Bioremediation of a heating oil-contaminated aquifer
quantification of processes by chemical, biological, and stable isotope analyses

Doctoral Thesis

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Bioremediation of a Heating Oil-Contaminated Aquifer - Quantification of Processes by Chemical, Biological, and Stable Isotope Analyses

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
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presented by

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Zürich 2000
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The accidental release of petroleum hydrocarbons (PHC) such as gasoline, diesel, and heating oil is a common source of ground water contamination. Since dissolved PHC concentrations in ground water downgradient of a contamination (i.e. PHC plume) are often above drinking water standards, remediation actions have to be taken. Among numerous remediation strategies, monitored natural attenuation (MNA) has found special interest since no excavation of the contaminated material is necessary. MNA relies on physical, chemical, and biological processes to reduce the mass, toxicity, mobility, volume or concentrations of contaminants in soil and ground water. Biological processes (intrinsic bioremediation) are most favorable because they ideally lead to the degradation of PHC to harmless compounds (i.e. carbon dioxide, water, and biomass). The main aim of this thesis was to develop and apply methods to assess and characterize processes involved in intrinsic bioremediation.

Intrinsic bioremediation was investigated at an aquifer in Studen, Switzerland which was contaminated with heating oil. After a partial removal of the PHC by physical means (i.e. pumping and excavation of contaminated material), the authorities decided to remediate the site by MNA. Indigenous microbial populations had developed that were capable of degrading heating oil and there was no immediate threat to drinking water wells downgradient.

Within the contaminated area the aquifer exhibited reduced conditions. Dissolved inorganic carbon (DIC) increased from 7.6 mM at an upgradient well to 13.9 mM in the contaminated area. The decrease of oxidants and the
increase of reduced species is a valuable sign of ongoing bioremediation. However, the DIC concentrations detected in the field were higher than the concentrations expected based on oxidants and reduced species. Stable carbon isotope analysis of PHC, aquifer matrix and DIC revealed that about 88% of the produced DIC originated from (non-methanogenic) PHC mineralization, indicating the effectiveness of intrinsic bioremediation.

In order to further characterize intrinsic bioremediation, ground water was sampled and analyzed over a 14-month period. Chemical composition of the ground water along the center flow line did not change significantly over this time, as expected for a site with a stable contaminant plume. Spatially distinct stable carbon isotope ratios (δ¹³C) of DIC ranging from -16.5‰ to -4.4‰ were found, indicating the presence of different ongoing microbial processes. Microbial populations in the ground water were detected by fluorescent staining (DAPI) and by in situ hybridization with rRNA-specific probes. Microorganisms belonging to Archaea and Bacteria were found in ground water within the contaminated area. The archaeal population at the Studen site consisted almost exclusively of organisms related to the species *Methanosaeta concilii*, an organism known to generate energy only by aceticlastic methanogenesis. In combination with the stable isotope and ground water chemical data, these results support the hypothesis that the terminal PHC-degradation step in contaminated aquifers is preferentially aceticlastic methanogenesis and not CO₂ reduction.

Sulfate reduction is an important process at various PHC-contaminated field sites. However, little is known about the role of sulfate-reducing bacteria in PHC-contaminated aquifers. A convenient tool to study the activity of these bacteria in aquifers is the stable sulfur isotope ratio (δ³⁴S). During microbial sulfate reduction δ³⁴S is altered i.e. stable sulfur isotopes are fractionated. The extent of fractionation depends on various environmental conditions (e.g. temperature, sulfate concentration, carbon source) and therefore simple isotopic balances as shown with DIC and δ¹³C are not applicable. Batch experiments were performed with different sulfate-reducing bacteria and toluene as the sole carbon source in order to better understand δ³⁴S fractionation in PHC-contaminated aquifers. The observed
fractionations (20‰ to 47‰) were in the same range as was reported for other strains grown on different carbon sources.

In this study it was demonstrated that the combination of geochemical, biological and stable isotope analyses, contribute to a better assessment and characterization of important microbial degradation processes in PHC-contaminated aquifers remediated by MNA.
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Zusammenfassung


Die Untersuchungen des mikrobiellen Abbaus von MKW erfolgten in einem Grundwasserleiter in der Gemeinde Studen (Kanton Bern), welcher mit Heizöl kontaminiert war. Ein Teil der Verschmutzung konnte durch das Abtragen des kontaminierten Erdreiches sowie mit Grundwasserpumpen und Öladscheidern entfernt werden. Da einerseits die im Untergrund
vorhandene mikrobielle Population fähig war, das Heizöl abzubauen und andererseits keine unmittelbare Gefährdung für die Öffentlichkeit bestand, beschlossen die verantwortlichen Behörden, diesen Standort mittels MNA zu sanieren.

Im Grundwasser innerhalb der kontaminierten Zone herrschen auf Grund der ablaufenden mikrobiellen Prozesse reduzierende Verhältnisse. Die Konzentration des gelösten anorganischen Kohlenstoff (Dissolved Inorganic Carbon, DIC) nimmt dabei um ~ 6.3 mM zu. Die Abnahme der Elektronenakzeptoren und die Zunahme der reduzierten Spezies im Grundwasser innerhalb der Kontamination sind wertvolle Indikatoren für die ablaufenden biologischen Prozesse. Bilanzen, welche auf Grund dieser chemischen Parameter erstellt wurden, unterschätzten aber die effektiv produziert Menge DIC. Mit der Analyse der stabilen Kohlenstoffisotopen-Verhältnisse ($\delta^{13}$C; entspricht $^{13}$C/$^{12}$C Verhältnis) von MKW, Untergrundmatrix und DIC konnte bewiesen werden, dass ungefähr 88% des gebildeten Kohlenstoffes aus dem mikrobiellen Abbau von MKW stammen.

methanogenen Bedingungen vorzugsweise die Disproportionierung von Acetat und nicht die Reduktion von Kohlenstoffdioxid ist.

In verschiedenen MKW-kontaminierten Grundwasserleitern ist die Sulfatreduktion ein wichtiger anaerober Metabolismus. Trotz dieser Tatsache weiß man bis heute wenig über die Rolle von sulfatreduzierenden Bakterien in MKW-verschmutzten Grundwässern. Ein geeignetes Instrument für die In situ-Untersuchung dieser Mikroorganismen sind stabile Schwefelisotope. Während der mikrobiellen Sulfatreduktion erfährt das stabile Schwefelisotopen-Verhältnis ($\delta^{34}$S; entspricht $^{34}$S/$^{32}$S Verhältnis) eine Veränderung (Fraktionierung). Die Größe der Fraktionierung hängt von verschiedenen Umweltbedingungen ab, zum Beispiel von Temperatur, Sulfatkonzentration und Art der Kohlenstoffquelle. Aus diesem Grunde kann keine einfache Isotopenbilanz analog zur DIC-Bilanzierung mittels $\delta^{13}$C erstellt werden. Laborexperimente mit verschiedenartigen sulfatreduzierenden Bakterien und Toluol als einziger Kohlenstoffquelle wurden durchgeführt, um die $\delta^{34}$S Fraktionierung unter bestimmten Umweltbedingungen besser abschätzen zu können. Früher beschriebene sulfatreduzierende Kulturen, die in Gegenwart von einfachen physiologischen Kohlenstoffverbindungen inkubiert wurden, zeigten Fraktionierungen in demselben Bereich (20% bis 47%).

In dieser Studie wurde gezeigt, dass sich die biologischen Abbauprozesse in einem Grundwasserleiter gut charakterisieren und quantifizieren lassen, indem biologische und chemische Parameter mit $\delta^{13}$C- und $\delta^{34}$S-Werten verknüpft werden.
1.1 Significance of petroleum hydrocarbons

The 20th century can without doubt be named the century of petroleum hydrocarbons. Modern lifestyle and the world's economy depend to a large extent on the availability of the 'black gold' and its derivatives. Fuels derived from crude oil supply more than half of the world's total supply of energy (OECD, 1998). Gasoline, kerosene, and diesel are used as fuel for cars, tractors, trucks, aircrafts, and ships. Heating oil and natural gas are used to heat homes and commercial buildings as well as to generate electricity. Crude oil products are the basic materials used in manufacturing of synthetic fibers for clothing and in plastics, paints, fertilizers, insecticides, soaps, and synthetic rubber (Speight, 1998).

It is a common assumption that crude oil generation is associated with the deposition of organic detritus. Crude oil is assumed to be the product arising from microbial decay, physical and chemical transformation of plant and animal debris that was incorporated into sediments under various environmental conditions over extended periods of time (Tissot and Welte, 1984). However, questions remain regarding the details of transformation and mechanisms by which crude oil is expelled from the source sediment and accumulates in the reservoir rock (Speight, 1998).
The majority of crude oil reserves identified to date are located in few very large fields, known as giants. In fact, approximately three hundred of the largest oil fields contain almost 75% of the presently known available crude oil. Although most of the world’s nations produce at least minor amounts of oil, the primary areas of oil production are in the Persian Gulf, North and West Africa, the North Sea, and the Gulf of Mexico (Speight, 1998). In addition, of the ninety oil-producing nations five Middle Eastern countries possess almost 70% of the currently known oil reserves (Speight, 1998).

Figure 1-1: Refinery production worldwide. (Adapted from U.S. Department of Energy, 1994, International energy annual report (Burger, 1997))

The chemical composition of crude oil varies with the location of its origin (Testa and Winegardner, 1991). Crude oil is a mixture of many different compounds consisting almost exclusively of carbon (83-87%) and hydrogen (10-14%). Nitrogen (0.1-2%), oxygen (0.05-1.5%), sulfur (0.05-6%) and metals (Ni and V; < 1000 ppm) are also found in crude oil and are
important constituents, which influence the oil’s behavior during refining operations (Speight, 1998). Most hydrocarbon components of crude oil are lighter than water and due to their apolar nature largely immiscible with water (Eastcott et al., 1988).

Crude oil is refined by different chemical and physical processes, resulting in a large variety of products (Fig. 1-1). In 1999, approximately 74.56 million barrels (1 barrel = 158.8 liters) were consumed world wide per day (EIA, 2000).

1.2 Petroleum hydrocarbons as contaminants

Given the millions of barrels of oil that are shipped around the world and stored in various containment, it is inevitable that oil or oil products are spilled. Spills and leaks present an acute risk to humans, animals, and the environment (Burger, 1997). Big spills that occur during transportation of crude oil (e.g. the Exxon Valdez accident in Prince William Sound, Alaska 1989) receive worldwide attention and extensive media coverage (Swannell et al., 1996). However, many small spills happen during crude oil recovery, transport, and refining. They are often ignored by both the public and government agencies because their effects seem small and inconsequential. Nevertheless, every year about 400 million liters of oil are spilled (Burger, 1997).

This study focuses on spills of liquid petroleum hydrocarbon (PHC) products such as gasoline, diesel fuel, and heating oil. Together they account for about 70% of the oil products (Burger, 1997). They are among the substances that most frequently contaminate ground water. In 1993, the U.S. Environmental Protection Agency (EPA) reported 318’000 confirmed releases and expected another 100’000 confirmed releases over the next several years within the U.S. (EPA, 1996b). In 1997, Swiss authorities estimated the total number of contaminated sites to be between 3000 to 4000 (Aepli Elsenbeer et al., 1997). About 60% of these sites are PHC contaminated (Hofmann, 1995).
Movement and fate of PHC-compounds in the environment is controlled by physical-chemical properties of the compounds and the physical, chemical, and biological nature of the subsurface matrix, through which the compounds are migrating. Spilled PHC percolate through the pores of the unsaturated zone until they reach an impermeable layer or the ground water table. PHC float on the ground water table due to their lower density than water and spread typically in ground water flow direction (Fig. 1-2). If the ground water table is fluctuating, PHC are distributed also vertically in the so-called smear zone.

Figure 1-2: Conceptual model of a PHC-contaminated aquifer.
Some PHC-compounds are fairly water-soluble (e.g. benzene 1.79 g/l, toluene 0.579 g/l, ethylbenzene 0.135g/l, p-xylene 0.221 g/l (Eastcott et al., 1988)). These better water-soluble compounds dissolve more rapidly than other PHC-compounds wherever PHC comes into contact with ground water (Wiedemeier et al., 1999). This process leads to the formation of a contaminant plume of dissolved PHC-compounds in ground water flow direction (Fig. 1-2). The PHC-compounds with higher solubility, e.g., benzene, toluene, ethylbenzene, and xylene (BTEX) are harmful for the environment due to their toxicity (e.g., benzene is a known carcinogen) and therefore regulated by national legislation (Bundesrat, 1998; EPA, 1996a).

1.3 Remediation strategies for aquifers

Remediation of PHC-contamination historically focused on techniques such as excavation and disposal or treatment of contaminated material, or pumping of ground water and subsequent treatment to remove dissolved contaminants (pump and treat) (Bowlen and Kosson, 1995; Langwaldt and Puhakka, 2000). Nowadays, there are more remediation strategies applied to clean up or confine PHC-spills in the subsurface (for a recent comprehensive review see Riser-Roberts (1998)).

Generally, the remediation methods are subdivided into in situ methods and ex situ methods. During in situ remediation the contaminated subsurface material is treated while remaining in its natural location, whereas ex situ strategies require the excavation of the contaminated subsurface material. Ex situ treatments are further distinguished whether the treatment is performed directly at the site (on site treatments) or in a facility away from the site (off site treatments).

1.3.1 Ex situ remediation techniques

Ex situ techniques require the contaminated subsurface material to be excavated. An exception is the pump and treat method, where only the contaminated ground water is pumped to the surface and treated on site.
Because PHC-contaminated material has to be brought to the surface, handled, and transported, all ex situ methods entail worker exposure to PHC-vapors. Excavation may leave a clean site, but not necessarily clean subsurface material. The overall costs of an ex situ treatment including excavation, transport, and storage can be quite high (Dineen, 1991; Mackay and Cherry, 1989). In general, an ex situ treatment is more suitable for a confined, smaller site with a confined plume than for a large site with a more diffuse plume (Cole, 1994). Selected common ex situ treatments and their advantages and limitations are listed in Tab. 1-1.

**Table 1-1:** Selected ex situ techniques for PHC-contaminated aquifers. Hazardous landfill and incineration are used exclusively off site. Land farming and soil washing can be applied on and off site, whereas pump and treat techniques are only possible on site.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Procedure</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Hazardous landfill</strong></td>
<td>Excavated soil is deposited at a landfill</td>
<td>Excavation technically simple</td>
<td>Subsurface material is lost The problem is just transported</td>
</tr>
<tr>
<td><strong>Incineration (Burning)</strong></td>
<td>Excavated soil is burned in e.g. cement factories or special ovens</td>
<td>Contaminants are destroyed</td>
<td>Subsurface material is lost Not applicable for big volumes Extremely high costs</td>
</tr>
<tr>
<td><strong>Soil washing</strong></td>
<td>Sand and gravel fraction is separated and cleaned. Loam and humic fraction is burned or deposited</td>
<td>Contaminants are concentrated</td>
<td>Soil matrix is lost Not applicable for all types of soils and volumes</td>
</tr>
<tr>
<td><strong>Land farming</strong></td>
<td>Soil is amended with microorganisms and / or nutrients</td>
<td>Soil stays intact Reuse of soil possible</td>
<td>Large space required Regulatory limitations for the reuse of soil</td>
</tr>
<tr>
<td><strong>Pump and treat</strong></td>
<td>Ground water is pumped to the surface where contaminants are removed Clean ground water is reintroduced to the aquifer</td>
<td>Little disturbance of the aquifer</td>
<td>Facilities needed to treat large volumes of water Regulatory restriction for the reintroduction of treated water</td>
</tr>
</tbody>
</table>
Table 1-2: Selected in situ techniques for PHC contaminated aquifers. The methods are often used in combination and in variations under other treatment names.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Procedure</th>
<th>Advantage</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil Vapor Extraction (SVE)</td>
<td>Soil gas is removed by pumping along with the volatile contaminants</td>
<td>Reduced exposition of the environment to volatile contaminants</td>
<td>Only applicable at sites with volatile PHC (gasoline) and relatively low ground water level</td>
</tr>
<tr>
<td>Air sparging</td>
<td>Injection of air into the water saturated aquifer</td>
<td>Volatile PHC are purged from the ground water</td>
<td>Very local effect</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxygen is introduced to the ground water</td>
<td>To be used in conjunction with vacuum recovery system</td>
</tr>
<tr>
<td>Soil flushing</td>
<td>Mobilization of contaminants by addition of chemicals (e.g. surfactants) through an infiltration well Circulation via extraction well</td>
<td>Quick removal of contaminants without soil disturbance</td>
<td>Preferential flowpaths prevent even distribution Treatment of large volumes of contaminated water</td>
</tr>
<tr>
<td>Bioaugmentation</td>
<td>Addition of specially adapted microorganisms to the ground water</td>
<td>Addition of biomass</td>
<td>Has not been proven useful for contaminated ground water systems</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Introduction of lacking enzyme systems to aquifer biota</td>
<td></td>
</tr>
<tr>
<td>Engineered in situ bioremediation</td>
<td>Support of microorganisms by addition of e.g. O₂, NO₃⁻, P through an infiltration well Circulation via extraction well</td>
<td>Support of indigenous microorganisms</td>
<td>Preferential flowpaths prevent even distribution Limited O₂ solubility Use of NO₃⁻ limited by regulatory constraints</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Physical removal of contaminants by circulation (Pump and Treat)</td>
<td></td>
</tr>
<tr>
<td>Reactive walls ‘Funnel and gate’</td>
<td>Walls across ground water flow path containing material to clean the passing water</td>
<td>Relatively simple installation</td>
<td>Wells are subject to clogging</td>
</tr>
</tbody>
</table>
1.3.2 In situ remediation techniques

In situ treatment of contaminated sites offers an alternative to the traditional approach to site remediation involving excavation and redisposal. In situ techniques are often applied to sites were an excavation would be impossible, or where commercial operations have to stay in service. The main advantage of in situ techniques is the non-disturbing approach. In situ remediation strategies take advantage of either the physical properties of the contaminants and/or aim to support PHC-degrading microorganisms (Tab. 1-2). In situ methods are hampered by the length of time needed for the clean up. Remediation may take years accompanied by periodic monitoring. The prolonged duration of in situ remediation increases the costs for the maintenance (e.g. chemicals, equipment).

All ‘engineered’ clean up methods (in situ and ex situ) have in common that the public perception is usually good because ‘something is done’.

1.3.3 Monitored natural attenuation of petroleum hydrocarbons in aquifers

Natural attenuation refers to the observed reduction in contaminant concentrations as contaminants migrate from the source. In practice, natural attenuation also is referred to as intrinsic remediation, intrinsic bioremediation, natural restoration, or passive bioremediation. Natural attenuation is an in situ remediation strategy, which relies solely on natural occurring processes. The U.S. EPA defines monitored natural attenuation as follows (EPA, 1997):

The term monitored natural attenuation refers to the reliance on natural attenuation processes (within the context of a carefully controlled and monitored site cleanup approach) to achieve site-specific remedial objectives within a time frame that is reasonable compared to that offered by other more active methods. The ‘natural attenuation processes’ that are at work in such a remediation approach include a variety of physical, chemical, or biological processes that under favorable conditions, act without human intervention to reduce the mass, toxicity, mobility, volume, or con-
centration of contaminants in soil and groundwater. These in-situ processes include biodegradation; dispersion; dilution; sorption; volatilization; and chemical or biological stabilization, transformation, or destruction of contaminants.

In this thesis the term monitored natural attenuation (MNA) is used to describe all the processes that act to reduce the concentrations of contaminants in aquifers. The term intrinsic bioremediation is used to refer to the component of MNA brought about by biological degradation mechanisms.

1.3.4 Processes involved in monitored natural attenuation

1.3.4.1 Advection

Advective transport refers to the transport of solutes by the bulk movement of ground water. Advection is the most important process driving the downgradient migration of dissolved contaminants in the saturated subsurface.

1.3.4.2 Dispersion

Hydrodynamic dispersion is further divided into molecular diffusion and mechanical dispersion. Molecular diffusion can be ignored in areas with relatively high ground water velocities. The dominant process contributing to dispersion is mechanical dispersion. As defined by Domenico and Schwartz (1990), mechanical dispersion is mixing that occurs as a result of local variations in ground water velocity. Dispersion results in reduced contaminant concentrations and introduces contaminants into pristine portions of the aquifer.

1.3.4.3 Sorption

Sorption is the process by which dissolved contaminants partition from the ground water and adhere to particles comprising the aquifer matrix. Sorption of dissolved PHC results in retardation of the contaminant relative to the average ground water flow velocity and an apparent reduction in dissolved PHC concentration in ground water (Stuart et al., 1991).
1.3.4.4 Dilution (Recharge)

Water, e.g. precipitation percolating through the unsaturated zone dilutes the ground water. The additional water will also contribute to the dilution of the plume. The influx of electron-acceptor-charged water might alter geochemical processes, and in some cases facilitate additional biodegradation.

1.3.4.5 Volatilization

Contaminants are removed from ground water by volatilization and subsequent outgassing through the unsaturated zone. Different factors affect the volatilization of PHC, e.g. the concentration, the Henry’s law constant or the diffusion coefficient of the compound.

1.3.4.6 Intrinsic bioremediation

Indigenous soil microorganisms are capable of degrading PHC-constituents to CO₂ and water. Therefore intrinsic bioremediation is the most beneficial process of MNA because it is the only process that effectively reduces the mass of PHC (Fig. 1-3).

![Diagram](http://example.com/diagram.png)

**Figure 1-3:** Simplified scheme of microbial PHC mineralization.

It appears that PHC-degrading bacteria are ubiquitous in ground water systems (Chapelle, 1999). Direct counts of subsurface bacteria with fluorescence microscopy yielded an overall average of $10^6$ microorganisms per
gram dry soil, whereas numbers of free living bacteria in ground water have been reported to range from $10^4$ to $10^6$ cells/ml (review of Anderson and Lovley (1997). Nowadays, it is accepted that many PHC-compounds found in gasoline, diesel, or heating oil can be degraded under aerobic as well as anaerobic conditions (Heider et al., 1999). However, it is important to note that the contaminants have to be available for the microorganisms (i.e. dissolved in ground water) in order to allow optimal activities of the relevant microorganisms (Holliger and Zehnder, 1996).

Although aerobic biodegradation is considered to be the most efficient process to mineralize PHC (Atlas and Bartha, 1992), the natural rate of O$_2$ supply within the saturated zone is often not sufficient to meet the O$_2$ demand of aerobic microbial respiration. Therefore, PHC-contaminated aquifers usually become anoxic (Baedecker et al., 1993; Landmeyer et al., 1996; Vroblesky and Chapelle, 1994). Various field and laboratory studies have shown that the mineralization of PHC does not rely exclusively on O$_2$ as an oxidant but can also be coupled to the reduction of NO$_3^-$, Fe(III), SO$_4^{2-}$ and CO$_2$ (Fig. 1-3, for recent reviews see Anderson and Lovley (1997) and Heider et al. (1999)). The energy yield obtained by the microorganisms is different for each oxidant (Tab. 1-3).

**Table 1-3:** Redox potential ($E_h^o$) for selected reactions. The energy released during electron transfer decreases with decreasing $E_h^o$. $E_h^o$ in mV at pH=7 and T=25°C (Data adapted from Wiedemeier et al. (1999)).

<table>
<thead>
<tr>
<th>Reactions</th>
<th>$E_h^o$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$</td>
<td>+820</td>
</tr>
<tr>
<td>$2NO_3^- + 12H^+ + 10e^- \rightarrow N_2 + 6H_2O$</td>
<td>+740</td>
</tr>
<tr>
<td>$MnO_2(s) + HCO_3^- + 3H^+ + 2e^- \rightarrow MnCO_3(s) + 2H_2O$</td>
<td>+520</td>
</tr>
<tr>
<td>$FeOOH(s) + HCO_3^- + 2H^+ + e^- \rightarrow FeCO_3(s) + 2H_2O$</td>
<td>-50</td>
</tr>
<tr>
<td>$SO_4^{2-} + 9H^+ + 8e^- \rightarrow HS^- + 4H_2O$</td>
<td>-220</td>
</tr>
<tr>
<td>$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$</td>
<td>-240</td>
</tr>
</tbody>
</table>
Figure 1-4: Conceptualization of electron acceptor zones in the subsurface. $O_2$, $NO_3^-$, Fe(III), and $SO_4^{2-}$ stand for aerobic degradation, denitrification, iron (III) reduction and sulfate reduction, respectively.
Microorganisms always use the most favorable available oxidant to degrade PHC, i.e. the oxidant with the highest $E^0_h$. As oxidants are consumed microbial activity shifts to populations capable to use electron acceptors with lower redox potentials. This results in a succession of redox conditions (aerobic respiration → denitrification → manganese (IV) reduction → iron (III) reduction → sulfate reduction → methanogenesis) at field sites with bacterial consortia adapted to those specific redox conditions (Fig. 1-4).

![Diagram](image)

**Figure 1-5:** Relative importance of BTEX biodegradation mechanisms at 38 sites contaminated with PHC (adapted from Wiedemeier et al. (1999)).

Wiedemeier et al. (1999) collected data from 38 different PHC-contaminated sites and calculated the relative importance of BTEX biodegradation mechanisms (Fig. 1-5). Although this figure represents only a rough estimate of the relative importance of each mechanism, it is clear that anaerobic biodegradation mechanisms, and specifically sulfate reduction and
methanogenesis, are the most important biodegradation mechanisms at many PHC-contaminated sites.

### 1.3.5 Lines of evidence to verify intrinsic bioremediation & monitoring

Even though it is established that PHC-contaminated sites can undergo intrinsic bioremediation, a careful site evaluation and assessment is necessary to apply MNA as a remediation technique (Chapelle, 1999). Several lines of evidence are typically required to verify MNA (U.S. National Research Council, 1993):

1) Documented loss of contaminants from the site.
2) Laboratory assays showing that microorganisms in site samples have the potential to transform the contaminants under the expected site conditions.
3) Evidence showing that biodegradation is actually occurring in the field.

The first line of evidence involves using historical contaminant data to show the behavior of the contaminant plume i.e. expanding, stable, or shrinking plume. The second line of evidence is usually achieved with microcosms inoculated with site material in order to show that the indigenous microorganisms are capable of degrading site contaminants at a particular rate (Bregnard et al., 1996; Höhener et al., 1998). The third line of evidence might include documentation of, e.g., the depletion of electron acceptors ($O_2$, $NO_3^-$, $SO_4^{2-}$), the increase of reduced species ($Fe^{2+}$, $CH_4$) and dissolved inorganic carbon (DIC), or stable carbon isotope analysis of DIC (see Chapter 2).

The progress of natural attenuation has to be monitored periodically once the method is applied to a site. Monitoring typically includes measurement of data required for the third line of evidence. These data allow to assess and interpret changes in ground water composition over prolonged time. Furthermore, monitoring should include data on the behavior of the plume over time in order to be able to react to unexpected changes in ground water conditions such as, e.g., a change in ground water flow direction. In
case MNA fails to perform as anticipated, a contingency 'back up' remediation strategy should be available (EPA, 1997).

1.3.6 Advantages and limitations of monitored natural attenuation

MNA is, like all in situ methods, non-intrusive and can therefore be applied to sites where excavation is impossible or not a feasible option. Intrinsic bioremediation is the only process that actually leads to an effective loss of PHC-mass. The method is often less costly than other available remediation techniques and can easily be used in conjunction or as follow up to other methods (Cho et al., 1997; Wiedemeier et al., 1999).

However, there are also some limitations to MNA. The time frame to reach remediation goals is usually short for engineered remediation techniques whereas MNA requires more time. Because of the extended time needed, legal considerations have to be made in regard to responsibilities and monitoring. Even though MNA is not a 'walk away' method, public acceptance might be more difficult to achieve than for an engineered method. Further, MNA may be subject to natural and anthropogenic changes in local hydrogeologic conditions such as changes in ground water flow direction or chemical composition of the ground water. Therefore a contingency remediation strategy must be available. As for any method, aquifer heterogeneity often complicates site characterization.

1.4 Scope and outline of thesis

In this thesis a concept was developed and adapted for the demonstration of the efficiency of intrinsic bioremediation in PHC-contaminated aquifers. The concept includes chemical, biological, and stable isotope analyses. The combination of the obtained data allows to assess and characterize the relevant processes during intrinsic bioremediation. Studies were performed in batch experiments under controlled laboratory conditions and at a PHC-contaminated field site. The field site was located in Studen, Canton Bern,
where spilled heating oil contaminated the aquifer. In Chapters 2 to 4, the following research questions were addressed and discussed. Chapter 5 contains a more general discussion of various topics related to MNA.

**Chapter 2:** What is the hydrologic and hydrogeologic situation in Studen? Are ground water composition, alkalinity balances and dissolved organic carbon balances reliable tools to monitor intrinsic bioremediation? Can stable carbon isotopes be used to determine the efficiency of intrinsic bioremediation?

**Chapter 3:** Do chemical, biological, and stable carbon isotope data change over time? How can specific microbial processes be assessed and characterized in contaminated aquifers?

**Chapter 4:** Which are the factors that influence the fractionation of stable sulfur isotopes during sulfate reduction? Is the stable sulfur isotope fractionation an indicator for the rate of sulfate reduction at field sites?
Intrinsic bioremediation of a petroleum hydrocarbon-contaminated aquifer and assessment of mineralization based on stable carbon isotopes


Abstract

This study presents a stepwise concept to assess the in situ microbial mineralization of petroleum hydrocarbons (PHC) in aquifers. A new graphical method based on stable carbon isotope ratios ($\delta^{13}$C) was developed to verify the origin of dissolved inorganic carbon (DIC). The concept and the isotope method were applied to an aquifer in Studen, Switzerland, in which more than 34'000 liters of heating oil were accidentally released. Chemical analyses of ground water revealed that in this aquifer locally, anaerobic conditions prevailed, and that PHC mineralization was linked to the consumption of oxidants such as $O_2$, $NO_3^-$, and $SO_4^{2-}$ and the production of reduced species such as $Fe^{2+}$, $Mn^{2+}$, $H_2S$ and $CH_4$. However, alkalinity and DIC balances showed a quantitative disagreement in the link between oxidant consumption and DIC production, indicating that chemical data alone may not be a reliable assessment tool. $\delta^{13}$C ratios in DIC have been used before for bioremediation assessment, but results were reported to be negatively influenced by methanogenesis. Using the new graphical method to display $\delta^{13}$C
data, it was possible to identify anomalies found in methanogenic monitoring wells. It could be shown that 88% of the DIC produced in the contaminated aquifer originated from microbial PHC mineralization. Thus, the new graphical method to display $\delta^{13}C$ ratios appears to be a useful tool for the assessment of microbial hydrocarbon mineralization in a complex environment.

2.1 Introduction

Contamination of soil and ground water by the accidental release of petroleum hydrocarbons (PHC) is a common problem for drinking water supplies (U.S. National Research Council, 1993). At petroleum spill sites, PHC usually migrate vertically downward through the unsaturated zone due to the force of gravity and then laterally along the ground water table (Boulding, 1995). Capillary forces and ground water table fluctuations result in the vertical smearing of the PHC phase in the vicinity of the ground water table. The zone where PHC are found as a free phase is designated as the source area (ASTM, 1995). More soluble PHC components such as monoaromatic hydrocarbons are continuously released from the source area into the ground water, creating a contaminant plume within the aquifer (Bedient et al., 1994). Field evidence suggests that the extension of many plumes reaches a steady-state distribution due to natural attenuation (Eganhouse et al., 1993; Davis et al., 1994; McAllister & Chiang, 1994; Borden et al., 1995). Natural attenuation of PHC in soil and ground water may include a number of processes such as biodegradation by naturally-occurring microorganisms (intrinsic bioremediation), sorption, dispersion, advection, dissolution, and volatilization (Rifai et al., 1995). Biodegradation is the only process that leads to a reduction of the total mass of PHC, but to what extent it contributes to natural attenuation is often difficult to quantify.

Ideally, biodegradation results in complete mineralization of PHC, forming only carbon dioxide, water, and biomass. Although aerobic biodegradation is considered to be the most efficient process to mineralize PHC
(Atlas & Bartha, 1992), the natural rate of \( O_2 \) supply within the saturated zone is often not sufficient to meet the \( O_2 \) demand of aerobic microbial respiration. Therefore, PHC contaminated aquifers usually become anoxic (Baedecker et al., 1993; Bennett et al., 1993; Vroblesky & Chapelle, 1994; Landmeyer et al., 1996). Various field and laboratory studies have shown that the mineralization of PHC does not rely exclusively on \( O_2 \) as an oxidant but can also be coupled to the reduction of \( \text{NO}_3^- \), Mn(IV), Fe(III), \( \text{SO}_4^{2-} \) and \( \text{CO}_2 \) (for recent reviews see Holliger & Zehnder, 1996 and Krumholz et al., 1996).

The validation of intrinsic bioremediation as an effective remediation strategy requires the demonstration of mineralization of the contaminants in situ (Madsen et al., 1991; U.S. National Research Council, 1993; Hunkeler, 1997). Supporting evidence typically includes the decrease of contaminant concentrations, the formation of carbon dioxide, the consumption of oxidants, the formation of reduced species, and the formation of biomass. More recently, stable carbon isotopes ratios were used to monitor biodegradation of PHC (Van de Velde et al., 1995; Landmeyer et al., 1996; Aggarwal et al., 1997; Conrad et al., 1997). However, a reliable demonstration of mineralization should include several lines of evidence (Madsen et al., 1991; U.S. National Research Council, 1993; Hunkeler, 1998).

In this paper, we present a detailed concept to assess intrinsic bioremediation at a heating oil-contaminated site (Studen, Switzerland). The concept includes the following steps: 1) a natural gradient tracer experiment is performed to characterize the ground water transport at the site, 2) the ground water chemistry along the plume centerline is examined and the dominant microbial and geochemical processes occurring in the aquifer are identified, 3) DIC and alkalinity changes in the ground water are measured and subsequently compared with calculated values based on stoichiometric considerations, and finally 4) Stable carbon isotope ratios (\( \delta^{13}C \)) are used to identify the sources of the produced DIC. The balance of the stable carbon isotopes of the DIC is used to discriminate between DIC produced by mineralization and carbonate dissolution, respectively (Carothers & Kharaka, 1980; Boutton, 1991; Conrad et al., 1997). The results of our study demon-
strate that intrinsic bioremediation is a major process in the removal of PHC at this site.

2.2 Field Site Description

2.2.1 Site and remediation history

In an urban area in Studen, Switzerland, a spill of more than 34’000 liters of heating oil was discovered in 1993 (Fig. 2-1). Preliminary observations (unpublished data) indicated that an indigenous microbial population had developed following the spill, possibly degrading PHC. Due to these results and the fact that there was no immediate threat to drinking water wells downgradient, the cantonal water protection authorities decided to limit engineered remediation effort to the physical removal of the free PHC phase by pumping and to monitor natural attenuation processes.

Typical of many polluted sites, the Studen site contains inherent obstacles to the establishment of a quantitative assessment of intrinsic bioremediation. Primarily, it is not known exactly how much heating oil was spilled or when the spill occurred. Secondly, the site is located in an urban area, and part of the contaminated zone is inaccessible due to buildings and underground utilities. Finally, most monitoring wells were installed as part of an emergency physical remediation plan. Therefore, both the diameter of the wells and the length and depth of the screened portions vary. In accessible areas, small trenches were excavated down to the ground water table to localize the contamination. Before refilling the trenches with clean soil, 11 wells were installed using prefabricated concrete pipes (0.6 to 0.8 m diameter) which were screened in the lowest meter. These wells are designated S1 - S11 (Tab. 2-1). Between 1993 and 1996, about 34’000 liters of heating oil were recovered using floating pumps. After June 1996, no further engineered remediation actions were undertaken.
2.2 Field Site Description

Source area (estimated)

- Monitoring wells (bold characters: sampled on June 17, 1996)

- Water table on June 17, 1996 (m.a.s.l.)

- Leak discovered in 1993

- Buildings, above water table

- Roads

Figure 2-1: Location and map of source area and monitoring wells at the Studen site.
In January 1993 during the emergency assessment and later in May 1996, several monitoring wells were drilled in less accessible areas in order to estimate the size of the source area and of the plume (wells P1 - P17 and P20 - P28, respectively). The wells were equipped with either PVC piezometer tubes or steel tubes (P12 - P17). All tubes were screened in the saturated part of the aquifer. The zone where the wells contained heating oil in free phase in 1993 was assumed to represent the source area (Fig. 2-1 and Tab. 2-1). The source area remained stable after 1993. In May 1996, PVC piezometer tubes screened in their lower parts were placed into five of the concrete wells, and the void space was filled with clean gravel and topped with loam. These wells are designated PS (Tab. 2-1).

2.2.2 Hydrogeological characteristics

The field site (Fig. 2-1) is located at 435 m above sea level in the Berner Seeland, one of the major gravel aquifers in the Alpine Foreland Basin (Biaggi et al., 1994). The aquifer consists of unconsolidated glaciofluvial outwash deposits. Drilling core material at the site consisted of 3 - 8 m of interbedded layers of poorly sorted silt, sand and gravel. The screened sections of most of the wells are in zones of gravel or sandy gravel. During the drilling of the boreholes P10, P13, P15, P25 and P26 at the north eastern part of the site, a zone of silt was found at the ground water table that extends into the saturated zone by up to 2 m and is underlain by sandy gravel. Only the screens of P25 and P26 reach this sandy gravel zone. The unconfined aquifer is underlain at 20 - 25 m depth by an aquitard, which consists of molasse sediments (Biaggi et al., 1994).

Hydraulic conductivities were determined by small single well pumping tests and were between $1.0 \times 10^{-4}$ and $9.3 \times 10^{-3}$ m s$^{-1}$ (Merkt & Stebler, 1998). The porosity of the aquifer was assumed to correspond to that of a nearby similar geological formation, which was on average 0.19 (Jussel et al., 1994).
### Table 2-1: Overview of all monitoring wells.

<table>
<thead>
<tr>
<th>Observation well</th>
<th>Diameter</th>
<th>Free phase 1993</th>
<th>Free phase 1996</th>
<th>Ground water table a) m.a.s.l. c)</th>
<th>Bottom of well b) m.a.s.l. c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 -&gt; PS1 d)</td>
<td>60 - 11.5</td>
<td>-</td>
<td>-</td>
<td>433.20</td>
<td>432.49</td>
</tr>
<tr>
<td>S2</td>
<td>60</td>
<td>-</td>
<td>+</td>
<td>433.16</td>
<td>432.63</td>
</tr>
<tr>
<td>S3 -&gt; PS3 d)</td>
<td>80 - 11.5</td>
<td>+</td>
<td>+</td>
<td>433.16</td>
<td>432.65</td>
</tr>
<tr>
<td>S4 -&gt; PS4 d)</td>
<td>60 - 11.5</td>
<td>+</td>
<td>+</td>
<td>433.14</td>
<td>432.45</td>
</tr>
<tr>
<td>S5 -&gt; PS5 d)</td>
<td>80 - 11.5</td>
<td>+</td>
<td>+</td>
<td>433.09</td>
<td>432.69</td>
</tr>
<tr>
<td>S6</td>
<td>80</td>
<td>+</td>
<td>+</td>
<td>433.07</td>
<td>430.93</td>
</tr>
<tr>
<td>S7</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>433.08</td>
<td>432.50</td>
</tr>
<tr>
<td>S8</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>433.04</td>
<td>432.89</td>
</tr>
<tr>
<td>S9 -&gt; PS9 d)</td>
<td>80 - 11.5</td>
<td>+</td>
<td>+</td>
<td>433.07</td>
<td>432.63</td>
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<td>+</td>
<td>+</td>
<td>433.11</td>
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<td>S11</td>
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<td>+</td>
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<td>-</td>
<td>-</td>
<td>433.11</td>
<td>431.70</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>433.16</td>
<td>432.21</td>
</tr>
<tr>
<td>P3</td>
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<td>-</td>
<td>-</td>
<td>433.02</td>
<td>430.77</td>
</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>433.06</td>
<td>430.94</td>
</tr>
<tr>
<td>P5</td>
<td>14.5</td>
<td>+</td>
<td>+</td>
<td>433.06</td>
<td>431.93</td>
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<td>433.05</td>
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<td>-</td>
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</tr>
<tr>
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<td>-</td>
<td>-</td>
<td>433.00</td>
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<tr>
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<td>5.5</td>
<td>+</td>
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</tr>
<tr>
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<td>-</td>
<td>-</td>
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<td>P28</td>
<td>11.5</td>
<td>-</td>
<td>-</td>
<td>432.85</td>
<td>429.07</td>
</tr>
</tbody>
</table>

**a)** measured on June 17, 1996  
**b)** all wells are screened the entire length in the ground water  
**c)** meters above sea level  
**d)** concrete wells were equipped with PVC piezometer tubes in May 1996  
**e)** not measured  
**f)** monitoring well is not accessible anymore
2.2.3 Hydrologic characteristics

The ground water table at the site is generally 2 - 4 m below the surface and slopes in a north-easterly direction with a gradient of about 0.15 m per 100 m (Fig. 2-1). From 1993 to 1996, the maximum fluctuation of the ground water table was 0.4 m. The temperature of the ground water changed seasonally between a minimum of 9.5°C in March and a maximum of 11.5°C in September. The main source of recharge to the Berner Seeland aquifer is precipitation as well as the river Alte Aare and the Aare-Hagneck channel, located 2.5 and 15 km, from the site, respectively (Biaggi et al., 1994). Recharge from precipitation was estimated to be 0.44 m year\(^{-1}\) (Biaggi et al., 1994). At the site recharge is lower because water collected on roofs and roads is discharged into the sewer system. The annual precipitation measured at the site was 1.08 m in 1995 and 0.58 m in 1996. During the natural gradient tracer experiment from August to December 1996, precipitation was only 0.19 m.

2.3 Field and Laboratory Methods

2.3.1 Natural gradient tracer experiment

On August 14, 1996, after geochemical sampling in June 1996, a natural gradient tracer experiment was started by the injection of KI, NaBr, and H\(_3\)BO\(_3\) in the wells P2, PS3 and PS9, respectively. These tracers (all from Siegfried AG, Zofingen, Switzerland) were selected because they are considered to be conservative (Davis et al., 1980; Gelhar et al., 1992), not retarded by the residual oil and the background concentrations of these compounds in the aquifer of Studen are low (< 6 \(\mu\)M). Three different injection wells were chosen to observe the transport characteristics at the site along the plume centerline and along its expected borders. Tracer solutions were prepared by mixing concentrated stock solutions of the tracers with water from a water supply well located about 1 km upgradient of the site in the same aquifer. The solutions were then injected into the saturated zone.
through the injection wells during a 24 to 26 hour period at the following rates: 4.61 min\(^{-1}\) in P2 (5 kg KI) and 9.31 min\(^{-1}\) in PS3 and PS9 (10 kg NaBr and 10 kg H\(_3\)BO\(_3\), respectively). Large injection volumes were chosen to avoid the sinking of the tracer clouds due to the difference in density between the tracer solution and the ambient ground water (LeBlanc et al., 1991). Assuming a porosity of 0.19 and no mixing with ambient ground water, the initial volume of the aquifer occupied by the tracer solutions was about 74 m\(^3\) at PS3 and PS9 and 37 m\(^3\) at P2. Samples were taken at monitoring wells shown in Fig. 2-2A&B before and immediately after completion of solute injection then every 3 to 4 days during the initial two weeks and every week during the following months. The breakthrough curves of the tracers in selected wells were interpolated and integrated. Mean flow velocities were calculated by dividing the distance from the injection well to the monitoring well by the time at which 50% of the tracer mass had passed this well (Matthess & Ubell, 1983).

2.3.2 Ground water sampling techniques

Ground water samples were collected using downhole submersible electrical pumps (Whale Superline 991, Munster Simms Engineering, Bangor, Northern Ireland) with teflon tubing (Maagtechnic, Dübendorf, Switzerland). At least two well volumes were removed before samples were taken.

Ground water samples for the analysis of dissolved monoaromatic hydrocarbons were collected in 40 ml glass vials, immediately acidified with 32% HCl to pH 2 and closed without head space with a Teflon-lined screw cap. Oxygen concentration, temperature, electrical conductivity, and pH were determined immediately at the site using the appropriate electrodes (O\(_2\): Clark type electrode, WTW Oxi 96; temperature and conductivity: WTW LF 325; pH: WTW 95; all from WTW, Weilheim, Germany) fitted to a flow cell. Samples for analysis of dissolved species except for S(-II) were filtered in the field immediately after sampling using 0.22 μm polyvinylidenefluoride filters (Millipore, Bedford, USA). Samples for analysis of cations and dissolved metals were acidified with 0.1% distilled concentrated
HNO₃; samples for S(-II) analysis were fixed using zinc acetate solution (APHA, 1989). For alkalinity determinations and gas analysis, 117 ml serum bottles were filled and closed without head space using butyl rubber stoppers. Within 24 hours after sampling, a 20 ml head space of N₂ was introduced simultaneously with the removal of 20 ml of liquid, which was used for alkalinity determination. Gases were measured in the resulting head space after shaking followed by 12 hours of equilibration at 7°C (Bossard et al., 1981). For stable carbon isotope analysis of the DIC, glass bottles (1000 ml) were filled with unfiltered ground water and closed without head space using rubber stoppers. Within 24 hours after sampling, the DIC was precipitated as BaCO₃ by addition of 10 ml of a CO₂-free 2 M NaOH and 40 ml of a CO₂-free 1.2 M BaCl₂ solution. After more than 12 hours of equilibration, the precipitate was filtered under a stream of N₂ and dried at 105°C for 12 h.

2.3.3 Chemical analysis

Total petroleum hydrocarbons were determined by infrared spectroscopy (APHA, 1989). Samples for dissolved monoaromatic hydrocarbons were analyzed using a HP 5972A MS in selected ion monitoring mode interfaced to a HP 5890A Series II HRGC and a Tekmar LSC 2000 autosampler purge-and-trap system (Hewlett-Packard Ltd., USA and Tekmar, Cincinnati, USA). Monoaromatic hydrocarbon results reported are the average of two independent measurements.

Concentrations of Cl⁻, Br⁻, NO₂⁻, NO₃⁻, SO₄²⁻, PO₄³⁻ as well as NH₄⁺, Na⁺, K⁺, Ca²⁺ and Mg²⁺ were determined with a Dionex DX-100 ion chromatograph (Dionex, Sunnyvale, USA). H₃BO₃ was determined spectrophotometrically using the method with azomethine-H as described in Fresenius et al. (1988). Dissolved Fe and Mn were quantified by atomic absorption spectroscopy in an air-acetylene flame (Varian SpectrAA 400, Varian Techtron, Springvale, Australia). It was assumed that the dissolved Fe and Mn consisted mainly of Fe(II) and Mn(II) respectively, because the solubility of Fe(III) is low at pH < 6 (Stumm & Morgan, 1996). The terms Fe(II), Mn(II), Ca(II) and Mg(II) correspond to the sum of all dissolved species with the redox state +II. S(-II) was measured colorimetrically according to
standard methods (APHA, 1989) and represents the sum of H$_2$S, HS$^-$ and S$^{2-}$. Alkalinity was measured by potentiometric titration using Gran plots for graphical determination of the end point (Stumm & Morgan, 1996). Charge balances were calculated to assure the accuracy of the analyses (Fritz, 1994). The deviation between the sums of negative and positive charges was generally less than 10% (Concentrations of Cl$^-$, PO$_4^{3-}$, NH$_4^+$, Na$^+$, and K$^+$ used in charge balances are not shown in Tab. 2-2).

The head space technique was used to quantify CO$_2$, N$_2$O and CH$_4$ as described elsewhere (Bossard et al., 1981). The partial pressures of the gases were determined by gas chromatography (Carlo Erba Model 8000, Rodano, Italy) on a HayeSep D column using N$_2$ as carrier and a Carlo Erba thermal conductivity detector. Concentrations of dissolved gases were calculated according to Bossard et al. (1981) based on Henry’s Law using the following Henry constants which are corrected for 7°C: CO$_2$: 0.0594 mol l$^{-1}$ atm$^{-1}$ (Stumm & Morgan, 1996); N$_2$O: 0.0449 mol l$^{-1}$ atm$^{-1}$ (Weiss & Price, 1980); CH$_4$: 0.00206 mol l$^{-1}$ atm$^{-1}$ (Yamamoto et al., 1976); C$_2$H$_6$: 0.0032 mol l$^{-1}$ atm$^{-1}$ (Schwarzenbach et al., 1993). DIC concentrations were calculated from alkalinity and pH (Stumm & Morgan, 1996).

### 2.3.4 Stable carbon isotope analysis

All measured $^{13}$C/$^{12}$C ratios are reported in the delta notation ($\delta^{13}$C) referenced to the VPDB standard (Coplen, 1996). The $\delta^{13}$C value is defined as:

$$\delta^{13}C = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \cdot 1000$$

where $R_{\text{sample}}$ and $R_{\text{standard}}$ are the carbon isotope ratios in the sample and the standard, respectively. For stable carbon isotope analysis of the DIC, the dried BaCO$_3$ was converted to CO$_2$ at 90°C in an automated acid bath preparation system using H$_3$PO$_4$ and then measured on a Fisons-Prism isotope-ratio mass spectrometer (Fisons, Middlewich, Cheshire, UK). For stable carbon isotope analysis of the carbonates in the aquifer material, a sample of
mixed aquifer material from the drilling cores of P20 and P28 was ground, and the δ\(^{13}\)C was determined identically to the precipitated BaCO\(_3\). The δ\(^{13}\)C of the PHC was measured using the free phase collected from S6 and P16. About 10 µl of the oil were combusted in an evacuated quartz tube at 950°C during 3 h using 1 g CuO as an oxidant. The produced CO\(_2\) was cryogenically purified on a vacuum line and analyzed. The analytical reproducibility of all δ\(^{13}\)C measurements was < 0.2‰.

### 2.3.5 Stable carbon isotope balances

The increase of DIC concentrations in wells of the contaminated site relative to uncontaminated wells upgradient was assessed using stable carbon isotope balances. As the ground water flowing through the source area becomes enriched in DIC, the δ\(^{13}\)C\(_{\text{inc}}\) is expected to change relative to the contributions of each of the DIC\(_{\text{inc}}\) sources. This is expressed by the following two mass balance equations for DIC\(_{\text{meas}}\) and δ\(^{13}\)C\(_{\text{meas}}\)*DIC\(_{\text{meas}}\) (equations 2 and 3):

\[
\text{DIC}_{\text{meas}} = \text{DIC}_{\text{bg}} + \text{DIC}_{\text{inc}} \tag{2}
\]

\[
\delta^{13}\text{C}_{\text{meas}} \times \text{DIC}_{\text{meas}} = \delta^{13}\text{C}_{\text{bg}} \times \text{DIC}_{\text{bg}} + \delta^{13}\text{C}_{\text{inc}} \times \text{DIC}_{\text{inc}} \tag{3}
\]

<table>
<thead>
<tr>
<th>DIC(_{\text{meas}})</th>
<th>measured DIC concentration (mol l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIC(_{\text{bg}})</td>
<td>measured DIC concentration upgradient (background P20) (mol l(^{-1}))</td>
</tr>
<tr>
<td>DIC(_{\text{inc}})</td>
<td>DIC increase (mol l(^{-1}))</td>
</tr>
<tr>
<td>δ(^{13})C(_{\text{meas}})</td>
<td>δ(^{13})C of DIC(_{\text{meas}}) (‰)</td>
</tr>
<tr>
<td>δ(^{13})C(_{\text{bg}})</td>
<td>δ(^{13})C of DIC(_{\text{bg}}) (‰)</td>
</tr>
<tr>
<td>δ(^{13})C(_{\text{inc}})</td>
<td>δ(^{13})C of DIC(_{\text{inc}}) (unknown) (‰)</td>
</tr>
</tbody>
</table>

The mass balance (equation 3) has the form of a linear equation. When the product of δ\(^{13}\)C\(_{\text{meas}}\)*DIC\(_{\text{meas}}\) is plotted versus DIC\(_{\text{inc}}\), a straight line is obtained with a slope equal to the δ\(^{13}\)C\(_{\text{inc}}\) and an intercept of δ\(^{13}\)C\(_{\text{bg}}\)*DIC\(_{\text{bg}}\).
according to equation 3. Hence, the slope yields an overall average of the unknown isotopic ratio of the produced DIC in the contaminated zone.

| 0.002 - 0.010 mM Br⁻ | 0.004 - 0.010 mM H₃BO₃ |
| 0.010 - 0.100 mM Br⁻ | 0.010 - 0.050 mM H₃BO₃ |
| 0.100 - 1.000 mM Br⁻ | 0.050 - 0.700 mM H₃BO₃ |

Source area

Lines connecting wells along plume centerline

Figure 2-2: A: Distribution of Br⁻ 94 days after the injection into PS3 (arrow). Sampled monitoring wells shown only. In the insert, a typical example of a breakthrough curve is shown (at Br⁻ PS5).

B: Distribution of H₃BO₃ 94 days after the injection into PS9 (arrow). Sampled monitoring wells shown only.
2.4 Results

2.4.1 Natural gradient tracer experiment

Bromide tracer that was injected into PS3 was observed in PS4 immediately after the completion of the injection of the tracer solution. After 10 days it appeared in PS5, where concentrations increased until day 85 (Fig. 2-2A, insert). As a selected example, the concentrations of bromide after 94 days are presented in Fig. 2-2A. At that time, the center of mass of the bromide tracer had passed PS5 (Fig. 2-2A, insert), and the front had already reached P27. The borate tracer injected in PS9 was first observed after 7 days simultaneously in P5, P23, P24 and P25. The concentration began to increase in S8 after 27 days. The concentration maximum of borate 94 days after the injection was found in S8, whereas it was no longer detected in P25 (Fig. 2-2B). The iodide tracer injected in P2 disappeared completely from the injection well within 41 days. A breakthrough was observed only in P6 with first appearance after 107 days and a maximum concentration of 113 μM on day 126. In addition, iodide concentrations larger than the background concentration of 6 μM were observed occasionally in P1 (day 63 and 83) and in PS9 (day 26 and 34). Iodide was not observed in any other wells. Based upon these results we determined a general ground water flow direction of approximately 55° east.

The natural gradient tracer experiment was also used to calculate approximate residence times and mean flow velocities of the ground water and the dissolved species. The mean residence time of the bromide between PS3 and P5 was 125 days. The estimated residence time in the entire source area was approximately 165 days. Mean flow velocities determined by analysis of the breakthrough curves ranged from 0.3 m day⁻¹ (PS3 -> PS4) to 0.8 m day⁻¹ (PS9 -> P5).
Figure 2-3: Concentrations of monoaromatic hydrocarbons in ground water samples of wells along the plume centerline, measured on July 16, 1997. 1: Sum of p- and m-xylene; 2: Ethylbenzene; 3: o-Xylene; 4: Toluene; 5: Benzene; 6: Sum of 3- and 4-Ethylbenzene; 7: 2-Ethylbenyene; 8: n-Propylbenzene; 9: Isopropylbenzene; 10: 1,2,4-Trimethylbenzene; 11: 1,3,5-Trimethylbenzene; 12: 1,2,3-Trimethylbenzene; 13: Tetramethylbenzenes.
2.4.2 PHC

Dissolved monoaromatic hydrocarbons were found within the source area at a maximal total concentration of 123 μg l\(^{-1}\) in PS5 (sum of compounds shown in Fig. 2-3). The corresponding maximal total petroleum hydrocarbon concentration measured was 1 mg l\(^{-1}\) in PS5. No dissolved hydrocarbons were found outside of the source area. The concentrations of individual dissolved monoaromatic hydrocarbons measured in the source area along the plume centerline (see below) followed a similar trend each except for toluene, which was already depleted in P5 (Fig. 2-3).

2.4.3 Temporal variability of ground water composition

During the 41 month period in which detailed analysis of the chemical parameters in ground water at Studen were made (January 1995 – May 1998), concentrations of important parameters remained fairly constant. As an example, we show NO\(_3^\text{-}\) concentrations in 3 wells at the Studen site and in a well of drinking water facility located 1 km upgradient of the site (Fig. 2-4). In all 4 wells the NO\(_3^\text{-}\) concentrations remained constant suggesting steady-state conditions. At the drinking water facility and in P20 the average NO\(_3^\text{-}\) concentration and its standard deviation over the observed time period was 0.410 mM ± 0.014 mM and 0.278 mM ± 0.021 mM, respectively. The wells within (PS4) and just outside of the source area (P8) show consistently low NO\(_3^\text{-}\) concentrations (average of 0.008 mM ± 0.008 mM and 0.065 mM ± 0.014 mM, respectively. Fig. 2-4) indicating an increased but stable denitrification activity.

2.4.4 Ground water chemistry along the plume centerline

Based on the observations of the tracers and the distribution of the free phase, the transect P20 - PS3 - PS4 - PS5 - P5 - P25 - P27 was chosen as a streamline representing the plume centerline (Fig. 2-2A). Of the seven selected wells, one is located upgradient of the source area (P20), four are within the source area (PS3, PS4, PS5, P5) and two are downgradient of the source area (P25 and P27).
Table 2-2: Chemical analysis of ground water samples collected June 17, 1996. Well names refer to Fig. 2-1; T = Temperature; Alk = Alkalinity; DIC = Dissolved Inorganic Carbon.

<table>
<thead>
<tr>
<th>Well</th>
<th>T °C</th>
<th>pH</th>
<th>Alk</th>
<th>DIC</th>
<th>O₂</th>
<th>NO₃⁻</th>
<th>SO₄²⁻</th>
<th>S(II)</th>
<th>Fe(II)</th>
<th>Mn(II)</th>
<th>CH₄</th>
<th>Mg²⁺</th>
<th>Ca²⁺</th>
<th>δ¹³C</th>
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<td>0.021</td>
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</table>

a) not measured
All values of chemical parameters measured on June 17, 1996, which represents a typical profile encountered at the Studen site, are reported in Tab. 2-2. Values in wells, which represent the plume centerline are plotted against the distance of the respective wells from P20 (Fig. 2-5). Oxidants (\(O_2\), \(NO_3^-\), \(SO_4^{2-}\)) were consumed within the source area and reached background concentrations again downgradient (Fig. 2-5). In the monitoring wells within the source area, dissolved Fe(II) and Mn(II) were detected. Concentrations of S(-II) were above detection limit only in PS3 (0.012 mM) and PS4 (0.003 mM). \(CH_4\) was found in PS3, PS4, PS5 and P25 (Fig. 2-5). The \(N_2O\) concentration was below 0.005 mM; \(NO_2^-\) and \(PO_4^{3-}\) concentrations were below the detection limit of 0.002 mM in all measured monitoring wells. Alkalinity, DIC and Ca(II) were elevated within the source area. The Mg(II) concentration showed no changes over the length of the plume centerline. In all wells along the plume centerline, the \(\delta^{13}C\) of the DIC was found to be more negative than upgradient in P20 (Fig. 2-5). The most negative \(\delta^{13}C\) was measured in PS3 at the upgradient border of the source area.

Figure 2-4: \(NO_3^-\) concentrations in selected wells from January 1995 to May 1998. \(\times\): Drinking water facility 1 km upstream of the site; \(\bullet\): P20; \(\diamond\): P8; \(\square\): PS4.
2.4.5 Stable carbon isotope balances

As shown in Tab. 2-3, different DIC sources have distinct isotopic ratios. Non-methanogenic PHC mineralization yields DIC with a negative $\delta^{13}C (-27.8\%)$ (measured $\delta^{13}C$ of the Studen oil), carbonate dissolution yields DIC with a $\delta^{13}C$ of around zero (-1.1%, measured in aquifer material from Studen), and methanogenesis yields DIC with a positive $\delta^{13}C (+38\%)$, see Tab. 2-3).

From all the wells printed in bold in Fig. 2-1, the product of $\delta^{13}C_{\text{meas}}$ *DIC$_{\text{meas}}$ is plotted versus DIC$_{\text{inc}}$ in Fig. 2-6 according to equation 3. The slopes of possible different sources of DIC$_{\text{inc}}$ are shown in the insert of Fig. 2-6 as vector A (non-methanogenic PHC mineralization; slope = -27.8%), vector B (carbonate dissolution; slope = -1.1%), and vector C (methanogenic PHC mineralization; slope = +38%).

A least square linear regression analysis of data measured on June 17, 1996 from monitoring wells labeled in bold in Fig. 2-1 was performed. Data from wells with methane concentrations < 0.1 mM yield a slope of -24.4% ($R^2 = 0.96$). Wells having methane concentrations > 0.1 mM (PS4 and PS5) show, as expected, a deviation from vector A towards more positive values (Fig. 2-6). In wells containing no or little methane, all DIC$_{\text{inc}}$ can be attributed either to non-methanogenic hydrocarbon mineralization (fraction $f_h$) or carbonate dissolution (fraction $f_c$). Based upon the distinct $\delta^{13}C$ values of these processes equation 4 is established.

$$f_h \times (-27.8\%) + f_c \times (-1.1\%) = -24.4\%$$

Considering the fact that $f_h + f_c = 1$, equation 4 yields $f_h = 0.88$, signifying that in the average over all wells, 88% of the DIC increase originates from non-methanogenic PHC mineralization and 12% from carbonate dissolution.
Table 2-3: Stoichiometric equations of selected processes involved in hydrocarbon mineralization.

<table>
<thead>
<tr>
<th>Process</th>
<th>Contribution to DIC</th>
<th>Contribution to alkalinity</th>
<th>$\delta^{13}C$ of DIC$_{inc}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microbial hydrocarbon mineralization</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.68 $&lt;$CH$_{1.85}$&gt; + O$_2$</td>
<td>0.68 CO$_2$ + 0.63 H$_2$O</td>
<td>+ 0.68</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- 27.8 $^d$</td>
</tr>
<tr>
<td>0.85 $&lt;$CH$_{1.85}$&gt; + NO$_3^-$ + H$^+$</td>
<td>0.85 CO$_2$ + 0.5 N$_2$ + 1.29 H$_2$O</td>
<td>+ 0.85</td>
<td>+ 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- 27.8 $^d$</td>
</tr>
<tr>
<td>0.34 $&lt;$CH$_{1.85}$&gt; + MnO$_2$(s) + 2 H$^+$</td>
<td>0.34 CO$_2$ + Mn$^{2+}$ + 1.31 H$_2$O</td>
<td>+ 0.34</td>
<td>+ 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- 27.8 $^d$</td>
</tr>
<tr>
<td>0.17 $&lt;$CH$_{1.85}$&gt; + FeOOH(s) + 2 H$^+$</td>
<td>0.17 CO$_2$ + Fe$^{2+}$ + 1.66 H$_2$O</td>
<td>+ 0.17</td>
<td>+ 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- 27.8 $^d$</td>
</tr>
<tr>
<td>1.37 $&lt;$CH$_{1.85}$&gt; + SO$_4^{2-}$ + 2 H$^+$</td>
<td>1.37 CO$_2$ + H$_2$S + 1.26 H$_2$O</td>
<td>+ 1.37</td>
<td>+ 2</td>
</tr>
<tr>
<td>1.37 $&lt;$CH$_{1.85}$&gt; + 0.74 H$_2$O</td>
<td>0.37 CO$_2$ + CH$_4$</td>
<td>+ 0.37</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ 38 $^e$</td>
</tr>
<tr>
<td><strong>Geochemical processes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCO$_3$(s) + 2 H$^+$</td>
<td>CO$_2$ + Ca$^{2+}$ + H$_2$O</td>
<td>+ 1</td>
<td>+ 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- 1.1 $^f$</td>
</tr>
<tr>
<td>CO$_2$ + Fe$^{2+}$ + H$_2$O</td>
<td>FeCO$_3$(s) + 2 H$^+$</td>
<td>- 1</td>
<td>- 2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Fe$^{2+}$ + H$_2$S</td>
<td>FeS(s) + 2 H$^+$</td>
<td>0</td>
<td>- 2</td>
</tr>
</tbody>
</table>

- $^a$ All species are given in the form in which they exist at the reference point of the alkalinity titration (pH=4.3). Thus the number of protons that are produced or consumed corresponds to alkalinity consumption or production. The species printed bold were used to quantify the processes.
- $^b$ Moles per mole stoichiometric turnover of species printed bold.
- $^c$ It was assumed that hydrocarbons in heating oils have an average H/C ratio of 1.85 (denoted as $<$CH$_{1.85}$>). Biomass formation is assumed to be negligible.
- $^d$ The $\delta^{13}C$ of DIC produced by non-methanogenic microbial hydrocarbon mineralization assumed to be not subject to any fractionation (Hunkeler, 1997; Aggarwal et al., 1997).
- $^e$ Methanogenic PHC mineralization is reported to enrich $\delta^{13}C$ of DIC up to +38‰ (Grossman, 1997).
- $^f$ Measured $\delta^{13}C$ of carbonate in a mixed sample of the aquifer material from P20 and P28.
- $^g$ Depends on $\delta^{13}C$ of the precipitating DIC and fractionation factor between the DIC and the FeCO$_3$ (Deines et al., 1974; Carothers et al., 1988).
2.4.6 Equilibria of geochemical processes

Saturation calculations for minerals that can potentially undergo precipitation or dissolution were performed as described in Hunkeler (1998) in order to identify geochemical reactions which might influence the concentrations of DIC, Fe(II), Mn(II), Ca(II) and S(-II). The ground water at the Studen site is saturated or slightly supersaturated with respect to CaCO$_3$ (calcite). No distinct differences were observed in the calcite saturation between monitoring wells in the source area to wells outside of the source area. All monitoring wells were undersaturated with respect to MnCO$_3$ (rhodocrosite). PS4, PS5 and P5 were the only wells that were supersaturated with respect to FeCO$_3$ (siderite).

![Ground water chemistry graph](image)

**Figure 2-5:** Ground water chemistry along the plume centerline on June 17, 1996.
2.5 Discussion

2.5.1 Natural gradient tracer experiment

The determination of the flow direction based on hydraulic heads at sites with small gradients and a free phase of PHC in the monitoring wells is subject to considerable uncertainty (Bedient et al., 1994). Therefore, a tracer experiment using conservative tracers in three different injection wells was performed to get a better understanding of transport within, and downgradient of the source area. A general flow direction at this site of approximately 55° east was obtained from the bromide and borate tracer injections. The trajectory of the center of the iodide cloud could not be detected, probably because it travelled to the northeast of all wells. This flow path would be consistent with predictions based on hydraulic heads (Fig. 2-1).

2.5.2 Influence of physical processes on concentration profiles

The concentrations of measured species in the ground water may be influenced by mixing with pristine ground water, which can occur by two ways: either by vertical and lateral mixing in the aquifer (dispersive mixing) or during sampling within the monitoring well. When interpreting the chemical data along the plume, one has to be aware that they are significantly influenced by the screen lengths and the vertical extension of the plume. Incorrect assessment of vertical concentration profiles can lead to false interpretation of plume lengths, plume directions and even source zone location (Martin-Hayden & Robbins, 1997).

At our site, most monitoring wells were drilled by the cantonal authorities to allow an initial survey of the extension of the contaminated zone and reach to different depths (Tab. 2-1). Wells PS3, PS4 and PS5 had a screened length of less than 0.7 m below the ground water table, whereas wells P5, P20, P25 and P27 had a screened length of 2.08 m, 2.80 m, 3.99 m, 3.72 m respectively below the ground water table. Thus the chemical data presented in Figs. 3 and 5 may partially reflect the screen length but these data give a qualitative insight into the respective processes.
2.5.3 Dissolved monoaromatic hydrocarbons

We focused our attention on monoaromatic hydrocarbons because of their toxicity and their higher water solubilities compared to other hydrocarbons present in heating oil (Eganhouse et al., 1996). Dissolved monoaromatic hydrocarbons are therefore the oil constituents that most likely form a plume. The elevated concentrations along the plume centerline show that monoaromatic hydrocarbons dissolve from the residual PHC pool into the water phase within the source area. However, contrary to the non-reactive tracers, monoaromatic hydrocarbons are not found in monitoring wells downgradient of the source area (P23, P25, P27). In fact, a decrease of hydrocarbon concentration within the source area can be observed from PS5 to P5 (Fig. 2-3). It must be noted that disappearance of hydrocarbons alone is not a proof of intrinsic bioremediation (MacDonald & Rittmann, 1993). Other natural attenuation effects such as dilution, sorption, or preferential flow paths can lead to a diminishing hydrocarbon concentration. At Studen, the disappearance of hydrocarbons is probably due to a number of processes including dilution, sorption, and biodegradation. Eganhouse et al. (1996) showed selective biodegradation of single monoaromatic compounds at another site. Whether the same is also true for the Studen site remains open and is subject to further studies.

2.5.4 Microbiological and geochemical processes

The monitoring in the source zone showed decreased levels of oxidants and increased levels of reduced species, DIC, and alkalinity within the source area with respect to the background concentrations. This indicates increased microbial activity within the source area. Changes in concentrations of species measured in P5, P25, and P27 towards background concentrations (Fig. 2-5) may be partly explained by the mixing of plume water with pristine ground water during sampling as discussed above.

Based on the observed concentrations of oxidants and reduced species at the Studen site and saturation calculations, we propose that the microbial and geochemical processes shown in Tab. 2-3 are the most significant ones in our aquifer. We are fully aware that other processes also take place in the
aquifer; however, the chosen processes seem to be the dominant, quantifiable processes. Similar processes have been shown to be relevant in ground water biogeochemistry in laboratory studies (Hess et al., 1996; Hunkeler et al., 1998) and other field sites (Herczeg et al., 1991; Hunkeler, 1997). Production of DIC from naturally occurring DOC is excluded for quantitative reasons, since the observed increases of up to 7 mM DIC is far higher than the DOC concentrations observed in the aquifer (0-0.22 mM, Kozel, 1992). The ground water that enters the site is saturated with respect to CaCO$_3$ (calcite) but undersaturated with respect to FeCO$_3$ (siderite), MnCO$_3$ (rhodocrosite) and FeS (amorphous iron sulfide). The observed increase in dissolved Ca(II) within the source area is due to the dissolution of CaCO$_3$. The large formation of CO$_2$ within the source area causes significantly lower pH values in these monitoring wells (Tab. 2-2). The pH drop leads to the dissolution of calcite to reach saturated conditions again.

2.5.5 Stable carbon isotope balances

Stable carbon isotope ratios in inorganic carbon have been used previously to verify non-methanogenic petroleum hydrocarbon mineralization by showing that the inorganic carbon has the same isotopic ratio (-27‰ to -30‰) as the PHC (Van de Velde et al., 1995; Aggarwal et al., 1997). However, at sites where methanogenesis occurs, the $\delta^{13}$C in DIC were reported to range from around -30‰ up to +11.9‰ (Landmeyer et al., 1996; Conrad et al., 1997), adding serious difficulties to the proving of PHC mineralization. Conrad and co-workers (1997) suggested the use of $^{14}$C to correct for this, however, $^{14}$C-analyses are costly. We developed a graphical method (Fig. 2-6) based on $\delta^{13}$C in DIC only which allows one to display the data from different non-methanogenic and methanogenic monitoring wells from a heterogeneous field site. Samples from wells with methane concentrations > 0.6 mM (PS4 and PS5) deviate clearly from the trend of all other wells. The wells with < 0.1 mM methane are all lying on a straight line having the slope of -24.4‰ in Fig. 2-6, although their DIC concentrations may have been influenced by mixing processes due to the different screen lengths of the wells. The mixing of a water sample containing high DIC concentrations
with a pristine water sample containing background DIC concentrations (e.g. from deep aquifer strata) has the effect that the data point in Fig. 2-6 moves up along the slope towards the intercept. Thus, mixing with pristine ground water during sampling does not affect the slope of equation 3 and the conclusions drawn from it. However, it has to be stated that the graphical method yields only a defined slope in an aquifer where the relative contributions of mineralization and carbonate dissolution are constant. Also, there is concern that in very shallow monitoring wells, the volatilization of CO₂ might add some uncertainty, since lighter isotopes tend to volatilize more easily (Stumm & Morgan, 1996).

![Graph](image)

**Figure 2-6:** \( \delta^{13}C_{\text{meas}} \times \text{DIC}_{\text{meas}} \) plotted versus \( \text{DIC}_{\text{inc}} \) for wells whose names are in bold characters in Fig. 2-1 on June 17, 1996. Vectors indicating the source of \( \text{DIC}_{\text{inc}} \): A - \( \text{DIC}_{\text{inc}} \) produced by non-methanogenic PHC mineralization, B - \( \text{DIC}_{\text{inc}} \) produced by carbonate dissolution, C - \( \text{DIC}_{\text{inc}} \) produced by methanogenic PHC mineralization.

The assessment of the slope yielded in Fig. 2-6 allowed to conclude that PHC mineralization is the predominant process of DIC production in
this aquifer under the assumption that natural organic matter is not considered as a source of DIC. Similar contributions of PHC mineralization and carbonate dissolution were reported by Revesz et al. (1995) at a field site contaminated with crude oil. However, in aquifers where larger quantities of particulate or dissolved natural organic matter are oxidised to DIC, the assessment of PHC mineralization has to include also $^{14}$C data, since the $\delta^{13}$C in natural organics and in PHC are similar (Baedecker et al., 1993; Conrad et al., 1997).

### 2.5.6 DIC and alkalinity balances

These balances are based on a comparison of calculated increases due to stoichiometric considerations with observed increases (Hunkeler, 1997). The calculated DIC increase was determined using the measured consumption of oxidants and production of reduced species and Ca(II) and the stoichiometric equations shown in Tab. 2-3. The observed DIC increase is the measured difference in DIC concentration. The calculated alkalinity increase and observed alkalinity increase accompanying microbial hydrocarbon mineralization were determined analogously. In order to quantify the proposed processes, the following assumptions had to be made: i) steady-state conditions were established, ii) the oxidants were used to mineralize hydrocarbons exclusively and iii) no gas exchange between the unsaturated and the saturated zone occurred. The biases of the first and second assumptions are considered to be only minor: the chemical concentrations show no trends towards increased or decreased concentrations with only minor variations (Fig. 2-4) and the ground water in the vicinity of the site has a low content in dissolved organic matter (DOC < 0.22 mM, Kozel, 1992). However, the extent of gas exchange between the unsaturated and the saturated zone is presently unknown.

DIC and alkalinity balances were established for the flow paths between P20 and the monitoring wells in the plume centerline. In the shallow wells (PS3, PS4 and PS5) mixing with pristine ground water during sampling can be neglected and therefore the flow path from P20 to PS4 was selected to illustrate DIC and alkalinity balances (Fig. 2-7). The calculated
DIC increase based on the stoichiometric factors (Tab. 2-3) and the measured species in ground water was only about one third of the observed DIC increase (Fig. 2-7). On the other hand, the observed alkalinity increase is smaller than the calculated alkalinity increase (Fig. 2-7).

![Figure 2-7: DIC and alkalinity balances along the flowpath P20 - PS4.](image)

Considering the balances, a number of processes listed in Tab. 2-3 have to be discussed in detail. Fe(III) reduction is underestimated as the microbiologically produced Fe(II) reacts e.g. with S(-II) or CO$_3$$^{2-}$ and precipitates as FeS or FeCO$_3$. The Fe(II) concentration found in ground water is therefore likely to be lower than the actual produced Fe(II) concentration. The reduction of 1 mol Fe(III) produces 0.17 mol DIC and 2 mol alkalinity, whereas the precipitation of Fe(II) consumes 1 mol DIC and 2 mol alkalinity (Tab. 2-3). The fate of the sulfur of SO$_4^{2-}$ after its reduction to H$_2$S is unknown.
Possible reactions are precipitation with metals, reoxidation, or transport to the unsaturated zone. The turnover of $\text{SO}_4^{2-}$ is therefore underestimated when only the concentrations in the ground water are compared. The reduction of $\text{SO}_4^{2-}$ contributes to the alkalinity balance and is the major contributor to the DIC balance per mol turnover (Tab. 2-3). However, precipitation of $\text{H}_2\text{S}$ affects only the alkalinity balance. The gas exchange might also play a significant role. Aerobic hydrocarbon mineralization and methanogenesis may be underestimated because the amounts of $\text{CH}_4$ escaping and $\text{O}_2$ entering the system through the unsaturated zone are unknown. Both processes would lead to an increase in the calculated DIC without affecting alkalinity (Tab. 2-3) and were observed in an aquifer contaminated by crude oil (Revesz et al., 1995).

We assume that all of the above mentioned processes are involved in the production of the unaccounted DIC. Similar discrepancies in calculated production and measured increase of DIC and alkalinity were observed in all monitoring wells along the plume centerline, as well as at other field sites (Bennett et al., 1993; Hunkeler, 1997). In order to further improve the mass balance to be used as a reliable assessment tool, the gaseous and solid phases must be considered as well.

2.5.7 Conclusions

Despite the fact that the site in Studen contains inherent obstacles to the establishment of a quantitative assessment of intrinsic bioremediation, we were able to gather strong evidence for microbial PHC mineralization by the measurement of different independent lines of evidence. The natural gradient tracer test gave solid information about the direction and the velocity of the ground water transport at the site. Hydrocarbon analysis showed the presence of dissolved monoaromatic hydrocarbons in the source area, but concentrations in wells situated downgradient of the source area were below detection limit. However, absolute concentrations of dissolved species have to be interpreted with great care since they tend to depend on the vertical extension of the plume and on the screen length of the monitoring wells (Martin-Hayden & Robbins, 1997). The monitoring of hydrocarbons,
oxidants, reduced species and DIC in the ground water along and perpendicular to the plume centerline reflected microbiological activity and geochemical reactions in the aquifer. Oxidants were decreased and reduced species, Ca(II), alkalinity, and DIC were increased within the source area. But DIC and alkalinity balances in shallow monitoring wells, which were not affected by dilution, revealed that not all processes had been correctly accounted for. This disagreement indicates that the quantification of PHC mineralization is underestimated when only concentrations in the ground water are used for the calculation of the balances. The new graphical method to interpret stable carbon isotope ratios of the DIC was used instead to prove the microbial mineralization of PHC. It was shown that 88% of the produced DIC originates from non-methanogenic PHC mineralization at this site. These results indicate enhanced biological activity in the source area and its borders. The conclusion drawn from $\delta^{13}C$ data has the advantage of not being affected by the differences in the screen lengths of the wells. In summary, the presented data show that a heating oil-contaminated aquifer with a low $O_2$ background concentration can potentially be treated by intrinsic bioremediation. Further studies are being undertaken to better understand the vertical profile of dissolved species and to quantify additional processes such as precipitation and dissolution reactions.

Acknowledgement

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Characterizing intrinsic bioremediation in a petroleum hydrocarbon-contaminated aquifer by combined chemical, isotopic, and biological analyses


Abstract

Chemical, isotopic, and biological parameters were evaluated over a one year period to characterize microbial processes associated with intrinsic bioremediation in a petroleum hydrocarbon-contaminated aquifer located in Studen, Switzerland. Chemical parameters measured included oxidants such as O\textsubscript{2}, NO\textsubscript{3}\textsuperscript{-} and SO\textsubscript{4}\textsuperscript{2-}, reduced species such as Fe\textsuperscript{2+} and CH\textsubscript{4}, and dissolved inorganic carbon (DIC). Stable carbon isotope analyses of DIC were used to differentiate between different processes that contribute to DIC production. Microbial populations were identified by sequence analysis of archaeal 16S rDNA and in situ hybridization using a general DNA binding dye (DAPI) and specific probes targeting the domain Archaea (Arch915) and Bacteria (Eub338), as well as the species Methanoseta concilii (Rocl11) and Methanospirillum sp. (Rocl2). Ground water exhibited reduced conditions and elevated concentrations of DIC within the contaminated zone. Spatially distinct values of δ\textsuperscript{13}C ranging from -16.51\% to
-4.44‰ were found, indicating the presence of different ongoing microbial processes. Detected microbial populations (% of DAPI-stained cells) within the contaminated zone belonged to Archaea (9 ± 2% to 31 ± 13%) and Bacteria (13 ± 3% to 32 ± 13%). In wells with methanogenic activity, *Methanoseta concilii* accounted for up to 26% of all DAPI-detected microorganisms. These results demonstrate that this novel combination of chemical, isotopic, and biological analysis provides valuable insights that can be used for the characterization of microbial processes in contaminated aquifers.

### 3.1 Introduction

Accidentally released petroleum hydrocarbons (PHC) are a common source of ground water contamination worldwide. Over the past decades, a variety of techniques have been proposed for the remediation of PHC-contaminated aquifers (for a recent review, see Riser-Roberts, (1998)). Among them, intrinsic bioremediation has become a popular and widely accepted remediation technique (Chapelle, 1999). During intrinsic bioremediation, PHC are ideally converted to carbon dioxide, methane, and water by naturally-occurring microorganisms under site-specific environmental conditions (Head, 1998). However, decreasing contaminant concentrations in an aquifer may also be attributed to sorption, dilution, volatilization, and abiotic degradation (Rifai et al., 1995).

Methods for confirming microbial activity in contaminated aquifers may include chemical analyses for monitoring the depletion of substrate (PHC) (Reinhard et al., 1997) or oxidants (Thorstenson et al., 1979; Borden et al., 1995; Hunkeler et al., 1999b), detection of metabolites (Beller et al., 1995), measurement of stable carbon isotope ratios (Landmeyer et al., 1996; Bolliger et al., 1999), microcosm studies (Wilson et al., 1983; Chaîneau et al., 1995; Bregnard et al., 1996), or assessment of microbial communities present within the aquifer (Dojka et al., 1998; Shi et al., 1999).
At contaminated sites, microbial activity is often limited by the availability of suitable oxidants (e.g. $O_2$, $NO_3^-$, $SO_4^{2-}$) (McAllister and Chiang, 1994). Since microbial populations need time to adapt to the highly specific conditions in contaminated aquifers, different redox processes evolve over time, establishing natural redox gradients at contaminated sites (Smith et al., 1991; Anderson and Lovley, 1997). Eventually, near-steady-state conditions are often reached as concentrations of oxidants and metabolic products (e.g., $CO_2$ and $CH_4$) remain invariable over time and space, which is indicative of specific microbial processes in distinct regions of the aquifer (Baedecker et al., 1993; Bennett et al., 1993). However, temporal and spatial steady-state conditions may not be achieved if ground water recharge by rainfall is a significant source of oxidants (Vroblesky and Chapelle, 1994).

Stable carbon isotope ratios ($\delta^{13}C$) of dissolved inorganic carbon (DIC) were used in several studies to assess microbial activity (Van de Velde et al., 1995; Landmeyer et al., 1996; Aggarwal et al., 1997; Conrad et al., 1997; Hunkeler et al., 1999b). Three different processes contribute to an increase in DIC, each of them providing a different $\delta^{13}C$ signature. These processes are carbonate dissolution, non-methanogenic PHC mineralization, and methanogenic PHC mineralization. Only a minimal isotopic fractionation (change of the isotopic ratio due to preferential use of one of the involved isotopes) is associated with carbonate dissolution and non-methanogenic PHC mineralization (Grossman, 1997). In contrast, methanogenesis is a fractionating process that yields a DIC enriched in $^{13}C$ in the range of 50% to 90% for $CO_2$ reduction and 40% to 60% for acetate fermentation (Whiticar et al., 1986). The latter is believed to be the dominant biotic source of $CH_4$ in freshwater environments (Whiticar et al., 1986; Revesz et al., 1995). Utilizing the difference in $\delta^{13}C$ signatures of PHC and carbonate, both Revesz et al. (1995) and Bolliger et al. (1999) demonstrated at two different PHC-contaminated aquifers that over 80% of DIC produced originated from the non-methanogenic mineralization of PHC.

Biological parameters are often used as indicators to demonstrate a potential for PHC mineralization (Chaîneau et al., 1995; Bregnard et al., 1996; Hess et al., 1997). Increases in cell numbers and/or biomass of total or
specific populations in contaminated aquifers relative to non-contaminated sites indicate the availability of nutrient sources such as PHC (Dojka et al., 1998; Zarda et al., 1998; Shi et al., 1999). For example, Dojka et al. (1998) applied comparative sequence analysis of a 16S rDNA library to samples from an anaerobic aquifer contaminated with PHC and chlorinated solvents. The authors found a large microbial diversity with many sequences being affiliated with different phylogenetic lineages of the domains Bacteria and Archaea.

Chemical, isotopic, and biological analyses provide valuable, but very specific insights into processes associated with intrinsic bioremediation of PHC-contaminated aquifers. However, due to both the complexity of natural ecosystems and the inherent limitations of each method discussed, a comprehensive characterization can usually not be attained by applying one method alone. In recent years, it has become well accepted that several lines of evidence are necessary to reliably characterize microbial activity during bioremediation (Madsen et al., 1991; U.S. National Research Council, 1993).

The aim of this study was to use a novel combination of chemical, isotopic, and biological analyses to characterize intrinsic bioremediation in a PHC-contaminated aquifer in space and time. More specifically, we wanted to investigate the variability of oxidants, stable carbon isotope ratios, and microbial communities in the contaminated zone of the aquifer. Of particular interest was the identification of microbial species prevailing in the aquifer methanogenic zone, for which we employed sequence analysis of archaeal 16S rDNA and the in situ hybridization technique.

### 3.2 Experimental Procedures

**3.2.1 Field Site Description and Sampling Procedures**

A spill of more than 34 m³ of heating oil from a leaking underground pipe was discovered in 1993 in an urban area in Studen, Switzerland. The unconfined aquifer underlying the site consists of unconsolidated glacioflu-
vial outwash deposits. The ground water table is generally between 2 and 4 meters below ground surface. Typical of many polluted sites, the Studen site contains inherent obstacles for a rigorous assessment. For example, it is not known exactly how much heating oil was spilled or when exactly the spill occurred. Engineered remediation was limited to the physical removal of free-phase PHC by pumping and partial excavation of contaminated soil. A detailed site description is in Bolliger et al. (1999).

Data presented in this study were collected from wells located along an approximate center flow line for ground water at the site. Of the six wells along the center flow line, two wells are located outside (P20 upgradient and P25 downgradient) of the contaminated zone (defined here as the zone where free-phase PHC was observed in wells, Fig. 3-1). Three wells (PS3, PS4, PS5) are located within the contaminated zone, and another well (P5) is located at the downgradient fringe. Wells PS3, PS4, and PS5 were drilled to ~ 3.7 m below ground surface and penetrate the ground water to a depth.

Figure 3-1: Map of the Studen site showing the heating oil-contaminated zone and selected monitoring wells along the center flow line.
of ~ 0.7 m. Wells P20, P5, and P25 were drilled to 5.3 m, 4.50 m, and 6.60 m below ground surface and penetrate the aquifer to a depth of 2.8 m, 2.1 m, and 4.0 m, respectively. Dissolved petroleum hydrocarbons were detected within the contaminated zone at a maximum concentration of 1 mg l⁻¹ in PS5, 123 μg l⁻¹ thereof were attributed to monoaromatic hydrocarbons (Bolliger et al., 1999).

Ground water samples were collected using downhole submersible electrical pumps (Whale Superline 991, Munster Simms Engineering, Bangor, Northern Ireland) with teflon tubing (Maagtechnic, Dübendorf, Switzerland). At least two volumes of water contained within the well casing were removed before samples were taken. Oxygen concentration, temperature, and electrical conductivity were determined immediately at the site using appropriate electrodes (O₂ Clark type electrode WTW Oxi 96, and temperature and conductivity: WTW LF 325; all from WTW, Weilheim, Germany) fitted into a flow cell. Samples for analysis of anions were filtered immediately after sampling using 0.22 μm polyvinylidenefluoride filters (Millipore, Bedford, USA). For pH and alkalinity determinations, serum bottles (117 ml) were filled and closed without head space using butyl rubber stoppers. For stable carbon isotope analysis of DIC, one liter glass bottles were filled with unfiltered ground water and closed without head space using rubber stoppers. For biological analyses, two sterile 50 ml tubes were each filled with 40 ml ground water. For the generation of a 16S rDNA library, ground water was collected from PS5 on August 25, 1998 in a one liter Ruttner bottle.

3.2.2 Chemical Analyses

Concentrations of anions were determined using a Dionex DX-100 ion chromatograph (Dionex, Sunnyvale, CA, USA). Within 24 hours after sampling, a 20 ml head space of N₂ was introduced into the serum bottles coupled with the removal of 20 ml of liquid, which was used for alkalinity determination. Alkalinity was measured by potentiometric titration using Gran plots for graphical determination of the end point (Stumm and Morgan, 1996). Methane concentrations were measured in the resulting serum
bottle head space after shaking followed by 12 hours of equilibration at 4°C (Bossard et al., 1981). The pH was measured in the laboratory with a MP 225 pH meter equipped with a InLab407 electrode (both Mettler-Toledo, Schwerzenbach, Switzerland). DIC concentrations were calculated from alkalinity and pH (Stumm and Morgan, 1996).

### 3.2.3 Stable Carbon Isotope Analyses

Within 24 hours after sampling, DIC was precipitated as BaCO₃ by adding 10 ml of CO₂-free 2 M NaOH and 40 ml of CO₂-free 1.2 M BaCl₂ solution. The precipitate was filtered and dried at 105°C for 12 h. The dried BaCO₃ was converted to CO₂ at 90°C in an automated acid bath preparation system using H₃PO₄ and then measured on a Fisons-Prism isotope-ratio mass spectrometer (Fisons, Middlewich, Cheshire, UK). All measured ¹³C/¹²C ratios are reported in the delta notation (δ¹³C), referenced to the VPDB standard (Coplen, 1996). The δ¹³C value is defined as:

\[
\delta^{13}C \,[\text{‰}] = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000
\]  

where \( R_{\text{sample}} \) and \( R_{\text{standard}} \) are the carbon isotope ratios in the sample and the standard, respectively. The analytical error of the δ¹³C measurements was < 0.2‰.

### 3.2.4 Biological Analyses

Microorganisms were harvested by centrifugation of two 40-ml-samples at 6,000 rpm for 15 min and resuspended in 500 μl of phosphate buffered saline (PBS; 0.13 M NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, pH 7.2). After centrifugation at 13,000 rpm for 1 min, cells were washed in 500 μl of sterile distilled water and fixed in 1 ml of 4% paraformaldehyde in PBS on ice for 3 h (Hahn et al., 1992). Thereafter, cells were washed twice in PBS and stored in 80 μl of 50% ethanol in PBS at -20°C.

Before application to slides, 5 μl of the paraformaldehyde fixed cell suspension were dispersed in 95 μl of 0.1% sodium pyrophosphate in PBS by mild sonification for 3 min in a sonification bath (Zarda et al., 1997). Ten
µl were subsequently spotted onto gelatin-coated slides (0.1% gelatin, 0.01% KCr(SO₄)₂) dried at 45°C for 30 min, and finally dehydrated in 50, 80, and 96% ethanol for 3 min each.

Cy3-labeled oligonucleotide probes Eub338 (Amann et al., 1990), Arch915 (Stahl and Amann, 1991), and Rotcl1 and Rotcl2 (Zepp Falz et al., 1999) (MWG Biotech, Ebersberg, Germany) were hybridized under standard conditions in the presence of 20% (Arch915 and Rotcl2) or 30% (Eub338 and Rotcl1) formamid and DAPI (4',6-diamidino-2-phenylindole, final conc. 20 ng ml⁻¹) at 42°C for 2 h (Zarda et al., 1997). After hybridization and washing, slides were mounted with Citifluor solution (Citifluor, Canterbury, UK) and examined with a Zeiss Axiolpho microscope fitted for epifluorescence with a high pressure mercury bulb (50 W) and filter sets 02 (Zeiss; G365, FT395, LP420) and HQ-Cy3 (AHF Analysentechnik, Tübingen, Germany; G 535/50, FT 565, BP 610/75) at 400 x magnification (Zeiss Plan-Neofluar 40x/1.30 oil).

Bacteria and Archaea were analyzed by image analysis in 14 images per sample with up to 400 cells per image (Schönholzer et al., 1999). Cell numbers and cell sizes were determined based on measurements of area and perimeter for each microorganism or bacterial agglomerate. After subsequent determination of fiberlength and fiberwidth (Anonymous, 1992; Russ, 1995), biovolumes were calculated (Bloem et al., 1995).

One-µl of cells suspended in 80 µl of water (not fixed in paraformaldehyd) was used as template in a PCR-based approach amplifying the 16S rRNA gene of all organisms belonging to the domain Archaea. Genes of 16S rRNA were amplified by PCR on a MJR PTC-200 thermal cycler (MJ Research, Watertown, MA, USA), as previously described (Grosskopf et al., 1998). Amplified fragments were cloned as described in Tonolla et al. (1999) and 16S rDNA sequences deposited in the EMBL/GenBank databases with accession numbers AJ249242 to AJ249244.

The phylogenetic positions of the clones were determined with EMBL/GenBank databases searches using FASTA (Pearson and Lipman, 1988). Sequences of clones and selected microorganisms were aligned using the CLUSTAL W program (version 1.6) (Thompson et al., 1994) and a phylo-
3.3 Results and Discussion

3.3.1 Chemical Analyses

Ground water collected from monitoring wells located along the center flow line at the Studen site (Fig. 3-1) exhibited a chemical zonation that is typical for contaminated aquifers undergoing intrinsic bioremediation (Anderson and Lovley, 1997). Concentrations of oxidants such as $O_2$, $NO_3^-$, $SO_4^{2-}$ were depleted, whereas concentrations of reduced species such as $Fe^{2+}$ and $CH_4$ were elevated within the contaminated zone (wells PS3, PS4, PS5, P5) relative to background concentrations (well P20) (Tab. 3-1). Since oxygen concentrations were already low at the background well P20, little mineralization could be attributed to aerobic respiration (Bolliger et al., 1999). Nitrate was almost completely consumed by the time ground water reached well PS3 (Tab. 3-1), indicating that the nitrate reduction zone was located upgradient of PS3. Although $Fe^{2+}$ concentrations were generally low along the center flow line, a slight increase in $Fe^{2+}$ concentrations was observed within the contaminated zone (Tab. 3-1). This result, however, has to be interpreted with caution, since quantitative measurements of dissolved $Fe^{2+}$ are influenced by a variety of geochemical reactions, which may remove $Fe^{2+}$ permanently from the aqueous phase (Baedecker et al., 1992). Sulfate concentrations decreased from well P20 to wells PS3 and PS4, and it was completely consumed by the time ground water reached PS5 (Fig. 3-2 A). Measured $CH_4$ concentrations in PS5 and PS4 were near saturation (Yamamoto et al., 1976), indicating methanogenic conditions in this portion of the aquifer (Fig. 3-2 B). Methane was also detectable in PS3 and P5, although in lower concentrations. These results indicate that the wells PS3, PS4, and P5 were located in a transition zone where conditions for both sulfate reduction and methanogenesis are found. In well PS5, however, conditions entirely favored methanogenesis.
Table 3-1: Chemical and isotopic ground water parameters along the central flow line

<table>
<thead>
<tr>
<th>Well</th>
<th>Sampling Date</th>
<th>Temp [°C]</th>
<th>pH</th>
<th>Alk. [mM]</th>
<th>DIC [mM]</th>
<th>O₂ [mM]</th>
<th>NO₃⁻ [μM]</th>
<th>Fe²⁺ [μM]</th>
<th>SO₄²⁻ [mM]</th>
<th>CH₄ [μM]</th>
<th>δ¹³C [%o]</th>
<th>RSD %a</th>
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</thead>
<tbody>
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<td>10.8</td>
<td>7.01</td>
<td>6.55</td>
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<td>0.277</td>
<td>1.9</td>
<td>0.372</td>
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<td>6.99</td>
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<td>0.00</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>12.4</td>
<td>7.08</td>
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<td>7.26</td>
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<td>7.6%</td>
<td>4.5%</td>
<td>-</td>
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<td>16.4%</td>
<td>-</td>
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<td>-</td>
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<td>0.228</td>
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<td>1.5%</td>
<td>-</td>
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<td>39.1%</td>
<td>12.3%</td>
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<td>8.82</td>
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<td>0.04</td>
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<td>0.8%</td>
<td>-</td>
<td>32.9%</td>
<td>161.2%</td>
<td>12.5%</td>
<td>115.5%</td>
<td>3.0%</td>
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a) Relative Standard Deviation
3.3 Results and Discussion

Microbial mineralization processes led to an increase in DIC concentrations within the contaminated zone of the site (Fig. 3-2 C). The pH decreased within the contaminated zone, as was expected from the production of CO$_2$ by PHC mineralizing microorganisms (Tab. 3-1).

Figure 3-2: Selected chemical parameters along the center flow line. A: Sulfate; B: Methane; C: DIC; D: $\delta^{13}$C of DIC

Alkalinity, pH, and DIC concentrations as well as the concentrations of oxidants and reduced species remained fairly invariable in space and time during the observation period. Although ground water temperature varied seasonally, temperature variations did not appear to affect any of the chemical parameters measured (Tab. 3-1). Large relative standard deviations (RSD) were calculated for CH$_4$ concentration in PS3 and sulfate concentrations in PS3 and PS4 (Tab. 3-1). Since these wells are within a transition zone of sulfate reduction and methanogenesis, these fluctuations may be attributed to a difference in sensitivity of these processes to varying environmental parameters or to a gradual shift from almost exclusively sulfate reducing conditions towards more methanogenic conditions. Similar observations were made by Baedecker et al. (1993) at the downgradient edge of a body of crude oil, where concentrations of CH$_4$ and Fe$^{2+}$ increased more than an order of magnitude during a 5 year observation period, indicating that this aquifer became more reducing at that specific location. Nevertheless, at the Studen site the general stability of the measured chemical parameters suggests stable biological conditions with specific microbial processes in distinct regions of the aquifer during the observation period.

### 3.3.2 Stable Carbon Isotope Analyses

Stable carbon isotope ratios of DIC upgradient (well P20) and downgradient (well P25) of the contaminated zone were similar and thus were assumed to represent the background $\delta^{13}$C of DIC in the Studen aquifer (Tab. 3-1). Wells within the contaminated zone showed higher DIC concentrations and a different $\delta^{13}$C compared to the background values. In well PS3, $\delta^{13}$C was always more negative than the background value (Fig. 3-2 D). Therefore, non-methanogenic degradation of heating oil ($\delta^{13}$C = -27.8‰, Bolliger et al., 1999) was responsible for the mineralization of PHC upgradient of well PS3. In wells PS4, PS5, and P5, $\delta^{13}$C values were more positive than in well PS3, indicating methanogenesis as an ongoing process in the vicinity of these wells. This observation was supported by the presence of CH$_4$ in these wells (Fig. 3-2 B). The most positive $\delta^{13}$C value was measured in well PS5, coinciding with the highest measured CH$_4$ con-
centrations. These results suggest that the highest activity of methanogenesis is located in the vicinity of well PS5. Similar isotopic patterns were previously obtained at other PHC-contaminated field sites (Baedecker et al., 1993; Landmeyer et al., 1996; Hunkeler et al., 1999b).

Small variations over time in $\delta^{13}C$ values were observed in wells outside of the contaminated zone (P20 and P25) (Tab. 3-1). Within the contaminated zone, variations were larger, possibly due to environmental parameters that are known to influence metabolic activity such as groundwater fluctuations that may alter oxidant or substrate availability (Beeman and Suflita, 1990). Moreover, the $\delta^{13}C$ of DIC is an cumulative measurement, which reflects the sum of different biogeochemical reactions.

A considerably more negative $\delta^{13}C$ value was obtained in PS5 during sampling in March 23, 1999, while $\delta^{13}C$ values obtained in other wells on that day showed reasonable agreement with prior measurements at the respective locations (Fig. 3-2 D). We are uncertain why the $\delta^{13}C$ value obtained in PS5 was more negative on that day than on any other day measured, in particular if we consider the corresponding high CH$_4$ and DIC concentrations measured in PS5 on the same day (Fig. 3-2 B&C). However, similar observations were previously made by Landmeyer et al. (1996) in a jet fuel contaminated aquifer under seasonally variable environmental conditions.

At Studen, a progressive shift of the $\delta^{13}C$ of the DIC along the center flow line towards more positive values indicated that PHC mineralization was coupled to the depletion of oxidants and the simultaneous increase of CH$_4$.

3.3.3 Biological Analyses

Total cell numbers detected by automated image analysis after DAPI-staining revealed large differences in samples from wells along the center flow line (Fig. 3-3). The largest numbers were detected in ground water from wells located within the contaminated zone, being at least one order of magnitude higher than those of the background well P20 (below detection limit, < $10^4$ cells ml$^{-1}$).
In samples collected along the center flow line on March 23, 1998, cells hybridizing to probe Eub338, which identified members of the domain Bacteria, accounted for 13 ± 3 to 32 ± 13% of DAPI-stained cells (Fig. 3-3). Probe Arch915, targeting members of the domain Archaea, detected between 9 ± 2 and 31 ± 13% of DAPI-stained cells (Fig. 3-3). The relative abundance of cells belonging to the domain Bacteria were in the same range as has been reported for anoxic lake sediments (13 to 32% in Studen compared to 12 to 20% in lake sediments), whereas the population of Archaea in Studen was significantly larger (9 to 31% compared to 5 to 7%) (Zepp Falz et al., 1999). Our data are in agreement with observations made at another PHC and chlorinated solvent contaminated site where a high abundance of cells belonging to the domain Archaea was also detected (Dojka et al., 1998). We are aware that the obtained ground water samples may not exactly represent actual, active communities in the subsurface, as was previ-
ously discussed by Alfreider et al. (1997). However, a study performed at a crude oil-contaminated site concluded that an average of 15% of the total bacterial population may be suspended in ground water (Bekins et al., 1999).

Figure 3-4: Phylogenetic relationship with known methanogenic species of the 16S rRNA sequences retrieved from well PS5 on August 25, 1998.

Sequence analysis of several clones of a 16S rDNA library from microbial cells obtained from well PS5 showed that sequences related to Methanoseta concilii and Methanospirillum sp. were present in the contaminated aquifer material (Fig. 3-4). In situ hybridization with probes targeting cells belonging to Methanoseta concilii (Rotcl1) and Methanospirillum sp. (Rotcl2) confirmed these data because both detected significant numbers of cells. Probe Rotcl1 identified 10 ± 4% to 26 ± 10% of the cells detected with DAPI (Fig. 3-3), whereas results for probe Rotcl2 were mostly below detection limit (< 10^4 cells ml^-1, not shown). The data from sequence analysis of archael 16S rDNA and subsequent in situ hybridization therefore suggest that the archael population at the Studen site mainly consists of organisms related to Methanoseta concilii (Fig. 3-3 & Fig. 3-4).
Biological parameters in well PS5 were measured over time because of this well’s stable methanogenic conditions and the presence of large total cell numbers. In samples from well PS5, small variations were observed in total cell numbers and microorganisms detected by the probe Eub338 (Fig. 3-5 A). On the other hand, cell numbers detected with probes Arch915 and
3.3 RESULTS AND DISCUSSION

Rotcl11 remained fairly stable during the observation period. Similar results were obtained for average cell volumes (Fig. 3-5 B). Although similar in number, cells belonging to the domain Bacteria had less volume than cells belonging to the domain Archaea, indicating that their growth conditions may not have been very favorable. As expected, the volume of cells detected by probe Rotcl11 is closely related to that of cells detected by probe Arch915 (Fig. 3-5 B).

It was previously hypothesized that the terminal step of PHC degradation in the methanogenic zone of a contaminated aquifer is aceticlastic methanogenesis (Revesz et al., 1995; Dojka et al., 1998). Our results clearly strengthen this hypothesis. In well PS5, a high CH₄ concentration was measured, the δ¹³C of DIC was more positive than the background (as is expected for methanogenic activity), and data obtained by sequence analysis and in situ hybridization indicated that the microbial population was almost exclusively dominated by one species only, which is capable of producing CH₄ (*Methanoseta concilii*). *Methanoseta*, however, are known to generate energy only through aceticlastic methanogenesis.

Thus, the here presented novel combination of chemical, isotopic and biological analyses allows detailed insights and provides valuable information for the understanding and characterization of biogeochemical interactions and processes related to intrinsic remediation of PHC-contaminated aquifers.

Acknowledgements

The authors thank J.P. Clément (Amt für Gewässerschutz und Abfallwirtschaft, Kanton Bern) for his cooperation at the field site. Special thanks to P. Höhener and B. Kofmel for their support in the laboratory and during sample collection. This work was supported by the Swiss National Science Foundation (Priority Programme Environment).
Sulfur isotope fractionation during microbial sulfate reduction by toluene degrading bacteria


Abstract

Sulfate-reducing bacteria contribute considerably to the mineralization of petroleum hydrocarbons (PHC) in contaminated environments. Stable sulfur isotope fractionation during microbial sulfate reduction was investigated in batch experiments using various sulfate concentrations with different toluene-degrading, sulfate-reducing bacteria. The marine strain Desulfobacula toluolica, the fresh water strain PRTOL1, and an enrichment culture from a heating oil-contaminated site were tested for their fractionation behavior under conditions resembling those of a contaminated environment.

The observed sulfate reduction rates ranged from 0.494 ± 0.019 to 0.007 ± 0.002 μmol cm⁻³ d⁻¹. The obtained enrichment factors (ε) for the fractionation of stable sulfur isotopes during sulfate reduction by the studied microorganisms are between 19.8 ± 1.8 and 46.9 ± 4.1‰. These enrichment factors are similar to those reported previously for mesophilic sulfate reducing bacteria, although 46.9‰ is one of the highest enrichment factors ever
measured in a pure culture. Toluene has obviously no major effect on the stable sulfur isotope fractionation even though it is more difficult to degrade than lactate or ethanol, which had been used in most previous studies. Surprisingly, there was no significant correlation between sulfate concentrations and enrichment factors. Moreover, each of the studied organisms showed a different trend when enrichment factors were compared to sulfate reduction rates; D. toluolica had a higher enrichment factor at higher rates, PRTOL1 displayed lower enrichment factors at higher rates, whereas the enrichment culture showed no trend at all. The obtained results allow a better understanding of stable sulfur isotope ratios measured at PHC contaminated environments.

4.1 Introduction

Sulfate reduction is an important anaerobic metabolism at various petroleum hydrocarbon (PHC)-contaminated field sites (e.g. Reinhard et al., 1997; Thierrin et al., 1995; Vroblesky et al., 1996). During dissimilatory sulfate reduction, bacteria reduce sulfate (SO\textsubscript{4}\textsuperscript{2-}) to sulfide (H\textsubscript{2}S) in order to obtain energy for the oxidation of carbon sources. In PHC-contaminated sites, sulfate-reducing microorganisms contribute to the mineralization of organic contaminants to CO\textsubscript{2} and water. Therefore, they play an important role in the detoxification of PHC-polluted environments.

Sulfur in natural environments consists largely of two stable isotopes, \textsuperscript{32}S (95.02% natural abundance) and \textsuperscript{34}S (4.21% natural abundance) (Hoefs, 1997). Dissimilatory reduction of sulfate by sulfate-reducing bacteria results in the enrichment of \textsuperscript{32}S in produced sulfide and the depletion of \textsuperscript{32}S in the remaining SO\textsubscript{4}\textsuperscript{2-} pool (e.g. Harrison and Thode, 1958). This isotope fractionation is due to the lower bond strength of \textsuperscript{32}S-O as compared to that of \textsuperscript{34}S-O in the sulfate molecule; the \textsuperscript{32}S-O bond is preferentially broken during the reduction process by sulfate-reducing bacteria. In laboratory studies isotopic fractionation of up to 46.0% has been observed for pure cultures of mesophilic sulfate-reducing bacteria (Kaplan and Rittenberg, 1964).
Sulfur isotope fractionation during sulfate reduction has been observed in various ground water studies (e.g., Alewell and Giesemann, 1996; Robertson and Schiff, 1994) as well as at different contaminated sites, e.g., at a waste disposal site in Great Britain (Bottrell et al., 1995), and at a former lignite mining area in Germany (Asmussen and Strauch, 1998). Differences in isotopic composition between sulfate and sulfide were attributed to microbial activity within these different environments. Unfortunately, little is known about the stable sulfur isotope fractionation in PHC-contaminated environments subject to sulfate reduction.

Several laboratory pure culture studies were performed to explain sulfur isotope fractionation observed in nature. Using resting-cell and chemostat experiments, it was found that the degree of sulfur isotope fractionation by the mesophilic Desulfovibrio desulfuricans is inversely related to the specific sulfate reduction rate (sSRR, mass sulfate cell$^{-1}$ time$^{-1}$) (Chambers et al., 1975; Harrison and Thode, 1958; Kaplan and Rittenberg, 1964). Several studies indicated that sSRR is related to factors such as sulfate concentration, temperature, and/or carbon source (Harrison and Thode, 1958; Kaplan and Rittenberg, 1964; Kemp and Thode, 1968). Experiments with various sulfate-reducing bacteria carried out at different temperatures using lactate as carbon source showed that stable sulfur isotope fractionation is in the range between 0 and 40‰, independent of growth-temperature (Böttcher et al., 1999). Most pure culture studies for investigation of stable sulfur isotope fractionation were designed to obtain maximum growth rates. Therefore, high sulfate concentrations and readily available carbon sources such as lactate and ethanol were used in most studies. However, such conditions are rarely encountered in PHC-contaminated aquifers, where metabolic activity is often electron acceptor (here SO$_4^{2-}$) limited and carbon sources are complex PHC-constituents or their metabolic breakdown products.

In the present study, we report on sulfur isotope fractionation observed in batch cultures grown under conditions resembling those in sulfate-reducing PHC-contaminated environments. For this purpose the marine strain Desulfobacula toluolica (Rabus et al., 1993), the fresh water strain PRTOL1
(Beller et al., 1996), and an enrichment culture from a PHC-contaminated site (Bolliger et al., 1999) were grown at three different sulfate concentrations using toluene as sole carbon source. Stable sulfur isotope ratios ($^{34}\text{S} / ^{32}\text{S}$) were measured in sulfate and sulfide. Enrichment factors were calculated and related to studied organisms, initial sulfate concentrations, and sulfate reduction rates.

4.2 Material and Methods

4.2.1 Organisms and cultivation

*D. toluolica* (Rabus et al., 1993) was obtained from Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany (DSMZ 7467). Active cultures of the toluene-degrading, sulfate-reducing bacterium PRTOL1 (Beller et al., 1996) were kindly provided by D.R. Boone and H.R. Beller. The inoculum for the enrichment culture was obtained from a heating oil-contaminated aquifer in Studen, Switzerland (Bolliger et al., 1999) and maintained under sulfate-reducing conditions with toluene as sole carbon source.

*D. toluolica* was cultivated in a basal medium as described in Rabus et al. (1993), whereas freshwater organisms (PRTOL1 and enrichment culture) were grown in basal media described in Beller et al. (1996). For the experiments sulfate was added as Na$_2$SO$_4$ (MicroSelect, Fluka). The media were supplemented with trace element mixture (EDTA-chelated for *D. toluolica*, non-chelated for PRTOL1 and enrichment culture), selenite-tungstate solution, bicarbonate solution, vitamin mixture, vitamin B$_{12}$ solution, and sulfide solution as described in Widdel and Bak (1992). Toluene (purum, Fluka) was added as dilute solution (2% v/v) in an inert lipophilic solvent used as carrier phase (Mineral oil, MicroSelect; Fluka) to maintain a nearly constant toluene concentration in the aqueous phase of about 0.25 mM during the experiments (Rabus et al., 1993). Thirty ml of carrier phase was added per liter of medium. The final pH of the media was approximately 7.1. The media were inoculated with 5% (v/v) pregrown cultures. Bacteria
were cultured in 120 ml serum bottles with a small headspace (90% N₂, 10% CO₂) at 28°C in the dark.

**4.2.2 Microcosm experiments**

Three different initial sulfate concentrations (about 0.3, 1, and 3 mM) were used to inoculate microcosms containing the freshwater organisms PRTOL1 and the enrichment culture. Strain *D. toluolica* was inoculated with ten times more sulfate (about 3, 10, and 30 mM) in order to match sulfate concentrations typically encountered by this strain in marine environments. Control experiments were performed with either no toluene added to the carrier phase but inoculated with bacteria and with no bacteria inoculated but with toluene in the carrier phase. Sulfate concentration was monitored during incubation, and at certain intervals microcosms were analyzed in triplicates. Experiments were terminated when either the initially supplied sulfate was consumed or the reduction of sulfate ceased.

**4.2.3 Chemical analysis**

After vigorous shaking, 0.2 ml of medium was removed from the microcosms and immediately dispensed in 4.8 ml 20 mM zinc acetate solution for sulfide analysis. Cline reagent (0.5 ml) was immediately added and 20 min later absorbance at 670 nm was measured by spectrophotometry (Cline, 1969). An additional 2 ml of medium were withdrawn for sulfate measurement and counting of bacterial numbers. After centrifugation (10 min at 13000 rpm), the supernatant was used for anion measurement by ion chromatography (IC-100, Dionex) whereas the remaining pellet was further treated for determination of bacterial numbers (see below).

**4.2.4 Bacterial numbers**

The remaining pellet was fixed in 4% paraformaldehyde in phosphate buffered saline (PBS, Sambrook et al., 1989) over night and washed with 50% (v/v) ethanol/PBS. Cell suspensions were stored at −20°C in 50% (v/v) ethanol/PBS.
Before application to slides, the paraformaldehyde fixed cell suspensions were sonicated mildly 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 96% ethanol for 3 min each. The cells were stained with DAPI (4',6-diamidino-2-phenylindole, final concentration 200 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 AxioIphot microscope fitted for epifluorescence with a high pressure mercury bulb as described in detail by Zarda et al. (1997). Cells were counted in twenty images per sample. The mean of the cell numbers counted at the begin and at the end of an experiment were used for the calculation of the sSRR.

4.2.5 Isotope Analysis

For isotopic measurements sulfide was precipitated as ZnS by addition of 5 ml 1 M zinc acetate solution to the microcosm. 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 then precipitated as BaSO\(_4\) by adding 5 ml 1.2 M BaCl\(_2\) solution to the filtrate and the precipitate recovered on a 0.45 μm HVLP membrane filter (Millipore). Both filters were dried at 80°C over night. Excess mineral oil was removed from filtrates using hexane.

For stable sulfur isotope ratio measurements approximately 700 μg of BaSO\(_4\) or 300 μg of ZnS were weighted in tin cups. Vanadium pentoxide was added as catalyst in the amount of about two times the weight of the sample. Sulfur isotopes were subsequently measured on a FISONS OPTIMA mass spectrometer coupled in continuous-flow with a Carlo Erba Elemental analyzer. The system was calibrated using the international standards IAEA NZ1, IAEA NZ2, and NBS127 (IAEA, 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 according to:
\[ \delta^{34}S \left[\% e \right] = \left( \frac{\delta^{34}S}{\delta^{32}S} \right)_{\text{sample}} / \left( \frac{\delta^{34}S}{\delta^{32}S} \right)_{\text{V-CDT}} - 1 \} \cdot 1000 \] (1)

The isotope enrichment factor (\(\varepsilon\)) is a measure in per mill for the fractionation of stable isotopes during a reaction and allows for easy comparison of different reactions. In a closed system, enrichment factors can be determined using Rayleigh distillation equations (Mariotti et al., 1981). In this study enrichment factors were derived from the slope (\(\varepsilon\)) of the linear regression of measured \(\delta^{34}S\) values of sulfate and sulfide according to (Böttcher et al., 1999):

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

\[ \delta^{34}S(\text{H}_2\text{S}) = \delta^{34}S(\text{SO}_4^{2-})_0 - \varepsilon \frac{(f \ln f)}{(1-f)} \] (3)

In these equations, \(f\) denotes the fraction of unreacted sulfate, and \(\delta^{34}S(\text{SO}_4^{2-})_0\) represents the initial isotopic composition of dissolved sulfate. Measured sulfide data [\(\delta^{34}S(\text{H}_2\text{S})\)] were corrected for the initial sulfide concentration in the microcosms and its isotopic composition.

### 4.3 Results and Discussion

Strains *D. toluolica*, PRTOL1, and the enrichment culture from the heating oil-contaminated aquifer in Studen, Switzerland, reduced sulfate in microcosms inoculated with toluene as sole carbon source and sulfate as sole electron acceptor (Fig. 4-1). Note that *D. toluolica* was inoculated with 10 times higher initial sulfate concentrations than PRTOL1 and the enrichment culture in order to match sulfate concentrations in marine environments.
Sulfate utilization during microcosm incubation for three different organisms. Zero-order sulfate reduction rates (SRR) were obtained from the slopes of linear regressions of the measured sulfate concentrations versus time. Data from PRTOL1 and enrichment culture inoculated with 0.3 mM initial sulfate concentration were also fitted assuming first-order kinetics (dotted lines).

* Note that sulfate concentrations for *D. toluolica* were 10 times larger than displayed in figure.

*D. toluolica* reduced nearly all of the added sulfate in batches inoculated with 3.7 and 10.3 mM sulfate (Fig. 4-1). Sulfide produced during sulfate reduction can be toxic or inhibitory for microorganisms (Madigan et al., 2000). Obviously, sulfide concentrations in these batches stayed below...
inhibitory levels. However, reduction of sulfate ceased in batches inoculated with 29 mM sulfate after the reduction of about 18 mM sulfate. Sulfate concentration remained constant for about 45 days while ~10.5 mM sulfate was still in solution (not shown). Presumably, the threshold concentration of sulfide was reached for *D. toluolica* and caused the reaction to stop. The last sample was taken after 81 days (not shown) but was not included in the rate calculation because of the inhibition.

Strain PRTOL1 was able to utilize most of the sulfate in batches inoculated with 0.3 and 1.1 mM sulfate, but in batches inoculated with 3.1 mM initial sulfate concentration only about 25% of the sulfate was reduced over a period of 98 days. The concentration remained constant for another 20 days when the last sample was taken (Fig. 4-1). The organism PRTOL1 is highly sensitive to sulfide with inhibition reported in the range of 1 to 3 mM (Beller et al., 1996). During the experiments sulfide was not precipitated by means of ferrous iron as proposed by Beller et al. (1996). Therefore, sulfide accumulated and stopped further sulfate reduction after day 98.

The enrichment culture was able to reduce the supplied sulfate in all three experiments with the concentrations of 0.3, 1, and 4 mM sulfate, respectively (Fig. 4-1). Sulfate concentrations remained unchanged in controls inoculated without bacteria and in controls inoculated with bacteria but with no toluene added to the lipophilic carrier phase (not shown).

We calculated zero order sulfate reduction rates (SRR; mass sulfate volume\(^{-1}\) time\(^{-1}\)) using linear regression analysis (Fig. 4-1). SRR varied depending on the different strains. *D. toluolica* reduced sulfate more than an order of magnitude faster than the studied freshwater cultures (Tab. 4-1). However, at low initial sulfate concentration (3.7 mM) the rate significantly decreased for *D. toluolica*; nevertheless, it was still bigger than rates measured for PRTOL1 and enrichment culture incubated with the same initial sulfate concentration (Tab. 4-1). Different SRR for the studied organisms may be explained by differences in cell densities of the respective inoculum. Batches of pregrown cultures had different cell densities and therefore bacterial numbers in the microcosms at the beginning of the experiment varied among the different strains (Tab. 4-1).
Table 4.1: Compilation of measured sulfate reduction rates (SRR) and specific sulfate reduction rates (sSRR).

| Initial \( \text{SO}_4^{2-} \) concentration (mM) | Desulfobacula toluolica enrichment culture | PRTOL 1 | 
|---|---|---|---|
| 29.0 | 0.449 ± 0.004 | 9.0 ± 0.1 | 0.007 ± 0.002 |
| 10.3 | 0.049 ± 0.019 | 12.0 ± 0.013 | 0.013 ± 0.003 |
| 3.7 | 0.024 ± 0.003 | 12.0 ± 0.014 | 0.014 ± 0.003 |

Error \(^a\): 95% confidence interval.

\(^a\) 95% confidence interval.
The lowest rate was obtained in the experiment of PRTOL1 incubated with 3.1 mM sulfate. Probably, produced sulfide did not only stop the reaction, but acted as an inhibitor at smaller concentrations.

Sulfate reduction in the experiments of PRTOL1 and the enrichment culture incubated with 0.3 mM sulfate followed first-order kinetics (Fig. 4.1). Rate constants of $0.069 \pm 0.005$ day$^{-1}$ and $0.053 \pm 0.007$ day$^{-1}$ were obtained for PRTOL1 and the enrichment culture, respectively. These rate constants are an order of magnitude larger than rate constants measured for toluene degradation in a sulfate reducing aquifer (Thierrin et al., 1995). High toluene concentrations and other environmental factors (e.g. temperature) observed at the site may prevent a rapid degradation of toluene compared to our batch experiments conducted under optimized conditions. To allow a direct comparison of rates between all our experiments, zero-order rates were also computed for PRTOL1 and the enrichment culture inoculated with 0.3 mM sulfate. However, only the first twelve respectively nine data points were considered for this calculation (Fig. 4.1).

Habicht and Canfield (1997) emphasized that it is the sSRR and not the SRR that controls fractionation. The sSRR obtained in this study (Tab. 4.1) are comparable to or faster than those measured in other experiments. For example Kaplan and Rittenberg (1964) obtained $7.5 \cdot 10^{-16}$ to $1.1 \cdot 10^{-14}$ mol cell$^{-1}$ day$^{-1}$ with cultures of Desulfovibrio desulfuricans grown on various carbon substrates, the same organisms grown in continuous cultures on lactate exhibited a sSRR of $2.4 \cdot 10^{-17}$ to $9.6 \cdot 10^{-15}$ mol cell$^{-1}$ day$^{-1}$ in a study of Chambers et al. (1975) and Böttcher et al. (1999) reported a sSRR $6.0 \cdot 10^{-15}$ mol cell$^{-1}$ day$^{-1}$ for a thermophilic gram-negative bacterium grown at 60°C. However, differences of sSRR between different studies should not be over-emphasized. Even though sSRR are an important value to compare results, one has to be aware that the sSRR is a calculated value effected by the uncertainty of the determination of the SRR and of the numbers of bacteria. Various methods were applied to measure SRR (e.g. sulfide production, sulfate consumption) and total bacterial numbers (e.g. direct counts, optical density). In addition, individual experiments were designed differently, e.g., experiments were performed in chemostats (Chambers et al., 1975),
whereas other experiments performed in microcosms were terminated after the utilization of less than 5% sulfate (Kaplan and Rittenberg, 1964).

During sulfate reduction the isotopic composition of the remaining sulfate and the produced sulfide changed in our microcosms as expected for a reaction in a closed system limited in sulfate (Thode, 1988). The combined evaluation of the measured isotope data for sulfate and sulfide demonstrates the expected linear relationship according to Equations 2 and 3 (Fig. 4-2). The enrichment factor (ε) corresponds to the slope of the linear regression of the measured δ³⁴S values (Tab. 4-2). It is important to emphasize that enrichment factors reported in this study were calculated from 10 to 25 independent data points, therefore, reliable 95% confidential intervals could be calculated (Tab. 4-2).

Table 4-2: Summary of obtained enrichment factors (ε).

<table>
<thead>
<tr>
<th>Initial (\text{SO}_4^{2-}) concentration</th>
<th>data points</th>
<th>ε</th>
<th>Error a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>mM</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Desulfobacula toluolica</td>
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<td>21</td>
<td>32.6</td>
</tr>
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<td></td>
<td>10.3</td>
<td>15</td>
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</tr>
<tr>
<td></td>
<td>3.7</td>
<td>14</td>
<td>24.9</td>
</tr>
<tr>
<td>PRTOL 1</td>
<td>3.1</td>
<td>21</td>
<td>46.9</td>
</tr>
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<td></td>
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</tr>
<tr>
<td></td>
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</tbody>
</table>

a) 95% confidence interval

Enrichment factors observed in this study are similar to those reported by other authors for different strains and under different conditions (e.g. Böttcher et al., 1999; Chambers et al., 1975; Kaplan and Rittenberg, 1964).
However, it is noteworthy that the highest previously measured enrichment factor in a pure culture was 46.0% (Kaplan and Rittenberg, 1964). The enrichment factor obtained in our experiments for the batch of PRTOL1 inoculated with 3.1 mM sulfate is the second pure strain showing a fractionation of about 46%.

Figure 4-2: Sulfur isotope fractionation in sulfate and sulfide during sulfate reduction by the studied organisms with toluene as sole carbon source at 28°C. $\delta^{34}S(SO_4^{2-})$ values (closed symbols) are plotted versus $-\ln f$ according to equation 2 whereas $\delta^{34}S(H_2S)$ (open symbols) values are plotted versus $\{(f \ln f) / (1-f)\}$ according to equation 3. Data are fitted using a linear regression analysis.
No significant correlation was found between initial sulfate concentrations in the different experiments and obtained enrichment factors (Tab. 4-2). This is in contrast to a study by Harrison and Thode (1958) in which enrichment factors decreased for initial sulfate concentrations below 0.6 mM. We did not observe such an effect both in experiments where sulfate concentration dropped below 0.6 mM during the experiment nor in experiments where 0.3 mM sulfate was initially added (Fig. 4-2).

![Figure 4-3: Obtained enrichment factors for all experiments versus (A) sulfate reduction rate (SRR) and (B) specific sulfate reduction rate (sSRR). Symbols indicate the studied organisms (● Desulfobacula toluolica; ■ PRTO1; ○ enrichment culture). Error bars show the 95% confidence interval.](image)

To allow for a comparison between different studies, enrichment factors have to be related to the sSRR (e.g. Chambers et al., 1975; Habicht and Canfield, 1997; Kaplan and Rittenberg, 1964). In several studies using either lactate or ethanol as carbon sources, it was shown that the fractionation is an inverse function of the sSRR. (Chambers et al., 1975; Harrison and Thode, 1958; Kaplan and Rittenberg, 1964; Kemp and Thode, 1968). In this study with toluene as carbon source there is no obvious correlation.
between fractionation factors and either SRR or sSRR (Fig. 4-3 A&B). Besides, each strain exhibited a different trend; *D. toluolica* showed higher enrichment factors at higher rates, PRTOLI showed higher enrichment factors at lower rates, and no trend was observed for the enrichment culture (Fig. 4-3 A&B). In order to judge the fractionation of the here studied organisms in respect to SRR and sSRR, more data over a wider range of SRR and bacterial numbers would be required.

### 4.4 Conclusions

Sulfate-reducing microorganisms discriminate between $^{34}\text{SO}_4^{2-}$ and $^{32}\text{SO}_4^{2-}$ during dissimilatory reduction of sulfate. The resulting isotope fractionation varies depending on reduction rates and factors influencing the reduction rate such as sulfate concentration, carbon source, organisms or temperature. In this study toluene served as sole carbon source. Different organisms reduced sulfate at different SRR most likely due to varying bacterial densities in the inoculum. The sSRR showed closer agreement and varied only within about an order of magnitude. Enrichment factors obtained from the different organisms were independent of the initial sulfate concentration and are in the same range as was previously reported for organisms grown with lactate and ethanol as carbon sources. Thus, neither the complex carbon source toluene nor the type of sulfate reducing bacteria seemed to have a major effect on the process of fractionation. We were unable to observe a significant correlation between enrichment factor and SRR or sSRR, as was previously reported for other pure culture experiments with ethanol or lactate as sole carbon source. Interestingly, the studied organisms show completely different individual trends in this respect. However, in this study only a small range of SRR was covered and relatively few data points were available for comparison.

Even though batch experiments can not replace field evaluations, we hypothesize that in PHC-contaminated environments in which relatively easily degradable hydrocarbon compounds such as toluene are present
enrichment factors between 20 and 46‰ may be obtained, independent of the prevailing sulfate concentrations.

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5.1 Engineered versus intrinsic bioremediation in Studen

In Studen it was decided to use MNA because indigenous microbial populations were shown to be capable of degrading the spilled heating oil. More importantly, no drinking water wells were located downgradient of the spill and therefore there was no immediate threat to public health (Chapter 2). Engineered in situ bioremediation (i.e. addition of $O_2$, $NO_3^-$ and nutrients, see Tab. 1-2) could have been also a possible remediation option for the Studen site. However, in retrospective it was a good choice not to use engineered in situ remediation for the following reasons. i) The costs of such a remediation can add up significantly over the years due to the maintenance of the installation and the continuous injection of oxidants and nutrients. ii) The site in Studen is located in an urban area. The quality of life of local residents would be significantly hampered by installations needed for engineered in situ remediation such as pumps, pipes, and above ground mixing tanks. It is therefore questionable if the perception of an in situ technique would have been as favorable as it is today for MNA. iii) The subsurface in Studen is very heterogeneous. There are many preferential
flow paths, therefore the injected water is not likely to reach all regions of
the aquifer (Gabriel and Hug, 1997).

A positive side effect of engineered in situ bioremediation with circu-
lating ground water is the continuous removal of heating oil from the aqui-
fer by physical means (Hunkeler et al., 1999b). For any in situ remediation
strategy it is important to remove as much free phase PHC from the subsur-
face as possible. In Studen, free floating PHC was removed by ground water
pumps with oil separators, which resulted in the removal of ~34'000 liters
heating oil.

It is speculative if the site in Studen would have been remediated faster
if engineered in situ bioremediation had been applied. Fact is that seven
years after the accident, i.e. after seven years of MNA, the plume seems to
be stable and biodegradation of PHC occurs without external addition of
oxidants. In Studen, history to date indicates that the regulatory agencies
made a wise decision to choose a passive remediation technology with
respect to costs, applicability of MNA, and the acceptance by residents.

5.2 Time frame of monitored natural attenuation

One of the major limitations of MNA is the uncertain time frame of the
remediation (Wiedemeier et al., 1999). Up to date it is hard to predict when
certain clean up criteria will be met. However, this information is very
important for stakeholders (i.e. authorities, community) when MNA is dis-
ussed as a remediation strategy (EPA, 1997). It should be possible to esti-
mate the duration of a remediation with a detailed 3D-model. In order to be
able to create such a model the following information on volume, composi-
tion, and hydrogeology need to be available.

Without the knowledge of the volume of the remaining contaminants
in the subsurface, an accurate time frame prediction is not possible. The
amount of PHC in the subsurface and its chemical composition must be
determined either by estimation from PHC contents in soil obtained by drill-
ing or by evaluating historic data. In addition, partitioning tracers such as
$^{222}$Rn have been shown to be a valuable tool to quantify PHC in contaminated aquifers (Hunkeler et al. 1997).

The composition of the fuel mixture has to be known and considered for a long-term prediction. More soluble PHC components (e.g. BTEX) are dissolved more rapidly according to Raoult's law (Schluep, 2000). Less soluble components (e.g. polyaromatic hydrocarbons) might dissolve more slowly or do not dissolve at all because they are strongly sorbed to the soil matrix. The resulting continuous change in dissolved PHC compounds might alter the degradation rates because some of the heavier PHC are more difficult to degrade or not even bioavailable (Howard et al., 1991).

Furthermore, the hydrology and hydrogeology of the aquifer need to be accurately assessed and detailed depth resolved ground water data are needed to establish an accurate 3D-model (Chapelle, 1999; Merkt and Stebler, 1998).

5.3 Vertical extension of the plume

In PHC-contaminated aquifers, the pool of spilled PHC is floating on the ground water table and therefore tends to spread laterally (Cole, 1994). The zone where PHC are found as a free phase on the ground water table is designated source area (ASTM, 1995). Below the source area a plume of dissolved PHC develops, which is typically larger than the source area (Fig. 1-2; Wiedemeier et al., 1999). The plume usually exhibits reduced conditions due to the microbial activity involved in the degradation of dissolved PHC compounds (Chapter 1 & 2; Anderson and Lovley, 1997; Baedecker et al., 1993). In Chapter 2 the assessment of intrinsic bioremediation processes in a plume is described. Here the implication of the vertical extension of the plume on the assessment at the Studen site is discussed. Sulfate concentrations along the plume centerline serve as a selected example to substantiate this discussion (Fig. 5-1, excerpt from Fig. 2-5). Sulfate concentrations in ground water decrease gradually in the contaminated area, and in well PS5 all sulfate is consumed. However, sulfate concentrations as high as the back-
ground values (well P20) are measured in well P5 and further downgradient in well P25 and well P27 (Fig. 5-1). Several explanations are possible to explain this unexpected concentration increase downgradient of well PS5.

Figure 5-1: Sulfate concentrations along the plume centerline on June 17, 1996. (Excerpt from figure 5, chapter 2). Ground water flow direction is from left to right.

Dispersion contributes to the mixing of reduced ground water with pristine ground water along the fringes of the plume. However, dispersion perpendicular to the main flow direction is generally too small to explain the high sulfate concentrations in well P5 and well P25 (Gelhar et al., 1992). Recharge can be another cause of ground water dilution and a source of additional oxidants (Vroblesky and Chapelle, 1994). This is unlikely at the Studen site because the ground water table is 3 to 4 meters below ground and precipitation is only 0.44 m year⁻¹ (Biaggi et al., 1994). In addition, large parts of the site are not affected by infiltration due to buildings and roads. Last and most likely, the increase in sulfate concentration is an artifact of sampling due to the design of the monitoring wells (Tab. 2-1). Wells
PS3, PS4, and PS5 penetrate the aquifer < 1 meter, whereas wells P5, P25, and P27 have a screened length of 2.08, 3.99 and 3.72 meters, respectively. The samples from a particular well represent a flux-weighted average concentration of the screened section of the well. Because of the limited vertical extension of plumes it seems likely that during sampling in deep monitoring wells reduced ground water of the plume is mixed with pristine ground water from the depth (Martin-Hayden and Robbins, 1997). However, it is noteworthy that the result of the stable carbon isotope balances as presented and discussed in Section 2.5.5 is not affected by this effect.

In Studen great care was taken to optimize the location of new installed wells. However, the vertical extension of the wells did not receive enough attention. For future investigations and monitoring, it is unfortunate to have such long monitoring wells exactly downgradient of the source area.

However, there are techniques available to circumvent the problem of lacking vertical resolution. In order to probe only a section of a long monitoring well, packers can be installed at a certain depth interval (Barczewski and Marschall, 1989). Unfortunately, the obtained samples are often affected by vertical flow outside of the well. Another method uses two pumps simultaneously at various pumping rate ratios to obtain samples. The vertical concentration profile is calculated from measured concentrations and applied pumping rate ratios (Thullner et al., 2000). Packers and two pump techniques are limited by the time needed for installation and verification before sampling. A more convenient and often used method is the installation of bundle-piezometers. For bundle-piezometers one larger well is drilled and several small wells reaching each to a different depth are then installed and allow so to obtain depth resolved samples. Furthermore, especially at larger sites, several wells can be installed in the same area each screened at a different depth interval. In conclusion, a pattern of wells optimized in location and depth is desirable when MNA is applied at a contaminated site.
5.4 Limits of alkalinity- and DIC-balances

In Chapter 2 it was mentioned that some relevant oxidants and reduced species might be added (e.g. O₂, SO₄²⁻) or removed (e.g. Fe²⁺, H₂S, CH₄) from the ground water system due to various processes such as dissolution, chemical reactions or outgassing through the unsaturated zone. For example, in contaminated aquifers with a high sulfate content in its matrix (e.g. gypsum), it is impossible to determine the exact amount of sulfate effectively turned over during sulfate reduction because sulfate is continuously released from minerals. The product of sulfate reduction, sulfide, is also not a reliable parameter for quantification due to its volatility and reactivity (e.g. precipitation with Fe²⁺ as FeS (Furrer et al., 1996)). Therefore the assessment of intrinsic bioremediation by alkalinity- and DIC-balances calculated from concentrations of oxidants (i.e. O₂, NO₃⁻, SO₄²⁻) and reduced species (i.e. Fe²⁺, Mn²⁺, H₂S and CH₄) is hampered and might lead to wrong conclusions (Fig. 2-7; Hunkeler, 1997).

5.5 Assessment of bioremediation by stable isotope analyses

The problems discussed in Section 5.4 can at least be partially solved by stable isotope methods. Stable isotopes of the same element differ only in their mass, therefore they have almost identical chemical properties (Hoefs, 1997). For this reason stable isotopes are also considered as in situ tracers. The bond strength to neighboring atoms differs slightly due to the different masses of stable isotopes (Hoefs, 1997). Isotope fractionations (i.e. alteration of the stable isotope ratio) occurs either during chemical processes when the breaking of a bond is the rate-limiting step or during physical processes where the mass is of importance (Clark and Fritz, 1997). During biological reactions the stable isotope ratio is often changed.
5.5.1 Stable carbon isotope balance for DIC

It is possible to quantify contributions of additional sources to DIC by stable carbon isotope ratios as shown in Chapter 2. Here assumptions and limitations, which have to be considered when applying this method, are further discussed.

In order to establish a stable carbon isotope balance the following conditions must be met. i) The different sources that contribute to DIC must be known, ii) each source must have a clearly distinguishable stable carbon isotope ratio, and iii) the stable isotope ratio of a source must remain constant during DIC formation.

\[
\begin{align*}
\text{PHC mineralization} & \quad -27.8 \%o \\
\text{Methanogenesis} & \quad \text{Methane} -60 \%o \\
\text{DIC in ground water} & \quad -14.4 \%o \\
\text{Carbonate dissolution} & \quad -1.1 \%o \\
\text{DIC in plume} & \quad -16 \text{ to } -18 \%o
\end{align*}
\]

**Figure 5-2:** Sources and sinks of DIC with $\delta^{13}C$ of DIC as observed in Studen. Methanogenesis at the Studen site produced methane with a $\delta^{13}C$ of approximately -60\%. The remaining DIC is therefore enriched in $^{13}C$, however, to what extent depends on the relevant methanogenic process i.e. CO$_2$ reduction or acetate fermentation (see chapter 3 for more detail).

In Studen there are three different sources of DIC, i.e. PHC mineralization, carbonate dissolution, and DIC already present in the ground water
(Fig. 5-2). Any kind of organic matter in the aquifer would be an additional source of DIC. Fortunately, there is little organic matter present in the Studen aquifer.

Each of these three sources contributing to the DIC concentration observed in the plume in Studen have a clearly distinguishable δ¹³C-value (Fig. 5-2). Organic matter has typically not a clear defined δ¹³C-value (Clark and Fritz, 1997). Therefore, when organic matter is present in an aquifer, further methods such as ¹⁴C-analysis are needed to distinguish between the different sources as shown by Conrad et al. (1997).

The isotopic balance is only valid if the stable carbon isotope ratios of the different sources are not altered during the formation of DIC (i.e. carbonate dissolution and microbial mineralization of PHC). Generally, during carbonate dissolution the δ¹³C is increasingly fractionated with higher temperatures (Hoefs, 1997). However, at field sites ground water temperature is low, which leads only to minor fractionations (< 1‰, Clark and Fritz, 1997). Until recently it was assumed that the δ¹³C of PHC is not changed during microbial mineralization to DIC independent of prevailing redox conditions, with the exception of methanogenesis, which produces large fractionations (~60‰) (Aggarwal et al., 1997; Grossman, 1997; Whiticar et al., 1986). However, Meckenstock et al. (1999) and Ahad et al. (2000) showed that toluene, as a selected PHC, is fractionated during the microbial degradation independent of prevailing redox conditions. The observed fractionations are rather small (~2‰) so that they do not significantly influence the stable carbon isotope balance at field sites and therefore have no implication on the stable carbon isotope balance. In view of the large fractionations occurring during methanogenesis, samples containing methane have to be omitted from analysis as shown in Fig. 2-6 and require specific interpretation.

Because all three conditions for a reliable stable carbon isotope balance were met in Studen, it was possible to calculate the contributions of PHC mineralization and carbonate dissolution to total DIC as shown in Chapter 2 (Fig. 2-6).
5.5.2 Stable sulfur isotopes – fractionation by sulfate-reducing bacteria

Sulfate reduction is a dominant process at many different PHC-contaminated sites (Wiedemeier et al., 1999). However, relatively little is known about the efficiency of these bacteria and the factors which influence their activity. During the microbial reduction of sulfate, stable sulfur isotopes are fractionated (Thode, 1988). Because stable sulfur isotope fractionation is not constant (Harrison and Thode, 1958) it is not possible to apply a similar isotopic balance as for the determination of produced DIC with stable carbon isotopes. More laboratory and field studies are necessary to obtain a better understanding of the relevant processes and their effect on stable sulfur isotope fractionation. Once a better knowledge is obtained on how the fractionation is influenced by environmental parameters, stable sulfur isotope data may be used to determine the rate of sulfate reduction as shown in Chapter 4. Furthermore, stable sulfur isotope analysis with knowledge of the relevant fractionation processes would allow to quantify the contribution of sulfate-reducing bacteria to PHC-degradation in aquifers with sulfate-rich minerals.

5.6 Lessons learned for other pollutants

MNA can not only be used for PHC-contaminated aquifers but also for other pollutants which are preferably treated in situ (EPA, 1997). Fuel additives such as MTBE (methyl tert-butyl ether) are another category of substances of concern at PHC-contaminated sites. They are added to fuel in order to achieve a more complete combustion of gasoline hydrocarbons, which in turn should help to keep ozone concentrations low in urban air. However, at each gasoline spill, MTBE is introduced into the environment. Unfortunately, in situ biodegradation of MTBE has been minimal or limited at best (EPA, 1999). The here presented concept to assess and characterize bioremediation can be applied to PHC-contaminated sites where MTBE is present. However, the concept is focused on compound mixtures such as
heating oil and not on specific single compounds thereof. Therefore, in order to selectively assess only MTBE-degradation at a PHC-contaminated field site, new methods have to be developed. For example compound specific stable carbon isotope measurements could be such a method if the $\delta^{13}$C of MTBE is altered during degradation.

Chlorinated solvents such as perchloroethene (PCE) and trichloroethene (TCE) are another important group of frequent ground water contaminants. TCE is the most frequent detected ground water contaminant at hazardous waste sites in the U.S. (U.S. National Research Council, 1996). TCE is a suspected carcinogen and therefore of similar toxicity as benzene, which is a known carcinogen. Chlorinated solvents have a higher density than water (Dense non-aqueous-phase-liquid, DNAPL) in contrast to PHC, which are lighter than water (Light non-aqueous-phase-liquid, LNAPL). The difference in this specific physical-chemical property has far reaching consequences for the behavior of these two substance classes in the subsurface (Wiedemeier et al, 1999). LNAPL float on the ground water table as shown in Fig. 1-2 whereas DNAPL being denser than water penetrate the water table and migrate into the saturated zone. Migration of DNAPL is strongly influenced by changes in the texture of the subsurface. DNAPL spread laterally where changes in matrix permeability inhibit vertical migration. This leads to the formation of several small DNAPL pools in the aquifer rather than one large pool floating on the ground water table as for LNAPL (Wiedemeier et al., 1999). Chlorinated solvents can be mineralized by microorganisms and are therefore amenable to MNA (EPA, 1997). However, unlike PHC, which can be oxidized by microorganisms under either aerobic or anaerobic conditions, many chlorinated solvents degrade only under a specific range of the redox potential (Wiedemeier et al., 1999).

Some aspects of the concept presented in this thesis can be applied or modified for sites that are contaminated with chlorinated solvents. A detailed assessment of the hydrogeology of the site is of critical importance. Tracer experiments and aquifer characterization as presented in Chapter 2 are necessary to apply MNA for chlorinated solvents. An accurate understanding of ground water redox conditions as shown in Chapters 2 and 3 is
crucial to predict biodegradation of chlorinated solvents. The biological methods presented in Chapter 3 can be modified by using different probes and combined with chemical and stable isotope analyses allow to characterize microbial populations present at chlorinated solvent-contaminated sites. Stable carbon isotope ratios are already used to monitor the progress of the biodegradation of specific chlorinated compounds in contaminated aquifers (Bloom et al., 2000; Hunkeler et al., 1999a).

Even though MNA is for some pollutants a very slow and tedious process, it is often the only applicable method at many sites. However, it is important to emphasize that MNA is not always an appropriate remediation technique for all contaminants and is restricted to sites where contaminants are attenuated satisfactorily. Nevertheless, MNA is the natural reference case (self-healing capacity of nature), by which every other method should be evaluated.


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