Anaerobic biodegradation of BTEX using Mn(IV) and Fe(III) as alternative electron acceptors

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Abstract Anaerobic BTEX biodegradation was tested in batch experiments using an anaerobic sediment as inoculum under Fe(III) and Mn(IV) reducing conditions. All BTEX were degraded under the conditions tested, specially under Mn(IV) reducing conditions, where benzene was degraded at a rate of 0.8 µmol l⁻¹d⁻¹, significantly much faster than Fe(III) reducing conditions. Under Fe(III) reducing conditions, ethylbenzene was the compound that degraded at the faster rate of 0.19 µmol l⁻¹d⁻¹. Mn(IV) reducing conditions are energetically more favourable than Fe(III), therefore, BTEX were more rapidly degraded under Mn(IV) reducing conditions. These results represent the first report of the degradation of benzene with Mn(IV) as the final electron acceptor. Amorphous manganese oxide is a natural widely distributed metal in groundwater, where it can be microbiologically reduced, leading to the degradation of monoaromatic compounds.

Keywords Anaerobic conditions; BTEX degradation; Fe(III) reduction; Mn(IV) reduction

Introduction Petroleum fuels are crude distillates that comprise a complex mixture composed predominantly of paraffins, cycloparaffins and aromatic compounds, together with additives such as MTBE (Bowlen and Kosson, 1995; Puig-Grajales et al., 2000). The BTEX (benzene, toluene, ethylbenzene and xylene isomers) compounds are primary constituents of petroleum-distillate fuels, and are chemicals of environmental concern as they are relatively soluble, toxic, carcinogenic, and can be very mobile in the environment (Dean, 1985). Depending on type and concentration of electron acceptors present in the environment (e.g. nitrate, sulphate, carbon dioxide), pH conditions and oxidation/reduction potential, anaerobic biodegradation can occur under denitrifying, Fe(III) reducing, sulphate reducing, or methanogenic conditions. Another less common anaerobic degradation mechanism such as manganese reduction might predominate, if the physical and chemical conditions in subsurface favour the use of this electron acceptor by microorganisms (Madsen, 2002). Nonetheless, environmental conditions and microbial competition ultimately will determine which processes will predominate (Vargas et al., 1998).

Metals are abundant in terrestrial, estuarine, marine environments (e.g. Fe and Mn up to 51 and 0.9 g/kg, respectively) and groundwater, where the oxidized forms of iron, Fe(III), and manganese, Mn(IV), accumulate mainly in the form of a variety of hardly soluble oxides and hydroxides (Ehrlich, 1996). The complete oxidation of organic compounds through the dissimilatory reduction of Mn or Fe oxides constitutes the most recently discovered and least explored of the major types of anaerobic respiration in nature (Thamdrup, 2000).

Previous studies have indicated that anaerobic BTEX degradation might be stimulated in aquifer sediments by making alternative electron acceptors available for microbial reduction (Burland and Edwards, 1999; Elshahed et al., 2001). For example, it was demonstrated...
in aquifers containing significant amounts of Fe(III) that anaerobic benzene degradation occurred simultaneously with the reduction of Fe(III) to Fe(II) (Lovley et al., 1996). The aim of this work was to demonstrate the anaerobic biodegradation of BTEX, in batch experiments, using high energy electron acceptors like amorphous Mn(IV) and Fe(III), and anaerobic sediment with a long hydrocarbon contamination history.

Methods

Preparation of Mn(IV) and Fe(III) amorphous crystals
Amorphous Mn(IV) (Vernadite, MnO₂) oxide was prepared by mixing equal amounts of 0.4 mol l⁻¹ KMnO₄ and 0.4 mol l⁻¹ MnCl₂ and adjusting the pH to 10 by adding NaOH (Langenhoff et al., 1996; Lovley and Phillips, 1988). Amorphous Fe(III) (goethite, FeOOH) oxide was prepared by neutralizing a solution of 0.4 mol l⁻¹ FeCl₃ with NaOH (Langenhoff et al., 1996; Lovley and Lonergan, 1990). The metal oxide suspensions were washed three times by centrifugation and resuspended in distilled water. Finally, the metal oxides were suspended in basal medium.

Inoculum source
Anaerobic Rhine River sediment and associated water was collected at 1.2 m depth alongside the banks of the river near Wageningen, The Netherlands. Sediment was collected with a hand auger and placed into 1-litre plastic jar. The jar was filled as completely as possible with sediment without any remaining space in the jar. This sediment was chosen as inoculum because it has been previously reported to be able to degrade aromatic compounds (Cervantes et al., 2001; Puig et al., 2000).

Microcosms biodegradability assays
Phosphate (20 mmol l⁻¹) buffered basal mineral medium (pH 7.0) was prepared as previously described (Puig-Grajales et al., 2000). Biodegradability assays were conducted in 124 ml serum bottles. Mineral medium was cooled in a stream of N₂. All media were dispensed into the serum bottles, after being flushed with He, at a final volume of 50 ml (74 ml as headspace), and then inoculation took place by adding 10 g/l (dry weight) of previously homogenized sediment. The serum bottles were sealed with Viton stoppers and aluminium crimps and were flushed with helium gas and incubated overnight at 30°C to allow the biological consumption of residual oxygen. Mn(IV) and Fe(III) (20 mmol l⁻¹) were separately added as the final electron acceptor according to Lovley et al. (1996) and Puig-Grajales et al. (2000).

Benzene (64 µmol l⁻¹), toluene (54 µmol l⁻¹), ethylbenzene (47 µmol l⁻¹), o-, m- and p-xylene (47 µmol l⁻¹) were added as the only carbon and energy sources, at concentrations equivalent to 5 mg/l. All the bioassay serum bottles were incubated in a dark room at 30°C and 100 rpm, and were manually shaken before sampling to ensure the homogeneous distribution of the compounds. Sterile and abiotic controls were prepared in the same manner as the experimental bottles and were used as a control for abiotic removal mechanisms. Sterile controls were performed in the presence of sediment that was autoclaved twice for 40 min at 120°C, in different days prior to the addition of BTEX. Concentration of BTEX and reduction of the corresponding electron acceptor were monitored over time as described below.

Analytical procedures
The decrease in BTEX concentrations was measured routinely in the headspace by gas chromatography (GC) [Agilent, series 6850]. Headspace samples of 100 µl were injected onto a column (HP-1, capillary 30 m x 320 µmol l⁻¹ x 0.25 µmol l⁻¹) and analyzed with a
flame ionization detector. The column temperature was 150°C, and helium (3.0 mL per min) was used as a carrier gas. The temperatures of the injection port, oven, and detector were 240, 150 and 250°C respectively. Standards were prepared with known concentrations of BTEX and the same amount of basal medium (50 mL) and therefore reflecting the equilibrium in BTEX concentration between the headspace and mineral medium. BTEX were added 2 h before analysis and were kept at 30°C until GC analysis.

The Mn(II) and Fe(II) production were estimated routinely by measuring their accumulation using a colorimetric test kit from Hach (limit of detection 0–10 mg/L). Samples were collected in an anaerobic chamber with N₂-H₂ (90%:10%) atmosphere to avoid Mn(II) or Fe(II) oxidation. Previous to analysis all samples were filtered with a Nalgene syringe filter (25 µm) with a surfactant free cellulose acetate membrane (0.45 µ) in order to eliminate the sediment and insoluble Fe or Mn.

**Chemicals**
The aromatic compounds used in this study included benzene, toluene, ethylbenzene, and o-, m-, and p-xylene, all 99% purity from Aldrich Chemical (Milwaukee).

**Results and discussion**

**Biodegradation of BTEX with Fe(III) as final electron acceptor**
The anaerobic biodegradation of individual BTEX compounds was tested using Fe(III) [20 mmol l⁻¹] as final electron acceptor. The results obtained are summarized in Table 1 and in Figure 1A for benzene. In all the abiotic controls the BTEX concentrations did not decrease significantly. Actually, less than 2% of endogenous reduction occurred in all instances and all ratios were corrected from the endogenous and autoclaved controls.

All BTEX compounds were biodegraded under Fe(III) reducing conditions with anaerobic Rhine river sediment as inoculum (Table 1). The ethylbenzene was the most rapidly biodegraded compound at a rate of 0.19 µmol l⁻¹d⁻¹ after 240 days of incubation, being 3.1 times faster than benzene degradation rate, 0.08 µmol l⁻¹d⁻¹, after 710 days incubation period. According to these results, benzene was the most difficult compound to be biodegraded followed by p-xylene, which was degraded at a rate of 0.11 µmol l⁻¹d⁻¹ after 445 days of incubation period. Toluene, o- and m-xylene were degraded at similar rates. The degradation of BTEX was linked to Fe(III) reduction as soluble Fe(II) was produced and accumulated in the medium as shown in Table 1 and Figure 1A. The soluble Fe(II) production was nearly stoichiometric in all cases.

**Biodegradation of BTEX with Mn(IV) as final electron acceptor**
All BTEX were biodegraded in presence of Mn(IV) as a final electron acceptor using Rhine river sediment as inoculum. In all the abiotic controls the BTEX concentrations remained without any significant change and all rates were also corrected for the endogenous control.

**Table 1** Biodegradation of BTEX under Fe(III) reducing conditions using Rhine-river sediment as inoculum (∆, standard deviation, n = 6)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µmol l⁻¹)</th>
<th>Incubation (d)</th>
<th>Lag phase (d)</th>
<th>Degradation rate (µmol l⁻¹/d)</th>
<th>Measured iron reduced/substrate ratio (mol/mol)</th>
<th>Predicted iron reduced/substrate ratio (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>64.0</td>
<td>710</td>
<td>65</td>
<td>0.08</td>
<td>29.52 ± 3.54</td>
<td>30</td>
</tr>
<tr>
<td>Toluene</td>
<td>54.3</td>
<td>320</td>
<td>44</td>
<td>0.17</td>
<td>43.61 ± 5.23</td>
<td>36</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>47.1</td>
<td>240</td>
<td>18</td>
<td>0.19</td>
<td>58.73 ± 8.54</td>
<td>42</td>
</tr>
<tr>
<td>o-xylene</td>
<td>47.1</td>
<td>275</td>
<td>44</td>
<td>0.16</td>
<td>61.41 ± 7.60</td>
<td>42</td>
</tr>
<tr>
<td>m-xylene</td>
<td>47.1</td>
<td>280</td>
<td>44</td>
<td>0.17</td>
<td>58.60 ± 8.03</td>
<td>42</td>
</tr>
<tr>
<td>p-xylene</td>
<td>47.1</td>
<td>445</td>
<td>44</td>
<td>0.11</td>
<td>61.45 ± 7.90</td>
<td>42</td>
</tr>
</tbody>
</table>
Benzene was the compound degraded at the fastest rate, 0.83 µmol l⁻¹d⁻¹ after 77 days incubation period without lag phase (Figure 1B), whereas o-xylene was degraded at the lowest rate, 0.17 µmol l⁻¹d⁻¹ after 260 days incubation period. Toluene degradation rate, 0.60 µmol l⁻¹d⁻¹ was almost three times faster when comparing to the rest of the compounds at the same conditions. As in the case with Fe(III), degradation of BTEX was linked to Mn(IV) reduction as soluble Mn(II) was produced and accumulated in the serum bottles as shown in Table 1 and Figure 1B. The soluble Mn(II) production was nearly stoichiometric in all cases.

In general, the degradation rates of the BTEX were higher in presence of Mn(IV) when used as final electron acceptor. The most significant cases were for benzene and toluene degraded at 10 and 3.5 times faster under Mn(IV) reducing conditions, respectively. In the same way, a similar fashion was observed for the lag phases which were longer when Fe(III) was used as final electron acceptor. These results indicate some differences in the populations of BTEX-degrading microorganisms present in the sediment. When Mn(IV) is present in sediments, Fe(III) is generally excluded, due to preferential utilization of Mn(IV) by the microorganisms (Lovley, 1997). In fact Mn(IV) is considered thermodynamically more favourable, influencing the degradation of compounds which are considered as recalcitrant under other conditions (Langenhoff et al., 1996).

The lack of any functional groups in the aromatic ring was a characteristic associated with the recalcitrance of aromatic compounds in anaerobic environments (Schink, 1988). However, with the exception of benzene, one or more BTEX compound biodegradation has been demonstrated under the following conditions: methanogenesis, sulfate, nitrate, Fe(III) and Mn(IV) reduction (Anderson et al., 1998; Edwards and Grbic-Galic, 1994; Kazumi et al., 1997; Langenhoff et al., 1996; Rabus and Widdel, 1995). Even when a number of recent studies have demonstrated that anaerobic benzene biodegradation can occur (Anderson et al., 1998; Burland and Edwards, 1999; Caldwell and Sufiita, 2000; Coates et al., 1996; Kazumi et al., 1997; Lovley et al., 1995, 1996), benzene has often been observed to be resistant to microbial degradation under anoxic conditions (Alvarez and Vogel, 1995; Langenhoff et al., 1996), indicating that benzene biodegradation under anaerobic conditions is not ubiquitous in the environment.

The first report about in situ anaerobic oxidation of benzene was in sediments from the Fe(III)-reducing zone of petroleum-contaminated aquifer located in Bemidji, Minnesota (Anderson et al., 1998). The capacity for benzene degradation was associated with increased numbers of microorganisms of the genera Geobacter, which are know to oxidize organic compounds to carbon dioxide with reduction of Fe(III) (Anderson et al., 1998; Rooney-Varga et al., 1999). Caldwell and Sufiita (2000) proposed that phenol and benzoate are intermediates of the anaerobic benzene biodegradation under methanogenic, sulfate- and Fe(III)-reducing conditions.

Table 2 Biodegradation of BTEX under Mn(IV) reducing conditions using Rhine-river sediment as inoculum (±, standard deviation, n = 6)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µmol l⁻¹)</th>
<th>Incubation (d)</th>
<th>Lag phase (d)</th>
<th>Degradation rate (µmol l⁻¹/d)</th>
<th>Measured manganese reduced/substrate ratio (mol/mol)</th>
<th>Predicted manganese reduced/substrate ratio (mol/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>64.0</td>
<td>77</td>
<td>0</td>
<td>0.83</td>
<td>14.83 ± 1.3</td>
<td>15</td>
</tr>
<tr>
<td>Toluene</td>
<td>54.3</td>
<td>80</td>
<td>5</td>
<td>0.60</td>
<td>21.22 ± 1.9</td>
<td>18</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>47.0</td>
<td>186</td>
<td>24</td>
<td>0.20</td>
<td>28.50 ± 2.7</td>
<td>21</td>
</tr>
<tr>
<td>o-xylene</td>
<td>47.0</td>
<td>260</td>
<td>23</td>
<td>0.17</td>
<td>28.81 ± 3.2</td>
<td>21</td>
</tr>
<tr>
<td>m-xylene</td>
<td>47.0</td>
<td>186</td>
<td>27</td>
<td>0.26</td>
<td>28.81 ± 2.7</td>
<td>21</td>
</tr>
<tr>
<td>p-xylene</td>
<td>47.0</td>
<td>186</td>
<td>47</td>
<td>0.18</td>
<td>28.81 ± 3.0</td>
<td>21</td>
</tr>
</tbody>
</table>
Toluene is an easily biodegraded compound that is degraded at high rates under methanogenesis, nitrate-, sulfate-, Fe(III)- and Mn(IV)-reducing conditions (Evans et al., 1991; Langenhoff et al., 1996; Lovley and Lonergan, 1990; Rabus et al., 1993). More recently, Cervantes et al. (2001) reported toluene biodegradation with quinones and humus as terminal electron acceptors, representing a novel form of microbial respiration. Toluene biodegradation proceeds via reaction with a fumarate molecule to form a benzylsuccinate derivative (Elshahed et al., 2001). Ethylbenzene and xylene isomers biodegradation have been reported previously under methanogenesis, nitrate- and sulphate-reducing conditions (Champion et al., 1999; Elshahed et al., 2001; Harms et al., 1999; Seyfried et al., 1994). Similar to toluene, the activation of these aromatic compounds involves the conversion to their corresponding benzylsuccinic derivatives.

To date, with the exception of toluene, BTEX biodegradation under Mn(IV)-reducing conditions has not been investigated. In our experiments all BTEX were degraded under Fe(III) and Mn(IV) reducing conditions. To our knowledge this is the first report about benzene, ethylbenzene and xylene isomers biodegradation coupled to Mn(IV) reduction.

Manganese and iron are natural widespread metals that may enhance the biodegradation of monoaromatic hydrocarbons like BTEX, specifically benzene, which was rapidly degraded reducing Mn(IV) to Mn(II). Manganese and iron are energetically and kinetically more favourable than other anaerobic conditions. Moreover, the use of a solid metal oxide like Mn(IV) or Fe(III) as a final electron acceptor represents a promising and low cost alternative for the bioremediation of fuel contaminated sites, like groundwater and sediments, where Fe and Mn can be found (Thamdrup, 2000). An important difference and advantage of Mn over Fe lies in the relative tendency of the later to form insoluble sulphide precipitates. By this way Fe is continuously removed from the environment, while Mn is
rarely precipitated (Nealson and Myers, 1992). Such difference may impact the role of these two metals in the case of marine and freshwater sediment contamination.

Conclusions
The results of the present study have important ecological implications. It was demonstrated that biological activity significantly stimulated the degradation rate of BTEX coupled to the reduction of Mn(IV) and Fe(III). According to these results, it is important to highlight the biodegradation potential of historically polluted sediments, indicating long term enrichment of hydrocarbon-degrading microorganisms after prolonged exposure to aromatic hydrocarbon pollutants.

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References


