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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 (Gibson & Subramanian 1984). 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 (RIWA 1993). Most of these compounds were shown to be mutagenic or carcinogenic (Zedeck 1980).

Research on the microbial degradation of aromatics has mainly focused on aerobic transformation reactions (Gibson & Subramanian 1984; Smith 1990). 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 (Fuchs et al. 1994; Grbić-Galić 1990; Smith 1994). Degradation of toluene by pure cultures has been reported under sulfate-reducing (Rabus et al. 1993), iron-reducing (Lovley & Lonergan 1990) and denitrifying conditions (Altenschmidt & Fuchs 1991; Dolfing et al. 1990; Evans et al. 1991; Schocher et al. 1991), and parts of degradation pathways were elucidated (Altenschmidt & Fuchs 1992; Beller et al. 1992; Evans et al. 1992). 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 (Lovley et al. 1994), and with sulfate as electron acceptor (Lovley et al. 1995). In

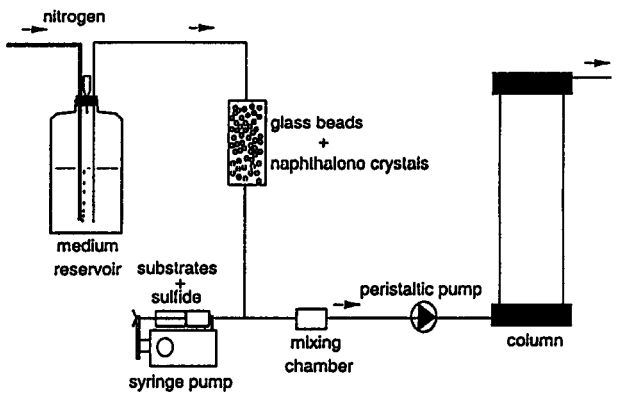

Figure 1. Schematic diagram of the column system.

mixed cultures, derived from ferulic acid-degrading sewage sludge enrichments, benzene degradation was shown under methanogenic conditions (Grbić-Galić & Vogel 1987; Vogel & Grbić-Galić 1986). 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 (Edwards & Grbić-Galić 1992b).

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 (Mihelcic & Luthy 1988a; Mihelcic & Luthy 1988b; Mihelcic & Luthy 1991). This degradation was also demonstrated in soil-slurry systems (Al-bashir et al. 1990). 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 (Figure 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).

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 $Na_2HPO_4.2H_2O$; 0.063 g $NaHCO_3$; 0.11 g $CaCl_2.2H_2O$; 0.1 g $MgCl_2.6H_2O$; 0.027 g NH_4Cl; 0.0085 g Na_2SO_4, and 0.1 ml of a trace element solution (Holliger et al. 1993). 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 metal-oxides 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 (Knowles 1982). 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 (Figure 2). 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]naphthalene, 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.

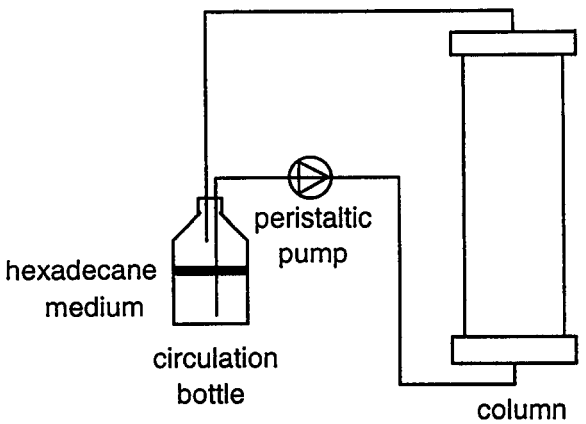

Figure 2. Schematic diagram of the circulating column system.

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 (Lovley & Phillips 1986).

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 (Burdige & Nealson 1985). 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).

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.

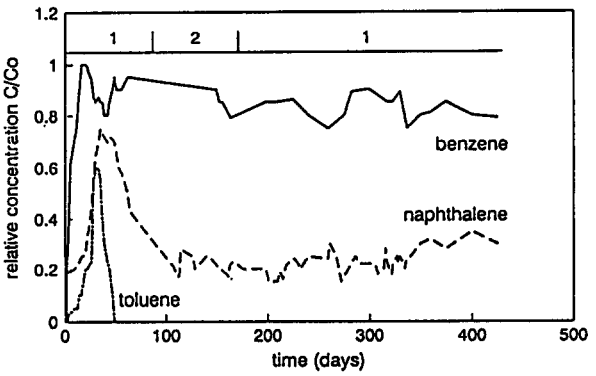

Figure 3. Behaviour of toluene, benzene and naphthalene in the presence of sulfate. C/Co is effluent concentration relative to influent concentration. (1): no benzoate added, (2): addition to the medium of 5 μM benzoate

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 (Figure 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

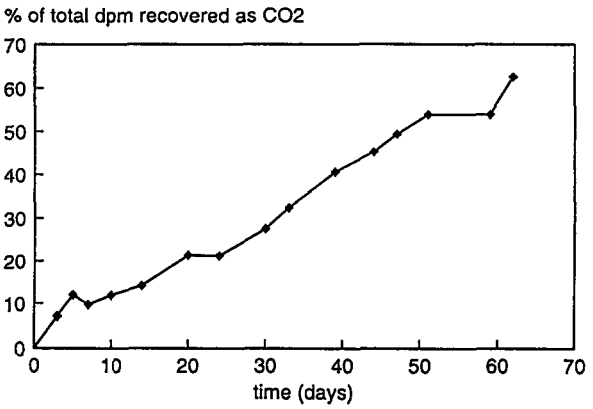

Figure 4. Production of $^{14}CO_2$ from [^{14}C]naphthalene in the presence of sulfate in a recirculating system.

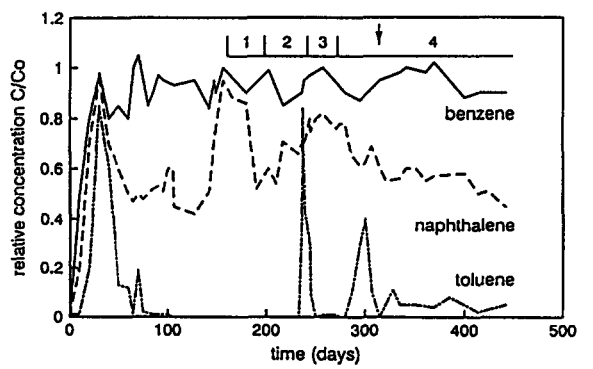

Figure 5. Behaviour of toluene, benzene and naphthalene in the presence of manganese. C/Co is effluent concentration relative to influent concentration. Additional substrates at (1): 50 μM benzoate, (2): 250 μM benzoate, (3): 50 μM phenol: (4): 50 μM lactate. Between day 100 and 225 toluene was omitted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

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).

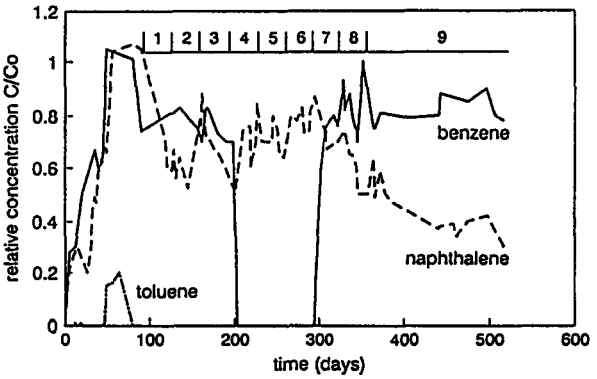

Figure 6. Behaviour of toluene, benzene and naphthalene in the presence of nitrate. C/Co is effluent concentration relative to influent concentration. Additional substrates at (1): 5 μM benzoate: (2): 50 μM benzoate, (3): 250 μM benzoate, (4): 50 μM acetate, (5): 50 μM lactate, (6): 150 μM lactate, (7):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

Manganese-reducing column

Toluene showed a partial breakthrough (Figure 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 (Figure 6). 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 (Bosma et al. 1988) tetrachloroethene (de Bruin et al. 1992) and hexachlor-cyclohexanes (Middeldorp et al. 1996). 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.

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 (Aihara 1992).

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. ($\Delta G = \Delta G° + RT \ln K$). Data used from Weast (1971–1972) and Stumm & Morgan (1981).

	Toluene c_{in}=25μM	Benzene c_{in}=25μM	Naphthalene c_{in}=25μM	Naphthalene c_{in}=200μM
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 (Stumm & Morgan 1981), 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.

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 (Fuchs et al. 1994, Smith 1994). 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 (Langenhoff 1996). 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 (Edwards 1992b; Lovley 1994; Lovley 1995). 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 (Edwards et al. 1992a). 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 (Mihelcic & Luthy 1988a; Mihelcic & Luthy 1988b).

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 (Figure 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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