ANAEROBIC DEGRADATION OF TOXAPHENE BY THE ISOLATED MICROORGANISM *DEHALOSPIRILLUM MULTIVORANS*

Steffen Ruppe[1], Anke Neumann[2], Walter Vetter[1]

[1]Friedrich-Schiller-University Jena, Department of Food Chemistry. Dornburger Str. 25, DE-07743 Jena, Germany

[2]Friedrich-Schiller-University, Department of Applied and Ecological Microbiology Philosophenweg 12, DE-07743 Jena, Germany

Introduction

Toxaphene (Camphechlor, Melipax) has been used in high quantities as a pesticide since 1945[1,2]. Because of its persistence in the environment, the bioaccumulative behavior of some compounds of technical toxaphene (CTTs) in the aquatic food webs, as well as its toxicity to fish and higher animals, toxaphene was banned in many countries some twenty years ago. In the environment, the complex technical mixture is significantly changed. In sediment, soil, and anoxic sewage sludge hexa- and a heptachlorobornanes were the major residues, and the two major CTTs were identified as B6-923 and B7-1001[3,4]. The principal pathway of toxaphene degradation appears to be Cl→H substitution, primarily at carbons with geminal Cl atoms (*gem*-Cls). An active participation of microorganisms has been suggested in the degradation of toxaphene[5]. To obtain more information on the role of microorganisms in the anaerobic degradation of toxaphene, we attempted to use suspensions of the single, strictly anaerobic gram negative bacterium *Dehalospirillum multivorans*[6]. The potential of *D. multivorans* for the anaerobic degradation of organohalogens was demonstrated for chloroethenes and chloropropenes[7].

Material and Methods

Chemicals

Individual CTTs were from Promochem (Wesel, Germany) and Dr. Ehrenstorfer (Augsburg, Germany), or were produced from environmental samples. The CTTs are abreviated using AV-codes[8].

Cultivation of D. multivorans

The bacterium was routinely grown on anaerobic media with 40 mM pyruvate and 40 mM fumarate and 0.2 % yeast extract[6]. The medium was inoculated with ~10 % of a culture grown on 40 mM formate and 5 mM PCE as energy source and incubated at 25 °C and 200 rpm in a water bath shaker[6]. The bacteria grew within less than 24 h to cell densities (OD_{578}) of about 1.0 which is corresponding to ~0.22 mg cell protein per mL.

Preparation of Cell Suspensions

The bacteria were harvested in the late logarithmic growth phase under anoxic conditions by centrifugation at 8,000 rpm for 10 min at 4 °C. The pellets were resuspended in 100 mL 0.1 M Tris-HCl (pH 7.5). For each series of experiments, 4.5 mL of the cell suspension and 0.5 mL of the electron donor formate (40 mM) were dispensed into 35-70 10 mL vials, 5 µL of CTTs in *n*-hexane were added and the vials were stoppered with teflon-lined septa. The samples were kept anaerobically in a glove box (95 % N_2 and 5 % H_2). Heat-inactivated samples were prepared by incubating the closed tubes for 10 min at 95

°C. After that, CTTs were added by injection through the septa. Duplicates of samples, sample blanks and spiked controls without bacterial culture were analyzed for each sample extracted at each period.

Sample Preparation and Analysis

Vials were opened, the internal standard perdeuterated a-HCH (a-PDHCH) was added and the entire sample was immediately extracted with 2x 10 mL *n*-hexane (ultrasonic bath, 5 min). The combined *n*-hexane extracts were filtered through Na_2SO_4, and the volume was adjusted to 2 mL (technical toxaphene) or 1 mL (individual CTTs). 1 µL of each extract was analyzed by GC/ECD and GC/ECNI-MS as described elsewhere[9].

Results and Discussion

Incubation of technical toxaphene (Melipax) with freshly grown cells of *D. multivorans* allowed to study the degradation of CTTs in dependence on the reaction time. The starting sample showed the known complex CTT pattern characterized by a high number of mainly octa - and nonachlorobornanes. After seven days, B6-923 was the most abundant CTT in the sample. Otherwise, the CTT pattern was similar to that of the starting sample. After 16 days, the degradation process resulted in a more or less quantitative elimination of all octa- through decachloro CTTs. B6-923 and B7-1001 were the dominant degradation products, which is in agreement with literature data. B7-1001 was the only recalcitrant heptachloro CTT in our samples, while additional, yet unknown heptachlorobornanes were previously described in a contaminated sediment[10]. After 41 days, the late eluting CTTs were almost quantitatively eliminated and the concentration of the early eluting CTTs (including B6-923 and B7-1001) was also reduced.

Degradation of single compounds shown at the example of B8-806

Degradation of several individual CTT standards was also investigated in this work. Other investigators have shown that degradation of both B8-806 and B8-809 (i. e. Toxicant A) leads to B6-923[11,12]. The structurally similar diastereomers co-elute on DB-5/HP-5-columns. The two precursors of B6-923 possess two *gem*-Cls, and one of each has to be substituted with H atoms to result in B6-923. One of the *gem*-Cls is on C2, i. e. a secondary carbon in the 6-membered-ring (C1-C6), while the other is located at C8, i. e. a primary carbon of the bridge (C7-C9). Here we attempted to detect intermediate products of this degradation process. To avoid insecurities caused by mixed reactions of the two compounds in Toxicant A, neat B8-806 was used in this study (Figure 1a,d).

After six hours of treatment with *D. multivorans*, an unknown heptachlorobornane was detected by GC/ECNI-MS as an intermediate product while B8-806 was quantitatively eliminated (Figure 1b).

The lack of any B7-515 (Toxicant B; B8-806 minus Cl on C8, Figure 1g) in the sample demonstrates that the initial rapid reductive dechlorination of B8-806 occurs at the secondary carbon C2 and not at the primary carbon C8 (Figure 1e). Cl→H exchange at one of the *gem*-Cls on C2 may produce two products. Elimination of the 2-*endo*-Cl on B8-806 results in B7-1473 (Figure 1e) and elimination of the 2-*exo*-Cl yields B7-1461 (Figure 1h). Further reduction of the intermediate heptachlorobornane to B6-923 clarified that the substitution took place at 2-*endo* (Figure 1c). Therefore, the intermediate CTT of the B8-806-degradation to B6-923 must be B7-1473 (Figure 1e).

Additionally, a minor hexachlorobornane next to B6-923 was detected in the samples (Figure 1c). This hexachlorobornane is most likely B6-913 (Figure 1i) previously identified by Fingerling et al.[12] which was also identified as a minor peak in the elimination experiment with Melipax. This also suggests that the minor heptachlorobornane is B7-1461 (Figure 1h).

D. multivorans degraded B8-806 within a few hours which is essentially faster than degradation of B6-923. Furthermore, substitution of the 2-*endo*-Cl (B8-806 → B7-1473) was ~20-fold faster (6 h vs. 5 days, Figure 1) than reduction at C8 (B7-1473 → B6-923).

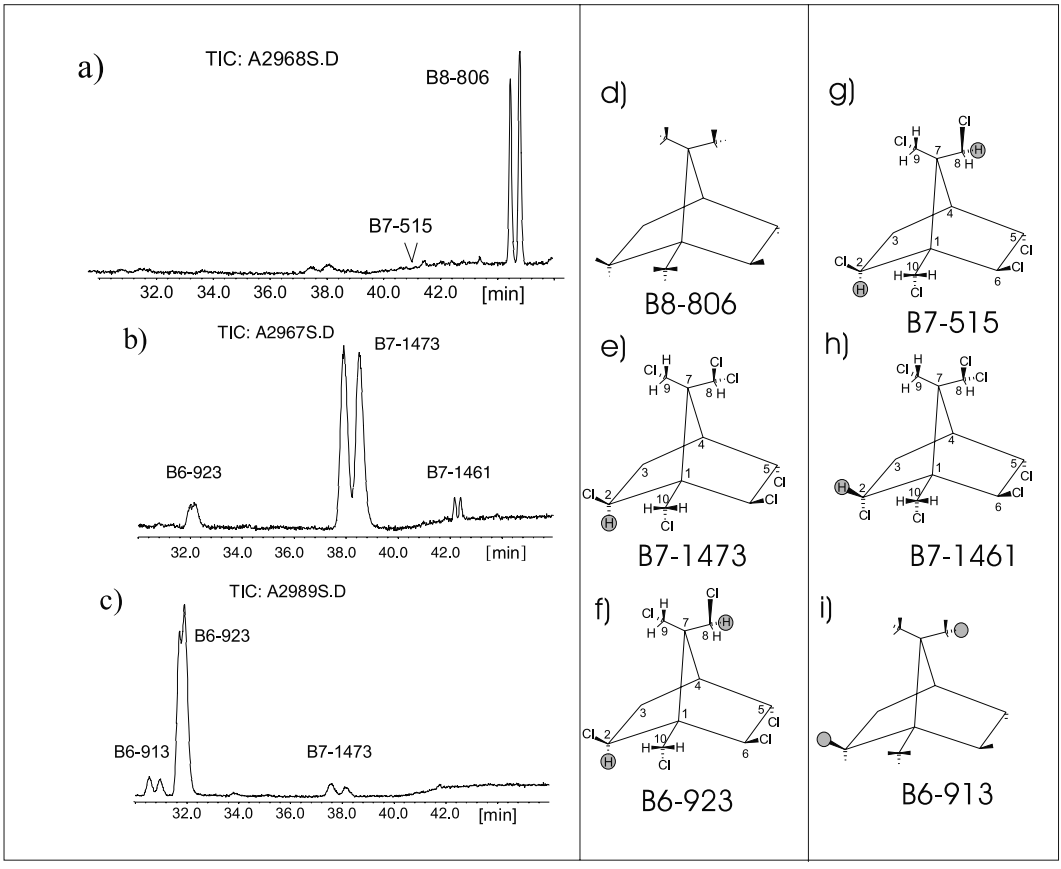

Figure 1. Degradation of 2,2,5-endo,6-exo,8,8,9,10-octachlorobornane (B8-806) by D. multivo-rans. Left panel: GC/ECNI-MS multiple ion chromatograms (enantioselective b-BSCD column) of the degradation after (a) 0 hours (starting sample); (b) six hours; and (c) five days. Right two panels: structure of (d) B8-806; (e) 2-exo,3-endo,6-exo,8,9,9,10-heptachlorobornane (B7-1473) - the intermediate identified in this study; (f) 2-exo,3-endo,6-exo,8,9,10-hexachlorobornane (B6-923). Further structures on display are (g) B7-515; (h) B7-1461 identified in this study; (i) B6-913.

The comparably slow process in degradation of one of the *gem*-Cls on primary carbons puts new light on the degradation of B8-1414 and other CTTs that are at the same time intermediates of the toxaphene degradation. Degradation of a (geminal) Cl atom at the primary carbon C8 of B9-1679 results in B8-1414. Assuming that reduction at the primary carbons C8 and C10 (both are found on B9-1679) occurs with a comparable reaction speed, the degradation of B8-1414 in toxaphene will be partly compensated by its formation from B9-1679. Consequently, B8-1414 shams to be more stable than it actually is in the degradation of toxaphene. Furthermore, we also found some degradation of B6-923. After eight days (14 days) *D. multivorans* eliminated 60 % (70 %) of the initial B6-923 concentration, respectively. However, the process was much slower than for CTTs with *gem*-Cls, and this confirms the importance of this strucutural feature on the stability of CTTs.

OTHER HALOGENATED POPs OF CONCERN

Comparison lab-conditions with natural processes. In a recent study with naturally contaminated sediments in southern US, four unknown heptachloro CTTs were detected[10]. Our revisiting of the data confirmed that two of them (hepta #A and #D in ref. 10) are identical with B7-1473 and B7-1461, i. e. the intermediate products of B8-806 identified in this study. This suggests that the remaining two unknown heptachlorobornanes (hepta #B and #C in ref. 10) are most likely intermediates of the dechlorination of B8-809 (i. e. B7-1458 and B7-1470). Vetter and Maruya also reported that hepta #A and #B were less stable than #C and #D in fish (longnose gar)[10]. The high abundance of the intermediate products hepta #A - #D in the naturally contaminated sediments after a long period of contamination justifies the prediction that the natural microflora is not/no more efficient in processing the high amounts of toxaphene. The processing of toxaphene with *D. multivorans* was much more effective than it was in previous experiments with anoxic sewage sludge[9]. Therefore, our use of the effective *D. multivorans* illustrated that the CTT degradation is highly depending on the types of active microorganisms present in natural environments. A different mixture of microbes as well as the chemical conditions in the soil (suitable electron donors, pH, and redox potential) may thus be one if not the determining parameter that will have an impact on the degree of toxaphene degradation.

In addition to mechanistic findings (much faster degradation of gem-Cls at secondary than on primary carbons, see above) the method described in this work is also suitable to identify and to elucidate the structure of previously unknown but environmentally relevant CTTs such as B7-1473 and B7-1461.

References

1. Saleh M. A. Rev. Environ. Contam. Toxicol. 1991, 118, 1-95.
2. Vetter W. and Oehme M. Toxaphene. Analysis and Environmental Fate of Congeners. In: The Handbook of Environmental Chemistry, Vol. 3, Part K, New Types of Persistent Halogenated Compounds; J. Paasivirta, ed., Springer, 2000, pp. 237-287.
3. Miskimmin B.M., Muir D.C.G., Schindler D.W., Stern G.A. and Grift N.P. Environ. Sci. Technol. 1995, 29, 2490-2495.
4. Stern G.A., Loewen M.D., Miskimmin B.M., Muir D.C.G. and Westmore J. B. Environ. Sci. Technol. 1996, 30, 2251-2258.
5. Mirsatari S.G., McChesney M.M., Craigmill A.C., Winterlin W.L. and Seiber J.N. J. Environ. Sci. Health 1987, B22/6, 663-690.
6. Scholz-Muramatsu H., Neumann A., Meßmer M., Moore E. and Diekert, G. Arch. Mircobiol. 1995, 163, 48-56.
7. Neumann A., Siebert A., Trescher T., Reinhardt S., Wohlfahrt G. and Diekert G. Arch. Microbiol. 2002, 177, 420-426.
8. Andrews P. and Vetter W. Chemosphere 1995, 31, 3879-3886.
9. Vetter W. and Kirchberg D. Environ. Sci. Technol. 2001, 35, 960-965.
10. Vetter W. and Maruya K.A. Environ. Sci. Technol. 2000, 34, 1627-1635.
11. Khalifa S., Mon T.R., Engel J.L. and Casida J.E. J. Agric. Food Chem. 1974, 22, 653-657.
12. Fingerling G., Hertkorn N. and Parlar H. Environ. Sci. Technol. 1996, 30, 2984-2992.