. Dried and acidified sediments were analyzed for organic carbon (OC or TOC [total organic carbon]) and total nitrogen (TN) using a Carlo Erba CHN analyzer.

3. RESULTS AND DISCUSSION

3.1. The Studied Sediments. Nearly 4.4% of the total organic carbon of the LCP sediment was petroleum (5,950 μg/g dry weight; Table 1). The gas chromatogram of the LCP hydrocarbons was dominated by the UCM of branched and cyclic compounds (Fig. 2) indicative of petroleum hydrocarbons and comprising ~95% of the hydrocarbon content. The remaining ~5% of the hydrocarbons are long-chain n-alkanes (>C_{25} and maximizing at C_{31}) derived primarily from vascular plants including *Spartina* but potentially also terrigenous plants on the adjacent uplands. The concentration of long-chain *n*-alkane vascular plant hydrocarbons is ~300 μg/g. Hydrocarbons in the Sapelo sediment were some 34-fold lower in concentration (175 μg/g) and in the absence of any visible UCM in the gas chromatogram appeared to be predominately biogenic and of vascular plant origin. Remarkably, the levels of biogenic hydrocarbons in the two sediments were within a factor of 2 of each other.

If indeed the relative abundances of long-chain *n*-alkanes indicate that the vascular (*Spartina* plus terrigenous species) plant component of the LCP sediment is ~2-fold that of the Sapelo sediment, then this might account for the higher %OC of the LCP sediment (Table 1). In spite of efforts to remove root material from the sediments before analysis, we believe that the LCP sample did contain more root fragments than the Sapelo sediment. The C/N ratio of TOC_{LCP} was also higher than that of $TOC_{Sapelo,}$ also consistent with more *Spartina*-derived material in the former since *Spartina* tissues have a C/N of ~ 40 vs. ~ 7 for benthic diatoms (Middelburg et al., 1997) that may be more

abundant in the Sapelo sediment than in the LCP sediment. The ~ 2 ‰ depletion of ^{13}C of TOC_{LCP} compared to TOC_{Sapelo} (Table 2) suggests further that the LCP sample might contain relatively more non-*Spartina*, vascular plant derived OC (i.e., ^{13}C-depleted) than the Sapelo sample. Stable carbon isotope systematics of salt marsh sediments in the southeastern U.S. are complex, however, as observed in the Sapelo salt marsh first by Haines (1976) and subsequently by Fogel et al (1989). The C_4 pathway of carbon fixation by *Spartina* gives it a $\delta^{13}C$ of about −13 to −12 ‰, while other marsh macrophytes using the C_3 pathway (e.g., *Juncus roemerianus* and *Salicornia virginica*) have $\delta^{13}C$ values of −26 to −23 ‰. Any organic matter derived from adjacent uplands that accretes in the marsh would carry with it primarily a C_3 vascular plant $\delta^{13}C$ value of −28 to −25 ‰. Benthic diatoms have $\delta^{13}C$ values of −26 to −23 ‰, and suspended particulate organic carbon (POC) in tidal water that floods the *Spartina* stands have $\delta^{13}C$ values of −23 to −20 ‰.

Unexpectedly, TOC_{LCP} was enriched in ^{14}C compared to TOC_{Sapelo} (Table 2) despite the LCP sediment being significantly contaminated with ^{14}C-free petroleum hydrocarbons ($\Delta^{14}C$ <− 950 ‰ based on LCP total and adductable hydrocarbon fractions; Table 2). Because of this petroleum component SLE_{LCP} is strongly ^{14}C-depleted ($\Delta^{14}C$ − 380 ‰). By contrast, the SLE_{sapelo} is modern ($\Delta^{14}C$ + 23 ‰), but its enrichment relative to TOC_{Sapelo} means that TOC must contain a significant proportion of non-extractable ^{14}C-depleted carbon. An explanation for a relative enrichment in ^{14}C of TOC at the LCP site is that we included more modern root material in this sample as root fragments were difficult to completely remove. An alternate explanation for the depletion in ^{14}C of TOC at Sapelo is that this sampling site was nearer to a major tidal

creek and thus was more frequently flooded by tidal incursions than the LCP site. More-frequent flooding might result in accretion of a greater proportion of ^{14}C-depleted terrigenous material in the Sapelo marsh. Soil organic matter ranges in ^{14}C-age from modern to several thousands of years (Trumbore, 2000; Raymond and Bauer, 2001), and organic carbon exported by rivers is significantly depleted with respect to present-day atmospheric $\Delta^{14}C$-CO_2 depending on proportions of modern plant litter ($\Delta^{14}C$ values of +150 to +310 ‰; Richter *et al*., 1999; Trumbore, 2000; Raymond and Bauer, 2001), relict soil horizons and fossil (sedimentary kerogen or petroleum residues) carbon (Raymond and Bauer, 2001).

3.2. Sediment PLFAs

The concentration of PLFAs (Table 1) in the LCP sediments was 20.6 μg/g and in the Sapelo sediment was 41.1 μg/g. In both sediments, PLFAs comprised about 0.35% of TOC. PLFAs in both sediments ranged from $C_{14} - C_{30}$ (Fig. 3 shows the PLFA distribution in the LCP sediment), including straight-chained and branched monoenoic, and saturated components. Among these, *iso*- and *anteiso*-branched monoenoic and saturated C_{15} and C_{17} acids, 10-methyl-C_{16} (10-Me-C_{16}) and monoenoic C_{16} (e.g., $C_{16:1\omega7c}$) and C_{18} (e.g., $C_{18:1\omega7c}$) acids are characteristic of many bacteria (Ratledge and Wilkinson, 1988; Kaneda, 1991), especially Gram-negative sulfate-reducing bacteria (Taylor and Parkes, 1983; Edlund *et al*., 1985; Dowling *et al*., 1986; Kohring *et al*., 1994; Vainshtein *et al*., 1992), and iron(III)-reducing bacteria (Moule and Wilkinson, 1987; Nichols *et al*., 1992; Coleman *et al*., 1993; Teece *et al*., 1999; Venkateswaran *et al*., 1999; Zhang *et al*., 2003). In salt marsh sediments inhabited by the tall form of *Spartina*, sulfate- and

Fe(III)-reducing bacteria are abundant (10^6 to 10^8 cells/ml) and anaerobic decomposition predominates over aerobic decomposition (Lowe *et al*., 2000; Kostka *et al*., 2002a,b; Gribsholt *et al*., 2003; Furukawa *et al*., 2004).

Of the other major $<C_{20}$ fatty acids, $C_{14:0}$ may derive from benthic algae (mainly diatoms living on the sediment surface), while $C_{16:0}$ and $C_{18:0}$ are from many and diverse sources. The $>C_{20}$ long-chain, even-carbon predominant fatty acids ($C_{24:0}$ $C_{26:0}$, $C_{28:0}$) and α-hydroxy acids are of vascular plant origins. Our analyses of *Spartina* leaves found $C_{16:0}$, $C_{18:3}$ and $C_{18:2}$, whereas roots and rhizomes contain a more complex fatty acid distribution, consisting of $C_{16:0}$, $C_{18:3}$, $C_{18:2}$, $C_{18:1\omega9}$, $C_{18:1\omega11}$ and long-chain even-carbon predominant C_{22}-C_{28} *n*-alkanoic and α-hydroxy acids. The most notable difference between *Spartina* fatty acids and the marsh sediment fatty acids is the absence of C_{18}-polyunsaturated fatty acids (PUFAs) in the latter, possibly because detrital *Spartina*-derived PUFAs have been diagenetically transformed to $C_{18:1}$ and $C_{18:0}$ in the sediments (Kawamura *et al*. 1980).

3.3. Radiocarbon of PLFAs. Individual (or composites) of Sapelo and LCP PLFAs were isolated (as PL-FAMEs) by preparative gas chromatography (e.g., Fig. 3) and analysed for their $\Delta^{14}C$ and $\delta^{13}C$ compositions (Table 3 and Fig. 4). When possible, individual compounds were analyzed but, in some cases, similar compounds had to be combined into one sample to obtain enough material for ^{14}C analysis or because isomers eluted too closely to be isolated separately. *Iso*- and *anteiso*-C_{15} were collected together to give a single branched-$C_{15:0}$ sample (br-15:0 in Table 3), as were $C_{16:1\omega9}$ and $C_{16:1\omega7}$ to give a single $C_{16:1}$ isolate (16:1), *iso*- and *anteiso*-C_{17} plus several $C_{17:1}$ isomers to give a

branched-C_{17} (br-17:0), and $C_{18:1\omega11}$ plus $C_{18:1\omega9}$ to give a $C_{18:1}$ (but predominately $C_{18:1\omega11}$) composite (18:1).

For PLFAs, $\Delta^{14}C$ values (corrected for derivatization; cumulative errors are shown in Table 3) ranged from +4 ‰ for $C_{28:0}$ to +206 ‰ for $C_{16:0}$. All PLFAs were enriched in ^{14}C compared to their respective TOCs and SLEs. There must, therefore, be a non-PLFA component of TOC and the SLEs that is depleted in ^{14}C. Branched-$C_{15:0}$, $C_{16:1}$, 10-Me-$C_{16:0}$ and branched-$C_{17:0}$ were depleted in ^{14}C relative to $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$. This difference could reflect incorporation of some ^{14}C-depleted carbon into the bacterial membrane components versus ^{14}C-enriched modern carbon fatty acids that derive from photoautotrophy by benthic algae and *Spartina*.

Several PLFAs had surprisingly high $\Delta^{14}C$ values, e.g., $C_{14:0}$, $C_{16:0}$ and $C_{18:0}$, at both sites and $C_{18:1}$ at Sapelo. There are two possibilities for these results - either laboratory processing added material enriched in ^{14}C or the results reflect a natural process that results in enriched values for these compounds.

Processing of any sample, from collection in the field through conversion to the solid carbon form used for AMS analysis, affords many opportunities for the addition of contaminant carbon to a sample. This can be a particular problem for the smallest samples. Studies of general laboratory contamination have found that quantities of carbon added are generally small (<1-3 μg C) and have $\Delta^{14}C$ values less than 1 (Pearson *et al*., 2000; Currie *et al.* 2000). At NOSAMS, results are corrected for introduction of this carbon. Evidence from the present study indicates that any contamination not accounted for by the above correction could serve to reduce the observed ^{14}C content, not enrich it. Measurements of methylpalmitate before and after isolation by PCGC (+59 and

+35 ‰, respectively) confirm the expected trend. A more serious issue may be the contamination by ^{14}C-labelled tracer of surfaces and equipment used to prepare the samples. This is unlikely here because ^{14}C-labelled C_{16} and C_{18} have never been used in either laboratory in which samples were processed, and a general laboratory contamination would have had the most pronounced effect on the smallest samples and perhaps on all compounds isolated as well. In fact, the most enriched PLFAs here are those isolated in the highest concentrations and for which larger amounts of carbon were analyzed by AMS (Table 3).

Natural processes could result in the observed trends. Plant material synthesized from atmospheric CO_2 over the last 50 years reflects atmospheric radiocarbon values ranging from 0 to up to +800 ‰. Material that averages inputs from several years or decades can reflect any value in this range depending on the amount of material present from a particular period. However, we measured $\Delta^{14}C$ values of +71 and +98 ‰ for *Spartina* leaf and root tissues, and +69 and +104 ‰ for DIC at the sampling locations in Sapelo and LCP, respectively, similar to atmospheric $\Delta^{14}C$ of ~ +70 ‰ at the time these samples were collected (Levin and Hesshaimer, 2000; Levin and Kromer, 2004).

3.4. Isotopic Distinction Between LCP and Sapelo PLFAs

There is a small, but we believe real, isotopic separation between LCP and Sapelo samples, and between short-chain bacterial vs. long-chain vascular (terrigenous and *Spartina*) plant PLFAs. On a dual-isotope plot of $\delta^{13}C$ values vs. $\Delta^{14}C$ values (Fig. 5), PLFAs clustering in the lower left corner are the bacterial membrane fatty acids (br-15:0, 15:0, 16:1, 10-Me-16, and br-17:0) isolated from the LCP sediment. The same PLFAs

from the Sapelo sediment are enriched in both ^{14}C and ^{13}C and thus plot above and to the right of the LCP group. Long-chain PLFAs ($C_{24:0}$ $C_{26:0}$, $C_{28:0}$, and α-hydroxy acids) from Sapelo tend to be enriched in ^{13}C but depleted in ^{14}C relative to the same compounds at the LCP site, again consistent with relatively more abundant terrigenous component. The remaining compounds ($C_{14:0}$, $C_{16:0}$, $C_{18:0}$) are markedly enriched in radiocarbon and must originate from contemporary sources, possibly *Spartina*, although their $\delta^{13}C$ values are ambiguous. Note, however, that the separation between LCP and Sapelo PLFAs on the basis of their $\Delta^{14}C$ values is greater than based on $\delta^{13}C$ values. The ^{14}C approach is free from potential isotope fractionation effects on $\delta^{13}C$ values associated with carbon metabolism, assimilation, and resynthesis as fatty acids (see reviews by Hayes, 1993, 2001), and the range of $\Delta^{14}C$ values in carbon sources for sediment bacteria is much greater than the range of $\delta^{13}C$ values.

3.5. Fossil Carbon in Bacterial PLFAs at the LCP Site. The premise of this study was that the ^{14}C content of bacterial PLFAs at the contaminated LCP site would be reduced by incorporation of radiocarbon-"dead" fossil carbon, presumably resulting from microbial breakdown of the contaminating petroleum and flow of fossil carbon into bacterial biomass (i.e., membrane fatty acids). Our results indicate that the bacterial PLFAs from a severely contaminated site demonstrate the incorporation of carbon from a petroleum source. We can estimate the fraction of fossil carbon in the LCP bacterial PLFAs using a simple isotope mass balance

$$1 = f_{modern} + f_{fossil} \quad (1)$$

$$\Delta^{14}C_{PLFA} = f_{modern}(\Delta^{14}C_{modern}) + f_{fossil}(\Delta^{14}C_{fossil}). \quad (2)$$

where f_{modern} is the fraction of modern carbon cycling in the marsh sediments, independent of whether it is algal or *Spartina*-derived, f_{fossil} is the fraction of OC derived from degraded petroleum, $\Delta^{14}C_{PLFA}$ is the radiocarbon content of the specific PLFA and $\Delta^{14}C_{modern}$ and $\Delta^{14}C_{fossil}$ are radiocarbon contents of appropriate endmembers.

The choice of the fossil endmember is relatively straightforward, and for this calculation we use – 968 ‰, the value we measured for the urea-adducted UCM in the LCP sediment (Table 2). The modern endmember is more problematic, but a reasonable value is + 100 ‰. A $\Delta^{14}C$ value of +101 ± 12 ‰ is the average value we measured for the five bacterial membrane PLFAs (br-15:0, 15:0, 16:1, 10-Me-16, and br-17:0) at the Sapelo site (Table 3; vs. +25 ± 19 ‰ for the same PLFAs at the LCP site). In addition, we measured $\Delta^{14}C$ values of +98 ‰ of total organic carbon in *Spartina* roots, +36 and +69 ‰ for Sapelo and LCP α-hydroxy acids, and +69 and +104 ‰ for pore water DIC at the sampling sites (Tables 2 and 3). We do not have porewater $\Delta^{14}C_{DOC}$ value for our sample sites, but data from elsewhere indicate that DOC in salt marshes is modern, with $\Delta^{14}C_{DOC}$ values ranging between –4 and +110 ‰ (mean +48 ‰; Raymond and Hopkinson, 2003). Thus, we have chosen +100 ‰ as the modern endmember, yielding a f_{fossil} between 6 and 10% (Fig. 6) for the presumed bacterial PLFAs. Figure 6 also shows f_{fossil} estimates obtained with modern $\Delta^{14}C$ values of +50 ‰ and +200 ‰. Thus the overall range of f_{fossil} could be between 2 and 17 %, but probably is more of the order of 6-10%.

This calculation surely oversimplifies the situation because there are undoubtedly multiple carbon sources with a continuum of $\Delta^{14}C$ values in marsh sediments. Organic matter that has been remobilized from continental areas, either as soils or eroded rocks,

will add ^{14}C-depleted (sometimes termed "pre-aged") material to accreting marsh sediments. In this context, it is interesting to note that the hydrocarbon fraction of the Sapelo sediment has a $\Delta^{14}C$ value of − 537 ‰ indicating that it contains a significant fraction of old carbon that is not apparent from the gas chromatogram (Fig. 2). However, if carbon sources of intermediate ^{14}C content were important sources of crbon to PLFAs, a depletion in ^{14}C for the Sapelo PLFAs might also be observed, and this seems not to be the case.

Since we measured two isotopes, we also tried a ^{13}C mass balance. However, this approach proved to be less instructive because $\delta^{13}C$ values of possible carbon sources were not sufficiently different ($\delta^{13}C \sim -17$ ‰ for C_4-*Spartina*, ~ –33 ‰ for C_3-land plants, ~ –25 ‰ for C_3-marsh macrophytes and benthic diatoms, and ~ –27 ‰ for petroleum hydrocarbons at LCP) to yield meaningful a result.

Bacterial assimilation of organic matter in aquatic environments has been shown using coupled stable (C and N) isotope and radiocarbon approaches to preferentially utilize young and by inference reactive organic matter (Cherrier *et al*., 1999; Raymond and Bauer, 2001; McCallister *et al*., 2004). The $\Delta^{14}C$ values we observed in both the Sapelo and LCP sediments are consistent with this interpretation. On the other hand, depleted $\Delta^{14}C$ values for bacterial PLFAs at the LCP site indicate that where fossil carbon is present and under the appropriate environmental conditions, at least some of it can be degraded sufficiently to a labile form that can be assimilated by sediment bacteria.

The proportion of fossil carbon in bacterial PLFAs in the contaminated LCP marsh sediment (6-10%) is considerably smaller than has been observed in two other recent studies, but the conditions of those investigations are considerably different from

our study. Petsch *et al*. (2001) grew bacteria aerobically in the laboratory on macromolecular organic matter from the 365-million year old (Late Devonian) New Albany Shale as their sole carbon source. Measured $\Delta^{14}C$ values for $C_{16:0}$, $C_{18:0}$, $C_{18:1+2}$ and cyclopropyl $C_{17:0 + 19:0}$ were between and −922 and −711 ‰. Kerogen in the shale had a $\Delta^{14}C$ value !990 ‰. By isotopic mass balance, Petsch et al. estimated from 74 to 94% of the carbon in the PLFAs was derived from kerogen. In a more recent study, Pearson *et al.,* (2005) measured substantial ^{14}C depletions (−418 and −227 ‰) for PLFAs (*anteiso*-C_{15}, $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and $C_{26:0}$) isolated from sediments from the Guaymas Basin hydrothermal environment. These results indicated that the PLFAs likely originated from a complex consortium of bacteria, including mat *Beggiatoa* spp., that have transformed hydrothermal petroleum-derived carbon into biomass, with 46-100% of bacterial carbon possibly deriving from hydrothermal petroleum. The markedly lower incorporation of fossil carbon in our marsh sediment compared to these two investigations may simply reflect the fact that in the marsh, many other sources of carbon with a range of ages and presumably biological labilities are present and substantially more abundant than the fossil component. These other carbon forms surely cycle much faster and are thus incorporated more readily than their fossil counterpart.

Our conclusion that fossil carbon is being metabolized and incorporated into bacterial biomass in LCP marsh sediments we studied also contrasts with a similar study in intertidal marsh sediments on the coast of Massachusetts. Slater et al (2005) measured natural abundance ^{14}C in PLFAs in a sediment core at the site of the 1969 West Falmouth oil spill (Reddy et al., 2002; White et al., 2005), but found no evidence of incorporation of fossil carbon into PLFAs in sediments buried at depth where petroleum was present.

It was suggested that microbial metabolism of petroleum residues at depth in West Famlouth sediment might be limited by the unavailability of electron acceptors, such as sulfate, or to relatively extreme seasonal variations in temperature, both of which could slow rates of hydrocarbon degradation relative to more labile carbon sources. In the salt marsh sediments of southeast Georgia, on the other hand, temperatures are considerably higher and oscillating redox conditions in near-surface sediments (Aller 1994; Sun et al., 2002) may allow a higher rate of mixed of aerobic and anaerobic microbial metabolism of petroleum in the upper ~5 cm of tidally-flooded sediment we sampled. Thus petroleum-derived carbon is detected in the bacterial PLFAs at the LCP site.

4. SUMMARY

Our radiocarbon results indicate that petroleum in southeast Georgia marsh sediments degrades to labile metabolic products that can be assimilated by bacteria to produce biomass and ^{14}C-depleted membrane polar lipids. Incorporation of petroleum-derived carbon into bacterial biomass represents 6-10 % of the carbon uptake at present. However, since the oil has been in the LCP sediment for decades and is presumably already weathered and degraded to a certain degree, is it possible that degradation and uptake might be greater for a "fresher" petroleum substrate? Nonetheless, it is clear that a small but significant fraction of fossil carbon is assimilated by salt marsh bacteria even in the presence of a much larger pool of presumably more-labile and faster-cycling "natural" carbon substrates. Since the PLFAs most affected by incorporation of a radiocarbon "dead" fossil component are biosynthesized by a wide variety of bacteria, we can not unequivocally conclude which bacterial functional groups incorporate labile

metabolites. Nonetheless, the importance of sulfate and iron reduction in the marsh sediments coupled with PLFAs common in sulfate-reducing and iron-reducing bacteria (branched C_{15} and C_{17} acids, 10-Me-C_{16}, $C_{16:1}$ and $C_{18:1}$) showing the greatest ^{14}C-depletions in the oiled sediment relative to the control - strongly suggests that at the least these microbial groups are involved in some way.

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Table 1. Bulk composition of the Sapelo and LCP sediments.

	Sapelo	LCP
OC (%)	5.3	13.6
C/N(a)	10.2	17.4
THC (μg/g)	175	5950
THC/OC (μg/mg)	3.3	43.8
PLFA (μg/g)	20.4	41.0
PLFA/OC (μg/mg)	0.38	0.30

Table 2. Stable carbon ($\delta^{13}C$) and radiocarbon ($\Delta^{14}C$) isotope data for bulk Sapelo and LCP materials.

	$\delta^{13}C$ (‰)[a]		$\Delta^{14}C$ (‰)		μmol C [b]		NOSAMS OS number [c]	
	Sapelo	LCP	Sapelo	LCP	Sapelo	LCP	Sapelo	LCP
Sediment TOC	-18.9	-20.7	-3 ± 6	+57 ± 24	33	113	21237	21238, 26385, 26413
SLE	-23.6	-25.7	+23 ± 8	-380 ± 7	7	7	22150	22152
Hydrocarbons	-25.9	-27.4	-537 ± 8	-950 ± 2	7	12	24396, 24398, 26666	26656, 26658
Hydrocarbon-adducts	nd [d]	-27.6	nd	-968 ± 2	nd	11	nd	26643
Hydrocarbon-nonadducts	nd	-27.1	nd	-172 ± 11	nd	21	nd	26667
Spartina roots	-13.8	nd	+98 ± 5	nd	134	nd	48049	nd
DIC (tall *Spartina*)	-0.3	-3.0	+69 ± 3	+104 ± 3	97	52	32087	32085

[α] $\delta^{13}C$ (‰) values are ± 0.1 ‰

[b] μmol carbon analyzed by AMS

[c] NOSAMS assession (OS) numbers; multiple numbers indicate replicate analyses from the same extract

[d] Not determined

Table 3. Stable carbon ($\delta^{13}C$) and radiocarbon ($\Delta^{14}C$) isotope data for Sapelo and LCP PLFAs.

		$\delta^{13}C$ (‰)[b]		$\Delta^{14}C$ (‰)		µmol C [c]		NOSAMS OS number [d]	
PLFA	symbol[a]	Sapelo	LCP	Sapelo	LCP	Sapelo	LCP	Sapelo	LCP
14:0	a	-25.2	-28.6	+139 ± 17	+122 ± 25	5	5	37317	33712
br-15:0 [e]	b	-23.6	-24.8	+ 95 ± 29	+47± 6	4	18	24397, 33717, 33715	24368
15:0	c	nd [f]	nd	+ 94 ± 25	+4 ± 25	3	4	32981	33714
16:1 [e]	d	-25.4	-32.7	+122 ± 9	+7 ± 10	11	7	24385	32972
16:0	e	-25.6	-27.6	+206 ± 7	+180 ± 7	19	20	24378	23708, 47044
10-me 16:0	f	-26.9	-27.9	+101 ± 10	+36 ± 18	14	7	24380	33704
br-17:0 [e]	g	-23.6	-25.6	+93 ± 9	+31 ± 10	23	7	24377	32975
18:1 [e]	h	-27.2	-28.4	+160 ± 11	+92 ± 7	19	24	24379	24376
18:0	i	-17.5	-23.3	+180 ± 11	+261 ± 10	8	9	32971	32970
22:0	j	-23.0	nd	+88 ± 24	nd	5	nd	33711	nd
24:0	k	-22.3	-24.9	+84 ± 11	+125 ± 20	7	6	32973	33705
26:0	l	-22.7	-24.2	+43 ± 22	+75 ± 11	6	7	33710	32974
28:0	m	-22.4	nd	+12 ± 9	nd	19	nd	32953	nd
α-hydroxy	n	-23.2	-24.7	+36 ± 9	+69 ±10	12	9	32965	32969

[a] Symbols in Figs. 3, 4 and 5

[b] $\delta^{13}C$ (‰) values are ± 0.1 ‰

[c] µmol carbon analyzed by AMS

[d] NOSAMS assession (OS) numbers; multiple numbers indicate replicate analyses from the same extract

[e] Composited samples were: br-15:0 (*iso* - + *anteiso* -15:0); 16:1 (16:1ω9 + 16:1ω7); br-17:0 *(iso* - + *anteiso* -17:0 + several 17:1 isomers); 18:1 (18:1ω11 + 18:1ω9)

[f] Not determined

Figure Captions

Fig. 1. Analytical protocol for isolating hydrocarbon and PLFA fractions.

Fig. 2. Gas chromatograms of total hydrocarbons from the LCP and Sapelo sediments.

Fig. 3. Gas chromatograms of PLFAs from the LCP sediment and selected PCGC fractions.

Fig. 4. $\Delta^{14}C$ and $\delta^{13}C$ for PLFAs and α-hydroxy acids. Data and compound abbreviations are given in Table 3. The dashed lines are only viewing aids. Solid squares (■) for LCP and open circles (○) for Sapelo.

Fig. 5. Cross-plot of $\delta^{13}C$ and $\Delta^{14}C$ for PLFAs from LCP and Sapelo sediments. PLFA identifications are in Table 3 – lower case and solid squares (■) for LCP, upper case and open circles (○) for Sapelo.

Fig. 6. Calculated proportions of fossil carbon (as % fossil carbon) in bacterial PLFAs from LCP, assuming different $\Delta^{14}C$'s for modern C.

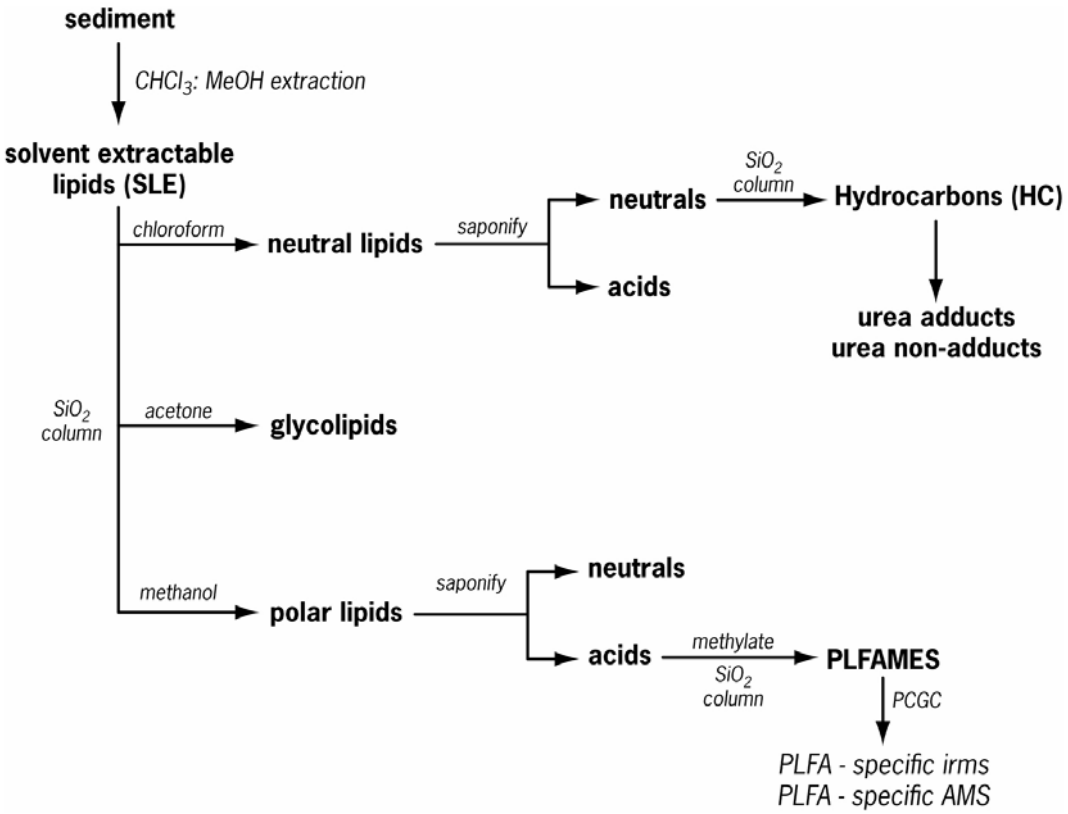
sediment
$CHCl_3$: MeOH extraction
solvent extractable lipids (SLE)
chloroform
neutral lipids
saponify
neutrals
acids
SiO_2 column
Hydrocarbons (HC)
urea adducts
urea non-adducts
SiO_2 column
acetone
glycolipids
methanol
polar lipids
saponify
neutrals
acids
methylate
SiO_2 column
PLFAMES
PCGC
PLFA - specific irms
PLFA - specific AMS

Fig 1

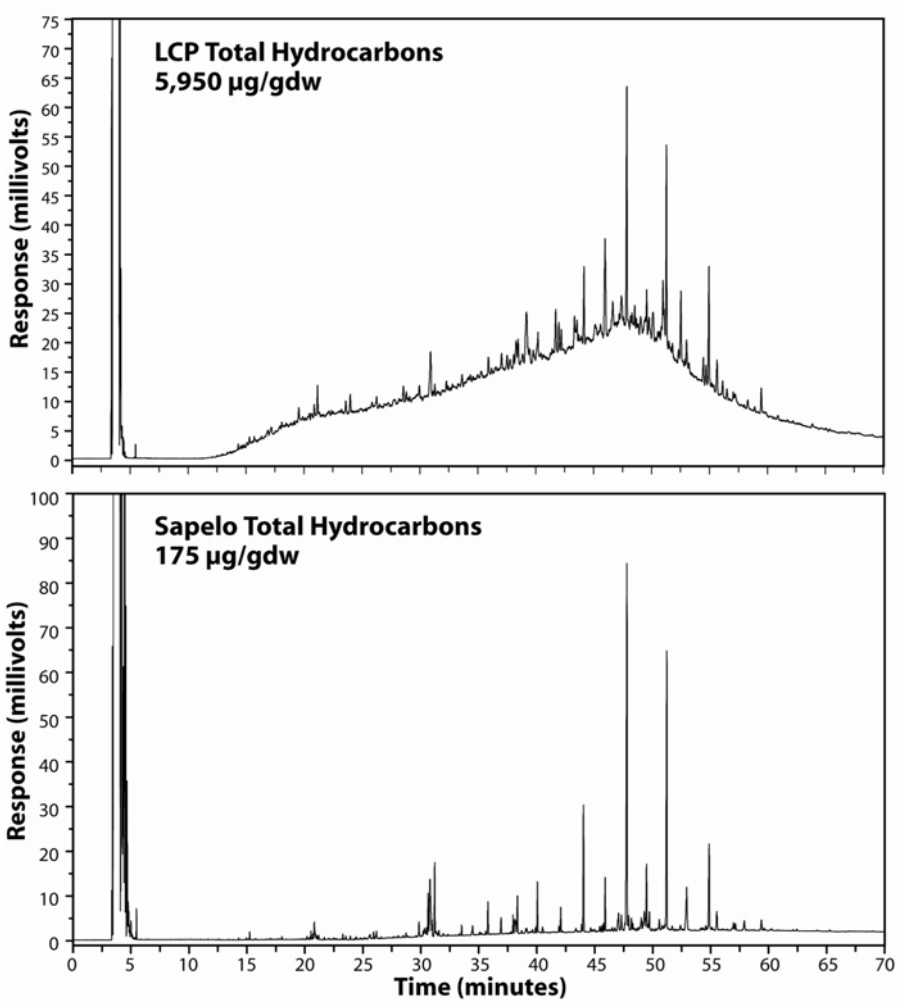
LCP Total Hydrocarbons
5,950 µg/gdw
Response (millivolts)
Sapelo Total Hydrocarbons
175 µg/gdw
Response (millivolts)
Time (minutes)

Fig 2

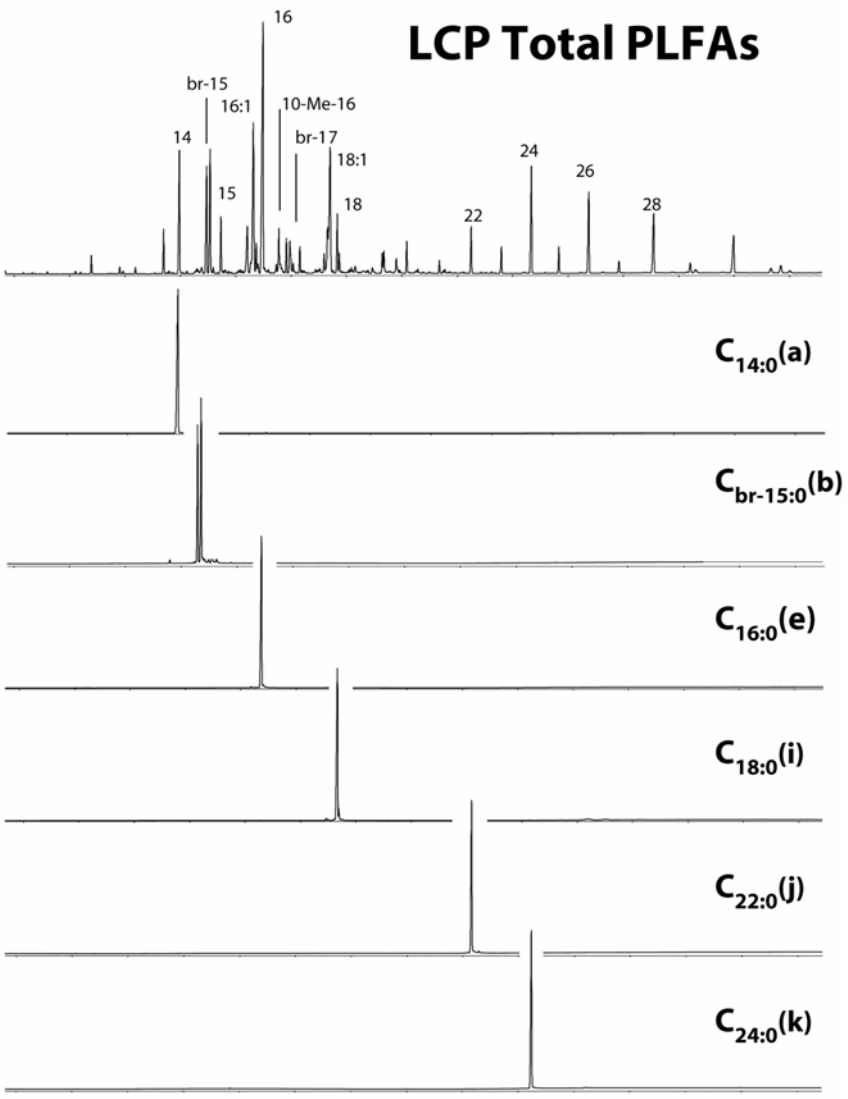
LCP Total PLFAs
16
br-15
16:1
10-Me-16
14
br-17
18:1
24
26
15
18
22
28
$C_{14:0}$(a)
$C_{br-15:0}$(b)
$C_{16:0}$(e)
$C_{18:0}$(i)
$C_{22:0}$(j)
$C_{24:0}$(k)

Fig 3

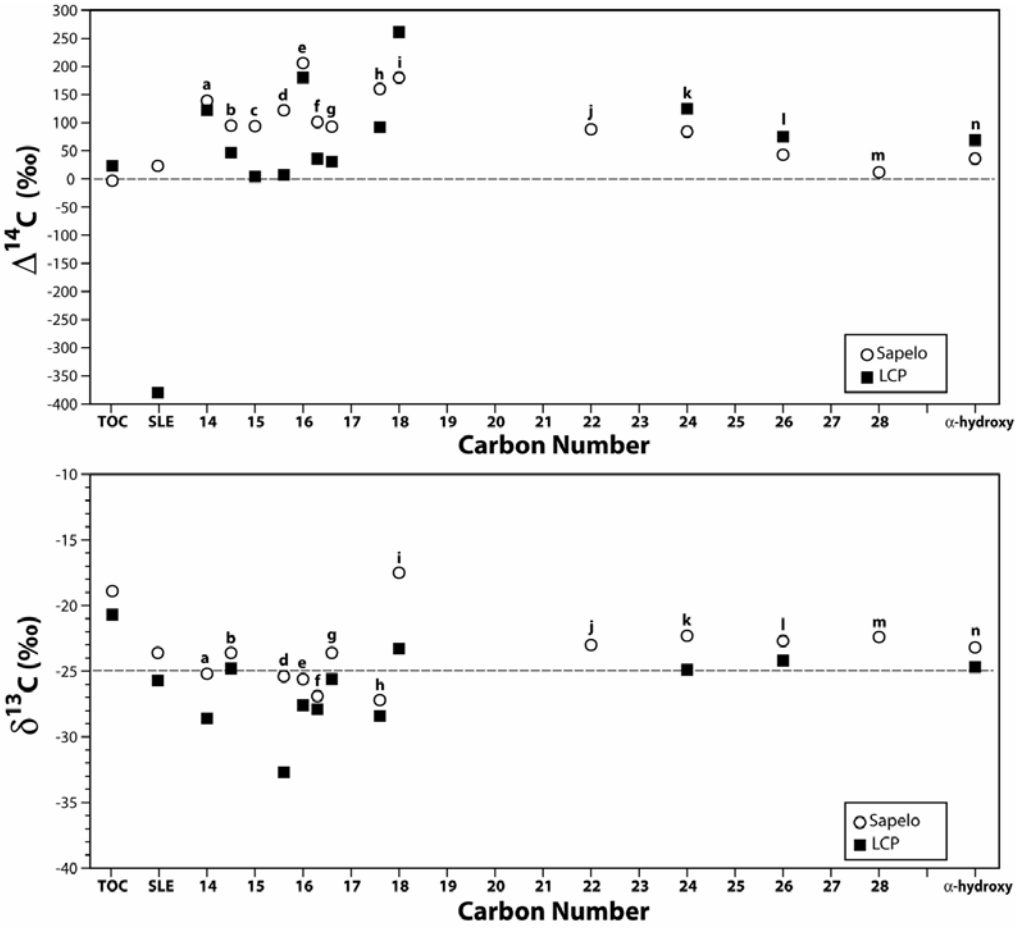

Fig 4

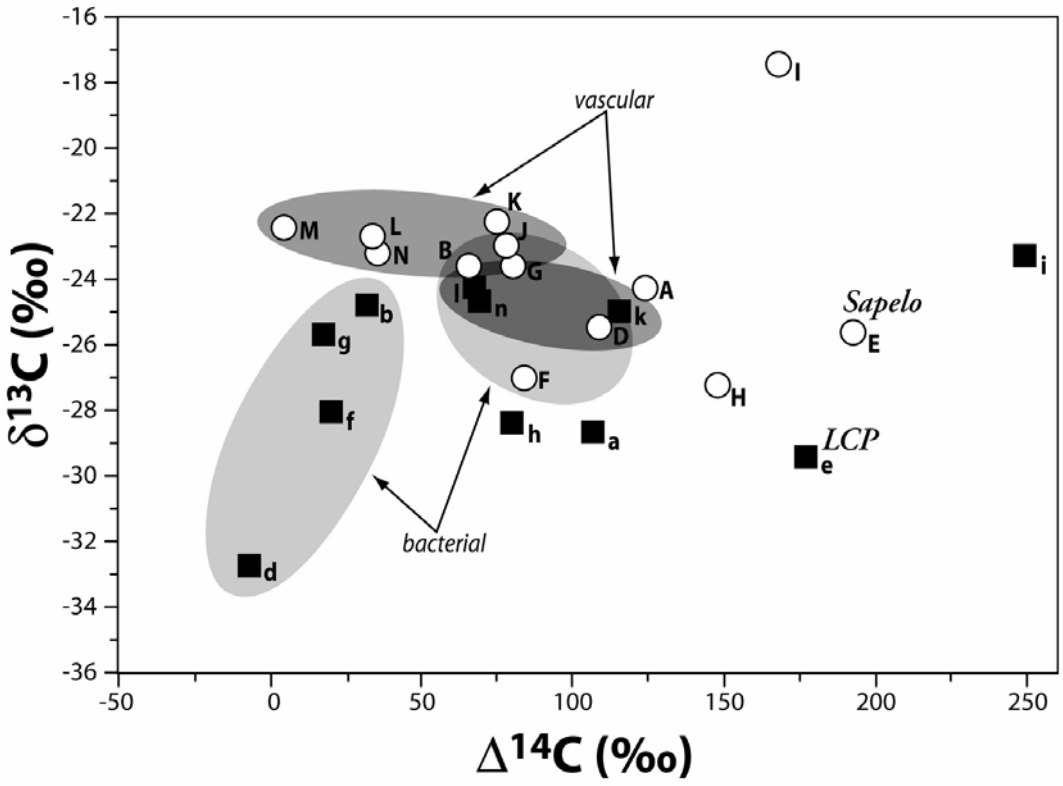
-16
-18
-20
-22
-24
-26
-28
-30
-32
-34
-36
-50
0
50
100
150
200
250
δ13C (‰)
Δ14C (‰)
vascular
bacterial
Sapelo
LCP
M
L
N
K
J
B
G
I
n
A
k
D
F
H
l
i
E
b
g
f
h
a
e
d

Fig 5

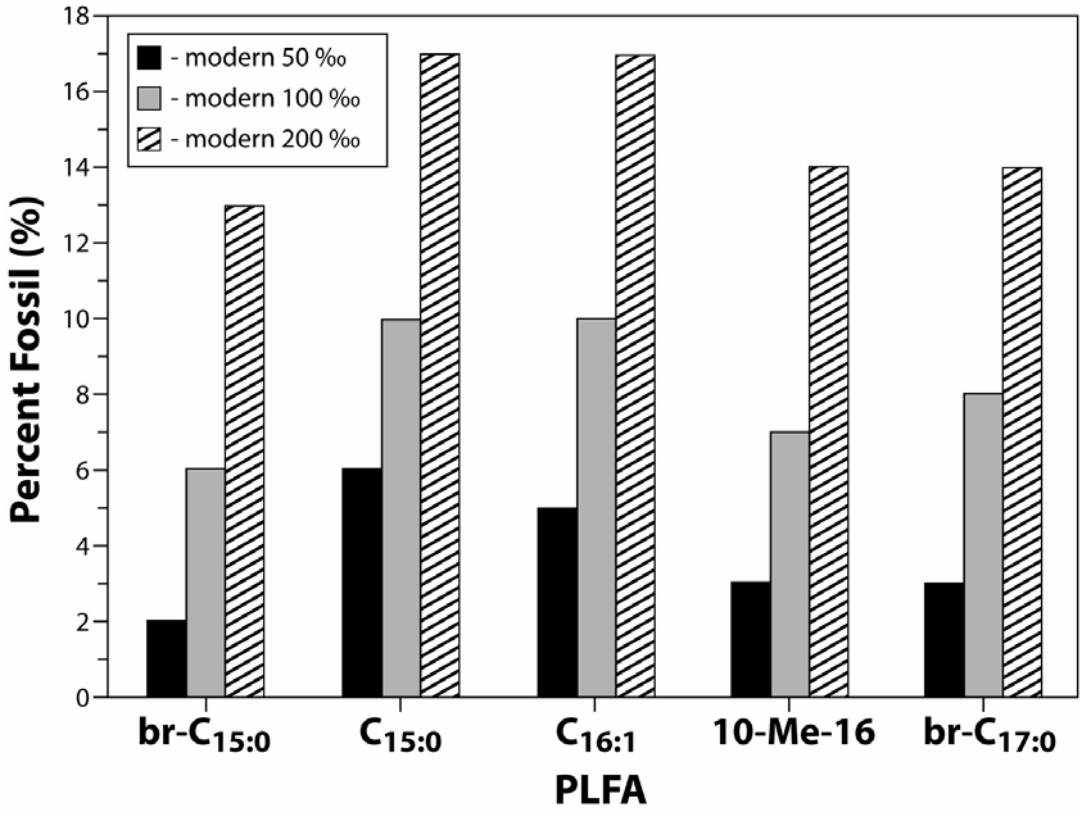
- modern 50 ‰
- modern 100 ‰
- modern 200 ‰
Percent Fossil (%)
0
2
4
6
8
10
12
14
16
18
br-C15:0
C15:0
C16:1
10-Me-16
br-C17:0
PLFA

Fig 6