Studies on bacterial activities during degradation of mineral oil compounds

Dissertation

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1 Introduction

Organic groundwater contaminants

Chlorinated solvents and petroleum hydrocarbons are the main contributors to environmental pollution. They derive from various sources of contamination, e.g. gasworks plants, landfill leachates, accidental petroleum or fuel spills, mineral oil processing factories, and dry-cleaning facilities (US-EPA, 1999). Among them, the most abundant organic pollutants in the USA and Western Europe by far are the chlorinated hydrocarbons tetrachloroethene (PCE), trichloroethene (TCE), and dichloroethene isomers (DCEs) (Arneth et al., 1989; Schiedeck et al., 1997). They are of great concern because of their toxicity. Moreover, vinyl chloride (VC), an intermediate of PCE degradation, accumulates in the environment and has the potential to damage liver and the nervous system; it is also a carcinogen for human beings (Mohn and Tiedje, 1992; Holliger et al., 1999). Similar to chlorinated aliphatics, the monoaromatic hydrocarbons benzene, toluene, ethylbenzene, and xylene isomers (BTEX) belong to the list of priority pollutants defined by the US-Environmental Protection Agency (US-EPA, 1998). These putatively mutagenic and carcinogenic substances make up the bulk (>50 wt%) of the water-soluble fraction of gasoline (Coleman et al., 1984), and they may enter the environment via fuel spills. Maximum tolerable levels of single BTEX compounds and polyaromatic hydrocarbons (PAH) dissolved in the groundwater have been defined (US-EPA, 2002). If chemical spills containing aromatic hydrocarbons enter an aquifer, the BTEX compounds are barely retained. They migrate with the groundwater flow and form contaminant plumes. Their relatively high solubility and mobility in the subsurface frequently endangers drinking water supplies. The more hydrophobic polyaromatic constituents of mineral oils scarcely spread out. Instead, they remain adsorbed either to the source itself or to the soil surface.

Bacterial degradation of aromatic compounds

US statistics occasionally reported that 85 to 90% of petroleum contaminant plumes have reached steady state or are receding (Rice et al., 1995; Mace et al., 1997). Based on the results of these and other previous studies, it became apparent that the key process that accounts for the removal of BTEX from groundwater, is biodegradation by autochthonous petroleum hydrocarbon-degrading microbial communities (Norris, 1994; Wiedemeier et al., 1999). PAHs with up to five aromatic rings were removed from soil (Kanaly and

Harayama, 2000) and complete transformation, e.g. of benzo(a)pyrene, to CO_2 under aerobic conditions was detected (Kanaly et al., 1997). O_2 acts as a cosubstrate for enzymes activating BTEX compounds and PAH-derivatives in mono- or dioxygenase reactions (Gibson and Subramanian, 1984; Cerniglia, 1992; Borden et al., 1994). In sediments that are heavily contaminated with organic pollutants, aerobic degradation quickly depletes the groundwater of O_2. Consequently, the majority of the contaminants will then need to be degraded anaerobically without the utilization of oxygen. Even if O_2 is continuously recharged with the groundwater, it is unlikely that aerobic biodegradation will significantly contribute to *in situ* biodegradation (Christensen et al., 2001). The solubility of O_2 in water is limited, and moreover chemical reactions, such as the oxidation of Fe(II) or sulfide, may trap O_2 before it even reaches the zone where biodegradation is active (Lee et al., 1988).

Within the last 20 years, the ecological implications of anaerobic processes in contaminant biodegradation have become apparent. Interest has grown in investigating bacteria that are able to degrade contaminants using electron acceptors alternative to O_2. Bouwer and McCarthy developed a conceptual model dealing with the sequential appearance of predominant terminal electron accepting processes in contaminated aquifers. In the presence of organic material, the O_2 partial pressure rapidly drops, and thereby gives way for the start of denitrification reactions. After depletion of nitrate, the terminal electron acceptors consecutively used are Fe(III), sulfate, and CO_2. The series from O_2 to CO_2 is accompanied with an increase in water-dissolved H_2 and a decrease in the redox potential (Bouwer and McCarthy, 1984; Lovley et al., 1994). The relative importance of the respective electron acceptors in BTEX contaminated aquifers was rated in a US study comprising 38 field cases. The study revealed that sulfate is by far the most used electron acceptor, followed by CO_2, nitrate, and Fe(III); as expected, O_2 plays a secondary role as electron acceptor in the field (Wiedemeier et al., 1999). The theoretical model of redox zonation, however, has to be considered a simplification of natural conditions. Due to the heterogeneity of sediments and alternating groundwater levels, the terminal electron accepting processes of BTEX degradation in anoxic aquifers may be more variable with respect to time and spatial resolution than the conceptual model of Bouwer and McCarthy concedes (Vroblesky and Chapelle, 1994).

Contaminant degradation under anoxic conditions

The enrichment and isolation of bacteria growing with putatively toxic substrates was facilitated by culturing approaches with non-aqueous carrier phases for aromatic hydrocarbons. In the meantime cultures oxidizing various

aromatic substrates have been described. Bacterial strains degrading ethylbenzene, toluene, xylene isomers, and naphthalene were isolated with either nitrate, Fe(III), or sulfate as electron acceptors (Lovley et al., 1993; Anders et al., 1995; Rabus and Widdel, 1995; Beller et al., 1996; Galushko et al., 1999; Harms et al., 1999; Meckenstock, 1999a; Müller et al., 1999; Rockne et al., 2000; Coates et al., 2001a; Kniemeyer et al., 2003). Also, an enrichment culture degrading both 2-methylnaphthalene and naphthalene was described (Annweiler et al., 2000; Meckenstock et al., 2000). Recently, pure cultures of benzene-degrading bacteria coupled to nitrate-reduction and enrichment cultures degrading benzene under various redox conditions were reported (Coates et al., 2001b; Ulrich and Edwards, 2003). Another group extensively investigated the degradation potential of bacteria from anoxic harbor sediments regarding PAHs, and documented mineralization of naphthalene, phenanthrene, or fluorene by autochthonous bacterial communities (Coates et al., 1997). PAH-utilization by microbes is – in addition to the toxicity – limited by the dissolution kinetics of the fairly hydrophobic molecules. An online resource is available at http://arjournals.annualreviews.org/doi/suppl/10.1146/annurev.micro.56.012302.160749, listing further aromatic compounds used as growth substrates under anoxic conditions. Despite the often slow growth and difficult cultivation of anaerobic bacteria, pathways of anaerobic aromatic hydrocarbon degradation have been extensively investigated during the last years (Fig. 1). Anaerobic biodegradation of aromatic hydrocarbons has been most intensely examined with toluene. Systematic studies performed with the denitrifying toluene degraders *Thauera aromatica* and *Azoarcus* sp. revealed that degradation is initiated by addition of fumarate to the methyl group to form benzylsuccinate. The first enzymatic step is proposed to be the abstraction of a hydrogen atom from the methyl group by a radical-catalyzed enzyme reaction (Biegert et al., 1996; Beller and Spormann, 1997, 1998). Later on, this particular reaction type has been found to be remarkably widespread in bacterial degradation of aromatic hydrocarbons under anoxic conditions (Spormann and Widdel, 2000). Another degradation pathway was discovered for ethylbenzene under denitrifying conditions where degradation proceeds via hydroxylation of the ethyl side chain by the molybdenum enzyme ethylbenzene dehydrogenase to 1-phenylethanol, which is followed by a subsequent oxidation to acetophenone (Rabus and Widdel, 1995; Ball et al., 1996; Kniemeyer and Heider, 2001). Recently, evidence for fumarate addition reactions to ethylbenzene under sulfate-reducing conditions was obtained in environmental studies and in studies with a sulfate-reducing pure culture (Elshahed et al., 2001; Kniemeyer et al., 2003). In contrast to toluene and ethylbenzene, the first activation reaction in anaerobic benzene degradation

remains to be elucidated. Based on metabolites identified, it might be either a carboxylation or a hydroxylation reaction (Caldwell and Suflita, 2000; Phelps et al., 2001). Metabolite studies on a benzene-degrading sulfate-reducing enrichment culture with ^{13}C-labeled substrates showed that the carboxyl group of benzoate did not derive from bicarbonate in the medium but from the benzene itself (Caldwell and Suflita, 2000) (Fig. 1).

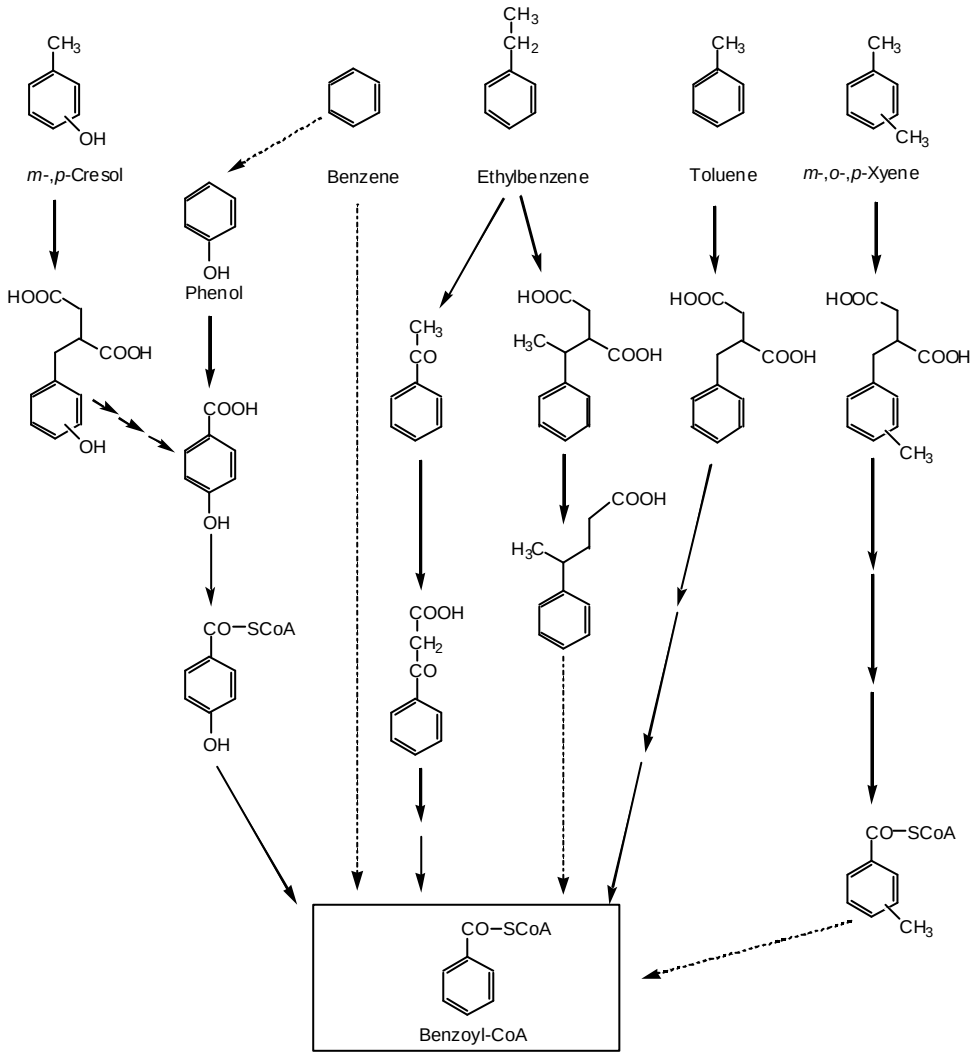

Fig. 1. Peripheral pathways for anaerobic transformation of some groundwater contaminants to the central intermediate benzoyl-CoA. Broken arrows indicate proposed pathways (modified from (Boll et al., 2002)).

Importance of the quantification of *in situ* biodegradation

Apart from interest in the characterization of bacterial degradation processes present in specific habitats, quantification of biodegradation in the subsurface is of major importance in the context of risk assessment, management of contaminated land, and bioremediation measures. Natural attenuation, an

alternative bioremediation concept, is already practiced in the US, and is in the meantime also accepted in Germany. Natural attenuation is based on intrinsic processes contributing to cleanup of environmental contaminations, as there are dispersion, dilution, sorption, evaporation, chemical transformations, or biodegradation (Wiedemeier et al., 1999). Successful (bio-) remediation means reduction of the amount of contaminants in groundwater and optimally to get to drinking water quality. Requirements for natural attenuation are an existing microbial degradation potential and the performance of concomitant monitoring measures at the respective site (US-EPA, 1999). Recommendations to prove biodegradation in the field were established by the US National Research Council (NRC). These recommendations include (i) documented loss of contaminants, (ii) laboratory assays showing bacterial degradation potential, (iii) evidence for biodegradation in the field (e.g., via computer-based models) (NRC, 1993). Fulfillment of these points can – at best – give qualitative indications for bacterial degradation at a specific site. Thus, for the prediction of the future behavior of a contaminant plume, it is essential to distinguish between the different biotic and abiotic reactions affecting concentrations, and, if possible, to balance the extent of the different processes. Since biodegradation is the main process leading to sustainable contaminant reduction, its qualitative and quantitative assessment is a major requirement for groundwater monitoring operations.

Various methods to determine *in situ* biodegradation of aromatic hydrocarbons

A number of different qualitative and semi-quantitative approaches are applied to determine bacterial contaminant degradation in the environment. Monitoring of contaminant concentrations and the simultaneous depletion of electron acceptors in a contamination plume give strong indications of biodegradation (Wiedemeier et al., 1999). The amount of O_2 depleted may be taken to characterize the extent of aerobic biodegradation activities. Analogously, changes in the concentrations of nitrate, Fe(II), sulfate, and methane are indicative of anaerobic respiration processes. The utilization of electron acceptors corresponds to the sum of all degradation reactions in the aquifer, not exclusively to the amount of environmental contaminants degraded. Nevertheless, measuring the concentrations of electron acceptors possibly provides information on limitations for biodegradation.

Another method that is often applied is the evaluation of the aerobic biodegradation potential in microcosm studies using contaminated material from the field site. However, at most sites contaminated with aromatic hydrocarbons there is no O_2 available. The evaluation of anaerobic microbial processes degrading mineral oil compounds in microcosms is time-consuming

and experiments are sometimes difficult to reproduce (Lovley, 2001). Laboratory results from aerobic and anaerobic microcosm experiments cannot be translated directly to the field. Further, while anoxic material is transferred into culture bottles, the anaerobic consortia which probably perform the key role in contaminant degradation *in situ* might be disturbed, or contact with trace amounts of oxygen might lead to inhibition of bacterial growth (Wiedemeier et al., 1999).

Molecular approaches, such as PCR or fluorescence *in situ* hybridization (FISH), can be used to detect contaminant-degrading microorganisms in soils and sediments (Amann et al., 1995; Fennell et al., 2001). Apart from showing the mere presence of certain bacteria, the *in situ* detection of genes that are coding for characteristic degradative enzymes is performed, which provides information on the theoretical intrinsic degradation potential for certain compounds at the site (Löffler et al., 2000; Stapleton et al., 2000).

A method that allows quantitative assessment of biodegradation of single compounds is based on results of reductive dehalogenation studies. PCE degradation is known to produce a series of daughter products upon stepwise dechlorination. These daughter products accumulate in the environment and can be taken as indicators of partial biodegradation (Vogel and McCarthy, 1985; Bradley and Chapelle, 1996; Gossett and Zinder, 1996). The principle of indicator metabolites was transferred to studies with BTEX compounds, even though aromatic compounds are, in general, degraded without release of recalcitrant intermediates. However, signature metabolites of bacterial BTEX degradation could be detected in contaminated aquifers and provide a qualitative proof of their degradation (Beller et al., 1995; Beller and Edwards, 2000). Specific metabolites of anaerobic degradation pathways, e.g. succinate adducts, were detected in recent studies with batch cultures and in contaminated aquifers (Beller et al., 1995; Elshahed et al., 2001; Beller, 2002; Gieg and Suflita, 2002; Phelps et al., 2002).

Compound-specific isotope analysis

Promising progress has been made within the last 20 to 25 years regarding the analysis of compound-specific stable isotope compositions, which opens new perspectives for the quantification of biodegradation *in situ*. Single compound isotope analysis is performed by isotope ratio-monitoring gas chromatography mass spectrometry (IRM-GC-MS), which consists of a gas chromatograph connected via a combustion line to an isotope mass spectrometer (Hayes et al., 1990). Stable isotope analysis of organic compounds has been used for a long time and for various purposes, e.g., to trace the origin of natural gas reservoirs (Clark and Fritz, 1997; Hoefs, 1997), to investigate CO_2-fixation by plants (O'Leary, 1984), and to characterize biochemical reactions (Northrop, 1981).

Today it is possible to measure traces of organic substances in compound mixtures with respect to their stable isotope compositions (For a review see Lichtfouse, 2000). Similar to plants, autotrophic bacteria and archaea produce pronounced isotope effects upon CO_2-fixation (Preuss et al., 1989; Zhang et al., 2002), namely, that the compounds consisting of lighter isotopes are turned over preferentially. As a consequence, all organic carbon of biochemical origin, including aromatic hydrocarbons, is depleted in ^{13}C. In one of the first publications on isotope fractionation in the context of contaminant degradation, Stahl investigated the aerobic biodegradation of crude oil in seawater under defined conditions and analyzed the carbon isotope composition of the residual, non-degraded contaminant fraction (Stahl, 1980). In this study's findings, degradation of the aromatic fraction was not linked to concomitant isotope shifts; however, isotope fractionation occurred upon degradation of the aliphatic fraction. More recent investigations on isotope fractionation during biodegradation of contaminants have focused mainly on BTEX and chlorinated solvents. Studies performed on stable isotope analysis as an indicator of microbial biodegradation in aquifers have been reviewed by Grossman (Grossman, 2002).

Determination of stable isotope fractionation

Carbon, hydrogen, nitrogen, oxygen and sulfur are major constituents of organic matter and have at least two stable isotopes that can be separated analytically by their mass differences. The lighter isotope is always the more abundant species. Except for hydrogen where the relative mass of deuterium is twice the mass of ^{1}H, the chemical and physical properties of stable isotopes are nearly identical. Since the zero-point energy is slightly higher for the lighter isotope, their chemical bonds are easier to break than those of the heavier isotopes (Bigeleisen and Wolfsberg, 1958). Kinetic isotope effects result from the different reaction velocities of molecules consisting of heavy or light stable isotopes. The compounds with the lighter isotopes are preferentially transformed, and this results in an unequal distribution of isotopes between substrates and reaction products. Pronounced kinetic isotope effects emerge only during the rate-limiting step of a reaction. Secondary isotope effects may occur if a heavier neighboring atom changes the chemical properties of the site of the reaction but does not directly participate in the reaction.

The most commonly used mathematical description of isotope fractionation processes is the Rayleigh equation $R_t/R_0 = (C_t/C_0)^{(\alpha - 1)} = f^{(\alpha - 1)}$ (Rayleigh, 1896; Mariotti et al., 1981) where R_t is the $^{13}C/^{12}C$ isotope signature at time t, C_0 is the initial concentration of the substrate, C_t/C_0 is the fraction f of substrate remaining at time t, and α is the isotope fractionation factor. The kinetic isotope fractionation factor α is a constant for a reaction at given experimental

conditions and can be obtained from experiments if $\ln(R_t/R_0)$ is plotted over $\ln(C_t/C_0)$ for the sampling points *t*. A common notation of isotope fractionation is the isotope enrichment factor ε, which is defined as $\varepsilon = (\alpha - 1) \times 1{,}000$ (Clark and Fritz, 1997).

Hydrogen isotope analysis is less sensitive than that of carbon isotopes, due to the natural abundance of deuterium which is about 50 times lower than the abundance of ^{13}C (Hoefs, 1997). Therefore, hydrogen isotope analysis requires higher compound concentrations compared to that of carbon isotopes (Meier-Augenstein, 1999; Hunkeler et al., 2001). On the other hand, the hydrogen isotope effects usually are around two orders of magnitude higher than carbon isotope effects (Hilkert et al., 1999). These characteristics make $^{2}H/^{1}H$ isotope analysis most appropriate to detect small biodegradation activities of compounds present at higher amounts.

Isotope fractionation in the course of contaminant degradation

In the course of BTEX degradation in the environment, stable isotope fractionation occurs as a result of microbial activity, since abiotic reactions (sorption to surfaces, dilution, and volatilization) have been demonstrated not to be coupled with significant carbon isotope fractionation in the field (Meckenstock et al., 1999c; Slater et al., 2000; Vieth et al., 2002). The early findings by Stahl, who could not detect isotope fractionation during degradation of aromatic compounds were confirmed in later studies in which aerobic degradation of toluene by undefined enrichment cultures did not lead to carbon isotope fractionation (Sherwood Lollar et al., 1999). Isotope fractionation during anaerobic degradation of toluene was first studied with the sulfate-reducing bacterial strain TRM1 (Meckenstock et al., 1999b), and significant ^{13}C isotope enrichment was observed. Other anaerobic strains using nitrate, sulfate, or ferric iron as electron acceptors, and fermenting bacteria were investigated regarding isotope fractionation during toluene degradation as well (Meckenstock et al., 1999c, Ahad et al., 2000; Ward et al., 2000). The resulting isotope fractionation factors are nearly identical (Meckenstock et al., 1999c). This is a convincing result, since the first enzyme reaction in anaerobic toluene degradation seems to use the same mechanism in all anaerobic bacteria. A carbon isotope fractionation factor significantly greater than the factors determined under anoxic conditions was obtained during aerobic toluene degradation with a pure culture of *Pseudomonas putida* strain mt-2, in contrast to Stahl's previous findings (Meckenstock et al., 1999c). Studies on carbon isotope fractionation with the denitrifying strain EBN1 revealed a fractionation factor that was in the same range with factors determined during anaerobic toluene degradation, although the initial reaction type is different

(Vieth et al., 2001; Griebler, unpublished results). Laboratory studies with anaerobic benzene-degrading cultures showed pronounced carbon and hydrogen isotope fractionation (Mancini et al., 2003) under denitrifying, sulfate-reducing, and methanogenic conditions. Laboratory experiments with the sulfate-reducing enrichment culture N47 degrading naphthalene or 2-methylnaphthalene revealed a small but significant $^{13}C/^{12}C$ fractionation (Griebler, unpublished results). No carbon isotope effects could be detected in degradation experiments with polyaromatic hydrocarbons such as fluoranthene (Trust Hammer et al., 1995). However, in these experiments the extent of degradation was possibly too small for a reliable determination of isotope shifts upon degradation. Lack of significant $^{13}C/^{12}C$ fractionation was reported also for aerobic degradation of 2-methylphenanthrene (Mazeas et al., 2002) and crude oil fractions (Mansuy et al., 1997).

Objectives

This work focused on the anaerobic bacterial degradation of the aromatic hydrocarbons toluene, *m*-, and *o*-xylene, and on isotope fractionation linked to these biodegradation processes.

The aims of the present study were:

- to evaluate the potential use of the adsorber resin Amberlite-XAD7 as a substrate carrier for the cultivation of bacteria with BTEX or naphthalene
- to characterize an *o*-xylene-degrading sulfate-reducing bacterium, *Desulfotomaculum* strain OX39
- to determine key processes of stable carbon and hydrogen isotope fractionation during contaminant degradation
- to examine the effects of temperature, growth rates, and redox conditions on the degree of fractionation during aerobic and anaerobic degradation of various aromatic hydrocarbons

References

Ahad, J.M.E., Sherwood Lollar, B., Edwards, E.A., Slater, G.F., and Sleep, B.E., 2000. Carbon isotope fractionation during anaerobic biodegradation of toluene: Implications for intrinsic bioremediation. Environ. Sci. Technol. 34, 892-896.

Amann, R., Ludwig, W., and Schleifer, K.-H., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. FEMS Microbiol. Rev. 59, 143-169.

Anders, H.J., Kaetzke, A., Kampfer, P., Ludwig, W., and Fuchs, G., 1995. Taxonomic position of aromatic-degrading denitrifying pseudomonad

strains K172 and KB740 and their description as new members of the genera *Thauera,* as *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov, respectively, members of the beta subclass of the proteobacteria. Int. J. Syst. Bacteriol. 45, 327-333.

Annweiler, E., Materna, A., Safinofski, M., Kappler, A., Richnow, H.H., Michaelis, W., and Meckenstock, R.U., 2000. Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. Appl. Environ. Microbiol. 66, 5329-5333.

Arneth, J.D., Milde, G., Kerndorff, H., and Schleyer, R., 1989. Waste deposit influences on groundwater quality as a tool for waste type and site selection for final storage quality. *In* The Landfill. - Lecture notes in earth sciences. Baccini, P. (ed). Berlin: Springer Verlag.

Ball, H.A., Johnson, H.A., Reinhard, M., and Spormann, A.M., 1996. Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J. Bacteriol. 178, 5755-5761.

Beller, H.R., Ding, W.-H., and Reinhard, M., 1995. Byproducts of anaerobic alkylbenzene metabolism useful as indicators of *in situ* bioremediation. Environ. Sci. Technol. 29, 2864-2870.

Beller, H.R., Spormann, A.M., Sharma, P.K., Cole, J.R., and Reinhard, M., 1996. Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl. Environ. Microbiol. 62, 1188-1196.

Beller, H.R., and Spormann, A.M., 1997. Benzylsuccinate formation as a means of anaerobic toluene activation by the sulfate-reducing strain PRTOL1. Appl. Environ. Microbiol. 63, 3729-3731.

Beller, H.R., and Spormann, A.M., 1998. Analysis of the novel benzylsuccinate synthase reaction for anaerobic toluene activation based on structural studies of the product. J. Bacteriol. 180, 5454-5457.

Beller, H.R., and Edwards, E.A., 2000. Anaerobic toluene activation by benzylsuccinate synthase in a highly enriched methanogenic culture. Appl. Environ. Microbiol. 66, 5503-5505.

Beller, H.R., 2002. Analysis of benzylsuccinates in groundwater by liquid chromatography/tandem mass spectrometry and its use for monitoring *in situ* BTEX biodegradation. Environ. Sci. Technol. 36, 2724-2728.

Biegert, T., Fuchs, G., and Heider, J., 1996. Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur. J. Biochem. 238, 661-668.

Bigeleisen, J., and Wolfsberg, M., 1958. Theoretical and experimental aspects of isotope effects in chemical kinetics. Adv. Chem. Phys. 1, 15-76.

Boll, M., Fuchs, G., and Heider, J., 2002. Anaerobic oxidation of aromatic compounds and hydrocarbons. Curr. Opin. Chem. Biol. 6, 604-611.

Borden, R.C., Gomez, C.A., and Becker, M.T., 1994. Geochemical indicators of intrinsic bioremediation. Ground Water 33, 180-189.

Bouwer, E.J., and McCarthy, P.L., 1984. Modeling of trace organics biotransformation in the subsurface. Ground Water 22, 433-440.

Bradley, P.M., and Chapelle, F.H., 1996. Anaerobic mineralization of vinyl chloride in Fe(III)-reducing aquifer sediments. Environ. Sci. Technol. 30, 2084-2086.

Caldwell, M.E., and Suflita, J.M., 2000. Detection of phenol and benzoate as intermediates of anaerobic benzene biodegradation under different terminal electron-accepting conditions. Environ. Sci. Technol. 34, 1216-1220.

Cerniglia, C.E., 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3, 351-368.

Christensen, T.H., Kjeldsen, P., Bjerg, P.L., Jensen, D.L., Christensen, J.B., Baun, A., Albrechtsen, H.-J., and Heron, G., 2001. Biogeochemistry of landfill leachate plumes. Appl. Geochem. 16, 659-718.

Clark, I., and Fritz, P., 1997. Environmental isotopes in hydrogeology. Boca Raton, Florida: Lewis Publishers.

Coates, J.D., Woodward, J., Allen, J., Philip, P., and Lovley, D.R., 1997. Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. Appl. Environ. Microbiol. 63, 3589-3593.

Coates, J.D., Bhupathiraju, V.K., Achenbach, L.A., McInerney, M.J., and Lovley, D.R., 2001a. *Geobacter hydrogenophilus, Geobacter chapellei* and *Geobacter grbiciae*, three new, strictly anaerobic, dissimilatory Fe(III)-reducers. Int. J. Syst. Evol. Microbiol. 51, 581-588.

Coates, J.D., Chakraborty, R., Lack, J.G., O´Connor, S.M., Cole, K.A., Bender, K.S., and Achenbach, L.A., 2001b. Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas.* Nature 411, 1039-1043.

Coleman, W.E., Munch, J.W., Streicher, R.P., Ringhand, H.P., and Knopfler, F.C., 1984. The identification and measurement of components in gasoline, kerosene, and No. 2 fuel oil that partition into the aqueous phase after mixing. Arch. Environ. Cont. Toxicol. 13, 171-178.

Elshahed, M.S., Gieg, L.M., McInerney, M.J., and Suflita, J.M., 2001. Signature metabolites attesting to the *in situ* attenuation of alkylbenzenes in anaerobic environments. Environ. Sci. Technol. 35, 682-689.

Fennell, D.E., Carroll, A.B., Gossett, J.M., and Zinder, S.H., 2001. Assessment of indigenous reductive dechlorinating potential at a TCE-contaminated site using microcosms, polymerase chain reaction analysis, and site data. Environ. Sci. Technol. 35, 1830-1839.

Galushko, A., Minz, D., Schink, B., and Widdel, F., 1999. Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. Environ. Microbiol. 1, 415-420.

Gibson, D.T., and Subramanian, V., 1984. Microbial degradation of aromatic hydrocarbons. *In* Microbial degradation of organic compounds. Gibson, D.T. (ed). New York: Dekker, pp. 181-252.

Gieg, L.M., and Suflita, J.M., 2002. Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum-contaminated aquifers. Environ. Sci. Technol. 36, 3755-3762.

Gossett, J.M., and Zinder, S.H., 1996. Microbiological aspects relevant to natural attenuation of chlorinated ethenes. *In* Proceedings of the Symposium on Natural Attenuation of Chlorinated Organics in Ground Water. Dallas, Texas.

Grossman, E.L., 2002. Stable carbon isotopes as indicators of microbial activity in aquifers. *In* Manual of environmental microbiology. Hurst, C.J. (ed). Washington D.C.: ASM Press.

Harms, G., Zengler, K., Rabus, R., Aeckersberg, F., Minz, D., Rossello-Mora, R., and Widdel, F., 1999. Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl. Environ. Microbiol. 65, 999-1004.

Hayes, J.M., Freeman, K.H., Po, B.N., and Hoham, C.H., 1990. Compound-specific isotope analysis. A novel tool for reconstruction of ancient biogeochemical processes. Adv. Org. Geochem. 16, 1115-1128.

Hilkert, A.W., Douthitt, C.B., Schlüter, H.J., and Brand, W.A., 1999. Isotope ratio monitoring gas chromatography/ mass spectrometry of D/H by high temperature conversion isotope ratio mass spectrometry. Rapid Commun. Mass Spectrom. 13, 1226-1230.

Hoefs, J., 1997. Stable isotope geochemistry. Berlin, Germany: Springer Verlag.

Holliger, C., Wohlfarth, G., and Diekert, G., 1999. Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol. Rev. 22, 383-398.

Hunkeler, D., Andersen, N., Aravena, R., Bernasconi, S.M., and Butler, B.J., 2001. Hydrogen and carbon isotope fractionation during aerobic biodegradation of benzene. Environ. Sci. Technol. 35, 3462-3467.

Kanaly, R., Bartha, R., Fogel, S., and Findlay, M., 1997. Biodegradation of [^{14}C]benzo[a]pyrene added in crude oil to uncontaminated soil. Appl. Environ. Microbiol. 63, 4511-4515.

Kanaly, R.A., and Harayama, S., 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J. Bacteriol. 182, 2059-2067.

Kniemeyer, O., and Heider, J., 2001. Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron-sulfur/heme enzyme. J. Biol. Chem. 276, 21381-21386.

Kniemeyer, O., Fischer, T., Wilkes, H., Glöckner, F.O., and Widdel, F., 2003. Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. Appl. Environ. Microbiol. 69, 760-768.

Lee, M.D., Thomas, J.M., Borden, R.C., Bedient, P.B., Ward, C.H., and Wilson, J.T., 1988. Biorestoration of aquifers contaminated with organic compounds. Crit. Rev. Environ. Control 18, 29-84.

Lichtfouse, E., 2000. Compound-specific isotope analysis. Application to archaeology, biomedical sciences, biosynthesis, environment, extraterrestrial chemistry, food science, forensic science, humic substances, microbiology, organic geochemistry, soil science and sport. Rapid Comm. Mass Spectrom. 14, 1337-1344.

Löffler, F.E., Sun, Q., Li, J., and Tiedje, J.M., 2000. 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. Appl. Environ. Microbiol. 66, 1369-1374.

Lovley, D.R., Giovannoni, S.J., White, D.C., Champine, J.E., Phillips, E.J.P., Gorby, Y.A., and Goodwin, S., 1993. *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. Arch. Microbiol. 159, 336-344.

Lovley, D.R., Chapelle, F.H., and Woodward, J.C., 1994. Use of dissolved H_2 concentrations to determine distribution of microbially catalyzed redox reactions in anoxic groundwater. Environ. Sci. Technol. 28, 1205-1210.

Lovley, D.R., 2001. Anaerobes to the rescue. Science 293, 1444-1446.

Mace, R.E., Fisher, R.S., Welch, D.M., and Parra, S.P., 1997. Extent, mass, and duration of hydrocarbon plumes from leaking petroleum storage tank sites in Texas. Austin, Texas: Bureau of Economic Geology, University of Texas at Austin.

Mancini, S.A., Ulrich, A.C., Lacrampe-Couloume, G., Sleep, B.E., Edwards, E.A., and Sherwood Lollar, B., 2003. Carbon and hydrogen isotopic fractionation during anaerobic biodegradation of benzene. Appl. Environ. Microbiol. 69, 191-198.

Mansuy, L., Philp, R.P., and Allen, J., 1997. Source identification of oil spills based on the isotopic composition of individual components in weathered oil samples. Environ. Sci. Technol. 31, 3417-3425.

Mariotti, A., Germon, C., Hubert, P., Kaiser, P., Letolle, R., Tardieux, A., and Tardieux, P., 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. Plant Soil 62, 413-430.

Mazeas, L., Budzinski, H., and Raymond, N., 2002. Absence of stable carbon isotope fractionation of saturated and polycyclic aromatic hydrocarbons during aerobic bacterial biodegradation. Org. Geochem. 33, 1259-1272.

Meckenstock, R.U., 1999a. Fermentative toluene degradation in anaerobic defined syntrophic cocultures. FEMS Microbiol. Lett. 177, 67-73.

Meckenstock, R.U., Annweiler, E., Warthmann, R., Schink, B., Michaelis, W., and Richnow, H.H., 1999b. $^{13}C/^{12}C$ stable isotope fractionation of toluene by anaerobic degradation: A new method to monitor biological degradation *in situ*? *In* Novel approaches for bioremediation of organic pollution. Fass, R., Flashner, Y., and Reuveny, S. (eds). New York: Kluwer Academic Publishers, pp. 219-227.

Meckenstock, R.U., Morasch, B., Warthmann, R., Schink, B., Annweiler, E., Michaelis, W., and Richnow, H.H., 1999c. $^{13}C/^{12}C$ isotope fractionation of aromatic hydrocarbons during microbial degradation. Environ. Microbiol. 1, 409-414.

Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B., 2000. Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Appl. Environ. Microbiol. 66, 2743-2747.

Meier-Augenstein, W., 1999. Applied gas chromatography coupled to isotope ratio mass spectrometry. J. Chromatogr. A 842, 351-371.

Mohn, W.W., and Tiedje, J.M., 1992. Microbial reductive dehalogenation. Microbiol. Rev. 56, 482-507.

Müller, J.A., Galushko, A., Kappler, A., and Schink, B., 1999. Anaerobic degradation of *m*-cresol by *Desulfobacterium cetonicum* is initiated by formation of 3-hydroxybenzylsuccinate. Arch. Microbiol. 172, 287-294.

Norris, R.D., 1994. In-situ bioremediation of soils and groundwater contaminated with petroleum hydrocarbons. *In* Handbook of bioremediation. Norris, R.D. (ed). Boca Raton, Florida: Lewis Publishers, pp. 17-37.

Northrop, D.B., 1981. The expression of isotope effects on enzyme-catalyzed reactions. Annu. Rev. Biochem. 50, 103-131.

NRC, 1993. *In situ* bioremediation: when does it work? Washington, DC: National Research Council (NRC).

O'Leary, M.H., 1984. Carbon isotope fractionation in plants. Phytochemistry 20, 553-567.

Phelps, C.D., Zhang, X., and Young, L.Y., 2001. Use of stable isotopes to identify benzoate as a metabolite of benzene degradation in a sulphidogenic consortium. Environ. Microbiol. 3, 600-603.

Phelps, C.D., Battistelli, J., and Young, L.Y., 2002. Metabolic biomarkers for monitoring anaerobic naphthalene biodegradation *in situ*. Environ. Microbiol. 4, 532-537.

Preuss, A., Schauder, R., Fuchs, G., and Stichler, W., 1989. Carbon isotope fractionation by autotrophic bacteria with three different carbon dioxide fixation pathways. J. Biosci. 44, 397-402.

Rabus, R., and Widdel, F., 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163, 96-103.

Rayleigh, J.W.S., 1896. Theoretical considerations respecting the separation of gases by diffusion and similar processes. Philos. Mag. 42, 493-498.

Rice, D.W., Grose, R.D., Michaelsen, J.C., Dooher, B.P., MacQueen, D.H., Cullen, S.J. et al., 1995. California Leaking Underground Fuel Tank (LUFT) Historical Case Analyses. Sacramento, California: California State Water Resources Control Board.

Rockne, K.L., Chee-Sanford, I.C., Sanford, R.A., Hedlund, B.P., Staley, I.T., and Strand, S.E., 2000. Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. Appl. Environ. Microbiol. 66, 1595-1601.

Schiedeck, T., Grathwohl, P., and Teutsch, G., 1997. Literaturstudie zum natürlichen Rückhalt / Abbau von Schadstoffen im Grundwasser. Lehrstuhl für angewandte Geologie, Universität Tübingen (im Auftrag der Landesanstalt für Umweltschutz Baden-Württemberg).

Sherwood Lollar, B., Slater, G.F., Ahad, J., Sleep, B., Spivack, J., Brennan, M., and MacKenzie, P., 1999. Contrasting carbon isotope fractionation during biodegradation of trichloroethylene and toluene: implications for intrinsic bioremediation. Org. Geochem. 30, 813-820.

Slater, G.F., Ahad, J.M.E., Sherwood Lollar, B., Allen-King, R., and Sleep, B., 2000. Carbon isotope effects resulting from equilibrium sorption of dissolved VOCs. Anal. Chem. 72, 5669-5672.

Spormann, A.M., and Widdel, F., 2000. Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. Biodegradation 11, 85-105.

Stahl, W.J., 1980. Compositional changes and $^{13}C/^{12}C$ fractionations during the degradation of hydrocarbons by bacteria. Geochim. Cosmochim. Acta 44, 1903-1907.

Stapleton, R.D., Sayler, G.S., Boggs, J.M., Libelo, E.L., Staufer, T., and Macintyre, W.G., 2000. Changes in subsurface catabolic gene frequencies during natural attenuation of petroleum hydrocarbons. Environ. Sci. Technol. 34, 1991-1999.

Trust Hammer, B., Mueller, J.G., Coffin, R.B., and Cifuentes, L.A., 1995. The biodegradation of fluoranthene as monitored using stable carbon isotopes. *In* Monitoring and verification of bioremediation. Hinchee, R.R., Douglas, G.S., and Ong, S.K. (eds), pp. 233-239.

Ulrich, A.C., and Edwards, E.A., 2003. Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures. Environ. Microbiol. 5, 92-102.

US-EPA, 1998. Carcinogenic effects of benzene: an update. Washington, DC: National Center for Environmental Assessment, Office of Research and Development, U.S. Environmental Protection Agency, pp. 1-45.

US-EPA, 1999. Use of monitored natural attenuation at superfund, RCRA corrective action, and underground storage tank sites. *In* OSWER directive 9200, pp. 4-17P.

US-EPA, 2002. National primary drinking water regulations. Washington, DC: Office of Water, U.S. Environmental Protection Agency, pp. 424-425.

Vieth, A., Kästner, M., Morasch, B., Meckenstock, R.U., and Richnow, H.H., 2001. Charakterisierung des biologischen Abbaus von BTEX im Grundwasser über Isotopenfraktionierung - Feldstudien. TerraTech 37-41.

Vieth, A., Voskamp, M., Kopinke, F.D., Meckenstock, R.U., and Richnow, H.H., 2002. Isotope fractionation as a tool to document the in-situ biodegradation of organic pollutants in contaminated aquifers. *In* Third International Conference on Water Resources and Environment Research. Schmitz, G.H. (ed). Dresden, Germany: Eigenverlag des Forums für Abfallwirtschaft und Altlasten e.V., pp. 198-202.

Vogel, T.M., and McCarthy, P.L., 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. 49, 1080-1083.

Vroblesky, D.A., and Chapelle, F.H., 1994. Temporal and spatial changes of terminal electro-accepting processes in a petroleum hydrocarbon-contaminated aquifer and the significance for contaminant biodegradation. Water Resour. Res. 30, 1561-1570.

Ward, J.A.M., Ahad, J.M.E., Lacrampe-Couloume, G., Slater, G.F., Edwards, E.A., and Sherwood Lollar, B., 2000. Hydrogen isotope fractionation during methanogenic degradation of toluene: Potential for direct verification of bioremediation. Environ. Sci. Technol. 34, 4577-4581.

Wiedemeier, T.H., Rifai, H.S., Newell, C., J., and Wilson, J.T., 1999. Natural attenuation of fuels and chlorinated solvents in the subsurface. New York: John Wiley & Sons, Inc.

Zhang, C.L., Ye, Q., Reysenbach, A.-L., Götz, D., Peacock, A., White, D.C. et al., 2002. Carbon isotopic fractionations associated with thermophilic bacteria *Thermotoga maritima* and *Persephonella marina*. Environ. Microbiol. 4, 58-64.

2 The use of a solid adsorber resin for enrichment of bacteria with toxic substrates and to identify metabolites: degradation of naphthalene, *o*-, and *m*-xylene by sulfate-reducing bacteria

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Abstract

Anaerobic sulfate-reducing bacteria were enriched from contaminated aquifer samples with naphthalene, *o*-, and *m*-xylene as sole carbon and energy source in the presence of Amberlite-XAD7, a solid adsorber resin. XAD7 served as a substrate reservoir maintaining a constantly low substrate concentration in the culture medium. In equilibration experiments with XAD7, the aromatic hydrocarbons needed up to 5 days to achieve equilibrium between the water and the XAD7 phase. The equilibrium concentration was directly correlated with the amount of added substrate and XAD7. In the enrichments presented here, XAD7 and aromatic hydrocarbons were adjusted to maintain substrate concentrations of 100 µM *m*-, or *o*-xylene, or 50 µM naphthalene. After five subsequent transfers, the three cultures were able to grow with higher substrate concentrations in the absence of XAD7 although they grew best with lower hydrocarbon concentrations. Two new xylene-degrading cultures were obtained that could not utilize toluene as carbon source. *o*-Xylene was degraded anaerobically by a culture, which could also oxidize *m*-xylene but not *p*-xylene. Eighty-three percent of the electrons from *o*-xylene oxidation were recovered in the produced sulfide, indicating a complete oxidation to CO_2. Another sulfate-reducing enrichment culture oxidized *m*-xylene completely to CO_2 but not *o*-, or *p*-xylene. A naphthalene-degrading sulfate-reducing enrichment culture oxidized naphthalene completely to CO_2. Metabolites of naphthalene degradation were recovered from the XAD7 phase and subjected to GC/MS analysis. Besides the metabolites 2-naphthoic acid and decahydro-2-naphthoic acid which were identified by the mass spectrum and coelution with chemically synthesized reference compounds, the reduced 2-naphthoic acid derivatives 5,6,7,8-tetrahydro-2-naphthoic acid and octahydro-2-naphthoic acid were tentatively identified by their mass spectra. Cultivation of bacterial cultures in the presence of XAD7 and subsequent derivatization and extraction of metabolites directly from the solid XAD7 resin provides a

new method for the isolation of sensitive bacteria and identification of metabolites.

Introduction

Aromatic hydrocarbons in the environment mostly originate from anthropogenic sources like mineral oil spills or gasworks plants and to a minor extent, from biological production in anoxic sediments or, e.g. termite nests (Jüttner and Henatsch, 1986; Jüttner, 1988; Chen et al., 1998). Due to their environmental importance as groundwater contaminants, there has been interest in microbial degradation of benzene, toluene, ethylbenzene, xylene (BTEX), and polycyclic aromatic hydrocarbons (PAH). Numerous aerobic bacteria have been isolated that can use aromatic hydrocarbons as carbon and energy source and the degradation pathways have been elucidated (Gibson and Subramanian, 1984; Cerniglia, 1992; Harwood and Parales, 1996). However, hydrocarbon contaminated aquifers become usually anoxic, with a redox gradient along the groundwater flow path, and the major part of the contaminants is degraded within the anoxic zone of the plume (Christensen et al., 1994). This implies that anaerobic bacteria degrade these substrates and several strains have been isolated with toluene as the sole carbon and energy source and nitrate, ferric iron, manganese or sulfate as the electron acceptors (Dolfing et al., 1990; Lovley and Lonergan, 1990; Evans et al., 1991; Rabus et al., 1993). Fermentative toluene degradation by enrichments and a defined syntrophic co-culture has been demonstrated (Edwards and Grbic-Galic, 1994; Ficker et al., 1999; Meckenstock, 1999). Some of the denitrifying toluene degraders can grow as well with *m*- or *p*-xylene, and also a denitrifying trimethylbenzene-degrading enrichment culture has been reported (Häner et al., 1995; Häner et al., 1997). Ethylbenzene is degraded by denitrifying bacterial strains (Ball et al., 1996). Also marine sulfate-reducing strains that can degrade *o*-, or *m*-xylene, respectively, have been described (Harms et al., 1999).

Anaerobic degradation of non-substituted aromatic hydrocarbons like benzene or naphthalene has been shown for microcosm experiments with various electron acceptors (Milhelcic and Luthy, 1988; Langenhoff et al., 1996; Coates et al., 1997; Rockne and Strand, 1998; Weiner and Lovley, 1998; Burland and Edwards, 1999) and transferable naphthalene-degrading cultures have been reported (Bedessem et al., 1997; Zhang and Young, 1997; Galushko et al., 1999; Meckenstock et al., 2000; Rockne et al., 2000). One difficulty in culturing BTEX- and PAH-degrading bacteria is, to some extent, due to the chemical stability of aromatic hydrocarbons which makes them difficult to metabolize in the absence of molecular oxygen. Another limiting factor is the toxicity of these compounds. Substrate concentrations high enough to support growth

may cause toxic effects and toxic metabolites can accumulate in the growth medium. On the other hand, elucidation of novel degradation pathways usually starts with identification of metabolites, which are frequently found in the growth medium.

Here, we present the enrichment of new sulfate-reducing freshwater bacteria degrading BTEX compounds or polycyclic aromatic hydrocarbons in the presence of the solid adsorber resin Amberlite-XAD7. The resin served as a substrate reservoir and kept the hydrocarbon concentration below toxic levels. XAD7 adsorbed compounds could be recovered from the solid phase and subsequent analysis with GC/MS revealed high yields of metabolites.

Material and Methods

Enrichment procedure

Inocula were taken from a soil column filled with gravel from an 8 m depth of a PAH-contaminated aquifer near Stuttgart, Germany (Meckenstock et al., 1999). The column was operated for several months at 16°C with a mixture of naphthalene (20 µM), benzene (150 µM), toluene (300 µM) and *o*-, *m*-, *p*-xylene (60 µM each) as carbon and electron source and sulfate as electron acceptor. The medium was bicarbonate-buffered, pH 7.2, with 3 mM ferrous chloride as the reducing agent. After degradation of naphthalene, toluene, *o*-, and *m*-xylene was established in the column, enrichments cultures were started with 3 ml samples containing solid material withdrawn from sampling ports with a syringe.

Growth conditions

Amberlite-XAD7 (Fluka, Buchs, Switzerland) was washed five times with ethanol (99.8%) and five times with distilled water. Traces of ethanol were removed by lyophilizing and drying for 2-3 days at 90°C. XAD7 (0.3 g) was autoclaved in an empty 100 ml serum bottle filled half with bicarbonate-buffered (30 mM) freshwater medium, pH 7.2–7.4, reduced with 1 mM sulfide (Widdel and Bak, 1992). The basal medium consisted of 1.0 g/l NaCl, 0.4 g/l $MgCl_2$ * 6 H_2O, 0.2 g/l KH_2PO_4, 0.25 g/l NH_4Cl, 0.5 g/l KCl, 0.15 g/l $CaCl_2$ * 2 H_2O. Sodium-sulfate (10 mM) was added as electron acceptor, the headspace flushed with N_2/CO_2 (80/20), and the bottles sealed with Viton rubber stoppers (Maag Technic, Dübendorf, Switzerland). Crystalline naphthalene (5-10 mg) was added before closing the bottle. *o*-Xylene, and *m*-xylene (10 µl) were injected with a syringe through the stopper and the medium was allowed to equilibrate for 3–4 days. $FeCl_2$ (3 mM) was added to *o*-xylene-degrading cultures in order to scavenge produced sulfide. Cultures were incubated at 30°C in the dark. Subsequent transfers were performed with 5 ml inoculum into 50 ml of medium.

Electron balance

Every electron balance was determined as the mean of three independent growth experiments. For assessment of the electron balance of the naphthalene-degrading culture, a naphthalene crystal of about 1 mg was added to 50 ml medium and incubated under sterile conditions for 2-3 weeks. After the crystal was completely dissolved, the medium was inoculated with 5 ml of a pre-culture and sulfide and naphthalene concentrations were monitored. *o*-Xylene or *m*-xylene (2 µl, respectively) were injected as a liquid through the stoppers to 50 ml medium and the bottles were inoculated with 5 ml pre-culture.

XAD7 adsorption experiments

XAD7 (0.3 to 1 g) was added to empty 100 ml serum bottles and autoclaved. Double distilled water (50 ml) was added, and the bottles sealed with Viton rubber stoppers. Different amounts of pure xylene were added with a syringe. Naphthalene was added as a crystal to the XAD7-containing bottles. The bottles were closed with Viton stoppers, autoclaved, and 50 ml water was added through the stoppers with a syringe. Three replicates were incubated at 30°C.

Analytical procedures

Samples for determination of growth parameters were taken with a syringe through the stopper. Growth and sulfate reduction were monitored by measuring OD_{578} and sulfide production (Cline, 1969). Each data point for sulfide or hydrocarbon analysis was determined in duplicates with a mean measurement error smaller than 5%. Hydrocarbon concentrations were determined by high-performance liquid chromatography (HPLC) on a Beckman System Gold equipped with a C_{18}-reversed phase column and a UV-detector (206 nm). Eluent was acetonitrile/ammonium phosphate buffer, pH 3.5, (70/30). Samples for HPLC analysis (250 µl) were added to 1 ml ethanol (p. a., 99.8%) and precipitates were removed by centrifugation (5 min, 15,000 × g).
To analyze metabolites of cultures grown with naphthalene in the presence of XAD7, the medium was decanted from the serum flasks, and the remaining XAD7 washed several times with distilled water. XAD7 was lyophilized and 100 mg was directly subjected to metabolite derivatization with 1 ml ethereal diazomethane solution to methylate carboxylic acids and aromatic hydroxyl groups (Fales et al., 1973). After 90 min at room temperature, the liquid was separated from the solids and the XAD7 was extracted once more with 1 ml diethyl ether. The solvent was removed from the combined extracts by a gentle stream of nitrogen and derivatized products dissolved in hexane. Silica gel column chromatography (1 × 10 cm, 70-230 µm mesh size) was applied to separate remaining naphthalene from the polar metabolites. Naphthalene was

eluted first with hexane/dichloromethane (80:20) and the remaining metabolites with diethyl ether. The diethyl ether fraction was analyzed with GC and GC/MS.

Gas chromatographic analyses were performed with a Carlo Erba Fractovap 4160, equipped with a 60 m capillary column (DB-5, J and W Scientific, 0.32 mm ID, 0.25 µm film thickness) and a flame ionization detector (FID). Hydrogen was used as carrier gas, and the temperature program was 80°C (3 min isothermal), 80-300°C (4°C/min), 300°C (10 min isothermal). The injection mode was on column.

GC/MS measurements were performed with a HP 6890 gas chromatograph coupled with a Quattro II mass spectrometer (Micromass, Altrincham, UK). Helium was used as carrier gas and GC conditions were the same as described above. The following MS conditions were applied: ionization mode: EI^+; ionization energy: 70 eV; source temperature: 180°C; mass range: m/z 50-400. For identification of metabolites, instrumental library searches applying the NIST / NIH / EPA mass spectral data base (US National Institute of Standards and Technology / US National Institutes of Health / US Environmental Protection Agency) and co-injection with chemically synthesized reference compounds were applied (Meckenstock et al., 2000).

Results

Anaerobic enrichment cultures were set up with different aromatic hydrocarbons as sole carbon and energy source, sulfate as electron acceptor, and Amberlite-XAD7 as a substrate reservoir. A systematic investigation of the XAD7 adsorption kinetics showed that an equilibrium concentration of *o*-xylene in the aqueous phase was achieved after approximately 5 days (Fig. 1A). The same was true for naphthalene when the closed culture bottles were prepared by autoclaving XAD7 together with solid naphthalene and subsequent addition of water (Fig. 1C). The hydrocarbon equilibrium concentrations in the aqueous phase were linearly dependent on the amount of *o*-xylene or naphthalene added to the bottles, indicating that the XAD7 phase was not saturated in the applied ranges (Fig. 1B,C).

In our enrichment cultures, the amounts of XAD7 and aromatic hydrocarbons were adjusted to a low equilibrium concentration in the aqueous phase of 100 µM for *m*- and *o*-xylene, and 50 µM for naphthalene as determined by HPLC analysis of the growth medium. Thus, toxic effects due to high substrate concentrations were avoided and the substrate was continuously replenished upon consumption. Nevertheless, substrate degradation decreased the aqueous concentration of, e.g., *o*-xylene to 3-5 µM during the exponential growth phase. After 3 months of incubation, a faint turbidity appeared in the

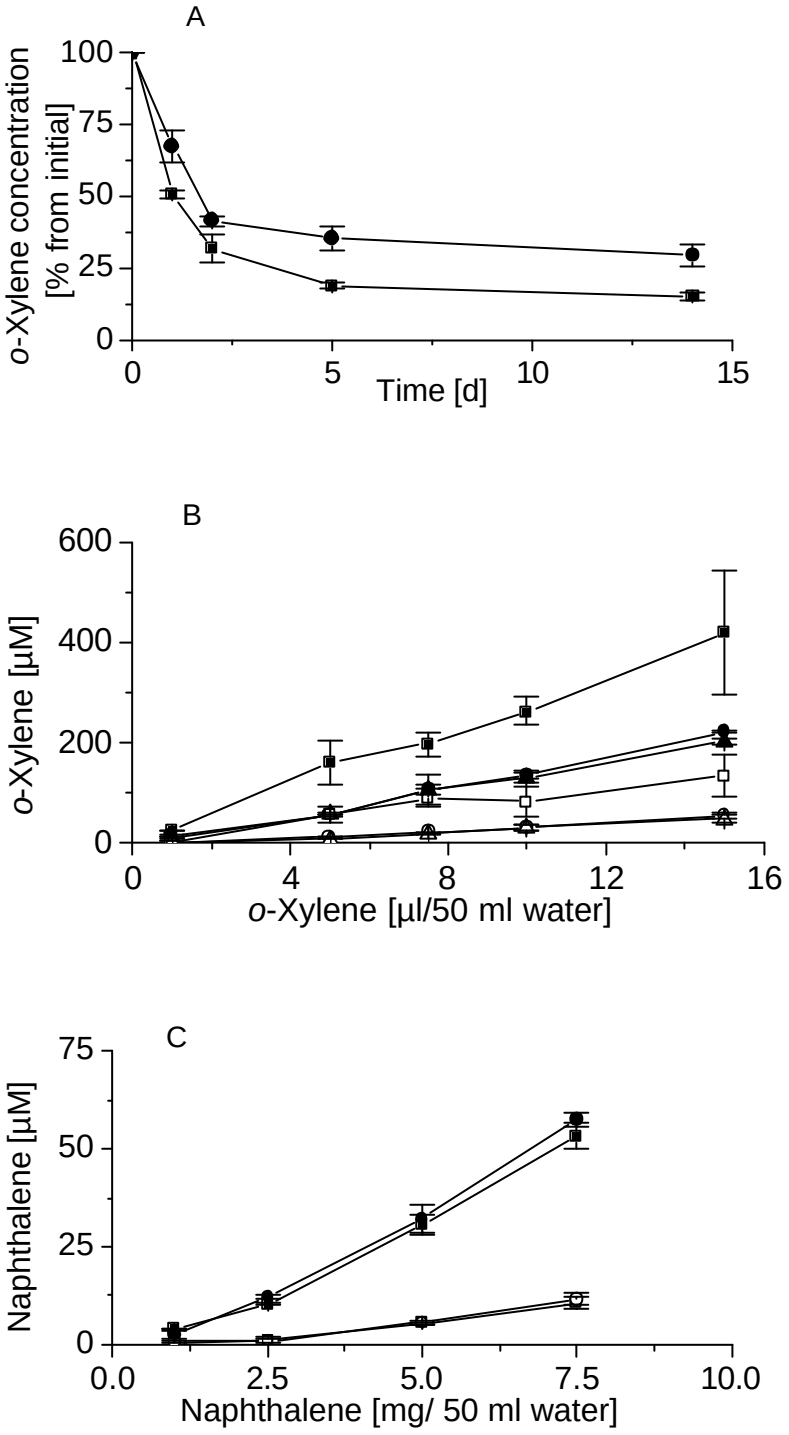

Fig. 1. Adsorption of aromatic hydrocarbons to the solid adsorber resin Amberlite-XAD7 under growth conditions. Data points are mean concentrations in the aqueous phase (n = 3). (A) Adsorption kinetics of 5 µl (■) or 10 µl (●) *o*-xylene from the aqueous to the XAD7 phase in 50 ml water. (B) *o*-Xylene equilibrium concentrations in the aqueous phase with 0.3 g (closed symbols) or 1 g XAD7 (open symbols) per 50 ml water. Samples were taken after 5 (■), 13 (●), and 22 days (▲) of incubation. (C) Naphthalene equilibrium concentrations in the aqueous phase with 0.3 g (closed symbols) or 1 g XAD7 (open symbols) per 50 ml water. Samples were taken after 5 (■), and 17 days (●) of incubation.

cultures with naphthalene, *o*-, and *m*-xylene. Growing cultures could be transferred without sediment under the respective cultivation conditions at 4-week intervals. There was no growth with XAD7 alone in the absence of a further substrate. Enrichments with the same substrates were also tried with the aromatic hydrocarbons only or with heptamethylnonane as carrier phase but growth of bacteria could not be observed under these conditions.

An *o*-xylene degrading culture was enriched in the presence of XAD7 plus 3 mM $FeCl_2$ to scavenge produced sulfide. After six transfers, the culture could be grown at low *o*-xylene concentrations (ca 200 µM) in the absence of XAD7. Product analyses of three replicate growth experiments showed a mean electron recovery of 83 ± 14% in the produced sulfide, indicating that *o*-xylene was oxidized completely to CO_2 (Fig. 2A). The culture could also utilize *m*-xylene but neither toluene nor *p*-xylene was degraded within the observation period of 60 days.

A *m*-xylene degrading culture was enriched in the absence of iron. The culture did not oxidize toluene, *o*-, or *p*-xylene within 60 days of observation. An electron balance showed a recovery of 97 ± 6% of the electrons from *m*-xylene in the produced sulfide (Fig. 2B).

The naphthalene-degrading culture grew best with solid naphthalene in the presence of XAD7 at pH 7.4, as indicated by an increase in cell density (OD_{578}) and by sulfide production. After 5 subsequent transfers, the organisms adapted to higher naphthalene concentrations and grew as well in the absence of XAD7 with solid naphthalene (maximal solubility ca 200 µM at 23°C) (Fig. 2C). In order to achieve a proper electron balance, the culture was grown in the absence of XAD7 with low concentrations of naphthalene. Quantitative analysis of the reaction products revealed that 88 ± 11% of the electrons from naphthalene oxidation appeared in the produced sulfide, indicating that naphthalene was oxidized completely to CO_2.

In order to analyze metabolites of anaerobic naphthalene degradation, the bacteria were cultivated in the presence of XAD7 and metabolites were extracted from XAD7 particles. GC/MS analysis of the extracts exhibited a number of metabolite peaks in the total ion current with a high signal-to-noise ratio (Fig. 3). 2-Naphthoic acid was identified by its mass spectrum and by coelution with the commercially available reference compound, and was the major metabolite in all naphthalene grown cultures. In addition, a tetrahydro-2-naphthoic acid derivative was found in all cultures. GC retention time and the mass spectrum were not identical with the commercially available 1,2,3,4-tetrahydro-2-naphthoic acid, and the compound was therefore tentatively identified as 5,6,7,8-tetrahydro-2-naphthoic acid. Moreover, one octahydro-2-naphthoic acid was tentatively identified by its mass spectrum. Decahydro-2-naphthoic acid isomers were identified as further metabolites by comparison with the synthesized reference compound (Meckenstock et al., 2000).

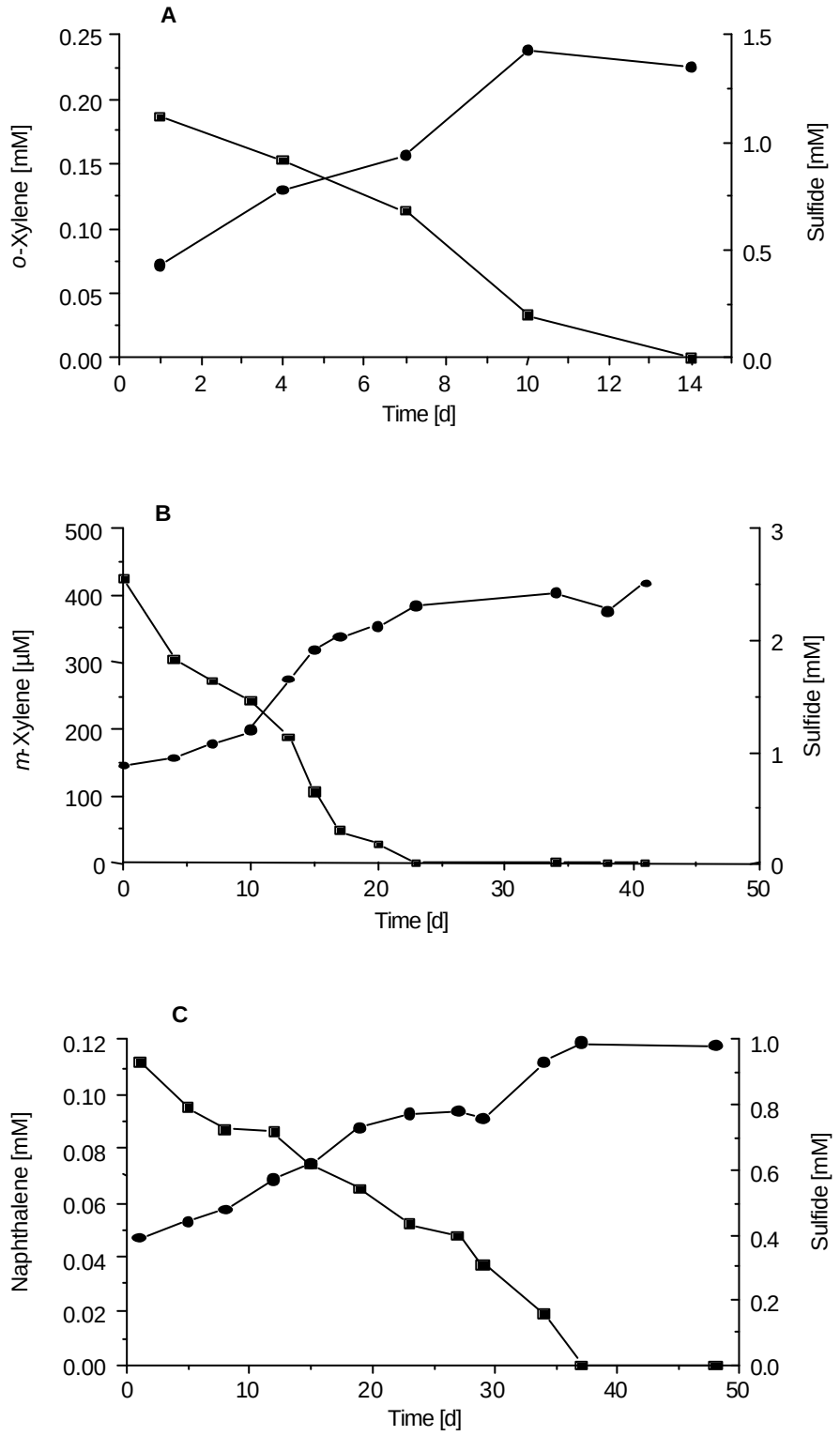

Fig. 2. Anaerobic degradation of aromatic hydrocarbons by three different sulfate-reducing enrichment cultures in the absence of XAD7. Substrate (■) and sulfide concentrations (●) were monitored during the time course of the experiment. Representative experiments of three replicates are shown with (A) *o*-xylene, (B) *m*-xylene, and (C) naphthalene as growth substrate.

Discussion

Cultivation of anaerobic BTEX- or PAH-degrading microorganisms is difficult due to the toxicity of the substrates, and requires careful adjustment of the cultivation conditions. In the present study we used the adsorber resin Amberlite-XAD7 to adsorb aromatic compounds in an equilibration phase before inoculation. Thus, introduced bacteria faced rather low actual concentrations in the growth medium, i.e. 100 µM *m*- and *o*-xylene, or 50 µM naphthalene instead of 2 mM xylene or 200 µM naphthalene if the same amount of substrate was added in the absence of XAD7. Three different sulfate-reducing bacterial cultures were enriched with the respective substrates to almost homogeneous microscopic appearance. So far, the cultures refused to grow in deep agar or liquid dilution series and could not be purified.

Substrate bound to the resin is in equilibrium with the medium and is released when the concentration in the liquid drops upon consumption by microorganisms. This allows growing bacteria to reasonable densities avoiding toxic concentrations of aromatic hydrocarbons. Evaluation of the adsorption kinetics of hydrocarbons to XAD7 revealed that under the described cultivation conditions it took about 5 days before equilibrium between the aqueous and the XAD7 phase established and the bottles could be inoculated. Due to the linear correlation between the amount of added hydrocarbon and the equilibrium concentration, a suitable substrate concentration can be calculated and adjusted. This is important especially in the initial enrichment phase, the most critical step when working with sensitive anaerobic bacteria. When reproducible growth was established after several transfers our cultures could also be grown also in the absence of XAD7 at limited hydrocarbon concentrations. Nevertheless, growth was always better in the presence of XAD7.

The initial enrichments were also attempted with the respective hydrocarbons only or in the presence of heptamethylnonane as a carrier phase. However, in our experiments we were only successful with XAD7. Nevertheless, several other anaerobic and aerobic cultures have been isolated on BTEX or PAH with heptamethylnonane as a carrier phase which has the same effect as XAD7, providing the cultures with low hydrocarbon concentrations (Rabus, et al., 1993; Harms, et al., 1999; Galushko, et al., 1999, Déziel et al., 1999). Besides maintaining a low substrate concentration, the culture may benefit from the presence of XAD7 by adsorbing potentially toxic metabolites. Metabolites carrying functional groups like carboxylic acids or phenolic compounds are easily trapped on the high surface area (450 m^2/g) of the hydrophilic polymer as we could demonstrate by the extraction of naphthalene degradation products from the XAD7 phase. Such metabolites carrying carboxylic groups

are not likely to enter a heptamethylnonane phase at neutral pH and could be a constant strive for the cultures.

Originally designed to remove polar compounds from non-aqueous phase liquids the polyacrylic ester XAD7 is weakly polar and wettable. Due to a true density of ca 1.07 g/l, it sinks to the bottom of the culture bottle. The XAD7 resin is chemically stable, non-toxic, and resistant to microbial degradation due to its polymeric structure. Heptamethylnonane has a certain water solubility and might exhibit additional stress or toxic effects to sensitive bacteria. When growth of our cultures was established after several transfers they grew also with heptamethylnonane as the carrier phase.

Solid materials are sometimes used in biotechnology to support growth of bacteria in fixed bed reactors. Solid materials can influence the growth of organisms leading to higher cell counts (Giard and Vedros, 1981) or, i.e. production of spores (Weber et al., 1999). We cannot exclude that the presence of XAD7 in the initial enrichments had similar stimulating effects because the bacteria colonized the solid surface. Nevertheless, in our experiments it is more likely that the physical properties of the resin like adsorption of toxic substrates and metabolites were the decisive factors for the organism.

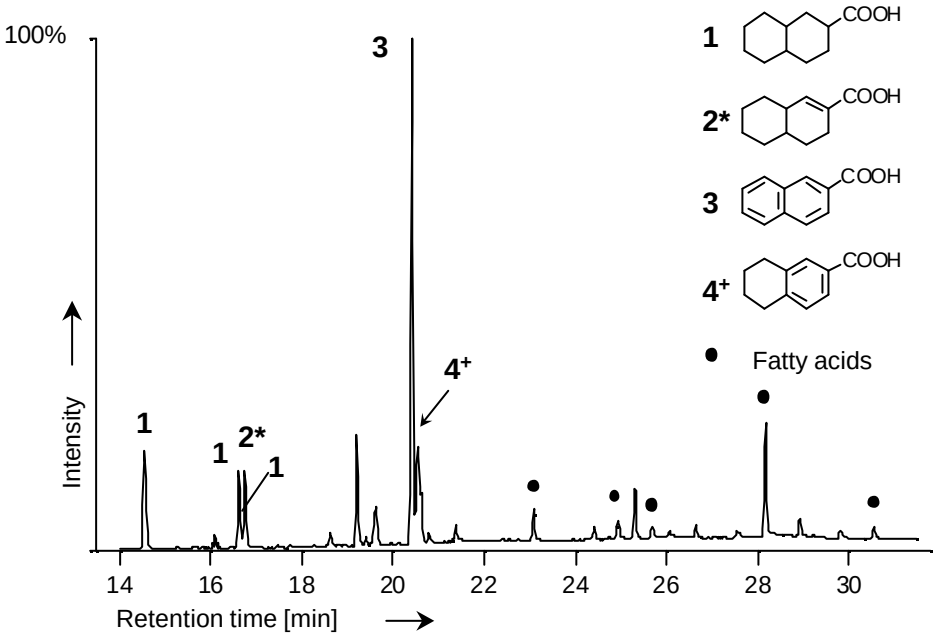

Fig. 3. Partial GC/MS total ion current of an extract recovered from the XAD7 phase of an anaerobic naphthalene degradation experiment. Peaks are numbered according to the given structures. Identical numbers indicate peaks of different isomers of the respective compounds. Due to the derivatization, carboxylic acids are present in the extract as methyl esters. (*), tentatively identified by the mass spectrum; the position of the double bond was not determined. (+), tentatively identified by the mass spectrum.

Degradation products adsorbed to the XAD7 resin and were recovered from the solid phase for GC/MS analysis with high yields. Processing of XAD7 samples according to our method is simple and less time consuming than extraction from liquid culture supernatants although the number of metabolites we identified was not higher compared to the extraction of the liquid samples. Nevertheless, applying the XAD7 method we confirmed the metabolites of anaerobic naphthalene degradation described in earlier studies (Meckenstock, et al., 2000; Zhang and Young, 1997). The appearance of reduced naphthoic acid derivatives could be reproduced and confirms that the aromatic ring system of 2-naphthoic acid is destabilized by a reductive attack as it is known for the anaerobic benzoyl-CoA degradation pathway (Harwood et al., 1999; Koch et al., 1993; Koch and Fuchs, 1992).

Anaerobic degradation of xylene is postulated to proceed by addition of fumarate to one of the methyl groups, analogous to toluene degradation (Heider et al., 1999). This was suggested by the production of methylbenzylsuccinate during *o*- and *m*-xylene degradation (Beller and Spormann, 1997; Krieger et al., 1999; Wilkes et al., 2000). However, xylene degradation seems to be highly specific for the different isomers as the known xylene-degrading organisms can utilize only either *o*-, *m*-, or *p*-xylene. Until recently, there was no anaerobic bacterial culture known to grow with *o*-xylene. Harms et al., (1999) isolated two sulfate-reducing, marine bacterial strains that grew with toluene and *o*- or *m*-xylene, respectively, but not with any other xylene isomer. Cultivation of xylene-degrading anaerobic bacteria seems to be more difficult than the cultivation of toluene degraders, as indicated by the limited number of isolates.

Our new freshwater cultures are the first xylene-degrading anaerobic bacteria that are not able to utilize toluene. The *m*-xylene degrading culture could use only *m*-xylene but none of the other xylene isomers or toluene. The *o*-xylene degrading culture oxidized *o*-xylene and *m*-xylene but neither toluene nor *p*-xylene. Until now, most anaerobic xylene-degrading cultures have been isolated with toluene as a carbon source and thus, it appeared likely that the degradation pathways for toluene and xylene are similar if not identical. The fact that our xylene-degrading cultures cannot grow with toluene indicates that anaerobic xylene metabolism is not simply a side reaction of toluene degradation. Nevertheless, the initial activation reaction in anaerobic xylene degradation might be similar to toluene activation, i.e. addition of fumarate to the methyl group.

In the present study, we show that the solid adsorber resin XAD7 is useful in the enrichment of sensitive bacteria with toxic substrate. XAD7 can serve as a substrate reservoir maintaining constantly low concentrations below toxic levels and providing the bacteria with sufficient amounts of substrate to support growth. Organic compounds produced by the microorganisms are

trapped on the resin preventing the accumulation of potentially toxic metabolites in the culture medium. The same metabolites can be easily recovered from the XAD7 particles to investigate the degradation pathways.

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References

Ball, H.A., Johnson, H.A., Reinhard, and M., Spormann, A.M., 1996. Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J. Bacteriol. 178, 5755-5761.

Bedessem, M.E., Swoboda-Colberg, N.G., and Colberg, P.J.S., 1997. Naphthalene mineralization coupled to sulfate-reduction in aquifer-derived enrichments. FEMS Microbiol. Lett. 152, 213-218.

Beller, H.R., and Spormann, A.M., 1997. Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. J. Bacteriol. 179, 670-676.

Burland, S.M., and Edwards, E.A., 1999. Anaerobic benzene biodegradation linked to nitrate reduction. Appl. Environ. Microbiol. 65, 529-533.

Cerniglia, C.E., 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3, 351-368.

Chen, J., Henderson, G., Grimm, C.C., Lloyd, S.W., and Laine, R.A., 1998. Termites fumigate their nests with naphthalene. Nature 392, 558-559.

Christensen, T.H., Kjeldsen, P., Albrechtsen, H.-J., Heron, G., Nielsen, P.H., Bjerg, P.L., and Holm, P.E., 1994. Attenuation of landfill leachate pollutants in aquifers. Crit. Rev. Environ. Sci. Technol. 24, 119-202.

Cline, J.D., 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. 14, 454-458.

Coates, J.D., Woodward, J., Allen, J., Philip, P., and Lovley, D.R., 1997. Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. Appl. Environ. Microbiol. 63, 3589-3593.

Déziel, A., Comeau, Y., and Villemur, R., 1999. Two-liquid-phase bioreactors for enhanced degradation of hydrophobic/toxic compounds. Biodegradation 10, 219-233.

Dolfing, J., Zeyer, J., Binder-Eicher, P., and Schwarzenbach, R.P., 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. 154, 336-341.

Edwards, E.A., and Grbic-Galic, D., 1994. Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Appl. Environ. Microbiol. 60, 313-322.

Evans, P.J., Mang, D.T., Kim, K.S., and Young, L.Y., 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57, 1139-1145.

Fales, H.M., Jaouni, T.M., and Babashak, J.F., 1973. Simple method for preparing ethereal diazomethane without resorting to codistillation. Anal. Chem. 45, 2302-2303.

Ficker, M., Krastel, K., Orlicky, S., and Edwards, E., 1999. Molecular characterization of a toluene-degrading methanogenic consortium. Appl. Environ. Microbiol. 65, 5576-5585.

Galushko, A., Minz, D., Schink, B., and Widdel, F., 1999. Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. Environ. Microbiol. 1, 415-420.

Giard, R.L., and Vedros, N.A., 1981. Biphasic resin systems for growing concentrated cultures of microorganisms and its application to the cultivation of *Neisseria gonorrhoeae*. Appl. Environ. Microbiol. 41, 846-847.

Gibson, D.T., and Subramanian, V., 1984. Microbial degradation of aromatic hydrocarbons. *In* Microbial degradation of organic compounds. Gibson, D.T. (ed). New York: Dekker, pp. 181-252.

Häner, A., Höhener, P., and Zeyer, J., 1995. Degradation of *p*-xylene by a denitrifying enrichment culture. Appl. Environ. Microbiol. 61, 3185-3188.

Häner, A., Höhener, P., and Zeyer, J., 1997. Degradation of trimethylbenzene isomers by an enrichment culture under N_2O-reducing conditions. Appl. Environ. Microbiol. 63, 1171-1174.

Harms, G., Zengler, K., Rabus, R., Aeckersberg, F., Minz, D., Rossello-Mora, R., and Widdel, F., 1999. Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl. Environ. Microbiol. 65, 999-1004.

Harwood, C.S., and Parales, R.E., 1996. The *b*-ketoadipate pathway and the biology of self-identity. Annu. Rev. Microbiol. 50, 553-590.

Harwood, C.S., Burchardt, G., Herrmann, H., and Fuchs, G., 1999. Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. FEMS Microbiol. Rev. 22, 439-458.

Heider, J., Spormann, A.M., Beller, H.R., and Widdel, F., 1999. Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol. Rev. 22, 459-473.

Jüttner, F., 1988. Benzene in the anoxic hypolimnion of a freshwater lake. Naturwissenschaften 75, 151-153.

Jüttner, F., and Henatsch, J.J., 1986. Anoxic hypolimnion is a significant source of biogenic toluene. Nature 323, 797-798.

Koch, J., Eisenreich, W., Bacher, A., and Fuchs, G., 1993. Products of enzymatic reduction of benzoyl-CoA, a key reaction in anaerobic aromatic metabolism. Eur. J. Biochem. 211, 649-661.

Koch, J., and Fuchs, G., 1992. Enzymatic reduction of benzoyl-CoA to alicyclic compounds, a key reaction in anaerobic aromatic metabolism. Eur. J. Biochem. 205, 195-202.

Krieger, C.J., Beller, H.R., Reinhard, M., and Spormann, A.M., 1999. Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azoarcus* sp. strain T. J. Bacteriol. 181, 6403-6410.

Langenhoff, A.A.M., Zehnder, A.J.B., and Schraa, G., 1996. Behaviour of toluene, benzene, and naphthalene under anaerobic conditions in sediment columns. Biodegradation 7, 267-274.

Lovley, D.R., and Lonergan, D.J., 1990. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl. Environ. Microbiol. 56, 1858-1864.

Meckenstock, R.U., 1999. Fermentative toluene degradation in anaerobic defined syntrophic cocultures. FEMS Microbiol. Lett. 177, 67-73.

Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B., 2000. Anaerobic naphthalene-degradation by a sulfate-reducing enrichment culture. Appl. Environ. Microbiol. 66, 2743-2747.

Meckenstock, R.U., Morasch, B., Warthmann, R., Schink, B., Annweiler, E., Michaelis, W., and Richnow, H.H., 1999. $^{13}C/^{12}C$ isotope fractionation of aromatic hydrocarbons during microbial degradation. Environ. Microbiol. 1, 409-414.

Milhelcic, J.R., and Luthy, R.G., 1988. Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. Appl. Environ. Microbiol. 54, 1182-1187.

Rabus, R., Nordhaus, R., Ludwig, W., and Widdel, F., 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. 59, 1444-1451.

Rockne, K.J., Chee-Sanford, J.C., Sanford, R.A., Hedlund, B.P., Staley, J.T., and Strand, S.E., 2000. Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. Appl. Environ. Microbiol. 66, 1595-1601.

Rockne, K.L., and Strand, S.E., 1998. Biodegradation of bicyclic and polycyclic aromatic hydrocarbons in anaerobic enrichments. Environ. Sci. Technol. 32, 3962-3967.

Weber, F.J., Tramper, J., Rinzema, A., 1999. A simplified material and energy balance approach for process development and scale-up of *Coniothyrium minitans* conidia production by solid-state cultivation. Biotechnol. Bioeng. 65, 447-458.

Weiner, J.M., and Lovley, D.R., 1998. Rapid benzene degradation in methanogenic sediments from a petroleum-contaminated aquifer. Appl. Environ. Microbiol. 64, 1937-1939.

Widdel, F., and Bak, F., 1992. Gram-negative mesophilic sulfate-reducing bacteria. *In* The prokaryotes. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York: Springer Verlag, pp. 3352-3378.

Wilkes, H., Boreham, C., Harms, G., Zengler, K., and Rabus, R. , 2000. Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria. Org. Geochem. 31, 101-115.

Zhang, X., and Young, L.Y., 1997. Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Appl. Environ. Microbiol. 63, 4759-4764.

3 Degradation of *o*- and *m*-xylene by a novel sulfate reducer belonging to the genus *Desulfotomaculum*

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Abstract

A strictly anaerobic bacterium, strain OX39, was isolated with *o*-xylene as substrate and sulfate as electron acceptor from an aquifer at a former gasworks site contaminated with aromatic hydrocarbons. Apart from *o*-xylene, strain OX39 grew with *m*-xylene and toluene that all three were oxidized completely to CO_2. Induction experiments indicated that *o*-xylene, *m*-xylene, and toluene degradation were initiated by different specific enzymes. Methylbenzylsuccinate was identified in supernatants of cultures grown with *o*- and *m*-xylene, and benzylsuccinate was detected in supernatants of toluene-grown cells thus indicating that degradation was initiated in all three cases by fumarate addition to the methyl group. Strain OX39 is sensitive towards sulfide and depends on Fe(II) in the medium to scavenge the sulfide produced. Analysis of the PCR-amplified 16S rRNA gene revealed that strain OX39 can be assigned to the Gram-positive endospore-forming sulfate reducers of the genus *Desulfotomaculum* and is the first hydrocarbon-oxidizing bacterium in this genus.

Introduction

Petroleum hydrocarbons are among the most abundant groundwater contaminants and can derive from gasworks plants, landfill leachates, and accidental fuel spills (US-EPA, 1999). The monoaromatic hydrocarbons benzene, toluene, ethylbenzene, and xylene isomers (BTEX) are putatively mutagenic or carcinogenic substances and make up more than 50% by weight of the water-soluble gasoline fraction (Coleman et al., 1984). Due to their relatively high solubility, they are mobile with the groundwater flow and form contaminant plumes in aquifers. The degradation potential of anaerobic bacteria *in situ* regarding aromatic hydrocarbons was investigated in several studies and a decrease of BTEX compounds could be demonstrated under denitrifying, Fe(III)-reducing, sulfate-reducing, and methanogenic conditions (Kazumi et al., 1997; Reinhard et al., 1997; Gieg et al., 1999; Phelps and Young, 2001). Numerous anaerobic bacterial cultures have been enriched in the past, and pure strains have been isolated. Of all BTEX compounds, toluene has been

studied most extensively with respect to its anaerobic degradation. Pure cultures of toluene-degrading bacteria were isolated which use NO_3^-, Fe(III), or SO_4^{2-} as electron acceptors (Lovley and Lonergan, 1990; Seyfried et al., 1994; Zhou et al., 1995; Beller et al., 1996). Under anoxic conditions, degradation of toluene proceeds via addition of fumarate to the methyl group (Biegert et al., 1996; Beller and Spormann, 1997a; Kane et al., 2002).

Consumption of *o*-xylene was discovered simultaneously with toluene degradation in methanogenic consortia (Edwards and Grbic-Galic, 1994). Later, transformation of *o*-xylene to *o*-methyl homologues of benzylsuccinate by toluene-degrading strains was reported, however no further oxidation steps could be observed (Beller and Spormann, 1997b). So far, only one anaerobic *o*-xylene-degrading bacterium, the sulfate-reducing strain oXyS1, was isolated from marine sludge (Harms et al., 1999). Apart from degradation of *o*-xylene, strain oXyS1 can grow with further aromatic substrates such as toluene, *o*-ethyltoluene, and *o*-toluic acid. Isolation efforts with *m*-xylene as carbon source led to some denitrifying cultures (Rabus and Widdel, 1995; Hess et al., 1997) and to one sulfate-reducing strain mXyS1 (Harms et al., 1999). Strain mXyS1 degrades further aromatic substrates, such as toluene, *m*-ethyltoluene, *m*-isopropyltoluene, and *m*-toluic acid but no compounds with methyl groups in *o*-position. Several denitrifying strains isolated with toluene were later found to grow with *m*-xylene as well, e.g. *Azoarcus* strain T and *Azoarcus tolulyticus* strain Td15 (Fries et al., 1994; Seyfried et al., 1994). *p*-Xylene, the third xylene isomer, has not been found yet to be degraded by pure cultures under anoxic conditions. However, one study recorded degradation of *p*-xylene by a bacterial enrichment when nitrate was provided as the electron acceptor (Häner et al., 1995). Neither pure cultures nor enrichments have been documented so far that couple xylene degradation to Fe(III) reduction.

Most sulfate-reducing bacteria degrading aromatic hydrocarbons were enriched from contaminated marine or brackish environments with high concentrations of sulfate, and were classified as members of the δ subclass of the Proteobacteria (Widdel and Rabus, 2001; Kniemeyer et al., 2003). Even though widespread in sediments and soils (Fry et al., 1997; Stubner and Meuser, 2000; Detmers et al., 2001), no anaerobic spore-forming strains have been isolated with aromatic hydrocarbons so far. Probably, enrichment procedures under laboratory conditions favor strains that grow faster than spore formers (Widdel, 1992).

To our knowledge, we present here the first anaerobic bacterium degrading both *o*- and *m*-xylene. This strain is also the first member of the genus *Desulfotomaculum* that uses aromatic hydrocarbons as growth substrates.

Materials and Methods

Purification and culture conditions

Strain OX39 was enriched from aquifer material of a former gasworks site near Stuttgart, Germany. The site was contaminated with mono- and polycyclic hydrocarbons (PAH), as well as heterocyclic compounds. Bacteria were cultivated at 30°C in bicarbonate-buffered freshwater mineral medium, pH 7.4, with sulfate (10 mM) as electron acceptor as described before (Morasch et al., 2001). The medium was prepared under an atmosphere of N_2/CO_2 (80:20) and reduced with Na_2S (1 mM) (Widdel and Bak, 1992). A sterile, anoxic $FeCl_2$ solution was added to a final concentration of 3 mM, and precipitated as black ferrous sulfide.

Bacteria were grown in 120 ml serum bottles half filled with mineral medium and sealed tightly with Viton rubber stoppers (Maag Technic, Dübendorf, Switzerland). Aromatic substrates were injected with micro-syringes directly through the rubber stoppers. In order to keep concentrations in the water phase low but to provide a substrate reservoir, 0.3 g adsorber resin Amberlite-XAD7 (Fluka, Buchs, Switzerland) was added per bottle, and the concentration of aromatic hydrocarbons stabilized at a level of 30 – 60 µM as described before (Morasch et al., 2001). Instead of Amberlite-XAD7, in some experiments, 1 ml 2,2,4,4,6,8,8,-heptamethylnonane (Aldrich, Milwaukee, WI) was used as a non-aqueous carrier liquid amended with 1.5% xylene. Bottles with heptamethylnonane were shaken gently twice a week.

The most abundant morphological cell type in the enrichment culture was isolated via liquid dilution series in bicarbonate-buffered freshwater medium with *o*-xylene as carbon source and in the presence of Amberlite-XAD7. Purity of strain OX39 was checked by phase-contrast microscopy. Absence of contaminants was proven in Bacto AC broth complex medium (3.5 g/l and 0.35 g/l; Difco; BD diagnostics, Franklin Lakes, NJ), and in freshwater medium with yeast extract (0.01%), lactate (5 mM), pyruvate (5 mM), and glucose (1 mM). Pure cultures were transferred every two to three months.

Growth conditions

Substrate utilization was monitored as increase in sulfide concentrations over time (Cline, 1969) and by microscopic investigation of cell density. The pH optimum was determined with 50 ml cultures growing with 7.5 µl of *o*-xylene in the presence of 0.3 g XAD7 at pH values adjusted between 6.6 and 7.8 with HCl (1 M) or Na_2CO_3 (1 M). The temperature optimum was determined over a range of 14 to 36°C in two parallel incubation series with *m*-xylene as substrate.

Growth substrates were added to culture bottles from aqueous stock solutions. Liquid aromatic compounds were injected with syringes through the rubber stoppers, and solid PAHs were added as crystals before flushing the headspace

with N_2/CO_2. Every substrate was tested in duplicate or triplicate. *o*-Xylene as growth substrate was used as a positive reference and a control was run without addition of substrate.

H_2 utilization was checked after injection into the gas phase of pregrown cultures (50 ml/bottle), and was monitored as sulfide increase over time.

For determination of electron recoveries and for induction experiments, three parallel cultures per substrate were incubated with *m*-xylene, *o*-xylene, or toluene. Substrates were added as anoxic stock solutions in mineral medium to achieve defined initial substrate concentrations from 50 to 200 µM. Substrate concentrations were checked by HPLC (see below) concomitant with sulfide formation.

Alternative electron acceptors tested were $Fe(OH)_3$ (15 mM), fumarate (7.5 mM), sulfite (2 mM), thiosulfate (2 mM), nitrate (2 or 10 mM), or sulfur (spoon tip). Usage of electron acceptors was checked microscopically as growth or by ion chromatography (DX10; Dionex, Sunnyvale, CA).

Preparation of cell-free extracts

One day before cell harvest, culture bottles were shaken gently. FeS precipitates settled over night and remained in the bottles when suspended bacteria were carefully transferred into centrifuge tubes. Bacteria were harvested by centrifugation (8,300 × g, 30 min, 10°C; HiCen 21; Herolab, Wiesloch, Germany), and the pellet was washed in Tris/HCl (50 mM, pH 7.5). For preparation of cell-free extracts, washed cells were suspended in the same buffer containing 20 mM EDTA and incubated after addition of 50 µg lysozyme /ml for 2 h at room temperature (lysozyme from egg white; Merck, Darmstadt, Germany). Cells were broken after lysis by ultrasonication (Sonoplus homogenisator HD 2200 equipped with MS73; Bandelin, Berlin, Germany, tip diameter 3 mm, 6 × 20 pulses of 0.4 s and amplitude of 60 µm). Efficiency of disruption was controlled microscopically. Cell debris was removed by centrifugation (20,000 × g, 5 min). Supernatants were stored at –20°C. Cell-free extracts for enzyme assays were prepared under N_2 atmosphere in buffers reduced with H_2 (10^5 Pa) plus palladium-charcoal (1 mg/l), and with dithioerythritol (2.5 mM).

Protein concentrations were determined according to (Bradford, 1976) (Bio-Rad, Munich, Germany).

Enzyme assays

Carbon monoxide dehydrogenase (EC 1.2.99.2), formate dehydrogenase (EC 1.2.1.2), and 2-oxoglutarate dehydrogenase (EC 1.2.7.3) were measured by analyzing the reduction of benzyl viologen (2 mM) photometrically over time at 578 nm. Assays were performed in anoxic 1.5 ml cuvettes closed with butyl rubber stoppers at 30°C. All solutions were added from anoxic stock solutions with precision syringes. *Desulfobacter* sp. strain "Zu" was used as positive

reference in 2-oxoglutarate dehydrogenase tests (Schauder et al., 1986; Spormann and Thauer, 1988).
Strain OX39 was checked for desulfoviridin by alkalinization and subsequent exposition to UV light (Postgate, 1959).

Analysis and sampling

BTEX concentrations were determined by high-performance liquid chromatography (HPLC; Bischoff Chromatography, Leonberg, Germany) with a C_{18} reversed phase column (Prontosil; length, 200 mm; inside diameter, 3 mm; film thickness, 3 µm; Bischoff, Leonberg, Germany) at 30°C and UV-detection at 210 nm using a mixture of acetonitrile (Chromasol V super gradient grade; Fluka, Buchs, Switzerland) and Millipore water (70:30, vol/vol) as eluent. Culture samples were diluted 1:5 with ethanol (99.9% gradient grade) and centrifuged (20,000 × g, 5 min) to remove precipitates.
Putative intermediates of toluene and xylene degradation were extracted from culture supernatants adjusted to pH < 1.5 with either dichloromethane or diethyl ether. Metabolites were converted to methyl esters with a mix of trimethylchlorosilane (Fluka, Buchs, Switzerland) and methanol (1:8) at 75°C for 1 h. For examination of molecules of lower molecular masses, carboxylic groups were silylated with N,O-bistrimethylsilyltrifluoro-acetamide (Merck, Darmstadt, Germany) for 6 h at 60°C. For mass spectroscopic analysis, the reaction mixture was dried under a stream of N_2 and dissolved afterwards in ethyl acetate (for methylated compounds) or cyclohexane (for silylated compounds). The GC was equipped with a flame ionization detector (Trace GC; Thermo Finnigan; San Jose, CA) and a fused silica capillary column (DB-5; length, 30 m; inside diameter, 0.25 mm; film thickness, 0.25 µm; Agilent, Palo Alto, CA). The temperature program was as follows: 4 min at 43°C, increase to 280°C at a rate of 4°C/min. Carrier gas was helium at a flow rate of 2 ml/min. The GC was calibrated before with fluorotributylamine as tuning gas. Mass spectrometric analysis was performed for fragments between 50 and 400 m/z using a Trace-DSQ MS unit (Thermo Finnigan). Identity of substances was confirmed by comparison either with published chromatograms or with reference compounds.
All chemicals used were of analytical grade (Merck, Darmstadt, Germany, or Fluka, Buchs, Switzerland, if not mentioned otherwise). Gases were purchased from Messer Griesheim (Krefeld, Germany).

Morphological characterization

Gram staining was performed using *Acetobacterium woodii* and *Desulfovibrio vulgaris* as positive and negative references, respectively (Bartholomew and Mittwer, 1952).
Spores were observed by phase contrast microscopy either in fresh wet mounts at 400-fold magnification or after spore staining (Schaeffer and Fulton, 1933).

Cultures were pasteurized in half filled 20 ml tubes sealed with butyl rubber stoppers for 10 min at 80°C. After pasteurization the culture was transferred into 50 ml mineral medium and incubated at 30°C.

Analysis of the 16S rRNA gene

Total genomic DNA was extracted from cells grown to stationary phase and harvested by centrifugation (Sambrook and Russel, 2001). The 16S rRNA genes were amplified by PCR using three different sets of primers, i.e., (1) 27f and 1492r (numbers indicate the corresponding positions in the *Escherichia coli* 16S rRNA gene), (2) 27f and 1087r, and (3) 530f and 1525r (Lane, 1991). PCR products were ligated into the vector pGEM-T (Promega, Mannheim, Germany) and transformed into *E. coli* JM109 supercompetent cells, as described by the manufacturer (Promega). Several clones from each PCR reaction were sequenced. For DNA sequencing, infrared dye 800-labeled M13 sequencing primers (MWB Biotech, Ebersberg, Germany) were used in combination with a Thermosequenase sequencing kit and 7-deaza-GTP (Amersham Bioscience, Freiburg, Germany). Sequencing was performed on a LI-COR 4200 sequencer (MWG Biotech). Sequence alignments, comparisons, and phylogenetic trees were accomplished using the ARB program package and the 16S rRNA gene database (db_Dez00.arb) provided with it (www.arb-home.de) (Strunk and Ludwig, 1996). Additional sequences from bacteria of the *Desulfotomaculum* group were imported from the EMBL database (www.ebi.ac.uk). Phylogenetic trees were constructed using either maximum parsimony, Phylip distance matrix, or maximum likelihood strategies. Highly variable regions were excluded with a 50% filter of invariance. The 16S rRNA sequence of strain OX39 was deposited in the EMBL database (www.ebi.ac.uk) under the accession number AJ577273.

Results

Isolation

Strain OX39 was enriched from contaminated aquifer material with *o*-xylene as substrate and sulfate as electron acceptor in presence of Amberlite-XAD7. After several transfers, growth with *o*-xylene became significantly faster and one cell type clearly predominated. Since isolation efforts in agar medium did not succeed, the strain was isolated with *o*-xylene as carbon source in liquid dilution series.

Substrate utilization

Degradation of *o*-xylene, *m*-xylene, and toluene was coupled to sulfate reduction. Stoichiometric formation of sulfide proved that these compounds were mineralized completely to CO_2. The mean electron recovery in three

independent growth experiments was 99.8 ± 16.9% with *o*-xylene, 95.1 ± 4.6% with *m*-xylene (Fig. 1), and 85.6 ± 13.3% with toluene.

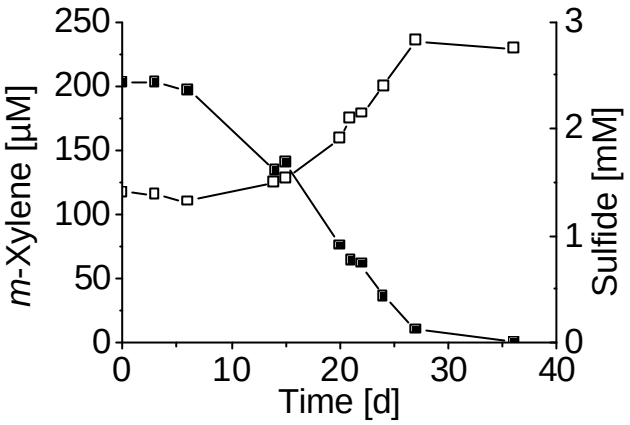

Fig. 1. Substrate utilization by strain OX39. Degradation of *m*-xylene (■) and concomitant production of sulfide (□) were monitored over time.

Apart from *o*-xylene, *m*-xylene, and toluene, further compounds were tested as substrates. The range of substrates utilized was very limited (Table 1). Strain OX39 used neither acetate and lactate, nor ethanol, methanol, or glucose. In an additional set of experiments, several substrates were supplied at lower concentrations to better reflect the concentrations bacteria are probably facing in aquifers (Table 1), but neither increase in cell number nor sulfide formation was found. Aromatic hydrocarbons other than toluene, *o*-xylene, and *m*-xylene were not degraded. Benzylsuccinate, an intermediate of toluene degradation, could not be used by the bacteria when added as substrate to the culture. *o*-Toluic acid and *m*-toluic acid were degraded, and degradation of benzoate started after a lag phase of more than 60 days. If H_2 was added with a syringe to pregrown cultures no stoichiometric H_2S formation was observed. Strain OX39 could not be transferred with hydrogen as electron source even if acetate (1 mM) was supplied as carbon source.

Key enzymes

Strain OX39 showed CO-dehydrogenase activity of 76 µmol/(min*mg protein) that was strictly proportional to the protein amounts added. Specific activity of formate dehydrogenase was in the same range (61 µmol/(min*mg protein)). Neither 2-oxoglutarate dehydrogenase activity nor desulfoviridin was detected in cell-free extracts.

Pathway of xylene degradation

In order to elucidate the pathway of toluene, *o*-xylene, and *m*-xylene degradation, culture supernatants were checked for specific metabolites. Benzylsuccinate and the respective methylated derivatives could be extracted and identified by GC-MS (Fig. 2D, E, F) as their mass fragments were in

Table 1. Substrate utilization by strain OX39 based on monitoring of sulfide production over 100 days.

Compound	Amount [µl/50 ml]	Concentration [mM]	Utilization
Benzene	1[a]	0.22[b]	-
Ethylbenzene	1.5[a]	0.24[b]	-
Toluene	2[a]	0.38[b]	+
m-Xylene	2[a]	0.32[b]	+
o-Xylene	2[a]	0.32[b]	+
p-Xylene	1.5[a]	0.24[b]	-
Naphthalene	Spoon tip[a]	n.d.	-
1-Methylnaphthalene	1[a]	0.14[b]	-
2-Methylnaphthalene	Spoon tip[a]	n.d.	-
1,2-Dimethylnaphthalene	1[a]	0.13[b]	-
1,3-Dimethylnaphthalene	1[a]	0.13[b]	-
1,4-Dimethylnaphthalene	1[a]	0.13[b]	-
Phenol	Spoon tip[a]	n.d.	-
m-Cresol	Spoon tip[a]	n.d.	-
o-Cresol	Spoon tip[a]	n.d.	-
p-Cresol	Spoon tip[a]	n.d.	-
Benzylsuccinate	Spoon tip	n.d.	-
m-Toluic acid		1	+
o-Toluic acid		1	+
Benzoate		1	+
Acetate		10; 2	-
Formate		5; 2	-
Lactate		10; 1	-
Propionate		5	-
D,L-Malate		5	-
Pyruvate		10; 1.5	-
Fumarate		5; 0.5	-
Succinate		5; 0.5	-
Ethanol		10	-
Methanol		10; 2	-
Glucose		1	-

[a]0.15 g XAD7 was added as non-liquid carrier phase to reduce the concentration in the medium and was allowed to equilibrate with the compound for one week before inoculation. [b]Concentration was calculated. *n.d.*: not determined.

agreement with published spectra (Krieger et al., 1999). Further compounds found in culture supernatants were methylphenylitaconic acids (Fig. 2G, H), *o*-toluic acid, *m*-toluic acid, and benzoate (Fig. 2N, O, P). In order to identify further intermediates of degradation, cells were cultivated with *o*-xylene and *o*-xylene-d_{10} supplied as growth substrates at equal amounts. 2-Methylbenzylsuccinate, 2-methylphenylitaconic acid, and *o*-toluic acid were found also as the respective deuterated compounds. Mass signals of 2-methylbenzoylsuccinic acid methyl ester (m/z = 264) and deuterated 2-methylbenzoylsuccinic acid-d_8 methyl ester (m/z = 272) were absent which in analogy to anaerobic toluene degradation might be the subsequent intermediates. However, in supernatants of *o*-xylene/*o*-xylene-d_{10}–grown cultures two substances eluted in the GC shortly after each other at 30.93 min and 31.06 min and had compound mass signals of m/z = 214 and 206 (Fig. 3). The molecular mass of m/z = 206 is that of 3-*o*-toluoyl propionic acid (Fig. 2M; CAS number 6939-36-2; not commercially available) and m/z = 214 the respective deuterated compound of putative 3-*o*-toluoyl propionic acid-d_8 which might have formed by decarboxylation of 2-methylbenzoylsuccinic acid. While the unfragmented compounds differed by 8 m/z, the major fragments, probably composed of the aromatic ring and parts of the alkyl chain, had differences of 7 m/z between deuterated and non-deuterated fragmentation patterns (Fig. 3). In culture supernatants of *o*-xylene-grown strain OX39, the first peak with m/z = 214 corresponding to tentatively identified 3-*o*-toluoyl propionic acid-d_8 could not be detected. *o*-Phthalic acid was also found in small amounts in extracts of *o*-xylene/*o*-xylene-d_{10}-grown cells, however, the deuterated analogue *o*-phthalic acid-d_4 was absent. Extracts of *m*-xylene and benzoate-grown bacteria showed traces of *o*-phthalic acid as well. Interestingly, salicylic acid (Fig. 2S) was identified in extracts of supernatants of toluene-grown cells. In addition, in *m*-xylene-grown cultures methylsalicylic acid (Fig. 2R) was tentatively identified. No methylsalicylic acid could be detected in *o*-xylene-grown or *o*-xylene/*o*-xylene-d_{10}-grown culture extracts.

Dimethylnaphthalenes, which are structural analogous to xylenes, were not used as growth substrates. Bacteria utilized *m*- and *o*-xylene in the presence of 1,2- 1,3-, or 1,4-dimethylnaphthalene that made up 6% of the total substrate. After growth, cultures were extracted for GC-MS. In two of these extracts with *m*-xylene as growth substrate and either 1,2- or 1,4-dimethylnaphthalene, compounds were found with m/z at 200 and a fragmentation pattern similar to that of naphthoic acid methylester plus 14 mass units (Fig. 4) (Meckenstock et al., 2000) and agreed in its major fragments with silylated 7-methylnaphthoic acid (Phelps et al., 2002). These are strong indications that dimethylnaphthalenes can be co-metabolized with *m*-xylene

as major substrate by strain OX39. However, succinate adducts of dimethylnaphthalenes were not detected.

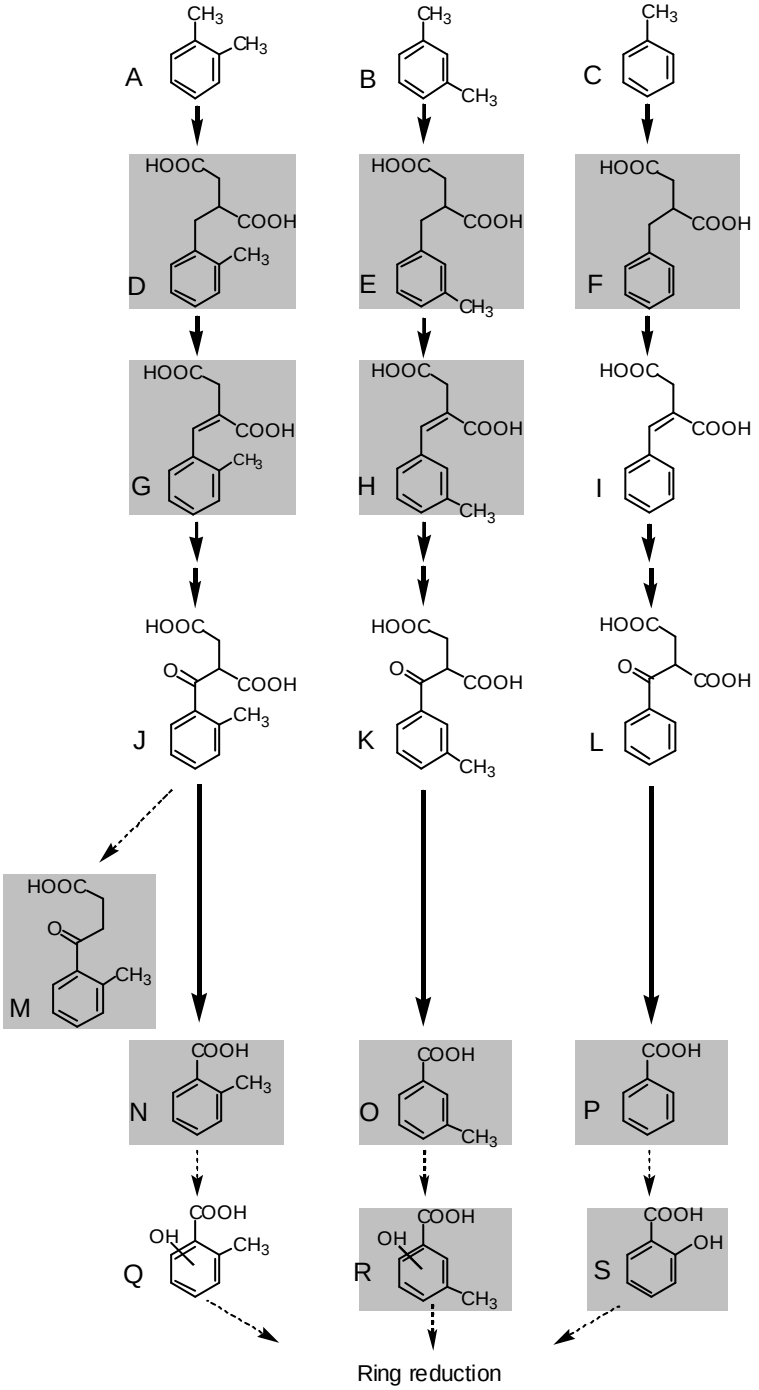

Fig. 2. Proposed pathways for the anaerobic degradation of *o*-xylene (A), *m*-xylene (B), and toluene (C) by strain OX39. The metabolites identified in culture supernatants are highlighted in grey: 2-methylbenzylsuccinic acid (D), 3-methylbenzylsuccinic acid (E), benzylsuccinic acid (F), 2-methylphenylitaconic acid (G), 3-methylphenylitaconic acid (H), phenylitaconic acid (I), 2-methylbenzoylsuccinic acid (J), 3-methylbenzoylsuccinic acid (K), benzoylsuccinic acid (L), 3-*o*-toluyl propionic acid (M), *o*-toluic acid (N), *m*-toluic acid (O), benzoic acid (P), methylsalicylic acid (Q, R), and salicylic acid (S). Broken arrows indicate pathways proposed on the basis of metabolites detected.

Induction experiments with substrate mixtures

If *m*- and *o*-xylene were simultaneously supplied as substrates at equal amounts to bacteria precultured with the same mixture, *m*-xylene was degraded preferentially (Fig. 5A). Several days after depletion of *m*-xylene, *o*-xylene degradation started. When the addition of the substrate mixture was repeated, *o*-xylene degradation was inhibited again as long as *m*-xylene was present (Fig. 5A). In experiments in which small concentrations of toluene (15 to 20 µM) were injected directly into cultures growing either with *m*-xylene or *o*-xylene, *m*-xylene degradation was not affected whereas *o*-xylene degradation was inhibited. Soon after depletion of *m*-xylene, strain OX39 started to degrade toluene (Fig. 5B).

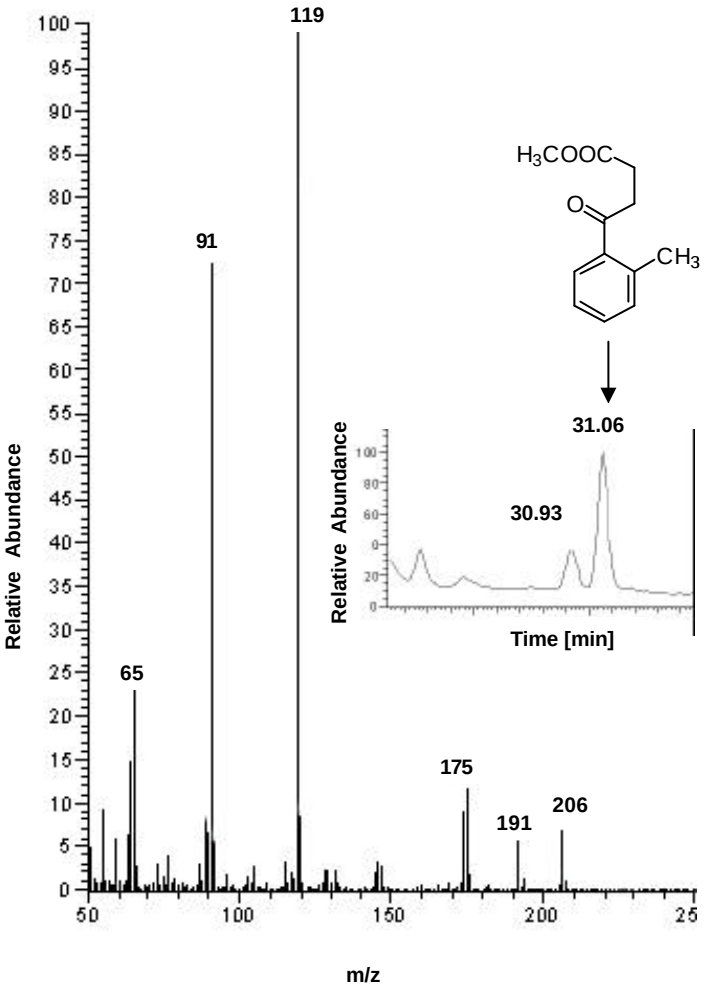

Fig. 3. GC-MS fragmentation pattern of tentatively identified 3-*o*-toluoyl propionic acid methyl ester extracted from culture supernatant of cells grown with *o*-xylene and *o*-xylene-d_{10} in equal amounts. The insert shows the GC chromatogram of tentatively identified 3-*o*-toluoyl propionic acid-d_8 eluting at 30.93 minutes and tentatively identified 3-*o*-toluoyl propionic acid eluting at 31.06 minutes.

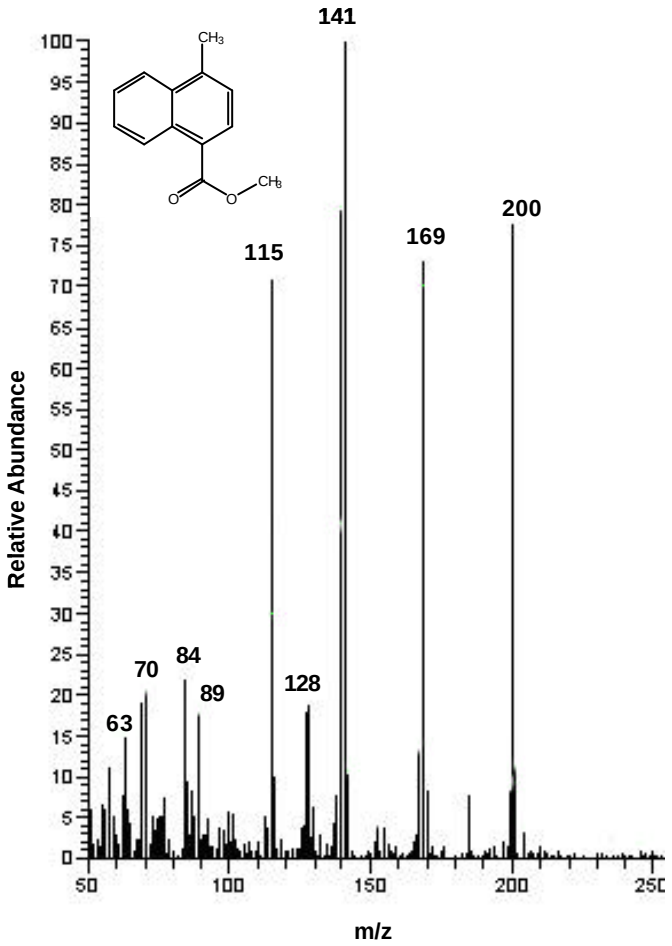

Fig. 4. GC-MS fragmentation pattern of tentatively identified 1-methyl-4-naphthoic acid methyl ester extracted from culture supernatant of cells grown with *m*-xylene in the presence of traces of 1,4-dimethylnaphthalene.

To examine induction of OX39 for single compounds, cultures were pregrown with either toluene, *m*-xylene, or *o*-xylene until the substrate was completely degraded. Subsequently, every culture was fed with one of the three substrates, respectively (Fig. 5C). If strain OX39 was cultivated again with the same substrate, the culture utilized this compound fastest. Degradation of *m*-xylene was also fast if the culture was pregrown with *o*-xylene. Initiation of *m*-xylene degradation was slower if cells were adapted to toluene and then fed with *m*-xylene, and 100 µM of substrate was degraded within one month. In contrast, transfers from *m*-xylene or toluene to *o*-xylene showed little degradation activity within 30 days. Similarly, toluene degradation by *o*-xylene-pregrown cultures was poor. Toluene degradation by *m*-xylene-grown cells started after a lag-phase and subsequently the degradation rate was significantly lower than by toluene-grown cultures. The same was true for bacteria transferred from toluene to *m*-xylene. If small concentrations of *p*-xylene were supplied together with *m*-xylene *p*-xylene was not degraded, but degradation of *m*-xylene was not inhibited (data not shown).

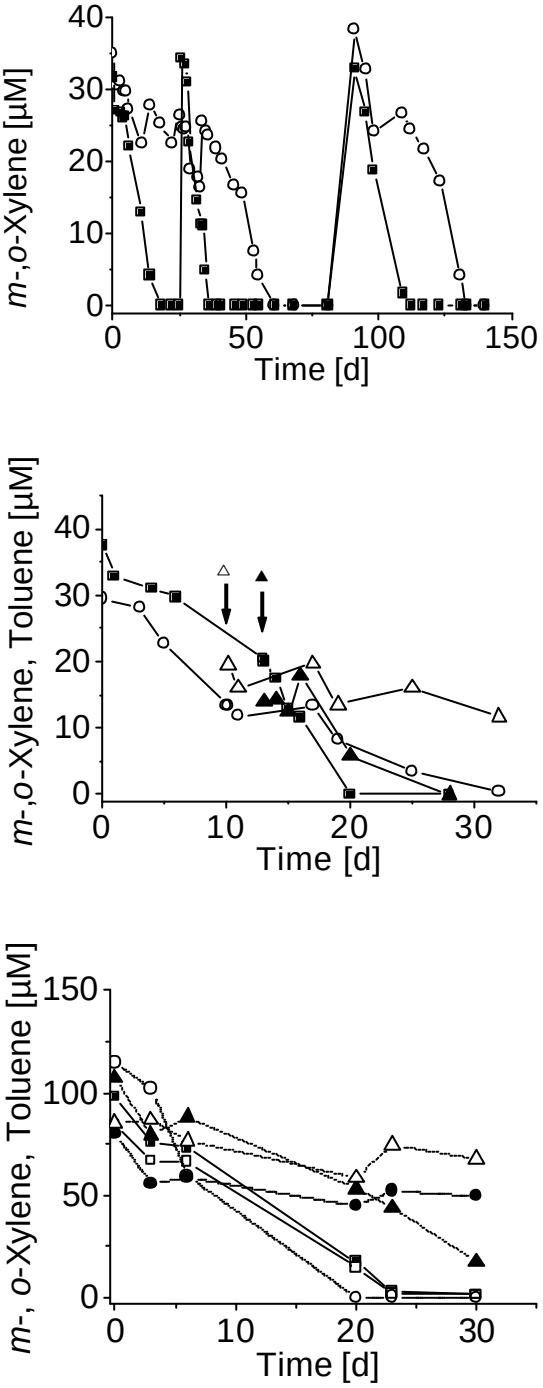

Fig. 5. Effect of co-substrates on substrate utilization by strain OX39. A.) Degradation of *m*- (■) and *o*-xylene (○) supplied as a mixture in equal amounts. B) Effect of toluene addition to growing cultures on degradation of *m*- (■) and *o*-xylene (○). The arrows depict the point of toluene injection to *m*-xylene (▲) and *o*-xylene (△) degrading cultures. The experiment was performed in two parallels. C) Induction experiment with cultures of strain OX39 pregrown either with *m*-xylene or *o*-xylene until substrate depletion and subsequently re-fed with one of the three substrates. *m*-Xylene-pregrown cells cultivated with *m*-xylene (■), *o*-xylene (●), or toluene (▲). *o*-Xylene-pregrown cells cultivated with *m*-xylene (□), *o*-xylene (○), or toluene (△). Each curve was taken as representative of four growth experiments performed.

Morphology

Cells of strain OX39 were rods, 7-8 µm long and 1.5 µm in diameter, motile, and stained Gram-positive (Fig. 6A). Endospores were formed preferentially after periods of starvation or after cultivation at slightly elevated temperature. Spores were located in the center of the cells (Fig. 6B). Several months after pasteurization, the strain started again to utilize *o*-xylene.

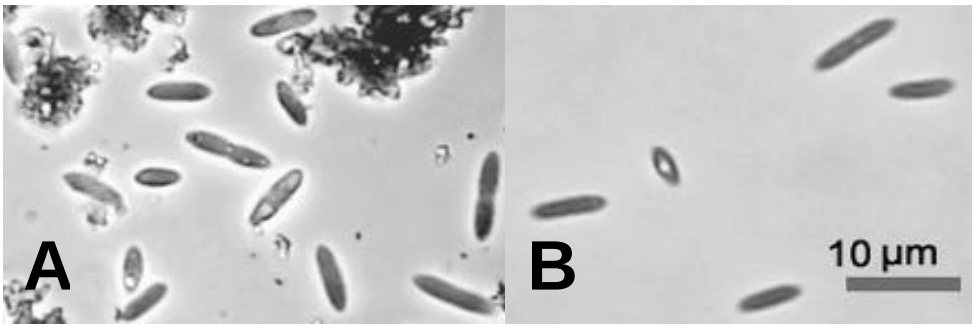

Fig. 6. Phase contrast micrographs of strain OX39. A) Bacteria grown with *o*-xylene in sulfide-reduced medium with 3 mM Fe(II). B) Cells of strain OX39 with one bearing an endospore.

Growth conditions

The optimum temperature for metabolic activity was between 25 and 30°C. At temperatures below 20°C or higher than 30°C growth was significantly slower. At 34°C more cells contained endospores and tended to form chains of four or more cells. At 36°C, sulfide production ceased and the number of uneven-shaped cells increased. However, cultures started to grow again after several weeks at 36°C if they were incubated again at 30°C.

Strain OX39 grew at pH 6.5 to 7.8 with *o*-xylene as carbon source. Conditions between pH 7.4 and 7.6 were most favorable.

Growth was absolutely dependent on the presence of Fe(II). Usually, $FeCl_2$ was added to the culture bottles to a final concentration of 3 mM, to scavenge free sulfide which inhibited growth. However, 300 µM of Fe(II) was found to be sufficient to support utilization of 1 mM *o*-xylene (with 0.15 g XAD7 present). Cells did not grow with Fe(II)-concentrations of 64 µM or less. The cell shape became more and more irregular during incubation without Fe(II).

When the culture was transferred into bottles where sulfate was replaced by sulfur, sulfite, thiosulfate, $Fe(OH)_3$, or fumarate the cells did not grow at all with *o*-xylene supplied as carbon source. With nitrate as alternative electron acceptor, the bacteria used up the remnant sulfate from the previous bottle but neither accumulation of nitrite nor decrease in nitrate could be found.

Phylogenetic classification based on the 16S rRNA gene

The phylogenetic relationship based on the analysis of the 16S rRNA gene is shown in Fig. 7. Strain OX39 belongs to the branch of endospore-forming sulfate reducers.

Discussion

Based on sequence analysis of its 16S rRNA gene, strain OX39 belongs to the branch of endospore-forming sulfate reducers. The most similar strain is *Desulfotomaculum* sp. strain R-AcetonA170 which was isolated from anoxic bulk soil of a paddy rice field. This strain degrades, e.g., acetate, acetone, and benzoate, but has not been tested for degradation of aromatics yet (Wind et al., 1999). Further relatives of these two strains are mainly represented by sequences from uncultured clones (Stubner and Meuser, 2000). Two of these (WCHB1-20 and 1-89) have been isolated from the methanogenic zone of an aquifer in the U.S. contaminated with jet fuel and chlorinated solvents (Dojka et al., 1998). Another close relative in that group is known from a co-culture capable to covert phenol to benzoic acid under anoxic conditions (Letowski et al., 2001). Due to their endospores members of the genus *Desulfotomaculum* are more resistant towards heat, drought, and exposition to air than vegetative cells of other genera. The spore formers' capability of adaptation to fluctuating environmental conditions together with their abundance in aquifer systems suggests that strain OX39 and affiliated strains play an important role in degradation of environmental contaminants *in situ*.

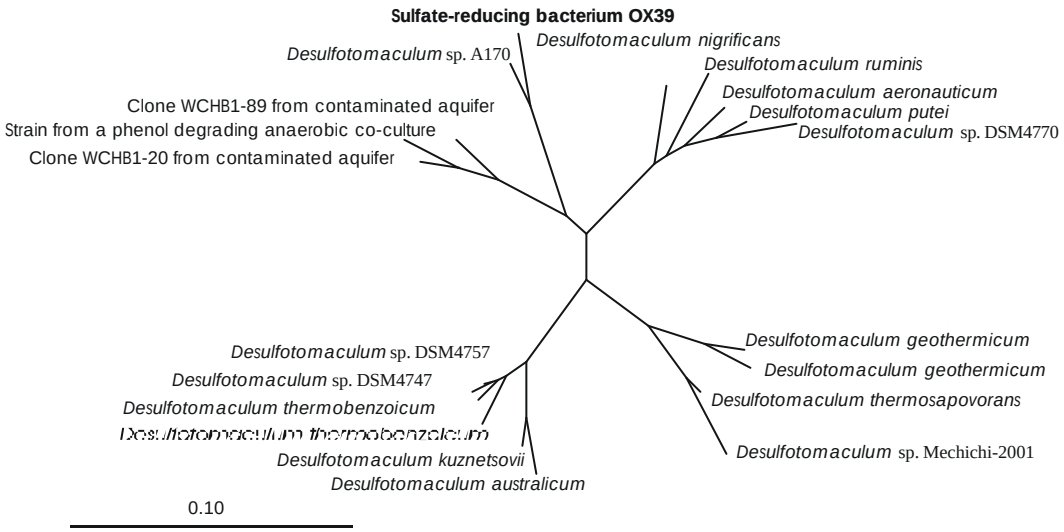

Fig. 7. Phylogenetic affiliation of strain OX39. The unrooted tree was constructed with the maximum likelihood method omitting the hypervariable regions of the 16S rRNA gene with a filter of invariance set at 50%.

The substrate range of strain OX39 turned out to be remarkably limited and within more than hundred days bacteria did not start to use organic acids such as acetate or lactate which are common carbon sources for sulfate-reducing bacteria. The only compounds found to be degraded were the aromatic hydrocarbons *m*-xylene, *o*-xylene, and toluene and certain assumed intermediates in the respective degradation pathways. Also, lag times were fairly long before growth started when transferred from xylenes to toluene or benzoate. When strain OX39 was offered one of the three substrates *m*-xylene, *o*-xylene, and toluene, the fastest degradation was obtained with the substrate that the culture degraded before. Here, the necessary enzyme was still induced to a certain extent. In contrast, most changes to other substrates slowed down degradation significantly. The capability for *m*-xylene degradation was regained relatively fast suggesting that the induction time for the *m*-xylene degrading enzyme was shortest. Thus, strain OX39 might be a sulfate-reducing bacterium that strictly depends on aromatic hydrocarbons for growth, or it migth be extremely slow in adapting to new substrates.

Growth experiments with strain OX39 and a mixture of two xylene isomers showed that *m*-xylene and *o*-xylene are not degraded by the same enzyme. *m*-Xylene was the preferential substrate, and *o*-xylene was not degraded in the presence of *m*-xylene. It is likely that *m*-xylene suppressed the induction of *o*-xylene degrading enzymes. After *m*-xylene degradation, the lag-phase lasted several days until oxidation of *o*-xylene started. Strain OX39 is the first anaerobic bacterium shown to degrade two xylene isomers. Xylene degradation by other pure cultures was restricted to either the *o*- or the *m*-isomer: The sulfate-reducing strain oXyS1 degraded exclusively substrates with methyl groups in *o*-position, and strain mXyS1 used exclusively substrates with methyl groups in *m*-position (Harms et al., 1999). These findings propose that *o*- and *m*-xylene degradation are two rather different capabilities which are both combined in strain OX39.

From mass spectrometric identification of metabolites extracted from culture supernatants of strain OX39, it appears plausible that degradation of toluene, *m*-xylene, and *o*-xylene proceeds via fumarate addition to one of the methyl groups to form (methyl-)benzylsuccinate. So far, the benzylsuccinate synthase reaction is the only initial reaction reported for degradation of methylated aromatics, e.g., toluene, *m*-cresol, *p*-cresol, *m*-xylene, *o*-xylene, and 2-methylnaphthalene, by sulfate-reducing, denitrifying, and Fe(III)-reducing bacteria (Beller and Spormann, 1997a, b; Leuthner et al., 1998; Krieger et al., 1999; Müller et al., 1999; Annweiler et al., 2000; Müller et al., 2001; Kane et al., 2002). Further steps in benzylsuccinate degradation detected by enzymatic assays are a CoA-transferase reaction yielding benzylsuccinyl-CoA and a subsequent dehydrogenase reaction leading to phenylitaconic acid (Leutwein and Heider, 1999, 2001, 2002). The subsequent degradation steps have been

proposed on the basis of molecular investigations of the *bbs* operon (Leuthner and Heider, 2000) where the authors hypothesize that succinyl-CoA is cleaved from benzoylsuccinyl-CoA to be recycled for further addition reactions and results in the formation of the central intermediate benzoyl-CoA. In contrast, recent investigations on anaerobic degradation of *n*-hexan proposed a decarboxylation reaction prior to stepwise β oxidation (Widdel and Rabus, 2001; Wilkes et al., 2002). In our studies however, putative 3-*o*-toluolyl propionic acid detected in supernatants of strain OX39 might probably be a dead end metabolite that derives from spontaneous decarboxylation of 2-methylbenzoylsuccinic acid rather than a transient metabolite of the *o*-xylene degradation. Further investigations are required here. Metabolite extraction from culture supernatants of strain OX39 did not exhibit intermediates with reduced ring systems, as would be produced by benzoyl-CoA reductase and subsequent reaction steps (Harwood et al., 1999). Contrarily, salicylic acid showed up in toluene- and benzoate-grown cultures, as did tentatively identified methylsalicylic acid when strain OX39 was grown with *m*-xylene. These substances may either be dead-end metabolites excreted by the bacterial cells, or they may be metabolites resulting from a hydroxylation reaction at the aromatic ring performed prior to ring reduction.

A recent study presented experiments with *Azoarcus* sp. strain T capable of mineralizing both toluene and *m*-xylene. The bacterium used the same enzyme for addition of fumarate to *m*-xylene and to toluene. A mutant deleted in the *bss* gene which codes for benzylsuccinate synthase was unable to grow with both substrates, but grew with benzoate (Achong et al., 2001). After the gene was restored, the capability of the denitrifying bacterium to initiate *m*-xylene and toluene degradation was recuperated. Our results with strain OX39 indicate that toluene, *o*-xylene, and *m*-xylene are attacked each by one specific (set of) enzymes.

Phelps and Young recently reviewed anaerobic degradation of BTEX and complex mixtures of substrates inhibiting each other's degradation (Phelps and Young, 2001). In the environment, contaminations typically are composed of several individual compounds and bacterial degradation might be inhibited. In this context, degradation of all three xylene isomers was found to be inhibited by addition of increasing concentrations of toluene in the environment (Barbaro et al., 1992). Our findings underline that toluene can interfere with xylene degradation.

Apart from interfering with substrate degradations, simultaneous presence of several compounds might lead to bacterial cometabolism, meaning conversion of compounds that do not favor growth and energy conservation of the respective bacterium but are turned over by unspecific side activities. The substrate range of benzylsuccinate synthase of *Azoarcus* sp. strain T was

investigated in assays with partially purified enzyme, and it was found that fumarate was added also to xylenes, mono-fluortoluenes, benzaldehyde, and others. However, most of these compounds were metabolized by the enzyme but were not used for growth by the bacterial strain (Beller and Spormann, 1999). In our studies on cometabolism of *m*-, and *o*-xylene with various dimethylnaphthalenes tentatively identified methylnaphthoic acids appeared in the culture supernatants. The putative product of dimethylnaphthalene degradation indicates that strain OX39 is able to transform two ring compounds cometabolically.

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References

Achong, G.R., Rodriguez, A.M., and Spormann, A.M., 2001. Benzylsuccinate synthase of *Azoarcus sp.* strain T: Cloning, sequencing, transcriptional organization, and its role in anaerobic toluene and *m*-xylene mineralization. J. Bacteriol. 183, 6763-6770.

Annweiler, E., Materna, A., Safinofski, M., Kappler, A., Richnow, H.H., Michaelis, W., and Meckenstock, R.U., 2000. Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. Appl. Environ. Microbiol. 66, 5329-5333.

Barbaro, J.R., Barker, J.F., Lemon, L.A., and Mayfield, C.I., 1992. Biotransformation of BTEX under anaerobic, denitrifying conditions: Field and laboratory observations. J. Contam. Hydrol. 11, 245-272.

Bartholomew, J.W., and Mittwer, T., 1952. The Gram stain. Bacteriol. Rev. 16, 1-29.

Beller, H.R., and Spormann, A.M., 1997a. Benzylsuccinate formation as a means of anaerobic toluene activation by the sulfate-reducing strain PRTOL1. Appl. Environ. Microbiol. 63, 3729-3731.

Beller, H.R., and Spormann, A.M., 1997b. Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. J. Bacteriol. 179, 670-676.

Beller, H.R., and Spormann, A.M., 1999. Substrate range of benzylsuccinate synthase from *Azoarcus* sp. strain T. FEMS Microbiol. Lett. 178, 147-153.

Beller, H.R., Spormann, A.M., Sharma, P.K., Cole, J.R., and Reinhard, M., 1996. Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl. Environ. Microbiol. 62, 1188-1196.

Biegert, T., Fuchs, G., and Heider, J., 1996. Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur. J. Biochem. 238, 661-668.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Cline, J.D., 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. 14, 454-458.

Coleman, W.E., Munch, J.W., Streicher, R.P., Ringhand, H.P., and Knopfler, F.C., 1984. The identification and measurement of components in gasoline, kerosene, and No. 2 fuel oil that partition into the aqueous phase after mixing. Arch. Environ. Cont. Toxicol. 13, 171-178.

Detmers, J., Schulte, U., Strauss, H., and Kuever, J., 2001. Sulfate reduction at a lignite seam: Microbial abundance and activity. Microbial Ecol. 42, 238-247.

Dojka, M.A., Hugenholtz, P., Haack, S.K., and Pace, N.R., 1998. Microbial diversity in a hydrocarbon- chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64, 3869-3877.

Edwards, E.A., and Grbic-Galic, D., 1994. Anaerobic degradation of toluene and o-xylene by a methanogenic consortium. Appl. Environ. Microbiol. 60, 313-322.

Fries, M.R., Zhou, J., Chee-Sanford, J., and Tiedje, J.M., 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60, 2802-2810.

Fry, N.K., Fredrickson, J.K., Fishbain, S., Wagner, M., and Stahl, D.A., 1997. Population structure of microbial communities associated with two deep, anaerobic, alkaline aquifers. Appl. Environ. Microbiol. 63, 1498-1504.

Gieg, L.M., Kolhatkar, R.V., McInerney, M.J., Tanner, R.S., Harris Jr., S.H., Sublette, K.L., and Suflita, J.M., 1999. Intrinsic bioremediation of petroleum hydrocarbons in a gas condensate-contaminated aquifer. Environ. Sci. Technol. 33, 2550-2560.

Häner, A., Höhener, P., and Zeyer, J., 1995. Degradation of *p*-xylene by a denitrifying enrichment culture. Appl. Environ. Microbiol. 61, 3185-3188.

Harms, G., Zengler, K., Rabus, R., Aeckersberg, F., Minz, D., Rossello-Mora, R., and Widdel, F., 1999. Anaerobic oxidation of *o*-xylene, *m*-xylene, and

homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl. Environ. Microbiol. 65, 999-1004.

Harwood, C.S., Burchhardt, G., Herrmann, H., and Fuchs, G., 1999. Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. FEMS Microbiol. Rev. 22, 439-458.

Hess, A., Zarda, B., Hahn, D., Häner, A., Stax, D., Höhener, P., and Zeyer, J., 1997. *In situ* analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl. Environ. Microbiol. 63, 2136-2141.

Kane, S.R., Beller, H.R., Legler, T.C., and Anderson, R.T., 2002. Biochemical and genetic evidence of benzylsuccinate synthase in toluene-degrading, ferric iron-reducing *Geobacter metallireducens*. Biodegradation 13, 149-154.

Kazumi, J., Caldwell, M.E., Suflita, J.M., Lovley, D.R., and Young, L.Y., 1997. Anaerobic degradation of benzene in diverse anoxic environments. Environ. Sci. Technol. 31, 813-818.

Kniemeyer, O., Fischer, T., Wilkes, H., Glöckner, F.O., and Widdel, F., 2003. Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. Appl. Environ. Microbiol. 69, 760-768.

Krieger, C.J., Beller, H.R., Reinhard, M., and Spormann, A.M., 1999. Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azoarcus* sp. strain T. J. Bacteriol. 181, 6403-6410.

Lane, D.J., 1991. 16S/23S rRNA sequencing. In Nucleic acid techniques in bacterial systematics. Stackebrandt, E., and Goodfellow, M. (eds). London: John Wiley & Sons Ltd., pp. 115-175.

Letowski, J., Juteau, P., Villemur, R., Duckett, M.F., Beaudet, R., Lepine, F., and Bisaillon, J.G., 2001. Separation of a phenol carboxylating organism from a two-member, strict anaerobic co-culture. Can. J. Microbiol. 47, 373-381.

Leuthner, B., and Heider, J., 2000. Anaerobic toluene catabolism of *Thauera aromatica*: the *bbs* operon codes for enzymes of β oxidation of the intermediate benzylsuccinate. J. Bacteriol. 182, 272-277.

Leuthner, B., Leutwein, C., Schultz, H., Hörth, P., Haehnel, W., Schlitz, E. Schägger, H, and Heider, J., 1998. Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol. Microbiol. 28, 615-628.

Leutwein, C., and Heider, J., 1999. Anaerobic toluene-catabolic pathway in denitrifying *Thauera aromatica*: activation and β-oxidation of the first intermediate, (R)-(+)-benzylsuccinate. Microbiology 145, 3265-3271.

Leutwein, C., and Heider, J., 2001. Succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase: an enzyme of the anaerobic toluene catabolic pathway in denitrifying bacteria. J. Bacteriol. 183, 4288-4295.

Leutwein, C., and Heider, J., 2002. (R)-Benzylsuccinyl-CoA dehydrogenase of *Thauera aromatica*, an enzyme of the anaerobic toluene catabolic pathway. Arch. Microbiol. 178, 517-524.

Lovley, D.R., and Lonergan, D.J., 1990. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl. Environ. Microbiol. 56, 1858-1864.

Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B., 2000. Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Appl. Environ. Microbiol. 66, 2743-2747.

Morasch, B., Annweiler, E., Warthmann, R.J., and Meckenstock, R.U., 2001. The use of a solid adsorber resin for enrichment of bacteria with toxic substrates and to identify metabolites: degradation of naphthalene, *o*-, and *m*-xylene by sulfate-reducing bacteria. J. Microbiol. Meth. 44, 183-191.

Müller, J.A., Galushko, A., Kappler, A., and Schink, B., 1999. Anaerobic degradation of *m*-cresol by *Desulfobacterium cetonicum* is initiated by formation of 3-hydroxybenzylsuccinate. Arch. Microbiol. 172, 287-294.

Müller, J.A., Galushko, A., Kappler, A., and Schink, B., 2001. Initiation of anaerobic degradation of *p*-cresol by formation of 4-hydroxybenzylsuccinate in *Desulfobacterium cetonicum*. J. Bacteriol. 183, 752-757.

Phelps, C.D., and Young, L.Y., 2001. Biodegradation of BTEX under anaerobic conditions: a review. Adv. Agronom. 70, 329-357.

Phelps, C.D., Battistelli, J., and Young, L.Y., 2002. Metabolic biomarkers for monitoring anaerobic naphthalene biodegradation in situ. Environ. Microbiol. 4, 532-537.

Postgate, J., 1959. A diagnostic reaction of *Desulphovibrio desulphuricans*. Nature 163, 481-482.

Rabus, R., and Widdel, F., 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163, 96-103.

Reinhard, M., Shang, S., Kitanidis, P.K., Orwin, E., Hopkins, G.D., and Lebron, C.A., 1997. *In situ* BTEX biotransformation under enhanced nitrate- and sulfate-reducing conditions. Environ. Sci. Technol. 31, 28-36.

Sambrook, J., and Russel, D.W., 2001. Molecular cloning. A laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schaeffer, A.B., and Fulton, M., 1933. A simplified method of staining endospores. Science 77, 194.

Schauder, R., Eikmanns, B., Thauer, R.K., Widdel, F., and Fuchs, G., 1986. Acetate oxidation to carbon dioxide in anaerobic bacteria via a novel pathway not involving reactions of the citric acid cycle. Arch. Microbiol. 145, 162-172.

Seyfried, B., Glod, G., Schocher, R., Tschech, A., and Zeyer, J., 1994. Initial reactions in the anaerobic oxidation of toluene and *m*-xylene by denitrifying bacteria. Appl. Environ. Microbiol. 60, 11, 4047-4052.

Spormann, A.M., and Thauer, R.K., 1988. Anaerobic acetate oxidation to CO_2 by *Desulfotomaculum acetoxidans*. Arch. Microbiol. 150, 374-380.

Strunk, O., and Ludwig, W., 1996. ARB: a software environment for sequence data. Munich: Technical University Munich, Germany.

Stubner, S., and Meuser, K., 2000. Detection of *Desulfotomaculum* in an Italian rice paddy soil by 16S ribosomal nucleic acid analyses. FEMS Microbiol. Ecol. 34, 73-80.

US-EPA, 1999. Use of monitored natural attenuation at superfund, RCRA corrective action, and underground storage tank sites. *In* OSWER directive 9200, pp. 4-17P.

Widdel, F., 1992. The Genus *Desulfotomaculum*. *In* The procaryotes. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). Berlin: Springer Verlag, pp. 1792-1799.

Widdel, F., and Bak, F., 1992. Gram-negative mesophilic sulfate-reducing bacteria. *In* The prokaryotes. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York: Springer Verlag, pp. 3352-3378.

Widdel, F., and Rabus, R., 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr. Opin. Biotechnol. 12, 259-276.

Wilkes, H., Rabus, R., Fischer, T., Armstroff, A., Behrends, A., and Widdel, F., 2002. Anaerobic degradation of *n*-hexane in a denitrifying bacterium: further degradation of the initial intermediate (1-methylpentyl)succinate via C-skeleton rearrangement. Arch. Microbiol. 177, 235-243.

Wind, T., Stubner, S., and Conrad, R., 1999. Sulfate-reducing bacteria in rice field soil and on rice roots. System. Appl. Microbiol. 22, 269-279.

Zhou, J., Fries, M.R., Chee-Sanford, J.C., and Tiedje, J.M., 1995. Phylogenetic analysis of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus tolulyicus* sp. nov. Int. J. Syst. Bacteriol. 45, 500-506.

4 Stable hydrogen and carbon isotope fractionation during microbial toluene degradation: mechanistic and environmental aspects

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Abstract

Primary features of hydrogen and carbon isotope fractionation during toluene degradation were studied to evaluate if the analysis of isotope signatures can be used as a tool to monitor biodegradation in contaminated aquifers. D/H hydrogen isotope fractionation during microbial degradation of toluene was measured by gas chromatography. Per-deuterated toluene-d_8 and nonlabeled toluene were supplied in equal amounts as growth substrates, and kinetic isotope fractionation was calculated from the shift of the molar ratios of toluene-d_8 and nondeuterated toluene. The D/H isotope fractionation varied slightly for sulfate-reducing strain TRM1 (slope of curve [*b*] = -1.219), *Desulfobacterium cetonicum* (*b* = -1.196), *Thauera aromatica* (*b* = -0.816), and *Geobacter metallireducens* (*b* = -1.004) and was greater for the aerobic bacterium *Pseudomonas putida* strain mt-2 (*b* = -2.667). The D/H isotope fractionation was 3 orders of magnitude greater than the $^{13}C/^{12}C$ carbon isotope fractionation reported previously. Hydrogen isotope fractionation with nonlabeled toluene was 1.7 and 6 times less than isotope fractionation with per-deuterated toluene-d_8 and nonlabeled toluene for sulfate-reducing strain TRM1 (*b* = -0.728) and *D. cetonicum* (*b* = -0.198), respectively. Carbon and hydrogen isotope fractionation during toluene degradation by *D. cetonicum* remained constant over a growth temperature range of 15 to 37°C but varied slightly during degradation by *P. putida* strain mt-2, which showed maximum hydrogen isotope fractionation at 20°C (*b* = -4.086) and minimum fractionation at 35°C (*b* = -2.138). D/H isotope fractionation was observed only if the deuterium label was located at the methyl group of the toluene molecule which is the site of the initial enzymatic attack on the substrate by the bacterial strains investigated in this study. Use of ring-labeled toluene-d_5 in combination with nondeuterated toluene did not lead to a significant D/H isotope fractionation. The activity of the first enzyme in the anaerobic toluene degradation pathway, benzylsuccinate synthase, was measured in cell-free extracts of *D. cetonicum* with an initial activity of 3.63 mU (mg of protein)$^{-1}$. The D/H isotope fractionation (*b* = -1.580) was 30% greater than in growth

experiments with *D. cetonicum*. Mass spectroscopic analysis of the product benzylsuccinate showed that H atoms abstracted from the toluene molecule by the enzyme were retained in the same molecules after the product was released.

Our findings revealed that the use of deuterium-labeled toluene was appropriate for studying basic features of D/H isotope fractionation. Similar D/H fractionation factors for toluene degradation by anaerobic bacteria, the lack of significant temperature dependence, and the strong fractionation suggest that analysis of D/H fractionation can be used as a sensitive tool to assess degradation activities. Identification of the first enzyme reaction in the pathway as the major fractionating step provides a basis for linking observed isotope fractionation to biochemical reactions.

Introduction

Biological isotope fractionation leads to unequal distribution of heavier and lighter isotopes between the educts and products of a reaction. In most cases, the lighter isotopes are preferentially used and the heavier isotopes are enriched in the residual substrate fraction. This is a well-known phenomenon for CO_2 fixation during photosynthesis (O'Leary, 1980) and methanogenesis (Krzycki et al., 1987; Fogel and Cifuentes, 1993) and for methane oxidation (Coleman et al., 1981; Whiticar and Faber, 1986; Blair and Aller, 1995). In recent years, isotope fractionation has been described for bacterial degradation of environmental contaminants, such as chlorinated hydrocarbons (Ertl et al., 1996; Hunkeler et al., 1999; Bloom et al., 2000) and aromatic compounds (Lebedew et al., 1969; Stahl, 1980; Meckenstock et al., 1999b; 1999c; Ahad et al., 2000; Wilkes et al., 2000). In addition, $^{13}C/^{12}C$ isotope fractionation of chlorinated and aromatic hydrocarbons was demonstrated in contaminated aquifers (Dempster et al., 1997; Hunkeler et al., 1999; Richnow and Meckenstock, 1999), and this finding was taken as an indication of microbial degradation. Assessment of isotope fractionation was therefore discussed as a method to monitor biological degradation directly in the aquifer. However, in natural environments factors like redox conditions and temperature can vary and may influence isotope fractionation. To assess the contributions of bacterial degradation activities *in situ* to natural attenuation, further understanding of possible influences of physical and chemical parameters on isotope fractionation has to be attained.

In addition to carbon isotope fractionation of organic contaminants, some studies reported on chlorine (Sturchio et al., 1998) or deuterium/hydrogen isotope fractionation (Coleman et al., 1981; Ward et al., 2000). Kinetic D/H isotope fractionation has been shown to be 2 orders of magnitude greater than carbon isotope fractionation (Coleman et al., 1981). However, the single-

compound isotope analysis by gas chromatography-combustion-isotope ratio monitoring mass spectrometry (GC-C-IRMS), which is the standard method for carbon isotope analysis (Hayes et al., 1990; Merritt et al., 1994), became available for hydrogen fractionation only recently (Hilkert et al., 1999), and hydrogen isotope analysis is still more expensive and less precise than carbon isotope analysis.

Here, we describe a method for investigating D/H isotope fractionation during bacterial toluene degradation by gas chromatography (GC). The depletion of lighter toluene species and the enrichment of labeled toluene in the residual fraction were determined to assess hydrogen isotope fractionation. The effects of different electron acceptors or temperatures on isotope fractionation were checked, and the major fractionating step in anaerobic bacterial toluene degradation was identified. Furthermore, the proposed reaction mechanism of benzylsuccinate synthase was confirmed by D/H isotope analysis with GC and mass spectroscopy.

Methods

Strains and Cultivation

Sulfate-reducing strain TRM1 was isolated in our laboratory (Meckenstock, 1999a), and *Desulfobacterium cetonicum* DSM 7267, *Thauera aromatica* strain K172 (=DSM 6984), and *Geobacter metallireducens* strain GS-15 (=DSM 7210) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *Pseudomonas putida* strain mt-2 was a kind gift from J. R. van der Meer, Dübendorf, Switzerland.

Anaerobic strains were cultivated in carbonate-buffered freshwater mineral medium, pH 7.2 to 7.4 (Widdel and Bak, 1992). This medium was prepared in the absence of oxygen under a N_2-CO_2 atmosphere (80:20) and was reduced with Na_2S (1 mM). Either Na_2SO_4 (10 mM) or $NaNO_3$ (10 mM) was added as an electron acceptor. $FeCl_2$ (3 mM) was added to the medium for strain TRM1 in order to scavenge the sulfide produced. The same freshwater medium was used to cultivate *Geobacter metallireducens*, but $FeCl_2$ (1 mM) was added as a reducing agent instead of sulfide, and Fe(III) citrate (50 mM) was used as an electron acceptor. Aerobic bacteria were cultivated in mineral medium M9 (Sambrook et al., 1989). Serum bottles (120 and 500 ml) were half filled with mineral medium and tightly sealed with Viton rubber stoppers (Maag Technic, Dübendorf, Switzerland). Toluene was injected into the culture bottles with syringes through the stoppers and was allowed to equilibrate overnight before inoculation. All substrates, including nonlabeled toluene, per-deuterated toluene-d_8 (Fluka, Buchs, Switzerland), ring-deuterated toluene-d_5, and methyl-deuterated toluene-d_3 (Campro Scientific, Berlin, Germany), were

analytical grade. Growth experiments were performed in triplicate at 30°C unless indicated otherwise. Aerobic cultures were shaken at 100 strokes min^{-1}.

Analysis and Sampling

Growth of sulfate-reducing bacteria and *G. metallireducens* was monitored by measuring the formation of sulfide or Fe(II) (Cline, 1969; Stookey, 1970). Growth of all other strains was determined by measuring the increase in optical density at 578 nm. Toluene concentrations were analyzed by high-performance liquid chromatography (HPLC; System Gold; Beckman, Fullerton, Calif.) with a C_8 reversed-phase column (GROM-SIL; ODS-5 ST; length, 250 mm; particle size, 5 μm; Grom, Herrenberg, Germany) and UV detection (206 nm) using acetonitrile-100 mM ammonium phosphate buffer (pH 3.5) (70:30, vol/vol) as the eluent. Culture samples (250 μl) were diluted 1:5 with ethanol (99.9%, p. a.) and centrifuged (14,000 × g, 5 min) to remove precipitates.

Isotope analysis

D/H isotope fractionation in experiments with mixtures of deuterium-labeled toluene and nonlabeled toluene could be assessed by separation of the different toluene species in a GC capillary column. GC-C-IRMS is sensitive enough to detect changes in the deuterium ratio in the per mille range and was used to analyze D/H isotope fractionation in experiments with nonlabeled toluene. $^{13}C/^{12}C$ isotope fractionation was also measured by GC-C-IRMS.

Liquid samples (2 to 7 ml) for GC analysis were taken with a syringe through the stoppers and were extracted with 0.3 ml of pentane (Suprasolve; Merck, Darmstadt, Germany). Aliquots (4 μl) of the pentane phase were analyzed in three replicates with a GC equipped with a flame ionization detector (Carlo Erba Instruments, Milan, Italy). The GC was equipped with a fused silica capillary column (optima δ-3; length, 60 m; inside diameter, 0.32 mm; film thickness, 0.35 μm; Macherey-Nagel, Düren, Germany). Baseline separation of the different toluene species was achieved at 60°C and 80 kPa with a N_2 flow rate of 1.5 ml min^{-1}. Samples were injected into the heated injector (200°C) with a split of 1:15.

$^{13}C/^{12}C$ isotope ratios were determined by GC-C-IRMS. The system consisted of a GC (HP-5890; Hewlett-Packard Co, San Diego, Calif.) which was connected via a combustion unit (GC-combustion interface; Finnigan, Bremen, Germany) to an isotope mass spectrometer (Finnigan MAT 252; Finnigan). The GC was equipped with a fused silica capillary column (BPX-5; length, 50 m; inside diameter, 0.32 mm; film thickness, 0.5 μm; SGE, Darmstadt, Germany). The temperature program consisted of 2 min at 40°C, followed by a linear increase to 180°C at a rate of 6°C min^{-1}. Samples were injected splitless at 250°C.

D/H isotope ratios were determined with an Isochrome GC-C-IRMS system (Micromass, Manchester, United Kingdom) consisting of a GC unit which was connected via a chromium furnace to a mass spectrometer (Isoprime,

Manchester, United Kingdom). The temperature of the furnace was adjusted to 1,800°C. Samples were injected splitless at 20°C in a KAS4 cooled on column injector (Gerstel, Germany), and subsequently the injector was heated to 300°C at a rate of 12°C s^{-1}; the temperature was kept at 300°C for 5 min. The GC conditions were the same as those described above for the determination of carbon isotope ratios.

Calculations

Calculations were based on the Rayleigh equation for closed systems, which was developed to describe distillation processes (Rayleigh, 1896) and was adapted for isotope fractionation (equation 1) (Hoefs, 1997). D/H isotope fractionation with labeled toluene was determined by calculating the GC peak areas of pentane-extracted toluene species. The hydrogen isotope ratio (R_t) was the quotient of the concentrations ([toluene-d_8]/[toluene]) at time t. If the logarithms of the isotope ratios (R_t/R_0) are plotted over the respective logarithms of the concentrations (c_t/c_0), according to equation 2 (Hoefs, 1997; Meckenstock et al., 1999c; Richnow and Meckenstock, 1999), the kinetic isotope fractionation factor (α) could be determined from the slopes of the curves (b) with $b = 1/\alpha - 1$ (equation 3). c_t and c_0 were the total toluene concentration at time t and time zero, respectively. When only nondeuterated toluene was supplied in isotope fractionation experiments, isotope ratios (R_t) were determined from the common d values according to equations 4 and 5 (Meckenstock et al., 1999b). The international PDB and SMOW standards were used to calculate d values from GC-C-IRMS analysis. d_0 was the initial isotope signature of the substrate.

$$R_t/R_0 = (c_t/c_0)^{(1/a-1)} \quad (1)$$

$$\ln(R_t/R_0) = (1/a - 1) \times \ln(c_t/c_0) \quad (2)$$

$$b = 1/a - 1 \quad (3)$$

$$d_t = \left(\frac{R_{sample} - R_{std}}{R_{std}} \right) \times 1{,}000 \quad (4)$$

$$(d_t + 1{,}000)/(d_0 + 1{,}000) = R_t/R_0 \quad (5)$$

The fractionation factor (α) is a nonlinear function of b given in $\alpha = 1/(b+1)$ and not defined in $b = -1$. For carbon isotope fractionation during toluene degradation, b usually was between -0.001 and b = -0.01, a range where α is almost linearly dependent on b. The slopes of D/H isotope fractionation were within a range of -5 to $b = 0$. For b approaching -1, α diverged against $\pm \infty$ and became an abstract term for quantitative descriptions. It is therefore more illustrative to describe D/H fractionation by the slope, which is used

throughout this paper. The slope decreases with increasing isotope fractionation. The results of $^{13}C/^{12}C$ isotope fractionation experiments are given in the common α notation.

Benzylsuccinate synthase assay

A culture of *D. cetonicum* (0.8 liter; optical density at 578 nm, 0.3) was harvested in the late exponential growth phase. The cells were washed in anoxic potassium phosphate buffer (50 mM, pH 7.5) which had been supplemented with NaCl (175 mM) and $MgCl_2$ (25 mM), reduced with H_2 (10^5 Pa)-palladium-charcoal (1 mg $liter^{-1}$) (Merck, Darmstadt, Germany) and titanium(III) citrate (1 mM), and filtered. Cells were suspended in 3 ml of buffer and broken in a French pressure cell (9 MPa) in the presence of 2 mM fumarate. Cell debris was removed by centrifugation (14,000 × g, 5 min). The cell extract was diluted to a volume of 6 ml with buffer. One milliliter of a solution containing 0.5 mM toluene-d_8 and 0.5 mM toluene (in reduced potassium phosphate buffer) was added to the diluted extract, and the preparation was mixed.

The benzylsuccinate synthase test was performed at 30°C in 8-ml glass vials sealed with Viton rubber stoppers. At each sampling time, 500 µl of buffer was injected into the reaction vial and mixed. Subsequently, a 500-µl sample was removed with a syringe through the stopper, and aliquots (200 µl) of the sample were transferred into two 1.7-ml glass vials. The reaction was stopped with ice-cold 1M NaOH (20 µl) and the mixture was overlaid with 100 µl pentane (Suprasolve) containing 2.5 mM ethylbenzene (Fluka, Buchs, Switzerland) as an internal standard. A control assay was run without cell extract. Toluene-d_8/toluene ratios and overall toluene concentrations were determined by GC as described above. The aqueous phase remaining from the extraction was diluted fivefold with ethanol, and benzylsuccinate was analyzed by high-performance liquid chromatography as described above with acetonitrile-ammonium phosphate buffer (30:70) as the eluent. Protein concentrations were determined with a protein assay kit (Bio-Rad, Munich, Germany). The identity of benzylsuccinate in the extracts was confirmed by coelution with a benzylsuccinate reference (Sigma, Deisenhofen, Germany).

Mass spectroscopic analysis

Benzylsuccinate was converted to its methyl ester with trimethylchlorosilane (Supelco, Bellefonte, Pa.) in methanol (8:1, vol/vol) at 60°C for 1 h. The reaction mixture was extracted with dichloromethane for a mass spectrometric analysis performed with a quadrupole system (MSD; Hewlett-Packard Co). The GC was equipped with a fused silica capillary column (DB-1; length, 30 m; inside diameter, 0.32 mm; film thickness, 0.25 µm; J&W scientific, Forsholm, Calif.). The injection mode was splitless. The temperature program was as follows: 2 min at 60°C, followed by an increase to 250°C at a rate of 4°C min^{-1}.

Results

Separation of isotopic toluene species

A method was developed to determine D/H isotope fractionation upon toluene degradation by GC analysis. Batch cultures were grown with mixtures of deuterated toluene-d_8 and nonlabeled toluene (50:50, vol/vol). The nondegraded residual toluene fraction in the cultures was extracted with pentane and analyzed by GC. The different toluene species were separated by GC; the elution time for toluene-d_8 was 13.8 min, and this compound was followed by methyl-labeled toluene-d_3 (13.95 min), ring-labeled toluene-d_5 (14.0 min), and nonlabeled toluene (14.2 min). During growth with a mixture of per-deuterated toluene-d_8 and nonlabeled toluene, the different bacterial strains degraded nonlabeled toluene faster, and consequently the hydrogen isotope ratios R_t of [toluene-d_8] to [toluene] increased substantially, as illustrated by a degradation experiment with *D. cetonicum* (Fig. 1).

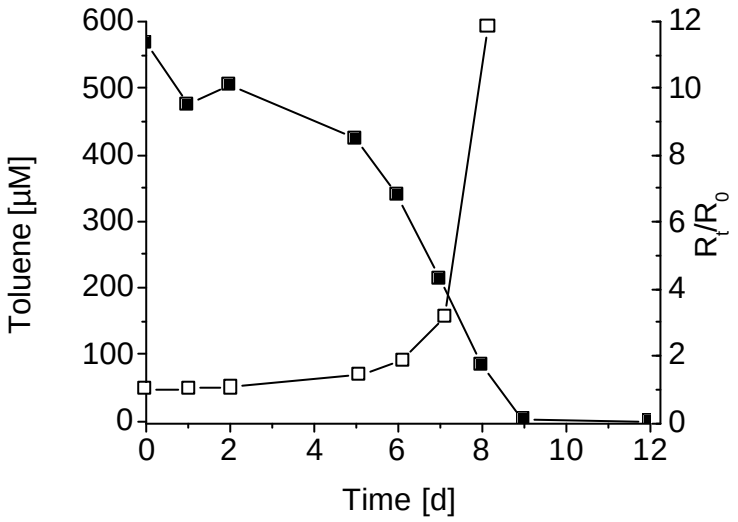

FIG. 1. Substrate conversion during growth of *D. cetonicum* with a mixture of 50% toluene-d_8 and 50% nonlabeled toluene as the sole carbon and energy source. Toluene concentrations (■) and related toluene-d_8/toluene ratios in the residual nondegraded substrate fraction (□) were monitored over time. R_t is the isotope ratio calculated as follows: [toluene-d_8]/[toluene].

D/H isotope fractionation factors for various bacterial strains cultivated with toluene

The D/H isotope fractionation factors obtained in growth experiments with different bacterial strains were all of the same order of magnitude. The greatest fractionation of toluene degradation was observed in growth experiments with the aerobic bacterium *P. putida* strain mt-2 with a fractionation of $b = -2.667 \pm 0.163$. The D/H isotope fractionation by anaerobic bacteria was slightly less

with b = -1.219 ± 0.254 for sulfate-reducing strain TRM1, b = -1.196 ± 0.075 for *D. cetonicum*, b = -1.004 ± 0.077 for *G. metallireducens*, and b = -0.816 ± 0.133 for *T. aromatica* (Fig. 2). All of the anaerobic strains showed similar degrees of isotope fractionation, although they used different electron acceptors.

D/H isotope fractionation of toluene with the natural deuterium abundance

D. cetonicum and strain TRM1 were grown with nonlabeled toluene, and the D/H isotope compositions were determined by GC-C-IRMS. D/H isotope fractionation of b = -0.728 was obtained for strain TRM1 with nonlabeled toluene; this fractionation was 1.7 times less than the fractionation observed when toluene-d_8 and nonlabeled toluene were supplied. In experiments with *D. cetonicum* grown with nonlabeled toluene, the D/H isotope fractionation was b = -0.198, or six times less than the value obtained in growth experiments with per-deuterated toluene-d_8 and nonlabeled toluene (50:50) (b = -1.196) (Fig. 2), indicating that isotope fractionation with deuterated toluene was not identical to fractionation with unlabeled toluene but was of the same order of magnitude.

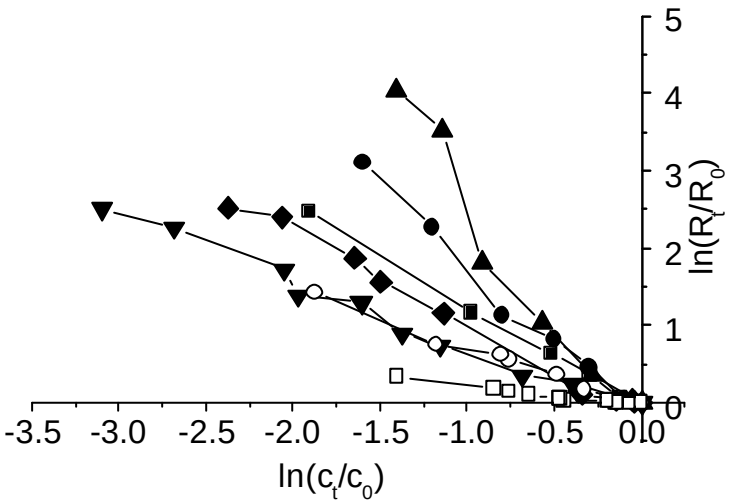

FIG. 2. D/H isotope fractionation during degradation of a mixture of toluene-d_8 and nonlabeled toluene in batch cultures. Representative curves from three replicates are shown for *P. putida* strain mt-2 (▲), strain TRM1 (●), *D. cetonicum* (■), *G. metallireducens* (◆), and *T. aromatica* (▼). For *D. cetonicum* (□) and strain TRM1 (○) D/H isotope fractionation was determined in batch cultures grown with nonlabeled toluene, and the natural abundance of deuterium was measured by GC-C-IRMS.

Temperature dependence of isotope fractionation

To assess effects of temperature on isotope fractionation, bacterial D/H isotope fractionation upon toluene degradation was investigated in growth experiments performed with the aerobic bacterium *P. putida* strain mt-2 and

per-deuterated toluene-d_8 and nonlabeled toluene in equal amounts at five different temperatures between 15°C and 35°C. The highest isotope fractionation value was obtained at 20°C, with b = -4.086 ± 0.127; the value decreased to b = -2.138 ± 0.667 at 35°C (Fig. 3A). With the anaerobic bacterium *D. cetonicum,* the D/H isotope fractionation differed between b = -1.092 ± 0.239 at 18°C and b = -1.260 ± 0.009 at 37°C, a difference which was not significant within the range of the standard deviation (Fig. 3A).

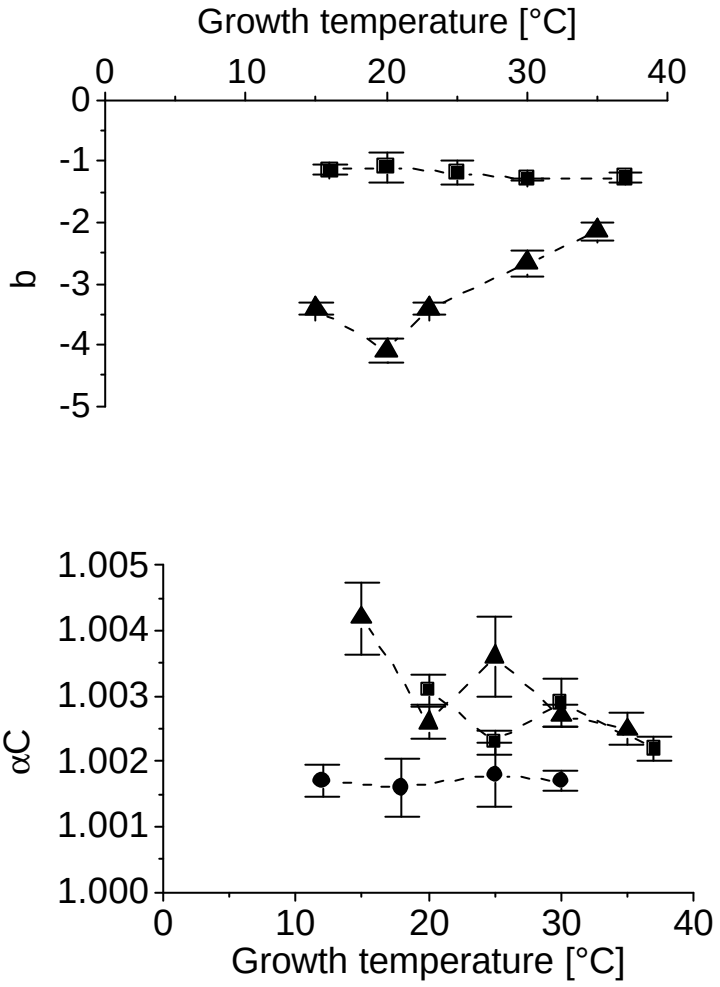

FIG. 3. Influence of temperature on D/H isotope fractionation (A) and $^{13}C/^{12}C$ isotope fractionation (B) during toluene degradation by *D. cetonicum* (■), sulfate-reducing strain TRM1 (●), and *P. putida* strain mt-2 (▲). (A) D/H isotope fractionation factors were determined by the slope (b = 1/α -1) of the double logarithmic plot of equation 2. For D/H isotope fractionation, each data point represents the average fractionation factor calculated from three independent growth experiments; the error bars indicate the standard deviations. (B) Carbon isotope fractionation factors were calculated from the regression curves in a double logarithmic plot of equation 2. Each data point represents a $^{13}C/^{12}C$ isotope fractionation factor (αC). The error bars indicate the reliability of αC calculated from the slopes (b = 1/α -1) of the regression curves.

Temperature effects on carbon isotope fractionation were analyzed in growth experiments with *P. putida* strain mt-2, *D. cetonicum*, and strain TRM1 cultivated with nonlabeled toluene. Similar to D/H isotope fractionation, there was a slight decrease in the $^{13}C/^{12}C$ isotope fractionation factors with decreasing temperatures for *P. putida* strain mt-2 from $\alpha C = 1.0042 \pm 0.0006$ at 15°C to $\alpha C = 1.0025 \pm 0.0003$ at 35°C. The $^{13}C/^{12}C$ isotope fractionation factors for the anaerobic organisms *D. cetonicum* and TRM1, however, did not vary with temperature within the observed standard deviations (Fig. 3B).

Identification of the fractionating step

We examined whether the observed strong D/H isotope fractionation during bacterial toluene degradation could be attributed to the first enzyme reaction of the toluene degradation pathway or to other parameters, like substrate diffusion to the cells or substrate uptake. Therefore, different toluene species with deuterium labels either at the aromatic ring (toluene-d_5) or at the methyl group (toluene-d_3) were used as growth substrates in equal amounts with per-deuterated toluene-d_8 or nondeuterated toluene. The three bacterial strains used initiate degradation with an attack on the methyl group; this has been proven for *P. putida* strain mt-2 (Shaw and Harayama, 1992) and *D. cetonicum* (Müller, et al., 1999) and is assumed for strain TRM1. *D. cetonicum* (Fig. 4), sulfate-reducing strain TRM1, and *P. putida* strain mt-2 showed strong D/H isotope effects (b = - 1.251, b = - 1.280, and b = - 4.218, respectively) (Table 1) if methyl-deuterated toluene-d_3 was used in combination with nonlabeled

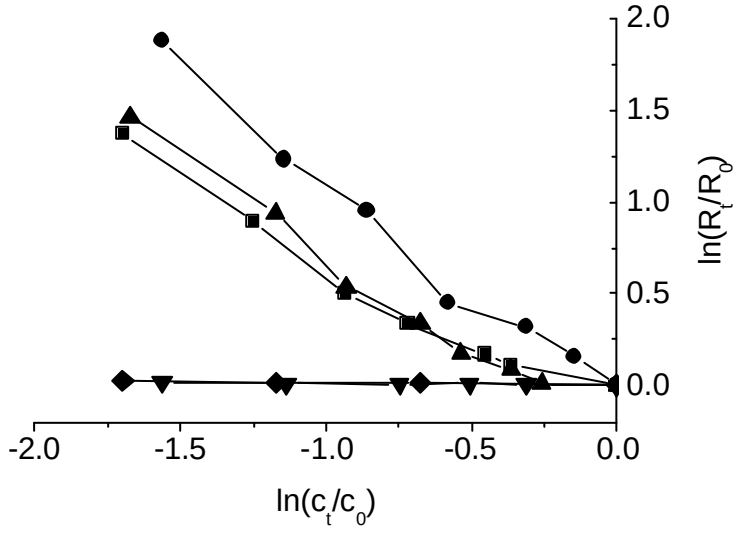

FIG. 4. D/H isotope fractionation during degradation of various combinations of selectively deuterated toluene species. Representative curves from three replicates are shown for *D. cetonicum* grown with equal amounts of toluene-d_8 and toluene-d_5 (■), with toluene-d_3 and nonlabeled toluene (●), with toluene-d_8 and nonlabeled toluene (▲), with toluene-d_5 and nonlabeled toluene (◆), and with toluene-d_8 and toluene-d_3 (▼). The curves were plotted by using equation 2.

toluene. When ring-deuterated toluene-d_5 was used in combination with nonlabeled toluene, the D/H isotope fractionation factors for *D. cetonicum*, strain TRM1, and *P. putida* mt-2 were negligible within the analytical errors. In addition, no D/H isotope fractionation within the standard deviations could be demonstrated for the three strains if methyl-deuterated toluene-d_3 was provided in equal amounts with per-deuterated toluene-d_8. When ring-deuterated toluene-d_5 was used in combination with per-deuterated toluene-d_8, the D/H isotope fractionation factors were b = -0.679 for *D. cetonicum*, b = -0.917 for strain TRM1, and b = -2.696 for *P. putida* strain mt-2, and thus slightly lower than the values obtained in experiments with toluene-d_3 and nonlabeled toluene.

TABLE 1. D/H isotope fractionation during growth of *D. cetonicum*, sulfate-reducing strain TRM1, and *P. putida* strain mt-2 with mixtures (50:50) of two selectively deuterated toluene species.

	D. cetonicum		strain TRM1		*P. putida* strain mt-2	
Substrate mixture	b	αD	b	αD	b	αD
Toluene-d_8 and toluene-d_3	-0.002 ± 0.003[a]	1.002 ± 0.003	0.167 ± 0.219	0.857 ± 0.148	-0.016 ± 0.003	1.016 ± 0.007
Toluene-d_3 and nonlabeled toluene	-1.251 ± 0.034	-3.991 ± 0.331	-1.280 ± 0.080	-3.572 ± 0.922	-4.218 ± 0.125	-0.311 ± 0.015
Toluene-d_8 and toluene-d_5	-0.679 ± 0.115	3,117 ± 2,947	-0.917 ± 0.336	12.005 ± 3.379	-2.696 ± 0.163	-0.590 ± 0.057
Toluene-d_5 and nonlabeled toluene	-0.005 ± 0.004	1.005 ± 0.004	-0.012 ± 0.005	1.042 ± 0.042	-0.079 ± 0.041	1.088 ± 0.048
Toluene-d_8 and nonlabeled	-1,196 ± 0.075	-5.090 ± 1.990	-1.219 ± 0.254	-4.566 ± 4.145	-2.667 ± 0.163	-0.600 ± 0.652

[a]Mean ± standard deviation based on three independent growth experiments.

Benzylsuccinate synthase assay with *D. cetonicum*

The activity and D/H isotope fractionation of benzylsuccinate synthase were determined in discontinuous enzyme assays at 30°C. About 80 µM toluene was consumed, and 70 µM benzylsuccinate was produced (Fig. 5). At the beginning of the experiment the enzyme activity in the cell extracts was 3.63 mU (mg of

protein)$^{-1}$, but the activity decreased to zero within 180 min. The maximum toluene turnover rate determined was 12% of the *in vivo* degradation activity (29.3 nmol min^{-1} mg of protein^{-1}). The D/H isotope fractionation obtained in the benzylsuccinate synthase assay was b = -1.580 with 50% toluene-d_8 and 50% nonlabeled toluene as the substrates, or 30% greater than the fractionation by growing *D. cetonicum* cells (Fig. 6). Mass spectroscopic analysis revealed that benzylsuccinate-d_8 and nondeuterated benzylsuccinate were produced. The maximum rate of nondeuterated benzylsuccinate production was 4.4 nmol min^{-1} mg of protein^{-1} and was 11 times higher than the rate of production of benzylsuccinate-d_8 (0.4 nmol min^{-1} mg of protein^{-1}). The mass peaks m/z 243 (benzylsuccinate-d_7) and m/z 237 (benzylsuccinate-d_1) could not be detected.

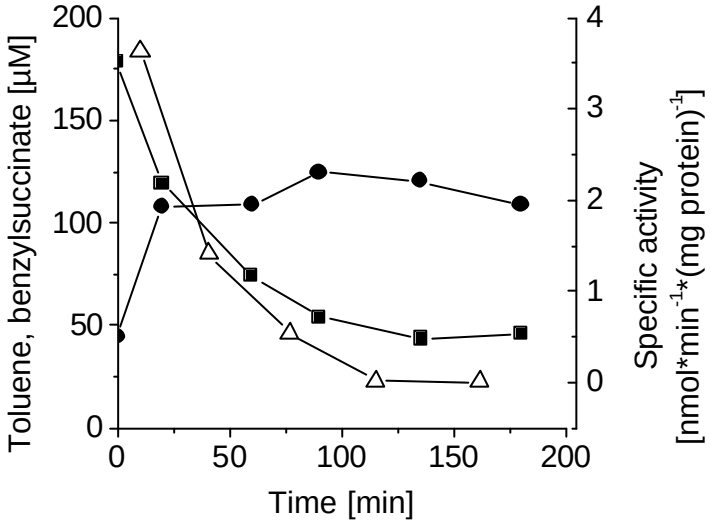

FIG. 5. Discontinuous benzylsuccinate synthase activity assay with *D. cetonicum* cell extract. The total concentrations of toluene-d_8 and nonlabeled toluene (■) and of benzylsuccinate (●) shown are means based on two samples per time point. The specific benzylsuccinate synthase activity (△) was calculated by determining the toluene degradation rate between two adjacent sampling points.

Discussion

Mechanistic aspects of hydrogen and carbon isotope fractionation were studied to assess whether isotope fractionation could serve as a tool to estimate biological degradation in contaminated aquifers. Therefore, isotope fractionation was investigated with several bacterial strains using different electron acceptors during toluene degradation. The influence of growth temperature was determined, and the fractionating step in anaerobic toluene degradation was identified.

Hydrogen isotope fractionation was studied by a method in which the strains were grown on mixtures of different deuterated toluene species under defined laboratory conditions. Thus, the basic questions of bacterial isotope fractionation could be determined by GC analysis without using the more expensive and time-consuming technique of single-compound isotope analysis by GC-C-IRMS.

Many biochemical reactions are known to cause significant partitioning of isotopes between substrates and products. Isotope effects not only are due to sheer mass differences but also are due to the impact of additional neutrons on the mechanism and velocity of a biochemical reaction. Direct involvement of a bond which is substituted with a heavy isotope is known as a primary isotope effect. The dissociation energy needed to cleave a heavy isotope-substituted bond is higher and is reflected in a decrease in the reaction rate (O'Neil, 1986). Weaker, secondary isotope effects occur when the heavy-atom isotope is located close to the bond but not directly involved in the reaction mechanism.

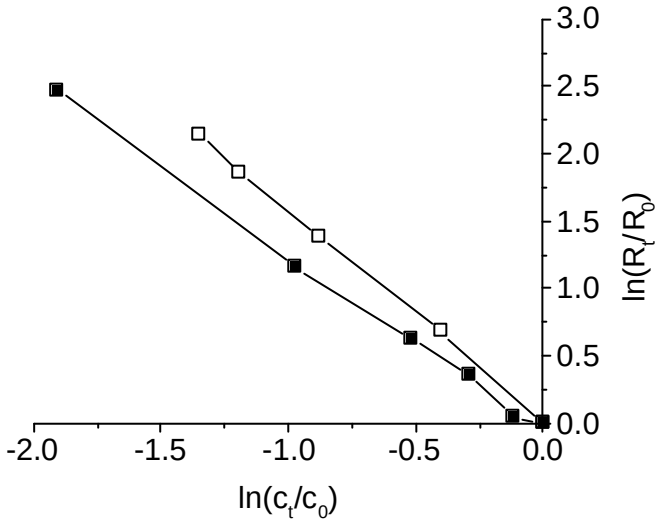

FIG. 6. Toluene D/H isotope fractionation in the cell-free benzylsuccinate synthase reaction (□) and in a growth experiment (■) with *D. cetonicum*. Equal amounts of toluene-d_8 and nonlabeled toluene were used in both experiments.

D/H isotope fractionation factors for various bacterial strains cultivated with toluene

In contrast to carbon isotope fractionation, which was almost the same for all of the anaerobic strains tested (Meckenstock et al., 1999c), the 100- to 1000-times greater hydrogen isotope fractionation factors varied among the different species within the same order of magnitude. As the mass of deuterium is 100% greater than the mass of an hydrogen atom, deuterium isotope effects are more pronounced. Small differences in the toluene-degrading enzymes are

multiplied by these effects and are reflected in the variations in the D/H isotope fractionation factors for the bacterial strains studied. These variations in D/H isotope fractionation are unfavorable for quantitative evaluation of biological degradation at natural sites by analysis of hydrogen isotope ratios. Nevertheless, as the hydrogen isotope fractionation was at least 100-fold greater than the corresponding carbon isotope fractionation, D/H isotope ratios might be promising indicators for detection of low bacterial activities in natural environments.

D/H isotope fractionation of toluene of natural deuterium abundance

D/H isotope fractionation by the sulfate-reducing organisms strain TRM1 and *D. cetonicum* grown with toluene-d_8 and nonlabeled toluene was compared to fractionation when nonlabeled toluene was used as sole carbon source, in order to determine the reliability of using labeled toluene species in D/H isotope fractionation studies. The isotope fractionation values determined for growth with nonlabeled toluene were 1.7 and 6 times lower than the values determined for growth with toluene-d_8 and nonlabeled toluene for strain TRM1 and *D. cetonicum*, respectively. The reason for this might be a different reactivity of a methyl group labeled with three deuterium atoms compared to the reactivity of nonlabeled toluene molecules which have only one deuterium atom at the methyl group. In the case of per-deuterated toluene-d_8 the primary isotope effect resulting from the cleavage of a C—D bond is enhanced by secondary isotope effects caused by two additional deuterium atoms in the methyl group. For statistical reasons the natural occurrence of toluene with two or three deuterium atoms at the methyl group is negligible.

Another factor contributing to the differences in the results of isotope fractionation between nonlabeled toluene and toluene-d_8-nonlabeled toluene is the two types of isotope analysis used. Determination of isotope fractionation by GC is based on separation of methyl-labeled toluene and non-methyl-labeled toluene. Therefore, every deuterium of the labeled molecule is directly or indirectly subject to fractionation. GC-C-IRMS analysis of toluene for natural deuterium abundance detects every deuterated toluene molecule without taking into account the localization of the deuterium atoms. Statistically 0.8% of all toluene molecules carry a deuterium atom. The probability that the deuterium label is located at the methyl group is $^3/_8$, which means that five-eights of the molecules detected are not subject to fractionation. Thus, the isotope fractionation factors determined with labeled compounds should be two to three times greater than the factors obtained with nonlabeled toluene because of the different methods used, which indeed was the case.

The relative concentrations of deuterium-labeled molecules did not influence isotope fractionation; this conclusion was reached because the natural

abundance of deuterium in nonlabeled toluene was 4 orders of magnitude lower than the abundance in experiments performed with deuterated toluene. The results indicate that the use of deuterium-labeled toluene to study D/H isotope fractionation is a valid technique, although the fractionation obtained is not identical to fractionation obtained with nonlabeled toluene.

Temperature dependence of isotope fractionation

In geosciences, temperature is a well-known parameter that affects the degree of isotope fractionation (O'Neil, 1986; Hoefs, 1997). We examined whether temperature alters the extent of isotope fractionation during toluene degradation. No significant dependence on temperature for hydrogen or carbon isotope fractionation was observed for the anaerobic bacteria *D. cetonicum* and strain TRM1. In contrast, hydrogen isotope fractionation by *P. putida* strain mt-2 increased with decreasing temperatures and showed the greatest fractionation at 20°C. No clear effect of temperature on $^{13}C/^{12}C$ carbon isotope fractionation by *P. putida* strain mt-2 could be found. The reason why the temperature effect on isotope fractionation was so small might be due to the temperature range of mesophilic bacterial activity (10°C to 40°C), a more limited range than that of geochemical processes. It has been shown that undefined cultures of methane-oxidizing bacteria exhibit greater $^{13}C/^{12}C$ isotope fractionation at 30°C ($\alpha C = 1.025$) than at 11.5°C ($\alpha C = 1.013$) (Coleman et al., 1981), although thermodynamics suggests that the fractionation decreases with increasing temperature. However, since in this study an undefined mixture of methanotrophic bacteria was used, the observed effect might have been due to different subpopulations with alternate enzyme systems enriched at the two different temperatures. Our results showed that variations in temperature should not significantly affect isotope fractionation in contaminated anoxic aquifers. There was also no correlation between toluene degradation rates and isotope fractionation because growth of our strains was closely linked with the growth temperature, e.g., the generation time of the sulfate-reducing strain TRM1 decreased from 25.5 days at 12°C to 5 days at 30°C (data not shown), but the $^{13}C/^{12}C$ isotope fractionation factors were identical ($\alpha C = 1.0017$).

Identification of the fractionating step

Isotope fractionation may be caused by transport of the substrate to the cell, by uptake into the cell, or by the first enzyme reaction or a subsequent enzyme reaction in the degradation pathway. Growth experiments with selectively deuterated toluene species as carbon sources were performed to identify which of the processes mentioned above is relevant for fractionation. *P. putida* strain mt-2, *D. cetonicum*, and strain TRM1 all initiate toluene degradation with an enzymatic attack on the methyl group (Worsey and Williams, 1975; Shaw and Harayama, 1992; Biegert et al., 1996; Beller and Spormann, 1997; Müller et al.,

1999). The greatest hydrogen isotope fractionation effects were observed when the bacteria grew with a mixture of methyl-labeled toluene-d_3 or per-deuterated toluene-d_8 and nonlabeled toluene. If the deuterium label was located at the aromatic ring or if toluene-d_3 was used in combination with per-deuterated toluene-d_8, isotope fractionation was not detectable. These findings indicate that higher molecular masses did not influence processes such as transport to the cells and substrate uptake and that the D/H isotope fractionation determined was not due to the differences in the overall molecular masses. Significant fractionation occurred only when the methyl group was labeled, which shows that the initial enzymatic attack at the methyl group was the major step which led to hydrogen isotope fractionation.

Benzylsuccinate synthase assay with *D. cetonicum*

Benzylsuccinate synthase in a cell extract of *D. cetonicum* exhibited a 30% greater D/H isotope fractionation with per-deuterated toluene-d_8 and nonlabeled toluene than benzylsuccinate synthase in growing cells exhibited, confirming that the benzylsuccinate synthase reaction is the major isotope-fractionating step. This is consistent with previous reports which showed that isotope fractionation decreases when the supply of the enzyme with educts is less than saturation (O'Neil, 1986; Peterson and Fry, 1987; Fogel and Cifuentes, 1993).

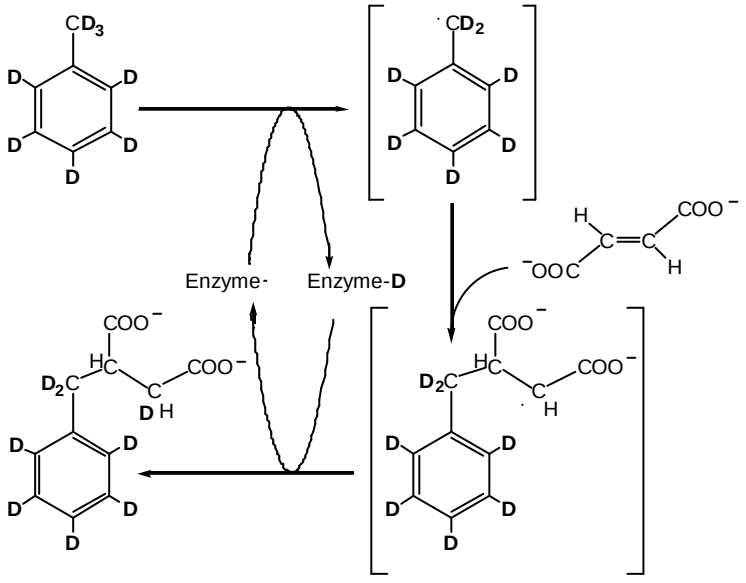

FIG. 7. Reaction mechanism of benzylsuccinate synthase as proposed by Heider et al. (Heider et al., 1999). The reaction is shown for per-deuterated toluene-d_8 as substrate. The deuterium radical subtracted from one molecule of toluene-d_8 by the glycyl radical enzyme is transferred from the enzyme to the same molecule to generate benzylsuccinate-d_8.

It has been proposed that benzylsuccinate synthase is a glycyl radical enzyme which abstracts one hydrogen radical from toluene in a primary step and returns the identical hydrogen to the benzylsuccinate molecule produced (Fig. 7) (Beller and Spormann, 1998; Leuthner et al., 1998; Heider et al., 1999). To study the benzylsuccinate synthase mechanism, the products of the enzyme assay performed with per-deuterated toluene-d_8 and nondeuterated toluene in equal amounts were analyzed by mass spectroscopy. Benzylsuccinate-d_8 and nonlabeled benzylsuccinate were detected, and nondeuterated benzylsuccinate was produced 11 times faster than benzylsuccinate-d_8. Not even traces of benzylsuccinate-d_7 or benzylsuccinate-d_1 masses were detectable, which had to be expected if a hydrogen was attached to the glycyl radical enzyme before substrate binding and was exchanged with a hydrogen atom from benzylsuccinate before product release. Absence of these masses derived from transfer of hydrogen radicals to other substrate molecules was predicted from the proposed reaction mechanism (Fig. 7) (Heider et al., 1999). A similar experiment was performed earlier with the toluene-degrading, denitrifying strain T and with toluene-d_3 and nondeuterated toluene as substrates (Beller and Spormann, 1998). However, the hydrogen isotope fractionation effects described above were not considered. Unless the differently deuterated toluene species are turned over completely, the distribution of benzylsuccinate-d_3 and benzylsuccinate should be about 90:10 because of the differing turnover rates. Consequently, the masses of benzylsuccinate-d_1 and benzylsuccinate-d_2 that are produced by a possible alternative enzyme mechanism must be considered extremely low. The distributions of benzylsuccinate species are evident only if the different reaction rates of benzylsuccinate synthase with deuterated or nondeuterated substrates are known and taken into account.

In this study, we showed that the first enzymatic reaction in anaerobic toluene degradation is the major process leading to hydrogen isotope fractionation. No significant effects of temperature or changes in the composition of the bacterial community are expected as long as anoxic conditions prevail in the aquifer. Isotope fractionation during aerobic degradation of toluene could be influenced by growth temperatures. The D/H isotope fractionation during toluene degradation was 3 orders of magnitude greater than the $^{13}C/^{12}C$ isotope fractionation for the same bacterial strains described previously, and analysis of hydrogen isotope fractionation in natural environments might be considered an appropriate tool for detecting low bacterial benzene-toluene-ethylbenzene-xylene degradation activities at contaminated sites.

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References

Ahad, J.M.E., Sherwood Lollar, B., Edwards, E.A., Slater, G.F., and Sleep, B.E., 2000. Carbon isotope fractionation during anaerobic biodegradation of toluene: Implications for intrinsic bioremediation. Environ. Sci. Technol. 34, 892-896.

Beller, H.R., and Spormann, A.M., 1997. Benzylsuccinate formation as a means of anaerobic toluene activation by the sulfate-reducing strain PRTOL1. Appl. Environ. Microbiol. 63, 3729-3731.

Beller, H.R., and Spormann, A.M., 1998. Analysis of the novel benzylsuccinate synthase reaction for anaerobic toluene activation based on structural studies of the product. J. Bacteriol. 180, 5454-5457.

Biegert, T., Fuchs, G., and Heider, J., 1996. Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur. J. Biochem. 238, 661-668.

Blair, N.E., and Aller, R.C., 1995. Anaerobic methane oxidation on the Amazon shelf. Geochim. Cosmochim. Acta. 59, 3707-3715.

Bloom, Y., Aravena, R., Hunkeler, D., Edwards, E.A., and Frape, S.K., 2000. Carbon isotope fractionation during microbial degradation of trichloroethene, *cis*-1,2-cichloroethene, and vinyl chloride: Implications for assessment of natural attenuation. Environ. Sci. Technol. 34, 2768-2772.

Cline, J.D., 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. 14, 454-458.

Coleman, D.D., Risatti, B., and Schoell, M., 1981. Fractionation of carbon and hydrogen isotopes by methane-oxidizing bacteria. Geochim. Cosmochim. Acta 45, 1033-1037.

Dempster, H.S., Sherwood Lollar, B., and Feenstra, S., 1997. Tracing organic contaminants in groundwater: a new methodology using compound-specific isotopic analysis. Environ. Sci. Technol. 31, 3193-3197.

Ertl, S., Seibel, F., Eichinger, L., Frimmel, F.H., and Kettrup, A., 1996. Determination of the $^{13}C/^{12}C$ isotope ratio of organic compounds for the biological degradation of tetrachloroethene (PCE) and trichloroethene (TCE). Acta Hydrochim. Hydrobiol. 24, 16-21.

Fogel, M.L., and Cifuentes, L.A., 1993. Isotope fractionation during primary production. *In* Org. Geochem. Engel, H.M., and Macko, S.A. (eds). New York: Plenum Press, pp. 73-97.

Hayes, J.M., Freeman, K.H., Po, B.N., and Hoham, C.H., 1990. Compound-specific isotope analysis. A novel tool for reconstruction of ancient biogeochemical processes. Adv. Org. Geochem. 16, 1115-1128.

Heider, J., Spormann, A.M., Beller, H.R., and Widdel, F., 1999. Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol. Rev. 22, 459-473.

Hilkert, A.W., Douthitt, C.B., Schlüter, H.J., and Brand, W.A., 1999. Isotope ratio monitoring gas chromatography/ mass spectrometry of D/H by high temperature conversion isotope ratio mass spectrometry. Rapid Commun. Mass Spectrom. 13, 1226-1230.

Hoefs, J., 1997. Stable isotope geochemistry. Berlin: Springer Verlag.

Hunkeler, D., Aravena, R., and Butler, B.J., 1999. Monitoring microbial dechlorination of tetrachloroethene (PCE) in groundwater using compound-specific stable carbon isotope ratios: Microcosm and field studies. Environ. Sci. Technol. 33, 2733-2738.

Krzycki, J.A., Kenealy, W.R., DeNiro, M.J., and Zeikus, J.G., 1987. Stable isotope fractionation by *Methanosarcina barkeri* during methanogenesis from acetate, methanol, or carbon dioxide-hydrogen. Appl. Environ. Microbiol. 53, 2597-2599.

Lebedew, W.C., Owsjannikow, W.M., Mogilewskij, G.A., and Bogdanow, W.M., 1969. Fraktionierung der Kohlenstoffisotope durch mikrobiologische Prozesse in der biochemischen Zone. Angew. Geol. 15, 621-624.

Leuthner, B., Leutwein, C., Schultz, H., Hörth, P., Haehnel, W., Schlitz, Schägger, H. and Heider, J., 1998. Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol. Microbiol. 28, 615-628.

Meckenstock, R.U., 1999a. Fermentative toluene degradation in anaerobic defined syntrophic cocultures. FEMS Microbiol. Lett. 177, 67-73.

Meckenstock, R.U., Annweiler, E., Warthmann, R., Schink, B., Michaelis, W., and Richnow, H.H., 1999b. $^{13}C/^{12}C$ stable isotope fractionation of toluene by anaerobic degradation: A new method to monitor biological degradation *in situ*? *In* Novel approaches for bioremediation of organic pollution. Fass, R., Flashner, Y., and Reuveny, S. (eds). New York: Kluwer Academic Publishers, pp. 219-227.

Meckenstock, R.U., Morasch, B., Warthmann, R., Schink, B., Annweiler, E., Michaelis, W., and Richnow, H.H., 1999c. $^{13}C/^{12}C$ isotope fractionation of

aromatic hydrocarbons during microbial degradation. Environ. Microbiol. 1, 409-414.

Merritt, D.A., Brand, W.A., and Hayes, J.M., 1994. Isotope ratio-monitoring gas chromatography mass spectrometry - Methods for isotopic calibration. Org. Geochem. 21, 573-583.

Müller, J.A., Galushko, A., Kappler, A., and Schink, B., 1999. Anaerobic degradation of *m*-cresol by *Desulfobacterium cetonicum* is initiated by formation of 3-hydroxybenzylsuccinate. Arch. Microbiol. 172, 287-294.

O'Leary, M.H., 1980. Determination of heavy-atom isotope effects on enzyme-catalysed reactions. *In* Enzyme kinetics and mechanism. Purich, D.L. (ed). New York: Academic Press, pp. 83-103.

O'Neil, J.R., 1986. Theoretical and experimental aspects of isotopic fractionation. Rev. Mineral. 16, 1-40.

Peterson, B.L., and Fry, B., 1987. Stable isotopes in ecosystem studies. Annu. Rev. Ecol. Syst. 18, 293-320.

Rayleigh, J.W.S., 1896. Theoretical considerations respecting the separation of gases by diffusion and similar processes. Philos. Mag. 42, 493-498.

Richnow, H.H., and Meckenstock, R.U., 1999. Isotopen-geochemisches Konzept zur *in situ* Erfassung des biologischen Abbaus in kontaminiertem Grundwasser. TerraTech 5, 38-41.

Sambrook, J., Fritsch, E.F., and Maniatis, R., 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Shaw, J.P., and Harayama, S., 1992. Purification and characterisation of the NADH:acceptor reductase component of xylene monooxygenase encoded by the TOL plasmid pWW0 of *Pseudomonas putida* mt-2. Eur. J. Biochem. 209, 51-61.

Stahl, W.J., 1980. Compositional changes and $^{13}C/^{12}C$ fractionations during the degradation of hydrocarbons by bacteria. Geochim. Cosmochim. Acta 44, 1903-1907.

Stookey, L.L., 1970. Ferrozine - a new spectrophotometric reagent for iron. Anal. Chem. 42, 779-781.

Sturchio, N.C., Clausen, J.L., Heraty, L.J., Huang, L., Holt, B.D., and Abrajano, T.A., 1998. Chlorine isotope investigation of natural attenuation of trichloroethene in an aerobic aquifer. Environ. Sci. Technol. 32, 3037-3042.

Ward, J.A.M., Ahad, J.M.E., Lacrampe-Couloume, G., Slater, G.F., Edwards, E.A., and Sherwood Lollar, B., 2000. Hydrogen isotope fractionation during methanogenic degradation of toluene: Potential for direct verification of bioremediation. Environ. Sci. Technol. 34, 4577-4581.

Whiticar, M.J., and Faber, E., 1986. Methane oxidation in sediment and water column environments - isotope evidence. Adv. Org. Geochem. 10, 759-768.

Widdel, F., and Bak, F., 1992. Gram-negative mesophilic sulfate-reducing bacteria. *In* The prokaryotes. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York: Springer Verlag, pp. 3352-3378.

Wilkes, H., Boreham, C., Harms, G., Zengler, K., and Rabus, R., 2000. Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria. Org. Geochem. 31, 101-115.

Worsey, M.J., and Williams, P.A., 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (arvilla) mt-2: Evidence for a new function of the TOL plasmid. J. Bacteriol., 7-13.

5 Carbon and hydrogen stable isotope fractionation during aerobic bacterial degradation of aromatic hydrocarbons

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Abstract

$^{13}C/^{12}C$ and D/H stable isotope fractionation during aerobic degradation was determined for *Pseudomonas putida* strain mt-2, *Pseudomonas putida* strain F1, *Ralstonia pickettii* strain PKO1, and *Pseudomonas putida* strain NCIB 9816 grown with toluene, xylenes, and naphthalene. Different types of initial reactions used by the respective bacterial strains could be linked with certain extents of stable isotope fractionation during substrate degradation.

Text

Intrinsic microbial degradation is an important process in contaminant elimination in polluted aquifers, which can be used for the sustainable cleanup of contaminated sites. However, cost-effective remediation strategies like natural attenuation require a profound knowledge of the microbial degradation processes in the subsurface. Although biodegradation of aromatic hydrocarbons by aerobic and anaerobic bacteria has been investigated in detail in laboratory systems (Gibson and Subramanian, 1984; Widdel and Rabus, 2001) the assessment at field sites remains difficult. Stable carbon isotope analysis is one approach to quantify microbial activities *in situ*. For laboratory cultures, isotope fractionation has been shown to occur during degradation of aromatic hydrocarbons, such as toluene (Meckenstock et al., 1999; Ahad et al., 2000; Morasch et al., 2001), or chlorinated hydrocarbons, such as trichloroethene (TCE) (Ertl et al., 1996; Sherwood Lollar et al., 1999; Bloom et al., 2000). In addition, in contaminated field sites carbon isotope fractionation could be observed and was interpreted to be indicative of microbial degradation *in situ* (Hunkeler et al., 1999; Richnow and Meckenstock, 1999). For toluene as a model compound, it has been demonstrated that isotope fractionation is caused mainly by the first enzyme reaction in the degradation pathway whereas transport to and into the cell appears not to be relevant for fractionation. The extent of isotope fractionation is considered to be independent of differences in the growth kinetics of the bacteria (Morasch et al., 2001). Isotope fractionation during anaerobic degradation of toluene was in

the same order of magnitude for denitrifying, iron(III)-reducing, sulfate-reducing, and fermenting bacteria (Meckenstock et al., 1999; Ahad et al., 2000), probably because, in these cases degradation was initiated by benzylsuccinate synthase. This finding suggests that, in anoxic environments, isotope fractionation could be applied to assess biological degradation quantitatively, as has been worked out recently for several aquifers (Richnow et al., 2001; Richnow et al., 2002).

The objective of this study was to examine whether carbon and hydrogen isotope fractionation could be used to quantify intrinsic biodegradation as well in oxic environments. Previous studies with the aerobic bacterium *Pseudomonas putida* strain mt-2 showed an extent of isotope fractionation similar to anaerobic toluene degrading strains (Meckenstock et al., 1999), whereas isotope fractionation during toluene degradation by undefined aerobic microbial communities was not detected (Sherwood Lollar et al., 1999). Therefore, we started a systematic investigation on the effects of different oxygenase enzymes and stable isotope fractionation.

P. putida strain mt-2 (Morasch et al., 2001), *Ralstonia pickettii* strain PKO1 (J. J. Kukor, Rutgers University, N.J.), and *P. putida* strain F1 (A. M. Cook, Konstanz, Germany) were taken as representatives of bacteria using different toluene degradation pathways in polluted aquifers. Strains were grown in 200 ml batch cultures with 15 µl of toluene, *m*-xylene, or *p*-xylene (analytical grade quality; Fluka, Buchs, Switzerland) as described previously (Morasch et al., 2001). Cultures for isotope fractionation experiments were inoculated with 5% (vol/vol) of pre-cultures (optical density at 578 nm [OD_{578}] = 0.25), and growth was monitored by measuring the OD_{578}. Hydrocarbon concentrations were determined by high-performance liquid chromatography (HPLC) (Morasch et al., 2001). During degradation of toluene by *P. putida* strain mt-2 (methyl monooxygenase), the $^{13}C/^{12}C$ isotope ratios in the nondegraded toluene were determined by gas chromatography-combustion-isotope ratio monitoring mass spectrometry (GC-C-IRMS) (Morasch et al., 2001) and shifted from $d\,^{13}C$ = -29.18‰ ± 0.35‰ to more positive values of $d\,^{13}C$ = -23.22‰ ± 0.32‰ after 80% of the substrate was degraded (equation 4) (Fig. 1). Analysis of the carbon isotope ratios $\ln(R_t/R_0)$ and the respective concentrations $\ln(c_t/c_0)$ according to the Rayleigh equation for closed systems (equation 1) (Rayleigh, 1896; Hoefs, 1997) resulted in an isotope fractionation factor αC = 0.9967 ± 0.0003 (Fig. 2A). R_t and c_t are the isotope ratio $^{13}C/^{12}C$ and the residual substrate concentration at time t and R_{Std} is the isotope ratio of an international standard (Vienna PDB).

For direct comparison between the bacterial pure cultures, isotope fractionation will be referred to as “enrichment factor e" (equation 2), which is indicated as e_n in experiments with nonlabeled toluene. During toluene

degradation by *P. putida* strain mt-2 the enrichment factor was e_n = -3.3 ± 0.3 for $^{13}C/^{12}C$ isotope fractionation (Table 1).

$$\frac{R_t}{R_0} = \left(\frac{c_t}{c_0}\right)^{(aC-1)} \quad (1)$$

$$e = (a-1)\times 1{,}000 \quad (2)$$

$$\ln\left(\frac{R_t}{R_0}\right) = \ln\left[\frac{\left(\frac{c_t}{c_0}\right)}{(1+R_t)/(1+R_0)}\right]\times(aH-1) \quad (3)$$

$$d^{13}C[‰] = \left(\frac{R_{sample}}{R_{std}} - 1\right)\times 1{,}000 \quad (4)$$

P. putida strain mt-2 also degrades *m*- and *p*-xylene by using the same enzyme as toluene (methyl monooxygenase) (Suzuki et al., 1990). The enrichment factors for carbon isotope fractionation obtained during degradation of *m*- and *p*-xylene were slightly smaller than those of toluene (Table 1).

Toluene degradation by *R. pickettii* strain PKO1 (ring monooxygenase) resulted in a carbon isotope fractionation that was three times lower than that with strain mt-2. The third toluene-degrading strain, *P. putida* strain F1, hydroxylated the substrate with a toluene-dioxygenase to the corresponding catechol. The concomitant $^{13}C/^{12}C$ isotope fractionation was insignificant. The same was observed with *P. putida* strain NCIB 9816 in degradation experiments with naphthalene (DSM 8368; Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Strain NCIB 9816 attacks naphthalene by a dioxygenase reaction similar to that of strain F1. It was grown in batch culture with 150 to 200 µM dissolved naphthalene as a carbon source. Naphthalene was transferred into the culture bottles as solid crystal and autoclaved in 180 ml of H_2O. After cooling, the stock solutions for mineral medium M9 were added. The carbon isotope ratio in the residual naphthalene did not change during degradation over time and the isotope fractionation was not significant (Fig. 2A, Table 1).

Hydrogen isotope fractionation during degradation of aromatic hydrocarbons was investigated by cultivating the same four bacterial strains with a mixture of nondeuterated toluene and per-deuterated toluene-d_8 (8 µl each) or with a mixture of nonlabeled naphthalene and per-deuterated naphthalene-d_8, (3 mg each). The ratio of residual labeled and nonlabeled substrate in the culture

during growth was analyzed in pentane extracts of liquid samples (2 to 7 ml) by GC (Morasch et al., 2001). Naphthalene and per-deuterated naphthalene-d_8 were separated by GC to the baseline at a constant oven temperature of 140 °C. Isotope fractionation factors were calculated according to equation 3 with the isotope ratio R_t = [deuterated substrate]/[substrate] (Bigeleisen and Wolfsberg, 1958). For a very high abundance of the heavier isotope, as was used in the experiments for hydrogen isotope fractionation, equation 1 changes to equation 3 (Bigeleisen and Wolfsberg, 1958).

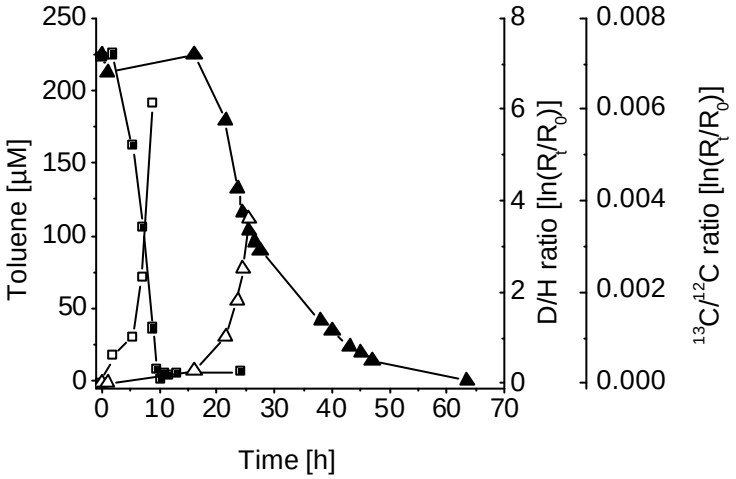

Fig. 1. Isotope shifts during degradation of toluene and a toluene-toluene-d_8 mixture by *P. putida* strain mt-2. The decrease in toluene (■) and concomitant increase in ^{13}C (R = $^{13}C/^{12}C$) in the remaining substrate (□) and decrease in toluene-toluene-d_8 concentration (▲), and concomitant increase in deuterium R_t = [toluene-d_8]/[toluene] in the residual nondegraded substrate fraction (△) were monitored over time. In every case, data are shown for one representative experiment out of three performed.

Enrichment factors derived from equation 3 are depicted as e_l. Degradation of half of the substrate mixture by *P. putida* strain mt-2 (methyl monooxygenase) was accompanied by a 40-fold enrichment of per-deuterated toluene-d_8, correlated with a near-total depletion of nonlabeled toluene (Fig. 1). The D/H isotope fractionation determined in the first growth phase was e_l = -905 ± 71. *R. pickettii* strain PKO1 (ring monooxygenase) and *P. putida* strain F1 (ring dioxygenase) degraded the toluene-toluene-d_8 mixture resulting in a D/H isotope fractionation more than 10 and 50 times stronger than the carbon isotope fractionation by the same strains, but still 50 and 30 times lower, respectively, than the D/H fractionation by *P. putida* strain mt-2. *P. putida* strain NCIB 9816 (ring dioxygenase) grown with naphthalene-naphthalene-d_8 showed a D/H isotope fractionation that was twice as strong as the D/H isotope fractionation by *P. putida* strain F1 (Fig. 2B, Table 1).

Of the four strains examined, *P. putida* strain mt-2 showed by far the highest $^{13}C/^{12}C$ and D/H isotope fractionation during aerobic toluene degradation. The methyl monooxygenase of this bacterium exhibits similarities in its amino acid sequence to the methane monooxygenase of *Methylosinus trichosporium* OB3b (Worsey and Williams, 1975; Harayama et al., 1992). Oxygen bound as

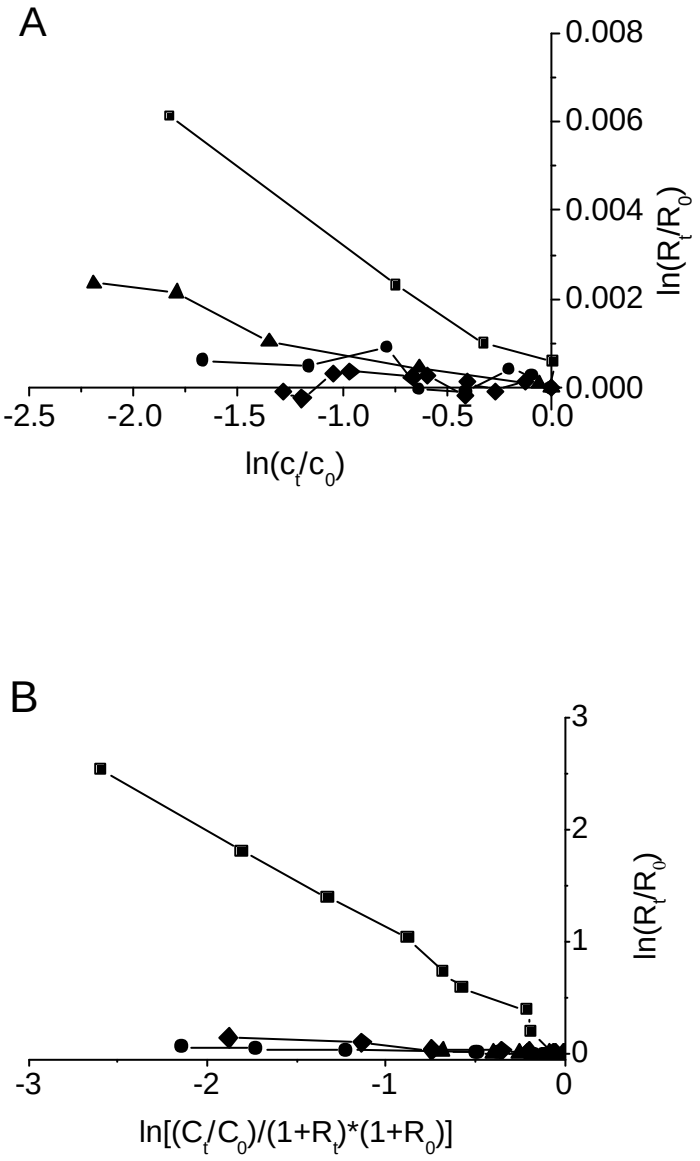

Fig. 2. Carbon (A) and hydrogen (B) stable isotope fractionation during degradation of toluene by *P. putida* strain mt-2 (■), *R. pickettii* strain PKO1 (▲), and *P. putida* strain F1 (●) and isotope fractionation during degradation of naphthalene by *P. putida* strain NCIB 9816 (◆). $^{13}C/^{12}C$ carbon isotope fractionation was plotted according to equation 1. D/H isotope fractionation was determined with equation 3 during growth with equal amounts of per-deuterated toluene-d_8 and toluene and per-deuterated naphthalene-d_8 and naphthalene.

an oxene group to the catalytic iron of methane monooxygenase is proposed to lead to a homolytic C—H bond cleavage of the substrate. The reaction is associated with strong isotope effects during methane conversion and minor effects during ethane and propane conversion (Brazeau and Lipscomb, 2000). The authors assume that the reaction mechanism with methane differs slightly from that with other substrates. Similarly, in our study, the xylene monooxygenase of *P. putida* strain mt-2 caused strong hydrogen and carbon isotope fractionation. Minor differences in the enzymatic reactions with toluene, *m*-, and *p*-xylene might be the reason for the variations in the respective $^{13}C/^{12}C$ isotope fractionation factors. Cytochrome P450 is another well-known example of an enzyme that dissociates a C—H bond and inserts oxygen into benzylic or aliphatic substrates (Yoshizawa et al., 2000). In an experimental study, Manchester et al. (Manchester et al., 1997) found the strongest isotope effects with *p*-xylene and toluene, which showed six times higher rate constants than deuterated *p*-xylene and toluene.

Toluene degradation by *R. pickettii* strain PKO1 is catalyzed by a monooxygenase reaction hydroxylating the carbon 3 of the aromatic ring (Olsen et al., 1994). The amino acid sequence of this toluene-3-monooxygenase is remarkably similar to that of toluene-4-monooxygenase of *Pseudomonas mendocina* strain KR1, an enzyme studied in great detail (Whited and Gibson, 1991). The poor hydrogen isotope fractionation of strain PKO1 is probably due to an enzymatic reaction with the π-electron system of the aromatic substrate. The hydroxylation presumably is a two-step process starting with an electrophilic attack of iron-bound oxygen on the aromatic ring and the formation of a C—O σ-bond. In a faster second step, the hydrogen atom bound to this carbon atom is released as a proton, and the electrons are used to reconstitute the aromaticity of the carbon ring skeleton (Carey and Sundberg, 1990). The weak carbon and hydrogen isotope fractionation observed here might be due to secondary isotope effects caused by atoms of the substrate molecule not directly contributing to the reaction.

P. putida strains F1 and NCIB 9816 both attack toluene through an initial dioxygenase reaction on the aromatic ring (Jeffrey et al., 1975; Yeh et al., 1977; Carredano et al., 2000). Initially, π-electrons of the aromatic substrate are attracted by activated oxygen, which is bound to a catalytic iron center (Subramanian et al., 1979). Primary isotope effects do not accompany this reaction because there is no distinct bond cleavage involved. The first reaction product of toluene and naphthalene oxidation is a *cis*-dihydrodiol, which is dehydrogenated in a subsequent step to form a catechol derivative (Gibson et al., 1970). The terminal oxygenase units of toluene and naphthalene dioxygenase of strains F1 and NCIB 9816 both are hexamers ($\alpha_3\beta_3$) with catalytic mononuclear ferrous iron centers (Gassner et al., 1995; Torok et al., 1995; Kauppi et al., 1998). The amino acid sequences of their subunits suggest

that they derived from a common ancestor (Harayama et al., 1992) and share the same reaction mechanism (Gibson and Parales, 2000). Accordingly, $^{13}C/^{12}C$ isotope fractionation was very weak during toluene degradation by *P. putida* strain F1, and was not significant during naphthalene degradation by *P. putida* strain NCIB 9816. The more pronounced secondary D/H isotope fractionation effects of *P. putida* strain NCIB 9816 might be caused by individual features of the naphthalene degradation mechanism.

Table 1. $^{13}C/^{12}C$ and D/H isotope fractionation by different aerobic bacterial strains during growth with aromatic hydrocarbons.[a]

Strain	**Enzyme mechanism**	**Carbon source**	$e\mathbf{C}_n$	$e\mathbf{D}_l$
***P. putida* strain mt-2**	Methyl monooxygenase	Toluene	-3.3 ± 0.3	-905 ± 71
	Methyl monooxygenase	*m*-Xylene	-1.7 ± 0.04	ND[b]
	Methyl monooxygenase	*p*-Xylene	-2.3 ± 0.3	ND[b]
***R. pickettii* strain PKO1**	Ring monoooxygenase	Toluene	-1.1 ± 0.2	-16 ± 5.3
***P. putida* strain F1**	Ring dioxygenase	Toluene	-0.4 ± 0.3	-28 ± 10
***P. putida* strain NCIB 9816**	Ring dioxygenase	Naphthalene	-0.1 ± 0.2	-65 ± 13

[a]Stable carbon isotope fractionation is given as the enrichment factor, e_n, and was calculated with equations 1 and 2. Hydrogen isotope fractionation with mixtures (50:50) of per-deuterated and nonlabeled substrate was determined with equation 3. The enrichment factor for labeled substances, e_l, was determined by equation 2. Mean values ± standard deviation are given based on three independent growth experiments with fivefold isotope analysis per data point.
[b]ND, not determined.

Recently, Hunkeler et al. (2001) found relatively small amounts of $^{13}C/^{12}C$ and D/H isotope fractionation during benzene degradation by *Acinetobacter* sp. and *Burkholderia* sp. (Hunkeler et al., 2001). Others observed a small $^{13}C/^{12}C$ isotope fractionation during benzene degradation by an aerobic mixed culture that was enriched from groundwater of a petrochemical site (Stehmeier et al., 1999). The small isotope fractionation factors and the increase in $d^{13}C$ for the initial mono- or dioxygenase reactions at the benzene ring that were documented in these papers are in agreement with our observations that

oxygenases acting on the π-electron system of the aromatic ring produce only minor isotope effects.

The present study and previous investigations allow some preliminary conclusions to be drawn about when stable isotope fractionation could be used to assess biodegradative activities in contaminated groundwater. Consistent carbon isotope fractionation during toluene degradation by anaerobic microorganisms allows a quantitative assessment of bacterial degradation under anoxic conditions. Hydrogen isotope effects are stronger than carbon isotope effects by two or three orders of magnitude, but are also more variable between different types of anaerobic bacteria. Small extents of degradation are detectable, but quantification *in situ* might be difficult. However, D/H and $^{13}C/^{12}C$ isotope fractionation in oxic zones of contaminated aquifers could be used only as a qualitative indicator of biodegradation. The differences in isotope fractionation between bacterial strains with different oxygenase reactions preclude a quantitative assessment of *in situ* biodegradation based on isotope fractionation in oxic zones. Nevertheless, combined measurement of carbon isotope fractionation for *in situ* quantification and hydrogen isotope fractionation as a positive indicator of bacterial activities could be a valuable tool to assess biodegradation in the environment.

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References

Ahad, J.M.E., Sherwood Lollar, B., Edwards, E.A., Slater, G.F., and Sleep, B.E., 2000. Carbon isotope fractionation during anaerobic biodegradation of toluene: Implications for intrinsic bioremediation. Environ. Sci. Technol. 34, 892-896.

Bigeleisen, J., and Wolfsberg, M., 1958. Theoretical and experimental aspects of isotope effects in chemical kinetics. Adv. Chem. Phys. 1, 15-76.

Bloom, Y., Aravena, R., Hunkeler, D., Edwards, E.A., and Frape, S.K., 2000. Carbon isotope fractionation during microbial degradation of trichloroethene, *cis*-1,2-cichloroethene, and vinyl chloride: Implications for assessment of natural attenuation. Environ. Sci. Technol. 34, 2768-2772.

Brazeau, B.J., and Lipscomb, J.D., 2000. Kinetics and activation thermodynamics of methane monooxygenase compound Q formation and reaction with substrates. Biochemistry 39, 13503-13515.

Carey, F.A., and Sundberg, R.J., 1990. Advanced organic chemistry. New York: Plenum Press.

Carredano, E., Karlsson, A., Kauppi, B., Choudhury, D., Gibson, D.T., Eklund, H., and Ramaswamy, S., 2000. Substrate binding site of naphthalene 1,2-dioxygenase: Functional implications of indole binding. J. Mol. Biol. 296, 701-712.

Ertl, S., Seibel, F., Eichinger, L., Frimmel, F.H., and Kettrup, A., 1996. Determination of the $^{13}C/^{12}C$ isotope ratio of organic compounds for the biological degradation of tetrachloroethene (PCE) and trichloroethene (TCE). Acta Hydrochim. Hydrobiol. 24, 16-21.

Gassner, G.T., Ludwig, M.L., Gatti, D.L., Correll, C.C., and Ballou, D.P., 1995. Structure and mechanism of the iron-sulfur flavoprotein phthalate dioxygenase reductase. FASEB J. 9, 1411-1418.

Gibson, D.T., and Subramanian, V., 1984. Microbial degradation of aromatic hydrocarbons. *In* Microbial degradation of organic compounds. Gibson, D.T. (ed). New York: Dekker, pp. 181-252.

Gibson, D.T., and Parales, R.E., 2000. Aromatic hydrocarbon dioxygenases in environmental biotechnology. Curr. Opin. Biotechnol. 11, 236-243.

Gibson, D.T., Cardini, G.E., Maseles, F.C., and Kallio, R.E., 1970. Incorporation of oxygen-18 into benzene by *Pseudomonas putida.* Biochemistry 9, 1631-1635.

Harayama, S., Kok, M., and Neidle, E.L., 1992. Functional and evolutionary relationships among diverse oxygenases. Annu. Rev. Microbiol. 46, 565-601.

Hoefs, J., 1997. Stable isotope geochemistry. Berlin: Springer Verlag.

Hunkeler, D., Aravena, R., and Butler, B.J., 1999. Monitoring microbial dechlorination of tetrachloroethene (PCE) in groundwater using compound-specific stable carbon isotope ratios: Microcosm and field studies. Environ. Sci. Technol. 33, 2733-2738.

Hunkeler, D., Andersen, N., Aravena, R., Bernasconi, S.M., and Butler, B.J., 2001. Hydrogen and carbon isotope fractionation during aerobic biodegradation of benzene. Environ. Sci. Technol. 35, 3462-3467.

Jeffrey, A.M., Yeh, H.J.C., Jerina, D.M., Patel, T.R., Davey, J.F., and Gibson, D.T., 1975. Initial reactions in the oxidation of naphthalene by *Pseudomonas putida.* Biochemistry 14, 575-584.

Kauppi, B., Lee, K., Carredano, E., Parales, R.E., Gibson, D.T., Eklund, H., and Ramaswamy, S., 1998. Structure of an aromatic-ring-hydroxylating dioxygenase - naphthalene 1,2-dioxygenase. Structure 6, 571-586.

Manchester, J.I., Dinnocenzo, J.P., Higgins, L., and Jones, J.P., 1997. A new mechanistic probe for cytochrome P450: An application of isotope effect profiles. J. Am. Chem. Soc. 119, 5069-5070.

Meckenstock, R.U., Morasch, B., Warthmann, R., Schink, B., Annweiler, E., Michaelis, W., and Richnow, H.H., 1999. $^{13}C/^{12}C$ isotope fractionation of

aromatic hydrocarbons during microbial degradation. Environ. Microbiol. 1, 409-414.

Morasch, B., Richnow, H.H., Schink, B., and Meckenstock, R.U., 2001. Stable hydrogen isotope fractionation during microbial toluene degradation: Mechanistic and environmental aspects. Appl. Environ. Microbiol. 67, 4842-4849.

Olsen, R.H., Kukor, J.J., and Kaphammer, B., 1994. A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. J. Bacteriol. 176, 3749-3756.

Rayleigh, J.W.S., 1896. Theoretical considerations respecting the separation of gases by diffusion and similar processes. Philos. Mag. 42, 493-498.

Richnow, H.H., and Meckenstock, R.U., 1999. Isotopen-geochemisches Konzept zur *in situ* Erfassung des biologischen Abbaus in kontaminiertem Grundwasser. TerraTech 5, 38-41.

Richnow, H.H., Gehre, M., Kästner, M., Morasch, B., and Meckenstock, R.U., 2001. Characterisation of microbial *in situ* degradation of aromatic hydrocarbons. *In* Innovative methods in support of bioremediation. Magar, V.S., Vogel, T.M., Aelion, M., and Leeson, A. (eds). Columbus, Ohio: Battelle press, pp. 99-108.

Richnow, H.H., Vieth, A., Kästner, M., Gehre, M., and Meckenstock, R.U., 2002. Isotope fractionation of toluene: A perspective to characterise microbial *in situ* degradation. Sci. World J. 2, 7227-7234.

Sherwood Lollar, B., Slater, G.F., Ahad, J., Sleep, B., Spivack, J., Brennan, M., and MacKenzie, P., 1999. Contrasting carbon isotope fractionation during biodegradation of trichloroethylene and toluene: implications for intrinsic bioremediation. Org. Geochem. 30, 813-820.

Stehmeier, L.G., Francis, M.M., Jack, T.R., Diegor, E., Winsor, L., and Abrajano, T.A., 1999. Field and in vitro evidence for in-situ bioremediation using compound-specific $^{13}C/^{12}C$ ratio monitoring. Org. Geochem. 30, 821-833.

Subramanian, V., Liu, T.-N., Yeh, W.K., and Gibson, D.T., 1979. Toluene dioxygenase: Purification of an iron-sulfur protein by affinity chromatography. Biochem. Biophys. Res. Commun. 91, 1131-1139.

Suzuki, M., Hayakawa, T., Shaw, J.P., Rekik, M., and Harayama, S., 1990. Primary structure of xylene monooxygenase: Similarities to and differences from the alkane hydroxylation system. J. Bacteriol. 172, 1690-1695.

Torok, D.S., Resnick, S.M., Brand, J.M., Cruden, D.L., and Gibson, D.T., 1995. Desaturation and oxygenation of 1,2-dihydronaphthalene by toluene and naphthalene dioxygenase. J. Bacteriol. 177, 5799-5805.

Whited, G.M., and Gibson, D.T., 1991. Toluene-4-monooxygenase, a three-component enzyme system that catalyzes the oxidation of toluene to *p*-cresol in *Pseudomonas mendocina* KR1. J. Bacteriol. 173, 3010-3016.

Widdel, F., and Rabus, R., 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr. Opin. Biotechnol. 12, 259-276.

Worsey, M.J., and Williams, P.A., 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (arvilla) mt-2: Evidence for a new function of the TOL plasmid. J. Bacteriol. 124, 7-13.

Yeh, W.K., Gibson, D.T., and Liu, T.-N., 1977. Toluene dioxygenase: A multicomponent enzyme system. Biochem. Biophys. Res. Commun. 78, 401-410.

Yoshizawa, K., Kagawa, Y., and Shioto, Y., 2000. Kinetic isotope effects in a C-H bond dissociation by the iron-oxo species of cytochrome P450. J. Phys. Chem. 104, 12365-12370.

6 Stable isotope fractionation caused by glycyl radical enzymes during bacterial degradation of aromatic compounds

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Abstract

Stable isotope fractionation was studied during degradation of *m*-xylene, *o*-xylene, *m*-cresol, and *p*-cresol with two pure cultures of sulfate-reducing bacteria. Degradation of all four compounds is initiated by a fumarate addition reaction by a glycyl radical enzyme, analogous to the well-studied benzylsuccinate synthase reaction in toluene degradation. The extent of stable carbon isotope fractionation caused by these radical type reactions was between ε = -1.5‰ and ε = -3.9‰ which is in the same order of magnitude as data provided before for anaerobic toluene degradation. Based on our results, an analysis of isotope fractionation should be applicable for the quantification of *in situ* bioremediation of all contaminants degraded by glycyl radical enzyme mechanisms that are smaller than 14 carbon atoms. In order to compare the carbon isotope fractionation upon degradation of various substrates differing in the number of their carbon atoms, intrinsic enrichment factors ($\varepsilon_{intrinsic}$) were calculated. A comparison of intrinsic enrichment factors ($\varepsilon_{intrinsic}$) referring to the single carbon atoms of the molecule where the benzylsuccinate synthase reaction took place with compound specific enrichment factors (ε) elucidated that both varied on average to the same extent. Despite variations during degradation of different substrates, the range of isotope enrichment factors found for glycyl radical reactions was reasonably narrow to propose rough estimates of biodegradation *in situ* using an average enrichment factor if no fractionation factor is available for single compounds.

Introduction

Many biochemical reactions are known to cause fractionation of stable isotopes. Molecules consisting of lighter isotopes are utilized preferentially and consequently heavier molecules are enriched in the residual substrate pool. Well-known examples are autotrophic CO_2 fixation by plants (O'Leary, 1984) and bacterial methanogenesis (Krzycki et al., 1987). Within the last years, several studies investigated stable isotope fractionation during bacterial degradation of contaminants such as toluene (Meckenstock et al., 1999),

benzene (Mancini et al., 2003), and chlorinated hydrocarbons (Hunkeler et al., 1999) that were associated with a significant enrichment of ^{13}C in the residual substrate fraction. These findings opened the perspective to assess contaminant degradation at polluted sites qualitatively, under certain conditions even quantitatively, and to outline the future development of the contamination and its potential impact on the environment and drinking water supply. This concept has been applied successfully in several field experiments so far (Hunkeler et al., 1999; Sherwood Lollar and Slater, 2001; Meckenstock et al., 2002; Richnow et al., 2003).

Basic features of microbial stable isotope fractionation were examined for anaerobic and aerobic bacterial cultures of toluene as a model substrate (Morasch et al., 2001). It was demonstrated that the first enzyme reaction of toluene degradation is the rate-limiting step in degradation and that this reaction is also the key process leading to isotope fractionation. Isotope effects due to transport of toluene to and into the cells were negligible. Furthermore it was shown that various aerobic and anaerobic degradation reactions led to characteristic degrees of fractionation (Morasch et al., 2002). Vice versa, it is not possible, to deduce the type of the underlying reaction mechanism from the extent of fractionation because fractionation factors can vary significantly between identical reactions. Studies dealing with stable isotope fractionation of particular reaction mechanisms provided information about the span of isotope fractionation effects caused by these mechanisms. Based on theoretical considerations and calculations it was proposed that the rate-limiting step in the benzylsuccinate synthase reaction is the addition of fumarate to the benzyl radical (Himo, 2002).

Stable isotope fractionation during degradation reactions initiated by glycyl radical enzymes is of particular interest because this reaction mechanism seems to be predominant in anaerobic degradation of contaminants such as aromatic hydrocarbons, alkyl-phenols, and alkanes. Independent of the respective electron acceptor employed, anaerobic bacterial toluene degradation proceeds via benzylsuccinate formation in all cases investigated, so far (Spormann and Widdel, 2000; Gibson and Harwood, 2002). The enzyme mechanism was first described for anaerobic toluene degradation by denitrifying bacteria where the enzyme benzylsuccinate synthase catalyses the addition of a fumarate molecule to the methyl group to form benzylsuccinate (Biegert et al., 1996). Fumarate addition and subsequent activation and β oxidation converts the former methyl group to a carbonylic function that acts as an entry port for single electrons in the subsequent ring reduction (Boll et al., 2002). The glycyl radical formed in benzylsuccinate synthase and related enzymes derives from 5´-deoxyadenosyl that is a cleavage product of *S*-adenosylmethionine (SAM) (Frey, 2001). The enzyme reaction withdraws one hydrogen atom from a

highly conserved cystein residue of the enzyme. This thiyl radical then takes one hydrogen atom from the methyl group of the aromatic substrate, which later is transferred from the cystein residue to the C_β of the succinyl side chain of the produced benzylsuccinate (Frey, 2001; Krieger et al., 2001) (Fig. 3).

We investigated stable isotope fractionation in degradation pathways initiated by a glycyl radical mechanism with two bacterial strains. *Desulfobacterium cetonicum*, a toluene-degrading sulfate-reducer, was shown to degrade also *m*- and *p*-cresol via fumarate addition (Müller et al., 1999, 2001). Enzyme activities in cell-free extracts showed that the cresols are probably not converted by the toluene-degrading benzylsuccinate synthase, but it remains open if the two different cresol isomers are attacked by the same enzyme.

Degradation of *m*- and *o*-xylene by the sulfate-reducing strain OX39 proceeds also via fumarate addition, which was confirmed by the identification of methylbenzylsuccinate derivatives in culture supernatants (Morasch, unpublished results). Induction experiments with strain OX39 with *m*-xylene, *o*-xylene, and toluene showed that every substrate needed a specific enzyme for degradation (Morasch, unpublished results).

The objective of the present study was to systematically investigate stable carbon isotope fractionation during radical enzyme reactions of aromatic compounds which are prominent groundwater contaminants. The study should elucidate if this type of reaction produces a consistent isotope fractionation during degradation. For better comparison of isotope fractionation of the various substrate molecules, data of the intrinsic isotope fractionation at the molecular site of reactions are provided.

Material and Methods

Cultivation of bacteria

The sulfate-reducing strain OX39 was isolated from soil of a site contaminated with BTEX and PAH near Stuttgart, Germany (Morasch, unpublished results). *D. cetonicum* (DSM 7267) was taken from the lab culture collection.

Bacteria were cultivated at 30°C, in bicarbonate-buffered freshwater (strain OX39) or brackish (*D. cetonicum*) mineral medium, pH 7.4, with sulfate, 10 mM, as electron acceptor. The medium was prepared under an atmosphere of N_2/CO_2 (80:20) and reduced with Na_2S (1mM) (Widdel and Bak, 1992). A sterile, anoxic $FeCl_2$ solution was added to the medium of strain OX39 to a final concentration of 3 mM.

Strains were grown in 120 ml serum bottles half-filled with mineral medium and tightly sealed with viton rubber stoppers (Maag Technic, Dübendorf, Switzerland). Cultures for isotope fractionation experiments were inoculated with 6 ml of pre-cultures. *m*-Xylene and *o*-xylene were injected directly into the culture bottles with micro-syringes through the rubber stoppers. *m*-Cresol

and *p*-cresol were added from aqueous stock solutions (100 mM). Metabolic activity was monitored by following sulfide production (Cline, 1969).

Hydrocarbon analysis

Xylene concentrations were determined by high-performance liquid chromatography (HPLC, Bischoff Chromatography, Leonberg, Germany) with a C_{18} reversed phase column (Prontosil, 200 x 3 mm, 3 µm film thickness, Bischoff, Leonberg, Germany) at 30°C and UV-detection at 210 nm using a mix of acetonitrile (Chromasol V super gradient grade, Fluka, Buchs, Switzerland) and demineralized water (70:30, vol/vol) as eluents. Cresol concentrations were determined using a mix of acetonitrile and ammonium phosphate buffer (100 mM, pH 3.5) (50:50, vol/vol) as eluent. Culture samples were diluted 1:5 with ethanol (99.9 % gradient grade) and centrifuged (20,000 × g, 5 min) to remove precipitates before analysis.

Isotope analysis

$^{13}C/^{12}C$ stable carbon isotope ratios were determined by isotope-ratio-monitoring gas chromatography mass spectrometry (IRM-GC-MS). The system consisted of a GC (HP-5890, Hewlett-Packard Co., San Diego, Ca.) connected via a combustion unit (GC-combustion interface, Finnigan, Bremen, Germany) with an isotope mass spectrometer (Finnigan MAT 252, Finnigan). Samples were measured as described earlier (Morasch et al., 2001).

Calculations

The $^{13}C/^{12}C$ isotope ratios of the substrate were calculated as relative $\delta^{13}C$ values [‰] according to equation (1), where R_{sample} is the $^{13}C/^{12}C$ isotope ratio of the sample and R_{std} the isotope ratio of the international Pee Dee Belemnite standard (Hoefs, 1997). Kinetic isotope fractionation factors αC were calculated using equation (2), which is derived from the Rayleigh equation for closed systems (Rayleigh, 1896; Hoefs, 1997). C_t/C_0 is the fraction of the substrate remaining in the sample at time t. If $\ln(R_t/R_0)$ is plotted over $\ln(C_t/C_0)$, for the time intervals t the slope of the linear regression curve gives the kinetic isotope fractionation factor αC as (αC - 1). A convenient expression of stable isotope fractionation is the enrichment factor ε that can be retrieved directly from αC using equation 3 (Clark and Fritz, 1997).

$$d^{13}C[‰] = \left(\frac{R_{sample}}{R_{std}} - 1 \right) \times 1{,}000 \qquad (1)$$

$$\ln\left(\frac{R_t}{R_0} \right) = \ln\left(\frac{C_t}{C_0} \right) \times (aC - 1) \qquad (2)$$

$$e = 1{,}000 \times (aC - 1) \qquad (3)$$

Results and Discussion

Stable $^{13}C/^{12}C$ isotope fractionation was determined during growth of *D. cetonicum* with *m*- and *p*-cresol that had initial $\delta^{13}C$ signatures of -29.32 ± 0.36‰ and –27.50 ± 0.36‰, respectively. Anaerobic *m*-cresol degradation caused a strong increase in $\delta^{13}C$ in the residual substrate fraction which is shown in a representative experiment where degradation of 68% of the initial 1030 µM led to an isotope shift from -29.04 ± 0.47‰ to –24.53 ± 0.41‰ (Fig. 1A). The average isotope enrichment factor ε = -3.9 ± 0.5‰ for anaerobic *m*-cresol degradation was determined in four parallel experiments (Fig. 2). Anaerobic degradation of *p*-cresol produced a shift in $\delta^{13}C$ from –27.11 ± 0.17‰ to –23.44 ± 0.25‰ during degradation of 89% of initial 1052 µM (data not shown).

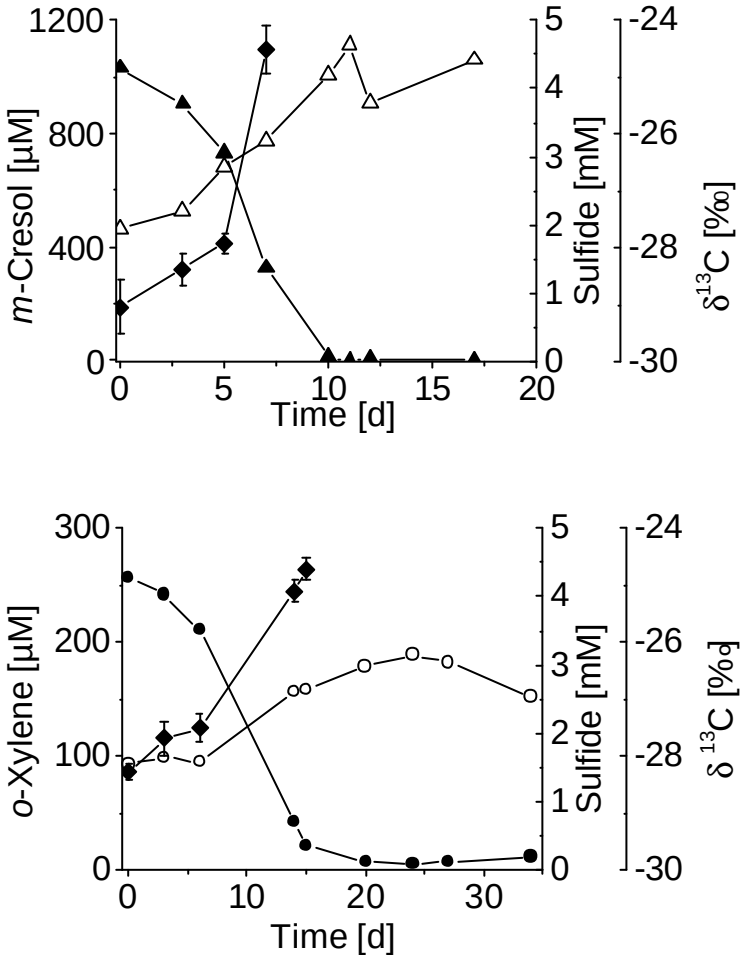

Fig. 1. ^{13}C enrichment in the residual substrate fraction during anaerobic degradation of (A) *m*-cresol (▲) by *D. cetonicum* and (B) *o*-xylene (●) by the sulfate-reducing strain OX39. Changes in sulfide concentration (△), and $\delta^{13}C$ (◆) were monitored over time. The carbon isotope composition is presented as an average of six individual measurements with error bars indicating the standard deviation. The diagram shows one representative experiment out of four, and three parallels for *m*-cresol and *o*-xylene, respectively.

The smaller increase in $\delta^{13}C$ per amount of substrate degraded resulted in an average enrichment factor of ε = -1.6 ± 0.1‰ calculated from the results of three parallel growth experiments (Fig. 2). Other isotope fractionation factors for anaerobic cresol degradation are not available from the literature, yet. For comparison, the isotope fractionation produced during toluene degradation by *D. cetonicum* was ε = -2.2 ± 0.4‰ (Morasch et al., 2001).

Stable carbon isotope fractionation was determined during degradation of *m*- and *o*-xylene of strain OX39 in three independent growth experiments per substrate. The initial $\delta^{13}C$ carbon signatures of the substrates were –27.66 ± 0.44‰ for *m*-xylene and –28.19 ± 0.06‰ for *o*-xylene. During degradation of 82% of the initial *m*-xylene (203 µM), the ^{13}C isotope became enriched to –24.81 ± 0.16‰ in one of three growth experiments (data not shown). The *m*-xylene degradation experiments yielded an average enrichment factor of ε = -1.8 ± 0.2‰ (Fig. 2). Degradation of an initial *o*-xylene concentration of 257 µM by strain OX39 resulted in a $\delta^{13}C$ shift from –28.28 ± 0.13‰ to –24.72 ± 0.19‰ when 85% of the substrate was used (Fig. 1B). The average enrichment factor of these experiments was ε = - 1.5 ± 0.1‰ (Fig. 2) and thus similar to the factor found for degradation of the *m*-xylene isomer. In previous degradation experiments using a sediment column filled with contaminated aquifer material under sulfate-reducing conditions, a carbon isotope fractionation by ε = -1.1‰ for *o*-xylene was determined which was lower than the enrichment factor reported here (Richnow et al., 2003). Furthermore, carbon isotope fractionation of ε = -0.9 ± 0.1‰ was found for degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture (Griebler, unpublished results) which also initiates degradation via fumarate addition (Annweiler et al., 2000).

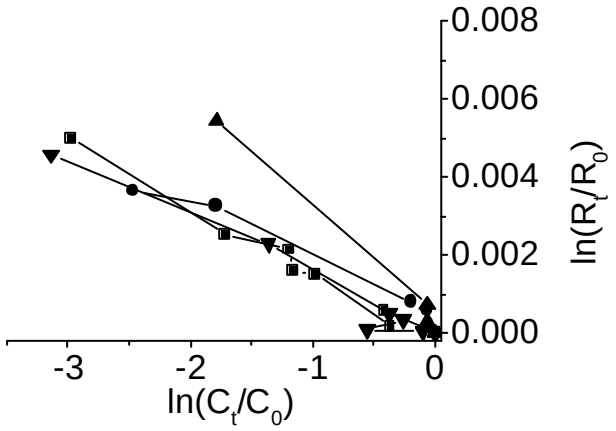

Fig. 2. Stable carbon isotope fractionation during degradation of *m*-xylene (■) and *o*-xylene (●) by strain OX39, and *m*-cresol (▲) and *p*-cresol (▼) by *D. cetonicum*. $^{13}C/^{12}C$ isotope data are plotted according to equation (2).

By far the most information on isotope fractionation by glycyl radical reactions is available concerning toluene degradation under various redox conditions. In a comparative study, carbon isotope fractionation factors determined for bacterial pure cultures using Fe(III), NO_3^-, or SO_4^{2-} as electron acceptors were found to be between ε = -1.8‰ and ε =-1.7‰ (Meckenstock et al., 1999). These results where in the same order as what we retrieved for *p*-cresol, *m*-, and *o*-xylene. Another study reported on isotope fractionation upon toluene degradation of ε = -0.8‰ and ε = -0.5‰ by sulfate-reducing and methanogenic enrichment cultures, respectively, which were significantly lower than those measured in our experiments (Ahad et al., 2000).

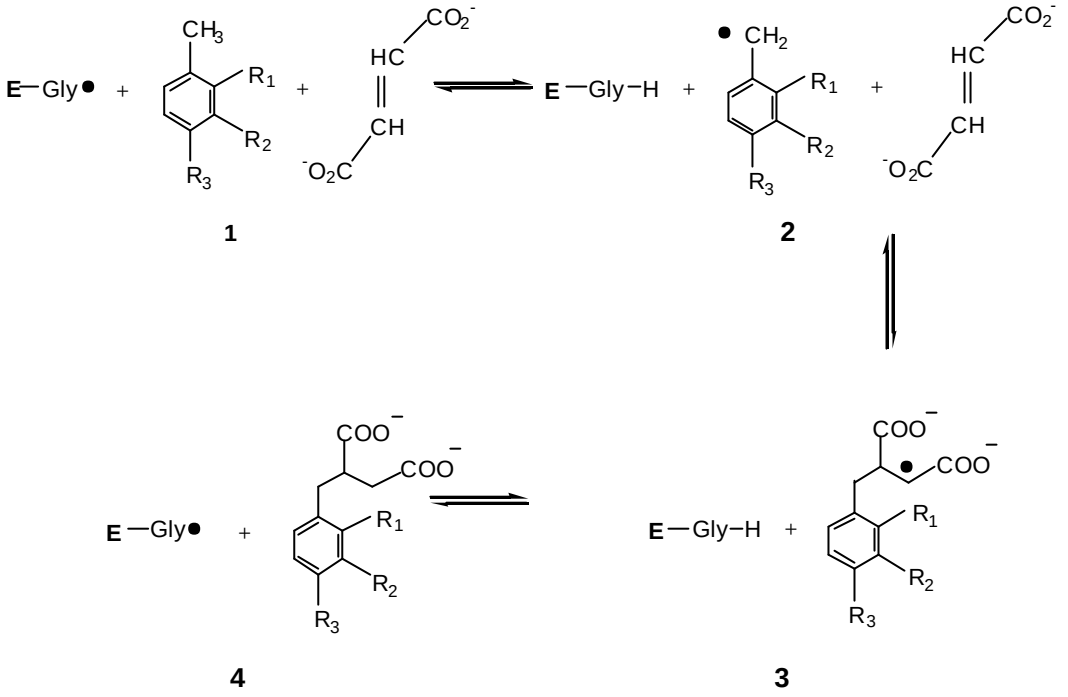

Fig. 3. Reaction mechanism proposed for benzylsuccinate synthase. (Modified after Frey, 2001 (Frey, 2001) for *m*-, *o*-xylene, and *m*-, or *p*-cresol, with R_1, R_2, R_3 = H, CH_3, OH, respectively.)

Summarizing the data available on isotope fractionation during degradation reactions employing glycyl radical mechanisms the lowest fractionation (ε = -0.5‰) was found for toluene degradation under methanogenic conditions, and the strongest one (ε = –3.9‰) for degradation of *m*-cresol by *D. cetonicum*. Most enrichment factors obtained were about ε = -1.8‰. It should be emphasized that isotope fractionation in the radical reactions of the benzylsuccinate synthase type involves just one methyl carbon atom at the reactive site. However, determination of enrichment factors by compound specific isotope analysis (CSIA) gives overall $\delta^{13}C$ values of the entire molecule. This discrepancy can be overcome by calculating site-specific isotope fractionation factors $\alpha_{intrinsic}$, which refer to the atom of the target molecule

where the enzymatic reaction takes place. The following formula was deduced from the Rayleigh equation 2 to consider also the number *n* of carbon atoms of the substrate molecule:

$$\alpha_{intrinsic} = 1/\left(\left(\frac{n}{a}\right) - (n-1)\right) \qquad (4)$$

This allows the normalization of isotope fractionation of aromatic compounds with different numbers of carbon atoms in order to study specific reactions in detail. However, the comparison of intrinsic enrichment factors ($\varepsilon_{intrinsic}$) upon reactions of the benzylsuccinate synthase type in table (1) shows that the different intrinsic factors ($\overline{e_{\text{intrinsic}}} = -12.3 \pm 5.8‰$) vary to the same extent as the overall enrichment factors ($\bar{e} = -1.67 \pm 0.86‰$), which are 51% and 47% standard deviation, respectively (Table 1).

Table 1. Comparison of compound-specific carbon isotope enrichment factors (ε) to the intrinsic isotope enrichment calculated for the carbon atoms at the molecular site of the reaction ($\varepsilon_{intrinsic}$) of the respective substrate. Average enrichment factors ε and $\varepsilon_{intrinsic}$ ± standard deviations were calculated from the sum of all data presented in the table.

Bacterial strain	**Substrate**	e	$e_{intrinsic}$	**Reference**
Thauera aromatica	Toluene	-1.7	-11.9	(Meckenstock et al., 1999)
Geobacter metallireducens	Toluene	-1.8	-12.6	(Meckenstock et al., 1999)
Sulfate-reducing strain TRM1	Toluene	-1.7	-11.9	(Meckenstock et al., 1999)
Desulfobacterium cetonicum	Toluene	-2.2	-15.4	(Morasch et al., 2001)
Sulfate-reducing enrichment culture	Toluene	-0.8	-5.6	(Ahad et al., 2000)
Methanogenic enrichment culture	Toluene	-0.5	-3.5	(Ahad et al., 2000)
Sulfate-reducing strain OX39	*m*-Xylene	-1.8	-14.4	This study
Sulfate-reducing strain OX39	*o*-Xylene	-1.5	-12.0	This study
Desulfobacterium cetonicum	*m*-Cresol	-3.9	-27.3	This study
Desulfobacterium cetonicum	*p*-Cresol	-1.6	-11.2	This study
Sulfate-reducing enrichment culture	2-Methylnaphthalene	-0.9	-9.9	(Griebler, unpublished results)
Average		-1.67 ± 0.86	-12.3 ± 5.8	

Obviously, the specific properties of every single enzyme catalyzing the same type of reaction but taking other substrates influence the degree of isotope fractionation. Thus, the use of the intrinsic enrichment factor does not lead to additional gain of information.

Nevertheless, from the enzyme reactions known so far one can assume an average intrinsic fractionation factor and could speculate on the maximal molecular size that would still allow a measurable isotope fractionation of the whole molecule in benzylsuccinate type reactions in the field. Taking an average inaccuracy of isotope analysis of ± 0.5‰ at the detection limits, the absolute isotope shift needed to detect reliably isotope fractionation would be about 2‰ if about 90% of the substrate was degraded. This would correlate to an overall enrichment factor of ε = -0.895‰. Taking the average intrinsic enrichment factor from table 1 the presumptions would be fulfilled for molecules such as, e.g., toluene with seven carbon atoms ($\varepsilon_{overall}$ = -1.76‰) or xylene ($\varepsilon_{overall}$ = -1.54‰). The borderline would be at molecules of 13 carbon atoms with $\varepsilon = -0.949$‰. Beyond 14 carbon atoms the overall enrichment factor ($\varepsilon = -0.881$‰) is probably too low to allow significant isotope shifts upon degradation to be analyzed. 2-Methylnaphthalene with 11 carbon atoms is close to the analytical limits, but isotope fractionation can be measured under ideal conditions.

In situ, enrichment of heavier isotopes along a groundwater flow can be taken as qualitative evidence for biodegradation. In addition, the extent of bacterial degradation can be quantified combining the isotope signatures determined in the field with the laboratory-derived fractionation factors and using the Rayleigh equation 2 (Meckenstock et al., 2002; Richnow et al., 2003). The enrichment factors for *m*-cresol, *p*-cresol, *m*-xylene, and *o*-xylene from this study were used together with assumed changes in the isotope signatures between $\delta^{13}C$ = –30‰ and $\delta^{13}C$ = –20‰ to calculate the portion of substrate remaining compared to the initial concentration (Fig. 4). The percentage of biodegradation is defined as according to equation 5 (Richnow et al., 2003).

$$B[\%] = 100 \times (1\text{-}C_t/C_0) \qquad (5)$$

To give an impression of how accurate an average fractionation factor would be we used $\bar{e} = -1.67 \pm 0.86$‰ from table 1 to calculate the extent of biodegradation based on assumed isotope shifts. At small isotope shifts of 1‰ in the field, the extent of biodegradation would be 45.5 ± 16.15% (Fig. 4).

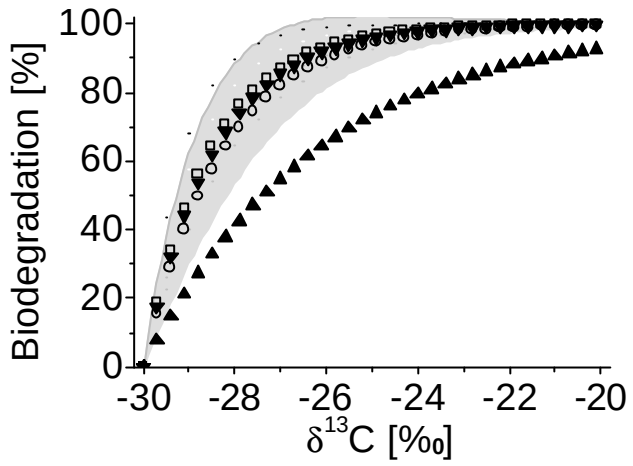

Fig. 4. Calculation of the percentage of bacterial biodegradation *B*[%] based on assumed $\delta^{13}C$ [‰] isotope shifts. Curves with symbols are based on the isotope enrichment factors determined here for *D. cetonicum* degrading *m*-cresol (▲), *p*-cresol (▼), and OX39 degrading *m*-xylene (○), and *o*-xylene (□). The error range of biodegradation calculated for the average glycyl radical type enrichment factor is marked in grey.

The error becomes smaller with increasing extent of biodegradation, e.g., an isotope shift of 5‰ results in an average biodegradation of 95.1 ± 6.87% (Fig. 4). Thus, it might be possible to apply an average enrichment factor to calculate the biodegradation of methylated compounds by glycyl radical enzymes in contaminated aquifers if the shifts in $\delta^{13}C$ are sufficiently high.

Acknowledgements

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References

Ahad, J.M.E., Sherwood Lollar, B., Edwards, E.A., Slater, G.F., and Sleep, B.E., 2000. Carbon isotope fractionation during anaerobic biodegradation of toluene: Implications for intrinsic bioremediation. Environ. Sci. Technol. 34, 892-896.

Annweiler, E., Materna, A., Safinofski, M., Kappler, A., Richnow, H.H., Michaelis, W., and Meckenstock, R.U., 2000. Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. Appl. Environ. Microbiol. 66, 5329-5333.

Biegert, T., Fuchs, G., and Heider, J., 1996. Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur. J. Biochem. 238, 661-668.

Boll, M., Fuchs, G., and Heider, J., 2002. Anaerobic oxidation of aromatic compounds and hydrocarbons. Curr. Opin. Chem. Biol. 6, 604-611.

Clark, I., and Fritz, P., 1997. Environmental isotopes in hydrogeology. Boca Raton, Florida: Lewis Publishers.

Cline, J.D., 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. 14, 454-458.

Frey, P.A., 2001. Radical mechanisms of enzymatic catalysis. Annu. Rev. Biochem. 70, 121-148.

Gibson, J., and Harwood, C.S., 2002. Metabolic diversity in aromatic compounds utilization by anaerobic microbes. Annu. Rev. Microbiol. 56, 345-369.

Himo, F., 2002. Catalytic mechanism of benzylsuccinate synthase, a theoretical study. J. Phys. Chem. 106, 7688-7692.

Hoefs, J., 1997. Stable isotope geochemistry. Berlin, Germany: Springer Verlag.

Hunkeler, D., Aravena, R., and Butler, B.J., 1999. Monitoring microbial dechlorination of tetrachloroethene (PCE) in groundwater using compound-specific stable carbon isotope ratios: Microcosm and field studies. Environ. Sci. Technol. 33, 2733-2738.

Krieger, C.J., Roseboom, W., Albracht, S.P.J., and Spormann, A.M., 2001. A stable organic free radical in anaerobic benzylsuccinate synthase of *Azoarcus* sp. strain T. J. Biol. Chem. 276, 12924-12972.

Krzycki, J.A., Kenealy, W.R., DeNiro, M.J., and Zeikus, J.G., 1987. Stable isotope fractionation by *Methanosarcina barkeri* during methanogenesis from acetate, methanol, or carbon dioxide-hydrogen. Appl. Environ. Microbiol. 53, 2597-2599.

Mancini, S.A., Ulrich, A.C., Lacrampe-Couloume, G., Sleep, B.E., Edwards, E.A., and Sherwood Lollar, B., 2003. Carbon and hydrogen isotopic fractionation during anaerobic biodegradation of benzene. Appl. Environ. Microbiol. 69, 191-198.

Meckenstock, R.U., Morasch, B., Kästner, M., Vieth, A., and Richnow, H.H., 2002. Assessment of bacterial degradation of aromatic hydrocarbons in the environment by analysis of stable carbon isotope fractionation. Water, Air and Soil Pollution: Focus 2, 141-152.

Meckenstock, R.U., Morasch, B., Warthmann, R., Schink, B., Annweiler, E., Michaelis, W., and Richnow, H.H., 1999. $^{13}C/^{12}C$ isotope fractionation of aromatic hydrocarbons during microbial degradation. Environ. Microbiol. 1, 409-414.

Morasch, B., Richnow, H.H., Schink, B., and Meckenstock, R.U., 2001. Stable hydrogen isotope fractionation during microbial toluene degradation: Mechanistic and environmental aspects. Appl. Environ. Microbiol. 67, 4842-4849.

Morasch, B., Richnow, H.H., Schink, B., Vieth, A., and Meckenstock, R.U., 2002. Carbon and hydrogen stable isotope fractionation during aerobic bacterial degradation of aromatic hydrocarbons. Appl. Environ. Microbiol. 68, 5191-5194.

Müller, J.A., Galushko, A., Kappler, A., and Schink, B., 1999. Anaerobic degradation of *m*-cresol by *Desulfobacterium cetonicum* is initiated by formation of 3-hydroxybenzylsuccinate. Arch. Microbiol. 172, 287-294.

Müller, J.A., Galushko, A., Kappler, A., and Schink, B., 2001. Initiation of anaerobic degradation of *p*-cresol by formation of 4-hydroxybenzylsuccinate in *Desulfobacterium cetonicum*. J. Bacteriol. 183, 752-757.

O'Leary, M.H., 1984. Carbon isotope fractionation in plants. Phytochemistry 20, 553-567.

Rayleigh, J.W.S., 1896. Theoretical considerations respecting the separation of gases by diffusion and similar processes. Philos. Mag. 42, 493-498.

Richnow, H.H., Annweiler, E., Michaelis, W., and Meckenstock, R.U., 2003. Microbial *in situ* degradation of aromatic hydrocarbons in a contaminated aquifer monitored by carbon isotope fractionation. J. Contam. Hydrol. 65, 101-120.

Sherwood Lollar, B., and Slater, G.F., 2001. Stable carbon isotope evidence for intrinsic bioremediation of tetrachloroethene and trichloroethene at area 6, Dover Air Force Base. Environ. Sci. Technol. 35, 261-269.

Spormann, A.M., and Widdel, F., 2000. Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. Biodegradation 11, 85-105.

Widdel, F., and Bak, F., 1992. Gram-negative mesophilic sulfate-reducing bacteria. *In* The prokaryotes. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York: Springer Verlag, pp. 3352-3378.

7 Discussion and outlook

Improved cultivation techniques for anaerobic bacteria with toxic substrates

The results obtained show that the utilization of Amberlite-XAD7 in batch experiments is promising, both for the cultivation of bacteria and for the investigation of their degradation pathways. Since high concentrations of aromatic hydrocarbons may be toxic for bacteria, the adsorber resin is used to keep concentrations in the aqueous phase of the culture bottles low. Simultaneously, the adsorber resin provides a carbon source, which is adsorbed to the resin's surface. Using Amberlite-XAD7 in bacterial cultures was set up for growth with nonpolar substrates such as BTEX compounds and naphthalene. Appropriate dissolution kinetics of aromatic hydrocarbons from Amberlite-XAD7 into the aqueous phase governs its function as a suitable substrate reservoir. Toluene, xylene isomers, and naphthalene were dissolved from the particles at a sufficient rate to keep the bacteria steadily growing. The sulfate-reducing *Desulfotomaculum* strain OX39 was isolated via a liquid dilution series in the presence of XAD7 with *o*-xylene as substrate. Later on, the resin was used for cultivation of strain OX39. Apart from strain OX39, further enrichment cultures were obtained when Amberlite-XAD7 was used, namely a sulfate-reducing culture growing with benzene (Safinowski, unpublished results), a *p*-xylene-utilizing enrichment (Morasch, unpublished results), and the highly enriched naphthalene-degrading culture N47 (Meckenstock et al., 2000). Additionally, Amberlite-XAD7 was shown to be an effective tool in tracing the substrate degradation in bacterial batch cultures, since metabolites released by the bacteria into the water phase were adsorbed to the resin surface. Thereby, it was possible to recover four organic acids emerging during anaerobic naphthalene degradation from Amberlite-XAD7. This is a powerful advantage compared to nonpolar liquid carrier phases such as heptamethylnonane or paraffin oil (Rabus et al., 1993). Cultivation approaches with non-liquid carrier phases may offer further potentials. For instance, several adsorber resins were tested in batch culture experiments regarding their adsorption capacities towards *o*-xylene and the more polar methyl *tert*-butyl ether (MTBE) with promising results (Bi, 2003). At present, experiments are being conducted in which bacteria are enriched with MTBE and *tert*-butyl alcohol (TBA) as carbon sources under anoxic conditions with various electron acceptors and two different types of adsorber resins as substrate reservoirs.

Degradation of aromatic hydrocarbons by Desulfotomaculum strain OX39

Classical cultivation approaches that sought for isolating and cultivating sulfate-reducers from the environment mostly led to an enrichment of non spore-forming bacteria. Spore-formers have been isolated only in rare cases. High concentrations of carbon sources in batch cultures favor fast-growing strains; therefore, the slow-growing *Desulfotomaculum spp*. are probably outcompeted. Nevertheless, members of the genus *Desulfotomaculum* have been demonstrated to be abundant in bulk soil or other oligotrophic habitats. Investigations on the bacterial composition of various environments with the application of PCR probes shed light on the occurrence of spore-forming bacteria in anoxic habitats with low carbon concentrations or periodically alternating redox conditions. Based on molecular studies, spore-forming sulfate reducers are considered to be the prevalent bacteria in bulk soil and deep aquifers (Fry et al., 1997; Stubner and Meuser, 2000). However, it is difficult to obtain results about physiological interactions during bacterial contaminant degradation by molecular techniques.

Strain OX39 oxidized toluene, *m*-xylene, and *o*-xylene completely to CO_2. Thus, it is the first known member of the genus *Desulfotomaculum* that degrades aromatic hydrocarbons. Surprisingly, the degradation pathways of the three aromatic hydrocarbons were highly substrate-specific and differed in a – yet – unidentified reaction step, most probably involving the glycyl radical enzymes which initiate their degradation. Changes from one substrate to the other led to considerable lag-phases. Presence of certain substrates interfered with the biodegradation of other substrates, for instance, mixtures of *m*- and *o*-xylene were not simultaneously degraded, and the presence of toluene inhibited *o*-xylene degradation. Cometabolism of dimethylnaphthalenes, which are substrate analogous of xylenes, with *m*- or *o*-xylene was not proven unambiguously, because methyl-naphthoic acid was tentatively identified (Phelps, 2002) but signal metabolites such as succinate-adducts (Beller, 2002) were not detected. The side activity of benzylsuccinate synthase was documented *in vitro,* e.g., for mono-fluortoluenes and benzaldehyde that were not used as growth substrates by *Azoarcus sp.* strain T (Beller and Spormann, 1999). It is worth considering that strain OX39 was isolated from an aquifer contaminated with complex mixtures of BTEX and PAH, where inhibitory effects or cometabolism may occur (Phelps and Young, 2001). In particular cases, toluene was reported to suppress the degradation of *m*- and *o*-xylenes (Barbaro et al., 1992; Meckenstock, unpublished results), and, in sulfide-rich groundwater, toluene was preferentially degraded along the flow path, followed by a decrease in *o*-xylene, naphthalene, *m*- and *p*-xlyene, trimethylbenzenes, ethylbenzene, and benzene (Davis et al., 1999). This

indicates that the link between degradation studies in microcosm experiments and community analysis can be promising to characterize bacterial types responsible for contaminant degradation in the environment and interference of various substrates with each other upon biodegradation.

It was confirmed by metabolite extraction from culture supernatants that strain OX39 degrades toluene via benzylsuccinate and the central intermediate benzoyl-CoA. Evidence that degradation of *m*- and *o*-xylene by strain OX39 proceeds via analogous steps was given by the detection of methyl-benzylsuccinates and methyl-phenylitaconic acids. The currently supposed pathway for degradation of xylene isomers was recently questioned by Elshahed et al., who detected further putative metabolites in enrichment cultures and in environmental samples (Elshahed et al., 2001). Namely, *m*-carboxybenzylsuccinic acid and isophthalic acid were found in extracts of *m*-toluic acid-grown batch cultures. A degradation pathway was proposed that includes a second addition of fumarate to the remaining methyl group. However, these compounds might either be transient metabolites or dead-end products of xylene-degradation. In our experiments with strain OX39 grown with either *m*-xylene or mixtures of *o*-xylene-d_{10} and *o*-xylene, no indications of the intermediates (iso-)phthalic acid or any carboxybenzylsuccinic acids were found, although *m*- and *o*-toluic acid were confirmed as degradation products.

It has still to be elucidated whether *m*-xylene and *o*-xylene are utilized via the central intermediate benzoyl-CoA, like toluene, or if they undergo further degradation with an additional methyl group, starting with methyl-benzoyl-CoA. Widdel and Rabus emphasized that methyl groups in the meta-position would not interfere with β oxidation steps, whereas methyl groups in the ortho- (or para-) position had to be transferred in a way similar to alkane degradation under anaerobic conditions (Rabus et al., 2001; Widdel and Rabus, 2001). Metabolite extraction from cultures of strain OX39 and culture supernatants did not exhibit intermediates with reduced ring systems, as would be produced by benzoyl-CoA reductase and subsequent reaction steps (Harwood et al., 1999). Contrarily, salicylic acid showed up in toluene- and benzoate-grown cultures, as did methyl-salicylic acid when strain OX39 was grown with *m*-xylene. These substances may either be dead-end metabolites excreted by the bacterial cells, or they may be metabolites resulting from a hydroxylation reaction at the aromatic ring performed prior to ring reduction.

Basic principles of isotope fractionation

Hydrogen isotope fractionation was assessed by using gas chromatography to monitor the utilization of selectively deuterated toluene species upon degradation. The GC method is competitive to IRM-GC-MS concerning

sensitivity, reproducibility, costs, and availability. Growth experiments with different bacterial strains, different substrates, and at different temperatures provided a large data set of hydrogen isotope fractionation factors. Investigations of basic principles of isotope fractionation, such as the influence of the substrate's molecular mass, temperature, fractionating step, and the role of the initial enzyme reaction were necessary to answer questions on the biochemistry of aromatic hydrocarbon degradation and to evaluate the application of the isotope fractionation concept in the environment. Isotope fractionation factors obtained for degradation of deuterated toluene species were comparable to isotope fractionation factors during degradation of nonlabeled substrates – with a natural deuterium abundance of 0.15‰ – which illustrated that this approach via GC was appropriate. However, the mathematical description of isotope fractionation is valid under natural conditions if the isotope ratio does not change significantly over time or if the absolute amount of the heavier compound is very small (Rayleigh, 1896; Bigeleisen and Wolfsberg, 1958). Consequently, a modified type of Rayleigh equation was used that considers the initial ratio of labeled and nonlabeled substrates:

$$\ln\left(\frac{R_t}{R_0}\right) = \ln\left(\left(\frac{C_t}{C_0}\right)\times\left(\frac{1}{(1+R_t)/(1+R_0)}\right)\right)\times\left(\frac{1}{aC}-1\right)$$

A table with recalculated fractionation factors is provided by (Hunkeler et al., 2002) (see Appendix).

Isotope fractionation during different enzyme reactions

Isotope fractionation during bacterial degradation of contaminants is caused mainly by enzymatic turnover and not by transport mechanisms. This could be shown with selectively deuterated toluene species and especially in benzylsuccinate synthase assays with cell-free extracts of *Desulfobacterium cetonicum*. It was confirmed that deuterium labels led to isotope shifts only if they were located at the site of the enzymatic reaction. Carbon and hydrogen isotope fractionation was studied with aerobic bacteria, using different enzymatic mechanisms to initiate toluene degradation. The extent of fractionation in the different mechanisms was found to range from very strong to insignificant. If the rate-limiting step was a reaction involving a homolytic cleavage of a carbon-hydrogen bond at the methyl group, isotope fractionation was strong. In contrast, oxygenase reactions attacking the toluene molecule at the π-electrons of the aromatic ring led to characteristically weak carbon and hydrogen isotope fractionation. The range of isotope fractionation during contaminant degradation initiated by glycyl radical type reactions was in the same order of magnitude, but it varied up to a factor of eight with different

substrates and under different conditions. To lay the groundwork for a better comparison of carbon isotope fractionation of substrates with different numbers of carbon atoms, undergoing similar enzymatic mechanisms, the calculation of carbon isotope fractionation for the molecular site of the reaction ($\alpha_{intrinsic}$) is under discussion.

Quantification of contaminant degradation in the environment based on stable isotope fractionation

The isotope fractionation factors obtained in laboratory experiments provide the basis to quantify biodegradation of individual contaminants in the field. Possible changes in the degree of isotope fractionation due to variations in temperature and redox conditions can be excluded since the results from batch experiments showed constant isotope fractionation under anoxic conditions independent from growth temperatures and electron acceptors used. These findings laid the foundation for the qualitative assessment of *in situ* contaminant degradation with laboratory-derived isotope fractionation factors.

The first step towards determination and characterization of bacterial degradation in the environment was the conduction of column experiments under sulfidogenic conditions with toluene and *o*-xylene as substrates (Meckenstock et al., 1999). The concentrations of toluene and *o*-xylene were found to decrease in a gradient from the inlet over various sample ports along the column. Simultaneously, the monitored proportion of the heavier carbon isotope in the remnant substrate was found to increase. The amount of biodegradation was calculated using the laboratory-derived isotope fractionation factors obtained from anaerobic laboratory batch cultures, observed isotope shifts, and the initial concentration at the inlet of the column (Meckenstock et al., 1999).

Successful quantifications of contaminants in the field were performed in several studies. Richnow et al. investigated an aquifer contaminated with tar oil in the city of Hamburg, Germany (Richnow et al., 2002). All analyzed compounds decreased in a gradient along the groundwater flow path, and some of them showed significant isotope fractionation. The extent of biodegradation was calculated for toluene, which was degraded to more than 95 percent in most downstream groundwater wells. Interactions of contaminants with the aquifer matrix did not affect their isotope composition to a significant extent. Indane and indene exhibited constant isotope signatures along the groundwater flow path, although their concentrations also decreased in steep gradients. Further efforts towards the quantification of biodegradation were made for a petroleum spill resulting from a tanker truck accident near Ingolstadt, Germany, (Meckenstock et al., 2002), a landfill leachate at Vejen, Denmark, with significant BTEX contents (Vieth et al., 2001; Richnow et al.,

2003), and for contaminations with chlorinated hydrocarbons (Hunkeler et al., 1999). Fractionation factors during reductive dehalogenation of PCE and TCE were recently applied to determine the *in situ* biodegradation of chlorinated ethenes in the environment (Sherwood Lollar and Slater, 2001; Song et al., 2002). Strong isotope shifts in the residual substrate fraction along the groundwater flow path, combined with the typical pattern of parent and daughter compounds such as TCE, *cis*-DCE, and vinyl chloride, indicated the occurrence of biodegradation (Wiedemeier et al., 1999; Sherwood Lollar and Slater, 2001). However, the TCE and DCE partially originated directly from the contaminant, and thereby complicated the assessment of biodegradation by isotope signatures (Vieth, unpublished results). Nevertheless, biodegradation of PCE and TCE was found to be higher than 40 percent in particular field cases (Sherwood Lollar and Slater, 2001; Vieth, unpublished results).

Quantification efforts at an industrial site in the Netherlands showed that benzene underwent isotope fractionation under Fe(III)-reducing, sulfate-reducing, and methanogenic conditions. The laboratory-derived isotope fractionation factor used was determined before in batch studies with a methanogenic enrichment culture (Mancini et al., 2002). This study illustrated that anaerobic benzene degradation was linked to significant isotope enrichment effects, although the reaction must be located at the aromatic ring. The initial mechanism of anaerobic benzene degradation remains to be elucidated. However, in accordance with predictions, slight but significant isotope enrichments were detected for naphthalene degradation by the sulfate-reducing enrichment culture N47 (Griebler, unpublished results).

The most comprehensive study so far, to quantify biodegradation in the environment, was performed at a tar oil contaminated former gasworks site near Stuttgart, Germany. Degradation of benzene, toluene, xylenes, naphthalene, and 1-methylnaphthalene was quantified along the groundwater flow. In addition, qualitative evidence of degradation was given for ethylbenzene, 2-methylnaphthalene, and benzothiophene (Griebler, unpublished results).

Until now, the technique is not applicable at sites with multiple contaminant sources, such as many industrial areas with a long history of contamination, e.g. former gasworks plants. Moreover, a quantitative assessment is limited, so far, to anoxic environments. Under oxic conditions, different degradation pathways contribute to the overall biodegradation and may be linked to isotope enrichments reaching from minor to large. Isotope fractionation of aromatic hydrocarbons can be taken only as a positive indicator of biodegradation. Lacking isotope fractionation in the field is no sufficient proof to indicate the absence of biodegradation.

The quantification of contaminant degradation in the environment based on stable isotope analysis might become a routine application for spills with BTEX or chlorinated hydrocarbons. Moreover, further substances of environmental concern are investigated in respect to their isotope fractionation upon biodegradation and a potential *in situ* quantification, e.g., the fuel additives MTBE and TBA. As various types of enzymatic reactions lead to a significant degree of isotope fractionation the spectrum of compounds whose degradation can be monitored via isotope analysis might be even wider. Combined approaches of stable isotope analysis and an identification of metabolites specific for particular contaminants can help to assess degradation of compounds with smaller extents of isotope fractionation.

References

Barbaro, J.R., Barker, J.F., Lemon, L.A., and Mayfield, C.I., 1992. Biotransformation of BTEX under anaerobic, denitrifying conditions: Field and laboratory observations. J. Contam. Hydrol. 11, 245-272.

Beller, H.R., and Spormann, A.M., 1999. Substrate range of benzylsuccinate synthase from *Azoarcus* sp. strain T. FEMS Microbiol. Lett. 178, 147-153.

Beller, H.R., 2002. Analysis of benzylsuccinates in groundwater by liquid chromatography/tandem mass spectrometry and its use for monitoring in situ BTEX biodegradation. Environ. Sci. Technol. 36, 2724-2728.

Bi, E., 2003. Evaluation of sorbents for enrichment of bacteria with *tert*-butyl alcohol (TBA) and methyl-*tert*-butyl ether (MTBE). Institute for Geoscience, Center for Applied Geoscience (ZAG). Tübingen: Eberhard-Karls-Universität Tübingen.

Bigeleisen, J., and Wolfsberg, M., 1958. Theoretical and experimental aspects of isotope effects in chemical kinetics. Adv. Chem. Phys. 1, 15-76.

Davis, G.B., Barber, C., Power, T.R., Thierrin, J., Patterson, B.M., Rayner, J.L., and Wu, Q., 1999. The variability and intrinsic remediation of a BTEX plume in anaerobic sulphate-rich groundwater. J. Contam. Hydrol. 36, 265-290.

Elshahed, M.S., Gieg, L.M., McInerney, M.J., and Suflita, J.M., 2001. Signature metabolites attesting to the *in situ* attenuation of alkylbenzenes in anaerobic environments. Environ. Sci. Technol. 35, 682-689.

Fry, N.K., Fredrickson, J.K., Fishbain, S., Wagner, M., and Stahl, D.A., 1997. Population structure of microbial communities associated with two deep, anaerobic, alkaline aquifers. Appl. Environ. Microbiol. 63, 1498-1504.

Harwood, C.S., Burchhardt, G., Herrmann, H., and Fuchs, G., 1999. Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. FEMS Microbiol. Rev. 22, 439-458.

Hunkeler, D., Aravena, R., and Butler, B.J., 1999. Monitoring microbial dechlorination of tetrachloroethene (PCE) in groundwater using compound-specific stable carbon isotope ratios: Microcosm and field studies. Environ. Sci. Technol. 33, 2733-2738.

Hunkeler, D., Meckenstock, R.U., and Richnow, H.H., 2002. Quantification of isotope fractionation in experiments with deuterium-labeled substrate. Appl. Environ. Microbiol. 68, 5205-5207.

Mancini, S.A., Lacrampe-Couloume, G., Jonker, H., Van Breueklen, B.M., Groen, J., Volkering, F., and Sherwood Lollar, B., 2002. Hydrogen isotopic enrichment: An indicator of biodegradation at a petroleum hydrocarbon contaminated field site. Environ. Sci. Technol. 36, 2464-2470.

Meckenstock, R.U., Annweiler, E., Warthmann, R., Schink, B., Michaelis, W., and Richnow, H.H., 1999. $^{13}C/^{12}C$ stable isotope fractionation of toluene by anaerobic degradation: A new method to monitor biological degradation *in situ*? *In* Novel approaches for bioremediation of organic pollution. Fass, R., Flashner, Y., and Reuveny, S. (eds). New York: Kluwer Academic Publishers, pp. 219-227.

Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B., 2000. Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Appl. Environ. Microbiol. 66, 2743-2747.

Meckenstock, R.U., Morasch, B., Kästner, M., Vieth, A., and Richnow, H.H., 2002. Assessment of bacterial degradation of aromatic hydrocarbons in the environment by analysis of stable carbon isotope fractionation. Water, Air and Soil Pollution: Focus 2, 141-152.

Phelps, C.D., and Young, L.Y., 2001. Biodegradation of BTEX under anaerobic conditions: a review. Adv. Agronom. 70, 329-357.

Phelps, C.D., Battistelli, J., and Young, L.Y., 2002. Metabolic biomarkers for monitoring anaerobic naphthalene biodegradation *in situ*. Environ. Microbiol. 4, 532-537.

Rabus, R., Nordhaus, R., Ludwig, W., and Widdel, F., 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. 59, 1444-1451.

Rabus, R., Wilkes, H., Behrends, A., Armstroff, A., Fischer, T., Pierik, A.J., and Widdel, F., 2001. Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: Evidence for (1-methylpentyl)succinate as initial product and for involvement of an organic radical in *n*-hexane metabolism. J. Bacteriol. 183, 1707-1715.

Rayleigh, J.W.S., 1896. Theoretical considerations respecting the separation of gases by diffusion and similar processes. Philos. Mag. 42, 493-498.

Richnow, H.H., Vieth, A., Kästner, M., Gehre, M., and Meckenstock, R.U., 2002. Isotope fractionation of toluene: A perspective to characterise microbial *in situ* degradation. Sci. World J. 2, 7227-7234.

Richnow, H.H., Meckenstock, R.U., Ask, L., Baun, A., Ledin, A., and Christensen, T.H., 2003. *In situ* biodegradation determined by carbon isotope fractionation of aromatic hydrocarbons in an anaerobic landfill leachate plume (Vejen, Denmark). J. Contam. Hydrol. 59-72.

Sherwood Lollar, B., and Slater, G.F., 2001. Stable carbon isotope evidence for intrinsic bioremediation of tetrachloroethene and trichloroethene at area 6, Dover Air Force Base. Environ. Sci. Technol. 35, 261-269.

Song, D.L., Conrad, M.E., Sorenson, K.S., and Alvarez-Cohen, L., 2002. Stable carbon isotope fractionation during enhanced *in situ* bioremediation of trichloroethene. Environ. Sci. Technol. 36, 2262-2268.

Stubner, S., and Meuser, K., 2000. Detection of *Desulfotomaculum* in an Italian rice paddy soil by 16S ribosomal nucleic acid analyses. FEMS Microbiol. Ecol. 34, 73-80.

Vieth, A., Kästner, M., Morasch, B., Meckenstock, R.U., and Richnow, H.H., 2001. Charakterisierung des biologischen Abbaus von BTEX im Grundwasser über Isotopenfraktionierung - Feldstudien. TerraTech 37-41.

Widdel, F., and Rabus, R., 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr. Opin. Biotechnol. 12, 259-276.

Wiedemeier, T.H., Rifai, H.S., Newell, C., J., and Wilson, J.T., 1999. Natural attenuation of fuels and chlorinated solvents in the subsurface. New York: John Wiley & Sons, Inc.

8 Summary

1. Adsorber resin Amberlite-XAD7 was applied in bacterial batch cultures to enrich, isolate, and cultivate bacteria that use putatively toxic substrates. Adsorption to the resin surface substantially reduced the initial concentrations of *o*-xylene and naphthalene in the water phase within five days. As a result, toxic effects of aromatic hydrocarbons towards bacteria could be avoided and, simultaneously, a substrate reservoir could be provided.
2. Among other cultures, strain OX39 was enriched and isolated from contaminated aquifer material, in the presence of XAD7 and with *o*-xylene as a substrate. Strain OX39 is a rod-shaped Gram-positive endospore-forming sulfate-reducing bacterium. Analysis of the 16S rRNA gene showed its phylogenetic affiliation with the genus *Desulfotomaculum*. Strain OX39 is sensitive towards sulfide and depends on the presence of Fe(II) in the medium. The optimum pH for growth was around 7.4, and the optimum temperature for growth between 25 and 30°C. The upper temperature limit was found to be at 36°C, at which point sulfide production ceased. Electron acceptors alternative to sulfate could not be used. The substrate range was found to be limited to toluene, *m*- and, *o*-xylene, as well as their degradation products benzoic acid, *m*-, and *o*-toluic acid, respectively. Toluene, *m*-xylene, and *o*-xylene are completely oxidized to CO_2. By extraction of signature metabolites from culture supernatants, it was confirmed that the degradation of toluene, *m*-xylene, and *o*-xylene proceeds via fumarate addition to one methyl group. Thus, strain OX39 is the first aromatic hydrocarbon-using member of the genus *Desulfotomaculum*.
3. For measurement of $^2H/^1H$ isotope fractionation upon bacterial toluene degradation by gas chromatographic analysis an alternative approach was developed in order to investigate the basic features of isotope fractionation. Methyl group-deuterated toluene-d_3, ring-deuterated toluene-d_5, completely-deuterated toluene-d_8, and nonlabeled toluene were supplied as substrates in pairs of equal amounts. The ratio of both substrates was determined upon growth (R_t = [toluene-d_8]/[toluene]) and used to calculate the hydrogen isotope fractionation. The ratio of the two concentrations, as analyzed by GC, was reproducible ± 2% standard deviation. Results acquired with selectively-deuterated toluene species could be validated with nonlabeled toluene by hydrogen isotope fractionation using IRM-GC-MS.
4. Upon degradation of toluene under sulfate-reducing conditions, carbon and hydrogen isotope fractionation were found to be independent of growth temperatures. In contrast, carbon and hydrogen isotope fractionation upon

aerobic toluene degradation by *P. putida* strain mt-2 was found to vary with growth temperatures. The isotope fractionation was not influenced by degradation rates.

5. Isotope enrichment factors for toluene determined in growth experiments with bacterial pure cultures taking nitrate, Fe(III), or sulfate as electron acceptors were very close to each other. Significantly higher isotope enrichment was observed during toluene degradation under oxic conditions. Therefore, important prerequisites for quantification of contaminant degradation *in situ* might be fulfilled only under anoxic conditions, as in absence of O_2 isotope enrichment might be considered independent of temperature changes and of redox conditions.
6. Growth experiments using several combinations of selectively-deuterated toluene species revealed that the main process leading to isotope fractionation was the first enzyme reaction, whereas substrate diffusion to the cells and substrate uptake by the bacteria was negligible. Three bacterial strains which initiate toluene degradation with an enzyme reaction at the methyl group showed pronounced fractionation effects if one toluene species with a deuterated methyl group was supplied in combination with another toluene species with a nondeuterated methyl group, i.e., methyl-group-labeled toluene-d_3/nonlabeled toluene or ring-labeled toluene-d_5/fully-deuterated toluene-d_8. In contrast, during degradation of toluene-d_3/toluene-d_8 and toluene-d_5/nonlabeled toluene no isotope effects were observed. Influence of additional masses – by deuterium labels – on the bacterial isotope enrichment was not detectable. Direct comparison of stable hydrogen isotope fractionation upon anaerobic toluene turnover by benzylsuccinate synthase to toluene degradation in growth experiments revealed slightly stronger fractionation in the enzyme assay, confirming that this enzyme reaction was the key step for isotope fractionation.
7. Systematic investigations of stable carbon isotope fractionation during enzyme reactions of the glycyl radical type, with different substrates and various bacteria, illustrated that the range of isotope enrichment caused by this mechanism was narrow. An average enrichment factor of all single factors determined for this reaction type so far may be applicable to quantify overall biodegradation of contaminants degraded via this pathway in the environment.
8. Under oxic conditions, degradation of toluene can proceed via different pathways. One bacterial degradation strategy targets a carbon-hydrogen bond of the methyl group in a monooxygenase reaction, a mechanism accompanied by large isotope enrichment effects that varied just slightly between different methyl-benzenes turned over by the same methyl monooxygenase. Other strains use mono- and dioxygenase reactions attacking the aromatic ring. New

bonds are formed between π-electrons of the substrate and activated oxygen which is bound to catalytic iron centres of oxygenase enzymes. These reactions led to characteristically weak or insignificant hydrogen and carbon isotope fractionation. The big differences in isotope enrichment for aerobic degradation of aromatic hydrocarbons preclude quantitative applications in the environment. However, isotope shifts can be taken as positive indications for *in situ* biodegradation.

9 Zusammenfassung

1. Zur Anreicherung, Isolierung und Kultivierung von Bakterien, die potentiell toxische Substrate verwerten, wurde Adsorberharz Amberlite-XAD7 den Kulturflaschen zugesetzt. Die Anfangskonzentrationen von *o*-Xylol und Naphthalin in der wässrigen Phase verringerten sich in Gleichgewichtsexperimenten innerhalb von fünf Tagen signifikant durch eine Adsorption dieser Stoffe an die Oberfläche des Amberlite-XAD7. Eine unter Umständen toxische Wirkung aromatischer Kohlenwasserstoffe auf die Bakterien wurde durch die Verwendung des Adsorberharzes verhindert und darüber hinaus konnte für die Bakterien ein Substratvorrat zur Verfügung gestellt werden.
2. Neben weiteren Bakterienkulturen wurde Stamm OX39 in Gegenwart von XAD7 mit *o*-Xylol als Substrat aus kontaminiertem Bodenmaterial angereichert und isoliert. Stamm OX39 ist ein stäbchenförmiger, Gram-positiver, endosporenbildender Sulfatreduzierer. Die Analyse seines 16S rRNA-Gens zeigte die phylogenetische Zugehörigkeit zum Genus *Desulfotomaculum*. Stamm OX39 ist empfindlich gegenüber freiem Sulfid und deshalb auf die Zugabe von Eisen(II) in ihr Mineralmedium angewiesen. Der bevorzugte pH für das Wachstum lag im Bereich von 7,4 und die optimale Wachstumstemperatur zwischen 25°C und 30°C. Die obere Temperaturgrenze, an der Stamm OX39 die Sulfidproduktion einstellte, war bei 36°C. Abgesehen von Sulfat konnte kein weiterer Elektronenakzeptor verwendet werden. Wachstumssubstrate von Stamm OX39 beschränkten sich auf Toluol, *m*-Xylol und *o*-Xylol, sowie auf deren Abbauprodukte Benzoesäure, *m*- und *o*-Tolylsäure. Toluol, *m*-Xylol und *o*-Xylol wurden vollständig zu CO_2 oxidiert. Der Abbauweg beginnt in allen drei Fällen mit einer Additionsreaktion von Fumarat an die Methylgruppe, was durch die Identifizierung von Schlüsselmetaboliten in Kulturüberständen gezeigt werden konnte. Stamm OX39 ist somit das erste dem Genus *Desulfotomaculum* angehörende Bakterium, welches aromatische Kohlenwasserstoffe verwertet.
3. Zur Messung der $^2H/^1H$ Isotopenfraktionierung im Zuge des bakteriellen Toluolabbaus wurde ein auf Gaschromatographie basierendes Verfahren entwickelt. Es ermöglichte die Untersuchung grundlegender Prinzipien der bakteriellen Isotopenfraktionierung. Methylgruppendeuteriertes Toluol-d_3, ringdeuteriertes Toluol-d_5, volldeuteriertes Toluol-d_8 und unmarkiertes Toluol wurden paarweise als Substrate zu jeweils gleichen Teilen zugegeben. Die $^2H/^1H$ Isotopenfraktionierung während des Wachstums wurde als Änderung des Verhältnisses beider Substrate zueinander zu verschiedenen Zeitpunkten definiert (R_t = [Toluol-d_8]/[Toluol]). Das Verhältnis beider Toluolspezies war

innerhalb von ± 2% ihrer Standardabweichung zuverlässig reproduzierbar. Die Ergebnisse aus Experimenten mit spezifisch markierten Toluolmolekülen wurden mit der Isotopenfraktionierung beim Abbau von unmarkiertem Toluol – analysiert per IRM-GC-MS – auf ihre Gültigkeit überprüft.

4. Die Kohlenstoff- und Wasserstoffisotopenfraktionierung beim Toluolabbau war unter sulfatreduzierenden Bedingungen unabhängig von der Wachstumstemperatur. Im Gegensatz dazu variierten beim aeroben Toluolabbau durch *P. putida* Stamm mt-2 sowohl Kohlenstoff- als auch Wasserstoffisotopenfraktionierung mit der Wachstumstemperatur. Die Isotopenfraktionierung war unabhängig von den Abbauraten.
5. Isotopen-Anreicherungsfaktoren für Toluol wurden in Wachstumsversuchen mit verschiedenen Bakterienstämmen, die Nitrat, Fe(III) oder Sulfat als Elektronenakzeptoren verwendeten, ermittelt und waren miteinander vergleichbar. Deutlich höhere Anreicherungsfaktoren ergaben sich für den aeroben Toluolabbau mit *P. putida* Stamm mt-2. Da die Isotopenfraktionierung nur unter anoxischen Bedingungen unabhängig von der Temperatur und den verwendeten Elektronenakzeptoren war, ist die Abwesenheit von O_2 eine Grundvoraussetzung für die Quantifizierung des Schadstoffabbaus in der Umwelt.
6. Wachstumsexperimente, mit deuterierten Toluolspezies in verschiedenen Kombinationen zeigten, dass die erste Enzymreaktion der wichtigste Prozess war, der zu einer Isotopenfraktionierung führte. Die Diffusion des Substrats zur Zelle sowie die Substrataufnahme in die Zelle waren bezüglich ihrer Fraktionierungseffekte vernachlässigbar. Drei Bakterienstämme, bei welchen der Toluolabbau mit einer Reaktion an der Methylgruppe beginnt, zeigten eine starke Isotopenfraktionierung, wenn Toluolspezies mit markierter Methylgruppe in Kombination mit Toluol, das an der Methylgruppe nicht markiert war, verwendet wurden. Substratpaare mit methylgruppendeuteriertem Toluol-d_3/unmarkiertem Toluol oder ringdeuteriertem Toluol-d_5/vollständig deuteriertem Toluol führten zu einer ausgeprägten Isotopenfraktionierung. Im Gegensatz dazu waren in Abbauversuchen mit den Substratpaaren Toluol-d_3/Toluol-d_8 und Toluol-d_5/unmarkiertes Toluol keine Isotopeneffekte messbar. Isotopenfraktionierungseffekte durch die zusätzlichen Massen der Deuteriumatome war beim bakteriellen Abbau aromatischer Kohlenwasserstoffe nicht nachweisbar. Die $^2H/^1H$ Isotopenfraktionierung wurde in zellfreiem Extrakt für den anaeroben Toluolumsatz durch das Enzym Benzylsuccinatsynthase bestimmt. Vergleiche ergaben eine stärkere Isotopenfraktionierung im Enzymtest als in den entsprechenden Wachstumsexperimenten. Dieses Ergebnis bestätigt, dass die initiale Enzymreaktion der entscheidende Schritt ist, der zu einer Isotopenfraktionierung führt.

7. Eine systematische Untersuchung der Fraktionierung stabiler Kohlenstoffisotope wurde mit verschiedenen Bakterienstämmen und unterschiedlichen Substraten durchgeführt, die immer mit derselben Enzymreaktion starteten. Sämtliche Enzymreaktionen, die durch ein Glycyl-Radikal katalysiert wurden, verursachten Isotopenanreicherungsfaktoren in einem engen Bereich. Die Berechnung des durchschnittlichen Anreicherungsfaktors für diesen Reaktionstyp legt nahe, dass eine Gesamtquantifizierung für alle Schadstoffe, die in der Umwelt über diesen Abbauweg umgesetzt werden, durchgeführt werden könnte.
8. Unter oxischen Bedingungen gibt es verschiedene Abbauwege für Toluol. Eine Abbaustrategie der Bakterien greift an einer C-H-Bindung der Methylgruppe an und führt dort eine Hydroxylgruppe ein. Diese Methylmonooxygenase-Reaktion bewirkte deutliche Isotopenanreicherungseffekte. Die $^{13}C/^{12}C$ Isotopenfraktionierung variierte nur gering zwischen verschiedenen Methyl-Benzolen, die vom gleichen Enzym umgesetzt wurden. Andere Bakterienstämme benutzen Mono- bzw. Dioxygenasen, die am aromatischen Ring angreifen. Neue Bindungen entstehen zwischen den π-Elektronen des Substrats und aktiviertem Sauerstoff, gebunden an katalytische Eisenzentren der Oxygenasen. Diese Reaktionen führten zu charakteristisch schwachen bis nicht-signifikanten Wasserstoff- und Kohlenstoffisotopeneffekten. Diese deutlichen, mechanistisch bedingten Unterschiede beim aeroben Abbau aromatischer Kohlenwasserstoffe schließen eine Quantifizierung *in situ* aus. Jedoch kann eine Verschiebung in der Isotopenzusammensetzung einzelner Substanzen als Hinweis für ihren Bioabbau gelten.

10 References

Achong, G.R., Rodriguez, A.M., and Spormann, A.M., 2001. Benzylsuccinate synthase of *Azoarcus sp.* strain T: Cloning, sequencing, transcriptional organization, and its role in anaerobic toluene and *m*-xylene mineralization. J. Bacteriol. 183, 6763-6770.

Ahad, J.M.E., Sherwood Lollar, B., Edwards, E.A., Slater, G.F., and Sleep, B.E., 2000. Carbon isotope fractionation during anaerobic biodegradation of toluene: Implications for intrinsic bioremediation. Environ. Sci. Technol. 34, 892-896.

Amann, R., Ludwig, W., and Schleifer, K.-H., 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. FEMS Microbiol. Rev. 59, 143-169.

Anders, H.J., Kaetzke, A., Kampfer, P., Ludwig, W., and Fuchs, G., 1995. Taxonomic position of aromatic-degrading denitrifying pseudomonad strains K172 and KB740 and their description as new members of the genera *Thauera*, as *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov, respectively, members of the beta subclass of the proteobacteria. Int. J. Syst. Bacteriol. 45, 327-333.

Annweiler, E., Materna, A., Safinofski, M., Kappler, A., Richnow, H.H., Michaelis, W., and Meckenstock, R.U., 2000. Anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture. Appl. Environ. Microbiol. 66, 5329-5333.

Arneth, J.D., Milde, G., Kerndorff, H., and Schleyer, R., 1989. Waste deposit influences on groundwater quality as a tool for waste type and site selection for final storage quality. *In* The Landfill. - Lecture notes in earth sciences. Baccini, P. (ed). Berlin: Springer Verlag.

Ball, H.A., Johnson, H.A., Reinhard, M., and Spormann, A.M., 1996. Initial reactions in anaerobic ethylbenzene oxidation by a denitrifying bacterium, strain EB1. J. Bacteriol. 178, 5755-5761.

Barbaro, J.R., Barker, J.F., Lemon, L.A., and Mayfield, C.I., 1992. Biotransformation of BTEX under anaerobic, denitrifying conditions: Field and laboratory observations. J. Contam. Hydrol. 11, 245-272.

Bartholomew, J.W., and Mittwer, T., 1952. The Gram stain. Bacteriol. Rev. 16, 1-29.

Bedessem, M.E., Swoboda-Colberg, N.G., and Colberg, P.J.S., 1997. Naphthalene mineralization coupled to sulfate-reduction in aquifer-derived enrichments. FEMS Microbiol. Lett. 152, 213-218.

Beller, H.R., Ding, W.-H., and Reinhard, M., 1995. Byproducts of anaerobic alkylbenzene metabolism useful as indicators of *in situ* bioremediation. Environ. Sci. Technol. 29, 2864-2870.

Beller, H.R., Spormann, A.M., Sharma, P.K., Cole, J.R., and Reinhard, M., 1996. Isolation and characterization of a novel toluene-degrading, sulfate-reducing bacterium. Appl. Environ. Microbiol. 62, 1188-1196.

Beller, H.R., and Spormann, A.M., 1997a. Benzylsuccinate formation as a means of anaerobic toluene activation by the sulfate-reducing strain PRTOL1. Appl. Environ. Microbiol. 63, 3729-3731.

Beller, H.R., and Spormann, A.M., 1997b. Anaerobic activation of toluene and *o*-xylene by addition to fumarate in denitrifying strain T. J. Bacteriol. 179, 670-676.

Beller, H.R., and Spormann, A.M., 1998. Analysis of the novel benzylsuccinate synthase reaction for anaerobic toluene activation based on structural studies of the product. J. Bacteriol. 180, 5454-5457.

Beller, H.R., and Spormann, A.M., 1999. Substrate range of benzylsuccinate synthase from *Azoarcus* sp. strain T. FEMS Microbiol. Lett. 178, 147-153.

Beller, H.R., and Edwards, E.A., 2000. Anaerobic toluene activation by benzylsuccinate synthase in a highly enriched methanogenic culture. Appl. Environ. Microbiol. 66, 5503-5505.

Beller, H.R., 2002. Analysis of benzylsuccinates in groundwater by liquid chromatography/tandem mass spectrometry and its use for monitoring *in situ* BTEX biodegradation. Environ. Sci. Technol. 36, 2724-2728.

Bi, E., 2003. Evaluation of sorbents for enrichment of bacteria with *tert*-butyl alcohol (TBA) and methyl-*tert*-butyl ether (MTBE). Institute for Geoscience, Center for Applied Geoscience (ZAG). Tübingen: Eberhard-Karls-Universität Tübingen.

Biegert, T., Fuchs, G., and Heider, J., 1996. Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. Eur. J. Biochem. 238, 661-668.

Bigeleisen, J., and Wolfsberg, M., 1958. Theoretical and experimental aspects of isotope effects in chemical kinetics. Adv. Chem. Phys. 1, 15-76.

Blair, N.E., and Aller, R.C., 1995. Anaerobic methane oxidation on the Amazon shelf. Geochim. Cosmochim. Acta. 59, 3707-3715.

Bloom, Y., Aravena, R., Hunkeler, D., Edwards, E.A., and Frape, S.K., 2000. Carbon isotope fractionation during microbial degradation of trichloroethene, *cis*-1,2-cichloroethene, and vinyl chloride: Implications for assessment of natural attenuation. Environ. Sci. Technol. 34, 2768-2772.

Boll, M., Fuchs, G., and Heider, J., 2002. Anaerobic oxidation of aromatic compounds and hydrocarbons. Curr. Opin. Chem. Biol. 6, 604-611.

Borden, R.C., Gomez, C.A., and Becker, M.T., 1994. Geochemical indicators of intrinsic bioremediation. Ground Water 33, 180-189.

Bouwer, E.J., and McCarthy, P.L., 1984. Modeling of trace organics biotransformation in the subsurface. Ground Water 22, 433-440.

Bradford, M.M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Bradley, P.M., and Chapelle, F.H., 1996. Anaerobic mineralization of vinyl chloride in Fe(III)-reducing aquifer sediments. Environ. Sci. Technol. 30, 2084-2086.

Brazeau, B.J., and Lipscomb, J.D., 2000. Kinetics and activation thermodynamics of methane monooxygenase compound Q formation and reaction with substrates. Biochemistry 39, 13503-13515.

Burland, S.M., and Edwards, E.A., 1999. Anaerobic benzene biodegradation linked to nitrate reduction. Appl. Environ. Microbiol. 65, 529-533.

Caldwell, M.E., and Suflita, J.M., 2000. Detection of phenol and benzoate as intermediates of anaerobic benzene biodegradation under different terminal electron-accepting conditions. Environ. Sci. Technol. 34, 1216-1220.

Carey, F.A., and Sundberg, R.J., 1990. Advanced organic chemistry. New York: Plenum Press.

Carredano, E., Karlsson, A., Kauppi, B., Choudhury, D., Gibson, D.T., Eklund, H., and Ramaswamy, S., 2000. Substrate binding site of naphthalene 1,2-dioxygenase: Functional implications of indole binding. J. Mol. Biol. 296, 701-712.

Cerniglia, C.E., 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation 3, 351-368.

Chen, J., Henderson, G., Grimm, C.C., Lloyd, S.W., and Laine, R.A., 1998. Termites fumigate their nests with naphthalene. Nature 392, 558-559.

Christensen, T.H., Kjeldsen, P., Albrechtsen, H.-J., Heron, G., Nielsen, P.H., Bjerg, P.L., and Holm, P.E., 1994. Attenuation of landfill leachate pollutants in aquifers. Crit. Rev. Environ. Sci. Technol. 24, 119-202.

Christensen, T.H., Kjeldsen, P., Bjerg, P.L., Jensen, D.L., Christensen, J.B., Baun, A., Albrechtsen, H.-J., and Heron, G, 2001. Biogeochemistry of landfill leachate plumes. Appl. Geochem. 16, 659-718.

Clark, I., and Fritz, P., 1997. Environmental isotopes in hydrogeology. Boca Raton, Florida: Lewis Publishers.

Cline, J.D., 1969. Spectrophotometric determination of hydrogen sulfide in natural waters. Limnol. Oceanogr. 14, 454-458.

Coates, J.D., Woodward, J., Allen, J., Philip, P., and Lovley, D.R., 1997. Anaerobic degradation of polycyclic aromatic hydrocarbons and alkanes in petroleum-contaminated marine harbor sediments. Appl. Environ. Microbiol. 63, 3589-3593.

Coates, J.D., Bhupathiraju, V.K., Achenbach, L.A., McInerney, M.J., and Lovley, D.R., 2001a. *Geobacter hydrogenophilus, Geobacter chapellei* and *Geobacter grbiciae*, three new, strictly anaerobic, dissimilatory Fe(III)-reducers. Int. J. Syst. Evol. Microbiol. 51, 581-588.

Coates, J.D., Chakraborty, R., Lack, J.G., O´Connor, S.M., Cole, K.A., Bender, K.S., and Achenbach, L.A., 2001b. Anaerobic benzene oxidation coupled to nitrate reduction in pure culture by two strains of *Dechloromonas*. Nature 411, 1039-1043.

Coleman, D.D., Risatti, B., and Schoell, M., 1981. Fractionation of carbon and hydrogen isotopes by methane-oxidizing bacteria. Geochim. Cosmochim. Acta 45, 1033-1037.

Coleman, W.E., Munch, J.W., Streicher, R.P., Ringhand, H.P., and Knopfler, F.C., 1984. The identification and measurement of components in gasoline, kerosene, and No. 2 fuel oil that partition into the aqueous phase after mixing. Arch. Environ. Cont. Toxicol. 13, 171-178.

Davis, G.B., Barber, C., Power, T.R., Thierrin, J., Patterson, B.M., Rayner, J.L., and Wu, Q., 1999. The variability and intrinsic remediation of a BTEX plume in anaerobic sulphate-rich groundwater. J. Contam. Hydrol. 36, 265-290.

Dempster, H.S., Sherwood Lollar, B., and Feenstra, S., 1997. Tracing organic contaminants in groundwater: a new methodology using compound-specific isotopic analysis. Environ. Sci. Technol. 31, 3193-3197.

Detmers, J., Schulte, U., Strauss, H., and Kuever, J., 2001. Sulfate reduction at a lignite seam: Microbial abundance and activity. Microbial Ecol. 42, 238-247.

Déziel, A., Comeau, Y., and Villemur, R., 1999. Two-liquid-phase bioreactors for enhanced degradation of hydrophobic/toxic compounds. Biodegradation 10, 219-233.

Dojka, M.A., Hugenholtz, P., Haack, S.K., and Pace, N.R., 1998. Microbial diversity in a hydrocarbon- chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64, 3869-3877.

Dolfing, J., Zeyer, J., Binder-Eicher, P., and Schwarzenbach, R. P., 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. 154, 336-341.

Edwards, E.A., and Grbic-Galic, D., 1994. Anaerobic degradation of toluene and *o*-xylene by a methanogenic consortium. Appl. Environ. Microbiol. 60, 313-322.

Elshahed, M.S., Gieg, L.M., McInerney, M.J., and Suflita, J.M., 2001. Signature metabolites attesting to the *in situ* attenuation of alkylbenzenes in anaerobic environments. Environ. Sci. Technol. 35, 682-689.

Ertl, S., Seibel, F., Eichinger, L., Frimmel, F.H., and Kettrup, A., 1996. Determination of the $^{13}C/^{12}C$ isotope ratio of organic compounds for the biological degradation of tetrachloroethene (PCE) and trichloroethene (TCE). Acta Hydrochim. Hydrobiol. 24, 16-21.

Evans, P.J., Mang, D.T., Kim, K.S., Young, L.Y., 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. 57, 1139-1145.

Fales, H.M., Jaouni, T.M., and Babashak, J.F., 1973. Simple method for preparing ethereal diazomethane without resorting to codistillation. Anal. Chem. 45, 2302-2303.

Fennell, D.E., Carroll, A.B., Gossett, J.M., and Zinder, S.H., 2001. Assessment of indigenous reductive dechlorinating potential at a TCE-contaminated site using microcosms, polymerase chain reaction analysis, and site data. Environ. Sci. Technol. 35, 1830-1839.

Ficker, M., Krastel, K., Orlicky, S., and Edwards, E., 1999. Molecular characterization of a toluene-degrading methanogenic consortium. Appl. Environ. Microbiol. 65, 5576-5585.

Fogel, M.L., and Cifuentes, L.A., 1993. Isotope fractionation during primary production. *In* Org. Geochem. Engel, H.M., and Macko, S.A. (eds). New York: Plenum Press, pp. 73-97.

Frey, P.A., 2001. Radical mechanisms of enzymatic catalysis. Annu. Rev. Biochem. 70, 121-148.

Fries, M.R., Zhou, J., Chee-Sanford, J., and Tiedje, J.M., 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60, 2802-2810.

Fry, N.K., Fredrickson, J.K., Fishbain, S., Wagner, M., and Stahl, D.A., 1997. Population structure of microbial communities associated with two deep, anaerobic, alkaline aquifers. Appl. Environ. Microbiol. 63, 1498-1504.

Galushko, A., Minz, D., Schink, B., and Widdel, F., 1999. Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. Environ. Microbiol. 1, 415-420.

Gassner, G.T., Ludwig, M.L., Gatti, D.L., Correll, C.C., and Ballou, D.P., 1995. Structure and mechanism of the iron-sulfur flavoprotein phthalate dioxygenase reductase. FASEB J. 9, 1411-1418.

Giard, R.L., and Vedros, N.A., 1981. Biphasic resin systems for growing concentrated cultures of microorganisms and its application to the cultivation of *Neisseria gonorrhoeae*. Appl. Environ. Microbiol. 41, 846-847.

Gibson, D.T., and Parales, R.E., 2000. Aromatic hydrocarbon dioxygenases in environmental biotechnology. Curr. Opin. Biotechnol. 11, 236-243.

Gibson, D.T., Cardini, G.E., Maseles, F.C., and Kallio, R.E., 1970. Incorporation of oxygen-18 into benzene by *Pseudomonas putida*. Biochemistry 9, 1631-1635.

Gibson, D.T., and Subramanian, V., 1984. Microbial degradation of aromatic hydrocarbons. *In* Microbial degradation of organic compounds. Gibson, D.T. (ed). New York: Dekker, pp. 181-252.

Gibson, J., and Harwood, C.S., 2002. Metabolic diversity in aromatic compounds utilization by anaerobic microbes. Annu. Rev. Microbiol. 56, 345-369.

Gieg, L.M., Kolhatkar, R.V., McInerney, M.J., Tanner, R.S., Harris Jr., S.H., Sublette, K.L., and Suflita, J.M., 1999. Intrinsic bioremediation of petroleum hydrocarbons in a gas condensate-contaminated aquifer. Environ. Sci. Technol. 33, 2550-2560.

Gieg, L.M., and Suflita, J.M., 2002. Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum-contaminated aquifers. Environ. Sci. Technol. 36, 3755-3762.

Gossett, J.M., and Zinder, S.H., 1996. Microbiological aspects relevant to natural attenuation of chlorinated ethenes. *In* Proceedings of the Symposium on Natural Attenuation of Chlorinated Organics in Ground Water. Dallas, Texas.

Grossman, E.L., 2002. Stable carbon isotopes as indicators of microbial activity in aquifers. *In* Manual of environmental microbiology. Hurst, C.J. (ed). Washington D.C.: ASM Press.

Häner, A., Höhener, P., and Zeyer, J., 1995. Degradation of *p*-xylene by a denitrifying enrichment culture. Appl. Environ. Microbiol. 61, 3185-3188.

Häner, A., Höhener, P., and Zeyer, J., 1997. Degradation of trimethylbenzene isomers by an enrichment culture under N_2O-reducing conditions. Appl. Environ. Microbiol. 63, 1171-1174.

Harayama, S., Kok, M., and Neidle, E.L., 1992. Functional and evolutionary relationships among diverse oxygenases. Annu. Rev. Microbiol. 46, 565-601.

Harms, G., Zengler, K., Rabus, R., Aeckersberg, F., Minz, D., Rossello-Mora, R., and Widdel, F., 1999. Anaerobic oxidation of *o*-xylene, *m*-xylene, and

homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl. Environ. Microbiol. 65, 999-1004.

Harwood, C.S., and Parales, R.E., 1996. The *b*-ketoadipate pathway and the biology of self-identity. Annu. Rev. Microbiol. 50, 553-590.

Harwood, C.S., Burchardt, G., Herrmann, H., Fuchs, G., 1999. Anaerobic metabolism of aromatic compounds via the benzoyl-CoA pathway. FEMS Microbiol. Rev. 22, 439-458.

Hayes, J.M., Freeman, K.H., Po, B.N., and Hoham, C.H., 1990. Compound-specific isotope analysis. A novel tool for reconstruction of ancient biogeochemical processes. Adv. Org. Geochem. 16, 1115-1128.

Heider, J., Spormann, A.M., Beller, H.R., and Widdel, F., 1999. Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol. Rev. 22, 459-473.

Hess, A., Zarda, B., Hahn, D., Häner, A., Stax, D., Höhener, P., and Zeyer, J., 1997. *In situ* analysis of denitrifying toluene- and *m*-xylene-degrading bacteria in a diesel fuel-contaminated laboratory aquifer column. Appl. Environ. Microbiol. 63, 2136-2141.

Hilkert, A.W., Douthitt, C.B., Schlüter, H.J., and Brand, W.A., 1999. Isotope ratio monitoring gas chromatography/ mass spectrometry of D/H by high temperature conversion isotope ratio mass spectrometry. Rapid Commun. Mass Spectrom. 13, 1226-1230.

Himo, F., 2002. Catalytic mechanism of benzylsuccinate synthase, a theoretical study. J. Phys. Chem. 106, 7688-7692.

Hoefs, J., 1997. Stable isotope geochemistry. Berlin, Germany: Springer Verlag.

Holliger, C., Wohlfarth, G., and Diekert, G., 1999. Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol. Rev. 22, 383-398.

Hunkeler, D., Aravena, R., and Butler, B.J., 1999. Monitoring microbial dechlorination of tetrachloroethene (PCE) in groundwater using compound-specific stable carbon isotope ratios: Microcosm and field studies. Environ. Sci. Technol. 33, 2733-2738.

Hunkeler, D., Andersen, N., Aravena, R., Bernasconi, S.M., and Butler, B.J., 2001. Hydrogen and carbon isotope fractionation during aerobic biodegradation of benzene. Environ. Sci. Technol. 35, 3462-3467.

Hunkeler, D., Meckenstock, R.U., and Richnow, H.H., 2002. Quantification of isotope fractionation in experiments with deuterium-labeled substrate. Appl. Environ. Microbiol. 68, 5205-5207.

Jeffrey, A.M., Yeh, H.J.C., Jerina, D.M., Patel, T.R., Davey, J.F., and Gibson, D.T., 1975. Initial reactions in the oxidation of naphthalene by *Pseudomonas putida.* Biochemistry 14, 575-584.

Jüttner, F., and Henatsch, J.J., 1986. Anoxic hypolimnion is a significant source of biogenic toluene. Nature 323, 797-798.

Jüttner, F., 1988. Benzene in the anoxic hypolimnion of a freshwater lake. Naturwissenschaften 75, 151-153.

Kanaly, R., Bartha, R., Fogel, S., and Findlay, M., 1997. Biodegradation of [^{14}C]benzo[a]pyrene added in crude oil to uncontaminated soil. Appl. Environ. Microbiol. 63, 4511-4515.

Kanaly, R.A., and Harayama, S., 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J. Bacteriol. 182, 2059-2067.

Kane, S.R., Beller, H.R., Legler, T.C., and Anderson, R.T., 2002. Biochemical and genetic evidence of benzylsuccinate synthase in toluene-degrading, ferric iron-reducing *Geobacter metallireducens*. Biodegradation 13, 149-154.

Kauppi, B., Lee, K., Carredano, E., Parales, R.E., Gibson, D.T., Eklund, H., and Ramaswamy, S., 1998. Structure of an aromatic-ring-hydroxylating dioxygenase - naphthalene 1,2-dioxygenase. Structure 6, 571-586.

Kazumi, J., Caldwell, M.E., Suflita, J.M., Lovley, D.R., and Young, L.Y., 1997. Anaerobic degradation of benzene in diverse anoxic environments. Environ. Sci. Technol. 31, 813-818.

Kniemeyer, O., and Heider, J., 2001. Ethylbenzene dehydrogenase, a novel hydrocarbon-oxidizing molybdenum/iron-sulfur/heme enzyme. J. Biol. Chem. 276, 21381-21386.

Kniemeyer, O., Fischer, T., Wilkes, H., Glöckner, F.O., and Widdel, F., 2003. Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. Appl. Environ. Microbiol. 69, 760-768.

Koch, J., and Fuchs, G., 1992. Enzymatic reduction of benzoyl-CoA to alicyclic compounds, a key reaction in anaerobic aromatic metabolism. Eur. J. Biochem. 205, 195-202.

Koch, J., Eisenreich, W., Bacher, A., and Fuchs, G., 1993. Products of enzymatic reduction of benzoyl-CoA, a key reaction in anaerobic aromatic metabolism. Eur. J. Biochem. 211, 649-661.

Krieger, C.J., Beller, H.R., Reinhard, M., and Spormann, A.M., 1999. Initial reactions in anaerobic oxidation of *m*-xylene by the denitrifying bacterium *Azoarcus* sp. strain T. J. Bacteriol. 181, 6403-6410.

Krieger, C.J., Roseboom, W., Albracht, S.P.J., and Spormann, A.M., 2001. A stable organic free radical in anaerobic benzylsuccinate synthase of *Azoarcus* sp. strain T. J. Biol. Chem. 276, 12924-12972.

Krzycki, J.A., Kenealy, W.R., DeNiro, M.J., and Zeikus, J.G., 1987. Stable isotope fractionation by *Methanosarcina barkeri* during methanogenesis

from acetate, methanol, or carbon dioxide-hydrogen. Appl. Environ. Microbiol. 53, 2597-2599.

Lane, D.J., 1991. 16S/23S rRNA sequencing. *In* Nucleic acid techniques in bacterial systematics. Stackebrandt, E., and Goodfellow, M. (eds). London: John Wiley & Sons Ltd., pp. 115-175.

Langenhoff, A.A.M., Zehnder, A.J.B., and Schraa, G., 1996. Behaviour of toluene, benzene, and naphthalene under anaerobic conditions in sediment columns. Biodegradation 7, 267-274.

Lebedew, W.C., Owsjannikow, W.M., Mogilewskij, G.A., and Bogdanow, W.M., 1969. Fraktionierung der Kohlenstoffisotope durch mikrobiologische Prozesse in der biochemischen Zone. Angew. Geol. 15, 621-624.

Lee, M.D., Thomas, J.M., Borden, R.C., Bedient, P.B., Ward, C.H., and Wilson, J.T., 1988. Biorestoration of aquifers contaminated with organic compounds. Crit. Rev. Environ. Control 18, 29-84.

Letowski, J., Juteau, P., Villemur, R., Duckett, M.F., Beaudet, R., Lepine, F., and Bisaillon, J.,G., 2001. Separation of a phenol carboxylating organism from a two-member, strict anaerobic co-culture. Can. J. Microbiol. 47, 373-381.

Leuthner, B., Leutwein, C., Schultz, H., Hörth, P., Haehnel, W., Schlitz, E. Schägger, H., and Heider, J., 1998. Biochemical and genetic characterization of benzylsuccinate synthase from *Thauera aromatica*: a new glycyl radical enzyme catalysing the first step in anaerobic toluene metabolism. Mol. Microbiol. 28, 615-628.

Leuthner, B., and Heider, J., 2000. Anaerobic toluene catabolism of *Thauera aromatica*: the *bbs* operon codes for enzymes of β oxidation of the intermediate benzylsuccinate. J. Bacteriol. 182, 272-277.

Leutwein, C., and Heider, J., 1999. Anaerobic toluene-catabolic pathway in denitrifying *Thauera aromatica*: activation and β-oxidation of the first intermediate, (R)-(+)-benzylsuccinate. Microbiology 145, 3265-3271.

Leutwein, C., and Heider, J., 2001. Succinyl-CoA:(*R*)-benzylsuccinate CoA-transferase: an enzyme of the anaerobic toluene catabolic pathway in denitrifying bacteria. J. Bacteriol. 183, 4288-4295.

Leutwein, C., and Heider, J., 2002. (R)-Benzylsuccinyl-CoA dehydrogenase of *Thauera aromatica*, an enzyme of the anaerobic toluene catabolic pathway. Arch. Microbiol. 178, 517-524.

Lichtfouse, E., 2000. Compound-specific isotope analysis. Application to archaeology, biomedical sciences, biosynthesis, environment, extraterrestrial chemistry, food science, forensic science, humic substances, microbiology, organic geochemistry, soil science and sport. Rapid Comm. Mass Spectrom. 14, 1337-1344.

Löffler, F.E., Sun, Q., Li, J., and Tiedje, J.M., 2000. 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. Appl. Environ. Microbiol. 66, 1369-1374.

Lovley, D.R., and Lonergan, D.J., 1990. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl. Environ. Microbiol. 56, 1858-1864.

Lovley, D.R., Giovannoni, S.J., White, D.C., Champine, J.E., Phillips, E.J.P., Gorby, Y.A., and Goodwin, S., 1993. *Geobacter metallireducens* gen. nov. sp. nov., a microorganism capable of coupling the complete oxidation of organic compounds to the reduction of iron and other metals. Arch. Microbiol. 159, 336-344.

Lovley, D.R., Chapelle, F.H., and Woodward, J.C., 1994. Use of dissolved H_2 concentrations to determine distribution of microbially catalyzed redox reactions in anoxic groundwater. Environ. Sci. Technol. 28, 1205-1210.

Lovley, D.R., 2001. Anaerobes to the rescue. Science 293, 1444-1446.

Mace, R.E., Fisher, R.S., Welch, D.M., and Parra, S.P., 1997. Extent, mass, and duration of hydrocarbon plumes from leaking petroleum storage tank sites in Texas. Austin, Texas: Bureau of Economic Geology, University of Texas at Austin.

Manchester, J.I., Dinnocenzo, J.P., Higgins, L., and Jones, J.P., 1997. A new mechanistic probe for cytochrome P450: An application of isotope effect profiles. J. Am. Chem. Soc. 119, 5069-5070.

Mancini, S.A., Lacrampe-Couloume, G., Jonker, H., Van Breueklen, B.M., Groen, J., Volkering, F., and Sherwood Lollar, B., 2002. Hydrogen isotopic enrichment: An indicator of biodegradation at a petroleum hydrocarbon contaminated field site. Environ. Sci. Technol. 36, 2464-2470.

Mancini, S.A., Ulrich, A.C., Lacrampe-Couloume, G., Sleep, B.E., Edwards, E.A., and Sherwood Lollar, B., 2003. Carbon and hydrogen isotopic fractionation during anaerobic biodegradation of benzene. Appl. Environ. Microbiol. 69, 191-198.

Mansuy, L., Philp, R.P., and Allen, J., 1997. Source identification of oil spills based on the isotopic composition of individual components in weathered oil samples. Environ. Sci. Technol. 31, 3417-3425.

Mariotti, A., Germon, C., Hubert, P., Kaiser, P., Letolle, R., Tardieux, A., and Tardieux, P., 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. Plant Soil 62, 413-430.

Mazeas, L., Budzinski, H., and Raymond, N., 2002. Absence of stable carbon isotope fractionation of saturated and polycyclic aromatic hydrocarbons during aerobic bacterial biodegradation. Org. Geochem. 33, 1259-1272.

Meckenstock, R.U., 1999a. Fermentative toluene degradation in anaerobic defined syntrophic cocultures. FEMS Microbiol. Lett. 177, 67-73.

Meckenstock, R.U., Annweiler, E., Warthmann, R., Schink, B., Michaelis, W., and Richnow, H.H., 1999b. $^{13}C/^{12}C$ stable isotope fractionation of toluene by anaerobic degradation: A new method to monitor biological degradation *in situ*? *In* Novel approaches for bioremediation of organic pollution. Fass, R., Flashner, Y., and Reuveny, S. (eds). New York: Kluwer Academic Publishers, pp. 219-227.

Meckenstock, R.U., Morasch, B., Warthmann, R., Schink, B., Annweiler, E., Michaelis, W., and Richnow, H.H., 1999c. $^{13}C/^{12}C$ isotope fractionation of aromatic hydrocarbons during microbial degradation. Environ. Microbiol. 1, 409-414.

Meckenstock, R.U., Annweiler, E., Michaelis, W., Richnow, H.H., and Schink, B., 2000. Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Appl. Environ. Microbiol. 66, 2743-2747.

Meckenstock, R.U., Morasch, B., Kästner, M., Vieth, A., and Richnow, H.H., 2002. Assessment of bacterial degradation of aromatic hydrocarbons in the environment by analysis of stable carbon isotope fractionation. Water, Air and Soil Pollution: Focus 2, 141-152.

Meier-Augenstein, W., 1999. Applied gas chromatography coupled to isotope ratio mass spectrometry. J. Chromatogr. A 842, 351-371.

Merritt, D.A., Brand, W.A., and Hayes, J.M., 1994. Isotope ratio-monitoring gas chromatography mass spectrometry - Methods for isotopic calibration. Org. Geochem. 21, 573-583.

Milhelcic, J. R., Luthy, R. G., 1988. Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water systems. Appl. Environ. Microbiol. 54, 1182-1187.

Mohn, W.W., and Tiedje, J.M., 1992. Microbial reductive dehalogenation. Microbiol. Rev. 56, 482-507.

Morasch, B., Annweiler, E., Warthmann, R.J., and Meckenstock, R.U., 2001a. The use of a solid adsorber resin for enrichment of bacteria with toxic substrates and to identify metabolites: degradation of naphthalene, *o*-, and *m*-xylene by sulfate-reducing bacteria. J. Microbiol. Meth. 44, 183-191.

Morasch, B., Richnow, H.H., Schink, B., and Meckenstock, R.U., 2001b. Stable hydrogen isotope fractionation during microbial toluene degradation: Mechanistic and environmental aspects. Appl. Environ. Microbiol. 67, 4842-4849.

Morasch, B., Richnow, H.H., Schink, B., Vieth, A., and Meckenstock, R.U., 2002. Carbon and hydrogen stable isotope fractionation during aerobic

bacterial degradation of aromatic hydrocarbons. Appl. Environ. Microbiol. 68, 5191-5194.

Müller, J.A., Galushko, A., Kappler, A., and Schink, B., 1999. Anaerobic degradation of *m*-cresol by *Desulfobacterium cetonicum* is initiated by formation of 3-hydroxybenzylsuccinate. Arch. Microbiol. 172, 287-294.

Müller, J.A., Galushko, A., Kappler, A., and Schink, B., 2001. Initiation of anaerobic degradation of *p*-cresol by formation of 4-hydroxybenzylsuccinate in *Desulfobacterium cetonicum*. J. Bacteriol. 183, 752-757.

Norris, R.D., 1994. In-situ bioremediation of soils and groundwater contaminated with petroleum hydrocarbons. *In* Handbook of bioremediation. Norris, R.D. (ed). Boca Raton, Florida: Lewis Publishers, pp. 17-37.

Northrop, D.B., 1981. The expression of isotope effects on enzyme-catalyzed reactions. Annu. Rev. Biochem. 50, 103-131.

NRC, 1993. *In situ* bioremediation: when does it work? Washington, DC: National Research Council (NRC).

O'Leary, M.H., 1980. Determination of heavy-atom isotope effects on enzyme-catalysed reactions. *In* Enzyme kinetics and mechanism. Purich, D.L. (ed). New York: Academic Press, pp. 83-103.

O'Leary, M.H., 1984. Carbon isotope fractionation in plants. Phytochemistry 20, 553-567.

Olsen, R.H., Kukor, J.J., and Kaphammer, B., 1994. A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PKO1. J. Bacteriol. 176, 3749-3756.

O'Neil, J.R., 1986. Theoretical and experimental aspects of isotopic fractionation. Rev. Mineral. 16, 1-40.

Peterson, B.L., and Fry, B., 1987. Stable isotopes in ecosystem studies. Annu. Rev. Ecol. Syst. 18, 293-320.

Phelps, C.D., and Young, L.Y., 2001a. Biodegradation of BTEX under anaerobic conditions: a review. Adv. Agronom. 70, 329-357.

Phelps, C.D., Zhang, X., and Young, L.Y., 2001b. Use of stable isotopes to identify benzoate as a metabolite of benzene degradation in a sulphidogenic consortium. Environ. Microbiol. 3, 600-603.

Phelps, C.D., Battistelli, J., and Young, L.Y., 2002. Metabolic biomarkers for monitoring anaerobic naphthalene biodegradation *in situ*. Environ. Microbiol. 4, 532-537.

Postgate, J., 1959. A diagnostic reaction of *Desulphovibrio desulphuricans*. Nature 163, 481-482.

Preuss, A., Schauder, R., Fuchs, G., and Stichler, W., 1989. Carbon isotope fractionation by autotrophic bacteria with three different carbon dioxide fixation pathways. J. Biosci. 44, 397-402.

Rabus, R., Nordhaus, R., Ludwig, W., Widdel, F., 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. 59, 1444-1451.

Rabus, R., and Widdel, F., 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch. Microbiol. 163, 96-103.

Rabus, R., Wilkes, H., Behrends, A., Armstroff, A., Fischer, T., Pierik, A.J., and Widdel, F., 2001. Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: Evidence for (1-methylpentyl)succinate as initial product and for involvement of an organic radical in *n*-hexane metabolism. J. Bacteriol. 183, 1707-1715.

Rayleigh, J.W.S., 1896. Theoretical considerations respecting the separation of gases by diffusion and similar processes. Philos. Mag. 42, 493-498.

Reinhard, M., Shang, S., Kitanidis, P.K., Orwin, E., Hopkins, G.D., and Lebron, C.A., 1997. *In situ* BTEX biotransformation under enhanced nitrate- and sulfate-reducing conditions. Environ. Sci. Technol. 31, 28-36.

Rice, D.W., Grose, R.D., Michaelsen, J.C., Dooher, B.P., MacQueen, D.H., Cullen, S.J., Kastenberg, W.E., Everett, L.G., and Marino, M.A., 1995. California Leaking Underground Fuel Tank (LUFT) Historical Case Analyses. Sacramento, California: California State Water Resources Control Board.

Richnow, H.H., and Meckenstock, R.U., 1999. Isotopen-geochemisches Konzept zur *in situ* Erfassung des biologischen Abbaus in kontaminiertem Grundwasser. TerraTech 5, 38-41.

Richnow, H.H., Gehre, M., Kästner, M., Morasch, B., and Meckenstock, R.U., 2001. Characterisation of microbial *in situ* degradation of aromatic hydrocarbons. *In* Innovative methods in support of bioremediation. Magar, V.S., Vogel, T.M., Aelion, M., and Leeson, A. (eds). Columbus, Ohio: Battelle press, pp. 99-108.

Richnow, H.H., Vieth, A., Kästner, M., Gehre, M., and Meckenstock, R.U., 2002. Isotope fractionation of toluene: A perspective to characterise microbial *in situ* degradation. Sci. World J. 2, 7227-7234.

Richnow, H.H., Annweiler, E., Michaelis, W., and Meckenstock, R.U., 2003. Microbial *in situ* degradation of aromatic hydrocarbons in a contaminated aquifer monitored by carbon isotope fractionation. J. Contam. Hydrol. 65, 101-120.

Richnow, H.H., Meckenstock, R.U., Ask, L., Baun, A., Ledin, A., and Christensen, T.H., 2003. *In situ* biodegradation determined by carbon

isotope fractionation of aromatic hydrocarbons in an anaerobic landfill leachate plume (Vejen, Denmark). J. Contam. Hydrol. 59-72.

Rockne, K.L., and Strand, S.E., 1998. Biodegradation of bicyclic and polycyclic aromatic hydrocarbons in anaerobic enrichments. Environ. Sci. Technol. 32, 3962-3967.

Rockne, K.L., Chee-Sanford, I.C., Sanford, R.A., Hedlund, B.P., Staley, I.T., and Strand, S.E., 2000. Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. Appl. Environ. Microbiol. 66, 1595-1601.

Sambrook, J., Fritsch, E.F., and Maniatis, R., 1989. Molecular cloning. A laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sambrook, J., and Russel, D.W., 2001. Molecular cloning. A laboratory manual. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Schaeffer, A.B., and Fulton, M., 1933. A simplified method of staining endospores. Science 77, 194.

Schauder, R., Eikmanns, B., Thauer, R.K., Widdel, F., and Fuchs, G., 1986. Acetate oxidation to carbon dioxide in anaerobic bacteria via a novel pathway not involving reactions of the citric acid cycle. Arch. Microbiol. 145, 162-172.

Schiedeck, T., Grathwohl, P., and Teutsch, G., 1997. Literaturstudie zum natürlichen Rückhalt / Abbau von Schadstoffen im Grundwasser. Lehrstuhl für angewandte Geologie, Universität Tübingen (im Auftrag der Landesanstalt für Umweltschutz Baden-Württemberg).

Seyfried, B., Glod, G., Schocher, R., Tschech, A., and Zeyer, J., 1994. Initial reactions in the anaerobic oxidation of toluene and *m*-xylene by denitrifying bacteria. Appl. Environ. Microbiol. 60, 11, 4047-4052.

Shaw, J.P., and Harayama, S., 1992. Purification and characterisation of the NADH:acceptor reductase component of xylene monooxygenase encoded by the TOL plasmid pWW0 of *Pseudomonas putida* mt-2. Eur. J. Biochem. 209, 51-61.

Sherwood Lollar, B., Slater, G.F., Ahad, J., Sleep, B., Spivack, J., Brennan, M., and MacKenzie, P., 1999. Contrasting carbon isotope fractionation during biodegradation of trichloroethylene and toluene: implications for intrinsic bioremediation. Org. Geochem. 30, 813-820.

Sherwood Lollar, B., and Slater, G.F., 2001. Stable carbon isotope evidence for intrinsic bioremediation of tetrachloroethene and trichloroethene at area 6, Dover Air Force Base. Environ. Sci. Technol. 35, 261-269.

Slater, G.F., Ahad, J.M.E., Sherwood Lollar, B., Allen-King, R., and Sleep, B., 2000. Carbon isotope effects resulting from equilibrium sorption of dissolved VOCs. Anal. Chem. 72, 5669-5672.

Song, D.L., Conrad, M.E., Sorenson, K.S., and Alvarez-Cohen, L., 2002. Stable carbon isotope fractionation during enhanced *in situ* bioremediation of trichloroethene. Environ. Sci. Technol. 36, 2262-2268.

Spormann, A.M., and Thauer, R.K., 1988. Anaerobic acetate oxidation to CO_2 by *Desulfotomaculum acetoxidans*. Arch. Microbiol. 150, 374-380.

Spormann, A.M., and Widdel, F., 2000. Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. Biodegradation 11, 85-105.

Stahl, W.J., 1980. Compositional changes and $^{13}C/^{12}C$ fractionations during the degradation of hydrocarbons by bacteria. Geochim. Cosmochim. Acta 44, 1903-1907.

Stapleton, R.D., Sayler, G.S., Boggs, J.M., Libelo, E.L., Staufer, T., and Macintyre, W.G., 2000. Changes in subsurface catabolic gene frequencies during natural attenuation of petroleum hydrocarbons. Environ. Sci. Technol. 34, 1991-1999.

Stehmeier, L.G., Francis, M.M., Jack, T.R., Diegor, E., Winsor, L., and Abrajano, T.A., 1999. Field and in vitro evidence for in-situ bioremediation using compound-specific $^{13}C/^{12}C$ ratio monitoring. Org. Geochem. 30, 821-833.

Stookey, L.L., 1970. Ferrozine - a new spectrophotometric reagent for iron. Anal. Chem. 42, 779-781.

Strunk, O., and Ludwig, W., 1996. ARB: a software environment for sequence data. Munich: Technical University Munich, Germany.

Stubner, S., and Meuser, K., 2000. Detection of *Desulfotomaculum* in an Italian rice paddy soil by 16S ribosomal nucleic acid analyses. FEMS Microbiol. Ecol. 34, 73-80.

Sturchio, N.C., Clausen, J.L., Heraty, L.J., Huang, L., Holt, B.D., and Abrajano, T.A., 1998. Chlorine isotope investigation of natural attenuation of trichloroethene in an aerobic aquifer. Environ. Sci. Technol. 32, 3037-3042.

Subramanian, V., Liu, T.-N., Yeh, W.K., and Gibson, D.T., 1979. Toluene dioxygenase: Purification of an iron-sulfur protein by affinity chromatography. Biochem. Biophys. Res. Commun. 91, 1131-1139.

Suzuki, M., Hayakawa, T., Shaw, J.P., Rekik, M., and Harayama, S., 1990. Primary structure of xylene monooxygenase: Similarities to and differences from the alkane hydroxylation system. J. Bacteriol. 172, 1690-1695.

Torok, D.S., Resnick, S.M., Brand, J.M., Cruden, D.L., and Gibson, D.T., 1995. Desaturation and oxygenation of 1,2-dihydronaphthalene by toluene and naphthalene dioxygenase. J. Bacteriol. 177, 5799-5805.

Trust Hammer, B., Mueller, J.G., Coffin, R.B., and Cifuentes, L.A., 1995. The biodegradation of fluoranthene as monitored using stable carbon isotopes.

In Monitoring and verification of bioremediation. Hinchee, R.R., Douglas, G.S., and Ong, S.K. (eds), pp. 233-239.

Ulrich, A.C., and Edwards, E.A., 2003. Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures. Environ. Microbiol. 5, 92-102.

US-EPA, 1998. Carcinogenic effects of benzene: an update. Washington, DC: National Center for Environmental Assessment, Office of Research and Development, U.S. Environmental Protection Agency, pp. 1-45.

US-EPA, 1999. Use of monitored natural attenuation at superfund, RCRA corrective action, and underground storage tank sites. *In* OSWER directive 9200, pp. 4-17P.

US-EPA, 2002. National primary drinking water regulations. Washington, DC: Office of Water, U.S. Environmental Protection Agency, pp. 424-425.

Vieth, A., Kästner, M., Morasch, B., Meckenstock, R.U., and Richnow, H.H., 2001. Charakterisierung des biologischen Abbaus von BTEX im Grundwasser über Isotopenfraktionierung - Feldstudien. TerraTech 37-41.

Vieth, A., Voskamp, M., Kopinke, F.D., Meckenstock, R.U., and Richnow, H.H., 2002. Isotope fractionation as a tool to document the in-situ biodegradation of organic pollutants in contaminated aquifers. *In* Third International Conference on Water Resources and Environment Research. Schmitz, G.H. (ed). Dresden, Germany: Eigenverlag des Forums für Abfallwirtschaft und Altlasten e.V., pp. 198-202.

Vogel, T.M., and McCarthy, P.L., 1985. Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride, and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. 49, 1080-1083.

Vroblesky, D.A., and Chapelle, F.H., 1994. Temporal and spatial changes of terminal electro-accepting processes in a petroleum hydrocarbon-contaminated aquifer and the significance for contaminant biodegradation. Water Resour. Res. 30, 1561-1570.

Ward, J.A.M., Ahad, J.M.E., Lacrampe-Couloume, G., Slater, G.F., Edwards, E.A., and Sherwood Lollar, B., 2000. Hydrogen isotope fractionation during methanogenic degradation of toluene: Potential for direct verification of bioremediation. Environ. Sci. Technol. 34, 4577-4581.

Weber, F.J., Tramper, J., and Rinzema, A., 1999. A simplified material and energy balance approach for process development and scale-up of *Coniothyrium minitans* conidia production by solid-state cultivation. Biotechnol. Bioeng. 65, 447-458.

Weiner, J.M., and Lovley, D.R., 1998. Rapid benzene degradation in methanogenic sediments from a petroleum-contaminated aquifer. Appl. Environ. Microbiol. 64, 1937-1939.

Whited, G.M., and Gibson, D.T., 1991. Toluene-4-monooxygenase, a three-component enzyme system that catalyzes the oxidation of toluene to *p*-cresol in *Pseudomonas mendocina* KR1. J. Bacteriol. 173, 3010-3016.

Whiticar, M.J., and Faber, E., 1986. Methane oxidation in sediment and water column environments - isotope evidence. Adv. Org. Geochem. 10, 759-768.

Widdel, F., 1992a. The Genus *Desulfotomaculum*. *In* The procaryotes. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). Berlin: Springer Verlag, pp. 1792-1799.

Widdel, F., and Bak, F., 1992b. Gram-negative mesophilic sulfate-reducing bacteria. *In* The prokaryotes. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H. (eds). New York: Springer Verlag, pp. 3352-3378.

Widdel, F., and Rabus, R., 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr. Opin. Biotechnol. 12, 259-276.

Wiedemeier, T.H., Rifai, H.S., Newell, C., J., and Wilson, J.T., 1999. Natural attenuation of fuels and chlorinated solvents in the subsurface. New York: John Wiley & Sons, Inc.

Wilkes, H., Boreham, C., Harms, G., Zengler, K., and Rabus, R. , 2000. Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria. Org. Geochem. 31, 101-115.

Wilkes, H., Rabus, R., Fischer, T., Armstroff, A., Behrends, A., and Widdel, F., 2002. Anaerobic degradation of *n*-hexane in a denitrifying bacterium: further degradation of the initial intermediate (1-methylpentyl)succinate via C-skeleton rearrangement. Arch. Microbiol. 177, 235-243.

Wind, T., Stubner, S., and Conrad, R., 1999. Sulfate-reducing bacteria in rice field soil and on rice roots. System. Appl. Microbiol. 22, 269-279.

Worsey, M.J., and Williams, P.A., 1975. Metabolism of toluene and xylenes by *Pseudomonas putida* (arvilla) mt-2: Evidence for a new function of the TOL plasmid. J. Bacteriol., 7-13.

Yeh, W.K., Gibson, D.T., and Liu, T.-N., 1977. Toluene dioxygenase: A multicomponent enzyme system. Biochem. Biophys. Res. Commun. 78, 401-410.

Yoshizawa, K., Kagawa, Y., and Shioto, Y., 2000. Kinetic isotope effects in a C-H bond dissociation by the iron-oxo species of cytochrome P450. J. Phys. Chem. 104, 12365-12370.

Zhang, C.L., Ye, Q., Reysenbach, A.-L., Götz, D., Peacock, A., White, D.C. et al., 2002. Carbon isotopic fractionations associated with thermophilic bacteria *Thermotoga maritima* and *Persephonella marina*. Environ. Microbiol. 4, 58-64.

Zhang, X., and Young, L.Y., 1997. Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Appl. Environ. Microbiol. 63, 4759-4764.

Zhou, J., Fries, M.R., Chee-Sanford, J.C., and Tiedje, J.M., 1995. Phylogenetic analysis of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus tolulyicus* sp. nov. Int. J. Syst. Bacteriol. 45, 500-506.

11.1 Quantification of Isotope Fractionation in Experiments with Deuterium-Labeled Substrate

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Isotope analysis is a potentially sensitive method to trace in situ degradation of organic contaminants. In a recent paper, Morasch et al. (3) investigated the mechanism of isotope fractionation during toluene biodegradation using deuteriumlabeled toluene. The authors overlooked that the Rayleigh equation that is normally used to evaluate isotope fractionation at natural abundance level (2) is not applicable to studies with labeled substrate, particularly if large isotope fractionation occurs. For several of their experiments they obtained negative hydrogen isotope fractionation factors (see Table 1 in reference 3), which contradict the definition of the fractionation factor (see below). Since labeled compound will likely be used in further investigations to study isotope fractionation, it is important to demonstrate why the commonly used Rayleigh equation is usually not applicable in such studies and to provide an alternative method to quantify isotope fractionation.

The magnitude of isotope fractionation is normally characterized by the fractionation factor, which is defined as follows for kinetic isotope fractionation:

$$a = \frac{H/L}{dH_p/dL_p} \tag{1}$$

where H and L are the concentrations of the substrate with heavy and light isotopes, respectively, at a given time and dH_p and dL_p are increments of product with heavy or light isotopes, respectively, that appear in an infinitely short time (instantaneous product). In some studies, the fractionation factor is defined by the inverse ratio (2). Since all terms in equation 1 are positive, α has to be positive. For mass balance reasons,

$$\begin{aligned} dH_p &= -dH \\ dL_p &= -dL \end{aligned} \tag{2}$$

Combining equations 1 and 2 and rearrangement leads to

$$\frac{dL}{L} = a \times \frac{dH}{H} \tag{3}$$

Integration of equation 3 from L_0 to L and H_0 to H gives

$$\ln\frac{L}{L_0} a \times \ln\frac{H}{H_0} \quad \text{or}$$

$$\frac{H}{H_0} = \left(\frac{L}{L_0}\right)^{1/a} \tag{4}$$

Dividing both sides by L/L_0 yields

$$\frac{R}{R_0} = \left(\frac{L}{L_0}\right)^{(1/a-1)} \tag{5}$$

where R and R_0 are the isotope ratios (H/L) at a given time t and at time zero, respectively. The fraction of substrate that has not reacted yet, f, at time t is given by

$$f = \frac{L+H}{L_0+H_0} = \frac{L\times(1+R)}{L_0\times(1+R_0)} \tag{6}$$

Equations 5 and 6 are analogous to those given by Bigeleisen and Wolfsberg (1), except that here they were derived without any specific assumption about the reaction kinetics and using a different definition of α and f.

The crucial point is that L/L_0 in equation 5 can only be approximated by f if either (i) the concentrations of the heavy isotopes, H and H_0, are small, as common for studies at natural abundance level, or (ii) $1 + R \approx 1 + R_0$. In the first case, the first expression for f in equation 6 approaches L/L_0; in the second case, the

second expression can be approximated by L/L_0. If one of these two conditions is fulfilled, equation 5 can be simplified to

$$\frac{R}{R_0} = f^{(1/a-1)} \qquad (7)$$

which corresponds to the Rayleigh equation as used by the authors of the study (3). However, in the experiments with labeled compound presented in the study, condition i is not fulfilled since the compound with deuterium accounts for 50% of the total toluene concentration. Condition ii is not fulfilled either. For example, for the experiment illustrated in Fig. 1 in reference 3, R_0 is 1 and R varies between 1 and about 12 and thus, the assumption that $1 + R \approx 1 + R_0$ holds true is not valid. In other experiments, even higher R values of up to about 54 were observed (see Fig. 2 in reference 3).
By combining equations 5 and 6, an accurate equation is obtained that relates R, R_0, f, and α:

$$\ln\frac{R}{R_0} = \left(\frac{1}{a} - 1\right) \times \ln\frac{f}{(1+R)/(1+R_0)} \qquad (8)$$

This equation can be used to determine α by plotting $\ln(R/R_0)$ versus $\ln\{f/[(1 + R)/(1 + R_0)]\}$. Applying this approach to the data of the experiment with *Desulfobacterium cetonicum* (as given in Fig. 1 in reference 3), an α value of approximately 2.7 is obtained instead of -5.09. The value of 2.7 is only an approximation, since the data for the calculation were estimated from Fig. 1 in reference 3. The calculated value is in the typical range for primary hydrogen isotope effects. Using the correct equation, the introduction of an uncommon parameter to characterize isotope fractionation becomes unnecessary and the data can be discussed in a framework consistent with a large number of studies on isotope fractionation during enzymatic reactions.

REFERENCES

1. **Bigeleisen, J., and M. Wolfsberg.** 1958. Theoretical and experimental aspects of isotope effects in chemical kinetics. Adv. Chem. Phys. **1:** 15–76.
2. **Mariotti, A., J. C. Germon, P. Hubert, P. Kaiser, T. Letolle, A. Tardieux, and P. Tardieux.** 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrifi-cation processes. Plant Soil **62:**413–430.
3. **Morasch, B., H. H. Richnow, B. Schink, and R. U. Meckenstock.** 2001. Stable hydrogen and carbon isotope fractionation during microbial toluene degradation: mechanistic and environmental aspects. Appl. Environ. Microbiol. **67:** 4842–4849.

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Authors' Reply

The comments by Dr. Hunkeler provide a valuable extension of our results on basic features of isotope fractionation. Indeed, the fractionation factor α_n (n for natural abundance) calculated in Morasch et al. (3) is valid to describe isotope fractionation of carbon and hydrogen isotopes only at low abundance of the heavier isotopes (^{13}C and D, respectively) as presented in the manuscript. For experiments at elevated abundances of the heavier isotope, the isotope fractionation factor α_l (l for labeled compounds) should be calculated using equation 3 as given by Bigeleisen and Wolfsberg (1) and mentioned by Dr. Hunkeler.
In Morasch et al. (3), we used the slope b of a linear regression of the data in a double logarithmic plot of $\ln(R_t/R_0)$ versus $\ln f$ ($f = C_t/C_0$, fraction of substrate remaining [C_t, substrate concentration at time A; C_0, substrate concentration at time zero]) to evaluate the extent of isotope fractionation (equation 2). In experiments with substrates of natural isotope composition, b can be converted directly to the

fractionation factor α_n or the enrichment factor ε with $b = 1/\alpha - 1$ or $e = b \times 1{,}000$, because equations 2 and 1 approximate equation 3 at low abundances of the heavier isotope (1, 2).

What is the consequence of using equation 2 instead of equation 3 also in experiments with deuterium-labeled compounds at elevated abundance? In this case, the slope b calculated by equation 2 becomes a fitting parameter of the data

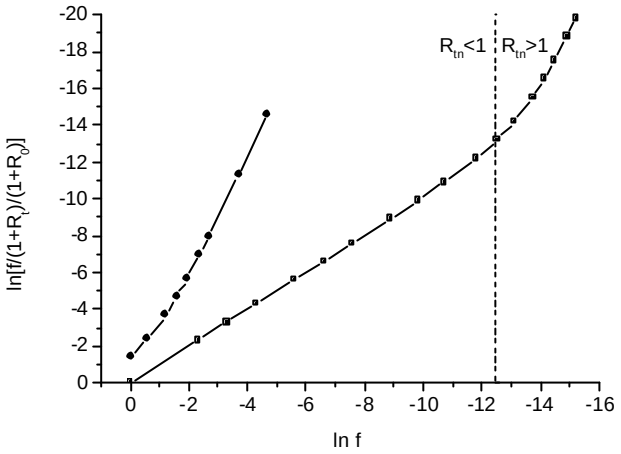

FIG. 1. Simulated hydrogen isotope fractionation experiment for toluene degradation by strain TRM1 as calculated with equation 3 versus equation 1 and $\alpha_l = \alpha_n = 3.3$. The calculations start with $R_{0n} = 0.0001$ for the simulation of an experiment with natural abundance of deuterium (■) and with $R_{0l} = 1$ for the simulation of an experiment with labeled compounds (●). R_t runs from R_0 to infinity. At natural abundance of the heavier isotope $\ln\{f/[(1 + R_t)/(1 + R_0)]\}$ approximates $\ln f$ because R_t and R_0 are very small and equation 3 approximates equation 1. Therefore, the slopes of the curves in the range of $\ln f \rightarrow -12$ show the deviation in the description of isotope fractionation simulated with equation 1 or 3 for natural abundance (■) and labeled compounds (●). The dashed line depicts the isotope ratio of $R_{tn} = 1$.

which differs by a constant value from the slope b' calculated via equation 3, where $\ln(R_t/R_0)$ is plotted versus $\ln\{f/[(1 + R_t)/ (1 + R_0)]\}$. The difference in using the two equations is depicted in Fig. 1 for a hypothetical experiment where the isotope ratio R_t runs from R_0 to infinity and $\ln f$ is plotted versus $\ln\{f/[(1 + R_t)/(1 + R_0)]\}$. For natural abundance of deuterium, the slope of $\ln f$ versus $\ln\{f/[(1 + R_t)/(1 + R_0)]\}$ equals 1, indicating that the two terms are almost identical as long as R_t is smaller than 1. If R_t is larger than 1, the slope of the curve changes and approximates another constant value. Note that the slope of the curve for $R_t \rightarrow 1$ is similar to the slope of a simulated experiment using labeled compounds with $R_0 = 1$ if the same fractionation factor is applied (Fig. 1). The extent of fractionation is only hypothetical since one could hardly run a real degradation experiment over such enormous concentration ranges. Nevertheless, it shows that the difference in the calculations using equations 1 and 2 or 3 depends on the isotope ratio R_t being larger or smaller than 1. This property of the calculations becomes especially important if isotope fractionation of elements such as chlorine is studied, where the natural abundances of the heavier and lighter isotopes are almost equal ($R_0 = 1$).

In our experiments the concentrations were usually in the range of $0 < \ln f < -4$. Here, the difference of the slopes for labeled compounds and for natural abundance reveals the systematic difference between the two ways of calculation. Figure 1 also shows that the curve for the labeled compounds is not exactly a straight line. However, the experimental error of the isotope analysis is usually much larger than the error by fitting the data with a linear regression, but the description of the data set with equation 3 would certainly improve the interpretation. With respect to the data produced by Morasch et al., the systematic difference in the description of the data set with equation 2 or 3 results in the same interpretation of the isotope fractionation experiments with deuterium-labeled compounds. The direct comparison of the obtained isotope fractionations of b and α clearly shows the relation of isotope fractionation and enzyme mechanisms. The major difference in the use of the two equations is that the absolute value of the commonly used isotope fractionation factor α can only be calculated from equation 3.

TABLE 1. D/H isotope fractionation factors α or ε and fitting parameter *b* obtained from studies with nonlabeled (α_n) or labeled toluene (α_l)[a]

Strain	Substrate mixture	α_n	α_l	ε_l	b
Desulfobacterium cetonicum	Toluene	1.247	1.247	-198.1	-0.198
	Toluene-d_8 and toluene-d_3		0.996 ± 0.005	4.016 ± 5.04	-0.002 ± 0.003
	Toluene-d_3 and nonlabeled toluene		3.772 ± 1.084	-734.9 ± 76.19	-1.251 ± 0.034
	Toluene-d_8 and toluene-d_5		2.058 ± 0.090	-514.1 ± 21.25	-0.679 ± 0.115
	Toluene-d_5 and nonlabeled toluene		1.009 ± 0.017	-8.920 ± 16.69	-0.005 ± 0.004
	Toluene-d_8 and nonlabeled toluene		3.244 ± 0.261	-691.7 ± 24.80	-1.196 ± 0.075
TRM1	Toluene	3.672	3.650	-726.0	-0.728
	Toluene-d_8 and toluene-d_3		0.885 ± 0.142	129.9 ± 181.3	0.167 ± 0.219
	Toluene-d_3 and nonlabeled toluene		3.384 ± 0.170	-704.5 ± 14.85	-1.280 ± 0.080
	Toluene-d_8 and toluene-d_5		2.070 ± 0.233	-516.9 ± 54.38	-0.917 ± 0.336
	Toluene-d_5 and nonlabeled toluene		1.014 ± 0.012	-13.81 ± 11.67	-0.012 ± 0.005
	Toluene-d_8 and nonlabeled toluene		3.276 ± 0.281	-694.7 ± 26.18	-1.219 ± 0.254
Thauera aromatica	Toluene-d_8 and nonlabeled toluene		2.543 ± 0.567	-606.8 ± 87.68	-0.816 ± 0.133
Geobacter metallireducens	Toluene-d_8 and nonlabeled toluene		2.550 ± 0.187	-607.8 ± 28.76	-1.004 ± 0.077
Pseudomonas putida strain mt-2	Toluene-d_8 and toluene-d_3		1.005 ± 0.0004	-4.98 ± 0.40	-0.016 ± 0.003
	Toluene-d_3 and nonlabeled toluene		22.96 ± 4.368	-956.4 ± 8.29	-4.218 ± 0.125
	Toluene-d_8 and toluene-d_5		13.65 ± 2.452	-926.7 ± 13.16	-2.696 ± 0163
	Toluene-d_5 and nonlabeled toluene		1.098 ± 0.031	-89.25 ± 25.71	-0.079 ± 0.041
	Toluene-d_8 and nonlabeled toluene		17.78 ± 13,46	-943.8 ± 42.58	-2.667 ± 0.163

[a] α_n was calculated with equation 1, α_l and ε_l were calculated with equation 3, and *b* was calculated with equation 2. ε_l is calculated as $\varepsilon_l = (1/\alpha_l - 1) \times 1{,}000$. Average isotope fractionation factors for experiments with mixtures of labeled toluene species α_l, the respective fitting parameter *b*, and the standard deviations result from three independent growth experiments. Original data were taken from reference 3. Fractionation at natural deuterium abundance was obtained only from a single growth experiment and is not given with a standard deviation.

We have recalculated the isotope fractionation factors of the experiments with labeled compounds published in Morasch et al. (3) using equation 3 (Table 1). The recalculated data may provide the reader with fractionation factors comparable to those published in other studies. However, the data show also that an α_n obtained at a natural abundance of the heavier isotope is not necessarily identical with the α_l obtained in labeling experiments.

In summary, for experiments with defined conditions the use of deuterium-labeled compounds is an elegant way to overcome the problem of limited availability of isotope mass spectrometers for D/H analysis and to reduce analysis costs for basic studies of isotope fractionation. Isotope fractionation factor α should be calculated from labeling experiments with equation 3 but are difficult to relate to isotope fractionation occurring at natural abundance of hydrogen isotopes (3).

$$\ln(R_t/R_0) = (1/a_n - 1) \times \ln f \quad (1)$$

$$\ln(R_t/R_0) = b \times \ln f \quad (2)$$

$$\ln(R_t/R_0) = (1/a_l - 1) \times \ln \{f/[(1 + R_t)/(1+R_0)]\} \quad (3)$$

REFERENCES

1. **Bigeleisen, J., and M. Wolfsberg.** 1959. Theoretical and experimental aspects of isotope effects in chemical kinetics. Adv. Chem. Phys. **1:**15–76.
2. **Mariotti, A., C. Germon, P. Hubert, P. Kaiser, R. Letolle, A. Tardieux, and P. Tardieux.** 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. Plant Soil **62:**413–430.
3. **Morasch, B., H. H. Richnow, B. Schink, and R. U. Meckenstock.** 2001. Stable carbon and hydrogen isotope fractionation during microbial toluene degradation: mechanistic and environmental aspects. Appl. Environ. Microbiol.
67:4842–4849.

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11.2 Publications

Published

Meckenstock, R.U., Morasch, B., Warthmann, R., Schink, B., Annweiler, E., Michaelis, W., and Richnow, H.H., 1999. $^{13}C/^{12}C$ isotope fractionation of aromatic hydrocarbons during microbial degradation. Environ. Microbiol. 1, 409-414.

Morasch, B., Annweiler, E., Warthmann, R.J., and Meckenstock, R.U., 2001. The use of a solid adsorber resin for enrichment of bacteria with toxic substrates and to identify metabolites: degradation of naphthalene, *o*-, and *m*-xylene by sulfate-reducing bacteria. J. Microbiol. Meth. 44, 183-191.

Richnow, H.H., Gehre, M., Kästner, M., Morasch, B., and Meckenstock, R.U., 2001. Characterisation of microbial *in situ* degradation of aromatic hydrocarbons. *In* Innovative methods in support of bioremediation. Magar, V.S., Vogel, T.M., Aelion, M., and Leeson, A. (eds). Columbus, Ohio: Battelle press, pp. 99-108.

Vieth, A., Kästner, M., Morasch, B., Meckenstock, R.U., and Richnow, H.H., 2001. Charakterisierung des biologischen Abbaus von BTEX im Grundwasser über Isotopenfraktionierung - Feldstudien. TerraTech, 37-41.

Morasch, B., Richnow, H.H., Schink, B., and Meckenstock, R.U., 2001. Stable hydrogen isotope fractionation during microbial toluene degradation: Mechanistic and environmental aspects. Appl. Environ. Microbiol. 67, 4842-4849.

Meckenstock, R.U., Morasch, B., Kästner, M., Vieth, A., and Richnow, H.H., 2002. Assessment of bacterial degradation of aromatic hydrocarbons in the environment by analysis of stable carbon isotope fractionation. Water, Air and Soil Pollution: Focus 2, 141-152.

Morasch, B., Richnow, H.H., Schink, B., Vieth, A., and Meckenstock, R.U., 2002. Carbon and hydrogen stable isotope fractionation during aerobic bacterial degradation of aromatic hydrocarbons. Appl. Environ. Microbiol. 68, 5191-5194.

Morasch, B., Griebler, C., Vieth, A., Richnow, H.H., and Meckenstock, R.U., 2002. Quantifizierung des mikrobiellen Schadstoffabbaus in kontaminierten Grundwasserleitern über Kohlenstoff- und Wasserstoff-Isotopenfraktionierung. *In* 3. Symposium "Natural Attenuation - Umsetzung, Finanzierung, Perspektiven". Bremen, pp. 31-39.

In preparation

Morasch, B., Richnow, H.H., Vieth, A., Schink, B., and Meckenstock, R.U., Stable isotope fractionation caused by glycyl radical enzymes during bacterial degradation of aromatic compound. Submitted to Appl. Environ. Microbiol.

Morasch, B., Schink, B., Tebbe, C.C., and Meckenstock, R.U., Degradation of *o*- and *m*-xylene by a novel sulfate reducer belonging to the genus *Desulfotomaculum*. In preparation.

Meckenstock, R. U., Morasch, B., Griebler, C., and Richnow H. H. Analysis of stable isotope fractionation as a tool to monitor biodegradation in contaminated aquifers. Submitted to J. Contam. Hydrol.

11.3 Curriculum vitae

	Barbara Morasch
Adresse	Im Klösterle 16, 87700 Memmingen
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Schulausbildung

1980 - 1984	Grundschule Brettheim
1984 - 1993	Gymnasium Gerabronn, Abitur

Studium

1993 - 1999	Studium der Biologie an der Universität Konstanz Mündliche Diplomprüfung bei Prof. Dr. B. Schink und Prof. Dr. P. M. H. Kroneck. Diplomarbeit am Lehrstuhl von Prof. Dr. B. Schink: *Grundlagen zur Kohlenstoff- und Wasserstoffisotopen-fraktionierung beim bakteriellen Toluolabbau,* betreut von Dr. Rainer U. Meckenstock.
1999 - 2003	Promotion an der Universität Konstanz am Lehrstuhl für Mikrobielle Ökologie und Mikrobiologie von Prof. Dr. B. Schink unter Betreuung von Dr. Rainer U. Meckenstock. Seit Oktober 2001 Weiterführung der Doktorarbeit am Zentrum für Angewandte Geowissenschaften der Eberhard-Karls-Universität Tübingen.

11.4 Abgrenzung der Eigenleistung

Die vorliegende Arbeit wurde im Rahmen des DFG-Schwerpunktprogramms Nr 546 „Geochemische Prozesse mit Langzeitfolgen in anthropogen beeinflussten Sicker- und Grundwässern“ angefertigt. Der Antrag wurde von Prof. Dr. Bernhard Schink und Dr. Rainer U. Meckenstock gestellt. Das Programm lief bis 2001.

Die in dieser Arbeit dargestellten Experimente wurden von mir selbständig durchgeführt. Ausgenommen sind die in Kapitel zwei dargestellten Arbeiten zur Aufklärung des Naphthalinabbauwegs, welche Dr. Eva Annweiler vom Institut für Biogeochemie und Meereschemie der Universität Hamburg durchgeführt hat.

Stamm OX39, der in Kapitel drei beschrieben wird, ist von Dr. Rainer U. Meckenstock am Lehrstuhl für Mikrobielle Ökologie und Mikrobiologie der Universität Konstanz isoliert worden. Alle weiteren Experimente zur Charakterisierung und zum Schadstoffabbau habe ich selbst durchgeführt. Dr. Christoph Tebbe aus Braunschweig hat die 16S rRNA zur phylogenetischen Einordnung von Stamm OX39 analysiert.

Dr. Hans Hermann Richnow und Dr. Andrea Vieth haben am Umweltforschungszentrum Leipzig-Halle GmbH die Isotopenmessungen mittels IRM-GC-MS für die Kapitel vier bis sechs durchgeführt.

Die Publikation für Kapitel zwei wurde von Dr. Rainer U. Meckenstock geschrieben. Alle weiteren Manuskripte habe ich mit Unterstützung von Dr. Rainer U. Meckenstock und Prof. Dr. Bernhard Schink verfasst.

11.5 Danksagung

Diese Arbeit wurde am Lehrstuhl für Mikrobielle Ökologie und Mikrobiologie der Universität Konstanz von Prof. Dr. Bernhard Schink unter der Betreuung von Dr. Rainer Meckenstock durchgeführt. Von Oktober 2001 an forschte ich dafür am Zentrum für Angewandte Geowissenschaften der Universität Tübingen.

Mein Dank gilt Herrn Schink für die Möglichkeit, meine Doktorarbeit an seinem Lehrstuhl anzufertigen, für das gründliche und schnelle Lesen der Manuskripte und für viele wertvolle - mitunter kritische - Anmerkungen.

Rainer Meckenstock danke ich für die gute Betreuung über viele Jahre, für sein Interesse und seine Motivation - und natürlich für die unzähligen Ideen und Anregungen.

Bei Stefan Haderlein möchte ich mich für die Unterstützung meiner Arbeit in Tübingen bedanken.

Hans Hermann Richnow und Andrea Vieth danke ich für das Durchführen der Isotopenanalysen und die gute Zusammenarbeit über viele Jahre, sowie Christoph Tebbe für die 16S-Analyse von Stamm OX39.

Viele Kollegen in Konstanz und in Tübingen haben mit ihrer Hilfsbereitschaft zum Gelingen dieser Arbeit beigetragen, besonders hervorheben möchte ich Sascha Galuschko, Christian Griebler und Hans-Peter Arp.

Meinen Eltern und Freunden danke ich herzlich für ihre Unterstützung während der letzten Jahre.