4-Methylphenol produced in freshwater sediment microcosms is not a bisphenol A metabolite

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\textbf{Highlights}

- Partial disappearance of bisphenol A (BPA) observed in anoxic sediment microcosms.
- 4-Methylphenol (4-MP) detected as putative degradation intermediate.
- Experiments using \textsuperscript{13}C-labeled BPA demonstrated that 4-MP was not derived from BPA.
- The formation of 4-MP to suggest BPA degradation must be carefully interpreted.

\textbf{Graphical Abstract}

\textbf{Abstract}

4-Methylphenol (4-MP), a putative bisphenol A (BPA) degradation intermediate, was detected at concentrations reaching 2.1 mg L\textsuperscript{-1} in anoxic microcosms containing 10 mg L\textsuperscript{-1} BPA and 5 g of freshwater sediment material collected from four geographically distinct locations and amended with nitrate, nitrite, ferric iron, or bicarbonate as electron acceptors. 4-MP accumulation was transient, and 4-MP degradation was observed under all redox conditions tested. 4-MP was not detected in microcosms not amended with BPA. Unexpectedly, incubations with \textsuperscript{13}C-labeled BPA failed to produce \textsuperscript{13}C-labeled 4-MP suggesting that 4-MP was not derived from BPA. The detection of 4-MP in live microcosms amended with lactate, but not containing BPA corroborated that BPA was not the source of 4-MP. These findings demonstrate that the transient formation of 4-MP as a possible BPA degradation intermediate must be interpreted cautiously, as microbial activity in streambed microcosms may generate 4-MP from sediment-associated organic material.

\textbf{1. Introduction}

Bisphenol A, 2,2-bis(4-hydroxyphenyl)propane (BPA) is used to manufacture polycarbonate, epoxy resins, flame retardants, and
lacquer coatings on food cans, as well as other products (Staples et al., 1998). The estimated global production exceeds 5.2 Mt per yr, making BPA one of the highest production volume chemicals in the world (The Dow Chemical Company, 2013). As a consequence of high-capacity use, BPA has been detected in environmental systems including surface waters, sediments, and groundwater (Bolz et al., 2001; Heemken et al., 2001). Bearing structural resemblance to estrogens, BPA can bind to the estrogen receptor and is considered weakly estrogenic. The fate of BPA in the environment has been studied in recent years. Several aerobic BPA degraders have been reported that use BPA as the sole source of carbon and energy (Lobos et al., 1992; Spiwacks et al., 1994; Ike et al., 1995; Kang and Kondo, 2002; Kolvenbach et al., 2007; Fischer et al., 2010). Based on the identification of intermediates, a few different aerobic BPA degradation pathways have been proposed (Spiwacks et al., 1994; Kolvenbach et al., 2007; Fischer et al., 2010). A recent survey of 107 soil samples demonstrated aerobic BPA degradation in 85 samples, and 26 BPA-degrading isolates were obtained belonging to the genera Pseudomonas, Klebsiella, Pandoraea, Alcaligenes, Enterobacter, Serratia, Bacillus, Bordetella, and Sphingomonas (Matsumura et al., 2009). These findings suggest that diverse bacterial groups are capable of degrading BPA under aerobic conditions.

A significant mass of BPA resides in anoxic sediments (Bolz et al., 2001; Heemken et al., 2001), but very little is known about the fate of BPA under anoxic conditions. Several studies investigating microbial BPA degradation under anoxic conditions have concluded that BPA is recalcitrant and undergoes "little or no" biodegradation in the absence of oxygen (Kang and Kondo, 2002, 2005; Voordecker et al., 2002). No microbial BPA degradation was observed in anoxic microcosms established with freshwater sediment (Yang et al., 2003), marine sediments (Yang and Kookana, 2003), and soil (Yang and Kookana, 2005). Halogenated BPA were reductively dehalogenated to BPA in estuarine sediment microcosms, but no further degradation was observed under different redox conditions (Voordecker et al., 2002). Experimental evidence supporting anaerobic BPA degradation is scarce. Chiou (2010) reported a 14% loss of BPA concentration after a 120 d incubation period with anoxic river sediment. Similarly, Kang and Kondo (2002) reported a 10% loss of initial BPA added to anoxic river water microcosms, and (Patterson et al., 2010) reported BPA removal under denitrifying conditions. At best, these studies demonstrated BPA disappearance but BPA degradation intermediates and end-products were not identified or quantified. To predict the environmental fate of BPA in anoxic environments, more detailed studies of BPA degradation under anoxic conditions are needed. This study detected 4-methylphenol (4-MP) in anoxic sediment microcosms as a possible BPA degradation intermediate but experiments using 13C-labeled BPA implicated that 4-MP was derived from another source, presumably sediment-associated organic matter, emphasizing the need for careful results interpretation regarding the environmental fate of BPA or similar phenolic compounds.

2. Materials and methods

2.1. Chemicals

BPA (>99% purity) was purchased from Sigma Aldrich (St. Louis, MO), 4-MP (>98% purity) was purchased from Acros Organics (Fair Lawn, NJ), and L-tyrosine (99% purity) was purchased from MP Biomedicals (Chicago, IL). Specifically labeled versions of BPA, 2,2-bis[4-hydroxyphenyl][1,3-13C2]propane (13C2-BPA) and 2,2-bis[4-hydroxyphenyl][13C6]propane (13C6-BPA) were synthesized by condensation of [1,3-13C2] and [13C6]acetone, respectively, with five equivalents of phenol in the presence of a cation exchange resin catalyst, Amberlyst 15, which was purchased from Rohm and Haas (Philadelphia, PA) (Singh, 1992). The labeled acetone (>99% purity) was purchased from Cambridge Isotopes Laboratories (Andover, MA). Aromatic ring labeled BPA, 2,2-bis[4-hydroxy[13C5]phenyl]propane (13C6-2-BPA, >99% purity) was also purchased from Cambridge Isotopes Laboratories.

2.2. Microcosm setup

Microcosms were established in 60 mL serum bottles closed with black butyl rubber stoppers (Geo-Microbial Technologies, Ochelata, OK). Sediment samples were collected from four geographically distinct locations (latitude, longitude), including the Third Creek (35.949284, -83.939861), the Partnach Gorge (47.459198, 11.127777), the Neckar River (48.780193, 9.245422), and the Hainbach Creek (48.776559, 9.300439). The Third Creek location has a history of contamination of chlorinated solvents, the Neckar River flows along industrial areas, and the Partnach Gorge and Hainbach Creek are considered pristine water bodies. Each serum bottle received 5 g of sediment material (wet weight) and reduced (0.2 mM L-cysteine and 0.2 mM sodium sulfide) mineral salts medium to achieve a total volume of 30 mL in each vessel as well as a single addition of 2 mM of lactate as a readily fermentable substrate to ensure rapid establishment of anoxic conditions. BPA was added to the microcosms to a final aqueous phase concentration of 10 mg L\(^{-1}\) (44 µM). Autoclaved control microcosms with BPA, and live microcosms without BPA, electron acceptor, or lactate were established for each sediment sample. All manipulations were performed inside an anoxic chamber (Coy Laboratories Products, Ann Arbor, MI) containing a nitrogen/hydrogen (97/3; v/v) atmosphere. The microcosms were incubated at room temperature in the dark without shaking. To establish different redox conditions, electron acceptors including nitrate (2 mM), nitrite (0.5 mM), amorphous ferric oxyhydroxide (FeOOH, 10 mM, nominal concentration), ferric citrate (5 mM), sulfate (10 mM), and bicarbonate (30 mM) were added from anoxic, sterilized stock solutions at the concentrations indicated in parentheses. FeOOH was prepared following an established procedure (Lovley and Phillips, 1988). Electron acceptors were replenished by syringe when depleted. To test BPA degradation under anoxic conditions, microcosms were established using the same mineral salts medium except that the bicarbonate buffer system was replaced with HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (10 mM, pH 7.0) and the reductants and lactate were omitted. These microcosms were incubated in the dark, and the headspace was purged with filter-sterilized air every second day. Additional microcosms were prepared with the Third Creek sediment to access the biodegradability of 4-MP. Ten mg L\(^{-1}\) of 4-MP and 2 mM of lactate were added and electron acceptors were replenished as above.

2.3. Analytical procedures

The amount of BPA associated with the sediments was determined after sampling by an ultrasonic solvent extraction method (Xu et al., 2008) with some modifications. In brief, 5 g of sediment was mixed with 10 mL of acetone/ethyl acetate (50:50, v/v), sonicated at 20 kHz for 15 min using a Branson Sonifier 250 (Branson Ultrasonic, Danbury, CT), and centrifuged at 5000 g for 10 min. The extracts from three consecutive solvent extractions were combined and evaporated to dryness under a stream of filtered (0.25 µm) nitrogen. The residue was dissolved in 10 mL water/acetoniitrile (50:50, v/v) and subjected to HPLC analysis. The adsorption capacity of BPA on Third Creek sediment was also evaluated. Triplicate 60 mL vessels that received 5 g of autoclaved sediment,
30 mL of mineral salt medium and 300 µg (10 mg L\(^{-1}\)) of BPA each were incubated for 5 d. The slurry was removed from the vessels and transferred into 30 mL glass centrifuge tubes. The supernatants obtained after centrifugation at 5000 g for 10 min and analyzed by HPLC.

An Agilent 1200 Series HPLC equipped with a diode array detector (DAD) and fluorescence detector (FLD) was used for the detection and quantification of BPA, its potential metabolites, and L-tyrosine. Separation was achieved on an Agilent Eclipse XDB C18 column (4.6 mm × 150 mm, 5 µm) using isocratic elution with 1 mL min\(^{-1}\) of acetonitrile/water (50:50, v/v). The FLD was set at excitation and emission wavelengths of 226 and 310 nm, respectively. The DAD recorded the entire UV/Vis absorption spectra of peaks ranging from 190 to 400 nm. Ferric and ferrous iron were measured using the ferrozine assay (Lin et al., 2009), and other electron acceptors were monitored by ion chromatography using a Dionex ICS 2100 system equipped with an 4 mm hydroxide-selective anion-exchange column (Sunnyvale, CA).

To identify putative BPA transformation products, liquid–liquid extraction coupled with GC–MS was used. Three water-immiscible solvents including dichloromethane, ethyl ether, and ethyl acetate were employed for separation and purification of the metabolites. For metabolite extraction, a 2 L Pyrex glass vessel received 1 L of bicarbonate-buffered medium and 20 g of Third Creek sediment, and was incubated inside the anoxic chamber. After 30 d of incubation, 18 mL of sample was withdrawn from the vessel, and mixed with 2 mL of each solvent. The mixtures were shaken for 1 h and allowed to settle for 10 min. The organic phases were separated using a glass pipette and evaporated under a stream of nitrogen. The residues were dissolved in 100 µL acetonitrile for GC–MS and HPLC analyses. Compound identity was confirmed by comparing HPLC retention times, UV/Vis absorption spectra, and mass spectra with authentic standards.

3. Results and discussion

3.1. BPA associated with sediment

No BPA was detected in extracts from the untreated sediment samples indicating that no extractable BPA was associated with any of the sediments used in this study. Following the addition of BPA, 20–50% sorbed to the solids depending on the origin of the sediment (Table 1). The ultrasonic solvent extraction method efficiently desorbed BPA from the sediments with recoveries of 96–100% (Table 1).

3.2. Fate of BPA in sediment microcosms

Aqueous phase BPA concentration profiles in microcosms established with the Third Creek sediment demonstrated an initial decrease in BPA concentrations, which was expected based on the sorption experiments (Fig. 1). Under oxic conditions, aqueous phase BPA was completely degraded within 1 week of incubation. Aerobic BPA degradation has been demonstrated previously (Kang et al., 2002; Ying et al., 2003; Sarmah and Northcott, 2008; Matsumura et al., 2009) and these findings indicate that microorganisms capable of oxygen-dependent BPA catabolism are present in freshwater sediments. In the absence of oxygen, none of the live microcosms showed a statistically significant loss of aqueous phase BPA compared to negative control incubations (\(p > 0.05\), Fig. 1), although the mean concentrations were slightly lower than those of the negative control incubations.

Interestingly, a putative BPA transformation product was detected in all microcosms except those amended with sulfate or incubated under oxic conditions (Fig. 2). The same peak was observed in BPA-amended microcosms established with the other three freshwater sediment materials after a 1 month incubation period under the same conditions. The transformation product was not detected in live control microcosms without BPA suggesting that this compound was a possible BPA transformation product. The formation of this putative BPA metabolite did not coincide with a decrease in aqueous BPA concentrations, which remained near 5 mg L\(^{-1}\). We initially concluded that the rates of BPA desorption from the sediment exceeded the BPA degradation rates, or that sorbed BPA was preferentially degraded.

Table 1

<table>
<thead>
<tr>
<th>Sampling location</th>
<th>Third Creek</th>
<th>Partnach Gorge</th>
<th>Neckar River</th>
<th>Hainbach Creek</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Adsorption</td>
<td>53 (4)</td>
<td>22 (3)</td>
<td>34 (3)</td>
<td>50 (4)</td>
</tr>
<tr>
<td>% Recovery</td>
<td>98 (1)</td>
<td>99 (1)</td>
<td>97 (2)</td>
<td>100 (3)</td>
</tr>
</tbody>
</table>

Values in parenthesis represent the standard deviations of three replicates.
After a 1 year incubation period, each vessel was sacrificed to determine the total amount of BPA remaining (Table 2). In the microcosms incubated under oxic conditions, no BPA was detected associated with the sediment indicating complete removal. In contrast, BPA was detected in all microcosms incubated without oxygen. A 2-tailed t-test was used to determine the statistical significance of the discrepancies between live treatments and negative controls (p < 0.01, Table 2) suggesting a process that reduced the amount of extractable BPA was present in live incubations only. For example, aging and the formation of covalently bound residues can affect the extraction efficiency (Northcott and Jones, 2000; Fent et al., 2003; Nowak et al., 2011). Alternatively, reactive mineral-mediated (Lin et al., 2009) or microbial BPA transformation may have occurred.

### Table 2

Mass balance of BPA after 1 year of incubation period.

<table>
<thead>
<tr>
<th>Third Creek sediment</th>
<th>Partnach Gorge sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BPA-S</strong></td>
<td><strong>BPA-L</strong></td>
</tr>
<tr>
<td>Nitrate</td>
<td>107.1</td>
</tr>
<tr>
<td>Nitrite</td>
<td>103.9</td>
</tr>
<tr>
<td>Sulfate</td>
<td>111.7</td>
</tr>
<tr>
<td>Methanogenic</td>
<td>120.0</td>
</tr>
<tr>
<td>FeOOH</td>
<td>108.3</td>
</tr>
<tr>
<td>FeCit</td>
<td>104.3</td>
</tr>
<tr>
<td>Negative</td>
<td>115.9</td>
</tr>
<tr>
<td>Neckar River sediment</td>
<td></td>
</tr>
<tr>
<td><strong>BPA-S</strong></td>
<td><strong>BPA-L</strong></td>
</tr>
<tr>
<td>Nitrate</td>
<td>123.4</td>
</tr>
<tr>
<td>Nitrite</td>
<td>129.0</td>
</tr>
<tr>
<td>Sulfate</td>
<td>131.4</td>
</tr>
<tr>
<td>Methanogenic</td>
<td>126.7</td>
</tr>
<tr>
<td>FeOOH</td>
<td>128.2</td>
</tr>
<tr>
<td>FeCit</td>
<td>116.7</td>
</tr>
<tr>
<td>Negative</td>
<td>132.0</td>
</tr>
</tbody>
</table>

The unit is μg. Values in parenthesis represent the standard deviations of duplicate samples. BPA-S represents the total amount of BPA extracted from the solid phase, and BPA-L represents that of BPA extracted from the liquid phase. Discrepancy was obtained by subtracting BPA-S and BPA-L from the initial amount (300 μg) added to each bottle.

3.3. Identification of a putative BPA metabolite

In order to identify the putative BPA degradation intermediate, liquid–liquid extraction procedures were evaluated. Among the solvents used for the extraction, ethyl ether showed the best extraction yields. The GC–MS analysis of the extract identified the putative metabolite as 4-MP (Fig. 3). The identity of 4-MP was confirmed by comparing the HPLC retention times, UV–Vis absorption spectra, and mass spectra with authentic 4-MP. The maximum 4-MP concentrations detected in the Third Creek sediment microcosms ranged from 6.3 × 10^−1 to 20.8 × 10^−3 mg L^−1 depending on the electron acceptor provided. A hypothetical pathway to explain the formation of 4-MP from BPA is shown in Fig. SM1 of Supplementary Material (SM). The oxidation of one of the phenol rings can lead to a cationic intermediate, which undergoes a methyl shift (i.e., Wagner–Meerwein rearrangement). Subsequent C–C bond cleavage and rearomatization can form the putative intermediate 4-MP. To test if this pathway contributes to 4-MP formation in the microcosms, 13C2–BPA with only methyl carbons labeled, 13C3–BPA with all non-aromatic carbons labeled, and 13C12–BPA with all aromatic carbons labeled were added to Third Creek sediment microcosms. Material labeled only outside of the aromatic rings was not commercially available, and 13C2–BPA and 13C12–BPA were synthesized from phenol and 13C-acetone with yields of 90–92%. The formation of 4-MP was observed in the microcosms supplied with 13C-labeled BPA; however, no label was detected in 4-MP indicating that BPA was not the source of 4-MP. A series of control experiments demonstrated that 4-MP did not originate from laboratory plastic materials (e.g., syringes) chemicals used for medium preparation, and implicated the sediments as possible sources. To assess if the sediments were the source of 4-MP, microcosms without organic amendments and electron acceptors were monitored. None of these microcosms produced 4-MP. Another set of microcosms received 2 mM of lactate but no BPA or electron acceptors. Unexpectedly, after a 1 month incubation period, the formation of 4-MP was observed in these microcosms. This finding indicated that 4-MP formation from the sediment required the addition of an electron donor (e.g., lactate). No 4-MP was detected in the lactate stock solution or the sediment, indicating that 4-MP was formed during microcosm incubation.

4-MP was not detected in the live microcosms amended with sulfate (Fig. 4) suggesting that (i) 4-MP was not generated from the sediment-associated organic matter in the presence of sulfate.

![Fig. 3. Full scan GC–MS chromatogram and the mass spectrum of 4-MP (inset). The arrow indicates the retention time of 4-MP.](Image)
or that (ii) 4-MP was rapidly degraded in the presence of sulfate and evaded detection. The microcosms amended with 4-MP showed that this molecule was degraded under all redox conditions tested (Fig. 5), as has been demonstrated previously (Bak and Widdel, 1986; Tschech and Fuchs, 1987; Lovley and Lonergan, 1990; Rabus et al., 1993; Londry et al., 1997). The rates of 4-MP degradation varied depending on the electron acceptor provided, and the degradation was significantly faster in nitrate-compared to sulfate-amended microcosms (Fig. 5). This observation suggested that 4-MP formation from sediment organic matter was repressed in the presence of sulfate, possibly due to sulfide toxicity (Gibson, 1990).

Several anaerobic intestinal bacteria such as Lactobacillus sp. (Yokoyama and Carlson, 1981) and Clostridium difficile (D’Ari and Barker, 1985) can form 4-MP from l-tyrosine via decarboxylation in the absence of oxygen, but 4-MP formation from soils or sediments has not been reported. Furthermore, l-tyrosine was not observed in any microcosm (detection limit of 50 μg L⁻¹) (data not shown). Lactate addition and microbial activity were essential for 4-MP formation in the microcosms, suggesting microbial biomass or sediment-associated organic matter as possible sources of 4-MP; however, the detailed formation mechanism(s) remain to be determined. Since the formation of 4-MP was observed in microcosms established with sediments collected from different geographical locations, it is likely that the mechanisms and pathways leading to 4-MP formation are common to freshwater sediments. The ¹³C-labeling experiments excluded BPA as a source of 4-MP, and our findings emphasize that the detection of possible aromatic BPA degradation products such as 4-MP in sediments or sediment-derived microcosms must be interpreted carefully to avoid erroneous conclusions regarding the fate of BPA or similar compounds.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2014.09.008.

References


Radar, P., Stäuber, F., 2008. Detection and identification of aromatic BPA degradation products such as 4-MP in sediments or sediment-derived microcosms must be interpreted carefully to avoid erroneous conclusions regarding the fate of BPA or similar compounds.


