

# Cometabolic Transformation of Mono- and Dichlorobiphenyls and Chlorohydroxybiphenyls by Methanotrophic Groundwater Isolates

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Mixed and pure methanotrophic cultures, previously isolated from an uncontaminated groundwater aquifer, were screened for their abilities to degrade a range of ortho-chlorinated mono- and dichlorobiphenyls when grown under conditions promoting expression of the soluble methane monooxygenase. Only the mixed culture and the pure type II strain CSC1 were found to degrade 2- and 4-chlorobiphenyl; 2,4-dichlorobiphenyl; and 4-hydroxy-2-chlorobiphenyl. Substrate disappearance in the mixed culture was consistently 8-10% higher than in incubations with strain CSC1, and observed biomass transformation capacities ranged from 0.05 to 0.5 mg of PCB/mg of protein. The main products found in both pure and mixed culture incubation extracts were identified as hydroxylated chlorobiphenyls. Additionally, products resulting from ring cleavage of the biphenyl, presumably generated by the heterotrophs present in the mixed culture, were observed. The release of chloride or dechlorinated intermediates was not observed in pure culture incubations. *Methylobionas* sp., a type I methanotroph, was unable to mediate hydroxylation of the chlorinated biphenyls. These results indicate that methanotrophs may be able to contribute to the initial degradation of lesser chlorinated biphenyls and that methanotrophic-heterotrophic interactions may play an important role in the further mineralization of these substrates.

## Introduction

The release and distribution of polychlorinated biphenyls (PCBs) in the environment via atmospheric sources or accidental spills have contributed to the recognition of PCBs to be among the most abundant chlorinated hydrocarbons in the global environment, particularly in the hydrosphere (1, 2). Since the early 1970s, research on their environmental fate has culminated in a partial understanding of the relative contributions and abilities of anaerobic methanogenic communities and aerobic heterotrophic bacteria to mediate PCB biotransformations and/or mineralization.

Under anaerobic methanogenic conditions, river and lake sediments as well as suspended cultures have been demonstrated to reductively dechlorinate industrial chlorobiphenyl (Aroclor) mixtures and individual highly chlorinated biphenyl congeners (3-6). However, the dechlorination process is often incomplete and results in the accumulation of mono- and dichlorobiphenyls. The most frequently encountered congeners are 2-chlorobiphenyl, and 2,2', 2,4', 2,6-, 2,4-, and 2,3-dichlorobiphenyls. The relative accumulation of the ortho-chlorinated congeners has been shown to be dependent on both the

microbial inoculum used and the substitution pattern of the chlorobiphenyl congener considered (3, 7).

Most of the ortho-chlorinated congeners are less rapidly degraded by dioxygenase-harboring aerobic heterotrophic bacteria, which generally oxidize the aromatic ring in the 2,3-position, presumably due to steric hindrance by the ortho-substituent (8). Yet, some of the congeners in Aroclor mixtures lacking open 2,3-positions have been reported to be degraded by aerobic biphenyl degraders, indicating the existence of another enzymatic mechanism to circumvent the steric problems indicated earlier (8-10). Aerobic heterotrophic bacteria containing NADH-dependent hydroxybiphenyl monooxygenases have been shown to grow on and oxidize certain ortho- and para-hydroxylated biphenyls, but were found to be unable to hydroxylate the chlorinated analogues, including chlorobiphenyls and hydroxylated chlorobiphenyls (10, 11). An hydroxylated phenyl moiety was found to be required for enzyme activity (10, 12).

Methanotrophic bacteria, or methane-utilizing methylotrophs, often can be found on the interface between aerobic and anaerobic strata where they are associated with stable sources of methane such as those generated by methanogens. Since they represent up to 8% of the aerobic bacterial population and are abundant in lakes and aquifer sediments (13), metabolic activities of methanotrophs potentially have a significant influence on catalyzing transformations of xenobiotic organic compounds (14, 15). Central to methane catabolism by methanotrophs is the methane monooxygenase (MMO), which converts methane to methanol and requires oxygen and a source of reducing power (NAD(P)H) for activity (13, 16). Whereas all methanotrophs isolated to date have been shown to express the particulate form of the enzyme (pMMO), some have been found to express the soluble MMO (sMMO) as well (16-19). In those methanotrophs where both forms are present (types II and X), their respective expression has been shown to be dependent on the concentration of copper in the culture medium, such that under copper-limiting conditions sMMO is expressed and pMMO is not (16, 19, 20).

The methanotrophic cometabolic oxidation of TCE and other alkyl halide contaminants has been studied under different environmental conditions using both mixed and axenic isolates from soil and aquifer samples (21-26). Methanotrophs have been found to insert one oxygen from dioxygen in (chlorinated) alkenes, alkanes, and aromatic compounds, resulting in the accumulation of hydroxylated intermediates. Whereas alkyl halides have been found to be transformed by methanotrophs harboring either or both forms of the enzyme (17, 27-29), only the soluble form has been shown to oxidize some monoaromatic (27, 30) and polycyclic aromatic hydrocarbons (29-33). Recently, a type I methanotroph isolated from a TCE-contaminated aquifer was found to express the sMMO and it oxidized both TCE and naphthalene (34).

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Table 1. Characteristics of Moffett Naval Air Station Methanotrophic Isolates

inoculum	classification	MMO	TCE oxidation rates	inclusions
MM1	type II	soluble/particulate	Cu <sup>2+</sup> /EDTA dependent	yes
strain CSC1	type II	soluble/particulate	Cu <sup>2+</sup> /EDTA dependent	yes
<i>Methylomonas</i> sp.	type I	particulate	Cu <sup>2+</sup> /EDTA dependent	no

Because of the limited activities of aerobic heterotrophs and anaerobic methanogenic populations against ortho-chlorinated PCB congeners, and the apparent relaxed substrate specificities of sMMO, the objective of this study was to assess methanotrophic activity against a range of lesser chlorinated ortho- and para-substituted chloro- and hydroxybiphenyls. The mixed and pure type I and type II cultures were previously isolated from the uncontaminated Moffett Naval Air Station aquifer and were characterized for their trichloroethylene (TCE) -degrading abilities under different environmental conditions (24, 35). Aside from *Methylomonas* sp. MM2, which mediated TCE oxidation by pMMO, both other inocula expressed sMMO. In this paper, the hydroxylation of mono- and dichlorobiphenyls and hydroxylated chlorobiphenyls is reported under conditions promoting methanotrophic activity and expression of sMMO.

#### Materials and Methods

The inocula used in this study were as follows: (i) a mixed culture (MM1) containing one type II methanotroph and 3-4 uncharacterized heterotrophs; (ii) the pure type II methanotroph, strain CSC1, isolated from (i); and (iii) a pure type I methanotroph, *Methylomonas* sp. MM2, which was derived from the same aquifer sample but not from (i). Their characteristics are summarized in Table 1. Purity of the strains for heterotrophic contamination was tested routinely by plating on tryptone-glucose medium.

Classification of both pure cultures and the methanotroph in the mixed culture, as type I and type II methanotrophs, was based on the intracytoplasmic membrane ultrastructure and enzymatic characteristics (24). Whereas both the mixed culture and strain CSC1 cross-reacted during 16S rRNA signature probe hybridization with soluble MMO from *Methylosinus trichosporium* OB3b, *Methylomonas* sp. MM2 did not (36; Tsien and Hanson, unpublished observation). The presence of pMMO in *Methylomonas* sp. MM2 was based on cell fractionation studies, which revealed CH<sub>4</sub> oxidation in the membrane fraction, and was inferred from the characteristic orders of magnitude lower TCE oxidation rates when a metal chelating agent was added to the medium as compared to when it was omitted (24).

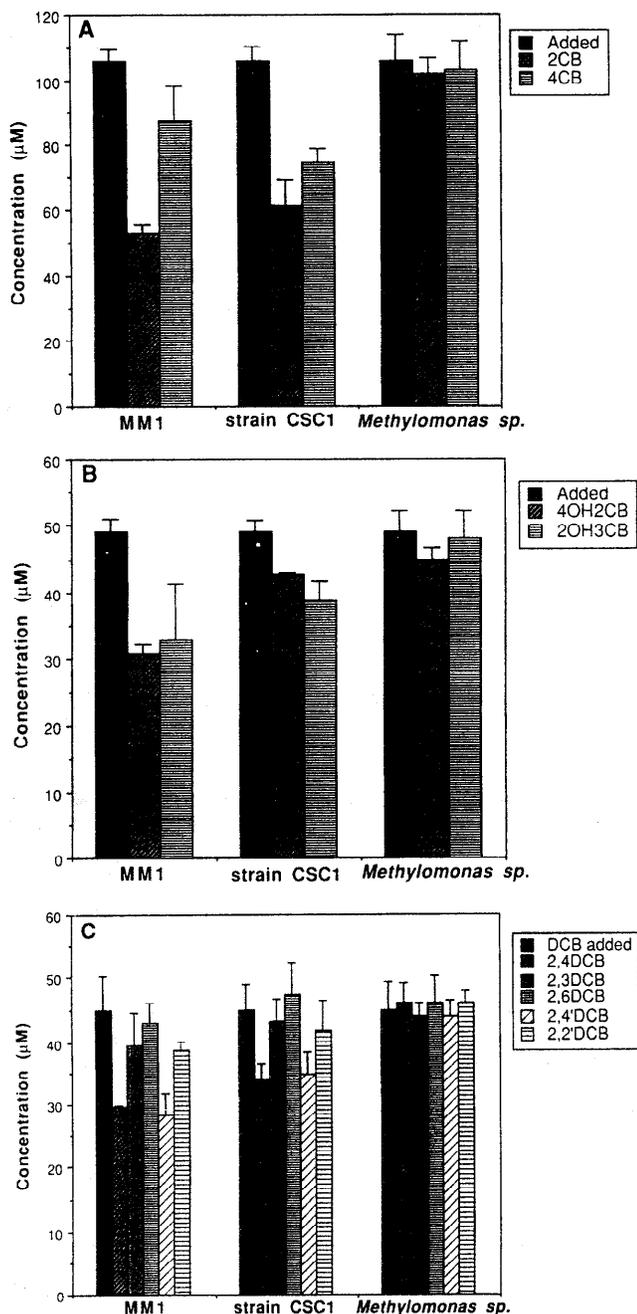
All inocula were grown in continuously stirred reactors (1 L) containing Whittenbury medium under a continuous flow of approximately 35% methane in air at ambient temperature (24, 37) to an optical density (OD) of 1.4-1.6 (0.4-0.6 g of protein L<sup>-1</sup>). Whittenbury medium does not contain copper, but contains metal-ion chelating NaEDTA, and has been shown to result in the highest TCE transformation rates by all the above cultures tested (24). Aliquots (30 mL) of these cell suspensions were dispensed in 125-mL Erlenmeyer flasks, capped with Mininert screwcap valves, and subsequently spiked with either 49 mM of hydroxy-PCBs, 106 mM of monochlorinated PCBs, or 45 mM of the dichlorinated PCB congeners, added from

methanol stock solutions. The flasks were incubated at room temperature on a rotary shaker (200 rpm) for 20 days and spiked daily with alternating pulses of 15 mL of methane as an inducing substrate for methane monooxygenase activity and with 15 mL of air. The alternating pulse of methane, which provides a source of reducing power for the MMO, was not expected to have an effect on enzyme activity, as the inocula were not actively growing during the incubation period and both the methanotroph in the mixed culture and strain CSC1 were shown to contain lipid inclusions as a source for reducing power (24). Henry and Grbić-Galić (34) showed, using the same cultures, that TCE transformation capacity was maintained for 15 h under methane-starvation conditions. Additionally, in the current study all incubations were spiked with 2 mM formate (final concentration) at regular time intervals. The addition of formate as a source of reducing power was previously shown to increase TCE oxidation rates in similar incubation studies with *Methylomonas* sp. MM2, but had no effect in mixed culture and CSC1 incubations (35).

The cells were not actively growing during the time of incubation, rather they were maintained as resting cell suspensions. Total cell protein content in the incubation vessel (35 mL) remained constant during the experiment and were 4.8 ± 0.6, 20 ± 3, and 16 ± 2 mg for *Methylomonas* sp, mixed culture MM1, and strain CSC1, respectively. For each compound studied, two autoclaved and chemical controls (without inoculum) were monitored along with the two replicate live incubations. A 5-mL sample was removed from each replicate as a time zero point.

After 20 days, each replicate was split in two 15-mL aliquots and subjected to either a neutral or an acidic (pH 2) extraction with 2 vol of hexane:acetone (9:1). The extracts were dried over excess Na<sub>2</sub>SO<sub>4</sub> and concentrated in dodecane to 1 mL, using a rotavapor apparatus (Brinkmann Instruments, Westbury, NY). The dodecane fractions were analyzed on a gas chromatograph (Model 5890 A, Hewlett-Packard Co., Palo Alto, CA), equipped with both electron capture (ECD) and flame ionization (FID) detectors and a 30-m DB-5 capillary column (J&W Scientific, Folsom, CA). The injection mode was split/splitless (split ratio 10), and the injector and both detector temperatures were at 275 and 280 °C, respectively. The temperature program ran from 90 to 190 °C at a rate of 5 °C min<sup>-1</sup>, with an initial and final holding time of 5 min. The flow rate of the carrier gas (H<sub>2</sub>) was set at 2 mL min<sup>-1</sup>, with a column head pressure of 10 kPa.

Those samples showing the appearance of metabolites were subjected to analyses on a mass spectrometer (Model TSQ 70 MAT, Finnigan Instruments, San Jose, CA) operated at 70 eV, which was interfaced to a gas chromatograph (Model 3400, Varian Associates, Walnut Creek, CA). The temperature program and column were identical to those described earlier. UV-visible spectra were obtained on a diode array spectrophotometer (Model 8451A, Hewlett-Packard Co., Palo Alto, CA). The scanning range was from 190 to 600 nm.



**Figure 1.** Degradation of monochlorobiphenyls (A), hydroxylated monochlorobiphenyls (B), and dichlorobiphenyls (C) by mixed and axenic groundwater isolates.

### Results and Discussion

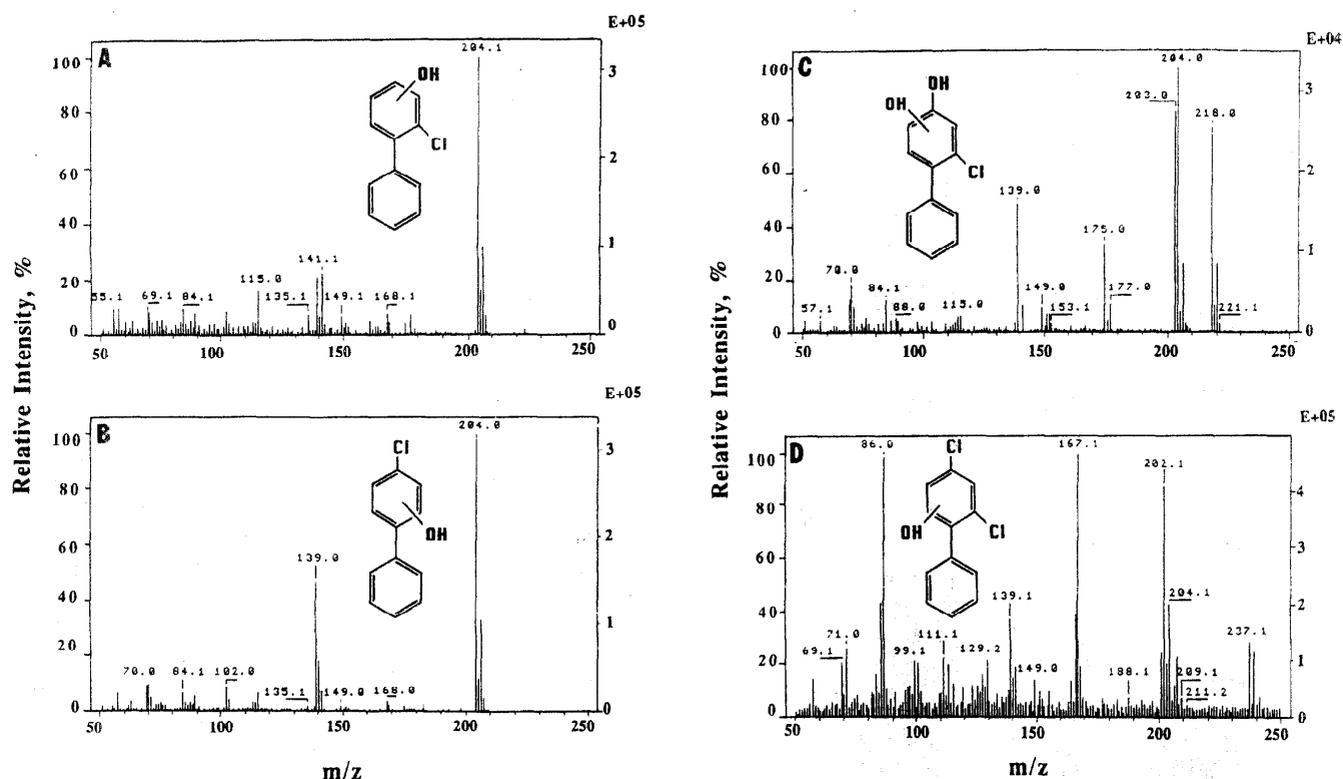
The results from screening of the groundwater isolates on the chlorobiphenyl and chlorohydroxybiphenyl isomers are presented in Figure 1. All values are corrected for recoveries in the chemical (99%) and autoclaved (94–99%) controls. Thus, both mixed culture MM1 and the type II strain CSC1 were able to degrade 2- and 4-CB, 4-hydroxy-2-chlorobiphenyl (4OH2CB), 2-hydroxy-3-chlorobiphenyl (2OH3CB), and 2,4- and 2,4'-DCB. No significant disappearance (<15%) was observed in incubations with 2,3-, 2,6-, and 2,2'-dichlorobiphenyls for either inoculum. Substrate disappearance was mostly found to be higher in mixed culture incubations (30–51%) than in incubations with strain CSC1 (24–42%) alone, except in the case of 4CB.

The observed biomass transformation capacities of the inocula ranged from 0.05 to 0.5 mg of PCB/mg of protein

and were the highest for 2CB. The differences between the transformation capacities of the mixed and pure CSC1 cultures against the chlorobiphenyls tested were not statistically different, except in the cases of 4CB (higher for strain CSC1) and 4OH2CB (higher for MM1). This difference can possibly be explained by the removal of toxic intermediates, formed from the initial oxidation of the chlorobiphenyls by the heterotrophic bacteria in the mixed culture. The oxidation of TCE by these cultures was reportedly severely impaired under methane-starvation conditions, due to a buildup of highly reactive epoxide intermediates (35). Incubations with the pure type I methanotroph *Methylomonas* sp. strain MM2 did not result in the disappearance of any of the chlorobiphenyl isomers. Overall, the biomass transformation capacities were orders of magnitude higher than those reported for the same cultures during TCE oxidation ( $\mu\text{g}$  of TCE/mg of cell dry weight): values on the order of 0.15, 0.37, and 1.5 were calculated for strain CSC1, *Methylomonas* sp., and MM1, respectively (24, 35). These differences have to be interpreted carefully, since (i) the complete degradation of TCE was achieved during the time of incubation, (ii) the initial substrate-to-biomass ratio was much lower than in the chlorobiphenyl incubations, and (iii) the air/methane/formate substrate amendments were different.

The main products found in both pure and mixed culture incubations with 2CB, 4CB, 4-hydroxy-2-chlorobiphenyl, and 2,4-DCB were identified as hydroxylated chlorobiphenyls (UV-visible spectrum;  $\lambda_{\text{max}}$  246 nm, pH 7), based on mass spectral analysis (Figure 2A–D). The ions and interpretation of the fragmentation patterns of the products are given in Table 2. The loss of a proton resulting from a benzylic C-H cleavage ( $[M - 1]^+$ ) or of hydrogen due to molecular thermolysis ( $[M - 2]^+$ ), as well as strong ions resulting from the loss of CO and CHO are characteristic for alcohols (38). The formation of hydroxylated intermediates was the result of methanotrophic oxidation, since (i) they were found not only in the mixed culture incubation extracts but also in incubations with the pure methanotrophic culture CSC1 isolated from the latter (Table 3), and (ii) the range of chlorobiphenyl substrates oxidized was nearly identical for both inocula (Figure 1). Though the heterotrophic population present in the mixed culture has not been characterized, heterotrophic hydroxybiphenyl monooxygenases studied to date have been shown to be unable to hydroxylate chlorinated biphenyl analogues (10, 11). No transformation products were found in either the killed or the chemical controls, indicating that the hydroxylation did not result from chemical oxidation.

The formation of phenolic compounds resulting from methanotrophic oxidation of chlorinated and nonchlorinated aromatic compounds has been demonstrated earlier, using either whole cell suspensions (14, 15, 30, 31) or cell extracts (27, 29, 32, 33). Thus, monohalogenated benzenes were oxidized to both ortho- and para-hydroxylated halophenols using the type II methanotroph, *Methylococcus capsulatus* Bath (27). The position of the hydroxyl group on the chlorobiphenyl metabolites used in this study could not be inferred from mass spectral data alone. Incubations of the chlorobiphenyls and hydroxylated chlorobiphenyls with the type I methanotroph *Methylomonas* sp. did not result in the formation of hydroxylated intermediates. This is consistent with previous findings that bacteria expressing particulate MMO have a more



**Figure 2.** Mass spectra and suggested chemical structure of the hydroxylated metabolites from 2-chlorobiphenyl (A), 4-chlorobiphenyl (B), 4-hydroxy-2-chlorobiphenyl (C), and 2,4-dichlorobiphenyl (D) after incubation with strain CSC1. (The position of the hydroxyl group could not be inferred from mass spectral data alone.)

**Table 2. Major Fragmentation Ions and Proposed Structure of Products from Transformation of Chlorobiphenyls by Mixed and Axenic Type II Methanotrophic Cultures**

substrate <sup>a</sup>	major ions ( <i>m/z</i> , intensity)	empirical formula (MW)
2CB	204 (100), (M) <sup>+</sup> ; 168 (10), (M - 35) <sup>+</sup> loss of chlorine; 141 (22), (M - 63) <sup>+</sup> loss of CO and Cl <sup>-</sup> ; 115 (18), (M - 89) <sup>+</sup> loss of -CH=CH-; 102 (10), (M - 102) <sup>+</sup> loss of -CH <sub>2</sub> -	C <sub>12</sub> H <sub>9</sub> OCl (204)
4CB	204 (100), (M) <sup>+</sup> ; 168 (5), (M - 35) <sup>+</sup> loss of chlorine; 139 (50), (M - 63) <sup>+</sup> loss of CO and Cl <sup>-</sup> ; 115 (18), (M - 89) <sup>+</sup> loss of -CH=CH-; 102 (10), (M - 102) <sup>+</sup> loss of -CH <sub>2</sub> -	C <sub>12</sub> H <sub>9</sub> OCl (204)
4OH2CB	218 (80), (M - 2) <sup>+</sup> ; 204 (100), (M - 16) <sup>+</sup> ; loss of OH; 175 (38), (M - 43) <sup>+</sup> loss of CO and CH <sub>3</sub> <sup>+</sup> ; 139 (50), (M - 81) <sup>+</sup> loss of CO, OH, and chlorine	C <sub>12</sub> H <sub>9</sub> O <sup>2</sup> Cl (220)
2,4DCB	237 (28), (M - 1) <sup>+</sup> ; 202 (88), (M - 36) <sup>+</sup> loss of one chlorine; 167 (100), (M - 71) <sup>+</sup> loss of both chlorines; 139 (38), (M - 99) <sup>+</sup> loss of both chlorines and CO; 86 (98), (M - 152) <sup>+</sup>	C <sub>12</sub> H <sub>8</sub> OCl <sub>2</sub> (238)

<sup>a</sup> Abbreviations: CB, chlorobiphenyl; DCB, dichlorobiphenyl; 4OH2CB, 4-hydroxy-2-chlorobiphenyl.

**Table 3. Cometabolic Transformation Products Found in Mixed and Axenic Culture Incubations with Chlorobiphenyls**

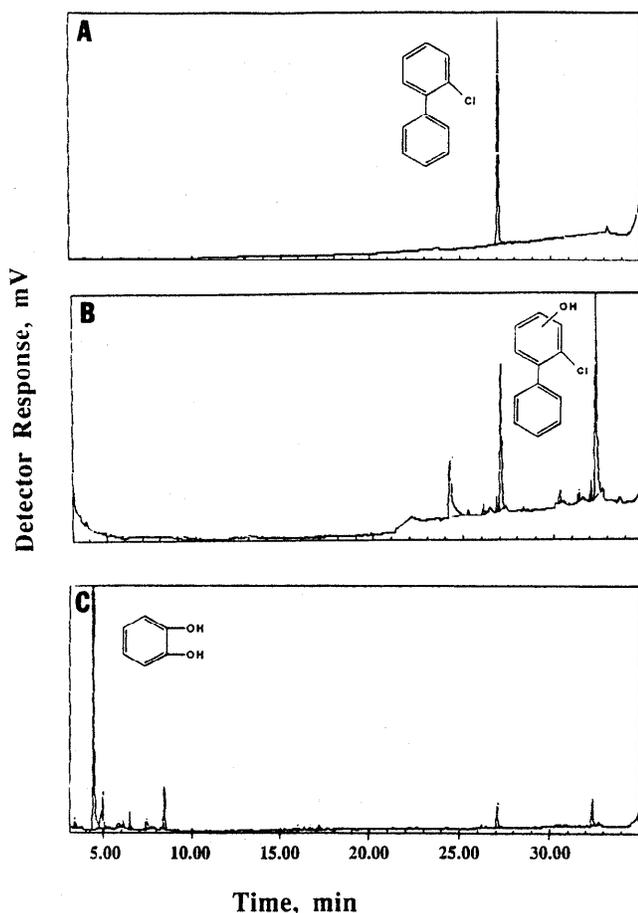
congener	products and metabolites	
	mixed culture MM1	strain CSC1
2CB	hydroxyCB, catechol, acid <sub>x</sub> <sup>a</sup>	hydroxyCB
4CB	hydroxyCB, catechol, acid <sub>x</sub>	hydroxyCB
2,4DCB	acid <sub>x</sub>	hydroxyDCB
2,4'-DCB	hydroxyDCB <sup>b</sup>	ND <sup>c</sup>
4OH2CB	dihydroxyCB, acid <sub>x</sub>	dihydroxyCD
2OH3CB	acid <sub>x</sub>	ND

<sup>a</sup> Abbreviations: CB, chlorobiphenyl; DCB, dichlorobiphenyl; 4OH2CB-, 4-hydroxy-2-chlorobiphenyl; 2OH3CB, 2-hydroxy-3-chlorobiphenyl; acid<sub>x</sub>, acid-extractable metabolites. <sup>b</sup> Based on selected ion monitoring (SIM)-MS analysis (M)<sup>+</sup>, (M + 2)<sup>+</sup>, (M + 4)<sup>+</sup>. <sup>c</sup> ND, not determined.

restricted enzyme specificity and are unable to catalyze the oxidation of aromatic compounds.

Both chlorinated and nonchlorinated products were found in the acidic extracts of mixed culture incubations with 2- and 4-chlorobiphenyl and with chlorinated hydroxybiphenyls (Table 3). Mass spectral analysis identi-

fied one of the products as catechol (M<sup>+</sup> 112), based on comparison of the mass fragmentation pattern to that of an authentic standard. This observation is exemplified in typical gas chromatographic traces for incubations of both inocula with 2CB (Figure 3). Whereas the neutral extract of incubations with strain CSC1 only shows the accumulation of a hydroxylated chlorobiphenyl, acidic extracts of mixed culture incubations indicate the additional accumulation of ring cleavage products. Ring cleavage of aromatic compounds requires dioxygenase activity, which has not been detected in methanotrophic bacteria, but is a rather common trait in aerobic heterotrophic bacteria (39). Thus, further degradation of the hydroxylated intermediates has to be ascribed to the heterotrophic bacteria present in the mixed culture, as these products were not found in incubations with strain CSC1. The role of heterotrophic bacteria in mediating further degradation of methanotrophic transformation products has been investigated by Uchiyama et al. (43) using trichloroethylene (TCE); mixed methanotrophic/heterotrophic cultures completely or partially degraded the metabolites accumulated from TCE by the pure



**Figure 3.** Gas chromatographic (FID) traces of 2-chlorobiphenyl (2CB) incubations at time zero (A), after 20-day incubation with strain CSC 1 (B) and with mixed culture MM1 (C). The peaks for 2CB and the hydroxylated product in panel C are deceptively small due to normalization of the detector signal response to the highest peak. Refer to Figure 1 for actual concentrations.

methanotrophic culture. In the current study, the release of inorganic chloride as evidence for the mineralization of the chlorobiphenyls was not observed during either mixed or axenic culture incubations with all substrates tested.

The mechanism of ring cleavage has not been elucidated, but does not follow the meta-ring fission pathway postulated for heterotrophic degradation of chlorobiphenyls, due to the absence of formation of both the yellow-pigmented ( $\lambda_{\max}$  (pH 7) = 410 nm,  $\lambda_{\max}$  (pH 2) = 340 nm) meta-ring cleavage product and chlorobenzoate intermediates in the incubation medium (40–42). However, the addition of 0.1 N NaOH to neutral extracts from 2CB, 4CB, and 2C4BPOL incubations resulted in the instantaneous formation of highly water-soluble, yellow-pigmented compounds ( $\lambda_{\max}$  418–442 nm, pH 10). Spectroscopic characterization of these compounds precluded their identification as dienone-type meta-ring cleavage products, due to the absence of a hypsochromic shift upon acidification (i.e., keto/enol tautomerization). Rather, a hypochromic effect was observed. Further investigations are required to elucidate the origin and structure of these products, as well as their possible relation to the oxidation of chlorobiphenyls by methanotrophic inocula.

The analytical methods and extraction procedures used in this study influenced the metabolites that were detected and identified. For example, it has been inferred that the sMMO oxidizes aromatic compounds via the transient

formation of aromatic epoxides (13, 27, 31). However, possible degradation mechanisms of the aromatic carbon-carbon double bond epoxide would result in (i) bond cleavage under basic conditions (pH > 9.0, + H<sub>2</sub>O/OH<sup>-</sup>), (ii) isomerization to phenols under neutral conditions (pH 7.0, + H<sub>2</sub>O), and (iii) formation of keto/enol tautomers under acidic conditions (pH 2.0, + 2 H<sub>2</sub>O) (44). Thus, only hydroxylated products and the parent compound would be extracted into hexane:acetone from neutral and acidified medium and be detected by the GC method used; ketones would surely rearomatize to phenols under these conditions (45). The epoxide, if formed as a precursor, would not be detected due to the aforementioned degradation reactions.

### Conclusions

This study constitutes the first report on cometabolic transformations of lesser chlorinated biphenyls by mixed and pure type I and II methanotrophic inocula maintained on methane as a carbon source, under conditions favoring methanotrophic growth and maximum expression of sMMO. These findings contribute to our understanding of the possible fate of biologically more recalcitrant chlorobiphenyl congeners in the environment on the interface between aerobic and anaerobic zones. From this study, it appears that *o*-, *p*-, and *o,p*-substituted congeners were preferentially oxidized, though no mechanism has been put forth. The hydroxylated metabolites generated are more water soluble than their parent compounds, which may facilitate their migration and pose an environmental problem if they are more toxic than the parent compounds. This problem may be offset if the metabolites are further degraded to ring cleavage products by aerobic heterotrophic microorganisms, the potential of which has been indicated in mixed culture incubations. Therefore, future studies should address mass balance questions and chlorobiphenyl transformation kinetics to determine the transient nature of metabolite accumulation.

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