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Field-scale ^{13}C-labeling of phospholipid fatty acids (PLFA) and dissolved inorganic carbon: tracing acetate assimilation and mineralization in a petroleum hydrocarbon-contaminated aquifer

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Abstract

This study was conducted to determine the feasibility of labeling phospholipid-derived fatty acids (PLFA) of an active microbial population with a ^{13}C-labeled organic substrate in the denitrifying zone of a petroleum hydrocarbon-contaminated aquifer during a single-well push-pull test. Anoxic test solution was prepared from 500 l of groundwater with addition of 0.5 mM Br^- as a conservative tracer, 0.5 mM NO_3^-, and 0.25 mM [2-^{13}C]acetate. At 4, 23 and 46 h after injection, 1000 l of test solution/groundwater mixture were sequentially extracted. During injection and extraction phases we measured Br^-, NO_3^- and acetate concentrations, characterized the microbial community structure by PLFA and fluorescent in situ hybridization (FISH) analyses, and determined $^{13}C/^{12}C$ ratios in dissolved inorganic carbon (DIC) and PLFA. Computed first-order rate coefficients were 0.63 ± 0.08 day^{-1} for NO_3^- and 0.70 ± 0.05 day^{-1} for acetate consumption. Significant ^{13}C incorporation in DIC and PLFA was detected as early as 4 h after injection. At 46 h we measured $\delta^{13}C$ values of up to 5614‰ in certain PLFA (especially monounsaturated fatty acids), and up to 59.8‰ in extracted DIC. Profiles of enriched PLFA and FISH analysis suggested the presence of active denitrifiers. Our results demonstrate the applicability of ^{13}C labeling of PLFA and DIC in combination with FISH to link microbial structure and activities at the field scale during a push-pull test. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Petroleum hydrocarbon; Phospholipid fatty acid; ^{13}C-labeling; Whole-cell hybridization; Stable carbon isotope; Denitrification; Push-pull test

1. Introduction

Microbial activities in soils and groundwater are characterized by complex interactions between different microbial populations, availability and characteristics of electron acceptors, carbon and energy sources, nutrients, and physical and chemical properties of the environment [1,2]. Typically, studies on microbial communities in natural environments have focused either on their structure or on their metabolic function. However, linking structure and function is important for understanding microbial community dynamics in natural environments, which remains a challenge, particularly at the field scale [3–5].

Only a small fraction of soil and subsurface microorganisms can be characterized by conventional cultivation techniques [6], thus current knowledge of microbial community structures is often based on either of two main culture-independent methodologies: nucleic acid-based molecular approaches and phospholipid fatty acid (PLFA) analysis. Molecular techniques such as fluorescent in situ hybridization (FISH) and community DNA fingerprinting are widely used to characterize microbial communities [6–8]. Likewise, analysis of microbial PLFA extracted from soils, sediments, or water samples has been used to assess community structure and dynamics in a variety of environments [9–11]. The analysis of PLFA profiles was also used to detect changes in microbial communities that occurred in response to petroleum hydrocarbon (PHC) contamination [12,13], and to infer the presence of active metabolic groups in such environments [14]. Several studies have employed molecular and PLFA analyses in combination to characterize microbial communities [11,13,15].

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Approaches to study microbial functions include analyses of the metabolism of certain substrates in laboratory-grown pure cultures (e.g. see the review on anaerobic metabolism of hydrocarbons by Heider et al. [16]), or in simple consortia obtained by enrichment of environmental samples [17]. However, these results are difficult to extrapolate to natural environments, because such experiments are usually performed under controlled laboratory conditions, which may not necessarily reflect natural conditions. Moreover, complex interactions between different populations are often not considered [18]. On the other hand, microbial activities determined at the field scale provide information on consumption rates and potential metabolic pathways [19], but information on the identity of the microorganisms carrying out a particular process is usually lacking. A method for field-scale activity measurement in aquifers is the so-called push-pull test (PPT) that has been used for the in situ quantification of microbial activities in PHC-contaminated aquifers [20]. This method is based on the injection of a test solution that contains a conservative tracer and one or more reactants in an aquifer through a well. After an incubation period, the mixture of groundwater and test solution is extracted from the same well and analyzed to determine reactant consumption [21,22]. So far, PPTs have been employed to quantify several microbial processes in PHC-contaminated aquifers including aerobic respiration, denitrification, sulfate reduction and methanogenesis [20,23,24], and degradation of PHC constituents under nitrate- and sulfate-reducing conditions [25].

A way to link microbial functions with structure is the method of ^{13}C labeling of biomarker molecules [4,26]. Microorganisms that assimilate a ^{13}C-labeled compound incorporate the label in their macromolecules (e.g. PLFA, amino acids, nucleic acids), thus providing direct evidence of utilization of a specific substrate. This approach has been successfully applied for linking specific populations within complex microbial communities with substrate usage through ^{13}C-enrichment of PLFA biomarkers in soil, sediment, and aquifer microcosm experiments [4,5,27,28]. Recently, C assimilation and C flux within different trophic levels of the food web were analyzed in situ in the intertidal zone using ^{13}C-labeled carbonate [29] (for further information on ^{13}C-labeling of biomarkers in microbial ecology see the review by Boschker and Middelburg [30]). Although the degradation of ^{13}C-labeled organic matter such as algae and acetate has been studied at the field scale in other ecosystems [31,32], to our knowledge, no attempt has been made to follow the degradation of an organic molecule using the PLFA biomarker approach directly at the field scale in a natural ecosystem such as an aquifer.

The objective of this study was to determine the feasibility of detecting ^{13}C-incorporation in PLFA derived from suspended aquifer microorganisms upon degradation of an organic carbon source, i.e. acetate. To this end we performed a PPT, in which [2-^{13}C]acetate was injected into the denitrifying zone of a PHC-contaminated aquifer. We determined consumption rates of acetate and NO_3^-; we also measured ^{13}C-enrichments in the PLFA of suspended microbial populations and in dissolved inorganic carbon (DIC), and characterized the suspended bacterial community structure by combining PLFA and FISH analyses.

2. Materials and methods

2.1. Field site description

The study was conducted in a heating oil-contaminated aquifer in Studen, Switzerland, which was characterized in detail by Bolliger et al. [33]. In 1993, a spill from a leaking underground heating oil pipe was discovered at the site. Engineered remediation was limited to the removal of free-phase heating oil ($\sim$34 m^3) by partial excavation of contaminated soil and by pumping until 1996. At that time engineered remediation was terminated and monitored natural attenuation was selected as the follow-up remediation strategy.

The 20–25-m-thick unconfined aquifer consists of unconsolidated glaciofluvial outwash deposits with interbedded layers of poorly sorted silt, sand and gravel. The groundwater table is generally 2–4 m below ground surface. Hydraulic conductivity ranges from 1.0×10^{-4} to 9.3×10^{-3} m s^{-1}, porosity is estimated at 0.19, and the average pore water velocity is $\sim$0.4 m day^{-1} [33].

The experiment presented in this study was performed in the summer of 2000 in monitoring well P8, which is located at the fringe of the contaminant plume (no free-phase PHC was ever detected in this well). Groundwater in well P8 exhibited a dissolved PHC concentration of 0.07 mg l^{-1}, a low O_2 concentration (0.009 mM), and was partially depleted of NO_3^- (0.069 mM) compared to the upgradient well P20 (0.258 mM NO_3^-), which suggests denitrifying conditions in the vicinity of P8 [33]. This conclusion was supported by results from a preliminary PPT conducted previously in P8, in which we observed substantial NO_3^- and acetate consumption (data not shown).

2.2. Field experiment and sample collection

From well P8, 500 l of groundwater were extracted and collected in a plastic container that was kept under N_2 atmosphere to avoid oxygen diffusion into the groundwater. Test solution was prepared by adding to this water Br^- as a non-reactive, conservative tracer (as NaBr, 0.5 mM final concentration), NO_3^- as electron acceptor (as KNO_3, 0.5 mM), and acetate as carbon source (as NaAc, 0.25 mM). The acetate employed was [2-^{13}C]acetate (Cambridge Isotope Laboratories, MA, USA) diluted 1:1 with unlabeled acetate (Fluka, Buchs, Switzerland). The theoretical, calculated $^{13}C/^{12}C$ ratio (expressed as $\delta^{13}C$)

of acetate in the test solution was ∼21250‰. Injection of the test solution by gravity drainage was completed within 0.57 h. Extraction of the groundwater/test solution mixture was performed sequentially in three steps: 100 l were recovered after 4 h, 400 l after 23 h and 500 l after 46 h, all at a constant flow rate of 6 l min^{-1} using a submersible pump (Grundfos MP-1, Grundfos Pumpen, Fällanden, Switzerland).

Samples were collected from background groundwater (before injection of the test solution), and during both the injection and extraction phase. Samples for dissolved species were filtered in the field through 0.45-μm polyvinylidene fluoride filters (Millipore, Bedford, MA, USA). Samples for pH and alkalinity were collected in 120-ml serum bottles closed without headspace with butyl rubber stoppers. For $\delta^{13}C$ analysis of DIC, unfiltered groundwater samples were collected in 1-l glass bottles closed with rubber stoppers without headspace. These samples were subsequently processed to precipitate DIC as $BaCO_3$ as described by Bolliger et al. [33]. Samples for PLFA extraction were collected in 10-l plastic containers, poisoned with HgCl (1.7 mM final concentration) and kept on ice to stop microbial activities and incorporation of [2-^{13}C]acetate until further processing. Within 10 h, these samples were filtered through glass fiber and 0.2-μm polyvinylidene fluoride filters (Millipore) to collect the suspended biomass, and kept at −20°C until PLFA extraction. For microbial cell counts and FISH analysis, samples of 50 ml were collected in plastic tubes, kept on ice during transport, and processed immediately after arrival in the laboratory.

2.3. Chemical analysis and calculation of in situ reaction rate coefficients

Concentrations of Br^-, NO_3^- and acetate were measured on a DX320 ion chromatograph (Dionex, Sunnyvale, CA, USA). Alkalinity was measured by potentiometric titration using Gran plots for graphical determination of the end point [34], and pH was measured in the laboratory with a MP 225 pH meter equipped with an InLab409 electrode (both Mettler-Toledo, Schwerzenbach, Switzerland). Concentrations of DIC were calculated from alkalinity and pH [34].

First-order reaction rate coefficients for the consumption of NO_3^- and acetate were calculated from extraction breakthrough curves using the method of Haggerty et al. [21]. This method assumes that an injected reactant is transformed within the aquifer according to the first-order type reaction $dC/dt = -kC_r$, where C_r is the reactive solute concentration, and the rate coefficient k can be determined from:

$$\ln\left(\frac{C_r^*(t^*)}{C_{tr}^*(t^*)}\right) = \ln\left[\frac{(1-e^{-kt_{inj}})}{kt_{inj}}\right] - kt^* \quad (1)$$

where C^* is relative concentration (i.e. measured concentration, C divided by concentration in the injected test solution, C_o), subscripts r and tr denote reactant and tracer, respectively, t^* is time elapsed since the end of the test solution injection, and t_{inj} is the duration of test solution injection. The 95% confidence intervals of k ($2\sigma_k$) were computed from the variance of the estimated k as described by Schroth et al. [23].

Stoichiometric ratios, SR (mol NO_3^- per mol acetate consumed) were calculated from extraction breakthrough curves for each sample point using:

$$SR = \frac{(C_{tr}^* - C_{NO_3}^*)C_{o,NO_3}}{(C_{tr}^* - C_{Ac}^*)C_{o,Ac}} \quad (2)$$

2.4. PLFA analysis

Total lipids were extracted from microbial biomass collected on the filters by a modified Bligh–Dyer method [35] and were further fractionated to neutral, glyco- and phospholipids by column chromatography on silica gel (ICT, Basel, Switzerland) as described previously [36]. The phospholipids were dried and derivatized into fatty acid methyl esters, separated by gas chromatography (Hewlett Packard HP 5890 series II equipped with a HP Ultra 2 capillary column) under MIDI™ standard conditions and quantified using a FID detector. PLFA with chain lengths of 9 to 20 carbon atoms were identified employing the MIDI™ Microbial Identification System using the TBSA40 peak library (MIDI, version 4.0). A mass spectrometer (GCQ Finnigan MAT, Bremen, Germany) was used for additional verification of peak identity. The nomenclature used for the PLFA 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 and PLFA

For $\delta^{13}C$ 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).

The $^{13}C/^{12}C$ measurements in PLFA were carried out on a Finnigan MAT 252 isotope ratio mass spectrometer (Finnigan, Bremen, Germany) coupled via combustion interface to a Hewlett Packard HP 5890 gas chromatograph, which was equipped with an HP Ultra 2 capillary column and operated as described by Abraham et al. [37]. The

column effluent was combusted to CO_2 on-line in an oxidation furnace (copper–nickel–platinum catalyst, 980°C). The combustion gas was dried and passed through a reactor with elemental copper (600°C) to remove surplus O_2 and reduce NO_x prior to $^{13}C/^{12}C$ ratio measurement.

All stable C-isotope data are reported using the standard δ notation:

$$\delta^{13}C\ (‰) = [(R_{sample}/R_{VPDB})-1]\times 10^3 \quad (3)$$

where R_{sample} 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 as described previously [37].

2.6. Fluorescence in situ hybridization

Biomass from 50 ml of groundwater was obtained by centrifugation, fixed and stored at −20°C as described by Bolliger et al. [38]. Before analysis, the samples were dispersed by mild sonication with a probe of 2 mm diameter during 1 min at 20% power (Sonifier B-12, Branson, Danbury, CT, USA). Aliquots of 10–20 μl were applied to glass slides, dried at room temperature and dehydrated by sequential immersion during 3 min in 50, 80 and 100% ethanol. The bacterial cells were stained with 4′,6-diamidino-2′-phenylindole (DAPI) (Sigma, Buchs, Switzerland) and hybridized with fluorescently labeled 16S rRNA-targeted nucleotide probes as described by Zarda et al. [39]. The probes used were: Eub338 targeting Eubacteria [40], Alf1b, Bet42a, Gam42a, and SRB385 for α-, β-, γ-, and δ-Proteobacteria, respectively [41,42]. Slides were mounted with Citifluor solution (Canterbury, UK) and analyzed with a Zeiss microscope equipped for epifluorescence using the appropriate filters at a 400× magnification [39].

3. Results

3.1. Consumption of electron acceptor and carbon sources

Breakthrough curves of Br^-, acetate and NO_3^- showed a sharp decline at the beginning of the extraction due to dilution of the test solution with native groundwater (Fig. 1a). Throughout the extraction phase, relative concentrations of NO_3^- and acetate were lower than relative Br^- concentration, indicating acetate and NO_3^- consumption during the test. Moreover, cumulative relative recovered masses of NO_3^- and acetate (obtained by integrating breakthrough curves in Fig. 1a) were lower than the relative recovered mass of Br^-, which also indicated consumption of reactants (Fig. 1b). Computed first-order rate coefficients (Eq. 1) were 0.63 ± 0.08 day^{-1} ($k \pm 2\sigma_k$) for NO_3^-

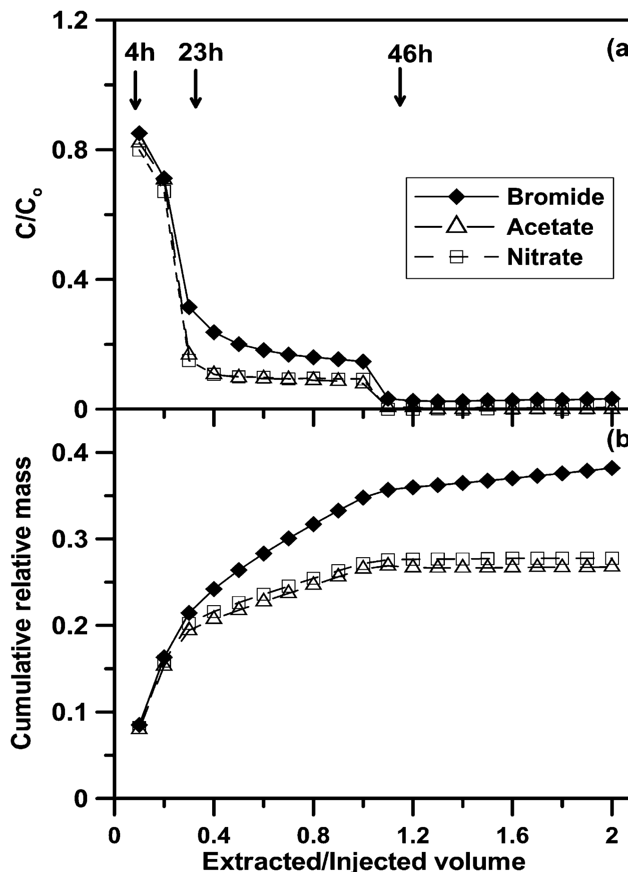

Fig. 1. Breakthrough curves showing (a) relative concentrations and (b) cumulative relative mass (i.e. cumulative mass extracted/total mass injected) recovered for Br^-, NO_3^- and acetate during the PPT vs. the relative cumulative extracted volume (cumulative volume extracted divided by the total injected volume of test solution). Arrows indicate the starting time of the three extractions.

and 0.70 ± 0.05 day^{-1} for acetate consumption, which is equivalent to a half-life of 1.1 day for NO_3^- and 1.0 day for acetate. Calculated stoichiometric ratios (Eq. 2) changed little during the PPT (data not shown); thus, we present only an average SR value, which was 2.3 ± 0.3 mol NO_3^- per mol acetate consumed.

3.2. Concentration and stable carbon isotope ratios of DIC

Measured DIC concentrations did not vary significantly during the experiment and ranged from 9.3 to 12.8 mM (data not shown). We were unable to accurately calculate the DIC produced during the test, because the amount of produced DIC was too small compared to the background DIC concentration. Assuming that the total amount of consumed acetate (39.5% of the injected acetate, Fig. 1b) was mineralized and no assimilation occurred, the theoretical maximum amount of produced DIC would be ∼198 mmol in 1000 l of extracted test solution/groundwater mixture, while ∼11 000 mmol of background DIC were present in the same volume. However, detectable ^{13}C-enrichments in extracted DIC were measured at early stages of the experiment (Fig. 2). The $\delta^{13}C$ value of the

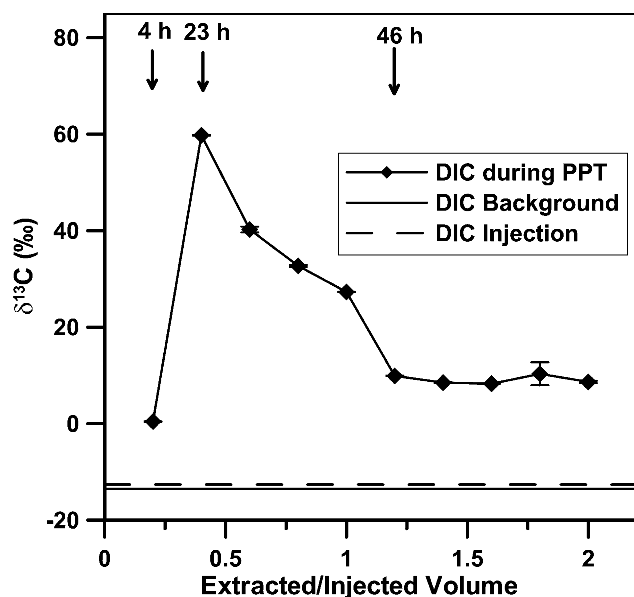

Fig. 2. Values of $\delta^{13}C$ measured in DIC during the experiment. The solid line at −13.5‰ is the $\delta^{13}C$ value measured in DIC of the background groundwater (before the experiment), and the dashed line is the $\delta^{13}C$ value measured in DIC of the injection solution.

background DIC was −13.48 ± 0.12‰ and already 4 h after the injection $\delta^{13}C$ values in extracted DIC increased to +0.45 ± 0.07‰. After 23 h the $\delta^{13}C$ value increased to +59.78 ± 0.10‰ and thereafter decreased rapidly because test solution was highly diluted by native groundwater towards the end of the extraction phase.

3.3. Analysis of PLFA profiles

Thirty-three PLFA were detected in the samples, but only 22 that were present in most of the samples were considered for this analysis. The typical chromatogram showed that the dominant compounds on the PLFA profile were 18:1ω9c (48.8%), 18:0 (7.3%), 18:1ω7c (6.9%), 16:0 (5.2%) and 18:2ω6c (5.5%) (Fig. 3). Unsaturated PLFA 16:1ω7c, 17:1ω8c, i18:1, 18:1ω6c, as well as terminal branched PLFA i17:0, a17:0, i18:0, i19:0 and saturated PLFA 17:0, 20:0 and cy19:0 were present in concentrations of 1–3%. We also detected traces of 10Me17:0 and 10Me18:0, some polyunsaturated fatty acids such as 18:3ω6c and 20:4ω6c, as well as saturated PLFA 14:0 and 15:0. A comparison of background groundwater samples with samples collected at 4, 23 and 46 h revealed that the PLFA profiles remained essentially identical during the test (data not shown).

3.4. Isotopic measurements of PLFA

The average $\delta^{13}C$ value of PLFA from the background samples was −28 ± 3.5‰ (Fig. 4). After 4 h of incubation, there was detectable ^{13}C-enrichment in several PLFA, and the majority was ^{13}C-enriched towards the end of the experiment. The highest ^{13}C-enrichment was found in the PLFA with chain length of 16 carbons. After 46 h of incubation the $\delta^{13}C$ values of these fatty acids were +5614‰ (16:1ω7c), +4196‰ (16:1ω5c), and +1154‰ (16:0). Terminal branched PLFA of 14 and 17 carbon chain lengths (i14:0, a14:0 and i17:0) were ^{13}C-enriched to a smaller extent ($\delta^{13}C$ ranging between +100 and 200‰), as compared to 10Me17:0 and 10Me18:0, which were not ^{13}C-enriched throughout the experiment.

We presented the $\delta^{13}C$ values of 18:1ω9c, 18:1ω7c and 18:1ω6c as one value for all three fatty acids, because these compounds could not be baseline-separated under the GC-IRMS chromatographic conditions used. As a consequence, in most of the samples it was not possible to obtain the $\delta^{13}C$ value for each individual peak (data not shown). However, in cases where separation was possible, we determined that the enrichment of the combined peak was mainly due to the enrichment of 18:1ω7c. For example, after 23 h of incubation, the $\delta^{13}C$ values were −9‰ for 18:1ω9c, +621‰ for 18:1ω7c and +118‰ for 18:1ω6c ($\delta^{13}C$ for the three fatty acids combined in this

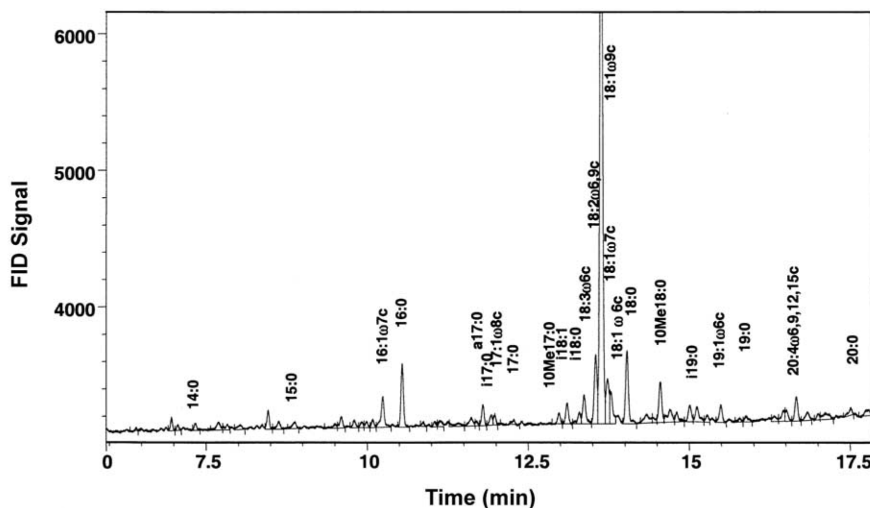

Fig. 3. A typical PLFA chromatogram as recorded by gas chromatography using a FID detector.

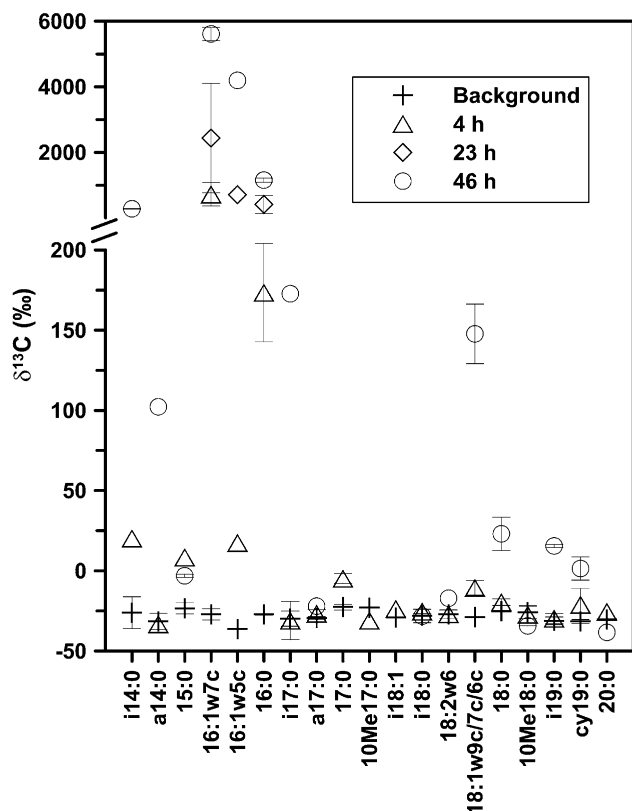

Fig. 4. $\delta^{13}C$ value of PLFA extracted form suspended bacteria in the groundwater before (BG) and during the experiment at 4, 23 and 46 h.

sample was +80‰). Note that we were unable to determine the $\delta^{13}C$ value of 20:4ω6c due to insufficient sample amounts. Fig. 4 also shows $\delta^{13}C$ values of i14:0, a14:0 and 16:1ω5c. The peaks of these PLFA were small and were therefore not identified by the MIDI system (Fig. 3), but they were subsequently positively identified by mass spectrometry.

3.5. In situ hybridization

Bacterial cell numbers in groundwater samples determined by DAPI staining were low throughout the test (10^5–10^6 bacteria ml^{-1}, data not shown). In background groundwater 37.3% of DAPI-stained bacterial cells hybridized with probe EUB338, and this number increased to 57.6% at 46 h (Table 1). The majority of EUB-hybridized cells were β-Proteobacteria (39–57% of EUB). The α-Proteobacteria represented 23–28% of EUB and the γ-Proteobacteria around 15% of EUB. Bacterial cells belonging to the δ-Proteobacteria (sulfate-reducing bacteria) were detected, but they represented less than 1% of Eubacteria.

4. Discussion

4.1. Quantification of acetate and NO_3^- consumption

Extraction phase breakthrough curves indicated NO_3^- and acetate consumption in well P8. Acetate degradation under nitrate-reducing conditions was chosen for this experiment, because it is generally a fast process and energetically the most favorable anaerobic respiration [1]. Computed first-order rate coefficients (k) of 0.70 ± 0.05 day^{-1} for acetate and 0.63 ± 0.08 day^{-1} for NO_3^- consumption in our experiment were in the same range as those obtained in the same aquifer under acetate-enhanced, sulfate-reducing conditions (with computed k values of 0.6 day^{-1} for acetate and 0.25 day^{-1} for sulfate consumption [43]). Similarly, our estimates of k are within the range of previously published rate coefficients (or rates) on NO_3^- consumption in other aquifers [44]. We have previously demonstrated that this method to determine k is highly reproducible and accurate [23,24].

The computed average stoichiometric ratio of 2.3 ± 0.3 mol NO_3^- per mol acetate consumed substantially exceeds the theoretical nitrate/acetate consumption ratio of 1.6, assuming complete acetate mineralization. This suggests that NO_3^- is used not only as terminal electron acceptor for acetate degradation, but also for degradation of other organic substrates present in the vicinity of well P8 (e.g. PHC or their metabolites). Abiotic reduction of NO_3^- by reduced species such as Fe(II) could also explain some of the NO_3^- consumption, but it is not usually considered to be very important in soils or aquifers [44]. Furthermore, some NO_3^- may have been assimilated by microorganisms for newly synthesized cell material.

4.2. Mineralization and assimilation of ^{13}C-labeled acetate

In this experiment, the $\delta^{13}C$ measured in the DIC revealed that a portion of the acetate was mineralized in the aquifer, already at early stages of the test (only 4 h after the injection). But an exact mass balance on [2-^{13}C]acetate could not be calculated in this case, because of the low precision in the computation of produced DIC as discussed before, and because we were unable to quantify the amount of acetate that was assimilated in new cell material of both attached and suspended microorganisms. On the other hand, the significant ^{13}C-enrichment detected in total DIC clearly demonstrated the high sensitivity of

Table 1
Community composition of groundwater samples as determined by FISH with the fluorescently labeled rRNA-targeted oligonucleotide probes EUB338, Alf1b, Bet42a, Gam42a and SRB385

Probe	Target	Relative abundance (% of DAPI)	
		BG	46 h
EUB338	Bacteria	37.3 ± 2.8	57.6 ± 2.2
Alf1b	α-Proteobacteria	10.7 ± 3.6	13.3 ± 3.1
Bet42a	β-Proteobacteria	21.4 ± 4.1	22.6 ± 1.2
Gam42a	γ-Proteobacteria	5.9 ± 1.7	8.2 ± 5.5
SRB385	δ-Proteobacteria	< 1	< 1

Samples are from the background (BG) groundwater (before injection) and 46 h after injection.

this technique, even when employed in situ in an open system such as an aquifer.

In spite of the low suspended bacterial biomass present in groundwater of well P8 (10^5–10^6 bacteria ml^{-1}), substantial ^{13}C incorporation in PLFA showed bacterial [2-^{13}C]acetate assimilation even at early stages during the experiment. Moreover, $\delta^{13}C$ values as high as $5614 \pm 204‰$ for 16:1ω7c after 46 h are a clear indication of the high sensitivity of the method. This suggests that the amount of labeled compound used in future experiments can be reduced.

4.3. Community structure and activity

The PLFA composition and the relative distribution of different bacterial groups analyzed by FISH remained fairly constant during the experiment, which indicated a stable composition of the suspended microbial community during the course of the experiment. This is an important result because the purpose of our experiment was the detection of metabolically active bacteria in the aquifer without substantially changing the original community composition. But we are aware that because of the experimental design we cannot be certain that the microbial community attached to the aquifer matrix remained unchanged. FISH analysis revealed an increase in the percentage of cells detected by the EUB338 probe during the experiment (from 37.3 to 57.6% of total DAPI counts), which may reflect an increase of bacterial activity and rRNA content rather than a change in community composition [45].

Denitrifying bacteria are a phylogenetically diverse group, mainly composed of Gram-negative bacteria, affiliated to α-, β-, and γ-Proteobacteria [46]. The FISH analysis of suspended bacterial cells collected during our experiment demonstrated that α-, β-, and γ-Proteobacteria accounted for 100% of eubacterial cells and that eubacteria were an important part of the microbial community (37–57% of the total DAPI counts) (Table 1) suggesting that they could be responsible for the denitrifying activities in this part of the aquifer.

The dominance of monounsaturated fatty acids, as observed in our experiment (monounsaturated PLFA represented over 65% of the total fatty acids) can be interpreted as an indication of a large population of Gram-negative bacteria within the microbial community [47]. However, previous studies acknowledged a weakness in using PLFA biomarkers to subdivide communities of Gram-negative bacteria because of the lack of dominant PLFA that could differentiate populations belonging to either α-, β-, or γ-Proteobacteria [13,14,28]. The ^{13}C-enrichment of mainly monounsaturated PLFA together with the importance of α-, β-, and γ-Proteobacteria suggested that these bacteria were responsible for [2-^{13}C]acetate assimilation in the aquifer during our experiment. This is in agreement with a previous study, in which we incubated denitrifying microcosms with toluene labeled with ^{13}C at the methyl group and sediments from this aquifer [28]. Results from this study revealed that only 16:1ω7c/t, 16:0, cy17:0, and 18:1ω7c were ^{13}C-enriched and a comparison of the ^{13}C-labeling profile of PLFA with that of pure cultures and supplementary FISH analysis enabled us to link toluene degradation to *Azoarcus* sp. (β-Proteobacteria) and related species.

Some PLFA that were only abundant in low relative amounts, e.g. i14:0, a14:0, 18:0, 18:2ω6c and 19:1ω6c, were ^{13}C-enriched towards the end of the experiment (46 h). The polyunsaturated PLFA 18:2ω6c is a marker for fungi [48], although it has also been found in some protozoa of marine or clinical origin [49,50]. This PLFA was significantly ^{13}C-enriched at 46 h, which might indicate a C transfer from bacteria to fungi or protozoa. Sequential enrichment of PLFA characteristic for different groups of organisms, such as Eubacteria, cyanobacteria, or Eukarya was also observed in another study [29].

In summary, in this study we linked acetate assimilation in situ in an aquifer to indigenous microorganisms through ^{13}C-labeling of microbial PLFA, while simultaneously providing quantitative information on substrate consumption. This is a first step to extend our previous work on linking substrate degradation to specific microbial populations in PHC-contaminated environments [18] to the field scale. We plan to perform future tests using the approach described in this paper in areas of the aquifer in which we expect the presence of bacteria that possess more distinctive PLFA biomarkers (for example sulfate-reducing or methanotrophic bacteria) and also extend the analysis to microorganisms attached to the aquifer matrix. In these zones the incorporation of ^{13}C in the biomarkers could provide an irrefutable link between function and structure of microbial communities [3,18].

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