Detection of microbial populations in petroleum hydrocarbon contaminated aquifers and isolation of denitrifying hydrocarbon degraders

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Detection of Microbial Populations in Petroleum Hydrocarbon Contaminated Aquifers and Isolation of Denitrifying Hydrocarbon Degraders

A dissertation submitted to the
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DOCTOR OF NATURAL SCIENCES

presented by
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1998
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Petroleum hydrocarbons are among the most frequently found contaminants in soil and groundwater. Since concentrations of dissolved hydrocarbons such as BTEX (benzene, toluene, ethylbenzene, xylene) in groundwater downgradient of the source zone are often above drinking water standards, remediation actions have to be taken. In situ bioremediation has found special interest since it leads, ideally, to a total mineralization of petroleum hydrocarbons without the need to excavate the contaminated material. It can be grouped into intrinsic and engineered in situ bioremediation. Intrinsic in situ bioremediation relies on the ability of naturally occurring microorganisms to degrade petroleum hydrocarbons under the given environmental conditions and is usually regarded as a plume control strategy. Engineered in situ bioremediation is often based on a pump and treat procedure, providing an external supply of oxidants and nutrients in order to increase degradation by indigenous microorganisms and thus to enhance the removal of petroleum hydrocarbons.

The first part of this thesis deals with laboratory aquifer columns, which were chosen as one-dimensional experimental model systems to evaluate the spatial distribution of petroleum hydrocarbon mineralization in an engineered in situ bioremediation. The results demonstrated that the infiltration of aerobic groundwater enriched with NO$_3^-$ and PO$_4^{3-}$ increased the DIC production and thus the mineralization along the entire flow path of the infiltrated water. Throughout the columns, there was a large increase in total bacteria compared to the cell numbers found prior to column operation.
The second part describes the microbial communities involved in petroleum hydrocarbon mineralization. The bacterial community structure in a laboratory aquifer column as well as in two field sites, which were both treated by intrinsic bioremediation, was analyzed by in situ hybridization. The laboratory column and the field sample were highly contaminated with petroleum hydrocarbon concentrations between 1.1 - 4.5 g kg\textsuperscript{-1}. Fluorescent probes yield perfect spatial resolution and can instantaneously be detected by epifluorescent microscopy. The investigations showed that in each aquifer a characteristic population had developed. However, the different aquifers showed similar trends in bacterial community structure. The total number of cells and the detectability rate by in situ hybridization were correlated with the level of contamination. In a field sample from a source area and in the laboratory aquifer column numbers were high with 2.4 \times 10^8 and 1.0 \times 10^9 cells g\textsuperscript{-1} aquifer material [dry wt.], respectively. The detection rate was very high in the laboratory column (>90%) as well as in the contaminated field sample (40%), but low in a non-contaminated aquifer (5-25%).

Petroleum hydrocarbon-degrading Azoarcus sp., which were isolated from a contaminated aquifer, could be detected in numbers between 1 and 2% of the total bacterial community in the laboratory aquifer column as well as in contaminated field material. This demonstrates that they are significant members of the indigenous microbiota. The assumption that the occurrence of Azoarcus sp. is correlated with petroleum hydrocarbon pollution could be supported by investigations of the bacterial community in a non-contaminated aquifer where no Azoarcus sp. could be detected. However, the function of Azoarcus sp. in contaminated aquifers has to be determined by further investigations in order to obtain a better understanding of the different petroleum hydrocarbon degradation pathways and to analyze the enzymes involved in petroleum hydrocarbon mineralization.

Der erste Teil dieser Doktorarbeit befasst sich mit Laboratoriums-Grundwasser-Säulen, welche als eindimensionale Modellsysteme ausgewählt wurden, um die Verteilung der Kohlenwasserstoff Mineralisierung in einer technischen Bioremediation zu untersuchen. Die Resultate zeigten, dass das Infiltrieren von aerobem Grundwasser, das mit Nitrat und Phosphat angereichert war, die Produktion von gelöstem anorganischen Kohlenstoff und somit die Mineralisation entlang des gesamten Fließweges erhöhte. Überall in den Säulen war die Bakterienzahl, verglichen zu den Anfangswerten, stark angestiegen.
ZUSAMMENFASSUNG.


Groundwater contamination by petroleum-derived hydrocarbons has become a widespread problem in industrialized countries. Leaking underground fuel tanks and accidents where such compounds are involved are a significant source of soil and groundwater contamination. Although many organic compounds in petroleum have an impact on human health, the relatively soluble compounds - benzene, toluene, ethylbenzene, and isomers of xylene (BTEX) - are of most concern due to their toxicity and, for benzene, carcinogenicity [Bitton and Gerba, 1984].

Figure 1.1: Illustration of a contaminated site.
When petroleum hydrocarbons are spilled into the subsurface, gravity causes a migration of the contaminants downward through the unsaturated zone as a distinct liquid (Fig. 1.1). This vertical migration is accompanied by lateral spreading due to capillary forces and the heterogeneity of the subsurface. As the petroleum hydrocarbons migrate downwards through the unsaturated zone, some residual liquid is trapped in the pore space. In addition to the migration of the liquid petroleum hydrocarbons, some of the petroleum hydrocarbons volatize, forming vapors which subsequently spread throughout the unsaturated zone [Norris et al., 1994].

Some of the petroleum hydrocarbons will reach the saturated zone and spread laterally on top of the water table. The zone with petroleum hydrocarbons in the non-aqueous phase is denoted as source area (Fig. 1.1). As the petroleum hydrocarbons encounter flowing groundwater, soluble compounds dissolve and spread due to advection, dispersion, and diffusion, leading to the formation of a contamination plume (Fig. 1.1). There the petroleum hydrocarbon concentrations in the groundwater are often above drinking water standards and may pose a health risk [Millner et al., 1992]. Therefore, the occurrence of petroleum hydrocarbon contamination frequently requires remediation actions.

Remediation strategies may consist of physical, chemical, and biological approaches. Physical procedures include excavation of material, flushing with water, and air sparging of volatile compounds of petroleum hydrocarbons, while chemical treatments often focus on the adsorption or precipitation of the petroleum hydrocarbons. Today, biological approaches are frequently used for the clean-up of sites contaminated with petroleum hydrocarbons [Davis et al., 1994; Borden et al., 1995]. These strategies rely on the collective ability of microorganisms (preferably indigenous organisms) to degrade the petroleum hydrocarbons under prevailing environmental conditions. While physical and chemical remediation techniques simply transfer the contaminant into other environments, bioremediation ideally leads to the complete degradation and detoxification of the contaminants at comparatively low costs [Norris et al., 1994].
1.1 Bioremediation strategies

Bioremediation may be achieved *ex situ* or *in situ* (off site and on site, respectively) (Tab. 1.1). In an *ex situ* process, the hydrocarbons together with the groundwater or soil, in which they reside, are removed and taken to a separate treatment system where conditions for treatment can better be optimized. However, an *ex situ* treatment may impart high costs for construction and maintenance of the treatment system. In an *in situ* process, the contaminants are treated without being removed [Atlas, 1981]. The decision for an *ex* or *in situ* bioremediation depends on a variety of site factors as well as on certain clean-up requirements. If a risk analysis proposes immediate action and thereby a reduction of a certain risk (e.g. for drinking water contamination) the material has to be excavated and treated *ex situ*. However, *ex situ* processes are hindered if the contaminated area is located under buildings and no excavation is possible. Another disadvantage of *ex situ* remediation is concerned with the extremely high costs (Tab. 1.1).

<table>
<thead>
<tr>
<th>characteristics</th>
<th><em>in situ</em> intrinsic</th>
<th><em>in situ</em> engineered</th>
<th><em>ex situ</em> engineered</th>
</tr>
</thead>
<tbody>
<tr>
<td>level of pollution</td>
<td>high for a long time</td>
<td>high for a long/medium time</td>
<td>immediate reduction</td>
</tr>
<tr>
<td>remediation time</td>
<td>very long</td>
<td>long</td>
<td>short</td>
</tr>
<tr>
<td>costs&lt;sup&gt;a&lt;/sup&gt;</td>
<td>none&lt;sup&gt;b&lt;/sup&gt;</td>
<td>low</td>
<td>high</td>
</tr>
</tbody>
</table>

<sup>a</sup> with respect to other clean-up technologies  
<sup>b</sup> except for monitoring

Table 1.1: Bioremediation technologies and their characteristics.
In situ bioremediation techniques are applied if the site allows this strategy and if costs should be low. They can be grouped into two different types: intrinsic and engineered (Tab. 1.1). Intrinsic in situ bioremediation relies on the ability of naturally occurring microorganisms to degrade petroleum hydrocarbons under the given environmental conditions without external intervention [Barker et al., 1987; Baedecker et al., 1993; Davis et al., 1994; Borden et al., 1995]. It is usually regarded as a plume control strategy [Rifai, 1995] since it leads to the stabilization of plumes while the contamination in the source zone is only removed slowly. Intrinsic bioremediation only requires extensive monitoring of the contamination plume to ensure that the compounds do not advance more quickly than the indigenous microorganisms can degrade them.

Engineered in situ bioremediation is often based on a pump and treat procedure (Raymond method), providing an external supply of oxidants and nutrients to the source zone and plume in order to increase degradation by indigenous microorganisms and thus to enhance the removal of petroleum hydrocarbons (Fig. 1.2). Furthermore, a hydraulic containment is frequently established to prevent the spreading of dissolved compounds [US National Research Council, 1993]. In order to allow a uniform distribution of the oxidants and nutrients, the subsurface has to be permeable and relatively homogeneous. The oxidants and nutrients are often dissolved in water and supplied using a groundwater extraction-injection scheme (Fig. 1.2). So far, O₂ and NO₃⁻ have been used as oxidants in engineered in situ bioremediation [Norris, 1994]. Since a pump and treat procedure is relative costly, there is nowadays a shift towards sparging procedures. Where biosparging is applicable, a small screened well has to be installed in the aquifer. Compressed air is forced out of the screen and moves radially outward and upward. This method can provide dissolved oxygen across the entire contamination area and it is much more cost effective than a pump and treat procedure (factor 1:25) [Semprini, 1997].
1.2 Demonstration of bioremediation

According to the US National Research Council (1993), the demonstration of biodegradation should consist of two steps:

(1) laboratory assays showing that microorganisms in site samples have the potential to transform the contaminants under the site conditions, and

(2) one or more pieces of evidence showing that the biodegradation is actually realized in the field.

The potential degradation abilities of microorganisms can be investigated in microcosm and column studies containing contaminated material from the considered site. With many physiological compounds, it can generally be assumed that microbial populations able to degrade them are ubiquitous. However, even if biodegradation is successfully demonstrated in laboratory experiments, it may not take place in the field or only at much slower rates (e.g. due to a limited availability of the carbon source, i.e. contaminant, or of the oxidants and nutrients).

A demonstration of biodegradation in the field is hampered by the high physical, chemical, and biological heterogeneity of the subsurface which makes it difficult to correlate changes in the contamination concentrations
with microbial activity. The consumption of oxidants as well as the decrease of petroleum hydrocarbon concentrations can therefore be due to both biotic as well as abiotic processes like precipitation, adsorption or a downstream transport by the groundwater.

1.3 Factors affecting bioremediation

Successful bioremediation is influenced by properties of the substrate (contaminant), the environment and the indigenous microbial community (Fig. 1.3). The contamination must be available for the organisms in non-toxic concentrations. For a complete substrate mineralization enough nutrients and electron acceptors have to be present. Hydrogeological aspects including groundwater flow and dispersivity of the aquifer have to be considered to optimize remediation activities. The most important factor in a bioremediation project is the microbial community. The indigenous population usually consists of organisms that are adapted to a variety of redox potentials and that are able to mineralize different substrates.

![Image](image.png)

**Figure 1.3:** Interrelationships of essential components that determine successful bioremediation [Hinchee, 1994].
1.3.1 Contaminant properties

**Biodegradability**: As a class, petroleum hydrocarbons are biodegradable [Gibson, 1984]. The highly soluble members are generally biodegraded more rapidly and to lower residual levels than are the less soluble members. Thus, monoaromatic compounds such as benzene, toluene, ethylbenzene, and the xylenes are more rapidly degraded than the compounds with two or more rings such as e.g. naphthalene. Under aerobic conditions all of these compounds are degradable [Atlas, 1981]. Many of them are also degradable under anaerobic conditions, though no degradation of several compounds such as isopropylbenzene and 1,2,3-trimethylbenzene (hemellitol) are observed. For some compounds a mineralization in enrichment cultures is described, but no pure culture able to use the substrate could be obtained yet. One possible explanation is that degradation of certain compounds may depend on the consorted action of different bacteria, which form syntrophic associations.

For the aerobic degradation of BTEX compounds 5 pathways are known up to now (Tab. 1.2). To some extent they may be considered as model systems for more complex aromatic compounds as the pathways include similar enzymatic steps (e.g. oxygenation and ring-cleavage reactions) [Duetz, 1996].

<table>
<thead>
<tr>
<th>pathway</th>
<th>strain</th>
<th>degradation of BTEX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>benzene, ethylb.</td>
<td>toluene, m-xylene, p-xylene, o-xylene</td>
</tr>
<tr>
<td>tod</td>
<td><em>P. putida</em> F1</td>
<td>complete, complete, partial^, partial^, partial^</td>
</tr>
<tr>
<td>TOL</td>
<td><em>P. putida</em> (pWW0)</td>
<td>no, complete, complete, complete, complete, no</td>
</tr>
<tr>
<td>G4</td>
<td><em>B. cepacia</em> G4</td>
<td>no, complete, no, no, no</td>
</tr>
<tr>
<td>KR1</td>
<td><em>P. mendocina</em> KR1</td>
<td>no, complete, partial, no, no</td>
</tr>
<tr>
<td>PK01</td>
<td><em>P. piketti</em> PK01</td>
<td>no, complete, no, no, no</td>
</tr>
</tbody>
</table>

1) Degradation to corresponding catechols

**Table 1.2**: The substrate range of different pathways for the degradation of monoaromatic hydrocarbons [Duetz, 1996].
Only one pathway is known to exist for the aerobic degradation of benzene. The complete pathway was reported for *Pseudomonas putida* F1 (*tod* pathway) [Gibson, 1968; Gibson et al., 1970]. The first enzyme, benzene dioxygenase, yields the characteristic intermediate *cis*-benzene dihydrodiol. The second intermediate, catechol, is ring-cleaved and further degraded through the *meta* pathway [Smith, 1994]. Toluene may also be converted through the *tod* pathway (Tab. 1.2). Also for *m* - and *p*-xylene only one aerobic mineralization pathway is known to exist. The so-called TOL pathway was discovered in *P. putida* mt-2 [Worsey and Williams, 1975]. The pathway involves a conversion of a methyl group to a carboxyl group, and a *meta* pathway, through which the toluates formed are converted to central metabolites. Toluene can be degraded through the pathways described above for benzene and *m* - and *p*-xylene (Tab. 1.2). In addition, three other pathways have been described (Fig. 1.4). The pathway in *P. mendocina* KR1 combines the approaches of the *tod* and the TOL pathway. It involves both direct oxygenation of the aromatic ring and oxidation of the methyl group [Whited and Gibson, 1991; Wright and Olsen, 1994]. The pathways in *Burkholderia cepacia* G4 and *P. pickettii* PKO1 are similar to the *tod* pathway with respect to 3-methyl catechol being the second intermediate. The first intermediates, however, are *o*-cresol [Shields et al., 1989] and *m*-cresol [Olsen et al., 1994], respectively, rather than toluene dihydrodiol.
Figure 1.4: Initial steps in five pathways for the aerobic metabolism of toluene, a: P. putida pWW0; b: P. putida F1; c: P. mendocina KR1; d: B. cepacia G4; e: P. pikettii PK01 [Duetz, 1996].

The anaerobic degradation and transformation reactions of monoaromatic compounds is well studied and documented (Fig. 1.4). The principle problem to overcome in aromatic degradation is the chemical stability of the aromatic ring. In all aerobic degradation pathways the highly reactive molecular oxygen is used for the initial attack on the aromatic nucleus as well as for the final dearomatising, ring splitting step.

A similary reactive cosubstrate is not available in anaerobic pathways. In particular, the actual dearomatising reaction in the different anaerobic pathways proceeds by reduction of the aromatic ring to non-aromatic cyclohexane-derivatives. Many bacteria involved in the anaerobic degradation
are facultatively anaerobic and frequently also degrade aromatics aerobically. However, the pathways as well as the aromatic substrate patterns are different under aerobically and under denitrifying conditions. All denitrifying bacteria known to degrade aromatic compounds belong to the β-subdivision of Proteobacteria. Denitrifying bacteria use the benzoyl-CoA pathway for anaerobic degradation of most aromatics, where the aromatic substrates are first transformed to benzoyl-CoA before the aromatic ring is reductively dearomatised (Fig. 1.5).

**Figure 1.5:** Anaerobic degradation of toluene to benzoyl-CoA in *T. aromatica* [Heider and Fuchs, 1997].

Ferric iron or Manganese(IV) reduction is a geochemically important alternative pathway of anaerobic respiration and has repeatedly been shown to be linked to anaerobic degradation of aromatics. Several bacterial species capable of ferric iron reduction are known, mainly from the γ- and δ-subdivision of Proteobacteria [Hunt et al., 1996; Lovley and Lonergan, 1990] (Fig. 1.6).

Sulfate reducing bacteria as an organism group degrade a very broad range of aromatic compounds, although the degradation capacity of individual species seems more restricted than within denitrifying or iron-reducing bacteria. All known aromate degrading species are members of either the δ-subdivision of Proteobacteria or Gram-positive, endospore-forming, sulfate-reducing bacteria [Edwards et al., 1992; Wilkes et al., 1996] (Fig. 1.6). All known aromatic degradation pathways in sulfate reducers lead to benzoyl-CoA as intermediate, but the mechanism of the benzoyl-CoA reduction in sulfate reducers has not been addressed yet.
Under methanogenic conditions, complete benzoate mineralization (for example) proceeds in two steps catalyzed by a fermentative and a methanogenic bacterium. The aromatic substrate spectrum utilized by a consortium must be a feature of the syntrophic fermentative bacteria. Methanogenic consortia appear to utilize even recalcitrant aromatics such as benzene [Vogel and Grbic'-Galic', 1986].

Anaerobic degradation of aromatic compounds is generally a two-phase process. In the first phase, the aromatic substrate is converted to one of a few central intermediates, which are suitable for reductive dearomatization, like for example benzoyl-CoA, the central intermediate in anaerobic degradation of the majority of aromatic compounds (Fig. 1.5). The second phase is initiated by the reduction of these intermediates to non-aromatic compounds, which are then further degraded to acetyl-CoA and CO₂ [Heider and Fuchs, 1997].

Toluene is one of the most readily degradable aromatic hydrocarbons under anaerobic conditions [Rueter et al., 1994]. Organisms capable of growing on toluene include denitrifying, iron- and sulfate-reducing bacteria and methanogenic consortia [Frazer et al., 1995] (Fig. 1.6). The pathway of toluene degradation has recently been elucidated for the denitrifying *Thauera aromatica*. The first step involves the condensation of the methyl group of toluene with fumarate to yield the intermediate benzylsuccinate [Biegert et al., 1996] (Fig. 1.5). This unusual reaction is catalyzed by the enzyme benzylsuccinate synthase, a highly oxygen-labile enzyme. Synthesis of benzylsuccinate is apparently exergonic enough to proceed efficiently in the forward direction. It has been established that benzylsuccinate is further oxidized to benzoyl-CoA, a reaction dependent on the presence of CoA and nitrate as oxidant. Toluene degradation via benzylsuccinate could also be detected in strain EbN1, which indicates that the condensation of toluene with fumarate seems to be a mechanism widely used in anaerobic bacteria to initiate toluene degradation [Ball et al., 1996].

Ethylbenzene is utilized as substrate by some denitrifying strains. For example, cells of EB1 as well as EbN1 grown on ethylbenzene convert ethylbenzene first to 1-phenylethanol, then acetophenone. Further degradation
to an unknown carboxylated product, probably 3-oxophenylpropionate, and benzoyl-CoA require the presence of CO₂ [Ball et al., 1996]. One of the predicted enzymes involved, a NAD-dependent 1-phenylethanol dehydrogenase, has been detected in ethylbenzene grown cells [Rabus, 1995].

Cometabolic conversion of o-xylene to the corresponding 2-methylbenzylsuccinate intermediate has been reported for a denitrifying toluene degrading isolate [Evans et al., 1992]. Pure cultures of denitrifying bacteria growing on m-xylene have been repeatedly obtained (Fig. 1.6). Although no accumulation of the possible intermediate 3-methylbenzylsuccinate has been reported, benzylsuccinate synthase or a similar enzyme may be involved in m-xylene degradation.

The non-substituted aromatic hydrocarbon benzene is extremely recalcitrant under anaerobic conditions, but clear evidence has been presented that they can be degraded anaerobically in mixed cultures or in sediments under methanogenic [Vogel and Grbic'-Galic',1986], iron- [Hunt et al., 1996] or sulfate-reducing [Weiner and Lovley, 1996] conditions (Fig. 1.6). Anaerobic benzene degradation does not seem to be stimulated by nitrate [Vogel and Grbic'-Galic',1986]. The metabolic pathway for benzene degradation is unknown. Phenol has been reported as possible intermediate in a benzene degrading methanogenic enrichment culture [Vogel and Grbic'-Galic',1986], but this could not be confirmed in later experiments.
Figure 1.6: Degradation of monoaromatic compounds under various redox-conditions (NO$_3^-$: denitrifying, Fe$_{3+}$: Fe(III)-reducing, SO$_4^{2-}$: sulfate-reducing conditions, CO$_2$: methanogenic conditions); Degradation reported in: ++ microcosms, +++ enrichment cultures, +++ pure cultures, - non degradable/not determined; References: a) Ball et al., 1996; b) Biegert et al., 1996; c) Dolfing et al., 1990; d) Edwards et al., 1992; e) Edwards and Grbic-'Galic', 1994; f) Fries et al., 1994; g) Hünner et al., 1995; h) Hünner, not published; i) Hunt et al., 1996; k) Hutchins et al., 1991; l) Lovley and Lovley, 1990; m) Lovley et al., 1994; n) Morgan et al., 1993; o) Rabus and Widdel, 1995a; p) Rabus and Widdel, 1996; q) Rabus et al., 1993; r) Rüeter et al., 1994; s) Vermace et al., 1995; t) Vogel and Grbic-'Galic', 1986; u) Weiner and Lovley, 1996; v) Wilkes et al., 1996; w) Wilson et al., 1986a.
Bioavailability: An important factor for a bioremediation is the accessibility of the contaminant by the microorganisms, that is its bioavailability [Mihelcic, 1993; Volkering et al., 1993]. Bioavailability depends on environmental conditions such as e.g. the structure of the soil matrix, but also on the solubility of the contaminant so that diffusive and advective transport can take place. Biodegradation cannot occur if the organic compound is not accessible for the organism or to extracellular enzymes that the organism might excrete. At a micropore scale, organic contaminants are often strongly sorbed onto the outer surface of soil particles or within small cracks or pores within the soil particles [Norris et al., 1994]. Diffusion both into and out of small cracks or pores is often a very slow process, sometimes in the order of months to years. It has frequently been observed that if contaminated soil is exposed to an appropriate bacterial community, partial degradation of the contaminant will occur rapidly, perhaps within hours. However, the less accessible contaminants contained within the microscopic fissures present may not be degraded within months [Beck and Kevin, 1995; Brown et al., 1995; Bosma et al., 1997].

Factors that influence the bioavailability change very strongly from site to site and must be evaluated carefully when considering the potential for bioremediation [Bregnard et al., 1998], or for remediation by any procedure for that matter. These complexities do not impact on bioremediation alone and are limitations to remediation in general.

Toxicity: Compounds that have been shown to be degradable by microorganisms in laboratory studies will not often be mineralized in the field because they may be present at concentrations that are toxic to the organisms. For example, phenol is readily degraded as a primary substrate by many microorganisms, but if it is present at 500 mg l⁻¹, it can be toxic to these same organisms, and thus not be biodegraded [McCarty, 1996]. This is the case with many contaminants. Frequently, contamination does not include a single compound, but rather a mixture of compounds. While the compounds that
may pose that greatest hazard to human health may not be at a concentration toxic to microorganisms, the other chemicals might create toxic conditions.

1.3.2 Environmental factors

Efficient biodegradation is dependent on basic environmental factors like nutrients and oxidants, pH values, temperature and moisture. Groundwater flow for example must be sufficient to deliver the required nutrients and oxidants according to the demand of the organisms, and the amended groundwater should sweep the entire area requiring treatment. The environment may also have a direct impact on the catabolic activity of the microorganisms involved in biodegradation.

Nutrients: For biological growth coupled to mineralization of organic contaminants appropriate nutrients must be present and available to the microorganisms. Some nutrients must be accessible in macro-amounts, others in micro- or in trace-amounts, respectively. Micro-quantities are required of sulphur, magnesium, iron, molybdenum, cobalt, and nickel, as needed for the formation of key proteins and enzymes within the cells. Other nutrients like Mo, Ni, Fe, Mn, and Co are necessary just in trace amounts. Generally, these elements are sufficiently abundant in the environment.

To permit production of nucleic acids and proteins, the microorganisms require carbon, nitrogen and phosphorus in macro-amounts. Since bioremediation considers heavily contaminated environments, it can be presumed that sufficient carbon is available. The specific nutritional requirements are not easily predictable. If the total hydrocarbon amount is converted to cell material, however, it can be assumed that nutrient requirements of carbon to nitrogen to phosphorus ratios are in the order of 100:10:1 [Norris et al., 1994].

Nitrogen can be added in the form of ammonia or nitrate. NH$_4^+$ is preferentially used as nitrogen source by microorganisms. However, NH$_4^+$ can adsorb at mineral surfaces [Bjerg and Christensen, 1993] or it can be oxidized if the groundwater contains O$_2$ (or H$_2$O$_2$). Looking at engineered in situ bioremediations, usually NO$_3^-$ is added in form of nitrate salts.
Phosphorus can be added as orthophosphate, organic phosphorus or polyphosphates [Aggerwal et al., 1991; Mills and Frankenberger, 1994]. Orthophosphate is readily available for microorganisms. However, orthophosphate strongly adsorbs to surfaces or precipitates with Ca(II) or Fe(II), and therefore reaches only zones of the aquifer that are close to the injection well. Furthermore, precipitation of orthophosphate can lead to plugging of the aquifer. Polyphosphates do not adsorb at mineral surfaces or precipitate, but they must be hydrolyzed before the phosphorus is available for microorganisms. Polyphosphates are therefore suitable to provide phosphorus at greater distances from injection wells.

**Oxidants:** Oxidants used in oxidative energy-producing processes are of high significance for the microorganisms.

The amount of available molecular oxygen must be sufficient to satisfy the relevant stoichiometry for hydrocarbon biodegradation [Wilson et al., 1986b]. Stoichiometrically, approximately 3 kg of molecular oxygen are required to convert 1 kg of hydrocarbons to carbon dioxide and water [Hutchins and Wilson, 1991]. Thus, for the bioremediation of 1 kg of aquifer material containing 10 g kg⁻¹ hydrocarbon compounds, a minimum of 3.1 m³ of aerobic water containing 10 mg l⁻¹ O₂ must be supplied. But at many sites there may be also a very high abiotic oxygen demand due to the presence of hydrogen sulfide, Fe²⁺ or other readily oxidizable compounds, making it difficult to increase the reduction potential into the aerobic range (> 0.82 V; Tab. 1.3) [Wilson et al., 1986a]. With highly contaminated surface soils, molecular oxygen may be introduced by frequent turnover or mixing of soil, but in contaminated groundwater, other methods for introducing molecular oxygen must be employed such as air sparging [Brown, 1994], bioventing [Hinchee et al., 1994] or the addition of hydrogen peroxide [Brown et al., 1984]. Hydrogen peroxide, which decomposes to oxygen and water by microbial catalases or metal oxide catalysts [Pardieck et al., 1992], is completely soluble in water. Practical considerations, including toxicity towards bacteria, limit hydrogen peroxide concentrations to 100 - 1000 ppm.
HalfReaction $E^\circ$

<table>
<thead>
<tr>
<th>oxidized species</th>
<th>reduced species</th>
<th>$E^\circ$ (volts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$O_2 + 4H^+ + 4e^-$</td>
<td>$\Rightarrow 2H_2O$</td>
<td>+ 0.82</td>
</tr>
<tr>
<td>$2NO_3^- + 12H^+ + 10e^-$</td>
<td>$\Rightarrow N_2 + 6H_2O$</td>
<td>+ 0.74</td>
</tr>
<tr>
<td>$MnO_2(s) + HC_03^- + 3H^+ + 2e^-$</td>
<td>$\Rightarrow MnC_03(s) + 2H_2O$</td>
<td>+ 0.52</td>
</tr>
<tr>
<td>$FeOOH(s) + HCO_3^- + 2H^+ + e^-$</td>
<td>$\Rightarrow FeC_03(s) + 2H_2O$</td>
<td>- 0.05</td>
</tr>
<tr>
<td>$SO_4= + 9H^+ + 8e^-$</td>
<td>$\Rightarrow HS^- + 4H_2O$</td>
<td>- 0.22</td>
</tr>
<tr>
<td>$CO_2 + 8H^+ + 8e^-$</td>
<td>$\Rightarrow CH_4 + 2H_2O$</td>
<td>- 0.24</td>
</tr>
</tbody>
</table>

$values$ $are$ $for$ $aqueous$ $solutions$ $with$ $pH = 7, [HCO_3^-] = 0.001M, [Cl^-] = 0.001M$

Table 1.3: Standard reduction potentials at 25°C for some redox couples that are important electron acceptors in microbial respiration [Stumm and Morgan, 1981].

Bioremediation using oxidants other than molecular oxygen is potentially advantageous for overcoming the difficulty in supplying molecular oxygen for aerobic processes [Essaid et al., 1995; Thierrin et al., 1995]. Due to the high solubility in water, attractive alternatives to oxygen are nitrate [Frazer et al., 1995; Kuhn et al., 1988; Milhelcic and Luthy, 1988], sulfate [Edwards et al., 1991; Beller et al., 1992], and carbon dioxide [Grbic'-Galic' and Vogel, 1987]. These oxidants are inexpensive and nontoxic to microorganisms. The overall degradation efficiency can potentially be increased by using other oxidants than oxygen. Nitrate salts for example are much more soluble in water than molecular oxygen (92 g NaNO$_3$ l$^{-1}$ or 1.33 M NO$_3^-$, 10 mg O$_2$ l$^{-1}$ or 0.31 mM). Comparing the water solubilities and the half-reactions for molecular oxygen and nitrate reduction,

$$2NO_3^- + 12H^+ + 10e^- \Rightarrow N_2 + 6H_2O \quad (1)$$

$$O_2 + 4H^+ + 4e^- \Rightarrow 2H_2O \quad (2)$$

it is evident that the reducing equivalents that can be introduced into the aquifer using saturated sodium nitrate solution is approximately 50 times higher than with a saturated oxygen solution [Norris et al., 1994].

Anaerobic degradation of aromatic petroleum hydrocarbons has been demonstrated with a variety of redox conditions, including reduction of nitrate, iron(III) and manganese(IV) oxides, sulfate, and carbon dioxide (Fig. 1.6). In aquifers contaminated with biodegradable organic compounds,
oxidants tend to be used successively in order of decreasing free energy yield. Molecular oxygen is the preferred oxidant, followed by nitrate, manganese(IV) and iron(III) oxides (MnO$_2$ and FeOOH, respectively), sulfate, and carbon dioxide (Tab. 1.3).

1.3.3 Microorganisms

It is assumed that anaerobic degradation of aromatic compounds is restricted to the activities of bacteria. It must be remembered, however, that the metabolic capacities of anaerobic fungi have only recently begun to attract interest. Anaerobic aromatic ring degradation has not yet been reported in members of the Archaea [Harwood and Gibson, 1997]. Microorganisms able to degrade aromatic hydrocarbons under different anaerobic conditions have been isolated and physiologically and phylogenetically characterized to various extents. Among BTEX, toluene has been studied most intensively as a substrate, especially in anaerobic pure cultures of denitrifying [Fries et al., 1994], iron(III)-reducing [Lovley and Lonergan, 1990], and sulfate-reducing [Rabus et al., 1993] bacteria (Fig. 1.6). Most of the described pure cultures, that were isolated under anaerobic conditions on a variety of aromatic hydrocarbons, were obtained under denitrifying conditions. The following section focuses on isolates able to mineralize monoaromatic hydrocarbons coupled to reduction of NO$_3^-$ and N$_2$O.
<table>
<thead>
<tr>
<th>species</th>
<th>strain</th>
<th>reference</th>
<th>benzoate</th>
<th>phenol</th>
<th>toluene</th>
<th>o-xylene</th>
<th>m-xylene</th>
<th>p-xylene</th>
<th>ethylbenzene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas spp.</td>
<td>T</td>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>nd</td>
</tr>
<tr>
<td>Thauera spp.</td>
<td>T1</td>
<td>3,6</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>S100</td>
<td>10</td>
<td>nd</td>
<td>+</td>
<td>+</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>S2</td>
<td>9</td>
<td>nd</td>
<td>+</td>
<td>+</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Thauera aromatic</td>
<td>K172</td>
<td>1,10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>ß-Proteobacterium</td>
<td>Eb1</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Azoarcus spp.</td>
<td>EbN1</td>
<td>8</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Azoarcus spp.</td>
<td>PbN1</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Azoarcus spp.</td>
<td>ToN1</td>
<td>8</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thauera spp.</td>
<td>mXyN1</td>
<td>8</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Azoarcus evansii</td>
<td>KB740</td>
<td>1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Azoarcus tolyticus</td>
<td>Tol-4</td>
<td>7, 11</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Azoarcus tolyticus</td>
<td>Td-15</td>
<td>7, 11</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Azoarcus tolyticus</td>
<td>XQ3</td>
<td>5</td>
<td>nd</td>
<td>nd</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>


Table 1.4: Pure cultures able to degrade aromatic hydrocarbons under denitrifying conditions.
Most of the described strains (Tab. 1.4) can be assigned to the genera *Thauera* or *Azoarcus*, respectively, which belong to the β-subdivision of Proteobacteria, and *Pseudomonas*, which belong to the γ-subdivision of Proteobacteria. However, many organisms described as *Pseudomonas* spp. were reclassified in later studies as members of the β-subdivision of Proteobacteria (e.g. strain K172 [Tschech and Fuchs, 1987; Anders et al., 1995]). Two denitrifiers isolated with toluene, strain T and Td-15, are also able to mineralize *m*-xylene [Dolfing et al., 1990; Fries et al., 1994] (Tab. 1.4) and all cultures isolated on *m*-xylene can use toluene as substrate [Rabus and Widdel, 1995; Hess et al., 1997]. Anaerobic degradation of benzene, *o*-xylene, and *p*-xylene was shown in enriched bacterial communities but not in pure cultures (Fig. 1.6, Tab. 1.4). Strain T1 was isolated on toluene but partial losses of *o*-xylene that were concomitant to the losses of toluene can also be observed in this culture [Evans et al., 1991]. Strain K172 is able to grow anaerobically on benzoate, toluene and phenol (Tab. 1.4). rDNA sequences of strain K172 show 97.8 % similarity to *Thauera selenatis*, which is characterized by a unique property: it reduces selenium oxyanions under anaerobic conditions [Rech and Macy, 1992]. Strain K172 was tested for selenate reduction by using benzoate or acetate as an electron donor, but no selenate reduction was detected. Therefore, strain K172 was classified as a new species in the genus *Thauera*, as *Thauera aromatica* [Anders et al., 1995].

Strains EbN1, ToN1, and mXyN1 are denitrifying bacteria which were isolated on ethylbenzene, toluene, and *m*-xylene, respectively [Rabus and Widdel, 1995]. These three isolates were the first cultures of denitrifying bacteria shown to grow anaerobically with crude oil as the sole source of organic substrates. Strain mXyN1 shows a rDNA sequence similarity of 99.6 % to *Thauera aromatica* K172, but has not been classified yet [Anders et al., 1995].

Recently eight strains (Tol-4, Td-1, Td-2, Td-3, Td-15, Td-17, Td-19, and Td-21) were isolated on toluene under denitrifying conditions from different habitats like petroleum-contaminated sediment and soil, home compost pile, soil from industrial area and from a landfill, noncontaminated
organic soil and even from contaminated marine sediments [Zhou et al., 1995]. The results of studies of the 16S rRNA gene sequences of these organisms, their cellular fatty acid compositions, and some of their physiological and morphological characteristics revealed that they were closely related to the novel nitrogen-fixing bacterial genus *Azoarcus* including for example *A. indigens* VB32, *Azoarcus* spp. BH72, *Azoarcus* spp. S5b2, and *A. communis* SWub3 (Fig. 1.7). Most of these strains were isolated from roots of the salt- and flood-tolerant grass *Leptochloa fusca* (L.) Kunth, called Kallar grass. They can grow anaerobically on malic acid with nitrate but not on toluene [Hurek and Reinhold-Hurek, 1995]. These previously described isolates exhibit nitrogenase activity, which is a key feature of the genus *Azoarcus* [Reinhold-Hurek et al., 1993]. In contrast to the previously described members of the genus *Azoarcus* which were reported to be aerobic, the eight new isolates are able to grow on toluene under strictly anaerobic, denitrifying conditions. The ability to fix nitrogen, the physiological, biochemical, and morphological features strongly supported the conclusion based on the results of the 16S rRNA sequence analyses that the eight isolates are members of the genus *Azoarcus*. However, the toluene-degrading ability of these organisms under denitrifying conditions, their non-rhizosphere niche, several physiological, biochemical, and nutritional differences, and differences of 3 to 8% between their 16S rRNA sequences and the sequences of other *Azoarcus* species suggested that the new isolates are members of a new species in the genus *Azoarcus*. Hence, they were named *Azoarcus tolulyticus* with the type strain A. tolulyticus Tol-4 [Zhou et al., 1995].

An investigation of the fatty acid composition, the quinone type and an analysis of the 16S rRNA gene sequences showed that strain KB740 [Braun and Gibson, 1984] also belongs to the genus *Azoarcus* and is closely related to strain ToN1, which has not been validly described yet. Since strain KB740 differs in many properties from the other members of the genus *Azoarcus*, it was described as a new species in the genus *Azoarcus*, as *A. evansii* with strain KB740 as type strain [Anders et al., 1995]. Strain pF6, a bacterium able to degrade pyridine under both aerobic and anaerobic conditions, shows a similarity value of 100% to 16S rRNA gene sequences of *A. evansii* KB740.
[Rhee et al., 1997], but BTEX mineralization capacities of this isolate has not been investigated. Another Azoarcus spp. isolate, strain 22Lin, is able to grow on benzoate but not on alkylbenzenes or monoterpenes. It was isolated on cyclohexane-1,2-diol under denitrifying conditions [Harder, 1997].

Figure 1.7: Phylogenetic tree of selected species belonging to the genera Thauera and Azoarcus and some closely related strains.
1.4 Molecular detection methods

In the past, studies on microorganisms often focused on attempts to isolate and subsequently characterize the organisms able to degrade certain compounds of the contaminations [Anders et al., 1995; Fries et al., 1994; Lovley and Lonergan, 1990; Rabus et al., 1993; Rabus and Widdel, 1995; Schocher et al., 1991; Zhou et al., 1995], to unravel catabolic pathways involved in biodegradation [Chee-Sanford et al., 1996; Migaud et al., 1996; Seyfried et al., 1994; Zeyer and Kocher, 1988], and to analyze populations and catabolic potentials of isolates in artificial model systems [Hess et al., 1997]. In natural environments, studies on these microorganisms are often impeded by the fact that many microorganisms are difficult to isolate or even resist cultivation, which is an essential prerequisite to characterization by traditional laboratory methods. Studies on the significance of specific isolates in the contaminated soil or aquifer material, on their occurrence and distribution at the field site, and on their biomass, bioactivity, or biodiversity are largely hampered by the lack of sensitive methodology for their detection and quantification in situ [Brock, 1987].

Today, molecular biological techniques are available that have the potential to solve a number of problems encountered with the detection and identification of microorganisms adequately. These techniques are unbiased by the limitations of culturability because molecules rather than microorganisms are detected. They have demonstrated the restrictions of culture-dependent detection protocols which often underestimate total numbers of bacteria as well as the diversity of the bacterial community (see [Amann et al., 1995] for review). The analysis of uncultured microbial communities is based on the principles of nucleic acid hybridization reactions in which stable double-stranded nucleic acid molecules (hybrids) are formed from complementary single-stranded molecules (target and probe).

Up to date, two general approaches, the ex situ and the in situ approach, respectively, are commonly used to detect target sequences by hybridization techniques. In the ex situ approach the hybridization reaction is performed on
extracted and purified target molecules (DNA or rRNA). The techniques include membrane- or liquid hybridization assays in which the nucleic acids are directly used as targets. Hybridization assays may also be performed on amplification products of the target sequences generated by the polymerase chain reaction (PCR) [Hahn et al., 1994]. The PCR technique is based on the thermocyclic amplification of target sequences on DNA by the extension of specifically annealed primers by a thermostable DNA polymerase (e.g. Taq polymerase) [Mullis and Faloona, 1987; Saiki et al., 1988]. Results of detection methods, however, which include an extraction and/or amplification of target sequences may be influenced by a number of factors. For example, the cellular physiology of the target organism and the choice of the nucleic acid extraction procedure may result in a selective lysis of the cells [Moré et al., 1994; Picard et al., 1992]. Moreover, the presence of contaminating substances may inhibit both direct hybridization assays as well as PCR amplification [Liesack et al., 1991; Tebbe and Vahjen, 1993]. The quality of PCR-based results may further be influenced by the choice of the primers [Rainey et al., 1994] or the selective amplification of, for example, rrn operons [Farrelly et al., 1995] or by artifacts, e.g. the formation of chimeras [Brakenhoff et al., 1991; Kopczynski et al., 1994; Liesack et al., 1991; Shuldiner et al., 1989]. Furthermore, detection methods which include an extraction of the target do not yield any information on the exact localization of the target organism in its habitat though first reports on in situ PCR have been published [Hodson et al., 1995; Tolker-Nielsen et al., 1997].

The in situ approach is based on the microscopic detection of labeled probes hybridized to target sequences in fixed microbial cells still remaining in the environment (see [Amann et al., 1995; Stahl and Amann, 1991] for review). Consequently, this technique also enables to retrieve information on the localization of the target organism. Because naturally amplified molecules like ribosomal RNAs are used as target an artificial amplification of the target sequences can be avoided.

The following part describes the basic components of the in situ hybridization technique putting major emphasis on its use for the in situ analysis of bacterial populations in terrestrial habitats including aquifers.
Initially, three components of the \textit{in situ} hybridization technique are discussed: i) the selection of a target sequence, ii) the design, synthesis and labeling of a probe, and iii) the general application of the \textit{in situ} hybridization technique in terrestrial environments.

\subsection*{1.5 Target molecules}

Studies on the composition of microbial communities should focus on targets which allow an identification of the organisms based on their evolutionary relationships. The identification of microorganisms by defined nucleic acid sequences should therefore target genes of high phylogenetic significance. Up to now, the most elaborated data bases for primary structures of informative macromolecules have been compiled for ribosomal RNA molecules (rRNA). Phylogenetic characterization of microorganisms has been done on the basis of sequence comparison of all three rRNAs (5S, 16S and 23S rRNA) [Woese, 1987; Woese et al., 1990], though only 16S and 23S rRNA molecules are of appropriate size for broad phylogenetic analyses.

Ribosomal RNA molecules or their genes are almost perfect targets for the detection of microorganisms by molecular methods. They are ubiquitously distributed, functionally and evolutionary homologous in all organisms, and extremely conserved in secondary and tertiary structure. They seem to lack artifacts of lateral gene transfer between organisms and provide sufficient sequence information to permit statistically significant comparisons. Furthermore, they are naturally amplified with up to several ten thousands of copies per cell and, important for hybridization assays, they are single-stranded in some regions [Olsen et al., 1986].

Comparative analysis of rRNA molecules revealed various levels of conservation throughout the primary structure. Beside the presence of several 15-20-nucleotide regions with little or no variation, several regions with high variation have been identified [Lane, 1991]. Sequence comparison showed that the major areas of idiosyncrasy are not distributed randomly across the entire molecule, but are rather clustered into specific variable regions [Höpfl et al., 1989; Woese et al., 1983]. In addition to these regions, highly variable
insertions can be found in the rRNA of certain microorganisms [Roller et al., 1992]. Because major differences are clustered in variable regions it is not always necessary to determine the entire nucleic acid sequence in order to obtain suitable signature sequences for more or less closely related groups of organisms [Kohne et al., 1986; Stahl and Amann, 1991].

1.6 Probes and labeling strategies

Usually single- or double-stranded DNA, single-stranded RNA or oligonucleotides are used as probes (Fig. 1.8). Due to the quite small size of conserved and variable regions on rRNA, small synthetic oligonucleotides (15 to 40 bases) are the ideal probes for taxonomic groups, though the applicability of labeled polynucleotides with a length of several hundred nucleotides, either PCR products (double-stranded DNA) or in vitro transcripts (single-stranded RNA) as probes has also been demonstrated [Ludwig et al., 1994; Trebesius et al., 1994]. Up to now, many oligonucleotide probes with different specificities between subspecies- and domain-level have been designed and used in the identification and detection of microorganisms (see [Amann et al., 1995] for review). Oligonucleotides complementary to more highly conserved sequences are used as probes to identify organisms at the domain level [DeLong et al., 1989; Giovannoni et al., 1988], whereas those targeting variable regions in which even closely related species have large differences within a few nucleotides, serve as probes for narrower target groups (Fig. 1.9) [Hess et al., 1997; Zepp et al., 1997].
The specificity of the hybridization reaction is controlled by the hybridization conditions, e.g. the temperature and ionic strength of the buffer [Stahl and Amann, 1991]. Thereby, conditions can be established that allow hybrid-formation only between complementary sequences. A proper hybridization can usually be better controlled for short oligonucleotide probes than for longer probes (e.g. in vitro transcripts) because instability of a hybrid introduced by a single mismatched base pair is much higher for short hybrids than for long ones. Oligonucleotide probes allow discrimination between targets only differing in one base (Fig. 1.9) [Manz et al., 1992; Wallace et al., 1979]. The potentially high specificity of short, monolabeled oligonucleotide probes, however, is linked to a generally lower sensitivity as compared to longer probes like in vitro transcripts or PCR amplification products since the latter can carry more label per molecule [Matthews and Kricka, 1988].
probe Azo644

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. toluyliticus Tol-4</td>
<td>GCTTGTGACT-\text{GCACGGCTAGAGTACGCG}-AGAGGGGGGT</td>
</tr>
<tr>
<td>A. toluyliticus Td-15</td>
<td>GCTTGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
<tr>
<td>A. toluyliticus Td-17</td>
<td>GCTTGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
<tr>
<td>A. evansii KB740</td>
<td>GCTTGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
<tr>
<td>Azoarcus spp. EbN1</td>
<td>GCTTGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
<tr>
<td>A. indigens VB32</td>
<td>GCTTGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
<tr>
<td>Azoarcus spp. BH72</td>
<td>GCTTGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
<tr>
<td>Thauera aromatica K172</td>
<td>GCTTGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
<tr>
<td>Isolate Os-ac-16</td>
<td>GCTTGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
<tr>
<td>Zoogloea ramigera</td>
<td>GATGGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
<tr>
<td>Rhodocyclus tenuis</td>
<td>GCTTGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
<tr>
<td>Alcaligens eutrophus</td>
<td>GCTTGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
<tr>
<td>Burkholderia solanacearum</td>
<td>GCTTGTGACT-\text{GCA}[\ldots]\text{CA}-AGAGGGGGGT</td>
</tr>
</tbody>
</table>

Figure 1.9: Specificity of probe Azo644 checked on aligned 16S rDNA sequences of target bacteria and selected non-target organisms; = represents identical bases as in A. toluyliticus Tol-4.

Nucleic acid labels fall into two broad categories, radioactive and non-radioactive labels. In the last decade, increased attempts have been made to substitute radioisotopes like $[^3H]$, $[^32P]$, $[^35S]$ or $[^125I]$ by non-radioactive reporter groups. Most of the recently developed systems use reporter groups detected by optical, luminescence, fluorescence or precipitating systems [Urdea et al., 1988]. Detection of target sequences by complementary probes can be accomplished by direct or indirect labeling methods. In the direct systems, probes are covalently linked with the signal-generating reporter group such as, for example, fluorescent dyes [Amann et al., 1990 a/b; DeLong et al., 1989]. In indirect systems, probes are modified by universal reporter groups, e.g. digoxigenin or biotin, which are detected by an additional, non-covalent interaction with a high-affinity binding partner coupled with the signal-generating reporter group, e.g. antibody/enzyme or antibody/fluorescent dye conjugates [Hahn et al., 1994; Zarda et al., 1991].
1.7 Detection of bacteria in terrestrial environments by *in situ* hybridization

During the last years, the *in situ* hybridization technique has intensively been used to analyze bacterial community structure in different environments, such as aquatic systems [Alfreider et al., 1996; Wagner et al., 1994], sediments [Ramsing et al., 1993; Spring et al., 1993], and soils [Fischer et al., 1995; Hahn et al., 1992; Zarda et al., 1997]. In addition to the quite laborious method of using probes labeled with radioisotopes and subsequent autoradiography [Giovannoni et al., 1988; Mirza et al., 1994; Prin et al., 1993], microbial cells can specifically be detected by several non-radioactive detection methods using probes labeled with fluorescent dyes [Amann et al., 1990; Amann et al., 1991; Fischer et al., 1995], enzymes [Amann et al., 1992; Schönhuber et al., 1997] or non-radioactive reporter molecules like biotin [Lebaron et al., 1997; Lim et al., 1993] or digoxigenin [Fischer et al., 1995; Zarda et al., 1991]. Cells hybridized to fluorescent probes can directly be examined by epifluorescence microscopy when attached to filters [Bowden, 1977; Hobbie et al., 1977] or slides [Amann et al., 1990; Hahn et al., 1992] (Fig. 1.5). Those in solution can eventually be quantified by flow cytometry [Christensen et al., 1995; DeLeo and Baveye, 1996; Lange et al., 1997; Thorsen et al., 1992; Wallner et al., 1993; Wallner et al., 1995]. Cells hybridized to enzyme- or digoxigenin-labeled probes are examined by brightfield microscopy. Enzyme activity in cells is shown by substrate precipitation, whereas binding of digoxigenin-labeled probes is detected via antibody/alkaline phosphatase conjugates and substrate precipitation.

All labeling strategies enable to detect hybrids in whole cells in the environment even though cells are masked by or entrapped in aggregates of dense particles such as plant material [Hahn et al., 1993 b; Hahn et al., 1997] or soil minerals [Fischer et al., 1995; Hahn et al., 1992; Hönerlage et al., 1995; Schleifer et al., 1992]. Detectability of bacterial cells, however, is often impaired by problems caused by constituents of the matrix or by the physiological condition of the target organisms.
1.7.1 Influence of the matrix on detectability

The most prominent influence of the matrix on detectability is given by potential autofluorescence because autofluorescent signals of even low amounts of contaminating organic material can be brighter than potential hybridization signals, in particular when bacterial cells are attached to organic material [Hahn et al., 1992; Hahn et al., 1993 b]. Difficulties with autofluorescence of organic material can partially be overcome by the use of advanced detection technology, e.g. by confocal laser scanning microscopy [Assmus et al., 1995; Manz et al., 1995]. An alternative approach depends on the availability of high-quality images. It is based on an optimization of the sample preparation ensuring an even dispersion of the target cells in thin layers and the concomitant use of probes labeled with highly contrasting dyes. Detection of these bacteria therefore requires reliable methods to release cells attached to or entrapped in aggregates [Fry, 1990; Kingsley and Bohlool, 1981; Zarda et al., 1997]. Dispersal of aggregates and dissociation of microorganisms from particles can be effected by the choice of the dilution buffer [Bakken, 1985] or the addition of surfactants [Bohlool and Schmidt, 1973; Reyes and Schmidt, 1979], hydrolyzed gelatin [Kingsley and Bohlool, 1981] or chelating resins to the dilution buffer [Herron and Wellington, 1990; Hopkins et al., 1991 a/b]. Often ultrasonic treatments are used to enhance homogenization of soil samples [Evans et al., 1991; Kämpfer et al., 1993] without adverse effects on the number of living or fixed bacteria [Hess et al., 1997; Lindahl and Bakken, 1995; Zarda et al., 1997].

With respect to autofluorescence, green-fluorescent dyes such as 5[6]-carboxy-fluorescein-N-hydroxysuccinimide-ester (FLUOS, Boehringer) are usually superior to red-fluorescent dyes like tetramethylrhodamine isothiocyanate (TRITC, Boehringer). However, the signal intensity obtained with these dyes is usually lower which consequently reduces the sensitivity of detection. This reduction is avoided by the application of the red-fluorescent dye Cy3 Reactive Dye (Cy3, Amersham) which is a very photostable carbocyanine dye and produces bright signals due to a high molar extinction coefficient (150000 M⁻¹cm⁻¹) and a high quantum yield [Mujumdar et al.,
In combination with the concomitant use of optimized filter systems (e.g. HQ-Cy3 (AHF Analysentechnik, Tübingen, Germany; G 535/50, FT 565, BP 610/750), Cy3 allows a reliable differentiation of probe-conferring signals from autofluorescence of organic material [Zarda et al., 1997; Zepp et al., 1997] and consequently detection yields increase significantly in both aquatic as well as terrestrial environments [Glöckner et al., 1996; Zarda et al., 1997]. In bulk soil, for example, the detection yield increased from 1% of the total number of bacteria determined after staining with the fluorescent, DNA intercalating dye 4',6-diamidino-2-phenylindole (DAPI) [Hahn et al., 1992] to approx. 40% of the DAPI-stained cells using an optimized sample preparation and a Cy3-labeled oligonucleotide probe targeting members of the domain Bacteria [Zarda et al., 1997].

Alternative labeling and detection strategies relying on enzyme assays can also help to circumvent problems caused by the autofluorescence of the matrix [Bochenek and Hirsch, 1991; Edman et al., 1988]. The application of peroxidase-labeled probes or digoxigenin-labeled probes, detected via antibody/alkaline phosphatase conjugates, is not restricted by background signals of, for example, plant material [Bochenek and Hirsch, 1991; van de Wiel et al., 1990]. This detection strategy consequently allows the reliable differentiation of probe-conferring signals from autofluorescence of organic material [Hahn et al., 1993 b; Zepp et al., 1997]. However, in contrast to the relatively small fluorescent oligonucleotide probes with a molecular weight of approx. 6500, the use of large molecules such as enzyme-labeled oligonucleotides (molecular weight of approx. 50000), in vitro transcript probes or the antibody/enzyme conjugates with a molecular weight of approx. 200 000 is often restricted due to the limited permeability of the cells [Amann et al., 1992; Hahn et al., 1994; Hahn and Zeyer, 1994; Zarda et al., 1991]. In addition to this restriction, other drawbacks of these protocols such as the diffuse images of stained cells and the incompatibility of some assays with DAPI staining may hamper the use of these non-standard assays for the in situ detection of bacteria [Fischer et al., 1995; Hönerlage et al., 1995].
1.7.2 Influence of the physiological condition of the target organism on detectability

Because the signal intensity obtained from an individual cell by hybridization with rRNA targeted probes depends on the content of ribosomes [DeLong et al., 1989; Poulsen et al., 1993], *in situ* hybridization can most reliably be used on physiologically active bacteria such as e.g. syntrophic organisms [Harmsen et al., 1996 a/b], bacterial endosymbionts [Amann et al., 1991; Embley et al., 1992; Finlay et al., 1993] or those grown under optimized laboratory conditions [Amann et al., 1990 a/b; Amann et al., 1992; Zarda et al., 1991]. High detection yields are also obtained in nutrient-rich environments such as activated sludge with 89% of DAPI-stained bacteria [Wagner et al., 1994 a/b] or lake snow with 55-100% [Weiss et al., 1996]. Smaller fractions of the natural microbial communities are detected in oligotrophic habitats like biofilms in drinking water (50%) [Kalmbach et al., 1997], drinking water (70% of surface-associated and 40% of planktonic cells) [Manz et al., 1993], the winter cover and pelagic zone of a high mountain lake (40-81%) [Alfreider et al., 1996], bacterioplankton (35-67%) [Hicks et al., 1992], or in earthworm casts (37%) [Fischer et al., 1995].

Many terrestrial habitats are regarded as nutritionally poor environments to which bacteria may adapt by the formation of resting or dormant cells such as dwarf cells, cysts or spores [Roszak and Colwell, 1987]. Because the signal intensity obtained by hybridization with rRNA targeted probes depends on the content of ribosomes, detectability may therefore be influenced by a low cellular rRNA content of dormant cells. However, even though the amount of target sequences in dormant cells such as e.g. spores is usually lower than in vegetative cells [Quiros et al., 1989; Setlow, 1994], their amount should still be sufficient for detection. Due to alterations in cell wall structure of spores which increase their resistance to adverse environmental conditions, their restricted permeability is therefore considered to be a major factor preventing the optimal use of *in situ* hybridization.

Detectability can be improved by adding nutrients to activate the organisms [Hahn et al., 1992]. For biofilms in drinking water, for example,
the addition of nutrients results in a large increase in detectability from 50 to 80% of DAPI-stained bacteria [Kalmbach et al., 1997]. Differences in detectability of bacteria by in situ hybridization may therefore reflect differences in activity due to the availability of energy sources.

Vegetative cells of Gram-negative and many Gram-positive organisms are usually permeable for fluorescent oligonucleotides after fixation in paraformaldehyde and/or ethanol [Amann et al., 1990 a/b; Braun-Howland et al., 1992; Hahn et al., 1992]. Problems with permeability can be extremely pronounced in dormant cells which often contain additional permeability barriers and therefore need special permeabilization conditions even for fluorescent oligonucleotide probes [Amann et al., 1995; Fischer et al., 1995; Hahn et al., 1994; Hahn and Zeyer, 1994; Zepp et al., 1997]. Many different procedures have been developed with the aim to increase the permeability of vegetative and dormant cells of bacteria (see [Zarda et al., 1997] for procedures). For pure cultures, the permeabilization procedures work reasonably well. In terrestrial environments such as e.g. bulk soil, however, none of these procedures resulted in an increase in detectability of bacteria [Zarda et al., 1997].

1.8 Outline of the thesis

The aim of this thesis is to investigate the processes involved in petroleum hydrocarbon mineralization in contaminated aquifers and to describe structures of microbial communities in aquifers treated by engineered and intrinsic in situ bioremediation.

Part I: The mineralization of petroleum hydrocarbons was investigated in laboratory columns simulating engineered in situ bioremediation under field conditions. Microbial degradation of contaminating petroleum hydrocarbons was analyzed by observing changes in groundwater chemistry. Concentration profiles of O₂, NO₃⁻, NO₂⁻, dissolved inorganic and organic carbon (DIC and DOC, respectively), protein, and total residual hydrocarbons were measured. Based on the oxidant consumption and petroleum
hydrocarbon degradation growth of microorganisms along the infiltration path could be proposed.

4. Specific detection
The bacterial community structure and the importance of the isolates in contaminated aquifers was investigated in the laboratory columns as well as in the field site [chapter 3, 4, 5].

3. Probe design
Two fluorescent (Cy3) labeled oligonucleotide probes targeting the 16S rRNA of the isolates and closely related organisms were designed [chapter 3].

1. Enrichment and isolation
From the contaminated aquifer material [chapter 2] denitrifying strains were isolated on toluene and m-xylene, respectively [chapter 3].

2. Sequencing of rRNA of isolates
The isolates were phylogenetically characterized by comparative 16S rRNA analysis and further differentiated from each other by analysis of partial 23S rRNA sequences [chapter 3].

Figure 1.10: Flow chart of the principal phases of the rRNA approach for the analysis of microbial populations.

Part II: Fig. 1.10 gives an overview on the concept of this thesis. Microorganisms able to degrade certain aromatic hydrocarbons under denitrifying conditions were isolated from contaminated aquifer material using enrichment and isolation techniques. After designing specific probes for the isolates and closely related organisms their importance during bioremediation of petroleum hydrocarbon contaminated aquifers was investigated. Since field conditions are extremely heterogenous with many unknown factors influencing the microbial population, we first characterized the distribution of certain bacterial groups during a simulation of engineered bioremediation using laboratory aquifer columns, in which the number of unknown parameters was lower than at the corresponding field site.
Subsequently, the structure of the microbial community at different contaminated field sites was studied.
Bioremediation of a diesel fuel contaminated aquifer: simulation studies in laboratory aquifer columns

Abstract

The *in situ* bioremediation of aquifers contaminated with petroleum hydrocarbons is commonly based on the infiltration of groundwater supplemented with oxidants (e.g. O₂, NO₃⁻) and nutrients (e.g. NH₄⁺, PO₄³⁻). These additions stimulate the microbial activity in the aquifer and several field studies describing the resulting processes have been published. However, due to the heterogeneity of the subsurface and due to the limited number of observation wells usually available, these field data do not offer a sufficient spatial and temporal resolution. In this study, flow-through columns of 47 cm length equipped with 17 sampling ports were filled with homogeneously contaminated aquifer material from a diesel fuel contaminated *in situ* bioremediation site. The columns were operated over 96 days at 12°C with artificial groundwater supplemented with O₂, NO₃⁻ and PO₄³⁻. Concentration profiles of O₂, NO₃⁻, NO₂⁻, dissolved inorganic and organic carbon (DIC and DOC, respectively), protein, microbial cells and total residual hydrocarbons were measured. Within the first 12 cm, corresponding to a mean groundwater residence time of < 3.6 hours, a steep O₂ decrease from 4.6 to <0.3 mg l⁻¹, denitrification, a production of DIC and DOC, high microbial cell numbers and a high removal of hydrocarbons were observed. Within a distance of 24 to 40.5 cm from the infiltration, O₂ was below 0.1 mg l⁻¹ and a denitrifying activity was found. In the presence and in
the absence of $O_2$, $n$-alkanes were preferentially degraded compared to branched alkanes. The results demonstrate that i) infiltration of aerobic groundwater into columns filled with aquifer material contaminated with hydrocarbons leads to a rapid depletion of $O_2$, ii) $O_2$ and $NO_3^-$, respectively, can serve as oxidants for the mineralization of hydrocarbons, and iii) the modelling of redox processes in aquifers has to consider denitrifying activity in presence of $O_2$.

2.1 Introduction

The contamination of soils and aquifers with petroleum hydrocarbons is a major environmental problem. Spilled hydrocarbons are transported by gravity through the unsaturated zone and are usually floating on groundwater, from where they disperse horizontally along the groundwater gradient and vertically within the capillary fringe. Removal of floating hydrocarbons by pumping is always incomplete. Due to capillary forces and sorption processes, residual hydrocarbons are trapped in pores and act as a continuing source of potentially toxic organic contaminants.

In situ bioremediation has been increasingly considered as a novel technology to clean up residual hydrocarbon contaminations in soils and aquifers. In a number of field studies, indigenous microbial populations were stimulated by infiltrating groundwater supplemented with $O_2$, $NO_3^-$ and selected nutrients [Battermann, 1983; Hutchins and Wilson, 1991; Gersberg et al., 1993; Kämpfer et al., 1993; Downs et al., 1994; Nelson et al., 1994; Hunkeler et al., 1995]. To mineralize the hydrocarbons, microorganisms require electron acceptors (e.g. $O_2$, $NO_3^-$, $Mn^{(II, IV)}$, $Fe^{(III)}$, $SO_4^{2-}$, $CO_2$). For thermodynamic reasons, $O_2$ is preferentially consumed, and since the rate of $O_2$ consumption often exceeds the rate of $O_2$ supply, the subsurface turns anoxic. Attempts to increase the supply rate of $O_2$ are hampered by the poor aqueous solubility of $O_2$. A supply of the highly soluble $H_2O_2$ to produce $O_2$ by catalytic decomposition can lead to toxic effects, to an oversaturation with $O_2$ and to a clogging of the aquifer by $O_2$ bubbles [Spain et al., 1989; Hinchee et al., 1991; Pardieck et al., 1992]. Although petroleum hydrocarbons are most readily degraded under aerobic conditions [Atlas and Bartha, 1992], it
has been demonstrated that a complete mineralization of a number of hydrocarbons also occurs under denitrifying [Zeyer et al., 1986], iron-reducing [Lovley and Lonergan, 1990], sulfate-reducing [Aeckersberg et al., 1991] and methanogenic conditions [Edwards and Grbic-Galic, 1994]. This opens the attractive opportunity to supply e.g. NO$_3^-$ to the contaminated zone. This oxidant has a similar redox potential as molecular oxygen [Stumm and Morgan, 1981], it has a high solubility, it is not adsorbed by the solid matrix of the aquifer, it can be converted by many microorganisms, it can simultaneously serve as oxidant and nitrogen source and it is cheap. Infiltration of NO$_3^-$, however, may not be tolerated by the authorities since NO$_3^-$ is a groundwater contaminant itself and may lead to the production of other groundwater contaminants like NO$_2^-$, N$_2$O and NH$_4^+$. Due to the limited access to the subsurface, studies on bioremediation processes are difficult in field systems. Therefore, a number of authors tried to simulate the processes in laboratory aquifer columns. However, previously published laboratory aquifer column studies did not deal with residual hydrocarbons, but with easily degradable substrates such as lactate or acetate as carbon and energy source for microbial growth [Cobb and Bouwer, 1991; Von Gunten and Zobrist, 1992; Von Gunten and Zobrist, 1993]. Moreover, localization of aerobic and denitrifying processes were not reported in studies where aromatic hydrocarbons were supplied [Kuhn et al., 1985; Kuhn et al., 1988; Chen et al., 1992]. Recently, we reported on a field study of an in situ bioremediation of a diesel fuel contaminated aquifer in Menziken, Switzerland [Hunkeler et al., 1995]. Groundwater supplemented with O$_2$ (320 μM) and NO$_3^-$ (1020 μM) as electron acceptors and NH$_4$H$_2$PO$_4$ (75 - 125 μM) as nutrient was infiltrated and the oxidants were almost completely consumed at a monitoring well located 20 m downstream of the infiltration. A tracer experiment with chloride, bromide and fluorescein revealed that this distance corresponded to a mean groundwater residence time of 22 hours. No wells were available in between and therefore the localization of hydrocarbon mineralization and oxidant consumption processes could not be achieved. Furthermore, accurate carbon and nitrogen mass balances and electron balances could not be
established, since the field site is an open system in which certain mass fluxes (e.g. transport of soil gases) can only be estimated. The remediation processes at Menziken were simulated in laboratory aquifer columns equipped with many sampling ports and filled with aquifer material from the field site. The aim of this project was i) to study the localization of oxidant consumption, hydrocarbon mineralization and growth of microorganisms along the infiltration path, and ii) to establish carbon, nitrogen and electron balances of the mineralization process.

2.2 Materials and methods

2.2.1 Experimental set-up

Two laboratory aquifer columns were constructed of Plexiglas cylinders (length 47 cm, inner diameter 5 cm, wall thickness 0.5 cm). A scheme of the set-up is shown in Fig. 2.1A. At the bottom, a Plexiglas sieve plate covered with a nylon net (mesh 70 µm) was placed 2 cm above the column inlet. The outlet of the columns was made of stainless steel tubing placed in a rubber stopper. Sampling ports were made of stainless steel hypodermic needles (ID 1.5 mm, Unimed, Geneva, Switzerland) placed into GC septa (Injection Rubber Plugs Part. No. 201-35584, Shimadzu, Japan) that were fitted into 4.8 mm borings. Sampling ports were positioned starting at 1.5 cm from the sieve plate with a vertical distance of 1.5 cm (first six ports) and 3 cm, respectively (Fig. 2.1A). Prior to column operation, an experiment was conducted by filling the column with stagnant anoxic water, and no O₂ permeation was observed over 3 days. Aquifer material from the contaminated site at Menziken was excavated, sieved (<4.5 mm) and analyzed as reported by [Bregnard et al., 1996]. Dried material consisted of <1% silt and clay (<0.02 mm), 13% fine sand (0.02-0.2 mm) and 86% coarse sand (0.2-4.5 mm). The material having a field capacity of approximately 13.5% water by volume contained weathered hydrocarbons (825 mg kg⁻¹). It was additionally spiked with fresh diesel fuel (Esso, Switzerland) to yield a total hydrocarbon concentration of 1100 mg kg⁻¹. The material was homogenized by vigorous stirring and filled into the columns. After allowing the material to settle within the first 2 days, a
total porosity of 0.14 was calculated from the volume and the weight of the column fillings. The columns were operated with an artificial groundwater at 12 ± 0.5°C (in situ temperature) in upright position under upflow conditions (flow rates of 8.8 ± 0.5 ml h⁻¹). The artificial groundwater consisted of 6000 µM NaHCO₃, 1610 µM NaN₃, 100 µM MgSO₄·7H₂O in distilled water and it was autoclaved at 120 °C. Sterile solutions of CaCl₂·2H₂O (250 µM final conc.), HCl (1000 µM) and KH₂PO₄ (10 µM) were added after autoclaving. The artificial ground water contained 4.4 ± 0.6 mg O₂ l⁻¹ at the sampling port located in front of the bottom sieve plate of the columns (Fig. 2.1A). Two identical columns were operated over 96 days. During that time, a total of 8 to 11 concentration profiles were measured in intervals of 4 to 14 days, and comparable concentration profiles were observed in both columns. Transport parameters were characterized by adding 1 mM of NaBr to the artificial groundwater at day 22 and 55 of operation. Breakthrough curves were obtained by measuring Br⁻ and analyzed according to [Brenner, 1962]. They yielded effective porosities of 0.080 ± 0.002, average groundwater residence times of 12.1 ± 0.2 hours in the columns and longitudinal dispersivities of 4 ± 1.3 cm. At day 96, the aquifer material was extruded from one column and sliced into 10 samples, as shown in Fig. 2.1A (dashed lines). All data including profiles of dissolved parameters shown in the results section were obtained from this column.

2.2.2 Chemicals and analytical methods

All chemicals were purchased from Fluka (Buchs, Switzerland) or Merck ABS (Dietikon, Switzerland) at the highest purity available and were used as received. Filtrations were done using 0.2 µm Millipore PTFE filters (Millipore, Volketswil, Switzerland). Microbial cells were removed from aquifer solids by vortexing 1 g of aquifer material in 10 ml filtered artificial groundwater containing 0.1 % Na₄P₂O₇ [Balkwill et al., 1988]. The aquifer solids were allowed to settle down for 2 minutes, and then, the cells in the supernatant were fixed, stained with DAPI and directly counted with a Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) as reported by [Hahn et al., 1992]. Protein concentrations were determined by
the Bradford method [Sedmak and Grossberg, 1977] with bovine serum albumin as standard. Unless stated otherwise, groundwater samples from the columns were filtered. For the measurement of Ca\(^{2+}\), dissolved iron, dissolved manganese and dissolved organic carbon (DOC), samples were acidified with 0.1% distilled HNO\(_3\). O\(_2\) concentrations were measured in unfiltered samples by the azide modification of the Winkler method [APHA, 1989] adapted to sample volumes of 4 ml. All procedures for O\(_2\) measurement, including the transfer from the sampling ports of the columns into vials, were carried out under a stream of N\(_2\) gas preventing contamination with O\(_2\) from air. Concentrations of NO\(_3^-\), SO\(_4^{2-}\), Cl\(^-\) and Br\(^-\) were determined by a Dionex DX-100 ion chromatograph equipped with an IonPac AS4A-SC column using an eluent of 1.8 mM Na\(_2\)CO\(_3\) and 1.7 mM NaHCO\(_3\). Ca\(^{2+}\) was determined by a Dionex DX-100 ion chromatograph equipped with an IonPac AS12A-SC column using an eluent of 20 mM CH\(_4\)SO\(_3\). NH\(_4^+\), NO\(_2^-\) and HS\(^-\) were measured colorimetrically [APHA, 1989] and dissolved iron and manganese were quantified by atomic absorption spectroscopy according to standard methods [APHA, 1989]. DOC was measured in a Horiba TOC-analyzer by infrared detection of CO\(_2\) after catalytic oxidation at 900°C. Before measurement, the acidified samples were vigorously bubbled with N\(_2\) to ensure complete stripping of CO\(_2\). Dissolved N\(_2\)O and CH\(_4\) were quantified using the head space technique described by [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 (length 1.8 m, inner diameter 3.2 mm, Alltech Inc., Deerfield Ill.) at 70°C with N\(_2\) as carrier (flow 19 ml min\(^{-1}\)) and a Carlo Erba thermal conductivity detector (220°C). Concentrations of dissolved gases were calculated from Henry's law and the following Henry constants corrected for 12°C: N\(_2\)O: 0.0374 M atm\(^{-1}\) [Weiss and Price, 1980] and CH\(_4\): 0.00177 M atm\(^{-1}\) [Yamamoto et al., 1976]. Alkalinity was measured by potentiometric titration using Gran plots for graphical determination of the end point [Almgren et al., 1983]. pH was measured in unfiltered and unstirred samples with a pH electrode. Dissolved inorganic carbon (DIC = H\(_2\)CO\(_3\) + HCO\(_3^-\)+ CO\(_3^{2-}\)) was calculated from alkalinity and pH with equations and constants taken from [Stumm and
Morgan, 1981]. For stable carbon isotope analysis, 150 ml of sample was collected under N₂ in a flask containing 5 ml of a CO₂-free solution of NaOH (1.5 M) and DIC was precipitated by adding 2 g of BaCl₂, filtered and dried. The BaCO₃ was converted to CO₂ on a vacuum line by adding H₃PO₄. The $^{13}$C/$^{12}$C ratios were measured with an isotope-ratio mass spectrometer and are reported in the usual delta ($\delta^{13}$C) notation referenced to the PDB standard (Belemnite from Pee Dee formation [Boutton, 1991]). Hydrocarbons were extracted in a soxhlet apparatus using dichloromethane (15 hours at 55°C) and then analyzed by capillary gas chromatography. Total hydrocarbons were quantified by infrared spectroscopy (IR) or by integrating GC-chromatograms. These methods were described in detail by [Bregnard et al., 1996].

2.3 Results

2.3.1 Consumption of oxidants

Changes over time of oxidant concentration profiles (O₂, NO₃⁻) are shown in Fig. 2.1 B+C. At the infiltrating end of the column, the O₂ gradient was steep during early column operation and then gradually got more gentle towards the end of the experiment (Fig. 2.1B). At 12 cm column depth, which corresponds to a mean residence time of the groundwater of 3.6 ± 0.1 h, concentrations of O₂ never exceeded 0.3 mg l⁻¹. In this study, the zone from 0 to 12 cm is designated as aerobic zone. Between 12 and 24 cm column depth, O₂ concentrations were between 0.1 and 0.3 mg l⁻¹, and therefore, this zone is designated as microaerobic zone. At sampling ports beyond 24 cm column depth, O₂ concentrations were always below detection limit (<0.1 mg l⁻¹), and therefore, this zone is designated as anaerobic zone. NO₃⁻ profiles showed distinct fluctuations within the first 27 days of column operation, with the highest consumption rates on day 27 (Fig. 2.1C). From day 41 to 90, NO₃⁻ profiles became more and more stable (Fig. 2.1C). Arithmetic means of oxidant concentration profiles (O₂, NO₃⁻) are shown in Fig. 2.2A. The mean NO₃⁻ consumption rate over the entire experiment was higher in the aerobic and anaerobic zones than in the microaerobic zone (Fig. 2.2A).
Figure 2.1: Schematic of column set-up and sampling ports (A). Solid circles indicate sampling ports used for chemical measurements. $O_2$ was measured in all of the first 8 ports. Changes over time of concentration profiles of $O_2$ (B), $NO_3^-$ (C), $NO_2^-$ (D), DOC (E) and DIC (F) along the column. The profiles shown are a representative selection out of 8-11 profiles measured over 96 days. The profiles were measured on the following days:
2.3.2 Production of reduced inorganic compounds

NO$_2^-$ concentrations increased in all zones of the column (Fig. 2.1D and 2.2A). High NO$_2^-$ concentrations were found at day 15 and from day 62 to 90 (Fig. 2.1D). Low NO$_2^-$ concentrations were observed between day 19 and 27. N$_2$O concentrations measured in the column effluent ranged from 11 to 12 µM between day 5 to 27 and day 62 to 90 and were below 2 µM in between (data not shown). Mean NH$_4^+$ concentrations increased from 2 ± 2 µM in the infiltrating water to 7 ± 4 µM in the column effluent. Concentrations of dissolved iron and manganese measured on day 62 were below detection limit (5 µM) on all sampling points. H$_2$S and CH$_4$ could not be detected in the effluent (detection limits 1 and 2 µM, respectively). During the 96 days of column operation, a formation of gas bubbles in the aquifer material in the column was visible. Most bubbles were located in the anaerobic zone. In the effluent of the column, partial pressures of O$_2$, CH$_4$, CO$_2$ and N$_2$O were <0.003 atm. We concluded that the gas formed in the column consisted mainly of N$_2$.

2.3.3 Dissolved organic and inorganic carbon

An increase in DOC concentrations was usually observed in the aerobic and anaerobic zone (Fig. 2.1E), whereas a slight decrease was often observed in the microaerobic zone. However, in the first 27 days of the experiment, DOC concentration peaks sometimes appeared in all zones (Fig. 2.1E); later on, concentration profiles stabilized. DIC concentrations increased slightly more in the aerobic and the anaerobic zone than in the microaerobic zone (Fig. 2.1F and 2.2B).
Figure 2.2: Arithmetic means of 8-11 concentration profiles of $O_2$, $NO_3^-$, and $NO_2^-$ (A), $\Delta$DIC (=increase of DIC relative to concentration in inlet) and DOC (B). The final profiles (after an incubation of 96 days) of cell numbers, proteins and total hydrocarbons ($C_{HC}$, measured by IR) in the aquifer material are shown in C.
2.3.4 Removal of hydrocarbons

Concentrations of total hydrocarbons (measured by IR) in 10 slices of aquifer material after 96 days of column operation are shown in Fig. 2.2 C. GC-chromatograms of aquifer material samples before and after 96 days of column operation are shown in Fig. 2.3 A-D. The concentrations of total hydrocarbons and individual components are given in Tab. 2.1. Highest removal of hydrocarbons was observed in the sample taken nearest from the column inlet. In that sample, concentrations of $n$-alkanes and $i$-alkanes decreased to 14 % and 28 % of initial concentrations, respectively. The ratio of $n$-alkanes to $i$-alkanes decreased from 0.97 to 0.47 (Tab. 2.1).

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a) unresolved complex mixture

Table 2.1: Quantification of diesel fuel components by GC chromatograms

In the anaerobic zone, concentrations of $n$-alkanes and $i$-alkanes decreased to 47 % and 85 % of initial concentrations, respectively (Fig. 2.3 and Tab. 2.1), and the ratio of $n$-alkanes to $i$-alkanes decreased also significantly. A distinct removal of UCM (Unresolved Complex Mixture)
compounds was observed in the aerobic zone (Fig. 2.3 and Tab. 2.1), whereas no preferential removal of UCM compounds relative to total hydrocarbons was detectable in the microaerobic and anaerobic zone (Tab. 2.1).

**Figure 2.3**: GC analysis of the aquifer material at the start of the experiment (A) and after 96 days in slice 0-2.25 cm (B), slice 13.5-19.5 cm (C) and slice 31.5-40.5 cm (D). UCM: Unresolved Complex Mixture, 11-26: carbon numbers of n-alkanes, f: farnesane (2,6,10-trimethylododecane), \( \cdot \)-C\textsubscript{16}: \( C_{16} \)-isoprenoid alkane (2,6,10-trimethyltridecane), n: norpristane (2,6,10-trimethylpentadecane), pr: pristane (2,6,10,14-tetramethylpentadecane), and ph: phytane (2,6,10,14-tetramethylhexadecane), u: unknown impurities (partially identified as phthalates).

### 2.3.5 Growth of microorganisms

At the beginning of column operation, the aquifer material contained \((2.5 \pm 1.7) \times 10^7\) indigenous microorganisms g\(^{-1}\) aquifer material [dry wt.], as counted directly using the DAPI staining technique. After 96 days of operation, highest cell numbers \((10.5 \pm 2.5) \times 10^7\) cells g\(^{-1}\) aquifer material [dry
wt.] were found in the sample closest to the column inlet (Fig. 2.2C). Cell numbers decreased with increasing column depth and reached a plateau beyond 8 cm. The protein concentrations increased from initially 0.1 mg protein g\(^{-1}\) up to 0.33 mg g\(^{-1}\) near the column inlet (Fig. 2.2C). The ratio of protein to cells had an almost constant value of \((1.7 \pm 0.3) \times 10^{-12} \text{g protein cell}^{-1}\) beyond 8 cm column depth, but was considerably higher within the first 8 cm of the column.

### 2.3.6 Carbon and nitrogen mass balances

After 96 days of column operation, carbon and nitrogen mass balances were calculated as follows: Total hydrocarbons (measured by IR) and proteins were analysed in all 10 slices of aquifer material in the column (Fig. 2.1A), and weighted averages for the aerobic, microaerobic and anaerobic zone were calculated.

<table>
<thead>
<tr>
<th>Microbiologically mediated electron donating processes:</th>
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<tr>
<td><strong>Production of DIC(_\text{min}^a)</strong></td>
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<tr>
<td>(&lt;\text{CH}_2&gt;^b + 2 \text{H}<em>2\text{O} \Rightarrow \text{DIC}</em>{\text{min}^a} + 6 \text{e}^- + 6 \text{H}^+)**</td>
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<tr>
<td><strong>Production of Biomass</strong></td>
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<tr>
<td>(&lt;\text{CH}_2&gt;^b + \text{H}_2\text{O} \Rightarrow \text{Biomass-C}^c,d + 2 \text{e}^- + 2 \text{H}^+)**</td>
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<th>Microbiologically mediated electron accepting processes:</th>
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<tr>
<td><strong>Reduction of O(_2^a)</strong></td>
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<tr>
<td>(\text{O}_2^a + 4 \text{e}^- + 4 \text{H}^+ \Rightarrow 2 \text{H}_2\text{O})**</td>
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<td><strong>Reduction of NO(_3^-) to NO(_2^-)</strong></td>
</tr>
<tr>
<td>(\text{NO}_3^- + 2 \text{e}^- + 2 \text{H}^+ \Rightarrow \text{NO}_2^a + \text{H}_2\text{O})**</td>
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<td><strong>Reduction of NO(_3^-) to N(_2)</strong></td>
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<tr>
<td>(\text{NO}_3^- + 4 \text{e}^- + 5 \text{H}^+ \Rightarrow 0.5 \text{N}_2\text{O}^a + 2.5 \text{H}_2\text{O})**</td>
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<td><strong>Reduction of NO(_3^-) to N(_2)</strong></td>
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<tr>
<td>(\text{NO}_3^- + 5 \text{e}^- + 6 \text{H}^+ \Rightarrow 0.5 \text{N}_2 + 3 \text{H}_2\text{O})**</td>
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<tr>
<td><strong>Production of Proteins</strong></td>
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<tr>
<td>(\text{NO}_3^- + 8 \text{e}^- + 10 \text{H}^+ \Rightarrow \text{Protein-N}^c,e + 3 \text{H}_2\text{O} + 4 \text{H}^+)**</td>
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\(a\) quantified as total production or consumption over 96 days  
\(b\) \(<\text{CH}_2> = \text{hydrocarbons}\)  
\(c\) quantified as weighted average of sliced samples analyzed after 96 days  
\(d\) the redox state of biomass was assumed to be 0  
\(e\) the redox state of nitrogen in proteins was assumed to be -III

**Table 2.2:** Scheme of processes used for the quantification of electron balances.
The elemental composition of hydrocarbons was considered as 86 % (w/w) carbon and 14 % (w/w) hydrogen (=\text{CH}_2, \text{see Tab. 2.2}). Proteins were considered to consist of 51 % (w/w) carbon and 20 % (w/w) nitrogen (Balkwill et al., 1988). Production or consumption of dissolved species were quantified by multiplying concentration changes of the averaged concentration profiles (Fig. 2.2 A, B) across aerobic, microaerobic and anaerobic zones times the total volume of groundwater flowing through the column in 96 days. The results were termed total production or total consumption of species. Total DIC production was separated in DIC originating from mineralization (DIC_{min}) and DIC originating from carbonates (DIC_{carbonate}) as shown in the section $^{13}C$ balance, equation 1.

In the carbon mass balance, 94 % of the organic carbon that was initially present (total hydrocarbons and biomass carbon) could be recovered (Fig. 2.4), when 4 fractions were quantified: Total hydrocarbons, biomass carbon, integrated production of DOC and of DIC_{min}. The loss of 6 % of the organic carbon may be attributed to the wash out of dissolved, volatile hydrocarbons, which were not quantified by the DOC analysis. In the aerobic and microaerobic zone, the decrease of total hydrocarbons was not balanced by the increase of products (biomass, DOC and DIC_{min}, Fig. 2.4). In the anaerobic zone, the increase of products exceeded the decrease in hydrocarbons.

In the nitrogen balance, only 36.7 % of the total consumption of NO$_3$-N (22.3 mMoles) could be analytically recovered: 22.7 % was washed out as NO$_2$-N, 1.1% as N$_2$O-N, and < 0.5 % as NH$_4^+$-N. At the end, 12.4% of the NO$_3$-N was found in proteins.
2.3.7 Electron balance

Based on the analytical data, a scheme of microbially mediated processes was established that should allow to couple the oxidation of hydrocarbons to the consumption of oxidants. Two electron donating processes (oxidation of hydrocarbons to $\text{DIC}_{\text{min}}$ and protein carbon, respectively) are balanced with 5 electron accepting processes for each zone of the column (Tab. 2.2, Fig. 2.5). The production of DOC was assumed not to be coupled to electron transfer reactions. Furthermore, it was assumed that production of $\text{N}_2$ by denitrification accounted for the missing nitrogen in the nitrogen balance. Overall, a reasonable balance between electron donating and accepting processes was found (Fig. 2.5). Note that more electrons were consumed by reduction of $\text{NO}_3^-$ than by reduction of $\text{O}_2$. This was observed even in the aerobic zone.
### Figure 2.5: Summary of processes considered for electron balances$^a$.

**$^{13}$C balance**

The origin of inorganic carbon produced in the column was traced by measuring $^{13}$C/$^{12}$C ratios in substrates and products and by using the balance equations (1) and (2):
\[
\text{DIC}_{\text{in}} + \text{DIC}_{\text{min}} + \text{DIC}_{\text{carbonate}} = \text{DIC}_{\text{out}} \quad (1)
\]
\[2\]
\[
\partial^{13}C_{\text{in}} \cdot \text{DIC}_{\text{in}} + \partial^{13}C_{\text{min}} \cdot \text{DIC}_{\text{min}} + \partial^{13}C_{\text{carbonate}} \cdot \text{DIC}_{\text{carbonate}} = \partial^{13}C_{\text{out}} \cdot \text{DIC}_{\text{out}}
\]

where:
\[
\text{DIC}_{\text{in}} = \text{concentration of DIC in column inlet (5.24 mM, experimentally determined)}
\]
\[
\text{DIC}_{\text{min}} = \text{unknown concentration of DIC originating from mineralization}
\]
\[
\text{DIC}_{\text{carbonate}} = \text{unknown concentration of DIC originating from carbonates}
\]
\[
\text{DIC}_{\text{out}} = \text{concentration of DIC in column outlet (6.15 mM, experimentally determined)}
\]
\[
\partial^{13}C_{\text{in}} = \text{isotope ratio of DIC in column inlet (-4.64%, experimentally determined)}
\]
\[
\partial^{13}C_{\text{min}} = \text{isotope ratio of carbon in petroleum hydrocarbons (-28%, [Boutton, 1991])}
\]
\[
\partial^{13}C_{\text{carbonate}} = \text{isotope ratio of DIC of carbonates from the column (+0.7%, experimentally determined)}
\]
\[
\partial^{13}C_{\text{out}} = \text{isotope ratio of DIC in outlet (-7.82%, experimentally determined)}
\]

Solving equations 1 and 2 yields the two unknown concentrations of \(\text{DIC}_{\text{min}}\) (0.84 mM) and of \(\text{DIC}_{\text{carbonate}}\) (0.07 mM). The concentration of \(\text{DIC}_{\text{carbonate}}\) calculated in this way corresponded well with the increase of the concentration of \(\text{Ca}^{2+}\) in the column (0.067 mM). In this study, the inflowing and outflowing water was always undersaturated with respect to calcite (calculations with stability constants given in [Stumm and Morgan, 1981]). \(\text{Ca}^{2+}\) liberated from carbonates may not be quantitatively washed out of columns, but be adsorbed to the aquifer matrix or undergo ion exchange processes [Bjerg and Christensen, 1993]. However, the good agreement of the carbonate dissolution calculated on the basis of \(\text{Ca}^{2+}\) concentrations with the \(^{13}\text{C}\) isotopic balance suggests that after 60 days of column operation, the sorption and ion exchange processes of \(\text{Ca}^{2+}\) had reached steady state, and that the quantification of \(\text{DIC}_{\text{carbonate}}\) was correct.
2.4 Discussion

2.4.1 Localization of \( O_2 \)-and \( NO_3^- \)-consuming processes

The results demonstrate that \( O_2 \) concentrations in the infiltrating artificial groundwater decreased to \(<0.1 \text{ mg l}^{-1}\) within mean residence times of 7.2 hours, and that no breakthrough of \( O_2 \) occurred within 96 days. In aquifers, mean groundwater flow velocities are often below \( 10 \text{ m day}^{-1} \) [De Marsily, 1986]. According to the results of this study, \( O_2 \) would be consumed within a distance of \(<3 \text{ m}\) from an infiltration well in petroleum hydrocarbon contaminated aquifers. This is consistent with many findings at field sites [Battermann, 1983; Hutchins and Wilson, 1991; Gersberg et al., 1993; Kämpfer et al., 1993; Downs et al., 1994; Nelson et al., 1994; Hunkeler et al., 1995] and illustrates the difficulties to supply \( O_2 \) beyond a certain distance from an infiltration well. The results demonstrate furthermore that \( NO_3^- \) concentrations decreased on the average from \( 1610 \text{ \mu M} \) to \( 660 \text{ \mu M} \), and that a decrease of \( NO_3^- \) concentrations was observed in all zones of the column irrespective of the \( O_2 \) concentrations of the bulk solution. 36.7% of the \( NO_3^- \) consumed in the columns was found in \( NO_2^- \), \( N_2O \), \( NH_4^+ \) and proteins. The low \( NH_4^+ \) concentrations in the column \(<7 \text{ \mu M}\) suggest that obligate anaerobic microorganisms known to use dissimilatory reduction of \( NO_3^- \) to \( NH_4^+ \) for respiration were of little importance. The electron balance (Fig. 2.5) supports the assumption that the rest of the \( NO_3^- \) consumed in the column was converted to \( N_2 \). In summary, this means that denitrifying mineralization was the major \( NO_3^- \) consuming process. The electron balance (Fig. 2.5) illustrates that already in the aerobic zone, the main consumption process of \( NO_3^- \) was reduction of \( NO_3^- \) to \( N_2 \) and \( NO_2^- \). Thus, denitrification occurred in this zone in spite of the presence of \( O_2 \) in bulk solution.

2.4.2 Coupling of oxidant consumption with hydrocarbon mineralization

The electron balance strongly indicates that the oxidants consumed in the column were mainly used for mineralization of hydrocarbons to DIC\(_{\text{min}}\). The carbon and \(^{13}\text{C}\) balances give further evidence that DIC originated mainly from the mineralization of hydrocarbons. Without proper balances, it often
can not be assessed whether total DIC produced in the column originated from mineralization of contaminants or e.g. from the dissolution of carbonates.

In the aerobic zone of the column, more hydrocarbons were removed than in the microaerobic and anaerobic zone (Fig. 2.2C, 2.3 and 2.4). This could either be due to biodegradation or due to transport. Linear and isoprenoid alkanes with neighbouring retention times in the GC chromatogram (e.g. $n$-C$_{17}$ and pristane) have the same physico-chemical properties (e.g. solubility, octanol-water partitioning) and thus should show a similar transport behaviour. However, $i$-alkanes are less biodegradable due to steric effects [Watkinson and Morgan, 1990]. The decrease of the ratio of $n$-alkanes to $i$-alkanes is therefore a strong indication for that $n$-alkanes are mineralized rather than transported [Pritchard and Costa, 1991]. Microbial degradation of $n$-alkanes occurred in the aerobic as well as in the anaerobic zone of the column. Mineralization of $n$-[1-14C]hexadecane to 14CO$_2$ under denitrifying conditions was confirmed in a microcosm study using the contaminated aquifer material from the same site [Bregnard et al., 1996].

### 2.4.3 Role of oxidants

In the aerobic degradation of hydrocarbons, O$_2$ plays a dual role: It is a co-substrate in initial transformation reactions by oxygenases [Watkinson and Morgan, 1990] and it also serves as the final electron acceptor for mineralization. Facultative denitrifying microorganisms can substitute NO$_3^-$ and even NO$_2^-$ or N$_2$O for O$_2$ as terminal electron acceptor [Tiedje, 1988]. However, a substitution for O$_2$ as the co-substrate of oxygenases is not possible, and yet unidentified alternative enzymes have to catalyze the initial oxidation of hydrocarbons. In the absence of O$_2$, denitrifying hydrocarbon degraders are known to exhibit slow growth on some hydrocarbons as sole carbon source [Häner et al., 1995; Rabus and Widdel, 1995] but frequently grow faster on partially oxidized transformation products of hydrocarbons such as alcohols or fatty acids [Schocher et al., 1991; Seyfried et al., 1994]. The largest increase of DOC concentrations was observed right at the column
inlet, where aerobic conditions existed. It is not known whether this DOC consisted of hydrocarbons or of transformation products from aerobic mineralization. Future studies should identify this organic carbon compounds to answer the question whether aerobic organisms generate partially oxidized transformation products that serve as substrates for denitrifying mineralization.

2.4.4 O$_2$-threshold for denitrification

For facultatively denitrifying microorganisms it has been demonstrated that the O$_2$ concentration is the dominant regulator for the expression of enzymes for the reduction of NO$_3^-$, NO$_2^-$ and N$_2$O in the respiratory chain. Most of these organisms synthesize the denitrifying enzymes only when O$_2$ concentrations are below 0.2 to 0.67 mg O$_2$ l$^{-1}$ [Tiedje, 1988]. This concentration range is frequently regarded as an O$_2$-threshold for denitrification [Tiedje, 1988]. In this study, denitrifying processes were observed in the presence of mean O$_2$ concentrations above this reported O$_2$-threshold.

The O$_2$-threshold concept has been applied in numerical models describing the aerobic and denitrifying mineralization of contaminants in aquifers [Kinzelbach et al., 1991; Schäfer et al., 1994]. The problem of "aerobic denitrification" is accounted for in the model formulations using mobile and immobile regions [Kinzelbach et al., 1991]. This concept allows aerobic mobile regions close to anaerobic immobile regions and therefore, it is possible to have anaerobic processes in cells of the model where there is still oxygen available in the mobile water. Microelectrode studies [Jørgensen and Revsbech, 1983] confirm such concepts by showing that the O$_2$ concentration in stagnant water or within soil aggregates can drop from 10 to < 0.1 mg l$^{-1}$ within a distance of 0.5 mm. The data presented in our study are suited to validate the model concept proposed by [Kinzelbach et al., 1991].
2.5 Summary and conclusions

The study demonstrates that the infiltration of aerobic, NO$_3^-$ containing artificial groundwater into laboratory aquifer columns filled with aquifer material contaminated with hydrocarbons results in the consumption of oxidants and a transformation of hydrocarbons to dissolved inorganic and organic carbon and bacterial biomass. Although the spatial penetration of O$_2$ was limited, removal of hydrocarbons and production of DIC occurred within the entire column length. A preferential degradation of $n$-alkanes compared to $i$-alkanes, but only a small removal of total hydrocarbons was observed in the anaerobic zone that prevailed at distances $> 24$ cm from the infiltration. Denitrification was observed not only under anaerobic conditions, but also in the presence of O$_2$ in the bulk solution. The results underline the beneficial role of NO$_3^-$ addition for aquifer bioremediation and have implications for the design of appropriate transport and reaction models.
In situ analysis of denitrifying, toluene and m-xylene degrading bacteria in a diesel fuel contaminated laboratory aquifer column

Abstract

A diesel fuel contaminated aquifer was bioremediated in situ by the injection of oxidants (O\textsubscript{2} and NO\textsubscript{3}\textsuperscript{-}) and nutrients into the contaminated zone in order to stimulate microbial activity. After 3.5 years of remediation, an aquifer sample was excavated and the material was used (i) to isolate bacterial strains able to grow on selected hydrocarbons under denitrifying conditions, and (ii) to construct a laboratory aquifer column in order to simulate the aerobic and denitrifying remediation processes. Five bacterial strains isolated from the aquifer sample were able to grow on toluene (strains T\textsubscript{2,3,4,6,10}) and nine bacterial strains grew on toluene and m-xylene (strains M\textsubscript{3,7,9,12}). Strains T\textsubscript{2,3,4,6,10} were cocci and strains M\textsubscript{3,7,9,12} were rods. The morphological and physiological differences were also reflected in small sequence variabilities in domain III of the 23S rRNA and on the 16S rRNA. Comparative sequence analysis of the 16S rRNA of one isolate of each group (T\textsubscript{3} and M\textsubscript{3}) revealed a close phylogenetic relationship of both groups of isolates to the genus Azoarcus. Two 16S rRNA-targeted oligonucleotide probes (Azo644 and Azo1251) targeting the isolates, bacteria of the A. tolulyticus group and A. evansii were used to investigate the significance of hydrocarbon degrading Azoarcus sp. in the laboratory aquifer column. The total number of bacteria in the column determined after DAPI staining was in the range of 5.8 x 10\textsuperscript{8} to
1.1 x 10^9 cells g⁻¹ aquifer material [dry wt.]. About 1% (anaerobic zone of the column) to 2% (aerobic zone of the column) of these bacteria were detectable by using a combination of probes Azo644 and Azo1251, demonstrating that hydrocarbon degrading *Azoarcus* sp. are significant members of the indigenous microbiota. More than 90% of the total number of bacteria were detectable by using probes targeting higher phylogenetic groups. Approximately 80% of these bacteria belonged to the β-subdivision and 10 to 16% to the γ-subdivision of the Proteobacteria. Bacteria of the α-subdivision of Proteobacteria were only present in high numbers (10%) in the aerobic zone of the column.

### 3.1 Introduction

Diesel fuel contaminated soils and aquifers can be partially remediated by pumping hydrocarbons occurring in free phase back to the soil surface or by stripping the subsurface with air [Bouwer, 1992]. Residual hydrocarbons, however, are often trapped in cracks and pores of the subsurface, and they may be removed by *in situ* bioremediation. This technique is usually based on the infiltration of water supplemented with oxidants (e.g. O₂, NO₃⁻) and/or nutrients (e.g. NH₄⁺, PO₄³⁻) to stimulate the catabolic activity of microorganisms in the subsurface and thereby the biodegradation of the hydrocarbons [Gersberg et al., 1993; Hunkeler et al., 1995; Hutchins and Wilson, 1991; Kämpfer et al., 1993; Nelson et al., 1994].

An *in situ* bioremediation was applied in a diesel fuel contaminated aquifer in Menziken, Switzerland [Hunkeler et al., 1995]. Ground water supplemented with O₂ (329 μM) and NO₃⁻ (1020 μM) as electron acceptors and NH₄H₂PO₄ (75 - 125 μM) as nutrient was infiltrated into the contaminated zone. At a monitoring well located 20 m downstream of the infiltration, the oxidants were found to be almost completely depleted [Hunkeler et al., 1995]. However, under field conditions, the hydrocarbon mineralization and oxidant consumption could only be analyzed with poor spatial resolution, and carbon mass, nitrogen and electron balances could not be established accurately. Therefore, after running the *in situ* bioremediation for 3.5 years it was decided to excavate some of the contaminated aquifer...
material and to simulate the processes in laboratory aquifer columns [Hess et al., 1996]. These columns had a length of 40.5 cm and they were operated under continuous flow conditions with an artificial groundwater containing O$_2$ (4.4 mg l$^{-1}$) and NO$_3^-$ (1610 µM). After a few days operation, distinct redox zones were established. Within 24 cm, the concentration of O$_2$ decreased from 4.4 mg l$^{-1}$ to a value below the detection limit of 0.1 mg l$^{-1}$. Denitrification was found to occur over the entire length of the column, regardless of the presence of O$_2$. Data on the evolution of dissolved inorganic carbon and the ratio of n-alkanes to i-alkanes suggested that biodegradation of hydrocarbons (initial hydrocarbon concentration of 1100 µg g$^{-1}$) occurred over the entire column length [Hess et al., 1996].

Diesel fuel consists of a large variety of hydrocarbons that can be degraded under aerobic and partially under anaerobic conditions [Bregnard et al., 1996]. With respect to groundwater pollution, aromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylene (BTEX) are of major concern since they are highly soluble in water and rather toxic. Diesel fuel contains on average about 1.35 mg toluene and 1.43 mg m- plus p-xylene g$^{-1}$ [Millner et al., 1992]. During the last years, many pure cultures of bacteria have been obtained that are able to degrade toluene under aerobic and anaerobic, that is, denitrifying [Dolfing et al., 1990; Evans et al., 1991; Fries et al., 1994; Rabus and Widdel, 1995; Schocher et al., 1991], iron-reducing [Lovley and Lonergan, 1990], and sulfate-reducing [Rabus et al., 1993] conditions. However, the significance of these isolates in their natural habitat, i.e. the contaminated soil and aquifer material, is not known.

The aim of our study was to investigate the significance of bacterial isolates in the diesel fuel contaminated laboratory aquifer column. These isolates were obtained from the excavated aquifer material from Menziken and had the capacity to degrade toluene and/or m-xylene under denitrifying conditions. The studies included i) the molecular characterization of the isolates by comparative sequence analysis of the 16S rRNA, and ii) the use of specific rRNA targeted oligonucleotide probes to enumerate the isolates in the diesel fuel contaminated laboratory aquifer column by $in situ$ hybridization.
3.2 Materials and methods

3.2.1 Isolation and growth conditions

Toluene and m-xylene degrading isolates were obtained from denitrifying enrichment cultures inoculated with excavated material of a diesel fuel contaminated aquifer (Menziken, Switzerland) [Hunkeler et al., 1995]. Samples of the aquifer material (10 g) were added to 50 ml of oxygen-free basal medium [Widdel and Bak, 1992], supplemented with 1.4 mM Na$_2$SO$_4$, 5 mM KNO$_3$, 1 ml l$^{-1}$ non-chelated trace element mixture SL10 [Widdel and Bak, 1992], 1 ml l$^{-1}$ selenite-tungstate-, and 0.5 ml l$^{-1}$ vitamin solution [Tschech and Fuchs, 1987], 15 ml l$^{-1}$ NaHCO$_3$ solution and either 0.19 mM toluene or 0.16 mM m-xylene at a pH of 7.2. The cultures were incubated at 28°C for 10 weeks. Isolates were obtained after aerobic incubation of serial dilutions of the enrichment cultures on agar plates containing basal medium supplemented with 0.1% pyruvate and 0.05% KH$_2$PO$_4$. They were subsequently grown and maintained in liquid culture under denitrifying conditions with either 0.19 mM toluene or 0.16 mM m-xylene, respectively. The cultures were incubated at 25°C on a rotary shaker (100 rpm). Growth of all isolates on both substrates was also tested under aerobic conditions.

3.2.2 Molecular characterization of isolates

Cells of well grown cultures of the isolates (3 ml) were harvested by centrifugation at 14 000 x g for 1 minute. Cells were resuspended in 100 μl of distilled water and lysed by the addition of 50 μl of proteinase K (6 mg ml$^{-1}$; Appligene, Basel, Switzerland) and 1.5 μl of 10% SDS and incubation for 30 minutes at 37°C. Nucleic acids were extracted with phenol/chloroform, precipitated with ethanol, dried and resuspended in distilled water [Sambrook et al., 1989].

Phylogenetic characterization was based on sequence comparison of approximately 1450 bp of the 16S rRNA of two isolates, T$_3$ and M$_3$, respectively, and of a 240 bp fragment of the 23S rRNA of all 14 strains obtained. The 16S rRNA was amplified by PCR using oligonucleotide primer
FGPS6 (5'GGAGAGTTAGATCTTGGCTCAG) containing an internal BgII site [Bosco et al., 1992] and primer 1510 (5'GTGCTGCAGGGTTACCTTGTTACGACT) extended with a PstI site [Embley et al., 1988]. The 23S rRNA fragment was amplified with primers 23InsV (5'CACAGATCTMADCGTAGNCGA1WGG) extended with a BgIII site [Roller et al., 1992] and 23InsR (5'CACAAGCTTTGTGCGGTGTTTNBGGTA) extended with a HindIII site [Roller et al., 1992]. Amplification by PCR was performed in a total volume of 100 µl containing 10 µl 10 x PCR buffer (500 mM KCl, 25 mM MgCl₂, 200 mM Tris/HCl, pH 8.4, 0.1% Triton 100), 2 µl dNTPs (each 10 mM in 10 mM Tris/HCl, pH 7.5), 0.2 µl Taq polymerase (Appligene, Basel, Switzerland, 5 U µl⁻¹), 3 µl of both primers (100 ng) and the DNA (approx. 100 ng). Thirty rounds of temperature cycling (Crocodile II Thermocycler, Appligene) at 95°C, 50°C and 70°C for 30 seconds each, were followed by a final seven minute incubation at 70°C.

The amplification products were treated with proteinase K (Appligene, final concentration 60 ng per 100 µl) at 37°C for 15 minutes [Crowe et al., 1991], extracted with phenol/chloroform, and precipitated with ethanol [Sambrook et al., 1989]. Subsequently, the DNA was cleaved with restriction enzymes PstI (Appligene) and BgIII (Promega, Catalys, Wallisellen, Switzerland) for 16S rRNA and BgIII and HindIII (Promega) for 23S rRNA fragments and cloned into pGEM-3zf (Promega) by standard methods [Sambrook et al., 1989]. The DNA was transformed into E. coli DH5α and plasmid preparation was performed by using the alkaline lysis method [Sambrook et al., 1989].

Cloned amplification products were sequenced using the Thermo Sequenase™ fluorescent labeled primer cycle sequencing kit (Amersham, Zurich, Switzerland) according to the manufacturers instructions. The sequences of the isolates were compared to the 16S rRNA sequences of EMBL Nucleotide Sequence Database by FASTA analysis of the sequence analysis GCG package (GCG program manual 1994, Madison, WI, USA).
3.2.3 Probes and stains

Oligonucleotide probes targeting rRNA of Bacteria (Eub338) [Amann et al., 1990], the α-, β-, and γ-subdivision of Proteobacteria (Alf1b, Bet42a, and Gam42a) [Manz et al., 1992], Gram-positive bacteria with a high G+C DNA content (HGC69a) [Roller et al., 1994] and probes Azo644 (5’GCCGTACTCTAGCCGTGC, position 644 - 661 on the 16S rRNA according to the E. coli numbering, [Brosius et al., 1981]) and Azo1251 (5’CGCGCTTTGGCAGCCCT, position 1251 - 1267 on the 16S rRNA according to the E. coli numbering) were synthesized with a primary amino group at the 5'-end (MWG Biotech, Ebersberg, Germany). The fluorescent dye Cy3 Reactive Dye (Amersham) was covalently bound to the amino group of the oligonucleotide probe, and the dye-oligonucleotide conjugate (1:1) was purified from unreacted components and stored at -20°C at concentrations of 25-30 ng μl⁻¹ [Amann et al., 1990].

The DNA intercalating dye 4’6-diamidino-2-phenylindole (DAPI, Sigma, Buchs, Switzerland) was stored in a 1 mg ml⁻¹ solution at -20°C [Porter and Feig, 1980]. DAPI staining was performed by the addition of DAPI to the hybridization reaction (final concentration of 20 ng μl⁻¹) and always used as a control staining to detect all bacteria present in the preparation.

3.2.4 Column operation and analytical procedures

A detailed description of the column operation and analytic protocols is given in [Hess et al., 1996]. After 96 days of operation the column was disconnected and the aquifer material was frozen and stored at -20°C until further use.

3.2.5 Cell fixation and pretreatment

For whole cell hybridization, the frozen aquifer core was dissected into pieces of 2.5 or 5 cm length (Fig. 3.3). This material and cells of pure cultures, respectively, were fixed in 3 volumes of 4% paraformaldehyde in phosphate-buffered saline (PBS, composed of 0.13 M NaCl, 7 mM Na₂HPO₄
and 3 mM NaH$_2$PO$_4$, pH 7.2 in water [Hahn et al., 1992] for 3 hours at 4°C. Fixed samples were washed with 50% ethanol in PBS and stored in 50% ethanol in PBS at -20°C [Fischer et al., 1995].

Before application to slides, 40 µl of the aquifer homogenate was mixed with 960 µl of 0.1% sodium pyrophosphate, and the material was dispersed with an ultrasonic treatment probe (2 mm diameter; Sonifier B-12, Branson, Danbury, Connecticut) at 20% for 1 minute. Twenty µl were subsequently applied to each well (8 mm in diameter) on gelatin-coated slides (0.1% gelatin, 0.01% KCr(SO$_4$)$_2$) and allowed to air dry [Amann et al., 1990]. After dehydration in 50, 80, and 96% ethanol for 3 minutes each, the preparations were treated with lysozyme (Fluka, Buchs, Switzerland, 1 mg corresponding to 37320 U dissolved in 1 ml of distilled water) at 37°C for 10 minutes [Fischer et al., 1995; Hönerlage et al., 1995], followed by a final dehydration in 50, 80, 96% ethanol.

3.2.6 Whole cell hybridization

Hybridizations were carried out in 8 µl of hybridization buffer (0.9M NaCl, 20mM Tris/HCl, 10 mM EDTA, 0.01% SDS, pH 7.2) in the presence of 10 to 30% formamide (Alf1b = 10%, HGC69a, Azo644 and Azo1251 = 20%, Eub338, Bet42a, and Gam42a = 30%), 1 µl of the probe (25-30 ng µl$^{-1}$), and 1 µl of the DAPI solution (200 ng µl$^{-1}$) at 40°C for 2 hours. After hybridization, the slides were washed in buffer containing 20 mM Tris/HCl, 10 mM EDTA, 0.01% SDS and either 440, 308, or 102 mM NaCl depending on the formamide concentration during hybridization (10, 20 and 30%, respectively) for 15 minutes at 48°C, subsequently rinsed with distilled water, and air-dried.

Samples were mounted with Citifluor solution (Citifluor, Canterbury, UK) and examined with a Zeiss Axiophot microscope (Zeiss, Oberkochen, Germany) fitted for epifluorescence detection with a high-pressure mercury bulb and filter sets 02 (DAPI) and HQ Cy3 (AHF Analysen Technik, Tübingen, Germany) (Cy3). Microorganisms were counted at 1000 x magnification in 20 fields covering an area of 0.01 mm$^2$ each [Fischer et al., 1995].
3.3 Results and discussion

3.3.1 Characterization of isolates

Five toluene (T_{2,3,4,6,10}) and nine m-xylene (M_{3,7,9,12}) degrading isolates were obtained under denitrifying conditions from the excavated aquifer material from Menziken. Under denitrifying conditions, isolates M_{3,7,9,12} grew on both toluene and m-xylene, while isolates T_{2,3,4,6,10} could only grow on toluene. Under aerobic conditions, all isolates were able to grow on toluene, but none of the isolates grew on m-xylene. T_{2,3,4,6,10} grew as cocci, while M_{3,7,9,12} grew as rods.

![Sequence comparison of domain III, beginning at the primer InsV, of the 23S rRNA of isolates T_{2,3,4,6,10} (T) and M_{3,7,9,12} (M).](image-url)
The morphological and physiological differentiation between isolates T2.3.4.6,10 and M3.7.9.12 was also reflected on the rRNA level. Sequence comparison of domain III of the 23S rRNA of all isolates revealed identical sequences within the respective groups which may indicate the multiple isolation of the same organisms. Between the isolates of each group, however, only similarity values of 93% were found (Fig. 3.1). Large differences were also obtained by comparative sequence analysis of the complete 16S rRNA of representative isolates of each group (T3 and M3, respectively). Here, a similarity value of only 96.2% was obtained (Tab. 3.1).

3.3.2 Probe design

Based on 16S rRNA sequences of isolates T3 and M3, 16S rRNA targeted oligonucleotide probes (Azo644 and Azo1251, respectively) were designed using a database containing 16S rRNA sequences of about 4000 bacteria and the ARB probe design program (kindly provided by Dr. W. Ludwig, TU Munich, Germany). Probe Azo644 was designed to detect both toluene degrading and toluene and m-xylene degrading isolates, whereas probe Azo1251 was designed to differentiate between both isolates. A search for target sequences for both probes in the EMBL Genbank showed identical sequences on 16S rRNA of A. toluolyticus (strains Tol-4, Td-1, Td-15, Td-17, Td-19 and Td-21), A. evansii KB740 and A. sp. pF6. An identical sequence to the target sequence of probe 644 was also detected on the 16S rRNA of the β-Proteobacterium PbN1, while the identical target sequence of probe 1251 was found on 16S rRNA of β-Proteobacterium ToN1. Several strains showed target sequences with one mismatch to the probes. On A. toluolyticus Td-2 and Td-3, as well as on A. denitrificans this mismatch represented a weak change which allows a detection similar to target sequences without a mismatch. In contrast, the mismatch on A. sp. BH72, Th. aromatica K172, and β-Proteobacteria EbN1 and mXylN1 represented a strong change allowing a differentiation between this sequence and the target sequence at high stringency hybridization conditions [Neef et al., 1996].
The applicability of both probes for whole cell hybridization and their specificity was demonstrated by whole cell hybridization on fixed cells of the isolates, *A. tolulyticus* Td-15, *A. indigens* VB32, *Th. aromatica* K172 and several reference organisms which were shown to contain target sequences similar to those of the isolates by the ARB program (*Alcaligenes* sp. (Genbank accession no. L31650), *Burkholderia cepacia* DSM 50181 (L28675), *Comamonas testosteroni* DSM 50251 (M11224), *Nitrosomonas europaea* ATCC 25978 (M96399) (Tab. 3.2).

<table>
<thead>
<tr>
<th>Organisms</th>
<th>probe Azo644 (18 bp)</th>
<th></th>
<th>probe Azo1251 (17 bp)</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>hybridization</td>
<td>mismatches</td>
<td>hybridization</td>
<td>mismatches</td>
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<tr>
<td>T2,3,4,6,10</td>
<td>+</td>
<td>0</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td>M3-7,9-12</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td><em>Azoarcus tolulyticus</em> Td-15</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td><em>Azoarcus indigens</em> VB32</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>3</td>
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<tr>
<td><em>Thauera aromatica</em> K172</td>
<td>-</td>
<td>1</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><em>Comamonas testosteroni</em></td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><em>Alcaligenes</em> sp.</td>
<td>-</td>
<td>3</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><em>Nitrosomonas europaea</em></td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>3</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em></td>
<td>-</td>
<td>4</td>
<td>-</td>
<td>4</td>
</tr>
</tbody>
</table>

(+) indicates a positive signal and (-) a negative signal

**Table 3.2:** Whole cell hybridization with probes Azo644 and Azo1251 on cells of the isolates T2,3,4,6,10 and M3-7,9-12 and some reference bacteria.

At highly stringent hybridization conditions using 30% formamide, isolates T2,3,4,6,10 and M3-7,9-12 as well as *A. tolulyticus* Td-15 were detected by whole cell hybridization with Cy3-labeled probe Azo644. Probe Azo1251, however, only hybridized to isolates M3-7,9-12 and *A. tolulyticus* Td-15, but not to isolates T2,3,4,6,10 (Tab. 3.2, Fig. 3.2). None of the reference organisms nor *A. indigens* VB32 or *Th. aromatica* K172 showed hybridization signals to one of the probes under these conditions. These results demonstrate the applicability of whole cell hybridization with probes Azo644 and Azo1251 for the detection of isolates T2,3,4,6,10 and M3-7,9-12. The specificity of the
probes for the detection of isolates T_{2,3,4,6,10} and M_{3-7,9-12}, however, is restricted because hybridization results on bacteria of the *A. tolylyticus* group as well as *A. evansii* suggest a more general specificity for hydrocarbon degrading *Azoarcus* sp..

![Figure 3.2: Epifluorescence micrographs of mixtures of isolates T_{3} (cocci) and M_{3} (rods) after whole cell hybridization with Cy3-labeled probes Azo644 (a) and Azo1251 (b). Bar represents 5 μm.](image)

### 3.3.3 Characterization of microbial populations in the column

Whole cell hybridization using a combination of both probes Azo644 and Azo1251 was subsequently used to study the significance of populations of the isolates T_{2,3,4,6,10} and M_{3-7,9-12} as well as the closely related bacteria of the *A. tolylyticus* group and *A. evansii* in the laboratory aquifer column simulating the processes at Menziken. Data on the O_{2}, NO_{3}⁻ and NO_{2}⁻ concentrations in this column were reported in detail by Hess et al. [Hess et al., 1996]. The first 12 cm of the column was aerobic with an O_{2} concentration > 0.1 mg l⁻¹, while the zone from 24 to 40.5 cm was anaerobic with an O_{2} concentration below the detection limit of 0.1 mg l⁻¹. The zone in between had an O_{2} concentration around 0.1 mg l⁻¹ and was termed microaerobic. Total bacterial numbers determined after DAPI staining were highest in the aerobic (1.1 x 10⁹ cells g⁻¹ aquifer material at 1.5 cm column depth) and the anaerobic (9.1 x 10⁸ cells g⁻¹ aquifer material at 32 cm column depth).
depth) zone of the column (Fig. 3.3c). In the aerobic zone, the large number of cells may be due to favourable environmental conditions since O$_2$ and NO$_3^-$ consumption as well as hydrocarbon mineralization were high (Fig. 3.3b). In the anaerobic zone the increase in cell numbers could be correlated to the increase in NO$_3^-$ consumption. In the microaerobic zone of the column bacterial numbers were low (5.8 x 10$^8$ cells g$^{-1}$ aquifer material). This result is again in agreement with the chemical data which showed that O$_2$ was almost depleted and the NO$_3^-$ consumption was low (Fig. 3.3b).

Whole cell hybridization with probe Eub338 targeting all bacteria revealed that between 86 and 99% of the DAPI-stained bacteria were detected throughout the whole column (Fig. 3.3c). The highest percentages were obtained in the aerobic zone with 99%, the lowest in the anaerobic zone with 86%. The large percentage of cells detectable by whole cell hybridization reveals the presence of metabolically active cells containing sufficient amounts of rRNA for detection and, at the same time, a sufficient permeability or permeabilization of the cells, which is important for the in situ detection of cells by whole cell hybridization with oligonucleotide probes [Amann et al., 1995]. Comparably high percentages of cells detectable by whole cell hybridization have been reported for nutrient-rich environments such as activated sludge with up to 89% [Wagner et al., 1994; Wagner et al., 1993, Wagner et al., 1994] or lake snow with 55-100% [Weiss et al., 1996]. In environments supplying less eutrophic conditions such as e.g. in drinking water, 70% of surface-associated and 40% of planktonic cells could be detected [Manz et al., 1993]. In the winter cover and pelagic zone of a high mountain lake, the percentage of cells detectable by whole cell hybridization resembled 40-81% [Alfreider et al., 1996], that in bacterioplankton 35-67% [Hicks et al., 1992] and that in earthworm casts up to 37% [Fischer et al., 1995]. Therefore the large percentage of cells detectable by whole cell hybridization also suggests that the aquifer column offers favourable growth conditions.
Figure 3.3: Column set-up (a), concentrations of $O_2$, $NO_3^-$, and $NO_2^-$ averaged over 96 days in the ground water and residual hydrocarbons after 96 days in the column (initial concentration was 1100 µg hydrocarbons g$^{-1}$) (b) and results of in situ hybridization (c, d).
In this laboratory aquifer column, bacteria of the β-subdivision of Proteobacteria were numerically dominant (Fig. 3.3c). In the aerobic zone, between 80 and 87% of the bacteria detectable by whole cell hybridization belonged to the β-subdivision of Proteobacteria. These numbers decreased to 42% in the middle of the column and then increased to 66% in the anaerobic zone (Fig. 3.3c). The remaining bacteria mainly belonged to the γ-subdivision of Proteobacteria with numbers between 10% in the aerobic and 16% in the anaerobic zone of the column, while bacteria of the α-Proteobacteria were detected in large amounts (10%) only in the aerobic zone (Fig. 3.3d). Bacteria with a high G+C DNA content were detected only in low numbers.

It was reported that the microbial communities in the aquatic environments were also dominated by bacteria of the β-subdivision of Proteobacteria, e.g. 27-42% of the cells in lake snow [Weiss et al., 1996] or 6.5-116% in the winter cover and pelagic zone of the mountain lake [Alfreider et al., 1996]. In earthworm casts cell numbers of bacteria of the α- and γ-subdivision of Proteobacteria exceeded numbers of bacteria of the β-subdivision of Proteobacteria by far [Fischer et al., 1995].

Our target organisms, i.e. the isolates, bacteria of the A. toluolyticus group and A. evansii, were detected by whole cell hybridization with a combination of probes Azo644 and Azo1251 throughout the whole column (Fig. 3d). Their population was higher in the aerobic zone of the column close to the inlet (2%) than in the rest of the column (1%). Compared to genus-specific detection of e.g. Acinetobacter which is present in numbers less than 8% of the total bacterial community in activated sludge with enhanced biological phosphate removal [Wagner et al., 1994], the detection of hydrocarbon degrading Azoarcus sp. in numbers between 1 and 2% suggests that these Azoarcus sp. may play an important role during bioremediation in the diesel fuel-contaminated aquifer material.

Because of the extreme differences between column and field conditions, an extrapolation of the population data obtained in our laboratory aquifer column (scale 40 cm, very limited heterogeneity) to the field (scale larger than 20 m, considerable spatial heterogeneity) is difficult. More
information on the catabolic activity of the hydrocarbon degrading *Azoarcus* population in columns and the field will probably be obtained when recent advances in studies on the anaerobic toluene mineralization pathway [Crowe et al., 1991; Migaud et al., 1996] will lead to the identification of the respective genetic information. These sequences will allow hopefully the design of probes directed against the mRNA of crucial catabolic enzymes and hence the determination of the *in situ* activity of microbial populations in the subsurface.
Analysis of bacterial community structures in a heating oil contaminated aquifer treated by intrinsic bioremediation

Abstract

This study presents the analysis of the bacterial community structure in a heating oil contaminated aquifer in Studen, Switzerland, which is treated by intrinsic bioremediation. The zone where petroleum hydrocarbons were found in free phase (source area, maximum concentration 123 μg l⁻¹) was 90 m long and 25 m wide. Oxidants such as O₂, NO₃⁻, and SO₄²⁻ were depleted, whereas reduced species such as Fe²⁺, Mn²⁺, and CH₄ as well as dissolved inorganic carbon (DIC) and Ca²⁺ were elevated. The observed changes in groundwater composition in the source area indicated an active microbial population. In two wells drilled at the border of the source area concentration profiles of ions and petroleum hydrocarbons in the porewater of the core material were measured. In both wells the petroleum hydrocarbon concentrations were below detection limit.

A characterization of the indigenous microbial population in the two wells showed moderately active organisms. Cell numbers were about 10⁷ to 10⁸ cells g⁻¹ aquifer material [dry wt.]. 10-20% of the cells were detectable by in situ hybridization with oligonucleotide probes. The population mainly consisted of members of the β- and γ-subdivision of Proteobacteria, but also members of the α-subdivision of Proteobacteria, gram-positive bacteria with high G+C DNA content, and Planctomycetes could be detected. One well showed very high numbers of protozoa (0.8-4 *10⁶ cells g⁻¹aquifer material [dry wt.]). The absence of Azoarcus tolulyticus could be confirmed by PCR with specific primers.
4.1 Introduction

In situ bioremediation is a technology often used for the clean-up of petroleum contaminated sites. It has found special attention because it ideally leads to complete mineralization of the petroleum hydrocarbons at reasonable costs. In situ bioremediation techniques can be grouped into two different types [U.S. National Research Council, 1993]: engineered and intrinsic. Intrinsic bioremediation is a relatively new option in which no active measures besides the removal of the source and the free floating oil phase are taken. Intrinsic in situ bioremediation relies on the ability of naturally occurring microorganisms to degrade petroleum hydrocarbons without external intervention [Barker et al., 1987; Baedecker et al., 1993; Davis et al., 1994; Borden et al., 1995]. It requires extensive monitoring to ensure that the contaminants do not migrate faster than the indigenous microorganisms can degrade them. Intrinsic bioremediation or natural attenuation are the terms now commonly applied to the process of establishing evidence that a contaminant will be degraded or otherwise be rendered harmless by natural processes [Madsen et al., 1991; U.S. National Research Council, 1993]. Intrinsic bioremediation is not only a biodegradation process. Physical and chemical phenomena such as dispersion, sorption, and abiotic transformations are often important as well [Rifai et al., 1995]. However biodegradation is most often the primary mechanism for contaminant destruction since it leads to a reduction of the total mass of hydrocarbons [MacDonald and Rittmann, 1993].

In an urban area in Studen, Switzerland, a spill of > 30 metric tons of heating oil was discovered in 1993, polluting one of the most important gravel aquifers in Switzerland. Preliminary observations indicated that an indigenous microbial population had developed, possibly degrading petroleum hydrocarbons [Bolliger et al., 1998]. Due to these results and the fact that no drinking water wells downstream were threatened, the authorities decided to limit remediation to the physical removal of the free phase by pumping and to monitor the natural attenuation processes. Previous
investigations [Bolliger et al., 1998] demonstrate that intrinsic bioremediation is a major process in the removal of petroleum hydrocarbons at this site and that an active microbial community has developed. The aim of this study was now to characterize the microbial community in this aquifer by in situ hybridization.

### 4.2 Materials and methods

#### 4.2.1 Site characterization

The field site is located in Studen, Canton Berne, Switzerland, at 440 m above sea level. In January 1993, a heating oil spill was discovered. The contamination was caused by a leaking underground pipe between a storage tank and a heating system. An unknown amount of heating oil had percolated into the subsurface over an unknown period of time. After the discovery of the contamination, remediation measures were undertaken by the cantonal water authorities. 11 wells were installed, which were screened in the lowest meter. Between 1993 and 1996, about 34'000 l of heating oil were recovered using floating pumps. After June 1996, no further active remediation actions were undertaken.

In January 1993 and May 1996, several monitoring wells were drilled in less accessible areas in order to estimate the size of the source area and the plume. All tubes were screened in the saturated part of the aquifer. The zone where the wells contained heating oil in free phase in 1993 is designated source area (Fig. 4.1). The source area remained stable after 1993.

The oil covered a zone of about 90 m length and 25 m width, extending below several buildings. The water table at the spill 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. 4.1). From 1993 to 1996, the maximum fluctuation of the water table was 0.4 m. The temperature of the groundwater changed seasonally between a minimum of 9.5°C in March and a maximum of 11.5°C in September.
Figure 4.1: Location and map of the site at Studen. Bold characters represent the two wells where the microbial community was analyzed.
4.2.2 Sampling

The excavated sandy-loamy material from the wells drilled in May 1996 was stored at -20°C in PVC liners wrapped in teflon bags filled with N₂. Two of the cores were used for porewater analysis and the characterization of the microbial community.

To analyze the porewater 6-9 g of the aquifer material were centrifuged for 10 minutes at 10'000 x g. The supernatant was transferred in 1 ml glass vials and closed with teflon-lined screw caps.

4.2.3 Chemical analysis

Concentrations of anions were determined by a Dionex DX-100 ion chromatograph equipped with an IonPac AS4A-SC column using an eluent of 1.8 mM Na₂CO₃ and 1.7 mM NaHCO₃. NH₄⁺ was determined by a Dionex DX-100 ion chromatograph equipped with an IonPac AS12A-SC column using an eluent of 20 mM CH₄SO₃. The detection limit was approximately 1 μM.

To measure the BTEX concentrations in the porewater, a mixture of 10 g aquifer material (ww.) and 1.5 ml distilled water was extracted with 1.5 ml pentane. Concentrations of benzene, toluene, ethylbenzene, o-, m-, and p-xylene, as well as 2-, 3-, 4-Ethyltoluene and 1,2,3-, 1,3,5-, 1,2,4-Trimethylbenzene were determined by injecting 1 μl of the extract by an autosampler into a Carlo Erba GC8000 gas chromatograph equipped with a flame ionization detector (Fisons Instruments) and a 2 m glass column (diameter 3 mm) packed with 5% SP 1200 and 5% bentone 34 on Supelcoport 100/120 mesh (Supelco Inc., Bellefonte, Pennsylvania, USA). The carrier gas was N₂, and the oven temperature was maintained at 100°C for 15 min. The chromatographic data were processed with Chrom-Card for Windows (Fisons Instruments).
4.2.4 Microbial analysis

For whole cell hybridization, the frozen aquifer cores were dissected into pieces of 10 cm length and some parts of it were fixed in 3 volumes of 4% paraformaldehyde in phosphate-buffered saline (PBS, composed of 0.13 M NaCl, 7 mM Na₂HPO₄ and 3 mM NaH₂PO₄, pH 7.2 in water) [Hahn et al., 1992] for 3 hours at 4°C. Fixed samples were washed with 50% ethanol in PBS and stored in 50% ethanol in PBS at -20°C [Fischer et al., 1995].

Before application to slides, 40 μl of the aquifer homogenate was mixed with 960 μl of 0.1% sodium pyrophosphate, and the material was dispersed with an ultrasonic treatment probe (2 mm diameter; Sonifier B-12, Branson, Danbury, Connecticut) at 20% for 1 minute. Ten μl were subsequently applied to each well (8 mm in diameter) on teflon coated slides, allowed to air dry, and dehydrated in 50, 80, and 96% ethanol for 3 minutes each [Amann et al., 1990].

Oligonucleotide probes targeting rRNA of Bacteria (Eub338) [Amann et al., 1990], the α-, β-, and γ-subdivision of Proteobacteria (Alf1b, Bet42a, and Gam42a) [Manz et al., 1992], Gram-positive bacteria with a high G+C DNA content (HGC69a) [Roller et al., 1994], Planctomycetes (Pla5a and Pla30a) [Zarda et al., 1997], Eukarya (Euk516) [Amann et al., 1990], and A. toluolyticus (Azo644) [Hess et al., 1997] were synthesized with the fluorescent dye Cy3 bound to the primary amino group at the 5'-end (MWG Biotech, Ebersberg, Germany). The dye-oligonucleotide conjugate (1:1) was stored at -20°C at concentrations of 25-30 ng μl⁻¹ [Amann et al., 1990].

DAPI staining (4’6-diamidino-2-phenylindole, Sigma, Buchs, Switzerland) was performed by the addition of DAPI to the hybridization reaction (final concentration of 20 ng μl⁻¹) and always used as a control staining to detect all bacteria present in the preparation [Hess et al., 1997].

Hybridizations were carried out as described previously [Hess et al., 1997; Zarda et al., 1997].

Samples were mounted with Citifluor solution (Citifluor, Canterbury, UK) and examined with a Zeiss Axiophot microscope (Zeiss, Oberkochen,
Germany) fitted for epifluorescence detection with a high-pressure mercury bulb and filter sets 02 (DAPI; Zeiss, Oberkochen, Germany) and HQ Cy3 (Cy3; AHF Analysen Technik, Tübingen, Germany). Microorganisms were counted at 1000 x magnification in 20 fields covering an area of 0.01 mm² each [Fischer et al., 1995].

For DNA isolation, which was then used for PCR, 3 ml of the cell suspension (Azoarcus tolulyticus Td-15 and A. indigens VB32) were centrifuged and resuspended in 100 µl H₂O. 50 µl Proteinase K (6 mg ml⁻¹; Appligene, Basel, Switzerland) and 1.5 µl 10 % sodium dodecyl sulfate (SDS) were added and incubated for 30 min at 37 °C. Nucleic acids were extracted with phenol / chloroform and precipitated with 96 % ethanol and 0.3 M sodium acetate at -80 °C [Sambrook et al., 1989]. The washed, dry DNA was resuspended in 50 µl H₂O and stored at -20 °C. To extract DNA from soil 6 g aquifer material were mixed with 20 ml 0.1 % pyrophosphate and shaken for 30 min. After centrifugation 0.4 g glas beats and 1 ml extraction buffer (200 mM Tris, pH 7.5; 1.5 % SDS; 10 mM EDTA; 1 % deoxycholate; NP-40; 0.5 mM thiourea; 10 mM DDT) were added and mixed in a bead beater (Braun, Germany) at 4000 rpm for 1 min. After centrifugation the supernatant was extracted with phenol / chloroform [Sambrook et al., 1989]. The DNA was then cleaned by sephadex-columns [Morgan et al., 1993], resuspended in 50 µl H₂O and stored at -20 °C.

For PCR primer Azo644 [Hess et al., 1997], specific for A. tolulyticus and the non-specific primer FGPS6 [Bosco et al., 1992] (MWG Biotech, Germany) were used. For the amplification 10 µl 10 x PCR-buffer (500 mM KCl, 25 mM MgCl₂, 200 mM Tris-HCl [pH=8.4], 0.1 % Triton X-100), 2 µl dNTP (each 10 mM in 10 mM Tris-HCl [pH=7.5]), 0.2 µl Taq polymerase (5 U/µl, Appligene), 4 µl of each of the two primers (app. 100 ng) and DNA (app. 100 ng) were mixed. 35 cycles for 30 sec at 95 °C, 30 sec at 56 °C and 1 min at 70 °C were performed, followed by 7 min at 70 °C.
4.3 Results

4.3.1 Chemical analysis

The two investigated cores (P22 and P25) were located beside and downstream, respectively, of the source area (Fig. 4.1) and showed no BTEX contamination in the porewater between 2.2 and 3.4 m depth (data not shown).

The analyses of ions in the porewater of the two cores showed maximum $\text{NO}_3^-$ concentrations (80-100 µM) as well as $\text{SO}_4^{2-}$ concentrations (550 µM in P22 and 1400 µM in P25) at a depth of 2.7 m below surface, which was just below the watertable at 2.5 m (Fig. 4.2 top). Average concentrations for $\text{NO}_3^-$ were between 20-30 µM and for $\text{SO}_4^{2-}$ 300-500 µM. $\text{NO}_2^-$ was below detection limit (<1 µM) in P22 and 6 µM in P25. $\text{PO}_4^{3-}$ was below detection limit in both cores. $\text{NH}_4^+$ was below 1 µM in P22 and between 100 - 400 µM in P25, with a maximum at 2.7 m depth.

4.3.2 Microbial analysis

The analysis of the microbial population by DAPI staining showed that the average cell numbers (average of depth 2.2-3.4 m below subsurface) were $1.9 \pm 1 \times 10^7$ cells g$^{-1}$ aquifer material [dry wt.] in P22 and $2.2 \pm 0.6 \times 10^8$ cells g$^{-1}$ aquifer material [dry wt.] in P25 (Fig. 4.2 bottom). In situ hybridization with probe Eub338 revealed a detectability of $20.4 \pm 9.6\%$ of all cells in P22 and $10.0 \pm 7.8\%$ in P25.

In P22 the population consisted mainly of organisms belonging to the β- and γ-subdivision of Proteobacteria (30% and 28%, respectively, of the bacteria detected by probe Eub338). Bacteria of the α-subdivision of Proteobacteria were detected only in small numbers (3%). Gram-positive bacteria with high G+C DNA content were present with just 2.5%, and 0.5% of the population were Planctomycetes. No eukaryotes could be found.
**Figure 4.2:** Summary of the results of the two cores P22 and P25. Top: concentrations of \( \text{NO}_3^- \) and \( \text{SO}_4^{2-} \) (in \( \mu \text{M} \)) in the porewater; bottom: total cell numbers (▲DAPI) and members of the domain Bacteria (●Eub338) in the aquifer material.

In P25 the highest numbers again could be found for the β-subdivision (12%) and γ-subdivision of Proteobacteria (6%). The amount of members of the α-subdivision of Proteobacteria was below detection limit. A more important group seemed to be the Planctomycetes with 4.5%. Gram-positive bacteria with high G+C DNA content were present at low numbers (0.2%). In this core Eukarya were present in high numbers \( \left( 4 \times 10^6 \text{ cells g}^{-1} \text{ aquifer material [dry wt.]} \right) \) (Fig. 4.3).
Organisms of the *Azoarcus tolulyticus* group, which are members of the β-subdivision of Proteobacteria, could not be detected in the two cores.

In addition to *in situ* hybridization, the absence of *A. tolulyticus* in the aquifer material at Studen could be confirmed by PCR with specific primer Azo644 and the universal primer FGPS6 (Fig. 4.4, left). No amplification product showed up after PCR with extracted DNA from the core material (amplified fragment would have a length of approximately 640 bp). Fig. 4.4, right, shows the results obtained with contaminated material from Hünxe, Germany (chapter 5), where *Azoarcus tolulyticus* could be detected by *in situ* hybridization as well as by the specific PCR. The specific detection of *Azoarcus* by PCR gives a qualitative statement about the presence or absence of these organisms. However, it is very sensitive with a detection limit of 3 cells g⁻¹ aquifer material [dry wt.], compared to *in situ* hybridization, where the limit is around 5000 cells g⁻¹ aquifer material [dry wt.] [Tsai and Olson, 1992].
4.4 Discussion

The amount of petroleum hydrocarbons in P25 was below detection limit in the porewater of the core material as well as in the groundwater, where no hydrocarbons were found outside the source area (Fig. 4.5) [Bolliger et al., 1998]. The elevated concentrations along the plume centerline show that monoaromatic hydrocarbons dissolve from the residual petroleum hydrocarbon pool into the water phase within the source area. However, monoaromatic hydrocarbons were not found in monitoring wells downstream of the source area (P25, P27).
Figure 4.5: Concentrations of BTEX compounds in groundwater samples of wells along the plume centerline, measured on July 16, 1996 [Bolliger et al., 1998].

The chemistry of the porewater in the core material (collected in May 1996) and the groundwater at the site (samples collected in June 1996) differed in some points. For example NO₃⁻ concentrations in the porewater were lower than in the groundwater (P22: 20 vs. 130 μM; P25: 30 vs. 160 μM), whereas SO₄²⁻ concentrations were higher in the porewater that in the groundwater (P22: 500 vs. 330 μM; P25: 315 vs. 265 μM) [Bolliger et al., 1998]. Concentration profiles of selected chemical parameters in the groundwater along a transection are shown in Fig. 4.6. Oxidants (O₂, NO₃⁻, SO₄²⁻) were consumed within the source area and reached background levels again downstream. In the monitoring wells within the source area, dissolved Fe(II) and Mn(II) were detected and alkalinity and DIC were elevated. This indicates increased microbial activity within the source area. Changes in
concentrations of species measured in P25 and P27 towards background values (Fig. 4.6) are partly explained by the mixing of plume water with pristine groundwater during sampling [Bolliger et al., 1998].

Figure 4.6: Groundwater chemistry along the plume centerline on June 17, 1996 [Bolliger et al., 1998].

Total cell numbers of bacteria in P22 as well as in P25 were similar to numbers found in the border zone of a xylene contaminated aquifer [Zarda et al., 1998]. The cell numbers detected in the core material from Studen (10^7-
10^8 cells g\(^{-1}\) aquifer material [dry wt.] were much lower than in the column studies (10^9 cells g\(^{-1}\) aquifer material [dry wt.]) [Hess et al., 1997], where an engineered in situ bioremediation was simulated. Some reasons for the observed differences in cell numbers are obvious. While in Studen the contamination is treated by intrinsic bioremediation (no oxidants and nutrients are added) the column studies simulated an engineered bioremediation where the microbial community is stimulated by the addition of oxidants and nutrients. Another reason for the lower cell numbers in Studen may be the lack of substrate in the investigated cores (hydrocarbons were below detection limit), whereas the column samples contained an initial hydrocarbon concentration of 1.1 g kg\(^{-1}\). In other highly contaminated aquifers containing 0.8-2.7 g hydrocarbon kg\(^{-1}\) originating from jet fuel 1.2-3.3 *10^8 cells g\(^{-1}\) aquifer material [dry wt.] were found by acridine orange direct counts [Sinclair et al., 1993]. In the same study non-contaminated sites were investigated and numbers in the range of 1.5 *10^7 up to 5 *10^8 cells g\(^{-1}\) aquifer material [dry wt.] could be detected.

The detectability of 10-20% of the total cell number by probe Eub338 is in the same range as previous results showed for the border zone between a non-contaminated and contaminated aquifer [Zarda et al., 1998]. Often high cell numbers correlate with high activity caused by ideal environmental conditions. In this study it is not the case. In P25 the cell number was higher with 2 *10^8 cells g\(^{-1}\) aquifer material [dry wt.] than in P22 (2 *10^7 cells g\(^{-1}\) aquifer material [dry wt.]), whereas the detection rate was lower (10% vs. 20%). The higher cell number in P25 could be due to groundwater transport out of the contaminated area, where the bacteria might be more numerous.

Whole cell hybridization with probe Euk516 showed high numbers of protozoa in P25 in the saturated as well as in the unsaturated zone (0.8-4 *10^6 cells g\(^{-1}\) aquifer material [dry wt.]). Slightly lower numbers were found in the border zone of a contaminated aquifer (1-2 *10^5 cells g\(^{-1}\) aquifer material [dry wt.] in the non-contaminated and 1-4 *10^5 cells g\(^{-1}\) aquifer material [dry wt.] in the contaminated zone) [Zarda et al., 1998].
A more detailed characterization of the microbial community by group specific probes turned out to be difficult due to the low detection rate. In both cores the largest group of detectable bacteria was the β-subdivision of Proteobacteria, similar to the results obtained in the column studies, although the percentage is different (>90% in the columns and 10-30% in the material from Studen).

In the core material at Studen a high percentage of the DAPI stained cells (6-28%) is represented by the γ-subdivision of Proteobacteria. Since most subdivisions are physiologically very heterogeneous, a higher phylogenetic resolution combined with detailed knowledge about physiological capacities would be necessary to attribute certain physiological activities to its constituent bacterial groups.

By whole cell hybridization no cells of the *Azoarcus tolulyticus* group could be detected, which could be confirmed by a PCR based detection method (Fig. 4.4). The absence of *Azoarcus tolulyticus* in cores P22 and P25 at Studen might be due to the lack of petroleum hydrocarbons in those cores and due to the oxidants available. Most of the described *Azoarcus* isolates mineralize hydrocarbons under aerobic and denitrifying conditions. However, analyses of the groundwater at Studen revealed low O₂ concentrations (<1 mg l⁻¹) [Bolliger et al., 1998] and low NO₃⁻ (20-30 μM) in the porewater. Therefore the growth conditions might not have been favourable for *Azoarcus*. A final proof of the assumption that *Azoarcus* sp. plays an important role during bioremediation in hydrocarbon-contaminated aquifers [Fries et al., 1997] might be important for designing more effective remediation strategies, but depends strongly on the availability of more information on the *in situ* catabolic activity of these organisms.
Analysis of bacterial and protozoan communities in an aquifer contaminated with monoaromatic hydrocarbons

Abstract

Bacterial and protozoan communities were examined in three cores (A, B and C) from an aquifer located at an abandoned refinery near Hünxe, Germany. Cores were removed along a transect bordering a plume containing various monoaromatic hydrocarbons. Monoaromatic hydrocarbons could not be detected in the unsaturated zone in any core and were present in large amounts only in the saturated zone in core C (between 280 and 42600 μM kg⁻¹ of core material [dry wt.]). In the saturated zones of cores A and B concentrations of monoaromatic hydrocarbons were much lower (between 30 and 190 μM kg⁻¹ of core material [dry wt.]). Xylene isomers accounted for 50-70% of monoaromatic hydrocarbons in all cores. The number of DAPI-stained bacteria was found to increase from the low-contaminated cores A and B (approx. 0.1 x 10⁸ cells and 0.2 x 10⁸ cells g⁻¹ of core material [dry wt.], respectively) to the high-contaminated core C (2.4 x 10⁸ cells g⁻¹ of core material [dry wt.]). The higher bacterial numbers in core C were found to coincide with a higher detection rate obtained by in situ hybridization (13-42% for core C as compared to 3-16% and 10-25% for cores A and B, respectively). Proteobacteria of the δ-subdivision (which include many sulfate-reducing bacteria) were the most predominant of the groups investigated (7-15% of DAPI-stained bacteria) and were followed by Proteobacteria of the γ- and β-subdivisions (4% and 1% of DAPI-stained bacteria, respectively). A similar proportion of bacteria assigned to the β-
subdivision of Proteobacteria was also be detected by a probe specific for hydrocarbon-degrading *Azoarcus* sp. The total numbers of protozoa and bacteria determined by direct counting occurred in a ratio of approx. $1 \times 10^3$, respectively, which was independent of depth or core examined. Most-probable-number (MPN) analysis combined with a subsequent classification of the culturable protozoa revealed that ciliates formed a minor proportion of the protozoan community (≤4%), and were confined exclusively to the surface layer (<1 m depth) in each core. The major component of the protozoan community was composed of nanoflagellates which increased in prevalence with depth. Naked amoebae became increasingly more encysted with depth, except in the high-contaminated core C where vegetative trophozoites were present in the saturated zone. The co-occurrence of bacteria and protozoa in association with high concentrations of monoaromatic hydrocarbons suggests the involvement of trophic interactions in the process of biodegradation.

5.1 Introduction

*In situ* bioremediation is widely considered to be an environmentally friendly and cost effective technology for the treatment of organically contaminated sites [MacDonald and Rittman, 1993; Hart, 1996]. The technology relies on the collective ability of microorganisms to degrade the contaminants under prevailing environmental conditions. Microbial degradation of organic contaminants such as petroleum-derived hydrocarbons is usually monitored by analyzing changes in chemical parameters including reductions in contaminants and oxidants, and increases in dissolved inorganic carbon and reduced species. In addition, measurements of the $^{13}\text{C}/^{12}\text{C}$ isotopic ratios in educts and products quite often allow mass balances of the catabolic processes to be established [Heitzer and Sayler, 1993; Hunkeler et al., 1997]. Microbiological monitoring of *in situ* bioremediation has been considered less reliable due to limited information on the requirements and capabilities of the indigenous microorganisms, and a lack of suitable methods for *in situ* determination of their abundance and activity [Brock, 1987].
Previous studies on bacterial populations in contaminated aquifers have largely been confined to techniques relying on the culturability of organisms as in the case with plate counts or most probable number techniques [Atlas and Bartha, 1992; Kämpfer et al., 1993; Song and Bartha, 1992; Dobbins et al., 1992]. However, these techniques only allow a low percentage of the total number of microorganisms to be assessed [Atlas and Bartha, 1992]. Many recent studies have focused on the isolation and characterization of bacteria capable of degrading specific contaminants [Fries et al., 1994; Rabus and Widdel, 1995; Zhou et al., 1995], on determining their catabolic pathways [Zeyer and Kocher, 1988; Chee-Sanford et al., 1996], or on determining the catabolic potential of natural populations in laboratory aquifer columns [Hess et al., 1996; Hess et al., 1997]. Consequently, the impact of natural communities of bacteria on the degradation of contaminants in the field remains poorly understood.

Within the last few years, it has been suggested that populations of subsurface protozoa might be a good indicator of in situ biodegradation activity [Madsen et al., 1991; Sinclair et al., 1993]. Since many protozoa are bacterivorous, it is thought that high numbers of protozoa are indicative of rapidly growing populations of bacteria [Novarino et al., 1997]. Comparatively large populations of sub-surface protozoa have been reported from various organically contaminated sites [Sinclair and Ghiorse, 1989; Madsen et al., 1991; Sinclair et al., 1993; Novarino et al., 1994/1997]. However, the influence of bacterivorous protozoa on the composition and hence degradative ability of the bacterial community at these sites is presently unknown. Since our knowledge of natural populations of microorganisms is largely derived from culture-dependent studies, it is likely that current information on the composition of bacterial and protozoan communities is biased by the limitations of culturability.

The present study examined the chemical environment in relation to the microbial community at a field site contaminated with monoaromatic hydrocarbons. The in situ hybridization technique (for review see [Amann et al., 1995]) was used in association with DAPI epifluorescence staining as a culture-independent direct counting method for determining in situ the
absolute abundance of bacteria and protozoa. Studies on bacterial community structure initially focused on the analysis of higher phylogenetic groups and on *Azoarcus* sp. which are considered to be involved in the biodegradation of hydrocarbons under oxic and denitrifying conditions [Hess et al., 1997]. A comparison was made between the enumeration of protozoa by *in situ* hybridization and by the culture-dependent most probable number (MPN) technique which also provided information on the composition of the protozoan community.

5.2 Materials and methods

5.2.1 Field site and sampling procedure

The field site was an area contaminated with monoaromatic hydrocarbons on the site of an abandoned refinery near Hünxe in the lower Rhine area of Germany (Fig. 5.1). The contaminated aquifer consisted of a calcite rich sand predominantly of grain size 0.2 to 1.0 mm and a fractional organic carbon content of 0.07% [Schäfer and Therrien, 1995]. A porosity of 25% was estimated for the saturated zone [Schäfer and Therrien, 1995]. The depth of the water table at different locations varied between 5.4 and 8 m and showed a yearly fluctuation of about 2 m. Regional flow direction was northwest at a head gradient of 0.07% and an average horizontal conductivity of 172 m day⁻¹ [Schäfer and Therrien, 1995]. Three remediation wells were installed in May 1988 to prevent further spreading of hydrocarbons (mainly xylene from a former xylene production plant) in the groundwater [Schäfer et al., 1994]. A total of 23 observation wells were also installed in the area surrounding the former xylene production plant. Groundwater from the contaminated area showed enhanced xylene concentrations but very low dissolved oxygen concentrations [Schäfer et al., 1994]. The area containing xylene in free phase was analyzed more precisely in February 1992 by sampling on a mesh having 10 x 10 m spacing (Fig. 5.1) [Schäfer et al., 1994]. This area was situated in the upstream part of the contaminated zone and served as a source for ongoing contamination. Active remediation by pumping was applied until November 1996. Microbial growth and xylene
dissolution during remediation were simulated using a three-dimensional numerical flow and transport model incorporating biochemical multispecies interactions [Schäfer and Therrien, 1995]. Xylene concentrations in wells in the downstream area were considered to be sufficiently low to stop the active remediation in 1996. Thereafter, the removal of hydrocarbons by intrinsic bioremediation was assumed to be in equilibrium with the leaching of contaminants from the source.

In March 1997, three cores A, B and C (diameter 3.6 cm; length 7.0 m) were taken along a transect bordering the xylene plume and northwest of the area with xylene in free phase (Fig. 5.1), using a pile driven coring device. *In situ* temperature was 12°C and no increase was observed due to the sampling procedure. At the time of sampling the water table was 5.4 m below the surface and the saturated thickness was about 15 m. Cores A, B and C showed a grey coloration below a depth of 6.6 m, 6.4 m and 6.2 m, respectively. Each core was sampled at 50 cm intervals above 4.5 m depth and at 10 cm intervals below this depth. Samples (approx. 10 g) for the analysis of volatile hydrocarbons were collected on site using gas-tight headspace flasks sealed with teflon coated rubber stoppers. For the analysis of microorganisms, core material (5 to 15 g) was added on site to 15 ml Falcon-tubes and either left unfixed or fixed with 5 ml of 4% paraformaldehyde solution. All samples were transferred on ice to the laboratory and then stored at either 4°C or -20°C prior to analysis.
**Figure 5.1.** Site map showing part of the abandoned refinery near Hünxe, Germany, and the location of the cores analyzed in this study (A, B, and C). Positions and designations (R41.2, R41.3, R41.4, and R6.2) of former wells are included for better comparability with the same report.
5.2.2 Chemical analysis

Pore water was obtained by centrifuging approx. 12 g of core material added to 5 ml plastic syringes which were plugged with silane treated glass wool (Supelco Inc., Bellefonte, USA). The filled syringes were placed in 15 ml Falcon tubes and centrifuged at 4°C and 2500 x g for 10 min. Concentrations of NO₃⁻, NO₂⁻, SO₄²⁻, PO₄³⁻, and Cl⁻ were analyzed in 15 μl samples of pore water by ion chromatography (Dionex DX-100 ion chromatograph equipped with an IonPac AS4A-SC column; Dionex, Sunnyvale, USA) using an eluent of 1.8 mM Na₂CO₃ and 1.7 mM NaHCO₃ [Hess et al., 1996]. Data from ion chromatography were analyzed using Chrom-Card for Windows (Fison Instruments, Rodano, Italy) [Hess et al., 1996].

For the analysis of volatile hydrocarbons, the volume of pore water in each flask was calculated from the dry weight of the core material, and an equal volume of pentane was injected [Häner et al., 1997]. After vortex-mixing for 1 min, water- and pentane-phases were allowed to separate. The pentane-phase was transferred to gas-tight glass vials which were then sealed with teflon-lined screw caps. One μl of each extract was injected into a Carlo Erba GC 8000 gas chromatograph equipped with a flame ionization detector (Fison Instruments). A glass column (diameter 3 mm; length 2 m) packed with 5% SP 1200 and 5% Bentone 34 on Supelcoport 100/120 mesh (Supelco Inc.) was used to resolve the hydrocarbons. Conditions were 100°C isothermal for 15 min and N₂ was used as the carrier gas. Data from gas chromatography were analyzed using Chrom-Card for Windows [Häner et al., 1997].

Less volatile hydrocarbons were analyzed in extracts of mixed material of each core from a depth of 5 to 7 m. Extracts were obtained from 6 g samples dried with 20% Na₂SO₄ by Soxhlet extraction with 35 ml CCl₄ at 55°C for 15 to 20 h [Bregnard et al., 1996]. The resulting extract was concentrated to a volume of 2 ml under a gentle stream of N₂ at 25°C. Two μl of extract were injected into a Fisons HRGC Mega II GC (Fison Instruments)
equipped with a flame ionization detector and a BGB-5 fused silica capillary column (length 3 m, inner diameter 0.32 mm) with a 5%-phenyl-methyl polysiloxane (0.25 μm) bonded phase (BGB Analytik AG, Zurich, Switzerland). The conditions for analysis were splitless injection with H₂ as the carrier gas and a column temperature of 40°C for 2 min followed by a temperature increase to 250°C at a rate of 3°C min⁻¹. Data were again analyzed using Chrom-Card for Windows with o-terphenyl as a calibration standard [Bregnard et al., 1996].

5.2.3 In situ hybridization

After 36 h of fixation in 4% paraformaldehyde, samples for in situ hybridization were washed twice with phosphate-buffered saline (0.13 M NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄; pH 7.2), resuspended in 98% ethanol to a final density of 0.6 g core material [dry wt.] per ml and stored at -20°C [Hahn et al., 1992]. These suspensions were then diluted tenfold in 0.1% pyrophosphate and thoroughly mixed. After 10 sec sedimentation, 10 μl of each dispersed sample was spotted onto gelatin-coated slides, dried at room temperature for at least 4 h, and finally dehydrated in 50%, 80% and 96% ethanol for 3 min in each.

Hybridizations were carried out with Cy3-labeled oligonucleotide probes as described previously [Zarda et al., 1997]. Detection levels for probes were the domains Bacteria (probe Eub338) and Eukarya (Euk516), bacteria of the α- (Alflb), β- (Bet42a), γ- (Gam42a), and δ- (SRB385 and SRB385Db) subdivisions of Proteobacteria, the high G+C Gram-positive bacteria (HGC69a), the Cytophaga-Flavobacterium cluster (CF319a) [Zarda et al., 1997], and hydrocarbon-degrading Azoarcus sp. (Azo644) [Hess et al., 1997]. Hybridizations were performed in 9 μl of hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, 5 mM EDTA, 0.01% SDS; pH 7.2) in the presence of 10% to 35% formamide, 1 μl of the probe (25 ng μl⁻¹), and 1 μl of a solution of the DNA intercalating dye 4',6-diamidino-2'-phenylindole (DAPI, 200 ng μl⁻¹, Sigma, Buchs, Switzerland) at 42°C for 2 h [Zarda et al., 1997]. After hybridization, the slides were washed in buffer at 48°C for 15 min, rinsed with distilled water and air-dried.
The slides were mounted with Citifluor solution and the preparations were examined with a Zeiss Axiophot microscope fitted for epifluorescence with a high-pressure mercury bulb (50 W) and filter sets 02 (Zeiss, Oberkochen, Germany; G 365, FT 395, LP 420) and HQ-Cy3 (AHF Analysentechnik, Tübingen, Germany; G 535/50, FT 565, BP 610/75). Organisms were counted at 1000 x magnification in randomly selected fields each covering an area of 0.01 mm². Twenty fields were examined for probe-confferred signals indicating specific bacterial groups. Hybridization signals obtained with probe Euk516 which was used to detect protozoa were examined in 300 fields. Bacterial and protozoan numbers were expressed per g core material [dry wt.] for DAPI-stained samples or as a % of DAPI counts for bacteria detected with specific oligonucleotide probes. Numbers were expressed as mean ± standard error.

5.2.4 MPN enumeration of protozoa

In addition to detection by in situ hybridization, protozoa were also analyzed by a modification of a most-probable number (MPN) technique [Rønn et. al., 1995]. Samples from 1 m-depth-intervals in each core (approx. 2 g) were weighed, suspended in a protozoan saline [Page, 1976] and vortex mixed. A three-fold dilution series was prepared from each suspension using a sterile soil extract diluted 1:4 with deionized water [Rønn et. al., 1995]. Dilution levels from 10¹ to 10⁵ with five replicates for each were prepared in a 96 multiwell plate and incubated at 21°C for 28 days. Plates were examined microscopically and each well scored for the presence or absence of ciliates, of flagellates, and of vegetative and encysted amoebae. An MPN for each sample was calculated from the number of positive endpoint dilutions [Clesceri et al., 1989] and expressed per g of core material [dry wt.]. The lower limit of detection for the technique was 0.8 protozoa g⁻¹ of core material [dry wt.] with an estimated standard error of 50% [Sinclair and Ghiorse, 1987].
5.3 Results

5.3.1 Environmental chemistry

Monoaromatic hydrocarbons could not be detected in any of the cores A, B and C above a depth of 4.8 m. Below this depth, monoaromatic hydrocarbons were detected in large amounts only in core C (between 280 and 42600 µM kg\(^{-1}\) of core material [dry wt.]) and in much lower amounts in cores A and B (between 0 and 190 µM kg\(^{-1}\) of core material [dry wt.]) (Fig. 5.2).

![Concentration profile graph](image)

**Figure 5.2.** Concentration profiles showing total aromatic hydrocarbons (●), total trimethylbenzenes (○) and m- (▼), p- (♦), and o-xylene (φ) in cores A, B, and C at depths between 4.5 and 7.0 m. Note the different scales used.

In core C, monoaromatic hydrocarbons comprised in order of decreasing concentrations o-xylene, 1,3,5-trimethylbenzene and/or 1,2,4-trimethylbenzene (not resolved on the gas chromatograph), 3-ethyltoluene, m-xylene, p-xylene, 4-ethyltoluene, 1,2,3-trimethylbenzene, ethylbenzene, toluene, and benzene. Xylene isomers accounted for 90±1% of monoaromatic...
hydrocarbons in core A, 77% in core B, and 50±5% in core C (with < 50% at a depth above 6 m and > 50% further down this core). Less volatile hydrocarbons were only detected in trace amounts in all three cores after Soxhlet extraction of a mixture of soil samples from below 5 m.

![Concentration profiles showing nitrate (•), nitrite (○), and sulfate (◊) in cores A, B, and C at depths between 5.0 and 7.0 m.](image)

**Figure 5.3.** Concentration profiles showing nitrate (•), nitrite (○), and sulfate (◊) in cores A, B, and C at depths between 5.0 and 7.0 m.

Nitrate concentrations in pore water from the saturated zones (Fig. 5.3) decreased from core A (between 6 and 135 μM) at the border of the plume towards core C (between 4 and 15 μM), lying inside the plume (Fig. 5.1). Along the depth profile of each core concentrations of nitrate and sulfate were generally found to decrease with depth. The concentrations of sulfate as well as of nitrite were highest in core C and lowest in core B (Fig. 5.3). Concentrations of sulfate ranged between 70 and 1090 μM in core A, between 210 and 490 μM in core B, and between 600 and 1800 μM in core C.
Concentrations of nitrite ranged between 1 and 22 μM in core A, between 2 and 10 μM in core B, and between 10 and 58 μM in core C.

5.3.2 Bacterial community structure

After DAPI staining, bacteria were detected in numbers between 4 and 8 x 10⁸ cells g⁻¹ of core material [dry wt.] in the non-contaminated surface layers in all cores (Fig. 5.4).

![Figure 5.4. Total number of bacteria determined after DAPI staining (•) and the percentage of cells detectable after hybridization with the Cy3-labeled probe Eub338 (<> in cores A, B, and C at depths between 0.1 m and 7.0 m.](image)

Numbers decreased within the first meter of each core to values between 0.1 and 1 x 10⁸ cells g⁻¹ of core material [dry wt.] and did not change significantly toward the saturated zone. In the saturated zone, a significant increase in bacterial numbers was only detected in contaminated core C with
up to $2.4 \times 10^8$ cells g$^{-1}$ of core material [dry wt.]. Numbers of bacteria in the low-contaminated cores A and B remained low (approx. $0.1 \times 10^8$ cells and $0.2 \times 10^8$ cells g$^{-1}$ of core material [dry wt.], respectively).

In situ hybridization with probe Eub338 designed to target all members of the domain Bacteria detected percentages of DAPI-stained cells of 3-16%, 10-25%, and 13-42% in cores A, B, and C, respectively. Low numbers of bacteria in cores A and B were reflected in a lower detection rate by in situ hybridization, which rendered the use of oligonucleotide probes difficult. Consequently, only spot checks were carried out for higher phylogenetic groups in these cores. During these checks, only members of the δ-subdivision of Proteobacteria were occasionally detected.

![Graph showing bacterial numbers (as % of DAPI counts) vs depth in core C](image)

**Figure 5.5.** Prevalence of bacterial groups belonging to the β-subdivision (●; Bet42a), γ-subdivision (Δ; Gam42a), and δ-subdivision of Proteobacteria (●; SRB385 and SRB385Db) and the genus Azoarcus (●; Azo644) in core C at depths between 4.5 and 7.0 m.
In the saturated zone of contaminated core C, 10-20% of DAPI-stained bacteria could be assigned to the phylogenetic groups investigated which represented up to 50% of the bacteria detectable by *in situ* hybridization (Fig. 5.5). Some 7-15% of DAPI-stained bacteria were detected with a combination of probes SRB385 and SRB385Db designed to detect members of the δ-subdivision of the Proteobacteria. Their number decreased slightly with increasing depth from 15% at a depth of 5 m to 10% at 5.4 m, and remained quite stable at this level (8% at 5.8 m, 10% at 6.2 m, 7% at 6.5 m, and 10% at 6.9 m). The analysis of bacteria belonging to the family *Desulfobacteriaceae* using probe SRB385Db and unlabeled probe SRB385 as a competitor showed a more pronounced decrease of cells with increasing depth. Their numbers decreased from 11% at a depth of 5 m to 7% (5.4 m), 6% (5.8 m), 5% (6.2 m), 3% (6.5 m) and finally 4% at a depth of 6.9 m. Populations of other higher phylogenetic groups remained relatively unchanged along this depth profile. Bacteria detected with probe Gam42a designed to detect members of the γ-subdivision of Proteobacteria accounted for 2-4% of DAPI-stained bacteria (Fig. 5.5). Those detected with probe Bet42a designed to detect members of the β-subdivision of Proteobacteria accounted for about 1% of DAPI-stained bacteria occurring between 5 and 6 m depth and decreased in prevalence below this depth (Fig. 5.5). Comparable numbers of bacteria were detected with probe Bet42a and with probe Azo644 which targeted hydrocarbon-degrading *Azoarcus* sp. belonging to the β-subdivision of Proteobacteria. *In situ* hybridization with probes designed to detect other higher phylogenetic groups such as the Gram-positive bacteria with a high DNA G+C content, the *Cytophaga-Flavobacterium* cluster, the α-subdivision of Proteobacteria, and the planctomycetes did not result in significant detection yields (<1% of DAPI-stained bacteria).

5.3.3 Protozoan community structure

Numbers of protozoa were detected in each core A, B, and C by *in situ* hybridization with probe Euk516 and by the MPN technique (Fig. 5.6). Although the actual numbers of protozoa detected by both methods differed
considerably (between 2 and 5 orders of magnitude higher using *in situ* hybridization), a similar trend was found between both sets of data. Numbers of protozoa were highest in the surface layer in each core (up to $10^5$ cells g$^{-1}$ of core material [dry wt.] as detected by *in situ* hybridization) and decreased sharply below about 1 m depth. Elevated numbers of protozoa approaching those found in the surface layer were only detected in the saturated zone in contaminated core C (Fig. 5.6). The ratio between total numbers of bacteria determined after DAPI-staining and protozoa detected with probe Euk516 was quite constant (core A: $0.7 \pm 0.4 \times 10^3$; core B: $1.7 \pm 0.9 \times 10^3$; core C: $1.2 \pm 0.7 \times 10^3$; average of cores A to C: $1.1 \pm 0.7 \times 10^3$).

**Figure 5.6.** Total number of protozoa as determined by *in situ* hybridization with the Eukarya-specific probe Euk516 (•) and by the MPN-technique (♂) in cores A, B, and C at depths between 0.1 and 7.0 m.
<table>
<thead>
<tr>
<th>Core Depth (m)</th>
<th>Total protozoa MPN (g⁻¹ dw.)</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ciliates</td>
<td>Flagellates</td>
</tr>
<tr>
<td>A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 - 0.3</td>
<td>300</td>
<td>2</td>
</tr>
<tr>
<td>1.0 - 1.6</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>3.0 - 3.5</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>4.0 - 4.5</td>
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<td>0</td>
</tr>
<tr>
<td>5.0 - 5.2</td>
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<td>0</td>
</tr>
<tr>
<td>6.0 - 6.2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>6.6 - 6.8</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 - 0.3</td>
<td>640</td>
<td>4</td>
</tr>
<tr>
<td>1.0 - 1.6</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>3.0 - 3.5</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>4.0 - 4.5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>5.0 - 5.2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>6.0 - 6.2</td>
<td>1100</td>
<td>0</td>
</tr>
<tr>
<td>6.6 - 6.8</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 - 0.3</td>
<td>1200</td>
<td>1</td>
</tr>
<tr>
<td>1.0 - 1.6</td>
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<td>0</td>
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<tr>
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</tr>
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<td>3</td>
<td>0</td>
</tr>
<tr>
<td>6.0 - 6.2</td>
<td>490</td>
<td>0</td>
</tr>
<tr>
<td>6.4 - 6.5</td>
<td>6</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 5.1. Prevalence of protozoa in core material from a BTEX contaminated site near Hünxe, Germany.
The analysis of the protozoan community using the MPN technique is based on the assignment of culturable protozoa to the morphologically distinct groups of ciliates, flagellates, and amoebae (Table 5.1). Ciliates accounted for 1-4% of the protozoan population and were confined to the surface layer above 1 m depth in each core. Flagellates and naked amoebae were found to co-occur in variable proportions to a depth of about 1.6 m below which the former were the predominant group in each core. Amoebae became increasingly more encysted with depth, except in contaminated core C, where vegetative trophozoites were also detected in the saturated zone (Table 5.1).

5.4 Discussion

5.4.1 Chemical characterization of the aquifer

The chemical data confirmed that cores A, B, and C were sampled in the border zone of the plume since large differences for monoaromatic hydrocarbons and for nitrate were observed between cores A and C. The highest concentrations of monoaromatic hydrocarbons (up to 40 mM kg⁻¹ of core material [dry wt.]) were found in core C where they exceeded the individual solubilities of the constituent hydrocarbons. The maximum solubility of the most prominent monoaromatic hydrocarbons found in core C ranged between 0.4 mM (trimethylbenzenes) and 1.7 mM (xylenes) [Schwarzenbach et al., 1993]. One kg of water-saturated core material was found to contain only about 0.14 l of water which corresponded to a water content of approx. 25%. This means that only a very small fraction of the hydrocarbons detected in core C can be solved in the pore water (approx. 200 μM kg⁻¹ of core material [dry wt.] and that a free xylene phase still exists in core C which probably serves as a source for further contamination [Schäfer et al., 1994]. Core C contained high concentrations of o-xylene, trimethylbenzene and ethyltoluene, in contrast to earlier findings which reported mainly p- and m-xylene in the plume [Schäfer et al., 1994]. The high
o-xylene concentrations could reflect the close proximity of the sampling sites to the former o-xylene production plant (Fig. 5.1).

The concentration of nitrate in each core was found to be inversely related to concentrations of monoaromatic hydrocarbons, suggesting that nitrate reduction and degradation of aromatics are coupled. Furthermore, dissolved oxygen concentrations were previously shown to be high outside the plume but low inside the plume [Schäfer and Therrien, 1995]. These observations together with the increased nitrite concentration in core C suggest that the resident microbial population uses monoaromatic hydrocarbons as their carbon and energy source under aerobic and nitrate reducing conditions. A decrease in sulfate concentrations was observed in the lower parts of cores A and C where the sediment also appeared greyish-black. This suggests that sulfate reductive assimilation may be taking place at a depth below about 6.5 m. This suggestion, however, is not supported by the sulfate profile in core B in which a decrease in sulfate concentration was not observed.

5.4.2 Bacterial community structure

The number of DAPI-stained bacteria increased from core A to core C and corresponded with increased levels of contaminants. The higher numbers of bacteria detected after DAPI-staining in the saturated zone of contaminated core C were found to coincide with a higher detection rate obtained with probe Eub338. The latter provides an indication for the presence of metabolically active cells which contain sufficient amounts of rRNA coupled with a sufficient cell permeability or permeabilization to permit their detection. The detection rate showed no direct correlation with the growth rate or activity of bacteria and varies according to the species examined and their consumption of nutrients and oxidants (for review see [Amann et al., 1995]). Nevertheless, it is likely that the increase in detectability of bacteria in the saturated zone of contaminated core C (as compared with the low-contaminated cores A and B) is due to the availability of monoaromatic hydrocarbons as growth substrates. The correspondingly low level of oxidants detected in core C provides support for this assumption.
Proteobacteria of the δ-subdivision comprising many sulfate-reducing bacteria were predominant in core C and provided support for the assumption of sulfate-reductive conditions. Due to the presence of sulfate-reducing bacteria in core C, the contribution of sulfate-reductive degradation might have been underestimated. However, further investigations would be necessary to quantify their contribution. A high portion of DAPI-stained bacteria (2-4%) was represented by the γ-subdivision of Proteobacteria. Since the γ-subdivision of Proteobacteria is physiologically very heterogeneous, a higher phylogenetic resolution would be necessary to attribute certain physiological activities to its constituent bacterial groups. Bacteria of the β-subdivision of Proteobacteria only accounted for approx. 1% of DAPI-stained cells. The latter findings were in contrast to those obtained in a laboratory aquifer column which was set up in order to simulate the aerobic and denitrifying remediation processes during degradation of petroleum-derived hydrocarbons. In this column up to 90% of DAPI-stained cells belonged to the β-subdivision of Proteobacteria [Hess et al., 1997]. Though many of the organisms detected in connection with aerobic [Fries et al., 1997a/b] or denitrifying [Hess et al., 1997] degradation of monoaromatic hydrocarbons belong to the β-subdivision of Proteobacteria, their impact on hydrocarbon-degradation at the field site in Hünxe may be impeded by adverse environmental factors such as low redox potentials. Lower numbers of bacteria belonging to the β-subdivision of Proteobacteria could also occur if these bacteria are less tolerant to high concentrations of aromatic hydrocarbons [Fries et al., 1997b] as, for example, those of the γ-subdivision of Proteobacteria [Inoue and Horikoshi, 1989].

The proportion of Azoarcus sp. detected in the saturated zone of contaminated core C was similar to that found in a laboratory aquifer column (1-2%) designed to simulate the aerobic and denitrifying remediation processes occurring during degradation of petroleum-derived hydrocarbons [Hess et al., 1997]. The genus Azoarcus has already been implicated in the biodegradation of monoaromatic hydrocarbons in a number of studies [Fries et al., 1997a/b; Hess et al., 1997]. Although, Azoarcus represents only a minor part of the total bacterial community at the field site, it does account
for more cells in the contaminated aquifer (2 x 10⁶ g⁻¹) than the model previously estimated for the total number of bacteria (max. 1.3 x 10⁶ g⁻¹) present [Schafer et al., 1994]. The percentage of hydrocarbon-degrading Azoarcus sp. detected accounted for up to 1.4% of the total bacterial community which demonstrates that they are significant members of the indigenous microbiota. Similar to the attribution of significant denitrification activity to Paracoccus sp. based on their 3.5% abundance [Neef et al., 1996], these results suggest that Azoarcus sp. may play an important role during bioremediation of hydrocarbon-contaminated aquifers. However, confirmation of this assumption depends on the availability of more information on the catabolic activity of the hydrocarbon-degrading Azoarcus populations both in column studies and in the field.

5.4.3 Protozoan community structure

Protozoa are integral members of the microbial community in groundwater aquifers [Sinclair and Ghiorse, 1987; Madsen et al., 1991; Sinclair et al., 1993; Novarino et al., 1994]. Numerically, they are second in importance only to bacteria [Novarino et al., 1997]. Evidence from the present study confirmed the discrepancy between culture-dependent and -independent methods with MPN counts between two and five orders of magnitude less than those using direct counting with eukaryotic probe Euk516. Interestingly though, both the MPN technique and probe Euk516 detected elevated numbers of protozoa in the surface layer in each core, which declined sharply with depth but again increased in the saturated zone in contaminated core C. These findings corresponded with published data from other hydrocarbon-contaminated aquifers [Sinclair and Ghiorse, 1987; Madsen et al., 1991; Sinclair et al., 1993].

The covariance between results from the MPN technique and probe Euk516 suggests some relative confidence in each approach. The MPN technique will probably remain an important alternative to direct counting methods until the latter have been sufficiently developed to provide information on protozoan taxa [Rønn et. al., 1995]. In a recent study, oligonucleotide probes were used to enumerate a particular nanoflagellate
species in a mixed population [Rice et al., 1997]. This demonstrates the possibility for developing molecular tools to investigate both the taxonomy and ecology of protozoa as well as of bacteria and their mutual influence in a variety of habitats by the same methods. Future studies in our laboratory will focus on the development of such tools.

Protozoan taxa comprising ciliates, flagellates and naked amoebae were identified during the present study. Ciliates formed a minor proportion of the protozoan community (≤ 4%) and were confined exclusively to the surface layer (<1 m depth) in each core. Similar findings from other aquifers have been attributed to straining by sediment on large protozoa over 20 μm in diameter [Sinclair and Ghiorse, 1987; Harvey, 1991]. The major component of the community was comprised of flagellates which increased in prevalence with depth and thereby confirmed earlier findings from other aquifers [Sinclair and Ghiorse, 1987; Novarino et al., 1994/1997]. The predominance of flagellates (particularly nanoflagellates of 2-3 μm in diameter) has been attributed to their optimal size for transport through the aquifer matrix [Harvey, 1995]. Naked amoebae became increasingly more encysted with depth except in core C where vegetative trophozoites were present in the saturated zone. Presumably, this was in response to elevated numbers of bacteria associated with contaminants in the saturated zone.

5.4.4 Toxicology

The occurrence of bacteria and protozoa in association with high concentrations of monoaromatic hydrocarbons raises questions concerning the tolerance of bacteria and protozoa to such contaminants. Bacteria react rather sensitively to high concentrations of monoaromatic hydrocarbons. Numbers of toluene- and phenol-degrading bacteria, for example, increased in MPN tubes by 1 to 1.5 orders of magnitude when the concentrations of toluene or phenol were reduced from 0.5 to 0.05 mM [Fries et al., 1997b]. These findings suggest that even low concentrations of monoaromatic hydrocarbons present in core C could be considered toxic for microorganisms, although only a minor portion of the aromatic hydrocarbons detected could be solved in the pore water (about 1.7 mM monoaromatic
hydrocarbons l⁻¹ according to Raoult's Law). In contrast, a toluene-degrading strain T1 has been shown to tolerate approx. 3 mM toluene [Evans et al., 1991] and a *Pseudomonas putida* strain has been found to grow in the presence of 50% (v/v) toluene of which max. 5.6 mM could be solved in the aqueous phase [Inoue and Horikoshi, 1989]. Due to their lower polarity, the monoaromatic hydrocarbons present at Hünxe (mainly o-xylene and trimethylbenzene) should be less toxic than toluene. This suggests that toxicity of monoaromatic hydrocarbons should not be a serious problem at Hünxe, at least not for bacteria.

Acute toxicity thresholds for ciliates exposed to a variety of hydrocarbons (including o-, m-, p-xylenes) were significantly lower than the concentrations measured during the present study [Rogerson et al., 1983]. Furthermore, conditions which are conducive to nitrate and sulfate reduction are normally associated with reduced oxygen levels. Protozoa are more sensitive to reduced oxygen levels than bacteria [Sinclair et al., 1993] though many are micro-aerophilic and may occupy niches adequately oxygenated from groundwater.

### 5.4.5 Interactions of bacteria and protozoa

Bacterivory is likely to be the major feeding strategy for protozoa in the Hünxe aquifer since protozoan and bacterial populations displayed a marked covariance. The populations of protozoa and bacteria determined by direct counting occurred in a ratio of approx. 1: 10³ respectively, which was independent of depth or core examined. The ratio of protozoa to bacteria has variously been reported as 1: 10¹-10⁵ [Sinclair and Ghiorse, 1989] and 1:10³ [Sinclair et al., 1993] from other aquifers. Presumably, these differences reflect heterogeneity in the physical composition in these aquifers or in the culturability of their microorganisms. Since the protozoan community in aquifers is normally dominated by heterotrophic flagellates with various feeding strategies [Novarino et al., 1997] it is also possible that heterogeneity in their populations contributes to differences observed between these aquifers. Estimates for feeding rates from batch culture studies suggest that flagellates require 10² bacteria whereas amoebae require 10³ bacteria per cell
division [Schnürer et al., 1986; Zwart and Darbyshire, 1992]. A growth requirement of $10^3$ bacteria per division has been observed for the flagellate Spumella sp. isolated from core C (unpublished data). Availability of bacteria is therefore unlikely to have been a limiting factor for the growth of either flagellates or amoebae in the Hünxe aquifer.

The role of protozoa in contaminant biodegradation is considered to be an indirect result of their ability to selectively graze on and control the biomass of aquifer bacteria [Madsen et al., 1991; Sinclair et al., 1993; Novarino et al., 1997]. This in turn creates a nutritional loop in which protozoa rapidly remineralize nutrients which sustain further bacterial growth [Coleman et al., 1977]. However, the growth of bacteria in porous media supplied with nutrients has frequently been observed to cause reductions in hydraulic conductivity due to bioclogging [Taylor and Jaffé, 1990; Vandeveire and Baveye, 1992; DeLeo and Baveye, 1997]. The progress of an engineered in situ bioremediation in aquifers, however, is dependent on a sufficient hydraulic conductivity to permit the pumping of metabolites through the contaminated zone [Sinclair et al., 1993]. The bacterial organic carbon content calculated for core C (0.04 mg C cm$^{-3}$ core material at a mean cell volume of 1 μm$^3$ and a carbon content of 10% of the wet weight) would be in the range of the value considered necessary for a ten-fold reduction of hydraulic conductivity (0.1 mg C cm$^{-3}$ core material) [Taylor and Jaffé, 1990]. Furthermore, the porosity of the aquifer material at Hünxe (0.25) is considerably smaller than the porosity of the aquifer material (0.35) used in the study by the same authors. This suggests that the bacterial community in core C may be sufficient to cause a reduction in hydraulic conductivity due to bioclogging during active bioremediation. The presence of bacterivorous protozoa in contaminated aquifers may restrict the potential for widespread bacterial bioclogging. The role of bacterivorous flagellates in limiting bacterial bioclogging in model aquifer columns is currently being investigated in our laboratory. Further studies should therefore evaluate the merits of incorporating a protozoan dimension into future bioremediation strategies.
Discussion and conclusions

6.1 Laboratory vs. field studies

Laboratory studies are essential to demonstrate the potential of bioremediation at a contaminated site. However, data obtained from the laboratory studies can be compared to field observations only to a limited extent. Microcosms, for example, are very defined, simply constructed and restricted systems, consisting of aquifer material, synthetic groundwater and a headspace (Fig. 6.1). These studies require very long incubation times of up to 400 days [Bregnard, 1997]. Therefore, it can not be predicted how the organisms adapt to the laboratory conditions. These may not any longer comprise the microbial population at the investigated field site. Aquifer columns are more complex with an increased number of unknown parameters, representing better the natural field conditions. But since in the field there are innumerable factors that can not be predicted or measured, laboratory studies are fundamental to give an idea of the situation in the field [Von Gunten and Zobrist, 1993].

Figure 6.1: Ecological systems, from left to right with decreasing complexity.
In the laboratory the use of small amounts of material and a destruction of the matrix lead to quite homogeneous conditions. However, the result is always some kind of selection under the given conditions because the heterogeneity of the field matrix can not be simulated completely. Even in a sandy aquifer with a quite homogeneous matrix there are niches with extreme conditions like no oxygen or exceeded salt concentrations. The microorganisms can develop specialists for each niche, resulting in a very diverse population.

In this thesis laboratory aquifer columns were chosen as one-dimensional experimental model systems to evaluate the spatial distribution of petroleum hydrocarbon mineralization. To investigate the effect of infiltration of groundwater amended with oxidants and nutrients, the columns were operated at the in situ temperature with artificial groundwater containing O₂, NO₃⁻, and PO₄³⁻. This experiment confirmed the localization of aerobic and denitrifying processes as a function of the mean groundwater residence time in the laboratory aquifer columns, DIC production attributable to each of these zones, the spatial distribution of hydrocarbon removal, and changes in microbial populations. The results demonstrated that the infiltration of aerobic groundwater enriched with NO₃⁻ and PO₄³⁻ increased the DIC production and thus the mineralization along the entire flow path of the infiltrated water. Throughout the columns, there was a large increase in total bacteria compared to the cell numbers found prior to column operation.

The strength of laboratory aquifer column studies is that mineralization rates coupled to different redox processes can be observed as a function of the mean groundwater residence time under continuous flow conditions in the contaminated aquifer. This allows the comparison with residence times observed at field sites and the development and validation of models describing the bioremediation processes in aquifers [Hunkeler et al., 1997]. The results of the laboratory column study demonstrate that O₂ concentrations in the infiltrating groundwater decreased to <0.1 mg l⁻¹ within mean residence times of 7.2 hours. In aquifers, mean groundwater flow velocities are often below 10 m day⁻¹ [De Marsily, 1986]. According to the results of the column
study, O₂ would be consumed within a distance of <3 m from an infiltration well in petroleum hydrocarbon contaminated aquifers. This is consistent with many findings at field sites [Kämpfer et al., 1993; Downs et al., 1994; Nelson et al., 1994; Hunkeler et al., 1995].

6.2 Evidence for petroleum hydrocarbon mineralization

Although laboratory studies might only give an indication of the processes and the microbial activity at a site they are necessary to obtain information on the efficacy of a bioremediation [US National Research Council, 1993]. The potential for a successful bioremediation is highly dependent on the contaminants being transformed. The most important factor is whether the microorganisms can use the contaminants as a source of carbon and energy under different redox conditions [Semprini, 1997]. Most petroleum hydrocarbons are examples of contaminants that can be utilized in this manner. In the subsurface there is a potential for mineralizing petroleum hydrocarbons under aerobic, denitrifying, Fe-reducing, sulfate-reducing, and methanogenic conditions.

In the laboratory aquifer columns used throughout this study an electron balance strongly indicated that the consumed oxidants were mainly used for mineralization of petroleum hydrocarbons to DIC. The carbon and ¹³C balances gave further evidence that DIC originated mainly from the mineralization of petroleum hydrocarbons. An analysis of the GC chromatograms showed that linear alkanes were preferentially degraded compared to isoprenoid alkanes, which can be explained by steric effects [Watkinson and Morgan, 1990].

In all of the described studies in this thesis, in the laboratory as well as at field sites, microbial communities able to mineralize petroleum hydrocarbons have developed, independent of the type of remediation (engineered and intrinsic in situ bioremediation).

The efficacy of a remediation project is often qualified by measuring different chemical parameters and their changes in time [Hunkeler, 1997]. However, oxidant concentration is not a reliable monitoring parameter since
oxidant consumption may not be coupled to petroleum hydrocarbon mineralization. Alkalinity measurements allow to verify petroleum hydrocarbon mineralization processes that are accompanied by alkalinity production or consumption, but it is influenced by other processes like precipitation of FeS or carbonate dissolution. Ratios of biodegradable to recalcitrant compounds can be measured to infer which petroleum hydrocarbon compounds are mineralized. For example linear (n-) and isoprenoid (i-) alkanes with neighbouring retention times in the GC chromatogram (e.g. n-C\textsubscript{17} and pristane) have the same physico-chemical properties (e.g. solubility, octanol-water partitioning) and thus should show a similar transport behaviour. The decrease of the ratio of n-alkanes to i-alkanes is therefore a strong indication for that n-alkanes are preferentially mineralized compared to i-alkanes [Watkinson and Morgan, 1990].

However, it may be difficult to interpret such data since initial ratios are often not available and since "recalcitrant hydrocarbons" may also be degraded. For a confirmation of biodegradation, other factors have to be included. In this thesis we focused on the microbial aspect of in situ bioremediation. In petroleum hydrocarbon contaminated aquifers where biodegradation was expected based on changes in groundwater chemistry, the presence of microorganisms able to mineralize petroleum hydrocarbons could be confirmed.

### 6.3 Specific detection of microorganisms

Studies on the ecology of bacterial communities are often hampered by the lack of convenient methodology for the detection and identification of specific bacteria in their habitat. Since many bacteria resist cultivation (less than 1% have been cultured so far [Amann et al., 1995; Pace, 1996]), specific enumeration of bacteria by methods such as selective plating and immunofluorescence which rely on the isolation of the target organisms usually underestimate number and diversity of bacterial populations in natural habitats. Today, however, molecular biological techniques are available that
have the potential to solve a number of problems encountered with the
detection and identification of bacteria adequately [Manz et al., 1992].

*In situ* hybridization is a valuable tool to analyze bacterial community
structure in many different environments. Though in principle the protocols
for pure cultures are applicable in the environment, the sensitivity of the
detection can be limited by both the amount of rRNA per cell and the
permeability of the cells for probes [Amann et al., 1995]. Detectability of
bacteria in nutrient-poor environments, however, may also be affected by
environmental factors such as e.g. the background fluorescence which may
severely hinder the detection especially of weak hybridization signals. In the
future, the sensitivity of the fluorescent *in situ* hybridization technique may
probably be increased significantly if recent developments in signal
amplification using horseradish peroxidase-labeled oligonucleotides and
tyramide signal amplification [Lebaron et al., 1997; Schönhuber et al., 1997]
can be applied to the specific detection of bacteria in the environment.

### 6.4 Microorganisms at contaminated sites

#### 6.4.1 Cell numbers and detection rate

The laboratory as well as the field studies described in this thesis
showed that at each site a characteristic microbial population had developed.
Although the presence of petroleum hydrocarbons has a selective effect on the
microorganisms (C-source, toxicity), which might lead to similar population
structures in petroleum hydrocarbon contaminated sites, it is just one
parameter among many that influence a population (e.g. oxidants, nutrient
limitations, hydrogeology). It is therefore difficult to compare the various
studies directly. However, the different sites showed similar trends in
bacterial community structure. The number of cells and the detectability rate
by *in situ* hybridization correlated with the level of contamination. In the
contaminated core C at Hünxe (chapter 5) and in the laboratory aquifer
column (chapter 3) numbers were high with $2.4 \times 10^8$ and $1.0 \times 10^9$ cells g$^{-1}$
aquifer material [dry wt.], respectively. The laboratory column and core C at
Hünxe were highly polluted with petroleum hydrocarbon concentrations between 1.1 - 4.5 g kg\(^{-1}\).

The detection rate was very high in the laboratory column (>90%) as well as in core C at Hünxe (40%), but low in non- or low-contaminated aquifers (25% in Studen and 5-25% in core A and B at Hünxe). In more favourable habitats like activated sludge, between 80-90% of the DAPI-stained cells could be detected by hybridization with probe Eub338 [Snaidr et al., 1997]. The detection rate provides a crude indication for the metabolic activity of a bacterial population, since only cells containing sufficient amounts of rRNA and, at the same time, with a sufficient permeability of the cells (discriminating dormant cells like spores). The detection rate in a mixed population allows, however, no direct correlation with the growth rate or activity of bacteria since it varies according to the species examined and their consumption of nutrients and oxidants (for review see [Amann et al., 1995]).

6.4.2 Bacteria

In Studen as well as in the column studies the largest group of detectable bacteria belonged to the β-subdivision of Proteobacteria (>90% in the columns and 10-30% in the material from Studen). This corresponds well with findings in other aquatic habitats like activated sludge [Snaidr et al., 1997] and drinking water distribution systems [Kalmbach et al., 1997] where an analysis of the community structure also revealed a dominance of the β-subdivision of Proteobacteria (41% and 90%, respectively). In the contaminated core C at Hünxe the amount of organisms belonging to the β-subdivision of Proteobacteria was much smaller with only 2.5-5% of detectable cells (chapter 5).

At Hünxe the redox potential seemed to have a strong influence on the microbial population structure. Based on the ion measurements we assumed the dominance of rather sulfate-reducing than denitrifying conditions at Hünxe, which agrees with the results of \textit{in situ} hybridization. A population mainly consisting of members of the δ-subdivision of Proteobacteria developed. \textit{In situ} hybridization with probe SRB385 and SRB385Db,
designed to detect members of the δ-subdivision of Proteobacteria, revealed rates between 7-15%. The petroleum hydrocarbon mineralization under sulfate-reducing conditions is well documented [Edwards et al., 1992; Rabus and Widdel, 1995b; Weiner and Lovley, 1996; Wilkes et al., 1992] (chapter 1, Fig. 1.6).

6.4.3 Protozoa

Whole cell hybridization with probe Euk516 showed high numbers of protozoa in P25 at Studen in the saturated as well as in the unsaturated zone (0.8-4 \times 10^6 cells g\(^{-1}\) aquifer material [dry wt.]). Slightly lower numbers were found in the border zone of a xylene contaminated aquifer (1-2 \times 10^5 cells g\(^{-1}\) aquifer material [dry wt.] in the non-contaminated and 1-4 \times 10^5 cells g\(^{-1}\) aquifer material [dry wt.] in the source zone) (chapter 5). In other aquifers the amount of protozoa was determined by a modified most-probable-number (MPN) technique and numbers between 1.4 \times 10^2 (uncontaminated) and 3.6 \times 10^4 (contaminated with jet fuel) cells g\(^{-1}\) aquifer material [dry wt.] were found [Sinclair et al., 1993]. Comparisons of the two techniques showed that protozoa numbers detected by in situ hybridization were 2-5 orders of magnitude higher than numbers found by MPN (chapter 5). Estimates for feeding rates from batch culture studies suggest that protozoa require 10^2 to 10^3 bacteria per cell division (Schnürrer et al. 1986; Zwart and Darbyshire, 1992). Availability of bacteria is therefore unlikely to have been a limiting factor for the growth in the investigated aquifers. The role of protozoa in biodegradation is considered to be an indirect result of their ability to selectively graze on and control the biomass of aquifer bacteria (Madsen et al. 1991; Sinclair et al. 1993; Novarino et al. 1997). This in turn creates a microbial loop in which protozoa rapidly remineralize nutrients which sustain further bacterial growth (Coleman et al., 1977).

6.4.4 Azoarcus sp.

In situ hybridization enables to analyze specific bacterial populations such as e.g. that of hydrocarbon-degrading Azoarcus sp. in terrestrial habitats
(e.g. in petroleum hydrocarbon contaminated aquifers). The detection of petroleum hydrocarbon-degrading Azoarcus sp. in numbers between 1 and 2% of the total bacterial community in laboratory aquifer columns, but also in contaminated aquifer material from core C at Hünxe (chapter 5), demonstrates that they are significant members of the indigenous microbiota. In addition to in situ hybridization, the presence of A. tolylyticus in aquifer material could be confirmed by PCR with specific primer Azo644 and the universal primer FGPS6 (chapter 4, Fig. 4.4). PCR, however, gives a qualitative statement about the presence or absence of Azoarcus and has a detection limit of 3 cells g⁻¹ aquifer material [dry wt.] (the limit for hybridization with probes is around 5000 cells g⁻¹ aquifer material [dry wt.] [Tsai and Olson, 1992]).

Similar to the attribution of significant denitrification activity to Paracoccus sp. based on their 3.5% abundance [Neef et al., 1996], these results suggest that Azoarcus tolylyticus may play an important role during bioremediation in petroleum hydrocarbon-contaminated aquifer material. The assumption that their occurrence is correlated with petroleum hydrocarbon pollution could be supported by investigations of the bacterial community in not or very low contaminated aquifer where no Azoarcus could be detected (chapters 4 and 5). A final proof of this assumption, however, depends on the availability of more information on the catabolic activity of the hydrocarbon-degrading Azoarcus populations in columns and the field.

Informations about the function and catabolic activity of Azoarcus sp. can probably be obtained if recent advances in studies on the anaerobic toluene mineralization pathway [Coschigano and Young, 1997; Egland et al., 1997; Harwood and Gibson, 1997; Chee-Sanford et al., 1996; Migaud et al., 1996] will lead to the identification of the respective genetic information. Messenger RNA (mRNA) of key enzymes may then either be used as target to detect functional groups of organisms or a specific function of a target organism [Hahn et al., 1993; Hönerlage et al., 1995]. If the amount of specific mRNA can be correlated to the enzyme activity, specific mRNA concentrations may be used to investigate catabolic activities of the target organisms and hence to determine the in situ activity of microbial populations in the subsurface.
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