Biodegradation **14:** 385–395, 2003.

Enrichment of a microbial culture capable of reductive debromination of the flame retardant tetrabromobisphenol-A, and identification of the intermediate metabolites produced in the process

Ziv Arbeli* & Zeev Ronen**
*Department of Environmental Hydrology and Microbiology, Institute for Water Science and Technologies, The Jacob Blaustein Institutes For Desert Research, Ben Gurion University of the Negev, Sede Boqer Campus, 84990, Israel (**author for correspondence: e-mail: zeevrone@bgumail.bgu.ac.il)*

Accepted 25 July 2003

Key words: brominated flame retardant, tetrabromobisphenol-A, reductive dehalogenation, metabolic pathway, enrichment culture, stimulation by sediment

Abstract

Tetrabromobisphenol-A is a reactive flame retardant used in the production of many plastic polymers. In previous research, it was demonstrated that anaerobic microorganisms from contaminated sediment debrominate tetrabromobisphenol-A to bisphenol-A, but an enrichment culture was not established. The current study was carried out to identify the intermediate metabolites in this process and to determine the factors facilitating enrichment of debrominating microorganisms. During the enrichment process in an anaerobic semi-continuous batch reactor, tetrabromobisphenol-A debromination gradually slowed down with concurrent accumulation of three intermediate products. These compounds were tentatively identified using GC-MS as tri-, di-, and mono-brominated bisphenol-A. GC-MS and HPLC analyses showed one dominant metabolite of dibromobisphenol-A, and NMR analysis identified it as 2,2′-dibromobisphenol-A. Addition of sterile sediment (15% wt/wt) to the reactor stimulated debromination of tetrabromobisphenol-A. Furthermore, different solid amendments such as surface soil and pulverized gray chalk from the site subsurface (100 m below ground) were also stimulating agents. We conclude that organic matter is involved in stimulation since the stimulation effect of the sediment, soil and gray chalk was abolished after it was heat-treated to 550 °C. Our study suggests that the debrominating culture requires some organic components found in the sediment, soil, and chalk in order to sustain activity and perhaps to survive. The possible mechanisms of stimulation by these solids are discussed.

Abbreviations: BBPA – bromobisphenol-A; BFR – brominated flame retardant; BPA – bisphenol-A; PCB – polychlorinated biphenyls; SCBR – semi-continuous batch reactor; TBBPA – tetrabromobisphenol-A

Introduction

Emerging evidence regarding distribution in the environment and toxicity, bioaccumulation and persistence qualities of brominated flame-retardants (BFRs) is of public concern (de Boer et al. 1998; Hooper & McDonald 2000; Renner 2000; de Wit 2000, 2002). BFRs are a group of chemicals used at relatively high concentrations in electronic equipment such as computers and television sets, in textiles, cars and many other applications. During the last few decades, the global consumption of brominated flame-retardants has grown dramatically due to the growth in the use of synthetic polymers, and the introduction of more rigorous fire safety requirements. Tetrabromobisphenol-A (TBBPA) [4,4′-isopropylidenebis(2,6-dibromophenol)] (see Table 3

* Present address: Laboratorio Ambiental, Instituto de Biotecnología – Edificio Manuel Ancizar, Universidad Nacional de Colombia, Bogotá, Colombia.

for chemical structure) is the most widely used and commercially important BFR. The annual global consumption of TBBPA is about 121,000 tonnes – roughly half of the total BFR consumption (de Wit 2002). TBBPA was found in sediment (Sellstrom & Jansson 1995), soil (Arnon 1999), and sewage sludge (Sellstrom & Jansson 1995), as well as in aquatic animals (Watanabe et al. 1983) and in human plasma (Thomsen et al. 2001). There is evidence that prolonged exposure of rats to TBBPA disturbs liver heme metabolism (Szymanska et al. 2000) as well as the neurological system (Eriksson et al. 1998). In vitro assays demonstrated that TBBPA was up to 25 times more potent for binding to human transthyretin (thyroid transport protein) than thyroxin (the native hormone) (Brouwer 1998).

Reductive dehalogenation is the first step in degradation of highly halogenated compounds such as polychlorinated dibenzodioxin PCDD and polychlorinated dibenzofurans PCDF (Wittich 1998), PCBs (Wiegel & Wu 2000) and TBBPA (Ronen & Abeliovich 2000). Some of the bacteria capable of reductive dehalogenation use the halo-organic compound as the final electron acceptor in a respiration process, which is coupled to ATP synthesis (Fantroussi et al. 1998; Holliger et al. 1999). Reductive dehalogenation of biarylhalides (halogenated compounds with two aromatic rings) is of special interest to us because TBBPA belongs to this group. Most of the studies on reductive dehalogenation of biarylhalides were done on PCBs (Wiegel & Wu 2000). Early enrichment attempts of PCB dechlorinating culture showed the necessity of the presence of sediment in the medium for dechlorination activity. Cutter et al. (1998) and Wu et al. (2000) demonstrated the enrichment of PCB dechlorinating cultures in a sediment-free medium but the role of sediment in sustaining the activity was not determined, and all reported attempts to isolate microorganisms that catalyze these reactions have been unsuccessful.

There are limited reports about microbial metabolism of TBBPA (de Wit 2000, 2002). Allard et al. (1987) demonstrated that TBBPA could undergo O-methylation by aerobic bacteria. Ronen and Abeliovich (2000) demonstrated for the first time that microbial mineralization of TBBPA occurs in a sequence of anaerobic-aerobic stages. This study showed that during the anaerobic stage, TBBPA is debrominated to bisphenol-A (BPA) [4,4′-isopropylidenediphenol], which is completely mineralized under aerobic conditions. However, debromination activity was lost after the first sub-culturing of the consortium and thus, enrichment of the anaerobic debrominating bacteria was impossible. In addition, the complete pathway leading to the anaerobic debromination of TBBPA was not described. Similarly, microorganisms from estuarine sediment from Arthur Kill (USA) could transform TBBPA to BPA (Voordeckers et al. 2002) but again, the intermediate metabolites were not identified and the culture was enriched only to a limited extent.

The current study had two main objectives: 1) to identify the intermediate compounds produced during anaerobic microbial debromination of TBBPA and to elucidate the pathway leading to complete debromination; and 2) to determine the factor that was limiting the continuous debromination of TBBPA, and thus to enrich the culture. In this work, we enriched the TBBPA debromination and identified the intermediate metabolites formed in the process. A slow dilution in a Semi- Continuous Batch Reactor (SCBR) and addition of sterile sediment was essential for enrichment of the debrominating culture. Elucidating the role of sediment in stimulating reductive dehalogenation of molecules such as TBBPA and PCBs merits further research.

Materials and methods

Culture methods

Sediment from the vicinity of an industrial complex in the northern Negev desert, Israel was used in this study (Ronen & Abeliovich 2000). Mineral medium was prepared according to Boyle et al. (1999) with some modifications: 900 ml of Solution A with (in grams) KCl, 1.3; KH_2PO_4, 0.2; NaCl, 21; resazurin, 0.001 autoclaved. After cooling it down the following sterile solutions were added: 1 ml of vitamin solution, 3 ml of trace element solution, 10 ml of salt solution, 100 μl of $NaSeO_3$ solution (40 mg/L) and 90 ml of $NaHCO_3$ solution (80 g/L). The vitamin solution (filter sterilised) contained (in mg/L): folic acid, 20; pyridoxine HCl, 100; riboflavin, 50; biotin, 20; thiamine, 50; nicotinic acid, 50; pantothenic acid, 50; vitamin B12, 1; *p*-aminobenzoic acid, 50; thiotic acid, 50. The trace element solution contained (in g/L): nitriloacetic acid, 2; $Fe(NH_4)(SO_4)_2{*}6H_2O$, 0.8; $MnSO_4{*}H_2O$, 1; $CoCl_2{*}6H_2O$, 0.2; $ZnSO_4{*}7H_2O$, 0.2; $CuCl_2{*}2H_2O$, 0.02; $NiSO_4{*}H_2O$, 0.02; $Na_2MoO_4{*}2H_2O$, 0.02; $Na_2WO_4{*}2H_2O$, 0.02; H_3BO_3, 0.02. The salt solution

contained (in g/L): NH_4Cl, 30; $CaCl_2{*}2(H_2O)$, 15; $MgCl_2{*}6(H_2O)$, 300.

The experiments (batch experiment and SCBR) were performed in an anaerobic chamber (Forma Scientific, Anaerobic system, Model 1025/1029 Marieta OH, USA) with an atmosphere of 94% N_2 and 6% H_2. Experimental flasks containing the medium, sealed with gas-permeable stoppers, were placed in the anaerobic chamber 24 h prior to the experiments. The pH was adjusted before inoculation (7.4), and after inoculation the flasks were sealed with a rubber stopper. Incubation was carried out at 30 °C without shaking in the anaerobic chamber and flasks were opened only for sampling.

Enrichment by serial dilutions was done in 125 ml serum bottles. Mineral medium (45 ml) was dispensed into serum bottles containing 5 g of sterile sediment and later 5 g of gray chalk. The bottles were sealed with Teflon septa and flushed with filtered sterile gas (80% N_2: 20% CO_2) for one hour, and the pH was adjusted to 7.4. The medium was reduced a few hours before inoculation with anoxic $Na_2S{*}9H_2O$ (36 mg/L).

Operation of the semi-continuous batch reactor

Sediment (15% wt/wt) was incubated anaerobically in the SCBR (250 ml flask) containing 110 ml mineral medium supplemented with TBBPA 90 μM (Aldrich, Milwaukee, Wis.) and glucose 1 g/L. Periodically, 21–23% of the liquid was removed and replaced with a fresh anaerobic medium. TBBPA and glucose were added to a final concentration of 90 μM and 0.5 g/L respectively (Table 1).

Batch experiments

120 ml flasks containing 40 ml of mineral medium (supplemented according to treatment) were placed in an anaerobic chamber 24 h before inoculation. In preliminary experiments, ethanol was found to support debromination and was preferred over glucose since it is a more selective carbon source. TBBPA and ethanol (final concentration of 90 μM and 0.2% vol/vol, respectively) were added just before inoculation (10 ml of SCBR culture). The SCBR was operated as follows: every 6 days, 25% of mixed slurry was replaced by mineral medium containing 10% (wt/wt) sterile gray chalk. TBBPA and ethanol were added to the final concentration of 55 μM and 0.1% (vol/vol) respectively, and the pH was adjusted to 7.4.

The following supplements (10% wt/wt) (all sterilized for 30 min. at 120 °C) were added to the cultures, one to each treatment batch, in order to test their influence on TBBPA debromination activity (Table 2): sediment from the site (wet weight), acid-purified sand (40–100 mesh) (BDH laboratory reagents), soil from Sede-Boqer (Negev desert, Israel), and pulverized (crushed and sieved below 0.5 mm) gray chalk and white chalk, (Wefer-Roehl et al. 2001). In addition, organic material was extracted from the gray chalk using methanol toluene mixture (1:1). The chalk was treated for 15 min. in an ultrasonic bath and then the solids were removed. The solvents were evaporated from the solids by drying the chalk for 48 h at 200 °C. We also compared the kinetics of TBBPA debromination at different concentrations of gray chalk (0, 2.5, 5, 10 and 20% wt/wt) and different initial concentrations of TBBPA (90, 180, 320 and 720 μM) with 10% (wt/wt) gray chalk. In order to test whether the stimulating factor is consumed over time, TBBPA (90 μM) and ethanol (0.1% vol/vol) were repetitively added to sediment slurry (20% wt/wt) in mineral medium when TBBPA had been converted to BPA.

Analysis

0.1 ml of 2N NaOH was added to a 2.9 ml slurry sample to facilitate desorption and desolution of TBBPA (recovery of 101.2 $\pm$ 10.2%). After centrifugation and filtration, the sample was analyzed for TBBPA and metabolites by HPLC (Ronen & Abeliovich 2000). Metabolites present in the SCBR effluent were extracted after acidification (pH 2.5) with ethyl acetate (1:1 vol/vol). The solvent was dried over Na_2SO_4 and then evaporated to dryness under a stream of N_2. Residues were dissolved in 100 μl of ethyl acetate and then analyzed and identified by GC-MS (Magnum, Finnigan Mat, San Jose, CA, USA). Separation was achieved using a DB-5 column (30 m $\times$0.25 mm ID.) with an initial temperature of 80 °C held for 4 min and then increased to 280 °C at a rate of 10 °C/min.

Dissolved organic carbon (DOC), Cl^-, Br^-, SO_4^{2-} and Ca^{2+} were extracted by water from the solids with double distilled water and sediment, soil (1/1 wt/wt), gray chalk or white chalk (1/10 wt/wt) by agitation (200 RPM) for 16 hours at 25 °C. The mixture was then centrifuged (8000 RPM for 10 min) and the supernatant was filtered (GF filter). DOC was analyzed using a Dohramann model DC-190 total-organic-carbon (TOC) analyzer (Rosemount Analytical, Santa Clara, CA) and ion concentrations were

Table 1. Stages in the operation of the semi continuous batch reactor (SBCR)

Stage	Culture exchanged (volume)	Medium added	TBBPA added (μM)	Glucose added (g/L)
1	Liquid (23%)	Mineral medium (MM)	90	0.5
2	Liquid (23%)	MM	120	0.5
3	Slurry (50%)	15% sterile sediment in MM	None	0.5
4	Slurry (21%)	15% sterile sediment in MM	90	0.5
5	Slurry (21%)	15% sterile sediment in MM	60	0.5

Table 2. Chemical composition of the solids used in stimulation experiments

Parameter	Hovav ediment[1]	Sede Boqer soil[1]	Gray chalk[2]	White chalk[2]
pH	7.49	7.87	7.77	8.01
		mg/kg		
DOC[3]	141	11	98	30
Cl^-	7,015	203	820	7,450
Br^-	766	10	0	0
SO_4^{2-}	4,722	1,214	1,700	840
Ca^{2+}	Not determined	658	457	188

[1]Parameters determined after extraction for 16 h (1:1) with double distilled water.
[2]Parameters determined after extraction for 16 h (1:10) with double distilled water.
[3]Dissolved organic carbon in the water extract.

determined by ion chromatography (4500i series, Dionex Co, Sunnyvale, CA).

NMR spectroscopy

The DiBBPA metabolite in the medium extract was purified by HPLC. The solvent was evaporated to dryness under a stream of N_2. Residues were dissolved in DMSO-d_6. ^{1}H-NMR spectra of the hydrogen substituent of the phenolic ring were obtained with a Bruker DMX-500 spectrometer at 300 °K.

Results

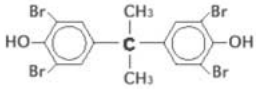
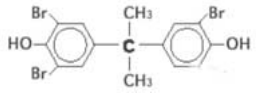
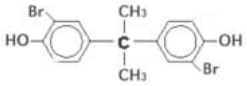
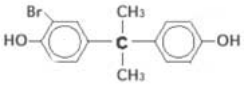
Debromination intermediates

During operation of the SCBR (Table 1), new peaks appeared in the HPLC chromatogram concurrently with the disappearance of TBBPA. These compounds had a shorter retention time, suggesting an increase in polarity relative to TBBPA. GC-MS analysis of ethyl acetate extract from the SCBR effluent revealed compounds with a base peak (in the mass spectra) of 451, 371 and 291. These base peaks suggest replacement of one, two, and three bromine atoms of TBBPA, respectively, by hydrogen atoms (Table 3). Because the bromine positions on TBBPA are symmetric (*ortho* on both sides of the OH group), only one isomer each of TriBBPA and MonoBBPA are expected. On the other hand, there are two possible isomers of DiBBPA (2,2′-dibromobisphenol-A and 2,6-dibromobisphenol-A). The GC-MS analysis did not indicate the presence of two dibromo intermediates. ^{1}H NMR spectra showed that there are three protons on each phenolic ring of the dibromo intermediate, which leads to the conclusion that there is one bromine on each ring (Figure 1). ^{1}H NMR spectra (DMSO-d_6, δ, ppm) of H_A, H_B and H_C on the aromatic ring (Figure 1) were 6.84 (d, ^3J = 7.2 Hz, 1H, H_A), 6.97 (d, d, ^3J = 7.2 Hz, ^4J = 1 Hz, 1H, H_B) and 7.23 (d, ^4J = 1 Hz, 1H, H_C), respectively. Thus, 2,2′-dibromobisphenol-A is the dibrominated intermediate product of the process.

Activity in a semi-continuous batch reactor (SCBR)

Initial attempts to enrich the TBBPA debrominating bacteria failed since debromination activity had been lost after re-inoculation (10% vol/vol) of the active culture into a fresh solid free medium under different

Table 3. Mass spectrum data of debromination metabolites. Each column contains the mass (*m/z*) of the most abundant fragments (in parentheses)

Compound	MW	Base peak ($-CH_3$)	-Br	-2Br	-3Br	-4Br	Structure
TBBPA	544	529	451	370	291	212	
			(5.0)	(4.1)	(9.5)	(4.9)	
TriBBPA	464	451	370	291	212		
			(6.1)	(8.9)	(8.5)		
DiBBPA	384	371	291	212			
			(5.2)	(5.5)			
MonoBBPA	304	291	212				
			(15.7)				

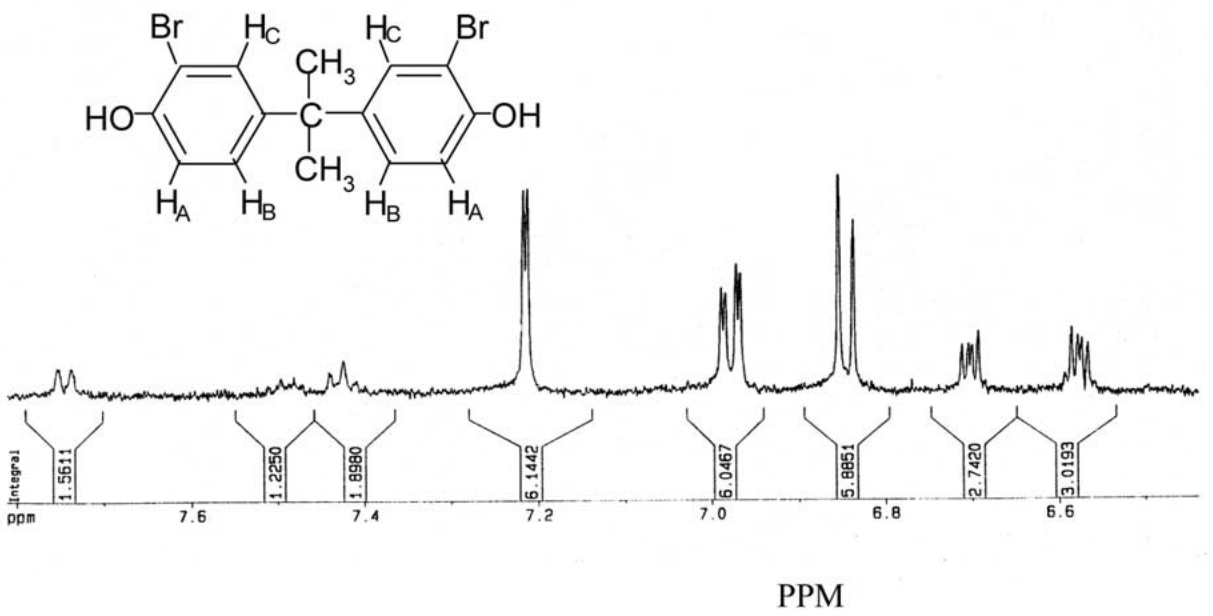


Figure 1. ^{1}H NMR spectra of 2,2′-dibromobisphenol-A produced during the reductive debromination of tetrabromobisphenol-A in the sediment SCBR (Figure 2).

conditions. Thus, we tried to enrich the culture using the SCBR (Table 1). The result was an accumulation of TBBPA and its intermediate metabolites – mainly TriBBPA and DiBBPA – concordant with a decrease in the accumulation of the final product, BPA (Figure 2). In an attempt to restore debromination activity, we changed the operation mode of the reactor (Table 1 stage 3 and on). The new operation mode restored debromination activity, and the concentration of TBBPA and its intermediate metabolites remained low for more than a month during which all the reactor volume was replaced (Figure 2 and Table 1). After 77 days, BPA the final product of TBBPA debromination – had accumulated to a level of 242 μM, indicating that the removal of TBBPA was indeed due to dehalogenation.

Gradual retardation of TBBPA debromination in 20% (wt/wt) sediment slurry was also observed in a batch experiment when TBBPA (90 μM) and ethanol as a carbon source (0.1% vol/vol) were added repetitively (Figure 3). Following the first addition, 91.6% of TBBPA was debrominated to BPA in 4 days. However, after the second addition, only 71.8% was converted to BPA in 6 days; and after the third addition only 41% was converted in a period of 8 days (Figure 3). The possibility that debromination was arrested due to product inhibition was rejected in the light of separate experiments which showed that BPA (400 μM) did not inhibit TBBPA debromination (Arbeli & Ronen, unpubl.).

Stimulation of TBBPA debromination

From the operation of the SCBR we concluded that sterile sediment stimulates TBBPA debromination activity. Thus, we tested different solid amendments in batch experiments. Sediment, crushed gray chalk, and soil from Sede Boqer appeared to stimulate debromination (Figure 4) while crushed white chalk from the same area and acid-purified sand had no apparent effect (Arbeli & Ronen, unpubl.). In order to determine if the stimulating factor is an organic component, we heat-treated the sediment, soil and gray chalk (550 °C for one hour). The heat-treated solids did not stimulate debromination (Figure 4). Likewise, no debromination was observed in medium amended with solvent extracted chalk, suggesting that an organic component, which was removed in the extraction process, is essential for debromination. The degree of stimulation depends on the concentration of gray chalk (Figure 5) and sediment (Arbeli & Ronen, unpubl.). Ten percent (wt/wt) gray chalk seems optimal for TBBPA debromination, while 20% (wt/wt) gray chalk, especially at low concentrations of TBBPA, might limit the bio-availability of TBBPA.

Kinetics of TBBPA debromination

In a batch experiment in which TBBPA was rapidly debrominated, the intermediate metabolites were rapidly consumed (Figure 6). The maximal rates of TBBPA debromination and BPA formation were nearly equal (33.12 $\pm$ 2.65 and 33.05 $\pm$ 4.53 μM/day, respectively). In some cases, 2,2′-DiBBPA concentration exceeded the concentration of TriBBPA (e.g., Figure 2) while the MonoBBPA concentration was always much lower (e.g., Figures 2, 3 and 6). The kinetics of debromination depended on the initial concentration of TBBPA. In increasing initial concentrations of TBBPA (90, 360 and 720 μM), both the lag phase ($\sim$0, $<$4 and $>$4 days, respectively) and the maximal debromination rate (159 $\pm$ 33.84, 221.31 $\pm$ 30.05 and 613 $\pm$ 18.37 μM Br^-/day, respectively) increased (Figure 7).

Enrichment the culture

After about 3 months of SCBR operation, the culture could be repeatedly transferred in serial 10-fold dilutions into mineral medium containing ethanol (0.2%) as a sole carbon and energy source and 10% (wt/wt) sterile sediment or, later (after the 4th transfer), gray chalk. In the first dilution transfer, only the 10^{-1} dilution showed debromination activity. With the following transfers, debromination activity was detected in lower dilutions: at the 8th transfer debromination activity was detected in the 10^{-8} dilution. This indicates that the TBBPA debrominating bacteria were enriched. After a few successful transfers, the culture could be repeatedly transferred (20% vol/vol) even to a sediment-free medium and retain debromination activity, albeit with a much slower debromination rate (lag phase $>$ 7 days and debromination rate of 9.71 $\pm$ 0.68 μM Br^-/day).

Discussion

Intermediate metabolites of TBBPA debromination were isolated and identified as TriBBPA, 2,2′-DiBBPA and MonoBBPA. The fact that the 2,6-dibromobisphenol-A isomer was not detected suggests that debromination is position-directed. If debromination of each of the three bromines on TriBBPA were

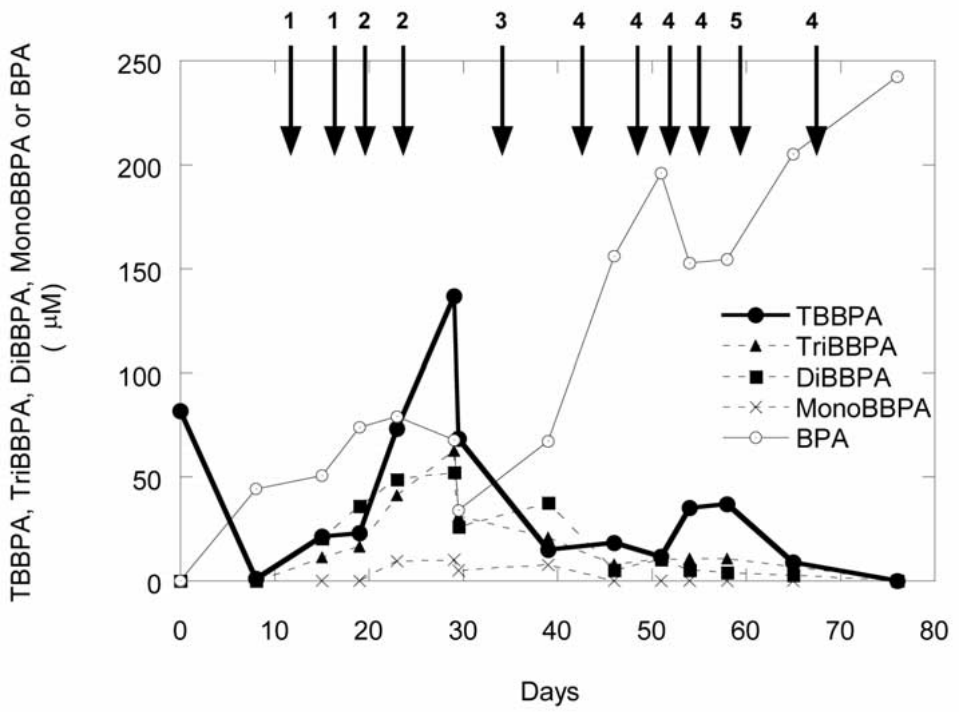


Figure 2. Fate of TBBPA and its debromination products in SCBR, initially inoculated with sediment (15% wt/wt), and run under the operating conditions described in Table 1. Arrows indicate the time of each treatment. Numbers above the arrows correspond to the stage number in Table 1.

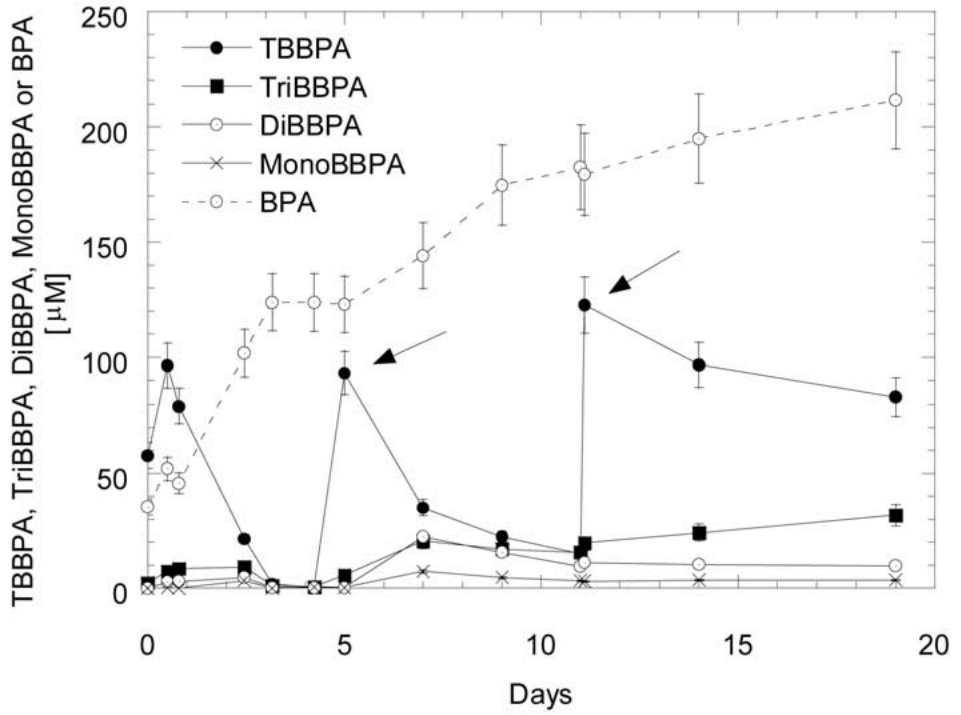


Figure 3. Change in debromination activity as a result of repetitive addition of TBBPA (90 μM) and ethanol (0.1%) to sediment slurry culture without replenishing the solids (time of addition is indicated by the arrows).

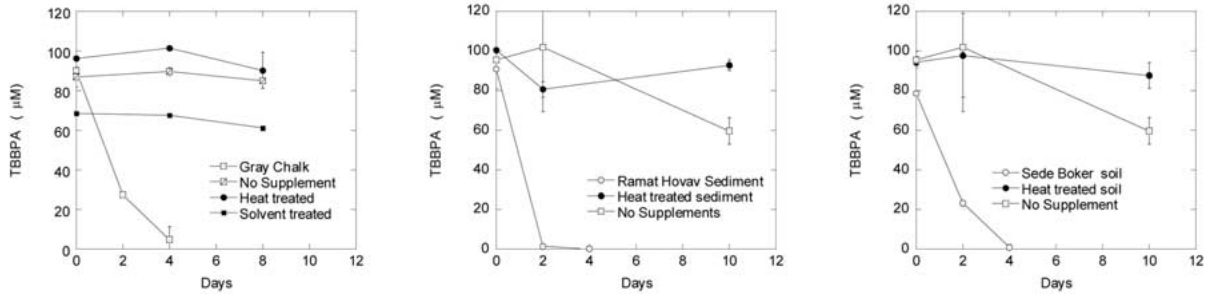


Figure 4. Stimulating effect of solid amendments (10% wt/wt) on debromination of TBBPA in batch cultures. Ethanol (0.2% vol/vol) was used as an electron and carbon source. Inoculum for cultures (20%) was taken from SCBR containing gray chalk (10% wt/wt).

equally favorable, we would expect the concentration of 2,6-dibromobisphenol-A to be 50% of the two dibrominated BPA isomers. Thus, either mainly (or only) 2,2′-dibromobisphenol-A is formed, or both isomers are formed but 2,6-dibromobisphenol-A is more rapidly debrominated to MonoBBPA. Both of these hypotheses can be explained by the electronegative property of bromine. The presence of a second bromine substitute on the phenolic ring induces a stronger positive dipole on the carbon of the first bromine and thus makes it more likely to undergo nucleophilic attack (an inductive acceptor effect). Although one could predict that the second bromine on the phenolic ring will stericly hinder an enzymatic attack, the electronegativity effect is dominant. The fact that the concentration of MonoBBPA was always much lower than that of TriBBPA and 2,2′-DiBBPA suggests that the steric effect may also play a role. Position- directed dehalogenation has been extensively reviewed for PCBs (Bedard & Quensen 1995; Dolfing & Beurskens 1995; Wiegel & Wu 2000), and appears to be common in reductive dehalogenation regardless of molecular size or halogen type (chlorine or bromine). 2,2′-dichlorobisphenol-A is the predominant end product of tetrachlorobisphenol-A dechlorination (Voordeckers et al. 2002), and *cis*-dichloroethene (cis-DCE) is usually the main product of reductive dechlorination of trichloroethene (TCE) (Rosner et al. 1997).

TBBPA debromination clearly depends on solids like sediment, soil and gray chalk (Figures 2–5). This observation is consistent with other studies on reductive dehalogenation of polyhalogenated compounds, mainly PCBs (Wiegel & Wu 2000). The fact that fresh sterile sediment was necessary to maintain debromination activity in SCBR (Figure 2 and Table 1), coupled with the observation that in batch experiments debromination slowed with two consecutive additions of TBBPA and carbon source (Figure 3), indicated

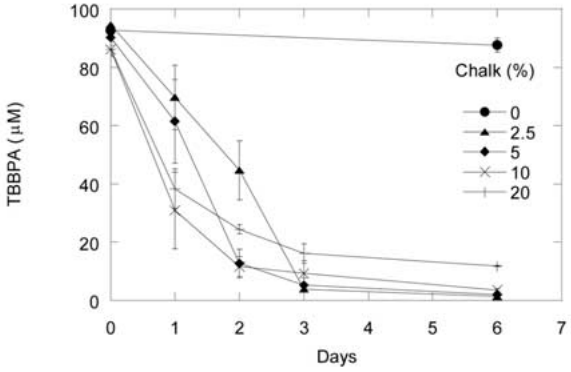


Figure 5. Effect of gray chalk concentration on TBBPA debromination in batch cultures. Ethanol (0.2% vol/vol) was used as an electron and carbon source. Inoculum for cultures (20%) was taken from SCBR containing gray chalk (10% wt/wt).

that the stimulating factor is being consumed over time. Our results, which show that heat-treated sediment, heat-treated soil, heat-treated gray chalk, and solvent-treated gray chalk do not stimulate TBBPA debromination, strongly suggest that the organic fractions of these solids are essential for debromination (Figure 4). Furthermore, the observation that gray chalk, but not white chalk, stimulates dehalogenation reinforces this hypothesis, because the main differences between gray and white chalk are the amount of organic matter present and its oxidation state: gray chalk has a six-fold organic carbon and a four-fold hydrogen index compared to white chalk (Wefer-Roehl et al. 2001). The degree of stimulation depends on the solid concentration and suggests that this factor is limiting (Figure 5).

The mechanism of stimulation by the solids was not identified. Hohnstock-Ashe et al. (2001) reported that pulverized sterile dolomite was essential for reductive dehalogenation of trichloroethene (TCE) in a microcosm. When yeast extract was amended instead

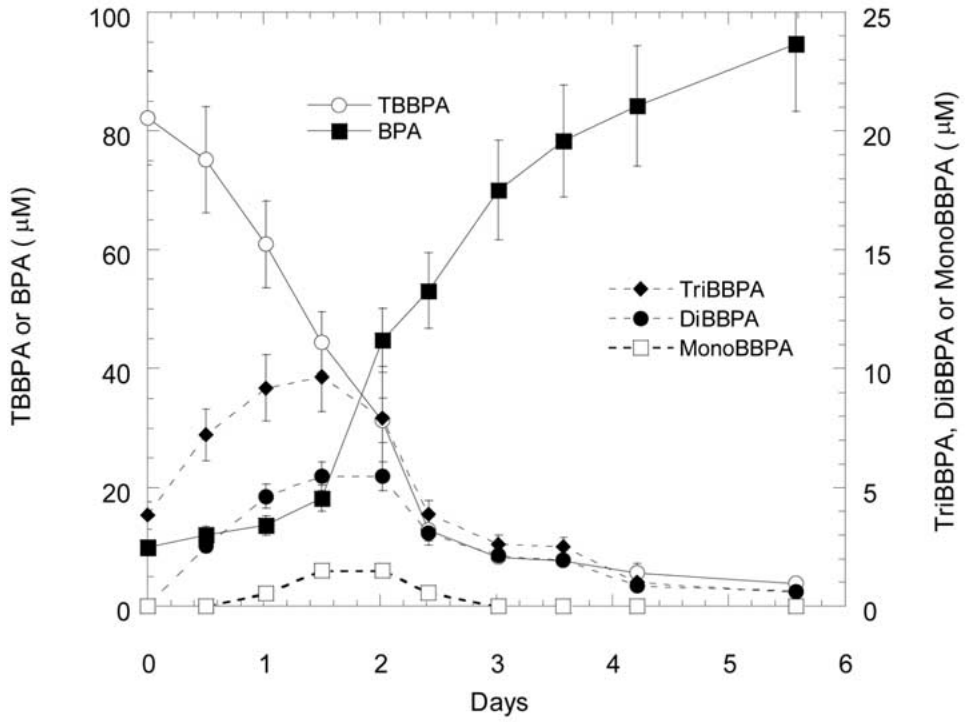


Figure 6. Kinetics of TBBPA debromination and formation of intermediate products in a batch culture containing 10% (wt/wt) gray chalk and supplemented with 0.2% (vol/vol) ethanol as an electron and carbon source. Inoculum for cultures (20%) was taken from SCBR containing gray chalk (10% wt/wt).

of dolomite, TCE was still dehalogenated. The authors concluded that pulverized dolomite supplied an electron donor, but when they provided the groundwater with the putative electron donor (hexadecane), no activity was obtained. In our culture, gray chalk without another carbon source did not stimulate TBBPA debromination. Thus, it is unlikely that gray chalk supplied both the electron donor and the carbon source to the debrominating bacteria. Reductive dehalogenating bacteria are often fastidious and the organic substrate of the solids might contain an important nutritional supplement for the debrominating bacteria that is consumed. Yet vitamin solution, yeast extract, pepton, trypton and spent medium of two different anaerobic mixed cultures (the indigenous population from the site and methanogenic culture from sediment of an anaerobic reservoir) could not supply this nutrient (Arbeli & Ronen, unpubl.).

The fact that stimulation of reductive dehalogenation by sediment and soil is more often observed in the case of the more hydrophobic molecule (e.g., PCBs: Wiegel & Wu 2000; 1,2,4-trichlorobenzene: Middeldorp et al. 1997) suggests that sorption of the molecule to the solids plays an important role. Wefer-Roehl et al. (2001) suggested that the higher sorption of the contaminants to gray chalk compared to white chalk (the same used in our study) is due to the higher concentration of organic mater in gray chalk, and its higher reductive state.

The effect of contaminant sorption on biodegradation depends on soil composition, the substrate property, and on the bacteria involved. A solid matrix can support microbial growth and biofilm formation. Strong sorption might decrease bio-availability and biodegradation (Alexander 2000), or increase bioavailability when the bacteria are also associated with the soil particles (Park et al. 2001; Fava & Piccolo 2002). Sorption of a toxic substrate to soil particles can reduce its toxic effect since the bacteria are exposed to lower concentrations of the toxin (Otte 1995). The fact that the lag phase increased when the initial concentration of TBBPA increased suggests that TBBPA is indeed toxic to the debrominating bacteria (Figure 7). Before enrichment in the SCBR, debromination activity had been lost following the first transfer (10% vol/vol) into a medium that contained sterile sediment.

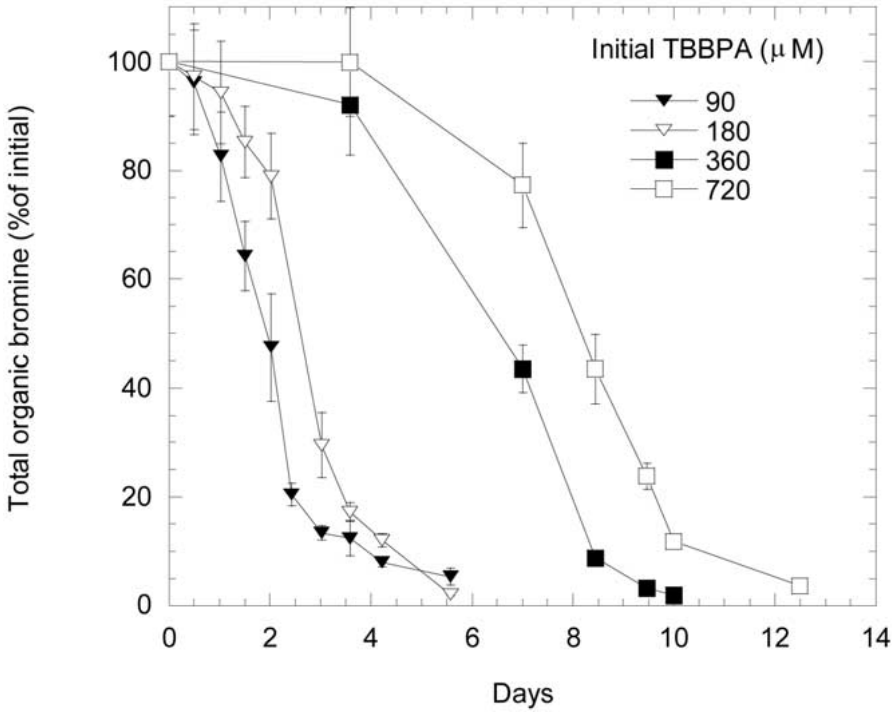


Figure 7. Effect of initial TBBPA concentrations on debromination in batch cultures (10% wt/wt gray chalk). The data presents the total organic bromine remaining on TBBPA and its debromination products. Ethanol (0.2% vol/vol) was used as an electron and carbon source. Inoculum for cultures (20%) was taken from SCBR containing gray chalk (10% wt/wt).

Similarly, low dilution (inoculation higher than 10% vol/vol) was essential for maintaining debromination activity of the solid-free culture. These two observations suggest that some minimum number of bacteria must be present for a successful inoculation, perhaps because of the toxicity of TBBPA.

The kinetics of debromination (Figures 6 and 7) as well as the gradual increase in the debrominating population in the enrichment culture, suggest that the TBBPA debrominating bacteria grow in mineral medium containing gray chalk, TBBPA, and ethanol as the sole carbon source. Whether growth is related to debromination is still unclear, but the fact that the debromination rate increases with the increase of initial TBBPA concentration hints that this might be the case. Debromination rates of our culture were much faster in comparison to the rate of dehalogenation of TBBPA in estuarine sediments (Voordeckers et al. 2002). This discrepancy is probably due to higher levels of dehalogenating bacteria in our enrichment culture, as well as to the addition of an electron donor to our cultures, probably a limiting factor in the estuarine sediments.

Conclusions

The current study resolved the metabolic pathway for the transformation of TBBPA to BPA. We were able to enrich the bacteria involved in reductive debromination of TBBPA. Sediment soil and gray chalk strongly stimulated debromination. However, we were also able to culture the bacteria in a solid-free mineral medium. Understanding the mechanism of stimulation by the sediment, soil, and gray chalk, and finding the exact factor that is essential for debromination, might facilitate the isolation of this bacterium. Furthermore, elucidation of these questions should have important implications in designing soil and sediment bioremediation processes.

Acknowledgements

We are grateful to Inna Kalikhman for the NMR analysis, and to Irene Urbanek Ruiz for her helpful comments on the manuscript. We also like to express our thanks to Ms. Zoe Grabiner for reading the manuscript.

References

Alexander M (2000) Aging, bioavailability, and overestimation of risk from environmental pollutants. Environ. Sci. Technol. 34: 4259–4265

Allard AS, Remberger M & Neilson AH (1987) Bacterial O-methylation of halogen-substituted phenols. Appl. Environ. Microbiol. 53: 839–845

Arnon S (1999) Transport of organic contaminants in desert soil-evaluation of contaminants flushing from a contaminated soil near Ramat-Hovav industrial park. M.Sc. thesis. Ben-Gurion University of the Negev, Sede-Boger Campus, Israel.

Bedard DL & Quensen JF (1995) Microbial reductive dechlorination of polychlorinated biphenyls. In: Young LY & Cerniglia CE (Eds) Microbial Transformation and Degradation of Toxic Organic Chemicals (pp 127–216). Wiley-Liss, New York

Boyle AW, Phelps CD & Young LY (1999) Isolation from estuarine sediment of a Desulfovibrio strain, which can grow on lactate coupled to the reductive dehalogenation of 2,4,6-tribromophenol. Appl. Environ. Microbiol. 65: 1133–1140

Brouwer A (1998) Structure-dependent multiple interaction of polyhalogenated aromatic hydrocarbons with the thyroid hormone system. Organohalogen Comp. 37: 225–228

Cutter L, Sowers KR & May HD (1998) Microbial dechlorination of 2,3,5,6-tetrachlorobiphenyl under anaerobic conditions in the absence of soil or sediment. Appl. Environ. Microbiol. 64: 2966–2969

de Boer JP, Wester G, Klamer HJC, Lewis WE & Boon JP (1998) Do flame retardants threaten ocean life? Nature 394: 28–29

de Wit CA (2000) Brominated Flame Retardants. Report 5065. Swedish Environmental Protection Agency

de Wit CA (2002) An overview of brominated flame retardants in the environment. Chemosphere 46: 583–624

Dolfing J & Beurskens JEM (1995) The microbial logic and environmental significance of reductive dehalogenation. Advances in Microbial Ecology 14: 143–206

Eriksson P, Jakobsson E & Fredriksson A (1998) Developmental neurotoxicity of brominated flame retardants, polybrominated diphenyl ether and tetrabromobisphenol A. Organohalogen Comp. 35: 375–377

Fantroussi SE, Naveau H & Agathos SN (1998) Anaerobic dechlorinating bacteria. Biotechnol. Prog. 14: 167–188

Fava F & Piccolo A (2002) Effect of humic substances on the bioavailability of polychlorinated biphenyls in a model soil. Biotechnol. Bioeng. 77: 204–211

Hohnstock-Ashe AM, Plummer SM, Yager RM, Baveye P & Madsen EL (2001) Further biogeochemical characterization of a trichloroethene contaminated fractures dolomite aquifer: Electron sources and microbial communities involved in reductive dehalogenation. Environ. Sci. Technol. 35: 4449–4456

Holliger C, Wohlfarth G & Diekert G (1999) Reductive dechlorination in the energy metabolism of anaerobic bacteria. FEMS Microbiol. Rev. 22: 383–398

Hooper K & McDonald TA (2000) The PBDEs: An emerging environmental challenge and another reason for breast-milk monitoring programs. Environ. Health Perspectives 108: 387–392

Middeldorp PJM, De Wolf J, Zehnder JB & Schraa G (1997) Enrichment and properties of a 1,2,4-trichlorobenzene-dechlorinating methanogenic consortium. Appl. Environ. Microbiol. 63: 1225–1229

Otte MP, Greer CW, Comeau Y & Samson R (1995) Effect of soil on the stimulation of pentachlorophenol biodegradation. In: Hichee RE, Hoeppel RE & Anderson DB (Eds) Bioremediation of Recalcitrant Organic Compounds. Bioremediation 3(7). Battele Press

Park JH, Kay D, Zhao X, Boyd SA & Voice TC (2001) Kinetic modelling of bioavailability for sorbed phase 2,4-Dichlorophenoxyacetic acid. J. Environ. Qual. 30: 1523–1527

Renner R. (2000) What fate for brominated fire retardants? Environ. Sci. Technol. 34: 222A–226A

Ronen Z & Abeliovich A (2000) Anaerobic-aerobic process for microbial degradation of Tetrabromobisphenol A. Appl. Environ. Microbiol. 66: 2372–2377

Rosner BM, McCarty PL & Spormann AM (1997) *In vitro* studies on reductive vinyl chloride dehalogenation by an anaerobic mixed culture. Appl. Environ. Microbiol. 63: 4139–4144

Sellstrom U & Jansson B (1995) Analysis of tetrabromobisphenol-A in product and environmental samples. Chemosphere 31: 3085–3092

Szymanaska JA, Piotrowski JK & Frydrych B (2000) Hepatotoxicity of tetrabromobisphenol A: Effects of repeated dosage in rats. Toxicology 142: 87–95

Thomsen C, Lundanes E & Becher G (2001) Brominated flame retardants in plasma samples from three different occupational groups in Norway. J. Environ. Monit. 3: 366–370

Voordeckers JW, Fennell DE, Jones K & Haggblom MM (2002) Anaerobic biotransformation of tetrabromobisphenol A, tetrachlorobisphenol A, and bisphenol A in estuarine sediments. Environ. Sci. Technol. 36: 696–701

Watanabe I, Kashimoto T & Tatsukawa R (1983) Identification of the flame retardant tetrabromobisphenol A in the river sediment and the mussel collected in Osaka. Bull. Environ. Contam. Toxicol. 31: 48–52

Wefer-Roehl A, Graber ER, Borisover MD, Adar E, Nativ R & Ronen Z (2001) Sorption of organic contaminants in a fractured chalk formation. Chemosphere 44: 1121–1130

Wiegel J & Wu Q (2000) Microbial reductive dehalogenation of polychlorinated biphenyls. FEMS Microb. Ecol. 32: 1–15

Wittich RM. (1998) Degradation of dioxin-like compounds by microorganisms. Appl. Microbiol. Biotechnol. 49: 489–499

Wu Q, Sowers KR & May HD (2000) Establishment of a polychlorinated biphenyl-dechlorinating microbial consortium, specific for doubly flanked chlorines, in a defined, sediment-free medium. Appl. Environ. Microbiol. 66: 49–53