LOW CHLORINATED TOXAPHENE IS MORE TOXIC IN ZEBRAFISH EMBRYO TEST THAN CONVENTIONAL TOXAPHENE

Kapp T[1], Kammann U[2], Vobach M[2], Vetter W[1]

[1] University of Hohenheim, Institute of Food Chemistry, Garbenstr. 28, D-70593 Stuttgart, Germany
[2] Federal Research Centre for Fisheries, Institute for Fishery Ecology, Palmaille 9, D-22767 Hamburg, Germany

Introduction

Toxaphene is an organochlorine pesticide which was produced on a million-ton scale by photochlorination of camphene or α-pinene extracted from lumber offal.[1-2] The resulting complex mixtures consist of >1000 compounds.[3] Because of its broad, non-systemic pesticidal activity it was extensively used on cotton, soy, tobacco, peanuts and many other crops.[2]

Main components of technical toxaphene are polychlorinated bornanes whose skeleton emerges during photochlorination by a Wagner-Meerwein rearrangement from the camphene precursors[4]. Some prominent compounds of technical toxaphene (CTTs) are shown below (**Fig. 1**).

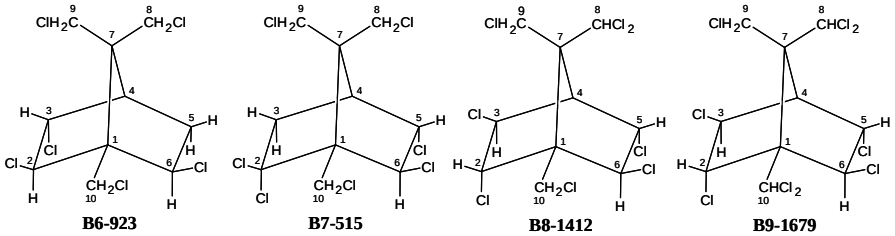

Figure 1: Structures of selected CTTs mentioned in this article. From left to right: 2-*exo*,3-*endo*,6-*exo*,8,9,10-hexachlorobornane (B6-923, Hx-Sed), 2,2,5-*endo*,6-*exo*,8,9,10-heptachlorobornane (B7-515, P-32), 2-*endo*,3-*exo*,5-*endo*,6-*exo*-8,8,9,10-octachlorobornane (B8-1412), and 2-*endo*,3-*exo*,5-*endo*,6-*exo*,8,8,9,10,10-nonachlorobornane (B9-1679, P-50).

Besides its insecticidal activity which was the main reason for application, toxaphene is extremely toxic to fish and aquatic invertebrates[1]. Consequently, toxaphene has also been used as a piscicide, especially in lakes located in Canada and the United States.[5] Although the acute toxicity to most mammals is relatively low, toxaphene was shown to be mutagenic in the Ames test.[6] Because of the comparatively high volatility and water solubility, toxaphene is highly mobile and can be transported over long distances.[7] As toxaphene is also distinctively bioaccumulated in aquatic biota, it is considered a persistent organic pollutant (POP). Toxaphene was banned worldwide with the Stockholm Convention treaty's entry into force in 2004.[8]

Transformation in soil or in sediment occurs slowly under anaerobic conditions, mostly by means of reductive dechlorination. The residue patterns resulting thereof are shifted towards lower chlorinated compounds when compared to the technical mixture.[4,9] As the toxaphene pattern accumulated by freshwater biota resembles the one of sediment more closely than that of commercial formulations, it is crucial to know how toxicity changes during environmental transformation of toxaphene. However, a problem in determination of toxic effects is the changing composition of residues found in different sample materials. Moreover, environmental samples comprising partially degraded toxaphene are usually contaminated with other POPs as well. Results on toxicity drawn from such samples cannot be attributed to toxaphene exclusively. By employing individual congeners in toxicity assessment one can avoid falsification of results by other harmful substances. However, the toxicity of single components or mixtures thereof can differ clearly from that of more complex mixtures.[10] Consequently, only insignificant toxicological data on altered toxaphene can be gathered this way.

The main objective of this work is the search for a synthetic approach to obtain mixtures resembling the residue pattern of partially transformed toxaphene as it is found in environmental samples. Once synthesized we tested the effects of the high and low chlorinated mixtures in the zebrafish embryo assay.[11] Zebrafish embryos have been successfully used to test the toxicity of environmentally relevant substances.[12-13]

Material and Methods

Synthesis of low and high chlorinated toxaphene. Photochlorination of camphene (90 %, Merck, Darmstadt, Germany) was conducted using UV-light emitted by a 150 W medium pressure mercury vapor lamp (TQ150, Heraeus Noblelight, Hanau, Germany), which was placed in a cooling jacket consisting of quartz glass. The reaction was carried out in quartz beakers (60 mm in height, 30 mm in diameter) held at room temperature by an external constant flow of water. In order to obtain low-chlorinated toxaphene products, 0.5 g camphene was dissolved in 15 mL dichloromethane (99.5 %). After addition of 5 mL SO_2Cl_2 (99%) the mixture was irradiated for 90 min under constant stirring. High-chlorinated toxaphene products were acquired by dissolving 0.5 g camphene in 20 mL SO_2Cl_2 and subsequent irradiation for 5 h. After the photoreactions, the mixture was added dropwise to ice-cooled water. CTTs were extracted with *n*-hexane and washed acid-free with water and aqueous sodium hydrogen carbonate solution. Finally, the extract was dried over anhydrous Na_2SO_4 and the solvent was removed using a rotary evaporator. The products gathered this way were clear yellow, oily liquids, whereas both color and viscosity increased with the degree of chlorination. An elemental combustion analysis of the products resulted in chlorine contents of 57.3 % and 68.8 % for the low and high chlorinated products, respectively. The corresponding mean empirical formulae derived from elemental analysis were $C_{10}H_{13}Cl_5$ for the low chlorinated mixture and $C_{10}H_{10}Cl_8$ for the high chlorinated mixture.

Reference standards. The chlorine content of authenthic Hercules Toxaphene® (lot number "x-16189-9", manufactured in 1978) was 68.9 % according to label and 68.4 % according to own combustion analysis. Individual standards (see Figure 1) were from Dr. Ehrenstorfer (Augsburg, Germany), LGC Promochem (Wesel, Germany), or isolated from environmental samples.[14]

GC/ECNI-MS. A CP-3800/1200 GC/MS equipped with a 30 m x 0.25 mm i.d. x 0.25 µm d_f Factor Four® CP-Sil 8ms capillary column (all items from Varian, Darmstadt, Germany) was used as recently described in details[15]. The GC oven temperature program started at 60 °C (held for 3 min), was then raised at 3 °C/min to 110 °C (held for 0.33 min) and was finally raised at 10 °C/min to 270 °C (held for 24 min). The split/splitless injector was operated in splitless mode for 2 min and kept at 230 °C. The transfer line temperature was maintained at 280 °C. Methane 4.5 was used as reagent gas at a pressure of 8.5 Torr in the ion source, which was kept at 200 °C. For measurements in both full scan and SIM mode, the scan time was set to 0.5 sec/cycle. The mass range covered by full scan mode was *m/z* 30-500. In SIM mode the following ions were monitored, covering the most abundant isotopic peaks of both the $[M\text{-}Cl]^-$ and $[M]^-$ ions of tetra- to decachlorobornanes: *m/z* 239.0, 240.0, 241.0, 242.0, 273.0, 274.0, 275.0, 276.0, 306.9, 307.9, 308.9, 309.9, 340.9, 341.9, 342.9, 343.9, 374.9, 375.9, 376.9, 377.9, 410.8, 411.8, 412.8, 413.8, 444.8, 445.8, 446.8, and 447.8. The SIM width was set to 0.5 u.

Zebrafish embryo test. The zebrafish (*Danio rerio*) embryo test was carried out basically according to DIN 38 415-T6.[12,16] Briefly, fertilized and well-developed eggs in the four to eight cell stage were selected for the test and placed in 24-well dishes (Nunc, Wiesbaden, Germany). In each well five eggs were exposed in 1 mL test solution containing 5-100 mg/L high or low chlorinated toxaphene and 1 % DMSO (v/v) in aerated artificial water according to DIN EN ISO 7346-1.[17] The artificial water contained 58.8 mg $CaCl_2$ x 2 H_2O, 24.6 mg $MgSO_4$ x 7 H_2O, 12.6 mg $NaHCO_3$ and 5.5 mg KCl in 5 L deionized water. Sixty eggs were used for each experiment. Incubation took place at 26 °C for 48 h. After 24 and 48 h eggs were monitored for coagulation and malformations using a stereo microscope. Results were expressed as percent of affected embryos (data range 0 to 100 %; effect rates below 11 % (lethal effects) and 9 % (non-lethal effects), respectively, considered as spontaneous. The tests were replicated twice consecutively including a negative and a positive control (3,4-dichloroaniline, 3.7 mg/L) in every batch. DMSO concentration was 1 % (v/v); negative controls were exposed to the solvent only. EC_{50} were calculated from dose-effect curves for all endpoints separately as well as for the sum of lethal and non-lethal affected embryos, using at least ten different experiments per substrate. A logistic curve was fitted to the observed effect percentages with the software GraphPad Prism® 4.0 (GraphPad Inc., San Diego, USA).

Results and Discussion

Synthesis of tailor-made toxaphene mixtures. Contrary to the technical synthesis of toxaphene, which occurred by exhaustive chlorination of camphene with chlorine gas under UV irradiation[1,2,4], the reaction products obtained in this work were synthesized using SO_2Cl_2 as chlorinating reagent. This way it was possible

to reproducibly control the degree of chlorination of the resulting mixtures by varying the reaction time or the concentrations of camphene and sulfuryl chloride, respectively. GC/MS measurements showed that the high chlorinated mixture mainly consisted of hepta- to nonachlorinated bornanes. Hexa- and deca-substituted bornane derivatives were present to a lesser extent. A similar pattern can be found in technical toxaphene. The selected congeners B6-923, B7-515, B8-1412 and B9-1679 could be identified in both technical toxaphene and the obtained high chlorinated mixture by their retention times and mass spectra (**Fig. 2b**).

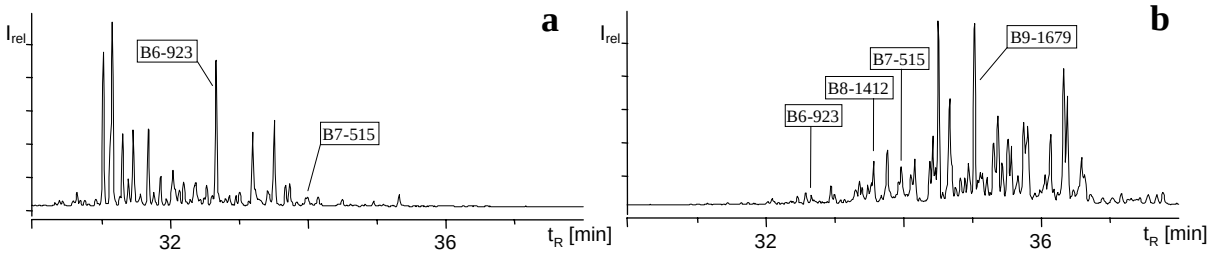

Figure 2: GC/ECNI-MS-SIM chromatograms of a) the low chlorinated reaction product and b) the high chlorinated reaction product

The similarity of the peak patterns is also in good match with the chlorine contents of the high chlorinated mixture (68.8 %) and Hercules Toxaphene® (68.4 %) determined by elemental analysis. The low chlorinated mixture showed a significantly different pattern from commercial toxaphene (**Fig. 2**). Mainly penta- and hexachlorobornanes, but also some heptachloro derivatives were identified (**Fig. 2a**). The most abundant hexachlorobornane in this mixture, B6-923, is an important congener in sediment samples, being a dead-end metabolite of many CTTs.[9] Most of the peaks eluting prior to B6-923 arise from pentachlorobornanes (**Fig. 2c**). These were also detected in sediment samples, although to a much lower degree.

Zebrafish assay with toxaphene mixtures of different degree of chlorination. Exposure to toxaphene led to lethal as well as to various non-lethal malformations in zebrafish embryos. The mode of action of low and high chlorinated toxaphene was similar albeit the toxic impacts differed significantly: the EC_{50} values for lethality (sum of lethal malformations) were 15.3 mg/L for low chlorinated toxaphene and 20.6 mg/L for high chlorinated toxaphene, respectively. The EC_{50} values for the corresponding non-lethal malformations were lower in most cases, and ranged from 5.6 to 28.3 mg/L. The most sensitive (occurring at lowest concentrations) non-lethal malformations were yolk sac edema and spinal malformations (**Fig. 3**). These malformations are not lethal to the embryo so that hatching will take place. However, the viability of malformed larvae is low, especially when a spinal deformation affects swimming. The toxic impact of toxaphene to zebrafish embryos was in the range of those of other environmentally relevant halogenated substances like e.g. aldrin and chlordane (lethal EC_{50} 2 - >10 mg/L, unpublished results). Comparing the EC_{50} values, low chlorinated toxaphene was more toxic than high chlorinated toxaphene. Both EC_{50} (sum of lethal and non-lethal malformations) are considerably lower for low chlorinated toxaphene. The difference between the two mixtures is significant because the 95% confidence intervals of the EC_{50} do not overlap (**Fig. 4**). Toxaphene induces a special malformation in embryonic development: In some experiments all embryos exhibited a clearly visible heartbeat but no circulation of the blood. Although this malformation has been mentioned before[17-18], it is quite a rare observation and may be typical for toxaphene and similar acting chemicals.

Conclusions. Results of this study indicate that environmentally transformed toxaphene is more toxic than higher chlorinated mixtures like technical toxaphene. This may explain the observation that in lakes where toxaphene has been used as a piscicide, a later resettling of fish stocks failed. In Hanson Lake, for instance, decades after the treatment with toxaphene, only one fish species is present nowadays.[19] This is all the more remarkable, as the half-life ($t_{1/2}$) of toxaphene in lakes was found to be rather short compared to that of other POPs like PCBs or DDT.[20] Hence, the toxic effects on fish have to abide although the level of toxaphene is strongly reduced. Especially low chlorinated toxaphene compounds are a conceivable cause for such observations.

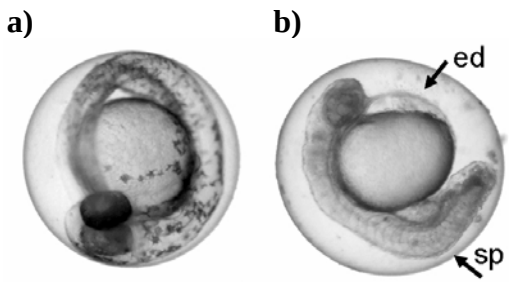

Figure 3: Zebrafish (*Danio rerio*) embryos after 48 h development; (a): normal development and (b) malformed embryo without pigmentation, exhibiting yolk sac edema (ed) and spinal deformation (sp) after treatment with low chlorinated toxaphene.

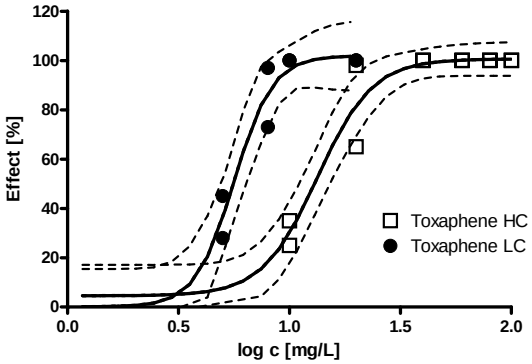

Figure 4: Malformations in zebrafish embryos caused by toxaphenes. Dose-effect curves and 95% confidence intervals of non-lethal endpoints (sum). (HC = high chlorinated, LC = low chlorinated)

Because of their unexpectedly high toxicity, specifically aquatic food should thus be subjected to a more extensive monitoring of these CTTs. Apart from this insight, the new synthetic approach featured in this work offers a possibility to obtain environmentally relevant compounds like penta- and hexachlorobornanes in a rather simple manner. By now, only one pentachlorobornane has been made commercially available.

References

1. Saleh MA, *Rev Environ Cont Toxicol* 1991;118:1.
2. Korte F, Scheunert I, Parlar H, *Pure Appl Chem* 1979; 51:1583.
3. Korytár P, van Stee LLP, Leonards PEG, de Boer J, Brinkman UAT,*J Chromatogr A* 2003;994:179.
4. Vetter W, Oehme M. 2000. In: Paasivirta J, ed, *The Handbook of Environmental Chemistry*, Vol 3, Part K ,Springer, Heidelberg, Germany, pp 237-287.
5. Voldner EC, Li YF, *Chemosphere* 1993;27:2073.
6. Hooper NK, Ames BN, Saleh MA, Casida JE, *Science* 1979;205:591.
7. Bidleman TF, Olney CE, *Nature* 1975;257:475.
8. United Nations Environment Programme. 2001. Stockholm Convention on Persistent Organic Pollutants. Geneva, Switzerland.
9. Maruya KA, Wakeham SG, Vetter W, Francendese L, *Environ Toxicol Chem* 2000;19:2198.
10. Calciu C, Chan HM, Kubow S. *Toxicology* 1997;124:153.
11. Roex EWM, de Vries E, van Gestel CAM, *Environ Pollut* 2002;120:355.
12. Kammann U, Biselli S, Huehnerfuss H, Reineke N, Theobald N, Vobach M, Wosniok W, *Environ Pollut* 2004;132:279.
13. Kammann U, Biselli S, Reineke N, Wosniok W, Danischewski D, Hühnerfuss H, Kinder A, Sierts-Herrmann A, Theobald N, Vahl HH, Vobach M, Westendorf J, Steinhart H, *J Soils Sed* 2005;5:225.
14. Vetter W, Klobes U, Krock B, Luckas B, Glotz D, Scherer G. *Environ Sci Technol* 1997;31:3023.
15. Gaul S, von der Recke R, Tomy G, Vetter W, *Environ Toxicol Chem* 2006;25:1283.
16. DIN 38 415–T6. 2001. German Standardization Organization. Beuth Vertrieb, Berlin, Germany.
17. DIN EN ISO 7346-1. 1998. German Standardization Organization. Beuth Vertrieb, Berlin, Germany.
18. Nagel R, *Altern Lab Anim* 2002;19:38-48 Suppl 1.
19. Vetter W, Bartha R, Stern GA, Tomy GT. *Environ Toxicol Chem* 1999;18:2775-2781.
20. Hickey JP, Batterman SA, Chernyak SM. *Arch Environ Contam Toxicol* 2006;50:97-110.