Activity and diversity of sulfate-reducing and methanogenic microorganisms in a petroleum-contaminated aquifer

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ACTIVITY AND DIVERSITY OF SULFATE-REDUCING AND METHANOGENIC MICROORGANISMS IN A PETROLEUM-CONTAMINATED AQUIFER

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Activity and Diversity of Sulfate-Reducing and Methanogenic Microorganisms in a Petroleum-Contaminated Aquifer

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Zurich 2003
Commit everything you do to the LORD. 
Trust him, and he will help you.

Befiehl dem HERRN deinen Weg und vertraue auf ihn, 
so wird er handeln.

Psalm 37, 5, The Bible
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CURRICULUM VITAE

DANK
The following chapters were published in refereed journals:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

Chapter 7:
Summary

Contamination of groundwater and soil with petroleum hydrocarbons (PHC) is a widespread environmental problem. Intrinsic bioremediation of polluted sites has recently evolved as an accepted technology. This method takes advantage of microorganisms that ideally convert hydrocarbons to carbon dioxide and water. Anaerobic microbial processes such as sulfate reduction and methanogenesis play an important role for the degradation of PHC at such sites. However, these processes are poorly understood.

The objective of this thesis was to assess rates of sulfate reduction and methanogenesis and to investigate microbial community structure of sulfate-reducing and methanogenic microorganisms in a PHC-contaminated aquifer. Field tests and laboratory microcosm studies were conducted and a combination of hydrological (single-well push-pull tests), chemical, molecular, and stable isotope tools was used. All studies were conducted in a PHC-contaminated aquifer in Studen, Switzerland or using sediment from this aquifer.

Microbial sulfate reduction was successfully assessed and quantified using single-well push-pull tests in combination with analysis of stable sulfur isotope fractionation. Computed first-order rate coefficients for sulfate reduction ranged from 0.043 to 0.130 day\(^{-1}\). To assess the activity of different groups of sulfate-reducing bacteria, we injected low-molecular weight organic acids (acetate, lactate, propionate, and butyrate) along with sulfate in a second series of push-pull tests. Low-molecular weight organic acids are typically consumed by specific groups of sulfate-reducing bacteria (Desulfobacter, Desulfovibrio, and Desulfobulbus). Microbial activities determined in these tests agreed well with fluorescence in situ hybridization that also detected the targeted groups of sulfate-reducing bacteria. In addition, denaturing gradient gel electrophoresis showed that the structure of the suspended bacterial community did not change during the push-pull tests. In all push-pull tests, sulfur isotope fractionation provided strong evidence for microbial sulfate reduction (enrichment factor \(\varepsilon = 16.1-25.7\%\)).

In a microcosm study, we incubated sediment from the Studen aquifer with low-molecular weight organic acids and identified the groups of sulfate-reducing bacteria that were selectively stimulated. Additions of lactate and propionate led to the enrichment of Desulfobulbus, while acetate, butyrate and citrate enriched for Desulfobacteriaceae, as determined by fluorescence in situ hybridization and phospholipid fatty acid analysis.
combined with multivariate statistical analysis. These results agreed with those from the push-pull experiments.

The influence of carbon sources (organic acids and PHC) on the extent of sulfur isotope fractionation was investigated in microcosms using a pure culture and enrichment cultures from the Studen aquifer. The average enrichment factor of all enrichment cultures ($\varepsilon = 23.0\%$) agreed well with $\varepsilon$ values determined in push-pull tests. Hence, sulfur isotope fractionation may be a useful tool to quantify microbial sulfate reduction at field sites.

Methanogenesis was investigated in a series of push-pull tests by injecting acetate, formate, $H_2 / CO_2$ or methanol to target different groups of methanogenic *Archaea*. Since these substrates had to be injected at concentrations much higher than in situ concentrations for push-pull tests to be evaluable, determined rates are denoted as potential rates of methanogenesis. These rates were highest for formate (1.86 mM d$^{-1}$), followed by $H_2$ (0.91 mM d$^{-1}$), acetate (0.38 mM d$^{-1}$), and methanol (0.11 mM d$^{-1}$). Substrate consumption and methane production in all tests agreed with the results of molecular methods. Sequencing of bands excised from denaturing gradient gels and fluorescence in situ hybridization detected the presence of acetate- (*Methanosaeta*) and formate or $H_2 / CO_2$- (e.g. *Methanomicrobiaceae*) consuming methanogens. However, no methanol-degrading *Archaea* were detected.

In conclusion, this thesis contributes to our understanding of biogeochemical processes in a PHC-contaminated aquifer undergoing intrinsic bioremediation. In particular, sulfate reduction and methanogenesis were quantified in situ, microbial community structure was assessed, and the extent of sulfur isotope fractionation during sulfate reduction for this environment was determined in field and microcosm studies. The combination of hydrogeological, chemical, molecular, and isotope methods yielded additional insights.
Zusammenfassung


Die Methanogenese wurde in einer Serie von Push-Pull Tests untersucht, in der Acetat, Format, H₂ / CO₂ oder Methanol injiziert wurden, welche von verschiedenen Gruppen methanogener Archaea verwendet werden. Da diese Substrate in Konzentrationen viel höher als ihre in-situ Konzentrationen injiziert werden mussten, damit die Push-Pull Tests auswertbar waren, bezeichnen wir die gemessenen Raten als potentielle Raten der Methanogenese. Diese Raten waren am höchsten für Format (1.86 mM d⁻¹), gefolgt von H₂ (0.91 mM d⁻¹), Acetat (0.38 mM d⁻¹) und Methanol (0.11 mM d⁻¹). Substratzehrung und Methanproduktion in allen Tests stimmten mit den Resultaten molekularer Methoden überein. Sequenzierung ausgeschnittener Banden aus „Denaturing Gradient“ Gelen und Fluoreszenz in situ Hybridisierung detektierten die Anwesenheit Acetat- (Methanosaeta) und Format- oder H₂ / CO₂- (z. B. Methanomicrobiaceae) verwendender methanogener Archaea. Allerdings konnten keine Methanol-degradierenden Archaea detektiert werden.

Introduction
1.1 Groundwater and soil contamination by petroleum hydrocarbons

Global economy and modern life heavily depend on the availability of petroleum hydrocarbons (PHC) as fuel for transportation, heating and energy production or as raw material for the production of synthetic fibers, dyes, fertilizers, pesticides, and other chemicals (Speight, 1999). More than half of the world’s energy demand is met by fuels derived from PHC (OECD, 1998). The current total world consumption of crude oil is approximately $12 \times 10^9$ liters per day (E.I.A., 2003).

The term “petroleum hydrocarbon” is a general expression describing pure or mixed compounds derived from crude oil by distillation and refinement. Petroleum hydrocarbon products such as gasoline, jet and diesel fuel or motor oils are complex mixtures of innumerable different compounds (e.g., Table 1-1) (Potter and Simmons, 1998). Based on their chemical structure, these compounds are classified into aliphatics and aromatics. Aliphatics are further divided into three main classes, alkanes, alkenes, and cycloalkanes (Table 1-1). Aromatics are divided into monoaromatic hydrocarbons, polynuclear aromatic hydrocarbons (PAHs), and heterocyclics. Of greatest environmental concern are the monoaromatic hydrocarbons (especially BTEX, Benzene, Toluene, Ethylbenzene, and Xylenes) due to their toxicity (e.g., benzene is a carcinogen) and high water solubility. But also PAHs are of concern due to their carcinogenicity. Handling, transport, and storage of PHC lead to a yearly worldwide spillage of 80 - 1200 million liters into the environment (Burger, 1997). In Switzerland, 3000 to 4000 contaminated sites are presently estimated to exist (Aepli Elsenbeer et al., 1997; BUWAL, 2003) of which about 60% are PHC-contaminated (Hofmann, 1995).

For cleanup of PHC-contaminated soils and aquifers, various ex situ and in situ remediation strategies have been developed. Among these, pump and treat, soil incineration, soil washing, soil vapor extraction, funnel-and-gate techniques with reactive barriers, and natural attenuation are only a few to be named (Bedient et al., 1999). The choice of the best remediation method is made based on the site characteristics, extent and type of pollution, risk potential, land use, and local laws (Dineen, 1991). Monitored natural attenuation (MNA) of PHC

### Table 1-1 Composition of diesel (average of five different fuels) (Gilbert and Calabrese, 1990; Hellmann, 1995; Milner et al., 1990; Propfe, 1990; Stone Jr., 1991)

<table>
<thead>
<tr>
<th>hydrocarbon group</th>
<th>% v/v</th>
</tr>
</thead>
<tbody>
<tr>
<td>aliphatics</td>
<td>71</td>
</tr>
<tr>
<td>alkanes</td>
<td>43</td>
</tr>
<tr>
<td>cycloalkanes</td>
<td>28</td>
</tr>
<tr>
<td>alkenes</td>
<td>0</td>
</tr>
<tr>
<td>aromatics</td>
<td>29</td>
</tr>
<tr>
<td>monoaromatics</td>
<td>17</td>
</tr>
<tr>
<td>PAHs</td>
<td>11</td>
</tr>
<tr>
<td>heterocyclics</td>
<td>3</td>
</tr>
</tbody>
</table>
spills has recently evolved as an accepted technology for the remediation of contaminated sites (Wiedemeier et al., 1999). The definition of MNA encompasses biological mineralization and physico-chemical effects such as dilution, sorption, and volatilization (EPA, 1999). However, only biological mineralization leads to a destruction of the contaminants. For this process the term “intrinsic bioremediation” was coined (Wiedemeier et al., 1999) and will be used throughout this study. Furthermore, the term mineralization will be used for complete destruction to CO₂ and H₂O, and degradation will be used for the sum of partial transformations and mineralization. Complex biogeochemical processes occur in PHC-contaminated aquifers undergoing intrinsic bioremediation and a diverse microbial population is involved in PHC degradation. Especially anaerobic processes such as sulfate reduction and methanogenesis play a major role.

1.2 Biological processes in PHC-contaminated aquifers undergoing intrinsic bioremediation

1.2.1 Redox zoning

When PHC are spilled onto soils, they will typically move downward through the unsaturated zone in a free phase by gravity until they reach the saturated zone. PHC are usually lighter than water so that they will float and spread laterally on the groundwater table. The

![Figure 1-1 Redox zones in a PHC-contaminated aquifer](image-url)
The zone in which free-phase PHC is present is denoted the source (Figure 1-1). Some of the PHC will dissolve in the groundwater and form the so-called plume. In general, both aerobic and anaerobic microbial processes contribute to the degradation of PHC in contaminated aquifers undergoing intrinsic bioremediation. Each process is associated with a certain energy yield (Table 1-2) and microorganisms that perform a process with a higher energy yield outcompete those that mediate processes with a lower energy yield (Stumm and Morgan, 1981).

Hence, different microbiological redox processes will partially exclude each other, resulting in a characteristic redox zone formation in a plume of dissolved PHC compounds (Figure 1-1). Typically, the first process in this sequence is aerobic respiration, having the highest energy yield under standard conditions (Table 1-2), followed by denitrification, Mn(IV) reduction, Fe(III) reduction, sulfate reduction, and methanogenesis. Each process is usually mediated by a different group of microorganisms. Since O₂ is toxic for most anaerobic microorganisms, and products of anaerobic microorganisms, e.g., sulfide, are toxic for the majority of microorganisms, the different processes further mutually exclude each other. The clear demarcations between the redox zones shown in Figure 1-1, however, represent an idealized picture since standard conditions rarely prevail in aquifers.

### 1.2.2 Spatially simultaneous occurrence of terminal electron-accepting processes

In recent years it has been frequently observed that two or more terminal electron-accepting processes may occur simultaneously in the same aquifer volume (Bekins et al., 1999; Cozzarelli et al., 2000; Jakobsen and Postma, 1999). Two reasons may explain this observation. On the one hand, conditions in the aquifer, e.g., electron acceptor concentrations, may be such that in-situ energy yields (ΔGᵢ values) of two processes become similar. For ex-
ample, Jakobsen and Postma (1999) have shown that the presence of low-reactivity Fe(III) (leading to low availability of Fe(III)) in combination with low sulfate concentrations may lead to similar in situ energy yields of sulfate and Fe(III) reduction and hence, their simultaneous occurrence. On the other hand, different processes may occur in different microniches (Figure 1-2) as has been hypothesized by Jakobsen and Postma (1999) and Bekins et al. (1999) for methanogenesis and Fe(III) or sulfate reduction. This is explained by a close association of different organisms, e.g., SRB and methanogens, in biofilms on the solid matrix and in pores. The process with the lower redox potential will occur in the smaller pores and more reduced microenvironments (Figure 1-2).

1.2.3 Importance of processes

In a survey of 38 PHC-contaminated aquifers, Wiedemeier et al. (1999) estimated the contribution of the redox processes shown in Figure 1-1 to BTEX biodegradation (Figure 1-3). Even though the relevance of a specific process depends on site characteristics such as electron acceptor availability, Figure 1-3 shows that anaerobic processes, in particular sulfate reduction and methanogenesis, may represent the most relevant biodegradation processes at many PHC-contaminated sites. Hence, the earlier assumption that aerobic processes play the most important role for PHC degradation is outdated (Wiedemeier et al., 1999).

Table 1-3 shows the stoichiometric equations of important processes involved in PHC mineralization and associated geochemical processes. Each process will be discussed in more detail below with emphasis on sulfate reduction and methanogenesis, the two processes that were investigated in closer detail in this thesis. Even though the separate discussion of the
processes suggests that they occur separately, in the “real world” they may occur simultaneously as discussed above, and a complicated web of interactions between the processes and the associated microorganisms exists (Figure 1-4). These interactions include, but are not limited to syntrophic consortia, involvement of solid phases, and reoxidation of reduced compounds.

1.2.4 Aerobic respiration

Almost all PHC components are degradable under aerobic conditions (Borden, 1994). Aerobic degradation is associated with the highest energy yield of all PHC degradation processes (Table 1-2). Therefore, PHC degradation with $O_2$ as electron acceptor is also more rapid than with any other electron acceptor (Wiedemeier et al., 1999). However, since $O_2$ consumption is fast and the aqueous solubility of $O_2$ is low (Table 1-4), $O_2$ supplies quickly become depleted in an organic-rich environment such as a PHC contaminant plume, leading to the establishment of anaerobic conditions (Chapelle et al., 2002).
Figure 1-4 Conceptual model of electron and carbon flow and important interactions of biogeochemical processes in a PHC-contaminated aquifer. Aerobic respiration, denitrification, iron and sulfate reduction and methanogenesis are presented in the order of decreasing redox potential. Fermenting organisms are active over the whole range of redox potentials. However, while methanogens depend on fermentation products for their metabolism (syntrophy), aerobes, nitrate reducers, Fe(III)- and sulfate-reducing bacteria may use fermentation products but also directly PHC components. For simplification, manganese reduction is not included in the picture and would be located between denitrification and Fe(III) reduction. Fe(III) stands for any kind of iron oxide, hydroxide or oxyhydroxide such as goethite and ferrihydrite.
Table 1-3  Stoichiometric equations of important processes involved in PHC mineralization

<table>
<thead>
<tr>
<th>Process</th>
<th>Stoichiometric equation</th>
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<tbody>
<tr>
<td>Microbial PHC mineralization&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Aerobic respiration</td>
<td>0.68 &lt;CH&lt;sub&gt;1.85&lt;/sub&gt;&gt; + O&lt;sub&gt;2&lt;/sub&gt; --&gt; 0.68 CO&lt;sub&gt;2&lt;/sub&gt; + 0.63 H&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td>Denitrification</td>
<td>0.85 &lt;CH&lt;sub&gt;1.85&lt;/sub&gt;&gt; + NO&lt;sub&gt;3&lt;/sub&gt;⁻ + H&lt;sup&gt;+&lt;/sup&gt; --&gt; 0.85 CO&lt;sub&gt;2&lt;/sub&gt; + 0.5 N&lt;sub&gt;2&lt;/sub&gt; + 1.29 H&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td>Iron(III) reduction</td>
<td>0.17 &lt;CH&lt;sub&gt;1.85&lt;/sub&gt;&gt; + FeOOH(s) + 2 H&lt;sup&gt;+&lt;/sup&gt; --&gt; 0.17 CO&lt;sub&gt;2&lt;/sub&gt; + Fe&lt;sup&gt;2+&lt;/sup&gt; + 1.66 H&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td>Sulfate reduction</td>
<td>1.37 &lt;CH&lt;sub&gt;1.85&lt;/sub&gt;&gt; + SO&lt;sub&gt;4&lt;/sub&gt;²⁻ + 2 H&lt;sup&gt;+&lt;/sup&gt; --&gt; 1.37 CO&lt;sub&gt;2&lt;/sub&gt; + H&lt;sub&gt;2&lt;/sub&gt;S + 1.26 H&lt;sub&gt;2&lt;/sub&gt;O</td>
</tr>
<tr>
<td>Fermentation&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.00 &lt;CH&lt;sub&gt;1.85&lt;/sub&gt;&gt; + 2H&lt;sub&gt;2&lt;/sub&gt;O --&gt; CH₃COOH + 1.85 H&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td>Methanogenesis (I)</td>
<td>CH₃COOH --&gt; CH₄ + CO₂</td>
</tr>
<tr>
<td>Methanogenesis (II)</td>
<td>CO₂ + 4H₂ --&gt; 2 H₂O + CH₄</td>
</tr>
<tr>
<td>Geochemical processes</td>
<td></td>
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<tr>
<td>Carbonate precipitation /</td>
<td>CaCO₃(s) + 2 H&lt;sup&gt;+&lt;/sup&gt; --&gt; CO₂ + Ca&lt;sup&gt;2+&lt;/sup&gt; + H₂O</td>
</tr>
<tr>
<td>dissolution</td>
<td></td>
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<tr>
<td>Siderite precipitation</td>
<td>CO₂ + Fe&lt;sup&gt;2+&lt;/sup&gt; + H₂O --&gt; FeCO₃(s) + 2 H&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>Iron monosulfide precipitation</td>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt; + H₂S --&gt; FeS(s) + 2 H&lt;sup&gt;+&lt;/sup&gt;</td>
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<sup>a</sup> Other processes not shown in this table include manganese reduction, acetogenesis, methanogenesis with other fermentation products such as methanol and formate, etc..

<sup>b</sup> It was assumed that PHC in heating oils have an average H/C ratio of 1.85 (denoted as <CH<sub>1.85</sub>>). Biomass formation was assumed to be negligible.

<sup>c</sup> Fermentation of PHC is only energetically favorable if the products, here acetate and H₂, are continuously consumed, e. g. by methanogenesis.

1.2.5 Denitrification

Nitrate is a good alternative electron acceptor to O₂ (Wilson and Bouwer, 1997), yielding only little less energy than O₂ (Zehnder and Stumm, 1988) (Table 1-2). In general, denitrifiers are facultatively anaerobic microorganisms (Borden, 1994), but even aerobic denitrification was observed in some instances (Wilson and Bouwer, 1997). High levels of O₂ inhibit denitrification. A variety of PHC compounds including many aromatic PHC are degradable under denitrifying conditions (Borden, 1994). Even benzene was recently demonstrated to be consumed in denitrifying enrichment cultures (Burland and Edwards, 1999). So far the only isolated anaerobic pure cultures growing on benzene as sole carbon sources are denitrifying cultures belonging to the genus *Dechloromonas* (Coates et al., 2001). Ambient concentrations of nitrate in PHC-contaminated aquifers (Table 1-4) are usually rapidly consumed, leading to
the onset of other anaerobic processes such as Fe(III), Mn(IV), and sulfate reduction, and methanogenesis (Wiedemeier et al., 1999).

Table 1-4  | Maximum concentrations [mM] of electron acceptors (O₂, NO₃⁻, SO₄²⁻; outside plume) and products of anaerobic processes (Mn(II), Fe(II), CH₄; inside plume) in PHC-contaminated aquifers

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<td>Reference</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Site</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O₂</td>
<td></td>
<td>0.018</td>
<td>0.166</td>
<td>0.156</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td></td>
<td>0.258</td>
<td>n. a.</td>
<td>4.386</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td></td>
<td>0.326</td>
<td>0.156</td>
<td>0.625</td>
</tr>
<tr>
<td>Mn(II)</td>
<td></td>
<td>0.055</td>
<td>n. a.</td>
<td>n. a.</td>
</tr>
<tr>
<td>Fe(II)</td>
<td></td>
<td>0.197</td>
<td>0.358</td>
<td>0.725</td>
</tr>
<tr>
<td>CH₄</td>
<td></td>
<td>0.684</td>
<td>0.100</td>
<td>0.093</td>
</tr>
</tbody>
</table>

*available Fe(III) at the Laurel Bay site was < 1 μmol g⁻¹, in glacial outwash aquifers values of 20-30 μmol g⁻¹ have been reported (Chapelle et al., 2002).*

**not available**

1.2.6 Iron / Manganese reduction

Many aromatic compounds including benzene, toluene, phenol, and p-cresol are degradable under Fe(III)-reducing conditions (Borden, 1994; Lovley et al., 1994). Iron(III)-reducing microorganisms of the Geobacter cluster were selectively enriched in benzene-degrading sediments (Rooney-Varga et al., 1999). While Mn(IV) reduction does in fact occur in PHC-contaminated aquifers (Williams et al., 1992), little is known about the role Mn(IV) reducers play in the degradation of single PHC components (Wiedemeier et al., 1999).

In contrast to other electron acceptors, which are usually transported to the contaminated zone via the advecting groundwater, Fe(III) and Mn(IV) are present in the solid phase as oxides or oxyhydroxides (Lovley, 1991) (Figure 1-4). To be able to reduce Fe(III) or Mn(IV), microorganisms have to be in direct contact with the mineral surface. Iron(III) reduction frequently results in high concentrations of Fe(II) in contaminated groundwater (Table 1-4) (Borden, 1994). The quantification of Fe(III) reduction in the environment is challenging be-
cause on the one hand, determination of the Fe(III) available to microorganisms may be ar-duous (Wiedemeier et al., 1999). Approaches using genetically engineered biosensor bacteria may help to overcome this problem (Loper and Henkels, 1997). On the other hand, Fe(II) readily precipitates with carbonate to form FeCO₃ (siderite) or with sulfide to form FeS (iron monosulfide) (Stumm and Morgan, 1981)(Figure 1-4), so that quantification of Fe(III) reduction based on measured Fe(II) concentrations in groundwater may underestimate the activity of Fe(III) reducers.

1.2.7 Sulfate reduction

In contrast to assimilatory sulfate reduction that is used by plants, fungi and most bacteria to incorporate reduced sulfur into various molecules, dissimilatory sulfate reduction is used for the generation of energy by a specific group of microorganisms, the sulfate-reducing bacteria (SRB) (Widdel, 1988). In this study, the term sulfate reduction will refer to the process of dissimilatory sulfate reduction. During this process, sulfate is used as electron acceptor and reduced to sulfide (S(-II)), here defined as the sum of S²-, HS⁻, and H₂S), which is excreted from the cell (Figure 1-5) (Madigan et al., 2003). The reduction of sulfate to S(-II) is coupled with a transport of eight electrons. In the cell, sulfate is activated with ATP (adenosine triphosphate), and APS (adenosine-5'phosphosulfate) is formed. This process is catalyzed by the enzyme ATP sulfurylase. Thereafter, APS is reduced to sulfite (SO₃²⁻) by the enzyme APS reductase with the release of AMP (adenosine monophosphate). The enzyme dissimilatory sulfite reductase (DSR) catalyzes the further reduction of sulfite to S(-II). Molybdate (MoO₄²⁻) as a structural analog of SO₄²⁻ inhibits the formation of APS and is frequently used as a competitive, specific inhibitor of sulfate reduction (Oremland and Capone, 1988).

Typical sulfate-reducing environments include marine sediments (Jørgensen, 1977), freshwater lake sediments (Cook and Kelly, 1992), petroleum reservoirs (Magot et al., 2000), and anaerobic aquifers (Bolliger et al., 1999; Bottrell et al., 1996). Sulfate reducers are often un-
welcome since $S(-II)$ is toxic to plants, animals, and humans (Widdel, 1988), may cause food spoilage (Postgate, 1984) and corrosion of steel material, reduction of oil quality, and adverse health effects on workers in oil reservoirs and drilling rigs (Magot et al., 2000). However, SRB are an important link in the global cycling of sulfur compounds, may be employed for the purification of waste waters, are involved in the formation of mineral deposits and metal sulfide ores (Postgate, 1984), and are responsible for the degradation of a wide range of contaminants at polluted sites (Ensley and Suflita, 1995).

In PHC-contaminated aquifers undergoing intrinsic bioremediation, sulfate reduction can be the most important process for PHC degradation (Figure 1-3) (Wiedemeier et al., 1999). In contrast to marine systems, where sulfate concentrations are high (28 mM), freshwater systems typically feature sulfate concentrations below 1-2 mM (Table 1-4) (Cook and Kelly, 1992). Therefore, sulfate reduction in freshwater systems is usually a first-order process with respect to sulfate concentration, while in seawater, it is independent of sulfate concentration and hence, zero-order (Cook and Kelly, 1992). Similar to Fe(III) reduction, quantification of sulfate reduction in contaminated aquifers based on measurements of $SO_4^{2-}$ consumption or $S(-II)$ production is often obscured by concurrent abiotic transformations of $SO_4^{2-}$ and $S(-II)$. For example, gypsum (CaSO$_4$) may dissolve from the aquifer matrix (Stumm and Morgan, 1981) or $S(-II)$ may precipitate in form of iron sulfides (Anderson and Lovley, 2000) (Figure 1-4).

1.2.8 Methanogenesis

Methanogenesis is mediated exclusively by a group of organisms belonging to the domain *Archaea* (Zinder, 1993). Only a restricted number of simple compounds supports methanogenic microorganisms, e.g., CO$_2$ as oxidant with H$_2$ as electron donor, acetate, methyl-type substrates (e.g., methanol) or formate (Zinder, 1993). The molecular mechanism of methanogenesis is of amazing complexity involving unique enzymes and coenzymes (Madigan et al., 2003) (Figure 1-6). Briefly, when CO$_2$ is reduced, it is first activated by a methanofuran-containing enzyme and subsequently reduced to a formyl group (-CH=O). The formyl group is then transferred to an enzyme containing methanopterin. After dehydration the formyl group is reduced to a methylene (=CH$_2$) and then to a methyl (-CH$_3$) group. The methyl group is transferred to an enzyme containing coenzyme M and there reduced to methane by the methyl reductase system. The last step of the reaction allows for energy conservation in methanogenesis. Methanogenesis from methyl-type substrates or acetate involves the transfer
of the methyl group to a corrinoid protein. The terminal step catalyzed by the enzyme methyl reductase is the same for all methanogens (Figure 1-6). Hence, 2-bromoethanesulfonate (BES), which inhibits the last step of methanogenesis, inhibits all known methanogens (Oremland and Capone, 1988).

Methanogenic microorganisms have been isolated from anaerobic digesters (Raskin et al., 1994), cattle rumen (Miller et al., 1986), rice fields (Joulian et al., 1998), oil wells (Ollivier et al., 1997), landfills (Fielding et al., 1988), and a range of extreme habitats (Garcia et al., 2000). They play important roles in anaerobic treatment of organic wastes, formation of biogas as an alternative source of energy (Oremland, 1988), and generation of \( \text{CH}_4 \) as a greenhouse gas (Wuebbles and Hayhoe, 2002). In the absence of other electron acceptors such as oxygen, nitrate, and sulfate, methanogens are involved in the terminal anaerobic breakdown of organic matter (Garcia et al., 2000).

Even though methane is the only thermodynamically stable organic compound in natural waters (Zehnder and Stumm, 1988), methane can be oxidized by anaerobic (Boetius et al., 2000) and aerobic methane oxidizers (Hanson and Hanson, 1996). Methane may also volatilize into the unsaturated zone. Hence, quantification of methanogenesis based on methane concentrations in groundwater may not be accurate.
1.3 Methods for quantification of biological processes in aquifers

Quantitative information on microbial processes in contaminated aquifers is needed to enhance our knowledge on microbial activities in the subsurface, to monitor the effectiveness of intrinsic bioremediation, and to calibrate the time demand of the bioremediation process. A wide variety of methods for the quantification of microbial processes has been developed and used, for example, laboratory-based methods such as batch and column experiments (Chapelle et al., 1996) and field-based methods such as two-well natural or forced gradient tracer tests, single-well injection or withdrawal tests with multiple observation wells, single-well push-pull tests, electron acceptor and dissolved inorganic carbon balances, and solute-transport modeling (Chapelle, 2001; Chapelle et al., 1996; Domenico and Schwartz, 1990; Hunkeler et al., 1999b). All laboratory-based methods may introduce uncertainties since sediment samples may not be representative, may become contaminated during sampling, and field conditions are not easily reproducible in the laboratory. Field methods may also be biased since the effects of microbial processes are not easily separated from abiotic processes such as hydrodynamic dispersion and sorption (Chapelle et al., 1996). However, field methods are generally considered more representative of actual subsurface conditions than laboratory-based methods because they target a larger volume of aquifer material than typically is investigated in batch or column studies (Istok et al., 1997). Nevertheless, laboratory methods allow for standardization and hence, are comparable to a greater extent than field studies.

One method developed to quantify situ microbial activities in the field is the single-well push-pull test (PPT), modified by Istok et al. (1997) from earlier methods for the quantification of physical aquifer properties and similar methods for investigation of microbial processes. To conduct a single-well push-pull test, the only on-site installation needed is a groundwater monitoring well. Monitoring wells are usually already installed throughout most contaminated sites for routine groundwater monitoring. In a PPT, a prepared test solution that contains a non-reactive, conservative tracer (e.g., bromide) and one or more reactive solutes (reactants, e.g., $SO_4^{2-}$) is injected ("pushed") into the aquifer through an existing well (Figure 1-7, "Injection"). During the following initial incubation period (i.e., a rest phase without pumping, "Incubation"), indigenous microorganisms consume reactants and generate metabolic products. Thereafter, the test solution/groundwater mixture is extracted ("pulled") from the same location ("Extraction"). Rates of microbial activities are then determined from an analysis of solute breakthrough curves obtained by measuring concentrations of tracer, reactants and/or metabolic products at the injection/extraction well during the extraction phase of
Injection Extraction

Figure 1-7 Single-well push-pull test

the test (Haggerty et al., 1998; Snodgrass and Kitanidis, 1998). Push-pull tests may be used to investigate site-scale variability of microbial processes (Schroth et al., 1998). If in PPTs various substrates or carbon sources are injected that are typically consumed by certain groups of microorganisms, information may be derived on the activity and diversity of these groups.

1.4 Diversity of microorganisms in anaerobic environments

1.4.1 Molecular methods to determine microbial community structure

To better understand the biogeochemical processes occurring in a PHC contaminant plume, it is essential that we comprehend the complex foodwebs and the community structure of the microbial population catalyzing PHC degradation (Figure 1-4). In this study, several methods were employed to investigate microbial community structures in field (water and sediment) and microcosm samples (Figure 1-8). These methods target different compartments of the microorganism: polymerase chain reaction with subsequent denaturing gradient gel electrophoresis (DGGE) involves extraction of DNA, fluorescence in situ hybridization targets ribosomal RNA, and by phospholipid fatty acid analysis the cell membrane constituents are analyzed. Hence, the three methods elucidate microbial community structure from different perspectives. The common advantage of these methods is that they may be used to directly investigate environmental microbial communities. In contrast, culture-based methods are biased because less than 1% of all microorganisms are currently culturable (Amann et al.,
Further advantages and disadvantages of the three methods are summarized in Table 1-5.

**Figure 1-8** Overview of molecular methods used in this study to determine microbial community structure in environmental samples

### 1.4.2 Denaturing gradient gel electrophoresis

Only ten years ago, denaturing gradient gel electrophoresis (DGGE) was first used for the analysis of complex environmental microbial communities (Muyzer et al., 1993). This method involves the extraction of DNA from environmental samples, PCR amplification, and the subsequent separation of DNA strands of the same length in a polyacrylamide gel containing a linearly increasing gradient of denaturants (Muyzer et al., 1993). Double stranded, helical DNA is loaded onto the gel and migrates towards the cathode in the applied electrical field. The denaturants in the gel will cause the DNA to denature. The electrophoretic mobility of the melted DNA, however, is by far lower than that of the helical DNA and hence, migration of the denatured molecule will halt. Fragments of DNA with the same length but different sequences will denature at different denaturant concentrations. Therefore, they will stop at different positions in the gel and be separated effectively. To increase the resolution of DGGE gels, a guanine (G)- and cytosine (C)-rich sequence is incorporated into one of the primers for PCR (GC clamp) (Muyzer and Smalla, 1998). The GC-clamp prevents the two DNA strands from complete dissociation into single strands during DGGE.
Table 1-5  Comparison of molecular methods that were used in this study to determine microbial community structures in environmental samples (Amann et al., 2001; Amann et al., 1997; Amann et al., 1995; Fuchs et al., 2000; Hodson et al., 1995; Muyzer et al., 1993; Muyzer and Smalla, 1998; Taylor and Parkes, 1985; Zelles, 1999)

<table>
<thead>
<tr>
<th>Method</th>
<th>DGGE</th>
<th>FISH</th>
<th>PLFA analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target molecules</td>
<td>DNA</td>
<td>rRNA</td>
<td>PLFA</td>
</tr>
<tr>
<td>Phylogenetic information</td>
<td>species level</td>
<td>genus or species level</td>
<td>biomarker PLFA, genus level at best, more specific in a specific environment</td>
</tr>
<tr>
<td>Quantitative information on community composition</td>
<td>semiquantitative at best</td>
<td>quantitative</td>
<td>semiquantitative at best</td>
</tr>
<tr>
<td>Monitoring community changes</td>
<td>good</td>
<td>good for few species, elaborate if many species are monitored</td>
<td>good</td>
</tr>
<tr>
<td>Evaluation of microbial community structure</td>
<td>good if limitations are considered</td>
<td>good but elaborate if many species are monitored</td>
<td>good but only a limited number of biomarkers exist</td>
</tr>
<tr>
<td>Possible bias can occur during these procedures / limitations of the method</td>
<td>•DNA extraction •PCR •electrophoresis •resolution only up to 500 base pairs •rare species are not detected</td>
<td>•permeabilization of cells •poor accessibility of target rRNA •low rRNA content of less active cells</td>
<td>•resolution of PLFA as phylogenetic marker not as good as rRNA or DNA •PLFA patterns of individual species may overlap •lack of information on distribution of PLFA across microbial phylogenotypes •PLFA do not contain all information in the lipids</td>
</tr>
<tr>
<td>Methods to overcome biases or limitations</td>
<td>•specific primers may show a higher sensitivity</td>
<td>•better fixation protocols •unlabeled helper probes binding adjacent to target site on the rRNA increase hybridization intensity •multiple labeling or in situ PCR to increase signal</td>
<td>•for some bacterial groups, e.g., SRB, a large PLFA data base exists •extraction of other lipids (archaeal lipids, non-ester-linked PLFA)</td>
</tr>
<tr>
<td>A priori assumption on the community</td>
<td>no a priori assumption necessary</td>
<td>a priori assumption has to be made on the community</td>
<td>no a priori assumption necessary</td>
</tr>
<tr>
<td>Time requirement</td>
<td>visualization of bands within 2-3 days possible, cloning more elaborate (within days - weeks), advantages for multiple samples</td>
<td>fast for detection of one phylotype (within hours), time-consuming for detection of a variety of organisms</td>
<td>elaborate extraction, results within several days</td>
</tr>
<tr>
<td>Downstream analyses</td>
<td>determination of sequences, design of new primers</td>
<td>none</td>
<td>none</td>
</tr>
</tbody>
</table>
1.4.3 Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) relies on the specific hybridization of fluorescently labeled oligonucleotide probes with intracellular rRNA (Amann et al., 1997). For FISH, cells have to be first permeabilized for the penetration of the probes into the cell. This is usually achieved using fixatives such as paraformaldehyde or ethanol. After immobilization of the permeabilized cells on filters or slides hybridization is achieved with oligonucleotide probes in an appropriate hybridization buffer and incubation at a temperature typically between 35 and 50°C. Thereafter, washing removes excessive probes and samples may be visualized using an epifluorescence microscope.

1.4.4 Phospholipid fatty acid analysis

Phospholipid fatty acid (PLFA) analysis is based on the extraction of lipids from cell membranes, e. g. of microorganisms in soil, using suitable solvents and the separation into different lipid classes. The PLFA are then derivatized to methyl esters and identified and quantified on a gas chromatograph (Zelles, 1999). The PLFA extraction method developed by Bligh and Dyer (1959) is the most widely used method (Bligh and Dyer, 1959; Zelles, 1999).

1.4.5 Diversity of sulfate-reducing bacteria

The SRB belong to four phylogenetic lineages, the gram-negative mesophilic SRB (e. g, Desulfobulbus, Desulfobacter, Desulfovibrio), gram-positive spore-forming SRB (Desulfomaculum), thermophilic bacterial SRB (Thermodesulfobacterium), and thermophilic archaeanal SRB (Archaeoglobus) (Castro et al., 2000). All gram-negative mesophilic SRB belong to the δ-Proteobacteria. In contaminated freshwater aquifers, only mesophilic gram-negative and gram-positive SRB will be of importance.

Only two decades ago, SRB were considered nutritionally very restricted. This point of view has changed dramatically over the past years (Ensley and Suflita, 1995). A wide variety of PHC contaminants including benzene, naphthalene, toluene, phenol, m- and p-cresol, xylenes, and hexadecane are degradable by SRB (Ensley and Suflita, 1995; Lovley et al., 1995; Zhang and Young, 1997). So far, two toluene-degrading sulfate-reducing pure cultures have been isolated, the marine organism Desulfobacula toluolica (Rabus et al., 1993), and the freshwater strain PRTOL1 (Beller et al., 1996), both members of the family Desulfobacteriaciae. SRB belonging to this family seem to be the most important PHC degraders since they were frequently isolated on PHC components or rRNA sequences related to them extracted.
from PHC-grown enrichment cultures (Beller et al., 1996; Harms et al., 1999; Koizumi et al.,
2002; Phelps et al., 1998; Rabus et al., 1993; So and Young, 1999). However, members of
Desulfotomaculum, Desulfobulbus, and Desulfovibrio were also found in PHC-contaminated
environments (Magot et al., 2000). Some of these may not directly degrade PHC but thrive on
organic acids derived from fermentation of PHC (Cozzarelli et al., 1994). Table 1-6 shows an
overview of the utilization of some important low-molecular weight organic acids by SRB. In
this thesis, these organic acids were employed in field and microcosm experiments as selec¬
tive stimulants of SRB (chapters 4 and 5). The larger portion of literature centers on SRB
from marine environments and much less is known on freshwater SRB, especially from PHC-
contaminated aquifers. However, SRB communities from marine and freshwater environ¬
ments have been shown to be distinctly different (Voordouw et al., 1992).

1.4.6 Diversity of methanogenic Archaea

The methanogenic Archaea are divided into five orders, the Methanobacteriales, Methano-
coccales, Methanomicrobiales, Methanosarcinales and the Methanopyrales (Madigan et al.,
2003). The latter is hypothermophile and hence, probably not relevant in PHC-contaminated
freshwater environments. Methanogenic Archaea may contribute significantly to contaminant
degradation in PHC-contaminated aquifers (Chapelle et al., 2002; Christensen et al., 1994;
Zengler et al., 1999). Even though methanogens cannot directly degrade PHC, in syntrophy
with fermenting organisms a broad range of contaminants has been reported to be degradable.
Even benzene, toluene, and hexadecane were mineralized under methanogenic conditions
(Grbic-Galic and Vogel, 1987; Weiner and Lovley, 1998; Zengler et al., 1999). Furthermore,
the formation of methane has been frequently observed in PHC-contaminated aquifers
(Bolliger et al., 1999; Chapelle et al., 2002; Gieg et al., 1999; Lu et al., 1999). Table 1-7
shows an overview of the utilization of important substrates by methanogenic Archaea.
### Table 1-6: Utilization of some carbon sources by selected mesophilic sulfate-reducing bacteria \(^a\)

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Substrate</th>
<th>Lactate</th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Propionate</th>
<th>Oxidation</th>
</tr>
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<tbody>
<tr>
<td><strong>Low-GC-gram positives</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td><em>Desulfotomaculum</em></td>
<td></td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>i/c</td>
</tr>
<tr>
<td><strong>Desulfovibrionaeae</strong></td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td><em>Desulfovibrio</em></td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>i</td>
</tr>
<tr>
<td></td>
<td><em>Desulfomicrobium</em></td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>i</td>
</tr>
<tr>
<td><strong>“Desulfobulbusaeae”(^b)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td><em>Desulfobulbus</em></td>
<td></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>i</td>
</tr>
<tr>
<td><strong>Desulfo bacteriaceae</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td><em>Desulfo bacter</em></td>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td><em>Desulfo cococcus</em></td>
<td></td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td><em>Desulfosarcina</em>(^*)</td>
<td></td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td><em>Desulfobacterium</em></td>
<td></td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td><em>Desulfonema</em></td>
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<td>+</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td><em>Desulfomonile</em>(^*)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td><em>Desulfo botulus</em>(^*)</td>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>i</td>
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<tr>
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<td><em>Desulfo arculus</em>(^*)</td>
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<td>-</td>
<td>(+)</td>
<td>+</td>
<td>(+)</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td><em>Desulforhabdus</em>(^*)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>c</td>
</tr>
<tr>
<td></td>
<td><em>Desulfo bacula</em></td>
<td></td>
<td>-</td>
<td>(+)</td>
<td>-</td>
<td>-</td>
<td>c</td>
</tr>
</tbody>
</table>

\(i\) = incomplete oxidation to acetate  
\(c\) = complete oxidation to CO\(_2\)  
(+) slow growth only  
+ at least one member of the genus is able to utilize the carbon source  
- no known members with ability to utilize carbon source  
* for these genera so far only one species is known  
\(^b\) family proposed by Rooney-Varga et al. (1998)
Table 1-7  Utilization of important substrates by selected methanogenic Archaea

<table>
<thead>
<tr>
<th>Family</th>
<th>Genus</th>
<th>Substrate</th>
<th>H₂ + CO₂</th>
<th>Formate</th>
<th>Acetate</th>
<th>Methanol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Methanobacteriales</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Methanobacterium</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Methanobrevibacter</td>
<td>+</td>
<td>+</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Methanosphaera</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td></td>
<td>Methanothermus</td>
<td>+</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Methanothermobacter</td>
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<td>+</td>
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<tr>
<td><strong>Methanococcales</strong></td>
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<tr>
<td></td>
<td>Methanococcus</td>
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<td>+</td>
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</tr>
<tr>
<td></td>
<td>Methanothermococcus</td>
<td>+</td>
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<tr>
<td></td>
<td>Methanocaldococcus</td>
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<tr>
<td></td>
<td>Methanotorris</td>
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<tr>
<td><strong>Methanomicrobiales</strong></td>
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<tr>
<td></td>
<td>Methanomicrobium</td>
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<td>+</td>
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<tr>
<td></td>
<td>Methanogenium</td>
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<td></td>
<td>Methanospirillum</td>
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<tr>
<td></td>
<td>Methanoplanus</td>
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<tr>
<td></td>
<td>Methanocorpusculum</td>
<td>+</td>
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<tr>
<td></td>
<td>Methanoculleus</td>
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<td>+</td>
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<tr>
<td></td>
<td>Methanofollis</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>Methanolacinia</td>
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<tr>
<td><strong>Methanosarcinales</strong></td>
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<tr>
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<td>Methanosarcina</td>
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<tr>
<td></td>
<td>Methanolobus</td>
<td>+</td>
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<tr>
<td></td>
<td>Methanohalobium</td>
<td>+</td>
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<tr>
<td></td>
<td>Methanococcoides</td>
<td>+</td>
<td></td>
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<tr>
<td></td>
<td>Methanohalophilus</td>
<td>+</td>
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<tr>
<td></td>
<td>Methanoseta</td>
<td>+</td>
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<tr>
<td>(former Methanothrix)</td>
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<tr>
<td></td>
<td>Methanosalsum</td>
<td>+</td>
<td></td>
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</tr>
</tbody>
</table>

*a adapted from Madigan (2003)

*b both H₂ and methanol are needed
1.5 Stable sulfur isotope fractionation during microbial sulfate reduction

1.5.1 Stable sulfur isotopes and the delta notation

Sulfur has four stable isotopes of which the two most abundant ones are $^{32}$S (95.02% natural abundance) and $^{34}$S (4.22%), whereas $^{33}$S (0.76%) and $^{36}$S (0.0136%) are more rare (Canfield, 2001). In sulfur isotope fractionation studies, usually only $^{32}$S and $^{34}$S were considered. The abundance of stable sulfur isotopes in a natural sample is given in the delta notation:

$$\delta^{34}S(\%) = \frac{R_{\text{sample}} - R_{\text{V-CDT}}}{R_{\text{V-CDT}}} \times 1000$$

(Equation 1-1),

where $R = \text{isotope ratio ($^{34}$S/$^{32}$S)}$, V-CDT = Vienna Canyon Diablo Troilite, the international standard for sulfur isotopes.

1.5.2 Mechanism of sulfur isotope fractionation

The reason for physicochemical isotope fractionation is the difference of bond strength between the lighter and the heavier isotope, e.g. the sulfur isotopes $^{32}$S and $^{34}$S (Figure 1-9). The potential energy minimum is lower for the heavier isotope than for the lighter isotope. Hence, it will take more energy to dissociate a chemical bond of a heavier isotope than that of a lighter isotope, in the same way as it is more difficult to lift a heavier weight than a lighter weight. Therefore, a bond of a heavy isotope is less easily broken so that in a chemical reaction, heavy isotopes react more slowly. This effect leads to the two kinds of physicochemical fractionations, equilibrium and kinetic (nonequilibrium) isotope fractionation (Clark and Fritz, 1997).

1.5.3 Equilibrium fractionation

Equilibrium fractionation is the isotope fractionation that occurs between two chemical species at equilibrium, defined as the state in which the forward and backward reaction rates are the same (Clark and Fritz, 1997). Statistically, since the bonds of the heavier isotope are stronger, they will also last longer, leading to a slower reaction rate of the heavier isotope as compared to the lighter isotope. Hence, both the forward and the backward reaction rates of the heavier isotope will be slower than those of the lighter isotope, leading to different equilibrium constants ($K$) for the isotope exchange reactions of the heavier ($K_h$) and the lighter
(K₀) isotope. Since the overall equilibrium constant for isotope exchange (K₀) is defined as the ratio of the equilibrium constant of the heavy isotope divided by the equilibrium constant of the light isotope (Kₒ/K₀), Kₒ will deviate from a value of 1, at which no fractionation occurs. A global equilibrium fractionation example is atmospheric CO₂, which is more enriched in $^{18}$O than H₂O in the oceans by the value of about 40%, in agreement with the theoretical value (Clark and Fritz, 1997).

Further conditions under which equilibrium fractionation occurs are that both reactant and product reservoirs and isotopes between reactant and product reservoirs are well mixed (i.e., the chemical reactions must have proceeded for a long enough time in both directions) (Clark and Fritz, 1997). However, most systems are not in equilibrium. In cases where the forward reaction rate is not much higher than the backward reaction rate, isotopic equilibrium may still be achieved. However, most microbial processes such as microbial sulfate reduction are unidirectional (no back reaction). Any isotope effect resulting from such processes will be due to kinetic isotope fractionation (Clark and Fritz, 1997).
1.5.4 Kinetic isotope fractionation and the Rayleigh distillation equation

During the course of a unidirectional chemical or biological reaction, the heavier isotope will react more slowly than the lighter isotope, resulting in kinetic isotope fractionation. Hence, in a closed system and for example during microbial $\text{SO}_4^{2-}$ reduction, the heavier isotope will accumulate in the residual reactant ($\text{SO}_4^{2-}$) and the lighter isotope will accumulate in the product ($\text{S}(-\text{II})$) (Figure 1-10). This process is described by the Rayleigh distillation equation (Clark and Fritz, 1997; Mariotti et al., 1981). As the reaction proceeds (decreasing residual fraction of $\text{SO}_4^{2-}$), residual $\text{SO}_4^{2-}$ becomes heavier (higher $\delta^{34}\text{S}$ values). At the same time, the instantaneously formed $\text{S}(-\text{II})$, which at each point of the reaction is lighter than the residual $\text{SO}_4^{2-}$, also becomes progressively heavier.

The constant distance of these two curves is a measure for the extent of isotope fractionation and called the enrichment factor, $\varepsilon$. The cumulatively produced $\text{S}(-\text{II})$ also becomes heavier during the course of the reaction, but in the end of the reaction (residual fraction $= 0$) it must have the same isotopic composition as the initial $\text{SO}_4^{2-}$.

Mathematically, the Rayleigh distillation equation is expressed as:

$$ R = R_0 f^{(\alpha-1)} = R_0 f^{\varepsilon/1000} \quad \text{(Equation 1-2)}, $$

where $R$ is the isotope ratio in a diminishing reservoir of the reactant, $R_0$ the initial isotope ratio, $f$ the remaining fraction of the reservoir, and $\alpha$ the fractionation factor for the reaction.

In delta notation, this equation takes the form:

$$ \ln \frac{\delta + 1000}{\delta_0 + 1000} = (\alpha - 1) \ln f = \frac{\varepsilon}{1000 \ln f} \quad \text{(Equation 1-3)}. $$
1.5.5 Factors that influence the extent of sulfur isotope fractionation

The extent of sulfur isotope fractionation, i.e. the $\varepsilon$ value, is not constant for different experimental conditions and microbial cultures. Values between -3 and 46% have been reported (Canfield, 2001). Factors influencing $\varepsilon$ values were discussed by Canfield (2001) in a recent review and are summarized in Table 1-8. Although this list suggests that the factors that influence the extent of sulfur isotope fractionation are well known, "these principles provide a guide for understanding the controls on isotope fractionation during sulfate reduction, but they do not provide a precise means by which fractionation can be predicted" (Canfield, 2001).

Table 1-8 Summary of factors that influence the enrichment factor ($\varepsilon$, extent of fractionation) during sulfur isotope fractionation (Canfield, 2001)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Influence on fractionation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-specific sulfate reduction rate (mol cell$^{-1}$ time$^{-1}$)</td>
<td>Lower rate $=&gt;$ higher fractionation</td>
</tr>
<tr>
<td>Sulfate concentration</td>
<td>Low concentration ($&lt;200$ $\mu$M) $=&gt;$ low fractionation ($\pm 0$), high concentration $=&gt;$ fractionations between 3 and 46 %</td>
</tr>
<tr>
<td>Carbon source concentration</td>
<td>Low concentration $=&gt;$ higher fractionation</td>
</tr>
<tr>
<td>Strain-specific properties</td>
<td>Variable and unknown (except complete and incomplete carbon source degradation)</td>
</tr>
<tr>
<td>Complete / incomplete carbon source degradation</td>
<td>Complete: higher fractionation, incomplete: lower fractionation</td>
</tr>
<tr>
<td>Energy yield ($\Delta G_0$) of the reaction</td>
<td>Higher energy yield $=&gt;$ lower fractionation</td>
</tr>
<tr>
<td>Metabolic state of the organism, “well-being”</td>
<td>Variable</td>
</tr>
<tr>
<td>$H_2$ as electron donor</td>
<td>Lower fractionation than with carbon substrates</td>
</tr>
<tr>
<td>Temperature</td>
<td>Influences cell-specific sulfate reduction rate, which in turn influences fractionation (see above)</td>
</tr>
<tr>
<td>Organisms with different growth temperature optima</td>
<td>Similar fractionations</td>
</tr>
</tbody>
</table>
1.5.6 Applications and the potential of stable isotope methods for determining rate coefficients

Stable isotope methods may be used to derive quantitative information on biological processes in the subsurface. For example, they were successfully used for the determination of the extent of biodegradation of a single compound (toluene) in a field situation when the isotope enrichment factor was known a priori (Meckenstock et al., 2002), or they may serve to assess the fate of chlorinated ethenes in the environment (Hunkeler et al., 1999a). Aggarwal et al. (1997) derived an equation to determine first-order rate coefficients ($k$) from isotope enrichment factors ($\varepsilon$) (Aggarwal et al., 1997):

$$\delta_t - \delta_0 = -\varepsilon k (t - t_0)$$

(Equation 1-4).

This equation was derived from the first-order rate law and the Rayleigh distillation equation. Sulfur isotope fractionation has been used qualitatively to indicate microbial sulfate reduction, but quantitative use such as proposed by Aggarwal et al. (1997) has the prerequisite that the enrichment factor is known and constant.

1.6 Scope and outline of thesis

The overall objective of this thesis was to evaluate microbial processes in a PHC-contaminated aquifer, in particular sulfate reduction and methanogenesis, by combining hydrological, chemical, molecular, microbiological, and stable isotope methods. Both field and laboratory methods were applied.

The field experiments were conducted in the PHC-contaminated aquifer in Studen, Kanton Bern, Switzerland, which was characterized in detail by Bolliger et al. (1999) (Figure 1-11). The push-pull field tests presented in chapters 2-4 were conducted in the sulfate-reducing well PS3 and tests described in chapter 7 in the methanogenic well PS5 (Figure 1-11). Inocula for the microcosm studies in chapters 5 and 6 were taken from well S6.

In particular, my objectives were:

in Chapter 2: to evaluate the feasibility of push-pull tests in combination with stable sulfur isotope analyses for the in-situ quantification of microbial sulfate reduction in a PHC-contaminated aquifer,

in Chapter 3: to provide a more detailed analysis of one of the push-pull tests presented in chapter 2,
in Chapter 4: to assess the activity and community structure of sulfate-reducing bacteria in a PHC-contaminated aquifer using macroscopic activity measurements (push-pull tests) in combination with molecular analyses (FISH, PCR-DGGE),

in Chapter 5: to characterize the SRB populations (using PLFA analysis and FISH) that were selectively stimulated when sediment from a PHC-contaminated freshwater aquifer was incubated in sulfate-reducing aquifer microcosms that were amended with various carbon sources (acetate, butyrate, propionate, lactate, and citrate),

in Chapter 6: to quantify and compare sulfur isotope fractionation in pure and enrichment culture microcosms amended with various carbon sources (naphthalene, 1,3,5-trimethylbenzene, heating oil, acetate, pyruvate, benzoate, and 3-phenylpropionate) under conditions that resemble freshwater field conditions in a PHC-contaminated aquifer,

in Chapter 7: to assess activity and community structure of methanogens in the anoxic zone of a PHC-contaminated aquifer using push-pull tests in combination with molecular analyses (FISH, PCR-DGGE),

in Chapter 8: to discuss the preceding chapters in a broader context with emphasis on rates of microbial processes in PHC-contaminated aquifers, advantages and limitations of the employed molecular methods to characterize microbial community structure, an evaluation of the dependence of the extent of stable sulfur isotope fractionation on various parameters with

![Map of the PHC-contaminated aquifer in Studen, Switzerland (Bolliger et al., 1999)](image-url)
the help of literature data, and a comparison of microcosm versus field studies with respect to rates of microbial activities, microbial community structures and sulfur isotope fractionation. Furthermore, an outlook with questions and approaches for future research is included.

1.7 References


In-Situ Assessment of Microbial Sulfate Reduction in a Petroleum-Contaminated Aquifer Using Push-Pull Tests and Stable Sulfur Isotope Analyses

2.1 Abstract

Anaerobic microbial activities such as sulfate reduction are important for the degradation of petroleum hydrocarbons (PHC) in contaminated aquifers. The objective of this study was to evaluate the feasibility of single-well push-pull tests in combination with stable sulfur isotope analyses for the in-situ quantification of microbial sulfate reduction. A series of push-pull tests was performed in an existing monitoring well of a PHC-contaminated aquifer in Studen (Switzerland). Sulfate transport behavior was evaluated in a first test. In three subsequent tests, we injected anoxic test solutions (up to 1000 L), which contained 0.5 mM bromide (Br\textsuperscript{-}) as conservative tracer and 1 mM sulfate (SO\textsubscript{4}\textsuperscript{2-}) as reactant. After an initial incubation period of 42.5 to 67.9 h, up to 1100 L of test solution/groundwater mixture was extracted in each test from the same location. During the extraction phases we measured concentrations of relevant species including Br\textsuperscript{-}, SO\textsubscript{4}\textsuperscript{2-} and sulfide (S(-II)), as well as stable sulfur isotope ratios ($\delta^{34}S$) of extracted, unconsumed SO\textsubscript{4}\textsuperscript{2-} and extracted S(-II). Results indicated sulfate reduction activity in the vicinity of the test well. First-order rate coefficients for sulfate reduction increased with groundwater temperature and ranged from $0.043 \pm 0.013 \text{ d}^{-1}$ to $0.130 \pm 0.015 \text{ d}^{-1}$. Isotope enrichment factors ($\epsilon$) computed from sulfur isotope fractionation of extracted, unconsumed SO\textsubscript{4}\textsuperscript{2-} ranged from $20.2 \pm 5.5\%$ to $22.8 \pm 3.4\%$. Together with observed fractionation in extracted S(-II), isotope enrichment factors provided strong evidence for microbially-mediated sulfate reduction. Thus, push-pull tests combined with stable sulfur isotope analyses proved useful for the in-situ quantification of microbial sulfate reduction in a PHC-contaminated aquifer.

Key words: bioremediation, microbial activity, sulfate reduction, single-well test, stable isotopes, petroleum hydrocarbons.

2.2 Introduction

Microbial sulfate reduction is an important metabolic activity in many petroleum hydrocarbon (PHC)-contaminated aquifers (Lovley, 1997; Wiedemeier et al., 1999). During dissimilatory sulfate reduction, bacteria reduce sulfate (SO\textsubscript{4}\textsuperscript{2-}) to sulfide (S(-II), defined here as the sum of $H_2S$, $HS^-$ and $S^2$). Concurrently, PHC and other indigenous organic compounds are oxidized and often mineralized to carbon dioxide (CO\textsubscript{2}) and water. Thus, microbial sulfate reduction contributes to the removal of PHC constituents from contaminated aquifers (Anderson and Lovley, 2000; Reinhard et al., 1997; Thierrin et al., 1993; Vroblesky et al.,
Over the last decade, it has become increasingly apparent that in-situ test methods are required to accurately assess subsurface microbial activities (Gillham et al., 1990; Madsen, 1991; Madsen, 1998). Recently, single-well injection-withdrawal tests, which we call “push-pull” tests, have been used for the in-situ quantification of microbial activities in PHC-contaminated aquifers (Istok et al., 1997; Reinhard et al., 1997; Schroth et al., 1998). In a push-pull test, a prepared test solution that contains a non-reactive, conservative tracer and one or more reactive solutes (reactants) is injected (“pushed”) into the aquifer through an existing well. During the following initial incubation period (i.e., a rest phase without pumping), indigenous microorganisms consume reactants and generate metabolic products. Thereafter, the test solution/groundwater mixture is extracted (“pulled”) from the same location. Rates of microbial activities are then determined from an analysis of solute breakthrough curves obtained by measuring concentrations of tracer, reactants and/or metabolic products at the injection/extraction well during the extraction phase of the test (Haggerty et al., 1998; Snodgrass and Kitanidis, 1998). So far, push-pull tests have been employed to quantify several microbial processes in PHC-contaminated aquifers including aerobic respiration, denitrification, sulfate reduction and methanogenesis (Istok et al., 1997), and degradation of PHC constituents under nitrate- and sulfate-reducing conditions (Reinhard et al., 1997). In addition, push-pull tests were used to assess spatial variability in aerobic respiration and denitrification (Schroth et al., 1998). However, despite their efforts Istok et al. (1997) were unsuccessful in determining rates of microbial sulfate reduction, as essentially none of the injected SO$_4^{2-}$ was consumed during their tests, possibly due to the relatively short incubation periods (~7 h) employed.

Quantification of microbial sulfate reduction based on reactant (SO$_4^{2-}$) consumption or product (S(-II)) formation may be obscured by concurrent abiotic transformations, e.g., by dissolution/precipitation of gypsum (CaSO$_4$, (Stumm and Morgan, 1981)), or by precipitation of S(-II) as iron sulfides (Anderson and Lovley, 2000). As a tool to discern microbial activity from abiotic transformations, stable isotope analyses have found increasing application in recent years. For example, sulfur in natural environments consists largely of two stable isotopes: $^{32}$S (95.02% natural abundance) and $^{34}$S (4.21% natural abundance) (Hoefs, 1997). Microbial sulfate reduction usually results in significant isotope fractionation, i.e., an enrichment of $^{34}$S in unconsumed SO$_4^{2-}$ coupled to an enrichment of $^{32}$S in produced S(-II) (Clark and Fritz, 1997; Hoefs, 1997; Krouse, 1980). Sulfur isotope fractionation in
groundwater was previously observed in forest hydrology studies (Alewell and Giesemann, 1996; Robertson and Schiff, 1994) as well as in contaminated aquifers, e.g. at a waste disposal site (Bottrell et al., 1995). Thus, sulfur isotope fractionation appears to be a valuable indicator for microbial sulfate reduction in various environments. Unfortunately, little is known about sulfur isotope fractionation in PHC-contaminated aquifers.

The main objective of this study was to evaluate the feasibility of push-pull tests in combination with stable sulfur isotope analyses for the in-situ quantification of microbial sulfate reduction in a PHC-contaminated aquifer. Sulfate reduction was quantified based on sulfate consumption observed during push-pull tests. In addition, stable sulfur isotope analyses of extracted, unconsumed $\text{SO}_4^{2-}$ and of extracted $\text{S}(-\text{II})$ were used to determine isotope enrichment factors, which served as indicators for microbial sulfate reduction.

2.3 Materials and Methods

2.3.1 Field site description

The study was conducted in a heating oil-contaminated aquifer in Studen, Switzerland (Fig. 2-1a), which was characterized in detail by Bolliger et al. (1999). 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 to 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 between 2 and 4 m below ground surface. Hydraulic conductivity ranges from $1.0 \times 10^{-4}$ to $9.3 \times 10^{-3}$ m s$^{-1}$, porosity is estimated at 0.19, and the average pore water velocity is $\sim 0.4$ m d$^{-1}$ (Bolliger et al., 1999).

Push-pull tests described in this paper were conducted in monitoring well PS3, which is located within the contaminant source zone (free-phase PHC present) (Fig. 2-1a). Well PS3 is constructed of 11.5 cm I.D. polyvinyl chloride casing and partially penetrates the aquifer to a depth of $\sim 1$ m below the ground water table. Compared to the uncontaminated, upgradient well P20 (Fig. 2-1a), groundwater in PS3 exhibited reduced conditions and contained up to 1 mg L$^{-1}$ dissolved PHC (Bolliger et al., 1999). Monitoring of geochemical parameters along a
Figure 2-1 (a) Site map of the heating oil-contaminated aquifer in Studen, Switzerland, and (b) concentrations of selected geochemical parameters along the center flow line as determined by Bolliger et al. (1999). Push-pull tests described in this paper were conducted in monitoring well PS3.
center flow line revealed that dissolved oxygen (not shown) and nitrate were almost completely depleted and SO$_4^{2-}$ was partially depleted by the time groundwater reached PS3 (Fig. 2-1b, adopted from Bolliger et al. (1999)). Conversely, methane (CH$_4$) concentrations increased considerably downgradient from PS3. Long-term monitoring for this site provided additional evidence that PS3 is located within a transition zone where both sulfate-reducing and methanogenic conditions are found (Bolliger et al., 2000).

2.3.2 Push-pull tests and sample collection procedures

Four push-pull tests, denoted PPT1 through PPT4, were performed over a nine-month period from August 1999 until April 2000. First, PPT1 was performed to evaluate sulfate transport behavior during a push-pull test. Then PPT2, PPT3 and PPT4 were performed to repeatedly quantify rates of microbial sulfate reduction. For each push-pull test, groundwater was first withdrawn from PS3 using a submersible pump (Grundfos MP-1, Grundfos Pumpen AG, Fällanden, Switzerland) and collected in 500-L plastic carboys. Test solutions were then prepared by adding bromide (Br$^-$, prepared from KBr, Fluka, Buchs, Switzerland) as non-reactive, conservative tracer and SO$_4^{2-}$ (prepared from K$_2$SO$_4$, Fluka) as reactant to achieve final concentrations of 0.5 mM Br$^-$ and 1.0 or 2.0 mM SO$_4^{2-}$ (Tab. 2-1). In PPT2, PPT3 and PPT4, the carboys were continuously sparged with nitrogen gas to minimize O$_2$ dissolution from air into test solutions during preparation and subsequent injection.

Table 2-1 Summary of experimental conditions during four push-pull tests performed to evaluate sulfate transport behavior (PPT1) and microbial sulfate reduction (PPT2, PPT3, and PPT4) in a PHC-contaminated aquifer.

<table>
<thead>
<tr>
<th>Test</th>
<th>SO$_4^{2-}$ injection concentration (mM)</th>
<th>Injection volume (L)</th>
<th>Injection duration (h)</th>
<th>Initial incubation period (h)</th>
<th>Total extracted volume (L)</th>
<th>Total test duration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPT1</td>
<td>2.0</td>
<td>190</td>
<td>0.92</td>
<td>1.1</td>
<td>380</td>
<td>4.1</td>
</tr>
<tr>
<td>PPT2</td>
<td>1.0</td>
<td>500</td>
<td>1.83</td>
<td>67.9</td>
<td>1000</td>
<td>73.0</td>
</tr>
<tr>
<td>PPT3</td>
<td>1.0</td>
<td>1000</td>
<td>2.83</td>
<td>44.1</td>
<td>1100</td>
<td>119.9</td>
</tr>
<tr>
<td>PPT4</td>
<td>1.0</td>
<td>1000</td>
<td>2.17</td>
<td>42.5</td>
<td>1100</td>
<td>117.3</td>
</tr>
</tbody>
</table>

For each push-pull test, injection of a specified volume of test solution into PS3 began at time $t = 0$ h and was completed within 0.92 to 2.83 h using gravity drainage (Tab. 2-1). In PPT1, continuous extraction began 1.1 h after the injection was terminated. Longer initial incubation periods were used in PPT2, PPT3 and PPT4 before continuous extraction was initiated in PPT2 and stepwise extraction over four consecutive days (batches of 200 to 300 L...
each per day) was employed in PPT3 and PPT4. In this fashion, we extracted between 380 and 1100 L of test solution mixed with native groundwater in each test at nearly constant flow rates. Total test duration varied from 4.1 h in PPT1 up to 119.9 h in PPT3 (Tab. 2-1).

Water samples for chemical/isotope analyses were obtained during the collection of groundwater in carboys (background concentrations), during the injection of test solutions (injection concentrations), and at regular intervals during the extraction phase of the push-pull tests. Samples for Br\(^-\) and SO\(_4^{2-}\) were filtered in the field using 0.45 \(\mu\)m polyvinylidene fluoride filters (Millipore, Bedford, USA) and stored in 12-mL plastic vials. Samples for alkalinity and pH were collected without headspace in 117-mL serum bottles using butyl rubber stoppers. All samples were stored at 4°C prior to analysis. Samples collected for dissolved O\(_2\), S(-II) and ferrous iron (Fe(II)) determination were analyzed immediately in the field (see below), as was groundwater temperature using an appropriate electrode (Cyberscan pH100, Eutech Cybernetics, Singapore) fitted to a flow cell.

Samples for sulfur isotope measurements of SO\(_4^{2-}\) (PPT2 - PPT4) were collected in 1-L glass bottles acidified with 2 mL of 32% HCl (Fluka). Sulfate was subsequently precipitated as BaSO\(_4\) by replacing 10 mL of sample with 10 mL of a 1.2 M BaCl\(_2\) solution. Samples for sulfur isotope measurements of S(-II) (PPT4 only) were collected in 25-L plastic carboys with 5 L of headspace. Immediately after collection samples were acidified using 180 mL of 1 M HCl and vigorously sparged with nitrogen gas for at least 30 min. During sparging, S(-II) was precipitated as Ag\(_2\)S in a gas trap. The trap contained 40 mL of 12.75 mM AgN\(_3\) buffered at pH = 4 using 100 mM acetic acid/20 mM sodium acetate (Moncaster and Bottrell, 1991).

### 2.3.3 Analytical methods

Bromide and SO\(_4^{2-}\) concentrations were determined using a DX-100 ion chromatograph system equipped with a conductivity detector (Dionex, Sunnyvale, CA, USA). Alkalinity was measured by potentiometric titration using Gran plots for graphical determination of the end point (Stumm and Morgan, 1981) and pH was measured in the laboratory with a MP 225 pH meter equipped with an InLab407 electrode (both Mettler-Toledo, Schwerzenbach, Switzerland). Dissolved inorganic carbon (DIC, sum of H\(_2\)CO\(_3\), HCO\(_3^-\) and CO\(_3^{2-}\)) concentrations were calculated from alkalinity and pH (Stumm and Morgan, 1981). Dissolved O\(_2\), S(-II) and Fe(II) were measured colorimetrically using a DR/890 colorimeter (Hach Co., Loveland, CO, USA) following standard protocols.
For stable sulfur isotope measurements BaSO₄ and Ag₂S were separately recovered on 0.45 µm HVLP membrane filters (Millipore). After drying at 80°C, ~ 0.7 mg BaSO₄ or Ag₂S were weighted in tin cups together with ~ 1.4 mg vanadium pentoxide (added as catalyst). Sulfur isotope ratios ($^{34}$S/$^{32}$S) were subsequently measured on an Optima mass spectrometer (Fisons, Middlewich, Chesire, UK) coupled in continuous-flow to an elemental analyzer (Carlo Erba Instruments, Milan, Italy). The system was calibrated using international standards IAEA NZ1, IAEA NZ2, and NBS127 (Gonfiantini et al., 1995). Analytical reproducibility of the measurements was ± 0.3‰. Data are reported in the conventional δ–notation relative to the Vienna-Canyon Diabolo Troilite (V-CDT) standard using:

$$\delta^{34}S(\%) = \frac{R_{\text{sample}} - R_{V-\text{CDT}}}{R_{V-\text{CDT}}} \times 1000$$

(Equation 2-1)

where $R_{\text{sample}}$ and $R_{V-\text{CDT}}$ are $^{34}$S/$^{32}$S sulfur isotope ratios in sample and V-CDT standard, respectively.

### 2.3.4 Determination of first-order rate coefficients

First-order rate coefficients for sulfate reduction were determined from sulfate consumption using the method of Haggerty et al. (1998). This method is based on an analysis of tracer and reactant transport in the alternating diverging/converging radial flow field surrounding a monitoring well during a push-pull test. The method assumes that the injected reactant is transformed within the aquifer according to the first-order type reaction $dC_r/dt = -kC_r$, where $C_r$ is the reactive solute concentration and $k$ is the rate coefficient. The method also assumes that the injected test solution is well mixed within the aquifer and that the advection-dispersion-sorption transport properties of tracer and reactant are similar. With these assumptions, the rate coefficient may be determined from (Haggerty et al., 1998):

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

(Equation 2-2)

where $C_r^*$ is relative concentration (i.e., measured concentration divided by the concentration in the injected test solution), 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 duration of the test solution injection. Hence, a plot of $\ln \left( C_r^*/C_{tr}^* \right)$ versus $t^*$ generates a straight line with a slope $-k$ and an intercept $\ln \left[ \left(1 - e^{kt_{ inj}}\right)/k t_{ inj} \right]$. A nonlinear least-squares routine was used to fit Eqn. (2-2) (both slope and intercept) to experimental breakthrough data to obtain
estimates of first-order rate coefficients for sulfate reduction. The 95% confidence interval for \( k (k \pm 2\sigma_k) \) was computed from the variance of the estimated \( k \), \( \sigma_k^2 \) using (Schroth et al., 1998):

\[
\sigma_k^2 = \sigma^2 \left[ \sum_{i=1}^{n} \left( \frac{1 - e^{kt_{inj}} + k t_{inj}}{k (e^{kt_{inj}} - 1)} - t_i \right) \right]^{-1}
\]

(Equation 2-3)

with \( i = 1 \) to \( n \), where \( n \) is the total number of observations, and \( \sigma^2 \) is the variance of errors in \( \ln (C_t/C^*_o) \).

### 2.3.5 Determination of isotope enrichment factors

Sulfur isotope fractionation was quantified by computing isotope enrichment factors, \( \varepsilon \) (in ‰). In a closed system enrichment factors can be determined by fitting Rayleigh distillation equations to experimental data (Mariotti et al., 1981). Specifically, enrichment factors of extracted, unconsumed \( SO_4^{2-} \) and extracted \( S(-II) \) may be determined from measured \( \delta^{34}S \) values using (Böttcher et al., 1999):

\[
\delta^{34}S(SO_4^{2-}) = \delta^{34}S(SO_4^{2-})_0 + \varepsilon \ln f
\]

(Equation 2-4)

\[
\delta^{34}S(S(-II)) = \delta^{34}S(SO_4^{2-})_0 - \varepsilon (f \ln f)/(1-f)
\]

(Equation 2-5)

where \( f \) is the fraction of extracted, unconsumed \( SO_4^{2-} \), and \( \delta^{34}S(SO_4^{2-})_0 \) is the initial isotope composition of sulfate in the injected test solution.

### 2.4 Results and discussion

#### 2.4.1 Push-pull tests

Breakthrough curves for \( Br^- \) and \( SO_4^{2-} \) showed a gradual decline in \( C^* \) as extracted test solution was increasingly diluted with native groundwater during PPT1 (Fig. 2-2). In addition, relative concentrations of \( SO_4^{2-} \) and \( Br^- \) were nearly identical throughout the extraction phase. This indicates that \( SO_4^{2-} \) transport behavior in general was similar to that of \( Br^- \) and that \( SO_4^{2-} \) sorption to aquifer solids in particular was negligible during PPT1 (Schroth et al., 2000). Thus, results from PPT1 confirmed the assumption of similar transport behavior for tracer and reactant, which is required for the accurate computation of rate coefficients in subsequent tests using the method of Haggerty et al. (1998). Moreover, by the end of PPT1 78% of the injected \( Br^- \) mass and 77% of the injected \( SO_4^{2-} \) mass was recovered (computed
by integrating solute breakthrough curves shown in Fig. 2-2). Recovery of similar relative solute masses clearly indicates that the total test duration (4.1 h, Tab. 2-1) was sufficiently short to prevent notable SO$_4^{2-}$ consumption during PPT1.

Extraction phase breakthrough curves for Br$^-$ and SO$_4^{2-}$ during PPT2, PPT3 and PPT4 (Fig. 2-3) showed gradual declines in $C^*$ similar to that observed during PPT1 (Fig. 2-2). However, relative SO$_4^{2-}$ concentrations were smaller than relative Br$^-$ concentrations during most of PPT2 - PPT4 extraction phases, which indicated that sulfate was consumed during those tests, presumably due to microbial activity. Relative concentrations of SO$_4^{2-}$ slightly larger than those of Br$^-$ were only observed in samples collected near the end of PPT2 - PPT4 extraction phases (Fig. 2-3). This is due to SO$_4^{2-}$ contained in native groundwater (~0.03 mM at the time the tests were conducted), which was extracted together with the injected test solutions. To account for SO$_4^{2-}$ contained in native groundwater, we computed corrected sulfate concentrations ($CSO_4^{2-}$) using Br$^-$ breakthrough curves as a measure of dilution of test solution with native groundwater and assuming constant SO$_4^{2-}$ background concentrations for each test (Schroth et al., 1998). As a consequence, relative $CSO_4^{2-}$ concentrations were smaller than relative Br$^-$ concentrations throughout PPT2 - PPT4 extraction phases (Fig. 2-3). Note that in PPT1 no correction of SO$_4^{2-}$ data was necessary due to the higher SO$_4^{2-}$ concentrations employed in this test.

During the extraction phases of PPT2, PPT3 and PPT4 we recovered between 25 and 31% of the injected Br$^-$ mass and between 20 and 23% of the injected SO$_4^{2-}$ mass. Differences between recovered relative Br$^-$ and SO$_4^{2-}$ masses ($21.3 \pm 7.2$ % on average for PPT2-PPT4)
Figure 2-3 Breakthrough curves for Br\(^-\) and SO\(_4^{2-}\) obtained during extraction phases of (a) PPT2, (b) PPT3, and (c) PPT4. Open symbols show corrected SO\(_4^{2-}\) concentrations (cSO\(_4^{2-}\)), which were computed using Br\(^-\) data as a measure of dilution between test solutions and native groundwater.
illustrate that total test durations (Tab. 2-1) were sufficiently long to allow notable sulfate consumption during these tests. Conversely, mass recovery of injected Br\(^{-}\) tracer in PPT2 - PPT4 (25 – 31\%) was poor compared to that in PPT1 (78\%). This is likely due to longer test durations in PPT2 – PPT4 (Tab. 2-1) in combination with a fairly high average pore water velocity (~ 0.4 m d\(^{-1}\)) at the site. Thus, during PPT2 – PPT4 a significant portion of test solution migrated beyond the radius of influence of PS3. However, it is important to note that no complete tracer mass recovery is required during push-pull tests for an accurate quantification of rate coefficients (Haggerty et al. (1998); see next section).

Sulfide increased from background concentrations (9.3 – 16.8 \(\mu\)M) to maximum concentrations ranging from 14.0 to 50.9 \(\mu\)M during PPT2 - PPT4 extraction phases (not shown). For each test, produced S\((-II)\) mass was computed by integrating measured S\((-II)\) concentrations after they were corrected for background and injected test solution S\((-II)\) concentrations. Produced S\((-II)\) mass recovered during PPT2 – PPT4 ranged from 2.3 to 14.9 mmol, which is much less than expected based on measured SO\(_4^{2-}\) consumption (41.4 – 50.0 mmol). Loss of produced S\((-II)\) during our tests was presumably due to FeS precipitation, as Fe\((II)\) concentrations up to 59.1 \(\mu\)M were measured during the extraction phases (solubility product for FeS\((s)\) is \(K_{sp} = 10^{-18.1}\) at 25 °C (Stumm and Morgan, 1981)). Increased Fe\((II)\) concentrations are commonly encountered within reduced zones of contaminated aquifers (e.g., Lovley (1997); Lovley and Anderson (2000)), often rendering S\((-II)\) data useless for the quantification of microbial sulfate reduction.

Other geochemical parameters did not vary significantly during PPT2 – PPT4 extraction phases (not shown). In particular, calculated DIC concentrations ranged from 11.1 to 13.2 mM during the extraction phases without an obvious trend and were even slightly smaller than DIC background concentrations (13.3 to 13.8 mM) in PS3. This is at least in part due to somewhat smaller DIC injection concentrations (9.7 to 13.3 mM, due to sparging of test solutions prior to injection) compared to DIC background concentrations. Moreover, based on measured SO\(_4^{2-}\) consumption and given the following reaction stoichiometry (Bolliger et al., 1999):

\[
1.37\text{CH}_{1.85} + \text{SO}_{4}^{2-} + 2\text{H}^{+} \rightarrow 1.37\text{CO}_{2} + \text{H}_{2}\text{S} + 1.26\text{H}_{2}\text{O} \quad \text{(Equation 2-6)}
\]

we would expect minimal DIC production during our tests (up to 68.5 mmol DIC in 1000 L of extracted test solution) compared to DIC contained in test solutions and native groundwater. Thus, DIC data obtained during our tests did not provide additional information for the quantification / verification of microbial sulfate reduction. This result contrasts results
for DIC analyses performed across entire contaminated sites, which often provide useful information on overall metabolic activities (e.g., Bolliger et al. (1999); Chapelle et al. (1996); Hunkeler et al. (1999)).

### 2.4.2 Quantification of microbial sulfate reduction

First-order rate coefficients for sulfate reduction were computed for PPT2, PPT3 and PPT4 based on Br\(^{-}\) and SO\(_4^{2-}\) breakthrough data (Fig. 2-3) using the method of Haggerty et al. (1998) (Fig. 2-4). Note that best fit lines in Fig. 2-4 are intentionally extended to the y-axis intercept as both slope and y-axis intercept were fitted (Eqn. 2-2), and that PPT2 results were unamenable for display due to the different extraction mode used in that test. Computed values of \( k \) ranged from 0.043 d\(^{-1}\) to 0.130 d\(^{-1}\) with 95% confidence intervals ranging from 0.010 d\(^{-1}\) to 0.015 d\(^{-1}\) (Tab. 2-2). Thus, values of \( k \) varied by less than a factor of three between the tests. On the other hand, variations in \( k \) were significant to the 95% confidence level and may at least in part be attributed to the difference in groundwater temperature at the time each test was conducted (Tab. 2-2). Specifically, values of \( k \) increased with increasing temperature in our tests, which is the generally expected response of microorganisms to changes in temperature within a specific interval suitable for growth (Madigan et al., 2003).

#### Table 2-2

<table>
<thead>
<tr>
<th>Test</th>
<th>First-order rate coefficient ( k \pm 2\sigma_k ) (d(^{-1}))</th>
<th>Isotope enrichment factor ( \varepsilon \pm 2\sigma_{\varepsilon} ) (%)</th>
<th>Groundwater temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPT2</td>
<td>0.130 ± 0.015</td>
<td>---(^b)</td>
<td>16.2</td>
</tr>
<tr>
<td>PPT3</td>
<td>0.085 ± 0.010</td>
<td>22.8 ± 3.4</td>
<td>12.5</td>
</tr>
<tr>
<td>PPT4</td>
<td>0.043 ± 0.013</td>
<td>20.2 ± 5.5</td>
<td>9.4</td>
</tr>
</tbody>
</table>

\(^a\) 95% confidence interval.

\(^b\) no \( \varepsilon \) computed, but isotope fractionation in SO\(_4^{2-}\) was qualitatively observed.

Values of \( k \) determined in our study agreed well with those determined by Chapelle et al. (1996), who obtained 0.02 ≤ \( k \) ≤ 0.08 d\(^{-1}\) for sulfate reduction by fitting a form of the advection-dispersion equation to sulfate concentrations measured across an entire PHC-contaminated site. In this context it is important to note that the \( k \) values we obtained represent local measurements made in the vicinity of a monitoring well known to be within a sulfate reducing zone rather than an average value across an entire contaminated site. On the
Chapter 2

other hand, we computed $0.003 \leq k \leq 0.004$ d$^{-1}$ for unamended and enhanced sulfate reduction in another gasoline-contaminated aquifer from data presented by Reinhard et al. (1997), who employed a test similar in design to ours for quantifying degradation rates of specific petroleum constituents. Obviously, many factors such as $\text{SO}_4^{2-}$ concentration and specific types and concentrations of substrates may contribute to differences in observed rates of microbial sulfate reduction. In addition, while not measured here, we would expect substantial variation in $k$ across our site due to spatial variability in microbial activities (“hot spots”). Existence of “hot spots” in contaminated aquifers has been extensively documented in the literature (e.g., Adrian et al. (1994); Chiang et al. (1989); Harvey et al. (1984); Schroth et al. (1998)).

2.4.3 Verification of microbial sulfate reduction

Increases in $\delta^{34}\text{S} (\text{SO}_4^{2-})$ compared to $\delta^{34}\text{S} (\text{SO}_4^{2-})_0$ were observed in samples collected during PPT2 – PPT4 extraction phases (Fig. 2-5, PPT2 data not shown). In Fig. 2-5, $\delta^{34}\text{S} (\text{SO}_4^{2-})$ is plotted against $f$, which was computed from experimental breakthrough data using $f = (C_r^s/C_{tr}^s)$. Note that $f > 0.65$ in all samples due to the limited amount of $\text{SO}_4^{2-}$ consumed during the tests (Fig. 2-5). To account for the isotope composition of background $\text{SO}_4^{2-}$, we computed corrected isotope ratios ($\delta^{34}\text{S} (\text{SO}_4^{2-})$) using $\text{Br}^-$ breakthrough data as a measure of dilution between test solutions and native groundwater (Fig. 2-5). By fitting the Rayleigh equation (Eqn. 2-4) to $\delta^{34}\text{S} (\text{SO}_4^{2-})$ data, we then obtained $\varepsilon$ values of $22.8 \pm 3.4\%$ for PPT3 and $20.2 \pm 5.5\%$ for PPT4 (Tab. 2-2). Note that we were unable to compute $\delta^{34}\text{S} (\text{SO}_4^{2-})$ data for PPT2 due to an unreliable measurement of the background $\text{SO}_4^{2-}$ isotope

![Figure 2-4 Determination of first-order rate coefficients of microbial sulfate reduction for PPT3 and PPT4. Lines show the best fit of Eqn. 2-2 to experimental data obtained using the method of (Haggerty et al., 1998).](image-url)
Assessment of sulfate reduction using push-pull tests

composition for the time this test was conducted. Consequently, no $\varepsilon$ value was computed for this test.

Enrichment factors computed for our tests agreed well with $\varepsilon$ values obtained by others for microbial sulfate reduction in different environments, e.g., $\varepsilon$ values of 16 to 42% (Habicht and Canfield, 1997), 19% (Böttcher et al., 1999), and 21.4 to 28% (Asmussen and Strauch, 1998). Moreover, $\varepsilon$ values we obtained were significantly larger than those commonly observed during physical / chemical transformations of $\text{SO}_4^{2-}$ (Krouse, 1980), and they were within a range that is indicative for microbial sulfate reduction (~20 to 40%, Clark and Fritz (1997)). Hence, stable sulfur isotope fractionation of extracted, un consumed $\text{SO}_4^{2-}$ provided strong evidence for microbial sulfate reduction during our tests. On the other hand, the magnitude of sulfur isotope fractionation depends on many environmental parameters (e.g., Böttcher et al. (1999); Clark and Fritz (1997)). Thus, a fully quantitative interpretation of $\varepsilon$ values obtained in natural systems such as

![Figure 2-5 Stable sulfur isotope ratios of remaining, unconsumed $\text{SO}_4^{2-}$ obtained during (a) PPT3 and (b) PPT4 extraction phases. Corrected isotope ratios ($\delta^{34}\text{S}(\text{SO}_4^{2-})$) were computed using Br data as a measure of dilution between test solutions and native groundwater. The solid lines represent Rayleigh distillation curves (Eqn. 2-4), which were fitted to $\delta^{34}\text{S}(\text{SO}_4^{2-})$ data using linear regression analyses.](image-url)
PHC-contaminated aquifers is not possible to date. Consequently, we cannot unequivocally exclude the possibility that a portion of injected $\text{SO}_4^{2-}$ may have been abiotically consumed during our tests. However, inorganic sulfate reduction is usually not significant in groundwaters (Clark and Fritz, 1997), and, at least for PPT1, $\text{SO}_4^{2-}$ precipitation did not appear to play a major role during the test (Fig. 2-2).

Concurrent to an increase in $\delta^{34}\text{S}(\text{SO}_4^{2-})$, a decrease in $\delta^{34}\text{S}(\text{S}(-\text{II}))$ was measured in samples collected during the extraction phase of PPT4 (not shown). This would be expected for a closed system, in which enrichment of $^{34}\text{S}$ in unconsumed $\text{SO}_4^{2-}$ must lead to a depletion of $^{34}\text{S}$ in produced S(-II). However, due to the small quantity of produced S(-II) recovered during the test and the relatively high S(-II) background concentration in native groundwater (see above), computation of $\varepsilon$ was not straightforward in this case. Depending on the (unknown) composition of extracted S(-II) (produced or background S(-II)), we computed hypothetical values of $\varepsilon$ by fitting Equation 5 to experimental data. Computed values of $\varepsilon$ ranged from $14.8 \pm 2.7\%$ (for the case that samples were composed of produced S(-II) only) to $30.1 \pm 6.5\%$ (for the case that samples were composed of 100% background S(-II) and variable amounts of produced S(-II)). For the case that samples were composed of 50% produced/50% background S(-II) we calculated $\varepsilon = 22.9 \pm 5.4\%$. The latter value of $\varepsilon$ in particular agreed well with values of $\varepsilon$ calculated from $\delta^{34}\text{S}(\text{SO}_4^{2-})$ data for PPT3 and PPT4 (Tab. 2-2), but an unbiased comparison between the different $\varepsilon$ values was obviously impossible. Nevertheless, the general observation of isotope fractionation in extracted S(-II) during PPT4 provided additional qualitative evidence for microbial sulfate reduction.

### 2.5 Summary and conclusions

Microbial sulfate reduction was quantified in-situ in a PHC-contaminated aquifer based on sulfate consumption observed during single-well push-pull tests. In three consecutive tests, calculated first-order rate coefficients for sulfate reduction varied by less than a factor of three, which could be explained in part by differences in groundwater temperature between the tests. Furthermore, we were able to determine rate coefficients with reasonable accuracy, which was indicated by relatively small 95% confidence intervals.

We demonstrated that quantification and/or verification of microbial sulfate reduction based on metabolic product formation (S(-II), DIC) was not feasible during our push-pull tests. On the other hand, we provided strong evidence for microbial sulfate reduction during our tests using stable sulfur isotope analyses. From measured isotope fractionation of
extracted, unconsumed SO$_4^{2-}$, we computed enrichment factors that suggested microbial activity as the major mechanism for SO$_4^{2-}$ consumption. Further evidence for microbial sulfate reduction during one push-pull test was obtained from stable isotope measurements of extracted S(-II), even though a straightforward analysis of $\delta^{34}$S(S(-II)) data was hampered by the small quantity of produced S(-II) compared to the high S(-II) background concentration. Further experiments will be required to interpret computed isotope enrichment factors resulting from microbial sulfate reduction in a more quantitative fashion. Nonetheless, push-pull tests combined with stable sulfur isotope analyses proved useful for the in-situ quantification of microbial sulfate reduction in a PHC-contaminated aquifer.

2.6 Acknowledgments

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2.7 References


Quantification of Microbial Sulfate Reduction in a Petroleum-Contaminated Aquifer

3.1 Abstract

Sulfate reduction is an important microbial process in many aquifers contaminated with petroleum hydrocarbons (PHC). In this paper, we present results of a field experiment conducted to quantify microbial sulfate reduction in a PHC-contaminated aquifer. Sulfate reduction was quantified based on sulfate consumption during a single-well “push-pull” test. An anoxic test solution (1000 L), which contained 0.5 mM bromide as conservative tracer and 1 mM sulfate as reactant, was injected (“pushed”) into an existing monitoring well within the sulfate reducing zone of the aquifer. After an initial incubation period of 42 h, 1100 L of test solution/groundwater mixture were extracted (“pulled”) from the same location during four consecutive days in batches of 200 to 300 L each. During extraction relevant species such as bromide, sulfate and sulfide were measured, as well as stable sulfur isotope ratios ($^{34}$S) of remaining, unconsumed sulfate. Results indicated sulfate reduction activity in the vicinity of the test well. A first-order rate coefficient for sulfate reduction of 0.089 d$^{-1}$ was obtained with a 95% confidence interval of $\pm$ 0.009 d$^{-1}$. In addition, sulfur isotope fractionation of remaining, unconsumed sulfate (enrichment factor $\varepsilon = 22.8 \pm 3.4\%$) provided strong evidence for microbially mediated sulfate reduction.

Key words: sulfate reduction, microbial activity, single-well push-pull test, sulfur isotope fractionation, bioremediation, petroleum hydrocarbons.

3.2 Introduction

Anaerobic microbial processes such as sulfate reduction play an important role in the degradation of petroleum hydrocarbons (PHC) during intrinsic bioremediation in contaminated aquifers (Lovley, 1997; Wiedemeier et al., 1999). Sulfate reduction in particular was found to significantly contribute to the removal of PHC at many field sites (Reinhard et al., 1997; Thierrin et al., 1993; Vroblesky et al., 1996; Wiedemeier et al., 1999). During dissimilatory sulfate reduction, bacteria reduce sulfate ($SO_4^{2-}$) to sulfide (defined here as the sum of $H_2S$, $HS^-$ and $S^{2-}$). Concurrently, PHC and other indigenous organic compounds are oxidized and in many cases mineralized to carbon dioxide (CO$_2$) and water. Thus, quantitative information on microbial sulfate reduction is needed to assess its contribution to overall PHC removal in contaminated aquifers.
In recent years, it has become increasingly apparent that in-situ test methods are required to accurately assess subsurface microbial activity (Madsen, 1991; Madsen, 1998). A method to quantify microbial activity in-situ, called the single-well “push-pull” test, was developed by Istok et al. (1997). In a push-pull test, a prepared test solution containing a non-reactive tracer and one or more reactive solutes (reactants) is injected (“pushed”) into the aquifer using an existing well. During the following incubation period, indigenous microorganisms consume reactants and generate metabolic products. Thereafter, the test solution/groundwater mixture is extracted (“pulled”) from the same location. Rates of microbial activities are then determined from an analysis of solute breakthrough curves obtained by measuring solute concentrations of tracer, reactants and/or products at the injection/extraction well during the extraction phase (Haggerty et al., 1998; Snodgrass and Kitanidis, 1998). So far, push-pull tests have been employed to quantify several microbial processes in PHC-contaminated aquifers including aerobic respiration, denitrification, sulfate reduction and methanogenesis (Istok et al., 1997), petroleum hydrocarbon degradation (Reinhard et al., 1997) and spatial variability in aerobic respiration and denitrification (Schroth et al., 1998). However, Istok et al. (1997) were unsuccessful in determining rates of microbial sulfate reduction, as essentially none of the injected sulfate was consumed during their tests, possibly due to relatively short incubation periods (~7 h) employed.

Quantification of microbial sulfate reduction based on sulfate consumption or sulfide production may be further obscured by concurrent abiotic transformations (e.g., dissolution/precipitation of gypsum (CaSO₄), precipitation of sulfide as FeS (Stumm and Morgan, 1981)). As a tool to discern microbial activity from abiotic transformations, stable isotope analyses have found increasing application in recent years. For example, microbial sulfate reduction generally results in significant isotope fractionation, i.e. enrichment of 32S in sulfide coupled to enrichment of 34S in the remaining, un consumed sulfate (Clark and Fritz, 1997; Hoefs, 1997; Krouse, 1980). Sulfur isotope fractionation in groundwater was previously observed in forest hydrology studies (Alewell and Giesemann, 1996; Robertson and Schiff, 1994) as well as in contaminated aquifers, e.g. at a waste disposal site (Bottrell et al., 1995) and in an aquifer polluted by agrochemicals (Moncaster et al., 2000). In most cases, differences in isotope composition between sulfate and sulfide were attributed to subsurface microbial activity. Thus, sulfur isotope fractionation appears to be a valuable indicator for microbial sulfate reduction in various environments. Nonetheless, little is known about sulfur isotope fractionation in PHC-contaminated aquifers.
In this paper, we present results of a push-pull test performed to quantify microbial sulfate reduction in a PHC-contaminated aquifer. Sulfate reduction was quantified based on sulfate consumption during the test using the method of Haggerty et al. (1998). Stable sulfur isotope analyses of remaining, unconsumed sulfate were used to determine an isotope enrichment factor, which served as qualitative indicator of microbial sulfate reduction.

3.3 Materials and Methods

3.3.1 Site description

The study was conducted at a heating oil-contaminated aquifer in Studen, Switzerland, which was characterized in detail by Bolliger et al. (1999). 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 (\( \sim 34 \text{ m}^3 \)) by partial excavation of contaminated soil and pumping until 1996. At that time engineered remediation was terminated and monitored natural attenuation was selected as follow-up remediation strategy.

The underlying, 20 to 25 m thick unconfined aquifer consists of unconsolidated glaciofluvial outwash deposits with interbedded layers of poorly sorted silt, sand and gravel. The ground water table is generally between 2 and 4 m below ground surface. Hydraulic conductivity ranges from \(1.0 \times 10^{-4}\) to \(9.3 \times 10^{-3}\) m s\(^{-1}\), porosity is estimated at 0.19, and average pore water velocity is \(\sim 0.4\) m d\(^{-1}\) (Bolliger et al., 1999). The push-pull test described in this paper was conducted in monitoring well PS3 within the contaminated zone of the aquifer. Well PS3 was constructed of 11.5 cm I.D. polyvinyl chloride casing and partially penetrates the aquifer to a depth of \(\sim 1\) m below the ground water table. Compared to an uncontaminated, upgradient well (P20), groundwater in PS3 exhibited reduced conditions in the presence of abundant petroleum hydrocarbons, which is typical of PHC-contaminated environments (Bolliger et al., 1999). Long-term monitoring for this site provided additional evidence that PS3 was located within a sulfate-reducing zone of the aquifer (Bolliger et al., 2000).

3.3.2 Push-pull test procedures

For the push-pull test described in this paper, 1000 L of groundwater were first withdrawn from PS3 using a submersible pump (Grundfos MP-1, Grundfos Pumpen AG, Fällanden, Switzerland) and collected in two 500 L plastic carboys. Test solution was then prepared by
adding bromide (Br\textsuperscript{-}, prepared from KBr, obtained from Fluka, Buchs, Switzerland) as conservative tracer and SO\textsubscript{4}\textsuperscript{2-} (prepared from K\textsubscript{2}SO\textsubscript{4}, Fluka) as reactant to achieve a final concentration of 0.5 mM Br\textsuperscript{-} and 1.0 mM SO\textsubscript{4}\textsuperscript{2-}. During preparation and subsequent injection, the carboys were constantly sparged with nitrogen gas to minimize O\textsubscript{2} dissolution from air into the test solution.

Injection of 1000 L of test solution into PS3 began at time $t = 0$ h and was completed in 2.83 h using gravity drainage. After the following initial incubation period of 42 h, test solution mixed with native groundwater was extracted during four consecutive days in batches of 200 to 300 L each. In this fashion, a total volume of 1100 L was extracted at a nearly constant flow rate of $\sim 4.2$ L/min prior to termination of the push-pull test at $t = 117$ h.

Water samples for chemical analyses were obtained during collection of groundwater in carboys (background concentrations), during injection of test solution (injection concentrations), and at regular intervals during the extraction phase of the push-pull test. Samples for Br\textsuperscript{-} and SO\textsubscript{4}\textsuperscript{2-} were filtered in the field using 0.22 µm polyvinylidene fluoride filters (Millipore, Bedford, USA) and stored in 12-mL plastic vials. Samples for alkalinity and pH were collected without headspace in 117-mL serum bottles using butyl rubber stoppers. Samples for sulfur isotope measurements of SO\textsubscript{4}\textsuperscript{2-} were collected in 1-L glass bottles acidified with 2 mL of 32% HCl (Fluka). All samples were stored at 4\textdegree C prior to analysis. Samples collected for dissolved O\textsubscript{2}, sulfide and Fe\textsuperscript{2+} determination were analyzed immediately in the field (see below), as was groundwater temperature using an appropriate electrode (Cyberscan pH100, Eutech Cybernetics, Singapore) fitted to a flow cell.

### 3.3.3 Analytical methods

Bromide and SO\textsubscript{4}\textsuperscript{2-} concentrations were determined using a Dionex DX-100 ion chromatograph system equipped with a conductivity detector (Dionex, Sunnyvale, CA, USA). Alkalinity was measured by potentiometric titration using Gran plots for graphical determination of the end point (Stumm and Morgan, 1981), and pH was measured in the laboratory with a MP 225 pH meter equipped with an InLab407 electrode (both Mettler-Toledo, Schwerzenbach, Switzerland). Dissolved inorganic carbon (DIC, sum of H\textsubscript{2}CO\textsubscript{3}, HCO\textsubscript{3}\textsuperscript{-} and CO\textsubscript{3}\textsuperscript{2-}) concentrations were calculated from alkalinity and pH (Stumm and Morgan, 1981). Dissolved O\textsubscript{2}, sulfide and Fe\textsuperscript{2+} were measured colorimetrically immediately after sampling using a DR/890 colorimeter (Hach Co., Loveland, CO, USA) following standard protocols.
For sulfur isotope measurements sulfate was precipitated as BaSO$_4$ by replacing 10 mL of sample with 10 mL 1.2 M BaCl$_2$, and was subsequently filtered using a 0.45 μm HVLP membrane filter (Millipore). After drying at 80°C, ~ 0.7 mg BaSO$_4$ were weighted in tin cups together with ~ 1.4 mg vanadium pentoxide (added as catalyst). Sulfur isotope ratios ($^{34}$S/$^{32}$S) were subsequently measured on a Fisons Optima mass spectrometer coupled in continuous-flow to a Carlo Erba Elemental analyzer (CE Instruments, Milan, Italy). The system was calibrated using international standards IAEA NZ1, IAEA NZ2, and NBS127 (Gonfiantini et al., 1995). Analytical reproducibility of the measurements was ± 0.3 %. Data are reported in the conventional δ–notation relative to the Vienna-Canyon Diabolo Troilite (V-CDT) standard using:

$$\delta^{34}S = \frac{R_{\text{sample}} - R_{\text{V-CDT}}}{R_{\text{V-CDT}}} \cdot 1000$$  \hspace{1cm} (Equation 3-1)

where $R_{\text{sample}}$ and $R_{\text{V-CDT}}$ are $^{34}$S/$^{32}$S sulfur isotope ratios in sample and V-CDT, respectively.

### 3.3.4 Data analysis

A first-order rate coefficient for sulfate reduction was determined from sulfate consumption using the method of Haggerty et al. (1998). This method is based on an analysis of tracer and reactive solute transport in the alternating diverging / converging radial flow field surrounding a monitoring well during a push-pull test. The method assumes that an injected reactive solute is transformed within the aquifer according to the first-order type reaction $dC_r/dt = -k C_r$, where $C_r$ is the reactive solute concentration and $k$ is the rate coefficient. The method also assumes that the injected test solution is well mixed within the aquifer, and that the advection-dispersion transport properties of tracer and reactant are identical. With these assumptions, the rate coefficient may be determined from (Haggerty et al., 1998):

$$\ln \left( \frac{C_r^{*}(t^{*})}{C_{tr}^{*}(t^{*})} \right) = \ln \left( \frac{1 - e^{-kt_{inj}}}{kt_{inj}} \right) - kt^{*}$$  \hspace{1cm} (Equation 3-2)

where $C^{*}$ is relative concentration (i.e., measured concentration divided by the concentration in the injected test solution), 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 duration of the test solution injection. Hence, a plot of $\ln \left( C_r^{*}/C_{tr}^{*} \right)$ versus $t^{*}$ generates a straight line...
with a slope \(-k\) and an intercept \(\ln \left(\frac{1-\frac{e^{kt_{\text{eq}}}}{1+e^{kt_{\text{eq}}}}} {kt_{\text{eq}}}\right)\). A nonlinear least-squares routine was used to fit Eqn. (3-2) (both slope and intercept) to experimental breakthrough data to obtain an estimate for the first-order rate coefficient for sulfate reduction. The 95% confidence interval for \(k\) \((k \pm 2\sigma_k)\) was computed from the variance of the estimated \(k\), \(\sigma_k^2\) using (Schroth et al., 1998):

\[
\sigma_k^2 = \sigma^2 \left[ \sum_{i=1}^{n} \left\{ \frac{1-e^{kt_{\text{eq}}}} {k\left(e^{kt_{\text{eq}}}-1\right)} - t^* \right\}^2 \right]^{-1}
\]  

(Equation 3-3)

with \(i = 1\) to \(n\), where \(n\) is the total number of observations, and \(\sigma^2\) is the variance of errors in \(\ln (C_r/C_r^*)\).

Sulfur isotope fractionation was quantified by computing an isotope enrichment factor \(\varepsilon\) (in per mill). In a closed system enrichment factors can be determined by fitting Rayleigh distillation equations to experimental data (Mariotti et al., 1981). Here, the enrichment factor of remaining, unconsumed \(\text{SO}_4^{2-}\) was determined from measured \(\delta^{34}\text{S}\) values using (Böttcher et al., 1999):

\[
\delta^{34}\text{S}(\text{SO}_4^{2-}) = \delta^{34}\text{S}(\text{SO}_4^{2-})_0 + \varepsilon \ln f
\]  

(Equation 3-4)

where \(f\) is the fraction of remaining, unconsumed \(\text{SO}_4^{2-}\), and \(\delta^{34}\text{S}(\text{SO}_4^{2-})_0\) is initial isotope composition of sulfate in the injected test solution.

### 3.4 Results

Background concentrations of selected geochemical parameters obtained during the initial collection of groundwater from PS3 confirmed reduced conditions in this zone of the aquifer (Tab. 3-1). In particular, \(\text{SO}_4^{2-}\) concentration in PS3 (0.03 mM) was much smaller than in the uncontaminated, upgradient well P20 (0.34 mM, not shown).

Apart from the addition of \(\text{Br}^-\) and \(\text{SO}_4^{2-}\), little difference in geochemical parameters was observed between PS3 background and the test solution during its injection into PS3 (Tab. 3-1). As expected, sulfide concentration declined and pH increased slightly in the test solution compared to background concentrations. This was due to the removal of sulfide and dissolved \(\text{CO}_2\) from solution as a consequence of continuous \(\text{N}_2\) sparging. Conversely, sparging with \(\text{N}_2\) effectively prevented additional dissolution of \(\text{O}_2\) in the test solution (Tab. 3-1).

Extraction phase breakthrough curves for \(\text{Br}^-\) and \(\text{SO}_4^{2-}\) showed a gradual decline in \(C^*\) as extracted test solution was increasingly diluted with time by native groundwater (Fig. 3-1a).
Measured $\text{SO}_4^{2-}$ concentrations were smaller than $\text{Br}^-$ concentrations during most of the extraction phase indicating that sulfate was consumed during the push-pull test, presumably due to microbial activity. However, near the end of the extraction phase $\text{SO}_4^{2-}$ concentrations were slightly larger than $\text{Br}^-$ concentrations (Fig. 3-1a). This is due to the fact that $\text{SO}_4^{2-}$ approached its background concentration (0.03 mM, Tab. 3-1) near the end of the extraction phase, whereas $\text{Br}^-$ approached a value of zero. To account for $\text{SO}_4^{2-}$ contained in native groundwater, we computed corrected sulfate ($^{\text{c}}\text{SO}_4^{2-}$) concentrations using the $\text{Br}^-$ breakthrough curve as a measure of dilution of test solution by native groundwater. Consequently, $^{\text{c}}\text{SO}_4^{2-}$ concentrations were smaller than $\text{Br}^-$ concentrations throughout the extraction phase (Fig. 3-1a).

Sulfate consumption during the push-pull test was also indicated by the progressive divergence of $\text{Br}^-$ and $\text{SO}_4^{2-}$ in cumulative relative mass recovery curves (Fig. 3-1b), which were obtained by integrating solute breakthrough curves (Fig. 3-1a). In Fig. 3-1b, cumulative relative mass recovered, $M'$ was computed from $M' = M/M_0$, where $M$ is cumulative mass of solute recovered with time, and $M_0$ is total mass of solute injected. By the end of the push-pull test, 25% of injected $\text{Br}^-$ mass was recovered, whereas 20% of injected $\text{SO}_4^{2-}$ mass was recovered when corrected for background concentration (see $^{\text{c}}\text{SO}_4^{2-}$, Fig. 3-1b).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PS3 background</th>
<th>Injected test solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{O}_2$ (mM)</td>
<td>0.011</td>
<td>0.009</td>
</tr>
<tr>
<td>$\text{Br}^-$ (mM)</td>
<td>0.00</td>
<td>0.50</td>
</tr>
<tr>
<td>$\text{SO}_4^{2-}$ (mM)</td>
<td>0.03</td>
<td>1.00</td>
</tr>
<tr>
<td>Sulfide (mM)</td>
<td>0.009</td>
<td>0.002</td>
</tr>
<tr>
<td>$\text{Fe}^{2+}$ (mM)</td>
<td>$&gt;0.059$</td>
<td>not measured</td>
</tr>
<tr>
<td>pH</td>
<td>6.72</td>
<td>6.84</td>
</tr>
<tr>
<td>Alkalinity (mM)</td>
<td>8.7</td>
<td>9.4</td>
</tr>
<tr>
<td>DIC (mM)</td>
<td>13.3</td>
<td>13.2</td>
</tr>
<tr>
<td>$\delta^{34}S$ (SO$_4^{2-}$)</td>
<td>10.0</td>
<td>10.6</td>
</tr>
</tbody>
</table>

$\text{DIC} = \text{dissolved inorganic carbon, computed from pH and alkalinity (Stumm and Morgan, 1981).}$
Figure 3-1 Breakthrough curves (a) and cumulative relative mass recovery curves (b) for bromide and sulfate obtained during the extraction phase of the push-pull test. Open symbols show corrected sulfate concentrations (\(\text{SO}_4^{2-}\)), which account for sulfate contained in native groundwater.
Using Br⁻ and ³⁵SO₄²⁻ breakthrough data, we computed a first-order rate coefficient for sulfate reduction of 0.089 d⁻¹ with a 95% confidence interval of ±0.009 d⁻¹ (Fig. 3-2). Breakthrough data obtained on the last day of extraction were excluded from this computation since both Br⁻ and ³⁵SO₄²⁻ concentrations were near their respective detection limit and large fluctuations in computed \( \ln \left( \frac{C_r}{C_i} \right) \) were observed (not shown). Note that the best fit line in Fig. 3-2 is intentionally extended to the y-axis intercept, since both slope and y-axis intercept were fitted in this approach (Eqn. 3-2).

Measured sulfide concentrations increased from 8.4 to 14.0 \( \mu \)M during the extraction phase of the push-pull test (Fig. 3-3). Contributions to measured sulfide concentration, which originated from native groundwater and injected test solution (the sum of which is labeled “pre-existing” sulfide in Fig. 3-3), were computed based on background and injection concentrations (Tab. 3-1) using Br⁻ breakthrough data as a measure of dilution. Next, we subtracted pre-existing from measured sulfide concentrations to obtain produced sulfide concentrations during the push-pull test (Fig. 3-3). Integration of curves displayed in Fig. 3-3 revealed that 2.3 mmol of produced sulfide were recovered during the test. In comparison, 50 mmol \( \text{SO}_4^{2-} \) were consumed during the same time. Other measured geochemical parameters

![Figure 3-2](image-url)

Figure 3-2 Regression plot for determination of first-order rate coefficient, obtained by fitting Eqn. 3-2 to the experimental data using a nonlinear least squares routine.
did not vary significantly during the extraction phase (not shown). In particular, DIC ranged between 12.1 and 13.1 mM during extraction without any obvious trend and was therefore in the range of the measured DIC background concentration in PS3 (Tab. 3-1).

An increase in the isotope ratio $\delta^{34}\text{S}$ of remaining, unconsumed $\text{SO}_4^{2-}$ was observed during the extraction phase of the push-pull test (Fig. 3-4). In Fig. 3-4, $\delta^{34}\text{S}(\text{SO}_4^{2-})$ is plotted against sulfate fraction $f$ with $f = (C_r / C_{tr})$ computed from experimental breakthrough data (Fig. 3-1a). To account for the isotope composition of $\text{SO}_4^{2-}$ present in native groundwater, we computed corrected isotope ratios $\delta^{34}\text{S}(\text{SO}_4^{2-})$ using Br$^-$ breakthrough data as a measure of dilution. By fitting the Rayleigh equation (Eqn. 3-4) to our experimental data, we then obtained an isotope enrichment factor of $\varepsilon = 22.8\%$ with a 95% confidence interval of ± 3.4% for $\delta^{34}\text{S}(\text{SO}_4^{2-})$ during the push-pull test.

![Figure 3-3](image)

**Figure 3-3** Breakthrough curves for measured, pre-existing and produced sulfide obtained during the extraction phase of the push-pull test. Pre-existing sulfide concentrations (representing sulfide contributions from native groundwater and injected test solution) were subtracted from measured concentrations to obtain produced sulfide concentrations.
3.5 Discussion and Conclusions

Sulfate reduction was quantified in-situ in a PHC-contaminated aquifer based on sulfate consumption observed during a single-well push-pull test (Fig. 3-1). Using the method of Haggerty et al. (1998), we determined a first-order rate coefficient of 0.089 d\(^{-1}\) with a 95% confidence interval of ± 0.009 d\(^{-1}\) (Fig. 3-2). This rate coefficient agrees well with findings of Chapelle et al. (1996), who determined 0.02 ≤ k ≤ 0.08 d\(^{-1}\) for sulfate reduction in another PHC-contaminated aquifer by fitting a form of the advection-dispersion equation to sulfate concentrations measured across the site. In this context it is important to note that the k value we obtained represents a local measurement in the vicinity of a test well known to be within a sulfate reducing zone rather than an average value across a contaminated site.

Poor overall mass recovery of injected tracer (25%) was obtained in our push-pull test, which may be attributed to the relatively long test duration (117 h) and fairly high average
pore water velocity (~ 0.4 m d^{-1}) at the site. Hence, a significant portion of the test solution migrated beyond the radius of influence of PS3 prior to extraction. It is important to note, however, that no complete tracer mass recovery is required during push-pull tests to obtain accurate estimates of $k$ (Haggerty et al., 1998). This differs from the frequently used method of moments (e.g., Istok et al. (1997)), in which a high tracer mass recovery is required to obtain accurate estimates of $k$. Witness to this conclusion is the small 95% confidence interval ($\pm \sim 10\%$ of the computed $k$ value) determined in our test.

We demonstrated that quantification of sulfate reduction based on metabolic product formation (sulfide, DIC) was not feasible during the push-pull test. Much less produced sulfide was recovered that expected based on sulfate consumption (Fig. 3-3), presumably due to FeS precipitation in Fe$^{2+}$-rich native groundwater (Tab. 3-1, Stumm and Morgan (1981)). Increased Fe$^{2+}$ concentrations are commonly encountered within reduced zones of contaminated aquifers (e.g., Lovley (1997); Lovley and Anderson (2000)). Moreover, due to the relatively high DIC background concentration and the slow rate of sulfate reduction, no increase in DIC was detected during our push–pull test. This differs from DIC analyses performed across entire contaminated sites, which often provide useful information on overall rates of metabolic activities (e.g., Bolliger et al. (1999); Chapelle et al. (1996); Hunkeler et al. (1999)).

Conversely, we feel confident to exclude sulfate precipitation (in form of CaSO$_4$) as a possible alternate mechanism contributing to the observed sulfate consumption. Cation exchange with potassium ions (1.5 mM, from the addition of KBr and K$_2$SO$_4$) contained in the test solution could result in an additional Ca$^{2+}$ concentration of 0.75 mM. Given the 4.0 mM background concentration of Ca$^{2+}$ in native groundwater (Bolliger et al., 1999), the maximum Ca$^{2+}$ concentration expected in test solution within the aquifer is 4.75 mM. If we consider a sulfate concentration of 1 mM, the ion activity product $[\text{Ca}^{2+}][\text{SO}_4^{2-}]$ is equal to $4.75 \times 10^{-6}$ M$^2$, which is more than a factor of 5 below the solubility product for CaSO$_4$ ($2.5 \times 10^{-5}$ M, Stumm and Morgan (1981)).

Strong evidence for microbial sulfate reduction during our test was obtained from isotope fractionation observed in remaining, unconsumed sulfate (Fig. 3-4). The obtained isotope enrichment factor ($\varepsilon = 22.8 \pm 3.4 \%$) agreed well with $\varepsilon$-values observed by others for microbial sulfate reduction in different environments (e.g., 16 to 42 %, Habicht and Canfield (1997)). Moreover, the $\varepsilon$-value we obtained was significantly larger than those commonly observed for physical / chemical transformations (Krouse, 1980).
In summary, combining a push-pull test with stable sulfur isotope analysis provided useful information for the in-situ quantification of microbial sulfate reduction in a PHC-contaminated aquifer. However, further tests will be required to verify the reproducibility of results for this method.

3.6 Acknowledgments

We wish to thank S. Bernasconi (ETH Zurich, Dept. of Geology) for performing the isotope analyses, and J.P. Clément (Amt für Gewässerschutz und Abfallwirtschaft, Canton Bern, Switzerland) for his cooperation at the field site. This study was funded by the Swiss National Science Foundation, Priority Program Environment, and by the Swiss Agency for the Environment, Forests and Landscape.

3.7 References


Activity and Diversity of Sulfate-Reducing Bacteria in a Petroleum Hydrocarbon-Contaminated Aquifer

J. Kleikemper, M. H. Schroth, W. V. Sigler, M. Schmucki, S. M. Bernasconi, and J. Zeyer
4.1 Abstract

Microbial sulfate reduction is an important metabolic activity in petroleum hydrocarbon (PHC)-contaminated aquifers. We quantified carbon source-enhanced microbial $\text{SO}_4^{2-}$ reduction in a PHC-contaminated aquifer using single-well push-pull tests and related the consumption of sulfate and added carbon sources to the presence of certain SRB genera. We also used molecular methods to assess suspended SRB diversity. In four consecutive tests, we injected anoxic test solutions (1000 L) containing bromide as conservative tracer, sulfate and either propionate, butyrate, lactate or acetate as reactants, into an existing monitoring well. After an initial incubation period, 1000 L of test solution/groundwater mixture was extracted from the same well. Average total test duration was 71 h. We measured concentrations of bromide, sulfate and carbon sources in native groundwater as well as in injection and extraction phase samples, and characterized the SRB population using fluorescence in situ hybridization (FISH) and denaturing gradient gel electrophoresis (DGGE). Enhanced sulfate reduction concomitant with carbon source degradation was observed in all tests. Computed first-order rate coefficients ranged from 0.19 to 0.32 d$^{-1}$ for sulfate reduction and from 0.13 to 0.60 d$^{-1}$ for carbon source degradation. Sulfur isotope fractionation in unconsumed sulfate indicated that sulfate reduction was microbially mediated. Enhancement of sulfate reduction due to carbon source additions in all tests and variability of rate coefficients suggested the presence of specific SRB genera and a high diversity of SRB. We confirmed this using FISH and DGGE. A large fraction of suspended bacteria hybridized with SRB-targeting probes SRB385 plus SRB385-Db (11 – 24% of total cells). FISH results showed that activity of these bacteria was enhanced by addition of sulfate and carbon sources during push-pull tests. However, DGGE profiles indicated that the bacterial community structure of the dominant species did not change during the tests. Thus, the combination of push-pull tests with molecular methods provided valuable insights into microbial processes, activities and diversity in the sulfate-reducing zone of a PHC-contaminated aquifer.

4.2 Introduction

Dissimilatory microbial $\text{SO}_4^{2-}$ reduction is an important metabolic activity in many reduced environments such as marine sediments (Jørgensen, 1977), anaerobic sludge (Manz et al., 1998) and contaminated aquifers (Lovley, 1997). This process is mediated by a metabolically
diverse group of microorganisms: the sulfate-reducing bacteria (SRB) (Odom and Singleton, 1993; Rabus et al., 1996; Widdel, 1988). Sulfate-reducing bacteria were found to grow on environmental contaminants such as petroleum hydrocarbon (PHC) constituents (e.g. benzene, toluene, ethylbenzene, xylenes, naphthalene, phenanthrene, alkanes) and halogenated compounds (Ensley and Suflita, 1995; Zhang and Young, 1997). A survey of 38 PHC-contaminated aquifers revealed that on average, $\text{SO}_4^{2-}$ reduction was responsible for 70% of PHC attenuation (Wiedemeier et al., 1999).

During the degradation of PHC, low-molecular weight organic acids such as acetate, propionate and butyrate are intermediates (Cozzarelli et al., 1994), which in turn may serve as carbon sources for SRB. Unfortunately, little is known about the role that such low-molecular weight organic acids may play in the community pathway of PHC degradation in contaminated aquifers. In marine sediments, organic acids derived primarily from fermentation serve as the SRB’s main carbon source (Parkes et al., 1989; Sørensen et al., 1981). Numerous studies have shown that all SRB genera preferentially degrade certain organic acids and cannot degrade others (Hanselmann et al., 1995; Kuever et al., 2001; Widdel, 1988; Widdel, 1992; Widdel and Bak, 1992). Only few SRB genera are known to readily degrade a wide range of organic acids, e.g., *Desulforhabdus amnigenus* is able to consume lactate, acetate, butyrate and propionate (Oude-Elferink et al., 1995). In general, lactate seems to be the most generic carbon source for SRB.

In the laboratory, microbial $\text{SO}_4^{2-}$ reduction was investigated using batch and column studies (Edwards et al., 1992; Hunkeler et al., 1998; Rueter et al., 1994; Thierrin et al., 1993). However, over the last decade it has become increasingly apparent that an accurate assessment of $\text{SO}_4^{2-}$ reduction in aquifers requires appropriate in situ test methods (Gillham et al., 1990; Madsen, 1991; Madsen, 1998). Recently, single-well “push-pull” tests (PPTs) have been used for the in-situ quantification of microbial activities in PHC-contaminated aquifers (Istok et al., 1997; Reinhard et al., 1997; Schroth et al., 1998). In a PPT, a test solution that contains a non-reactive, conservative tracer and one or more reactive solutes (reactants) is injected (“pushed”) into the aquifer through an existing well. During an initial incubation period (i.e., a rest phase without pumping), indigenous microorganisms ideally consume reactants and generate metabolic products. Thereafter, the test solution/groundwater mixture is extracted (“pulled”) from the same location and the concentrations of tracer, reactants and products are analyzed. Rates of microbial activities are then determined by comparing the breakthrough curves of tracer and reactants (Haggerty et al., 1998; Snodgrass and Kitanidis, 1998). Recently, we successfully employed PPTs to quantify microbial $\text{SO}_4^{2-}$ reduction.
concomitant with PHC degradation in a contaminated aquifer (Schroth et al., 2001). Stable sulfur isotope fractionation in extracted, unconsumed $\text{SO}_4^{2-}$ during those tests provided strong evidence that $\text{SO}_4^{2-}$ reduction was microbially mediated.

To our knowledge, no study has been published specifically addressing the diversity of SRB in PHC-contaminated aquifers. Nevertheless, this information is essential for our understanding of the biogeochemical processes that are intimately linked to bacterial diversity. Direct information on SRB communities may be obtained using laboratory molecular methods such as fluorescence in-situ hybridization (FISH) (Amann et al., 1995) or PCR with subsequent denaturing gradient gel electrophoresis (DGGE) (Muyzer and Smalla, 1998). These methods have been used previously to investigate SRB communities in marine sediments (Devereux et al., 1992), seawater (Teske et al., 1996) and anaerobic bioreactors (Wawer and Muyzer, 1995).

Unfortunately, we do not know whether the introduction of reactants during PPTs changes the microbial community in the subsurface. If it does, rate coefficients determined using this method may not reflect activities of the native community. Analysis of the suspended population during a PPT may provide some information on this issue, even though suspended and attached populations may be dissimilar (Alfreider et al., 1997).

The purpose of this research was to assess SRB diversity in a PHC-contaminated aquifer using macroscopic measurements of activities as well as molecular analyses. We quantified carbon source (acetate, propionate, butyrate and lactate) -enhanced microbial $\text{SO}_4^{2-}$ reduction using PPTs and related $\text{SO}_4^{2-}$ and carbon source consumption to the presence of certain SRB genera. These findings were compared with results from molecular analyses (FISH, PCR-DGGE), which we used to assess suspended SRB diversity and to monitor population changes of suspended bacteria during PPTs. Stable sulfur isotope analyses of extracted, unconsumed $\text{SO}_4^{2-}$ were used to determine isotope enrichment factors, which served as indicators of microbial $\text{SO}_4^{2-}$ reduction.

### 4.3 Materials and Methods

#### 4.3.1 Field site

The study was conducted in a heating oil-contaminated aquifer in Studen, Switzerland, which is undergoing remediation by monitored natural attenuation and was characterized in detail by Bolliger et al. (1999). Push-pull tests described in this paper were conducted in
monitoring well PS3, which is located within the contaminant source zone (free-phase PHC present). Well PS3 is constructed of 11.5 cm I.D. polyvinyl chloride casing and partially penetrates the aquifer to a depth of ~ 1 m below the ground water table. Groundwater in PS3 exhibited reduced conditions and contained up to 1 mg L⁻¹ dissolved PHC (Bolliger et al., 1999). Previous studies have shown that PS3 is located within a transition zone where both SO₄²⁻-reducing and methanogenic conditions are found (Bolliger et al., 1999; Bolliger et al., 2000).

4.3.2 Push-pull tests and sample collection procedures

To quantify rates of microbial SO₄²⁻ reduction concomitant with the degradation of either propionate, butyrate, lactate or acetate, we performed four PPTs (PPTₚₜ, PPTₚₜ, PPTₑₚ, PPTₑₛ) over a six-month period from May until October 2000 in similar fashion as those described in Schroth et al. (2001). Test solutions were prepared by collecting groundwater in 500-L plastic carboys and adding Br⁻ (as KBr) as non-reactive, conservative tracer and SO₄²⁻ (as K₂SO₄) as reactant to achieve final concentrations of ~0.5 mM Br⁻ and ~1.0 mM SO₄²⁻ (Tab. 4-1). As carbon sources we added either propionate, butyrate, acetate (prepared from their respective sodium salts), or lactate (prepared from a 50% DL-sodium lactate-solution) to achieve final concentrations of ~2.0 mM. In all PPTs, the carboys were continuously sparged with nitrogen gas to minimize O₂ dissolution from air into test solutions during preparation and subsequent injection.

For each PPT, injection of 1000 L of test solution into PS3 began at time \( t = 0 \) h and was completed within 0.72 to 1.67 h using gravity drainage. After an average initial incubation period of 22 h, we extracted a total of 1000 L of test solution/groundwater mixture (batches of 300 to 400 L each per day) during further incubation for two additional days. The average total test duration was 71 h.

Water samples for chemical/biological analyses were obtained during the collection of groundwater in carboys (background concentrations), injection of test solutions (injection concentrations), and at regular intervals during the extraction phases of the PPTs. Specifically, samples for the analysis of Br⁻, SO₄²⁻ and organic acids were filtered in the field using 0.45 µm polyvinylidene fluoride filters (Millipore, Bedford, MA) and stored in 12-mL plastic vials. Samples for the determination of CH₄ concentrations were collected without headspace in 117-mL serum bottles using butyl rubber stoppers. Samples for sulfur isotope
measurements in unconsumed $\text{SO}_4^{2-}$ were collected in 1-L glass bottles acidified with 2 mL of 32% HCl. All samples were stored at 4°C prior to analysis.

For total cell counts and FISH, 50 mL of unfiltered water was collected in sterile Falcon tubes. During PPT$_{ac}$, unfiltered water samples (250 mL each) were collected in sterilized glass bottles for use in subsequent PCR-DGGE analysis. All samples for biological analyses were immediately placed on ice until further processing in the laboratory.

Table 4-1  **Summary of experimental conditions during four push-pull tests performed to evaluate microbial $\text{SO}_4^{2-}$ reduction concomitant with carbon source degradation in a PHC-contaminated aquifer.**

<table>
<thead>
<tr>
<th>Test</th>
<th>Carbon source injected</th>
<th>Carbon source concentration (mM)</th>
<th>$\text{SO}_4^{2-}$ injection concentration (mM)</th>
<th>Br$^{-}$ injection concentration (mM)</th>
<th>Injection volume (L)</th>
<th>Injection duration (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPT$_{pr}$</td>
<td>propionate</td>
<td>1.89</td>
<td>0.92</td>
<td>0.47</td>
<td>1000</td>
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<tr>
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<td>1.02</td>
<td>0.51</td>
<td>1000</td>
<td>1.67</td>
</tr>
<tr>
<td>PPT$_{la}$</td>
<td>lactate</td>
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<td>0.53</td>
<td>1000</td>
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<td>PPT$_{ac}$</td>
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<td>1.15</td>
<td>0.58</td>
<td>1000</td>
<td>0.93</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Test</th>
<th>Initial incubation period (h)</th>
<th>Total extracted volume (L)</th>
<th>Total test duration (h)</th>
<th>Groundwater temperature (°C)</th>
<th>$\text{SO}_4^{2-}$ background concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPT$_{pr}$</td>
<td>21.9</td>
<td>1000</td>
<td>70.4</td>
<td>12.5</td>
<td>0.05</td>
</tr>
<tr>
<td>PPT$_{bu}$</td>
<td>24.6</td>
<td>1000</td>
<td>72.9</td>
<td>12.7</td>
<td>0.03</td>
</tr>
<tr>
<td>PPT$_{la}$</td>
<td>21.0</td>
<td>1000</td>
<td>70.0</td>
<td>13.1</td>
<td>0.04</td>
</tr>
<tr>
<td>PPT$_{ac}$</td>
<td>21.9</td>
<td>1000</td>
<td>71.3</td>
<td>14.5</td>
<td>0.02</td>
</tr>
</tbody>
</table>

### 4.3.3 Analytical methods

Bromide, $\text{SO}_4^{2-}$ and organic acid concentrations were determined using a DX-320 ion chromatograph system equipped with an electrical conductivity detector and an EG40 eluent gradient generator (Dionex, Sunnyvale, CA). The following KOH eluent gradient was used: 0-7 min: 1 mM KOH, 7-25 min: 1 mM to 25 mM KOH, 25-28 min: 25 mM to 60 mM KOH, 28-28.1 min: 60 mM to 1 mM KOH, 28.1-32 min: 1 mM KOH. Methane was quantified using the headspace method as described in Bolliger et al. (1999).
Stable sulfur isotope measurements were conducted as described previously (Schroth et al., 2001). Data are reported in the conventional δ-notation relative to the Vienna-Canyon Diabolo Troilite (V-CDT) standard using:

$$\delta^{34}S(\%o) = \frac{R_{\text{sample}} - R_{\text{V-CDT}}}{R_{\text{V-CDT}}} \times 1000 \quad \text{(Equation 4-1)}$$

where $R_{\text{sample}}$ and $R_{\text{V-CDT}}$ are $^{34}S/^{32}S$ sulfur isotope ratios in sample and V-CDT standard, respectively.

### 4.3.4 Determination of isotope enrichment factors

Sulfur isotope fractionation was quantified by computing isotope enrichment factors, ε (in %o). In a closed system enrichment factors can be determined by fitting Rayleigh distillation equations to experimental data (Mariotti et al., 1981). Specifically, enrichment factors of extracted, unconsumed $SO_4^{2-}$ may be determined from measured $\delta^{34}S$ values using (Böttcher et al., 1999):

$$\delta^{34}S(SO_4^{2-}) = \delta^{34}S(SO_4^{2-})_0 + \varepsilon \ln f \quad \text{(Equation 4-2)}$$

where $f$ is the fraction of extracted, unconsumed $SO_4^{2-}$ and $\delta^{34}S(SO_4^{2-})_0$ is the initial isotope composition of $SO_4^{2-}$ in the injected test solution. We corrected values of $\delta^{34}S(SO_4^{2-})$ to account for the isotope composition of background $SO_4^{2-}$ (using $Br^-$ breakthrough data as a measure of dilution between test solutions and native groundwater).

### 4.3.5 Determination of first-order rate coefficients

First-order rate coefficients for $SO_4^{2-}$ reduction and carbon source degradation were determined from $SO_4^{2-}$ and carbon source consumption using the method of Haggerty et al. (1998). This method is based on an analysis of tracer and reactant transport in the diverging / converging radial flow field surrounding a monitoring well during a PPT. Assuming a first-order type reaction $dC_r / dt = -kC_r$, where $C_r$ is reactant concentration and $t$ is time, the rate coefficient $k$ can be determined from (Haggerty et al., 1998):

$$\ln \left( \frac{C^*_r(t^*)}{C^*_{tr}(t^*)} \right) = \ln \left[ \frac{1 - e^{-kt^{*}}}{kt^{*}} \right] - k t^{*} \quad \text{(Equation 4-3)}$$

where $C^*$ is relative concentration (i.e., measured concentration divided by the concentration in the injected test solution), 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 duration.
of the test solution injection. A nonlinear least-squares routine was used to fit Eqn. (3) (both slope and intercept) to experimental breakthrough data to obtain estimates of first-order rate coefficients. The standard deviation of $k$ was computed from the variance of the estimated $k$ as described in Schroth et al. (1998).

4.3.6 Calculation of stoichiometric ratios

Theoretical stoichiometric ratios (mol carbon source per mol $SO_4^{2-}$) for incomplete carbon source degradation (to acetate) may be obtained from the following reactions:

Propionate: $\frac{4}{3} CH_3CH_2COO^- + SO_4^{2-} \rightarrow \frac{2}{3} CH_3COO^- + \frac{4}{3} HCO_3^- + HS^- + \frac{1}{3} H^+$

(Equation 4-4)

Butyrate: $\frac{2}{3} CH_3(CH_2)_2COO^- + SO_4^{2-} \rightarrow \frac{2}{3} CH_3COO^- + \frac{2}{3} HCO_3^- + HS^- + \frac{1}{3} H^+$

(Equation 4-5)

Lactate: $2 CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2 CH_3COO^- + 2 HCO_3^- + HS^- + H^+$

(Equation 4-6)

The theoretical stoichiometric ratio for acetate degradation was obtained from:

Acetate: $CH_3COO^- + SO_4^{2-} \rightarrow 2 HCO_3^- + HS^-$

(Equation 4-7)

Furthermore, we obtained theoretical stoichiometric ratios for complete carbon source degradation of propionate, butyrate and lactate by combining Eqns. (4-4)-(4-6) individually with (7).

Actual stoichiometric ratios were calculated individually for each water sample using measured concentrations of reactants and tracer. Alternatively, we calculated stoichiometric ratios from total masses recovered during the PPTs. In these calculations we assumed that added $SO_4^{2-}$ was used exclusively for the degradation of added carbon sources during the PPTs.

4.3.7 Cell counts and in situ hybridization

Cell counts and FISH were performed on samples collected during PPT$_{ia}$ and PPT$_{ac}$. Total cell counts were conducted using 4',6-diamidino-2'-phenylindole (DAPI) staining (Zarda et al., 1997). For in situ hybridization, we used the Cy3-labelled 16S rRNA oligonucleotide probes (all purchased from MWG Biotech, Ebersberg, Germany) EUB338 to target Bacteria (Amann et al., 1990b), Arch915 (Stahl and Amann, 1991) for Archaea, SRB385 (Amann et al., 1990a) plus SRB385-Db (Rabus et al., 1996) for SRB, DSV698 plus DSV1292 for Desulfovibrio, DSB985 for Desulfobacter and probe 660 for Desulfobulbus (Manz et al.,
Water samples for DAPI and FISH counts were processed within a few hours after sampling by centrifugation at 2500 \( \times \) g for 5 min and resuspension of the debris / cell-pellet in 1 ml of 4% paraformaldehyde in phosphate-buffered saline (130 mM NaCl, 7 mM \( \text{Na}_2\text{HPO}_4 \), 3 mM \( \text{NaH}_2\text{PO}_4 \)). Samples were further processed according to Zarda et al. (1997). Twenty \( \mu \)l from each fixed and dispersed sample was spotted onto ethanol-washed slides. Drying, hybridizations with oligonucleotide probes, DAPI staining and washing were performed under standard conditions (Zarda et al., 1997). Formamide concentrations in the hybridization mix were 30% for probe EUB338, 20% for Arch915, SRB385, SRB385-Db and DSB985, 35% for DSV698 and DSV1292 and 60% for probe 660. Sodium chloride concentrations in the wash buffer were 112 mM for probe EUB338, 250 mM for Arch915, SRB385, SRB385-Db and DSB985, 88 mM for DSV698 and DSV1292 and 15.6 mM for probe 660. The slides were mounted and visually detectable cells were counted according to Zarda et al. (1997). Counting results were corrected by subtracting autofluorescent cells.

### 4.3.8 DNA extraction and PCR-DGGE

To concentrate suspended bacterial cells, groundwater (250 ml) was filtered through 0.22 \( \mu \)m polyvinylidene fluoride-filters (Millipore, Bedford, MA), followed by storage of the filters in 1.5 mL of lysis buffer (50 mM Tris [pH 8], 50 mM EDTA, 50 mM NaCl) at \(-20^\circ\text{C}\). Following the addition of \( \sim 0.7 \) g glass beads (0.10 - 0.11 mm diameter) to the lysis buffer / filters, DNA was extracted by bead beating in a FastPrep 120 (Savant Instruments, Inc., Holbrook, NY) for 15 s at 4.5 m s\(^{-1}\). After brief centrifugation to settle the filter pieces, the buffer/DNA supernatant was transferred into a new tube, and the extraction of the filters was repeated with 0.5 ml of lysis buffer. Approximately 10 mg ml\(^{-1}\) lysozyme was added to the combined buffer / DNA solutions and the samples were incubated at room temperature for 10 min. One hundred \( \mu \)l of SDS (20%) and proteinase K (100 \( \mu \)g ml\(^{-1}\)) were added to each sample followed by incubation for 30 min at 37°C and 10 min at 55°C. DNA was further purified with chloroform-phenol extraction and isopropanol precipitation (Sambrook et al., 1989), and resuspended in 100 \( \mu \)l of water. Further cleanup of the DNA was performed using the QIAquick gel extraction kit (Qiagen AG, Basel, Switzerland). DNA was quantified by measuring absorbance at 260 nm and stored at \(-20^\circ\text{C}\).

The PCR of partial 16S rRNA genes was performed using two sets of primers. Primers UNIV 907 r-gc (5′-GC clamp-CCG TCA ATT CCT TTR AGT TT-3′) and SRB 385-f (5′CCT GAC GCA GCG ACG CCG-3′) (Amann et al., 1992) were used to amplify
approximately 523 bp of the 16S rRNA gene from SRB as well as some Gram positive bacteria and other δ-proteobacteria (Santegoeds et al., 1998). A second set of primers, BAC 968 f-gc (5'-GC clamp-AAC GCGAAGAAC CTT AC-3') and BAC 1401 r (5'-CGG TGT GTACAAGAC CC-3'), were used to amplify approximately 434 bp of the 16S rRNA gene sequence from most Bacteria (Felske et al., 1996). DGGE of PCR products was performed in a denaturing gradient of 30 to 55% at 200 V for 3 h as described previously (Sigler et al., 2001). DNA banding patterns were digitized and photographed using the GelDoc 2000 system and QuantityOne software (Bio-Rad Laboratories, Hercules, CA).

4.4 Results

4.4.1 Push-pull tests

Breakthrough curves for Br⁻, SO₄²⁻ and carbon sources showed a gradual decline in C* during PPT extraction phases as extracted test solution was increasingly diluted with native groundwater (Fig. 4-1a - d). Relative SO₄²⁻ and carbon source concentrations were smaller than relative Br⁻ concentrations during all PPT extraction phases. This difference is significant since the error of the IC measurements is smaller than 5%. To account for SO₄²⁻ contained in native groundwater (on average 0.035 mM), relative SO₄²⁻ concentrations shown were corrected using Br⁻ breakthrough curves as a measure of the dilution of test solution with native groundwater assuming constant SO₄²⁻ background concentrations for each test (Schroth et al., 1998). Acetate, propionate, butyrate and lactate concentrations were below the detection limit (~ 5 μM) in native groundwater of well PS3. During the extraction phases of all PPTs, we recovered 43 ± 7% (average ± standard deviation) of the injected Br⁻ mass and 33 ± 5% of the injected SO₄²⁻ mass (computed by integrating solute breakthrough curves shown in Fig. 4-1). Furthermore, 43% of propionate, 35% of butyrate, 39% of lactate and 21% of acetate were recovered. Acetate was also detected during the extraction phase of PPTₚᵣ (Fig. 4-1a). Concentrations of acetate linearly decreased from 40 μM in the beginning to 10 μM at the end of PPTₚᵣ. In all other PPTs, no intermediate organic acids were detected. Methane concentrations were 0.48 ± 0.61 mM in native groundwater of well PS3, 0.27 ± 0.19 mM in the injection solutions and remained essentially constant during all PPT extraction phases with concentrations at 0.45 ± 0.26 mM (not shown).
4.4.2 Rate coefficients and stoichiometric ratios

Computed values of $k$ for SO$_4^{2-}$ reduction ($k_{\text{sulfate}}$) were lowest for PPT$_{bu}$ and highest for PPT$_{la}$ and PPT$_{pr}$ (Tab. 4-2). Standard deviations ranged between 4.2 and 7.8% of $k_{\text{sulfate}}$. For carbon source degradation, $k$ values were highest for PPT$_{ac}$ and lowest for PPT$_{la}$ with intermediate values for PPT$_{pr}$ and PPT$_{bu}$ and standard deviations between 3.6 and 10.0% of the respective first-order rate coefficient.

Actual stoichiometric ratios changed little during the PPTs with only a small increase, therefore, only average values are displayed (Tab. 4-2). Per 1 mol SO$_4^{2-}$, $1.51 \pm 0.25$ mol propionate, $1.63 \pm 0.25$ mol butyrate, $0.97 \pm 0.15$ mol lactate and $3.49 \pm 0.51$ mol acetate were consumed. Similarly, when stoichiometric ratios were calculated from total masses recovered during the PPTs (see previous section), $1.49$ mol propionate, $1.66$ mol butyrate, $0.98$ mol lactate and $3.44$ mol acetate were consumed per 1 mol SO$_4^{2-}$. Actual stoichiometric ratios were higher than theoretical stoichiometric ratios assuming both complete and incomplete oxidation for all carbon sources.

![Figure 4-1 Extraction phase breakthrough curves for Br, SO$_4^{2-}$, propionate, butyrate, lactate and acetate during a) PPT$_{pr}$, b) PPT$_{bu}$, c) PPT$_{la}$ and d) PPT$_{ac}$.](image)

Figure 4-1 Extraction phase breakthrough curves for Br, SO$_4^{2-}$, propionate, butyrate, lactate and acetate during a) PPT$_{pr}$, b) PPT$_{bu}$, c) PPT$_{la}$ and d) PPT$_{ac}$. 
sources, except for lactate where the actual value was between the two theoretical values. If we assume complete oxidation of the carbon sources by SRB, the stoichiometry of the reactions would indicate that \( \text{SO}_4^{2-} \) reduction accounted for 41 ± 12% of degraded propionate, 25 ± 4% of butyrate, 69 ± 11% of lactate and 29 ± 5% of acetate. These numbers represent the minimum amount of carbon sources that were consumed by SRB when assuming that injected \( \text{SO}_4^{2-} \) was exclusively used for degradation of these carbon sources. If we assume incomplete oxidation of the carbon sources by SRB, the stoichiometry of the reactions would indicate that \( \text{SO}_4^{2-} \) reduction accounted for 96 ± 27% of the degraded propionate, 41 ± 7% of butyrate and 210 ± 33% of lactate. Assimilation of carbon and sulfur by SRB was not taken into account in these calculations, but was assumed to be low (Widdel, 1988).

### Table 4-2

First-order rate coefficients \( k \), stoichiometric ratios for \( \text{SO}_4^{2-} \) reduction concomitant with the degradation of carbon sources, and isotope enrichment factors during microbial \( \text{SO}_4^{2-} \) reduction in four push-pull tests.

<table>
<thead>
<tr>
<th>Test</th>
<th>First-order rate coefficient ( k ) ± SD ((\text{d}^{-1}))</th>
<th>Stoichiometric ratios(^a)</th>
<th>Isotope enrichment factor ( \delta ) ± SD ((%o))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{SO}_4^{2-} ) carbon source</td>
<td>s.r. ± SD</td>
<td>complete (^b)</td>
</tr>
<tr>
<td></td>
<td>actual</td>
<td>theoretical</td>
<td></td>
</tr>
<tr>
<td>PPT(_{pr})</td>
<td>0.29 ± 0.02</td>
<td>0.19 ± 0.02</td>
<td>1.51 ± 0.25</td>
</tr>
<tr>
<td>PPT(_{bu})</td>
<td>0.19 ± 0.01</td>
<td>0.14 ± 0.01</td>
<td>1.63 ± 0.25</td>
</tr>
<tr>
<td>PPT(_{la})</td>
<td>0.32 ± 0.03</td>
<td>0.13 ± 0.01</td>
<td>0.97 ± 0.15</td>
</tr>
<tr>
<td>PPT(_{ac})</td>
<td>0.24 ± 0.01</td>
<td>0.60 ± 0.06</td>
<td>3.49 ± 0.51</td>
</tr>
</tbody>
</table>

\( ^a \) expressed as mol carbon source utilized per mol \( \text{SO}_4^{2-} \)

\( ^b \) complete = complete oxidation to \( \text{CO}_2 \)

\( ^c \) incomplete = incomplete oxidation to acetate

### 4.4.3 Isotope enrichment factors

Sulfur isotope fractionation in unconsumed \( \text{SO}_4^{2-} \) was observed during extraction phases of all PPTs. Values of \( \delta^{34}\text{S} (\text{SO}_4^{2-}) \) were 10.7 ± 0.2\%o in test solutions (\( \delta^{34}\text{S} (\text{SO}_4^{2-})_0 \)) and increased up to 20.4 ± 5.0\%o on average during PPT extraction phases. Computed isotope enrichment factors (Eqn. 2) ranged from 16.1 ± 0.8 to 25.7 ± 1.8\%o (Tab. 4-2).
4.4.4 Cell counts and in situ hybridization

Total cell numbers in PPT$_{la}$ and PPT$_{ac}$ (DAPI) ranged from $6.6 \times 10^4$ to $1.51 \times 10^5$ cells ml$^{-1}$. Percentages of cells hybridizing with probe EUB338 ranged from 16 to 33% and with probe Arch915 from 27 to 44% of total (DAPI-stained) cells during PPT$_{la}$ (Fig. 4-2a) and PPT$_{ac}$ (Fig. 4-2b). Probes SRB385 and SRB385-Db together detected between 11 and 24% of total cells (Fig. 4-2). In both PPTs, lower percentages of Bacteria and SRB were determined in the background (BG) than during extraction phases.

Hybridizations with the genus-specific SRB probes showed that all of the targeted genera were present. Percentages of Desulfobulbus spp. ranged from 2.6 to 8.0% of total cells, Desulfovibrio spp. from 2.6 to 7.6% and Desulfo bacter spp. from 0.0 to 6.0%. Proportions of SRB genera changed little during both PPTs. However, Desulfovibrio spp. and Desulfobulbus spp. signals increased during PPT$_{la}$ and those of all three genera during PPT$_{ac}$ as compared to the background. The three investigated genera make up 30 to 59% of all SRB (as determined by probes SRB385 plus SRB385-Db) in PPT$_{ac}$ and 56 to 104% of all SRB in PPT$_{la}$.

4.4.5 PCR-DGGE

DGGE of PCR products generated from both primer sets resulted in distinct profiles, which exhibited approximately eight dominant bands each and an underlying smear of DNA that
represented non-dominant phylotypes (Fig. 4-3). Apart from slight band intensity differences, little change could be detected between DGGE profiles of DNA extracted from native groundwater of well PS3 (BG) and samples taken during PPT\textsubscript{ac}, regardless of the primer set used (as an example, profiles from the 1.0 extracted volume / injected volume sample are shown in Fig. 4-3).

4.5 Discussion

4.5.1 Push-pull tests

Lower relative \( \text{SO}_4^{2-} \) and carbon source concentrations compared to relative \( \text{Br}^- \) concentrations throughout all PPTs (Fig. 4-1a - d) indicated that \( \text{SO}_4^{2-} \) and carbon sources were consumed during those tests, presumably due to microbial activity. Differences between recovered relative \( \text{Br}^- \) and \( \text{SO}_4^{2-} \) masses (10.4 ± 2.3\% on average) and differences between relative \( \text{Br}^- \) and carbon source masses (propionate 9\%, butyrate and lactate 6\%, acetate 15\%) illustrate that total test durations (Tab. 4-1) were sufficiently long to allow detectable \( \text{SO}_4^{2-} \) and carbon source consumption during the tests. Conversely, mass recovery of injected \( \text{Br}^- \) tracer in the tests (36 – 52\%) was poor. This was due to a fairly high average pore water velocity (~ 0.4 m d\(^{-1}\)) at the site (Schroth et al., 2001). Thus, during the PPTs, a significant portion of test solution migrated beyond the radius of influence of PS3. However, it is important to note that complete tracer mass recovery is not required during PPTs for an accurate quantification of rate coefficients (Haggerty et al., 1998). Sulfide and Fe(II) concentrations were routinely measured in all PPTs according to Schroth et al. (2001). However, precipitation of these ions as FeS or FeCO\(_3\) obscures true sulfide and Fe(II) concentrations, hence rendering these data useless for quantification purposes.
4.5.2 Rate coefficients and stoichiometric ratios

We determined values of \( k_{\text{sulfate}} \) that were up to seven times higher than \( k_{\text{sulfate}} \) values obtained from PPTs in the same location, in which only \( \text{SO}_4^{2-} \) was added (Schroth et al., 2001). Therefore, addition of carbon sources in the current PPTs substantially enhanced microbial \( \text{SO}_4^{2-} \) reduction in the vicinity of well PS3. Hence, the rate coefficients we measured do not represent indigenous conditions, even though they are within a similar range as values reported elsewhere in the literature (for a discussion see Schroth et al. (2001)). A variety of environmental factors may evoke variations of \( k_{\text{sulfate}} \) between tests, e. g., groundwater temperature. However, in this case groundwater temperature variations cannot explain differences of \( k_{\text{sulfate}} \) since the temperature varied only by \( \pm 1.3^\circ\text{C} \) between tests (Tab. 4-1). Therefore, differences between \( k_{\text{sulfate}} \) in the PPTs seemed to be largely due to distinct carbon source degradability. Hence, \( k_{\text{sulfate}} \) values must be discussed in combination with rate coefficients for carbon sources (Fig. 4-4) and stoichiometries (Tab. 4-2). Lactate enhanced \( \text{SO}_4^{2-} \) reduction the most, hence, SRB preferred lactate over the other added carbon sources. This finding agrees with the results of other researchers who found that most SRB are able to grow on lactate (Hanselmann et al., 1995; Kuever et al., 2001; Widdel, 1988; Widdel, 1992; Widdel and Bak, 1992). Conversely, the acetate degradation rate coefficient was by far the highest of all carbon sources but \( \text{SO}_4^{2-} \) reduction accounted for only \(~30\%\) of total acetate degradation in PPT\(_{ac}\). Therefore, most of the acetate was probably consumed by Archaea that were an important part of the suspended population (Fig. 4-2). Their activity was indicated by the presence of \( \text{CH}_4 \).

If we assume complete degradation of the carbon sources by SRB, observed \( \text{SO}_4^{2-} \) consumption would only account for a part of the total carbon source consumption during the PPTs. Therefore, incomplete degradation of propionate, butyrate and lactate by SRB may have been a dominant degradation pathway (Eqns. (4-4)-(4-6)). But also...
fermentation processes (acetogenesis) may have been important for propionate, butyrate and lactate degradation. For example, even when we assume incomplete degradation of butyrate by SRB, this only accounts for 41% of total butyrate degraded during PPT_{bu}. Fermentation may well have been responsible for the missing 59% (Kleerebezem and Stams, 2000).

### 4.5.3 Isotope enrichment factors

Enrichment factors computed for our tests (Tab. 4-2) were in the same range as those determined previously in the same aquifer during PPTs without carbon source addition (22.8 ± 1.7‰ and 20.2 ± 2.8‰) (Schroth et al., 2001). However, in this study, variations in ε between tests were higher. Interestingly, our data also agreed with enrichment factors determined for a mixed, toluene-degrading, SO_{4}^{2-}-reducing batch culture for which the inoculum was collected from the same aquifer (19.8 - 28.2‰) (Bolliger et al., 2001). Furthermore, enrichment factors agreed well with ε values obtained by others for microbial SO_{4}^{2-} reduction in different environments and they were within a range that is indicative for microbial SO_{4}^{2-} reduction (for a discussion see Schroth et al. (2001)). Thus, these data clearly suggest that observed SO_{4}^{2-} consumption during the PPTs was microbially mediated.

### 4.5.4 In situ hybridization

Numbers of suspended cells associated with the domain Bacteria (EUB338, 16 – 33%) during PPT_{la} and PPT_{ac} were similar to Bacteria numbers determined in a previous study for the same aquifer (13 - 32%) (Bolliger et al., 2000). Archaea numbers, however, were higher (Arch915, 27-44% in our samples as compared to 9-31% in Bolliger et al. (2000)). Counts of SRB (SRB385 + SRB385-Db) detected in this study (11 – 24%) were higher than those detected by other authors using the same method in different environments, e.g. in activated sludge, anaerobic biofilms and bulk soil (1 - 12% of total bacteria) (Amann et al., 1992; Manz et al., 1998; Zarda et al., 1997). Reasons for this difference may include the environment we investigated and our choice of probes (SRB385 and SRB385-Db in combination), which may likely have resulted in higher detection rates. Moreover, we are aware that both probes also detect a range of other anaerobic bacteria (Manz et al., 1998). Thus, the numbers reported here likely overestimate true SRB numbers. Both for Bacteria and SRB and during both tests, differences between numbers of cells in extraction phase samples compared to background samples were in the same range. Therefore, the increase of the Bacteria numbers may have been due to an increase of SRB numbers. This agrees with an
increase of counts with the genera-specific SRB probes during the tests. Interestingly, counts
of Desulfovibrio (2.6 – 7.6%) and Desulfo bacter (0 – 6%) in this PHC-contaminated
environment were in the same range as was found in activated sludge, another freshwater
environment (2.8 – 5.2% and 1.8 %, respectively (Manz et al., 1998)). However, the same
authors detected Desulfobulbus numbers below the detection limit (< 0.1%), as compared to
2.6 – 8% in our samples, which may be due to the different environments examined.
Differences in cell counts between the tests, especially with regard to Desulfo bacter spp., are
difficult to interpret and may be caused by naturally occurring fluctuations of the populations
due to changes of environmental conditions.

4.5.5 PCR-DGGE

The presence of several bands in DGGE profiles indicated a diverse bacterial population in
groundwater near well PS3. Although we cannot statistically test the significance of this
observation, our results suggest that the suspended bacterial community of the dominant
species remained the same during PPTac (Fig. 4-3). At first, this finding does not seem to
agree with the FISH results as FISH indicated higher SRB and Bacterial activity during
extraction phases as compared to background samples. However, FISH detects the active
portion of the microbial population, which may change during a PPT (increase of RNA
content), while DGGE profiles reveal the population patterns, which appeared to remain unaltered.

4.5.6 Comparison of chemical data with molecular analyses

Although the carbon sources that we added in the current PPTs may not be important SRB
substrates in situ in this aquifer, their consumption in the tests may be related to the presence
of certain SRB genera and hence provide information on the bacterial community. Since most
SRB are unable to readily degrade all of the added carbon sources (Hanselmann et al., 1995;
Kuever et al., 2001; Widdel, 1988; Widdel, 1992; Widdel and Bak, 1992), substantial
enhancement of SO$_4^{2-}$ reduction in all of our tests as compared to tests without carbon source
addition (Schroth et al., 2001) suggests that a diverse SRB population is present. This agrees
with our results from FISH and DGGE. More specifically, consumption of acetate,
propionate and lactate coupled to SO$_4^{2-}$ reduction suggests the presence of Desulfo bacter,
Desulfobulbus and Desulfovibrio, respectively, because these genera were commonly found
to be associated with the degradation of the respective carbon sources in many different
environments (Parkes et al., 1993; Purdy et al., 1997; Sass et al., 1997) and also degrade them in pure culture (Widdel, 1988). Indeed, using FISH we demonstrated the presence of these three genera. Interestingly, acetate as a bacterial metabolite was detected during PPT_{pr}, suggesting incomplete degradation of propionate. This agrees with the presence of *Desulfobulbus*, an incomplete propionate oxidizer (Widdel, 1988). However, fermentation of propionate to acetate may also have occurred. Butyrate, on the other hand, is degraded by none of the three mentioned genera, suggesting the presence of additional SRB. Nevertheless, the comparison of results from molecular analyses with measurements of macroscopic activities is complicated by the fact that with the former we targeted only the suspended bacteria population, while the latter reflects both attached and suspended populations. Although we are aware that suspended microbial communities may not accurately reflect the overall microbial population (Alfreider et al., 1997), others have indicated that, in contaminated aquifers, the difference between structures of suspended and attached microbial populations may not be significant (Bekins et al., 1999). This will remain an issue of further study.

### 4.5.7 Conclusions

In this study, we presented a novel combination of single-well push-pull tests with molecular microbiological methods. Molecular and chemical data complimented each other and provided valuable insights into microbial processes and activities in the SO_{4}^{2-}-reducing zone of a PHC-contaminated aquifer. Sulfate-reducing bacteria from this freshwater environment were able to use a variety of organic carbon sources, which is indicative of a diverse SRB population, as many SRB are specialized on only a few carbon sources. Molecular data confirmed substantial diversity of suspended SRB. Activities of SRB were considerably enhanced by addition of organic carbon sources, which was corroborated by higher FISH detection rates during PPTs compared to native groundwater. Results from DGGE indicated that within the time frame of our experiments (4 days), the introduction of reactants during PPTs did not change the suspended microbial community of the dominant species. In future studies we will focus on community members responsible for PHC degradation and characterization of populations that are attached to the aquifer solid matrix.
4.6 Acknowledgements

We wish to thank J.P. Clément (Amt für Gewässerschutz und Abfallwirtschaft, Kanton Bern, Switzerland) for his cooperation at the field site. Helpful comments by two anonymous reviewers were greatly appreciated. This study was funded by the Swiss National Science Foundation, Priority Program Environment, and by the Swiss Agency for the Environment, Forests and Landscape (BUWAL).

4.7 References


Teske A., Wawer C., Muyzer G., and Ramsing N. B. (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-


Sulfate-Reducing Bacterial Community Response to Carbon Source Amendments in Contaminated Aquifer Microcosms

(http://www.elsevier.com/locate/femsecol)
5.1 Abstract

Microbial sulfate reduction is an important metabolic activity in many reduced habitats. However, little is known about the sulfate-reducing communities inhabiting petroleum-hydrocarbon (PHC)-contaminated freshwater aquifer sediments. The purpose of this study was to identify the groups of sulfate-reducing bacteria (SRB) selectively stimulated when sediment from a PHC-contaminated freshwater aquifer was incubated in sulfate-reducing aquifer microcosms that were amended with specific carbon sources (acetate, butyrate, propionate, lactate, and citrate). After two months of incubation, the SRB community was characterized using phospholipid fatty acid (PLFA) analysis combined with multivariate statistics as well as fluorescence in situ hybridization (FISH). Molybdate was used to specifically inhibit SRB in separate microcosms to investigate the contribution of non-SRB to carbon source degradation. Results indicated that sulfate reduction in the original sediment was an important process but was limited by the availability of sulfate. Substantially lower amounts of acetate and butyrate were degraded in molybdate treatments as compared to treatments without molybdate, suggesting that SRB were the major bacterial group responsible for carbon source turnover in microcosms. All of the added carbon sources induced changes in the SRB community structure. Members of the genus Desulfobulbus were present but not active in the original sediment but an increase of the fatty acids 15:1ω6c and 17:1ω6c and FISH results showed an enrichment of these bacteria in microcosms amended with propionate or lactate. The appearance of cy17:0 revealed that bacteria affiliated with the Desulfovacteriaceae were responsible for acetate degradation. Desulfovibrio and Desulfotomaculum spp. were not important populations within the SRB community in microcosms because they did not proliferate on carbon sources usually favored by these organisms. Metabolic, PLFA, and FISH results provided information on the SRB community in a PHC-contaminated freshwater environment, which exhibited stimulation patterns similar to other (e.g., marine) environments.

Keywords: Sulfate-reducing bacteria, petroleum-hydrocarbon contaminated aquifer, phospholipid fatty acid, fluorescence in situ hybridization, low-molecular weight organic acids, microcosm
5.2 Introduction

Dissimilatory microbial sulfate reduction is an important metabolic activity in many reduced environments such as marine sediments (Jorgensen, 1977), anoxic groundwater and soil (Alewell and Giesemann, 1996), anaerobic sludge (Manz et al., 1998; Oude-Elferink et al., 1998), and contaminated aquifers (Lovley, 1997). This activity is mediated by the metabolically diverse group of sulfate-reducing bacteria (SRB) (Odom and Singleton, 1993; Rabus et al., 1996; Widdel, 1988). Certain SRB are known to utilize environmental contaminants such as petroleum hydrocarbon (PHC) constituents (e.g. benzene, toluene, ethylbenzene, xylenes, polycyclic aromatic hydrocarbons, alkanes) or halogenated compounds directly as a source of carbon and energy (Ensley and Sufita, 1995; Zhang and Young, 1997). In addition, low-molecular weight organic acids such as acetate, propionate and butyrate are common metabolic intermediates in the degradation of PHC (Cozzarelli et al., 1994) and may also serve as carbon sources for SRB in contaminated environments (Rozanova et al., 1991). Acetate, propionate and butyrate are the principal organic acids in many anaerobic ecosystems (Balba and Nedwell, 1982; Parkes et al., 1989). In marine sediments, organic acids derived mostly from fermentation processes serve as the SRB’s main carbon source (Parkes et al., 1989; Sorensen et al., 1981) and lactate was identified to be an important carbon source for sulfate reduction in freshwater sediments (Cappenberg and Prins, 1974; Hordijk and Cappenberg, 1983). However, little is known about the role that such low-molecular weight organic acids may play in the community pathway of PHC degradation in contaminated aquifers.

In such aquifers, biogeochemical processes are intimately linked with the types of microorganisms present. Hence, information on identity and function of bacteria is essential to better understand these processes. Direct information on the identity of SRB communities may be obtained using laboratory molecular methods such as fluorescence in situ hybridization (FISH) (Amann et al., 1995) or the analysis of phospholipid fatty acids (PLFA) (Green and Scow, 2000). A large database of PLFA profiles was developed from pure culture studies (Aeckersberg et al., 1998; Dowling et al., 1986; Edlund et al., 1985; Kohring et al., 1994; Oude-Elferink et al., 1998; Parkes and Calder, 1985; Taylor and Parkes, 1983; Vainshtein et al., 1992). This database was successfully used to relate the presence or absence of specific PLFA to SRB community structure and dynamics in marine and brackish sediments and anaerobic sludge (Boschker et al., 1998; Oude-Elferink et al., 1998; Pelz et al., 2001b; Taylor and Parkes, 1985). However, we must be aware that yet undiscovered SRB
species and genera may inhabit anaerobic environments (Hines et al., 1999; Rooney-Varga et al., 1997). Only a few authors have characterized SRB communities in freshwater (Daly et al., 2000; Li et al., 1999) and direct information on SRB in PHC-contaminated freshwater environments is even more limited (Pelz et al., 2001b).

The purpose of this research was to characterize the SRB populations selectively stimulated when sediment from a PHC-contaminated freshwater aquifer was incubated in sulfate-reducing aquifer microcosms that were amended with specific carbon sources (acetate, butyrate, propionate, lactate, and citrate). These microcosms were incubated for two months and amended carbon source and sulfate concentrations were periodically monitored. In separate microcosms, the SRB were inhibited by molybdate treatment to investigate the contribution of other microorganisms to carbon source degradation (Oremland and Capone, 1988). Microbial community structure in the original sediment and the sulfate-reducing microcosms was investigated by FISH and PLFA analysis combined with multivariate statistical analysis. Metabolic, PLFA, and FISH data provided a more comprehensive picture of the SRB community in a sulfate-reducing, PHC-contaminated freshwater aquifer.

5.3 Materials and Methods

5.3.1 Sediment collection

Sediment for this study was collected from the bottom of a monitoring well in a PHC-contaminated aquifer in Studen, Switzerland (well S6) (Bolliger et al., 1999). Groundwater at this well was anoxic (3 μM oxygen), nitrate concentration was below the detection limit (< 1 μM) and sulfate concentration was 156 μM (Pelz et al., 2001a). The sediment in this well contained high amounts of PHC (11,500 mg kg⁻¹, determined by infra-red spectrometry), which represented approximately 30% of the total organic carbon (Pelz et al., 2001a). Sediment was recovered from the bottom of this well using a Peterson grab sampler and immediately transferred to 1-L glass bottles, which were constantly flushed with N₂ gas to maintain anaerobic conditions. The bottles were closed with butyl rubber stoppers and kept on ice during transport to the laboratory.

5.3.2 Microcosm experiments

For the construction of microcosms, 30 g of sediment was transferred to 117-mL serum bottles under N₂-atmosphere in an anaerobic glove box. Serum bottles were then closed using
butyl rubber stoppers. Anoxic medium was prepared and dispensed into serum bottles to obtain a total volume of ~100 mL sediment / medium mixture according to Bolliger et al. (2001); then bottles were crimp-sealed. A freshly prepared, anoxic, sterile-filtered FeSO₄ solution was added to each serum bottle to achieve a final concentration of 3 mM sulfate. Thereafter, microcosms were amended with sterile, anoxic solutions that contained either acetate, propionate, butyrate, lactate or citrate to achieve a final concentration of 3 mM. Additional microcosm series were used as controls: 3 mM sulfate- but not carbon-source amended (to account for consumption of endogenous carbon sources), autoclaved (to account for non-enzymatic processes, losses through sorption etc.), and 3 mM molybdate-treated (to account for non-SRB activity) microcosms were inoculated with sediment samples and incubated in parallel with the above-described treatments. All treatments were performed in duplicates and incubated statically at 25°C in the dark.

In all microcosms, sulfate and carbon source concentrations were periodically (every 7-10 days) measured over a two-month period. When concentrations of sulfate or carbon source were below ~0.5 mM, microcosms were replenished with sulfate or carbon source to achieve 3 mM final concentrations. Citrate was only once amended to microcosms because its concentration could not be monitored by the employed analytical method. After a 50-day incubation period, microcosms were sacrificed and 1-mL (total cell counts and FISH) and 20-ml (PLFA) aliquots of sediment sludge were used for community analyses.

5.3.3 Analytical methods

Concentrations of sulfate and amended carbon sources in the microcosms were quantified using a DX-100 ion chromatograph system (Dionex, Sunnyvale, CA, USA). For sulfate detection, the eluent was a bicarbonate buffer (1.8 mM Na₂CO₃ and 1.7 mM NaHCO₃). For carbon source (acetate, propionate, butyrate, lactate) measurements, a 5 mM Na₂B₄O₇ solution served as eluent. Using this method, the carbon sources all possessed the same retention time and could therefore not be distinguished from each other. Thus, while overall carbon source mineralization (oxidation to CO₂ and water) could be quantified, potential intermediates (e.g. acetate from incomplete oxidation of propionate) could not be discerned. Methane was measured but not quantified in the headspace of all microcosms using the headspace technique as described in Bolliger et al. (1999).
5.3.4 Total cell counts and in situ hybridization

Total cell counts and FISH analysis were performed only for one of each duplicate microcosm. Total cell counts were performed using 4', 6-diamidino-2'-phenylindole (DAPI) staining (Zarda et al., 1997). For FISH, we used the Cy3-labelled 16S rRNA oligonucleotide probes EUB338 targeting Bacteria (Amann et al., 1990b), Arch915 for Archaea (Stahl and Amann, 1991), SRB385 for δ-Proteobacteria including SRB (Amann et al., 1990a), SRB385-Db for Desulfobacteriaceae (Rabus et al., 1996), DSV698, DSV1292, DSD131, DSV214, and DSV407 for Desulfovibrio spp., DSB985 for Desulfobacter-like bacteria, probe 660 for Desulfobulbus spp. (Manz et al., 1998), and S-G-Dtm-0229-a-A-18 for Desulfotomaculum spp. (Hristova, 2000). The sediment sludge samples (1 mL) were fixed with paraformaldehyde or ethanol and stored according to Zarda et al. (1997). Except for probe S-G-Dtm-0229-a-A-18, for which the ethanol-fixed samples were used, paraformaldehyde-fixed samples were counted with all probes. Hybridizations with oligonucleotide probes as well as DAPI staining were performed under standard conditions (Zarda et al., 1997) in the presence of formamide (10% for DSV214; 15% for S-G-Dtm-0229-a-A-18; 20% for Arch915, SRB385, SRB385-Db, DSB985 and DSD131; 30% for EUB338; 35% for DSV698 and DSV1292; 50% for DSV407, and 60% for probe 660). After hybridization and washing (NaCl concentrations in the wash buffer were 500 mM for DSV214; 318 mM for S-G-Dtm-0229-a-A-18; 250 mM for Arch915, SRB385, SRB385-Db, DSB985 and DSD131; 112 mM for EUB338; 88 mM for DSV698 and DSV1292; 31.2 mM for DSV407, and 15.6 mM for probe 660), slides were mounted with Citifluor solution (Citifluor AF2, Citifluor Ltd., London, UK). Visually detectable cells were counted according to Zarda et al. (1997). Detection limit of hybridized cells was 1% of total (DAPI-stained) cells.

5.3.5 Extraction and analysis of PLFA

PLFA were extracted by a modified Bligh-Dyer method (Bligh and Dyer, 1959) as described previously (Abraham et al., 1998). The PLFA were methylated according to standard protocol (Abraham et al., 1998). Gas chromatography (Hewlett Packard HP 5890 series II equipped with a HP Ultra 2 capillary column and a flame ionization detector) was used to separate the PLFA methyl esters. Identification of PLFA was carried out using the MIDI system (MIDI Inc. version 4.0). Each sample was analyzed in duplicate.

PLFA nomenclature 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 double bond 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 anteisomethyl branching.

In order to detect differences in PLFA profiles between microcosms and the original sediment, centered principal component analysis (PCA) was applied to the data set (Macalady et al., 2000). This multivariate statistical method reduces large data sets by forming uncorrelated linear combinations of the observed variables (in our case, PLFA) resulting in several principal components (PC) which progressively explain less of the variability (variance) of the data (Dillon and Goldstein, 1984). Differences of PLFA abundance between microcosms were tested for their statistical significance using analysis of variance (ANOVA), which is a method of testing differences between group means by comparing sample variances.

5.3.6 Calculations of stoichiometric ratios

Theoretical stoichiometric ratios (mol carbon source per mol sulfate) for incomplete carbon source degradation to acetate were obtained from the following reactions:

Propionate: $\frac{4}{3} \text{CH}_3\text{CH}_2\text{COO}^- + \text{SO}_4^{2-} \rightarrow \frac{4}{3} \text{CH}_3\text{COO}^- + \frac{4}{3} \text{HCO}_3^- + \text{HS}^- + \frac{1}{3} \text{H}^+$

(Equation 5-1)

Butyrate: $\frac{2}{3} \text{CH}_3(\text{CH}_2)_2\text{COO}^- + \text{SO}_4^{2-} \rightarrow \frac{2}{3} \text{CH}_3\text{COO}^- + \frac{2}{3} \text{HCO}_3^- + \text{HS}^- + \frac{1}{3} \text{H}^+$

(Equation 5-2)

Lactate: $2 \text{CH}_3\text{CHOHCOO}^- + \text{SO}_4^{2-} \rightarrow 2 \text{CH}_3\text{COO}^- + 2 \text{HCO}_3^- + \text{HS}^- + \text{H}^+$

(Equation 5-3)

Citrate: $4 \text{COO}^-\text{CH}_2\text{COHCOO}^-\text{CH}_2\text{COO}^- + \text{SO}_4^{2-} + 8 \text{H}_2\text{O} \rightarrow 8 \text{CH}_3\text{COO}^- + 8 \text{HCO}_3^- + \text{HS}^- + 3 \text{H}^+$

(Equation 5-4)

The theoretical stoichiometric ratio for acetate degradation was obtained from:

Acetate: $\text{CH}_3\text{COO}^- + \text{SO}_4^{2-} \rightarrow 2 \text{HCO}_3^- + \text{HS}^-$

(Equation 5-5)

Furthermore, we obtained theoretical stoichiometric ratios for complete carbon source degradation of propionate, butyrate, lactate, and citrate from combining Eqns. (5-1)-(5-4) individually with Eqn. (5-5). Conversely, observed stoichiometric ratios were calculated as the ratio of the total amounts of sulfate consumed to carbon sources mineralized during the incubation period. In these calculations we assumed that added sulfate was used exclusively for the degradation of added carbon sources and that carbon sources were mineralized using
sulfate as sole electron acceptor. Assimilation of carbon and sulfur by SRB was not taken into account in these calculations because it was assumed to be low (Widdel, 1988).

## 5.4 Results

### 5.4.1 Sulfate and carbon source consumption

Sulfate was consumed over the 50-day period in all except the molybdate treatments and the autoclaved microcosm series (Table 5-1).

<table>
<thead>
<tr>
<th>Microcosm treatment</th>
<th>Measured data</th>
<th>Theoretical data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>$SO_4^{2-}$</td>
<td>carbon source</td>
</tr>
<tr>
<td></td>
<td>consumed</td>
<td>mineralized</td>
</tr>
<tr>
<td>[mmol]</td>
<td>[mmol]</td>
<td></td>
</tr>
<tr>
<td>acetate</td>
<td>$1.19 \pm 0.01$</td>
<td>$1.38 \pm 0.00$</td>
</tr>
<tr>
<td>propionate</td>
<td>$1.66 \pm 0.37$</td>
<td>$1.27 \pm 0.30$</td>
</tr>
<tr>
<td>butyrate</td>
<td>$1.80 \pm 0.15$</td>
<td>$0.80 \pm 0.00$</td>
</tr>
<tr>
<td>lactate</td>
<td>$1.80 \pm 0.00$</td>
<td>$1.45 \pm 0.00$</td>
</tr>
<tr>
<td>citrate</td>
<td>$1.29 \pm 0.14$</td>
<td>n. d.</td>
</tr>
<tr>
<td>sulfate only</td>
<td>$1.08 \pm 0.01$</td>
<td>—</td>
</tr>
<tr>
<td>acetate-molybdate</td>
<td>0</td>
<td>$0.55 \pm 0.01$</td>
</tr>
<tr>
<td>butyrate-molybdate</td>
<td>0</td>
<td>$0.03 \pm 0.03$</td>
</tr>
<tr>
<td>sterile-acetate</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

$a$ based on Eqns. (5-1)-(5-5)  
$n. d.$ = not determined

Total sulfate consumption was highest in butyrate-, lactate-, and propionate-amended microcosms (1.66 – 1.80 mmol) and lowest in those microcosms without carbon source...
amendment (1.08 mmol). The amended carbon sources were not mineralized in the autoclaved microcosms and low mineralization occurred in the butyrate-molybdate treatment (0.03 mmol butyrate). Note, however, that some butyrate fermentation to acetate may have occurred. This process would have been undetected due to the employed analytical method. A considerable amount of acetate was mineralized in the acetate-molybdate treated microcosms (0.55 mmol). However, much higher carbon source mineralization was observed in acetate, propionate, butyrate and lactate-amended microcosms. For these four treatments, observed stoichiometric ratios were closer to the theoretical (reaction equation-derived) values for complete than for incomplete carbon source oxidation (Table 5-1). Methane was detected in the headspaces of all microcosms except the autoclaved series (data not shown).

5.4.2 Total cell counts and in situ hybridization

Average total cell counts (DAPI) were highest in lactate, citrate and propionate-amended microcosms ($3.26 - 4.11 \times 10^9$ g$^{-1}$ dry sediment), while the original sediment and the molybdate-treated microcosms showed lowest cell numbers ($1.28 - 1.94 \times 10^9$ g$^{-1}$) (Table 5-2).

Numbers of cells hybridizing with the general Bacteria-specific probe (EUB338) were low in molybdate-treated microcosms and in the original sediment (6.1 - 7.6% of total DAPI-stained cells) and approximately five times higher in acetate, propionate, lactate, and butyrate amendments, and in microcosms without extra carbon source amendment (31.8 - 42.4%). A considerable abundance of Archaea (probe Arch915) was detected in acetate-amended microcosms (4.4%), in contrast to microcosms treated with molybdate and without extra carbon source amendment (< 1%). Cell counts with probes SRB385-Db and SRB385 followed approximately the same trend as results of probe EUB338. Counts were always higher with probe SRB385-Db than with SRB385. With these probes, we determined highest cell numbers in propionate-, lactate- or butyrate-amended microcosms (SRB385-Db and SRB385, 17.6-20.2% and 11.4-15.1%, respectively) and lowest cell numbers in the original sediment and in molybdate-treated microcosms (< 1 - 2.8%). The sum of cells hybridizing with both general SRB-probes (SRB385 plus SRB385-Db) relative to cells hybridizing with probe EUB338 ranged from 29% (molybdate - butyrate treatment) to 99% (lactate amendment). The genus Desulfobulbus (probe 660) was detected in several microcosms in significant amounts (propionate and lactate, 4.3 and 5.4%, respectively). Conversely, in all aquifer microcosms, cells belonging to specific subgroups within the Desulfobacteriaceae
(probe DSB985), Desulfovibrionaceae (probes DSV1292, DSV698, DSD131, DSV214, and DSV407), and Desulfotomaculum (S-G-Dtm-0229-a-A-18) were below the detection limit (< 1%).

<table>
<thead>
<tr>
<th>Microcosm treatment</th>
<th>Total numbers of DAPI-stained cells [10^9 g⁻¹ dry sediment]</th>
<th>% of DAPI-stained cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probe (target)</td>
<td>EUB338</td>
</tr>
<tr>
<td></td>
<td>(Bacteria) (Archaea) (8-Proteobacteria) (Desulfobacteriaceae) (Desulfobulbus)</td>
<td></td>
</tr>
<tr>
<td>S6</td>
<td></td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>acetate</td>
<td></td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>propionate</td>
<td></td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>butyrate</td>
<td></td>
<td>2.8 ± 0.6</td>
</tr>
<tr>
<td>lactate</td>
<td></td>
<td>4.1 ± 1.0</td>
</tr>
<tr>
<td>citrate</td>
<td></td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>sulfate only</td>
<td></td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td>acetate-molybdate</td>
<td></td>
<td>1.9 ± 0.5</td>
</tr>
<tr>
<td>butyrate-molybdate</td>
<td></td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

### 5.4.3 Phospholipid Fatty Acids

Using the MIDI-System, 91 ± 6% of the detected compounds were identified as PLFA. The PLFA profiles in microcosms were dominated by even-C-numbered PLFA (80% of total PLFA), while on average 20% of total PLFA were branched and 54% were monounsaturated. The most abundant compounds were 18:0 (21%), 18:1ω7c (9%), 18:1ω9c (9%), and 16:0 (9%). In the original sediment, 10me16:0 and 17:1ω6c were present while cy17:0, 15:1ω6c, and i17:1ω7c were lacking.
Table 5-3  Relative abundance of SRB-related PLFA (% relative to the total, 17 of a total of 69 PLFA are shown) detected in aquifer microcosms (values ± standard deviations of 4 measurements (two in each of the duplicate microcosms)).

<table>
<thead>
<tr>
<th>microcosm treatment</th>
<th>PLFA</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>i10:0</td>
<td>a12:0</td>
<td>15:1ω6c</td>
<td>15:0</td>
<td>16:1ω7c</td>
<td>16:0</td>
<td>10me16:0</td>
<td>a17:1</td>
</tr>
<tr>
<td>S6</td>
<td>2.3 ± 0.3</td>
<td>1.8 ± 0.2</td>
<td>b. d.**</td>
<td>1.5 ± 0.2b</td>
<td>8.5 ± 0.4</td>
<td>7.70 ± 0.1</td>
<td>3.1 ± 0.2a</td>
<td>1.2 ± 0.1</td>
<td>b. d.</td>
</tr>
<tr>
<td>acetate</td>
<td>1.5 ± 0.2</td>
<td>1.1 ± 0.1</td>
<td>b. d.</td>
<td>1.6 ± 0.2b</td>
<td>5.5 ± 1.2</td>
<td>10.0 ± 0.3</td>
<td>2.8 ± 0.8a</td>
<td>1.0 ± 0.1</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>propionate</td>
<td>1.6 ± 0.5</td>
<td>1.0 ± 0.3</td>
<td>0.4 ± 0.3**</td>
<td>2.3 ± 0.8b</td>
<td>5.6 ± 1.9</td>
<td>8.20 ± 1.3</td>
<td>1.8 ± 0.5b</td>
<td>1.5 ± 0.8</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>butyrate</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>b. d.</td>
<td>1.4 ± 0.2b</td>
<td>7.7 ± 0.9</td>
<td>10.8 ± 0.6</td>
<td>2.4 ± 0.4a</td>
<td>1.1 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>lactate</td>
<td>1.7 ± 0.4</td>
<td>1.2 ± 0.3</td>
<td>0.3 ± 0.3b</td>
<td>2.7 ± 0.7b</td>
<td>5.1 ± 0.9</td>
<td>8.40 ± 0.9</td>
<td>2.1 ± 0.7b</td>
<td>1.1 ± 0.6</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>citrate</td>
<td>1.4 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>b. d.</td>
<td>1.4 ± 0.4b</td>
<td>6.0 ± 1.4</td>
<td>10.1 ± 0.3</td>
<td>2.1 ± 0.6b</td>
<td>1.1 ± 0.4</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>sulfate only</td>
<td>1.4 ± 0.4</td>
<td>1.1 ± 0.4</td>
<td>b. d.</td>
<td>1.3 ± 0.6b</td>
<td>7.8 ± 3.9</td>
<td>8.30 ± 0.8</td>
<td>2.1 ± 0.4b</td>
<td>1.3 ± 0.3</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>acetate-m***</td>
<td>1.6 ± 0.3</td>
<td>1.2 ± 0.2</td>
<td>b. d.</td>
<td>0.4 ± 0.5b</td>
<td>6.0 ± 1.2</td>
<td>9.00 ± 0.8</td>
<td>2.4 ± 0.6a</td>
<td>2.5 ± 0.6</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>butyrate-m</td>
<td>1.0 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>b. d.</td>
<td>1.3 ± 0.3b</td>
<td>5.4 ± 1.9</td>
<td>9.00 ± 0.7</td>
<td>1.7 ± 0.4b</td>
<td>1.4 ± 0.4</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>

* Different letters (a, b, c, d) indicate significant differences (as determined by ANOVA analysis) between treatments (p = 0.05) for those PLFA which were microcosm-differentiating in the PCA.

** b. d. = below detection  *** m = molybdate
Principal component analysis of PLFA profiles using all of the detected 69 compounds did not result in a reasonable separation of microcosms into groups (not shown). Therefore, a second PCA was conducted using 16 PLFA (Table 5-3), which are known to be present in SRB (Aeckersberg et al., 1998; Dowling et al., 1986; Edlund et al., 1985; Kohring et al., 1994; Oude-Elferink et al., 1998; Parkes and Calder, 1985; Taylor and Parkes, 1983; Vainshtein et al., 1992), plus the ubiquitous 16:0. Figs. 5-1 and 5-2 show the first two components of this PCA represented by the two axes, which together explained 69% of the variance of the PLFA data. Microcosms that group closely together in Fig. 5-1 possess similar PLFA compositions. Based on Fig. 5-1, we distinguished three groups of microcosms: the first group contained the original sediment, the molybdate treatments and microcosms without carbon source amendments, the second the lactate and propionate microcosms and the third the acetate, butyrate, and citrate microcosms.

The factorial map in Fig. 5-2 shows the ordination scores of the PLFA. The compounds 17:1ω6c, 15:1ω6c, and 15:0 on the one hand and cy17:0 and 10me17:0 on the other hand separated along the first axis. In addition, these two PLFA groups separated from all other PLFA along both axes. Those PLFA in the factorial map in Fig. 5-2 that lie close to microcosms in Fig. 5-1 when the plot origins are superimposed are likely to have a high relative abundance in those microcosms. Hence, cy17:0 and 10me17:0 were likely to be abundant in acetate, butyrate and citrate treatments and 17:1ω6c, 15:1ω6c, 15:0, and possibly 17:1ω8c were dominant in microcosms amended with lactate and propionate. All other PLFA did not contribute substantially to the variability of PLFA profiles in microcosms. A closer look at the relative amounts of the mentioned PLFA in the microcosms (Table 5-3) reaffirms the PCA results. Significantly higher percentages ($p = 0.05$) of 17:1ω6c, 15:1ω6c, and 15:0 occurred in microcosms amended with lactate or propionate as compared to the other microcosm series (as indicated by different superscript letters in Table 5-3). In contrast, cy17:0 exhibited significantly higher percentages in acetate, butyrate, and citrate as compared to all other treatments. The PLFA 117:1ω7c, commonly found in Desulfovibrio (Edlund et al., 1985), was not detected in any of the microcosms.
Figure 5-1 Principal component analysis with 17 PLFA commonly found in SRB (Tab. 5-3). Each treatment is represented by four data points (two in each of the duplicate microcosms).

Figure 5-2 Factorial map of 17 SRB-related PLFA in the principal component analysis presented in Fig. 5-1.
5.5 Discussion

5.5.1 Sulfate and carbon source consumption

Sulfate reduction in the original sediment was more limited by the availability of sulfate than by that of organic carbon sources since substantial sulfate reduction occurred in microcosms with no extra carbon source amendment (Table 5-1). This concurs with the general concept that microbial activity in contaminated environments is typically electron acceptor-limited (McAllister and Chiang, 1994).

Molybdate completely inhibited sulfate reduction in our control microcosms. Consequently, lower total acetate and butyrate mineralization in molybdate treatments as compared to treatments without molybdate suggests that SRB were the major group responsible for the turnover of these carbon sources. This result is supported by FISH results (probes SRB385, SRB385-Db) showing that SRB were dominant members of the active bacterial community in the aquifer microcosms.

But FISH results also indicated the presence of Archaea in most treatments (probe Arch915, Table 5-2), which explains the methanogenic activity observed in all but the autoclaved microcosms. Considerable acetate consumption in acetate-molybdate treatments further indicated the presence of methanogens, since acetate could not have been mineralized by SRB in these treatments.

On the other hand, a comparison of carbon source turnover between molybdate treatments and respective treatments without molybdate indicates that the majority of sulfate reduction observed in the latter was coupled to the mineralization of amended rather than endogenous carbon sources. For example, considerably less acetate was mineralized in molybdate treatments than in treatments without molybdate (0.55 versus 1.38 mmol on average, Table 5-1). Hence, we may assume that on average at least 0.83 mmol of acetate was mineralized by SRB in treatments without molybdate. But mineralization of acetate by SRB is coupled to the reduction of an equal amount of sulfate (Eqn 5-5). Thus, at least ~70% of observed sulfate consumption in acetate-amended microcosms was coupled to acetate mineralization. Following the same line of reasoning, almost all of the observed sulfate consumption in butyrate-amended microcosms was coupled to butyrate mineralization. We would expect a similar behavior in propionate, lactate, and citrate microcosms, as these carbon sources are also easily degradable. Nevertheless, since we did not perform molybdate controls for propionate, lactate and citrate, we cannot unequivocally assess the contribution of amended
carbon source mineralization to overall sulfate consumption in these experiments. Likewise, the contribution of sulfate reduction to amended carbon source mineralization remains uncertain in these experiments. Hence, observed stoichiometric ratios (Table 5-1) for propionate, lactate, and citrate microcosms have to be interpreted with caution.

5.5.2 Community structure

Principal component analysis of PLFA profiles and FISH results indicated that no detectable changes of the SRB community were induced in control treatments as compared to the original sediment. In contrast to PLFA results, FISH data showed that the overall activity of SRB (probes SRB385-Db and SRB385) was enhanced in the control treatment with sulfate but no extra carbon source amendment (Table 5-2). Conversely, using PCA, carbon-source amended microcosms were separated into two groups that diverged from the original sediment (Fig. 5-1). Hence, selective stimulation of SRB upon carbon source addition occurred. Note that carbon source additions over the incubation period accounted for less than 5% of total carbon already present in the microcosms, i.e. sediment-borne organic matter and PHC. Consequently, carbon source additions represented a minor stimulation of the bacterial community in the microcosms. The PCA data suggested that at least two different sulfate-reducing keyplayers occurred in the microcosms.

The presence of 17:1ω6c in the original sediment suggested members of the genus Desulfobulbus (Oude-Elferink et al., 1998; Parkes et al., 1993), however, 15:1ω6c, a PLFA frequently found in this genus (Parkes et al., 1993), was lacking. Nevertheless, 15:1ω6c appeared in propionate- and lactate-amended microcosms. In the PCA (Fig. 5-1), this compound formed, together with 17:1ω6c, 15:0 and 17:1ω8c, a cluster of PLFA characteristic for the genus Desulfobulbus (Oude-Elferink et al., 1998; Parkes et al., 1993; Taylor and Parkes, 1983), revealing that Desulfobulbus proliferated upon lactate and propionate addition. This result was also corroborated by FISH results (probe 660, Table 5-2). Desulfobulbus is an incomplete oxidizer of lactate and propionate (Widdel, 1988), and our stoichiometric data (Table 5-1) agree with incomplete oxidation of these carbon sources. Hence, our results are in agreement with those from other researchers who also found stimulation of Desulfobulbus upon lactate and propionate additions to estuarine and marine sediments using PLFA analysis or 16S rRNA-targeted oligonucleotide probes (Parkes et al., 1993; Purdy et al., 1997; Taylor and Parkes, 1985).
The biomarker for *Desulfovibrio*, i17:1ω7c (Oude-Elferink et al., 1998; Vainshtein et al., 1992), was not found in the original sediment nor in any of the microcosms, and FISH analysis (probes DSV 698, DSV 1292, DSD131, DSV214, and DSV407) showed that specific members within this genus were not detected in any microcosms at our detection limit. This seems contradictory to the fact that in theory the difference between counts with probe SRB385 and probe 660 should be *Desulfovibrio*. However, care has to be taken since SRB385 also detects a range of other anaerobic bacteria (Manz et al., 1998). The five *Desulfovibrio* probes we used encompass 84% of all *Desulfovibrio* sequences in the database (Manz et al., 1998). Hence, if there had been *Desulfovibrio* in our microcosms they were either unaccounted for by the probes or they are unknown *Desulfovibrio* species, or indeed *Desulfovibrio* were not present and probe SRB385 detected non-target species. These results are surprising because *Desulfovibrio* was detected in water samples collected from the same aquifer but from a different well (Kleikemper et al., 2002). Spatial heterogeneity of bacterial community composition and/or activities or differences between attached and suspended bacteria may be a cause for this discrepancy. Nevertheless, several studies showed contradictory results concerning the importance of *Desulfovibrio* spp. at different sulfate-reducing marine and freshwater sites (Bak and Pfennig, 1991; Parkes et al., 1993; Purdy et al., 1997; Purdy et al., 2001; Trimmer et al., 1997). We suggest that *Desulfobulbus* spp. were the active lactate degraders instead of *Desulfovibrio* in our lactate-amended microcosms. Alternatively, *Desulfobulbus* may have mineralized propionate derived from fermentation of lactate (Purdy et al., 1997) in these microcosms.

Although cell counts were low, indicating low bacterial activities, approximately 25% of the total active *Bacteria* (probe EUB338) belonged to the *Desulfobacteriaceae* (probe SRB385-Db) in the original sediment. Although the presence of 10me16:0 in the original sediment may be indicative for members of the genus *Desulfobacter* (Dowling et al., 1986; Kohring et al., 1994; Vainshtein et al., 1992), cy17:0, which was also considered a specific biomarker for this genus (Dowling et al., 1986; Kohring et al., 1994), was lacking. This PLFA appeared in acetate-, butyrate- and citrate-amended microcosms after incubation and in the PCA, cy17:0 formed, together with 10me17:0, a second group of PLFA (Fig. 5-2) characteristic for *Desulfobacter*-like bacteria (Dowling et al., 1986; Kohring et al., 1994; Kuever et al., 2001; Oude-Elferink et al., 1998). Hence, *Desulfobacter*-like bacteria seemed to have proliferated upon acetate, butyrate and citrate additions. *Desulfobacter* spp. are specialized on acetate (Widdel, 1988) and probably mineralized acetate derived from
fermentation of butyrate and citrate in the respective microcosms. However, as opposed to PLFA data, detection of *Desulfobacter* spp. using FISH was < 1% in all treatments. Considering these data, we suggest that either *Desulfobacter* spp. were present but not active enough to show a strong fluorescence signal, or that an unknown, *Desulfobacter*-related SRB, which cannot be detected using probe DSB985, was responsible for acetate degradation. At this point we can rule out members of the genus *Desulfothermobacter* as acetate-degrading bacteria because this genus is known to possess cy17:0 but also 17:1ω6 in the same range (Oude-Elferink et al., 1998) and the latter PLFA was not detected in acetate, butyrate or citrate treatments. Furthermore, bacteria belonging to the genus *Desulfobacterium* were probably also not responsible for acetate degradation in microcosms since they do not contain cy17:0 (Vainshtein et al., 1992). In addition, *Desulfotomaculum*, a Gram-positive acetate degrader (Boschker et al., 1998; Widdel, 1992), was not detected by FISH using the specific probe (Hristova, 2000). Several other authors found differing results concerning the stimulation of *Desulfobacter*-type bacteria in marine or freshwater sediment slurries upon acetate addition (Boschker et al., 2001; Oude-Elferink et al., 1998; Parkes et al., 1993; Purdy et al., 1997; Taylor and Parkes, 1985). However, it is still unclear which conditions favor the dominance of *Desulfobacter*-type or other acetate-degrading SRB in the environment.

Numbers of *Archaea* were substantially lower in the sediment or in the microcosms presented here than in water samples collected from the same aquifer (Kleikemper et al., 2002). Again the cause may be spatially different communities and / or activities or differences between attached and suspended populations.

5.6 Conclusions

Care has to be taken when interpreting the presence of specific PLFA in the environment as indication for the presence of a specific population (Aeckersberg et al., 1998; Taylor and Parkes, 1985). However, the design of our aquifer microcosms minimized this problem as SRB were favored over other bacteria, and therefore changes in PLFA compositions most probably reflected changes in the SRB community. This study showed that SRB community changes in microcosms can be followed using statistically supported PLFA analysis in combination with FISH and metabolic data. Members of the genus *Desulfobulbus* were rather inactive in the original sediment but were stimulated upon propionate and lactate additions. The same is probably true for *Desulfobacter*-like-bacteria, which were stimulated in acetate-
amended microcosms. Conversely, we did not find evidence that Desulfovibrio was stimulated with lactate.

All carbon sources applied to the aquifer microcosms induced changes in the SRB community. Therefore, following the reasoning of Parkes et al. (1993), these carbon sources may not necessarily be present or used by the SRB in situ in this sediment. Instead, SRB may be directly involved in degradation of PHC constituents (Pelz et al., 2001b). We are aware of the problem that the dominant types of SRB in microcosms may change with increasing incubation time, especially on substrates that are incompletely oxidized (Parkes et al., 1993). However, the purpose of this study was not to investigate a population development time course. This could be the topic of future studies. Further information on SRB communities in contaminated aquifers may be obtained using DNA extraction and PCR-based approaches targeting specific SRB-genes (Deplancke et al., 2000; Karkhoff-Schweizer et al., 1995).

5.7 Acknowledgements

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5.8 References


Sulfur Isotope Fractionation during Growth of Sulfate-Reducing Bacteria on Various Carbon Sources

6.1 Abstract

Stable sulfur isotope fractionation during microbial sulfate reduction is a potential tool to estimate sulfate reduction rates. However, little is known about the influence of the utilized carbon source on the magnitude of sulfur isotope fractionation. To investigate this effect, both a pure culture (strain PRTOL1) and enrichment cultures from a PHC-contaminated aquifer were used and grown in microcosms on various carbon sources with an initial sulfate concentration of 1 mM. As sole carbon sources the PHC components naphthalene, 1,3,5-trimethylbenzene, and heating oil (enrichment culture) and the organic acids acetate, pyruvate, benzoate, and 3-phenylpropionate (enrichment culture and PRTOL1) were used. Sulfate reduction rates of all cultures ranged from $6 \pm 1$ nmol cm$^{-3}$ d$^{-1}$ (enrichment culture grown on 1,3,5-trimethylbenzene) to $280 \pm 6$ nmol cm$^{-3}$ d$^{-1}$ (enrichment culture grown on pyruvate). Cell-specific sulfate reduction rates ranged from $1.1 \times 10^{-14}$ mol cell$^{-1}$ d$^{-1}$ (PRTOL1 grown on pyruvate) to $1.5 \times 10^{-13}$ mol cell$^{-1}$ d$^{-1}$ (PRTOL1 grown on acetate). Sulfur isotope enrichment factors ($\varepsilon$) for the enrichment culture ranged from 15.4% (3-phenylpropionate) to 33.1% (1,3,5-trimethylbenzene) and for PRTOL1 from 30.3% (acetate) to 34.5% (pyruvate). The $\varepsilon$ values were constant down to sulfate concentrations of 0.2 mM, sometimes even below 0.1 mM. Cultures of PRTOL1 always showed higher $\varepsilon$ values than the enrichment culture when grown on the same carbon sources due to culture-specific properties. Higher $\varepsilon$ values were obtained when the enrichment culture was grown on PHC components than on organic acids, possibly due to lower energy yields (less negative $\Delta G^f$ values) of sulfate reduction with PHC than with organic acids. For strain PRTOL1, a weak inverse relationship between $\varepsilon$ values and cell-specific sulfate reduction rate could be shown, while no such relationship existed for the enrichment culture or when all data were combined. The average enrichment factor of all enrichment cultures (22.95%) agreed well with $\varepsilon$ values determined in field experiments at a PHC-contaminated site. Hence, the results of this study support the theory that sulfur isotope fractionation may be a useful tool to quantify microbial sulfate reduction at field sites.

6.2 Introduction

Microbial $\text{SO}_4^{2-}$ reduction is an important process in many contaminated aquifers (Wiedemeier et al., 1999). To accurately estimate its contribution to contaminant degradation,
in situ quantification of this process is essential (Madsen, 1991). Unfortunately, quantification of microbial $\text{SO}_4^{2-}$ reduction in contaminated aquifers based on measurements of $\text{SO}_4^{2-}$ consumption or $\text{S}(-\text{II})$ production is often obscured by concurrent abiotic transformations, e.g. by dissolution of gypsum ($\text{CaSO}_4$) from the aquifer matrix (Stumm and Morgan, 1981) or by precipitation of $\text{S}(-\text{II})$ in form of iron sulfides (Anderson and Lovley, 2000). Hence, alternative methods are needed to investigate rates of $\text{SO}_4^{2-}$ reduction. Stable isotope methods were successfully used to characterize and quantify biological processes in the subsurface (Hunkeler et al., 1999; Meckenstock et al., 2002). So far, sulfur isotope fractionation has been used qualitatively to indicate microbial $\text{SO}_4^{2-}$ reduction in contaminated aquifers (Alewell and Giesemann, 1996; Arneth and Hoefs, 1989; Asmussen and Strauch, 1998; Bottrell et al., 1995; Schroth et al., 2001). However, in order to quantitatively use sulfur isotope fractionation at a field site, the enrichment factor ($\varepsilon$) has to be known a priori with reasonable accuracy (Aggarwal et al., 1997).

While a maximum enrichment factor of $46.9\%_\text{o}$ has been observed in pure culture studies (Bolliger et al., 2001), the general range of $\varepsilon$ values reported in the literature for pure and mixed cultures was $-3 - 46.9\%_\text{o}$ (Bolliger et al., 2001; Canfield, 2001; Detmers et al., 2001; Habicht et al., 2002; Harrison and Thode, 1958). In petroleum hydrocarbon (PHC) contaminated aquifers, $\varepsilon$ values of $20 - 23\%_\text{o}$ were obtained during single-well push-pull tests (Schroth et al., 2001). These values were in good agreement with data from Bolliger et al. (2001), who investigated sulfur isotope fractionation (average $\varepsilon = 23.5\%_\text{o}$) by a toluene-degrading enrichment culture.

However, in a PHC-contaminated aquifer, SRB may not only directly degrade PHC components but also a range of organic acids which are metabolic products of fermenting bacteria. In first field experiments, $\varepsilon$ values with organic acids as carbon sources in a PHC-contaminated aquifer ranged from $16-26\%_\text{o}$ as determined using push-pull tests (Kleikemper et al., 2002b). These values were similar to those obtained with PHC components as carbon sources (Bolliger et al., 2001; Schroth et al., 2001).

The influence of different carbon sources on isotope fractionation by the same pure culture was investigated only by few early researchers with contradictory results (Kaplan and Rittenberg, 1964; Kemp and Thode, 1968). Detmers et al. (2001) hypothesized that if one species was tested for several carbon sources, then a relationship between $\varepsilon$ and cell-specific $\text{SO}_4^{2-}$ reduction rates might be shown. In previous studies, enrichment factors were found to be inversely related to cell-specific $\text{SO}_4^{2-}$ reduction rates when pure cultures of different
Desulfovibrio desulfuricans strains were grown on lactate varying temperature, lactate, and \( \text{SO}_4^{2-} \) concentrations (Chambers et al., 1975; Harrison and Thode, 1958; Kaplan and Rittenberg, 1964). Such a relationship, however, was not found in more recent studies (Brüchert et al., 2001; Detmers et al., 2001). Instead, strain-specific properties, complete or incomplete carbon source degradation (Brüchert et al., 2001; Detmers et al., 2001), and \( \text{SO}_4^{2-} \) concentration (Habicht et al., 2002) were found to be the most important factors influencing the extent of fractionation. Most of the studies published so far were conducted under optimal growth conditions. However, in PHC-contaminated freshwater environments, low \( \text{SO}_4^{2-} \) concentrations, low carbon source concentrations due to low solubility of most heating oil components in water (Wick et al., 2001), and complex carbon sources are frequently encountered.

This study presents an extension of the work of Bolliger et al. (2001) who investigated sulfur isotope fractionation of pure and enrichment cultures with toluene as sole carbon source and different \( \text{SO}_4^{2-} \) concentrations. Our objective here was to quantify and compare sulfur isotope fractionation in microcosms amended with various carbon sources under conditions that resemble freshwater field conditions in a PHC-contaminated aquifer (1 mM \( \text{SO}_4^{2-} \)). In particular, enrichment cultures from sediment of a PHC-contaminated aquifer were grown on naphthalene, 1,3,5-trimethylbenzene, heating oil, acetate, pyruvate, benzoate, and 3-phenylpropionate. A pure culture, strain PRTOL1 that had been isolated with toluene as sole carbon source from another PHC-contaminated freshwater aquifer by Beller et al. (1996), was used as model strain to explore the influence of carbon source on sulfur isotope fractionation by a single organism. PRTOL1 was incubated in parallel with the enrichment cultures on the same carbon sources (except for naphthalene, 1,3,5-trimethylbenzene, and heating oil on which PRTOL1 is not able to grow).

### 6.3 Materials and Methods

#### 6.3.1 Organisms and cultivation

Active cultures of the \( \text{SO}_4^{2-} \)-reducing bacterium PRTOL1 (Beller et al., 1996) were purchased from the Oregon Collection of Methanogens (Portland, OR). The inocula for the enrichment cultures were obtained from a PHC-contaminated aquifer in Studen, Switzerland (Bolliger et al., 1999) and maintained under \( \text{SO}_4^{2-} \)-reducing conditions with either acetate,
pyruvate, benzoate, 3-phenylpropionate, naphthalene, 1,3,5-trimethylbenzene, or weathered heating oil (recovered from the same site) as sole carbon sources.

The enrichment cultures and PRTOL1 were grown in basal media as described by Beller et al. (1996). For cultivation, $\text{SO}_4^{2-}$ was added as FeSO$_4$ (MicroSelect, Fluka, Switzerland). For subsequent microcosm experiments, $\text{SO}_4^{2-}$ was added as NaSO$_4$ (MicroSelect, Fluka, Switzerland) except of experiments with naphthalene, 1,3,5-trimethylbenzene and heating oil, where FeSO$_4$ had to be employed due to severe growth inhibition by S(-II) when NaSO$_4$ was used. The media were supplemented with non-chelated trace element mixture, selenite-tungstate solution, bicarbonate solution, vitamin mixture, vitamin B$_{12}$ solution, and S(-II) solution as described by Widdel and Bak (1992).

Aromatic hydrocarbons (naphthalene, 1,3,5-trimethylbenzene; purum, Fluka) were added as dilute solutions in an inert lipophilic solvent used as carrier phase (Mineral oil, MicroSelect; Fluka) to maintain nearly constant hydrocarbon concentrations in the aqueous phase of 0.1 mM during cultivation and microcosm experiments (Rabus et al., 1993). Three ml of carrier phase containing 0.69 mmol naphthalene or 0.79 mmol 1,3,5-trimethylbenzene were added per 100 ml of medium.

One ml of heating oil was added to the respective microcosms. Organic acids were added from anaerobic, autoclaved stock solutions to give final concentrations of 5 mM (acetate, pyruvate) or 1.0 mM (benzoate, 3-phenylpropionate). The addition of carbon sources in a mineral oil reservoir or at concentrations that would not be depleted if 1 mM SO$_4^{2-}$ was used ensured that carbon sources were non-limiting in our microcosm experiments. The enrichment cultures were grown on all carbon sources and strain PRTOL1 on organic acids. The final pH of the media was approximately 7.1. The media were inoculated with 10% (v/v) (naphthalene, 1,3,5-trimethylbenzene, heating oil) or 5% (v/v) (all other carbon sources) pre-grown cultures. Bacteria were cultured in 120 ml serum bottles with a headspace of approximately 17 ml (90% N$_2$, 10% CO$_2$) at 28°C inverted in the dark.

6.3.2 Microcosm experiments

Microcosm experiments were prepared in 120 ml serum bottles from basal media and substrates as described in the previous section. The initial SO$_4^{2-}$ concentration was approximately 1 mM in all microcosms. Two independent control experiments were performed for each set of microcosms. For the first control experiment we prepared microcosms as described above except that the carbon source was omitted. In the second
control experiment we prepared microcosms as described above except that culture inoculation was omitted. All experiments were conducted at 28°C in the dark. Sulfate concentrations were periodically monitored during the experiments, and at certain intervals sets of three microcosms per culture and employed carbon source were sacrificed and analyzed. Experiments were terminated when the initially supplied SO$_4^{2-}$ was consumed.

6.3.3 Chemical analyses

After vigorous shaking, 0.2 ml of medium was removed from the microcosms and immediately dispensed in 4.8 ml of 20 mM zinc acetate solution for S(-II) analysis. Cline reagent (0.5 ml) was immediately added and 20 min later absorbance was measured at 670 nm by spectrophotometry (Cline, 1969). An additional 2 ml of medium was withdrawn for SO$_4^{2-}$ measurement and counting of bacterial cell numbers. After centrifugation (10 min at 13000 rpm), the supernatant was used for anion (SO$_4^{2-}$, organic acids) measurement by ion chromatography (IC-320, Dionex) according to Kleikemper et al. (2002b). The remaining pellet was further treated for counting of bacterial cells (see below). Analytical reproducibility of the anion measurements was approximately ± 5% for SO$_4^{2-}$ and organic acids and ± 0.02 mM for S(-II).

6.3.4 Bacterial cell numbers

Bacterial cell numbers were determined in microcosms sacrificed at all sampling times of the respective experiments, and the arithmetic mean of these data was used to represent average cell numbers for each experiment. In experiments with naphthalene, 1,3,5-trimethylbenzene, and heating oil cell numbers were determined only at the first sampling point since FeS precipitates rendered cell counting under the microscope impossible at later times.

The remaining pellet (see previous section) was fixed overnight in 4% paraformaldehyde in phosphate buffered saline solution (PBS, Sambrook et al. (1989)) and then washed twice with PBS. Cell suspensions were stored at -20°C in 50% (v/v) ethanol / PBS. Before application to slides, the paraformaldehyde-fixed cell suspensions were centrifuged, the supernatant removed, and the pellet was dispersed in sodium pyrophosphate (0.1%) by mild sonication for 3 min in a sonication bath. Ten µl of suspension were subsequently spotted onto slides, dried at room temperature and finally dehydrated in 50, 80, and 100% ethanol for 3 min each. The cells were stained with DAPI (4',6-diamidino-2-phenylindole, final
concentration 500 ng ml\(^{-1}\)) at 42°C for 2 h. After staining and washing, slides were mounted with Citifluor solution (Citifluor, Caterbury, UK) and examined with a Zeiss Axiophot microscope fitted for epifluorescence with a high pressure mercury bulb as described in detail by Zarda et al. (1997). Cells were counted in 40 images per sample.

### 6.3.5 Isotope analyses

For isotope measurements S(-II) was precipitated as ZnS by addition of 5 ml 1M zinc acetate solution to the microcosms. Serum bottles were vigorously shaken before 1 ml of 2 M NaOH was added. The contents of the microcosms were then filtered using a 0.45 μm HVLP membrane filter (Millipore). Sulfate was subsequently precipitated as BaSO\(_4\) by first adding 2 ml 2 M HCl and then 5 ml 1.2 M BaCl\(_2\) solutions to the filtrate and the precipitate was recovered on a separate 0.45 μm HVLP membrane filter (Millipore). Both filters were dried at 60°C over night. Excess mineral or heating oil from the microcosms was removed from filtrates using hexane.

For stable sulfur isotope ratio measurements approximately 400-600 μg of BaSO\(_4\) or 150-200 μg of ZnS were weighted in tin cups. Vanadium pentoxide was added as catalyst in the amount of about twice the weight of the sample. Sulfur isotopes were subsequently measured on a FISONS OPTIMA mass spectrometer (Fisons, Middlewich, Chesire, UK) coupled in continuous-flow with a Carlo Erba elemental analyzer (CE Instruments, Milan, Italy). Data are reported in the conventional δ-notation relative to the Vienna-Canyon Diabolo Troilite (V-CDT) standard according to:

\[
\delta^{34}\!S(\%_o) = \left( \frac{[^{34}S/^{32}S]_{\text{sample}}}{[^{34}S/^{32}S]_{\text{V-CDT}}} - 1 \right) \times 1000
\]

(Equation 6-1)

The system was calibrated using the international standards IAEA-S1 (δ\(^{34}\!S = -0.3\%_o\)) and IAEA-S2 (δ\(^{34}\!S = 21.7\%_o\)) (Gonfiantini et al., 1995). The mean δ\(^{34}\!S\) value obtained for the international standard NBS127 was 20.4\%. Analytical reproducibility of the measurements was ± 0.3 \%o.

### 6.3.6 Determination of sulfate reduction rates

We computed SO\(_4^{2-}\) reduction rates (SRR, units of nmol cm\(^{-3}\) d\(^{-1}\)) for each culture and carbon source based on SO\(_4^{2-}\) consumption measured during the experiments assuming zero-
order kinetics. Thus, values of SRR were obtained from the slope of a straight line fitted to data of remaining $\text{SO}_4^{2-}$ concentration versus time using linear regression analysis.

For the enrichment culture grown on pyruvate and PRTOL1 incubated with 3-phenylpropionate we also computed first-order $\text{SO}_4^{2-}$ reduction rate coefficients ($k$, units of d$^{-1}$). For these computations we assumed a first-order-type reaction $dC/dt = -kC$, where $C$ is reactant (here $\text{SO}_4^{2-}$) concentration and $t$ is time. Hence, values of $k$ were obtained from the slope of a straight line fitted to data of $\ln C$ versus $t$ using linear regression analysis. Employing a generic linear regression tool we computed standard deviations for both SRR and $k$, and coefficients of determination ($R^2$) were used as a measure of goodness of the respective fits. Finally, we computed values of sSRR (mol cell$^{-1}$ d$^{-1}$) by dividing SRR values by average cell numbers previously determined for the respective cultures and carbon sources.

### 6.3.7 Determination of isotope enrichment factors

Stable isotope fractionation during a reaction is commonly quantified in terms of the fractionation factor $\alpha$ or the isotope enrichment factor $\varepsilon$ (in $\%$, e.g., Hoefs (1997)). These two measures of isotope fractionation are directly related by $\varepsilon = (\alpha-1) \times 1000$. Throughout this study we will use values of $\varepsilon$ to quantify stable sulfur isotope fractionation. In a closed system, values of $\varepsilon$ can be determined using Rayleigh distillation equations (Mariotti et al., 1981). Using linear regression analysis, values of $\varepsilon$ were obtained from the slope of a straight line simultaneously fitted to measured $\delta^{34}S$ values of remaining, unconsumed $\text{SO}_4^{2-}$ ($\delta^{34}S(\text{SO}_4^{2-})$) and accumulated S(-II) ($\delta^{34}S(\text{S}(-\text{II}))$) according to (Böttcher et al., 1999):

\[
\delta^{34}S(\text{SO}_4^{2-}) = \delta^{34}S(\text{SO}_4^{2-})_0 + \varepsilon \ln f \quad (\text{Equation 6-2})
\]

\[
\delta^{34}S(\text{S}(-\text{II})) = \delta^{34}S(\text{SO}_4^{2-})_0 - \varepsilon (f \ln f) / (1-f) \quad (\text{Equation 6-3})
\]

In Eq. 2 and 3, $f$ denotes the fraction of unconsumed $\text{SO}_4^{2-}$, and $\delta^{34}S(\text{SO}_4^{2-})_0$ represents the initial isotope composition of dissolved $\text{SO}_4^{2-}$ (equal to $8.1 \pm 1.5 \%$ in our experiments). Measured $\delta^{34}S(\text{S}(-\text{II}))$ data were corrected for the initial S(-II) concentration contained in the microcosms and its isotope composition. For a detailed derivation of Eq. 2 and 3 the reader is referred to Mariotti et al. (1981).
6.4 Results

6.4.1 Microbial sulfate reduction

Strain PRTOL1 and the enrichment culture consumed \( \text{SO}_4^{2-} \) in all microcosms amended with different substrates as sole carbon source and with \( \text{SO}_4^{2-} \) as sole electron acceptor (Fig. 6-1). Concomitantly, we observed production of \( \text{S}(-\text{II}) \) during these experiments. On the other hand, \( \text{SO}_4^{2-} \) concentrations remained unchanged in microcosms of the control microcosms during up to 132 d of incubation (not shown).

The enrichment culture consumed most of the \( \text{SO}_4^{2-} \) within \( \sim 25 \) d of incubation in microcosms amended with acetate, benzoate, and 3-phenylpropionate (Fig. 6-1a, c, d), and within \( \sim 6 \) d of incubation in microcosms amended with pyruvate (Fig. 6-1b). Conversely, \( \text{SO}_4^{2-} \) was consumed only within 100-130 d of incubation in microcosms amended with naphthalene, 1,3,5-trimethylbenzene, and heating oil (Fig. 6-1i-k). Using data presented in Fig. 6-1 we performed a mass balance on the sum of \( \text{SO}_4^{2-} \) and \( \text{S}(-\text{II}) \) in each sacrificed microcosm (Tab. 6-1). In microcosms amended with acetate, pyruvate, phenylpropionate, and benzoate close to 100% of the sum of initially added \( \text{SO}_4^{2-} \) and \( \text{S}(-\text{II}) \) was recovered, while in naphthalene, 1,3,5-trimethylbenzene, and heating oil microcosms, only 68-76% were recovered.

Strain PRTOL1 consumed only about 50% of \( \text{SO}_4^{2-} \) within 85 d of incubation in microcosms amended with acetate (Fig. 6-1e). Thereafter, \( \text{SO}_4^{2-} \) concentrations remained nearly constant for another 60 d (not shown). Conversely, nearly the entire \( \text{SO}_4^{2-} \) was consumed by PRTOL1 within 6 d in experiments with pyruvate (Fig. 6-1f), within 120 d with benzoate (Fig. 6-1g), and within 45 d with 3-phenylpropionate (Fig. 6-1h). In all PRTOL1 experiments, slightly more than 100% of the sum of initially added \( \text{SO}_4^{2-} \) and \( \text{S}(-\text{II}) \) was recovered in the sacrificed microcosms (Tab. 6-1).

6.4.2 Carbon source consumption

Concomitantly to \( \text{SO}_4^{2-} \) reduction and \( \text{S}(-\text{II}) \) production, organic acids were consumed in enrichment culture and PRTOL1 microcosms grown on acetate, benzoate, and 3-phenylpropionate, and presumably, also on pyruvate (not shown). Pyruvate concentrations were erratic for unknown reasons. Furthermore, we observed an evolution of acetate in enrichment culture microcosms grown on pyruvate, benzoate, 3-phenylpropionate and in
Figure 6-1  Concentrations of $\text{SO}_4^{2-}$ and S(-II) during microcosm experiments for (a-d, j-l) the enrichment cultures and (e-i) PRTOL1 grown on (a, e) acetate, (b, f) pyruvate, (c, g) benzoate, (d, h) 3-phenylpropionate, (i) naphthalene, (j) 1,3,5-trimethylbenzene, and (k) heating oil. Solid lines represent the fit of measured $\text{SO}_4^{2-}$ concentrations versus time used to compute zero-order $\text{SO}_4^{2-}$ reduction rates (SRR). Data of enrichment culture microcosms amended with pyruvate (b) and PRTOL1 microcosms amended with 3-phenylpropionate (h) were additionally fitted assuming first-order kinetics to obtain rate coefficients ($k$) (dotted lines).
PRTOL1 microcosms grown on benzoate (not shown). Usually, a large portion, but not all of the substrate degraded during microcosm experiments was accounted for by \( \text{SO}_4^{2-} \) reduction (Tab. 6-1). Increasing acetate concentrations to up to 0.46 mM were also observed in enrichment culture microcosms grown on naphthalene and heating oil, but not on 1,3,5-trimethylbenzene (not shown).

<table>
<thead>
<tr>
<th>Culture</th>
<th>Carbon source</th>
<th>% recovery of initially added ( \text{SO}_4^{2-} ) and ( \text{S}(-\text{II}) )^a</th>
<th>% of degraded carbon source accounted for by ( \text{SO}_4^{2-} ) reduction^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrichment cultures</td>
<td>acetate</td>
<td>109</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>pyruvate</td>
<td>98</td>
<td>106^c</td>
</tr>
<tr>
<td></td>
<td>benzoate</td>
<td>105</td>
<td>75^d</td>
</tr>
<tr>
<td></td>
<td>3-phenylpropionate</td>
<td>109</td>
<td>79^e</td>
</tr>
<tr>
<td></td>
<td>naphthalene</td>
<td>76</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>1,3,5-trimethylbenzene</td>
<td>68</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>heating oil</td>
<td>75</td>
<td>n.a.</td>
</tr>
<tr>
<td>PRTOL1</td>
<td>acetate</td>
<td>107</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>pyruvate</td>
<td>101</td>
<td>n.a.</td>
</tr>
<tr>
<td></td>
<td>benzoate</td>
<td>109</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>3-phenylpropionate</td>
<td>102</td>
<td>61</td>
</tr>
</tbody>
</table>

^a calculated by a mass balance of the sum of \( \text{SO}_4^{2-} \) and \( \text{S}(-\text{II}) \) in each microcosm

^b calculated by taking into account the stoichiometries of the respective reactions

^c value calculated from measured acetate since pyruvate concentrations were erratic; a theoretical (degraded pyruvate) : (produced acetate) ratio of 1:1 was assumed, resulting in an acetate : \( \text{SO}_4^{2-} \) stoichiometry of 4.00 (see Table 4)

^d a theoretical (degraded benzoate) : (produced acetate) ratio of 1:2 was assumed, resulting in a benzoate : \( \text{SO}_4^{2-} \) stoichiometry of 0.57 (see Table 4)

^e a theoretical (degraded 3-phenylpropionate) : (produced acetate) ratio of 1:3 was assumed, resulting in a 3-phenylpropionate : \( \text{SO}_4^{2-} \) stoichiometry of 0.44 (see Table 4)

^f n.a. = not available
6.4.3 Bacterial cell numbers

Bacterial cell numbers increased during experiments with the enrichment culture (Fig. 6-2a). In particular, increases in cell numbers of almost one order of magnitude were observed in enrichment culture experiments with acetate and pyruvate. For enrichment cultures grown on naphthalene, 1,3,5-trimethylbenzene, and heating oil, cell numbers are only available for the first data point (Tab. 6-2) since FeS precipitates in these microcosms at later time points made cell counts impossible.

An increase of cell numbers in cultures of PRTOL1 was only observed for the pyruvate-amended culture (Fig. 6-2b). In the experiment with PRTOL1 grown on acetate, cell numbers decreased with time, whereas in all other PRTOL1 experiments, cell numbers decreased with time and then increased again.

Average bacterial cell numbers for the experiments ranged from $6.7 \times 10^4$ to $1.8 \times 10^7$ (Tab. 6-2), associated with fairly large standard deviations ($\sigma_{\text{cells}}$, often > 50% of the mean ($\bar{x}$)).

### Table 6-2

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Average bacterial cell numbers</th>
<th>Carbon source</th>
<th>Average bacterial cell numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control cultures</td>
<td>$\bar{x}$ ± $\sigma_{\text{cells}}$</td>
<td>acetate</td>
<td>$6.2 \times 10^7$ ± $4.6 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>(cells cm$^{-3}$)</td>
<td>pyruvate</td>
<td>$1.8 \times 10^7$ ± $1.4 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>benzoate</td>
<td>$2.5 \times 10^6$ ± $1.5 \times 10^6$</td>
</tr>
<tr>
<td>PRTOL1</td>
<td></td>
<td>pyruvate</td>
<td>$1.6 \times 10^7$ ± $1.1 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>benzoate</td>
<td>$2.3 \times 10^5$ ± $1.2 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3-phenylpropionate</td>
<td>$1.9 \times 10^6$ ± $1.3 \times 10^6$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>naphthalene</td>
<td>$9.3 \times 10^5$ ± $6.7 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,3,5-trimethylbenzene</td>
<td>$1.1 \times 10^5$ ± $9.1 \times 10^4$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>heating oil</td>
<td>$1.7 \times 10^5$ ± $3.0 \times 10^4$</td>
</tr>
</tbody>
</table>

### Table 6-2

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Average bacterial cell numbers</th>
<th>Enrichment cultures</th>
<th>Average bacterial cell numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{x}$ ± $\sigma_{\text{cells}}$</td>
<td>acetate</td>
<td>$1.3 \times 10^7$ ± $1.0 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>(cells cm$^{-3}$)</td>
<td>pyruvate</td>
<td>$1.3 \times 10^7$ ± $1.1 \times 10^7$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>benzoate</td>
<td>$1.3 \times 10^5$ ± $1.2 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3-phenylpropionate</td>
<td>$2.0 \times 10^7$ ± $1.3 \times 10^5$</td>
</tr>
</tbody>
</table>

### Table 6-2

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Average bacterial cell numbers</th>
<th>R²</th>
<th>SRR ± $\sigma_{\text{SRR}}$</th>
<th>sSRR ± $\sigma_{\text{sSRR}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\bar{x}$ ± $\sigma_{\text{cells}}$</td>
<td></td>
<td>SRR ± $\sigma_{\text{SRR}}$</td>
<td>$R^2$</td>
</tr>
<tr>
<td></td>
<td>(cells cm$^{-3}$)</td>
<td></td>
<td>(nmol cm$^{-3}$ d$^{-1}$)</td>
<td></td>
</tr>
<tr>
<td>acetate</td>
<td>$6.2 \times 10^7$ ± $4.6 \times 10^5$</td>
<td>15</td>
<td>$35 \pm 2$</td>
<td>0.971</td>
</tr>
<tr>
<td>pyruvate</td>
<td>$1.8 \times 10^7$ ± $1.4 \times 10^7$</td>
<td>9</td>
<td>$280 \pm 6$</td>
<td>0.999</td>
</tr>
<tr>
<td>benzoate</td>
<td>$2.5 \times 10^6$ ± $1.5 \times 10^6$</td>
<td>12</td>
<td>$38 \pm 3$</td>
<td>0.936</td>
</tr>
<tr>
<td>3-phenylpropionate</td>
<td>$1.9 \times 10^6$ ± $1.3 \times 10^6$</td>
<td>15</td>
<td>$61 \pm 2$</td>
<td>0.989</td>
</tr>
<tr>
<td>naphthalene</td>
<td>$1.3 \times 10^5$ ± $6.7 \times 10^5$</td>
<td>15</td>
<td>$9 \pm 1$</td>
<td>0.887</td>
</tr>
<tr>
<td>1,3,5-trimethylbenzene</td>
<td>$1.1 \times 10^5$ ± $9.1 \times 10^4$</td>
<td>15</td>
<td>$6 \pm 1$</td>
<td>0.871</td>
</tr>
<tr>
<td>heating oil</td>
<td>$1.7 \times 10^5$ ± $3.0 \times 10^4$</td>
<td>15</td>
<td>$12 \pm 1$</td>
<td>0.936</td>
</tr>
<tr>
<td>PRTOL1</td>
<td>$1.3 \times 10^7$ ± $1.0 \times 10^5$</td>
<td>9</td>
<td>$19 \pm 2$</td>
<td>0.970</td>
</tr>
<tr>
<td>pyruvate</td>
<td>$1.3 \times 10^7$ ± $1.1 \times 10^7$</td>
<td>15</td>
<td>$143 \pm 8$</td>
<td>0.965</td>
</tr>
<tr>
<td>benzoate</td>
<td>$1.3 \times 10^5$ ± $1.2 \times 10^5$</td>
<td>15</td>
<td>$8 \pm 1$</td>
<td>0.900</td>
</tr>
<tr>
<td>3-phenylpropionate</td>
<td>$2.0 \times 10^7$ ± $1.3 \times 10^5$</td>
<td>9</td>
<td>$26 \pm 3$</td>
<td>0.953</td>
</tr>
</tbody>
</table>

*a Standard deviation, b Number of data points included in SRR computation.

c Coefficient of determination for linear regression analyses performed to obtain SRR.
6.4.4 Sulfate reduction rates

Sulfate reduction rates, determined from linear regression analyses of SO$_4^{2-}$ concentration versus time (Fig. 6-1), varied by up to a factor of 47 between different cultures and carbon sources (Tab. 6-2). In general, PRTOL1 and the enrichment culture degraded SO$_4^{2-}$ with rates in the same order of magnitude. Highest SRR were obtained for cultures grown on pyruvate (280 nmol cm$^{-3}$ d$^{-1}$ for the enrichment culture and 143 nmol cm$^{-3}$ d$^{-1}$ for PRTOL1), lowest values were observed for PRTOL1 grown on benzoate (8 nmol cm$^{-3}$ d$^{-1}$) or the enrichment culture grown on 1,3,5-trimethylbenzene (6 nmol cm$^{-3}$ d$^{-1}$). Furthermore, values of SRR were characterized by small standard deviations ($\sigma_{SRR}$, $\leq$ 10% of SRR in most cases), and coefficients of determination ($R^2$) were $> 0.9$ in all but three cases (Tab. 6-2).

Sulfate reduction in the enrichment culture incubated with pyruvate and PRTOL1 incubated with 3-phenylpropionate appeared to follow first-order kinetics (Fig. 6-1b, h) with SO$_4^{2-}$ concentrations declining exponentially. Using linear regression analyses (dashed lines in Fig. 6-1b,h), we obtained first-order rate coefficients $k = 0.398 \pm 0.019$ d$^{-1}$ for the enrichment culture, and $k = 0.018 \pm 0.002$ d$^{-1}$ for PRTOL1. Thus, values of $k$ were characterized by standard deviations $< 13\%$ of $k$, and coefficients of determination were $R^2$.
0.98 (number of data points, \( n = 15 \)) for the enrichment culture on pyruvate, and \( R^2 = 0.84 \) \( (n = 15) \) for PRTOL1 on 3-phenylpropionate.

Computed values of \( s_{\text{SRR}} \) ranged from \( 1.1 \times 10^{-14} \) to \( 1.5 \times 10^{-13} \) mol cell\(^{-1}\) d\(^{-1}\) and thus varied by a factor of up to 13 between experiments (Tab. 6-2). Differences in \( s_{\text{SRR}} \) values between the cultures were smaller than differences in SRR values. Values of \( s_{\text{SRR}} \) were in the same range for the enrichment cultures and PRTOL1.

### 6.4.5 Sulfur isotope fractionation

Values of \( \delta^{34}\text{S}(\text{SO}_4^{2-}) \) increased from \( 8.1\%_\circ \) \( (\delta^{34}\text{S}(\text{SO}_4^{2-})_0) \) to values of up to \( 85.0\%_\circ \) during the experiments (Fig. 6-3). Simultaneously to increases in \( \delta^{34}\text{S}(\text{SO}_4^{2-}) \), values of \( \delta^{34}\text{S}(\text{S}(\text{II})) \) increased and, in general, approached the initial isotope composition of \( \text{SO}_4^{2-} \) over the course of the experiments. The latter was not the case in the experiment with PRTOL1 grown on acetate in which \( \text{SO}_4^{2-} \) reduction had ceased prior to consumption of most of the supplied \( \text{SO}_4^{2-} \) (Fig. 6-1e). In this experiment, \( \delta^{34}\text{S}(\text{S}(\text{II})) \) remained substantially more negative than \( \delta^{34}\text{S}(\text{SO}_4^{2-})_0 \) (Fig. 6-3e).

Combined evaluation of measured sulfur isotope data revealed approximately linear relationships for all experiments when data was plotted as \( \delta^{34}\text{S}(\text{SO}_4^{2-}) \) versus \(-\ln f\) (Eq. 2) and \( \delta^{34}\text{S}(\text{S}(\text{II})) \) versus \((f \ln f)/(1-f)\) (Eq. 3, Fig. 6-3). Using linear regression analysis, we determined \( \varepsilon \) values that ranged from 15.4 to 34.5\%\( _{\circ} \) (Tab. 6-3).

Enrichment factors were computed from 21 to 27 independent data points each and were generally characterized by small standard deviations (\( \leq 8\% \) of \( \varepsilon \) in all cases), with \( R^2 \) values larger than 0.95 in all cases (Tab. 6-3). Enrichment factors were generally stable down to \( \text{SO}_4^{2-} \) concentrations of 0.2 mM, for the enrichment cultures in some cases even down to less than 0.1 mM (Fig. 6-3, \(-\ln f > 2.3\)). In a few instances, decreasing fractionation was found when \( \text{SO}_4^{2-} \) concentrations were below 0.066 mM (not shown). A weak relationship existed between \( \varepsilon \) and \( s_{\text{SRR}} \) for the PRTOL1 experiments \( (R^2 = 0.5) \), while no such relationship was apparent for the enrichment culture data or when all data were combined (Fig. 6-4) \( (R^2 < 0.5) \).
Table 6-3  Calculated sulfur isotope enrichment factors (ε) obtained during microbial $\text{SO}_4^{2-}$ reduction in microcosm experiments with various carbon sources.

<table>
<thead>
<tr>
<th>Culture</th>
<th>Carbon source</th>
<th>$n^a$</th>
<th>$\varepsilon \pm \sigma_\varepsilon^b$ (‰)</th>
<th>$R^2^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enrichment cultures acetate</td>
<td>27</td>
<td>18.5 ± 0.4</td>
<td>0.990</td>
<td></td>
</tr>
<tr>
<td>pyruvate</td>
<td>27</td>
<td>21.3 ± 1.2</td>
<td>0.950</td>
<td></td>
</tr>
<tr>
<td>benzoate</td>
<td>21</td>
<td>17.3 ± 0.7</td>
<td>0.976</td>
<td></td>
</tr>
<tr>
<td>3-phenylpropionate</td>
<td>24</td>
<td>15.4 ± 1.2</td>
<td>0.986</td>
<td></td>
</tr>
<tr>
<td>naphthalene</td>
<td>24</td>
<td>27.0 ± 0.8</td>
<td>0.981</td>
<td></td>
</tr>
<tr>
<td>1,3,5-trimethylbenzene</td>
<td>24</td>
<td>33.1 ± 1.3</td>
<td>0.970</td>
<td></td>
</tr>
<tr>
<td>heating oil</td>
<td>22</td>
<td>28.1 ± 1.1</td>
<td>0.972</td>
<td></td>
</tr>
<tr>
<td>PRTOL1 acetate</td>
<td>21</td>
<td>30.3 ± 0.5</td>
<td>0.995</td>
<td></td>
</tr>
<tr>
<td>pyruvate</td>
<td>27</td>
<td>34.5 ± 0.8</td>
<td>0.986</td>
<td></td>
</tr>
<tr>
<td>benzoate</td>
<td>21</td>
<td>28.8 ± 1.1</td>
<td>0.975</td>
<td></td>
</tr>
<tr>
<td>3-phenylpropionate</td>
<td>27</td>
<td>32.0 ± 0.9</td>
<td>0.983</td>
<td></td>
</tr>
</tbody>
</table>

---

$a$ Number of data points included in computation of $\varepsilon$ values.

$b$ Standard deviation.

c Coefficient of determination for linear regression analyses performed to obtain $\varepsilon$ values.

### 6.5 Discussion and Conclusions

#### 6.5.1 Microbial sulfate reduction

In our study $\text{SO}_4^{2-}$ was consumed during all but the two control experiments. However, even though acetate and $\text{SO}_4^{2-}$ were initially degraded and $\text{S}(-\text{II})$ was produced when **PRTOL1** was grown on acetate (Figs. 1e), $\text{SO}_4^{2-}$ reduction apparently ceased, with about half of the initially added $\text{SO}_4^{2-}$ remaining in aqueous solution. Since acetate was still present (not shown), substrate limitation can be excluded as a reason for cessation of $\text{SO}_4^{2-}$ reduction. During this experiment, we observed that cell numbers decreased (Fig. 6-2b) and on day 84, cells were not detectable anymore despite sample concentration. Beller et al. (1996) had observed that **PRTOL1** did not show growth on acetate, even though $\text{SO}_4^{2-}$ was reduced. In contrast to their results, we observed, at least initially, a decrease of the acetate concentration (not shown).
Sulfur isotope ratios in $\text{SO}_4^{2-}$ and $\text{S}(-\text{II})$ during microcosm experiments for (a-d, j-l) the enrichment cultures, (e-i) PRTOL1 grown on (a, e) acetate, (b, f) pyruvate, (c, g) benzoate, (d, h) 3-phenylpropionate, (i) naphthalene, (j) 1,3,5-trimethylbenzene, and (k) heating oil. Values of $\delta^{34}\text{S}(\text{SO}_4^{2-})$ (closed symbols) are plotted versus $-\ln f$ (Eq. 2), whereas values of $\delta^{34}\text{S}(\text{S}(-\text{II}))$ (open symbols) are plotted versus $(f \ln f)/(1-f)$ (Eq. 3). Solid lines represent the linear fit used to compute isotope enrichment factors ($\epsilon$).
Mass balances on SO\(_4^{2-}\) and S(-II) indicated that consumed SO\(_4^{2-}\) was entirely converted to S(-II) during many of our experiments (Fig. 6-1). However, in enrichment culture microcosms amended with naphthalene, 1,3,5-trimethylbenzene, and heating oil, mass balances indicated that significantly less than 100% of consumed SO\(_4^{2-}\) was converted to S(-II) (Tab. 6-1). Differences between the sum of initially supplied and recovered SO\(_4^{2-}\) and S(-II) were too large to be explained by analytical uncertainty alone. Possibly, sulfur may have been used in assimilatory processes during bacterial growth, or SO\(_4^{2-}\) was reduced to sulfur species other than S(-II) such as e.g. sulfite (Chambers and Trudinger, 1979; Madigan et al., 2003). Apart from the quantification of produced S(-II), however, no effort was made here to further elucidate the fate of initially supplied SO\(_4^{2-}\).

6.5.2 Carbon source consumption

Acetate production in some of the enrichment culture experiments indicated the presence of SRB that can only incompletely degrade the substrates to acetate. Strain PRTOL1 is known to degrade carbon sources completely to CO\(_2\) (Beller et al., 1996). Complete or incomplete degradation of carbon sources by SRB may have an influence on the extent of sulfur isotope fractionation (Detmers et al. (2001), see below).

In most experiments, SO\(_4^{2-}\) reduction did not account for all the carbon source degradation (Tab. 6-1). Hence, a certain proportion of the carbon was likely incorporated into biomass (Fig. 6-2) (Widdel, 1988) or possibly degraded to unidentified intermediates (e.g., benzylsuccinate (Beller et al., 1996)). In addition, some of the added acetate or acetate produced during incomplete degradation of the carbon sources in the enrichment culture microcosms may have been degraded by methanogenic microorganisms (Zinder, 1993). Indeed, CH\(_4\) was detected when headspace gas of some enrichment culture microcosms was sampled and analyzed.

6.5.3 Sulfate reduction rates

A wide range of SRR was obtained for the different experiments (Tab. 6-2). However, SRR depend to a large extent on the cell density in the respective microcosms. In our experiments, inoculants had different cell densities and initial (Fig. 6-2) and average bacterial cell numbers (Tab. 6-2) varied between different experiments, which may explain some of the observed variability in SRR. Interestingly, the first-order rate coefficients for SO\(_4^{2-}\) reduction determined in this study (\(k = 0.398 \pm 0.019 \text{ d}^{-1}\) for the enrichment culture grown on pyruvate,
and \( k = 0.018 \pm 0.002 \text{ d}^{-1} \) for PRTOL1 grown on 3-phenylpropionate) mark the upper and lower end of first-order rate coefficients that have been determined for \( \text{SO}_4^{2-} \) reduction in PHC-contaminated aquifers (0.02 - 0.32 d\(^{-1}\)) (Chapelle et al., 1996; Kleikemper et al., 2002b; Schroth et al., 2001).

Our sSRR values (1.1 x 10\(^{-14}\) to 1.5 x 10\(^{-13}\) mol cell\(^{-1}\) d\(^{-1}\), Tab. 6-2) are in the upper range of sSRR values obtained in previous studies (e.g., Chambers et al. (1975); Detmers et al. (2001); Kaplan and Rittenberg (1964)) and in the same range of sSRR values determined by Bolliger et al. (2001). However, caution is required when comparing sSRR values between various studies as discussed in Bolliger et al. (2001). The accuracy of sSRR values depends on the variability of the underlying parameters, i.e. SRR values and bacterial cell numbers. Cell numbers varied substantially during most of our experiments (Fig. 6-2). Consequently, computed average bacterial cell numbers, and hence, sSRR values, were associated with fairly large uncertainties (Tab. 6-2).

6.5.4 Sulfur isotope fractionation

Stable sulfur isotope ratios of unconsumed \( \text{SO}_4^{2-} \) and produced S(-II) changed during our experiments as expected for a reaction in a closed system (Fig. 6-4; Chambers and Trudinger (1979); Thode (1991)). In general, the range of \( \varepsilon \) values obtained in this study were comparable to those reported by other authors for various strains under different growth conditions (Bolliger et al., 2001; Böttcher et al., 1999; Chambers et al., 1975; Detmers et al., 2001; Kaplan and Rittenberg, 1964). The stable fractionations down to \( \text{SO}_4^{2-} \) concentrations of sometimes < 0.1 mM (Fig. 6-3) and decreasing fractionations below this value (not shown) agree with Habicht et al. (2002). Our results corroborate the observation that for freshwater strains as used in our study only a very low \( \text{SO}_4^{2-} \) concentration limits isotope fractionation and not, as previously thought, already concentrations below 1 mM (Harrison and Thode, 1958).

We will see that the variability of \( \varepsilon \) values in this study may be explained in terms of investigated organism and the influence of carbon source, which in turn is controlled by the variability of cell-specific \( \text{SO}_4^{2-} \) reduction rates, the energy yield of the reaction (\( \Delta G \) values), and complete or incomplete carbon source degradation.
**Influence of culture**

Cultures of PRTOL1 always showed higher $\varepsilon$ values than the enrichment cultures when both were grown on the same carbon source (Tab. 6-3). This is in agreement with Bolliger et al. (2001) who cultivated PRTOL1 and an enrichment culture derived from the same field site with toluene as sole carbon source. Two reasons may explain this result. Firstly, the enrichment cultures consisted of mixtures of different SRB, and different SRB are known to show a range of individual $\varepsilon$ values (Detmers et al., 2001). Hence the overall $\varepsilon$ of an enrichment culture will be an average value and therefore lower than that of a pure strain that tends to show high fractionation. Secondly, complete or incomplete carbon source degradation may have influenced $\varepsilon$ values as discussed below.

**Influence of cell-specific sulfate reduction rate**

Several authors have suggested that sulfur isotope fractionation is controlled by $s_{\text{SRR}}$ rather than $s_{\text{R}}$ (Chambers and Trudinger, 1979; Habicht and Canfield, 1997). Thus, $\varepsilon$ values should be related to values of $s_{\text{SRR}}$ when comparing results of different studies. A weak inverse relationship between sulfur isotope fractionation and $s_{\text{SRR}}$ was found for data from PRTOL1 ($R^2 = 0.49$, Fig. 6-4), which agrees with data from Bolliger et al. (2001) and tends to support the prediction of Detmers et al. (2001) that a correlation between $s_{\text{SRR}}$ and fractionation may be found if various substrates were...

![Graph](image)

**Figure 6-4** Calculated enrichment factors ($\varepsilon$) for all microcosm experiments as a function of cell-specific $\text{SO}_4^{2-}$ reduction rate ($s_{\text{SRR}}$). Error bars represent standard deviations in $\varepsilon$ and $s_{\text{SRR}}$ values.

$a$ data from Bolliger et al. (2001) were included for comparison.
tested for one organism. When data from all microcosm experiments were combined, there was no obvious correlation between $\varepsilon$ values and sSRR ($R^2 = 0.12$, Fig. 6-4). This agrees with more recent findings of Canfield et al. (2000) and Detmers et al. (2001), who concluded that there is little or no correlation between $\varepsilon$ and sSRR values for a broad range of SRB.

**Influence of energy yield**

This study is the first investigation of sulfur isotope fractionation when the PHC naphthalene, 1,3,5-trimethylbenzene and heating oil served as sole carbon sources. Interestingly, $\varepsilon$ values for these PHC compounds were very similar (27.0-33.1‰) and significantly larger (average: 29.4‰) ($p = 0.05$) than for enrichment cultures grown on organic acids (average: 18.1‰). Apart from the fact that probably different SRB communities were enriched on the different carbon sources (Kleikemper et al., 2002a; Parkes et al., 1993), the energy yields ($\Delta G_0$) of the respective reactions may influence the extent of isotope fractionation as has been suggested previously by Detmers et al. (2001). Higher energy yields were generally associated with low $\varepsilon$ values. However, we suggest that instead of using standard $\Delta G_0$ values (Detmers et al., 2001), it is be more appropriate to consider actual $\Delta G_f$ values under the conditions the reaction is occurring (Tab. 6-4). Values of $\Delta G_0$ for the enrichment cultures were not very different between organic acids and PHC compounds (except pyruvate, Tab. 6-4), but if actual $\Delta G_f$ values were taken into account, growth on the PHC compounds tended to yield less energy than growth on organic acids (Tab. 6-4). This explains in part the trend towards higher $\varepsilon$ values for the PHC.

**Influence of complete or incomplete carbon source degradation**

Several authors observed that completely degrading pure strains tended to show higher $\varepsilon$ values than incompletely degrading strains, due to generally lower energy yields (less negative $\Delta G_0$ values) of complete substrate oxidations (Brüchert et al., 2001; Detmers et al., 2001). While PRTOL1 is a completely degrading strain (Beller et al., 1996), the enrichment cultures grown on organic acids (except for acetate) likely also contained incompletely degrading SRB since acetate was produced in many of our experiments. The lower energy yields ($\Delta G_f$) for the complete oxidations of strain PRTOL1 as compared to incomplete carbon source degradation by the enrichment cultures is the second reason to explain the higher $\varepsilon$ values for strain PRTOL1 (Tab. 6-4, see above).
Table 6-4  Stoichiometric equations of the degradation of the carbon sources used in this study under SO$_4^{2-}$-reducing conditions and free-energy changes (kJ / mol degraded SO$_4^{2-}$) under standard conditions ($\Delta G_0$) and actual conditions ($\Delta G_f$). The stoichiometries of the reactions were derived from the measured SO$_4^{2-}$ and organic acid concentrations.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Culture</th>
<th>Stoichiometric reaction</th>
<th>$\Delta G_0$</th>
<th>$\Delta G_f$</th>
<th>$%$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>complete</td>
<td>PRTOL1 STOL enrichment</td>
<td>-48</td>
<td>-58</td>
<td>30.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH$_3$COO$^-$ + SO$_4^{2-}$ =&gt; 2HCO$_3^-$ + HS$^-$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>complete</td>
<td>PRTOL1 STOL enrichment</td>
<td>-106</td>
<td>-153</td>
<td>34.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH$_3$COCOO$^-$ + H$_2$O + 1.25SO$_4^{2-}$ =&gt; 3HCO$_3^-$ + 1.25HS$^-$ + 0.75H$^+$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>incomplete (1)</td>
<td>PRTOL1 STOL enrichment</td>
<td>-341</td>
<td>-558</td>
<td>21.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CH$_3$COCOO$^-$ + H$_2$O + 0.25SO$_4^{2-}$ =&gt; HCO$_3^-$ + 0.75H$^+$ + 0.25HS$^-$ + CH$_3$COO$^-$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzoate</td>
<td>incomplete (1)</td>
<td>PRTOL1 STOL enrichment</td>
<td>-43</td>
<td>-114</td>
<td>28.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C$_7$H$_5$O$_2^-$ + 4H$_2$O + 2.75SO$_4^{2-}$ =&gt; 5HCO$_3^-$ + 2.25H$^+$ + 2.75HS$^-$ + CH$_3$COO$^-$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>incomplete (2)</td>
<td>PRTOL1 STOL enrichment</td>
<td>-40</td>
<td>-144</td>
<td>17.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C$_7$H$_5$O$_2^-$ + 4H$_2$O + 1.75SO$_4^{2-}$ + 3HC0$_3^-$ + 2.25H$^+$ + 1.75HS$^-$ + 2CH$_3$COO$^-$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-Phenylpropionate$^d$</td>
<td>complete</td>
<td>PRTOL1 STOL enrichment</td>
<td>-51$^d$</td>
<td>-98$^d$</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>incomplete (3)</td>
<td>PRTOL1 STOL enrichment</td>
<td>-57$^d$</td>
<td>-154$^d$</td>
<td>15.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C$_6$H$_8$O$_2^-$ + 4H$_2$O + 5.25SO$_4^{2-}$ =&gt; 9HCO$_3^-$ + 2.75H$^+$ + 5.25HS$^-$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naphthalene$^e$</td>
<td>complete</td>
<td>STOL enrichment</td>
<td>-48</td>
<td>-101</td>
<td>27.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C$_8$H$_8$ + 6H$_2$O + 6SO$_4^{2-}$ =&gt; 10HCO$_3^-$ + 4H$^+$ + 6HS$^-$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,3,5-Trimethylbenzene</td>
<td>complete</td>
<td>STOL enrichment</td>
<td>-45</td>
<td>-85</td>
<td>33.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C$<em>9$H$</em>{12}$ + 3H$_2$O + 6SO$_4^{2-}$ =&gt; 9HCO$_3^-$ + 3H$^+$ + 6HS$^-$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heating oil$^f$</td>
<td>complete</td>
<td>STOL enrichment</td>
<td>-48</td>
<td>-74</td>
<td>28.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C$<em>{10}$H$</em>{16}$ + 13SO$_4^{2-}$ =&gt; 17HCO$_3^-$ + 4H$^+$ + 13HS$^-$ + H$_2$O</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ values of standard free energy are from Madigan et al. (2003) and Yaws (1999)

$^b$ calculated using the Nernst equation: $\Delta G_f = \Delta G_0 + RT \ln (c(products)/c(reactants))$ (e.g., Jakobsen and Postma (1999))

$^c$ numbers in brackets refer to number of acetate molecules produced per carbon source molecules degraded

$^d$ standard free energy of formation for 3-phenylpropionate was not available; a nominal value of -200 kJ/mol was assumed based on values for related compounds (Yaws, 1999). Hence, these values may only be compared with each other and not with the other values.

$^e$ even though some acetate was produced during naphthalene and heating oil degradation, the greater proportion of these carbon sources was degraded by a complete mechanism as calculated using measured SO$_4^{2-}$ and acetate concentrations

$^f$ heptadecane was chosen since it represents the average carbon number of heating oil (Lecomte and Mariotti, 1997)
In our enrichment cultures all carbon sources except acetate and 1,3,5-trimethylbenzene were - at least to some degree - incompletely degraded (production of acetate). Hence, \( \varepsilon \) values for these two substrates may be expected to be higher. Indeed, growth of cultures on 1,3,5-trimethylbenzene was associated with high \( \varepsilon \) values (33.08\%), explained by the comparatively low energy yield of the reaction (Tab. 6-4). However, while the energy yield for acetate was the lowest of all substrates (Tab. 6-4), \( \varepsilon \) was quite low (18.54\%, Tab. 6-3). Hence, the different SRB communities that developed on different carbon sources likely influenced \( \varepsilon \) values to some extent.

Influence of carbon source for strain PRTOL1

Growth of PRTOL1 on various carbon sources resulted in a narrow range of \( \varepsilon \) values (30.78 ± 2.52\%, average ± standard deviation, Table 3). More variable \( \varepsilon \) values were reported for PRTOL1 when grown on toluene and three different \( \text{SO}_4^{2-} \) concentrations (Bolliger et al., 2001) (\( \varepsilon = 32.1 - 46.9\% \)). Hence, for strain PRTOL1, \( \text{SO}_4^{2-} \) concentration seemed to have a greater influence on \( \varepsilon \) than carbon source type when the same range of sSRR values was considered. Only few other authors investigated sulfur isotope fractionation by the same strain grown on different carbon sources. For example, Kaplan and Rittenberg (1964) found that Desulfovibrio desulfuricans showed a higher fractionation on ethanol than on lactate, but Kemp and Thode (1968) found the reverse effect. The reason for this discrepancy may have been due to the different D. desulfuricans strains used in both studies (Kemp and Thode, 1968). Kaplan and Rittenberg (1964) explained their results in terms of the substrate-induced variability in sSRR, which also explains part of the variability in our data (Fig. 6-4). Nevertheless, experiments with similar sSRR, e.g. PRTOL1 grown on acetate and 3-phenylpropionate on the one hand or on toluene and benzoate on the other hand (Fig. 6-4) showed different \( \varepsilon \) values, suggesting that factors other than sSRR also influence the extent of sulfur isotope fractionation.

6.5.5 Relevance to field studies at contaminated sites

To use sulfur isotope fractionation for quantification of microbial sulfate reduction, the enrichment factor for a particular site has to be known (Aggarwal et al., 1997). In this study, the experiments that are probably most relevant for field studies in contaminated aquifers are the enrichment cultures grown on PHC components. However, SRB in the aquifer may not
only directly degrade PHC but also a range of organic acids, which are metabolic products of fermenting bacteria. Hence, our enrichment cultures grown on organic acids also carry relevance for field sites. In our experiments, we observed that enrichment cultures grown on PHC tended to show higher $\varepsilon$ values than on organic acids. However, the average $\varepsilon$ value of all enrichment cultures (23.0 $\pm$ 6.5‰) agreed well with values from field experiments (20.7‰ (Kleikemper et al., 2002b) or 21.5‰ (Schroth et al., 2001)) and with the average $\varepsilon$ of toluene-degrading enrichment cultures inoculated with material from the same aquifer (23.5 $\pm$ 4.3‰ (Bolliger et al., 2001)). Hence, SRB in the field may be using both fermentation products and PHC as carbon sources, leading to average $\varepsilon$ values.

Enrichment cultures grown on heating oil showed surprisingly high $\varepsilon$ values (28.10‰) compared to values generated in field experiments (21.5‰ $\pm$ 1.8‰ (Schroth et al., 2001)) in the same contaminated aquifer that the inoculum for the enrichment culture was obtained from. This discrepancy may be due to different conditions in field and microcosm experiments, of which temperature is the most eminent. In addition, the population in the field was possibly also growing on other substances (natural organic compounds) and during enrichment probably some specific SRB groups were selected for. Other authors previously observed that increasing temperature lead to increasing $\varepsilon$ values for natural SRB populations (Brüchert et al., 2001) and ascribed this to different SRB and associated fermenting bacteria being active at different temperatures.

In general, $\varepsilon$ values at PHC-contaminated sites seem to be higher than in other freshwater aquifer environments. For example, values of 9.7 - 15.5 were determined for uncontaminated aquifers (Robertson and Schiff, 1994; Strebel et al., 1990), while values of 9.5 – 21 were found for aquifers contaminated with landfill leachate, phenol, or with a multicomponent pollutant mixture (Asmussen and Strauch, 1998; Bottrell et al., 1995; Spence et al., 2001).

Our results show that due to the similar extent of sulfur isotope fractionation in microcosm and field studies, a relationship between $\varepsilon$ and the $\text{SO}_4^{2-}$ reduction rate coefficient such as proposed by Aggarwal et al. (1997) may be used for estimations of $\text{SO}_4^{2-}$ reduction rates in field settings. However, our results for growth of SRB on PHC compounds were all generated with microorganisms from the same site, hence, a comparison with other PHC-contaminated sites would be useful.
6.6 Acknowledgements

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6.7 References


Activity and Diversity of Methanogens in a Petroleum Hydrocarbon-Contaminated Aquifer

7.1 Abstract

Methanogenic activity was investigated in a petroleum hydrocarbon (PHC) -contaminated aquifer using a series of four push-pull tests with acetate, formate, H₂ + CO₂ or methanol to target different groups of methanogenic Archaea. Furthermore, the community composition of methanogens in water and sediment samples was explored using molecular analyses, i.e., fluorescence in situ hybridization (FISH) with probes specific for a range of methanogenic Archaea, denaturing gradient gel electrophoresis (DGGE) of 16S rDNA amplified with the Archaea-specific primer ARCH915, and sequencing of DNA from the dominant bands in DGGE profiles. Molecular analyses were subsequently compared with push-pull test data. To explore the contribution of other processes to observed substrate consumption, in particular Fe(III) reduction, another push-pull test was performed using 2-bromoethanesulfonate (BES) as a specific inhibitor of methanogens. Substrate consumption rates were 0.11 mM d⁻¹ for methanol, 0.38 mM d⁻¹ for acetate, 0.90 mM d⁻¹ for H₂, and 1.85 mM d⁻¹ for formate. Methane production was observed in all tests except of the test where BES was added. Consumption of the added substrates and CH₄ production during all tests suggested that at least three different physiologic types of methanogenic Archaea were present: H₂ + CO₂ / formate, acetate, and methanol utilizers. The presence of at least 15-20 bands in DGGE profiles indicated a diverse archaeal population. All clones were related to methanogenic Archaea. High potential H₂ and formate consumption rates agreed with the high diversity of methanogenic Archaea consuming these substrates (16S rDNA sequences related to Methanocaldococcus, Methanocorpusculum, Methanogenium, Methanospirillum, and Methanothermobacter). Considerable acetate consumption agreed with the presence of sequences related to the obligate acetate degrader Methanosaeata concilii. Using FISH, we detected Methanosaeata concilii (5-22% of total (DAPI-stained) microorganisms in water and sediment samples; probe Rotc11) and members of the Methanomicrobiaceae (1.4% of total microorganisms in one water sample, e.g. Methanocorpusculum, Methanogenium, Methanospirillum; probe MG1200). Counts with other Archaea-specific hybridization probes targeting Methanobacteriaceae, Methanosarcinaceae, and Methanospirillum were below 1%. DGGE profiles also suggested that the community composition of the dominant species was similar in water and sediment samples. The combination of hydrogeological and molecular methods in this study provided valuable information on the community structure and the potential activity of methanogens in a PHC-contaminated aquifer.
7.2 Introduction

Methanogenesis is a common and important process in many anaerobic environments, for example, in anaerobic digesters (Raskin et al., 1994a), cattle rumen (Miller et al., 1986), rice fields (Joulian et al., 1998), oil wells (Ollivier et al., 1997), landfills (Fielding et al., 1988), and a range of extreme habitats (Garcia et al., 2000). They play important roles in anaerobic treatment of organic wastes, formation of biogas as an alternative source of energy (Oremland, 1988), generation of CH₄ as a greenhouse gas (Wuebbles and Hayhoe, 2002), and degradation of contaminants in polluted soils and aquifers (Christensen et al., 1994; Zengler et al., 1999). In the absence of other electron acceptors such as oxygen, nitrate, and sulfate, methanogens are involved in the terminal anaerobic breakdown of organic matter (Garcia et al., 2000). Methanogenesis contributed considerably to the mineralization of petroleum hydrocarbons (PHC) in a contaminated aquifer when other electron acceptors were depleted (Chapelle et al., 2002). However, few studies so far focused on activity or diversity of methanogens in PHC-contaminated aquifers (Bolliger et al., 2000; Chapelle et al., 2002; Dojka et al., 1998; Watanabe et al., 2002), and to our knowledge no attempt has been made to link the presence of metabolic or phylogenetic types of methanogens to their activity in this environment. But such information is essential for our understanding of the biogeochemical processes occurring in contaminated aquifers and thus, for managing and monitoring the efficiency of bioremediation.

Methanogenic microorganisms are not able to directly degrade PHC components (Zengler et al., 1999). They catabolically rely on a restricted number of simple compounds, e.g., on CO₂ as oxidant with H₂ as electron donor or on acetate, methanol or formate (Zinder, 1993). Hence, they depend on other organisms such as fermenting or sulfate-reducing bacteria for their substrates. Methanogenic microorganisms all belong to the domain Archaea with different physiological types mostly belonging to different phylotypes (Zinder, 1993). For example, while most species of the Methanobacteriaceae and Methanomicrobiaceae prefer H₂ and CO₂ (or formate) as substrates for methanogenesis, Methanoseta, a genus within the Methanosarcinaceae, is known to generate energy only from acetate fermentation. Most of the other Methanosarcinaceae preferentially use methanol and related substrates for the generation of CH₄. Methanogens using H₂ and CO₂ contribute indirectly to PHC degradation by keeping H₂ pressure low enough so that fermentation of PHC becomes exergonic and fermenting organisms can grow (Garcia et al., 2000). Methanogens using acetate or methanol contribute directly to PHC degradation by cleaving end products of fermentations.
Previous investigations of methanogenic *Archaea* diversity in a range of different freshwater environments indicated that aceticlastic *Methanoseta* was present at many sites, and hence, probably an important *Archaea* species in these environments (Bolliger et al., 2000; Chapelle et al., 2002; Dojka et al., 1998; Watanabe et al., 2002; Zepp Falz et al., 1999). Zengler et al. (1999) demonstrated that Archaea related to *Methanoseta* spp. were involved in the anaerobic degradation of long-chain alkanes in a mixed culture enriched from anaerobic ditch sediment. Dojka et al. (1998) hypothesized that aceticlastic methanogenesis was the terminal step of hydrocarbon degradation in a PHC-contaminated aquifer but they inferred this from molecular data alone and not based on direct process measurements.

Several freshwater environments were also frequently found to host species of the H2- and CO2- or methanol-consuming *Methanomicrobiaceae* and *Methanosarcinaceae* (Watanabe et al., 2002; Zepp Falz et al., 1999). In particular, Zengler et al. (1999) detected clones related to *Methanospirillum* and *Methanoculleus*, both belonging to the *Methanomicrobiaceae*, in their alkane-degrading enrichment culture. Based on radiotracer methods and stable isotope data, Hansen et al. (2001) suggested that CO2 reduction was the dominant pathway of methanogenesis in a shallow sandy aquifer. Hence, a variety of different methanogenic microorganisms and activities were found in freshwater environments.

The diversity of methanogenic *Archaea* in the environment may be monitored using laboratory molecular methods such as fluorescence in situ hybridization (FISH) or denaturing gradient gel electrophoresis (DGGE) with subsequent cloning and sequencing of excised bands (Ficker et al., 1999; Raskin et al., 1994a; Roling et al., 2001). For example, using FISH, Ficker et al. (1999) found that 17% of total microorganisms in a toluene-degrading enrichment culture hybridized with a *Methanoseta*-specific hybridization probe and 2% hybridized with a *Methanospirillum*-specific probe. In samples from a sewage sludge digester, Raskin et al. (1994a) observed that *Methanoseta* spp. were abundant, while only few *Methanosarcina* spp. were visible. By assessing bands of DGGE gels, Röling et al. (2001) detected different archaeal community structures inside and outside the contaminant plume of a landfill leachate-polluted aquifer. However, when investigating microbial community structure in aquifers, it is important to keep in mind that attached and suspended communities may not be the same (Alfreider et al., 1997; Lehman and O'Connell, 2002). Hence, for an adequate community characterization it may be necessary to analyze both water and sediment samples.

Although methanogenic activity in the subsurface is difficult to monitor due to the volatility of CH4 and its preferred degradation by methanotrophs under both aerobic (Hanson
and Hanson, 1996) and anaerobic (Boetius et al., 2000) conditions, push-pull tests (PPTs) have been used to quantify methanogenesis in contaminated aquifers (Istok et al., 1997). In a PPT, a test solution that contains a non-reactive, conservative tracer and one or more reactive solutes (reactants) is injected (“pushed”) into the aquifer through an existing well. During an initial incubation period (i.e., a rest phase without pumping), indigenous microorganisms ideally consume reactants and generate metabolic products. Thereafter, the test solution/groundwater mixture is extracted (“pulled”) from the same location and the concentrations of tracer, reactants and products are analyzed. Rates of microbial activities are then determined by comparing the breakthrough curves of tracer and reactants (Haggerty et al., 1998; Snodgrass and Kitanidis, 1998).

Even if other dissolved electron acceptors such as nitrate and sulfate are not present, quantification of methanogenesis using PPTs may still be difficult since Fe(III) reduction which relies on solid Fe(III) minerals may occur simultaneously with methanogenesis in different microniches in the aquifer (Bekins et al., 1999). Fe(III) reduction is an important process in many contaminated environments (Cozzarelli et al., 2000; Lovley et al., 1994). Unfortunately, a specific inhibitor of dissimilatory Fe(III) reduction has not been reported so far (Oremland and Capone, 1988). However, if methanogenesis was inhibited in a PPT using 2-bromoethanesulfonate (BES) as a specific inhibitor, the contribution of processes other than methanogenesis to substrate degradation in PPTs may be estimated.

The objective of our study was to assess activity and diversity of methanogens in the anoxic zone of a PHC-contaminated aquifer. In four separate PPTs, we used acetate, formate, CO$_2$ + H$_2$, and methanol as substrates to examine the potential activity of different physiologic groups of methanogenic Archaea. Push-pull test data were then compared with molecular analyses (FISH, DGGE, and sequencing of excised DGGE bands) of the methanogen community composition in water and sediment samples. To explore the contribution of other processes, in particular Fe(III) reduction, to H$_2$ consumption as a selected process, another PPT was performed using BES as a specific inhibitor of methanogens (Oremland and Capone, 1988).
7.3 Materials and Methods

7.3.1 Field site

The study was conducted in a heating oil-contaminated aquifer in Studen, Switzerland, which is undergoing remediation by monitored natural attenuation (Bolliger et al., 1999). Push-pull tests described in this paper were conducted in monitoring well PS5, which is located within the contaminant source zone (free-phase PHC present). Well PS5 is constructed of 11.5 cm I.D. polyvinyl chloride casing and penetrates the aquifer to a depth of ~0.5 m below the ground water table. Groundwater in PS5 exhibited reducing conditions and contained up to 1 mg L\(^{-1}\) dissolved PHC (Bolliger et al., 1999). Previous studies have shown that PS5 is located within the methanogenic zone (Bolliger et al., 1999; Bolliger et al., 2000).

7.3.2 Push-pull tests and sample collection procedures

To assess methanogenic activity we performed four PPTs (PPTac, PPTfo, PPTH\(_2\), and PPTme) between September 2001 and October 2002 in similar fashion as described in Schroth et al. (2001) using the substrates acetate, formate, CO\(_2\) + H\(_2\) or methanol. To explore the contribution of Fe(III) reduction to substrate consumption, another PPT (PPTBES) assessing H\(_2\) consumption was performed in November 2002 in the presence of BES, a specific inhibitor of methanogens. In all PPTs, test solutions were prepared by collecting groundwater in 500-L plastic carboys and adding Br\(^-\) (as KBr) as a non-reactive, conservative tracer along with acetate, formate, H\(_2\) or methanol as reactants to achieve final concentrations of ~0.5 mM Br\(^-\) and ~2.0 mM acetate, formate or methanol (Table 1). Hydrogen in PPTH\(_2\) (0.61 mM) and PPTBES (0.39 mM) was added by sparging test solutions with H\(_2\) gas. Carbon dioxide was not added in PPTH\(_2\) or PPTBES since CO\(_2\) was present in the groundwater of PS5 at a concentration of 13.5 mM as dissolved inorganic carbon (DIC). In PPTBES, argon as a gas tracer (added by sparging) and ~2 mM BES were added to the test solution. In PPTH\(_2\), H\(_2\) was used to minimize O\(_2\) dissolution from air into test solutions during preparation and subsequent injection. In PPTBES, the carboys were continuously sparged with H\(_2\) and Ar simultaneously and in the remaining PPTs with N\(_2\) gas to keep test solutions anoxic.

For each PPT, injection of either 500 or 1000 L of test solution into PS5 began at time \(t = 0\) h and was completed within 0.57 to 1.85 h (Table 1) using gravity drainage. After an initial incubation period of 1.78 – 20.4 h, we extracted a total of 750 - 1000 L of test
solution/groundwater mixture during further incubation of up to two days. The average total test duration ranged from 4.17 h to 70.83 h. Preliminary tests had shown that test durations had to be varied due to different substrate degradation velocities.

Water samples for chemical analyses were obtained during the collection of groundwater in carboys (background concentrations), injection of test solutions (injection concentrations), and at regular intervals during the extraction phases of the PPTs. Specifically, samples for the analysis of Br⁻, organic acids or BES were filtered in the field using 0.45 μm polyvinylidenefluoride filters (Millipore, Bedford, MA) and stored in 12-mL plastic vials. For the analysis of methanol, unfiltered water was collected in 5 mL glass tubes with Teflon-coated screw caps. Samples for CH₄, H₂, and Ar analysis were collected without headspace in 117-mL serum bottles and closed using butyl rubber stoppers. All samples were stored at 4°C prior to analysis. Samples collected for dissolved O₂, S(-II) and ferrous iron (Fe(II)) determination were analyzed immediately in the field (see below).

Before and after the PPT series (i.e., in September 2001 and October 2002), groundwater from PS5 was sampled for biological analyses (total cell counts, FISH, and PCR-DGGE) by collecting 50 mL each of unfiltered water in sterile Falcon tubes. All samples for biological analyses were immediately placed on ice until further processing in the laboratory. After the PPT series, sediment samples were collected using a handheld hollow-stem auger (Humax, Switzerland) at a distance of ~30 cm from the well casing at a depth of 3-3.5 m below ground in the anoxic zone of the aquifer. Sediment samples were stored under N₂ atmosphere on ice during transport until immediate further processing in the laboratory.

### 7.3.3 Analytical methods

Bromide, acetate, and formate concentrations were determined using a DX-320 ion chromatograph (Dionex, Sunnyvale, CA, USA) as described in Kleikemper et al. (2002). BES, SO₄²⁻ and NO₃⁻ were quantified using a DX-100 ion chromatograph system (Dionex, Sunnyvale, CA, USA). Methane, H₂ and Ar concentrations were determined by gas chromatography (GC Carlo Erba Model 8000, Rodano, Italy) on a HayeSep D column with N₂ as carrier gas and a Carlo Erba thermal conductivity detector, using the headspace method as described in Bolliger et al. (1999). Methanol was quantified photometrically using alcohol oxidase coupled to peroxidase and 2,2’-azino-di-(3-ethyl)-benzthiazoline-6-sulfonic acid (ABTS) as described by Herzberg and Rogerson (1985). Dissolved O₂, S(-II), and Fe(II) were measured colorimetrically using a DR/890 colorimeter (Hach Co., Loveland, CO, USA).
following standard protocols. DIC concentrations were determined according to Bolliger et al. (1999).

7.3.4 Determination of zero-order rates

Zero-order rates for substrate degradation (in mM d⁻¹) in PPTfo were determined directly from substrate consumption using the method of Snodgrass and Kitanidis (1998). Since the data of all other PPTs did not fit a zero-order reaction, but rather a first-order type reaction, for these tests first-order rate coefficients (unit: d⁻¹) for substrate degradation were determined from substrate consumption using the method of Haggerty et al. (1998). To allow a comparison of substrate degradation between all tests, first-order rate coefficients were multiplied with the average substrate concentration during each test, resulting in zero-order substrate consumption rates (in mM d⁻¹).

7.3.5 Stoichiometries

In order to relate substrate degradation to CH₄ production in PPTs, the stoichiometries of the degradation reactions were taken into account. First, the total amount of degraded substrate was calculated from the differences of the bromide and substrate breakthrough curves (Tab. 7-2). Then, the theoretical stoichiometric amount of CH₄ that would be cumulatively produced from this calculated amount of degraded substrate was computed based on the following reaction equations (Madigan et al., 2003) (Eqns. 7-1-4):

\begin{align*}
\text{Acetate:} & \quad \text{CH}_3\text{COO}^- + \text{H}_2\text{O} \rightarrow \text{CH}_4 + \text{HCO}_3^- \quad (\text{Equation 7-1}) \\
\text{Formate:} & \quad 4\text{CHOO}^- + 4\text{H}^+ \rightarrow \text{CH}_4 + 3\text{CO}_2 + 2\text{H}_2\text{O} \quad (\text{Equation 7-2}) \\
\text{CO}_2 + \text{H}_2: & \quad \text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O} \quad (\text{Equation 7-3}) \\
\text{Methanol:} & \quad 4\text{CH}_3\text{OH} \rightarrow 3\text{CH}_4 + \text{CO}_2 + 2\text{H}_2\text{O} \quad (\text{Equation 7-4})
\end{align*}

7.3.6 Cell counts and in situ hybridization

An estimation of total cell numbers was accomplished using 4′,6-diamidino-2′-phenylindole (DAPI) staining (Zarda et al., 1997). For in situ hybridization, we used the Cy3-labelled 16S rRNA oligonucleotide probes (all purchased from MWG Biotech, Ebersberg, Germany) EUB338 to target Bacteria (Amann et al., 1990), Arch915 (Stahl and Amann, 1991) for Archaea, MG1200 for Methanomicrobiaceae, MB1174 for Methanobacteriaceae, MS1414 for Methanosarcinaceae, MX825 for Methanosacetaceae (Raskin et al., 1994b), Rotell1 for
Methanosaeta concilii, and Rotcl2 for Methanospirillum sp. (Endosymbiont of P. nasuta) (Zepp Falz et al., 1999).

Samples for FISH and DAPI counts were processed according to Zarda et al. (1997) with the following exceptions. Within a few hours after sampling, water samples were centrifuged at 2500 × g for 10 min and the debris / cell-pellet was resuspended in 1 ml of 4% paraformaldehyde in phosphate-buffered saline (PBS). Similarly, 2 g of sediment was fixed with 1.5 ml of 4% paraformaldehyde in PBS. Formamide concentrations in the hybridization mix were 10% for probe MG1200, 20% for Arch915, MX825, and Rotcl2, 30% for probe EUB338 and Rotcl1, and 35% for MB1174 and MS1414. Sodium chloride concentrations in the wash buffer were 440 mM for probe MG1200, 308 mM for Arch915, MX825, and Rotcl2, 100 mM for EUB338 and Rotcl1, and 80 mM for MB1174 and MS1414. The slides were mounted and visually detectable cells were counted according to Zarda et al. (1997).

### 7.3.7 DNA extraction and PCR-DGGE

To concentrate suspended bacterial cells, groundwater (100 ml) was filtered through 0.22 μm polyvinylidene fluoride-filters (Millipore, Bedford, MA), followed by storage of the filters in 1.5 mL of lysis buffer (50 mM Tris [pH 8], 50 mM EDTA, 50 mM NaCl) at -20°C. Two g of sediment samples were stored in 1.5 mL of lysis buffer (50 mM Tris [pH 9.5], 50 mM EDTA, 50 mM NaCl, and 5% SDS) at -80°C. Following the addition of ~ 0.7 g glass beads (0.10 - 0.11 mm diameter) to all samples, DNA was extracted by direct lysis in a FastPrep 120 bead beater (Savant Instruments, Inc., Holbrook, NY) for 15 s at 4.5 m s⁻¹ (filters) or for 30 s at 5.5 m s⁻¹ (sediment). Approximately 10 mg ml⁻¹ lysozyme was added to the buffer / DNA solutions and the samples were incubated at room temperature for 10 min. One hundred μl of SDS (20%) and proteinase K (100 μg ml⁻¹) were added to each sample followed by incubation for 30 min at 37°C and 10 min at 55°C. After brief centrifugation to settle the filter pieces or sediment, the supernatant was transferred into a new tube, and the extraction of the filters or sediment was repeated with 0.5 ml of lysis buffer. DNA was further purified with chloroform-phenol extraction and isopropanol precipitation (Sambrook et al., 1989), and resuspended in 100 μl of TE-buffer. DNA was quantified by measuring absorbance at 260 nm and stored at -20°C.

The PCR of partial (456 bp) archaeal 16S rRNA genes was performed using primers ARCH915-GC (5'-GC-clamp-AGGAATTGGGCAGCGAC-3') (Amann et al., 1995) and UNI-b-rev (5'-GACGGGGCGGTGTGT(A/G)CAA-3') (Bundt et al., 2001), modified
from Amann et al. (1995), as described in Pesaro and Widmer (2002). DGGE of PCR products was performed in a denaturing gradient of 30 to 60% at 75 V for 15 h as described previously (Sigler et al., 2001). DNA band patterns were digitized, photographed, and analyzed using the GelDoc 2000 system and QuantityOne software (Bio-Rad Laboratories, Hercules, CA). Lane background subtraction was conducted using the rolling disk method (disk size 2) and bands were detected with a sensitivity of 5.1. The similarity of bands was calculated using the dice coefficient method. Dominant bands containing DNA to be sequenced were excised and incubated for 4 h in 100 μl sterile water, followed by PCR as described above, however, with non-GC-clamped primers. PCR products were transformed into Escherichia coli DH5-α using the pGEM-T vector system according to manufacturer’s instructions (Promega Corp., Madison, WI) and commercially sequenced. Using the BLAST 2.0 algorithm, the derived sequences were compared to 16S rRNA gene sequences in the National Center for Biotechnology Information database (Altschul et al., 1997).

7.3.8 Nucleotide sequence accession numbers

The partial environmental 16S rRNA gene clone sequences recovered in this study have been deposited in the GenBank nucleotide sequence database under the accession numbers AY294408 - AY294415 (Tab. 7-3).

7.4 Results

7.4.1 Push-pull tests

In native groundwater of well PS5, acetate, formate, methanol, and H₂ concentrations were below the detection limit (< 5 μM for acetate and formate, ~6 μM for methanol and ~ 0.3 μM for H₂). During PPTs, groundwater temperature was 16.0 ± 0.3°C (Tab. 7-1), concentrations of O₂ were 4.2 ± 0.6 μmol L⁻¹, of SO₄²⁻ 14 ± 13 μmol L⁻¹, of NO₃⁻ 10 ± 15 μmol L⁻¹, of S(II) 8 ± 1 μmol L⁻¹, of Fe(II) 287 ± 24 μmol L⁻¹, and those of DIC were 14.7 ± 0.9 mmol L⁻¹. A calculation of the total amount of Fe(II) lost or produced during the PPTs showed that minor amounts of Fe(II) evolved (at most 18 mmol, PPTH₂) or disappeared (at most -17 mmol, PPTfo)(data not shown).
Breakthrough curves for Br⁻ and each substrate showed a decline in relative concentrations (C/C₀) during PPT extraction phases as extracted test solution was increasingly diluted with native groundwater (Fig. 7-la - e). Relative substrate concentrations were lower than relative Br⁻ concentrations during all PPT extraction phases. This difference is significant since the error of Br⁻ and substrate measurements was generally smaller than 5%. We recovered 44% (PPTac), 73% (PPTfo), 80% (PPTH₂), 49% (PPTme), and 31% (PPTBES) of the injected Br⁻ mass during the extraction phases of the PPTs (computed by integrating solute breakthrough curves shown in Fig. 7-1). Furthermore, 17% of acetate, 47% of formate, 28% of H₂ (8% in PPTBES), 43% of methanol and 30% of BES were recovered.

The computed zero-order degradation rate was highest for formate (1.859 mM d⁻¹) and lowest for methanol (0.111 mM d⁻¹) (Tab. 7-2). Standard deviations ranged from 1.8 to 6.4 % of zero-order degradation rates. If the rates are compared on the basis of stoichiometric CH₄ production (Eqns.
Figure 7-1 Extraction phase breakthrough curves for Br⁻, acetate, formate, H₂, and methanol in extraction phases during push-pull tests (a) PPTac, (b) PPTfo, (c) PPTH₂, (d) PPTme, and (e) PPTBES. C/C₀ is relative concentration, i.e., actual concentration divided by injected concentration. Note that the time scales for the PPTs were different (Tab. 7-1) and in PPTac (a), numbers on the x-axis have to be divided by 2. Ac⁻ = acetate, Fo⁻ = formate, Me⁻ = methanol.
formate still shows the highest rate (0.465 mM CH$_4$ d$^{-1}$), followed by acetate (0.379 mM CH$_4$ d$^{-1}$), H$_2$ (PPTH$_2$, 0.226 mM CH$_4$ d$^{-1}$), methanol (0.083 mM CH$_4$ d$^{-1}$), and H$_2$ (PPTBES, 0.042 mM CH$_4$ d$^{-1}$). For PPTBES, zero-order degradation rates calculated with Ar as conservative gas tracer were similar to those calculated with Br$^-$ (0.184 mM d$^{-1}$ as compared to 0.167 mM d$^{-1}$), hence, we have shown only data that was generated with Br$^-$. Methane concentrations were 0.67 ± 0.24 mM (average ± standard deviation of all tests) in native groundwater of well PS5 and 0.04 ± 0.04 mM in injection solutions. The CH$_4$ concentrations increased during extraction phases of all PPTs (Fig. 7-2a). After subtraction of the background CH$_4$, the total cumulative mass of produced CH$_4$ (computation according to Schroth et al. (1998)) ranged from -45 mmol (PPTBES) to +158 mmol (PPTac) (Fig. 7-2b, Tab. 7-2).

Formate was detected at initial concentrations of 72 and 30 μM during PPTH$_2$ and PPTBES, respectively, and concentrations declined during the tests (Fig. 7-1c, e). Organic acids other than those that were injected (except of formate in PPTH$_2$ and PPTBES) were not detected during PPT extraction phases.
### Table 7-2  Zero-order substrate degradation rates, cumulative substrate degraded, cumulative CH\(_4\) produced, and percentage of degraded substrate that was recovered as CH\(_4\) in five push-pull tests.

<table>
<thead>
<tr>
<th>Test</th>
<th>Zero-order degradation rate Value ± SD(^b)</th>
<th>Cumulative (total) substrate degraded mmol</th>
<th>Cumulative (total) CH(_4) produced mmol</th>
<th>Percentage of the degraded substrate that was recovered as CH(_4) %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPTac</td>
<td>0.379 ± 0.023 mM d(^{-1})</td>
<td>557</td>
<td>158</td>
<td>28</td>
</tr>
<tr>
<td>PPTfo(^b)</td>
<td>1.859 ± 0.119 mmol</td>
<td>268</td>
<td>44</td>
<td>65</td>
</tr>
<tr>
<td>PPTH(_2)</td>
<td>0.905 ± 0.016 mmol</td>
<td>157</td>
<td>74</td>
<td>188</td>
</tr>
<tr>
<td>PPTme</td>
<td>0.111 ± 0.005 mmol</td>
<td>58</td>
<td>26</td>
<td>60</td>
</tr>
<tr>
<td>PPTBES(^c)</td>
<td>0.167 ± 0.006 mmol</td>
<td>46</td>
<td>-49</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) SD = standard deviation  
\(^b\) Data for the formate test was fitted to a zero-order type reaction rather than to a first-order reaction.  
\(^c\) Zero-order degradation rate and cumulative substrate degraded in PPTBES refer to H\(_2\) degradation.

### 7.4.2 Cell counts and in situ hybridization

The total estimated cell number (DAPI) in water samples from PS5 was 1.12 ± 0.09 \(\times\) 10\(^5\) cells ml\(^{-1}\) at the beginning of the PPT series (2001) and 0.79 ± 0.20 \(\times\) 10\(^5\) cells ml\(^{-1}\) at the end of the series (2002). Percentages of cells hybridizing with probe EUB338 and Arch915 in water samples were 7.8 ± 1.8% and 35 ± 4.3% of total (DAPI-stained) microorganisms at the beginning and 22.5 ± 3.7% and 18.2 ± 6.4% at the end of the PPT series, respectively (Fig. 7-3). In sediment samples, 13.8 ± 3.0% of total microorganisms hybridized with probe EUB338 and 9.0 ± 3.3% with probe Arch915. Hybridizations with the genera-specific probes showed that the species *Methanosaeta concilii* (probe Rotcll) accounted for 22.3, 8.4, and 5.0% of total microorganisms in water (2001), water (2002), and sediment samples, respectively. The more general probe MX825 for *Methanosaeta* spp. detected 14.3, 6.5, and 2.5% of total microorganisms in water (2001), water (2002), and sediment samples. All other methanogenic *Archaea* that we probed for were below the detection limit of 1% (Zarda et al., 1997). One exception was the family *Methanomicrobiaceae* (probe MG1200) that accounted for 1.4% of total microorganisms in water samples after the PPT series (Fig. 7-3).
Figure 7-3 Percentage of total (DAPI-stained) cells hybridizing with fluorescent probes Arch915 (Archaea), EUB338 (Bacteria), Rotcll (Methanosaeta concilii), MX825 (Methanosetaeae), and MG1200 (Methanomicrobiaceae) in water samples recovered in September 2001 and October 2002 (i.e., before and after the PPT series) and sediment samples recovered in October 2002. Error bars indicate one standard deviation. Percentages of total cells hybridizing with MB1174, MS1414, and Rotcl2 were below the detection limit (1% of total cells).

7.4.3 PCR-DGGE

DGGE of PCR products resulted in distinct profiles, which exhibited 19 bands for each water sample and 12 bands for the sediment sample (Fig. 7-4). Profiles of water samples collected before (2001) and after (2002) the PPT series were 75% similar, while the sediment sample was only ~48% similar to both water samples. Especially in the lower part of the gel, some bands were present in water samples but not in the sediment sample. All sequenced clones (Table 3) were related to methanogenic Archaea with one representative of the order Methanococcales (Methanocaldococcus), 4 of the Methanomicrobiales (Methanocorpusculum, Methanogenium, Methanospirillum), one of the Methanosarcinales (Methanosaeta), and 2 of the Methanobacteriales (Methanothermobacter). Unfortunately, band No. 1 (Fig. 7-4) did not reamplify after excision in repeated attempts.
7.5 Discussion

7.5.1 Push-pull tests

Lower relative substrate concentrations compared to relative Br⁻ concentrations throughout all PPTs (Fig. 7-1a - e) indicated that substrates were consumed during those tests, presumably due to microbial activity. Differences between recovered cumulative relative Br⁻ and substrate masses showed that 27% of injected acetate, 26% of formate, 52% of H₂ (PPTH₂) or 23% of H₂ (PPTBES), and 6% of methanol were degraded in the respective tests. This illustrates that total test durations (Tab. 7-1) were sufficiently long to allow detectable substrate consumption during the tests.

Degradation rates determined in our study (Table 2) are 1-2 orders of magnitude higher than those published by Hansen et al. (2001) who found maximal rates of 0.011 mM d⁻¹. However, rates in that study were determined for a non-contaminated site where methanogenesis may be expected to be slower. Furthermore, since we added the substrates in concentrations higher than the indigenous level, the rates we measured do not represent indigenous conditions. Rather than determining indigenous degradation rates, our goal was to use the substrates to test for the activity of different groups of methanogens. Nevertheless, our rates were similar as those determined by Istok et al. Istok et al. (1997) using PPTs who found rates of 1.92-4.80 mM d⁻¹ for H₂ consumption and 0.048 mM d⁻¹ for CH₄ production in another PHC-contaminated aquifer.
<table>
<thead>
<tr>
<th>Clone</th>
<th>Order of methanogens</th>
<th>Closest identified relative (Database Accession No.)</th>
<th>% Identity</th>
<th>Database Accession No.</th>
<th>Utilized substrates</th>
<th>Targeted by FISH probe used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Methanomicrobiales</td>
<td>Methanocorpusculum bavaricum (AF509457)</td>
<td><strong>98</strong> (395/399)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>AY294415</td>
<td>formate, H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>MG1200</td>
</tr>
<tr>
<td>3</td>
<td>Methanococcales</td>
<td>Methanocaldococcus indiensis (AF547621)</td>
<td><strong>85</strong> (152/177)</td>
<td>AY294408</td>
<td>H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Methanomicrobiales</td>
<td>Methanocorpusculum bavaricum (AF509457)</td>
<td><strong>99</strong> (396/399)</td>
<td>AY294409</td>
<td>formate, H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>MG1200</td>
</tr>
<tr>
<td>5</td>
<td>Methanosarcinales</td>
<td>Methanosaeta concilii (X51423)</td>
<td><strong>98</strong> (452/457)</td>
<td>AY294410</td>
<td>acetate</td>
<td>MX825, RotCl1</td>
</tr>
<tr>
<td>6</td>
<td>Methanomicrobiales</td>
<td>Methanogenium caraci (M59130)</td>
<td><strong>89</strong> (409/457)</td>
<td>AY294411</td>
<td>formate, H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>MG1200</td>
</tr>
<tr>
<td>7</td>
<td>Methanobacteriales</td>
<td>Methanothermobacter thermoautotrophicus (X68717)</td>
<td><strong>84</strong> (181/215)</td>
<td>AY294412</td>
<td>formate, H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Methanomicrobiales</td>
<td>Methanospirillum hungatei (M60880)</td>
<td><strong>96</strong> (440/458)</td>
<td>AY294413</td>
<td>formate, H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>MG1200, RotCl2</td>
</tr>
<tr>
<td>9</td>
<td>Methanobacteriales</td>
<td>Methanothermobacter thermoautotrophicus (X68717)</td>
<td><strong>84</strong> (181/215)</td>
<td>AY294414</td>
<td>formate, H&lt;sub&gt;2&lt;/sub&gt; + CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>number of correctly aligned base pairs / total number of base pairs in the GenBank query. Total sequence length was 456-458 bp.
Differences in substrate consumption rates between tests may be evoked by variations of groundwater temperature, geochemical conditions, contributions of processes other than methanogenesis to substrate degradation, and distinct activities of different physiological groups of methanogens. Geochemical conditions remained fairly stable among PPTs (not shown), hence, they probably do not explain much of the variations in rates. Other processes possibly contributing to substrate consumption during PPTs are O₂, SO₄²⁻, NO₃⁻, and Fe(III) reduction and acetogenesis. For example, average concentrations of 4.2 μmol L⁻¹ O₂, 14 μmol L⁻¹ SO₄²⁻, and 10 μmol L⁻¹ NO₃⁻ were at maximum used for the degradation of 2.1, 14, and 6 μmol L⁻¹ of acetate. This would correspond to a total amount of 14.7 mmol acetate in 600 L of extracted volume (after that the acetate concentration was zero in PPTac), i.e. only about 2.6 % of total acetate that was actually degraded in PPTac (557 mmol). Hence, the contribution of electron acceptors other than CO₂ to substrate degradation except of Fe(III) was negligible.

Iron(III)-reducing bacteria are likely able to consume all of the added substrates (Straub and Buchholz-Cleven, 2001) and methanogenesis and Fe(III) reduction are known to occur simultaneously (Bekins et al., 1999). However, only minor amounts of Fe(II) evolved or disappeared during PPTs. For example, the observed production of 18 mmol Fe(II) in PPTH₂ may have been due to the consumption of 9 mmol H₂, which is only a fraction of the total H₂ that was consumed during this test (Table 2). However, a large uncertainty is associated with the Fe(II) balances due to high concentrations of Fe(II) in background water of PS5 and possible precipitation reactions of Fe(II), e.g. with S(-II) or CO₃²⁻. If we consider an Fe(II) concentration of 3×10⁻⁸ M and a S²⁻ concentration of 1.14×10⁻¹³ M (calculated for pH 6.7 and the sum of S²⁻, HS⁻, and H₂S = 8 μM), the ion activity product of [Fe(II)][ S²⁻] is equal to 3.4×10⁻¹⁷ M² which is 43 times higher than the solubility product of FeS (7.94×10⁻¹⁹ M²) (Stumm and Morgan, 1981). If we consider a CO₃²⁻ concentration of 2.64×10⁻⁶ M (for pH 6.7 and the sum of H₂CO₃, HCO₃⁻, CO₃²⁻ = 1.47×10⁻² M), the ion activity product of [Fe(II)][ CO₃²⁻] is equal to 7.9×10⁻¹⁰ M² which is 20 times higher than the solubility product of FeCO₃ (3.98×10⁻¹¹ M²) (Stumm and Morgan, 1981). Hence, the groundwater was supersaturated with respect to FeS and FeCO₃ and therefore, precipitation was likely. Although for these reasons the contribution of Fe(III) reduction to substrate consumption during PPTs remains unknown, the observed CH₄ production in the first four tests (Tab. 7-2) indicates that methanogenic Archaea contributed to a significant extent to substrate consumption during PPTs. Hence,
much of the variability in substrate consumption rates may have been due to different activities of different physiologic groups of methanogens.

The increase of CH₄ concentrations during all PPT extraction phases (Fig. 7-2a) was due to admixture of native groundwater containing CH₄ and CH₄ production (Fig. 7-2b, Tab. 7-2). A significant portion of the substrates was converted into CH₄ (Tab. 7-2) and recently conducted PPTs with ¹³C-labelled acetate or CO₂, where the label was recovered in CH₄, confirm these results (unpublished data). Part of the substrates, especially acetate, may have been degraded by other processes, e.g. Fe(III) reduction. We have no explanation for the >100% recovery of H₂ in CH₄ in PPTH₂.

Similar relative concentrations of Ar as compared to Br⁻ in PPTBES (not shown) showed that gases moved conservatively in our PPTs. Thus, H₂ disappearance during PPTH₂ and PPTBES was most likely due to microbial degradation and not to volatilization or diffusion. Relative BES concentrations were almost identical to Br⁻ concentrations in PPTBES (not shown), indicating that BES moved conservatively. This ensured that its inhibitory action on methanogens was in effect throughout this test. Moreover, BES concentrations during PPTBES remained above 0.1 mM (lowest concentration: 0.32 mM), the suggested lower limit for inhibition (Oremland and Capone, 1988). Inhibition of the terminal methylation reaction in methanogenic Archaea by BES (Oremland and Capone, 1988) may thus explain that CH₄ was not produced in this test (Tab. 7-2). Hence, PPTBES validates our calculations on CH₄ production in the first four PPTs (Tab. 7-2). In spite of the inhibition of CH₄ production in PPTBES, lower relative concentrations of H₂ as compared to Br⁻ (Fig. 7-1e) suggested that H₂ was degraded in this test but at a lower rate than in PPTH₂ (Tab. 7-2). Therefore, in PPTH₂, a considerable amount of H₂ may have been consumed by methanogens, corroborated by significant CH₄ production in this test (Tab. 7-2).

The evolution of formate upon injection of H₂ in PPTH₂ and PPTBES may be explained by the presence of hydrogen lyases both within methanogenic Archaea (Wu et al., 1993) and other bacteria (Woods, 1936). In both PPTs, 24% of total degraded H₂ was transformed into formate. The activity of hydrogen lyases is not inhibited by BES (Wu et al., 1993).

### 7.5.2 Microbial population analyses

Archaea were abundant in water (18-38% of total microorganisms) and sediment (9%; Fig. 7-3). The presence of 19 DGGE bands in water samples and 12 bands in the sediment sample indicated a diverse archaeal population near well PS5 (Fig. 7-4). Diverse and abundant
archaeal populations have been found in PHC-contaminated environments before (Chapelle et al., 2002; Dojka et al., 1998; Watanabe et al., 2002). All of the retrieved sequences were related to methanogenic *Archaea* with representatives of almost all orders of methanogens (Tab. 7-3) (Madigan et al., 2003). The closest unidentified relatives of most of our clones were uncultured methanogenic *Archaea* retrieved from contaminated, anaerobic freshwater environments, e.g. municipal wastewater sludge, oil- or TCE-contaminated groundwater, and toluene- or hexadecane-degrading enrichment cultures (Ficker et al., 1999; Lenczewski et al., 2003; Watanabe et al., 2002; Zengler et al., 1999). For example, clone 5 (*Methanosaeata*) was 100% identical with an uncultured organism from oil-contaminated groundwater from the bottom of a crude oil storage cavity (Watanabe et al., 2002). This suggests that similar archaeal communities establish in such contaminated environments.

Higher detection rates with the *Methanosaeta concilii*-specific probe Rotcll than with the more general probe MX825 that should detect all *Methanosaeta* spp. may be due to different accessibilities of the probe binding sites on the 16S rRNA or to different binding properties of the two probes (Fuchs et al., 1998). Hence, we refrained from comparing the results of two different hybridization probes in this study. Our inability to detect methanogenic *Archaea* other than *Methanosaeta* using FISH probes may have been due to a ribosome content in these cells that was too low for detection with FISH. This method may underestimate the abundance of slowly growing microorganisms with low rRNA contents (Watanabe et al., 2000). Only members of the *Methanomicrobiaceae* as detected by sequencing (Tab. 7-3) were also visualized in one water sample using FISH (Fig. 7-3). Nevertheless, FISH results suggested that *Methanosaeta concilii* was a dominant member of the microbial community both in water and sediment samples (Fig. 7-3). This agrees with DNA related to this species (band 5, Tab. 7-3) constituting a dominant band in DGGE profiles (Fig. 7-4) and with earlier research at the same site (Bolliger et al., 2000).

The higher percentages of *Archaea* in water as compared to sediment samples (Fig. 7-3) agrees well with other studies where frequently a higher percentage of methanogens was found free-living as compared to attached (Bekins et al., 1999; Godsy et al., 1992; Lehman and O’Connell, 2002). The reason for this remains unknown and deserves further research. However, *archaeal* community composition was 48% similar in water and sediment (Fig. 7-4). High similarity of attached and suspended microorganisms was shown previously for a freshwater system in terms of functional attributes (Worm et al., 2001) or using a culture-based approach for another PHC-contaminated aquifer (Bekins et al., 1999). Nevertheless, slight differences in banding patterns of water and sediment samples (Fig. 7-4; e.g., bands 2,
suggested that *Methanospirillum* (clone 8) seemed to favor the attached way of life and methanogens related to *Methanocorpusculum* (clone 2) and *Methanothermobacter* (clones 7 and 9) life in suspension. Other researchers have also observed differences in attached and suspended microbial communities (Alfreider et al., 2002; Lehman et al., 2001). Functionally, at least with respect to carbon source consumption (Tab. 7-3), *Methanospirillum*, *Methanocorpusculum*, and *Methanothermobacter* are similar but why they occupy different ecological niches remains speculative.

The higher relative numbers of *Archaea* in water samples before (35%) than after the test period (18%) were accompanied with only subtle changes in DGGE profiles (Fig. 7-4a and c, e.g., bands 7 and 9) and the detection of *Methanomicrobiaceae* in the latter water samples (Fig. 7-3). Such changes of the microbial community may be due to fluctuating environmental conditions (Chapelle et al., 2002).

### 7.5.3 Comparison of PPT data with molecular analyses

Since *Methanoseta* are known to generate energy only through aceticlastic methanogenesis (Whitman et al., 1992), the abundance of *Methanoseta concilii* in groundwater and sediment near well PS5 (Figs. 3, 4, Tab. 7-3; Bolliger et al. (2000)) suggests that acetate is the main substrate for methanogenesis in this aquifer. However, acetate was degraded more slowly than H₂ and formate in PPTs (Tab. 7-2). In addition, acetate accumulation in PPTBES, as one would expect when acetate-consuming methanogens are inhibited (Fukui et al., 2000), did not occur. On the contrary, sequencing data indicated that the majority of retrieved sequences represented formate- and H₂ + CO₂-consuming *Archaea* (Tab. 7-3). This agrees well with the observed high H₂ and formate degradation rates. Even though methanol was consumed during PPTme, no sequences related to methanol-degrading *Archaea* were found.

### 7.5.4 Conclusions

For the first time (to our knowledge), we investigated both activity and diversity of methanogens in a PHC-contaminated aquifer using PPTs and molecular analyses. Methane was produced from all of the substrates added in PPTs, which indicated the presence of at least three physiologic types of methanogenic *Archaea*. This was corroborated by DGGE and sequencing data. Two of the community members were also identified by FISH (*Methanoseta concilii* and members of the *Methanomicrobiaceae*). High H₂- and formate
consumption rates observed in PPTs agreed well with the high diversity of formate- and H₂-consuming methanogens and the inhibition of CH₄ production from H₂ when BES was added as a specific inhibitor of methanogens in a separate PPT. Considerable acetate consumption in PPTac agreed well with the presence of *Methanoseta concilii* as detected by FISH and 16S rDNA sequencing. Based on the DGGE profiles we hypothesize that while attached and suspended archaeal communities in PHC-contaminated aquifers may be similar with respect to the dominant species, with respect to minor species they may differ.

The combination of hydrogeological and molecular methods in this study provided valuable information on the community structure and the activity of methanogens in a PHC-contaminated aquifer. One method by itself could not have provided the full picture. Future studies will focus on the role of Fe(III) reduction and the direct linkage between activity and identity of PHC-degrading microorganisms.

### 7.6 Acknowledgements

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### 7.7 References


Herzberg G. R. and Rogerson M. (1985) Use of alcohol oxidase to measure the methanol produced during the hydrolysis of D-methyl-3-hydroxybutyric and L-methyl-3-hydroxybutyric acid. *Analytical Biochemistry* 149(2), 354-357.


Discussion
8.1 Rates of biological processes in petroleum-contaminated aquifers

8.1.1 Zero-order and first-order processes

Enzymatically catalyzed microbial processes are usually described using the Michaelis-Menten equation (e.g., Schwarzenbach et al., 1993; Voet and Voet, 1995; Fig. 8-1) with the parameters $V_{\text{max}}$ (fastest possible substrate removal rate) and $K_M$ (the concentration of substrate at which removal is half the fastest possible). For very low substrate concentrations ($c \ll K_M$), a process may be described as first-order and if $c \gg K_M$, a zero-order process may be assumed, which is independent of concentration. Hence, at low substrate concentrations, the slope of the curve may be assumed as constant and represents the first-order rate coefficient $k$, and the first-order rate law

$$\frac{dc}{dt} = -kc$$  \hspace{1cm} (Equation 8-1)

is valid.

In order to calculate first-order rate coefficients based on the breakthrough curves of single-well push-pull tests (Haggerty et al., 1998), $\ln \left( \frac{C_r}{C_c} \right)$ ($C_r$ = concentration of reactant, and $C_c$ = concentration of conservative tracer) versus $t^*$ (time since injection ended) is plotted. If such a plot yields a straight line-relationship (Fig. 8-2a), first-order kinetics may be assumed and $k$, the slope of the straight line be calculated. However, if the reactant concentration is too high, reaction kinetics according to the Michaelis-Menten equation may approach a zero-order process and first-order rate calculations (Haggerty et al., 1998) become invalid (Snodgrass and Kitanidis, 1998). Such a situation was encountered in a PPT conducted to assess methanogenesis in well PS5 (Fig. 1-11) using 2 mM formate as substrate.
(chapter 7), where the plot of ln \((C_r / C_c)\) and \(t^*\) was not linear (Figure 8-2b). At high formate concentrations in the beginning of the test, the enzyme complex that transforms formate was saturated with the substrate, resulting in apparent zero-order kinetics and the slope \((k)\) approaching zero. At lower formate concentrations towards the end of the test (higher \(t^*\)), the reaction approached first-order kinetics and \(k\) became approximately constant.

In order to compare rates or rate coefficients generated in two different PPTs, one in which the reaction followed zero-order and the other first-order kinetics (Fig. 8-2a, b), basically two strategies may be pursued: On the one hand, a lower substrate concentration could be employed in a repeated test to be able to calculate a meaningful \(k\). However, this may not be feasible if the reaction is very fast and the analytical detection limit of the reactant is reached. On the other hand, a zero-order rate for the initial part of the test may be calculated (Snodgrass and Kitanidis, 1998). First-order rate coefficients from other PPTs may then be converted to zero-order rates by multiplication with the average reactant concentration during the test as was done in chapter 7.

In order to successfully carry out PPTs for the quantification of microbial processes in the subsurface, concentrations of reactants in the injection solution must be higher than their concentrations in native groundwater (Istok et al., 1997). However, if the process is first-order, \(k\) values determined in PPTs are also valid for in situ conditions because at low substrate concentrations \((c << K_M, \text{ Fig. 8-1})\), \(k\) is constant and therefore valid for a range of concentrations.
8.1.2 Linking rates of electron acceptor consumption with overall PHC mass reduction

Rate coefficients for electron acceptor consumption are linked to PHC degradation by the stoichiometric equations shown in Table 1-1. For example, if sulfate reduction rate coefficients as determined by PPTs (chapter 2) are translated into PHC degradation and a sulfate concentration of 0.3 mM (as present in uncontaminated groundwater in P20, Fig. 1-11) is assumed, ideally up to 1 ml of heating oil per day and per liter of groundwater may be degraded by sulfate reduction alone. However, in order to calculate PHC mass reduction for the whole plume, uncertainties such as dissolution of CaSO₄ along the flow path and spatial and temporal variability of electron acceptor concentrations must be taken into account (Bolliger et al., 1999; Schroth et al., 1998; Stumm and Morgan, 1981; Vroblesky and Chapelle, 1994). Furthermore, PPTs are point measurements and not necessarily representative for the whole plume due to spatial and temporal variability of microbial processes (Adrian et al., 1994; Chiang et al., 1989; Schroth et al., 1998; Smith et al., 1991). In addition, for the Studen aquifer, the exact contributions of other electron-accepting processes to PHC degradation (aerobic respiration, denitrification etc.) would have to be evaluated. Especially a quantification of Fe(III) reduction would be very hard to achieve (Wiedemeier et al., 1999).

An overall estimation of plume dynamics from actual degradation rates necessitates the employment of groundwater models designed to predict intrinsic bioremediation. The presently most commonly used models are BIOPLUME and BIOSCREEN (Khan and Husain, 2003). However, in order to determine when a remediation goal is reached, the exact amount of PHC in the subsurface must be known, which is not the case for the Studen aquifer (Bolliger et al., 1999).

8.1.3 Comparison of rates determined by push-pull tests with those generated by other field methods

A comparison of rates of microbial processes as determined by single-well push-pull tests with other field methods has not yet been done systematically for the same aquifer. Other field methods to quantify microbial activities in the subsurface include two-well natural or forced gradient tracer tests, single-well injection or withdrawal tests with multiple observation wells, electron acceptor and dissolved inorganic carbon balances, and evaluation of supply and consumption of electron acceptors across a cross-section of an aquifer (Chapelle, 2001; Chapelle et al., 1996; Domenico and Schwartz, 1990; Hunkeler et al., 1999;
Discussion

Hunkeler et al., 2002). While electron acceptor balances may be used to calculate the relative contribution of different electron-accepting processes to PHC degradation, they cannot be used to compute rates (Hunkeler et al., 1999). Two-well forced and natural gradient tracer tests have the major disadvantage that the temporal resolution is limited to the residence time of water between the two wells. Furthermore, calculation of rates of microbial processes based on electron acceptor concentrations along an aquifer cross-section (Hunkeler et al., 2002) is prone to errors since for example, Fe(III) reduction and methanogenesis are usually underestimated due to precipitation of Fe(II) as FeCO₃ or FeS (Stumm and Morgan, 1981) or CH₄ volatilization (Adrian et al., 1994; Bolliger et al., 1999). In addition, at the investigated field site in Studen, the installed wells were too far apart to calculate meaningful rates of electron acceptor consumption based on electron acceptor concentrations. For example, along the flowpath P20 (uncontaminated) to PS3 (first contaminated well along the center flowline) (Fig. 1-11) almost all NO₃⁻ and sometimes more than 90% of SO₄²⁻ was already consumed (Bolliger et al., 2000). Hence, rates calculated for this flowpath would underestimate in situ rates, and the low concentrations of these electron acceptors in PS3 and downgradient wells (Bolliger et al., 2000) would make such rate calculations questionable due to analytical uncertainty. Finally, the water package sampled in one well is never the same water package that is sampled in the downgradient well and hence, temporal changes of electron acceptor input are difficult to account for.

8.1.4 Spatially and temporally simultaneous occurrence of different microbial redox processes

Microbial redox processes in PHC-contaminated aquifers may vary on a centimeter, and even millimeter scale (Bekins et al., 1999). Hence, rates as determined by PPTs in monitoring wells, which typically extend several tens of centimeters to meters into the groundwater, are a measure of the average microbial activity in a certain volume of the aquifer. To increase the spatial resolution of PPTs and to probe a smaller aquifer volume, packers may be used or the injected volume be reduced. However, if several microbial processes occur simultaneously in the same aquifer volume (Bekins et al., 1999, Jakobsen and Postma, 1999) they cannot easily be distinguished even with a high spatial resolution. Such a case was encountered in this thesis in PPTs during which different substrates were injected to target SRB (chapter 4) or methanogens (chapter 7). In both PPT series, the substrates were also consumed by non-target organisms, e.g. methanogens and Fe(III) reducers because usually more substrate was
Table 8-1  “Specific” inhibitors for a range of microbial processes (adapted from Oremland and Capone (1988) unless marked with a *).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target process</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Bromoethanesulfonate (BES)</td>
<td>Methanogenesis</td>
<td>Very high</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Methanogens, homoacetogens</td>
<td>Limited</td>
</tr>
<tr>
<td>Fluoroacetate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Acetate metabolism</td>
<td>High</td>
</tr>
<tr>
<td>Methyl fluoride&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Aceticlastic methanogenesis</td>
<td>High</td>
</tr>
<tr>
<td>Molybdate</td>
<td>Sulfate reduction</td>
<td>High</td>
</tr>
<tr>
<td>Selenate</td>
<td>Sulfate reduction</td>
<td>Lower than molybdate</td>
</tr>
<tr>
<td>Tungstate</td>
<td>Sulfate reduction</td>
<td>Lower than selenate</td>
</tr>
<tr>
<td>Nitrapyrin</td>
<td>Nitrification</td>
<td>Limited</td>
</tr>
<tr>
<td>*DL-glyceraldehyde</td>
<td>Autotrophic CO2 fixation</td>
<td>Limited</td>
</tr>
<tr>
<td>14C-carboxypentitol bisphosphate</td>
<td>Autotrophic CO2 fixation</td>
<td>Unknown</td>
</tr>
<tr>
<td>DCMU</td>
<td>Photosynthesis</td>
<td>Limited</td>
</tr>
<tr>
<td>Various antibiotics</td>
<td>Fungi, bacteria, or eucarya</td>
<td>Limited</td>
</tr>
<tr>
<td>Metal-chelating agents, nitrapyrin, acetylene</td>
<td>Aerobic methane oxidation</td>
<td>Limited</td>
</tr>
<tr>
<td>Methyl fluoride&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Aerobic methane oxidation</td>
<td>High</td>
</tr>
<tr>
<td>*Thiosulfate</td>
<td>Sulfide oxidation</td>
<td>Unknown</td>
</tr>
<tr>
<td>*Platinum(II), p-chloromercuribenzoate</td>
<td>Fe(III) reduction</td>
<td>Unknown, not tested for environmental communities</td>
</tr>
</tbody>
</table>

<sup>a</sup> due to high toxicity (MDL Information Systems, 1984-2001; World Health Organization, 1975) not suitable for field experiments

consumed than SO<sub>4</sub><sup>2-</sup> reduction or methanogenesis could account for. However, in situ quantification of Fe(III) reduction is rather challenging (Wiedemeier et al., 1999). To quantify the contribution of Fe(III) reduction to substrate (H<sub>2</sub>) consumption in PPTs targeting methanogens we used a specific - almost ideal (Oremland and Capone, 1988) (Table 8-1) - inhibitor of methanogens, 2-bromoethanesulfonate (chapter 7). Similarly, acetylene has been used successfully in other PPTs as an inhibitor of denitrification by inhibition of the N<sub>2</sub>O reductase in denitrifying microorganisms, leading to accumulation of N<sub>2</sub>O (Schürmann et al.,...
A compilation of inhibitors for various microbial processes that could be used for the assessment of microbial activities, e.g. using PPTs, is shown in Table 8-1 (Oremland and Capone, 1988).

8.2 Methods to determine microbial community structure

8.2.1 Problems encountered using molecular methods

Due to methodological differences and various advantages and drawbacks of the molecular methods used in this study (Table 1-2), those methods shed light on microbial community structures from different angles and hence, may give different pictures of microbial populations. For example, while using DGGE and sequencing we cloned methanogenic *Archaea* of four different orders of methanogens, FISH only detected representatives of two orders (chapter 7). Hence, the use of two or more methods to characterize microbial communities is strongly recommended (Amann et al., 1995).

As a supplement to chapter 4, we attempted to sequence the dominant DGGE bands (Fig. 8-3) of DNA that had been amplified using two primer sets targeting Bacteria or SRB. Since many bands did not reamplify at all, only a selection of bands could be sequenced (Fig. 8-3). In addition to SRB, the SRB primer pair also amplified gram-positive organisms and non-sulfate reducers from within the δ-Proteobacteria (Tab. 8-2, clones 6-7, 9-10) (Manz et al., 1998). Hence, in order to investigate SRB communities by DGGE and sequencing it is recommended either to use primers that are specific for SRB subgroups (Daly et al., 2000; Devereux et al., 1992) or target genes involved in the sulfate reduction pathway, e.g. the dissimilatory sulfite reductase (Wagner et al., 1998) or the adenosine-5'-'phosphosulfate reductase gene (Deplancke et al., 2000), which are conserved genes among SRB. The sequence database for these genes is expanding and

Figure 8-3 DGGE profiles (duplicate profiles on the left: SRB primer, right: bacterial primer) showing numbered locations of bands that were excised and sequenced (Table 8-2).
hence, will be a more and more useful tool to assess SRB communities in environmental samples. Interestingly, both the more general bacterial primer pair and the SRB primer pair amplified a *Desulfovibrio* sp. (clones 1 and 8), indicating that this SRB may have been an abundant organism in groundwater from the vicinity of well PS3. This finding agrees with the FISH results (chapter 4). *Syntrophus* sp. (clones 6 and 7) is a known syntroph of SRB and methanogens (Dojka et al., 1998), and hence, its occurrence matches the other results. The current results suggest that the overall bacterial community may not be dominated by SRB, but by other bacteria, e. g. *Cytophaga* spp. (clones 2,3) and/or members of the Bacillus/Clostridium group (clones 4,5,9,10). All sequences were related to anaerobic microorganisms and many of them were related to contaminant-degrading bacteria.

This example for DGGE and sequencing shows that even this established and widely used method may be elaborate but the results discontenting. Nevertheless, the method yielded conclusive results when other (archaeal) primers were used on DNA extracted from the same aquifer (chapter 7).

**Table 8-2** Closest identified relatives of environmental clones of bacteria when a general bacterial and an SRB-specific primer pair for PCR of DNA extracted from groundwater of well PS3 (Fig. 1-11) was used. Clone numbers refer to band numbers in Fig. 8-3.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Phylogenetic group</th>
<th>Primer pair</th>
<th>Closest identified relative (Database Accession No.)</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>δ-Proteobacteria</td>
<td>bacteria</td>
<td>Desulfovibrio sp. (U85468)</td>
<td>87 (313/357)*a</td>
</tr>
<tr>
<td>2</td>
<td>Cytophaga-Flexibacter-Bacteroides</td>
<td>bacteria</td>
<td>Cytophaga sp. (AJ240979)</td>
<td>93 (354/379)</td>
</tr>
<tr>
<td>3</td>
<td>Cytophaga-Flexibacter-Bacteroides</td>
<td>bacteria</td>
<td>Cytophaga sp. (AF195441)</td>
<td>91 (241/263)</td>
</tr>
<tr>
<td>4</td>
<td>Bacillus/Clostridium group</td>
<td>bacteria</td>
<td>Propionispira arboris (Y18190)</td>
<td>86 (309/357)</td>
</tr>
<tr>
<td>5</td>
<td>Bacillus/Clostridium group</td>
<td>bacteria</td>
<td>Johnsonella ignava (X87152)</td>
<td>87 (218/248)</td>
</tr>
<tr>
<td>6</td>
<td>δ-Proteobacteria</td>
<td>SRB</td>
<td>Syntrophus sp. (AF126282)</td>
<td>96 (392/407)</td>
</tr>
<tr>
<td>7</td>
<td>δ-Proteobacteria</td>
<td>SRB</td>
<td>Syntrophus acidotrophicus (U86447)</td>
<td>93 (307/330)</td>
</tr>
<tr>
<td>8</td>
<td>δ-Proteobacteria</td>
<td>SRB</td>
<td>Desulfovibrio sp. (AF193026)</td>
<td>94 (384/407)</td>
</tr>
<tr>
<td>9</td>
<td>Bacillus/Clostridium group</td>
<td>SRB</td>
<td>Bacillus sp. (ABO43848)</td>
<td>82 (238/290)</td>
</tr>
<tr>
<td>10</td>
<td>Bacillus/Clostridium group</td>
<td>SRB</td>
<td>Bacillus sp. (ABO43848)</td>
<td>83 (258/308)</td>
</tr>
</tbody>
</table>

* *number of correctly aligned base pairs / total number of base pairs in the GenBank query*
8.2.2 Linking structure and function of microbial communities

Linking structure and function of microbial communities is important for understanding microbial community dynamics in natural environments (Boschker et al., 2001; Boschker et al., 1998; Pelz et al., 1999). However, many studies on subsurface microbial communities either focused on structures or functions (e.g., Dojka et al., 1998; Hansen et al., 2001; Watanabe et al., 2000) and only few studies investigated and linked both (Bolliger et al., 2000; Chapelle et al., 2002; Pombo et al., 2002; Watanabe et al., 2002). In this thesis, another, non-molecular method, the single-well push-pull test (Istok et al., 1997), was used to investigate microbial community structure and to provide a link between activity and community structure of SRB or methanogens. For this purpose, SRB or methanogen subgroup-specific substrates were injected in separate PPTs and the rates of microbial substrate consumption determined. These activity data were compared with the presence or abundance of subgroups as determined by molecular methods (FISH, DGGE and sequencing). Furthermore, activity and community structure of SRB was also investigated in a laboratory microcosm study. Activity and molecular data agreed well with each other in all three studies (Tabs. 8-3 – 8-5). For example, acetate-, lactate-, propionate-, and butyrate-consuming SRB were detected using FISH and all of these organic acids degraded in PPTs (Tab. 8-3) or acetate- and H2-consumption rates were high and acetate- and H2-consuming methanogens abundant (Tab. 8-5). However, some disagreements were also observed. For example, while Desulfobacter-related PLFA were detected in microcosms amended with acetate, this genus was not detected using FISH (Tab. 8-4). Furthermore, while methanol was consumed in PPTs targeting methanol-consuming methanogens, methanol-consuming methanogens were not detected using molecular methods (Tab. 8-5). These results underline the importance to use at least two molecular methods when studying microbial community structure and to combine measurements of activities with investigations of microbial community structure.

The major drawback of using specific carbon sources or substrates to target subgroups of SRB or methanogens in PPTs method was that the “specific” substrates employed in the PPTs are consumed by several, sometimes overlapping subgroups of methanogens or SRB, and also other organisms such as Fe(III) reducers. Nevertheless, in combination with molecular methods such specific-substrate PPTs proved a useful tool to assess and link community structure and activity of microorganisms.
Table 8-3  Physiological and molecular analyses of sulfate-reducing bacteria in a field study in a PHC-contaminated aquifer (chapter 4)

<table>
<thead>
<tr>
<th>targeted SRB groups / genera</th>
<th>physiological analysis demonstrated activity</th>
<th>molecular analyses</th>
<th>FISH</th>
<th>DGGE and sequencing (preliminary results, no. of clones)</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfobacter</td>
<td>0.24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfotomaculum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfurhabdus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfovibrio</td>
<td>0.32</td>
<td>2.6 - 7.6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Desulfobulbus</td>
<td></td>
<td>2.6 - 8.0</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Desulfotomaculum</td>
<td>n. a.</td>
<td>n. a.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>some</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfobacteriaceae</td>
<td>encompassed in general probe (11 - 24%)³</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>propionate degrading SRB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfobulbus</td>
<td>0.29</td>
<td>2.6 - 8</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>some</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfobacteriaceae</td>
<td>encompassed in general probe (11 - 24%)⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>butyrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfotomaculum</td>
<td>0.19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>some</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfobacteriaceae</td>
<td>encompassed in general probe (11 - 24%)⁴</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n. a. = not analyzed
³ general probe = SRB385 + SRB385-Db

Table 8-4  Physiological and molecular analyses of sulfate-reducing bacteria in a microcosm study using sediment from a PHC-contaminated aquifer (chapter 5)

<table>
<thead>
<tr>
<th>targeted SRB groups / genera</th>
<th>physiological analysis demonstrated activity</th>
<th>molecular analyses</th>
<th>FISH</th>
<th>PLFA analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfobacter</td>
<td>1.19</td>
<td>-</td>
<td>x</td>
<td>no specific biomarker</td>
</tr>
<tr>
<td>Desulfotomaculum</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfurhabdus</td>
<td>encompassed in general probe (15.3%)⁵</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lactate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfovibrio</td>
<td>1.8</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Desulfobulbus</td>
<td></td>
<td>5.4</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Desulfotomaculum</td>
<td></td>
<td>-</td>
<td>no specific biomarker</td>
<td></td>
</tr>
<tr>
<td>some</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfobacteriaceae</td>
<td>encompassed in general probe (20.2%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 8-4 (continued)

<table>
<thead>
<tr>
<th>substrate</th>
<th>propionate-degrading SRB</th>
<th>butyrate-degrading SRB</th>
<th>citrate-degrading SRB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Desulfobulbus</td>
<td>Desulfotomaculum</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Desulfobacteriaceae</td>
<td>Desulfobacteriaceae</td>
<td>Desulfobacteriaceae</td>
</tr>
<tr>
<td></td>
<td>some</td>
<td>some</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>encompassed in general</td>
<td>encompassed in general</td>
<td>Desulfobacter-</td>
</tr>
<tr>
<td></td>
<td>probe (17.6%)</td>
<td>probe (17.8%)</td>
<td>related PLFA</td>
</tr>
<tr>
<td>propionate</td>
<td>1.66</td>
<td>1.8</td>
<td>1.29</td>
</tr>
</tbody>
</table>

*general probe = SRB385-Db

Table 8-5  Physiological and molecular analyses of methanogens in a field study in a PHC-contaminated aquifer (chapter 7)

<table>
<thead>
<tr>
<th>targeted methanogens</th>
<th>physiological analysis</th>
<th>molecular analyses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>demonstrated activity</td>
<td>FISH (%)</td>
</tr>
<tr>
<td></td>
<td>rate of substrate deg-</td>
<td>(total (DAPI-</td>
</tr>
<tr>
<td></td>
<td>radation (mmol d⁻¹)</td>
<td>stained) microor-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ganisms) no. of</td>
</tr>
<tr>
<td></td>
<td></td>
<td>clones</td>
</tr>
<tr>
<td>H₂ + CO₂ hydrogen-consuming methanogens</td>
<td>0.91</td>
<td>-</td>
</tr>
<tr>
<td>Methanobacteriales</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methanococcales</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methanomicrobiales</td>
<td>0.0 - 1.4</td>
<td>4</td>
</tr>
<tr>
<td>Methanosarcina</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>formate-consuming</td>
<td>1.86</td>
<td>-</td>
</tr>
<tr>
<td>methanogens</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Methanobacteriales</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methanococcales</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methanomicrobiales</td>
<td>0.0 - 1.4</td>
<td>4</td>
</tr>
<tr>
<td>acetate-consuming</td>
<td>0.38</td>
<td>-</td>
</tr>
<tr>
<td>methanogens</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Methanosaeta</td>
<td>5 - 22</td>
<td>1</td>
</tr>
<tr>
<td>Methanosarcina</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>methanol-consuming</td>
<td>0.11</td>
<td>-</td>
</tr>
<tr>
<td>methanogens</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Methanosphaera</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methanosarcinales</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Methanosaeta</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>


8.3 Limits of stable isotope methods for determining rate coefficients in the field

The equation developed by (Aggarwal et al., 1997),
\[
\delta_t - \delta_0 = -\varepsilon k (t - t_0) \tag{Equation 8-2},
\]
which relates the first-order rate coefficient \(k\) to the enrichment factor \(\varepsilon\) of a given reaction, may be applied to the example given by Strebel et al. (1990) for an anaerobic, sulfate-reducing aquifer. Using their \(\varepsilon\) value of 9.7‰, half-lives for sulfate reduction (67-88 years) are similar to those determined in their study with the first-order rate law (75-100 years) (Strebel et al., 1990). Hence, if \(\varepsilon\) is known and constant, equation 1-4 may be useful to quantify sulfate reduction. Aggarwal et al. (1997) proposed to use site-specific microcosm experiments to determine \(\varepsilon\) values; however, field conditions are not easily reproducible in the laboratory. Instead, push-pull tests may be used for this purpose since conditions during PPTs are more field-relevant. Nevertheless, if \(\varepsilon\) values are spatially variable throughout a site, e.g. due to substrate heterogeneity (Harrison and Thode, 1958; Kaplan and Rittenberg, 1964) or strongly varying sulfate concentrations (Habicht et al., 2002), deriving rate coefficients from equation 1-4 for the site would become questionable.

8.4 Parameters governing the extent of sulfur isotope fractionation

8.4.1 General considerations

The extent of sulfur isotope fractionation has been shown to depend on a range of parameters (Canfield, 2001a). These parameters were investigated more closely with the help of the available literature data (Bolliger et al., 2001; Böttcher et al., 1999; Brüchert et al., 2001; Canfield, 2001b; Canfield et al., 2000; Chambers et al., 1975; Detmers et al., 2001; Habicht and Canfield, 1996; Habicht and Canfield, 1997; Habicht et al., 2002; Harrison and Thode, 1958; Kaplan and Rittenberg, 1964; Kemp and Thode, 1968) and the data presented in chapter 6. The most important effects on \(\varepsilon\) will be discussed, which were complete vs. incomplete degradation, sulfate reduction rate, cell-specific sulfate reduction rate, sulfate and carbon source concentration, and free energy changes. It must be noted that not all of these parameters were available for all \(\varepsilon\) values, hence, Fig. 8-4 (a-e) does not in all cases present the same data sets. This unfortunately also made application of multivariate statistics to evaluate the parameters with respect to their influence on \(\varepsilon\) impossible.
Figure 8-4 Influence of (a) sulfate reduction rate (SRR), (b) log of the cell-specific sulfate reduction rate (sSRR; mol cell\(^{-1}\) d\(^{-1}\)), (c) log of the sulfate concentration \(c(SO_4^{2-})\), mM, (d) actual free-energy yield \(\Delta G_f\), kJ mol\(^{-1}\) \(SO_4^{2-}\), and (e) log of the carbon source concentration (mM), on the extent of sulfur isotope fractionation (enrichment factor \(\varepsilon\)). For some parameters the log is used for better visualization.
All plots distinguish between complete and incomplete carbon source degradation, which was found to be one of the main factors influencing $\varepsilon$ (Brüchert et al., 2001; Detmers et al., 2001). When plotting temperature versus $\varepsilon$, no overall relationship was found (not shown). However, for separate data sets, an influence of temperature on $\varepsilon$ had been observed and was explained in terms of the influence of temperature on the cell-specific sulfate reduction rate (Harrison and Thode, 1958; Kaplan and Rittenberg, 1964). To this point, the Nernst equation and hence, actual free energy changes ($\Delta G_f$), may be regarded as the “unifying” concept, which explains most of the factors that influence $\varepsilon$, i.e. standard free energy changes, temperature, substrate concentration, and cell-specific sulfate reduction rate, but not low sulfate concentrations and bulk sulfate reduction rates.

### 8.4.2 Sulfate reduction rate

The plot of sulfate reduction rate (SRR) versus $\varepsilon$ (Fig. 8-4 a) shows that at very high rates $\varepsilon$ is less variable, $\varepsilon$ values converge, and possibly a “boundary value” of ~22%o exists (Habicht and Canfield, 1997). The highest SRR values in Fig. 8-4 a were found in a system where sulfate was not limiting (Habicht and Canfield, 1997). Sulfate reduction is a multi-step process (Kemp and Thode, 1968) and the magnitude of sulfur isotope fractionation depends on which step becomes rate-limiting. At high cell-specific sulfate reduction rates and if sulfate is not limiting, the rate-limiting step may be the reduction of sulfate to sulfite (Rees, 1973). As an inorganic chemical reaction this process was associated with a fractionation of ~22%, which agrees with the observed “boundary value”. When $\varepsilon$ was plotted against log(SRR), no apparent relationship existed (not shown).

### 8.4.3 Cell-specific sulfate reduction rate

If $\varepsilon$ is plotted against the log of the cell-specific sulfate reduction rate (sSRR), a negative relationship for incomplete sulfate reducers exists whereas for complete degraders, no relationship is visible (Fig. 8-4 b). In Fig. 8-4 b, values determined for psychrophilic strains and strains grown at suboptimal temperature are omitted since they showed abnormally low fractionations due to changes in cell membranes or in the activities of the enzymes at low temperatures (Kemp and Thode, 1968). At low cell-specific sulfate reduction rates, the reduction of sulfite to sulfide, i.e. the last step in sulfate reduction, becomes rate-limiting and the overall fractionation will be the sum of the individual isotope fractionations of all sulfate reduction steps, in theory allowing for fractionations up to 50%o (Canfield, 2001a; Rees,
1973). At high specific rates, sulfate may become limiting so that the uptake of sulfate into
the cell, which is associated with a minimal isotope fractionation (~3%), becomes rate-
limiting (Habicht and Canfield, 1997; Rees, 1973; Thode, 1991).

8.4.4 Sulfate concentration

A plot of $\varepsilon$ versus the log of sulfate concentration (Fig. 8-4 c) clearly shows that at sulfate
concentrations below ~200 $\mu$M, fractionations approach zero, while at higher concentrations,
a large range of $\varepsilon$ values was observed. The situation in the cell at low sulfate concentrations
is similar as at high sulfate reduction rates: again the uptake of sulfate into the cell becomes
limiting, minimizing isotope fractionation (Thode, 1991) (Habicht et al., 2002; Rees, 1973).

8.4.5 Free energy changes

A plot of actual free energy changes ($\Delta G_f$, calculated by combining both reduction and
oxidation half reactions), calculated from standard free energy changes and actual
experimental conditions (temperature, reactant, and product concentrations) using the Nernst
equation (Schwarzenbach et al., 1993) over $\varepsilon$ shows a tendency towards higher fractionation
at higher values of $\Delta G_f$, i. e. smaller free energy yield (Fig. 8-4 d). A smaller energy yield
will cause a slow or inhibited transport of electrons to the sulfate-reducing enzymes, i. e. a
lower specific sulfate reduction rate, hence, isotope fractionation is expressed more strongly
(Brüchert et al., 2001). Complete degradation of carbon sources tends to yield less energy,
hence, $\varepsilon$ values for completely degrading strains tend to be higher than for incompletely
degrading strains (Fig. 8-4 d) (Brüchert et al., 2001; Detmers et al., 2001).

8.4.6 Carbon source concentration

A plot of $\varepsilon$ versus the log of the carbon source concentration shows that at low substrate
concentrations, fractionations tend to increase (Fig. 8-4 e). The situation in the cell at low
carbon source concentrations is similar as at low sulfate reduction rates: a lack of electron
donor leads to a slow or inhibited transport of electrons to the reducing enzymes, i. e. a
reduced energy yield and a lower cell-specific sulfate reduction rate (Brüchert et al., 2001),
hence, isotope fractionation is expressed more strongly (Harrison and Thode, 1958).
8.5 Microcosm versus field studies

8.5.1 Rate coefficients

In this thesis, first-order rate coefficients were determined for sulfate reduction in the field using PPTs and in laboratory microcosms, for which the inoculum was taken from the same site. Interestingly, $k$ values for both methods were within the same range ($0.043 - 0.32$ for PPTs and $0.053 - 0.398$ for microcosm experiments; see also Bolliger et al. (2001)). In another study, laboratory and field methods (natural gradient tracer test) conducted to determine toluene degradation rate coefficients also yielded similar results (Chapelle et al., 1996). Those authors recommended a combination of field and laboratory experiments since both are prone to numerous uncertainties. In fact, a great number of authors reported quite different rates for microbial processes measured in field or laboratory experiments (e.g., Chapelle and Lovley, 1990; Madsen, 1991; Stewart et al., 1993). However, PPTs have several advantages as compared to other field methods to determine rates of microbial activities (Istok et al., 1997), which may eliminate some of the uncertainties connected with other field methods.

8.5.2 Community structure of sulfate-reducing bacteria

The community structure of SRB was investigated using FISH and DGGE in a field study and using FISH and PLFA extraction in microcosm experiments. In the field study, we detected using FISH the SRB *Desulfobulbus*, *Desulfobacter*, and *Desulfovibrio* in water samples of the sulfate-reducing well PS3. In addition, by sequencing DGGE bands, the presence of *Desulfovibrio* was confirmed (Tab. 8-2). On the contrary, in microcosms only *Desulfobulbus* and members of the *Desulfbacteriaceae* were detected with FISH and PLFA analysis after selective stimulation with propionate or lactate and acetate, butyrate or citrate, respectively. Although there was some overlap, the differences of SRB community structures found in field and microcosm experiments may be due to the different sampling wells (PS3 for the field; S6 for the microcosm study), the different kinds of samples (field: water samples; microcosms: sediment samples), and the selective culture conditions in microcosms (defined medium, different temperature, carbon source additions). Nevertheless, the goal of the microcosm study was to investigate the SRB populations that could be selectively stimulated with different carbon sources. But in order to characterize indigenous microbial
communities, direct examination of field samples is most suitable (Pombo et al., 2002; Shi et al., 1999).

8.5.3 Sulfur isotope fractionation

The magnitude of sulfur isotope fractionation (enrichment factor $\varepsilon$) was surprisingly similar in field and microcosm experiments in this thesis (Tab. 8-6). In the microcosm study, however, we observed that enrichment cultures grown on PHC components tended to show higher $\varepsilon$ values ($29.4\%$) than on organic acids ($18.1\%$), which was explained by the different energy yields for those compound groups. Since field $\varepsilon$ values were similar to the overall average $\varepsilon$ values for the microcosm experiments (Tab. 8-6), SRB in the field may be using both fermentation products and PHC as carbon sources. Furthermore, enrichment cultures grown on heating oil showed higher $\varepsilon$ values ($28.1\%$) than in field experiments where heating oil served as carbon source ($21.5\%$; Schroth et al., 2001). This discrepancy may have been due to different conditions in field and microcosm experiments, e.g. temperature, the possible availability of other substances (natural organic compounds) in the field, and the selectivity of the enrichment medium.

<table>
<thead>
<tr>
<th>System</th>
<th>Study</th>
<th>$\varepsilon$ [‰]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microcosms</td>
<td>chapter 6</td>
<td>23.0 ± 6.5</td>
</tr>
<tr>
<td>Microcosms</td>
<td>Bolliger et al. (2001)</td>
<td>23.5 ± 4.3</td>
</tr>
<tr>
<td>Field</td>
<td>chapter 2</td>
<td>21.5 ± 1.8</td>
</tr>
<tr>
<td>Field</td>
<td>chapter 4</td>
<td>20.7 ± 3.9</td>
</tr>
</tbody>
</table>

8.6 Questions and approaches for future research

8.6.1 Quantification of Fe(III) and Mn(IV) reduction

Because quantification of Fe(III) and Mn(IV) reduction based on dissolved Fe(II) or Mn(II) concentrations may be obscured due to precipitation processes (Stumm and Morgan, 1981) and a determination of Fe(III) or Mn(IV) available to microorganisms is difficult (Wiedemeier et al., 1999), a high-priority research need is the quantification of Fe(III) and Mn(IV) reduction in anaerobic aquifers and the evaluation of the contribution of these processes to anaerobic PHC degradation. For this purpose, the Fe(III) or Mn(IV) available to microorganisms has to be quantified, e.g. using a biosensor organism (Loper and Henkels, 1997). Inhibitors of assimilatory Fe(III) reduction (Table 8-1) may also inhibit dissimilatory
Fe(III) reduction and hence, be useful for studying the latter process. However, before applications in the field are attempted, these compounds would have to be first tested in laboratory studies.

8.6.2 Evaluation of methods to determine rates of microbial activities

A systematic evaluation of a range of laboratory and especially field methods to quantify microbial processes in the subsurface including push-pull tests has not been done but may be useful to further evaluate the accuracy of these methods and to assess the reliability of rates determined using various methods.

8.6.3 Structure and function of microbial communities in field and laboratory experiments

Several new methods have been developed recently that link the activity of microorganisms in the environment to their identity and that could potentially be used directly in the field using push-pull tests. For example, a technique was developed to selectively isolate DNA from microorganisms incorporating bromodeoxyuridine (BrdU), a thymidine analog (Urbach et al., 1999). The major drawback of this method, however, is that not all organisms are able to incorporate BrdU. Furthermore, $^{13}$C-labeled carbon sources may be injected in PPTs and instead of phospholipid fatty acids (Boschker and Middelburg, 2002; Pombo et al., 2002), nucleic acids may be extracted and labeled and unlabeled DNA or RNA separated using equilibrium density gradient centrifugation (Manefield et al., 2002; Padmanabhan et al., 2003).

Using molecular techniques, the microbial community structure and active microorganisms in sulfate-reducing microcosms growing on different PHC compounds (chapter 6) may be assessed in order to increase our knowledge on microbial activities and communities, and hence, intrinsic bioremediation processes in PHC-contaminated aquifers.

Furthermore, suspended and attached microbial populations may be investigated at a high spatial resolution using molecular techniques and activity measurements with water and sediment from the same aquifer volume in order to evaluate the different functions and contributions to PHC degradation of microorganisms in these different habitats.
8.6.4 Sulfur isotope fractionation

In order to better understand the parameters that influence the extent of sulfur isotope fractionation the next reasonable step will be mechanistic studies with cell-free extracts and pure enzymes. Furthermore, the influence of excretion and cell-internal cycling of intermediates of sulfate reduction, e.g., sulfite and thiosulfate, may contribute to our understanding of sulfur isotope fractionation (V. Brüchert, personal communication).

8.6.5 Overall PHC mass reduction

Rates determined by push-pull tests in combination with electron acceptor and dissolved inorganic carbon balances, other monitoring tools, and aquifer modeling approaches may be a useful tool to calculate scenarios of overall PHC mass reduction in a PHC-contaminated aquifer. Such a multilevel approach may assist in estimating the time needed for bioremediation of specific contaminated sites undergoing monitored natural attenuation.

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