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Microbial O-Methylation of the Flame Retardant Tetrabromobisphenol-A

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We demonstrated the O-methylation of tetrabromobisphenol-A (TBBPA) [4,4′-isopropylidenebis (2,6-dibromophenol)] to its mono- and dimethyl ether derivatives by microorganisms present in different sediments. A most probable number assay of a marsh sediment suggested that up to 10% of the total aerobic heterotrophs may be capable of O-methylation. Although TBBPA dimethyl ether is not produced in industry, it has been detected in terrestrial and aquatic sediments. Our study supports the hypothesis that TBBPA dimethyl ether is a product of microbial O-methylation. The O-methylation of TBBPA, as well as its analog, tetrachlorobisphenol-A (TCBPA), was also demonstrated in cultures of two chlorophenol-metabolizing bacteria, *Mycobacterium fortuitum* CG-2 and *Mycobacterium chlorophenolicum* PCP-1. These strains also mediated the O-methylation of 2,6-dibromophenol and 2,6-dichlorophenol, analogs of TBBPA and TCBPA, to their corresponding anisoles, but 2,6-fluorophenol was not transformed. Due to the addition of hydrophobic methyl groups, O-methylated derivatives are more lipophilic, increasing the probability of bioaccumulation in the food chain. Future research regarding the toxicological effects of the O-methylated derivatives of TBBPA is recommended and will further elucidate potential risks to environmental and human health.

Introduction

Brominated flame-retardants are used extensively as additives for fire prevention in a wide range of consumer products, including plastics, textiles, and electronics (*1*). The most common brominated flame retardant, tetrabromobisphenol-A (TBBPA) [4,4′-isopropylidenebis (2,6-dibromophenol)], with an annual global market in the range of over 130 million kg in 2002 and over 170 million kg in 2004 (*2*), is predominantly used in the production of circuit boards. Because of their widespread use, brominated flame retardants have disseminated worldwide as environmental contaminants. TBBPA is recalcitrant and has been detected in soils and sediments around the world (*1, 3–7*).

TBBPA has been detected in aquatic and terrestrial biota in multiple studies (see refs *3* and *8* for reviews). Although the potential for TBBPA volatilization is low (*9*), human exposure to TBBPA is reflected in the detection of TBBPA in serum from computer technicians, electronic assembly workers, laboratory personnel, and the general population (*10–12*). It is unclear whether such exposure to the general population is the result of direct contact with TBBPA-containing products or indirect exposure to environmental contamination from terrestrial or atmospheric sources. Regardless of this uncertainty, the rising global market demand for TBBPA unfortunately ensures increasing contamination in the future. There is thus a heightened concern regarding the environmental fate and human exposure to TBBPA and other brominated flame retardants.

At high concentrations, TBBPA is acutely toxic to a variety of organisms (*9*). In particular, aquatic organisms such as fish, algae, and crustaceans are susceptible to TBBPA-induced toxicity. Evidence for low-dose toxicity is limited, although *in vitro* assays have linked TBBPA exposure to free radical production (*13*), cell death (*14*), and inhibition of neurotransmitter transport (*15*). Numerous studies have implicated TBBPA as a disruptor of thyroid and reproductive hormone function (*16–19*). Although knowledge of TBBPA toxicology is increasing, our understanding is still quite limited and many reports in the literature are conflicting (see ref (*8*)).

Given the widespread use of TBBPA in computers, plastics, electronics, and other consumer products, continued demand for the compound is expected. With the increasing use of TBBPA comes a heightened need to elucidate its environmental fate. Microorganisms are the major mediators for the cycling of halogenated organic compounds in the environment (*20*). It is therefore essential to fully delineate and understand the diverse microbial roles in biodegradation and biotransformation processes. Laboratory and field studies indicate that TBBPA degrades very slowly in the environment. Biodegradation of TBBPA under aerobic conditions has not been demonstrated. TBBPA is, however, reductively dehalogenated under anaerobic conditions to yield bisphenol A (*21–26*). Although bisphenol A is biodegradable under aerobic conditions (*24, 27–29*) it is highly recalcitrant in anaerobic sediments (*21*).

Biotransformation, an alternative to degradation, alters the compound without making substantial changes to the carbon skeleton of the substrate. Microbial O-methylation is one such biotransformation reaction commonly observed for many halogenated phenolic compounds (*30–32*). The microbial biotransformation products may differ greatly in their chemical characteristics (e.g., water solubility, partitioning onto solids) and more importantly their bioaccumulation potential and toxicity. Although some data exist on the accumulation and toxicology of TBBPA, little effort has been made to analyze potential metabolites that may exist in natural ecosystems. We thus set out to determine whether TBBPA and its analogs may be O-methylated in contaminated sediments and soils. Our study demonstrates that O-methylating microorganisms appear to be prevalent in these environments. O-Methylation of TBBPA and TCBPA and halogenated phenol analogs was also demonstrated with two soil bacteria, *Mycobacterium fortuitum* CG-2 and *Mycobacterium chlorophenolicum* PCP-1, previously shown to O-methylate chlorinated phenolics (*33*). Discerning the possible microbial metabolites of TBBPA, as well as the prevalence of the O-methylation reaction, is important for evaluating the environmental fate and health effects of brominated flame retardants.

Experimental Procedures

Substrates. The substrates utilized in this study were tetrabromobisphenol-A (TBBPA), tetrachlorobisphenol-A

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(TCBPA), 2,6-dibromophenol (DBP), 2,6-dichlorophenol (DCP), 2,6-difluorophenol (DFP), and tetrabromobisphenol-A dimethyl ether. With the exception of the dimethyl ether, the compounds were produced by Sigma Aldrich and of >98% purity.

Synthesis of TBBPA Dimethyl Ether. TBBPA (in acetone) was reacted with excess methyl iodide (CH_3I) and potassium carbonate (K_2CO_3) under reflux conditions following the method of Tulp (*34*). After reflux the reaction yield was determined by gas chromatography−mass spectrometry (GC−MS; see Analysis section). The acetone solution was decanted into a vial and evaporated to dryness, yielding TBBPA dimethyl ether crystals of >99% purity as determined by GC-MS. A stock solution of TBBPA dimethyl ether was prepared in acetone and utilized for further experiments.

Culture Conditions. *Mycobacterium chlorophenolicum* strain PCP-1 and *Mycobacterium fortuitum* strain CG-2 were originally isolated from chlorophenol-contaminated soil and sludge (*33, 35, 36*). The bacteria were grown in tryptic soy broth (30 g L^{-1}) at 28 °C under gentle agitation for 5 days. The cells were collected by centrifugation and resuspended to a density of 10^8 to 10^9 cells mL^{-1} in a basic mineral salts medium supplemented with 0.1% glucose as a carbon source. The mineral salts medium was composed of (in g L^{-1}): 2.9 K_2HPO_4, 2.1 KH_2PO_4, 2.0 NH_4Cl, 0.4 $MgSO_4$•$7H_2O$, 0.03 NaCl, 0.003 $CaCl_2$, and 0.001 $FeSO_4$•$7H_2O$. Halogenated substrates were added to a series of tubes from acetone stocks to give a final concentration of 50 μM. The acetone was evaporated and the tubes were inoculated with 2 mL of culture media. At various time points, these tubes were sacrificed and analyzed by GC−MS for metabolite formation.

Establishment of Sediment Microcosms. Sediments for two different sites, Kearny Marsh, NJ (collected in July 2004) and the Kymijoki River, Finland (collected in 2001) were stored at 4 °C until used for preparation of aerobic microcosms. Prior to setup, an acetone stock of TBBPA was added to the microcosm flasks to give a final nominal concentration of 10 μM and evaporated to dryness, leaving a coating of TBBPA on the vial. The microcosms were then filled with a slurry of 20% sediment and 80% distilled H_2O (1 mL sediment/4 mL water) and monitored for O-methylation over time.

MPN Assay. The number of O-methylating microbes g^{-1} sediment was determined for the Kearny Marsh sediment using a most probable number (MPN) assay (*37, 38*). A five replicate MPN series from 10^{-1} to 10^{-9} was prepared. Each tube contained TBBPA (20 μM) and 2 mL of diluted sediment in mineral salts media (see composition above) containing 0.1% yeast extract as a complex carbon source. After incubation at 28 °C for 45 days, the tubes with visual turbidity were scored positive for heterotrophs (total number of heterotrophs). The tubes were then extracted with 1 mL of hexane and analyzed for O-methylation using GC−MS. Based on detection limits for TBBPA and O-methyl derivatives and background signal in negative controls any conversion >1.0% of TBBPA to methylated forms was scored as positive for O-methylation. The most probable number of total heterotrophs and O-methylating microorganisms was then estimated using the most probable number calculator of Klee (*39*).

Analysis. Samples for analysis of TBBPA, TCBPA, and O-methyl derivatives by gas chromatography−mass spectrometry were extracted in hexane (1:1) for 1 h. The other substrates, 2,6-DBP, 2,6-DCP, and 2,6-DFP, were first acetylated with acetic anhydride in a carbonate buffer prior to extraction as described previously (*33*). These samples were analyzed on an Agilent HP 6890 GC−MS equipped with an HP-5MS column (30 m × 250 μm i.d., nominal film thickness 0.25 μm). The carrier gas was helium at a flow of 1 mL min^{-1}. The temperature ramp began at 60 °C for 1 min, then increased by 20 °C min^{-1} to a final temperature of 280 °C. Substrates and metabolites were identified through mass spectrometry equipped to scan from 35 to 600 m/z. The relative abundance of 2,6-DBP, 2,6-DCP, and 2,6-DFP (as acetyl derivatives) and their O-methylated metabolites was quantified from the total ion chromatograms. To quantify the relative abundance of TBBPA and its metabolites, selected ion monitoring (SIM) of key mass fragments was utilized: 529 (TBBPA), 543 (TBBPA monomethyl ether), and 557 (TBBPA dimethyl ether). The abundances of TBBPA and metabolites were determined from the SIM abundance corrected for the relative abundance of the selected ion in the total mass spectrum of each compound.

Results

Transformation of TBBPA and Production of Two Metabolites Mediated by Mycobacterium Strains. Since a variety of bacteria and fungi have previously been shown to O-methylate chlorinated phenols, we wanted to determine whether they may also mediate the transformation of the phenolic flame retardant, TBBPA. We therefore tested the ability of two *Mycobacterium* strains, CG-2 and PCP-1, to O-methylate TBBPA.

TBBPA was added to dense cultures (10^8 to 10^9 cells mL^{-1}) at a concentration of 50 μM. GC−MS analysis of cultures of *Mycobacterium fortuitum* CG-2 indicated the complete removal of TBBPA after 10 days. This loss of TBBPA correlated with the production of increasing concentrations of two distinct metabolites. A gas chromatogram of TBBPA and its metabolites, as well as mass spectra identifications, is shown in Figure 1a. Based on the bromine isotope pattern and key mass fragments, the metabolites were identified as the methyl and dimethyl ether derivatives of TBBPA and were identical to the chemically synthesized derivatives. The biotransformation of TBBPA with production of TBBPA mono- and dimethyl ethers by *M. fortuitum* strain CG-2 is shown in Figure 2a. Heat-killed controls showed no TBBPA loss or metabolite production, implicating the bacterial strain as the catalyst for O-methylation.

Cultures of *Mycobacterium chlorophenolicum* PCP-1 also mediated the transformation of TBBPA coupled to metabolite production but at a slower rate (Figure 2c). Mass spectral analysis again identified the two metabolites as TBBPA methyl and dimethyl ether, respectively.

Bacterial Mediated O-Methylation of TCBPA. TCBPA, the chlorine substituted analog of TBBPA, was similarly transformed by cultures of *Mycobacterium fortuitum* CG-2 and *Mycobacterium chlorophenolicum* PCP-1. The loss of TCBPA correlated to the production of two metabolites identified through mass spectral analysis of key fragments as TCBPA mono- and dimethyl ether, respectively (Figure 1b).

Strains CG-2 and PCP-1 O-methylated 50 μM TCBPA at a rate similar to that observed for TBBPA. After 10 days, *Mycobacterium fortuitum* CG-2 mediated the complete conversion of TCBPA into TCBPA monomethyl and dimethyl ether (Figure 2b). *Mycobacterium chlorophenolicum* PCP-1 also initiated the transformation of TCBPA into its methyl-ether derivatives (Figure 2d). No transformation of TCBPA was observed in sterile controls confirming the microbiological basis for the O-methylation reaction.

Effect of Halogen Substituents on O-Methylation. To determine the effect of steric changes of the halogen substituent on the rate of O-methylation, the TBBPA analogs 2,6-DBP, 2,6-DCP, and 2,6-DFP were tested with the two O-methylating *Mycobacterium* strains. *M. fortuitum* strain CG-2 mediated the transformation of 2,6-DBP coupled with the accumulation of a metabolite (Figure 3a) identified by mass spectral analysis as 2,6-dibromoanisole, the methylated derivative of 2,6-DBP. An identical conversion of 2,6-DCP

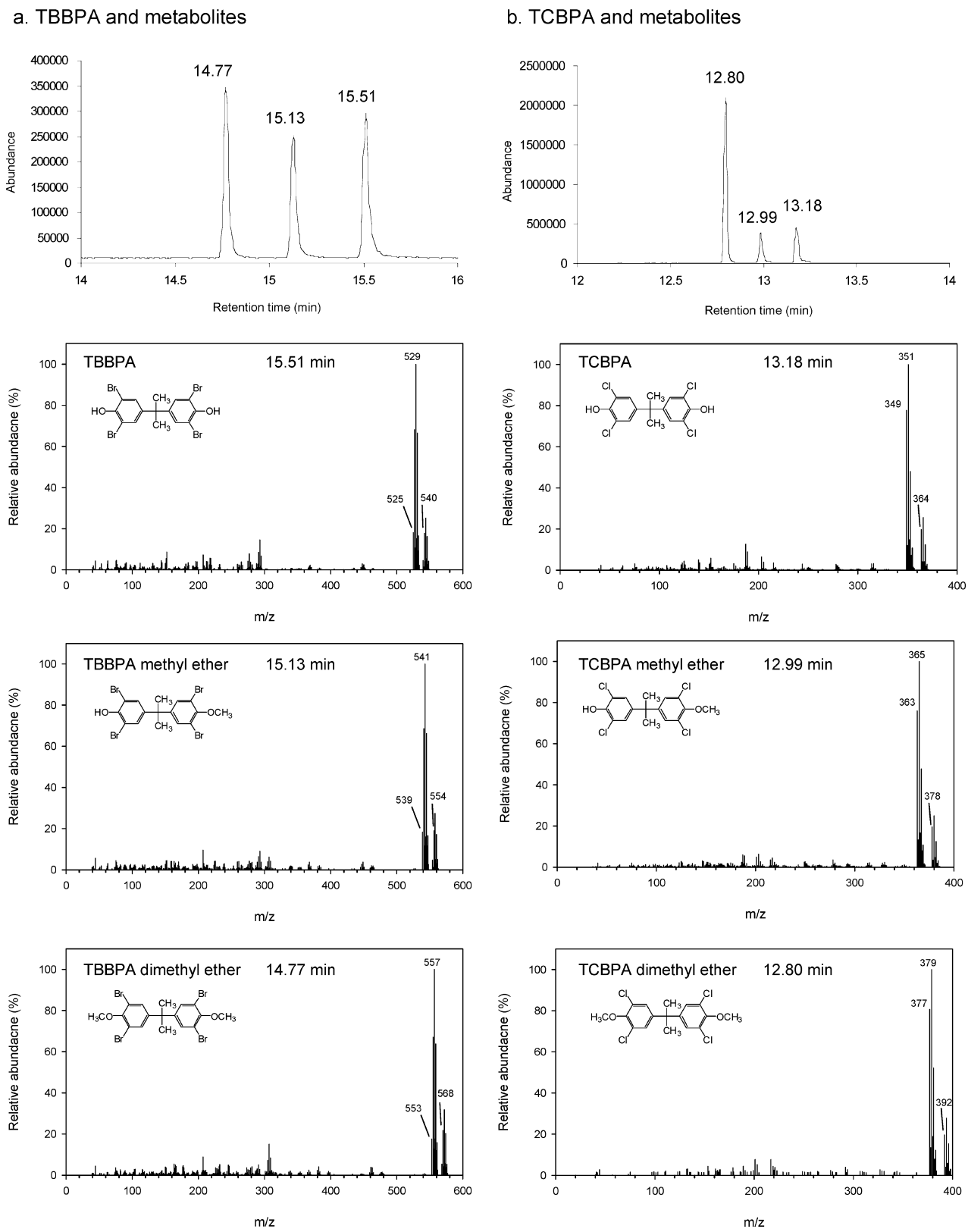

FIGURE 1. Gas chromatography and mass spectra of TBBPA (a) and TCBPA (b) and their O-methylated metabolites.

into 2,6-dichloroanisole was observed (Figure 3b). *M. cholorophenolicum* strain PCP-1 exhibited similar behavior, O-methylating both 2,6-DBP and 2,6-DCP to their corresponding anisoles (Figure 3d and e). The fluorinated derivative, 2,6-DFP, was not O-methylated by either strain (Figure 3c and f). No methylation of 2,6-DBP, 2,6-DCP, and 2,6-DFP was observed in sterile controls (data not shown).

O-Methylation of TBBPA in Sediment Microcosms. To determine the potential for microbial TBBPA O-methylation in the environment, microcosms were established using sediment from Kearny Marsh, New Jersey, and Kymijoki River, Finland and spiked with a TBBPA concentration of 10 μM. After 80 days, over 50% of the original TBBPA was transformed to TBBPA mono- and dimethyl

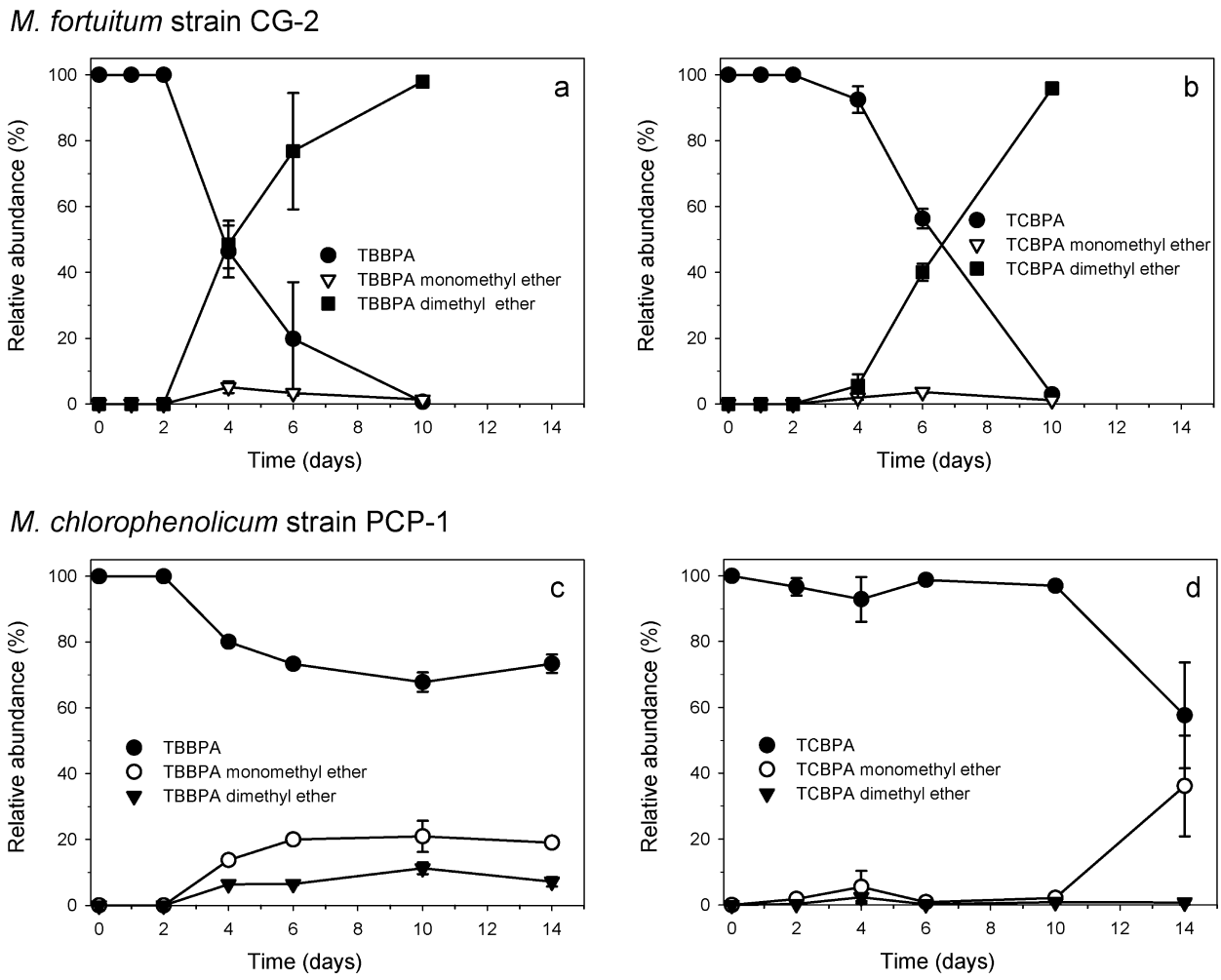

FIGURE 2. Conversion of TBBPA (a, c) and TCBPA (b, d) to mono- and dimethylated forms by *M. fortuitum* strain CG-2 (top) and *M. chlorophenolicum* strain PCP-1 (bottom). Data points are means of triplicate cultures ± one standard deviation.

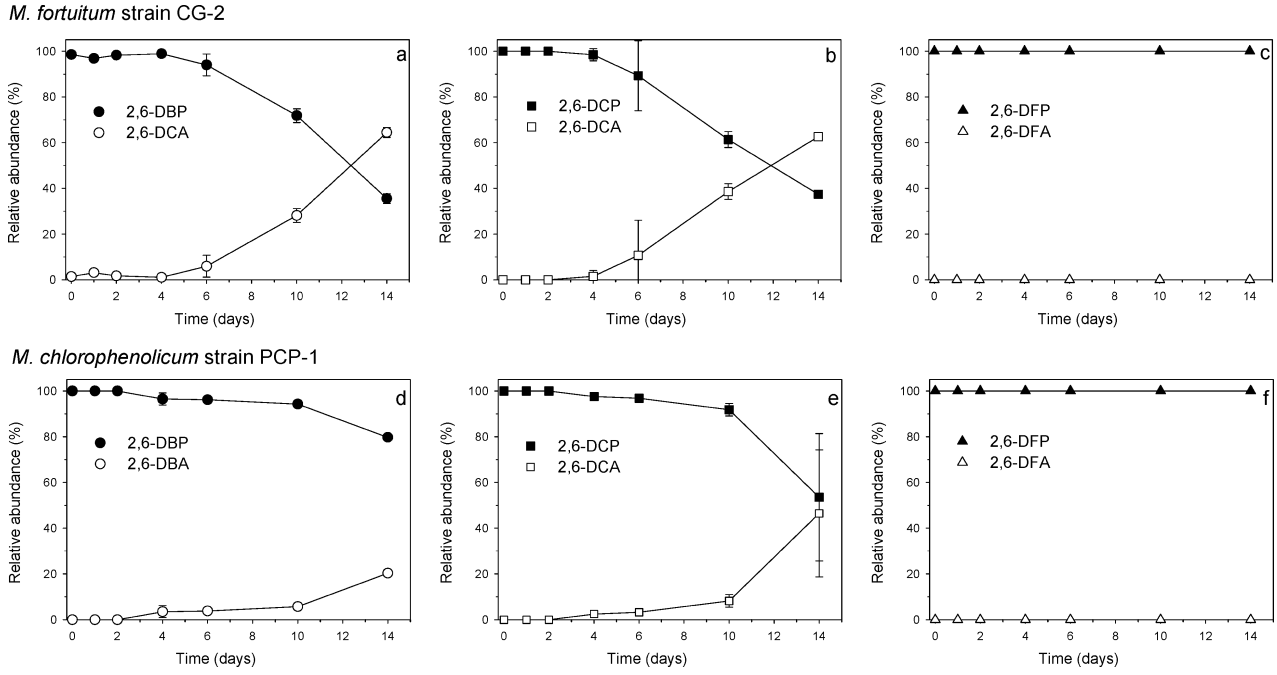

FIGURE 3. O-Methylation of 2,6-DBP (a, d), 2,6-DCP (b, e), and 2,6-DFP (c, f) to their corresponding anisoles by *M. fortuitum* strain CG-2 (top) and *M. chlorophenolicum* strain PCP-1 (bottom). Data points are means of triplicate cultures ± one standard deviation.

ether in the Kearny Marsh sediment (Figure 4a). Heat-killed, sterile controls showed no transformation over the 80-day period. After 60 days, approximately 10% of the TBBPA was converted to mono- and dimethylated deriva-

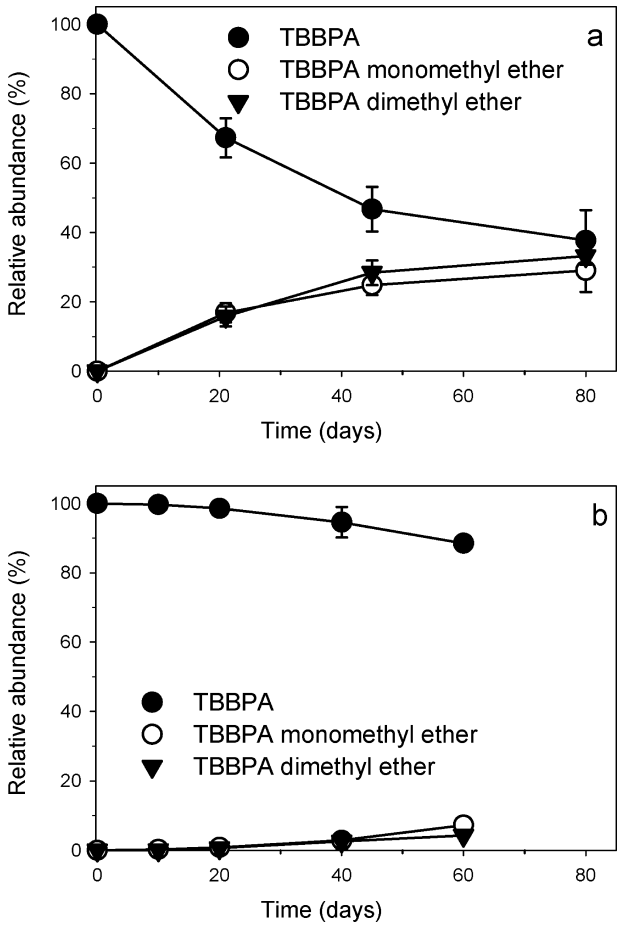

FIGURE 4. O-Methylation of TBBPA in Kearny Marsh sediment (a) and Kymijoki sediment (b) cultures. Data points are means of triplicate cultures ± one standard deviation.

tives in Kymijoki cultures (Figure 4b). Sterile controls showed no activity.

Enumeration of O-Methylating Microorgansism in Kearny Marsh Sediment. A 5-tube MPN assay was performed to estimate the abundance of O-methylating organisms in the Kearny Marsh sediment. The MPN test for O-methylation gave an estimate of 1.3×10^6 methylators g^{-1} soil (95% confidence limit 4.3×10^5 to 4.1×10^6). The total number of heterotrophs was estimated to be 1.1×10^7 g^{-1} soil (95% confidence limit 2.8×10^6 to 3.3×10^7), suggesting that up to 10% of the heterotrophs in the Kearny Marsh sediment may be capable of O-methylation.

Discussion

The abundant use of TBBPA and other brominated flame retardants has led to increasing environmental contamination. Although TBBPA is resistant to aerobic and anaerobic degradation, transformation reactions, such as reductive debromination and O-methylation, alter the compound without making changes to the carbon skeleton of the substrate and can be an alternative to degradation. Environmental and toxicological data concerning potential products of this process are sparse (*9*). Although TBBPA dimethyl ether is not produced in industry, it has been detected in samples of terrestrial and aquatic sediments, as well as biological samples (*5, 7, 40*). In these studies, it was hypothesized that the TBBPA dimethyl ether may be a product of microbial transformation. Allard et al. (*32*) first demonstrated that bacterial cultures were capable of O-methylating TBBPA although the reaction proceeded at a relatively slow rate. We show here that two *Mycobacterium* isolates known

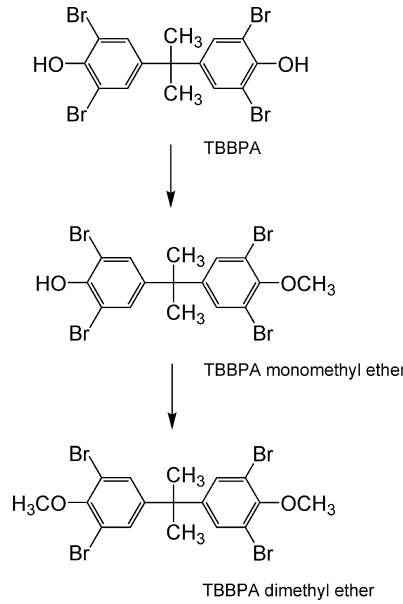

FIGURE 5. Sequential O-methylation of TBBPA to mono- and dimethylated derivatives.

for their ability to O-methylate chlorophenols transform both TBBPA and TCBPA to the corresponding mono- and dimethylated ethers (Figure 5). Additionally this study has demonstrated the microbially mediated O-methylation of TBBPA in sediment microcosms. This suggests that O-methylation of TBBPA may be an environmentally significant process.

Our data from Kearny Marsh suggest that TBBPA O-methylating microorganisms may be prevalent in the environment. In this sediment, approximately 10% of the total aerobic heterotrophs were capable of O-methylation. It thus seems likely that O-methylation will be a prominent reaction in contaminated sediments. Furthermore, previous studies have shown that a variety of bacteria and fungi mediate O-methylation of halogenated phenols (see ref (*31*) for review). In addition to TBBPA, numerous other halogenated anisoles of no known anthropogenic source have been detected in environmental and biological samples (*41–45*). The ubiquity of O-methylating microorganisms suggests that these compounds may also be products of biological O-methylation.

In the two *Mycobacterium* strains tested here, there seemed to be little difference in the rates of methylation of brominated versus chlorinated phenolics. It was previously demonstrated that the O-methylating enzyme(s) of many methylating bacteria prefer substrates with the hydroxyl group flanked by two halogen substituents, usually chlorine or bromine (*30, 32, 46*). For example, *Rhodococcus* strains did not O-methylate 2-chlorophenol or phenol (*30*). This preference for two bulky flanking substituents suggests a possible explanation for the lack of methylation of 2,6-DFP. Since the fluorine ion is the smallest of the halogens, the flanking substituents are likely too close in size to hydrogen to be recognized by the enzyme. The van der Waals radius of fluorine is 1.35 Å, quite similar to hydrogen's radius of 1.20 Å. The methyltransferase enzyme's suspected geometric selectivity also explains the similar methylation rates of 2,6-DBP and 2,6-DCP as the van der Waals radii of Br and Cl are 1.80 Å and 1.95 Å, respectively (*47*).

While we have established that TBBPA may be transformed under aerobic conditions to TBBPA mono- and dimethyl ether, there is no information on their fate in either aerobic or anaerobic environments. With the addition of two hydrophobic methyl groups, TBBPA dimethyl ether is more

lipophilic than its parent compound. This characteristic increases its potential for bioconcentration in fatty tissue. The presence of TBBPA dimethyl ether has been demonstrated in aquatic organisms such as fish and mussels in Japan (*7, 48*), as well as peregrine Falcon eggs in Greenland (*49*). A dimethoxylated polybrominated biphenyl, a compound similar in structure to TBBPA dimethyl ether, has been detected in the blubber of various marine mammals off the coast of Japan (*43*). Currently, little is known about the toxicology of TBBPA mono- and dimethylated ether. Given the suspected prevalence of bacterial O-methylation, additional environmental and toxicological data should be collected for these derivatives.

In summary, the biotransformation of TBBPA and its derivatives may prove to be important for health risk evaluation, as TBBPA is believed to induce a number of toxicological effects including cellular oxidative stress and neurotransmitter inhibition, while BPA is a suggested endocrine disruptor (*50–53*). The O-methylated derivatives are more lipophilic than TBBPA and therefore have a higher probability of bioaccumulating in fish and animal lipids and tissue. Further research regarding the microbial transformations of TBBPA and its products is needed for both environmental remediation and health assessments. Due to the recognized concern regarding the potential estrogenic effects of BPA and the completely unknown environmental and health effects of the O-methyl derivatives of TBBPA an evaluation of their toxicological effects is sorely needed.

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