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[14]C-Free Carbon Is a Major Contributor to Cellular Biomass in Geochemically Distinct Groundwater of Shallow Sedimentary Bedrock Aquifers

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Abstract Despite the global significance of the subsurface biosphere, the degree to which it depends on surface organic carbon (OC) is still poorly understood. Here, we compare stable and radiogenic carbon isotope compositions of microbial phospholipid fatty acids (PLFAs) with those of in situ potential microbial C sources to assess the major C sources for subsurface microorganisms in biogeochemical distinct shallow aquifers (Critical Zone Exploratory, Thuringia Germany). Despite the presence of younger OC, the microbes assimilated ^{14}C-free OC to varying degrees; ~31% in groundwater within the oxic zone, ~47% in an iron reduction zone, and ~70% in a sulfate reduction/anammox zone. The persistence of trace amounts of mature and partially biodegraded hydrocarbons suggested that autochthonous petroleum-derived hydrocarbons were a potential ^{14}C-free C source for heterotrophs in the oxic zone. In this zone, $\Delta^{14}C$ values of dissolved inorganic carbon ($-366 \pm 18‰$) and 11MeC16:0 ($-283 \pm 32‰$), an important component in autotrophic nitrite oxidizers, were similar enough to indicate that autotrophy is an important additional C fixation pathway. In anoxic zones, methane as an important C source was unlikely since the ^{13}C-fractionations between the PLFAs and CH_4 were inconsistent with kinetic isotope effects associated with methanotrophy. In the sulfate reduction/anammox zone, the strong ^{14}C-depletion of 10MeC16:0 ($-942 \pm 22‰$), a PLFA common in sulfate reducers, indicated that those bacteria were likely to play a critical part in ^{14}C-free sedimentary OC cycling. Results indicated that the ^{14}C-content of microbial biomass in shallow sedimentary aquifers results from complex interactions between abundance and bioavailability of naturally occurring OC, hydrogeology, and specific microbial metabolisms.

1. Introduction

In soils, organic carbon (OC) recently fixed products of photosynthesis are abundant and provide the major energy source for heterotrophs, which compose the major component of bacterial biomass (Högberg et al., 2001). In subsurface, the nature and availability of OC are likely critical for controlling microbial processes that can alter the chemical properties of the groundwater, degrade pollutants, and modify the mineral contents. For example, in shallow aquifers of Bangladesh, respiratory decomposition of surface OC anthropogenically or naturally advected to the groundwater shapes groundwater redox potential, which in turn controls the occurrence and mobility of toxic elements like arsenic (Anawar et al., 2011; Mailloux et al., 2013). In aquifers isolated from the input of labile, recent surface-derived OC, chemoautotrophic microorganisms using dissolved inorganic carbon (DIC) can make up an important fraction of the groundwater microbial community (Herrmann et al., 2015; Santoro et al., 2013). In such environments, heterotrophs can meet their metabolic demands by relying on ^{14}C-free sedimentary OC (Keller & Bacon, 1998; Mills et al., 2010; Seifert et al., 2011; Simkus et al., 2016) or by feeding on the chemoautotrophic microorganisms (Santoro et al., 2013).

The potential C sources for microbes living in groundwaters include DIC (i.e., through C fixation), dissolved OC (DOC) and methane (CH_4; Mills et al., 2010; Simkus et al., 2016). Their relative importance can be evaluated by comparing the natural abundance of [13]C ($\delta^{13}C$) and [14]C ($\Delta^{14}C$) in these sources with

microbial phospholipid-derived fatty acid (PLFAs). The $\delta^{13}C$ value of microbial PLFAs mainly depends on the $\delta^{13}C$ of assimilated C and any kinetic isotope effects associated with the C assimilation pathway (e.g., autotrophy vs. heterotrophy; van der Meer et al., 1998, 2001). However, the large variation observed in stable C isotope fractionation among microorganisms often prevents the unique identification of the C substrates. The comparison of $\Delta^{14}C$ of PLFAs with potential microbial C substrates is therefore an additional and useful tool (Mills et al., 2010; Petsch et al., 2001; Slater et al., 2005; Seifert et al., 2013; Simkus et al., 2016). As PLFAs are a major constituent of cellular membranes in active microorganisms, they are typically predominant and representative of active cells in groundwaters (Green & Scow, 2000; White et al., 1979) and thus particularly suitable for compound specific radiocarbon analysis. PLFAs common to all microorganisms (as C16:0 or C18:0) have often been used to estimate the in situ microbial biomass as they integrate the isotopic signal of the entire microbial community (Fang et al., 2004; Hurst et al., 2007). On the other hand, less abundant PLFAs that are more specific to a subset of microbes reflect the ^{14}C-content of the C source used by a phylogenetic group of bacteria. For example, the 10MeC16:0 is used as a biomarker for sulfate-reducing bacteria (SRBs; Taylor & Parkes, 1983), while the 11MeC16:0 is used for the nitrite-oxidizing bacteria (specifically *Nitrospira moscoviensis;* Lipski et al., 2001; Schwab et al., 2017). Previous carbon isotope studies in groundwaters used the $\Delta^{14}C$ of bulk PLFA or combined PLFAs (Mills et al., 2010; Simkus et al., 2016) to obtain sufficient C for radiocarbon measurements. Such results represent the average $\Delta^{14}C$ of C sources for the entire microbial community, but provide limited insights into interspecific interactions. Here, by comparing the stable and radiogenic carbon isotopes in potential microbial C sources with those of PLFAs common (e.g., C16:0) to all microorganisms, as well as PLFA specific to a subset of microbes (e.g., 10MeC16:0 or 11MeC16:0), we aimed to provide better insights into the cycling of C through microbes inhabiting the shallow subsurface.

Compound specific PLFA $\Delta^{14}C$ analyses have been reported to date for soils but not for pristine groundwaters for several reasons. First, the low biomass in groundwaters can make it very difficult and time-consuming to collect sufficient biomass for radiocarbon analyses. Sample size is a critical issue for compound specific radiocarbon analysis because extraneous C (C_{ext}) introduced by sample processing can make up a significant fraction of the analyzed C in very small samples (tens of micrograms C; Santos et al., 2010). Second, PLFA purification methods providing sufficient carbon for compound specific radiocarbon analysis take considerable effort and time, especially given the extra steps required to quantify C_{ext} introduced by processing. Most compound specific radiocarbon analysis measurements of PLFA to date have relied on preparative-gas chromatography (prep-GC) to separate lipids in sufficient purity and abundance (Eglinton et al., 1996; Eglinton et al., 1997; Kramer & Gleixner, 2006). However, this method requires multiple injections to collect enough samples for compound specific radiocarbon analysis and can result in contamination through column bleed that may require further purification steps. Here, we use a method combining large volume groundwater filtration (up to 10,000 L), PLFA purification by conventional chemical extraction, and subsequent semipreparative-high-performance liquid chromatography (prep-HPLC) to isolate microbial specific PLFAs in groundwater particulates for compound specific radiocarbon analysis. A detailed protocol of the prep-HPLC purification method as well as quantification of the C_{ext} introduced during HPLC and accelerator mass spectrometry (AMS) processing are in Schwab et al. (2019). Here, we discuss the $\Delta^{14}C$ values of varied PLFAs purified with this prep-HPLC purification method from biogeochemically distinct groundwaters and forest soils of recharge areas. We took advantage of the Hainich Critical Zone Exploratory (Hainich CZE), which provides infrastructure for sampling groundwaters with different physicochemical and lithological conditions (Kohlhepp et al., 2017), carbon isotopic compositions of DIC (Nowak et al., 2017), and microbial community structures and functions (Herrmann et al., 2015; Lazar et al., 2017; Nawaz et al., 2016; Opitz et al., 2014; Schwab et al., 2017). Based on PLFAs and 16S rRNA gene-targeted next generation sequencing, Schwab et al. (2017) showed that the microbial population in distinct aquifer assemblages of the Hainich CZE (Thuringia Germany) can be separated into three major biogeochemical zones, each characterized by specific physiological strategies or functions of the microorganisms caused by distinct surface-subsurface relationships. In this study, we used stable and radiogenic carbon isotope analyses of PLFAs and potential microbial sources to couple the diversity of microbial carbon sources to microbial functions in three wells representing these three biogeochemical zones. We aimed to better understand the heterogeneity of microbial C sources and metabolisms in relation to surface-subsurface relationships within these aquifer assemblages.
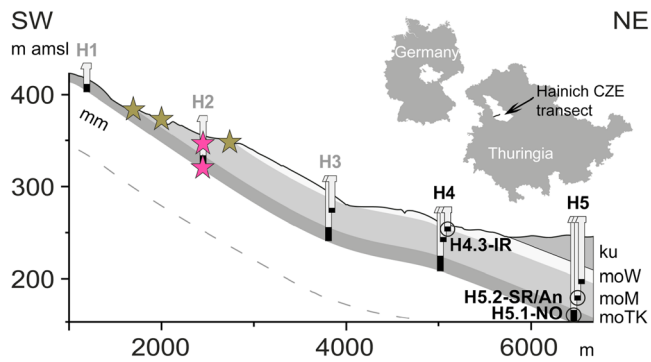
Figure 1. Schematic geological cross section of the Hainich monitoring well transect (without karst features). The wells sampled for this study are circled in black. The black colors in the wells indicate screen sections and accessed depths of the aquifer assemblages. The soil (yellow stars) and rock (pink stars) samples were collected in the groundwater recharge area. The soil samples were combined before analyses. Abbreviations: mm: Middle Muschelkalk; mo: Upper Muschelkalk; moTK: Trochitenkalk formation; moM & CB: Meissner formation with Cycloides-Bank; moW: Warburg formation; ku: Lower Keuper. Vertical exaggeration is 10 times modified from Küsel et al., 2016. CZE = Critical Zone Exploratory.

2. Site Selection and Sampling

2.1. Geological Setting and Site Selection

Located at the western margin of the Thuringian Basin (central Germany), the Hainich CZE of the Collaborative Research Centre AquaDiva includes a monitoring well transect that reaches two superimposed aquifer assemblages (Figure 1). The groundwater is hosted in fractured to karstic/fractured alternating sequences of limestones and mudstones of the Upper Muschelkalk lithostratigraphical subgroup (mo) that was deposited in an epicontinental sea during the Middle Triassic circa 240 Ma ago (Ockert & Rein, 2000). The lower limestone-dominated Trochitenkalk formation (moTK) contains one aquifer story inferred from minor confining beds (Kohlhepp et al., 2017). As used here, story represents a finely stratified geologic unit within the overall setting, which is dominated by layers of fractured limestone beds confined at the top and base by unfractured or poorly permeable mudstone layers (Kohlhepp et al., 2017). This aquifer, subsequently referred as HTL (Hainich transect lower aquifer assemblage), is used as a regional and prominent aquifer of the Upper Muschelkalk (Küsel et al., 2016). Intensively karstified limestone beds allow fast recharge with O_2-rich water from the uphill forested catchment area (Kohlhepp et al., 2017). The DIC radiocarbon values of HTL groundwaters reflect a mixture of modern CO_2 from mineralization of surface-derived OM and ^{14}C-free carbonate-C (Nowak et al., 2017). From this oxic aquifer, well H5.1 that has the largest distance to the surface recharge area (~85 m depth, lower topographic positions of the well transect on Figure 1), was sampled for compound specific radiocarbon analysis. The groundwater of this well is oxic to suboxic with averaged dissolved O_2 concentration of ~3 mg/L. Moderate concentrations of nitrate (~8 mg/L), and high concentrations of sulfate (~340 mg/L) and total sulfur (~110 mg/L) are common (Kohlhepp et al., 2017, and Figure 2). High sulfate concentration likely reflects the addition of rising sulfatic groundwaters from underlying Middle Muschelkalk formations (Kohlhepp et al., 2017). In the rest of the paper, this well is referred to as H5.1-NO to identify nitrite oxidation as a key biogeochemical process found in its groundwater (Herrmann et al., 2015; Schwab et al., 2017).

The aquifer assemblage overlaying the HTL is developed in the Meissner formation (moM). This aquifer assemblage, subsequently referred to as HTU (Hainich transect upper aquifer assemblage; Küsel et al., 2016), was further subdivided in nine partly connected or sealed aquifer stories within beds of alternating limestones and mudstones. From the HTU, we sampled wells H4.3 (aquifer story moM-8 at ~10 m depth; Kohlhepp et al., 2017) and H5.2 (aquifer story moM-3/4 at ~49 m depth) for compound specific radiocarbon analysis. Both aquifers are overlained by low permeability strata, which isolates them from the surface. Consequently, recharge surface waters originate from thick forest/agriculture soils located uphill on the aquifer transect (yellow stars in Figure 1). Recharge waters slowly percolate within the bedrock allowing for O_2 consumption, lowering of the redox potential and allowing chemical interaction with the bedrock to affect groundwater chemical composition (Kohlhepp et al., 2017; Küsel et al., 2016). Groundwaters sampled from both wells are anoxic (dissolved O_2 concentration < 0.02 mg/L = limit of detection; Figure 2) but have distinct biogeochemistry indicative of the importance of iron reduction in H4.3 (which we refer to here as H4.3-IR to emphasize iron reduction as a key biogeochemical process in this aquifer zone) and sulfate reduction/anammox metabolism in H5.2 (referred to below as H5.2-SR/An; Küsel et al., 2016; Kumar et al., 2017; Schwab et al., 2017). DIC radiocarbon values in these wells reflect not only carbonate dissolution, but also the addition of C from a ^{14}C-free source that has a ^{13}C-content consistent with a C_3 organic matter source (Nowak et al., 2017).

2.2. Groundwater, Soil, and Aquifer Host Rock Sampling

Groundwater particulate organic matter (POM) was sampled in May 2015. Prior to sampling, stagnant water was pumped from the wells, until the physicochemical parameters (temperature, pH, dissolved O_2 concentration, redox potential, and specific electrical conductivity) in pumped water reached steady state. Typically, ~9,000 L of groundwater was filtered on site using a submersible pump (SQ5-70 Grundfos,
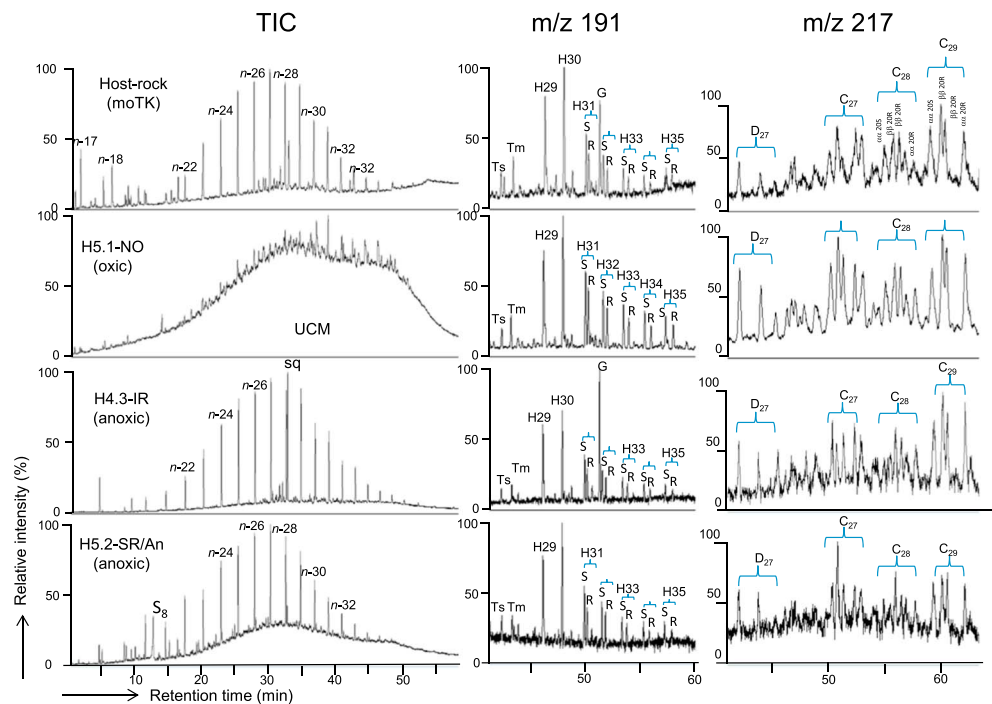
Figure 2. Total ion chromatograms (TICs) showing the *n*-alkane distributions and extracted mass chromatogram showing the hopane (m/z 191) and sterane (m/z 217) distributions, in aquifer host rock (moTK, pink star in Figure 1) and groundwaters in the different wells. The sample from the strata MoM was highly biodegraded and thus not presented here. Abbreviations: UCM, unresolved complex mixture; sq, squalene; S8, sulfur; *n*-x, x number of C in *n*-alkane; Ts, 18α(H)-22,29,30-trisnorhopane; Tm, 17α(H)-22,29,30-trisnorhopane; H29, 17α(H),21β(H)-30-norhopane; H30, 17α(H),21β(H)-hopane; brackets H31 to H35, 17α(H),21β(H) hopanes with the 22S and 22R configurations; brackets D27, diasteranes; brackets C27 to C29, regular steranes with ααS and ααR, and ββS and ββR configuration; G, gammacerane.

Denmark) connected to a stainless steel filter device (293 mm, Millipore, Merk, Germany) with a removable pre-combusted (5 hr at 500 °C) glass fiber filter (Sterlitech, USA, No. GF75293MM, diam. 293 mm) of fine porosity (0.3 μm) allowing a water flux of ~20 L/min. After filtration, the filters were wrapped in precombusted aluminum foil (5 hr at 500 °C) and stored in dry ice on site. In the laboratory, the filters were stored at −80 °C until analysis within the following month. The physicochemical parameters were monitored continuously during pumping in a flow-through cell equipped with a multimeter (Multi 3430, WTW, Xylem Analytics Germany), using probes Tetracon 925, FDO 925, Sentix 980, and ORP 900. To assess isotopic composition of surface OC sources, six topsoil samples were taken from approximately 5 to 10 cm depth below the surface in three forest sites located in the preferential recharge areas of the HTL and HTU (yellow stars in Figure 1). The soil samples were combined and sieved the same day (2.8 mm mesh size) to remove macroscopic roots, plant and animal residues, and rocks. The combined soil sample was then frozen and stored at −80 °C until analysis. Rock samples were collected from the two different strata (moTK and moM) of the two aquifer assemblages (pink stars in Figure 1).

2.3. Methane Sampling

Dissolved CH_4 and CO_2 in groundwater were extracted using a Liqui-Cel Membrane Contactor (Liqui-Cel, Membrana, Charlotte, NC, USA). Water was pumped at 4 L/min for an average of 210 min per well through a series of four particulate filters (178 μm and 40 μm stainless steel mesh, T-29595-39 and T-2959-35, Cole-Parmer, Vernon Hills, IL, USA, and 20 μm and 5 μm 5 in. polypropylene sediment depth filter, SD-25-0520 Flow-Pro and SD-25-0505 Hydronix, freshwatersystems.com, Greenville, SC, USA) and through the Liqui-Cel, where bulk dissolved gases were collected in preevacuated, preweighed 2 L stainless steel canisters with stainless steel bellows sealed valves (SS-4BG, Swagelok, Solon, Ohio, USA). Over the course of sampling each well, the steel canisters were replaced when the accumulated gas pressure reached 2.3 atm. To accumulate enough CH_4 and CO_2 for isotope analysis (≥ 0.3 mg C), three canisters of dissolved gas were filled on each of the three wells sampled. To prevent cross-contamination between well samples, all filters were replaced, and

the system was flushed with sample water for 30 min prior to sampling each new well. To inhibit microbial activity, the whole system was flushed with clean tap water for 15 min daily and stored dry.

3. Analytical Methods

3.1. Extraction and Isotope Measurement of DOC, DIC, Particulate OC, and Soil Organic Matter

Stable carbon isotope values of DIC (δ^{13}C) were measured by acidification and headspace extraction method described in Assayag et al. (2006). The extracted CO_2 was analyzed on an isotope ratio mass spectrometer (IRMS) coupled to a Gasbench II (Finnigan MAT Delta PLUS XL, Bremen, Germany) and a CTC PAL-80 autosampler. No δ^{13}C bias of the extracted CO_2 was reported for this method (Assayag et al., 2006). DIC radiocarbon (Δ^{14}C) values were determined by the method of Gao et al. (2014) and were measured by AMS at the Jena ^{14}C facility (Steinhof et al., 2004). The particulate OC (POC) were obtained by decalcifying a filter piece that was subsequently analyzed for POC δ^{13}C values using an elemental analyzer (EA; Thermo-Fisher, Bremen, Germany) interfaced to the DELTA V PLUS IRMS system via a Conflo III combustion interface (Thermo-Fisher, Bremen, Germany). The Δ^{14}C POC analyses were done at the WM Keck Carbon Cycle AMS facility at the University of California, Irvine. The combined dried soil sample was decalcified and sealed in silver precombusted (500 °C, 5 hr) capsules before δ^{13}C and Δ^{14}C analyses at the Max Planck Institute for Biogeochemistry (MPI-BGC) in Jena. The δ^{13}C values of the DOC in groundwater were measured by HPLC coupled with isotope mass spectrometry (MS) according to Scheibe et al. (2012).

3.2. Lipid Extraction

To minimize extraneous C contamination (C_{ext}), all glass (including filters) in contact with the samples during extraction and purification were baked at 500 °C for 5 hr to remove organic contaminants. PLFAs were extracted according a procedure described by Frostegård et al. (1993) and adapted to groundwater particulates by Schwab et al. (2017). Details of the PLFAs extraction procedure can be found in Schwab et al. (2019). In brief, the filters were extracted in a mixture of chloroform, methanol and water. A 5% aliquot of the Bligh-Dyer extract (BDE; Bligh and Dyer (1959)) was used for ^{14}C analyses. The rest was separated into the conventional neutral lipid, glycolipid and phospholipid fractions by liquid chromatography. The hydrocarbons (HCs) were isolated from the neutral lipids with hexane on a pre-activated silica column. After mild-alkaline hydrolysis and methylation of the phospholipid fraction (White et al., 1979), a 5% aliquot of the unsubstituted ester-linked PLFAs (EL-FAMEs) was used for compound identification and quantification by standard GC-MS and δ^{13}C analysis by GC combustion IRMS. The rest of the EL-FAMEs were eluted through an Ag-impregnated column to separate the saturated PLFAs from the unsaturated PLFAs. The saturated PLFAs were then purified individually by prep-HPLC for subsequent compound specific radiocarbon isotope analysis.

The HCs were extracted from ~2 kg of powdered rocks using soxhlet extraction with a mixture of DCM/MeOH (9/1). The HCs of the total lipids extract were then purified on a preactivated silica column. HCs were identified and quantified by GC-MS.

3.3. GC-MS

The PLFAs and the HCs were identified and quantified using a gas chromatograph (GC) coupled to a triple quadrupole mass spectrometer (TSQ-8000, Thermo-Fisher, Bremen, Germany). The GC was equipped with a TG-5silms capillary column (30 m, 0.25 mm, 0.25 μm film thickness). Helium was used as carrier gas at 1.2 ml/min. The PTV injector was operated in splitless mode at an initial temperature of 70 °C. Upon injection, the injector was heated to 280 °C at a rate of 14.5 °C/s, held at this temperature for 2 min, and then heated to 320 °C that was held for 5 min. For the PLFAs analysis, the GC oven was programmed to hold an initial temperature of 70 °C for 1 min, then heated at 2 °C/min until 250 °C, followed by 50 °C/min until 320 °C that was hold for 5 min. For the *n*-alkanes, the GC oven was programmed to hold an initial temperature of 80 °C for 1 min, then heated at 5 °C/min until 300 °C, followed by 50 °C/min until 320 °C, where the temperature was held for 5 min.

The *n*-alkanes in the HCs fraction were quantified using a standard mixture (*n*-C_{12} to *n*-C_{36}) measured in five different concentrations between 5 and 40 ng/μl. FAMEs were quantified either relative to an internal

standard (nonadecanoic acid-methyl ester, 19:0) added prior to GC analysis or relative to a standard mixture (Fame Mix, 37 components Supelco +10MeC16:0 and 10MeC18:0, LGC Standards GmbH, Germany) measured in five different concentrations between 5 and 40 ng/µl. The PLFAs were assigned by comparison with standards, published mass spectra (Lipski et al., 2001; Sinninghe Damsté et al., 2005) and from relative retention times. Positional isomers of monounsaturated FAMEs were determined by their dimethyl disulfide derivatives according the method of Dunkelblum et al. (1985). Standard nomenclature is used to describe PLFAs. The number before the colon refers to the total number of C atoms; the number(s) following the colon refers to the number of double bonds and their location (after the "ω") in the fatty acid molecule. The prefixes "Me," "cy," "i," and "a" refer to the methyl group, cyclopropane groups, and isobranched and anteiso-branched fatty acids, respectively.

3.4. Compound-Specific Stable Carbon Isotope Measurements

The stable C isotope composition of pre-purified PLFAs was measured using a GC combustion IRMS system (Deltaplus XL, Finnigan MAT, Bremen, Germany) at the MPI-BGC. Analyses were performed on the 5% aliquot. The GC (HP5890 GC, Agilent Technologies, Palo Alto USA) was equipped with a DB1-ms column (60 m, 0.32 mm ID, 0.25 µm film thickness, Agilent). The injector at 280 °C was operated in splitless mode with a constant flow rate of 1.2 ml/min. The oven temperature was maintained for 1 min at 70 °C, heated at 5 °C/min to 300 °C and held 15 min and then heated at 30 °C/min to 330 °C and held 3 min. Isotope values, expressed in the delta notation (‰), were calculated with ISODAT version software relative to the reference CO_2. Drift correction factor was determined using a reference mixture of FAMEs (C13:0 to C22:0) injected three times before and after two samples (six injections). The carbon isotopic composition of the reference FAMEs was determined off-line using a thermal conversion elemental analyzer (TC/EA; Thermo-Fisher, Bremen, Germany) interfaced to the DELTA V PLUS IRMS system via a Conflo III combustion interface (Thermo-Fisher, Bremen, Germany; Werner & Brand, 2001).

The contribution of the methyl carbon derived from the methanol in the FAME was removed by isotopic mass balance, with

$$\delta^{13}C_{fame} = \left[(N+1)\,\delta^{13}C_{measured} - \delta^{13}C_{MeOH}\right]/N$$

where N is the number of C atoms in the original fatty acid prior to esterification (Wakeham et al., 2006) and the $\delta^{13}C_{measured}$ is the stable carbon isotopic composition of the PLFA, respectively. The $\delta^{13}C_{MeOH}$ was -31.13 ± 0.030‰.

3.5. Compound-Purification by Prep-HPLC and Compound Preparation for $\Delta^{14}C$ Analyses

Details on the prep-HPLC purification method as well as method recovery and quantification of C_{ext} introduced during processing are in Schwab et al. (2019). Briefly, the saturated PLFAs were purified individually by 1200 series prep-HPLC system (Agilent Technologies, Waldbronn, Germany) on a C_{18} HPLC column (5 µm; 150 × 4.6 mm; EVO Phenomenex) with a preprogrammed of mixture of MeOH/water and acetonitrile. The different PLFAs were collected based on elution time using a fraction collector maintained at 10 °C. The elution times of the target PLFAs were determined previously from multiple injections with standards. A 1% aliquot of the prep-HPLC purified compound was run on GC-MS to test purity and recovery. The remaining was ultimately transferred into quartz reaction tubes. After solvent evaporation with N_2, cupric oxide wire was added and the tubes were evacuated and sealed on a vacuum line and the PLFAs were combusted as described in Kramer and Gleixner (2008). The evolved CO_2 was subsequently purified cryogenically on a vacuum extraction line, quantified, and sent to the WM Keck Carbon Cycle AMS facility at the University of California, Irvine, for graphitization and AMS measurement. The contribution of the methyl carbon derived from the methanol in the FAME was removed by isotopic mass balance

$$\Delta^{14}C_{fame} = \left[(N+1)\,\Delta^{14}C_{measured} - \Delta^{14}C_{MeOH}\right]/N$$

where N is the number of C atoms in the original fatty acid prior to esterification (Wakeham et al., 2006) and the $\Delta^{14}C_{measured}$ is the radiogenic carbon isotopic composition of the PLFA. The $\Delta^{14}C$ of MeOH used for fatty acids saponification was -997 ± 0‰.

3.6. Preparation and Measurement of Dissolved CH_4 and CO_2 for ^{14}C

Filled steel canisters were weighed to quantify the mass yield of dissolved gases collected. The canisters were then connected, one at a time, to a flow-through vacuum extraction line designed to sequentially extract CH_4 and CO_2 for ^{14}C and ^{13}C analysis (Pack et al., 2015). As sample gas is introduced to the vacuum line, (1) CO_2 is frozen out in a liquid nitrogen trap, (2) carbon monoxide is converted to CO_2 at 290 °C and frozen out in a second liquid nitrogen trap, and (3) CH_4 is oxidized to CO_2 in a 950 °C furnace with cupric oxide pellets. CH_4-derived CO_2 and sample CO_2 are purified on the vacuum line and yields are quantified manometrically. Triplicate samples from each sampled well, isolated separately, were eventually combined into one sample on the vacuum line and converted to graphite using the sealed-tube zinc reduction method (Xu et al., 2007).

Radiocarbon standards (modern biogas and ^{14}C-free gas) were extracted using the same methods to evaluate the procedural backgrounds of the sampling methods (Liqui-Cel), vacuum extraction, and graphitization. Consistent modern and dead $^{14}CH_4$ blanks (2.8 ± 0.9 and 2.0 ± 1.0 µg C, respectively) were determined for the Liqui-Cel system and for the vacuum extraction and graphitization processes. Modern and dead $^{14}CO_2$ blanks were determined as 11 ± 3 and 22 ± 5 µg C, respectively. These blanks are likely overestimated because the circulating setup of the laboratory experiment was difficult to keep airtight. Although $^{14}CO_2$ blanks were somewhat larger than those of CH_4, we assume their effects were negligible since total CO_2 yields in real samples were very large (50–70 mg C, or roughly 3,000 times larger than typical blank sizes). The observation that $^{14}CO_2$ data produced by the Liqui-Cel system agreed within the analytical error of ^{14}DIC, measured independently, validates this assumption. All ^{14}C data were mass corrected for sampling and processing blank effects where applicable. Alongside procedural and analytical standards, the graphite was measured for its $^{14}C/^{12}C$ ratio with AMS at the W.M. Keck Carbon Cycle Accelerator Mass Spectrometer facility at UC Irvine. Aliquots of the CH_4-derived CO_2 or the sample CO_2 were analyzed for $\delta^{13}C$ with IRMS (GasBench II coupled to Delta plus IRMS, Thermo-Fisher Scientific [Finnigan]).

Isotope fractionation is negligible for the laboratory vacuum extraction. However, based on independent tests, we determined the field Liqui-Cel extraction to impart a slight bias toward lighter isotopes, resulting in fractionations of <1‰ and < 1.5‰ for dissolved $\delta^{13}CH_4$ and $\delta^{13}CO_2$ samples, respectively. This has a small effect on the reported $\delta^{13}CH_4$ and $\delta^{13}CO_2$ values but is considered negligible for the conclusions of this work. Furthermore, any ^{13}C-fractionation imparted by the extraction system has no effect on ^{14}C values since all ^{14}C data is normalized to $\delta^{13}C = -25‰$ during AMS analysis by definition (Stuiver & Polach, 1977). The isotope separation between $\delta^{13}CO_2$ values produced from the Liqui-Cel and $\delta^{13}C$-DIC measured independently (~10‰) is comparable to expected and previously observed values produced from other Liqui-Cel membranes (Yokochi et al., 2018). This also implies a minimal fractionation effect (< 2‰) on $^{13}CO_2$ through the Liqui-Cel.

4. Results

4.1. HCs in Groundwaters and Aquifer Host Rocks

Because HCs in sedimentary aquifers constitute a potential C source for the microorganisms, we also assessed the nature of the HCs in groundwaters and host rocks of the aquifer (Figure 2). In those samples, the *n*-alkanes were observed over the range C_{18} to C_{35} and increased in abundance from C_{22} to C_{35}, with the C_{28-29} as prominent peak. The concentration of *n*-alkanes was in the ppm range in aquifer host rocks and in the parts per billion range in the groundwaters. The steranes (mass chromatograms of m/z 217) were dominated by the C_{29}-steranes and to a lesser extent by the C_{27}-steranes. The C_{27}-diasteranes were in lower concentrations. The triterpanes (mass chromatograms of m/z 191) showed high occurrence of αβ-hopanes from C_{30} to C_{35} with C_{30} as prominent peak, gammacerane, and extended hopanes (C_{31} to C_{35}). Since some of those compounds are derived from specific organisms and/or formed during specific physicochemical processes, they can be associated with particular biological sources, depositional conditions (pH, redox), or thermal maturation. Gammacerane is a known indicator of high salinity. High amounts of extended hopanes (C_{31} to C_{35}) and low diasteranes/steranes are often indicative of carbonates and evaporites as HC source rocks (Peters et al., 1986). The C_{27} and C_{29} steranes are abundant in planktons, algae, and higher plants (Volkman, 1986) and thus have been associated with shallow-water depositional environments (epicontinemtal sea) with both marine and terrestrial organic matter contributions. Alternatively, the
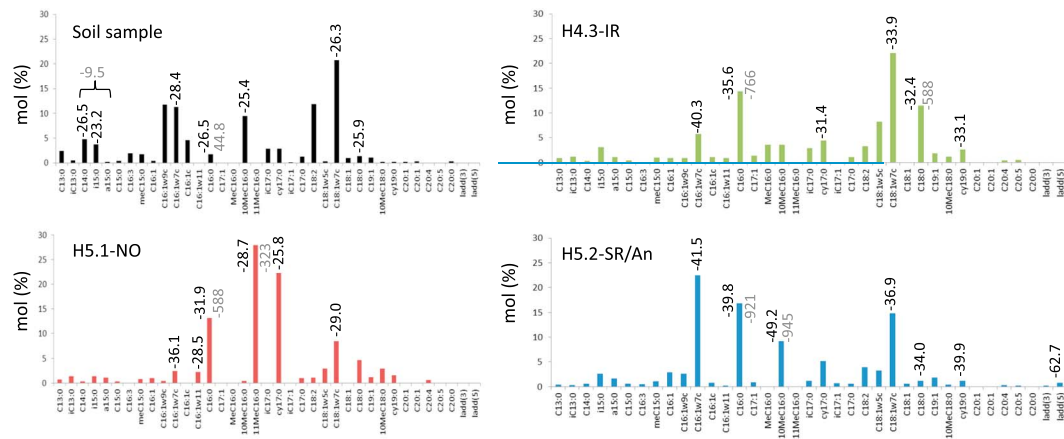
Groundwater physicochemistry in the different wells at the time of sampling

well name	biogeochemical zones[1]	depth (m)	water temperature [°C]	PH [-]	O_2 [mg L^{-1}]	NO_2^- [mg L^{-1}]	NH_4^+ [mg L^{-1}]	NO_3^- [mg L^{-1}]	SO_4^{2-} [mg L^{-1}]	TOC [mg L^{-1}]	Fe^{3++2+} [mg L^{-1}]	Mn_t [mg L^{-1}]	S_t [mg L^{-1}]
H5.1-NO	nitrite-oxidizing	84-88	11.4	7.3	3.1	0.05	0.0	7.7	337.2	3.3	0.006	0.004	109.6
H4.3-IR	iron-reducing	8.5-12.5	10.0	7.3	0.0	0.02	0.1	0.3	38.7	3.4	0.044	0.040	13.2
H5.2-SR/An	sulfate reducing/anammox	47-50	10.1	7.4	0.0	0.01	0.6	0.8	95.6	4.0	0.035	0.005	32.3

[1] Based on Schwab et al. (2017); Mn_t and S_t referred to total manganese and sulfur

Figure 3. Plot of the PLFA distributions (relative to the C19:0 internal standard) in combined soil sample and groundwater samples of the different zones. Samples are presented from the top to the bottom according to the decrease in the redox potential of the environment (Schwab et al., 2017). Major geochemical characteristics of the groundwater zones at the time of sampling are given in the table. The groundwater of the well H5.1-NO is oxic to suboxic, with nitrite oxidation (NO) identified as a dominant process. Note the abundance of 11MeC16:0 used as marker of autotrophic nitrite oxidizer *Nitrospira moscoviensis*. The groundwaters of the well H4.3-IR (iron reduction zone) and H5.2-SR/An (sulfate reduction/anammox) are anaerobic. In H5.2-SR/An, note the occurrence of [3]- and [5]-ladderane that are indicative of the presence of anammox. Consistent with a decrease of the redox potential, the increase of the relative abundance of the C16:0, C18:0, C16:1ω7c, C18:1ω7c, and 10MeC16:0 PLFAs from the well H51-NO to H52-SR/An suggested an increasing abundance of sulfur bacteria. The stable carbon isotope composition (δ^{13}C; ‰ vs. Vienna PeeDee Belemnite, VPDB) and radiogenic carbon content (Δ^{14}C) of important PLFAs are noted in black and gray, respectively. PLFA = phospholipid fatty acid.

predominance of Tm (17α(H)-22,29,30-trisnorhopane) over Ts (18α(H)-22,29,30-trisnorhopane; Ts/Ts + Tm ratio from 0.67 and 0.69) and the 22S/22S + 22R homohopane ratios of ~0.5 indicated organic matter transformation within the oil and gas maturity window (Peters et al., 2005).

In those samples, mature stereochemical distributions, low diasteranes concentration, low H_{29}/H_{30} hopane ratios, and similar *n*-alkane, hopane, and sterane distributions suggested similar source rock origins. Therefore, differences in HC molecular composition between samples likely reflected secondary processes, such as biodegradation or weathering. The samples of the aquifer host rocks revealed two distinct chromatograms of the HC fractions. The sample from the moM formation included a very large unresolved complex mixture (UCM) of branched and cyclic compounds relative to the *n*-alkanes, whereas the sample from moTK had a bimodal *n*-alkane distribution maximized at C_{17} and C_{29} with relative low UCM. Only this latter sample is shown in Figure 2. In both rock samples, the hopanes and steranes did not show apparent biodegradation. According to the biodegradation scale of Peters et al. (1986), the HCs in aquifer host rocks can be associated to moderately biodegraded oil. High UCM in oxic well H5.1-NO can be related to moderately biodegraded oil, whereas low UCM in the anoxic wells indicated slightly to nonbiodegraded oil (Figure 2). In all groundwater samples, the hopanes and steranes also did not show apparent biodegradation.

4.2. Distribution and Abundance of PLFAs in Groundwater and Soil

In surface soil sample and groundwater POM, 27 PLFAs were quantified (Table S1 in the supporting information) and 37 were identified (Table S2). They were considerable differences in PLFA distribution and concentration patterns across the sampling sites (Figure 3). The overall concentration of PLFA in surface soil was ~ 2.5 µg/g of dried soil. In groundwater, PLFA concentration was 2 orders of magnitude smaller,

ranging from ~ 30 ng/L of groundwater in wells H5.1-NO and H4.3-IR to ~ 60 ng/L of groundwater in well H5.2-SR/An. In soil, the major PLFAs were C16:1ω7c (11%), C16:1ω9c (11%), 10MeC16:0 (9%), C18:2 (12%), and C18:1ω7c (20%). In groundwaters, the major PLFAs were C16:0 (13%), 11MeC16:0 (27%), and cy17:0 (22%) in H5.1-NO; C16:0 (14%), C18:1ω7c (22%), and C18:0 (11%) in H4.3-IR; and C16:1ω7c (22%), C16:0 (17%), 10MeC16:0 (9%), and C18:1ω7c (15%) in H5.2-SR/An. The PLFA distributions showed three significant trends that can be related to decreasing redox potential and physiological adaptations of the microorganisms present in those groundwaters (Schwab et al., 2017).

1. An increase in the combined relative abundance of the C16:0, C18:0, C16:1ω7c, C18:1ω7c, and 10MeC16:0 PLFAs from 24% in H5.1-NO to 43% and 63% in H4.3-IR and H5.2-SR/An, respectively. This change could be related to an increasing occurrence of putative sulfate-reducing and sulfur-oxidizing bacteria consistent with the decreasing of redox potential of the groundwaters (Schwab et al., 2017). The 10MeC16:0 is produced in large proportions by SRB, as *Desulfobacter* (Dowling et al., 1986; Vainshtein et al., 1992). The predominance of C16:0, C16:1ω7c, and C18:1ω7c in sulfur-rich environments has been attributed to sulfide-oxidizing bacteria (Li et al., 2007).
2. A decrease in the relative abundance of the cy17:0 and 11MeC16:0. The later PLFA was only observed in the oxic well H5.1-NO and attributed to nitrite-oxidizing bacteria as *Nitrospira moscoviensis* (Schwab et al., 2017).
3. The presence in H5.2-SR/An of the PLFAs iC17:1, common in some SRB (Dowling et al., 1986; Vainshtein et al., 1992) and also of the [3]- and [5]-ladderane specific for the presence of anammox (Sinninghe Damsté et al., 2005).

4.3. Carbon Isotopes of the Microbial Sources: Dissolved Species, POC, Methane, and Total Extracted Lipids

The δ^{13}C value of POC in the forest soil was −26.5‰ (Table 1 and Figure 4). In groundwaters, POC was more ^{13}C-depleted, by ~2‰ in H5.1-NO and H5.2-SR/An and by ~7‰ in H4.3-IR. The δ^{13}C values of DIC (Table 1) were very similar in H5.1-NO (−11.7‰) and H4.3-IR (−11.2‰), but 2.4‰ more ^{13}C-depleted in H5.2-SR/An. The δ^{13}C values of methane were similar in H5.2-SR/An (−42.4‰) and H5.3 (−41.8‰) but were 2‰ more ^{13}C-enriched in H4.3-IR. The ^{14}C-content of DIC decreased from H5.1-NO (Δ^{14}C value = −366‰) to H5.2-SR/An (Δ^{14}C value = −721‰) over H4.3-IR (Δ^{14}C value = −532‰). The POC Δ^{14}C values showed the same trend as the DIC Δ^{14}C values, with the highest ^{14}C-content in H5.1-NO (Δ^{14}C value = −256‰), intermediate ^{14}C-content in H4.3-IR (Δ^{14}C value = −588‰), and lowest ^{14}C-content in H5.2-SR/An (Δ^{14}C value = −648‰). The POC of the surface soil had the highest ^{14}C-content (Δ^{14}C value = +26‰). The solvent extractable lipids (BDE) from the filters were significantly ^{14}C-depleted relative to both DIC and POC, but displayed the same trend, with higher ^{14}C-content in H5.1-NO (Δ^{14}C value = −793‰) and lower in H5.2-SR/An (Δ^{14}C value = −929‰). The Δ^{14}C values of methane in H4.3-IR, H5.2-SR/An and H5.3 were − 596‰, −973‰ and − 955‰, respectively. We assume the radiocarbon content of the rock and aquifer HC C sources is zero, that is, Δ^{14}C of −1000‰.

4.4. Carbon Isotopes in PLFAs in Groundwater POM and Soil

The δ^{13}C values of the dominant PLFAs ranged from −23.2‰ to −64.4‰ (Table 1 and Figure 4 and supporting information Table S3). On average the PLFAs isolated from groundwaters were more ^{13}C-depleted and depletion increased as redox potential decreased: forest soil sample (mean $\delta^{13}C_{PLFA}$ = −26.7‰), H5.1-NO (mean $\delta^{13}C_{PLFA}$ = −30.0‰), H4.3-IR (mean $\delta^{13}C_{PLFA}$ = −35.7‰) and H5.2-SR/An (mean $\delta^{13}C_{PLFA}$ = −43.7). The ^{13}C value of C16:0 followed a similar pattern (Table 1): forest soil, −26.5‰; H5.1-NO, −31.9‰; H4.3-IR, −35.6‰ and H5.2-SR/An, −39.8‰. The 10MeC16:0 showed the strongest decline in ^{13}C with the redox conditions from −25.4‰ in forest soil to −49.2‰ in H5.2-SR/An. In H5.1-NO, the δ^{13}C value of 11MeC16:0 was of −28.7‰. The most ^{13}C-depleted PLFA were the [3]- and [5]- ladderane (δ^{13}C value up to −64.4‰) in well H5.2-SR/An.

In surface soil, the PLFAs were the most ^{14}C-enriched (mean Δ^{14}C value = +60‰; Table 1 and Figure 4). In groundwater, all PLFAs measured were more ^{14}C-depleted and, as for the DIC and POC, the averaged PLFA ^{14}C-content decreased as follows: H5.1-NO (mean PLFAs Δ^{14}C value = −423‰), H4.3-IR (mean PLFAs Δ^{14}C value = −664‰), and H5.2-SR/An (mean PLFAs Δ^{14}C value = −929‰).

Table 1
$\delta^{13}C$ and $\Delta_{14}C$ Values of the Potential C sources (DIC, DOC, POC, BDE, CO_2 and CH_4) and PLFAs in the Different Wells Studied

PLFA-based redox zone[a]	Site/sample type	Potential C sources $\delta^{13}C$ (‰)										Potential C sources $\Delta_{14}C$ (‰)								
		DIC[b]	sd	DOC[b]	sd	POC	sd	CH_4	sd	CO_2	sd	DIC[c]	POC	±	BDE	±	CH_4	±	CO_2	±
	Forest soil					−26.5	1						26	26						
oxic-nitrite oxidizers	H5.1-NO	−11.7		−25.4		−28.4	1					−366	−256	3	−793	1.1	−596	3	−539	1
anoxic-iron reducers	H4.3-IR	−11		−25.1		−33.8	2	−39.8	2	−19.6	2	−532	−588	17	−905	0.9	−973	3.6	−751	1
anoxic-sulfate reducers/annamox	H5.2-SR/An	−9.1		−23.7		−28.4	3	−42.4	2	−18	2	−721	−648	2	−929	0.7	−955	3.6	−795	1
anoxic-sulfate reducers/annamox	H5.3-SR/An							−41.8	2	−18.5	2									

PLFA-based redox zone[a]	site/sample type	PLFA $\delta^{13}C$ (‰)										PLFA $\Delta_{14}C$ (‰)								
		C16:0	sd	10Me C16:0	sd	11Me C16:0	sd	a15:0/ i15:0	sd	C18:0	sd	C16:0	±	10Me C16:0	±	11Me C16:0	±	a15:0/ i15:0	±	C18:0	±
	Forest soil	−27	1									110	11								
oxic-nitrite oxidizers	H5.1-NO	−32	1	−25.4	1	−28.7	1	−23.2	2	−25.9	1	−562	9	−8	22	−283	32	77	12		
anoxic-iron reducers	H4.3-IR	−36	1	−45.1	1					−32.36	0	−753	41								
anoxic-sulfate reducers/annamox	H5.2-SR/An	−40	1	−49.2	0			−44.6	1	−33.97	1	−916	20	−942	22					−576	46

Note. DIC: dissolved inorganic carbon; DOC: dissolved organic carbon; POC: particulates organic carbon, BDE: Bligh-Dyer extract. [a]Based on Schwab et al. (2017). [b]Standard better than 0.2‰. [c]Standard better than 5‰. sd: Standard deviation n is in Table S3.
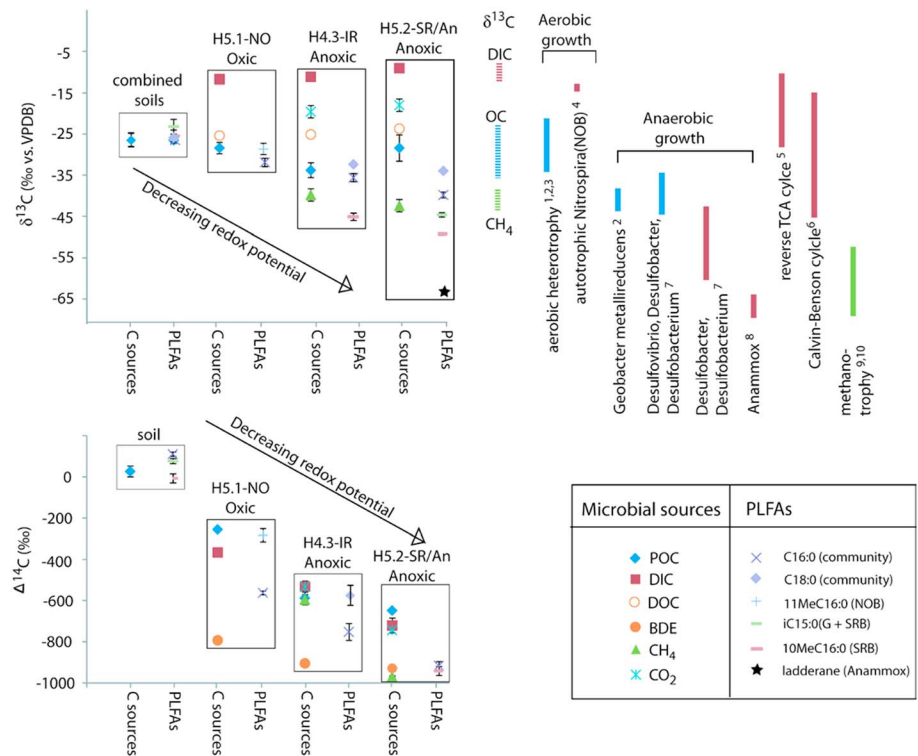
Figure 4. Plots of the stable carbon isotope composition (δ^{13}C; ‰ vs. Vienna PeeDee Belemnite, VPDB) and radiogenic carbon content (Δ^{14}C) of PLFAs and potential microbial C sources (DIC, POC, DOC, BDE, CH$_4$, and CO$_2$) in combined soil and groundwater samples (H5.1-NO, H4.3-IR, and H5.2-SR/An). The dashed colored bars show the range of measured δ^{13}C values of the potential microbial C sources (OC, DIC, and CH$_4$) in the sampled groundwaters. The solid colored bars show the range of PLFA δ^{13}C values expected to result from different microbes and metabolism pathways on these C sources. The C16:0 and C18:0 PLFAs are representative for the overall isotopic composition of the microbial community. The 11MeC16:0, 10MeC16:0, and ladderane are markers of autotrophic nitrite oxidizers (NOB), sulfate reducers (SRB), and anammox, respectively, as supported by their isotopic compositions. The Δ^{14}C and δ^{13}C values decreasing from soil to the sulfate reduction/anammox zone (H5.2-SR/An) demonstrated lower exchange between the surface and the groundwaters, resulting in decreasing input of recent C and increasing heterotrophy on ^{14}C-free sedimentary organic C. [1]Abraham et al. (1998), [2]Teece et al. (1999), [3]Pancost and Sinninghe Damsté (2003), [4]Lücker et al. (2010), [5]van der Meer et al. (1998), [6]Preuß et al. (1989), [7]Zhang et al. (2003), [8]Schouten et al. (2004), [9]Hayes (2001), [10]Jahnke et al. (1999). PLFA = phospholipid fatty acid; OC = organic carbon; DIC = dissolved inorganic carbon; POC = particulate OC; DOC = dissolved OC; BDE = Bligh-Dyer extract.

5. Discussion

5.1. Microbial Carbon Cycling by Subsurface Microorganisms

Soil PLFA had Δ^{14}C values reflecting the influence of contemporary atmospheric CO$_2$ (Graven, 2015; Levin et al., 2013) and recently fixed plant organic C (Seifert et al., 2013), indicating soil microorganisms rely mostly on C sources fixed from the atmosphere in the last years to decades. The soil PLFA δ^{13}C values (mean = −26.1‰) were typical for those reported in soil dominated by C$_3$ vegetation (Kramer & Gleixner, 2006) and consistent with PLFAs derived mainly from aerobic heterotrophs with $\Delta\delta^{13}$C$_{POC-PLFA}$ value between of about −7 to −6‰ (Abraham et al., 1998; Pancost & Sinninghe Damsté, 2003; Teece et al., 1999).

The three wells we sampled represent groundwaters from three distinct biogeochemical zones identified within the Hainich CZE by Schwab et al. (2017). One of these (H5.1-NO; zone 1) is oxic, while the other two (H4.3-IR and H5.2-SR/An) are anoxic, even though shallower than H5.1-NO (Figure 1). Well H4.3-IR (zone 2) has indications of Fe-cycling, while in H5.2-SR/An and H5.3 (zone 3) the presence of high abundance of anammox and of some sulfate reducers indicated N and S cycling. The Δ^{14}C values of all components measured (PLFAs, DIC, POC, BDE, CH$_4$ and CO$_2$) decreased from the oxic H5.1-NO through H4.3-IR to H5.2-SR/An (Figure 4). Below, we show that the cycling of ^{14}C-free C sources (potentially HCs or

sedimentary OC) in those groundwaters reflected different metabolic traits related to lower relative inputs of labile photosynthetic C from soil, consistent with lower availability of energetically favorable substrates (Schwab et al., 2017). It is unlikely that the high volume (10′000 L) sampling caused water mixing from different aquifer stories as pumping caused no changes in the major physicochemical parameters and minimal to no drawdown of the water table protecting the subsurface environments from artificial aeration and possible disturbance.

5.1.1. Origins of HCs in Groundwaters

The occurrence of trace amounts of mature HCs suggests that petroleum-derived compounds could be a source of ^{14}C-free C for the heterotrophic communities in those wells. The origin of these HCs is difficult to determine. Although the extremely low concentration clearly distinguished those sites from known petroleum contaminated aquifers where HCs concentrations are hundreds to thousands of micrograms per liter, matching HC distributions in groundwater and aquifer bedrocks suggested a similar and thus a natural origin. HCs can be native (autochthonous) from the Muschelkalk sediments or migrated from the underlying Permian/Jurassic formations, particularly from the Zechstein groups. Indeed, carbonates and evaporites of the Zechstein are expected the main source rocks of HCs in the Thuringian Basin, which represents an old oil and gas producing province in Eastern Germany (Karnin et al., 1996). Most HCs were generated during the early Late Cretaceous, when most of the basin experienced a subsidence period. During this time sediments of the Muschelkalk and Zechstein group were situated within the oil-gas window up to 2700 meter below ground level (Karnin et al., 1996). Such a basinal origin of the detected HCs is further supported by the predominance of thermally stable hopane and sterane isomers, and high amount of gammacerane and extended hopanes typically observed in mature oil derived from epicontinental carbonate/evaporite (e.g., Zechstein; Hartwig & Schulz, 2010). It is likely that those HCs are present in all the aquifer bedrocks (strata moTK and MoM; Figure 1) regardless of the sampling locations. However, secondary processes such as HC biodegradation or weathering are expected to greatly differ between locations. Therefore, as aquifer bedrocks were not sampled from the sediments in contact with the studied groundwaters (pink stars in Figure 1), secondary processes as HC biodegradation can only be estimated in the groundwater samples. In the oxic zone, the presence of moderately biodegraded HCs pointed to direct HCs assimilation by microorganisms, which could partially explain the ^{14}C-content of the PLFAs in this deeper groundwater.

Despite the presence of petroleum-derived complex HCs, which could support a thermogenic origin for methane in the aquifers, dissolved methane concentration and isotopic signatures lead us instead to infer a range of sources. In the upper anoxic aquifers, the evidence in 16S rRNA genes for methanogenic archaea, such as Methanobacteriales, Methanomicrobiales, and Methanosarcinales (Lazar et al., 2017), capable of acetoclastic or hydrogenotrophic methanogenesis, would suggest a contribution of archaeal methanogenic origin for the CH_4. However, the low CH_4 concentration, CH_4 $\delta^{13}C$ values around −40‰ and the $\delta^{13}C_{CO2-CH4}$ offset of 20‰ suggested the influence on CH_4 of additional processes such as (1) substrate depletion in a confined environment, (2) anaerobic CH_4 oxidation (AMO), or (3) contribution of thermogenic (petroleum-derived) CH_4 (Whiticar, 1999). In H4.3-IR, the similar $\Delta^{14}C$ values of CH_4 with its potential carbon substrates (POC and DIC) suggested archaeal methanogenic origin, though the low CH_4 ^{13}C value might indicate high substrate depletion of POC (Whiticar, 1999) that is expected in groundwaters that received very few inputs from surface organic matter. In H5.2-SR/An, the large $\Delta^{14}C$ offsets between CH_4 and its potential substrates as well as the enrichment of CH_4 $\delta^{13}C$ values suggested a major thermogenic methane origin in this groundwater. The process of AMO is carried out by syntrophic microbial communities of SRBs and AMO archaea, with the former generating a variety of highly diagnostic compounds that can be used as tracers of methanotrophy (Pancost et al., 2000; Sinninghe Damsté et al., 2005). However, the absence of such compounds as well as DNA from CH_4 oxidizing archaea (Lazar et al., 2017) in any groundwater POM and aquifer host rocks indicated that AMO is not a major process in these groundwaters. Direct CH_4 utilization by bacteria can thus not explain the low ^{14}C-content of the PLFAs in these shallower groundwaters.

5.1.2. Different ^{14}C-Content and Bioavailability of the Microbial C Sources

The wide range in ^{14}C-content of the different microbial carbon sources indicated that the total OC in those groundwaters was composed of fractions of varied ages and origins, and thus accessibility to the microbial communities (Figure 4). The HCs, which composed the oldest OM fractions, represented respectively a

Table 2

Calculated Proportion of DIC and Fossil-Derived C in Bacterial C16:0 PLFA of the Different Wells

H52-wr/an	f_{DIC} (%)	f_{fossil} (%)	Error[a] (%)	$\Delta^{14}C_{C16:0}$ (‰)	$\Delta^{14}C_{DIC}$ (‰)	$\Delta^{14}C_{fossil}$ (‰)
H5.1-NO	69	31	± 1	−562	−366	−1000
H4.3-IR	53	47	± 9	−753	−532	−1000
H5.2-SR/An	30	70	± 7	−916	−721	−1000

Note. $1 = f_{fossil} + f_{DIC}$; $\Delta^{14}C_{C16:0} = f_{DIC} * (\Delta^{14}C_{DIC}) + f_{fossil} * (\Delta_{14}C_{fossil})$. DIC = dissolved inorganic carbon; PLFA = phospholipid fatty acid.
[a]Estimation based on the analytical error of the accelerator mass spectrometry measurement.

parts per billion fraction of the total OC in these groundwaters. This implies that a large and more recent fraction of the total OC, which is not solvent extractable and thus potentially more polar and/or more strongly sorbed to matrix material (e.g., colloids), was not bioavailable for the microbial community. By decalcifying part of the filters for the POC $\Delta^{14}C$ analyses, we likely removed most of the acid-soluble compounds as PLFAs, carbohydrates, or sterols and thus may have concentrated more complex molecules as plant-derived suberin, cutin, and lignin, which reflect surface source materials and could be expected to be ^{14}C-enriched relative to PLFAs and HCs. Alternatively, as ^{14}C-free HCs are soluble in solvent, they will be preferentially concentrated in the BDE, which may explain its lower $\Delta^{14}C$ values. In soil, polyphenolic materials as lignins are thought to be more resistant to bacterial degradation due to their complex molecular structure and their close association with the mineral phase, particularly oxides (Mikutta et al., 2009; Trumbore, 2000). In groundwaters, those compounds are expected to predominate in soil-derived colloids and hence concentrate in the analyzed POC fractions; that is, substrates should accumulate that which consistently appeared resistant to the bacterial metabolism in those shallow aquifers.

Future inputs of fresh carbon supply at these sites may favor microbial metabolism of recent OM. However, the similarity of DIC $\Delta^{14}C$ and $\delta^{13}C$ values over 1 year of monitoring (Nowak et al., 2017) suggests that periodic oxidation of more recent OM is not a significant process, particularly in the anoxic wells where infiltration rate may be slow. Therefore, the presence of younger C source in those groundwaters appears not sufficient to ensure its microbial assimilation.

5.1.3. Well H5.1-NO, Oxic Zone

Consistent with the wide range of potential microbial carbon sources, the large isotopic offsets between the PLFAs indicated that the microbial C making up those lipids is derived from different sources and distinct metabolic pathways (Figure 4). The occurrence 11MeC16:0 suggested the presence of active *Nitrospira moscoviensis,* an obligate chemolithoautotrophic, nitrite-oxidizing bacterium (Ehrich et al., 1995; Lipski et al., 2001). Consistent with chemolithoautotrophic growth, and with DIC as the predominant C source, the $\Delta^{14}C$ values of 11MeC16:0 and DIC were not significantly different (given the large uncertainties for each analysis typically introduced when measuring radiocarbon in very small samples). The 17‰ $\delta^{13}C$ difference between DIC and 11MeC16:0 is in the range expected for autotrophic growth using the reverse TCA cycle for C assimilation (van der Meer et al., 1998) but is larger than the isotopic fractionation (2–6‰) observed in other autotrophic members of Nitrospirae (Lücker et al., 2010).

The radiocarbon content in the majority of living microbial biomass, estimated from the value in C16:0, was significantly lower than the potential C sources DIC and POC sampled at the same time in this well. Thus, additional C sources with lower ^{14}C-content are being used; possible sources are sedimentary OC or HCs, both of which were assumed to have zero radiocarbon (i.e., a $\Delta^{14}C$ value of ~−1000‰). Mass balance calculation assuming DIC as the other end-member source of C indicates that at least 31% ± 1% of the microbial lipid C represented by the C16:0 is derived from ^{14}C-free C sources (Table 2). The oxidation of ^{14}C-depleted OC could explain the ^{14}C-depletion of DIC relative to the POC pool. The occurrence of trace amounts of biodegraded HCs in this groundwater (Figure 2) pointed to possible heterotrophic bacterial assimilation of ^{14}C-free C derived from HCs. The other more recent microbial C sources may involve DOC, POC, and/or DIC. However, because C_3 plants, POC, DOC, and HCs had roughly the same $\delta^{13}C$ value range (e.g., −25‰ to −30‰) and because of the variable isotopic fractionations associated with differing biosynthetic production of PLFA from carbon substrates (Figure 4), the $\delta^{13}C$ values are unable to provide any resolution of those microbial carbon sources.

The $\Delta\delta^{13}C_{OM-C16:0}$ value of ~3.5‰ was in the range expected for heterotrophs (Hayes, 2001; Pancost & Sinninghe Damsté, 2003), whereas $\Delta\delta^{13}C_{DIC-C16:0}$ value of 20‰ exceeded the range for autotrophic growth using the reverse TCA cycle (van der Meer et al., 1998) but was consistent with autotrophic growth via the Calvin-Benson-Bassham cycle (Preuß et al., 1989). This cycle was previously indicated by quantitative PCR as an important C pathway in this groundwater (Herrmann et al., 2015).

5.1.4. Well H4.3-IR, Iron Reduction Zone

In anoxic environments, Fe-reducing microorganisms comprise a significant fraction of the microbial biomass as they can use oxidized iron as terminal electron acceptor for chemoautotrophic and heterotrophic growth. Consistent with the metabolic versatility of Fe-reducers, the $\Delta^{14}C$ values of C16:0 in well H4.3-IR that are representative of the overall community indicated microbial utilization of different C substrates falling between the BDE, DIC, and POC $\Delta^{14}C$ values (Figure 4). Mass balance calculations with DIC as the other end-member source indicated that ^{14}C-free sedimentary OC accounted for up to 47 ± 9% of the microbial lipid C represented by the C16:0 (Table 2). The close $\Delta^{14}C$ values of C18:0 with both DIC and POC did not allow us to differentiate between those substrates for the microbial lipid C represented in C18:0.

The occurrence of slightly to nonbiodegraded HCs in this groundwater indicated that HCs was not a major source of ^{14}C-free C (Figure 2). Furthermore, a major microbial C source from CH_4 was unlikely, since both C16:0 and C18:0 were enriched in ^{13}C in relation to CH_4 which was inconsistent with kinetic isotope effects associated with methanotrophy (Jahnke et al., 1999; Whiticar, 1999).

The ^{13}C-depletion of C16:0 and C18:0 relative to DIC ($\Delta\delta^{13}C$ ~22‰) and DOC ($\Delta\delta^{13}C$ ~8‰) consistently fell within the range of possible C isotope fractionations associated with autotrophy and heterotrophy, respectively (Londry et al., 2004; Pancost & Sinninghe Damsté, 2003; Preuß et al., 1989). Consistently, Herrmann et al. (2015) found that up to 17% of the microbial community in this well has the potential for chemolithoautotrophic CO_2 fixation via the Calvin-Benson cycle that has a reported range of carbon isotope fractionation between 5‰ to 36‰ (Londry et al., 2004; Preuß et al., 1989). Furthermore, the low ^{13}C values of 10MeC16:0 suggested autotrophy as primary C metabolism for this lipid (Figure 4; Londry et al., 2004), since heterotrophy is typically associated with low fractionation of ~3.5‰ (Hayes, 2001; Pancost & Sinninghe Damsté, 2003). This compound is abundant in Proteobacteria as *Desulfovibrio sp.* (Kohring et al., 1994; Taylor & Parkes, 1983; Vainshtein et al., 1992), *Desulfobulbus sp.* (Macalady et al., 2000; Parkes & Calder, 1985), or *Geobacter sp.* (Zhang et al., 2003), which have all been identified in this well (Nowak et al., 2017; Schwab et al., 2017). Since many species of these genera are capable of growing autotrophically with H_2 as well as different terminal electron acceptors (Lovley et al., 1993), it is possible that H_2-oxidizing, Fe-reducing, autotrophic bacteria are a source of the ^{13}C-depleted PLFAs in this groundwater.

5.1.5. Well H5.2-SR/An, Sulfate Reduction and Anammox Zone

The ladderanes indicated the presence of anammox bacteria (Figure 3; Sinninghe Damsté et al., 2005). Their occurrence was consistent with DNA- and RNA-based analyses (Schwab et al., 2017), and the presence of ammonium and nitrite, both of which are necessary for the anammox processes (Dalsgaard et al., 2005; Dalsgaard & Thamdrup, 2002). The ~52‰ depletion of ladderanes compared to DIC $\delta^{13}C$ values was within the range previously reported for anammox in the Black Sea (Figure 4), further suggesting that autotrophic carbon fixation pathways within the diverse group of anaerobic ammonium-oxidizing bacteria are similar (Schouten et al., 2004). In marine and freshwater environments, anaerobic ammonium oxidation has been demonstrated an important process controlling N distributions (Dalsgaard et al., 2005). Their presence in groundwater showed that ammonium removal from groundwater can occur through the action of naturally occurring bacteria.

The very low $\Delta^{14}C$ values of 10MeC16:0 and C16:0 in this well indicated that the C in these PLFAs is mainly (up to 70 ± 7%) derived from heterotrophs assimilating ^{14}C-free C from sedimentary OC (Table 2). The high relative abundance of those lipids (26%) suggested that active heterotrophs represented a substantial proportion of the prokaryotic community in this well. A major C origin from CH_4 was unlikely, since the C16:0 was enriched in ^{13}C in relation to CH_4, which is inconsistent with kinetic isotope effects associated with methanotrophy (Jahnke et al., 1999; Whiticar, 1999). Furthermore, the occurrence of slightly biodegraded HCs in this groundwater indicated that HCs was not a major source of ^{14}C-free C (Figure 2).

SRBs that produce high amounts of 10MeC16:0 (Kohring et al., 1994; Taylor & Parkes, 1983; Vainshtein et al., 1992) play an important role in the anaerobic oxidation of OM. They can metabolize a wide range of organic compounds as HCs or propionate, lactate, or acetate derived from the degradation of the OM (Glombitza et al., 2015; Kleikemper et al., 2002). Consistently, the presence of sulfur in the HC fractions (Figure 2) and the presence of SRBs such as *Syntrophus*, *Sulfuritalea*, *Desulfurivibrio*, *Desulfocapsa*, and *Sulfuricurvum* revealed by DNA sequencing (Schwab et al., 2017) supported an active sulfur cycling in the processes involved in the degradation of the OM in this well. However, the low ^{13}C values of C16:0 and

10MeC16:0 ($\Delta\delta^{13}C_{DOC-PLFA}$ up to $-20‰$) are apparently inconsistent with a purely heterotrophic C origin, since PLFAs from heterotrophs are typically 3‰ to 5‰ depleted relative to the C source (Pancost & Sinninghe Damsté, 2003). Additionally, reported C isotope fractionation between C substrates and PLFAs derived from heterotrophic SRBs are typically between $-9‰$ and $-12‰$ (Londry et al., 2004) and thus higher than those observed in this well. An explanation for the low $\delta^{13}C$ values of SRB-derived lipids (considering that AMO was not a major process in this well) is the assimilation of ^{13}C-depleted C from intermediate products during the anaerobic mineralization of ^{14}C-free OC. Recent studies on the C isotope composition of acetate in sedimentary pore water of deep subsurface showed that in the sulfate reduction zone acetate may be depleted up to 23‰ relative to the DOC and DIC (Heuer et al., 2009; Ijiri et al., 2012). Such ^{13}C-depleted acetates could serve as electron donors in the terminal steps of sedimentary OC mineralization (e.g., sulfate reduction) and thus partially explain the strong ^{13}C- and ^{14}C-depletion of the PLFAs in this well. As ammonium is typically produced during OM mineralization and used as substrate in the anammox process, such a mechanism could also explain the observed correlation between ammonium and the relative abundance of SRB and anammox-derived PLFAs previously observed in Schwab et al. (2017).

5.1.6. Preferential Bacterial Utilization of ^{14}C-Free C in the Shallow Aquifer

It is actually well established that microorganisms can assimilate recalcitrant old C (Petsch et al., 2001; Seifert et al., 2013; Simkus et al., 2016). However, the mechanisms controlling its incorporation into microbial biomass are still unclear. The general explanations lie in the availability and energetic favorability for heterotrophs to use naturally occurring organic matter, the limitation of electron acceptors, and the local microbial community structure. In these shallow groundwaters (10 and 50 m depth), we showed that despite the presence of larger amounts of more recent C sources, bacteria incorporated ^{14}C-free C in varying proportions depending on the local biogeochemistry, and physiological strategies of microorganisms. In environments where diffusive supply of electron acceptors is limited, it has been postulated that microorganisms optimize energy yields by metabolite exchange between microbes, which likely play a critical part in sedimentary OC cycling (Hoehler et al., 1994; Prokopenko et al., 2013). In line with those findings, it has been shown that in deep subsurface aquifers (1,200 m depth), methane cycling by a consortium of methanotrophic archaea and SRB results in significant incorporation of ^{14}C-free sedimentary OC in the overall bacterial communities (Simkus et al., 2016). In this shallow anoxic aquifer, we showed that incorporation of methane-derived C into the microbial biomass is not the dominant process for explaining the low ^{14}C-content of PLFAs, although up to 70% of the microbial C (as evidenced by C16:0 PLFA) was ^{14}C-free C. In deep anoxic sediments of the Mexican Pacific margin, cometabolisms between anammox and sulfur oxidizers have been shown an important process coupling the carbon, nitrogen, and sulfur cycles (Prokopenko et al., 2013). In those groundwaters, it may be envisaged that similar consortium between ammonium and sulfur bacteria plays a critical part in the incorporation of sedimentary ^{14}C-free C in the overall bacterial biomass linking the C, N, and S cycles.

6. Conclusion

Understanding carbon sources of subsurface microorganisms is fundamental to elucidate the role of microbes in carbon cycling and to preserve groundwater quality. Sedimentary organic matter represents a large potential source of carbon for subsurface microorganisms. However, this energy source is typically considered refractory, particularly when younger, and thus likely more labile organic C is present. Here, we compared the natural abundance of ^{13}C and ^{14}C in PLFAs derived from bacteria with the potential microbial C sources including DIC, dissolved POC, and methane in biogeochemically distinct shallow aquifers of the Hainich CZE. We showed that despite the presence of higher amounts of C derived from more recent C sources, bacteria incorporated ^{14}C-free C in varying proportions. The amount incorporated depended on local biogeochemistry, which was linked to physiological strategies of microorganisms inhabiting the groundwater environments, ranging from about 31% incorporation of ^{14}C-free C in the oxic zone to 47% in the iron reduction zone and 70% in the sulfate reduction/anammox zone. In oxic groundwater, the occurrence of the PLFA 11MeC16:0 (30% in total abundance) with isotopic values consistent with a predominant C source from DIC indicated the likely presence of chemolithoautotrophic *Nitrospira moscoviensis*. The $\delta^{13}C$ PLFA values in this groundwater were isotopically heavier than in the anoxic groundwaters, consistent with a major lipid origin from autotrophs using the reverse TCA cycle for C assimilation. In contrast, the $\delta^{13}C$ and $\Delta^{14}C$ values of PLFAs in anoxic groundwaters suggested that ^{14}C-free C, likely derived from ^{13}C-depleted

intermediates (i.e., acetate) produced during the anaerobic mineralization of sedimentary OC by sulfur bacteria, is the most important C source. Results of this study showed that sedimentary OC cycling in these shallow aquifers results of complex interactions between groundwater geochemistry and microbial communities but is not related to depth.

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