Anaerobic Degradation of Benzene, Toluene, Ethylbenzene, and o-Xylene in Sediment-Free Iron-Reducing Enrichment Cultures

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Monoaromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylene (BTEX) are widespread contaminants in groundwater. We examined the anaerobic degradation of BTEX compounds with amorphous ferric oxide as electron acceptor. Successful enrichment cultures were obtained for all BTEX substrates both in the presence and absence of AQDS (9,10-anthraquinone-2,6-disulfonic acid). The electron balances showed a complete anaerobic oxidation of the aromatic compounds to CO2. This is the first report on the anaerobic degradation of o-xylene and ethylbenzene in sediment-free iron-reducing enrichment cultures.

The degradation of aromatic hydrocarbons in contaminated aquifers is of general interest because some of the compounds are toxic or even carcinogenic. Due to anoxic conditions typically found in organic contaminant plumes, anaerobic degradation of aromatic compounds plays a major role for bioremediation processes, and increased knowledge on the anaerobic degradation of aromatic compounds has been acquired recently (8, 10, 29, 34, 36). Besides the reduction of soluble electron acceptors such as oxygen, nitrate, and sulfate, iron(III) reduction is thought to play a major role for the reduction of aromatic hydrocarbons at contaminated sites (13, 16). Biodegradation of benzene, toluene, ethylbenzene, and xylene (BTEX) compounds in the field under iron-reducing conditions in situ could be shown (11, 12, 25). However, since the first description of a pure culture of a dissimilatory iron-reducing microorganism growing with toluene as the sole carbon and energy source (19) little progress was made on the isolation of other iron-reducing bacteria that are able to degrade aromatic hydrocarbons (4). Iron-dependent degradation of some BTEX compounds could only be shown in contaminated sediments (1, 14, 26) in laboratory batch experiments containing aquifer matrix (21). A sediment-free benzene-oxidizing enrichment culture was reported which showed the evolution of CO2 from radiolabeled benzene as substrate (22, 26). No enrichments have been reported so far which are able to grow with ethylbenzene or xylene as the sole carbon and electron source and ferric iron as the electron acceptor.

We report here on the enrichment of different iron-reducing cultures, which are able to oxidize all BTEX compounds, respectively. The enrichment cultures were grown in a carbonate-buffered mineral medium (33) with a pH between 7.2 and 7.4. Sulfate (10 μM) was added as sulfur source, and FeCl3 (1 mM) was added as a reductant in order to eliminate traces of oxygen in the medium. The medium was anoxically transferred to 100-ml serum bottles in portions of 50 ml, the bottles were purged with an 80/20 (vol/vol) mixture of N2-CO2 gas and sealed with butyl rubber stoppers. Amorphous Fe(III) hydroxide was prepared as described in reference 20 and was added as the bulk electron acceptor to each bottle at a final concentration of 50 mM. Each bottle contained the solid adsorber resin Amberlite-XAD7 (0.3 g) (Fluka, Buchs, Switzerland) as a substrate reservoir, to keep hydrocarbon concentrations at a moderately low level during bacterial growth (24). The XAD7 was added to the bottles and autoclaved prior to the addition of the medium. One of the different BTEX compounds, i.e., benzene, toluene, ethylbenzene, or o-xylene, was injected through the stopper with a gastight syringe as the sole carbon and energy source at a nominal initial total concentration of about 1 mM, calculated for the volume of the aqueous phase without consideration of the adsorption to the XAD7 resin. The bottles were equilibrated for 1 week before incubation. All enrichments were inoculated with 5 ml of sludge, which had been taken from the bottom of a highly polluted groundwater monitoring well at a tar-oil-contaminated former gasworks site in Stuttgart, Germany. Some of the enrichment cultures contained AQDS (9,10-anthraquinone-2,6-disulfonic acid; 2 mM) as an additional agent to enhance microbial iron reduction (5, 18, 27, 35). All enrichments were cultivated independently in parallel, either with or without AQDS, at 30°C in the dark. Active enrichments were repeatedly transferred into fresh medium, and ferrous iron production was monitored. After four subsequent transfers the enrichments were sediment free and were taken for electron balance experiments (Fig. 1A to D). To this end, the enrichments (5 ml) were transferred to fresh medium which contained about 150 μM of one of the BTEX compounds and no XAD7 adsorber resin. The bottles were sealed with Viton rubber stoppers (Maag Technik, Dübendorf, Switzerland) and iron(II) production and BTEX degradation were measured over time. Each electron balance was determined in three parallel growth experiments. Sterile control
cultures were set up for each BTEX substrate without addition of enrichment cultures and without AQDS. The development of Fe(II) in the culture medium was measured with ferrozine (30). The BTEX concentrations in the bottles were measured with high-performance liquid chromatography (HPLC) and UV/visual detection at 210 nm. A Hyperchrome HPLC column (NC-03-200; 200 by 3.0 mm) with a filling of PRONTOSIL 120-3-C18-H (3.0 μm; Bischoff Chromatography, Leonberg, Germany) was used as the stationary phase, and a mixture of 70/30 (vol/vol) acetonitrile/water was used as the mobile phase at a constant flow rate of 0.6 ml/min. The concentrations of the aromatic hydrocarbons were calculated using external calibration with the pure substances.

In order to correct the BTEX concentrations measured in the aqueous phase for gas exchange with the headspace of the culturing bottles, the following values for dimensionless Henry's law constants at 30°C were used (calculated from data given in reference 28): 0.268 for benzene, 0.307 for toluene, 0.414 for ethylbenzene, and 0.253 for o-xylene.

Of special interest were the experiments with benzene-degrading enrichment cultures because benzene is known to be the most stable BTEX compound with respect to anaerobic degradation. The degradation of benzene to CO₂ by two different enrichment cultures could, however, be shown (Fig. 1A). Enrichment culture benz AQDS was cultivated in the presence of AQDS through all enrichment steps. It showed a lag phase of only 16 days and a complete degradation of benzene within 77 days of incubation. Culture benz K11, which was enriched without addition of AQDS, showed a lag phase of 61 days before degradation of benzene started. After 115 days the degradation slowed down, and it stopped after 162 days before complete exhaustion of benzene. The calculated electron recoveries are listed in Table 1. In contrast to former studies where a mineralization of benzene to CO₂ of 25% by a highly

FIG. 1. Iron(III) reduction by enrichment cultures growing with four different aromatic hydrocarbons as sole carbon and energy source and amorphous iron(III) hydroxide as the electron acceptor. The presented data are the means of triplicate experiments. Hydrocarbon concentrations (solid symbols) and ferrous iron concentrations (open symbols) are shown. As substrates (A) benzene, (B) toluene, (C) ethylbenzene, and (D) o-xylene were used. The control experiments were not inoculated (diamonds). Some enrichment cultures contained AQDS (triangles): (A) benz AQDS, (B) tol AQDS, (C) ebenz AQDS, and (D) ox AQDS. Parallel enrichments were performed without AQDS (circles): (A) benz K11, (B) tol K21, (C) ebenz K21, and (D) ox K11. The error bars represent the standard deviations of the three parallel experiments.
purified benzene-oxidizing enrichment culture was shown (17), the benzene-degrading enrichment cultures presented here showed recoveries of electrons from benzene oxidized in ferric iron reduced of 82% and 74%, respectively.

The degradation of toluene started immediately in both cultures without any significant lag phase as is indicated by the increase of the iron(II) concentration and the corresponding decrease in toluene concentration (Fig. 1B). In the culture tol AQDS, which was enriched in the presence of AQDS, the degradation appeared to be slightly faster than in culture tol K21 with amorphous ferric iron only. In both cultures, the toluene concentration was depleted already after 39 days of incubation and no further change in the Fe(II) concentration could be measured. The sterile control experiment showed no decrease in toluene concentration and no increase in Fe(II) over the whole period of the experiment.

Ethylbenzene was degraded by two different iron-reducing enrichment cultures (Fig. 1C). After relatively long lag phases of 61 days in case of the enrichment in the absence of AQDS (culture ebenz K21) and of 93 days with AQDS (culture ebenz AQDS), the substrate was used by both bacterial enrichments. After the lag phase, ethylbenzene was completely degraded to CO2 within 69 days (ebenz AQDS). Enrichment culture ebenz K21 did not show a complete depletion of the substrate within 162 days. In contrast to culture ebenz AQDS the degradation of ethylbenzene and also the increase in Fe(II) concentration were very slow.

The degradation of o-xylene by two different enrichment cultures started immediately after incubation of the bottles (Fig. 1D). With xylene, the reaction was much faster for the enrichment with AQDS than for the enrichment without AQDS. Already after 39 days, o-xylene was depleted with AQDS as electron shuttle (ox AQDS) whereas in culture ox K11, which was not supplemented with AQDS, the degradation was much slower and did not lead to a complete depletion even after 162 days of incubation. In the control culture, neither a decrease of o-xylene nor an increase of the Fe(II) concentration was observed.

Electron balances for the different experimental set ups were calculated for a complete oxidation of the aromatic hydrocarbons to CO2 (Table 1). In all eight cases, the electron balances were very similar and consistent with the general rule that about 70% to 90% of a hydrocarbon substrate is usually used to produce energy. Up to 30% of the hydrocarbon added to the cultures was probably used to produce biomass, as other studies of anaerobic degradation of hydrocarbons have shown (7, 15, 19). A significant accumulation of intermediates, i.e., an incomplete oxidation of the substrate, can be ruled out, because this would result in less ferrous iron than was measured in the cultures. For example, if acetate was produced instead of CO2 only 20 to 25% of the actual Fe(II) production would have been expected for the amount of substrate oxidized. In contrast to most earlier studies on the degradation of aromatic hydrocarbons by iron-reducing microbes (1, 14, 32, 36), we used sediment-free enrichment cultures and not microcosm studies. For that reason, we were able to exclude the influence of substances from the sediment samples, e.g., dissolved organic carbon. In the cultures supplied with AQDS the concentration of ferrous iron did not increase any further after the complete depletion of the aromatic hydrocarbons. This indicates that the AQDS was not used as carbon or energy source by the bacterial enrichment cultures.

This first report on the complete degradation of ethylbenzene and o-xylene under iron-reducing conditions supports the hypothesis that dissimilatory iron reduction may play an important role in the degradation of aromatic hydrocarbons in situ. The fact that we were able to enrich dissimilatory iron reducers degrading BTEX compounds other than toluene shows that the enrichment strategy presented here was very successful, probably due to the combination of two effects.

First, the concentration of the BTEX compounds was kept at a moderate level of about 60 μM due to the addition of the adsorber resin XAD7 (24), which reduces the cytotoxicity of the harmful BTEX substances. With time, a substrate pool adsorbed to the resin, equivalent to about 1 mM of total substrate if all the hydrocarbon would have been dissolved in the aqueous phase, was steadily released to the organisms leading to high cell densities in the bottles. This setup resembles more closely in situ conditions occurring at contaminated sites including those present at the specific site where the sludge was taken from, where usually low substrate concentrations are predominant (3, 9). In other studies, cultivation with low substrate concentrations resulted in the isolation of different bacterial strains compared to the cultivation in nutrient-rich media, probably because many microorganisms are adapted to nutrient-poor environments (2, 6).

Second, the addition of the electron-shuttling substance AQDS, which has already been described several times by different authors to accelerate iron reduction and substrate oxidation by iron-reducing bacteria (5, 18, 27, 35), proved useful for the enrichment of new iron-reducing microorganisms. Iron reduction and substrate utilization were also much faster in our set of experiments compared to the cultures without addition of AQDS. Recently, iron reduction by reduced sulfur species resulting from an internal sulfur cycle was reported (31). Although we have no indication that such processes occur in our assays we cannot rule out such mechanism, as we added 10 μM SO4^2- as sulfur source to our experiments.

In summary, our results demonstrate that BTEX-oxidizing iron-reducing microorganisms are naturally occurring at contaminated sites and can be enriched with the appropriate

<table>
<thead>
<tr>
<th>Enrichment culture</th>
<th>Electron equivalents calculated from measured concn of aromatic hydrocarbons (μM)</th>
<th>Electron equivalents recovered in ferrous iron measured (μM)</th>
<th>Calculated electron recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>benz K11</td>
<td>3,339</td>
<td>2,732</td>
<td>82 ± 20</td>
</tr>
<tr>
<td>benz AQDS</td>
<td>5,081</td>
<td>3,771</td>
<td>74 ± 9</td>
</tr>
<tr>
<td>tol K21</td>
<td>5,564</td>
<td>4,774</td>
<td>86 ± 12</td>
</tr>
<tr>
<td>tol AQDS</td>
<td>5,184</td>
<td>5,282</td>
<td>102 ± 4</td>
</tr>
<tr>
<td>ebenz K21</td>
<td>4,216</td>
<td>2,670</td>
<td>79 ± 3</td>
</tr>
<tr>
<td>ebenz AQDS</td>
<td>5,191</td>
<td>4,183</td>
<td>80 ± 8</td>
</tr>
<tr>
<td>ox K11</td>
<td>2,465</td>
<td>2,032</td>
<td>83 ± 22</td>
</tr>
<tr>
<td>ox AQDS</td>
<td>4,616</td>
<td>3,710</td>
<td>79 ± 8</td>
</tr>
</tbody>
</table>

* Shown are the percentages of electron equivalents recovered in ferrous iron measured from the averages of the triplicate cultures for the different electron balance experiments.
methods. However, it remains unclear what their ecological significance is compared to other respiratory classes of organisms because the presented data are derived from batch experiments and the results cannot be transferred to natural systems assuming similar microbial growth. We used single aromatic compounds as the sole carbon and electron source, which does not reflect the in situ situation at most sites contaminated with petroleum-derived hydrocarbons. At present, there are only limited data available about microbial degradation of mixtures of aromatic hydrocarbons that represent conditions that are closer to in situ conditions in contaminant aquifers (11, 23), and future research is needed to address the effects of multiple substrates on enrichment techniques and contaminant degradation.

REFERENCES


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