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Propylphenols are metabolites in the anaerobic biodegradation of propylbenzene under iron-reducing conditions

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Abstract

The metabolism of monoaromatic hydrocarbons by an iron-reducing bacterial enrichment culture originating from diesel-contaminated groundwater was examined using d_7-propylbenzene as a model hydrocarbon. Sequence analysis of the 16S rDNA gene showed that the dominant part (10 of 10 clones) of the enrichment culture consisted of a bacterium closely related to clones found in benzene-contaminated groundwater and to the iron-reducing β-proteobacterium, *Rhodoferax ferrireducens* (similarity values were 99.5% and 98.3%, respectively). In degradation studies conducted over 18 weeks, d_7-propylphenols were detected by gas chromatography–mass spectrometry (GC/MS) as intra-cellular metabolites concomitant with cell growth in the cultures. The amount of propylphenols increased during the exponential growth phase, and by the end of this phase 4×10^{-14} moles of ferric iron were reduced and 3×10^{-15} moles propylphenol produced for every cell formed. During the stationary growth phase the cell density was approximately 10^7 ml^{-1}, with significantly correlated amounts of propylphenols. Succinate derivates of propylbenzene or phenylpropanol previously shown to be the initial metabolites in the anaerobic degradation of alkylbenzenes could not be identified. This study is the first to report that oxidation of propylbenzene to propylphenols can initiate anaerobic propylbenzene degradation and that iron-reducing bacteria are responsible for this process. In addition, the study shows the importance of taking account of the metabolites adhering to solid phases when determining the extent of biodegradation, so as not to underestimate the extent of the process.

Introduction

In Sweden and many other countries, for example Japan and Norway, diesel and gasoline are stored on groundwater in underground vaults in granite bedrock (Roffey 1989). There are over 40 such storage vaults around Sweden, and the largest ones can hold over 150,000 m^3 of fuel. The storage vaults are located below the groundwater table, and water levels in the vaults are controlled by continuously pumping water out of them. This prevents the fuel from being transported to the surroundings. After decommissioning, the vaults are filled with groundwater. There will always be some fuel residues left in the emptied vaults and in fractures, connected to the vaults, in the granite. These residues may eventually be transported via the groundwater and spread in the environment. A large part of the hydrocarbons in diesel and gasoline are monoaromates and a major group of these is the alkylbenzenes. These are of environmental concern because of their toxicity and water solubility (Dean 1985). The fate of these hydrocarbons in the subsurface environments around vaults must therefore be thoroughly investigated.

Traditional ways of examining biodegradation in contaminated areas involve identifying and monitoring metabolites (Beller 2000). Many metabolites produced by the anaerobic degradation of alkylbenzenes have been identified in recent years. However, many studies of the anaerobic degradation of these hydrocarbons have mainly focused on environments where contamination redox plumes exist, for example in sediment, porous bedrock, and shallow aquifers (for example, Cozzarelli et al. 2001; Phelps & Young 1999). Biodegradation in fractured hard rock must be considered to be different from biodegradation in these environments, and it is very important to investigate the specific microbial processes and metabolites present in this ecosystem. The fuel does not penetrate the rock as it does in more porous bedrock and therefore no plumes are formed (Lorén et al. 2001). Groundwater in fractured rock quickly becomes oxygen depleted (Banwart et al. 1996), and anaerobic processes are thus the only ones important in biodegradation of contaminants. The groundwater is depleted in nitrate while sulphate and ferric iron are abundant (Hallbeck et al. 1998). It is an oligotrophic environment, meaning both that co-metabolism is probably not as widespread and that microbial diversity is not as great as in nutrient-rich waters.

In this study d_7-propylbenzene was used as a model monoaromate in detecting degradation metabolites under iron-reducing conditions by a characterised bacterial enrichment culture retained from granitic groundwater contaminated with diesel. Poorly ordered 2-line ferrihydrite (FeOOH) was used as the iron source since it exists on the surfaces of fractures in the granite around the storage vaults and is an important electron acceptor.

Materials and methods

Media and cultivation conditions

A bicarbonate-buffered medium modified from Widdel and Bak's (1992) 'multipurpose medium for sulfate-reducing bacteria' was prepared under anaerobic conditions. Except as otherwise indicated, all chemicals were purchased from VWR International (Göteborg, Sweden). All glassware was washed with methanol and hexane, and autoclaved prior to use.

The medium contained (l^{-1} distilled water): 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.1 g $MgCl \times 6H_2O$, 0.1g $CaCl_2 \times 2H_2O$, 0.05 g KNO_3, 0.1 g $MgSO_4 \times 7H_2O$, 0.05 mg Rezazurine. The medium was autoclaved and cooled under an N_2/CO_2 atmosphere (approximately 95/5 vol/vol). The following solutions were added according to Widdel & Bak (1992) except as otherwise indicated (l^{-1} distilled water): 1 ml non-chelated trace element mixture (Widdel et al. 1983), 1 ml selenite tungstate solution, 1 ml vitamin mixture, 1 ml thiamine solution, 1 ml vitamin B_{12} solution, and 30 ml 1M $NaHCO_3$; pH was then adjusted to between 7.0 and 7.2 in the medium. The medium was portioned into anaerobic flasks, sealed with butyl rubber stoppers (DUMA, Göteborg, Sweden) and aluminium screw caps, and placed in an anaerobic box (COY Laboratory Products, Grass Lake, MI, US) under an atmosphere of 1% H_2, 6% CO_2, and 93% N_2. Inside the anaerobic box, 100 ml (l^{-1} medium) of 0.1 M FeOOH was added to the medium as an electron acceptor. FeOOH was prepared by neutralising 0.1 M $FeCl_3$ to a pH of 7 with 1 M NaOH (Lovley & Phillips 1986). In some experiments (l^{-1} medium) 0.9 g $NaNO_3$ or 3 g Na_2SO_4 and 1.2 g Na_2S were added as electron acceptors, replacing FeOOH.

The medium was dispensed in 100-ml aliquots into serum bottles and sealed with nitrile rubber stoppers (Ulinco, Askim, Sweden) and aluminium clamps.

Sampling and source of bacteria

Water from a diesel-contaminated borehole (KM01) in Blädinge, Sweden (56° 50′ N, 14° 30′ E) was sampled. Close to KM01 there are now empty underground rock vaults that had been used to store diesel and gasoline for 30 years. Anaerobic groundwater was collected from the borehole using a Solinst discrete interval sampler, Model 425 (Solinst Canada Ltd., Georgetown, Ontario, Canada). The sampler was equipped with a sterile ice plug at the inlet to prevent contamination and was pressurised with N_2 to 1.2 bars to ensure anaerobic conditions. After sampling at 44 m, water from the sampler was immediately transferred into anaerobic serum bottles, stored at 4 °C, and within 24 h inoculated into the iron-reducing medium with no added electron donor. The culture was incubated at 20 °C for 7 months in the dark before it was used as the inoculum in this experiment.

Preparation of cultures

All transfers of bacteria to the bottles were made with N_2-flushed syringes and needles. The bottles were inoculated with bacteria to an initial cell number of 2×10^5 ml^{-1}. Sterile controls were prepared as growth medium without cells. As an electron donor, 4 μl of neat d_7-propylbenzene (C/D/N isotopes, Pointe-Claire, Quebec, Canada) was placed on a piece of Teflon (∅ 9 mm) with a Hamilton syringe and added to the bottles. The deuterium atoms were located on the propyl group. The samples were incubated in the dark at 22 °C.

Phylogenetic analysis

Ten ml of a culture was collected and treated with 5 ml of sterile oxalic acid (0.1%) for 20 min to dissolve and remove iron. The samples were filtrated using a sterilised 13-mm diameter filter holder (Millipore Corporation, Bedford, MA, US) provided with sterile 0.22-μm-pore-size PC filters (Osmonics, Minnetonka, MN, US). DNA was immediately extracted from the filter using the QIAamp DNA Mini Kit (QIAGEN Inc., Valencia, CA, US) following the manufacturer's instructions. Almost full-length 16S rDNA-genes were amplified by PCR with Taq polymerase (QIAGEN Inc., Valencia, CA, US) using the primers 27f and 1492r (Lane 1991; Weisburg et al. 1991, respectively). Amplification was performed with a GeneAmp PCR system 2400 (Applied Biosystems; Foster City, CA, US) with an initial denaturation step at 95 °C for 15 min, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, concluding with a final extension at 72 °C for 10 min. The PCR product was purified with a StrataPrep PCR Purification Kit (Stratagene Cloning Systems, La Jolla, CA, US) and cloned into *Escherichia coli* plasmids using a TOPO Cloning Kit (Invitrogen Life Technologies, Carlsbad, CA, US). Ten clones were sub-cultured, and the plasmids were extracted using the QIAprep Spin Miniprep Kit (QIAGEN Inc., Valencia, CA, US). Sequence reactions of 400 bases forward on the $5' \rightarrow 3'$ strand were performed using the ABI PRISM Big Dye Terminators v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, US) and the 357f primer (Lane 1991). The sequence reactions were separated by gel electrophoresis at Kiseq (Stockholm, Sweden), a laboratory accredited by the Swedish Board for Accreditation and Conformity Assessment (SWEDAC). One of the clones was completely sequenced using Lane's 357f and 1100r primers and the M13 (−21) and M13R primers included in the TOPO Cloning Kit.

The sequences were compared to the GenBank database and Ribosomal Database Project (RDP) databases using BLAST (www.ncbi.nlm.nih.gov/BLAST). Sequences were checked and aligned manually in the ARB software package (version 2.5B; O. Strunk and W. Ludwig, Technische Universität München, Munich, Germany).

Configuration of the degradation experiments

The experiment had a duration of 18 weeks. Triplicates of cultures and controls were harvested at weeks 0, 1, 2, 4, 8, 10, 12, 14, 16 and 18. The presence of metabolites, the ferrous iron concentration, and the total number of cells were determined in all bottles. At each sampling occasion, 50 ml were removed from the serum bottles in the anaerobic box. Of these, 5 ml were analysed for reduced iron and the total number of cells while the remaining 45 ml were analysed to detect metabolites. The cells were disrupted to make it possible to examine both intra- and extra-cellular metabolites. Introductory experiments had shown that no or very small amounts of metabolites were released into the medium.

Cell disruption, extraction, and derivatisation were done in clean serum bottles. To be able to compare metabolite production in the same bottle but at different occasions, for some of the samples extraction of the remaining content of the bottles was done directly in the original culture bottles two weeks after the first sampling. In this way it was possible to compare metabolite production in the same bottle over time and during different growth phases of the enrichment culture. It also made it possible to detect metabolites attached to the walls of the culture bottle. In these extractions, the piece of Teflon on which the propylbenzene had been added was included.

Metabolite detection

Metabolites were detected using a modified version of the protocol from Fogelqvist et al. (1980). Internal standard, 10 μl hexadecanoic acid (5 g l^{-1} in dichloromethane) was added to each 45-ml

sample. The sample was centrifuged in 50 ml PTFE tubes (Nalge Europe Ltd, Neerijse, Belgium) at 27,000 × g for 15 min (Sorvall RC-5B, Osterode, Germany), resulting in an iron pellet and a clear supernatant. The supernatant was filtered through a 0.22-μm Teflon filter (Millipore Corporation, Bedford, MA, US). The iron pellet was dissolved in 1 ml NaOH (0.1 M). The filter and the dissolved iron phase were transferred to a vial containing 0.1 ml tertiary-butyl ammonium hydrogen sulphate (0.4 M, Sigma-Aldrich, Stockholm, Sweden). The filtrate was extracted with 2 ml pentafluorobenzylbromide (350 μl l^{-1} in dichloromethane, Sigma-Aldrich, Stockholm, Sweden) by means of vigorous shaking for 20 min. One ml of this extracted phase was transferred to the vial with the filter and the iron phase, which was tightly sealed and treated with heat (70 °C) and ultra-sonication (Starsonic 90, Liarre, Bologna, Italy) for 20 min.

Of this solution, 1 μl was injected splitless into a Varian 3400 GC (Varian, Malmö, Sweden) equipped with a Varian Saturn 2000 MS. The injector (Varian 1061) temperature was 275 °C. Separation was done with a DB-5 quartz capillary column (RESTEK, Bellafonte, PA, US), 30 m × 0.25 mm (film thickness of 0.25 μm), programmed from 40 to 300 °C at a heating rate of 5 °C min^{-1} with an initial holding time of 1 min and a final holding time of 5 min. Helium was used as the carrier gas at a constant flow of 1 ml min^{-1}. Electron impact mass spectra were recorded from m/z 50 to 650 at a rate of 1 scan s^{-1} at a manifold temperature of 150 °C. The electron beam energy was 70 eV. Derivatised compounds could be detected by screening for the fragment of the pentafluorobenzyl group (m/z 181). The amounts of metabolites found were estimated using external standard curves for the respective non-deuterated compounds.

Total number of cells

The total number of cells in a sample was determined using the AODC method (Hobbie et al. 1977). Samples were mixed with equal amounts of oxalic acid (0.1 M) to dissolve any iron present and reduce clogging of the filters (Hallbeck & Pedersen 1990). Samples were filtered onto prestained Sudan black filters (0.2-μm pore size, Osmonics, Minnetonka, MN, US) and stained with 0.2 ml acridine orange (10 mg l^{-1}, Sigma-Aldrich, Stockholm, Sweden) for 5 min. The cells were counted in an epifluorescence microscope (Olympus BH-2, Olympus optical AB, Malmö, Sweden) using blue light (390–490 nm).

Ferrous iron

The amount of ferrous iron was determined spectrophotometrically (Beckman DU 7400, Beckman Instruments, Inc., Fullerton, CA, US) using a modified version of the Ferrozine method (Stookey 1970). The sample (0.1 ml) was diluted in 4.5 ml of 1 M HCl and extracted for 10 min. Then 0.4 ml of ferrozine (0.25%) and 3 ml of ammonium acetate (4 M) was added to the extract. The absorbance was measured spectrophotometrically at 562 nm and ferrous iron values were calculated from a standard curve.

Groundwater study

To confirm our results and to see whether bacteria *in situ* were able to perform the same metabolic processes as the enrichment culture, a freshly collected sample from KM01 was used to inoculate media containing sulphate, nitrate, and FeOOH as electron acceptors; the cultures were then incubated for 13 weeks. Sterile controls without inoccula were prepared. In this experiment, metabolites, reduced electron acceptors, and the total number of cells were also measured.

Sulphide was measured using Sulphide reagents 1 and 2, and nitrite using NitriVer3 as directed by the manufacturer (both were obtained from HACH Europe, Düsseldorf, Germany). Separate standard curves were made and the absorbances were measured spectrophotometrically with a Novaspec II spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden). In conducting the ferrous iron analysis, the sample was allowed to react for 15 instead of 10 min in 1 M HCl. The total number of cells was measured using the previously mentioned method. Phylogenetic analysis of the bacterial community from KM01 was performed by sequencing the 16S rDNA gene from the 10 clones, as described above.

Nucleotide sequence accession number

The nucleotide sequence from the enrichment culture clone reported in this paper has been deposited under Gen Bank no. AY686732.

Results

This study used a bacterial enrichment culture from contaminated groundwater in the vicinity of a diesel storage cavern in Sweden to investigate what metabolites iron-reducing bacteria produce during the anaerobic oxidation of propylbenzene.

Phylogenetic characterization of the enrichment culture

A phylogenetic characterisation of the enrichment culture showed that one bacterial species dominated in the culture. Partial sequencing of the 16S rDNA gene of the clones, performed with the forward primer from *E. coli* position 357, showed that all 10 clones examined from the enrichment culture were identical. Therefore, one of the clones was nearly completely sequenced (from *E. coli*, position 27–1492) and the sequence was compared to those of other bacteria in the GenBank database. The enrichment culture clone sequence was 99.5% affiliated with the clone ZZ9C20 previously found in benzene-contaminated groundwater (GenBank accesion no. AY214178, Alfreider & Vogt unpubl.). The sequence was also 98.8% affiliated with clone RB13C6 obtained from sediment in a monochlorobenzene-degrading bioreactor (GenBank accesion no. AF407412, Alfreider et al. 2002). Clone 1016 from the groundwater around an underground oil storage facility in Japan was also relatively similar (97.6%) to the enrichment culture clone (GenBank accesion no. AB030614, Watanabe et al. 2000). The closest identified species was the recently isolated iron-reducing β-proteobacterium, *Rhodoferax ferrireducens* (similarity value 98.3%) (GenBank accesion AF435948, Finneran et al. 2003).

Metabolite detection

GC/MS analysis of fatty acid and alcohol extracts from the cultures grown in the presence of propylbenzene for 18 weeks revealed three oxidized forms of propylbenzene. Metabolites of propylbenzene were never detected in the sterile controls. The three oxidized forms of propylbenzene eluted closely in the chromatogram (RT: 24.5, 25.6, and 26.0 min). The peaks corresponded to the isomers of d_7-propylphenol, i.e. *m*-, *o*-, and *p*-propylphenol, respectively. The compounds mainly fragmented to m/z 323, 289, and 181 in the MS. These dominant fragments in the *p*-propylphenol spectrum are shown in Figure 1A. To support the finding, non-deuterated *p*-propylphenol was derivatised and analysed. As shown in Figure 1B, a peak (RT: 25.9) with the dominant fragments of m/z 316, 287, and 181 was detected. This mass spectrum is comparable to the mass spectra of deuterated propylphenols, considering that the deuterium atoms weigh 1 mass unit more than do the hydrogen atoms (m/z 323 $-$ $7 = 316$, m/z $289 - 2 = 287$). The m/z 181 fragment represented the pentafluorobenzyl group. The isomers revealed no observable pattern, and therefore only the total amount of all three isomers of propylphenol is given. The isomers of propylphenol were regarded as having similar responses in the MS analysis. The metabolised forms of propylbenzene appeared in the cultures after 4 weeks of incubation, and after an initial increase the amount of metabolites plateaued from 6 weeks and thereafter for the duration the experiment (Figure 2A).

On some occasions, the extraction was also performed in the original culture bottles. Thirty times more phenols were found in extractions made in the original culture bottles after 6 weeks compared to extractions made with the supernatant in samples incubated 4 weeks, when the cells were in the exponential growth phase. In the samples incubated more than 6 weeks, on average 3.8 times more phenols (standard error, 0.7) were derivatised than in samples from the supernatants of the same cultures.

Microscopic studies

Cells with identical morphology were discovered in the cultures throughout the experiment. When the samples from the growth cultures were examined under the microscope it was observed that a great number of cells were closely associated with both the Teflon piece on which d_7-propylbenzene had been added and with the iron phase.

Microscopic studies of cells after the extraction procedure showed that 80–90% of the cells were disrupted. Prior to this study, growth cultures had been analysed without disrupting the cells, and no compound that could be classified as a metabolite of d_7-propylbenzene was detected.

Total number of cells

Cell growth in the enrichment culture was compared to formation of metabolites from

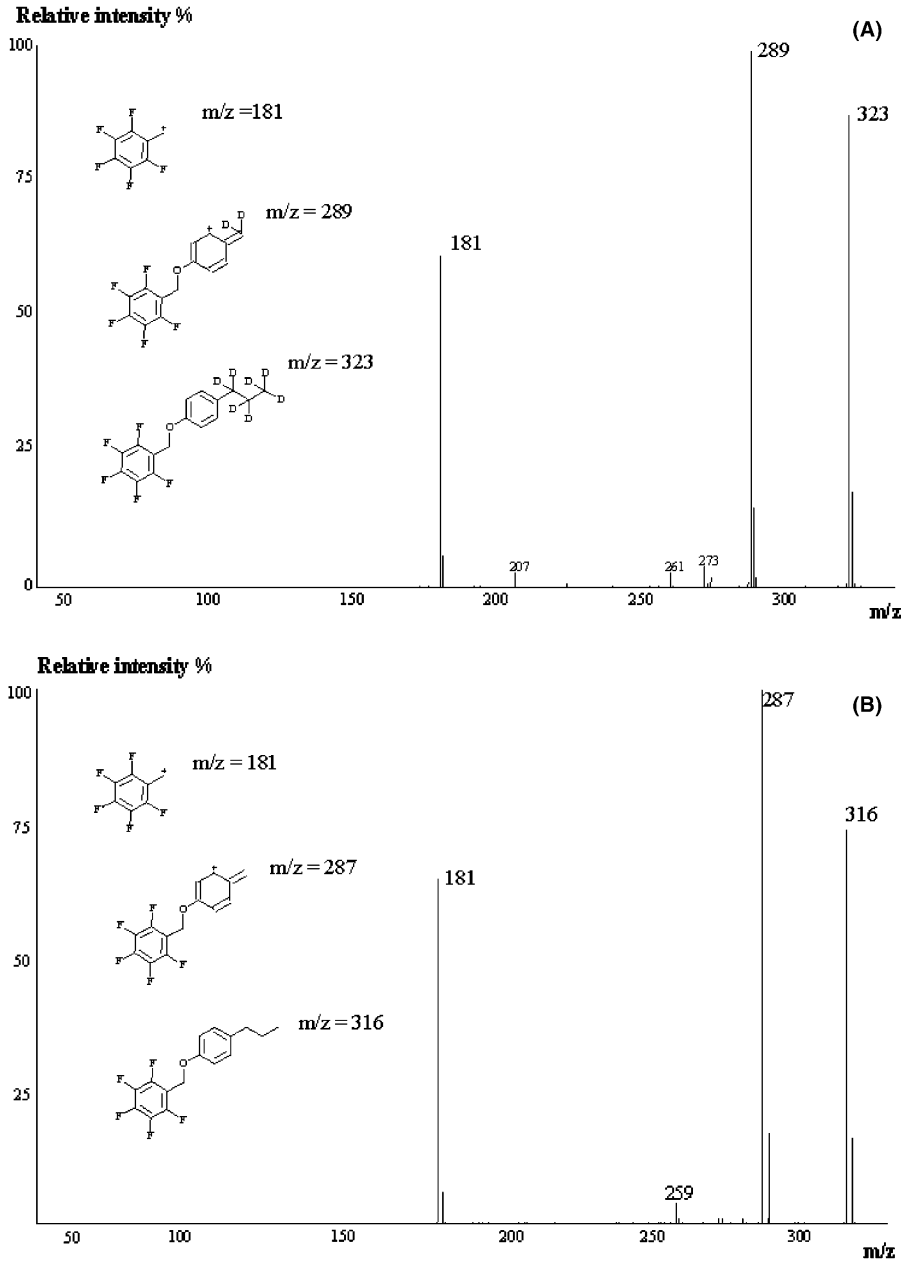

Figure 1. The dominant fragments and their structural formulae in the MS spectra from a metabolite: (A) deuterated *p*-propylphenol (RT: 26.0 min) detected in enrichment cultures amended with d_7-propylbenzene as electron donor and FeOOH as electron acceptor. To verify this metabolite, (B) non-deuterated *p*-propylphenol (RT: 25.9 min) was analysed in the same manner. The structures in the panels help illustrate why some fragments have higher *m*/*z*, as explained in the text.

propylbenzene. As mentioned before, the propylphenols reached a relatively constant level after 6 weeks of incubation. This occurred when the cultures had reached the stationary phase with a cell number of 10^7 ml^{-1}, which corresponds to five generations since inoculation (Figure 2B). As shown in Figure 3, a significant correlation (R: 0.92, $p < 0.001$) between the mean values of the number of cells and the amount of propylphenols was evident.

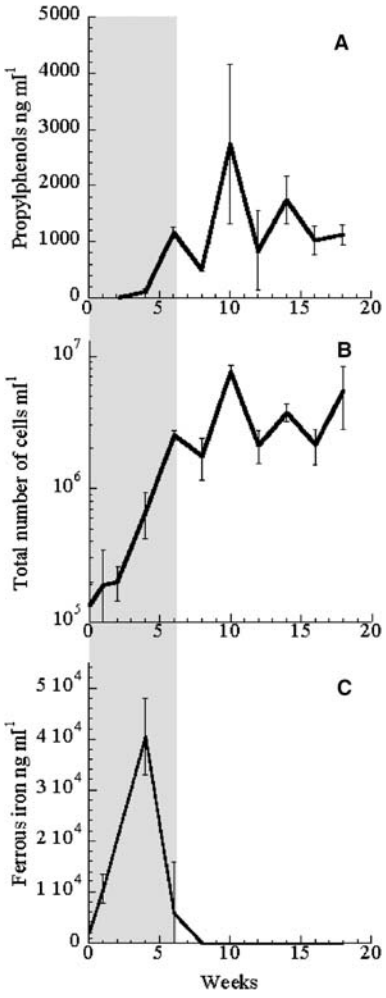

Figure 2. (A) Propylphenols (ng m^{-1}) (B) Total number of cells (ml^{-1}) and (C) Fe^{2+} (ng ml^{-1}) in medium plotted during the 18 weeks of the experiment with enrichment cultures amended with d_7-propylbenzene as electron donor and FeOOH as electron acceptor. The samples were incubated in the dark at 22 °C. The highlighted field represents the exponential growth phase. The amount of Fe^{2+} in sterile controls was subtracted from the amount of Fe^{2+} in cultures to eliminate abiotic factors. The standard error is given ($n = 3$).

Ferrous iron

The amount of ferrous iron in the cultures increased during the exponential phase, reaching 40,000 ng ml^{-1} (Figure 2C). When the cultures reached the stationary phase, the amount of ferrous iron decreased. During the 18 weeks of incubation a significant blackening of the iron phase in the cultures was observed. The controls remained unchanged in this regard.

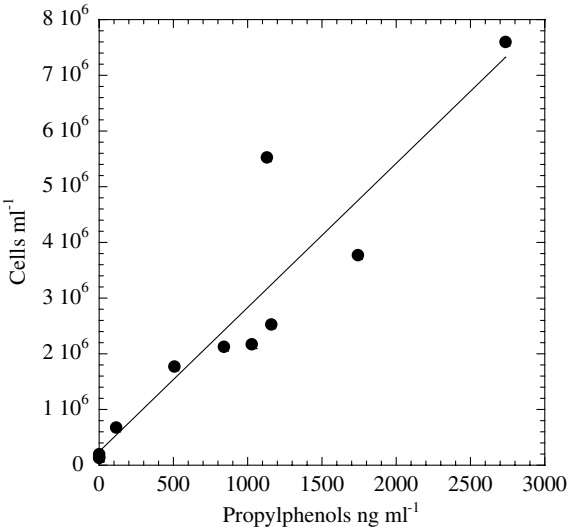

Figure 3. Correlation plot of the mean amounts of propylphenols and cell number in enrichment cultures amended with propylbenzene as electron donor and FeOOH as electron acceptor ($n = 3$). The Pearson's linear correlation coefficient, R, was 0.92 and probability, p, was < 0.001 at a 99% confidence level.

Groundwater study

To confirm that iron reducers were responsible for the oxidation of propylbenzene, a new sampling from KM01 was performed and the contaminated groundwater was used as inoccula in media containing sulphate, nitrate, and FeOOH as electron acceptors. In this experiment propylphenols were detected as metabolites only under iron-reducing conditions and with a concomitant increase of ferrous iron and the total number of cells (Table 1). Microscopic examination of these cultures revealed a more diverse cell morphology than was found in the original experiment using an enriched culture as the inoculum. Cell growth and nitrate reduction occurred in the nitrate-amended cultures but no propylphenols were present after 13 weeks. In the sulphate-amended medium no growth was observed. A chemical characterisation of the KM01 borehole showed depletion of oxygen and sulfate and the presence of ferrous iron and sulphide. Interestingly, of the 10 clones phylogenetically analysed, one clone found in KM01 was identical to the dominant bacterium in the propylphenol-producing enrichment culture.

Table 1. Results of the cultures inoculated with groundwater from KM01 after 13 weeks of incubation[a]

	FeOOH	Nitrate	Sulphate
Propylphenols (ng ml^{-1})	123 ± 52	–	–
Total number of cells (ml^{-1})	$5 \times 10^6 \pm 2 \times 10^6$	$6 \times 10^6 \pm 2 \times 10^6$	–
Reduced electron acceptor (ng ml^{-1})	23,000 ± 16,000[b]	15,000 ± 6000[c]	–[d]

[a] Data are means; the standard error is shown ($n = 3$).
[b] Fe^{2+} was measured.
[c] NO_2^- was measured.
[d] S^{2-} was measured.

Discussion

Metabolites from the degradation of propylbenzene in an iron-reducing enrichment culture were examined and a novel initiation reaction of the degradation process was found. Anaerobic bacteria oxidized d_7-propylbenzene to d_7-propylphenols during growth (Figures 1 and 2). When the anaerobic electron acceptors, sulphate, nitrate, and FeOOH, were evaluated, it became evident that iron reducers exclusively performed the hydroxylation step.

Phylogenetic analysis of the culture

In phylogenetically characterising the iron-reducing enrichment culture only one species was found. This result was also supported by the microscopy analyses, in which a homogenous population of uniform cell morphology was revealed in all cultures.

The bacterium in the cultures was closely affiliated to *R. ferrireducens* with a 98.8% similarity. This is the first time a bacterium of the genus *Rhodoferax* has been associated with the oxidation of monoaromatic hydrocarbons under anaerobic conditions. Furthermore, the bacterium was also closely related to 16S rDNA found in benzene-contaminated groundwater and in a monochlorobenzene-degrading bioreactor, and to clones from groundwater in the vicinity of an underground oil storage facility located in Japan (Alfreider & Vogt, unpublished; Alfreider et al. 2002; Watanabe et al. 2000). Hence, this suggests that propylphenol-producing bacteria exist in more ecosystems than just the one examined in this study.

Metabolite detection

In this study, hydroxylation of the aromatic ring carbon is used as initial oxidation step. The direct hydroxylation of the aromatic ring has previously been reported in the case of the anaerobic degradation of benzene (Caldwell & Suflita 2000; Vogel & Grbic-Galic 1986; Weiner & Lovley 1998). This strengthens the finding that phenols with or without alkyl chains can be considered to be indicators of the ongoing anaerobic microbial alteration of monoaromatic hydrocarbons. The ability anaerobically to oxidize monoaromatic hydrocarbons indeed seems to be widespread amongst bacteria. The most frequently studied and characterised toluene degraders, *Azoarcus strain T*, *Thauera aromatica*, and *Geobacter metallireducens*, are bacteria that are phylogenetically distantly related to *Rhodoferax* and to the clones closely related to the bacterium enriched in this study. These toluene degraders have been reported to possess the enzyme benzylsuccinate synthase, which catalyses the addition of fumarate to toluene (Beller & Spormann 1998; Kane et al. 2002). This addition reaction, yielding benzylsuccinate as the first intermediate in monoaromatic hydrocarbon degradation, has been reported for several alkylated benzenes including propylbenzene (Biegert et al. 1996; Elshahed et al. 2001; Martus & Püttman 2003). *T. aromatica* and *Azoarcus* sp. are also known to degrade ethylbenzene and propylbenzene via another degradation pathway that involves hydroxylation of the alkyl chain, resulting in 1-phenyletanol and 1-phenylpropanol, respectively (Ball et al. 1996; Rabus & Widdel 1995). None of these metabolites could be detected in this study.

The experimental step which used ultra-sonication to disrupt most of the cells made it possible to detect propylphenols as degradation products. These metabolites would not have been detected if only extra-cellular degradation products had been examined. The accuracy of the estimation of degradation capacities in contaminated areas would

increase if intra-cellular degradation products were included in the analysis.

Another important finding of this study was that the measured amount of metabolites was 4 times higher when extraction was conducted in the original culture bottles. Apparently, the cells and thus the metabolites adhered to solid surfaces in the culture bottles and also to the piece of Teflon. Microscopic examination of the Teflon pieces showed that they were covered with cells, and this supports the conclusion. This result once again showed the importance of surfaces in applied and environmental microbiology. To make accurate estimates of the extent of microbial degradation in a contaminated area, the estimated area of surfaces has to be taken into consideration.

Growth and ferrous iron production

The amount of propylphenols in a single culture bottle increased 30-fold in the first weeks of the experiment; this was during the exponential growth phase, indicating that propylphenols were produced during growth. Further support for this is presented in Figure 3, which shows how the amount of propylphenol strongly correlates with the cell number in the cultures.

Ferrous iron could be detected in the cultures during the exponential growth phase. By the end of exponential growth, 4×10^{-14} moles of ferric iron were reduced and 3×10^{-15} moles of propylphenols produced for every cell formed. In the stationary growth phase, no reduced iron could be detected, probably because non-extractable forms of iron minerals were formed. This happens during the extensive growth of iron-reducing bacteria, as reported by Fredrickson et al. (1998). When extraction time in the iron analysis was increased from 10 to 15 min, measurable amounts of ferrous iron could still be extracted for as long as 13 weeks, supporting this explanation. The iron phase blackened during incubation, as has previously been documented in the case of *R. ferrireducens*, suggesting that this is a special feature of this organism (Finneran et al. 2003).

Groundwater study

Phylogenetic analysis of the bacteria in the contaminated groundwater in KM01 found one clone identical to the bacterium in our cultures. Reproducible propylphenol production by bacteria directly from contaminated groundwater could be verified. Propylphenols were found only in cultures amended with iron as an electron acceptor. Even though there was substantial microbial growth in the cultures with nitrate as electron acceptor, no metabolites of propylbenzene were produced. In cultures to which sulphate was added as electron acceptor, no growth could be detected. This further proves that this iron-reducing bacterium inhabits contaminated groundwater and suggests that it might oxidise the aromatic ring *in situ*.

Exclusion of the enrichment step resulted in lower propylphenol production than in the enrichment experiment, because more than one bacterial species grew in the medium. The cell morphology in these cultures was also more diverse than in the enrichment experiments.

Conclusions

This study marks the first time propylphenols have been found as metabolites in the anaerobic degradation of propylbenzene. The propylbenzene oxidation was performed concomitant with cell growth and ferrous iron production under strictly iron-reducing conditions. Sequencing of the 16S rDNA showed that this was done by a bacterium closely related to *R. ferrireducens* (98.8% similarity).

It was also shown that the initial metabolites were retained inside the cells, and that without disruption of cells this metabolite would not have been detected. This implies that both intra- and extracellular metabolites must be taken into consideration when estimating the extent of biodegradation.

Finally, it was shown that the amount of metabolites was 4 times higher when surface bound bacteria were included in the metabolite extraction. Thus, degradation studies in the water phase only will underestimate the actual biodegradation capacity. Bacteria attached to surfaces such as rock fractures and particles will be responsible for most of the degradation in the environment.

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