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Field-scale isotopic labeling of phospholipid fatty acids from acetate-degrading sulfate-reducing bacteria

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Abstract

Isotopic labeling of biomarker molecules is a technique applied to link microbial community structure with activity. Previously, we successfully labeled phospholipid fatty acids (PLFA) of suspended nitrate-reducing bacteria in an aquifer. However, the application of the method to low energy-yielding processes such as sulfate reduction, and extension of the analysis to attached communities remained to be studied. To test the feasibility of the latter application, an anoxic test solution of 500 l of groundwater with addition of 0.5 mM Br^- as a conservative tracer, 1.1 mM SO_4^{2-}, and 2.0 mM [2-^{13}C]acetate was injected in the transition zone of a petroleum hydrocarbon-contaminated aquifer where sulfate-reducing and methanogenic conditions prevailed. Thousand liters of test solution/groundwater mixture were extracted in a stepwise fashion after 2–46 h incubation. Computed apparent first-order rate coefficients were 0.31 ± 0.04 day^{-1} for acetate and 0.34 ± 0.05 day^{-1} for SO_4^{2-} consumption. The δ^{13}C increased from $-71.03‰$ to $+3352.50‰$ in CH_4 and from $-16.15‰$ to $+32.13‰$ in dissolved inorganic carbon (DIC). A mass balance suggested that 43% of the acetate-derived ^{13}C appeared in DIC and 57% appeared in CH_4. Thus, acetate oxidation coupled to sulfate reduction and acetoclastic methanogenesis occurred simultaneously. The δ^{13}C of PLFA increased on average by 27‰ in groundwater samples and 4‰ in sediment samples. Hence, both suspended and attached communities actively degraded acetate. The PLFA labeling patterns and fluorescent in situ hybridization (FISH) analyses of sediment and groundwater samples suggested that the main sulfate-reducing bacteria degrading the acetate were *Desulfotomaculum acetoxidans* and *Desulfobacter* sp. in groundwater, and *D. acetoxidans* in sediment.

Keywords: Petroleum hydrocarbons; Phospholipid fatty acids; Isotopic labeling; Whole-cell hybridization; Stable carbon isotope; Push-pull test; Sulfate-reducing bacteria; Methanogenesis

1. Introduction

Linking microbial community structure with its activity in the environment remains a challenge in microbial ecology. Recently, several methods have been developed to tackle this problem (for a review, see [1]). Among these techniques, a particularly interesting approach is the use of stable isotopes combined with molecular tools. Organisms that actively metabolize a ^{13}C-labeled compound incorporate part of the label into their biomass. The identity of active organisms can then be unveiled by analyzing labeled biomarker molecules such as phospholipid-derived fatty acids (PLFA), DNA or RNA [2–5].

One of the advantages of PLFA or RNA labeling compared to DNA labeling is that microbial cells synthesize PLFA and RNA even when they are not replicating. Therefore, the turnover rate of the latter biomolecules is faster than DNA [5]. Lipids are particularly interesting

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in field-oriented research because in most bacteria they may account for 2–20% of the biomass [6]. Although the identification of certain microbial taxa from the analysis of PLFA derived from a complex microbial community can be ambiguous [7], the combination of PLFA analysis with other molecular tools can yield useful information [8–10]. Moreover, certain bacterial groups with specific biogeochemical activity such as sulfate-reducing bacteria (SRB) have been thoroughly characterized by PLFA profiling and were found to possess several characteristic PLFA biomarkers [11–13].

However, most of the studies on biomarker ^{13}C-labeling are normally performed in laboratory microcosms, and microbial activities measured under these conditions may not resemble the communities' activities at a particular field site [1]. Prolonged incubations could also produce a shift in microbial community composition by favoring microorganisms that readily degrade a substrate under laboratory conditions, but which are not very active in the field. Furthermore, laboratory studies can also neglect interactions between two or more different populations that are delicately balanced under natural conditions [14]. But there have been only a few attempts to use isotope labeling at the field scale using $H^{13}CO_3^-$ and PLFA analysis [15], several ^{13}C-labeled organic compounds and 16S rRNA gene analysis [16], and [2-^{13}C]acetate and PLFA analysis [7].

In our previous study, we demonstrated that it is possible to label PLFA at the field scale while determining the rate of substrate consumption in a petroleum hydrocarbon-contaminated aquifer using a push–pull test (PPT) [7]. This test consists of injecting a test solution that contains a conservative tracer and one or more reactants into an aquifer through a monitoring well. After an incubation period, the mixture of groundwater and test solution is extracted from the same well and analyzed to determine rates of reactant consumption [17]. In our previous study, we injected [2-^{13}C]acetate diluted 1:1 with unlabeled acetate into the nitrate-reducing zone of the aquifer. Nitrate-reducing bacteria were targeted in that experiment because nitrate reduction is energetically favorable process with high percentage of carbon assimilation. We found high enrichments in PLFA, in some cases of up to ~5000‰. But the labeled PLFA pattern was not used for identifying specific organisms, because bacteria that reduce nitrate are phylogenetically heterogeneous and lack characteristic PLFA that could be used as biomarker. Besides, in that experiment only bacterial communities suspended in groundwater were studied.

The feasibility of applying this method to slower but environmentally very important processes with lower percentage of carbon assimilation, like sulfate reduction and other energetically less favorable anaerobic respirations, thus still needed to be studied. Moreover, targeting microbial populations that possess recognized PLFA biomarkers, as in SRB, would allow specific identification of degrading populations. Finally, since attached microbial communities also contribute to the overall activity in an aquifer, the analysis of bacterial communities attached to the aquifer matrix is desirable.

The aim of the present study was therefore to evaluate whether we can identify directly in the field the populations of sulfate-reducing bacteria degrading ^{13}C-labeled acetate in the sulfate-reducing zone of a petroleum hydrocarbon-contaminated aquifer using the PLFA labeling approach combined with a PPT. We also investigated if other microorganisms or processes were involved in this degradation. In addition, we aimed at comparing suspended vs. attached microbial communities actively degrading the acetate.

2. Materials and methods

2.1. Field-site description

The study was conducted in a petroleum hydrocarbon-contaminated aquifer in Studen, Switzerland, which undergoes monitored natural attenuation and was characterized in detail by Bolliger et al. [18]. The work presented in this paper was performed in October 2002 in monitoring well PS3, which is located within the contaminant source zone (free-phase oil present). PS3 partially penetrates the aquifer to a depth of 3.5 m below ground surface. At the time the experiment was performed, the groundwater table was at 2.42 m depth. Groundwater in well PS3 exhibited reduced conditions (see Table 1) and dissolved petroleum hydrocarbons concentration of up to 1 mg/l [18]. Previous studies have shown that PS3 is located within a transition zone where both sulfate-reducing and methanogenic conditions are found [18,19].

Table 1
Chemical parameters in background groundwater of monitoring well PS3 and in the injection solution

	Background	Injection solution
pH	6.8	7.8
Alkalinity (mM)	8.4	9.5
DIC (mM)	12.4	10.0
O_2 (μM)	2.9	2.9
NO_3^- (μM)	2.0	2.0
Fe II (μM)	55.5	55.5
S^{2-} (μM)	12.8	3.4
SO_4^{2-} (mM)	0.15	1.10
CH_4 (mM)	0.23	0.01
Br^- (mM)	<0.01	0.50
Acetate (mM)	<0.01	2.01

2.2. Field experiment and sample collection

From well PS3, 500 l of groundwater were extracted and collected in a plastic container and were sparged with N_2 gas to avoid O_2 diffusion from air into the test solution during its preparation and injection. The test solution was prepared by adding to this water Br^-as a non-reactive, conservative tracer (as NaBr), SO_4^{2-} as electron acceptor (as K_2SO_4), and acetate as carbon source (as NaAc) (final concentrations listed in Table 1). We employed 20% methyl-labeled acetate ([2-^{13}C]acetate, 99% ^{13}C, Cambridge Isotope Laboratories, MA, USA); the remaining 80% was unlabeled acetate. The theoretical, calculated $^{13}C/^{12}C$ ratio (expressed as $\delta^{13}C$) of acetate in the test solution was ~9850‰. Injection of the test solution by gravity drainage at a depth of 3.3 m below ground surface was completed within 43 min. Extraction of the groundwater/test solution mixture was performed sequentially in four steps: 100 l were recovered after 2 h, 100 l after 22 h, 100 l after 25 h and 200 l after 46 h, all at a constant flow rate of ~5 l/min using a Grundfos MP-1 submersible pump (Grundfos Pumpen, Fällanden, Switzerland).

Samples were collected from background groundwater (during groundwater collection, after the first 150 l were pumped to avoid sampling the water of the well casing), and during both the injection and the extraction phase. All samples where kept on ice until further processing. Samples for Br^-, SO_4^{2-} and acetate were filtered in the field through 0.45 μm polyvinylidene fluoride filters (Millipore, Bedford, MA) and stored in 12 ml vials. Samples for pH, alkalinity, CH_4, and $\delta^{13}C$ analysis of dissolved inorganic carbon (DIC) were collected unfiltered in 120-ml serum bottles closed without headspace with butyl rubber stoppers to minimize loss of CH_4. The samples collected for CH_4 concentration measurements and $\delta^{13}C$ analysis of CH_4 were poisoned in the field immediately after collection with 0.2 ml of 10 M NaOH to stop any microbial activity. Samples for $\delta^{13}C$ analysis of DIC were poisoned in the field immediately after collection with 0.2 ml of CO_2-free 10 M NaOH and the DIC was precipitated as $BaCO_3$ by adding 4 ml of a CO_2-free 1.2 M $BaCl_2$ solution upon arrival to the laboratory. After more than 12 h of equilibration, the precipitate was filtered and dried at 105 °C for 12 h.

Samples for PLFA extraction were collected in 10-l plastic containers, poisoned with 10 ml of concentrated (37%) HCl and kept on ice until further processing. Within 10 h, these samples were filtered through glass fiber and 0.2 μm polyvinylidene fluoride filters (Millipore, Bedford, MA) to collect the suspended biomass, and the filters were kept at −20 °C until PLFA extraction. For microbial cell counts and FISH analysis, samples of 50 ml were collected in sterile falcon tubes, kept on ice during transport, and processed immediately after arrival to the laboratory. Before and after the PPT, we also collected sediment samples (3 cm diameter cores) using a Humax handheld hollow-stem auger (Max Hug, Luzerne, Switzerland) at a distance of ~30 cm from the well casing at a depth of 3–3.5 m below ground surface. Sediment samples were stored under N_2 atmosphere on ice until further processing in the laboratory.

2.3. Chemical analysis and calculation of apparent reaction rate coefficients

Concentrations of Br^-, SO_4^{2-} and acetate were measured by ion chromatography, alkalinity was measured by potentiometric titration and pH was determined with a pH electrode [7]. Concentrations of DIC were calculated from alkalinity and pH [20]. CH_4 was quantified by gas chromatography with a HayeSep N column (Restek, Bellefonte, PA) using N_2 as carrier gas and a FID detector, employing the headspace method as described in Bolliger et al. [18].

Apparent first-order rate coefficients for the consumption of SO_4^{2-} and acetate were calculated from extraction breakthrough curves using the method of Haggerty et al. [17]. This method assumes that an injected reactant is transformed within the aquifer according to the first-order reaction $dC/dt = -kC_r$, where C_r is the reactive solute concentration and k is the apparent first-order rate coefficient.

Stoichiometric ratios, SR (mol SO_4^{2-} per mol acetate consumed) were calculated from extraction breakthrough curves for each sampling point using:

$$\mathrm{SR} = \frac{\left(C^*_{\mathrm{Br}^-} - C^*_{\mathrm{SO}_4^{2-}}\right) C_{\mathrm{o,SO}_4^{2-}}}{\left(C^*_{\mathrm{Br}^-} - C^*_{\mathrm{Ac}}\right) C_{\mathrm{o,Ac}}}, \quad (1)$$

where C^* is relative concentration (i.e., concentration measured in the extracted samples divided by concentration in the injected test solution, C_o) of Br^-, SO_4^{2-} and acetate (Ac) [7].

2.4. PLFA analysis

Total lipids were extracted from microbial biomass collected on the filters and from 20 g wet weight of sediments by a modified Bligh–Dyer method [21] as described previously [22]. Lipids were then fractionated to neutral, glyco- and phospholipids by column chromatography on silica gel (ICT, Basle, Switzerland) as described previously [23]. The expected recovery efficiency of phospholipids from sediments with this method is 93 ± 5% [23]. Phospholipids were dried and derivatized into fatty acid methyl esters. Then they were separated by gas chromatography, identified, and their relative abundance calculated employing the MIDI™ System [7]. A mass spectrometer (GCQ Finnigan MAT, Bremen, Germany) was used for an additional

verification of peak identity. PLFA nomenclature used in this paper is in the form of A:BωC, where A designates the total number of carbons, B the number of double bonds and C the distance of the closest unsaturation from the aliphatic end of the molecule. The suffixes "-c" for *cis* and "-t" for *trans* refer to geometric isomers. The prefixes "i-" and "a-" refer to iso- and anteiso-methyl branching, and mid-chain methyl branches are designated by "Me-" preceded by the position of the branch from the acid end. A cyclopropyl ring is indicated as "cy-".

2.5. Determination of stable carbon isotope ratios in DIC, CH_4 and PLFA

For stable carbon isotope ratio analysis of DIC, dried $BaCO_3$ was converted to CO_2 at 90 °C in an automated acid bath preparation system and then measured on a Fisons-Prism isotope ratio mass spectrometer (Fisons, Middlewich, Cheshire, UK) in duplicates.

The stable carbon isotope ratio of CH_4 was determined using a Precon preparation device (Finnigan, Bremen, Germany). After injecting the sample through a septum into a helium stream, the gas passed a liquid-nitrogen trap and a chemical trap (based on NaOH) to remove all CO_2, then the CH_4 from the sample was combusted with 100% efficiency in an oven at 1000 °C to CO_2. The sample gas was collected in a second liquid-nitrogen trap, and later injected into a GC column (Poraplot Q) for separation of interfering gases such as N_2O. The gas was then passed to an isotope-ratio mass-spectrometer (Delta Plus XL, Finnigan, Bremen, Germany).

The stable carbon isotope ratio measurements in PLFA were carried out on a Finnigan Delta PLUS XL isotope ratio mass spectrometer (Finnigan, Bremen, Germany) coupled via combustion interface to a Hewlett Packard HP 5890 gas chromatograph, which was equipped with a HP Ultra 2 capillary column [7].

All stable carbon isotope ratios are reported using the standard δ-notation, $\delta^{13}C\ (‰) = (R_s/R_{VPDB}\text{-}1) \times 10^3$, where R_s and R_{VPDB} are the $^{13}C/^{12}C$ isotope ratios corresponding to the sample and the international Vienna PeeDee Belemnite standard, respectively (R_{VPDB} = 0.0112372 ± 0.0000090). The $\delta^{13}C$ values of PLFA were corrected for the methyl group introduced during derivatization [22]. Concentration of ^{13}C in any given compound and sample can be calculated from $\delta^{13}C$ and C_t (total C concentration, i.e., $^{13}C + {}^{12}C$) using the following equation:

$$^{13}C = C_t/\{1/[(\delta^{13}C/1000 + 1) * R_{VPDB}] + 1\}. \quad (2)$$

The relative ^{13}C incorporation into each PLFA is calculated with the following formula adapted from Boschker and Middelburg [24]:

$$\text{Relative } ^{13}C \text{ incorporation}(\%) = 100(F_t - F_0) * C_{PLFA}, \quad (3)$$

where F indicates the fraction of ^{13}C at the beginning of the experiment (0) and after a certain time (t) and C_{PLFA} is the PLFA concentration expressed in % of abundance. The fraction F can be calculated as: $F = R_s/(R_s - 1)$ and R_s is calculated from the measured $\delta^{13}C$ data.

2.6. Fluorescent in situ hybridization

Biomass from 50 ml of groundwater was obtained by centrifugation, fixed with paraformaldehyde and stored at −20 °C as described in Bolliger et al. [19]. Similarly, 2 g of sediment samples were fixed with 1.5 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS). Before analysis, 50 μl of sediment sample were diluted in 950 μl 0.01% pyrophosphate and dispersed by mild sonication with a probe of 2 mm diameter during 1 min at 20% power (VCX 500, Sonics, Newtown, CT). The bacterial cells were stained with 4′,6-diamidino-2′-phenylindole (DAPI) (Sigma, Buchs, Switzerland) and hybridized with fluorescent labeled 16S rRNA-targeted nucleotide probes as described in Zarda et al. [25]. The probes used were: Eub338 targeting Eubacteria [26], SRB385 [27] plus SRB385Db [28] for SRB of the δ-Proteobacteria, DSB985 for *Desulfobacter* [29] and Arch915 for Archaea [30]. Slides were mounted with Citifluor solution (Citifluor, Canterbury, UK) and analyzed with a Zeiss microscope equipped for epifluorescence using the appropriate filters at 1000× magnification [25].

3. Results

3.1. Consumption of sulfate and acetate

Breakthrough curves of Br^-, acetate and SO_4^{2-} showed a sharp decline at the beginning of the extraction due to dilution of the test solution with native groundwater (Fig. 1). Throughout the extraction phase, relative concentrations of SO_4^{2-} and acetate were lower than relative Br^- concentration, indicating acetate and SO_4^{2-} consumption during the test. Computed apparent first-order rate coefficients (k) were 0.31 ± 0.04 day^{-1} for acetate and 0.34 ± 0.05 day^{-1} for SO_4^{2-} consumption. By integrating the breakthrough curves, we calculated that during the extraction phase we recovered 31% of the injected mass of Br^-, 28% of acetate and 27% of SO_4^{2-}. A total of 29.7 mmol of acetate and 21.7 mmol of SO_4^{2-} were consumed during the test, which yields an overall SO_4^{2-}/acetate stoichiometric ratio of 0.73. The average stoichiometric ratio calculated for each sampling point (Eq. (1)) was 0.78 mol SO_4^{2-} consumed per mol of acetate consumed.

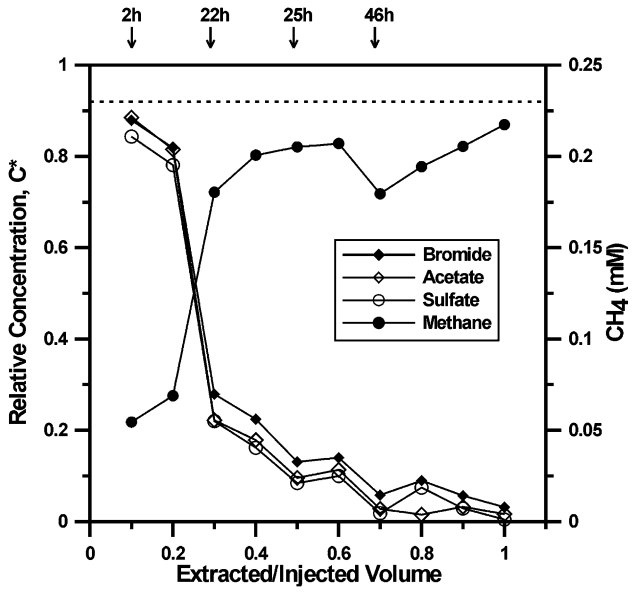

Fig. 1. Breakthrough curves showing relative concentrations (C^*, i.e., concentration measured in the extracted samples divided by concentration in the injected test solution) of Br^-, SO_4^{2-} and acetate during the push–pull test vs. the relative cumulative extracted volume (cumulative extracted volume divided by the total injected volume of test solution) and the measured CH_4 concentration. The dotted line represents the CH_4 background concentration. The arrows indicate elapsed time after the end of injection of the test solution when the sequential extractions started.

3.2. Concentration and stable isotopic composition of CH_4 and DIC

After 2 h of incubation, the concentration of CH_4 increased from 0.01 mM (injection concentration, Table 1) to 0.05 mM (Fig. 1). After 24 h, CH_4 concentration reached values similar to that of the background without further increase. The $\delta^{13}C$ value of the background CH_4 was $-71.03 \pm 0.11‰$ (Fig. 2(a)). The $\delta^{13}C$ value rose to +1107‰ after only 2 h, reaching a maximum after 22 h of incubation of +3352‰, then slowly decreased without reaching negative values even after almost 48 h of incubation.

Measured DIC concentrations in the extraction phase samples showed little variation with an average of 11.1 ± 0.6 mM (data not shown). The $\delta^{13}C$ was $-16.15 \pm 0.50‰$ in background DIC and $-13.61 \pm 0.43‰$ in injection solution DIC (Fig. 2(b)). Only 2 h after injection, the $\delta^{13}C$ DIC value increased to $-4.27 \pm 0.01‰$ and reached a maximum value of $37.12 \pm 0.13‰$ after 22 hours of incubation. Thereafter, $\delta^{13}C$ of DIC declined as a consequence of dilution of the injection solution with background groundwater containing unlabeled DIC.

Using Eq. (2) we estimated the concentration of ^{13}C present in DIC and CH_4 at any given point during the experiment using the concentration and $\delta^{13}C$ values of DIC and CH_4 in the injection solution, background

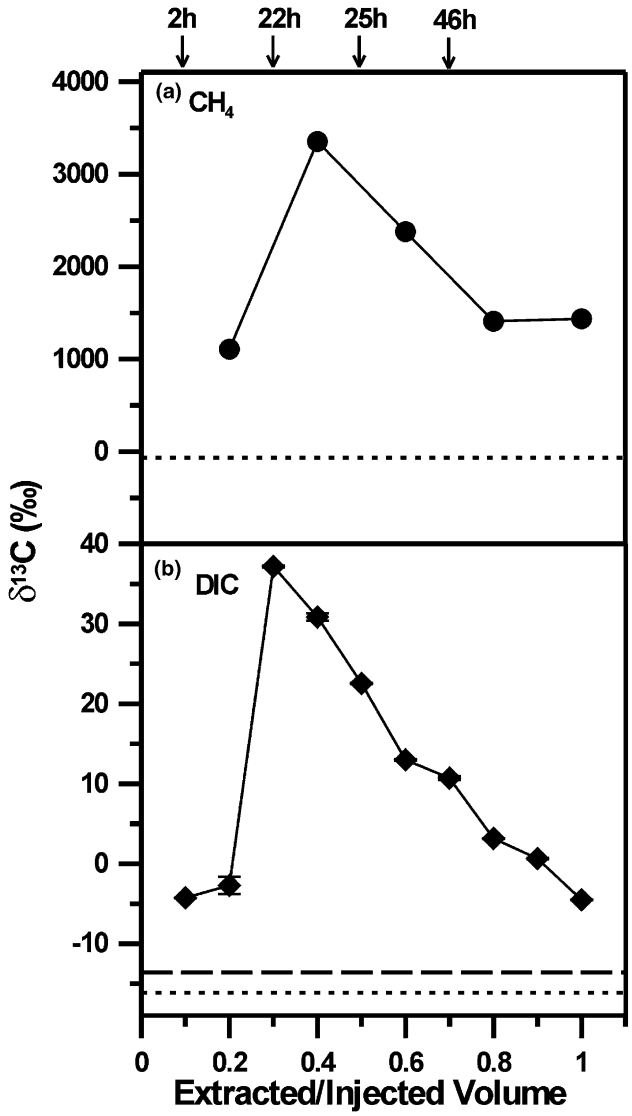

Fig. 2. Values of $\delta^{13}C$ measured in: (a) CH_4 and (b) dissolved inorganic carbon (DIC) during the experiment. The dotted lines represent the $\delta^{13}C$ background values and the dashed line the DIC $\delta^{13}C$ value in the injection solution. The arrows indicate the elapsed time after end of injection of the test solution. Error bars indicate standard deviations.

and in the samples during the experiment (data not shown). These data were used in a mass balance to determine the relative amount of ^{13}C in the CH_4 and DIC pools that was derived from the degradation of labeled acetate. The percentage of acetate-derived ^{13}C going to each of those pools was approximately 43% for DIC and 57% for CH_4, considering only the catabolic end products of acetate degradation.

3.3. PLFA relative abundance and stable isotopic composition

Profiles of relative PLFA abundance (a total of 34 PLFA were identified) were similar in sediment and

groundwater samples, although in the sediment the absolute amount of recovered biomass was smaller, since the volume of aquifer sampled is smaller compared to the water volume sampled, and as a consequence, very low abundance PLFA were difficult to detect (Fig. 3). Only very small profile variations were observed from the beginning to the end of the test in both groundwater and sediment samples.

The PLFA in groundwater samples got on average more enriched than those in sediment samples (Fig. 4). The average background $\delta^{13}C$ for PLFA in groundwater samples was $-35.7 \pm 8.2‰$, and after the experiment the average shifted to $-9.1 \pm 15.5‰$. The enrichments for each individual PLFA varied from 5‰ to 100‰ (Fig. 4(a)). On the other hand, in sediment samples the average PLFA $\delta^{13}C$ value increased from $-31.9 \pm 3.7‰$ to $-27.7 \pm 4.8‰$, with individual PLFA enrichments ranging from 0‰ to 16‰ (Fig. 4(b)). However, in order to compare which PLFA incorporated more label, it is important to relate the amount of ^{13}C incorporated into a PLFA to the relative PLFA concentration using Eq. (3). The PLFA in groundwater samples that on a relative basis incorporated more label were 16:1ω7c, 16:1ω5c, 16:0 and 18:2ω6c/18:1ω9c/18:1ω7c (we were unable to separate the latter three PLFA under the chromatographic conditions used in the $\delta^{13}C$ determination) (Fig. 5(a)). The PLFA 14:0, i15:0, a15:0, 10Me16:0, cy17:0, and 18:0 were also enriched but to a lesser degree. In the sediment samples, the only PLFA that showed substantial enrichment were 16:1ω7c, 16:0, 18:2ω6c/18:1ω9c/18:1ω7c and 18:0 (Fig. 5b).

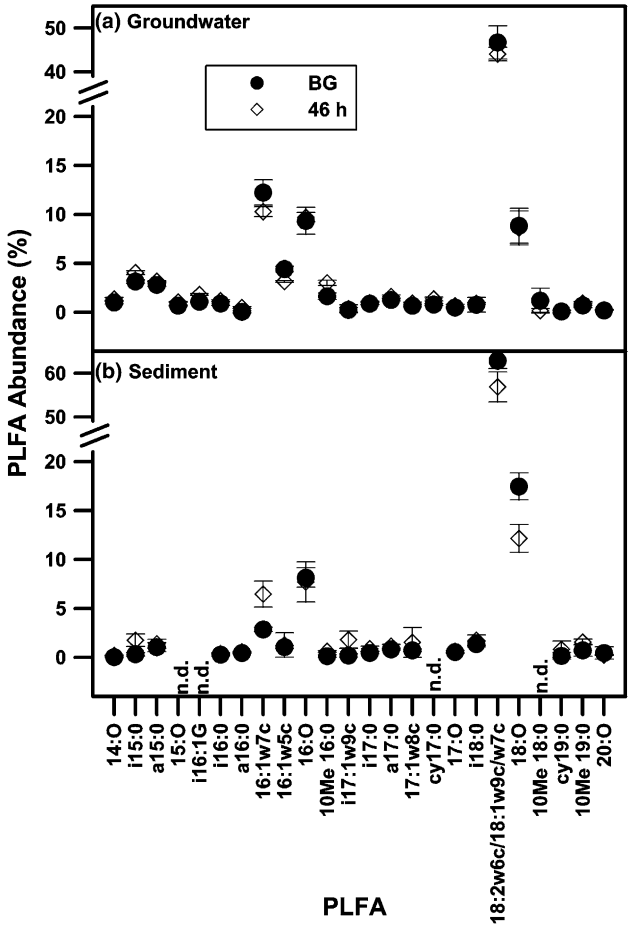

Fig. 3. PLFA abundance as % of total PLFA measured in: (a) groundwater and (b) sediment. n.d. = not detected. BG = Background. Error bars indicate standard deviations.

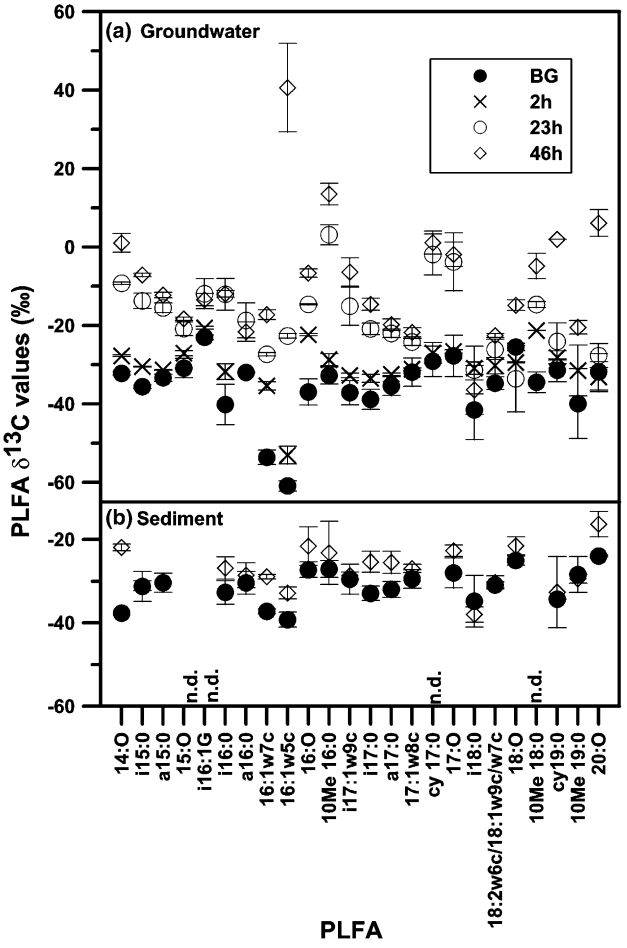

Fig. 4. Values of $\delta^{13}C$ measured in PLFA extracted form: (a) groundwater samples and (b) sediment samples. n.d. = not detected. BG = Background. Error bars indicate standard deviations.

3.4. Fluorescent in situ hybridization

Total cell numbers (DAPI-stained) were $4.7 \pm 0.2 \times 10^7$ cells cm^{-3} for groundwater samples and $1.3 \pm 0.2 \times 10^8$ cells g^{-1} (wet weight) in sediment samples. To compare the cell number of attached and suspended bacteria it is necessary to convert both numbers to bacteria per unit volume of aquifer material. Therefore, the number of cells cm^{-3} was multiplied by the average porosity (0.38 [31]) and the number of cells

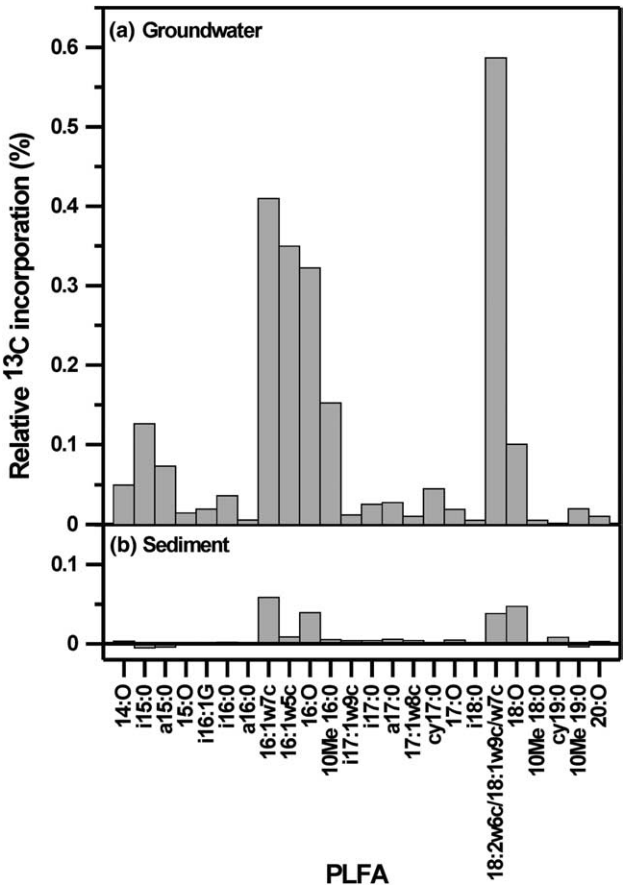

Fig. 5. Incorporation of ^{13}C into PLFA extracted from: (a) groundwater samples and (b) sediment samples.

per gram were multiplied by the average bulk density (1.64 g cm^{-3}). This calculation yields 1.8×10^7 suspended cells cm^{-3} and 2.1×10^8 attached cells cm^{-3}. Thus, roughly 8% of the microbial community is suspended. The predominant communities detected belonged to the Archaea and Eubacteria, and within the latter domain especially to the gram-negatice SRB (Table 2). The known acetate-utilizing SRB *Desulfobacter* comprised 9.2–15.4% of total cell numbers. Slightly lower percentages of *Desulfobacter* and archaeal communities were detected in attached compared to suspended communities. The percentage of the DAPI-stained cell that hybridized with the different probes remained fairly constant from the beginning to the end of the experiment, with two exceptions: for probe EUB 338 in groundwater samples and for probes SRB385 plus SRB385Db in sediment samples. In both cases, the percentage of DAPI-stained cells detected with the respective probes declined during the experiment.

4. Discussion

4.1. Substrate consumption

Low recovery of Br^- (31%), and therefore of injection solution, was due to a fairly high average pore water velocity (≈0.4 m day^{-1}) at the site [18]. However, the incubation time was chosen to allow detectable acetate and SO_4^{2-} consumption, as determined in a previous study [32].

Acetate and SO_4^{2-} were degraded at rates similar to what was found before in the same well with computed k of 0.31 ± 0.04 day^{-1} for acetate and 0.34 ± 0.05 day^{-1} for SO_4^{2-} (previously reported k values were 0.60 ± 0.06 day^{-1} for acetate and 0.24 ± 0.01 day^{-1} for SO_4^{2-} [32]). The computed k for SO_4^{2-} consumption were higher than previously reported by Schroth et al. [33] for the same well without any carbon substrate addition (k = 0.04 to 0.13 ± 0.01 day^{-1}). Similarly, in other aquifers k for SO_4^{2-} consumption without C addition was lower: 0.02–0.08 day^{-1} [34] and 0.04 day^{-1} [35]. The comparisons detailed above are an indication that sulfate reduction was enhanced by the addition of acetate; therefore acetate oxidation was at least in part coupled to sulfate reduction. In addition, previous studies in this aquifer have shown isotopic evidence that sulfate reduction measured during PPTs was attributable to microbial activity [32,36].

Stoichiometric calculations indicated that 0.73–0.78 mol SO_4^{2-} were consumed per mol of acetate. If we assume that all the SO_4^{2-} consumed was used to mineralize acetate at the theoretical 1:1 stoichiometric ratio, about one quarter of the acetate was degraded by other processes. However, we cannot disregard the possibility that SO_4^{2-} was also used as electron acceptor for oxidation of other organic substrates present in the aquifer (e.g., petroleum hydrocarbons). In that case, the proportion

Table 2
Community composition of groundwater and sediment samples as determined by FISH

Probe	Target	% of DAPI-stained microorganisms			
		Groundwater		Sediment	
		BG	After 46 h	BG	After 46 h
Eub338	Eubacteria	49.5 ± 3.5	39.9 ± 3.1	44.5 ± 0. 7	47.6 ± 8.5
SRB385 + SRB385Db	SRB of the δ-Proteobacteria	40.1 ± 3.5	41.9 ± 0.9	41.0 ± 0.9	23.5 ± 4.3
DSB985	*Desulfobacter*	12.0 ± 3.4	15.4 ± 4.6	10.5 ± 2.8	9.2 ± 1.5
Arch915	Archaea	33.1 ± 2.9	29.9 ± 3.7	27.3 ± 4.1	24.0 ± 3.6

of acetate degraded by other processes would be even higher; the latter appears corroborated by our isotopic analyses. The increase of $\delta^{13}C$ in DIC and CH_4 indicates that acetate was degraded concomitantly by at least two processes: (a) it was completely oxidized to CO_2 (presumably by SRB) and (b) it was reduced to CH_4. A mass balance of the ^{13}C content of the catabolic products of acetate revealed that approximately 43% of the absolute amount of ^{13}C derived from acetate degradation went to DIC and 57% to CH_4. The rapid and high ^{13}C-enrichment in CH_4 suggests that CH_4 is directly derived from the methyl group of acetate (which bears the label) and not from reduction of labeled CO_2. Therefore, we may speculate that a substantial part of the acetate was degraded by acetoclastic methanogenesis.

The two above-mentioned processes of acetate mineralization (oxidation and acetoclastic methanogenesis) were detected previously in this well, where stoichiometric calculations suggested that SO_4^{2-} reduction accounted for only 29% of acetate degradation [32]. Although the relative contribution of these processes is somewhat different in our study, the simultaneous consumption of SO_4^{2-} and ^{13}C-enrichment of DIC and CH_4 provides unequivocal evidence that acetate oxidation coupled with sulfate reduction and acetoclastic methanogenesis occurred simultaneously at high concentrations of both electron donor and SO_4^{2-}, at least during the short period of time that this experiment lasted. Our results thus add to a growing body of geochemical and microbiological evidence that several terminal electron-acceptor processes may occur in spatial proximity in a bulk volume of aquifer material [37–40].

4.2. Microbial community structure and its temporal stability

The addition of acetate in concentrations higher than normally measured for this environment could lead to an enrichment of microorganisms adapted to fast growth on acetate. Such enrichment would lead to a shift in the indigenous microbial community. However, PLFA patterns and FISH proportions in both attached and suspended bacteria remained almost identical from the beginning to the end of our experiment. This again shows, as in our previous studies [7,32], that there is no detectable change in the microbial community composition during the length of the experiment, and that the activities measured thus correspond to the potential activity of the microbial community present at that moment in the vicinity of the monitoring well.

We detected high percentages of gram-negative SRB and archaeal communities. Detection of *Desulfotomaculum* species was not attempted with probe S-G-Dtm-02929-a-A-18, because it cannot detect *Desulfotomaculum acetoxidans* [41], the only *Desulfotomaculum* species that could be suspected of acetate consumption in this well. Furthermore, using that probe we were not able to detect any *Desulfotomaculum* in groundwater from this aquifer in a previous study [32]. The decrease in detectability of DAPI-stained cells in some samples (Table 2) could be attributed to the difficulty of visualizing the cells in the samples. This was due to the high clay content in both groundwater and sediment samples, which interfered with the detection of microorganisms at the wavelength used to observe DAPI-stained cells.

4.3. Incorporation of ^{13}C into PLFA

In this study, the enrichment of PLFA extracted from groundwater samples varied from 0‰ to 100‰. The enrichment obtained is in the expected range, taking into account the results we obtained in our previous study (where we observed enrichments of up to 5000‰ in some PLFA [7]), the amount of label used, and the characteristics of the processes targeted in the present study. In our previous experiment ~25% of the acetate-C was ^{13}C in comparison to the present experiment with only ~10% as ^{13}C. Furthermore, nitrate reduction is energetically more favorable than sulfate reduction and therefore SRB incorporate lower relative amounts of carbon into their biomass. Growth yield on toluene, for example, is three times higher in *Pseudomonas* sp. strain K172 (a nitrate-reducing toluene-degrader) with a yield of 57 g cell mol^{-1} toluene [42] than in *Desulfobacula toluolica* PRTOL1 (a sulfate-reducing toluene-degrader) with a yield of 19 g cells mol^{-1} toluene [43].

The pattern of PLFAs that were labeled is different to any known acetate-oxidizing SRB PLFA profile. However, the specific labeling of just some fatty acids suggests that only a few genera are involved in the degradation of acetate. One of the PLFA that incorporated a high proportion of the label, 16:1ω7c, is an important PLFA in most acetate-oxidizing SRB [11] and is considered biomarker for *Desulfotomaculum acetoxidans* [11]. In addition, *D. acetoxidans* grown on acetate produces almost exclusively even-carbon straight-chain PLFA with 16:1ω7c and 18:1ω7c as the major monoenoic acids [11]. In our experiment, enrichment of monoenoic even-chain PLFA accounts for approximately 50% of total enrichment. A similar labeling pattern was found by Boschker et al. [2] after incubating estuarine sediments with ^{13}C-labeled acetate, and they concluded that the main acetate degrader in that sediment was *D. acetoxidans*. Hence, in our experiment, *D. acetoxidans* may have been an important acetate degrader.

On the other hand, 10Me16:0 that was also enriched and cy17:0 that was enriched to a lesser degree have not been detected in any *Desulfotomaculum* species, but have been found instead in high amounts in *Desulfobacter* species [11]. Furthermore, other branched uneven-carbon PLFA, such as i15:0 and a15:0, that were also

labeled in our experiment can account for 2 to 11% of PLFA in *Desulfobacter* grown in acetate but are not important in *D. acetoxidans* [11,44]. Hence, a ^{13}C-enrichment in those PLFA together with a strong presence of *Desulfobacter* detected by FISH suggests that among the suspended populations in this well also *Desulfobacter* utilizes the labeled acetate. In summary, our observations suggest that among the suspended communities of this well both *Desulfobacter* and *D. acetoxidans* play an important role in acetate degradation.

In sediment samples, the ^{13}C-incorporation was an order of magnitude lower than in groundwater samples (compare Fig. 5a and b). This could be explained if we consider that the total C present in attached bacteria is higher than the total C present in suspended bacteria in the same aquifer volume. Therefore, the label incorporated in the PLFA of attached biomass is diluted with more unlabeled C. Unfortunately, a precise estimate of relative contributions of suspended and attached communities to acetate degradation is not possible with the obtained data due to the experimental design. Nevertheless, both suspended and attached communities were active and degraded acetate. This contradicts previous observations by Alfreider et al. [45], who did not detect significant activity in suspended communities. However, their study was conducted in pristine aquifers with extremely low nutrient content. Other authors instead have found that suspended communities are denser and more active in contaminated aquifers than in pristine groundwater, with suspended communities ranging from 1 to 50% of the total aquifer community [46,47].

The enrichment pattern in sediment-derived PLFA indicates activity of only *D. acetoxidans*, because of its similarity with the results obtained by Boschker et al. [2]. In spite of the high abundance of *Desulfobacter*-like bacteria detected with FISH (~10% of total DAPI-stained bacteria), the latter group was probably not degrading a substantial amount of acetate, because PLFA biomarkers for gram-negative SRB were not significantly enriched. Another reason for the failure in detecting enrichment in *Desulfobacter* fatty acids could be that the recovery efficiency of PLFA from sediments is not high enough (expected efficiency is 93 ± 5% [23]), and with a low biomass sample some minor PLFA are likely not recovered. This could be avoided in future studies by extracting PLFA from bigger sediment samples.

In a previous microcosm study performed with sediment from the same aquifer, a *Desulfobacter*-like population increased in microcosms amended with acetate [48]. This was not the case in our field experiment, neither in the suspended nor the attached community. In the field both populations *Desulfobacter* and *D. acetoxidans,* competed effectively for acetate in the suspended microbial fraction at least during the length of the experiment. In contradiction, in the attached community *D. acetoxidans* appeared to dominate acetate degradation.

As observed from the isotopic data, an important part of the acetate was rapidly transformed to CH_4. This shows that the high percentage of Archaea detected with FISH (roughly 30% of total DAPI counts) were actively degrading acetate. One of the main populations of methanogens degrading acetate in this well could be *Methanosaeta concilii,* which is a known acetoclastic methanogen and was previously detected as an important component of the archaeal community in this aquifer [19,49].

5. Conclusions

Using a combination of techniques, we were able to follow acetate mineralization in the field through two different processes, oxidation coupled to sulfate reduction and acetoclastic methanogenesis. We demonstrated that this combination of techniques is appropriate to follow incorporation of C into bacteria that perform respiration with low-energy yield and do not incorporate high amounts of C into their biomass. We found unequivocal evidence that sulfate reduction and methanogenesis occurred simultaneously at high concentrations of both electron donor and SO_4^{2-}. We linked acetate degradation with the activity of suspended populations of *Desulfotomaculum acetoxidans* and *Desulfobacter* sp., and acetoclastic methanogens. Furthermore, within the attached community, only PLFA belonging to *D. acetoxidans* were labeled. Our results thus indicate that both suspended and attached microbial communities contribute to acetate degradation. Finally, the PLFA enrichment patterns observed in this study indicate that bacterial communities degrading acetate in the field differ from communities previously reported to be enriched with acetate in microcosm studies from the same aquifer, demonstrating that care has to be taken when extrapolating laboratory results to the field.

These results show that the isotope labeling technique is a powerful and sensitive tool to study processes at the field scale, which provides new insights on unraveling the delicately balanced interactions between microbial communities in natural habitats.

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