ELSEVIER

FEMS Microbiology Ecology 38 (2001) 123–131

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

# Tracing toluene-assimilating sulfate-reducing bacteria using $^{13}$C-incorporation in fatty acids and whole-cell hybridization

Oliver Pelz [a,*], Antonis Chatzinotas [b], Annatina Zarda-Hess [a], Wolf-Rainer Abraham [c], Josef Zeyer [a]

[a] *Institute of Terrestrial Ecology, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland*
[b] *Department of Rural Engineering, Swiss Federal Institute of Technology (EPF), Lausanne, Switzerland*
[c] *Division of Microbiology, GBF-National Research Centre for Biotechnology, Braunschweig, Germany*

Received 6 July 2001; received in revised form 28 September 2001; accepted 1 October 2001

First published online 8 November 2001

**Abstract**

Polar lipid-derived fatty acids (PLFA) commonly found in sulfate-reducing bacteria were detected in high abundance in the sediment harvested from a monitoring well of a petroleum-hydrocarbon (PHC)-contaminated aquifer. Aquifer microcosms were incubated under sulfate-reducing conditions with [methyl-$^{14}$C]toluene to determine the $^{14}$C-mass balances and with [methyl-$^{13}$C]toluene to follow the flow of carbon from toluene into biomarker fatty acids. An aliquot was used to establish an aquifer-derived toluene-degrading sulfate-reducing consortium, which grew well in liquid medium. Whole-cell hybridization using 16S rRNA-targeted oligonucleotide probes specific for different phylogenetic levels within the sulfate-reducing bacteria was applied in order to characterize the sulfate-reducing populations in the original sediment, the aquifer microcosms, and the aquifer-derived consortium. In the aquifer microcosms, the $^{14}$C quantification revealed that 61.6% of the [methyl-$^{14}$C]toluene was mineralized and 2.7% was assimilated. Following [methyl-$^{13}$C]toluene depletion ($<1$ μM), the highest $^{13}$C-enrichment was found in PLFA 16:1ω5c. In addition, biomarker fatty acids characteristic for the genera *Desulfobacter* and *Desulfobacula* (cy17:0 and 10Me16:0) were also $^{13}$C-enriched, contrary to those of other sulfate-reducing genera, e.g. *Desulfovibrio* and *Synthrophobacter* (i17:1ω7c), *Desulfobulbus* and *Desulforhabdus* (15:1ω6c and 17:1ω6c). Although hybridization detection rates remained low, indicating low bacterial activities, 43% (aquifer sediment) and 30% (aquifer microcosm) of the total active bacteria belonged to the Desulfobacteriaceae thus supporting the PLFA-based results. *Desulfobacter*-species (42%), which belong to the Desulfobacteriaceae, dominated the community of the consortium. Our study showed that carbon stable isotope analysis in combination with whole-cell hybridization could link toluene degradation in aquifer microcosms to the metabolic activity of the *Desulfobacter*-like populations. These populations could play an important role in the clean up of aromatic PHC-contaminated aquifers. 

*Keywords:* Petroleum-hydrocarbon-contaminated aquifer; Polar lipid fatty acid; Sulfate-reducing bacteria; Stable carbon isotope; Toluene; Whole-cell hybridization

## 1. Introduction

Microbial sulfate reduction is an important metabolic activity in many petroleum-hydrocarbon (PHC)-contaminated aquifers as contamination with mono-aromatic PHC, e.g. benzene, toluene, ethylbenzene, is of regulatory concern due to their solubility and toxicity [1]. Sulfate reduction can be coupled to the bacterial metabolism of mono-aromatic PHC, and thus has gathered increasing interest as an intrinsic remediation process [2,3]. Laboratory studies have already demonstrated the complete mineralization of mono-aromatic PHC by an unidentified, aquifer-derived consortium utilizing sulfate as the terminal electron acceptor [4]. The known mono-aromatic-PHC-utilizing, sulfate-reducing isolates are all affiliated within the family Desulfobacteriaceae (e.g. *Desulfobacula*) that comprises species of completely oxidizing sulfate-reducing bacteria [5].

Phylogenetic analyses based on 16S rRNA gene sequences divide the diverse group of sulfate-reducing bacteria in four distinct groups: Gram-positive spore-forming Eubac-

* Corresponding author. Present address: Product Safety, GUP/P - Z470, BASF AG, D-67056 Ludwigshafen, Germany. Tel.: +49 621 6058385.
*E-mail address:* oliver.pelz@basf-ag.de (O. Pelz).

0168-6496/01/$20.00 
PII: S0168-6496(01)00186-6

teria, Gram-negative mesophilic Eubacteria, thermophilic Eubacteria, and thermophilic Archaea [6]. Hybridization probes and polymerase chain reaction (PCR) primers directed toward different distinct groups within the sulfate-reducing bacteria have been developed, the majority of which target Gram-negative mesophilic bacteria [7–10]. Recently, however, hybridization probes specific for the Gram-positive spore-forming genus *Desulfotomaculum* and sub-groups within this genus have been designed [11]. Probing of sulfate-reducing marine enrichment cultures with 16S rRNA-targeted oligonucleotides has revealed that the majority of a mono-aromatic-PHC-degrading population of bacteria belonged to the family Desulfobacteriaceae [12,13]. The identities of sulfate-reducing populations responsible for the mono-aromatic-PHC degradation in contaminated freshwater aquifers are not well known. Thus, it remains unclear if mono-aromatic-PHC-mineralizing members of the Desulfobacteriaceae also play major roles in the mono-aromatic-PHC degradation in contaminated freshwater aquifers.

The lipid composition of the Gram-negative sulfate-reducing bacteria, especially polar lipid-derived fatty acids (PLFA), have been studied extensively and several uncommon PLFA, e.g. cy17:0, 10Me16:0, i17:1ω7c, 15:1ω6c and 17:1ω6c, have been suggested as specific biomarkers for the different groups of sulfate-reducing bacteria [14–18]. Although these PLFA may also be found in non sulfate-reducing bacteria (e.g. 10Me16:0 in actinomycetes), they are suitable to distinguish among the different groups of sulfate-reducing bacteria. They were applied as biomarkers in sulfate and carbon source-stimulated in situ studies to identify the active part of sulfate-reducing populations in marine environments [19,20]. Stable carbon isotope incorporation in PLFA was applied recently to provide evidence for the identity of the sulfate-reducing bacteria involved in acetate or propionate metabolism in anoxic sediments [21,22]. In these studies, PLFA analyses suggested that acetate or propionate added as $^{13}$C-labeled compounds were preferentially consumed by different, specialized groups of sulfate-reducing bacteria. However, nothing has been done to trace carbon fluxes during PHC degradation under sulfate-reducing conditions.

The goal of the present study was to combine [methyl-$^{13}$C]toluene incorporation in PLFA with 16S rRNA-targeted oligonucleotide probing to characterize the populations involved in toluene degradation in PHC-contaminated freshwater aquifers under sulfate-reducing conditions. The methods were applied to complex microbial communities of (i) freshly obtained sediment from a monitoring well of a PHC-contaminated aquifer, (ii) aquifer microcosms amended with toluene and sulfate, and (iii) a toluene-degrading, sulfate-reducing consortium.

## 2. Materials and methods

### 2.1. Set-up of microcosms

Sediment was taken with a grab sampler from the mud-pit of a monitoring well (S6) from a PHC-contaminated aquifer in Studen, Switzerland [23]. The groundwater at this monitoring well contained 3 μM oxygen, nitrate concentrations below the detection limit ($<1$ μM), and 156 μM sulfate. The sediment was characterized by high amounts of unsaturated PHC (9180 mg kg$^{-1}$) representing approximately 20% of the total organic carbon (stable carbon isotope ($\delta^{13}$C) value, $-26.7$‰) and low concentrations of mono-aromatic PHC, including 1.4 μM toluene [24]. Throughout the study, all aquifer sediment samples were immediately analyzed following harvest.

The aquifer microcosms were prepared in duplicate series and maintained (statically incubated in the dark at 12°C) as previously described, using the aquifer sediment, toluene (final concentration, 0.6 mM), either [methyl-$^{13}$C]toluene ($\delta^{13}$C, $+80.7 \pm 8.9$‰, Chemotrade, Leipzig, Germany), [methyl-$^{14}$C]toluene (7800 dpm ml$^{-1}$, Pathfinder Laboratories, St. Louis, MO, USA), or unlabeled toluene and $Na_2SO_4$ (final concentration, 10 mM) [24]. Two additional microcosm series were used as controls: autoclaved and biotic controls were inoculated with the aquifer sediment samples and incubated in parallel with the above described treatments. The autoclaved control (autoclaved twice on successive days, before sulfate and either unlabeled toluene or [methyl-$^{14}$C]toluene was added) was used to estimate non-enzymatic processes (sorption, losses, etc.). The biotic control was unautoclaved and was used to assess the behavior of the microbial community driven by the use of native organic carbon sources in the aquifer sediment and therefore, incubated under sulfate-reducing conditions, as described above, without toluene addition.

The inoculations with [methyl-$^{13}$C]toluene and [methyl-$^{14}$C]toluene were terminated after total depletion of toluene ($<1$ μM) and $^{14}$C quantification was performed as previously described [25] using a Tri-Carb 2200CA liquid scintillation analyzer (Packard instruments, Downers Grove, IL, USA). Due to high carbonate concentrations in sediments, two extractions of $^{14}CO_2$ were performed and it was not possible to extract and quantify $^{14}CH_4$ with our methods.

To determine changes in the community induced by incubation within the microcosms, aquifer sediment from the monitoring well and 1-ml sub-samples from the aquifer microcosm with unlabeled toluene were examined by staining of bacterial cells with 4,6-diamidino-2-phenylindole (DAPI) and whole-cell hybridization with Cy3-labeled 16S rRNA-targeting gene probes. The data from this study, e.g. concentrations of oxidants, reduced species, $\delta^{13}$C values and $^{14}$C quantification, were generated using a combination of triplicate measurements from each of the

duplicate samples. Statistical analyses were performed using the Student's *t*-test (one-tailed distribution, homoscedastic analysis ($P < 0.05$).

### 2.2. Enrichment of a toluene-degrading, sulfate-reducing consortium and cultivation of toluene-degrading strains

A toluene-degrading, sulfate-reducing consortium was enriched from the aquifer sediment because identities of sulfate-reducing populations responsible for the mono-aromatic-PHC degradation in contaminated freshwater aquifers are not well known. After toluene was mostly depleted in the aquifer microcosm, an aliquot of the sediment sludge (10% inoculum, v/v) was transferred to fresh liquid medium. The medium for the enrichment culture and its incubation conditions were identical to those described for the microcosms. As toluene degradation occurred, additional substrate was added by injecting pure toluene with a syringe (0.25 mM, final concentration). Sulfate was also replenished to a final concentration of 10 mM when concentrations decreased below 0.1 mM. Further enrichments were prepared by transferring only the liquid portion of the primary enrichment culture (10% inoculum, v/v) into fresh medium. A toluene-degrading, sulfate-reducing consortium was obtained, which grew well in liquid medium in the absence of aquifer sediment.

### 2.3. Analysis of toluene, oxidants and reduced species

Toluene, $O_2$, $CO_2$, and $CH_4$ were analyzed by standard methods using a Carlo Erba 8370 gas chromatograph (Rodano, Italy) equipped with a flame ionization detector or thermal conductivity detector and sulfate with a Dionex DX-100 ion chromatograph (Sunnyvale, CA, USA) as previously described [24]. Concentrations of dissolved $CH_4$ and $CO_2$ were calculated according to [23] based on the partial pressures of the gases in the headspace and Henry's Law using the Henry constants for $CH_4$ (0.00206 mol $l^{-1}$ $atm^{-1}$) and $CO_2$ (0.0594 mol $l^{-1}$ $atm^{-1}$).

### 2.4. Analysis of microbial community structure by whole-cell hybridization

The microbial community structure in samples of (i) aquifer sediment, (ii) aquifer microcosms and (iii) the consortium was analyzed by staining the bacterial cells with DAPI (Sigma, Buchs, Switzerland) and fluorescently labeled 16S rRNA-targeted oligonucleotide probes as described recently [24,26]. Whole-cell hybridization was performed using the Cy3-labeled oligonucleotide probes EUB338 (Bacteria) [7], Arch915 (Archaea) [27], LGC (low GC Gram-positive bacteria) [28], Gam42a (γ-Proteobacteria) [29], SRB385Db (Desulfobacteriaceae) [30], SRB385 (Desulfovibrionaceae) [31], 660 (*Desulfobulbus* spp.) [8], DSV698 (*Desulfovibrio* spp.), DSV1292 (*Desulfovibrio* spp.), and DSB985 (*Desulfobacter*-like bacteria) [9].

### 2.5. Extraction and analysis of PLFA

Total lipids were extracted from samples of (i) 15 g aquifer sediment, (ii) 20 ml sediment sludge of the aquifer microcosms, (iii) 50 ml culture of the consortium and (iv) 50 ml pure cultures of the two strains mentioned below, as described previously [32]. Lipids were further fractionated in neutral lipids, glycolipids and polar lipids by column chromatography on silica gel (ICT, Basel, Switzerland). The polar lipids were dried, and fatty acid methyl esters were generated and identified by gas chromatography according to a standard procedure [32]. The PLFA nomenclature was as previously described [24]. The $\delta^{13}C$ values of PLFA were measured on a 252 FinniganMAT isotope ratio mass spectrometer (Bremen, Germany) interfaced to a Hewlett Packard 5890 gas chromatograph, which was equipped with a Hewlett Packard HP Ultra 2 column [32].

To identify characteristic PLFA of toluene-degrading, sulfate-reducing bacteria, PLFA profiles of *Desulfobacula toluolica* (DSM 7467) [33] and the uncharacterized strain PRTOL1, isolated with toluene from fuel-contaminated soil [34], were analyzed. The medium and cultivation condition of these strains was as described above for the microcosms.

## 3. Results

### 3.1. Characterization of the PHC-contaminated aquifer

Whole-cell hybridization of the aquifer sediment revealed that besides the general Bacteria-specific probe (EUB338) only the probes SRB385Db and SRB385 could detect cells in significant amounts. Hybridization detection rates with EUB338 were low (14% of the total of DAPI-stained microorganisms), indicating low bacterial activities due to low cellular rRNA contents. 43% of the active and thus detectable microorganisms (microorganisms hybridized with probe EUB338) belonged to the Desulfobacteriaceae (Table 1). A considerable abundance of active archaeal cells (5%) was also detected in this sediment. The presence of active Archaea was also evidenced through relatively high amounts of dissolved $CH_4$ (between 0.06 and 0.08 mM), the product of methanogenic activity, in the aquifer microcosms at $T_0$ (Table 2).

The PLFA profile of the aquifer sediment was dominated by 16:0, 16:1ω7c, 18:1ω7c and characterized by even-numbered fatty acids (75%), which were 53% mono-unsaturated and 24% unbranched, saturated compounds (Fig. 1A). The two unusual compounds cy17:0 and 10Me16:0 were detected in relatively large amounts ($3.2 \pm 0.5\%$ and $6.8 \pm 1.0\%$). The averaged $\delta^{13}C$ values of all PLFA ($-28.9 \pm 2.8$ ‰) were closely related to the value of the total organic carbon from the aquifer sediment ($-26.7$ ‰). The $\delta^{13}C$ value of 16:1ω5c ($-40.3 \pm 3.8$ ‰)

Table 1
Relative cell amounts of microbial groups in freshly obtained aquifer sediment, the aquifer microcosm, and the aquifer-derived consortium obtained after DAPI-staining and whole-cell hybridization ($n = 30$; means ± S.D.) using specific rRNA-targeting probes

| Probe | Target | (% of DAPI-stained bacterial cells) | | |
|---|---|---|---|---|
| | | aquifer sediment | aquifer microcosm[a] | aquifer-derived consortium |
| EUB338 | Bacteria | 14 ± 7 | 30 ± 6 | 76 ± 9 |
| Arch915 | Archaea | 5 ± 2 | 4 ± 2 | 9 ± 5 |
| LGC | low GC Gram-positive bacteria | n.d.[b] | n.d. | 1 ± 2 |
| Gam42a | γ-Proteobacteria | n.d. | n.d. | 19 ± 7 |
| SRB385Db | Desulfobacteriaceae | 6 ± 3 | 9 ± 5 | 50 ± 11 |
| SRB385 | Desulfovibrionaceae | 1 ± 1 | 5 ± 5 | 6 ± 2 |
| DSB985 | *Desulfobacter*-like bacteria | n.q.[c] | n.q. | 42 ± 13 |
| DSV698 | *Desulfovibrio* spp. | n.q. | n.q. | 3 ± 4 |
| DSV1292 | *Desulfovibrio* spp. | n.q. | 1 ± 2 | 2 ± 4 |
| 660 | *Desulfobulbus* spp. | n.q | n.q. | 3 ± 1 |

[a]After toluene depletion.
[b]Not determined.
[c]Not quantifiable (< 0.5% abundance).

was significantly depleted, relative to the averaged value of all PLFA (Fig. 1B).

### 3.2. Characterization of the aquifer microcosm

Sulfate consumption in aquifer microcosms amended with toluene (8.7 ± 1.5 mM) was not significantly different from that of the biotic controls (6.7 ± 2.8 mM). A stoichiometric calculation shows that 4.5 M sulfate is required to degrade 1 M toluene, and thus, the degradation of 0.6 mM toluene in our microcosms accounted for 2.7 mM consumed sulfate (31% of the total of reduced sulfate) revealing that large amounts of native carbon were mineralized besides toluene (Table 2). During mineralization of native carbon sources and toluene, elevated $CO_2$ and $CH_4$ concentrations were observed, indicating that sulfate reduction and methanogenesis were both important terminal oxidation processes in the aquifer microcosms under the applied conditions. The $^{14}C$ quantification revealed that 61.6% of the toluene was mineralized to $CO_2$, whereas only 2.7% of the [methyl-$^{14}C$]toluene was assimilated into bacterial carbon (filter fraction) (Table 2).

Whole-cell hybridization of aquifer microcosm sub-samples revealed an increase in numbers of active cells detected by the EUB338 probe, which indicated a raise in bacterial activity and rRNA content. In comparison to the aquifer sediment, only small differences with respect to the structure of the sulfate-reducing community were determined (Table 1). In the aquifer microcosms, cells belonging to specific sub-groups within the Desulfovibrionaceae (detected by the probe DSV1292) were above the detection limit and accounted for 1% of the total DAPI-stained cells.

The relative abundance of PLFA from aquifer microcosms after toluene depletion in comparison to that from the aquifer sediment revealed dynamics within the compo-

Table 2
Metabolic activities and $^{14}C$-mass balances (means ± S.D.) in aquifer microcosms[a]

| Microcosms | Analysis of microcosms | | | | | $^{14}C$-Recovery at $T_t$ (%) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $SO_4^{-2}$ consumption[b] (mM) | $CO_2$ (mM)[c] | | $CH_4$ (mM)[c] | | volatile (e.g. $CO_2$ toluene) | | filter (e.g. biomass) | residue (e.g. metabolites) | stopper (e.g. sorbed toluene) | total |
| | | $T_0$ | $T_t$ | $T_0$ | $T_t$ | | | | | | |
| Toluene added | 8.7 ± 1.5 | 0.12 ± 0.00 | 0.33 ± 0.02 | 0.08 ± 0.02 | 0.52 ± 0.04 | 1.8 ± 0.0 | 61.6 ± 3.7 | 2.7 ± 0.2 | 7.2 ± 1.2 | 2.5 ± 0.3 | 74.0 ± 5.0 |
| Biotic control[d] | 6.7 ± 2.8 | 0.12 ± 0.00 | 0.28 ± 0.34 | 0.10 ± 0.02 | 0.17 ± 0.23 | – | – | – | – | – | – |
| Abiotic control[d] | 0.0 ± 0.0 | 0.24 ± 0.00 | 0.16 ± 0.08 | 0.06 ± 0.00 | 0.04 ± 0.00 | 28.5 ± 5.0 | 0.7 ± 0.0 | 25.3 ± 4.6 | 9.3 ± 0.1 | 24.7 ± 1.7 | 88.5 ± 1.4 |

$T_0$, time zero (freshly obtained sediment). $T_t$, time after total depletion of toluene.
[a]Microcosms were inoculated with freshly obtained sediment and incubated under sulfate-reducing conditions (50 days at 12°C) in mineral salts medium with 0.25 mM toluene.
[b]$SO_4^{-2}$ consumption per microcosm.
[c]Dissolved $CH_4$ and $CO_2$ were calculated according to [23] based on the % of the gases in the headspace and Henry's Law using the Henry constants for $CH_4$ and $CO_2$.
[d]Biotic controls were used for the determination of metabolic activities and abiotic controls for the $^{14}C$-mass balances.

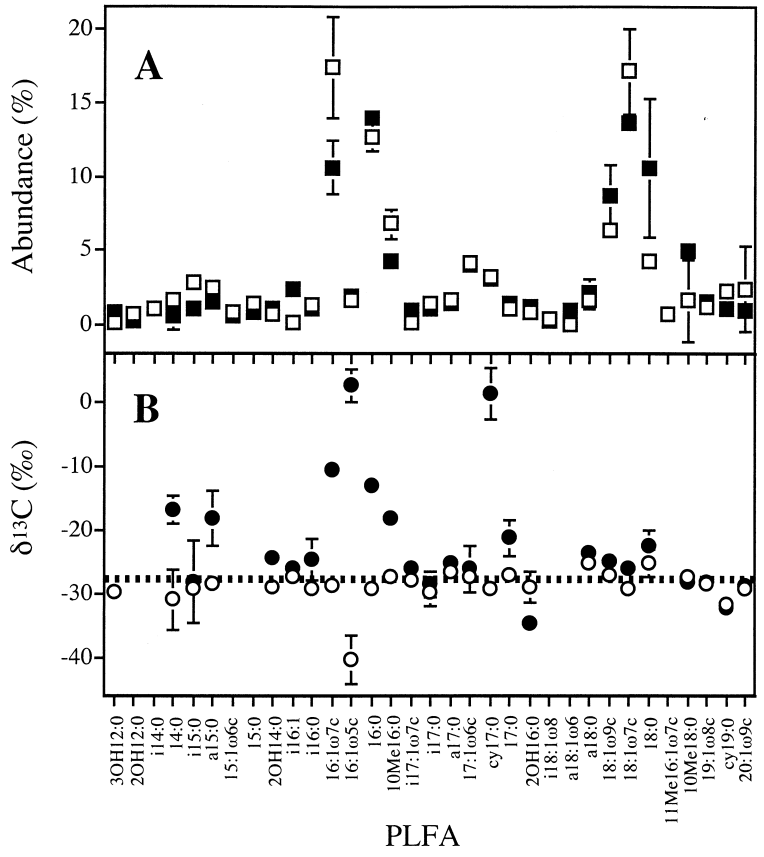


Fig. 1. Relative abundance (A) and $\delta^{13}C$ values (B) of PLFA from microcosms. The samples were analyzed from freshly obtained sediment of a monitoring well from a PHC-contaminated aquifer (□, ○) and after incubation in aquifer microcosms (■, ●) under sulfate-reducing conditions until total depletion of 0.6 mM [methyl-$^{13}$C]toluene (80.7‰). $\delta^{13}C$ values of 2OH-12:0, i14:0, 15:1ω6c, 15:0, i18:1ω8, a18:1ω8, and 11Me18:1ω7c were not determined because of the requirement to handle very low amounts of sample. Dashed line in (B) shows $\delta^{13}C$ of total organic carbon from the aquifer sediment.

sition of the microbial community (Fig. 1A). While the relative abundance of 3OH12:0, i16:1, 16:0, i17:1ω7c, i18:1ω6c, 18:0, and 19:1ω8c increased significantly, that of i15:0, 16:1ω7c, 10Me16:0, and cy19:0 decreased after depletion of toluene ($P < 0.05$).

In the [methyl-$^{13}$C]toluene-amended microcosms, it was possible to follow the flow of toluene carbon into bacterial biomass carbon through the determination of [methyl-$^{13}$C]toluene-labeled PLFA. After [methyl-$^{13}$C]toluene depletion, the $^{13}$C-incorporation profile showed that 48% of the total PLFA were $^{13}$C-enriched (14:0, a15:0, 2OH14:0, i16:0, 16:1ω7c, 16:1ω5c, 16:0, 10Me16:0, cy17:0, 17:0, 18:1ω9c, and 18:1ω7c; Fig. 1B). In particular, compounds with shorter chain length (C14–C17) were substantially more $^{13}$C-enriched relative to those with longer chain length. The two PLFA containing the highest amounts of $^{13}$C label were 16:1ω5c ($\delta^{13}C$, +2.5 ± 2.6‰) and cy17:0 ($\delta^{13}C$, +1.3 ± 3.9‰). The $\delta^{13}C$ values of the biomarkers for Gram-negative sulfate-reducing bacteria revealed [methyl-$^{13}$C]toluene incorporation in 10Me16:0 (−18.2 ± 0.9‰), contrary to a lack of $^{13}$C-incorporation for i17:1ω7c (−26.1 ± 0.5‰), and 17:1ω6c (−26.1 ± 3.6‰), following [methyl-$^{13}$C]toluene depletion.

### 3.3. Characterization of the aquifer-derived toluene-degrading consortium

The presence of toluene-degrading sulfate-reducing bacteria in the PHC-contaminated aquifer was demonstrated by the establishment of the aquifer-derived consortium grown under sulfate-reducing conditions with toluene as a sole carbon source. A positive correlation between bacterial activity and their detection by whole-cell hybridization was shown in the sediment-free liquid culture of the toluene-degrading consortium, where 85% of the DAPI-stained microorganisms were detected by the two general probes EUB338 (76%) and Arch915 (9%). This high detection rate was suitable for the application of the nested hybridization approach. The use of probes with different levels of phylogenetic resolution resolved the underlying community structure of the sulfate-reducing bacteria (Table 1). Cells belonging to specific sub-groups of the Desul-

fovibrionaceae accounted for approximately 5% (probes DSV698 and DS1292), whereas *Desulfobulbus* species were detected in the range of 3% (probe 660). However, the probe SRB385Db, detecting mainly Desulfobacteriaceae, hybridized with more than 50% of the DAPI-stained microorganisms. In addition, the hybridization with DSB985, which targets mainly *Desulfobacter* species, clearly revealed that the vast majority of SRB385Db-detectable microorganisms are affiliated to a distinct lineage within the Desulfobacteriaceae.

Mostly PLFA with even-numbered carbon chains (85%) characterized the PLFA profile of the consortium and unbranched (48%) and saturated mono-unsaturated (42%) compounds were dominating (Table 3). The compounds cy17:0 and 10Me16:0, found in the PLFA profile of the PHC-contaminated aquifer sediment and that of the aquifer microcosms, were also detected in considerable amounts in the consortium, 9 and 1%, respectively. The PLFA profiles of sulfate-reducing bacteria, cultivated with toluene as a sole substrate, are not known. To identify characteristic PLFA of toluene-degrading, sulfate-reducing bacteria, the PLFA profiles of *D. toluolica* and the uncharacterized strain PRTOL1 were analyzed. Although their PLFA profiles after growth on toluene were both characterized by mostly even-numbered compounds (90 and 80%, respectively), clear differences in the composition of individual PLFA revealed that these strains were not closely related to each other (Table 3). Unbranched, saturated PLFA (62%) but also 10Me16:0 (11%) and cy17:0 (1%), characterized the profile of *D. toluolica*, contrarily to that of strain PRTOL1, where the majority were mono-unsaturated PLFA (51%) with 16:1ω5c accounting for a relative abundance of 24%.

Table 3
Relative PLFA abundance (means ± S.D.) of the aquifer-derived consortium, *D. toluolica*, and strain PRTOL1[a]

| PLFA | Content (%) | | |
|---|---|---|---|
| | consortium | *D. toluolica* | PRTOL1 |
| 3OH12:0 | – | 5 ± 0 | – |
| i16:0[b] | – | 5 ± 2 | – |
| 16:1ω7c | 9 ± 1 | 4 ± 1 | 4 ± 1 |
| 16:1ω5c | 1 ± 1 | – | 24 ± 8 |
| 16:0 | 22 ± 4 | 38 ± 7 | 24 ± 2 |
| 10Me16:0 | 1 ± 1 | 11 ± 3 | – |
| a17:1[b] | – | – | 2 ± 1 |
| 17:1ω6c | – | – | 3 ± 0 |
| cy17:0 | 9 ± 3 | 1 ± 1 | – |
| 17:0 | 3 ± 1 | 3 ± 1 | 2 ± 1 |
| 18:1ω9c | 3 ± 0 | 3 ± 0 | 2 ± 1 |
| 18:1ω7c | 3 ± 0 | 3 ± 0 | 12 ± 2 |
| 18:1ω6c | – | – | 3 ± 3 |
| 18:1ω5c | – | – | 1 ± 0 |
| 11Me18:1ω7c[b] | – | – | 5 ± 2 |
| 18:0 | 21 ± 4 | 21 ± 4 | 5 ± 0.6 |

[a]Cultivation under sulfate-reducing conditions in mineral salt medium with 0.25 mM toluene as the sole substrate.
[b]Positional data for double bound and methyl branch based on GC retention time only (insufficient amounts were available for analysis).

## 4. Discussion

### 4.1. Community structure and activity in the PHC-contaminated aquifer

Although the bacterial activity in the aquifer sediment was low, SRB385Db-hybridizing microorganisms were detected in relatively high amounts, revealing that approximately 32% of the total of the active bacteria belonged to the Desulfobacteriaceae. Broad ranges of non-sulfate-reducing bacteria display 100% homologies within the target sequences of SRB385 and SRB385Db, which may result in an overestimation in particular of the Desulfobacteriaceae found in our aquifer sediment samples. Recently designed probes specific on the genus level and distinctive phylogenetic sub-groups within the sulfate-reducing bacteria overcome these drawbacks and should be applied in combination with both SRB385Db and SRB385, respectively, or in a nested hybridization approach [9]. However, using these specific probes, only microorganisms with a weak hybridization signal were detected in the aquifer sediment and the detection yields were below 0.5%, thus making further quantification within the Desulfobacteriaceae and Desulfovibrionaceae impossible.

The structure of the sulfate-reducing bacterial community in this sediment was also investigated by evaluating the PLFA profiles. PLFA have been used to monitor changes of specific groups of microorganisms within microbial communities and is an effective method of assessing bacterial distribution because this method is not affected by low bacterial activities [35]. The PLFA profiles were dominated by 16:1ω7c, 18:1ω7c, and 16:0, where the latter PLFA is ubiquitous and 16:1ω7c and 18:1ω7c are common constituents of Gram-negative bacteria [36]. The two biomarkers for *Desulfobacter*-like bacteria (*Desulfobacter* and the closely related genera, e.g. *Desulfobacula*) cy17:0 and 10Me16:0 have been previously used to investigate bacterial populations in marine sulfate-reducing sediments [14,37]. We determined the relative amount of *Desulfobacter*-like bacteria in the aquifer sediment according to a previous study [16]. This study quantified cell numbers of sulfate-reducing bacteria in environmental samples based on PLFA profiles from pure cultures of respective genera. Relatively large amounts of cy17:0 (3.2 ± 0.5%) and 10Me16:0 (6.8 ± 1.0%) indicated the presence of a large population of *Desulfobacter*-like bacteria representing at least 8–42% of the total bacterial population in the aquifer sediment. This result therefore provided additional information on the underlying Desulfobacteriaceae community, which could not be obtained by whole-cell hybridization. It must be stated though, that we only can estimate the relative abundance of these bacteria due to large variations of cy17:0 and 10Me16:0 in pure cultures (2–39

and 2–16%, respectively) and unknown levels of these compounds in *Desulfobacter* spp. grown under environmental conditions.

Other biomarkers of sulfate-reducing bacteria are 15:1ω6c and 17:1ω6c, which are major compounds in PLFA profiles of the genus *Desulfobulbus* [17,19] and *Desulforhabdus* (17:1ω6c) [16]. These PLFA were also detected in low amounts of approximately 0.8 and 4.1% of the total of PLFA, respectively. The combined marker (i17:1ω7c) for *Desulfovibrio* spp. and *Synthrophobacter* spp. [16,18] was not found in the aquifer sediment.

### 4.2. Toluene degradation linked to active sulfate-reducing populations in the aquifer microcosm

Since it was not possible to clearly characterize monoaromatic-PHC-degrading, sulfate-reducing populations in the contaminated freshwater aquifer, sediment from this aquifer was incubated with toluene and sulfate in microcosms to select for the activities of toluene-degrading sulfate-reducing bacteria. During dissimilatory sulfate reduction, bacteria obtain energy for the oxidation of carbon substrates and subsequent incorporation of substrate carbon into biomass. In our study, incubation of aquifer microcosms with [methyl-$^{14}$C]toluene revealed that not more than 2.7% of [methyl-$^{14}$C]toluene was incorporated into biomass (Table 2). Although these numbers seem realistic, it is possible that some of the labeled $^{14}CO_2$ may originate from $^{14}$C-labeled biomass that was mineralized during the incubation period. Small rates of toluene assimilation with sulfate as terminal electron acceptor are explained by small yields of free energy ($\Delta G^{0\prime} = -205$ kJ mol toluene), relative to that of e.g. nitrate ($\Delta G^{0\prime} = -3554$ kJ mol toluene) indicating that toluene is not a good growth-supporting substrate under the conditions of sulfate reduction [38]. However, the [methyl-$^{13}$C]toluene incorporation profile of PLFA from the aquifer microcosm showed significant $^{13}$C-enrichments in 48% of the total of PLFA, including enrichment of cy17:0 and 10Me16:0, biomarkers for *Desulfobacter*-like bacteria (Fig. 1B).

Although 10Me16:0 suggested the presence of *Desulfobacter* in a recent study investigating acetate consumption in anoxic sediments, $^{13}$C-acetate incorporation profiles were very different from PLFA profiles found in *Desulfobacter* spp., indicating that this genus was not the dominant acetate-consuming population [21,22]. The authors concluded that 10Me16:0 originated either from an inactive *Desulfobacter* population or from other genera containing this PLFA. However, the closely related genus *Desulfobacula* also possess cy17:0 and 10Me16:0 (e.g. *D. toluolica*: 1–3 and 11–18%, respectively; *Desulfobacula phenolica*: 4 and 17%, respectively) and includes toluene-degrading members (Table 3, [39]). Therefore we believe that under the current incubation conditions *Desulfobacter*-like bacteria were involved in the observed [methyl-$^{13}$C]toluene degradation.

The $^{13}$C-incorporation of cy17:0 and 10Me16:0 was not accompanied by an increase in concentration of these fatty acids after microcosm incubations. This may have been due to the fact that cy17:0 and 10Me16:0 are common constituents within different populations of *Desulfobacter*-like bacteria. The $^{13}$C-enrichment in cy17:0 and 10Me16:0 was therefore the result of a mixture of highly enriched PLFAs originating from the [methyl-$^{13}$C]toluene-assimilating population with those from other populations that assimilated native, unlabeled carbon substrates. In this manner, highly $^{13}$C-enriched PLFA (in all probability from toluene-degrading *Desulfobacter*-like bacteria) were diluted with unlabeled PLFA from non-toluene-assimilating relatives. The enrichments of 10Me16:0 and cy17:0 resulted from a relatively small [methyl-$^{13}$C]toluene-assimilating population within the *Desulfobacter*-like bacteria while the decrease of 10Me16:0 and constant amounts of cy17:0 following microcosm incubations indicated a decrease of population size of the non-toluene-assimilating populations.

A major fatty acid in *Desulfobulbus* spp. and *Desulforhabdus amnigenus* (17:1ω6c) [16] and the combined marker for the genera *Desulfovibrio* and *Synthrophobacter* (i17:1ω7c) were not $^{13}$C-enriched following [methyl-$^{13}$C]toluene depletion indicating that these sulfate-reducing bacteria were not involved in toluene metabolism under the applied conditions.

In the present study, the highest $^{13}$C-enrichment was found in 16:1ω5c, following [methyl-$^{13}$C]toluene depletion. In the freshly harvested sediment before [methyl-$^{13}$C]toluene incubation, this compound was significantly depleted (−40.3‰) relative to averaged values of the total of PLFA (−28.9‰), reflecting $CH_4$ assimilation because biogenic $CH_4$ has a depleted carbon isotopic signal (−65 to −50‰). This depleted isotopic signal supported previous suggestions that 16:1ω5 is characteristic for group I methanotrophs [40]. However, it has been shown that 16:1ω5 is also commonly found in other Gram-negative bacteria, including strain PRTOL1 (Table 3) and other genera of sulfate-reducing bacteria [14,16]. Thus, the $\delta^{13}C$ value of 16:1ω5c was mainly affected by $CH_4$ assimilation in the aquifer sediment and by [methyl-$^{13}$C]toluene assimilation in the aquifer microcosm. In each case two different bacterial populations were assumed to be active.

In summary, our results indicated that most likely *Desulfobacter*-like populations, or groups of yet unknown sulfate-reducing bacteria that also contain cy17:0 and 10Me16:0, consumed toluene and/or metabolites of the toluene degradation pathway. However, there is a chance that other populations, especially those containing 16:1ω5c were also involved in [methyl-$^{13}$C]toluene degradation. Although whole-cell hybridization revealed higher overall detectability of cells, the underlying sulfate-reducing populations of the microcosm community could not be resolved. As already mentioned this might be due to energy constraints but also to the fact that the used probes do not cover all existing sulfate-reducing populations.

### 4.3. Characterization of the sulfate-reducing, toluene-degrading, aquifer-derived consortium

Following whole-cell hybridization analysis to determine the most active toluene-degrading populations, a sulfate-reducing bacterial consortium was isolated from an aquifer microcosm. The high detectability rates of microorganisms hybridized with the probe EUB338 (76%) revealed overall high bacterial activities and the nested probe approach demonstrated that *Desulfobacter*-like bacteria were the most abundant toluene-degrading, sulfate-reducing population of this consortium, accounting for 42% of the total DAPI-stained microorganisms (Table 1). Many members of the genus *Desulfobacter* contain the target sequence of the probe DSB985, e.g. *Desulfobacter hydrogenophilus*, *Desulfobacter curvatus*, *Desulfobacter postgatei*, as do the toluene-degrading strains of other genera, e.g. *D. toluolica* and *D. phenolica* [9,39]. Sequences displaying 100% homology to the probe DSB985 have recently been obtained from complex microbial communities of contaminated marine environments, including a benzene-mineralizing consortium [12]. Therefore, we suggest that our consortium harbored an assemblage of different populations of *Desulfobacter*-like bacteria. Other bacteria, e.g. γ-Proteobacteria (19%), *Desulfovibrio* spp., and *Desulfobulbus* spp. (both 3% relative abundance) were also members of the consortium. Since toluene was the sole carbon source in this consortium, all detected bacteria were involved in toluene degradation or scavenged other organic compounds, e.g. cell debris, exudates.

### 4.4. Concluding remarks

Although naturally occurring anaerobic mono-aromatic-PHC degradation has the potential to remove significant quantities of these compounds from contaminated aquifers, studies of organisms involved and their activities are required before it will be possible to design rational strategies for accelerating bioremediation [1]. Our results show that *Desulfobacter*-like bacteria involved in the complete oxidation of mono-aromatic PHC must be considered when describing the bioremediation in PHC-contaminated aquifers under sulfate-reducing conditions. Although in some cases, PLFA analysis and whole-cell hybridization can be hampered due to a lack of biomarker specificity and bacterial activity constraints, a combination of both methods may be applied in aquifer microcosms, especially if more sensitive whole-cell hybridization techniques are applied [26]. In addition other methods, e.g. a combined microautoradiography and whole-cell hybridization should be tested [41]. Since sequences of only a few strains of mono-aromatic PHC-degrading sulfate-reducing bacteria are available [38], novel PHC-degrading isolates should be characterized to provide 16S rRNA sequence information for the generation of new oligonucleotide probes. Additional insights in the biochemistry of toluene degradation under sulfate-reducing conditions and especially gene sequences from involved enzymes are important and could form the basis for further specific primers targeting the mRNA in PCR- and hybridization-based approaches for the quantification of specific microbial activities [42].

## Acknowledgements

The contributions of G.T. Townsend for setting up the aquifer microcosms, C. Hesse for operating the IRMS, and W.V. Sigler for critically reviewing the manuscript are gratefully acknowledged. Grants from the Swiss National Science Foundation (Priority Programmes Biotechnology and Environment), and the Swiss Federal Office of Environment, Forests and Landscape (BUWAL) supported the study.

## References

[1] Lovley, D.R. (1997) Potential for anaerobic bioremediation of BTEX in petroleum-contaminated aquifers. J. Indust. Microbiol. Biotechnol. 18, 75–81.
[2] Anderson, R.T. and Lovley, D.R. (2000) Anaerobic bioremediation of benzene under sulfate-reducing conditions in a petroleum-contaminated aquifer. Environ. Sci. Technol. 34, 2261–2266.
[3] Pfiffner, S.M., Palumbo, A.V., Gibson, T., Ringelberg, D.B. and McCarthy, J.F. (1997) Relating ground water and sediment chemistry to microbial characterization at a BTEX-contaminated site. Appl. Biochem. Biotechnol. 63–65, 775–788.
[4] Edwards, E.A., Wills, L.E., Reinhard, M. and Grbic Galic, D. (1992) Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Appl. Environ. Microbiol. 58, 794–800.
[5] Widdel, F. and Bak, F. (1992) In: The Prokaryotes, 2nd edn. (Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H., Eds.), Vol. 1, pp. 583–624. Springer, New York.
[6] Castro, H.F., Williams, N.H. and Ogram, O. (2000) Phylogeny of sulfate-reducing bacteria. FEMS Microbiol. Ecol. 31, 1–9.
[7] Amann, R.I., Krumholz, L. and Stahl, D.A. (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172, 762–770.
[8] Devereux, R., Kane, M.D., Winfrey, J. and Stahl, D.A. (1992) Genus- and group-specific hybridization probes for determinative and environmental studies of sulfate-reducing bacteria. Syst. Appl. Microbiol. 15, 601–609.
[9] Manz, W., Eisenbrecher, M., Neu, T.R. and Szewzyk, U. (1998) Abundance and spatial organization of Gram-negative sulfate-reducing bacteria in activated sludge investigated by in situ probing with specific 16S rRNA targeted oligonucleotides. FEMS Microbiol. Ecol. 25, 43–61.
[10] Rabus, R. and Widdel, F. (1996) Utilization of alkylbenzenes during anaerobic growth of pure cultures of denitrifying bacteria on crude oil. Appl. Environ. Microbiol. 62, 1238–1241.
[11] Hristova, K.R., Mau, M., Zheng, D., Aminov, R.I., Mackie, R.I., Gaskins, H.R. and Raskin, L. (2000) *Desulfotomaculum* genus- and subgenus-specific 16S rRNA hybridization probes for environmental studies. Environ. Microbiol. 2, 143–159.
[12] Phelps, C.D., Kerkhof, L.J. and Young, L.Y. (1998) Molecular char-

acterization of a sulfate-reducing consortium which mineralizes benzene. FEMS Microbiol. Ecol. 27, 269–279.
[13] Rabus, R., Fukui, M., Wilkes, H. and Widdel, F. (1996) Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Appl. Environ. Microbiol. 62, 3605–3613.
[14] Dowling, N.J.E., Widdel, F. and White, D.C. (1986) Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulfate-reducers and other sulfide-forming bacteria. J. Gen. Microbiol. 132, 1815–1826.
[15] Kohring, L.L., Ringelberg, D.B., Devereux, R., Stahl, D.A., Mittelman, M.W. and White, D.C. (1994) Comparison of phylogenetic relationships based on phospholipid fatty acid profiles and ribosomal RNA sequence similarities among dissimilatory sulfate-reducing bacteria. FEMS Microbiol. Lett. 119, 303–308.
[16] Oude Elferink, S.J.W.H., Boschker, H.T.S. and Stams, A.J.M. (1998) Identification of sulfate reducers and *Syntrophobacter* sp. in anaerobic granular sludge by fatty-acid biomarkers and 16S rRNA probing. Geomicrobiol. J. 15, 3–17.
[17] Taylor, J. and Parkes, R.J. (1983) The cellular fatty acids of the sulfate-reducing bacteria, *Desulfobacter* sp., *Desulfobulbus* sp. and *Desulfovibrio desulfuricans*. J. Gen. Microbiol. 129, 3303–3310.
[18] Vainshtein, M., Hippe, H. and Kroppenstedt, R.M. (1992) Cellular fatty acid composition of *Desulfovibrio* species and its use in classification of sulfate-reducing bacteria. Syst. Appl. Microbiol. 15, 554–566.
[19] Parkes, R.J., Dowling, N.J.E., White, D.C., Herbert, R.A. and Gibson, G.R. (1993) Characterization of sulphate-reducing bacterial populations within marine and estuarine sediments with different rates of sulphate reduction. FEMS Microbiol. Ecol. 102, 235–250.
[20] Taylor, J. and Parkes, R.J. (1985) Identifying different populations of sulfate-reducing bacteria within marine sediment systems, using fatty acid biomarkers. J. Gen. Microbiol. 131, 631–642.
[21] Boschker, H.T.S., Nold, S.C., Wellsbury, P., Bos, D., De Graaf, W., Pel, R., Parkes, R.J. and Cappenberg, T.E. (1998) Direct linking of microbial populations to specific biogeochemical processes by $^{13}C$-labelling of biomarkers. Nature 392, 801–805.
[22] Boschker, H., de Graaf, W., Köster, M., Meyer-Reil, L.-A. and Cappenberg, T.E. (2000) Bacterial populations and processes involved in acetate and propionate consumption in anoxic brackish sediment. FEMS Microbiol. Ecol. 35, 97–103.
[23] Bolliger, C., Höhener, P., Hunkeler, D., Häberli, K. and Zeyer, J. (1999) Intrinsic bioremediation of a petroleum hydrocarbon-contaminated aquifer: assessment of mineralization based on stable carbon isotopes. Biodegradation 10, 201–217.
[24] Pelz, O., Chatzinotas, A., Andersen, N., Bernasconi, S.M., Hesse, C., Abraham, W.-R. and Zeyer, J. (2001) Use of isotopic and molecular techniques to link toluene degradation in denitrifying aquifer microcosms to specific microbial populations. Arch. Microbiol. 175, 270–281.
[25] Dolfing, J., Zeyer, J., Binder-Eicher, P. and Schwarzenbach, R.P. (1990) Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. 154, 336–341.
[26] Chatzinotas, A., Sandaa, R.A., Schönhuber, W., Amann, R., Daae Frida, L., Torsvik, V., Zeyer, J. and Hahn, D. (1998) Analysis of broad-scale differences in microbial community composition of two pristine forest soils. Syst. Appl. Microbiol. 21, 579–587.
[27] Stahl, D.A. and Amann, R.I. (1991) Development and Application of Nucleic Acid Probes. Wiley, New York.
[28] Meier, H., Amann, R., Ludwig, W. and Schleifer, K.-H. (1999) Specific oligonucleotide probes for in situ detection of a major group of Gram-positive bacteria with low DNA G+C content. Syst. Appl. Microbiol. 22, 186–196.
[29] Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.H. (1992) Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. Syst. Appl. Microbiol. 15, 593–600.
[30] Rabus, R. and Widdel, F. (1995) Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163, 96–103.
[31] Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R. and Stahl, D.A. (1990) Combination of 16S ribosomal RNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.
[32] Abraham, W.R., Hesse, C. and Pelz, O. (1998) Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage. Appl. Environ. Microbiol. 64, 4202–4209.
[33] Rabus, R., Nordhaus, R., Ludwig, W. and Widdel, F. (1993) Complete oxidation of toluene under strictly anoxia conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. 59, 1444–1451.
[34] Beller, H.R., Spormann, A.M., Sharma, P.K., Cole, J.R. and Reinhard, M. (1996) Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl. Environ. Microbiol. 62, 1188–1196.
[35] White, D.C. (1983) Analysis of microorganisms in terms of quantity and activity in natural environments. Symp. Soc. Gen. Microbiol. 34, 37–66.
[36] Ratlege, C. and Wilkinson, S.G. (1988) In: Microbiol. lipids (Ratlege, C. and Wilkinson, S.G., Eds.), Vol. 1, p. 963. Academic Press, London.
[37] Kusel, K., Pinkart, H.C., Drake, H.L. and Devereux, R. (1999) Acetogenic and sulfate-reducing bacteria inhabiting the rhizoplane and deep cortex cells of the sea grass Halodule wrightii. Appl. Environ. Microbiol. 65, 5117–5123.
[38] Heider, J., Spormann, A.M., Beller, H.R. and Widdel, F. (1999) Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol. Rev. 22, 459–473.
[39] Kuever, J., Könneke, M., Galushko, A. and Drzyzga, O. (2001) Reclassification of *Desulfobacterium phenolicum* as *Desulfobacula phenolica* comb. nov. and description of strain SaxT as *Desulfotignum balticum* gen. nov., sp. nov.. Int. J. Syst. Evol. Microbiol. 51, 171–177.
[40] Nold, S.C., Boschker, H.T.S., Pel, R. and Laanbroek, H.J. (1999) Ammonium addition inhibits C-13-methane incorporation into methanotroph membrane lipids in a freshwater sediment. FEMS Microbiol. Ecol. 29, 81–89.
[41] Lee, N., Nielsen, P.H., Andreasen, K.H., Juretschko, S., Nielsen, J.L., Schleifer, K.-H. and Wagner, M. (1999) Combination of fluorescent in situ hybridization and microautoradiography: a new tool for structure–function analyses in microbial ecology. Appl. Environ. Microbiol. 65, 1289–1297.
[42] Sayler, G.S., Layton, A., Lajoie, C., Bowman, J., Tschantz, M. and Fleming, J.T. (1995) Molecular site assessment and process monitoring in bioremediation and natural attenuation. Appl. Biochem. Biotechnol. 54, 277–290.