ANAEROBIC TRANSFORMATION OF COMPOUNDS OF TECHNICAL TOXAPHENE.

2. FATE OF COMPOUNDS LACKING GEMINAL CHLORINE ATOMS

STEFFEN RUPPE,† ANKE NEUMANN,‡ ERIC BRAEKEVELT,§ GREGG T. TOMY,§ GARY A. STERN,§ KEITH A. MARUYA,‖ and WALTER VETTER*‡#
†Department of Food Chemistry, Friedrich-Schiller-University Jena, Dornburger Strasse 25, D-07743 Jena, Germany
‡Department of Applied and Ecological Microbiology, Friedrich-Schiller-University Jena, Philosophenweg 12, D-07743 Jena, Germany
§Freshwater Institute, Department of Fisheries and Oceans, Winnipeg, Manitoba R2T 2N6, Canada
‖Institute of Food Chemistry, University of Hohenheim, Garbenstrasse 28, D-70599 Stuttgart, Germany
§Institute of Food Chemistry, University of Hohenheim, Garbenstrasse 28, D-70599 Stuttgart, Germany

(Received 18 April 2003; Accepted 31 July 2003)

Abstract—The major toxaphene metabolites in sediment and soils (2-exo,3-endo,6-exo,8,9,10-hexachlorobornane [B6-923] and 2-endoo,3-exo,5-exo,6-exo,8,9,10-heptachlorobornane [B7-1001]) were incubated with the isolated gram-negative bacterium Dehalospirillum multivorans. Within 14 d, biotransformation of B7-1001 was nearly quantitative, resulting in two penta- and six hexachlorobornanes, as well as one unsaturated hexachloro compound of technical toxaphene. The major transformation product (~50% of all metabolites) was identified as 2-exo,3-endo,5-exo,8,9,10-hexachlorobornane (B6-903). Abiotic dehydrochlorination of B7-1001 with methanolic KOH resulted in the formation and subsequent identification of the lone unsaturated compound as 2,5-exo,6-exo,8,9,10-hexachloroborn-2-ene. Thus, dehydrochlorination was found to be a minor process of the anaerobic transformation of toxaphene. Biotransformation of 70% of amended B6-923 within 14 d demonstrated that reductive dechlorination was not exclusively associated with geminal Cl atoms, as previously suggested. Three pentachlorobornanes were identified as transformation products, one of which was identical with a transformation product of B7-1001. This commonality unequivocally proves this metabolite to be 2-exo,3-endo,8,9,10-pentachlorobornane. Fifteen previously unknown metabolites of B6-923, B7-1001, and other toxaphene compounds identified in this study were detected in sediment from Lake Ontario (Canada), underscoring the importance of microbial toxaphene transformation in natural, aquatic environments.

Keywords—Toxaphene Anaerobic dechlorination Dehalospirillum multivorans Sediments

INTRODUCTION

The chloropesticide toxaphene (Camphechlor and Melipax) was produced by the chlorination of camphene since the late 1940s. Technical products consist of several hundred bicyclic components having an average composition of C₉₀H₄₂Cl₈ [1,2]. The global use was estimated at 0.45 to 1.33 x 10⁶ tons [1,2]. Because of its various fields of application, several environmental compartments were contaminated with toxaphene. Despite evidence of extensive transformation under reducing conditions (sediment, soil, and sewage sludge), several primarily lower-chlorinated compounds persist in artificial and naturally contaminated environments [3–9].

The toxaphene residue pattern in these media was usually dominated by 2-exo,3-endo,6-exo,8,9,10-hexachlorobornane (B6-923) and 2-endoo,3-exo,5-exo,6-exo,8,9,10-heptachlorobornane (B7-1001), ostensibly as a result of anaerobic transformation processes [5,10,11]. Furthermore, active participation of microorganisms in the reductive processing (Cl→H substitution, primarily at carbons with geminal Cl atoms [gem-Cls] [12]) of toxaphene has been demonstrated in several studies [6,11,13,14].

In a recent study, we used cultures of the single well-defined and strictly anaerobic gram-negative bacterium Dehalospirillum multivorans [14,15] to rapidly and selectively transform compounds of technical toxaphene (CTTs) [11]. Evidence was found that Cl→H substitution at gem-Cls on secondary carbons (especially at 2-exo-Cl) was preferred to primary carbons [11]. As was found in environmental samples, B6-923 and B7-1001, which lack gem-Cl atoms, were the major transformation products of both technical toxaphene and a number of higher-chlorinated CTTs. In addition, we produced evidence that these CTTs, previously thought of as dead-end metabolites [7], also were transformed, albeit slowly, by D. multivorans.

In the present study, we studied the fate of CTTs without gem-Cls by incubations with D. multivorans. We attempted to identify previously unknown pentachloro and hexachloro CTTs, and investigated their presence in freshwater sediment samples.

MATERIALS AND METHODS

Chemicals

Racemic standard solutions (1–10 ng/µl) of individual CTTs were purchased from LGC Promochem (Wesel, Germany) and Dr. Ehrenstorfer (Augsburg, Germany), or were isolated from environmental samples [11,12,16]. The technical product Melipax (1 kg) was found in a garden shed in Jena (Germany). Trichloroacetic acid and Tris-HCl were purchased from Fluka (Neu-Ulm, Germany), isooctane was purchased from Roth (Karlsruhe, Germany), n-hexane from LGC Promochem, and acetone from Baker (Deventer, The Netherlands) in the best quality available. The internal standard perdeuterated α-hexachlorocyclohexane was synthesized as described previously [17].
Ontario Lake sediment sample

A sediment core from the Mississauga Basin of Lake Ontario was taken in 1998. The first 15 cm were subsampled in 1-cm increments and dated using $^{210}$Pb. The 2- to 3-cm depth (1989–1992) showed a nearly complete transformation pattern, and was chosen for further analysis. The extraction and cleanup procedure is described elsewhere [18, 19].

Isolation of B8-1414, B7-1001, and B6-923

The B6-923 was isolated from a solution of the technical product Melipax. The B7-1001 and B8-1414 were isolated from a contaminated sediment sample from the southern United States [9]. Both samples were eluted with n-hexane from a 2.5-cm–inner diameter glass column filled with 60 g of silica gel (Merck, Darmstadt, Germany) activated for $>$16 h at 130°C. The B6-923 eluted in fraction 600 to 650 ml; B7-1001 and B8-1414 eluted in fraction 700 to 750 ml. The samples were evaporated to dryness under a gentle nitrogen stream, redissolved in approximately 500 µl of acetonitrile:H$_2$O (84:16, v/v), and 100 µl was successively injected into a reversed-phase high-performance liquid chromatography (HPLC) system (see below). Fractions from 0.2 min were collected and analyzed for the target CTTs. The bulk of B6-923 (6.2–6.4 min), B7-1001 (7.4–7.6 min), and B8-1414 (8.2–8.4 min) was collected. The B7-1001 was already pure (95%) after reversed-phase HPLC separation. Sufficient purities of B6-923 and B8-1414 (>95%) were obtained after normal-phase HPLC separation (mobile phase n-hexane) in the fractions from 10.6 to 11 and 4.0 to 4.2 min, respectively.

Hexachlorobornenes from dehydrochlorination of B7-1001

Isolated B7-1001 (0.8 µg) was added to a solution of KOH (0.5 g) in methanol (30 ml) [20]. The solution was stirred under reflux for 2 h. The cooled solution was extracted twice with 20 ml of n-hexane. Extracts were concentrated to 1 ml and were analyzed by gas chromatography–electron-capture detection and gas chromatography–electron-capture negative-ion mass spectrometry (GC-ECNIMS).

Cultivation of microorganisms

Bacteria were routinely grown in 1 L of anaerobic medium with 40 mM pyruvate and 40 mM fumarate, and yeast extract at 2 g/L [21]. The medium for D. multivorans was inoculated with 100 ml of a culture grown on 40 mM formate and 5 mM perchloroethene (2 ml of 0.25 M perchloroethene in hexadecane per 100 ml of medium). The medium for Escherichia coli (1 L) was inoculated with 10 ml of culture grown aerobically on Luria-Bertani media [22]. Additional details on our cultivation procedures are given elsewhere [11].

Preparation of cell suspensions

All manipulations were performed under anoxic and sterile conditions [11]. In brief, the bacteria were harvested by centrifugation, and pellets were resuspended in Tris-HCl and formate. Five milliliters of this cell suspension were dispensed into 10-ml vials, 5 µl of CTTs in n-hexane were added, and the vials were sealed with caps and Teflon®-lined septa (Macherey-Nagel, Düren, Germany) and kept anaerobically in a glove box (95% N$_2$ and 5% H$_2$) at 28°C [11]. Selected samples were stored in sealed glass beakers in a refrigerator (4°C) or in a climatic room at 37°C. Heat-inactivated samples were prepared by incubating the sealed tubes for 10 min at 95°C. Compounds of technical toxaphene (20–50 ng) were added by injection through the septa. Abiotic controls (chemical blank without bacteria) and unspiked (no toxaphene) live samples were prepared for all experiments (see quality assurance–quality control section). Protein concentrations (determined according to Bradford [23] as described in Neumann et al. [24]) are given below.

Sample preparation

At predetermined incubation times, vials were opened, an aliquot of the internal standard perdeuterated α-hexachlorocyclohexane was added, and entire samples were immediately extracted twice with 10 ml of n-hexane (5 min each in an ultrasonic bath). The n-hexane layers were separated, combined, and the volume adjusted to 1 ml [11]. One microliter of each extract was analyzed by gas chromatography–electron-capture detection and GC-ECNIMS (high-resolution mass spectrometry; 2 µL). Starting samples ($t = 0$ h) were spiked with toxaphene, shaken, and immediately extracted as shown above ($t < 3$ min).

Gas chromatography–electron-capture negative-ion mass spectrometry and high-resolution mass spectrometry

Analyses were performed with a Hewlett-Packard 5890 series II gas chromatograph (Waldbronne, Germany), fitted with a 60-m × 0.25-mm–inner diameter (0.25-µm film thickness) DB-5 capillary column (J&W, Mississauga, ON, Canada), connected by a heated transfer line maintained at 280°C to a Kratos concept high-resolution mass spectrometer (EBE geometry, where E is an electrostatic analyzer and B is a magnet; New York, NY, USA) using parameters described elsewhere [18]. Two abundant ions of the [M-Cl]$^-$ clusters of penta- through nonachlorobornanes were monitored in three time windows (Table 1).

Low-resolution GC-ECNIMS analyses were performed with a Hewlett-Packard 5890 gas chromatograph (Waldbronne, Germany) interfaced to a Hewlett-Packard 5989B mass spectrometer (moderating gas: methane). A 30-m × 0.25-mm–inner diameter (0.25-µm film thickness) column coated with 95% methyl and 5% phenyl polysiloxane phase (HP-5, Hewlett-Packard) was installed in the gas chromatography oven [11].

Gas chromatography–electron-capture detection

Analyses were performed with a dual gas chromatography–electron-capture detection system previously described in detail [11].

High-performance liquid chromatography

The HPLC system consisted of a 234 autosampler, a 306 pump, and a 201 to 202 fraction controller (Abimed Gilson, Langenfeld, Germany). An octadecylsilane LC-18-DB (Supelco, Bellefonte, PA, USA) in the reversed-phase HPLC mode and a polygosil 60 to 10 column (Macherey-Nagel) was used in the normal-phase HPLC mode.

Quality assurance–quality control

Quality assurance–quality control precautions taken during the present experiments mirrored those published previously [11]. Samples were prepared and analyzed in duplicate except for those amended with B8-1414, which were performed in triplicate. Differences among replicates varied between 0 and 30%. All results presented hereafter are mean values. Sample blanks and spiked controls without bacterial cultures were carried out for every series and over the same time range as the
pool remained after 7 d. At 37°C B7-1001 was formed by day 3, only 15 to 20% of the day 3 previous experiments [11]. Although a significant amount of toxaphene or individual congeners in spiked controls was <10%. The recovery of samples spiked with technical toxaphene or individual CTTs that were immediately reextracted ranged from 70 to 100% and the internal standard perdeuterated α-hexachlorocyclohexane was quantitatively recovered. Extractions were deemed quantitative by repeated extraction after treatment with H2SO4 [11].

RESULTS AND DISCUSSION

Recently, we demonstrated that the dominant heptachlorobornane of the anoxic transformation of toxaphene, B7-1001 [2,6,8,10–12,25], was partly degraded by Dehalospirillum multivorans [11]. In a set of experiments, we used the precursor B8-1414 and studied the formation and transformation of B7-1001 along with the influence of the incubation temperature (4, 28, and 37°C) on the rate of the transformation.

At 28°C (standard procedure [11]), about 95% of B8-1414 was transformed to B7-1001 within 3 d (Fig. 1). The transformation rate and yield were similar (∼10% difference) to previous experiments [11]. Although a significant amount of B7-1001 was formed by day 3, only 15 to 20% of the day 3 pool remained after 7 d. At 37°C, transformation of B8-1414 was much slower and the concentration of B7-1001 steadily increased with time, indicating that the equilibrium between formation and transformation was shifted to formation (Fig. 1). The occurrence of increased transformation activity at 28°C is consistent with temperature-dependent biotransformation of other chlorinated pollutants [26,27]. Thus, seasonal variations in CTT transformation rates can be expected under the wide range of ambient conditions found worldwide (e.g., temperate vs polar latitudes). For our work, an incubation temperature of 28°C showed the highest activity for CTT transformation, and was thus adopted as the standard condition for all future experiments.

Transformation of B7-1001

Incubation of this heptachlorobornane, which has no gem-Cls, resulted in a transformation rate of approximately 85% within 7 d and 98% within 14 d. These results are of the same order as those obtained during incubations with B8-1414 (see above). However, B7-1001 transformation was significantly slower than transformation of CTTs with gem-Cls [6,7,11]. Nine transformation products of B7-1001 were detected: six hexachlorobornanes, one unsaturated hexachloro compound, and two pentachlorobornanes (Fig. 2). Of the two commercially available hexachlorobornanes (B6-923 and B6-913), only the latter (B7-1001 minus 3-exo-Cl) could be formed and was detected in the samples (Fig. 3). The B6-913 was identified previously as a transformation product of B8-806 and other experiments. Loss of toxaphene or individual congeners in spiked controls was <10%. The recovery of samples spiked with technical toxaphene or individual CTTs that were immediately reextracted ranged from 70 to 100% and the internal standard perdeuterated α-hexachlorocyclohexane was quantitatively recovered. Extractions were deemed quantitative by repeated extraction after treatment with H2SO4 [11].

Table 1. Mass-to-charge (m/z) values used for gas chromatography–electron-capture negative-ion-high-resolution mass spectrometry–selected ion monitoring of penta- to nonachlorobornanes, hexachlorobornanes, and 13C-labeled mirex (internal standard)

<table>
<thead>
<tr>
<th>Congener group</th>
<th>Quantitation ion</th>
<th>Confirmation ion</th>
<th>SIM window*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C10H10Cl8</td>
<td>272.9585 [M-Cl +2]^-</td>
<td>270.9615 [M-Cl]^(-)</td>
<td>1</td>
</tr>
</tbody>
</table>

* SIM = selected ion monitoring window, with DB-5 column: 1, 10–20.5 min; 2, 20.5 min–26; 3, 26–35 min. The two abundant ions of the [M-Cl]^(-) clusters of penta through nonachlorobornane were monitored, except for hexachlorobornene (m/z 304.9225 [M-Cl]^- and 306.9196 [M-Cl +2]^(-)).
CTTs [7,11]. These results indicate that this compound is also a metabolite of important compounds such as B9-1679, B8-1414, and B8-1412 (Table 2).

The compound B6-627 (Fig. 3g) was identified by using the structure and retention data of Nikiforov et al. [28], who published relative retention indices and elution temperatures for B6-923, B6-627, and B7-1001 (Table 3). The detection of B6-627 in our incubations supports the hypothesis that it is formed from B7-1001 via elimination of 5-endo-Cl.

No standard was available to match the retention time of the dominant hexachlorobornane, which accounted for approximately 50% of the GC-ECNIMS response of all products in our samples. Previous experiments demonstrated that reductive dechlorination at the primary carbons C8, C9, and C10 was significantly lower than at secondary carbons [11]. Therefore, Cl→H exchange at positions on the six-membered ring (Fig. 3a, f, g, and i) appeared to be most plausible. Furthermore, the transformation pathway of B8-806 and B9-1025 suggested that endo-Cl's vicinal to C1 are readily eliminated [11]. Therefore, it follows that the dominant transformation product is 2-exo, 3-exo, 5-exo, 8, 9, 10-hexachlorobornane (B6-903) (Fig. 3f). Because B6-903 was only found as a trace compound in Melipax, the ratio B7-1001:B6-903 can be used to estimate the degree of transformation of B7-1001. Other candidates for major transformation products of B7-1001 are B6-589 (minus 6-exo-Cl) (Fig. 3i) and B6-627 (minus 5-endo-Cl) (Fig. 3g), whereas minor hexachlorobornanes may be formed by dechlorination at primary carbons.

The abundance of the unsaturated CTT Hx-Be2 (Fig. 2) increased by one order of magnitude from start to day 7, but only one half of the amount detected after 7 d was left after 14 d. Kimmel et al. [29] reported that technical toxaphene contains about 3% chlorocamphenes and 2% chlorobornenes (unsaturated compounds ~5%). Chlorocamphenes and chlorobornenes are isomers and cannot be unequivocally distinguished by GC-ECNIMS or gas chromatography-electron-ionization mass spectrometry [30]. Therefore, both bornene and camphene (via rearrangement) are possible hydrocarbon backbones for Hx-Be2. Unfortunately, infrared-spectroscopic characterization was not possible [29] because of the low amounts available. Turner et al. [20] produced chlorobornenes from B7-515 by treatment with KOH. Although three hexachlorobornenes are possible, only 2,2,5,8,9,10-hexachloroborn-5-ene (removal of the 6-exo-Cl) and 2,5-endo, 6-exo, 8, 9, 10-hexachloroborn-2-ene (removal of a Cl substituent at C2) were formed in the ratio 6:1 [20]. Our own treatment of B7-1001 with methanolic KOH resulted in equal amounts of two of the four possible hexachlorobornenes: Hx-Be1 and Hx-Be2 (Fig. 4a). From Turner et al. [20], it appears that only exo-Cl's are removed from chlorobornanes. Therefore, 3,5-exo, 6-endo, 8, 9, 10- and 2,5-endo, 6-exo, 8, 9, 10-hexachloroborn-2-ene are most likely the dehydrochlorination products of B7-1001 (Fig. 3). Note that International Union of Pure and Applied Chemistry nomenclature requires that the double bond is located between C2 and C3, regardless of the backbone numbering of the parent bornane.
Table 2. Structures and relative retention indices (RRIs) of individual compounds of technical toxaphene on a DB-5 column

<table>
<thead>
<tr>
<th>AV code</th>
<th>Other abbreviation</th>
<th>Metabolite of</th>
<th>RRI</th>
<th>IUPAC name or proposed structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>B5-483</td>
<td>—</td>
<td>B7-1001/B6-923</td>
<td>0.303</td>
<td>2-exo,3-endo,5-endo,9,10-Pentachlorobornane</td>
</tr>
<tr>
<td>—</td>
<td>Pe-Ba2</td>
<td>B6-923</td>
<td>0.289</td>
<td>Unknown pentachlorobornane</td>
</tr>
<tr>
<td>—</td>
<td>Pe-Ba3</td>
<td>B7-1001</td>
<td>0.301</td>
<td>Unknown pentachlorobornane</td>
</tr>
<tr>
<td>—</td>
<td>Hx-Be1</td>
<td>B7-1001</td>
<td>0.301</td>
<td>3,5-exo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>B6-903</td>
<td>—</td>
<td>B7-1001</td>
<td>0.301</td>
<td>Unknown hexachlorobornane</td>
</tr>
<tr>
<td>—</td>
<td>Hx-Be3</td>
<td>—</td>
<td>0.340</td>
<td>2-exo,5,endo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>B6-913</td>
<td>—</td>
<td>B7-1001/B7-1461/B8-806/</td>
<td>0.346</td>
<td>2-exo,5,endo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>—</td>
<td>Hx-Be4</td>
<td>—</td>
<td>0.347</td>
<td>Unknown hexachlorobornane</td>
</tr>
<tr>
<td>B7-1453</td>
<td>—</td>
<td>B7-1473/B7-515/B8-806/B8-809/</td>
<td>0.357</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>B6-923</td>
<td>—</td>
<td>Hx-Sed</td>
<td>0.397</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>—</td>
<td>Hx-Ba7</td>
<td>—</td>
<td>0.357</td>
<td>2-exo,5,endo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>B6-627</td>
<td>—</td>
<td>B7-1001</td>
<td>0.363</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>B7-1001</td>
<td>Hp-Sed</td>
<td>B8-1412/B8-1414/B9-1679/</td>
<td>0.366</td>
<td>2-exo,5,endo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>—</td>
<td>Hx-Ba8</td>
<td>—</td>
<td>0.385</td>
<td>Unknown hexachlorobornane</td>
</tr>
<tr>
<td>B8-1413</td>
<td>—</td>
<td>B8-806/</td>
<td>0.396</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Octachlorobornane</td>
</tr>
<tr>
<td>B7-1473</td>
<td>—</td>
<td>B8-806/</td>
<td>0.397</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Octachlorobornane</td>
</tr>
<tr>
<td>B8-1412</td>
<td>AES1</td>
<td>B9-1679</td>
<td>0.403</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Octachlorobornane</td>
</tr>
<tr>
<td>B7-1450</td>
<td>—</td>
<td>—</td>
<td>0.413</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Octachlorobornane</td>
</tr>
<tr>
<td>B7-515</td>
<td>—</td>
<td>P-32</td>
<td>0.419</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>B7-1474</td>
<td>—</td>
<td>—</td>
<td>0.422</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>B7-1440</td>
<td>—</td>
<td>—</td>
<td>0.422</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>B7-1461</td>
<td>—</td>
<td>B8-806/</td>
<td>0.431</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Hexachlorobornane</td>
</tr>
<tr>
<td>B8-531</td>
<td>—</td>
<td>P-39</td>
<td>0.468</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Octachlorobornane</td>
</tr>
<tr>
<td>B8-1414</td>
<td>—</td>
<td>B9-1679/</td>
<td>0.470</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Octachlorobornane</td>
</tr>
<tr>
<td>B8-1945</td>
<td>—</td>
<td>P-41</td>
<td>0.470</td>
<td>2-exo,3-endo,5-endo,6-endo,8,9,10-Octachlorobornane</td>
</tr>
<tr>
<td>B8-806</td>
<td>—</td>
<td>P-42a</td>
<td>0.474</td>
<td>2,2,5,endo,6,endo,8,9,10-Octachlorobornane</td>
</tr>
<tr>
<td>B8-809</td>
<td>—</td>
<td>P-42b</td>
<td>0.474</td>
<td>2,2,5,endo,6,endo,8,9,10-Octachlorobornane</td>
</tr>
<tr>
<td>B8-2229</td>
<td>P-44</td>
<td>B9-1025</td>
<td>0.479</td>
<td>2-exo,5,endo,6,endo,8,9,10-Octachlorobornane</td>
</tr>
<tr>
<td>B9-1679</td>
<td>P-50</td>
<td>—</td>
<td>0.512</td>
<td>2-exo,3-endo,5-endo,6,endo,8,9,10-Nonachlorobornane</td>
</tr>
<tr>
<td>B9-1025</td>
<td>P-62</td>
<td>—</td>
<td>0.603</td>
<td>2,2,5,endo,6,endo,8,9,10-Nonachlorobornane</td>
</tr>
</tbody>
</table>

**Notes:**
- **RRI =** \( t_r (\text{compound}) / t_r (\text{Be-1413}) + t_r (\text{Be-1679}) \), \( t_r = \) retention time.
- **AV =** Andrews and Vetter; IUPAC = International Union of Pure and Applied Chemistry.
- **Pe-Ba =** pentachlorobornane; Hx-Be = hexachlorobornene; Hx-Ba = hexachlorobornane; Hp-Ba = heptachlorobornane; Sed = sediment; P = Parlar number.
- **Structure not confirmed.
- **The suggested structures are based on \( t_r \) comparisons and known transformation pathways.
- **[11].**
- **[12].**

Comparisons of our gas chromatography retention times and ECNIMS spectra (ECNIMS of Hx-Be2 is dominated by the [M-Cl] \(^-\) ion and that of Hx-Be1 is dominated by the [M] \(^+\) ion) with data published by Saleh and Casida [31] clarified that both the minor product of KOH of treatment of B7-515 [20; see above] and Hx-Be2 are identical with 2,5-exo,6-exo,8,9,10-hexachloroborn-2-ene. Consequently, Hx-Be1 is most likely 3,5-exo,6-exo,8,9,10-hexachloroborn-2-ene.

Interestingly, Hx-Be2 also was detected as a metabolite of B7-1001 upon treatment with *D. multivorans* (Fig. 2). This answers the initial question that this compound is a bornone and not a camphene. Both hexachlorobornenes produced from B7-1001 by KOH treatment (Hx-Be1 and Hx-Be2) were detected in minute amounts in the technical product Melipax (Fig. 4b). Incubation experiments of Melipax (data not shown) led to a steady increase of the amount of Hx-Be1 and Hx-Be2 throughout the study (16 d). Therefore, Hx-Be1 also was formed by transformation of Melipax with *D. multivorans* but not from B7-1001. However, revisiting data from previous incubations of single octa- and nonachlorobornanes [11] with *D. multivorans* did not result in the identification of further chlorobornenes. This might be due to their low formation rates.

Table 3. Relative retention indices (RRIs) and elution temperatures of compounds of technical toxaphene (CTTs) used for the identification of B6-627

<table>
<thead>
<tr>
<th>CTT</th>
<th>RRI [28]</th>
<th>RRP</th>
<th>Ratio RRIgas study:</th>
<th>RRI [28]</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6-923</td>
<td>0.4222</td>
<td>0.3574</td>
<td>0.8465</td>
<td></td>
</tr>
<tr>
<td>B6-627</td>
<td>0.4290</td>
<td>0.3635</td>
<td>0.8474</td>
<td></td>
</tr>
<tr>
<td>B7-1001</td>
<td>0.4336</td>
<td>0.3665</td>
<td>0.8453</td>
<td></td>
</tr>
<tr>
<td>B8-1413</td>
<td>0.4494</td>
<td>0.3965</td>
<td>0.8823</td>
<td></td>
</tr>
<tr>
<td>B9-1025</td>
<td>0.5506</td>
<td>0.6035</td>
<td>1.0961</td>
<td></td>
</tr>
</tbody>
</table>

**Notes:**
- **Elution temperature 280°C.**
- **Elution temperature 240°C (this study).**
which would require a very large amount of parent chloroborne to exceed chlorobornene detection limits.

The selective formation of Hx-Be2 from B7-1001 by *D. multivorans* instead of the chemical dehydrochlorination indicates a highly specific interaction of the bacterial enzyme, due to electronic or steric reasons. Because chlorine atoms on the six-membered ring of B7-1001 are staggered in an *exo-* or *endo-*conformation, HCl elimination must occur in *cis*-orientation (H and Cl both in either *exo-* or *endo-*position). However, dehydrochlorination appears to be a minor pathway in the anaerobic transformation of toxaphene. This alternative mechanism of transformation likely occurs only in the case of low-chlorinated CTTs with special substructure such as the all-staggered *exo-* or *endo-*conformation on the six-membered ring.

**Transformation of B6-923**

The B6-923 carries neither gem-Cl's nor substituents in *endo-*positions vicinal to Cl, indicative of a high stability under anaerobic conditions (see above). Incubation with *D. multivorans* resulted in a significant transformation of B6-923 (20–50 ng; Fig. 5). Minor B6-923 transformation was observed with *E. coli* (data not shown). Heat-inactivated samples of *D. multivorans* reduced 20% of the initial amount of B6-923 (Fig. 5). Cell suspensions that were completely oxidized by treatment with trichloroacetic acid did not show any CTT transformation (data not shown). Therefore, heat-inactivated samples of *D. multivorans* still contain some reducing ingredients, such as redox-active cofactors (iron–sulfur clusters, corrinoids, flavine, cytochrome, and chinone) [14,15,32], which are likely responsible for the observed transformation of toxaphene [11].

The GC-ECD-high-resolution mass spectrometry enabled detection of three pentachlorobornanes (B5-465, Pe-Ba2, and B5-483) as transformation products of B6-923 (Fig. 6). The major product also has been detected during the transformation of B7-1001 (Fig. 3c). The only possible pentachlorobornane that can be formed from both B6-923 (minus 2-*exo*-Cl) and B7-1001 (minus 2-*endo*-Cl and minus 3-*exo*-Cl) is 5-*endo*,6-*exo*,8,9,10-pentachlorobornane (B5-483). Moreover, reanalysis of samples with high-resolution mass spectrometry enabled the detection of B5-465 in transformation experiments with B7-1453. As was shown above for B5-483, the only common metabolite of B6-923 and B7-1453 is 2-*exo*,3-*endo*,5-*exo*,9,10-pentachlorobornane (B5-465). Pentachlorobornenes were not detected in transformation experiments with B6-923. The results of the B6-923 transformation with *D. multivorans* were supported with results of the transformation experiments of technical toxaphene, which showed a decrease of the B6-923 concentration in the later period of this experiment [33].

**Penta- and hexachloro compounds in the environment**

Our incubation experiments with *D. multivorans* and individual CTTs (this study and that of Ruppe et al. [11]) resulted in the detection of approximately 20 previously unknown compounds that are potential CTT residues in anoxic sediment, soil, and sewage sludge. In this section we investigated if the same transformation products were found in environmental samples.

Several unknown heptachlorobornanes were detected in sediments from Terry/Dupree Creek (GA, USA), an estuarine marsh that received discharge from a former toxaphene plant [8,9]. Several heptachlorobornanes also were detected by Muir and Karlsson (D. Muir, Environment Canada, Burlington, ON, Canada, personal communication to W. Vetter). It is noteworthy that the heptachlorobornanes B7-1473, B7-1461, and B7-1470 [11], the pentachlorobornanes Pe-Ba3 and B5-483, as well as Hx-Be2 (this study) were present in Terry/Dupree
Creek samples [8]. Buser et al. [6] found relatively high amounts of the unsaturated compound X1 (compared to B6-923) in toxaphene transformation experiments with sewage sludge. Based on retention characteristics on a chiral stationary phase consisting of tert-butylidimethylsilylated β-cyclodextrin (β-BSCD), X1 and Hx-Be2 appear to be identical [6,8].

The CTT profile in the Lake Ontario sediment sample was dominated by the known major metabolites B6-923 and B7-1001 (Fig. 7). Interestingly, all transformation products of B7-1001 described herein were detected except B6-627 and Hx-Ba7 (Fig. 7). The elevated ratio B7-1001:B6-903 (see above) indicated that only small amounts of B7-1001 had been metabolized in the sample. In agreement with this, transformation products of B6-923 (B5-465 and Pe-Ba2) were not detected. The detection of four hexachlorobornanes including Hx-Be1 and Hx-Be2 supports our finding that dehydrochlorination is a minor transformation pathway of toxaphene in natural sediments.

Evaluation of the findings

This study and comparisons with natural environments clearly demonstrate that anoxic toxaphene transformation by a strict anaerobe (D. multivorans) generates many of the same residue congeners observed in reducing media in various environments (i.e., soils, sediments, and sewage sludge). It also follows that chemical conditions (suitable electron donors, pH, and redox potential; anoxic transformation of toxaphene was tested at a redox potential below ~ 110 mV [resazurin colorless]) and predominantly temperature are key factors that will impact the extent of toxaphene transformation. Although transformation of toxaphene was only tested with formate, it is known that D. multivorans can utilize a broad range of widespread electron donors (formate, pyruvate, lactate, and hydrogen) for both reductive dechlorination and growth [21]. Differences in these factors or conditions are the likely major reasons for the different toxaphene residue patterns found in experiments with anoxic sewage sludge [34], as well as in Canadian freshwater [25] and estuarine sediment from the southeastern United States [8,9].

We also identified more than 10 previously unknown CTT metabolites in a sediment sample from Lake Ontario. The greater recalcitrance of B6-923 and B7-1001 under reducing conditions compared with higher chlorinated CTTs also was confirmed in these incubations. Therefore, the selectivity of microorganisms to effect complete anaerobic biotransformation of toxaphene should primarily be tested with B6-923 and B7-1001, which are not readily transformed in sediment and soil but are metabolized by fish and other higher organisms [35,36]. Reducing the chlorine content of toxaphene residues in sediment will significantly reduce the likelihood of food web accumulation of CTTs.

Acknowledgement—We are grateful to G. Diekert and B. Luckas for supporting our work. We thank Chris Marvin for the sediment sample from Lake Ontario. W. Vetter acknowledges a donation in support of his research. S. Ruppe acknowledges a travel grant for measurements at the Freshwater Institute. We are grateful to C. Marvin (Environment Canada, Burlington, ON) for the donation of the sediment sample. K. Maruya acknowledges support from the Georgia Coastal Management Program (CIG NA070Z0113).

REFERENCES

15. Neumann A. 1998. Isolation and cloning of tetrachloroethene de-


