Biodegradation of toluene, benzene and naphthalene under anaerobic conditions.

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dr. C.M. Karssen,
in het openbaar te verdedigen
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Stellingen

1. Xenobiotica zijn niet zulke wereldvreemde verbindingen als in het algemeen wordt aangenomen.
   Fischer-Romero, C. et al. 1996. Tolumonas auensis gen.nov.,sp.nov., a toluene producing bacterium from anoxic sediments of a freshwater lake. Int. J. Syst. Bacteriol. 46:183-188.

2. Officieuze bacterienamen voor anaërobe tolueen-afbrekende bacteriën, zoals Tol2, strain T1, strain PRTOL1, duiden in het algemeen op weinig creativiteit van de wetenschapper.
   Frazer, A.C. et al. 1995. Toluene metabolism under anaerobic conditions: a review. Anaerobe 1:293-303.

3. De bewering van Zehnder en Stumm (1988) dat er destijds geen bacterie beschreven was die zijn energie uit de oxidatie van organische koolstofmolekulen en de reductie van mangaan of ijzer oxide kon halen, is niet terecht.
   Ghiorse, W.C. 1988. Microbial reduction of manganese and iron, p. 305-331. In: A.J.B. Zehnder (ed.), Biology of Anaerobic Microorganisms, John Wiley and Sons, New York.
   Lovley, D.R. et al. 1987. Anaerobic production of magnetite by a dissimilatory iron-reducing organism. Nature 330:252-254;
   Zehnder, A.J.B. and W. Stumm. 1988. Geochemistry and biogeochemistry of anaerobic habitats, p. 1-38. In: A.J.B. Zehnder (ed.), Biology of Anaerobic Microorganisms, John Wiley and Sons, New York.

4. De stellige uitspraak 'homocyclische aromatische koolwaterstoffen kunnen onmogelijk onder anaërobe condities worden afgebroken' is heden ten dage niet meer te rechtvaardigen.
   Prof. F. Wagner tijdens Dechema '91 te Karlsruhe, Duitsland.

5. De positieve werking van steeds populairdere, microbiële produkten zoals Yakult, Vifit etc., moet niet worden overschat door met name knoflook-etende personen.
   Naganawa, R. et al. 1996. Inhibition of microbial growth by ajoene, a sulfur-containing compound derived from garlic. Appl. Environ. Microb. 62:4238-4242.

6. Waardering is de beste motivatie!

7. De discussie omtrent het voorstel om werknemers voor minder dan het minimumloon te laten werken, lijkt weinig actueel gezien het aantal OIO's, AIO's, en NOPpers, dat dit al jaren -legaal- doet.
   Volkskrant, 25 januari 1997.

8. Invoering van het bursalenstelsel voor promovendi heeft niet alleen een negatief effect op hun positie, maar ook op het onderzoek.

9. Een verhuizing naar Engeland verduidelijkt hoe langzaam de totstandkoming van één Europa gaat.

10. Het toenemende gebruik van genetische probes geeft kleur aan de microbiologie.
Appl. Environ. Microbiol. 1996.

11. Reizen naar onbekende plaatsen is net als onderzoek; altijd vol verrassingen!

12. Er bestaan geen giftige stoffen, er zijn alleen giftige hoeveelheden.

13. De voorspelde schade, die werkgevers lopen als gevolg van sportblessures van hun werknemers, weegt niet op tegen de verhoogde fitheid en weerstand van sportieve werknemers.

14. Vrienden zijn onmisbaar!

Stellingen behorende bij het proefschrift "Biodegradation of toluene, benzene and naphthalene under anaerobic conditions" van Alette Langenhoff.
Wageningen, 4 april 1997

Ik wil graag iedereen bedanken die, op welke wijze dan ook, een bijdrage heeft geleverd gedurende de uitvoering van dit promotieonderzoek en het tot stand komen van dit proefschrift.

Aletta

Contents

Chapter 1

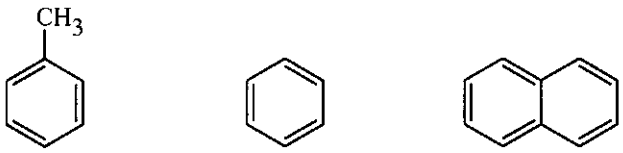
General Introduction

Aromatic compounds are widespread in nature. They can be of biological or of synthetical origin. The most important natural sources of aromatic compounds are poorly degradable polymers, such as lignin, condensed tannins and humus (27). Other natural sources of aromatics in plants contain only one or a few aromatic rings e.g., aromatic amino acids, flavonoids, tannins, etc. (60). Increased industrial activity has resulted in the release of a wide variety of aromatic pollutants in the environment. Aromatic hydrocarbons are important constituents of gasoline, crude oil and oil derivatives, are used as solvents and in wood preservation industries, and are produced by the pyrolysis of materials such as fossil fuels, saturated and unsaturated hydrocarbons and carbohydrates. They are widely distributed in soils and aquatic environments, due to leakage of industrial or sewage effluent and the use and disposal of petroleum products. High concentrations of aromatic hydrocarbons have been detected in the Netherlands. Initially, it was thought that the presence of certain aromatic pollutants in the environment was due to the activity of mankind only. However, there is evidence that an aromatic compound like toluene can be produced by bacteria as well (28, 37).

In the Netherlands, many areas exist that are heavily polluted with toxic aromatic compounds (67). Concentrations of up to 8 g aromatic hydrocarbons/kg dry weight were measured, e.g. in sediments of the river Ur, The Netherlands (58).

Most of the monoaromatic hydrocarbons are depressants of the central nervous system and can damage liver and kidneys. In addition, the polycyclic aromatic hydrocarbons (PAHs) are known carcinogens or mutagens (70). People have become increasingly more aware of the health risks caused by the presence of these toxic compounds in the environment, and removal of these compounds has to be taken care of.

In this chapter, general aspects of microbial transformations are given, and the knowledge about the anaerobic degradation of toluene, benzene, and naphthalene, compounds with different chemical structures (Fig. 1), is summarized. Various redox conditions are described, and an overview of one particular anaerobic condition, namely where Mn^{4+} functions as electron acceptor will be presented. The outline of this thesis is presented in the last section.



toluene benzene naphthalene

Fig. 1. Chemical structures of toluene, benzene and naphthalene.

Microbial transformation.

Aromatic compounds are known to be transformed by microorganisms. Biotransformation is one of the major processes determining their existence and persistence in the environment. In contrast to the often partial degradation by chemical transformations, a complete biological degradation (mineralization) under aerobic and anaerobic conditions is possible (11). Sometimes however, microbial transformations only result in small structural changes, which may lead to the formation of products that are hazardous to the environment as well.

Microbial transformation processes depend on the properties of the chemical compound, the involved microorganisms, and environmental factors. The structural, chemical and physical properties, and the available concentration will control microbial degradation besides the availability of other carbon- and energy sources, or electron donors, electron acceptors, essential nutrients, growth factors, sufficient moisture, and the absence of toxic compounds. Microbial activities depend further on environmental conditions such as temperature, pH, redox potential (Eh), salinity, availability of the aromatic compound etc. (33). Finally, the presence or absence of oxygen as direct oxidant markedly determines the range of possible metabolic pathways (71).

So far, much research has been done on the microbial degradation of aromatic hydrocarbons under aerobic conditions. Under these conditions, oxygen does not only serve as a terminal electron acceptor for the electrons released during metabolic reactions, but oxygen is also incorporated into the aromatic ring by mono- and dioxygenases. Numerous microorganisms have been isolated that mineralize aromatic hydrocarbons under aerobic conditions, and their degradation

pathways have been elucidated. However, due to soil characteristics and bacterial activity in soil, oxygen may become limiting in many polluted soils. As a consequence anaerobic bacteria dominate. These bacteria use terminal electron acceptors like, nitrate, Mn^{4+}, Fe^{3+}, sulfate or carbon dioxide. Since molecular oxygen can not be build into the aromatic ring under anaerobic conditions, different metabolic mechanisms are used for the degradation of aromatics.

Aromatic compounds possess an extremely high stability, because the aromatic structure provides the carbon-carbon bonds with an extra stabilization energy (1). Aerobic bacteria use molecular oxygen as the terminal electron acceptor, but also as a highly reactive cosubstrate to destabilize the aromatic structure. Under anaerobic conditions the function of oxygen as electron acceptor is taken over by alternative electron acceptors (nitrate, manganese, iron, sulfate or carbon dioxide). These alternatives cannot replace oxygen in its function as cosubstrate, and the first degradation steps differ from those under aerobic conditions.

Under anaerobic conditions, the stable structure of the aromatic compounds has to be activated by carboxylation, hydroxylation or CoA thioester formation. Intermediates have to be formed that are susceptible to reductive attack of the aromatic ring by dehydroxylation or transhydroxylations (Fig. 2) (30).

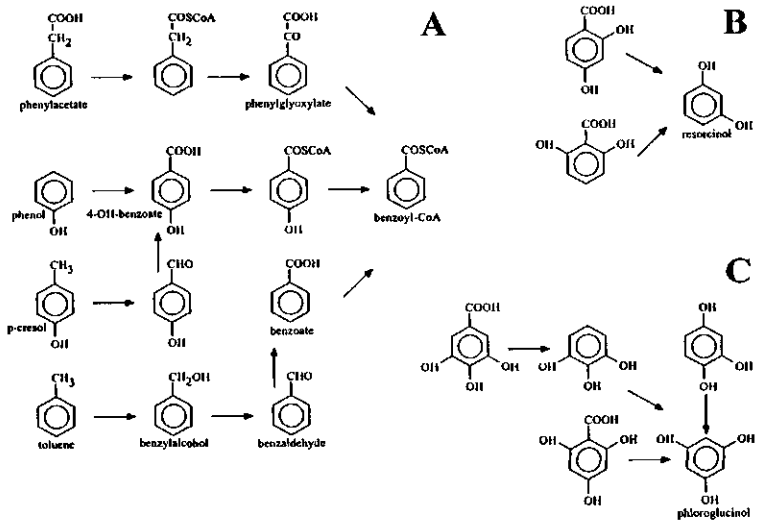


Fig. 2. Reactions leading to the formation of benzoyl-CoA (A), resorcinol (B), and phloroglucinol (C) (taken from Fuchs et al., 1994).

These intermediates are benzoyl-CoA (rather than benzoate), resorcinol and phloroglucinol. Compounds that enter directly one of the pathways mentioned (e.g., benzoate, resorcinol, and phloroglucinol), are degraded faster than compounds that first have to undergo additional transformations (60). Further transformation occurs by reductases. Finally, the formed non-cyclic compounds are converted into central metabolites using conventional pathways.

Ring substituents have their influence on the chemical stability of the aromatic compound. Aromatic compounds with a functional group (-OH, -COOH, $-NO_3$) e.g., benzoate, phenol and catechol, can be degraded anaerobically using various transformation reactions, e.g., reduction of the aromatic ring, dehydroxylation and dehydrogenation (30, 33). In homocyclic aromatic compounds (i.e., cyclic compounds with no oxygen present either in the ring structure or as a substituent) e.g., toluene, benzene and naphthalene, the chemical stability is much larger. This may partly explain why the anaerobic degradation of homocyclics is much less documented than heterocyclics.

The anaerobic degradation of the aromatic hydrocarbons toluene, benzene, and naphthalene.

The resonance energy of benzene is higher than that of naphthalene, indicating that the activation energy for benzene is higher than the energy needed to activate the ring structure of naphthalene (1). Toluene is less stable than these non-substituted aromatic compounds, because of the methyl group that destabilizes the aromatic nucleus, and as a consequence less energy is needed to activate the aromatic nucleus.

Anaerobic degradation of toluene has been shown with bacterial enrichment cultures under denitrifying conditions (36, 38, 49, 72), sulfate-reducing conditions (5, 22, 35) and methanogenic conditions (21, 34, 66, 69), and with pure bacterial cultures under denitrifying conditions (3, 19, 26, 61), iron-reducing conditions (44) and sulfate-reducing conditions (7, 57). No pure cultures have so far been isolated from methanogenic enrichments. Different degradation pathways have been postulated but more research is needed to confirm and complete them. In general,

these degradation pathways seem to be similar, regardless of the type of terminal electron acceptor (nitrate, manganese, iron, sulfate or carbon dioxide) involved.

Most of the research on the degradation pathway of toluene has been performed with denitrifying bacteria. The degradation of toluene in denitrifying bacteria mostly starts with an oxidation of the methyl group via benzylalcohol and benzaldehyde to benzoate and further to benzoyl-CoA (Fig. 3) (4, 9, 29, 61, 62).

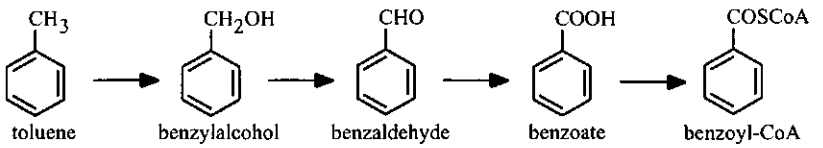


Fig. 3. Formation of benzoyl-CoA from toluene, measured under denitrifying conditions (taken from Altenschmidt and Fuchs, 1992).

Both a benzylalcohol dehydrogenase and a benzoyl-Coenzyme A reductase have been isolated from *Thauera* sp. strain K172 (8, 10). A different pathway was found in both a denitrifying bacterium (strain T1) (25), and a sulfate-reducing enrichment culture (6) (Fig. 4).

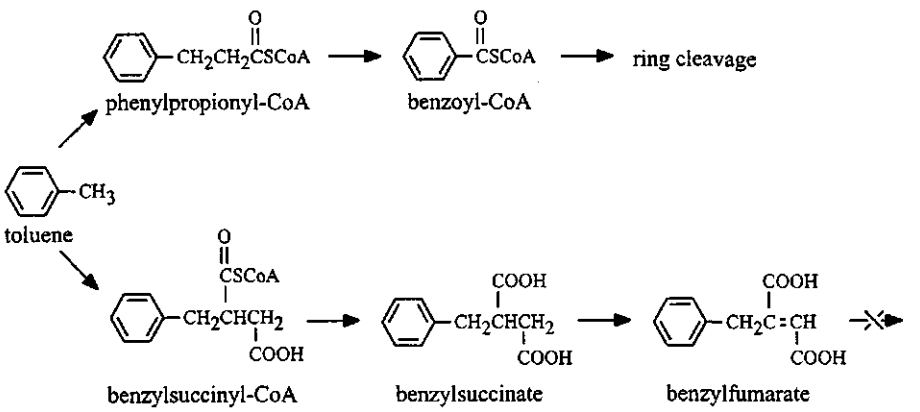


Fig. 4. Transformation of toluene in both a denitrifying bacterium and a sulfate-reducing enrichment culture. The upper pathway illustrates mineralization, and the lower pathway illustrates the formation of two dead-end products benzylsuccinate and benzylfumarate (taken from Evans et al., 1992).

About 80-85% of the toluene was transformed via an oxidative condensation of toluene with acetyl-CoA to phenylpropionyl-CoA, which underwent complete mineralization. The remaining toluene was transformed into two dead-end products: benzylsuccinate and benzylfumarate. These dead-end products were also formed by the sulfate-reducing strain PRTOL1 (7). In a methanogenic enrichment culture on toluene small amounts of *p*-cresol were detected (Fig. 5), formed via the oxidation of the aromatic ring (66).

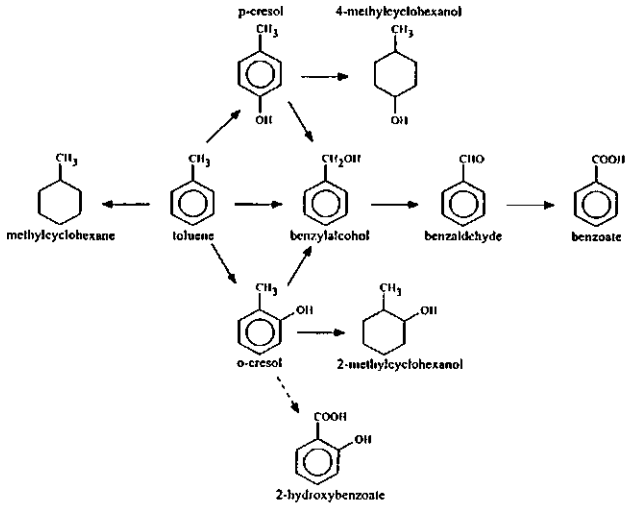


Fig. 5. Transformation of toluene with a methanogenic enrichment culture (taken from Grbić-Galić and Vogel, 1987).

The hydroxyl group was demonstrated to originate from water. Nonetheless, the conversion of toluene to *p*-cresol was slow and could therefore not account for the rapid metabolism of toluene in this culture. It was concluded that most of the toluene was degraded via the oxidation of the methyl group, resulting in the formation of benzoate, and that the conversion to *p*-cresol was only a minor pathway. These results demonstrate that the anaerobic biodegradation of toluene mainly starts with an attack of the methyl group.

Benzene has been considered to be persistent under anaerobic conditions for a long time, but evidence is emerging since the last decade that degradation is possible. For the first time benzene was demonstrated to disappear in a methano-

genic enrichment culture, derived from sewage sludge in which ferulic acid was degraded (34, 66). Complete anaerobic mineralization of benzene to CO_2 was found with aquifer-derived organisms (20), however it was not elucidated whether methanogenic or sulfate-reducing bacteria were involved. Only small amounts of methane were produced and the small changes in sulfate concentration were difficult to measure. With microbial consortia obtained from polluted sediments, the degradation of benzene was shown with iron (47, 48), and with sulfate as electron acceptor (42). So far, nothing is known about possible pathways, intermediates and the bacteria involved.

Knowledge about the anaerobic degradation of polycyclic aromatic hydrocarbons (PAHs) is scarce. Under denitrifying conditions, the mineralization of ^{14}C-naphthalene to $^{14}CO_2$ was found to coincide with the consumption of nitrate in a contaminated soil slurry (2). Naphthalene and acenaphthalene were also transformed in a soil-water system under denitrifying conditions. As these PAHs were not the only carbon sources in the tested system, their degradation could have been due to a cometabolic process (50, 51). The mineralization of [^{14}C]naphthalene and [^{14}C]phenanthrene to $^{14}CO_2$ under sulfate-reducing conditions was demonstrated with heavily contaminated sediment as inoculum, but no degradation was observed in less contaminated sediments (14). So far, no information is available on possible intermediates, on the degradation of higher molecular weight PAHs, and on the responsible bacteria.

Redox conditions.

Anaerobic bacteria that degrade aromatic hydrocarbons depend on the presence of and the capability to use other electron acceptors than oxygen (Table 1). The use of an electron acceptor with a high redox potential will provide the bacteria in theory more energy than the use of an electron acceptor with a lower redox potential. Whether a compound can be degraded in the presence of an electron acceptor partly depends on the amount of energy that is released in the oxidation/reduction reaction.

Table 1. Electron acceptors with their redox pair and redox potential (taken from Nealson and Myers, 1992).

Electron acceptor		Redox couple	Redox potential (mV)
Oxygen		O_2/H_2O	+ 820
Iron(III)	(aq)	Fe^{3+}/Fe^{2+}	+ 770
Nitrate		NO_3^-/NO_2^-	+ 430
Manganese(IV)	(s)	MnO_2/Mn^{2+}	+ 380
Nitrite		NO_2^-/NO	+ 350
Fumarate		Fumarate/Succinate	+ 33
Tetrathionate		$S_4O_6^{2-}/S_2O_3^{2-}$	+ 24
Iron(III)	(s)	Fe^{3+}/Fe^{2+}	+ 0
Sulfite		HSO_3^-/HS^-	- 110
Sulfate		SO_4^{2-}/HS^-	- 230
Carbon dioxide		CO_2/CH_4	- 240

Calculation of the Gibbs free energy changes (ΔG^o) of the oxidation of benzene, toluene and naphthalene coupled to the reduction of the different electron acceptors under standard conditions (25°C, 1 M, 1 atm and pH 7), demonstrate that all possible redox reactions are exergonic (Table 2). To obtain more reliable data the actual concentrations of the compounds, pH, and temperature have to be taken into account. This still does not predict a reaction to take place, but only shows whether thermodynamics allow a reaction to take place under the set conditions.

Reactions with electron acceptors that yield more energy are favoured over the ones that yield less energy (Table 1), with methanogenic conditions being the least favourable. The low energy yield under methanogenic conditions results in slow transformation reactions. Furthermore, fermenting bacteria can be involved in the initial degradation of aromatic hydrocarbons under methanogenic conditions (60). This interdependence of bacteria might explain why no pure cultures of methanogenic toluene degrading bacteria have been isolated so far. Furthermore, microorganisms do not always degrade a contaminant upon exposure. Often an adaptation phase is needed in which the compound is not degraded. Several mechanisms are proposed to explain this phenomena such as enzyme induction, growth of a biodegrading population, and genetic change to evolve new metabolic pathways. These processes occur much slower under lower energy yielding conditions, and adaptation of a methanogenic microbial population to changing growth substrates generally takes a long time. In contrast, in high energy yielding pro-

cesses like nitrate reduction, adaptation takes place faster, and nitrate-reducing bacteria degrade a wider variety of substrates (60).

Table 2. Free energy change ($\Delta G^{o'}$) of the overall-reactions of benzene, toluene and naphthalene at different redox conditions under standard conditions (25°C, 1 M, 1 atm and pH 7) in kJ/electron equivalent.
($\Delta G^{o'} = \Sigma\Delta G^{o'}_{f(products)} - \Sigma\Delta G^{o'}_{f(reactants)}$)
Data used from Weast, 1971-1972, and Stumm and Morgan, 1981.

	Toluene	Benzene	Naphthalene
CO_2	- 2.1	- 2.6	- 1,6
SO_4^{2-}	- 6.8	- 7.4	- 6.3
FeOOH*	- 40.1	- 40.7	- 39.6
MnO_2*	- 93.3	- 93.6	- 92.6
NO_3^-	- 99.8	-100.4	- 99.4
Fe^{3+}	-101.9	-102.5	-101.5
O_2	-106.3	-106.9	-105.9

* Solid-phase free energies were used

The sequence of electron acceptors shows that the reduction of metal oxides is energetically favourable over the reduction of sulfate and carbon dioxide. In sediments, sulfate-reduction and methanogenesis have been found to be inhibited by the presence of metal oxides (56). The degradation of aromatic hydrocarbons under methanogenic, sulfate- and nitrate-reducing conditions is well documented (16, 30). However, much less is known about the use of solid electron acceptors like iron and manganese oxide for the degradation of aromatic compounds. An iron-reducing bacterium *Geobacter metallireducens* strain GS-15 couples the oxidation of toluene, phenol and *p*-cresol to the reduction of Fe^{3+} oxide. In sediments amended with chelated Fe^{3+} forms the oxidation of benzene was demonstrated (48). In sediment slurries amended with amorphous manganese oxide the degradation of some aromatic compounds like benzoate, 4-hydroxybenzoate, aniline, 3-chlorobenzoate, 2,4-dichlorophenoxyacetic acid could be measured (52).

Manganese reduction.

Metals are abundant in terrestrial, estuarine and marine environments (e.g., Fe and Mn up to 51 and 0.9 g/kg, respectively) (18), where the oxidized forms of iron (Fe^{3+}) and manganese (Mn^{4+}) accumulate mainly in the form of a variety of hardly soluble oxides and hydroxides. It is postulated that these metals play an important role in the redox balance and carbon cycle via oxidation/reduction reactions. As shown in table 1, both iron and manganese may serve as an electron acceptor with a considerable release of energy. As they exist mainly as solids, the reduction of manganese oxide is energetically more favourable than the reduction of iron oxide. An advantage of these metal oxides, besides their favourable redox potential, is that they are not lost from the environment. The reduced forms (Mn^{2+} and Fe^{2+}) are oxidized in the anoxic zone, and various solid oxides are formed. These oxides are returned to the anoxic sediments by precipitation. With this metal cycle they can be reused as electron acceptor (Fig. 6). Since metal cycles occur in many sediments, the same metal can be used several times in subsequent reduction and oxidation reactions.

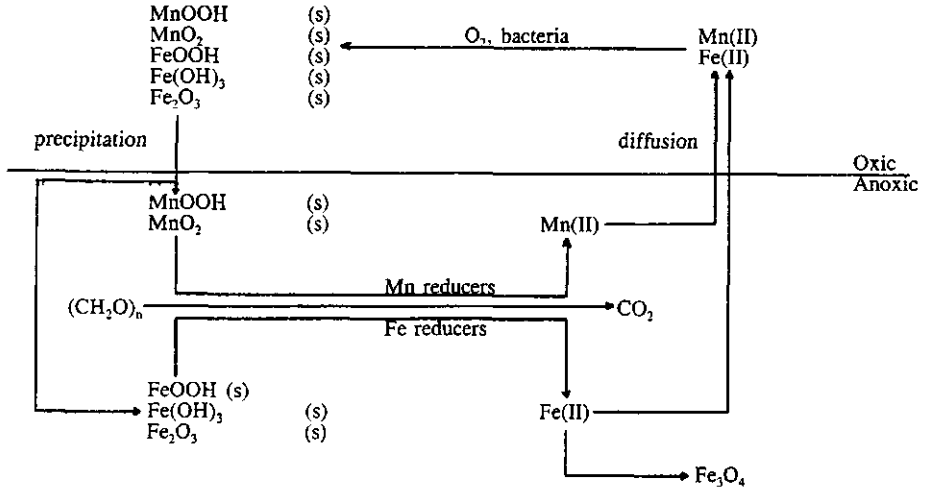


Fig. 6. Schematic representation of an iron and manganese cycle in natural environments. The reduced forms (Fe^{2+} and Mn^{2+}) are oxidized in the oxic zone by O_2, forming various solid oxides. These oxides precipitate into the anoxic zone (taken from Nealson and Myers, 1992).

Most scientific papers deal with the reduction of iron and manganese, because many iron-reducing bacteria can reduce manganese as well. The reduction mechanisms however, are different. Mutants of *Shewanella putrefaciens* MR-1 with an iron-reductase deficiency were still able to reduce manganese oxide (15). This demonstrated that at least one enzyme involved in the reduction of iron- or manganese oxide differ.

The mineralogy of the insoluble manganese oxides greatly affects their reactivity. Amorphous manganese oxides are reduced faster and function better as an electron acceptor than highly crystalline ones, due to a larger specific surface area of amorphous manganese oxides (39, 45, 56). Several dissimilatory manganese-reducing bacteria e.g., *Geobacter metallireducens* and *Shewanella putrefaciens*, reduce amorphous manganese oxide faster than more crystalline forms (39, 56). This effect varies among bacterial species; the rate of manganese reduction is in some microorganisms more affected by the crystallinity of the manganese oxide than in others (12, 55).

Microorganisms that use manganese oxides as electron acceptor possess unique physical and/or biochemical properties to deal directly with these solids. This may include the ability to solubilize manganese oxide, the ability to attach to the manganese oxide and directly transfer electrons to it, or the ability to transport manganese oxide into the cell as a solid. It was demonstrated that *Shewanella putrefaciens* required a physical contact with the insoluble manganese oxide (55). With the marine *Pseudomonas* strain BIII 88, it was found that this bacterium contains electron shuttles in the cell envelope (Mn^{2+}), that transport the reducing power across the cell envelope/manganese oxide particle interface (24).

The mechanism of manganese reduction can either be a direct (enzymatic) or an indirect process. Direct, dissimilatory manganese reduction is defined as the use of Mn^{4+} as external electron acceptor, coupled to organic matter oxidation in fermentation or anaerobic respiration (32). An example of a direct, dissimilatory manganese-reducing bacterium is *Shewanella putrefaciens* MR-1. It obtains energy for growth by using fermentation products such as H_2, formate and lactate as substrate and manganese oxide as terminal electron acceptor (53). Due to the reactivity of manganese oxide, it can also be reduced by organic or inorganic reductants produced and excreted by microorganisms. This process is called indirect

manganese reduction. An example of indirect manganese reduction is the reaction mediated by sulfate-reducers. The produced sulfide reacts outside the cell with Mn^{4+} reducing it to Mn^{2+} (13). This redox reaction is fast under standard conditions, and yields Mn^{2+} and elemental sulfur. Mn^{2+} is stable in the presence of sulfide, because the manganese sulfides are rather soluble and precipitate only at high concentrations (56). At low and neutral pH conditions, iron(II) fastly reduces manganese oxide (12, 45). In sediments with iron and manganese oxides, Mn^{4+} may be reduced seemingly before Fe^{3+}, even in the absence of manganese-reducing bacteria, since Fe^{2+} is immediately reoxidized by Mn^{4+}. Nitrite can spontaneously reduce manganese oxide as well (39). Various organic compounds can interact with manganese oxide, e.g., hydroquinones and phenolic compounds. Reactions with phenolic compounds occur mainly at low pH (46, 63, 64).

In nature, reduced manganese can be oxidized by O_2 to Mn^{4+} to form various oxides. When these fresh manganese oxides are formed, a variety of trace metals can be incorporated in these oxides (Table 3).

Table 3. Forms of manganese oxides in nature
Data used from Nealson, 1983.

Oxides and hydroxides	
Birnessite (δMnO_2)	$(Na,K,Ca)(Mg,Mn^{2+})Mn_6O_{14}.5H_2O$
Buserite	Na-Mn oxide hydrate
Hausmannite	Mn_3O_4
Hollandite	$(Ba,K)_{12}Mn_8O_{16}.xH_2O$
Manganite	MnOOH
Manganosite	$(Ba,K,Mn^{2+},Co)_2Mn_5O_{10}.xH_2O$
Pyrolusite (Rhamsdellite)	MnO_2
Pyrochroite	$Mn(OH)_2$
Todorokite	$(Na,K,Ca)(Mg,Mn^{2+})Mn_5O_{12}.xH_2O$
Iron and iron-silicates	
Jacobsite	$MnFeO_4$
Pyromanganite	$(Mn,Fe)SiO_3$
Rhodonite	$(Mn,Fe,Ca)SiO_3$
Carbonate	
Rhodochrosite	$MnCO_3$
Sulfide	
Albandite	MnS

This can be disadvantageous for sediments in which the formed oxides are reduced, and the toxic trace metals are released and become concentrated in the anoxic zone. In a carbonate-rich environment, the reduced manganese reacts with carbonate to form insoluble $MnCO_3$ (rhodochrosite). This is precipitated in strongly buffered manganese-reducing cultures and no longer available for further oxidation and reduction reactions (56).

Numerous manganese-reducing bacteria have been described, including aerobic and strictly anaerobic ones (23, 31, 40). Over 200 strains of manganese-reducing bacteria were isolated recently, and they were found to consist of a variety of different taxa (55). Of all isolated bacteria, only a few that couple anaerobic respiration-linked manganese reduction to organic carbon oxidation have been described and characterized. *Shewanella putrefaciens* (MR-1), formerly identified as *Alteromonas putrefaciens*, and some other *S. putrefaciens* strains have been isolated from lake and sea sediments. These facultative anaerobic bacteria use a wide range of electron acceptors, such as O_2, Fe^{3+}, Mn^{4+}, NO_3^-, NO_2^-, $S_2O_3^{2-}$, S^o, and fumarate, and can utilize lactate, pyruvate and some amino acids as substrate (56). The obligate anaerobe *Geobacter metallireducens* (GS-15) oxidizes a wide range of organic compounds, including organic pollutants like phenol, toluene and *p*-cresol, under iron-reducing conditions (41, 44). This bacterium was isolated from iron-rich sediment and is categorized in the δ-subclass of the Proteobacteria, which are closely related to *Desulfuromonas acetoxidans* (43). *G. metallireducens* degrades compounds like acetate, butyrate, propionate and ethanol with manganese oxide as electron acceptor. It can use nitrate or U(IV) as electron acceptor as well (44). Several *Bacillus* sp. have been isolated, but only one species (SG1) couples the reduction of manganese to growth with peptone as substrate (16).

The enzyme involved in the direct manganese reduction, the manganese reductase, has not yet been studied as extensively as the iron reductase. Manganese reduction has been found to be coupled to oxidative phosphorylation by using carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). In cultures of *S. putrefaciens* MR-1, the addition of CCCP resulted in inhibition of the oxidative phosphorylation, while manganese reduction was inhibited at the same time (53). In the same cultures, the presence of nitrate inhibited manganese reduction, just as in the case of iron reduction. This led to the hypothesis that nitrate reductase should

be the enzyme responsible for manganese reduction (31). However, the isolation of mutants deficient in either nitrate or manganese reduction, indicate the presence of different enzymes (17, 59).

In conclusion: manganese in the form of a manganese oxide is a solid electron acceptor, which can be found in different forms and can interact with many natural occurring oxidants and reductants. In some anaerobic sediments and soils, microbial catalyzed manganese reduction is a major process for the decomposition of naturally occuring organic matter and can play an important role in the degradation of organic contaminants. Little is known about the physiology of manganese reducing bacteria, and the enzymes involved in this metabolism.

Outline of this thesis.

The aim of the research presented in this thesis was to gain more insight in the possibilities and limitations of the degradation of homocyclic aromatic compounds under anaerobic conditions. Numerous soils and sediments that are polluted with aromatic hydrocarbons wait for their bioremediation. Toluene, benzene, and naphthalene were chosen as model compounds. With sediment column experiments, the behaviour of these aromatic compounds under five different redox conditions, methanogenic, sulfate-, iron-, manganese-, and nitrate-reducing conditions was studied (Chapter 2). The observed degradation of aromatics in these columns have been further elucidated. Chapter 3 describes attempts to enrich for naphthalene degrading sulfate-reducing bacteria. In chapter 4, the coupling between manganese reduction and toluene degradation is shown and the role of the solid electron acceptor and its influence on the degradation rate of toluene is studied in details. Chapter 5 deals with the characterization of the manganese-reducing, toluene degrading enrichment culture and its properties using physiological and rRNA techniques. Finally, the results obtained in this research are discussed in relation to their relevance for soil bioremediation technologies (Chapter 6).

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Chapter 2

Behaviour of Toluene, Benzene and Naphthalene under Anaerobic Conditions in Sediment Columns

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ABSTRACT

The biotransformation of toluene, benzene and naphthalene was examined in anaerobic sediment columns. Five columns filled with a mixture of sediments were operated in the presence of bicarbonate, sulfate, iron, manganese, or nitrate as electron acceptor. The columns were continuously percolated with a mixture of the three organic compounds (individual concentrations 25-200 μM) at 20°C.

Toluene was transformed readily (within 1 to 2 months) under all redox conditions tested. Benzene was recalcitrant over the test period of 375-525 days in all five columns. Naphthalene was partly transformed in the column with nitrate or manganese as electron acceptor present; the addition of benzoate had a positive effect in the column with nitrate. In the column with sulfate, the majority of the added naphthalene disappeared. No effect was observed after adding and omitting an easier degradable substrate. [^{14}C]naphthalene was used to confirm this disappearance to be the result of degradation; two third of the naphthalene was converted to CO_2.

INTRODUCTION

Aromatic hydrocarbons are widespread in nature and often contribute to polluted soils, sediments, and groundwater. Although part of the hydrocarbons is of biosynthetic origin, the majority is produced by the pyrolysis of organic material (15). The contamination of soil with aromatic hydrocarbons is seen on many industrial sites, especially those associated with petroleum industry. Concentrations of 8 g aromatic hydrocarbons/kg dry weight were measured in the sediment of the river Ur, The Netherlands (30). Most of these compounds were shown to be mutagenic or carcinogenic (37).

Research on the microbial degradation of aromatics has mainly focused on aerobic transformation reactions (15, 32). In these transformations, molecular oxygen has two functions: (i) as a terminal electron acceptor for the electrons released during metabolic reactions and (ii) as a direct oxidant of the aromatic ring. In many polluted environments, oxygen is limited and anaerobic processes prevail.

In the absence of oxygen, electron acceptors like nitrate, sulfate, bicarbonate and some metal-ions (e.g. iron and manganese) have taken over the function of oxygen as a terminal electron acceptor. Since these alternative electron acceptors cannot replace oxygen in its second function, the first reaction steps differ from those in the aerobic processes. Anaerobic degradation of homocyclic aromatic hydrocarbons has only been found recently (14, 16, 33). Degradation of toluene by pure cultures has been reported under sulfate-reducing (29), iron-reducing (22) and denitrifying conditions (3, 9, 13, 31), and parts of degradation pathways were elucidated (4, 5, 12). Only a few reports exist on the anaerobic degradation of benzene. With consortia obtained from sediments, the degradation of benzene was demonstrated under iron-reducing conditions (24), and with sulfate as electron acceptor (21). In mixed cultures, derived from ferulic acid-degrading sewage sludge enrichments, benzene degradation was shown under methanogenic conditions (17, 35). With aquifer-derived organisms a complete mineralization of benzene to CO_2 was demonstrated, although it was not clear whether this occurred under methanogenic or sulfate-reducing conditions (10).

Knowledge about the anaerobic degradation of polycyclic aromatic hydrocarbons (PAH) is scarce. In one study the lower molecular weight polycyclic compounds naphthalene and acenaphthene were degraded under denitrifying conditions in soil-water systems (26, 27, 28). This degradation was also demonstrated in soil-slurry systems (2). So far, nothing is known about possible pathways or intermediates. Although anaerobic bacteria have the ability to degrade homo- and polycyclic aromatic compounds, little is known about the most appropriate redox conditions for the biotechnological clean-up of anaerobic soils and sediments.

The objective of this study was to examine the anaerobic transformation of toluene, benzene and naphthalene in the presence of bicarbonate, sulfate, iron, manganese, or nitrate in soil percolation columns.

MATERIALS AND METHODS

Experimental set-up. The transformation studies were performed in five continuous-flow packed-bed columns (Fig. 1) during 375-525 days, with one electron acceptor per column. The columns were glass cylinders (60 ml volume, 15 cm length, 2.3 cm inside diameter) which were capped at the lower end by a standard fitting (Schott, Germany) and at the upper end with a viton stopper (Rubber B.V., Hilversum, The Netherlands).

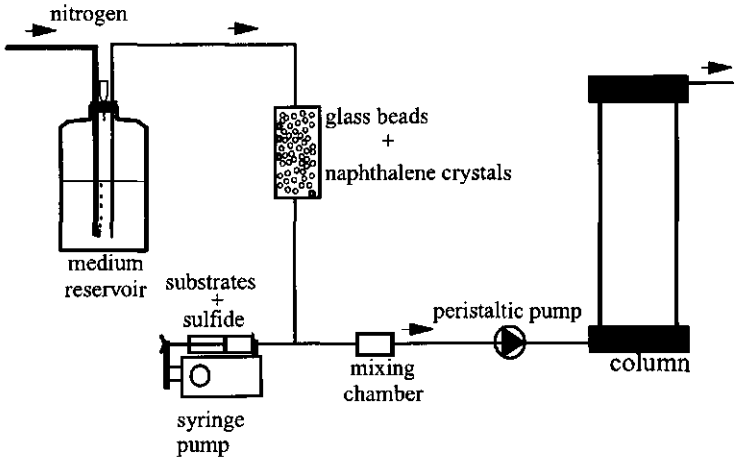

Fig. 1. Schematic diagram of the column system.

The wet-packed bed in the five columns consisted of a mixture of anaerobic soil and sediment polluted with (polycyclic) aromatic compounds and of granular sludge. Sediment from the river Rhine near Wageningen was used, because toluene, benzene, and naphthalene have been detected as contaminants in Rhine water. The same accounts for the use of polluted harbour sludge (Rotterdam and Zierikzee, The Netherlands) and soil polluted with PAH (DSM, De Staatsmijnen, Geleen, The Netherlands). In addition, granular sludge from an upflow anaerobic-sludge blanket reactor, used for the treatment of sugar beet wastewater (CSM, Centrale Suikermaatschappij, Breda, The Netherlands), was used because of its high density of anaerobic bacteria.

The columns were percolated continuously in an upflow mode under saturated conditions with an anaerobic medium containing per liter: 0.34 g KH_2PO_4; 1.07 g $Na_2HPO_4.2H_20$; 0.063 g $NaHCO_3$; 0.11 g $CaCl_2.2H_2O$; 0.1 g $MgCl_2.6H_20$; 0.027 g NH_4Cl; 0.0085 g Na_2SO_4, and 0.1 ml of a trace element solution (18). The medium was boiled, followed by cooling down under a N_2/CO_2 atmosphere (99.5/0.5%) to preserve anaerobic conditions. An excess of granular marble in the reservoir served as carbonate buffer in combination with the CO_2 in the gas phase. Mixtures of toluene, benzene, and naphthalene were added continuously with a syringe pump (Braun Medical, Utrecht, The Netherlands). Mixing of the aromatics and the medium occurred in a small mixing chamber (13 ml) just before the peristaltic pump. An influent concentration of 25 μM for each of the compounds was chosen. After 7 months of testing, the addition of naphthalene to the column with sulfate was changed. Medium was pumped through a glass column (15 ml) filled with glass beads and naphthalene crystals. This resulted in an influent naphthalene concentration of 200 μM.

Bicarbonate as electron acceptor was present in excess in the medium. Sulfate and nitrate were added as Na_2SO_4 and $NaNO_3$ via the syringe pump. Final concentrations were 10 mM each. In the columns with iron and manganese, amorphous Fe(III)- and Mn(IV)-oxide were mixed through the column material (approximately 5 mmol) and were re-added upon depletion. Toluene served as a positive control in these columns and its reappearance in the effluent was seen as a depletion of the iron- and manganese-oxide. This was done because it was not possible to measure the actual concentration of the oxides in the columns. The metaloxides were re-added by sluicing the columns into an anaerobic glovebox (Coy Laboratories Products, Toepffer GmbH, Göppingen, FRG), in which freshly made iron- or manganese-oxide was mixed through the material in the column. Reducing conditions were maintained by the addition of Na_2S via the syringe pump (0.4 mM final concentration). Because of possible inhibitory effects on denitrification processes, Na_2S was not used in the nitrate-reducing column (19). In the columns with iron and manganese, the added amorphous Fe(III)- and Mn(IV)-oxide were not significantly reduced by the sulfide because of their large excess in concentration.

The tubing in the system was either gastight neoprene or viton (Rubber B.V., Hilversum, The Netherlands). Viton was used from the point were the aromatics entered the system. The medium was pumped into the system by a peristaltic pump, equipped with acid-flex pump-tubes (Bran & Lubbe, Maarssen, The Netherlands).

The flow rate in the columns was 3.5 ml/h, which gave a retention time of the liquid in the packed-bed columns of 10 h. The experiments were done at 20°C in the dark.

[^{14}C]naphthalene study. A smaller column (10 cm length and 1.2 cm inner diameter giving a volume of 11 ml) was used to study the degradation of [^{14}C]naphthalene in the presence of sulfate in more detail. The wet-packed bed in this column was material from the larger column, in which disappearance of naphthalene had occurred in the presence of sulfate. Initially, the column was operated in the same way as the larger column. Upon breakthrough, followed by disappearance of the naphthalene, the column was operated as a closed circulating system for 60 days (Fig. 2).

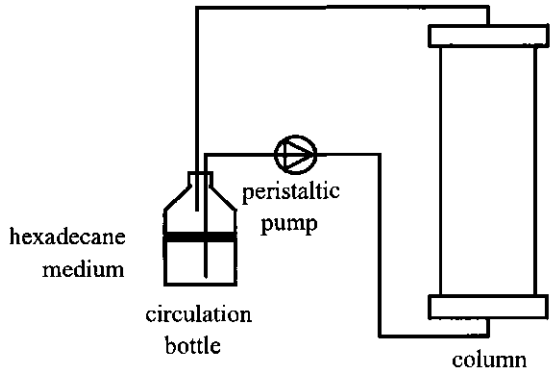

Fig. 2. Schematic diagram of the circulating column system.

Anaerobic medium (as described above plus 15 mM Na_2SO_4, 0.4 mM Na_2S and 0.5 mg/l resazurin) was circulated through the column. In the circulation bottle, a layer of hexadecane (1 ml) floated on top of the medium to create a two-liquid-phase system. The hexadecane layer contained both [^{12}C]- and [^{14}C]naph-

thalene, in a total concentration of 0.2 M with an activity of 3 μCi. This resulted in a naphthalene concentration in the liquid medium of about 35 μM. During the 60 days, a total of 75 μmol naphthalene was fed through the column. The experiment was performed at 20°C in the dark.

Preparation of Fe- and Mn-oxides. Amorphous iron-oxide, Fe(III)-oxyhydroxide, was made by neutralising a 0.4 M $FeCl_3$ solution with 1N NaOH until pH 7 (23).

Amorphous manganese(IV)-oxide was made by mixing equal amounts of 0.4 M $KMnO_4$ and 0.4 M $MnCl_2$ and adding 1N NaOH to obtain a pH 10 (7).

Thereafter, both metal-oxides were washed 4 times with demineralized water.

Addition of different substrates. The effect of several easily degradable carbon compounds on the transformation of toluene, benzene, or naphthalene was tested. Acetate, benzoate, lactate, and phenol were added at different time intervals via the syringe pump. Tested concentrations were 5-250 μM.

Sampling and analyses. The concentrations of toluene, benzene, and naphthalene were measured routinely. Samples were taken by allowing either the influent or effluent to flow into a gas-tight syringe. After centrifugation (13,000 rpm. for 3 min.) of the samples, they were analyzed on a High Performance Liquid Chromatograph (LKB, Bromma, Sweden). Samples (20 μl) were injected onto a Chromsep Chromspher PAH column (200x30 mm) at 25°C. The flow rate was 1 ml/min with an eluent of 55% acetonitrile and 45% nanopure water. All aromatic compounds were detected with an UV detector at 206 nm.

The production of $^{14}CO_2$ was measured routinely in 1 ml of medium withdrawn from the circulation bottle. 0.5 ml of medium was injected into 1 ml of 1.5 N NaOH and stripped with air (30 ml/min) for 5 min. To a third of this sample (0.5 ml), 4.5 ml scintillation liquid was added (Aqualuma Plus, Lumac, 3M, The Netherlands) and counted for 3 min in a scintillation counter (1211 Rackbeta, LKB). This measurement represented the total activity of the non-volatile compounds and CO_2. Another 0.5 ml medium was injected into 1 ml of 1.5 N HCl, stripped with air, and used for scintillation counting as previously described. This measurement represented the total activity of the non-volatile compounds. The $^{14}CO_2$-production was calculated as the difference between these two methods.

Chemicals. Toluene, benzene, naphthalene, acetate, benzoate, lactate, and phenol were purchased from E. Merck, Darmstadt, Germany. Naphthalene-1-^{14}C with a specific activity of 8.3 mCi/mmol was purchased from Sigma, St Louis, USA. All chemicals were of analytical grade and were used without further purification.

RESULTS

Methanogenic column. The column was operated for 525 days. After a partial breakthrough, toluene could not be detected in the effluent 2 months after start-up. The detection limit was 0.05 μM. No disappearance of benzene and naphthalene was observed, not even after the addition of acetate, benzoate, lactate, and phenol for a period of 20 to 40 days (results not shown).

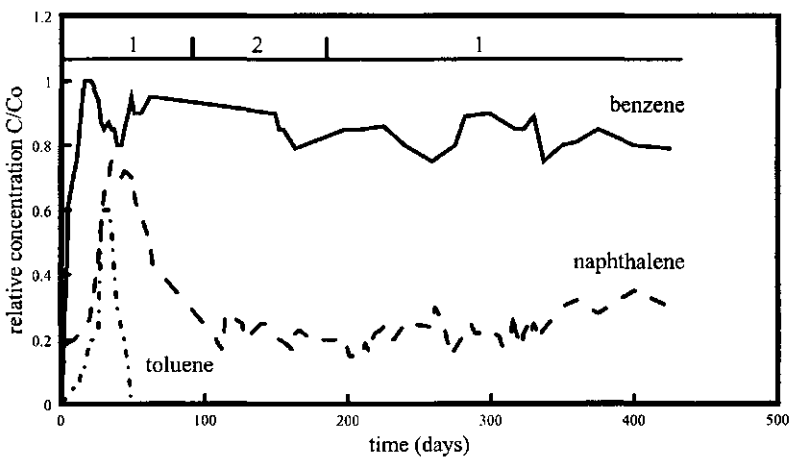

Fig. 3. Behaviour of toluene, benzene and naphthalene in the presence of sulfate. C/Co is effluent concentration relative to influent concentration. (1): no benzoate added, (2): addition to the medium of 5 μM benzoate.

Sulfate-reducing column. The behaviour of toluene, benzene, and naphthalene in the column with sulfate is shown in Figure 3. After a partial breakthrough of toluene, it was not detected in the effluent after 50 days of operation. From day 100 on toluene was omitted from the medium. Naphthalene also showed a partial breakthrough, followed by a steady decline. After 100 days, roughly 70 - 80% of the incoming naphthalene was removed. The addition or omittance of 5 μM benzoate did not seem to have any effect on the disappearance of naphthalene. The increase in influent concentration to 200 μM at day 200 did not result in an increase in the effluent concentration. No significant removal of benzene was observed during the 425 days of operation of the column.

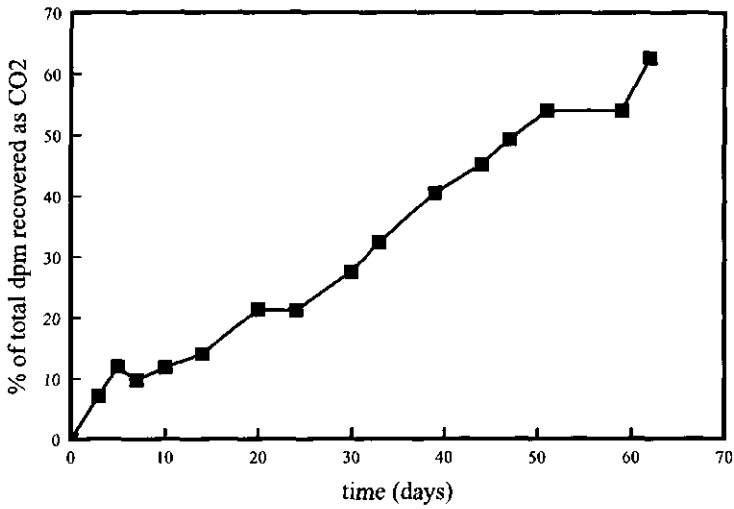

Fig. 4. Production of $^{14}CO_2$ from [^{14}C]naphthalene in the presence of sulfate in a recirculating system.

The second column with sulfate showed a fast breakthrough and disappearance of the naphthalene upon which radiolabeled naphthalene was added. Through circulation of the medium, the produced $^{14}CO_2$ accumulated in the system (Fig. 4). After 2 months, 60% of the added naphthalene was transformed to CO_2 (0.5 mmol).

Iron-reducing column. After a partial breakthrough, toluene was undetectable in the effluent after about 2 months of operation. At day 100 it was temporarily omitted from the medium. After 225 days, benzene and naphthalene were still not removed, and toluene was then re-added to check for microbial activity in the column. Within one week after this addition, toluene could no longer be detected in the effluent. The subsequent additions of easier degradable substrates (50-250 μM benzoate, phenol, and lactate) did not result in any disappearance of benzene or naphthalene during the 375 days of operation (results not shown).

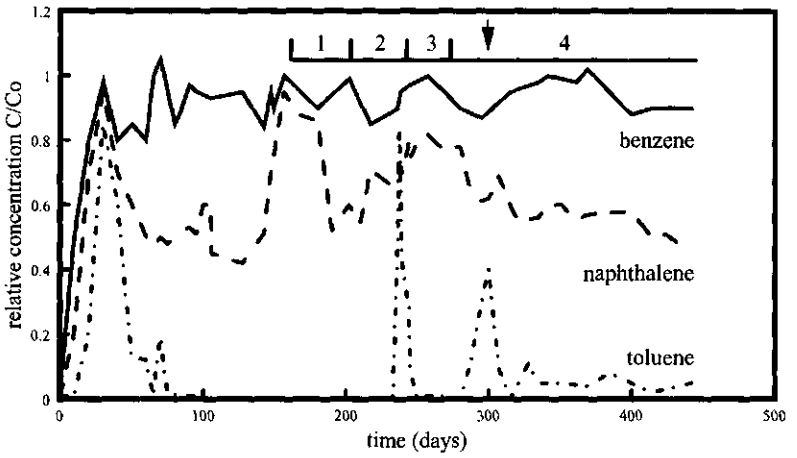

Fig. 5. Behaviour of toluene, benzene and naphthalene in the presence of manganese. C/Co is effluent concentration relative to influent concentration. Additional substrates at (1): 50 μM benzoate, (2): 250 μM benzoate, (3): 50 μM phenol: (4): 50 μM lactate. Between day 100 and 225 toluene was omitted from the influent. At day 300 (↓) 5 mmol MnO_2 was added to the column.

Manganese-reducing column. Toluene showed a partial breakthrough (Fig. 5). After 85 days, less than 2% was detected in the effluent. Similar to the operation of the column with iron, toluene was omitted from day 100 on and re-added at day 225. A similar pattern as in the first few months was observed, except for a faster removal of toluene after the breakthrough.

After readdition of manganese-oxide (5 mmol) at day 300, toluene was detected in the effluent (around 2 μM) for the remainder of the experiment. In contrast to benzene, part of the naphthalene disappeared. During the first 300 days, the naphthalene concentration in the effluent varied strongly (between 10 and 60% removal), but after the extra addition of Mn(IV)-oxide, a steady decline in the naphthalene concentration occurred. At the end of the experiment, around 60% of the incoming naphthalene was transformed. The effects of the additions of benzoate, phenol, and lactate were not conclusive.

Nitrate-reducing column. Also under denitrifying conditions, toluene showed a partial breakthrough. After 90 days of operation it was no longer detected in the effluent anymore and omitted from day 100 on (Fig. 6).

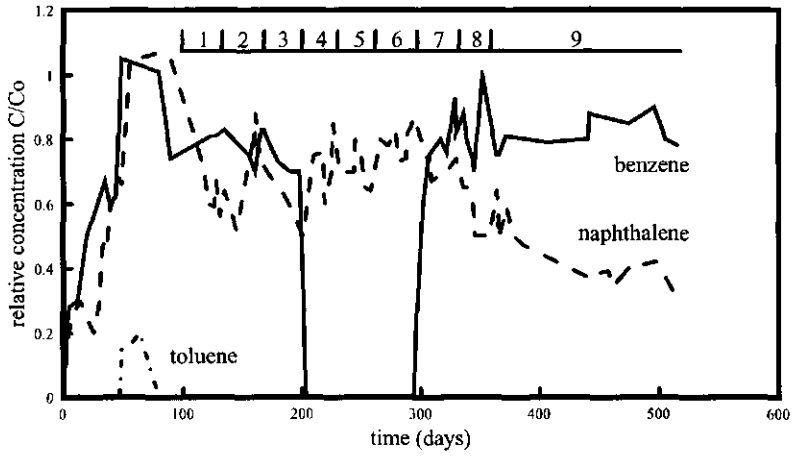

Fig. 6. Behaviour of toluene, benzene and naphthalene in the presence of nitrate. C/Co is effluent concentration relative to influent concentration. Additional substrates at (1): 5 μM benzoate: (2): 50 μM benzoate, (3): 250 μM benzoate, (4): 50 μM acetate, (5): 50 μM lactate, (6): 150 μM lactate, (7): 250 μM acetate, (8): 50 μM phenol, (9): 250 μM benzoate. Between day 200 and day 300 benzene was omitted from the column.

Benzene underwent no significant removal and therefore it was omitted between day 200 and 300. This was done to test a possible effect on the transformation of naphthalene. No effect was observed. A breakthrough of naphthalene was followed

by a removal of 10-50 % during the first 200 days. From day 300, a steady decline to 70 % removal at day 520 occurred in the presence of 250 μM benzoate. Previous additions of 5-250 μM benzoate, acetate, lactate, and phenol had no effect.

DISCUSSION

We have examined the behaviour of three aromatic hydrocarbons in flow-through sediment columns under different anaerobic conditions. Favourable conditions were created to succeed in biological transformations of the selected compounds. The experimental set-up allowed an easy change of the conditions to test different substrates. The packed-bed of the sediment columns provided aerobic and anaerobic microorganisms with a history of exposure to toluene, benzene, and naphthalene. Earlier, comparable experiments in our laboratory with anaerobic sediment columns resulted in transformation reactions of compounds like di- and trichlorobenzene (6) tetrachloroethene (8) and hexachlorocyclohexanes (25). In sediment column experiments, attention has to be paid to the adsorption of hydrophobic substrates to the column material. This to be sure that a decrease in effluent concentration is due to transformation and not a result of adsorption. The degree of adsorption of the aromatic hydrocarbons to the column material was studied in batch experiments. The results indicated a negligible adsorption (results not shown). In addition, we found that the three substrates showed a breakthrough in all tested columns. This also indicated little adsorption to the column material.

Bacteria can only use a compound for growth when the Gibbs free energy change (ΔG) is negative. Calculations of the ΔG-values of the oxidation of benzene, toluene, and naphthalene coupled to the reduction of the different electron acceptors in our experiments, demonstrate that under the conditions used, all possible redox reactions were exergonic (Table 1). So, all reactions are thermodynamically possible, with methanogenic and sulfate-reducing conditions being less favourable.

Table 1. Free energy change (ΔG) of the overall-reactions of benzene, toluene and naphthalene at different redox conditions under the used conditions (20°C, 25-200 μM, 1 atm and pH 6.7) in kJ/electron equivalent
($\Delta G = \Delta G^{\circ} + RT \ln K$).
Data used from Weast (1971-1972) and Stumm and Morgan (1981).

	Toluene $c_{in}=25\mu M$	Benzene $c_{in}=25\mu M$	Naphthalene $c_{in}=25\mu M$	Naphthalene $c_{in}=200\mu M$
CO_2	- 4.1	- 4.5	- 3.8	- 3.9
SO_4^{2-}	- 8.8	- 10.3	- 8.6	- 8.7
$FeOOH^*$	- 40.2	- 41.4	- 40.8	- 40.9
MnO_2^*	- 94.2	- 94.3	- 93.7	- 93.8
NO_3^-	-100.2	-104.3	-100.0	-100.1

* Solid-phase free energies were used

This table shows the free energy change of the different reactions after complete degradation of 1 μM substrate.
The concentration of CO_2 (present as granular marble $CaCO_3$) was calculated according to Stumm and Morgan (34), the maximum solubility of N_2 was used as the concentration of N_2 in the medium and the concentration of methane was 1 mM.

It can be predicted that benzene and naphthalene degradation under anaerobic conditions is more difficult than the degradation of toluene and that the reaction mechanisms will vary, due to the chemical properties of the aromatics. The chemical stability of the aromatic ring is determined by the presence of side-groups. Methyl- and hydroxylgroups drive electrons towards the aromatic ring and make the ring more susceptible to electrophilic substitution reactions. This, because less energy is needed to activate the ring structure. Benzene and naphthalene are therefore chemically more stable than toluene, and benzene is more stable than naphthalene (1).

Toluene was transformed relatively fast in the presence of bicarbonate, sulfate, iron, manganese, and nitrate as electron acceptor. This is in agreement with many other findings (14, 33). However, the transformation under manganese-reducing conditions is novel. In batch experiments with material taken from the column with manganese present, a decrease in toluene concentration coincided with an increase in Mn(II) concentration and we were able to enrich for toluene degrading manganese-reducing bacteria (20). This has not yet been documented

before.

Benzene was found to be recalcitrant under all conditions tested. Even after test periods of 375 to 525 days, no transformation of benzene was observed. This can only partly be explained by its high chemical stability because our findings are in contrast with those in three other studies, in which mineralization of benzene was found under anaerobic conditions (10, 21, 24). In the first study, over 90 % of the added [^{14}C]benzene could be recovered as $^{14}CO_2$. A specific electron acceptor for the oxidation of benzene could not be established. More than 80 % mineralization of [^{14}C]benzene was reported in the other two studies. The oxidation of benzene was coupled to the reduction of Fe(III) and sulfate, respectively. Previous anaerobic degradation studies with benzene, toluene, xylenes, and ethylbenzene added as mixtures, have shown that toluene and the xylenes were degraded, but that benzene and ethylbenzene were persistent (11). It was concluded that environmental conditions, like the presence of other substrates, are important for the anaerobic biodegradability of benzene. In our study, the presence of the more readily degradable toluene and naphthalene in the columns may have been of influence on the persistence of benzene.

Degradation of naphthalene was seen in the columns amended with sulfate, manganese and nitrate. In the presence of nitrate, a steady decrease in the naphthalene concentration was only found after the addition of 250 μM benzoate. Degradation of naphthalene did not occur with other tested substrates. Several explanations are possible: Benzoate can (i) serve as an electron donor, necessary for the reduction of the aromatic ring, (ii) have a positive effect as a possible intermediate in the degradation of naphthalene or (iii) act as an easily degradable substrate for growth. It has been shown before that naphthalene can be degraded under denitrifying conditions in soil-water systems. With excess nitrate, 4.5 mg/l naphthalene was degraded in batch-experiments to non-detectable levels ($<$0.01 mg/l) within two months (26, 27).

In the presence of manganese, part of the naphthalene was transformed. Whether this disappearance was influenced by the addition of easily degradable substrates is not yet clear. The decrease in effluent concentration beyond day 300 (Fig. 5) could have been due to the addition of fresh MnO_2 or to the presence of 50 μM lactate.

The disappearance of naphthalene was also found in the column with sulfate as electron acceptor. An eight-fold increase in the naphthalene concentration at day 200 had no effect on the effluent concentration. In contrast to the columns with nitrate and manganese, no effect was seen upon the addition or omission of an easily degradable substrate, in this case benzoate (5 μM). These experiments indicate the presence of an active naphthalene transforming microbial population. This was confirmed in the second column experiment, in which mineralization of naphthalene was proven. 60 % of the [^{14}C]naphthalene that had passed the column, could be recovered as $^{14}CO_2$.

In the described column experiments, the role of the electron acceptor has not been verified. With sulfate and nitrate as electron acceptor it was not possible to quantify the decrease in electron acceptor concentration, needed to transform the substrates, because of the low concentration of the aromatic substrates and the fluctuations in the influent and effluent concentrations. The reduced forms of the metal-oxides, Fe(II) and Mn(II), formed precipitates with sulfide and other compounds in the column and could not be measured in the effluent for that reason. This indicates that, for example in the column with sulfate, other electron acceptors like bicarbonate or oxidized metal-ions could have been involved in the degradation of naphthalene. However, this is not likely, because the columns with the electron acceptors bicarbonate and Fe(III) did not show a comparable degradation of naphthalene.

In conclusion, homocyclic aromatic compounds can be degraded anaerobically under favourable conditions. Proper environmental conditions like the presence of a suitable electron acceptor, nutrients, and other oxidizable compounds will be essential for the transformations to take place. Although these transformations are slow and unpredictable, anaerobic bioremediation processes do not require the addition of oxygen like in aerobic processes. This may decrease the bioremediation costs substantially. It is known that these aromatics are more susceptible to aerobic degradation and for bioremediation purposes it has to be evaluated whether lower degradation rates at lower costs under anaerobic conditions can compete with a faster, but more expensive, aerobic process.

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Chapter 3

Naphthalene Degradation under Sulfate Reducing Conditions:

Attempts to Enrich for Naphthalene Degrading Bacteria

Alette A.M. Langenhoff and Gosse Schraa

ABSTRACT

Naphthalene was degraded in continuously percolated, anaerobic sediment columns with sulfate as electron acceptor. Numerous attempts were made for further enrichment of sulfate-reducing, naphthalene degrading bacteria. Different naphthalene concentrations, inoculum sizes, medium compositions, extra additions etc. were used, but all attempts failed.

Naphthalene was shown not to be toxic for four strains of sulfate-reducing bacteria, *Desulforhabdus amnigenus* str. ASRB1, *Syntrophobacter fumaroxidans* str. MPOB, *Desulfitobacterium dehalogenans* and *Desulfomonile tiedjei*.

INTRODUCTION

The anaerobic degradation of various monoaromatic hydrocarbons has been extensively documented (7, 8, 20), but little is known about the degradation of polycyclic aromatic hydrocarbons (PAHs) in the absence of molecular oxygen. Under anaerobic conditions the aromatic hydrocarbons have to be activated via carboxylation, hydroxylation or CoA thioester formation, and need to be directed into a few central intermediates that are susceptible to reductive attack of the aromatic ring via dehydroxylations or transhydroxylations (7). The formed intermediates (benzoate, resorcinol and phloroglucinol) can be enzymatically attacked by reductases and the formed non-cyclic compounds are converted into central intermediates using conventional pathways (20). The degradation of PAHs like naphthalene, anthracene, phenanthrene and pyrene with other electron acceptors than molecular oxygen is thermodynamically possible, but the Gibbs free energy change under methanogenic and sulfate-reducing conditions is small. This suggests that these conditions are less favourable than nitrate-, manganese- or iron-reducing conditions (14).

Only a few reports exist on the anaerobic degradation of PAHs. Naphthalene (55 μM) and acenaphthalene (2.6 μM) were found to be transformed in the presence of other carbon sources under denitrifying conditions in 40 to 50 days in a soil-water system (16, 17). The degradation of naphthalene under denitrifying

conditions was also demonstrated in a contaminated soil slurry. The mineralization of [^{14}C]naphthalene to $^{14}CO_2$ coincided with the consumption of nitrate (2). Recently, the mineralization of [^{14}C]naphthalene and [^{14}C]phenanthrene was demonstrated under sulfate-reducing conditions with heavily contaminated sediments as inoculum. No degradation was observed in less contaminated sediments (5). We previously showed that naphthalene was degraded in laboratory-scale sediment columns (13). Two-third of the added [^{14}C]naphthalene was degraded to $^{14}CO_2$ in the presence of sulfate as electron acceptor.

Here we describe our attempts to enrich for, and isolate naphthalene degrading, sulfate-reducing bacteria.

MATERIALS AND METHODS

Medium and cultivation. Batch experiments were performed in 115-ml bottles, filled with 20 ml medium, designed to support sulfate-reducing bacteria. It contained per liter of demineralized water: 0.41 g of $KH_2PO_4.2H_2O$; 3.6 g of $NaHCO_3$; 0.44 g of NH_4CO_3; 0.11 g of $CaCl_2.2H_2O$; 0.1 g of $MgSO_4.4H_2O$; 0.3 g of NH_4Cl; 0.3 g of NaCl; 0.48 g of $Na_2S.9H_2O$; 2.56 g of Na_2SO_4; 0.0005 g of resazurin; 1 ml of acid trace element solution I (containing per liter: 1.5 g of $FeCl_2.4H_2O$; 0.1 g of $MnCl_2.4H_2O$; 0.12 g of $CoCl_2.6H_2O$; 0.07 g of $ZnCl_2$; 0.015 g of $CuCl_2$; 0.06 g of H_3BO_3; 0.25 g of $NiCl_2.6H_2O$; 1.4 g of HCl); 1 ml of alkaline trace element solution II (containing per liter: 0.4 g of NaOH; 0.02 g of Na_2SeO_3; 0.03 g of Na_2WO_4; 0.025 g of Na_2MoO_4) and 1 ml of vitamin solution (containing per liter: 2 mg of biotin; 10 mg of p-aminobenzoate; 10 mg of pantothenate; 50 mg of pyridoxine; 20 mg of nicotinamide; 20 mg of thiamine HCl; 10 mg of riboflavine; 10 mg of cyanocobalamine).

In an anaerobic glovebox (Coy Laboratories Products, Toeppfer GmbH, Göppingen, FRG), aliquots of stock solutions of $KH_2PO_4.2H_2O$, Na_2SO_4 and resazurin were made up with anaerobic demineralized H_2O to 90% of the final volume of the medium. The bottles were sealed with viton stoppers (Maag Technic AG, Dübendorf, Switzerland). The gas phase was changed to N_2/CO_2 and brought to 1.3 bar. The bottles were heat-sterilized at 121°C. To complete the medium,

three filter sterilized stock solutions (A, B and C) were added aseptically by syringe. Solution A contained 1 ml of trace element solution I, 1 ml of trace element solution II, and 1 ml of vitamin solution per 25 ml of demineralized water. Solution B contained $NaHCO_3$, NH_4HCO_3 and Na_2S, 20-fold the final concentration. Solution C contained $CaCl_2$, $MgSO_4$, NH_4Cl and NaCl, 40-fold the final concentration. 25 ml of solution A, 50 ml of solution B, and 25 ml of solution C were added per liter medium. The pH of the medium was usually between 6.5 and 6.8.

Material from an anaerobic sediment column in which the transformation of high concentrations of naphthalene (200 μM) in the presence of sulfate was observed (13), was used as inoculum. Approximately 1 gram of homogeneous column material was added to each of the batches. Naphthalene was the carbon- and energy source, and it was added to the batches in four ways. Naphthalene was added as an aqueous solution varying in concentration from 1 to 200 μM (saturation). To obtain higher final concentrations, the medium was completely saturated with naphthalene via the addition of naphthalene crystals. Before inoculation, the medium was filtered into empty, sterile, anaerobic bottles, to remove the crystals from the medium. A two-liquid-phase system of medium and hexadecane with naphthalene, was used to obtain a low concentration of naphthalene in the aqueous phase. For this, 0.2 ml of naphthalene dissolved in hexadecane (0.2 mM) was added to the batches. This resulted in a constant naphthalene concentration in the medium of 15 μM. A two-liquid-phase system with both [^{12}C]- and [^{14}C]-naphthalene in the hexadecane layer (0.2 M with an activity of 5 μCi/ml) was also used. A fourth way was to add naphthalene as an ethanol solution (50 mM of naphthalene in ethanol). Ethanol served thus as a second carbon- and energy source.

The bottles were incubated stationary in the dark at 30°C during at least one year. Every experiment was performed at least in duplicate and controls with inhibitors of microbial activity were taken along as well. 1 mM of sodium azide (an inhibitor of electron transport-linked respiration) and 15 mM of sodium molybdate (substrate analogue of sulfate and an inhibitor of sulfate-reducing bacteria) were used. Microbial activity in the batches was examined by measuring the naphthalene concentration, the production of sulfide and/or the production of $^{14}CO_2$.

Addition of fresh column material, column medium or column effluent. To approach the conditions of the column experiments in the batches, extra sediment (10 g) was added together with the inoculum, to a number of batches. This sediment was a freshly made mixture of soil, sediment and sludge as was used in the column with sulfate as electron acceptor, where the inoculum material originated from. Besides incubations with batch medium, column medium was used (13) or 50 % of the batch medium was replaced by column medium or effluent.

Addition of various compounds. Several batches were made with extra additions. Two grams of teflon beads (∅0.5 mm), glass beads (∅1.5 mm), bentonite (<0.5 mm), vermiculite, sterilized granular sludge from an upflow anaerobic sludge blanket reactor (CSM, Centrale Suikermaatschappij Breda, The Netherlands), sterilized Rhine river sediment (0.5 m depth, Wageningen, The Netherlands) or artificial sediment like precipitated aluminium phosphate (0.24 g/l of $AlCl_3.6H_2O$ added to phosphate rich medium) or sloppy agar (0.2 % w/v) was added to 115 ml bottles in a glovebox. The bottles were filled with anaerobic medium as described and inoculated with the column material.

Immobilization of the column material. Small amounts of the column material were used to be immobilized in κ-carrageenan (10). In an anaerobic glovebox, a solution of 3% κ-carrageenan in naphthalene-saturated medium was mixed with material from the sediment column. The medium composition was the same as previously described, except that $NaHCO_3$ was replaced by $KHCO_3$. Droplets were made with a syringe and needle, and coagulated in a 0.75 M KCl solution in naphthalene-saturated medium. After 1 hour of coagulation, the beads were used for further incubation in naphthalene-saturated medium.

Solid media. In addition to cultivation in liquid media, enrichments were also done in solid media. Both roll-tubes (medium with 2 % agar) (11) and soft agar shake tubes (medium with 1 % agar), with naphthalene (0.1 mM) as substrate and 0.1 g of column material as inoculum, were made.

Naphthalene toxicity. The toxicity of naphthalene to four sulfate-reducing bacteria was tested in batch experiments. *Desulforhabdus amnigenus* str ASRB1 (DSM 10338) (19), *Syntrophobacter fumaroxidans* str. MPOB (DSM 10017) (21), *Desulfitobacterium dehalogenans* (23) and *Desulfomonile tiedjei* (6) were grown in the previously described medium with 20 mM of propionate and 15 mM of sulfate,

20 mM of fumarate, 20 mM of pyruvate and 15 mM of sulfate, and 20 mM of pyruvate, respectively.

Various concentrations of naphthalene were added to a final medium concentration of 10, 50, 100 and 200 μM. The amount of inoculum varied from 0.25, 0.5, 1 to 2 % and the bottles were incubated at 37°C. Growth and the rate of substrate consumption (propionate, fumarate or pyruvate) were used to evaluate a toxic effect on the activity of the culture.

Sampling and analyses. The concentration of naphthalene in the batches was measured routinely. 0.5 ml of medium was taken with a syringe, centrifuged (13,000 rpm. for 3 min.) and analyzed on a High Performance Liquid Chromatograph (LKB, Bromma, Sweden). Samples (20 μl) were injected onto a Chromsep Chromspher PAH column (200x30 mm) at 25°C by using an autosampler (Spectra System AS1000). The mobile phase was 55% acetonitrile and 45% nanopure water at a flow rate of 1 ml/min. The eluted compounds were identified and quantified with a fluorescence detector (Fluor LC 304, Linear Instruments, Reno, Nevada, USA) at an excitation wavelength of 270 nm and an emission wavelength of 360 nm.

Organic acids (fumarate, pyruvate and propionate) were analyzed on a High Performance Liquid Chromatograph (LKB, Bromma, Sweden). Samples (20 μl) were injected onto a Chrompack organic acid column (300x6.5 mm) at 60°C by using an autosampler (Spectra System AS1000). The mobile phase was 0.01 N H_2SO_4 at a flow rate of 1 ml/min. The eluted compounds were identified and quantified by differential refractometry (LKB 2142 refractometer)

Sulfide was measured as described by Trüper and Schlegel (22).

The formation of $^{14}CO_2$ was measured as described in Chapter 4 (12).

Chemicals. Naphthalene, acetate, lactate, benzoate, propionate, fumarate, and pyruvate were purchased from E. Merck, Darmstadt, Germany. κ-Carrageenan Genugel X-028 was purchased from A/S Kobenhavns Pektinfabrik, Kopenhagen, Denmark. Naphthalene-1-^{14}C with a specific activity of 8.3 mCi/mmol was purchased from Sigma, St Louis, USA. All chemicals were of analytical grade and were used without further purification.

RESULTS & DISCUSSION

The goal of this work was to enrich for naphthalene degrading sulfate-reducing bacteria in batches from the previously described sediment column (13) and to study the degradation of naphthalene under sulfate-reducing conditions in more detail. The occurrence of naphthalene degradation in a sediment column with sulfate as electron acceptor was demonstrated in that work. The use of [^{14}C]naphthalene in a recycling column under similar conditions had resulted in the production of $^{14}CO_2$ (Chapter 2). As a consequence, one would expect to be able to transfer this activity to batches using the column material as inoculum. This procedure had been successful for the isolation of the tetrachloroethene degrading bacterium *"Dehalobacter restrictus"* (PER-K23) from a methanogenic sediment column in which tetrachloroethene was transformed to ethene (9).

A variety of incubation conditions has been tested, but none of them resulted in the enrichment of bacteria capable of degrading naphthalene in the presence of sulfate. The reason for this is unknown. One possibility is that naphthalene is toxic for anaerobic bacteria. At the time these experiments were done, no information was available about the toxicity of naphthalene to anaerobic bacteria.

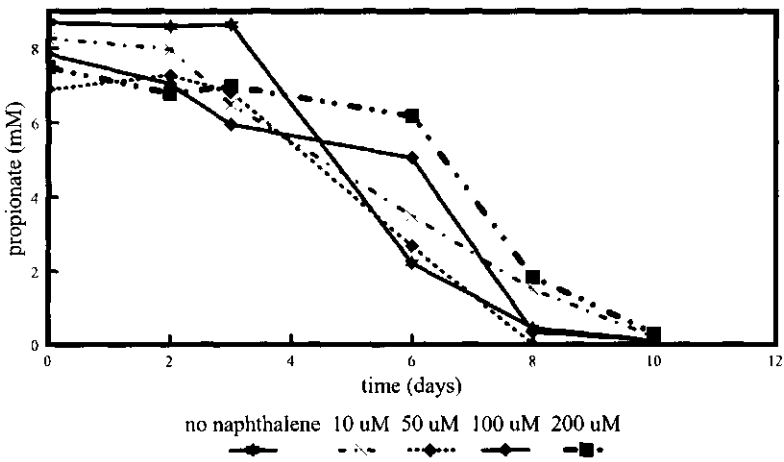

Fig. 1. Decrease in substrate concentration (propionate) during the growth of *Desulforhabdus amnigenus* in the presence of various concentrations of naphthalene (1% inoculum).

We performed toxicity tests with *Desulforhabdus amnigenus*, *Syntrophobacter fumaroxidans*, *Desulfitobacterium dehalogenans* and *Desulfomonile tiedjei*. The first two organisms had been isolated from granular sludge (19, 21). Granular sludge was also mixed through the sediment in our successful column experiments. The growth of the four sulfate-reducing bacteria was not affected by the presence of the different naphthalene concentrations. The batches with and without naphthalene showed more or less comparable growth of the four strains (Fig. 1). Even at the smallest inoculum size, the activity was maintained under all conditions tested (Table 1).

Table 1. The growth of four sulfate-reducing bacteria (*Desulforhabdus amnigenus*, *Syntrophobacter fumaroxidans*, *Desulfitobacterium dehalogenans* and *Desulfomonile tiedjei*) on their growth substrates propionate, fumarate, pyruvate and pyruvate, respectively, in the presence of various concentrations of naphthalene and with different amounts of inoculum size.

	Desulforhabdus amnigenus				***Syntrophobacter fumaroxidans***				***Desulfitobacterium dehalogenans***				***Desulfomonile tiedjei***			
concentration	**inoculum size (%)**				**inoculum size (%)**				**inoculum size (%)**				**inoculum size (%)**			
naphthalene	**0.25**	**0.50**	**1.0**	**2.0**	**0.25**	**0.5**	**1.0**	**2.0**	**0.25**	**0.5**	**1.0**	**2.0**	**0.25**	**0.5**	**1.0**	**2.0**
0 μM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10 μM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
50 μM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
100 μM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
200 μM	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ normal growth of the culture

These results demonstrate that the lack of transformation of naphthalene in our batch experiments with column material as inoculum is not likely to be due to toxicity. Toxic effects of naphthalene on aerobic bacteria have not been documented. Naphthalene crystals, resulting in relatively high naphthalene concentrations in the medium, were used to grow several *Pseudomonas* sp. without detrimental effects (24).

We used a variety of naphthalene concentrations in our enrichments, but we were never able to enrich for naphthalene degrading microorganisms. The addition of naphthalene dissolved in an ethanol solution, only resulted in the enrichment of ethanol degrading organisms. In the one-liquid-phase batches with naphthalene concentrations from 1 to 200 μM, no decrease of naphthalene was measured during 340 days (Fig. 2).

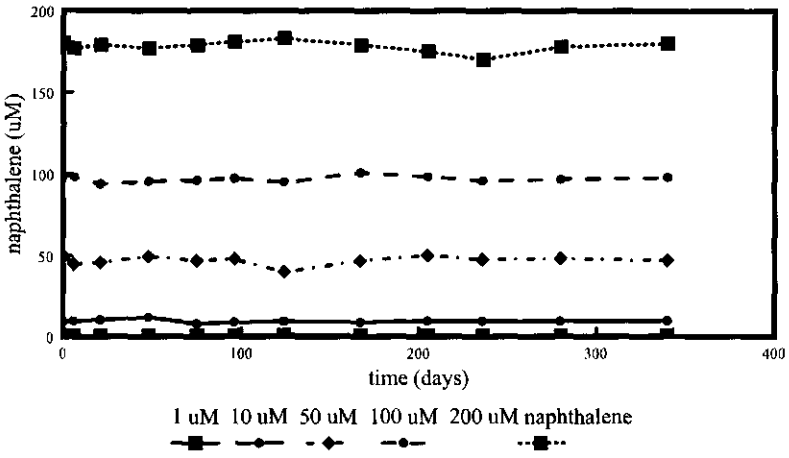

Fig. 2. Behaviour of naphthalene in one-liquid-phase batches with different concentrations of naphthalene as the only carbon- and energy source, and column material as inoculum.

In two-liquid-phase batches, it was not possible to measure a naphthalene decrease. We therefore followed the production of sulfide (Fig. 3) or $^{14}CO_2$. The batches with a high sulfide production were transferred into one-liquid-phase batches, but no decrease in naphthalene concentration could be measured after this transfer. The formation of sulfide in the first enrichments was probably due to the transformation of organic material present in the inoculum or the transformation of hexadecane. It is known that hexadecane can be degraded under sulfate-reducing conditions (1), although our controls without hexadecane showed the same sulfide production (data not shown). In batches with [^{14}C]naphthalene in a two-liquid-phase system, we could never detect the production of $^{14}CO_2$. This could be due to the persistence of

naphthalene or to a partial degradation, which would not result in CO_2 formation. Partial degradation would be in contrast to what we have seen in a previous sediment column.

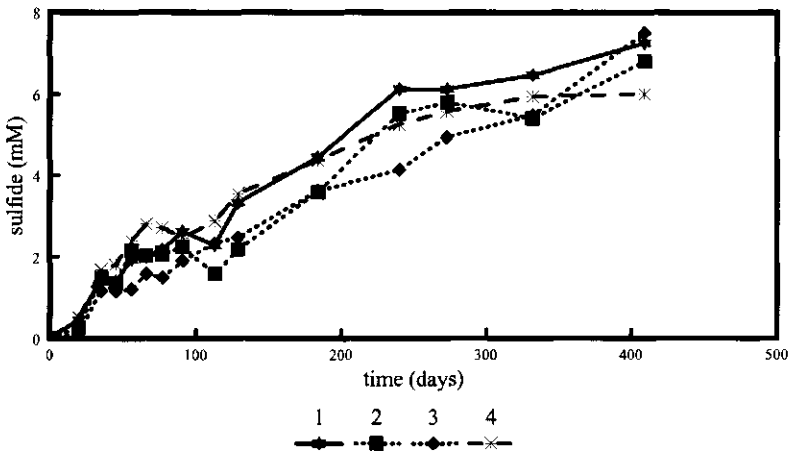

Fig. 3. Sulfide production in two-liquid-phase batches with naphthalene as substrate and column material as inoculum in quadruplicate.

Cysteine, in stead of sulfide, was used as a reducing agent in several experiments. Sulfide can react with metal ions present in the medium, forming insoluble complexes, and thus making them unavailable for bacteria. Combinations of sulfide (3mM) and cysteine (1 mM), and sulfide (1mM) and dithionite (0.17 mM) were tested as well, but none of these resulted in the enrichment of naphthalene degrading bacteria.

In the naphthalene degrading sediment column, the role of sulfate as electron acceptor was not verified, due to low concentrations and practical problems. Although it was not likely that other electron acceptors were involved in the degradation of naphthalene (13), other electron acceptors were tested in batch experiments as well. Only in the presence of oxygen a degradation of naphthalene in batches was found. With nitrate, amorphous manganese oxide, ferrous citrate, amorphous iron oxide, sulfite, thiosulfate or bicarbonate as electron acceptor no

degradation was observed.

A difference between the columns and the batches is the continuous flow of fresh medium with nutrients in the columns and the liquid/solid material ratio. The solid material in the columns can act as binding site for bacteria. In addition, some organic compounds, trace elements or other micronutrients from the sediment and/or granular sludge may be essential for the bacteria. It has been shown that the anaerobic degradation of polychlorinated biphenyls by enrichment cultures increased in the presence of sterile Rhine river sand (15) or Raritan river sediment (4). Sterile Rhine river sediment was also needed to maintain the mineralization of toluene by an enrichment culture that uses manganese oxide as ultimate electron acceptor (12). Furthermore, aluminium phosphate and sloppy agar were found to be necessary to cultivate gliding bacterial species, like filamentous sulfate reducers of the genus *Desulfonema* (25). The presence of two grams of teflon beads, glass beads, bentonite, vermiculite, granular sludge, Rhine river sediment or artificial sediment like precipitated aluminium phosphate or sloppy agar did not result in the enrichment of naphthalene degrading bacteria. This suggests that the inability to enrich for bacteria may not be due to the lack of a solid surface or the need of nutrients.

The addition of different organic substrates was tested, because lactate and benzoate had a positive effect on the transformation of naphthalene in sediment columns (13). The addition of acetate, lactate or benzoate (0.1 mM) had no positive effect on the lack of transformation of naphthalene in our batches.

To approach the conditions of the column experiments in the batches, extra sediment was added to the batches to obtain a lower liquid/solid material ratio. Furthermore, the column medium was used in stead of the normally used batch medium. Degradation of naphthalene could not be observed here either, not even when 50 % of the batch medium was replaced by column medium or by effluent of the sulfate-reducing column.

The columns had been operated at 20°C, whereas the batches were routinely incubated at 30°C. Incubation of batches at 20°C did also not result in the degradation of naphthalene.

Clustering the inoculum material by using κ-carrageenan can have a positive effect, when consortia of bacteria are needed to degrade naphthalene. Bringing the bacteria together may facilitate the diffusion of intermediates, thus increasing the degradation rate. Although the bacteria in the immobilized column material in the κ-carrageenan beads produced sulfide, no degradation of naphthalene was observed (Fig. 4). The produced sulfide was probably due to the transformation of organic material present in the inoculum, or the transformation of κ-carrageenan. κ-Carrageenan can be degraded under anaerobic conditions, although the solidified state makes it less available for microbial degradation (18). The production of sulfide indicates that bacteria were immobilized and were active, but that they had no ability to degrade naphthalene.

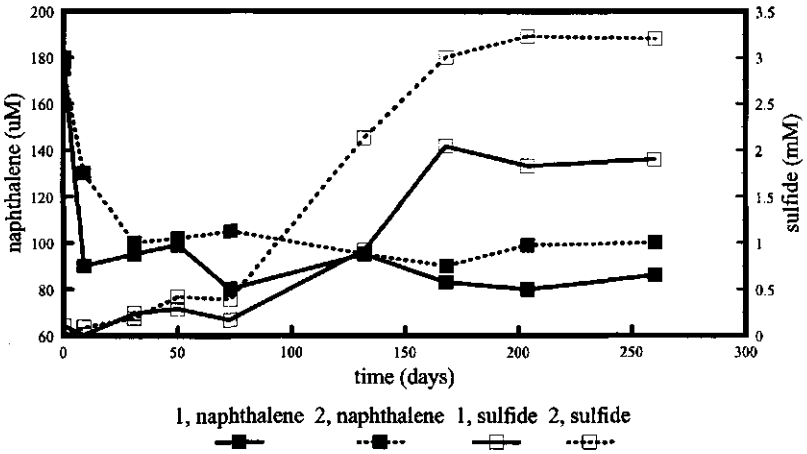

Fig. 4. The production of sulfide and the behaviour of the naphthalene concentration in the liquid in one-liquid-phase batches with immobilized column material as inoculum in duplicate.

Cultivation on solid agar resulted in the formation of black colonies on the agar. Transferring the colonies into liquid media or sloppy agar did not result in the enrichment of sulfate-reducing naphthalene degrading bacteria. The colonies may have grown on agar or impurities in the agar instead of on naphthalene.

Finally, it is known that microorganisms do not often degrade a contaminant upon exposure, but develop the capability to degrade the contaminant after prolonged exposure. This adaptation was demonstrated in a study with sediment from San Diego Bay. Naphthalene and phenanthrene were oxidized to carbon dioxide in sediments that were heavily contaminated with PAHs (33 mg/kg of sediment) but not in less contaminated sediments (4 mg/kg of sediment) (5). We performed batch experiments with heavily and less contaminated sludge and sediment originating from various locations, but were not able to transform naphthalene under a variety of anaerobic conditions.

In conclusion, we were not able to enrich for naphthalene degrading, sulfate-reducing bacteria, despite a variety of batch experiments (over 400) to create optimal conditions for degradation. It is frustrating, but the reason is not clear. It seems that selective enrichment for bacteria, responsible for the degradation of naphthalene, can fail to mimic the conditions that microorganisms require for their growth. Finding the reasons for failure, e.g. when unculturable organisms are involved (3), and finding microorganisms, is needed to obtain knowledge about the physiology and biochemistry of the anaerobic degradation of polycyclic aromatic hydrocarbons.

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Chapter 4

Microbial Reduction of Manganese Coupled to Toluene Oxidation

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ABSTRACT

Toluene degradation occurred in anaerobic flow-through sediment columns filled with contaminated sediment and sludge to which either amorphous or highly crystalline manganese oxide was added.

An enrichment culture from these sediment columns was able to grow on toluene under strictly anaerobic conditions in the presence of manganese oxide. The oxidation of toluene was coupled to the production of CO_2 and to the reduction of Mn(IV). Of the different manganese oxides tested, the rate was slowest with crystalline manganese oxide.

After several transfers of the enrichment culture, its ability to degrade toluene became less and was ultimately lost, unless sterilized Rhine river sediment was present in the medium.

Direct contact between the bacteria and the manganese oxide was found to be advantageous for a rapid toluene degradation. The degradation rate could further be increased by adding organic ligands such as oxalic acid or nitrilotriacetic acid.

INTRODUCTION

Leaks in underground fuel storage tanks, improper disposal techniques and spills of all types of petroleum products have led to a widespread toluene contamination of soil, sediment and groundwater. The growing awareness concerning the toxic and even suspected carcinogenic effect requires to reduce this contamination and to remediate the contaminated areas (9).

Under aerobic conditions toluene degradation proceeds rapidly and several aerobic toluene degrading bacteria have been isolated (16, 34, 35). However, in many polluted areas oxygen is limited and anaerobic processes prevail. Toluene degradation under anaerobic conditions has been demonstrated under methanogenic (11, 17, 36, 37), sulfate-reducing (2, 12), and nitrate-reducing conditions (19, 20, 27, 38) and several pure cultures of toluene degrading anaerobic bacteria have been described under sulfate-reducing (3, 32), iron-reducing (23), and nitrate-reducing (1, 10, 15, 33). In a previous study in our laboratory, we have observed the disap-

pearance of toluene in an anaerobic continuous flow sediment column in which Mn(IV), present as manganese oxide (MnO_2), was the dominant electron acceptor (21). Manganese is a widespread transition metal (about 5-10 times less abundant than iron) in our environment, and the different manganese oxides are potential electron acceptors for the oxidation of organic compounds in anaerobic sediments. Manganese reduction is energetically more favourable than iron reduction. The mineralogy of the insoluble oxides greatly influences their reactivity. Amorphous manganese oxides have a larger specific surface area than the crystalline forms, and this may make the manganese reduction to proceed faster (30). Except for that study, we are not aware of any reports on the anaerobic degradation of toluene under manganese-reducing conditions. A variety of manganese-reducing bacteria has been described, but none of them is able to degrade aromatic compounds (13, 22, 31). The manganese- and iron-reducing bacterium *Geobacter metallireducens* is able to degrade toluene with iron oxide as electron acceptor, but not with manganese oxide as electron acceptor (unpublished results). Only in sediment slurries amended with amorphous manganese oxide was the degradation of some aromatic compounds like benzoate, 4-hydroxybenzoate, aniline, 3-chlorobenzoate, and 2,4-dichlorophenoxyacetic acid observed (29).

In this study we report details on the degradation of toluene in sediment columns with three types of manganese oxide and on the enrichment and maintenance of a bacterial mixed culture that couples the oxidation of toluene to the reduction of Mn(IV).

MATERIALS AND METHODS

Sediment columns. The behaviour of toluene was studied in continuous-flow packed-bed columns as previously described (21). Three types of manganese oxide were used in three different columns: highly crystalline manganese oxide, amorphous manganese oxide and freeze dried amorphous manganese oxide (see below for details). These oxides (approximately 10 mmol/column) were mixed through the column material at the beginning of the experiment. Upon depletion, caused by reduction of the manganese oxide, fresh manganese oxide (5 mmol) was mixed

through the column material. These additions were done under strict anaerobic conditions in a glovebox.

A flow rate of 3.5 ml/h was used, which gave a column retention time of 10 h. The toluene concentration was 70 μM at the beginning of the experiments and was increased over a 110-day period to 300 μM. The columns were operated at 20°C in the dark.

The concentration of toluene was measured routinely in the dark. Samples were taken by allowing either the influent or the effluent to flow into a gas-tight syringe. After centrifugation (13,000 rpm. for 3 min) of the samples, they were analyzed by High Performance Liquid Chromatograph (HPLC).

Medium. For the enrichment cultures and for the batch experiments 115-ml bottles were used, filled with 20 ml of anaerobic medium. The medium contained per liter of demineralized water: 0.58 g of $NaH_2PO_4.2H_2O$; 2.5 g of $NaHCO_3$; 0.25 g of NH_4HCO_3; 0.11 g of $CaCl_2.2H_2O$; 0.1 g of $MgCl_2.6H_2O$; 0.1 g of KCl; 1.5 g of NH_4Cl; 0.4 g of $Na_2S.9H_2O$; 0.0005 g of resazurin; 1 ml of trace element solution (containing per liter: 15 mg of NTA; 1 mg of $FeSO_4.7H_2O$; 5 mg of $MnSO_4$-$.2H_2O$; 1 mg of $CoSO_4$; 1 mg of $ZnSO_4$; 0.1 mg of $CuSO_4.6H_2O$; 0.1 mg of $AlK(SO_4)_2$; 0.1 mg of H_3BO_3; 25 mg of Na_2MoO_4; 0.1 mg of $NiCl_2.6H_2O$; 10 mg of NaCl) and 1 ml of vitamin solution (containing per liter: 20 mg of biotin; 50 mg of *p*-aminobenzoate; 50 mg of pantothenate; 20 mg/l of folic acid; 50 mg of lipoic acid; 100 mg of pyridoxine; 50 mg of nicotinamide; 50 mg of thiamine-HCl; 50 mg of riboflavine; 1 mg of cyanocobalamine).

Media were prepared as described by Holliger (18): Aliquots of stock solutions of $NaH_2PO_4.2H_2O$ and resazurin were made up with anaerobic demineralized H_2O to 90% of the final volume of the medium in an anaerobic glovebox. The poorly soluble manganese oxide (0.1 g) was added to the bottles inside the glovebox as well. The bottles were sealed with viton stoppers (Maag Technic AG, Dübendorf, Switzerland) and outside the glovebox the gas phase was changed to N_2/CO_2 (80%/20%) and brought to 1.3 bar. The bottles were heat-sterilized at 121°C, before the aseptic addition by syringe of the rest of the medium from three filter-sterilized anaerobic stock solutions (A, B and C). Solution A contained 1 ml of trace element solution and 1 ml of vitamin solution per 25 ml of demineralized water. Solution B contained $NaHCO_3$, NH_4HCO_3 and Na_2S, 20-fold the final

concentration. Solution C contained $CaCl_2$ and $MgCl_2$, 40-fold the final concentration. Per liter medium, 25 ml of solution A, 50 ml of solution B and 25 ml of solution C were added. The pH of the medium was 6.7-7.0.

Toluene was added to the batches in two ways. A one-liquid-phase system was created via the addition of 2-6 ml of a toluene solution (5 mM, saturation) to give a final concentration in the liquid of 0.3 to 1 mM. Higher concentrations were not used because of possible inhibitory effects, but toluene was readded when depleted. A two-liquid-phase system of medium and hexadecane was used to create a constant low toluene concentration in the medium and a constant flux of toluene from the organic phase to the aquatic medium. To the batches, 0.5 ml of hexadecane containing 0.2 M toluene was added. This resulted in a concentration in the liquid phase of 190 μM. The bottles were incubated stationary in the dark at 30°C.

Chemicals. Toluene was purchased from Merck (Darmstadt, Germany) and was of analytical grade. [*ring*-UL-^{14}C]Toluene with a specific activity of 10.2 mCi/mmol was purchased from Sigma (St. Louis, MO, USA).

Highly crystalline manganese oxide (pyrulosite) with a specific surface area of less than 0.20 m^2/g was purchased from Aldrich (The Netherlands) and amorphous manganese oxide was made according to Burdige (6). The amorphous form had been identified as vernadite (δMnO_2), based on its X-ray diffraction pattern, its average oxidation state and its specific surface area ($\pm$ 180 m^2/g). The amorphous manganese oxide was washed 4 times by centrifugation and resuspended in demineralized water. Finally, the suspension was (freeze-dried and) stored anaerobically in bottles. Suspensions of amorphous manganese oxide in anaerobic medium were made for the addition to batch incubations.

Enrichment procedures. Material from each of the three sediment columns (2 g [wet weight]), in which toluene was transformed, served as inoculum for the primary enrichment cultures. Secondary enrichment cultures were prepared by transferring liquid and solids (MnO_2 + sediment; 10% [v/v]) to bottles containing fresh medium, after approximately 100 μmol of the toluene was transformed. A decrease in toluene concentration was measured routinely in the headspace by Gas Chromatograph (GC). The transfers were done every 30 to 60 days, depending on the transformation rate.

Subculturing the enrichment culture in the absence of sediment material after 4 to 5 transfers always resulted in the loss of the toluene transformation activity. Two grams of either teflon beads (∅0.5 mm), glass beads (∅1.5 mm), bentonite (∅ < 0.5 mm), vermiculite (∅ < 0.5 mm), granular sludge from an upflow anaerobic-sludge blanket reactor (CSM Breda, The Netherlands) or Rhine river sediment (from 0.5 m depth, Wageningen, The Netherlands) were added to the bottles in an anaerobic glovebox. The bottles were filled with anaerobic medium and amorphous MnO_2, sterilized, and inoculated with 10% (v/v) of an active enrichment culture. 2 ml of a toluene solution (5 mM) was added. The effect of the additions on the transformation activity was examined by GC during a period of at least 5 transfers (150 to 350 days).

Batch experiments. The coupling between the mineralization of toluene and the formation of Mn(II), the effect of direct contact between bacteria and manganese oxide and the effect of the addition of organic ligands were tested in batch experiments with enrichment cultures from the 12th generation or more. All experiments were performed at least in duplicate and controls with inhibitors for microbial activity were taken along. 1 mM of sodium azide (an inhibitor of electron transport-linked respiration) and 0.175% of formaldehyde (a general inhibitor of biological activity) both inhibiting manganese-reducing bacteria, were used (7).

The mineralization of toluene and the formation of Mn(II) was tested in two simultaneous batch experiments. In the first set of batches, the decrease in toluene concentration and the amount of Mn(II) formed was measured during the degradation of toluene in a two-liquid-phase system. A separate bottle was sacrificed for each Mn(II) measurement, because part of the produced Mn(II) will bind and adsorb to the remaining amorphous MnO_2, and also forms precipitates with metal ions present in the medium (8). In the second set of batches, the $^{14}CO_2$ production was examined. In two-liquid-phase batches [^{12}C]toluene and [*ring*-^{14}C]toluene were dissolved in hexadecane up to a total concentration of 0.2 M with an activity of 1 μCi/ml, and 0.5 ml was added to the batches. Routinely, 1 ml of medium was analyzed for the production of $^{14}CO_2$. Killed controls, controls without manganese oxide, and controls without toluene were taken along.

The effect of direct contact between the manganese-reducing bacteria and the manganese oxide surface was tested by adding amorphous manganese oxide to the

medium as described before or enclosed in a bacterial-filter package. The bacterial-filter package was a small, sealed package of 0.22 μm Durapore filter (Millipore, Etten-Leur, The Netherlands), which contained manganese oxide. This prevented the bacteria to be in direct contact with the solid manganese oxide. The bottles were inoculated with 10% (v/v) of an active enrichment culture, and toluene, in a hexadecane solution, was added as substrate. A decrease in toluene concentration in the headspace was measured routinely by GC.

The effect of an increased solubility of Mn(IV) on the toluene transformation rate was tested by the addition of organic ligands to the MnO_2. The effect of various concentrations of oxalic acid and nitrilotriacetic acid (NTA) on the solubility of manganese oxide was tested. Batches were made with 4 mM of oxalic acid or 2 mM of NTA and amorphous manganese oxide. The bottles were inoculated with 10% (v/v) of an active enrichment culture. Toluene, in a hexadecane solution, was added as substrate and its decrease was followed over time by GC.

Analytical procedures. The concentration of toluene in the column samples was measured by HPLC (LKB, Bromma, Sweden). Samples (20 μl) were injected onto a Chromsep Chromspher PAH column (200x30 mm) at 25°C. The flow rate was 1 ml/min with an eluent of 55% acetonitrile and 45% nanopure water. All aromatic compounds were detected with an UV detector at 206 nm.

The concentration of toluene in the batches was measured in the headspace by GC (Chrompack 438A, Chrompack Packard B.V., The Netherlands). Headspace samples of 200 μl were injected onto a capillary column (SIL 5CB; 10m * 0.53 mm, 2 μm bead size, Chrompack Packard B.V.) and analyzed with a flame ionization detector. The column temperature was 70°C.

Mn(II) and Mn(IV) were determined by Atomic Adsorption Spectroscopy (AAS). Solubilizing the different manganese complexes made it possible to distinguish between the different redox states of manganese (24). Both Mn(II) and Mn(IV) solubilize in a mixture of 0.25 N hydroxylamine-hydrochloride and 0.25 N hydrochloric acid, whereas in 0.5 N hydrochloric acid only Mn(II) solubilizes. After solubilising the manganese complexes in a separate bottle for each time measurement, the samples were filtered over an 0.45 μm filter and measured with AAS (Varian AAS 300/400 Spectroscopy). An air-acetylene flame and a wavelength of 279.5 nm (monochromatic light) were used. Concentrations of 0.02-5 mg

Mn/l could be measured with this method.

The production of $^{14}CO_2$ was measured in the liquid phase as previously described (21). The purged NaOH-treated samples accounted for the total activity of the non-volatile compounds, biomass, and CO_2. The HCl-treated samples accounted for the total activity of the non-volatile compounds, and biomass. The $^{14}CO_2$-production was calculated as the difference between these two measurements and corrections were made for the CO_2 concentration in the gas phase. Volatile [^{14}C]-compounds (other than CO_2) were calculated as the difference between unpurged NaOH-treated and purged NaOH-treated samples.

The trace element composition (Pb, B, Cr, Cu, Zn, Cd, Al, Mo, Ni, Co) of the medium with and without sterile Rhine river sediment was measured with Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES).

RESULTS

Sediment columns. Degradation of toluene occurred in the sediment columns in the presence of crystalline, amorphous and freeze-dried amorphous manganese oxide (Fig. 1). Following a partial breakthrough of toluene in the columns with crystalline and amorphous manganese oxide, between 80 and 95% of the incoming toluene was degraded after 100 days of operation. In the column with freeze-dried amorphous manganese oxide a partial breakthrough followed by degradation of toluene was observed as well, but not as fast as in the other two columns. In time, we increased step-wise the toluene concentration in the influent. The toluene degradation in the column with freeze-dried amorphous manganese oxide levelled off to 60% degradation during the first 60 days and decreased after this time period. Readditions of manganese oxide (5 mmol) at day 70 and day 110 to the column with freeze-dried amorphous manganese oxide led to a further toluene degradation.

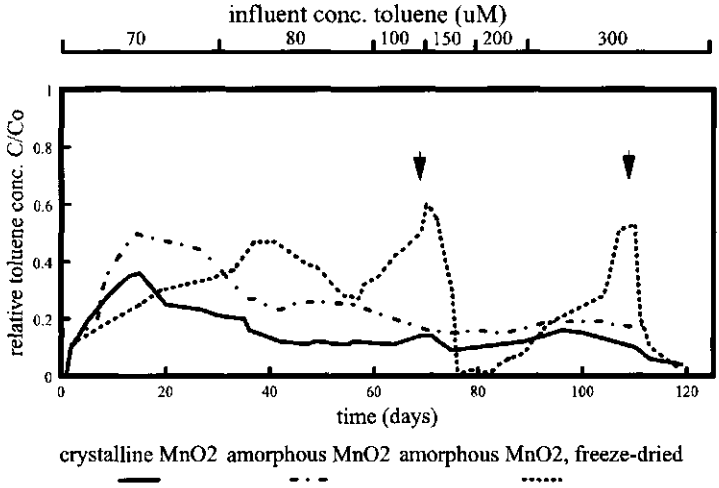

Fig. 1. Behaviour of toluene in anaerobic sediment columns with crystalline, amorphous or freeze-dried amorphous manganese oxide. C/Co is effluent concentration relative to influent concentration. On day 70 and day 110 (↓) 5 mmol of MnO_2 was added to the column. The influent toluene concentrations are given at the top of the figure.

Enrichment cultures. The first enrichment cultures degraded toluene in the presence of all three types of manganese oxide (Fig 2.). Upon transferring these first enrichments into fresh medium, 5 mM of bromoethanesulfonic acid (BrES), an inhibitor of methanogenesis, was added. The batches with amorphous MnO_2 and freeze-dried amorphous MnO_2 continued to degrade toluene, but no degradation was found with crystalline manganese oxide (results not shown).

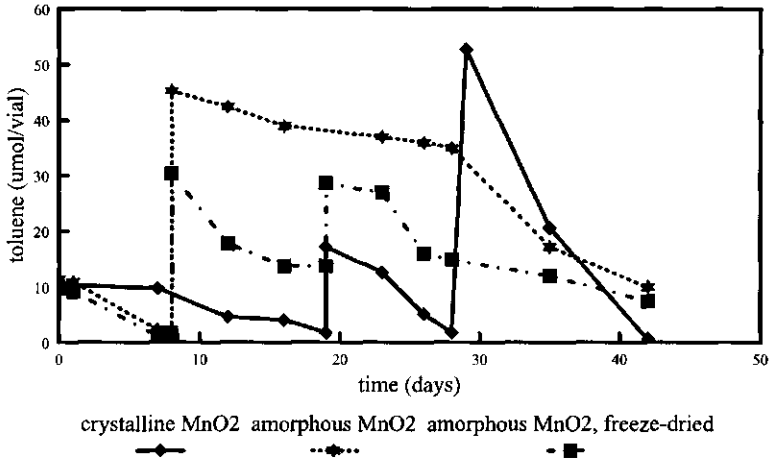

Fig. 2. Degradation of toluene by the first enrichment cultures with different types of manganese oxide.

Table 1. Effect of the presence of solid materials on the toluene degradation rate.

Addition	Transfer								
	1	2	3	4	5	6	7	8	9
none	+	+	+	±	-	nt	nt	nt	nt
glass beads	+	+	+	±	-	nt	nt	nt	nt
teflon beads	+	+	+	±	-	nt	nt	nt	nt
bentonite	+	+	+	±	-	nt	nt	nt	nt
vermiculite	+	+	+	±	-	nt	nt	nt	nt
granular sludge	+	+	±	±	-	nt	nt	nt	nt
Rhine river sediment	+	+	+	+	+	+	+	+	+

+ = degradation of 100 μmol of toluene within 30 days of incubation
± = degradation of 100 μmol of toluene between 30-120 days of incubation
- = no degradation of 100 μmol of toluene after 200 days of incubation
nt = not tested

Maintaining the degradation of toluene by the enrichment culture. The ability of the enrichment culture to degrade toluene decreased after several transfers. After the second transfer, lag phases of 80 days were observed as well as a decreasing toluene degradation rate, and after the fourth transfer degradation of toluene was not even observed after 200 days of incubation. The effect of different additions of solid materials was tested during 5 transfers (Table 1). The enrichment culture was able to maintain the activity only in the presence of sterile Rhine river sediment. In all cases, with sodium azide or formaldehyde killed controls and controls without manganese oxide did not show degradation of toluene.

Toluene oxidation versus manganese reduction. The degradation of toluene was coupled to the production of Mn(II) and $^{14}CO_2$ (Fig. 3). After 2 weeks of incubation, the toluene concentration had decreased from 135 to 95 μmol/vial, and 500 μmol/vial of reduced manganese and 110 μmol/vial of CO_2 were produced. Fifty percent of the toluene carbon was mineralized to CO_2, whereas 28% was transformed to non-volatile compounds and biomass, and 20% to volatile compounds (other than CO_2 or toluene). In batches without manganese oxide or toluene, no toluene oxidation or manganese reduction, respectively, was found.

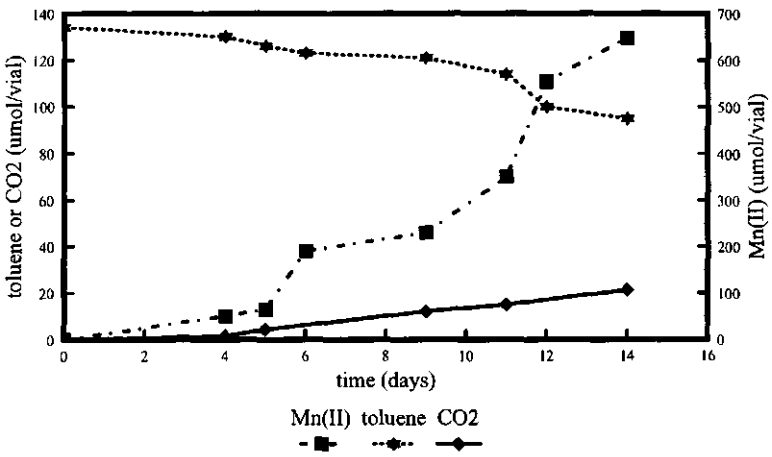

Fig. 3. Degradation of toluene and the production of Mn(II) and $^{14}CO_2$ in batch experiments with amorphous manganese oxide.

Direct contact between bacteria and manganese oxide. The necessity of the bacteria to be in contact with the insoluble manganese oxide, was tested by wrapping amorphous manganese oxide in a bacterial-filter package. This resulted in a slow degradation of toluene (Fig. 4). With the same amount of amorphous manganese oxide directly in the medium, toluene was degraded much faster. The use of highly crystalline manganese oxide showed a slow degradation of toluene, as already demonstrated.

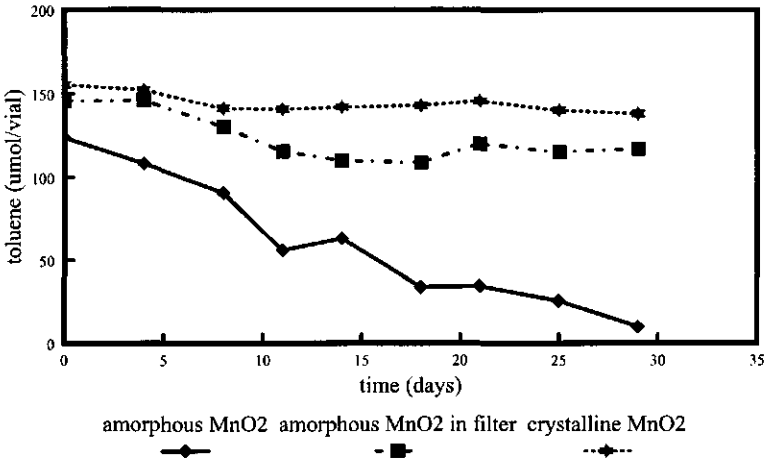

Fig. 4. Degradation of toluene in batch experiments with different types of manganese oxide.

Organic ligands. The solubility of manganese oxide can be increased by the addition of organic ligands like oxalic acid or NTA. The effect of various concentrations of oxalic acid and NTA on the solubility of amorphous manganese oxide was tested. The highest concentrations were found in the presence of 4 mM of oxalic acid or 2 mM of NTA, 12 mg Mn^{4+}/l and 75 mg Mn^{4+}/l, respectively. Without chelating agents, the soluble Mn concentration was 0.7 mg Mn^{4+}/l. These additions improved the degradation rate of toluene considerably (Fig. 5). In the incubations without chelating agents the degradation levelled off, most probably because the soluble Mn(IV) had been depleted after one week. In the presence of 4 mM of oxalic acid or 2 mM of NTA more soluble Mn(IV) was available and a higher degradation rate was maintained.

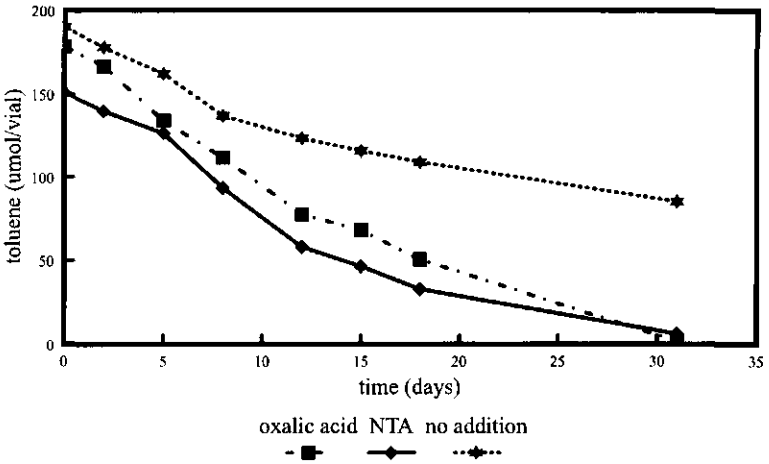

Fig. 5. Degradation of toluene in batch experiments with amorphous manganese oxide in the presence of 4 mM of oxalic acid or 2 mM of NTA.

DISCUSSION

Our results indicate that degradation of toluene under manganese-reducing conditions is possible. Toluene degradation was observed in flow-through sediment columns in the presence of crystalline manganese oxide, amorphous manganese oxide and freeze-dried amorphous manganese oxide. We did not verify whether manganese oxide indeed functioned as electron acceptor for toluene degradation in the columns. It was not possible to quantify the decrease in manganese oxide concentration, because the reduced form of Mn^{4+} formed precipitates with sulfide and other compounds in the column and could not be measured in the effluent for that reason. However, the finding that the gradual increase in toluene concentration in the effluent of the column with freeze-dried amorphous manganese oxide between day 57 and 70, and day 83 and 110, could be reversed by readding manganese oxide to the column, can only be explained when manganese oxide acts as electron acceptor. Opening the column and mixing the manganese oxide through the column material disturbed the microbial activity. This resulted in a temporary

increase of the toluene concentration in the effluent.

The type of manganese oxide in the enrichment cultures, strongly affected the degradation rate of toluene. In the primary enrichments, toluene was still degraded with all three types of manganese oxide. However, in secondary enrichments hardly any degradation of toluene was observed with crystalline manganese oxide as electron acceptor. This shows that the crystallinity of manganese oxide strongly affects the rate of reduction. Due to its larger specific surface area, a more amorphous form is reduced faster and functions better as an electron acceptor than a more crystalline one, [24, 25, 29]. Several dissimilatory manganese-reducing bacteria, e.g. *G. metallireducens* and *Shewanella putrefaciens* MR-1, reduce amorphous manganese oxide faster than a more crystalline form [24, 25]. However, this effect varies among bacterial species, with some being more sensitive to mineralogy than others [29, 32]. Amorphous manganese oxide was used for our further enrichments and batch experiments.

Despite the use of amorphous manganese oxide, we had difficulties to maintain the activity upon subculturing. The positive effect of the addition of sterile Rhine river sediment, which lasted at least 12 transfers, may be due to the presence of essential trace elements or nutrients. Analysis of the trace element composition in the medium with and without Rhine river sediment could not clarify this hypothesis. The concentrations of the trace elements in the medium were too low (<0.01 mg/l) to detect any differences. A comparable positive effect of the addition of river sediment on the degradation rate of xenobiotic compounds was found by others. The degradation rate of polychlorinated biphenyls by anaerobic enrichment cultures increased in the presence of sterile Rhine river sand (28) or sterile Raritan river sediment (5). In further experiments these authors demonstrated that replacement of the sediment by humic acids and a complex carbon and energy source can support this dechlorination as well (4). We added sterile Rhine river sediment standard to our medium to maintain active enrichment cultures. We did not add the sediment during batch experiments, because the enrichments could be used without any additions during 1 or 2 transfers.

The results from the batch experiments (Fig. 3) demonstrate that toluene was oxidized to carbon dioxide and that manganese oxide was the ultimate electron acceptor. The amount of CO_2 produced was less than expected, but this is because

part of the toluene has been used for cell synthesis (biomass) and because intermediates or dead-end products were formed (48% of the transformed toluene). The toluene consumption and the production of Mn(II) indicated that for each mol of toluene oxidized, ca. 14 mol of manganese oxide was reduced to Mn(II). This is less than expected, according the theoretical reaction 1, but this reaction does not include the formation of biomass and the partial transformation of toluene.

$$C_7H_8 + 18\ MnO_2 + 18\ H_2CO_3 \rightarrow 7\ CO_2 + 18\ MnCO_3 + 22\ H_2O \qquad \text{(reaction 1)}$$

The poor availability of manganese oxide is severely rate limiting in the toluene oxidation. The degradation rate was higher, when the enrichment culture was in direct contact with the solid manganese oxide. This indicates that the bacteria did not only use soluble manganese(IV) as electron acceptor, but also solid manganese oxide. They may have the ability to attach to the solid oxide. This will result in a direct and faster transfer of electrons. It has been proposed that manganese-reducing bacteria contain electron shuttles (Mn^{2+}) in the cell envelope, that transport the reducing power across the cell envelope/manganese oxide particle interface (14). The necessity of contact was also demonstrated in studies with *Shewanella putrefaciens* (formerly *Alteromonas putrefaciens* MR1). This bacterium grew on agar plates only in the manganese oxide rich overlay and not in the other agar layer. It was concluded, that for growth the bacterium requires physical contact with the insoluble manganese oxide.

Increasing the solubility of MnO_2 by the addition of organic ligands resulted in an increased toluene degradation rate. Lovley and coworkers found a similar effect with Fe(III) chelation. Toluene was more rapidly degraded in iron containing sediments when NTA or EDTA was added (25), and benzene was more rapidly degraded in iron-containing sediments when NTA, EDTA, humic acids or phosphates were added (26).

Microbial degradation of toluene with solid manganese oxide as electron acceptor is possible. Manganese is a widespread transition metal (about 5-10 times less abundant than iron), and manganese reduction is energetically more favourable than iron reduction. However, the degradation rate is low, due to the limited availability and solubility of the electron acceptor. Our results may be helpful for

further isolation studies of manganese- and iron-reducing bacteria which degrade xenobiotic compounds. For bioremediation purposes, the use of a solid electron acceptor like manganese or iron oxide that is not lost from the environmental compartment may be promising. Following reduction, it can be reoxidized and thereby returned to the sediments by precipitation, via which it can be reused as electron acceptor. Since these manganese and iron cycles occur in many sediments, manganese and iron oxide may be used numerous times.

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Chapter 5

Characterization of a Manganese-Reducing, Toluene Degrading Enrichment Culture

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ABSTRACT

A bacterial culture (LET-13) was enriched, which uses toluene as sole carbon and energy source, and manganese oxide as terminal electron acceptor. The culture is able to degrade a variety of substituted monoaromatic compounds like (*p*-hydroxy) benzylalcohol, (*p*-hydroxy) benzaldehyde, (*p*-hydroxy) benzoate, phenol, and the three isomers of cresol. Benzene, ethylbenzene, all xylenes and naphthalene were not degraded under the experimental conditions used.

Based on the results of growth experiments and the detection of intermediates, it is concluded that toluene is degraded via a methyl hydroxylation. A possible side reaction can lead to the formation of cresol.

All organisms in the culture look similar; motile rods, which are gram negative, oxidase negative and catalase negative. The culture was partly identified with phylogenetic analysis of cloned rDNA sequences. The phylogenetic analysis showed that at least two major groups of bacteria are present. One group of bacteria belongs to the *Bacteroides-Cytophaga* group, and one group consists of members of the ß-subclass of the *Proteobacteria*.

INTRODUCTION

Toluene is one of the non-oxygenated, monoaromatic compounds that are present in petroleum (others are e.g. benzene, ethylbenzene and xylenes). Due to leakages of petroleum storage tanks and spills at petroleum wells, these hydrocarbons contribute significantly to groundwater contamination. In anaerobic environments, they often seem to accumulate. Toluene is also known to be formed biologically (18) in anoxic freshwater sediments.

Most aromatic hydrocarbons are rapidly degraded under aerobic conditions; oxygen is involved in activation and fission of the aromatic ring and is the terminal electron acceptor. Information on the anaerobic microbial degradation of these compounds is emerging in the literature only since the last decade. Recently, we have published data about the bacterial degradation of toluene under manganese-reducing conditions (32). Such a degradation has never been demonstrated before.

Several manganese-reducing bacteria have been described in literature, but none of them can degrade aromatic compounds (33, 40). The manganese-reducing bacterium *Shewanella putrefaciens* MR-1 obtains energy for growth by using H_2, formate, or lactate as electron donor. The bacterium has been classified as a member of the γ-subclass of the *Proteobacteria* (39). *Geobacter metallireducens* degrades compounds like acetate, butyrate, propionate, and ethanol with amorphous manganese oxide as electron acceptor. *G. metallireducens* can degrade toluene with iron oxide as electron acceptor, but not with manganese oxide as electron acceptor (unpublished results). *G. metallireducens* has been categorized in the δ-subclass of the *Proteobacteria*, and is closely related to *Desulfuromonas acetoxidans* (34).

Toluene has been found anaerobically degradable under denitrifying (1, 2, 12, 15, 16, 21, 29, 31, 36, 46, 47, 52, 53), iron-reducing (35), sulfate-reducing (5, 6, 14, 26, 41) and methanogenic conditions (13, 25, 50, 51). Various bacteria have been isolated and parts of degradation routes have been elucidated. An overview of the anaerobic toluene metabolism is given by Frazer et al. (19).

Studies on the formation of intermediates in denitrifying bacteria (2, 46, 47), the sulfate-reducing bacterium Tol2 (41) and the iron-reducing bacterium *Geobacter metallireducens* (formerly known as GS-15) (35), suggest that the degradation proceeds through oxidation of the methyl group. Benzoyl-CoA is formed via benzylalcohol, benzaldehyde and benzoate, upon which ring fission occurs. Another pathway has been found for the denitrifying strain T1 (15) and in a sulfate-reducing enrichment culture (5). Toluene is first converted with acetyl-CoA to phenylpropionyl-CoA, which is further degraded to benzoyl-CoA. About 15% of the toluene would react with succinyl-CoA to form two dead-end products: benzylsuccinate and benzylfumarate. The same dead-end products have been detected with the sulfate-reducing bacterium strain PRTOL1 (6), but the main degradation pathway of toluene has not been clarified. Recently, the degradation of toluene in the denitrifying bacterium *Thauera aromatica* has been demonstrated to start with an initial addition of fumarate to the methyl group of toluene, to yield benzylsuccinate (9). A further degradation of benzylsuccinate to benzoyl-CoA is suggested to occur via ß-oxidation. Alternate pathways via a ring hydroxylation to cresol or a carboxylation to toluate have also been discussed, but no experimental data exist to support the occurrence of such a pathway (1, 35, 46). Small amounts of *p*-cresol

have been detected in a methanogenic enrichment culture with toluene, in which they seem to be formed via the oxidation of the aromatic ring (50). It was demonstrated that the hydroxyl group originated from water. However, the conversion of toluene to *p*-cresol is very slow, compared with the rapid breakdown of toluene in this culture.

Here we report the physiological and phylogenetical characterization of an anaerobic enrichment culture that mineralizes toluene in the presence of amorphous manganese oxide as electron acceptor. The culture has been maintained with toluene as sole carbon and energy source and with manganese oxide as electron acceptor for more than two years. Data on the use of alternate electron acceptors, the growth on possible intermediates and the identification of formed intermediates are discussed in this paper.

MATERIALS AND METHODS

Chemicals. All aromatics were purchased from E. Merck, Darmstadt, Germany and were of analytical grade. ^{13}C-toluene was purchased from ISOTEC Inc. (Miamisburg, Ohio, USA) and 99.3% was α-labelled.

Enrichment and cultivation. The enrichment culture had been obtained from sediment columns in which toluene was transformed under manganese-reducing conditions (32). Routine cultivations were carried out in 115-ml serum bottles with 20 ml of anaerobic medium and amorphous manganese oxide (δMnO_2) as electron acceptor, as described by Langenhoff et al. (32). In addition, 2 mM of nitrilotriacetic acid (NTA) was added to the medium to solubilize Mn(IV). Toluene was added from a heat sterilized water-saturated solution (2 ml), to obtain a medium concentration of 300 μM (18 μmol/bottle). Higher concentrations were not used because of inhibitory effects. Toluene was re-added when depleted. We have demonstrated in previous experiments that the addition of sterile Rhine river sediment was necessary to maintain the activity of the culture (32). In further experiments, we found that a concentrated supernatant of the Rhine river sediment could be used as well. The supernatant was prepared by shaking 40 g of sterile Rhine river sediment and 40 ml of manganese-reducing medium anaerobically at 30°C

overnight. This was followed by centrifugation and filter sterilization (0.22 μm Durapore filter, Millipore, USA). 2 ml of this Rhine river sediment supernatant was added standard to each of the batches. A gas phase of N_2/CO_2 (80%/20%), and a pressure of 1.3 bar were used. The bottles were incubated stationary in the dark at 30°C.

Transfer of the enrichment cultures (10% [v/v]) to bottles containing fresh medium was done after a total of approximately 100 μmol of toluene had been transformed. Transfers were normally made after 30 to 60 days, depending on the rate of transformation.

Purification. The manganese-reducing, toluene degrading bacterial culture was further attempted to purify using dilution series in liquid cultures and on solid media. Anaerobic agar roll tubes (28) or anaerobic agar plates were made with anaerobic medium with and without manganese oxide, 2% highly purified noble agar (Difco, Detroit, USA), and 0.3 mM toluene as substrate. The tubes or plates were inoculated with 0.1% (v/v) of the enrichment culture. The solidified agar in roll tubes or plates without manganese oxide was covered with a second agar layer in which amorphous manganese oxide was present. Both the tubes and the plates with one or a double layer of agar were incubated at 30°C in an anaerobic glovebox. The formation of clearing zones in the agar overlays (dark by the presence of manganese oxide) were used as indication for growth of manganese-reducing bacteria. This method has been described previously by Nealson (40), who found that manganese-reducing bacteria do not form visible colonies on solid media. Upon clearing of the agar, agar from the edges of these clearing zones was transferred to anaerobic manganese-reducing liquid medium.

Effect of temperature and pH. The temperature optimum for toluene degradation was determined in two-liquid-phase batches. A two-liquid-phase system of medium and hexadecane was used because of the low water solubility of toluene. Such a system creates a constant low toluene concentration in the medium and a constant flux of toluene from the organic phase to the aquatic medium. To 115-ml serum bottles with 20 ml of anaerobic manganese-reducing medium with NTA, 0.5 ml of hexadecane containing 0.2 M toluene was added. This resulted in a concentration in the liquid phase of 190 μM toluene (32). The bottles were inoculated with 10% [v/v] of the toluene degrading enrichment culture, and incubated at 4,

10, 20, 25, 30, 37, 43, and 55°C stationary in the dark.

The effect of pH on the toluene degradation rate was tested in similar two-liquid-phase batches. The pH was varied by applying different partial pressures of CO_2 in the gas phase and varying the concentration of phosphate buffer (K_2HPO_4 and $NaH_2PO_4.2H_2O$). It was confirmed that the pH at the end of each experiment was the same as the initial pH.

All experiments were performed in triplicate. Controls were made with 1 mM of sodium azide (an inhibitor of electron transport-linked respiration) and 0.175% of formaldehyde (a general inhibitor of biological activity). Both are known to inhibit manganese-reducing bacteria (11).

Electron microscopy. For negative staining, cells were fixed in 3% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M of sodium phosphate buffer pH 7.2. A formvar coated copper grid (150 mesh, ∅ 3 mm) was placed on a drop of cell suspension for 15 min. After washing the grid three times with distilled water, the grid was air-dried. After drying, the cells were contrasted with uranyl acetate and examined with a Jeol EM 1200 ex II transmission electron microscope.

Electron acceptor and donor utilization. Experiments were carried out in 115-ml serum bottles with 20 ml of anaerobic manganese-reducing medium with NTA, which had been inoculated with 10% [v/v] of the toluene degrading enrichment culture. Thiosulfate (20 mM), fumarate (20 mM), nitrate (20 mM), and oxygen were tested as electron acceptor with toluene in hexadecane (0.2 M) as substrate. Controls with manganese oxide as an electron acceptor and without electron acceptor were taken along as well.

Various possible intermediates in the degradation of toluene and different monoaromatic compounds were tested for their degradability by the enrichment culture. Compounds tested in one-liquid-phase batches were benzylalcohol, benzoate, *p*-hydroxy-benzylalcohol, *p*-hydroxy-benzaldehyde, *p*-hydroxy-benzoate, *o*-, *m*- and *p*-toluate, and phenol (all at 100 μM). Toluene, benzaldehyde, *o*-, *m*- and *p*-cresol, *o*-, *m*- and *p*-xylene, benzene, ethylbenzene, styrene, and naphthalene were tested in two-liquid-phase batches. A two-liquid-phase system was used because of the low water solubility of these compounds. 0.5 ml of hexadecane containing 0.2 M substrate was added. Controls were taken along to test for chemical interactions

between the aromatic substrates and manganese oxide. They were incubated under similar conditions, but without inoculum added.

Intermediates in the degradation of toluene were measured in one-liquid-phase batches. In time, liquid samples were taken and analyzed with HPLC and GC-MS. Higher concentrations of intermediates were obtained by adding 1 mM fluoro-acetate, an inhibitor of the tricarboxylic acid cycle. The inhibitor was added after 50 μmol of toluene was degraded (46).

Determination of intermediates was also done with [^{13}C]toluene. Cultures that had degraded 50 μmol of [^{12}C]toluene in a one-liquid-phase system, were fed [^{13}C]toluene. The formation of labelled intermediates was followed by GC-MS.

All experiments were performed at least in triplicate.

Isolation of nucleic acids. Nucleic acids were extracted from cultures that had degraded approximately 80 μmol of toluene. A 10-ml medium sample was centrifuged at 13,000 rpm for 20 minutes. The pellet was resuspended in 400 μl of autoclaved TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and transferred to a 1.5 ml Eppendorf tube. 200 μl of Tris/HCl buffered phenol (pH 8.0) was added together with 300 μl of glass beads (∅ 0.11 mm). The cells were disrupted by beat-beating during 5 min, using a cell homogenizer MSK (Braun, Melsungen, Germany) under CO_2-cooling, and subsequently centrifuged (15 min at 13,000 rpm). Nucleic acids were extracted from the aqueous phase with a phenol/chloroform/isoamylalcohol-mixture (25/24/1 [v/v/v]), and after centrifugation (15 min at 13,000 rpm), the aqueous phase was extracted by chloroform/isoamylalcohol (24/1 [v/v]). After centrifugation (15 min at 13,000 rpm), the nucleic acids were precipitated with 0.5 ml of isopropanol and 40 μl of 3 M sodium acetate (pH 5.2) at -70°C for 30 min. After centrifugation (15 min at 13,000 rpm), the nucleic acid pellet was washed with 70% ethanol, dried under vacuum and resuspended in 100 μl TE buffer. The quality of the extracts was analyzed by agarose gel-electrophoresis, followed by ethidium bromide staining (44). The DNA and rRNA extracts were used for dot blot hybridization, polymerase chain reaction (PCR), cloning, and temperature gradient gel electrophoresis (TGGE).

Dot blot hybridization. The sequences for the used 16S rRNA oligonucleotide probes and their target organisms are listed in table 1. All oligonucleotides for dot blot hybridizations were synthesized by Pharmacia (Uppsala, Sweden) and were 5' end labelled with [γ^{32}P]ATP (3000 Ci/mmol, Amersham, Little Chalfont, UK) (27).

Table 1. Summary of the oligonucleotide probes used in this study.

Probe	Target group	Sequence	Ref
EUB338	Bacteria	5'-GCTGCCTCCCGTAGGAGT-3'	(3)
ARC915	Archaea	5'-GTGCTCCCCCGCCAATTCCT-3'	(48)
BET42a	ß-subclass of Proteobacteria	5'-GCCTTCCCACTTCGTTT-3'	(37)
GAM42a	γ-subclass of Proteobacteria	5'-GCCTTCCCACATCGTTT-3'	(37)
SRB385	sulfate-reducing bacteria	5'-CGGCGTCGCTGCGTCAGG-3'	(3)

Dot blot hybridizations were performed on Hybond N+ filters (Amersham). Nucleic acid extracts of LET-13 were applied to the membrane with a Hybri.Dot manifold (Gibco BRL, Life Science Technologies, Gaithersberg MD, USA) and immobilized by UV light (4 min). *Escherichia coli* (*γ-Proteobacterium*), *Synthrophobacter wolinii* (DSM 2805, sulfate-reducing bacterium) and *Methanosaeta soehngenii* (DSM 2139, *Archaea*) were used as positive controls. All membranes were pretreated with 10 ml of hybridization buffer (0.5 mM phosphate buffer, 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumine, and 1 mM EDTA, pH 7.2) for 30 min. The probes were 5'-labelled with ^{32}P by using [γ-^{32}P]ATP and polynucleotide kinase. 1 μl (100 ng) of the probe was mixed with 2 μl of 10x kinase buffer (44), 1 μl of T4 polynucleotide kinase (Gibco BRL), 1.5 μl of [γ-^{32}P]ATP (3000 mCi/mmol, Amersham), and water to obtain a total volume of 20 μl. This mixture was incubated at 37°C for 30 min. The membranes were hybridized overnight at a temperature of 46°C and rinsed with 10 ml of 1 mM EDTA and 5x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). Finally, the membranes were washed in 10 ml of 1% SDS, 1x SSC at 48°C , sealed in polyethylene foil, and exposed to a phosphor storage screen for 2 hours. The screen was scanned for radioactive respons on a Phosphor Imager (Molecular Dynamics,

Sunnyvale, USA). The digital signals were processed by the manufacturers software (ImageQuant).

PCR amplification, isolation, cloning and sequencing of the 16S rRNA gene. The 16S-targeted polymerase chain reaction (PCR) was used to amplify 16S rRNA genes (1.5 kb fragments) of the bacteria present in the enrichment culture. Two eubacterial universal primers were used: the forward primer corresponding to *Escherichia coli* positions 8 to 27 (5' CACGGATCCAGAGTTTGATC/T(A/C)TG-GCTCAG) and the reverse primer corresponding to *E. coli* positions 1493 to 1510 (5' GTGCTGCAGGGTTACCTTGTTACGACT). The PCR assay (100 μl) contained amplification buffer (20 mM Tris/HCl pH 8.4, 50mM KCl), 3mM $MgCl_2$, 200μM each deoxynucleotide, 0.2μM each primer, 2.5 U Taq DNA polymerase (Life Technology) and 1 μg template DNA. The PCR involved an initial denaturation step of 4 min at 90°C, followed by 35 amplification cycles: 94°C for 45 s, 54°C for 45 s, and 68°C for 2 min. This was followed by a final 7 min incubation at 68°C.

The isolation, cloning and sequence analyses manipulations were done by using previously described procedures (44), with the following modifications. The amplification products were isolated and purified by agarose gel electrophoresis, excised from the gel and cleaned with glass milk (GlassMAX™, Gibco BRL). The fragments were cloned in a pGEM^T vector (Promega, Madison, WI, USA), and plasmid preparation was done with the Wizard kit (Promega). The positive clones were sequenced by using the dideoxy chain termination reaction (45), using an automated DNA sequencer model 373A (Licon, USA). The resulting sequences were compared with the 16S rRNA sequences available in literature and EMBL data bank by using a Fasta program. The sequences of clones I, II and III were deposited in the EMBL data bank.

Temperature gradient gel electrophoresis (TGGE). TGGE (Diagen TGGE system, Diagen GmbH, Düsseldorf, Germany) was used to obtain a band profile of the 16S rRNA fragments present in the DNA from the enrichment culture (17). The 16S rRNA fragments of the V6-V8 regions (0.4 kb) originated from direct amplification of the DNA extracted from the enrichment culture, and from the whole 16S rRNA cloned fragments. The primers used were universal eubacterial primers: the forward U968-GC primer (5' [GC-clamp] AACGCGAAGAACCT-

TAC 3') and the reverse primer L1401 (5' CGGTGTGTACAAGACCC 3'). The presence of the GC-clamp on one of the two primers is required to improve separation of the bands in the gel (38). The PCR assay contained the reagents in the same proportions as described before. PCR conditions were 25 cycles (94°C for 20 s, 56°C for 20 s, and 68°C for 40 s). A volume of 6 μl of the PCR products was used for the TGGE run.

The TGGE consisted of polyacrylamide gel (6% w/v acrylamide, 0.1% w/v bis-acrylamide, 8M urea, 20% v/v formamide, 2% v/v glycerol) and 1x TAE buffer (17). A temperature range (37°C to 46°C) at a constant voltage (120 V) for 15h was chosen. After the electrophoresis, the band profile on the gel was visualized by silver staining the gel as follows: a) 3 min fixing in 10% ethanol and 0.5% acetic acid; b) 10 min staining in 10% ethanol and 0.5% acetic acid and 0.2% $AgNO_3$; c) 2 to 3 min washing in demineralized water; d) developing in 1.5% NaOH, 0.1% formaldehyde and 0.01% $NaBH_4$, till good visualization of the bands; e) 5 min fixing in the same solution as in a); f) 5 min washing in demineralized water; g) 7 min treatment in a preservation solution containing 25% ethanol and 10% glycerol; h) cover the gel with cellophane and drying overnight in the oven at 60°C.

Other methods. Gram staining, and oxidase and catalase reactivity tests were performed by standard procedures (23).

Analytical procedures. Toluene, benzene, ethylbenzene, styrene, *o*-, *m*- and *p*-xylene were measured gaschromatographically by headspace analysis (32).

Benzylalcohol, benzaldehyde, benzoate, *o*-, *m*- and *p*-cresol, phenol, *p*-hydroxy-benzylalcohol, *p*-hydroxy-benzaldehyde, *p*-hydroxy-benzoate, *o*-, *m*-, and *p*-toluate, ethylbenzene, and naphthalene were measured by High Performance Liquid Chromatograph. Liquid samples (0.5 ml) were taken by syringe, centrifuged (13,000 rpm for 3 min) and analyzed on a HPLC (LKB, Bromma, Sweden). Samples (20 μl) were injected by using an autosampler (Spectra System AS1000) onto two Chromspher C8 columns (Chrompack, Bergen op Zoom, The Netherlands) connected in serie at roomtemperature. The mobile phase was 20% acetonitrile and 80% 10 mM H_2SO_4 at a flow rate of 0.6 ml/min. The eluted compounds were identified and quantified with an UV detector (LKB 2158 Uvicord SD, Bromma, Sweden) at 206 nm.

To identify and quantify aromatic intermediates by GC-MS, the samples were extracted (1:1) overnight in chloroform. The chloroform extracts were analyzed on a Hewlett-Packard 5890 series II gas chromatograph (Hewlett Packard, Amsterdam, The Netherlands) equipped with a mass selective detector (HP 5971A series mass selector). The samples (1 μl) were injected on a fused silica analytical column (HP5, 30 m x 0.25 mm) with an autosampler (HP, series 7673A). Oven temperature conditions for the separation were 40°C for 2 min, 10°C/min to 250°C, 250°C for 5 min. Data were acquired using either a full scan mode for identification of metabolites, or a selected ion monitoring mode (SIM) for quantification. The GC column did not allow the separation of *m*-cresol from *p*-cresol. Hence, when both compounds could be present, they were reported as summed parameter (*m*+*p*)-cresol.

RESULTS

Enrichment. A stable enrichment culture, capable of mineralizing toluene in the presence of amorphous manganese oxide, was obtained via repeated dilution and transfers in liquid media after two years of cultivation.

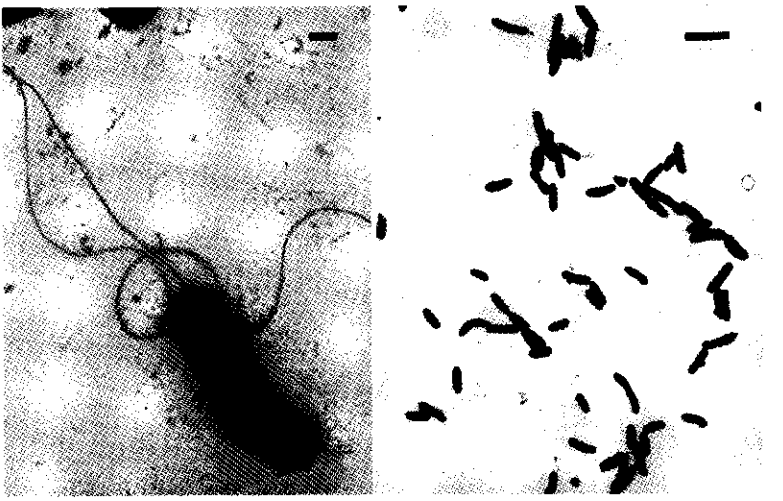

Fig. 1. Electron micrographs of culture LET-13; bars indicate 0.2 μm and 2 μm, respectively.

The culture, which will further be referred to as LET-13, consists of gram negative, motile rods, that are oxidase and catalase negative, and microscopically identical. The rods are about 1 to 2 μm long, and possess several peritrichious flagella (up to 6 μm in length), irregularly distributed around the cell (Fig. 1). LET-13 grows optimally between 25 and 35°C (Fig. 2A), whereas at 4 and 55 °C no growth was observed. The toluene degradation rate was maximal around pH 7.0 (Fig. 2B). No degradation of toluene was observed below pH 4.9 and above pH 8.8.

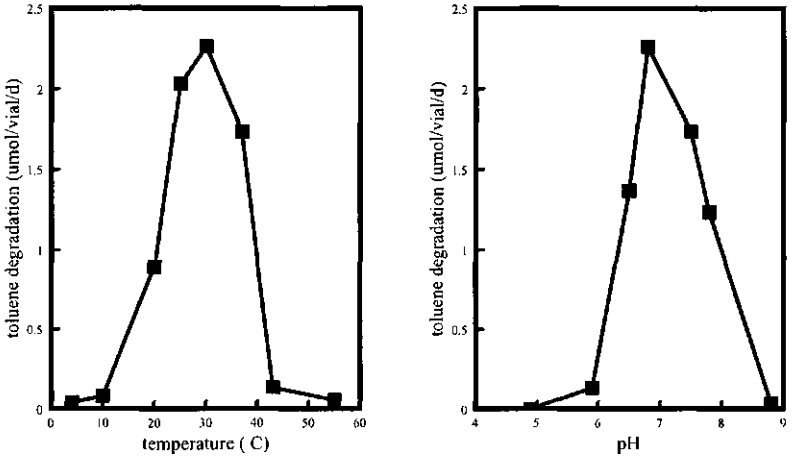

Fig. 2. Temperature (A) and pH (B) dependence of the toluene degradation rate of LET-13 in two-liquid-phase incubations with amorphous manganese oxide as electron acceptor.

Cultivation in agar roll tubes or on agar plates was not successful. Clearing zones, indicating the presence of manganese-reducing bacteria, did develop in the double-layer agar roll tubes and on the double-layer agar plates. However, no degradation of toluene was observed, after transferring the clearing zones into liquid medium, during 120 days of incubation.

Growth of LET-13 with alternate electron acceptors and donors. Nitrate and oxygen were both found to support growth of LET-13 with toluene as substrate. Transfer of these cultures into fresh medium with amorphous manganese oxide as electron acceptor resulted again in toluene degradation and manganese reduction. However, routine cultivation of LET-13 with nitrate or oxygen as electron acceptor and toluene as substrate, resulted after several transfers in enrich-

ment cultures, which could no longer use manganese oxide as electron acceptor. No degradation of toluene was observed with fumarate or thiosulfate as electron acceptor during 150 days of incubation.

LET-13 is able to oxidize and grow on benzylalcohol, benzaldehyde, benzoate, *o*-, *m*-, and *p*-cresol, phenol, *p*-hydroxy-benzylalcohol, *p*-hydroxy-benzaldehyde, and *p*-hydroxy-benzoate in the presence of manganese oxide. All cultures could be transferred successfully to fresh medium with the same aromatic compound or with toluene as substrate. No growth occurred with *o*-, *m*-, and *p*-xylene, *o*-, *m*-, and *p*-toluate, benzene, ethylbenzene, styrene and naphthalene. The incubations with the cresols were performed with nitrate instead of manganese oxide as electron acceptor, since the concentration of the cresols decreased rapidly in our controls. This was due to an abiotic reaction of manganese oxide with the cresols. All other tested compounds gave no chemical reaction with amorphous manganese oxide.

Detection of intermediates. With the presence of the tricarboxylic acid cycle inhibitor fluoro-acetate present in a manganese-reducing, toluene degrading culture LET-13, 30% of the toluene could be recovered as benzoate and less than 1% as *o*-cresol.

After the addition of [^{13}C]toluene to culture LET-13, small amounts of ^{13}C-labelled *o*-cresol (<1% of the added toluene) and ^{13}C-labelled (*m*+*p*)-cresol (1 to 3% of the added toluene) could be detected.

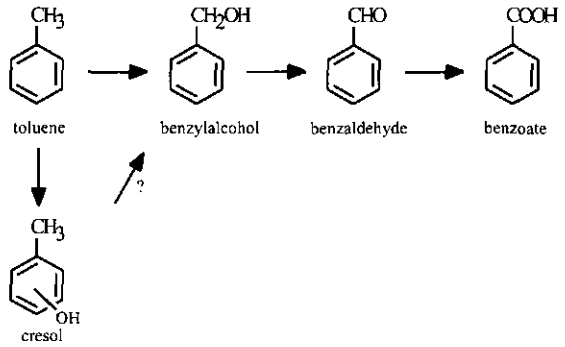

Fig. 3. Initial pathways in two proposed pathways for toluene metabolism in LET-13: an oxidation of the methyl group (upper branch), and an oxidation of the aromatic ring (lower branch) in the enrichment culture LET-13.

Since a large fraction of the produced cresols reacted abiotically with manganese oxide to undetectable products, we were unable to make a mass balance of the degradation of toluene and the accumulation of intermediates. 30% of the added toluene was transformed to benzoate, and in previous experiments (32) was shown that approximately 50% of the added toluene was completely mineralized. The initial steps in possible pathways for the toluene degradation in enrichment culture LET-13 are given in figure 3.

Dot blot hybridization of the enrichment. 16S rRNA isolated from LET-13 hybridized with a universal eubacterial probe (EUB338) and probes specifically for members of the ß-subclass (BET42a) and the γ-subclass (GAM42a) of the *Proteobacteria* (Fig. 4). No hybridization signal was found with the universal archaeal probe (ARC915) and the sulfate-reducing bacterial probe (SRB385). The 16S rRNA of the microorganisms that were used as positive controls gave hybridization signals as expected. Only *Escherichia coli* gave also a weak signal with BET42a.

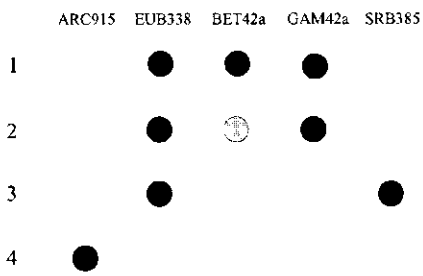

Fig. 4. Dot blot hybridization of 5 identical filters containing nucleic acids of 5 bacterial cultures. 1 μg of 16S rRNA extract from the following bacteria was bound to the filter; 1: LET-13 grown with toluene as substrate and manganese oxide as electron acceptor, 2: *Escherichia coli*, 3: *Synthrophobacter wolinii*, 4: *Methanosaeta soehngenii*.

DNA molecular analysis (TGGE, cloning, sequence analysis). The TGGE profile of the variable regions V6-V8 of LET-13 consisted of two strong bands (A and B) with a large difference in retention time and several weak bands in the proximity of these two bands (Fig. 5, line 1). PCR products from the clones I, II and III (Fig. 5, lines 2-3-4) gave bands with equal positions in the TGGE profile of

LET-13 (Fig. 5, band A, B, and A1). TGGE analyses of the 16S rRNA fragments corresponding to the variable regions V1-V3 and V4-V6 resulted in a TGGE profile with bands at comparable retention times as A, A_1 and B (data not shown).

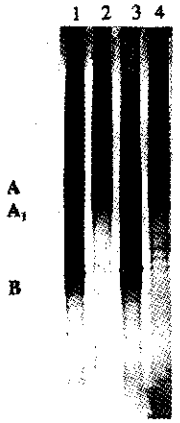

Fig. 5. Silver stained TGGE profile of product originating from V6-V8 regions 16S rRNA genes extracted from LET-13; 1: LET-13, 2: clone I, 3: clone II, 4: clone III. The strong bands are indicated as A and B, respectively, and a weak band as A_1.

The 16S rRNA gene sequence analysis was confined to the *Eubacterial* domain. The *Archaeal* domain was not analyzed since hybridization of the DNA with the archaebacterial probe did not reveal the presence of Archaea in LET-13. Nucleic acid sequence of 740 bp of clone I was determined. It comprised the variable regions V1-V4 and a long stretch of conserved region. From the comparison analysis, it belongs to the *Bacteroides/Cytophaga* branch group with homology levels between 70% to 80% with *Cytophaga* and *Bacteroides* genera. The nucleic acid sequence of two stretches of clone II, 550 bp comprising V3-V6 regions, and 500 bp comprising V7-V9 regions, were determined. The results from the comparison analysis of both stretches indicate members of the ß-subclass of the *Proteobacteria*. The highest homology (91%) was found with the genus *Azoarcus*. Two stretches (V1-V4 and V7-V9) of clone III that corresponded to a weak band A_1 close to the band A, showed homology values between 80 and 85% with *Cytophaga* and *Bacteroides* genera. Moreover, clones I and III showed a homology of more than 95%.

DISCUSSION

The newly enriched culture LET-13 is the first anaerobic bacterial enrichment culture that is able to couple the mineralization of toluene to the reduction of manganese oxide. We have demonstrated in earlier experiments (32) that the low degradation rate of toluene was mainly due to the use of the hardly soluble manganese oxide as electron acceptor (MnO_2). This rate could only be increased slightly by the addition of organic ligands. Routine transfers were done after 30 to 60 days of incubation, when approximately 100 μmol of toluene had been degraded. A culture was obtained that consisted only of short, motile, rod shaped bacteria. As all attempts to isolate a pure culture failed, we decided to proceed with a further characterization of the enriched culture LET-13, which stayed stable for more than two years.

Except for manganese (IV), enrichment culture LET-13 was also able to use nitrate and oxygen as electron acceptor. The degradation rate of toluene with these alternate electron acceptors was much higher than when manganese oxide was used. The redox potential of these electron acceptors is in the same range as Mn(IV), but nitrate and oxygen are more soluble and have a higher availability than Mn(IV). Thiosulfate and fumarate were not used as electron acceptors.

We have routinely tested cultivations of LET-13 on benzoate. Since benzoate was degraded faster than toluene, a faster growth of the bacteria would be the result. However, after cultivating four generations with benzoate, the bacteria had lost their toluene degrading activity completely.

The ability of LET-13 to degrade specific monoaromatic hydrocarbons, and the detection of large amounts of benzoate and minor amounts of *o*- and (*m*+*p*)-cresol as intermediates in the degradation of toluene, suggest the presence of at least two pathways. A major part of the toluene was degraded to benzoate, most probably via a methyl oxidation to benzylalcohol and benzaldehyde. We were not able to detect these compounds as intermediates, but both functioned as growth substrate. In a denitrifying, toluene degrading *Pseudomonas* strain, both compounds were found to be intracellular products (2). The oxidation of toluene via benzylalcohol and benzaldehyde has also been demonstrated in several other denitrifying strains (8, 20, 46, 47). Furthermore, benzylalcohol dehydrogenase and benzoyl-

CoA reductase have been purified from cell extracts of *Thauera aromatica* grown on toluene (7, 10). No information is available in the literature about the first enzyme that converts toluene into benzylalcohol, toluene methyl hydroxylase (1, 22). LET-13 is able to grow on *o*-, *m*- and *p*-cresol with nitrate as electron acceptor. The transformation of *p*-cresol has also been demonstrated before in a methanogenic enrichment culture (24), but could not be detected in a denitrifying and in a sulfate-reducing toluene degrading bacterium (1, 41). With toluene and manganese oxide, small amounts of *o*-cresol and (*m*+*p*)-cresol were found in the culture LET-13. No quantitative estimates could be made, since the cresols underwent a fast abiotic reaction with manganese oxide. The formation of polymeric oxidation products is the most logical result of the abiotic reaction of the cresols and manganese oxide (49).

The proposed pathways for toluene degradation by LET-13 as given in figure 3 have also been postulated by Lovley et al. (35) to occur in *G. metallireducens*, with iron as electron acceptor.

Two other pathways have been suggested. Toluene can be mineralized by an oxidative addition of acetyl-CoA to the methyl group of toluene, followed by ß-oxidation of the formed phenylpropionyl-CoA (5, 15). The addition of fumarate to the methyl group to form benzylsuccinate and benzoyl-CoA was demonstrated in the denitrifying bacterium *T. auromatica* (9). Since benzoate and cresol are no intermediates in these pathways, it is unlikely that these routes are significant pathways in LET-13.

Analyses of rRNA of LET-13 indicated the absence of methanogens and the presence of members of the ß- and γ-subclasses of the *Proteobacteria*. The high homology of the probes for the ß- and γ-subclasses of the *Proteobacteria* makes it with the used dot blot hybridizations difficult to distinguish between these two groups of bacteria. It has been shown in other studies that *E. coli* and other members of the γ-subclass of the *Proteobacteria*, gave a weak hybridization signal with BET42a while members of the ß-subclass can hybridize with GAM42a as well (37).

Sequence analyses of cloned rDNA of the enrichment culture resulted in at least 3 different sequences. Clone I and III are closely related (>95 % homology) and may be two copies of rDNA in the same bacterium. It is known that 16S

rRNA genes can differ in the same bacterium (30). In addition, sequence analyses showed that LET-13 contains two major bacterial types. One can be ascribed to the group of *Bacteroides-Cytophaga* and the other to the ß-subclass of the *Proteobacteria* (e.g. *Azoarcus*). Both *Bacteroides-Cytophaga* and *Azoarcus* can grow anaerobically, are motile rods and may possess flagella (4, 42). *Azoarcus* strains used to be characterized as strictly aerobic, but recently, anaerobic denitrifying, toluene degrading bacteria have been described as members of *Azoarcus* sp. (53). Since the level of homology of the two major groups of bacteria in LET-13 with known sequences for *Bacteroides-Cytophaga* and *Azoarcus* is less than 95%, it is likely that both bacteria in LET-13 belong to unknown genera. In fact, 95% is considered to be the homology value for attribution at the genus level (43). We could not clarify which type of bacterium is dominant in LET-13, nor which one is involved in the reduction of manganese oxide.

Several manganese-reducing bacteria have been described, but they are not closely related to the ones in LET-13. The manganese-reducing bacteria *Shewanella putrefaciens* and *Geobacter metallireducens* were classified as members of the γ-subclass and the δ-subclass of the *Proteobacteria*, respectively (34, 39). The latter was found to be closely related to *Desulfuromonas acetoxidans*.

We have demonstrated that LET-13 consists of two major groups of bacteria. The bacteria are related to *Bacteroides-Cytophaga* types and members of the ß-subclass of the *Proteobacteria*, but it is more likely that they belong to yet unknown genera. Whether each group of these bacteria in LET-13 performs one degradation pathway of toluene, or possesses the enzymes to perform both pathways is not known yet. Furthermore, it is possible that both groups of bacteria perform the mineralization of toluene in a syntrophic association.

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Chapter 6

General Discussion

In the last decades, a growing awareness of the health risks caused by the presence of toxic compounds in the environment has initiated the development of clean-up technologies. A number of technologies is in use for cleaning up contaminated soils, such as excavation followed by safe disposal or combustion. These conventional soil clean-up technologies require the replacement of the soil, disrupt the landscape, are costly (in the case of disposal) and may transfer contaminants to the air (in the case of combustion). For groundwater clean-up, pump-and-treat systems are used that withdraw large volumes of water to flush the contaminants from the aquifer solids. This technology is often inefficient and slow, and also incapable of restoring a good groundwater quality in a reasonable time. The hazards of these soil clean-up methods (e.g. transfer of contaminants to the air) and the limitations of groundwater clean-up methods have led to the development of bioremediation technologies.

Bioremediation is defined as a remedial measure that uses microorganisms to mineralize or immobilize hazardous contaminants or to transform them to less harmful compounds. Bioremediation can be divided into *in situ* bioremediation, in which the contaminated soil is treated in place, and *ex situ* bioremediation, in which the soil is excavated and treated either in an actively aerated bed (landfarming) or in a slurry-phase bioreactor. The advantage of an *in situ* process is that the contaminated soil does not need to be moved, and essentially remains undisturbed during treatment. This may make *in situ* bioremediation cheaper and safer than *ex situ* bioremediation.

In this chapter, principles of aerobic and anaerobic *in situ* bioremediation are given. The anaerobic *in situ* bioremediation of aromatic hydrocarbon-contaminated sites is emphasized and some prerequisites, limitations and drawbacks are discussed. Finally, control and evaluation of *in situ* bioremediation projects are discussed.

In situ bioremediation

Whether *in situ* bioremediation is an appropriate clean-up technique for a contaminated site depends on various factors. The presence of microorganisms, capable of degrading the pollutant, must be demonstrated either in field experiments or in laboratory tests with site-specific samples. In the absence of suitable microorganisms, microorganisms can be selected that are able to degrade the contaminants and these microorganisms can then be inoculated in the contaminated soil (43). The type of contaminant is important, e.g. low chlorinated and non-chlorinated aromatic compounds and alkanes are known to be aerobically well degradable. Much less is known about the degradation of the higher chlorinated compounds and homocyclic aromatics under anaerobic conditions. Knowledge of the degradation pathway in microorganisms gives insight whether a compound is mineralized or transformed into harmless compounds, or that a more toxic compound is formed. Environmental characteristics of a site affect bioremediation as well. A sufficient permeability of the soil is needed to transmit fluids, and a uniformity of the subsurface is important to predict chemical transport and fate of the contaminant, and to characterize the site (29). The site needs to have a steady groundwater flow, so the contaminant reaches the microorganisms in the subsurface. Additionally, the microorganisms require essential nutrients and electron acceptors/donors for growth; these may or may not be sufficiently present in the soil.

In situ bioremediation at a contaminated site can occur naturally when the site contains all the necessary microorganisms, nutrients and electron acceptors/donors. This selfpurification capacity is called intrinsic (*in situ*) bioremediation. In contrast, engineered *in situ* bioremediation can be used at sites where the selfpurification capacity is lacking or small. Environmental conditions are then manipulated to create better conditions for biodegradation. The activity of the microorganisms is increased by the addition of limiting nutrients and electron acceptors. The addition of oxygen is widely applied, because most aromatic contaminants are degraded by aerobic microorganisms. Another possibility is to stimulate the use of naturally occurring electron acceptors, such as metal oxides. The bioavailability of metal oxides in soil is often low but can be increased via the

addition of chelating agents (25). Finally, hydrophobic compounds such as PAHs are poorly soluble and adsorb strongly to soil particles. This low bioavailability results in a slow transformation of the contaminants in the soil. Increasing the bioavailability of PAHs by the addition of surfactants resulted in a faster transformation in laboratory experiments (44). However, knowledge and experience for a successful application of surfactants in the field, is still insufficient.

An engineered *in situ* bioremediation accelerates biodegradation rates in comparison to an intrinsic *in situ* bioremediation. This then requires less time for clean-up of a contaminated site. The shorter time to control and monitor the site, may then reduce costs of bioremediation. Consequently, intrinsic *in situ* bioremediation is a favourable option at those sites where biodegradation takes place naturally and the clean-up time is less important.

Aerobic *in situ* bioremediation

Engineered *in situ* bioremediation at sites contaminated with aromatic hydrocarbons is often based on the supply of oxygen, since most aromatic hydrocarbons can be degraded by aerobic microorganisms. Air, pure oxygen, or hydrogen peroxide are injected into the soil, or oxygenated water is pumped to the soil. The injection of air may be the simplest technique, but the injection of pure oxygen or H_2O_2 increases the amount of dissolved oxygen that is added to the soil from 8 mg/l to 40 mg/l and 100 mg/l, respectively (3). These higher concentrations of oxygen may reduce the time period needed for bioremediation and decrease the bioremediation costs, because less pumping is required. However, the supply of pure oxygen can cause problems due to its potential explosion hazard, and is therefore not widely used (3). Hydrogen peroxide is not hazardous, as it is rapidly transformed into O_2 and H_2O by microorganisms or by naturally occurring inorganic compounds, e.g. iron oxides (30). H_2O_2 is a highly reactive compound and its rapid decomposition can result in the production of oxygen above water saturation. Consequently, oxygen will form bubbles and these bubbles decrease the permeability of the soil (38). Small scale experiments have to clarify whether the addition of H_2O_2 is feasible for a specific site. The success of H_2O_2 addition for the

bioremediation of aromatic hydrocarbons has been demonstrated in several field experiments (4, 19, 30).

The first aerobic *in situ* bioremediation of a site contaminated with aromatic hydrocarbons was done at an oil pipeline spill in Pennsylvania in 1972 (29). Since then, *in situ* bioremediation has become an accepted technology for the clean-up of soils, contaminated with easily degradable petroleum products (27, 31, 36, 45). A recent, successful large scale *in situ* bioremediation has been demonstrated at the Exxon Valdez oil spill in Alaska in 1989 (31).

Sites contaminated with the gasoline compounds benzene, toluene, ethylbenzene, and xylenes (BTEX) are relatively easy to bioremediate under aerobic conditions. These compounds have a relatively high solubility in water compared to many other contaminants, and they can serve as carbon and energy source for many different aerobic bacteria. Higher molecular weight compounds, such as polycyclic aromatic hydrocarbons (PAHs), are much slower metabolized by bacteria. This is partly due to their complex structure, low solubility, and strong adsorption to soil. The use of white-rot fungi that degrade PAHs with extracellular enzymes (such as lignin peroxidase and manganese peroxidase) may then be a valuable alternative (40). These fungi need a primary growth substrate, such as cellulose or glucose, for the co-oxidation of aromatic hydrocarbons. The products of the co-oxidation are not further transformed by the fungi, and other microorganisms need to be present for a complete degradation of the contaminant. Thus, a mixed microbial population is required to remediate PAH-contaminated sites (40).

So far, current aerobic *in situ* bioremediation techniques are considered ineffective for the removal of most PAHs from contaminated soil within a reasonable time period (48), due to the low bioavailability of the PAHs. More success to degrade PAHs with less than four aromatic rings has been achieved with *ex situ* methods such as landfarming (48) or the use of bioreactors (22, 37). Bioreactors are expensive but effective, because optimal conditions are easier to maintain and a good mixing of soil, microorganisms, nutrients and electron acceptor is possible. The application of this method has not yet been studied extensively, and further research is needed to optimize its efficiency and to learn more about the economics of the process.

Anaerobic *in situ* bioremediation

The use of engineered anaerobic *in situ* bioremediation at sites contaminated with aromatic compounds is limited compared to aerobic *in situ* bioremediation. The limited use of anaerobic bioremediation is partly due to our lack of knowledge of the transformations of these compounds by anaerobic bacteria. Only in recent years has it become clear that anaerobic bacteria degrade aromatic hydrocarbons and that anaerobic transformations take place at contaminated sites (intrinsic bioremediation). The stimulation of these transformations has not yet been studied extensively and guidelines how to operate engineered anaerobic *in situ* bioremediation do not yet exist. The use of anaerobic *in situ* bioremediation processes is advantageous because the often difficult and costly supply of oxygen is not needed. Alternative electron acceptors, such as carbon dioxide, sulfate, iron, manganese, or nitrate have to be present at a contaminated site. Such extra additions might be more economical than the supply of oxygen (48). Based on results from the research described in this thesis and literature data, factors that influence anaerobic *in situ* bioremediation are discussed in the following section.

Factors affecting anaerobic *in situ* bioremediation

Although various studies have been performed to gain information on possible anaerobic transformation reactions of aromatic hydrocarbons (see Introduction) little is known about the microorganisms. The anaerobic degradation of aromatic compounds with a functional group, like phenol and cresol, has been demonstrated in several laboratory experiments and successful field bioremediation processes have been developed (20, 41). Knowledge on the degradation of non-oxygenated aromatic hydrocarbons is emerging only since the last few years (15, 17, 34), and almost nothing is known about the occurrence of anaerobic transformations at contaminated sites. Only a few *in situ* bioremediation studies with BTEX as pollutants and nitrate as electron acceptor are in progress (20, 33, 41).

Microorganisms do not always degrade a compound directly upon exposure, but often a lag-phase can be seen. During this lag-phase the compound seems to be

inert. Several mechanisms have been proposed to explain this phenomenon, including enzyme induction, growth of a degrading population, and genetic changes resulting in new metabolic pathways (23, 26). Adaptation may involve cooperative or symbiotic relationships of microorganisms in the degradation of a compound, and occurs not only within single microbial populations but also amongst diverse microbial populations. Adaptation was demonstrated in a study with sediment from San Diego Bay, California, USA. Naphthalene and phenanthrene were oxidized to carbon dioxide in sediments that were heavily contaminated with PAHs (33 mg/kg of sediment) within one month of incubation. In less contaminated sediments (4 mg/kg of sediment), mineralization had not started after 70 days of incubation (11). Apparently, heavily contaminated sediments contained adapted bacteria that were able to degrade the PAHs, whereas in the less contaminated sediments no adaptation had occurred. The use of contaminated material as inoculum, in which the microorganisms have been exposed to the pollutant for a long period of time, does not automatically result in the degradation of the pollutant. We studied the possible transformation of toluene, benzene, and naphthalene under different anaerobic conditions in columns filled with sediment, contaminated with those compounds (Chapter 2). The inoculum material contained aerobic and anaerobic microorganisms, with a history of exposure to these aromatics. We had expected that these microorganisms had been adapted to the contaminants and to anaerobic conditions, and that they could degrade them anaerobically. Immediate degradation was found for toluene under all conditions tested, while naphthalene was only degraded in the column amended with sulfate. After about 300 days, naphthalene also started to be degraded in the columns amended with manganese and nitrate. In the time-course of our experiments (350 to 500 days), microorganisms apparently have adapted to naphthalene degradation. We never observed degradation of benzene in any of our columns in the experimental period of 500 days, indicating that no microorganisms were adapted to benzene degradation. Caldwell (10) found in a study with benzene-contaminated sediment from various sites, both positive and negative results. Benzene degradation was demonstrated under methanogenic conditions with sediment from a gasoline contaminated aquifer near Empire, Michigan, while no degradation was found in sediment, originating from a landfill leachate impacted aquifer in Norman, Oklahoma during 3 years of incubation. With the sediment from Empire,

Michigan, and with sediment from a gasoline contaminated aquifer near Seal Beach, California, degradation of benzene was also demonstrated under sulfate-reducing conditions. No degradation was found with nitrate as electron acceptor during 2 years of incubation of all of the above sediments (21). These experiments demonstrate that microorganisms that degrade benzene in the absence of oxygen are not ubiquitously present at contaminated sites. Before anaerobic *in situ* bioremediation is chosen as a feasible technique, the presence of anaerobic benzene degrading microorganisms at that site must be demonstrated either in field experiments or in laboratory tests.

Another prerequisite may be the presence of a primary growth substrate, when the contaminant is degraded cometabolically. Since most aromatic hydrocarbons can serve as sole carbon and energy source for bacteria, a primary substrate may not be needed (Introduction). This was confirmed in the studies described in chapter 4 and 5; our enrichment culture LET-13 was able to mineralize toluene under manganese-reducing conditions without a primary growth substrate. Also naphthalene was degraded without the addition of an other substrate in a sediment column amended with sulfate (Chapter 2). Unfortunately, we were not able to enrich for naphthalene degrading bacteria in batch experiments (Chapter 3). No enrichment was observed in the absence of a primary growth substrate, but the addition of a primary growth substrate, such as acetate, lactate, or benzoate, did not result in the degradation of naphthalene in batch cultures either. Aerobic fungi are known to degrade aromatic hydrocarbons cometabolically (40). Such data do not exist on anaerobic fungi.

The column studies that we performed on the biodegradation of specific contaminants, took place under better conditions than can be expected in field situations. The columns were continuously percolated with the contaminants. This resulted in a saturation of the column material with the substrates, after which a breakthrough of the aromatics was observed (Chapter 2 and 4). From this point on, a certain concentration of contaminants remained in the aqueous phase, and should have been available for microorganisms. Biodegradation rates in the field are often much lower than found in laboratory-scale experiments. A limited bioavailability of the contaminant may contribute to this finding. Bacterial substrate uptake occurs mainly upon dissolution of the compound in the water phase (7). Most aromatic

hydrocarbons, and especially the polycyclic ones, are poorly soluble in water and may be present in so-called nonaqueous phase liquids (NAPLs). The degradation rate of compounds present in a NAPL is much lower than when they are dissolved in the aqueous phase, due to mass transfer limitations (1). Such a lower degradation rate of naphthalene was demonstrated at a naphthalene contaminated site (16). Aromatic hydrocarbons also tend to adsorb to soil particles, and desorption kinetics will influence the degradation rate. In addition, hydrophobic aromatic pollutants may form complexes with humic substances in the soil, which results in the formation of irreversibly bound residues (6). Making the contaminant less susceptible for microbial degradation. Finally, the contaminant can be present in micropores in the soil, in which the microorganisms cannot penetrate. The rate of diffusion of the compound towards the microorganisms will then strongly affect its rate of degradation.

The bioavailability of pollutants decreases with the 'age' of pollution (18), because of the chemical/physical interactions between the compounds and the humic substances in the soil as previously described. As a result, the rate of degradation will decrease in time and residual concentrations may occur. These findings suggest that bioremediation of a contaminated site might be more successful, when the clean-up starts as soon as possible after the contamination has occurred.

Microbial growth and activity is dependent on characteristics like pH, temperature, moisture and salinity. The temperature in the soil has a large effect on the degradation rate. During bioremediation, changes in degradation rates can be observed because of temperature changes in the soil during the seasons (1). Furthermore, the temperature of the soil is often below the temperature optimum of mesophilic bacteria and a low temperature of the soil is generally expected to have a negative influence on the degradation rate. However, it has been demonstrated that the degradation rate of toluene in a cold aquifer (5°C) was comparable with the degradation rate at higher temperatures (20°C) (9).

An important factor is the presence or delivery of the terminal electron acceptor and nutrients (mainly nitrogen and phosphorous). In our study, the continuous percolation of the columns with a balanced medium provided the microorganisms in the column with a constant supply of electron acceptor and nutrients. However, under field conditions, the competition for electron acceptor and

nutrients within the microbial population may limit the growth of the desired microorganisms and may result in a slow contaminant removal. Another obstacle is that electron acceptor and/or nutrients can be consumed by microorganisms, that are not involved in the degradation of the contaminant, before they reach the contaminated zone. A correct engineered delivery design should avoid this problem. Additionally, electron acceptors can react with compounds in the soil to form precipitates, e.g. metal sulfides. These precipitates can cause plugging of the soil, or can already cause plugging in the injection wells (42).

The electron acceptors that are present or added to the soil determine which types of microorganisms become dominant and consequently which degradation pathways are possible. In many soils, different electron acceptors are heterogeneously distributed. The distribution of electron acceptors results in the presence of various types of microorganisms in the soil that are heterogeneously distributed as well. Theoretically, a sequence of redox-mediated reactions should occur when more than one electron acceptor is available at a given site. The electron acceptor that will provide the microorganisms with the largest energy for growth will be used first (Introduction, Table 2). We did not observe this sequence in our experiments, because only one electron acceptor was added per column or batch, in stead of a mixture of electron acceptors. However, we did observe a 6 times higher degradation rate of toluene under manganese-reducing conditions than under methanogenic conditions in batch experiments. This may be the result of the difference in redox potential between the two electron acceptors. The use of a solid electron acceptor like manganese or iron oxide may have an other advantage besides its favourable redox potential. Upon being reduced, they can be reoxidized and thereby returned to the sediments by precipitation, after which they can be reused as electron acceptor. Since these manganese and iron cycles occur in many sediments (28), they can in theory be used numerous times during bioremediation. At low oxygen concentrations and in the presence of nitrate, it has been demonstrated that naphthalene is degraded with oxygenases as the first degrading enzymes and nitrate as the ultimate electron acceptor (12, 46).

The importance of nutrient addition was demonstrated with our enrichment culture LET-13 (Chapter 4). Sterile Rhine river sediment or its supernatant was needed to maintain the toluene degrading activity of the culture in batches. We

were not able to clarify which nutrients or trace elements were essential. Other contaminant degrading organisms are also known for their need of a specific addition. An anaerobic enrichment culture that dechlorinates PCBs needs the addition of Rharitan river sediment or humic acids (5, 8).

The concentration of the contaminant is important for the microorganisms. At high concentrations, toxic effects may occur. Toluene has been found to be toxic at a concentration of 0.5 mM to the sulfate-reducing strain Tol2 (32), and at 1 mM to our manganese-reducing enrichment culture LET-13 (Chapter 5), and to nitrate-reducing bacteria (2, 35). On the other hand, a low aqueous concentration can be below the concentration that is needed for the induction of the necessary enzymes.

The presence of mixtures of contaminants in the soil may influence the bioremediation rate. In laboratory-scale experiments, it was shown that in mixtures of benzene, toluene, ethylbenzene, and xylenes, compounds were degraded sequentially (17). Toluene was the first to be degraded, followed by xylene and ethylbenzene, while benzene was found to be persistent. However, in incubations with benzene only, degradation of benzene did occur (13). Although we also used mixtures of toluene, benzene and naphthalene in our column studies, we have never observed this phenomenon (Chapter 2). Toluene was the first compound to be degraded in all columns and omitting toluene did not result in the degradation of the two other compounds. This suggests that benzene and naphthalene degrading bacteria were not present. Degradation of naphthalene was also not observed in the presence or absence of benzene in the nitrate-reducing column. At contaminated sites, the contaminants are often present as mixtures. The degradability of a compound, present in a mixture with other contaminants, might be different than when the compounds are present as single contaminants.

Monitoring and evaluation of bioremediation

When all conditions and prerequisites for a successful *in situ* bioremediation technique have been accomplished, the degradation processes need to be controlled and evaluated to show and ensure that clean-up is taking place. Not all methods for

demonstrating biodegradation in laboratory experiments can be used in field experiments (39) or are equally successful in laboratory and field experiments.

The use of ^{14}C-labelled compounds is a common laboratory technique. Measuring $^{14}CO_2$ provides usually the most reliable results, but is only usable when the contaminant is completely mineralized. In our column studies, we could demonstrate that [^{14}C]naphthalene was mineralized by measuring the $^{14}CO_2$ production (Chapter 2). In field experiments, the capture of the produced $^{14}CO_2$ seems to be impossible. In Denmark, an anaerobic continuous field injection experiment with a radiolabelled compound has been performed. [^{14}C]Benzene was injected in a pollution plume, but no production of $^{14}CO_2$ could be measured (33). In the Netherlands, governmental organizations would never allow this type of experiments with (volatile) radiolabelled compounds that are hazardous and difficult to control.

Measuring the decrease in contaminant concentration gives an indication whether the contaminant is degraded, but this is usually not reliable enough. Artifacts, such as volatilization, off-site migration, adsorption to the soil, and other abiotic losses, must be taken into account in these measurements. Knowledge of the degradation pathway of a compound can be helpful. The pathway gives information whether a compound is mineralized or transformed into less harmful or more toxic compounds. Furthermore, the detection of intermediates can be used to monitor the degradation of the contaminant. In a recent aerobic field experiment, the production of 1,2-dihydroxy-1,2-dihydronaphthalene, an intermediate in the aerobic degradation of naphthalene, was used as evidence for a successful *in situ* bioremediation (47).

Measuring a decrease in electron acceptor concentration does not necessarily indicate that the contaminant is transformed. The added electron acceptor can be used for the degradation of the contaminant, but other organic or inorganic carbon compounds in the soil can also be oxidized with this electron acceptor. In addition, it is difficult to measure a decrease in electron acceptor concentration, when solid electron acceptors like iron and manganese oxide are used. Furthermore, the metals may after reduction be reoxidized again. The possible cycling of electron acceptors makes it difficult to use their decrease in concentration for the evaluation of contaminant removal. The type of oxide formed after oxidation, determines its

possible use as electron acceptor. A more amorphous form is reduced faster than a more crystalline one, and consequently the rate of contaminant oxidation depends on the type of metal oxide used. With amorphous metal oxides a higher degradation rate can be achieved than with crystalline ones (24).

Demonstration of the growth of contaminant degrading bacteria on agar plates will not be accurate either. Other organic material than the contaminant is present at the polluted site, and may serve as growth substrate for bacteria that grow on the plates. In addition, growth of bacteria on agar or impurities in the agar is also possible (Chapter 3). A more conclusive method is the demonstration of enzymes that are involved in the degradation of the compound. The presence and number of genes, coding for these enzymes, can be demonstrated by extraction of DNA from the site and the use of specific probes (14). For anaerobic degradation however, little is known about the responsible enzymes, and more research is needed before this method can be applied.

Knowledge about the success of degradation at field sites will often involve uncertainties, because none of the methods described is alone reliable enough to prove that bioremediation is occurring. For a good control, a combination of these methods has to be used and the results need to be interpreted carefully. Only then is it possible to evaluate whether an ongoing *in situ* bioremediation project is progressing towards successful completion.

Concluding remarks

An effective *in situ* bioremediation depends on the presence of the appropriate microorganisms. Laboratory experiments with site-specific samples can be used to demonstrate that degradation of a compound is possible at a particular site. One should realize that the conditions in laboratory experiments will often differ from those in the field, and that a decrease in contaminant concentration in the field has to be shown to demonstrate that the bioremediation is successful.

The results from the manganese-reducing, toluene degrading bacterial culture we enriched in this study, demonstrate that the bioremediation of toluene-contaminated sites is in principle possible when manganese oxides are present. However,

the required addition of complex compounds in laboratory experiments (e.g. sterile Rhine river sediment), indicates that the enriched bacteria have a specific nutrition need.

The naphthalene degrading activity that we observed in a sulfate-reducing sediment column, could not be transferred to batches. This illustrates the difficulties one encounters when trying to isolate bacteria that anaerobically degrade aromatic hydrocarbons.

The use of *in situ* bioremediation at a contaminated site must be based on sufficient knowledge of the presence of the required microorganisms, the contaminants, and the environmental conditions. At the moment, our knowledge on microorganisms that can degrade aromatic hydrocarbons anaerobically and the required conditions, is not sufficient to conclude that anaerobic *in situ* bioremediation of aromatic hydrocarbon contaminated sites can be successful (except for toluene). Insight in the microbiology (e.g. responsible bacteria, involved enzymes, enzyme regulation, degradation kinetics) is important to support the application of such a microbial technique.

The few studies done at anaerobic, contaminated sites indicate that aromatic hydrocarbons can be (partially) degraded anaerobically in nature, and that this degradation may be significant. Additional laboratory and field studies must help to establish the real potential of anaerobic microbial processes for the clean-up of polluted sites.

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Summary

Aromatic hydrocarbons are widespread in nature, due to increasing industrial activity, and often contribute to polluted soils, sediments, and groundwater. Most of these compounds are toxic at relatively high concentrations, but some are already carcinogenic at very low concentrations, e.g. benzene. A growing awareness of the health risks associated with contamination has directed research to the removal or degradation of such compounds. The use of microorganisms to degrade toxic compounds (bioremediation) is a relatively slow process compared to traditional, chemical methods, but it is a natural process, mostly very specific and low in costs. A review of the available information on the microbial degradation of aromatic compounds is given in chapter 1. The anaerobic degradation is emphasized, since in many polluted environments oxygen is limiting and anaerobic processes will prevail. In the absence of oxygen, compounds like nitrate, metal-ions (Fe^{3+} and Mn^{4+}), sulfate, and carbondioxide, have taken over the function of oxygen as a terminal electron acceptor. In addition, the first transformation reactions differ from those in aerobic processes. Oxygenases are no longer functioning and the degradation of oxygenated aromatic compounds, like benzoate and phenol, is known to occur via e.g. reduction, dehydroxylation and dehydrogenation of the aromatic ring. Information on the anaerobic degradation of mono- and polycyclic aromatic hydrocarbons without functional groups, like toluene, benzene, and naphthalene, is scarse. To gain more insight in the possibilities and limitations of the anaerobic degradation of these aromatic compounds, their behaviour in anaerobic sediment columns was followed. Toluene, benzene, and naphthalene were chosen as model compounds under methanogenic, sulfate-, iron-, manganese-, and nitrate-reducing conditions (Chapter 2). Toluene was transformed readily (within 1 to 2 months), while benzene was recalcitrant over the test period of 375-525 days under all redox conditions tested. Naphthalene was partly transformed in the column with nitrate or manganese as electron acceptor present; the addition of benzoate had a positive effect on the degradation of

naphthalene in the column with nitrate. In the column with sulfate, the majority of the added naphthalene disappeared. No effect on the degradation of naphthalene was observed after adding and omitting an easier degradable substrate. [^{14}C]naphthalene was used to confirm the disappearance to be the result of degradation; two third of the naphthalene was converted to CO_2.

Numerous attempts have been made for further enrichment of sulfate-reducing, naphthalene degrading bacteria (Chapter 3). Unfortunately, the observed degradation of naphthalene in a sediment column could not be obtained in batch cultures, despite the large variety of tested enrichment conditions (different naphthalene concentrations, inoculum size, medium composition, extra additions etc.). A toxic effect of naphthalene on sulfate-reducing bacteria could not be found.

Toluene degradation in the columns was demonstrated under all redox conditions tested. Chapter 4 describes the degradation of toluene in freshly started sediment columns, to which either amorphous or highly crystalline manganese oxide had been added. In batch experiments with material from these columns as inoculum, the degradation of toluene to CO_2 and the formation of biomass under manganese-reducing conditions was demonstrated. The oxidation of toluene was found to be coupled to the reduction of Mn(IV), and the rate of oxidation was found to be lower with the crystalline than with the amorphous manganese oxide. Upon successive transfers of the enrichment cultures, the toluene degrading activity would decrease in time. The activity could only be maintained in the presence of sterilized Rhine river sediment or its supernatant. Without the sediment, but in the presence of solids like teflon beads, glass beads, bentonite, vermiculite and sterilized granular sludge, the toluene degrading activity completely disappeared after 4 to 5 transfers. Furthermore, a direct contact between the bacteria and the manganese oxide was found to be advantageous for a rapid toluene degradation. The degradation rate could further be increased by adding organic ligands such as oxalic acid or nitrilotriacetic acid (NTA).

The highly purified enrichment culture LET-13, which degrades toluene with manganese oxide as electron acceptor, was obtained via repeated dilution series, and is described and characterized in chapter 5. LET-13 was able to degrade a variety of substituted monoaromatic compounds like (*p*-hydroxy) benzylalcohol, (*p*-hydroxy) benzaldehyde, (*p*-hydroxy) benzoate, cresol, and phenol. Benzene, ethylbenzene, xylene and naphthalene were not degraded under the experimental

conditions used. The degradation of toluene occurred via hydroxylation of the methyl group to benzoate, and a possible side reaction can lead to the formation of cresol.

All organisms in the culture look similar; motile rods which are gram negative, oxidase negative and catalase negative. The culture was partly identified with phylogenetic analysis of cloned rDNA sequences. The phylogenetic analysis showed that at least two major groups of bacteria are present. One group of bacteria belongs to the *Bacteroides-Cytophaga* group, and one group consists of members of the ß-subclass of the *Proteobacteria*.

Finally, the results from this research are discussed in relation to their relevance for soil bioremediation technologies.

Samenvatting

Aromatische koolwaterstoffen (zoals benzeen, ethylbenzeen, tolueen en naftaleen) zijn schadelijke stoffen die in het milieu belanden als gevolg van menselijke activiteiten. Ze vormen een belangrijke groep van verontreinigingen in grond, grondwater en in (haven)slib. De meeste van deze vluchtige verbindingen zijn pas toxisch, indien zij in een hoge concentratie voorkomen, maar enkele verbindingen, zolas bijvoorbeeld benzeen, zijn in een lage concentratie al kankerverwekkend. Efficiënte reinigingstechnieken zijn dan ook zeer gewenst.

Eén techniek is de afbraak door micro-organismen (bioremediatie). Ondanks het feit dat bioremediatie een relatief langzaam proces is, heeft het toch voordelen boven fysisch/chemische technieken. Het is een natuurlijk proces en tast de eigenschappen van de te reinigen bodems, die niet hoeven te worden verwijderd, nauwelijks aan. Een overzicht van het gedane onderzoek naar de microbiële afbraak van aromaten staat beschreven in hoofdstuk 1. Het benadrukt voornamelijk anaërobe afbraakprocessen, alhoewel het meeste onderzoek tot nu toe gericht is geweest op aërobe omzettingsreacties. Bij aërobe omzettingen treedt moleculaire zuurstof niet alleen op als elektronenacceptor, maar wordt het ook in de aromatische ring ingebouwd m.b.v. mono- en dioxygenase enzymen. Veel is inmiddels bekend over dit soort reacties en een groot aantal bacteriën, dat aromatische koolwaterstoffen volledig kan afbreken, is geïsoleerd. Een probleem dat in veel verontreinigingssituaties optreedt, is een gebrek aan zuurstof. Dit heeft tot gevolg dat aërobe micro-organismen niet meer actief zijn en dat alleen anaërobe afbraak kan plaatsvinden.

Anaërobe micro-organismen, waarbij zuurstof niet als elektronenacceptor fungeert, gebruiken elektronenacceptoren zoals nitraat, sommige metalen (Fe^{3+}, Mn^{4+}), sulfaat en kooldioxide. Tevens gebruiken zij andere enzymen dan oxygenases voor de eerste omzettingsreacties. Voor aromaten met een zuurstofhoudende zijgroep (zoals benzoaat en fenol) zijn omzettingsreacties zoals reductie van de aromatische ring, dehydroxylering en dehydrogenering bekend. Kennis over de

anaërobe omzettingsreacties van enkel- en meervoudige ringverbindingen zonder functionele groepen is echter nog schaars.

In dit proefschrift staan experimenten beschreven, die zijn uitgevoerd om meer inzicht te krijgen in de mogelijkheden en de limitaties van de anaërobe afbraak van aromatische koolwaterstoffen. In hoofdstuk 2 staan experimenten met anaërobe, continue doorstroomde sedimentkolommen beschreven. Tolueen, benzeen en naftaleen zijn hierbij gekozen als modelstoffen en het gedrag van deze verbindingen in de kolommen onder vijf verschillende redoxcondities (methanogene, sulfaat-, ijzer-, mangaan- en nitraatreducerende condities) is in de tijd gevolgd. Tolueen werd relatief snel afgebroken (binnen 1 tot 2 maanden) onder alle onderzochte condities. Benzeen werd gedurende het onderzoek in een periode van 375 tot 525 dagen in geen enkele van de vijf kolommen afgebroken. In de kolommen met nitraat of mangaanoxide als elektronenacceptor werd naftaleen gedeeltelijk afgebroken. De toevoeging van benzoaat in de kolom met nitraat kan hierbij een positieve invloed hebben gehad. Onder sulfaatreducerende condities werd naftaleen volledig afgebroken. De toevoeging van een makkelijker afbreekbare verbinding had hierbij geen positief of negatief effect op de afbraak. Met radioactief naftaleen is in een andere sedimentkolom aangetoond dat naftaleen voor ongeveer 60 % werd omgezet naar CO_2.

Nadat de afbraak van naftaleen in een sulfaatreducerende kolom was aangetoond, is materiaal van deze kolom gebruikt in batch-experimenten om sulfaatreducerende, naftaleen afbrekende bacteriën op te hopen (Hoofdstuk 3). Om onverklaarbare redenen is het echter niet gelukt om de afbraak van naftaleen ook in batches te bewerkstelligen. Dit ondanks de grote variatie in de geteste condities (verschillende concentraties naftaleen, verschillende hoeveelheden entmateriaal, mediumsamenstelling, toevoegingen zoals extra kolommateriaal, Rijnsediment enz.). Een eventueel effect van naftaleen op de groei van een aantal sulfaatreducerende bacteriën is getest bij verschillende naftaleen concentraties, maar de aanwezigheid van naftaleen bleek geen invloed te hebben op de groeisnelheid van deze bacteriën.

Zoals in hoofdstuk 2 staat beschreven, was tolueen onder alle geteste redoxcondities afbreekbaar. In hoofdstuk 4 worden vervolgexperimenten met tolueen als substraat beschreven, waarbij alleen mangaanoxide als elektronenacceptor is gebruikt. In nieuw opgestarte sedimentkolommen met kristallijn of

amorf mangaanoxide als elektronenacceptor, bleek er geen verschil in omzettingssnelheid van tolueen te zijn tussen de twee gebruikte mangaanoxiden. In batchexperimenten met entmateriaal uit de kolom, bleek echter dat een meer amorfe vorm van mangaanoxide als elektronenacceptor een snellere afbraak van tolueen gaf dan een kristallijne vorm. In verdere experimenten met amorf mangaanoxide is de afname van mangaanoxide (en de produktie van Mn^{2+}) en de produktie van CO_2 aangetoond.

Na een aantal keren te hebben overgeënt, bleek de afbraak van tolueen steeds langzamer te verlopen en uiteindelijk kwam deze na 4 à 5 generaties vrijwel stil te liggen. De activiteit kon alleen worden herkregen door de toevoeging van steriel Rijnsediment of het supernatant ervan. Andere toevoegingen zoals teflon- of glasparels, bentoniet, vermiculiet of steriel korrelslib hadden geen van allen een positieve invloed op de omzetting van tolueen. Verder is gebleken, dat een direct contact tussen de bacteriën en het slecht oplosbare mangaanoxide de afbraak van tolueen versnelde. Hetzelfde effect werd waargenomen als een chelerende verbinding, zoals oxaalzuur of NTA, aan het medium werd toegevoegd.

Na een groot aantal overentingen in vloeibaar medium is uiteindelijk de ophopingscultuur LET-13 verkregen, waarvan eigenschappen en karakteristieken staan beschreven in hoofdstuk 5. Een groot aantal aromatische verbindingen kan dienst doen als koolstof- en energiebron voor LET-13, nl. (*p*-hydroxy) benzylalcohol, (*p*-hydroxy) benzaldehyde, (*p*-hydroxy) benzoaat, cresol en fenol. Benzeen, ethylbenzeen, xyleen en naftaleen konden niet worden afgebroken. De afbraakroute van tolueen is gedeeltelijk opgehelderd. Tolueen wordt afgebroken door een methylhydroxylering via benzylalcohol en bezaldehyde naar benzoaat. De ozetting tot cresol is ook aangetoond, maar dit is slechts een nevenreactie in LET-13. De mangaanreducerende cultuur LET-13 bevat beweeglijke staafjes, van ongeveer 1,5 μm lengte, die gram-, oxidase- en katalase-negatief zijn. Met behulp van moleculaire technieken is aangetoond dat LET-13 ten minste twee verschillende groepen van bacteriën bevat. Eén van deze groepen bevat bacteriën die zijn in te delen bij de *Bacteroides-Cytophaga* groep, en de andere groep bestaat uit bacteriën die behoren bij de ß-subgroep van de *Proteobacteriën*.

In het laatste hoofdstuk van dit proefschrift worden de resultaten van dit onderzoek gerelativeerd en bediscussieerd in het kader van een mogelijke toepassing voor bodemreinigingsprocessen.

Curriculum Vitae

Alette Langenhoff werd geboren op 14 september 1966. Na het behalen van het VWO-diploma aan het Jacob Roelandslyceum in Boxtel, begon ze in 1984 met de studie Levensmiddelentechnologie aan de Landbouwuniversiteit Wageningen. Haar afstudeervakken waren Proceskunde en Industriële Microbiologie. Haar stages heeft ze gevolgd bij achtereenvolgens het Instituut voor Biochemie en Biotechnolgie, Technische Universiteit Braunschweig, Duitsland en het CFTRI (Central Food en Technology Research Institute) in Mysore, India. In augustus 1990 heeft ze het doctoraalexamen behaald.

Na twee tijdelijke banen bij de secties Proceskunde en Industriële Microbiologie van de vakgroep Levensmiddelentechnologie als resp. toegevoegd onderzoeker en practicum assistent, is ze van februari 1991 tot januari 1996 aangesteld geweest als assistent in opleiding (AIO) en toegevoegd onderzoeker bij de vakgroep Microbiologie van de Landbouwuniversiteit Wageningen. Tijdens dit onderzoek is ze werkzaam geweest aan de anaërobe afbraak van enkele aromatische verbindingen en de resultaten van dit onderzoek zijn beschreven in dit proefschrift.

Sinds 1 februari 1997 is zij werkzaam als onderzoeker bij het Department of Chemical Engineering and Chemical Technology, Imperial College, Londen, Engeland

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