Reductive dehalogenation of tetrabromobisphenol-A by sediment from a contaminated ephemeral streambed and an enrichment culture

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Abstract

This study was aimed at improving our understanding of the physiology of the microorganisms that reductively dehalogenate tetrabromobisphenol-A (TBBPA). Activity was followed in contaminated sediments from a polluted streambed as well as from fracture filling material underlying the stream. Reductive dehalogenation was observed in surface sediments but not in fracture filling samples from a depth of 3 m. Likewise, anaerobic microbial activity, represented by sulphate reduction, was much higher in the surface sediment. In the culture enriched from the surface sediment, optimal microbial debromination of TBBPA took place at a salinity of 2% and 3% NaCl, temperature of 30 °C, and pH of 7–8. Ethanol, pyruvate and the combination of hydrogen with acetate were the most suitable electron donors and carbon sources for this culture. Alternative electron acceptors like Fe^{3+}, SO_{4}^{2-}, NO_{3}^{-}, and 2,4,6-tribromophenol inhibited TBBPA debromination. The debrominating bacteria were heat sensitive (80 °C for 10 min) but were not inhibited by bromoethanesulphonate or molybdate. This study allowed optimisation of our culturing conditions, but was also important for understanding the factors which influence TBBPA debromination in situ.

Keywords: Brominated flame retardant; TBBPA; Reductive dehalogenation; Biodegradation; Bioremediation; Negev desert

1. Introduction

Tetrabromobisphenol-A (TBBPA) [4,4′-isopropylidenebis(2,6-dibromophenol)] is one of the most widely used brominated flame retardants (BFR) (de Wit, 2002). BFRs are a group of brominated organic compounds added to plastic polymers in electronic equipment, textiles, plastics and many other applications in order to render them non-flammable. TBBPA has been found in environmental samples, as well as in human plasma, and there is evidence of a possible toxic effect (de Wit, 2002; Alaee et al., 2003; Covaci et al., 2003; Sjodin et al., 2003; Watanabe and Sakai, 2003; Birnbaum and Staskal, 2004).

TBBPA was found to be a major contaminant in the vicinity of an industrial complex in the northern Negev desert of Israel (Arnon, 1996). The site is located above a fractured chalk aquifer, and some of the fractures are exposed in an ephemeral streambed (Nativ et al., 1999). The concentration of TBBPA in the soil adjacent to the stream was up to 450 mg kg⁻¹, and it was suggested that TBBPA could be transported by this stream into the Besor River toward the Mediterranean Sea, or into the groundwater, mainly through the fractures (Arnon, 1996; Arnon et al., 2006) (for map of site locations see Ronen and Abeliovich, 2000). Reductive dehalogenation of TBBPA as well as other degradation activities in the sediment of this ephemeral stream were previously described (Ronen and Abeliovich, 2000; Ronen et al., 2000; Sørensen et al., 2002).
Biochemical reactions are usually the main mechanism of contaminant removal from the environment. There are limited reports of microbial metabolism of TBBPA (de Wit, 2002). Recent studies have shown that microbial mineralisation of TBBPA occurs in a sequence of anaerobic–aerobic stages (Ronen and Abeliovich, 2000; Voordeckers et al., 2002; Arbeli and Ronen, 2003; Ravit et al., 2005). During the anaerobic stage, TBBPA is reductively debrominated to bisphenol-A (BPA) [4,4’-isopropylidenediphenol], which is completely mineralised under aerobic conditions. While aerobic bacteria that degrade BPA were isolated (Lobos et al., 1992; Ronen and Abeliovich, 2000), no bacteria that debrominate TBBPA have been isolated thus far. Moreover, stable reductive debromination of TBBPA by an anaerobic enrichment culture proved hard to establish, and was possible only in a semi-continuous batch reactor operated at high solid retention time, and by addition of sterile pulverized grey chalk, sediment or soil (Arbeli and Ronen, 2003).

The aim of this study was to get a greater insight into reductive debromination of TBBPA. Since the fractures and the streambed are the main conduits for possible migration of TBBPA, we initially concentrated on TBBPA debromination in the stream sediment and in the fracture filling from a depth of 3 m. We determined TBBPA activity by these two microbial communities. Additional experiments to evaluate the range of carbon source and electron donors used for TBBPA debromination were performed in situ.

2. Materials and methods

2.1. Sampling site

The study focused on sediments from a contaminated small ephemeral stream located in the vicinity of an industrial park in the northern part of the Negev Desert, Israel (Nativ et al., 1999; Ronen and Abeliovich, 2000). Superficial sediment samples were taken from the streambed. Sampling of the fracture filling material was possible due to excavation work carried out in order to improve water collection in this contaminated stream. Two distinct fracture filling types were observed: white and black. Samples of both were taken immediately after exposure of the fracture from a depth of 3 m.

2.2. Microbial activity in sediment and fracture filling

We evaluated sulphate reduction activity in microcosms augmented with acetate as an indication of total microbial activity. Sediment or fracture filling and water from the stream were placed in the anaerobic chamber (Forma Scientific, Anaerobic system, Model 1029, Marieta OH, USA) with an atmosphere of 94% N₂ and 6% H₂. After 24 h, sediment and water were mixed (1:10 wt/vol) and supplemented with acetate to a final concentration of 3 g l⁻¹, and 10 ml of the mixture was dispensed into 20 ml tubes. The tubes were sealed with butyl rubber septa, incubated at 30 °C without shaking, and at each sampling point three tubes were sacrificed for analysis. The headspace was sampled with a syringe (100 µl) for methane analysis (only for surface samples), after which the tubes were opened to determine the concentration of acetate and sulphate in the liquid phase. Dehalogenation of TBBPA by sediment and fracture filling microorganisms was determined as previously described (Ronen and Abeliovich, 2000).

2.3. Physiological characterization of the enrichment culture

2.3.1. Inoculation source and media

The enrichment culture was obtained from the stream sediment and maintained in a semi-continuous batch reactor (SCBR) as previously described (Arbeli and Ronen, 2003). Mineral medium was prepared according to Boyle et al. (1999) with some modifications (Arbeli and Ronen, 2003).

2.3.2. Experimental procedure

All experiments were run in triplicate and incubated at 30 °C without shaking. Experimental flasks (125 ml) containing the 40 ml mineral medium and 5 g sterile grey chalk sealed with gas-permeable cotton stoppers were placed in the anaerobic chamber 24 h prior to the experiments. Before inoculation, TBBPA (from 20 mg ml⁻¹ solution in 0.2 N NaOH) and ethanol (absolute grade) were added to final concentrations of 90 µM and 0.2% vol/vol, respectively, and the pH was adjusted to 7.4 ± 0.2. Ten ml of the SCBR slurry was added as an inoculum source (20% vol/vol) and the flasks were sealed with butyl rubber stoppers (Bellco glass Vineland, NJ, USA). Incubation was carried out at 30 °C without shaking in the anaerobic chamber and the flasks were opened (in the anaerobic chamber) only for sampling.

2.3.3. Physiological characterization

In all experiments, BPA production was compared rather than TBBPA depletion, since BPA is more polar and therefore has higher solubility in water. Additionally, appearance of BPA can be interpreted unambiguously as the product of debromination, while depletion of TBBPA can also occur due to sorption of TBBPA to the grey chalk.

To test the optimal conditions for TBBPA dehalogenation, cultures were incubated at different temperatures, NaCl concentrations, which replaced the usual NaCl content of 2.1% in the medium, and pHs. pH was adjusted to between 6 and 9 by addition of 1 N NaOH or 1 N HCl as required.

Experiments to evaluate the range of carbon source and electron donors used for TBBPA debromination were
performed in serum bottles (160 ml) containing 45 ml solid free mineral medium (without chalk) and inoculated with TBBPA debrominating culture (10% vol/vol) (debromination activity continued without chalk at a lower rate: Arbeli and Ronen, 2003). The bottles were sealed with Teflon-lined septa and flushed with gas (80% N₂:20% CO₂) for 1 h. The medium was chemically reduced a few hours before inoculation with anoxic Na₂S·H₂O (36 mg l⁻¹).

The following compounds were tested separately: ethanol (34.35 mM), pyruvate (17.5 mM), glucose (5.55 mM), acetate (17.5 mM), acetate and hydrogen (17.5 mM and 25% of the serum bottle atmosphere, respectively), acetate and formate (17.5 mM and 43.5 mM, respectively), citrate (5.2 mM), succinate (8.47 mM). Two controls were used: 1. with 10% (wt/vol) sterile grey chalk as the only added organic carbon, and 2. with no added organic carbon source. The pH was monitored after the addition of the carbon source (pH = 7.4 ± 0.2).

To study the influence of an alternative electron acceptor on debromination of TBBPA, chalk concentration was reduced to 1.5% and inoculation volume to 10%, in order to reduce interference from other possible electron acceptors from the chalk or the inoculum. The following inorganic electron acceptors were added in addition to TBBPA: Na₂SO₄ (40 mM), NaNO₃ (40 mM), Na₂SO₃ (40 mM), and amorphous Fe (250 mM, prepared as described by Lovley and Phillips (1986)). In addition we tested the influence of 2,4,6-tribromophenol (TBP) (30, 180 and 360 μM), on debromination of TBBPA. A culture with TBBPA as the only electron acceptor was used as a reference (possible other electron acceptors might have been present in the chalk). Ethanol (34.35 mM) was added as an electron donor and carbon source. Its concentration was followed in the reference, in treatments with a combination of TBBPA and sulphate, in a control without added electron acceptor and in a sterile control.

To test the influence of inhibitors of methanogenesis and sulphate reduction on TBBPA dehalogenation, bromoethanesulphonate (BES) (1 mM) and Na₂MoO₄ (20 mM) were added to the medium when indicated. To test if TBBPA dehalogenating bacteria can resist pasteurisation, the debrominating culture was heat treated at 80 °C for 10 min. TBBPA was then added to the culture to test for dehalogenation activity by the pasteurised culture.

2.4. Analysis

To facilitate desorption and dissolution of TBBPA and its metabolites, 0.1 ml of 2 N NaOH was added to 2.9 ml slurry sample (recovery of TBBPA was 101.2 ± 10.2%). After centrifugation and filtration, the sample was analysed for TBBPA and metabolites by HPLC (Ronen and Abeliovich, 2000). To extract TBBPA from environmental samples of sediment or fracture filling, 1 g of sample was mixed with 10 ml of hexane and acetone (1/1), and refluxed at 65 °C for 8 h. The solvent was dried over Na₂SO₄, filtered with glass wool and then evaporated to dryness under a stream of N₂. Residues were dissolved in 100 μl of ethyl acetate and then analysed by GC–MS as previously described (Ronen and Abeliovich, 2000). Acetate and methane were measured by gas chromatograph HP 5890 equipped with a packed column (80/120 carbopack BD/DA/4% CARBOWAX 20 M), as described by Arbeli (1998). SO₄²⁻ was analysed according to standard methods (APHA, 1992).

3. Results and discussion

3.1. Microbial activity by sediment and fracture filling

Fig. 1. Reduction of endogeneous sulphate and consumption of acetate by the microbial population of surface sediments (A); and white and black fracture filling (B). Autoclaved sample served as abiotic control.
the surface sediment than in the black or white fracture filling: sediment microcosms could reduce 43.5 mM sulphate in 9 d, while microcosms of black and white fracture filling could only reduce 51 mM and 37.1 mM, respectively, in 109 d (Fig. 1). In all microcosms, acetate consumption was nearly stochiometric coupled to sulphate reduction. Methanogenesis seems to be a minor process (measured only in the surface sediment microcosms): after 18 d of incubation, only 360 nmol methane was produced by 10 ml of microcosm slurry. This suggests that sulphate is indeed the ultimate electron acceptor of the microbial community in the sediment and in the fracture filling, and that sulphate reduction rate is a good indicator for the total activity of the anaerobic microbial community in these samples. It also implies that sulphate reducers might be the main competitors with the debrominating bacteria for available electron donors.

3.2. Optimal conditions for TBBPA debromination by the enrichment culture

Maximal rate of TBBPA debromination occurred at a salinity of between 2–3% NaCl, a temperature of 30 °C, and a pH of between 7–8 (Fig. 2). As with Marinobacter sp. strain DPUZ that was previously isolated from the same sediments (Sørensen et al., 2002), the debrominating bacteria are well adapted to the conditions in the sediment, particularly the salinity, which fluctuates greatly due to the high evaporative conditions in the desert, with an average close to that of seawater (Sørensen et al., 2002). This suggests that debromination of TBBPA could potentially occur at the site. It is noteworthy that BPA, the debromination product of TBBPA, was detected in sediment samples (data not presented). However, it is unclear whether it is indeed a product of debromination or another contaminant. There was no significant difference in debromination activity in the pH range of 7–8 (p > 0.4) and only a slight (but significant, p = 0.032) decrease in debromination rate at pH 6–6.5 (the chalk buffering capacity did not allow reduction of the medium pH below 6). Debromination was strongly inhibited at pH 8.5 and completely stopped at pH 9. The finding that low pH was only slightly inhibitory is noteworthy since the solubility of TBBPA is close to 0% at pH 7.5, ~24% at pH 8 and above 90% at pH 8.5 (for 100 mg l⁻¹ TBBPA) (Arnon et al., 2006). This might suggest that TBBPA can be dehalogenated when it is sorbed or when it is in a solid form. If this is so, the dehalogenation mechanism might be similar to reduction of crystalline iron. Similarly, Ballerstedt et al. (2004) had suggested that crystals of dioxins might undergo reductive dehalogenation. Nevertheless, neither of the studies (Ballerstedt et al., 2004, and this study) presents proof of this hypothesis.

3.3. Carbon source and electron donors

Besides ethanol, pyruvate, glucose, and the combination of hydrogen with acetate supported debromination of TBBPA. Slower debromination activity also took place when adding acetate together with formate. Acetate, succinate and citrate alone did not support debromination (Table 1). As expected, no debromination was observed in the two controls: (1) sterile grey chalk as the only added carbon source; or (2) without any organic carbon or electron source. Since the experiment was conducted with mixed culture, we cannot state categorically whether the compounds mentioned that supported debromination were the direct carbon source for the dehalogenating bacteria. However, the fastest debromination activity was obtained with pyruvate, ethanol and acetate with hydrogen. These substrates are commonly used by halorespiring bacteria (Holliger et al., 1999) and might also be used directly by the TBBPA debrominating bacteria. Glucose might need to be fermented and formate might need to be converted.
to hydrogen before being used by the dehalogenating bacteria.

### 3.4. Inorganic alternative electron acceptors

All the inorganic electron acceptors tested were inhibitory for TBBPA debromination (Fig. 3). Cultures that received no additional electron acceptor debrominated TBBPA faster than cultures that received \( \text{Fe}^{3+}, \text{SO}_4^{2-}, \text{NO}_3^- \) (89% of TBBPA was converted to BPA in 7 d in comparison with, respectively, 70%, 60% and 13%). Hence, the debrominating bacteria might have partially switched their metabolisms from debromination to the reduction of these inorganic electron acceptors, or they might have been exposed to higher competition with other bacteria in the culture for carbon and electrons (Townsend and Suflita, 1997). In addition, these inorganic electron acceptors or their reduction products, especially sulphide and nitrite, might be toxic. Of the three inhibition mechanisms mentioned above, our data about ethanol metabolisms during debromination (presented in Section 3.6 and Fig. 5) support the competition hypothesis, but the other two hypotheses cannot be excluded. Sulphite totally inhibits debromination of TBBPA (Fig. 3). Sulphite was often found to completely inhibit reductive dehalogenation in mixed and pure cultures or even cell extract, and it seems that the inhibition occurs at the enzymatic level (Townsend and Suflita, 1997). Our finding that debromination took place in the presence of sulphate is of major importance since both the sediment and the stream water are rich in sulphate.

### 3.5. 2,4,6-Tribromophenol as an alternative electron acceptor

Among other substances that were identified (by GC–MS) in the sediment from the site are 2,4,6-tribromophenol, pentabromotoluene, dibromobiphenyl, 3,3′,4,4′-tetrachlorobiphenyl and 1,3-bis-(2-bromoethyl) propanediol. Other haloorganic substances might inhibit TBBPA debromination if they are toxic to the debrominating bacteria or if the debrominating bacteria prefer these substances to TBBPA as an electron acceptor. We decided to check the influence of 2,4,6-tribromophenol (TBP) on TBBPA debromination because its structure is similar to that of TBBPA. Fig. 4 presents the kinetics of BPA production from TBBPA in the presence of 0, 30, 180 and 360 \( \mu \text{M} \) TBP. TBP inhibited TBBPA debromination and the extent of inhibition depended on TBP concentration. Cultures that did not receive TBP or received 30 \( \mu \text{M} \) TBP converted 75% of TBBPA to BPA in 4.5 and 15 d, respectively, while at higher concentration of TBP (180 or 360 \( \mu \text{M} \)), TBBPA debromination was completely stopped. In these experiments, TBP was debrominated (data not shown). 4-bromophenol was the main product of TBP debromination but phenol was also formed. Hence, the culture might preferred TBP as an electron acceptor, or TBP or its debromination products were toxic to the TBBPA debrominating microorganisms. Recently, Cupples et al. (2004) demonstrated that cis-1,2-dichloroethene (DCE) and vinyl chloride (VC) are competitive electron acceptors for the bacterium *Dehalococcoides* strain VS.

Interestingly, 2,4,6-trichlorophenol (TCP) was not dechlorinated by our enrichment culture. It is well known that reductive dechlorination is easier than reductive dehalogenation (Bedard and van Dort, 1998), since the aryl-bromine bond is weaker than the aryl-chlorine bond.

### Table 1

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>% of converted TBBPA</th>
</tr>
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<tbody>
<tr>
<td>Pyruvate</td>
<td>97.5 in 11 d</td>
</tr>
<tr>
<td>Ethanol</td>
<td>88.7 in 11 d</td>
</tr>
<tr>
<td>Acetate + H_2</td>
<td>88.2 in 17 d</td>
</tr>
<tr>
<td>Glucose</td>
<td>96.5 in 35 d</td>
</tr>
<tr>
<td>Acetate + formate</td>
<td>45.9 in 35 d</td>
</tr>
<tr>
<td>Acetate</td>
<td>n.d.</td>
</tr>
<tr>
<td>Succinate</td>
<td>n.d.</td>
</tr>
<tr>
<td>Citrate</td>
<td>n.d.</td>
</tr>
<tr>
<td>Grey chalk</td>
<td>n.d.</td>
</tr>
<tr>
<td>No carbon</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. No debromination in 35 d.

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**Fig. 3.** BPA formation from TBBPA by the microbial mixed culture in mineral medium supplemented with 1.5% grey chalk and with different alternative inorganic electron acceptors.

**Fig. 4.** BPA formation from TBBPA by the microbial mixed culture in mineral medium supplemented with 1.5% grey chalk and with different concentrations of TBP (in \( \mu \text{M} \)).
(Weast and Astle, 1980). Previously, Voordeckers et al. (2002) reported dehalogenation of TBBPA, 2,6-dibromo-phenol, tetrachlorobisphenol A and 2,6-dichlorophenol by the same sediment sample. It was not clear, however, if this was by the same bacteria or different bacteria from the same community.

3.6. Ethanol metabolism

To assess the importance of microbial competition in the presence of the different electron acceptors, we studied the metabolism of ethanol, which is the carbon source and electron donor used in this study (Sections 3.4 and 3.5). Cultures, which received both sulphate and TBBPA, were of special interest since sulphate is the dominant electron acceptor at the site. Indeed, these cultures oxidized ethanol to acetate faster than those cultures that received only TBBPA (Fig. 5). Moreover, acetate was completely consumed in the presence of sulphate while hardly any acetate was metabolised (within 10 d) when only TBBPA was present. For all other treatments the concentrations of ethanol and acetate were measured only in the beginning and at the end of the experiment (after 10 d—data not presented). In most treatments, (Fe$^{3+}$, SO$_2^-$/C0$_3$ and TBP at 30, and 180 µM) ethanol was not detected at the end of the experiment, while acetate accumulated to values of 29.92–31.25 mM, close to the theoretic concentration of ethanol in the beginning of the experiments (34.35 mM). When nitrate was supplied as an electron acceptor, the concentrations of ethanol and acetate at the end of the experiment were 0 and 1.21 mM, respectively. It seems that sulphate and nitrate have the strongest influence on carbon metabolism. This suggests that in the presence of sulphate and nitrate, sulphate and nitrate reducers, respectively, consume available carbon and electrons, which could have limited debromination activity. In the presence of sulphate, all the ethanol had been oxidized before BPA production was evident (Fig. 5). Nevertheless, attempts to relate metabolism of ethanol to TBBPA debromination might be misleading since the initial concentration of ethanol (34.35 mM) was 2 orders of magnitude higher than the initial concentration of bromine bound to BPA (360 µM), and since its oxidation products, acetate and hydrogen, can also be used as carbon source and electron donor, respectively (Table 1).

Surprisingly, when TBBPA was excluded from the medium (without sulphate) ethanol was consumed faster than in the TBBPA culture and acetate consumption was also detected, although slower than in the presence of sulphate (Fig. 5). When 360 µM TBP was supplied as an electron acceptor, ethanol was still detected at the end of the experiment (5.1 mM), while acetate concentration was lower than in the other treatments (22.98 mM). Previously we have shown that when TBBPA concentration increases, the time until debromination becomes evident also increases (Arbeli and Ronen, 2003). We suggested that this was a result of the toxicity of TBBPA to the debrominating bacteria. The observation that TBBPA and TBP slowed down ethanol and acetate metabolism suggests that they might be toxic to a wider range of bacteria or interfere in some other way with the metabolism of ethanol.

3.7. Pasteurisation and specific inhibitors

Debromination activity was lost after heat treatment for 10 min at 80 °C, indicating that the debrominating bacteria in these sediments do not form spores. Since both methanogenesis and sulphate reduction were detected in the sediment, we tested the influence of the specific inhibitors, BES and molybdate, respectively, on debromination. The results showed that neither of them inhibited debromination, indicating that sulphate reduction or methanogenesis are not essential for the process (Fig. 6).
In conclusion, debromination activity was observed in sediment samples but not in fracture filling samples. This might explain the big difference in the concentration of TBBPA in these samples. The data from this study strongly indicates that the microorganisms in the sediment are well adapted to the environmental conditions prevailing at the site. The reason for the absence of debromination activity in fracture filling samples is not clear, but is in accordance with the low activity of sulphate reduction. This last finding suggests that bioaugmentation might be essential for remediation of the fractures.

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References


