In-vitro biotransformation of chlorinated bornanes (toxaphene) in hepatic microsomes of marine mammals and birds. Influence on bioaccumulation and mutagenicity.

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Abstract
The *in*-vitro capacity of hepatic microsomal preparations to metabolise toxaphene-derived chlorinated bornanes (CHBs) increased in the order sperm whale < whitebeaked dolphin, albatross << harbour seal. The congeners CHB-26 and CHB-50 were persistent to oxidative biotransformation. The NCI mass spectra of a mono- and a tri-hydroxy metabolite of CHB-32, and a mono-hydroxy metabolite of CHB-62 were obtained. The number of peaks in the toxaphene residue in tissues of the same animals was inversely related to the *in*-vitro biotransformation capacity.

In the mutatox assay, toxaphene was sometimes directly mutagenic but metabolism did not appear to increase the mutagenicity.

1. Introduction

One of the compounds of which much is still unknown about its bioaccumulative potential in marine mammals and sea-birds, is the insecticide toxaphene. A hypothetical Arctic food web showed biomagnification factors (BMF) > 1 between successive trophic levels from algae to cetaceans on a lipid normalised basis, but the BMF from Arctic cod and char to ringed seal was only 0.1-0.2 [1]. Toxaphene has also been identified in liver of gadoid fish from the North Sea, although it has hardly been used in Western Europe [2]. This can possibly be explained by long-range atmospheric transport from tropical to colder areas [3-6]. Technical toxaphene is a very complex mixture primarily consisting of highly lipophilic chlorinated bornanes (CHBs) with low contributions of bornenes and bornadienes. The total number of CHBs present in toxaphene is estimated at ca. 600; most CHBs are Cl_7 and Cl_8 substituted [7-10].

Toxaphene can be metabolised by micro-organisms and laboratory mammals [7]. The *in-vitro* biotransformation of two CHB congeners by hepatic microsomal preparations of rat has also been reported [11]. Since *in-vivo* experiments with living marine predators are often not desirable for ethical, logistic and financial reasons, the application of such *in-vitro* assays offers unique possibilities to study the occurrence biotransformation in marine top predators. In this way, a lot of information can be obtained with liver preparations of only a few individuals.

Toxaphene was shown to be directly mutagenic in the AMES-test [7]. In case of the well-known carcinogenic compound benzo[a]pyrene, only some of the oxygenated metabolites formed are able to bind covalently to DNA, which may lead to a mutation and the subsequent development of a tumour [12]. Therefore the influence of *in-vitro* microsomal metabolism on the mutagenicity of toxaphene was also investigated in the mutatox®-test.

2. Materials and methods

Origin of samples:

- Sperm whale (*Physeter macrocephalus*): A male stranded alive near the Hague at 12th January 1995. Length 14.4 m; age: 15-25 years; estimated weight: 30 metric tonnes; thickness of blubber layer: 11-14 cm. A blubber sample was taken for analysis of the toxaphene residue.
- Whitebeaked dolphin (*Lagenorhynchus albirostris*): A pregnant female stranded alive at 24th January 1995 at Texel. Animal died at 14:15; samples frozen in liquid nitrogen by 18:00. Length: 2.5m; thickness of blubber (ventrally below sternum): 2 cm. Peculiarities: animal missed some teeth and lost milk. A blubber sample was taken for analysis of the toxaphene residue.
- Harbour seal (*Phoca vitulina*): A juvenile female found dead in the ferry harbour of Texel at 5th January 1994. Length: 1.10m; weight: 31 kg; thickness of blubber layer (ventrally below sternum): 4.0 cm. Cause of death: Body cavity was torn open, probably by the propeller of the ferry, since no indications of any scavening were visible. Blood poured out of the heart when this was punctured. Liver sample for chemical analysis of the residue.
- Albatross (*Diomedia imutabulis*): Homogenate of the livers of seven adult animals sampled at Midway (Hawaii) in April 1995. Samples of adipose tissue for chemical analysis of the toxaphene residue were taken from 5 other individuals in the same period at the same site.

Microsomal preparations: The liver was excised generally within some hours of death from animals that were found alive but died later on, and immediately homogenised on ice after the addition of half a volume of cold glycerol. The homogenate was frozen in liquid nitrogen and stored at -80°C for the preparation of microsomal fractions at a later stage. This was done by centrifugation; first at 10,000*g to remove the debris and subsequently at 100,000*g. The pellet containing the microsomes was resuspended in a cold 0.1 M phosphate buffer containing 1 mM EDTA, 1mM DTT (dithiotreitol) and 20% (v/v) glycerol, pH 7.6-7.8, depending on animal species.

The condition of the microsomal preparations was tested before use in the *in*-vitro assay with an EROD-assay [13]. Although this is only indicative for the activity of cytochrome P450 1A (CYP1A), the results were used as an indication for the general viability of the microsomal preparations in this study. Microsomal preparations with an EROD activity < 10 pmol*min^{-1}*mg^{-1} protein were discarded.

In-vitro biotransformation assays: 1ml of a 0.08 M phosphate buffer, containing 1 mM EDTA and 0.1ml of a microsomal suspension containing 10 mg protein/ml was added to a number of 25 ml Erlenmeyer flasks [14]. All assays were carried out in duplicate in a water bath at the body temperature of the experimental organism, with an incubation time of 90 min. Samples that received a supply of 0.1 ml 11mM NADPH as electron donor for the biotransformation reaction every 10 minutes were compared to reference samples that did not receive NADPH. Toxaphene mixtures were dissolved in acetone; 3ul of these solutions were added to the microsomal suspensions. The reaction was terminated by the addition of ice-cold methanol and compounds were extracted from the incubation mixture with hexane.

Analysis: For the determination of the parent compounds, the extracts were cleaned-up over alumina columns [15] and evaporated to 0.1-0.5 ml.

- Changes in the technical mixture were determined on an HP 5988A GC/NCI-MS (quadrupole, 200eV) equipped with a 50m * 0.21 mm * 0.20 µm CP-Sil 8 column. Ionisation gas CH_4. Only

selected masses were scanned, based on the occurrence of stable M-Cl ions. 1,2,3,4-tetrachloronaphthalene was used as an internal standard.

- Changes in a mixture containing the CHBs 26,32,50,62 and 69 (Dr. Ehrenstorfer cat. no. ZA 221001) were determined by GC-ECD on a HP 5880 GC equipped with a 50m *0.15mm* 0.30µm CP-Sil19 column and hydrogen as carrier gas. No results for CHB-69 are reported, because its peak was below the detection limit already in the reference samples. The remaining CHBs have the following structures:
  - CHB-26 (T2): 2-exo,3-endo,5-exo,6-endo,8b,8c,10a,10b-octachlorobornane
  - CHB-32 (ToxB): 2,2,5-endo, 6-exo, 8b,9c,10a-heptachlorobornane
  - CHB-50 (T12): 2-exo,3-endo,5-exo,6-endo,8b,8c,9c,10a,10b,-nonachlorobornane
  - CHB-62: 2,2,5,5,8b,8c,9c,10a,10b-nonachlorobornane.

  The original solvent cyclohexane was evaporated and the CHBs were redissolved in acetone prior to the *in-vitro* assays
- Full scan mass-spectra (magnet 500-250) of the metabolites of CHB-32 and CHB-62 were obtained by GC/NCI-MS (70eV) on a HP 5890 GC, equipped with a 25m*0.32mm CP-Sil 5 column, coupled to a VG Autospec Ultima Q mass spectrometer. Ionisation gas was CH_4. The extracts were just evaporated to 0.1 ml without a further clean-up over alumina, since this was shown to selectively retain the metabolites. Derivatisation with TMS before clean-up did not solve this problem.

Mutatox® assay: The standard assay was adapted to the use of the microsomal fractions prepared from marine animals instead of the standard S-9 fraction supplied by the manufacturer.

3. Results

The EROD activities of the different microsomal preparations were: sperm whale 17, albatross 21, whitebeaked dolphin 49, and harbour seal 159 pmol . mg^{-1} protein . min^{-1}.

The *in*-vitro capacity to alter the peak pattern of a technical mixture of the insecticide toxaphene increased in the order sperm whale < whitebeaked dolphin, albatross << harbour seal (fig.1). Since the number of peaks in the residues of environmentally exposed animals decreased in the same order, it appears that the results of the *in-vitro* assays are relevant for the bioaccumulation of toxaphene in environmentally exposed animals.

The congeners CHB- 26 and CHB-50 were persistent in all assays with the Parlar-mixture. In the harbour seal, both CHB-32 and CHB-62 were metabolisable, whereas in the whitebeaked dolphin and the albatross only CHB-32 was metabolised. The microsomes of the sperm whale failed to metabolise any of these four congeners.

The search for metabolites with harbour seal microsomes resulted in three compounds of which the mass-spectra were observed only after the addition of NADPH during the assay; i.e. when biotransformation could occur. Despite the use of negative chemical ionisation, the parent compounds of toxaphene do generally not show stable molecular ions [4]. Metabolite I was obtained from CHB-62. Since the two clusters with the highest masses of the parent compound were attributed to (M-2Cl) and (M-2Cl-HCl), both clusters of metabolite I were attributed to the (M-2Cl+O) and (M-2Cl-HCl+O) ions of a mono-hydroxy metabolite (Δm=16; fig. 2). Metabolites II and III were isolated from CHB-32 in two separate assays under different incubation conditions. Metabolite II showed two mass clusters that were attributed to the (M-Cl-HCl+O) and (M-Cl-2HCl+O) ions of a mono-hydroxy metabolite. Metabolite III showed two mass clusters which were attributed to the (M-2HCl+3O-$2H_2O$) and (M-3HCl+3O-$2H_2O$) ions of a tri-hydroxy metabolite.

Although the results of the mutatox®-assay on mutagenicity were not always unambiguous, the technical toxaphene mixture showed a direct mutagenic response in several cases, but biotransformation did not lead to an apparent increase of mutagenicity.

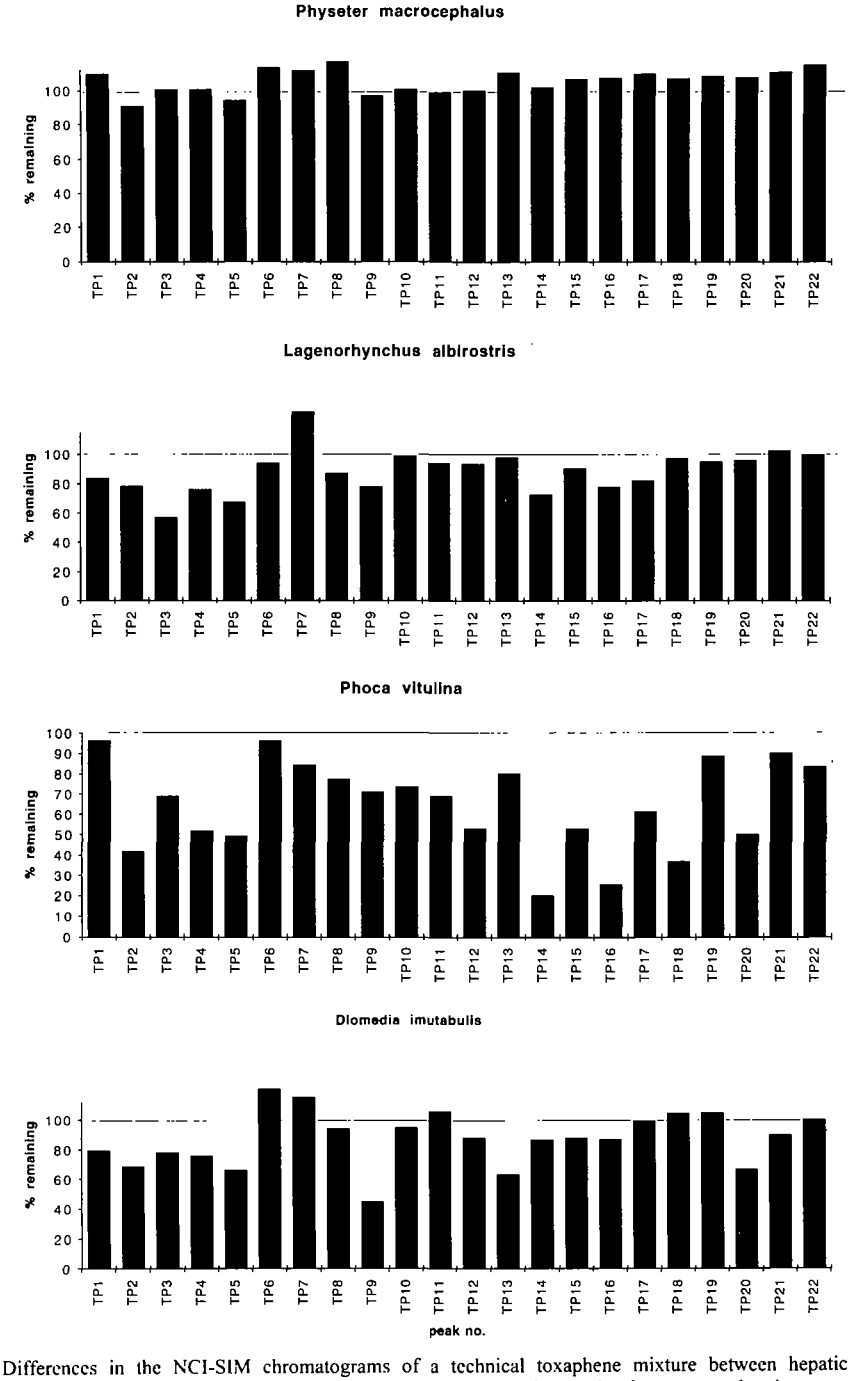

Fig.1: Differences in the NCI-SIM chromatograms of a technical toxaphene mixture between hepatic microsomal preparations that received NADPH during incubation, and reference samples that were incubated without NADPH. All results are means of duplicate sample pairs.

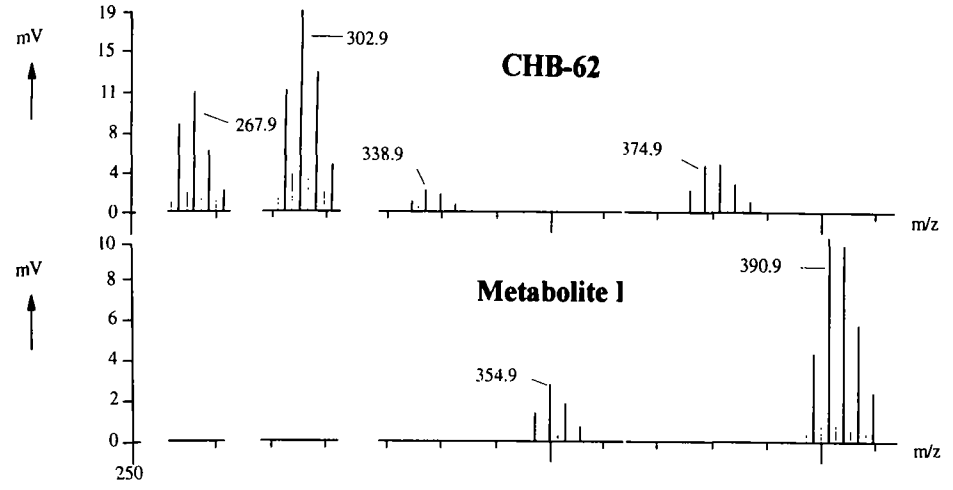

Fig. 2: NCI-mass-spectra of CHB-62 and its metabolite synthesised by harbour seal microsomes.

4. Discussion

CHB-26 and CHB-50 were persistent in all *in-vitro* assays and also appear to be generally present in residues extracted from environmental samples [10,16]. Both congeners possess one chlorine substituent at all lateral positions of the ring formed by the carbon atoms C-1 to C-6, and two Cl substituents at the methyl groups at C-8 and C-10 positions. Surprisingly, the unsubstituted methyl group at C-9 does apparently not lead to an easy degradability of CHB-26. When it is assumed that CHB-26 possesses the minimum Cl-substitution pattern for a persistent congener, than CHB-62 can be metabolised at the C-3 and the C-6 positions in the ring, whereas CHB-32 can be metabolised at the C-3 position in the ring and at the methyl groups at the C-8 and C-10 which both contain only one Cl-substituent. This is in agreement with the occurrence of a three-hydroxy metabolite in this study. The whitebeaked dolphin and the albatross were apparently unable to hydroxylate the unsubstituted positions in the ring of CHB-62. Therefore, the biotransformation of CHB-32 by these animals may have occurred at one of the methyl groups.

Based on the present results, it is advised to collect monitoring data on the concentrations of CHB-26 and CHB-50. These data should be supplemented with an indication of the total surface area of toxaphene-derived peaks in the residue [10].

Because of their globular structure with a strong resemblance to aldrin, it can be assumed that chlorinated bornanes are metabolised by isozymes of the cytochrome P450 2B subfamily (CYP2B) [17]. Thus, the activities of these enzymes might be good criteria to estimate the biotransformation capacity of species for CHBs. If this would be true, only a few aquatic top predators belonging to the order of the Carnivora [18] would be able to metabolise toxaphene to a high degree via an oxidative pathway, since gill-breathing organisms and cetaceans generally possess either no or very low and non-inducible levels of CYP2B [19-21]. Another metabolic pathway that might also be important for especially microbial metabolism is reductive dechlorination, which might occur for instance in the intestinal system of higher organisms, soils and sediments [7].

Technical toxaphene should be categorised as directly mutagenic. This is in contrast to the well-known mutagenic compound benzo[a]pyrene, of which only specific metabolites are able to bind covalently to DNA.

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6. References

[1]) Hargrave, B.T., D.C.G. Muir and T.F. Bidleman (1993): Toxaphene in amphipods and zooplankton from the Arctic Ocean. Chemosphere 27, 1949-1963.

[2]) Voldner, E.C. and Y.F. Li (1993): Global use of toxaphene. Chemosphere 27, 2073-2078.

[3]) Zell, M. and K. Ballschmiter (1980): Baseline studies of global pollution. Fres. Z. Anal. Chem. 300, 387-402.

[4]) de Boer, J. and P.G. Wester (1993): Determination of toxaphene in human milk from Nicaragua and in fish and marine mammals from the north-eastern Atlantic and the North Sea. Chemosphere 27, 1879-1890.

[5]) Wania, F. and D. Mackay (1993a): Global fractionation and cold condensation of low volatility organochlorine compounds in polar regions. Ambio 22, 10-18.

[6]) Wania, F. and D. Mackay (1993b): Modelling the global distribution of toxaphene: a discussion of feasability and desirability. Chemosphere 27, 2079-2094.

[7]) Saleh, M.A. (1991): Toxaphene: Chemistry, Biochemistry, Toxicity and Environmental Fate. Rev. Environ. Contam. Toxicol. 118, 1-85.

[8]) Vetter, W. (1993): Toxaphene, theoretical aspects of the distribution of chlorinated bornanes including symetrical aspects Chemosphere 26, 1079-1084.

[9]) Hainzl, D., J. Burhenne and H. Parlar (1994): HRGC-ECD and HRGC-NICI SIM quantification of toxaphene residues in selected marine organisms by environmentally relevant chlorobornanes as standard. Chemosphere 28, 237- 243.

[10]) de Boer, J., H.-J. de Geus and U.A.Th. Brinkman (1996): Multidimensional gas chromatographic analysis of toxaphene. Proc. 18th Intern. symp. on Capillary Chromatogr., Riva del Garda, Italy, May 24-24th.

[11]) Chandurkar, P.S. and F. Matsumura (1979): Metabolism of toxicant B and toxicant C of toxaphene in rats. Bull. Environ. Contam. Toxicol. 21, 539-547.

[12]) Thakker, D.R., H. Yagi, W. Levin, A.W. Wood, A.H. Conney and D.M. Jerina (1985): Polycyclic aromatic hydrocarbons: metabolic activation to ultimate carcinogens. In: Bioactivation of foreign compounds. Ed. M.W. Anders. Academic Press, Inc., Orlando, Florida and London, UK, 177-242.

[13]) Eggens, M.L. and F. Galgani (1992): Ethoxyresorufin O-deethylase (EROD) activity in flatfish: fast determination with a fluorescence plate-reader. Mar. Environ. Res. 33, 213-221.

[14]) Murk, A.J., D. Morse, J.P. Boon and A. Brouwer (1994): *In-vitro* metabolism of 3,3',4,4'-tetrachlorobiphenyl in relation to ethoxyresorufin-O-deethylase (EROD) activity in liver microsomes of several wildlife species. Eur. J. Pharmacol., Sect. Environ. Toxicol. Pharmacol. 270, 253-261.

[15]) Boon, J.P., I. Oostingh J. van der Meer and M.T.J. Hillebrand (1994): A model for the interpretation of chlorinated biphenyl concentrations in marine mammals. Eur. J. Pharmacol., Sect. Environ. Toxicol. Pharmacol. 270, 237-251.

[16]) Vetter, W., G. Scherer, M. Schlabach, B. Luckas and M. Oehme (1994): An unequivocal 1H NMR structural assignment of TOX8 and TOX9, the two most abundant toxaphene congeners in marine mammals. Fres. J. Anal. Chem. 349, 552-558.

[17]) Lewis, D.F.V., C. Ioannides and D.V. Parke (1986): Molecular dimensions of the substrate binding site of cytochrome P-448. Biochem. Pharmacol. 35, 2179-2185.

[18]) King, J.E. (1983): Seals of the world, British Museum of Natural History and Univ. Queensland Press, St Lucia, Queensland, Australia, 240 pp.

[19]) Stegeman, J.J. and P. Kloepper-Sams (1987): Cytochrome P-450 isozymes and monooxygenase activity in aquatic animals. Environ. Health Perspect. 71, 87-95.

[20]) Goksøyr, A. and L. Förlin (1992): The cytochrome P450 system in fish, aquatic toxicology and environmental monitoring. Aquat. Toxicol. 22, 287-312.

[21]) Boon, J.P., E. van Arnhem, S. Jansen, N. Kannan, G. Petrick, D.E. Schulz, J.C. Duinker, P.J.H. Reijnders and A. Goksøyr (1992): The toxicokinetics of PCBs in marine mammals with special reference to possible interactions of individual congeners with the cytochrome P450-dependent monooxygenase system - An overview-. In: Persistent Pollutants in Marine Ecosystems (a SETAC special publication). Eds. C.H. Walker and D.R. Livingstone, Pergamon Press, 119-159.