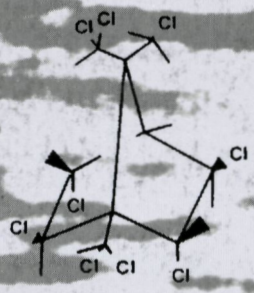
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De ontwikkeling van een in-vitro assay voor de bepaling van de invloed van biotransformatie op de bioaccumulatie en de mutageniteit van lipofiele organohalogeen-verbindingen in mariene toppredatoren

II. Toxafeen

Beleidsgericht ecologisch onderzoek van de Noordzee/Waddenzee

BEON

NIOZ
RIVO-DLO
RWS-RIKZ

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II. Toxafeen.

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Uitgebreide Samenvatting:

Doel van het onderzoek:

Het onderzoek binnen dit project beoogt een drietal doelen:

1. **Ontwikkeling van een *in-vitro* bioassay voor de oxydatieve biotransformatie van verschillende groepen van lipofiele organische microverontreinigingen in mariene topredatoren.**
2. **Validatie van de met de assay behaalde resultaten door vergelijking met residupatronen in prooi en predator als gevolg van natuurlijke blootstelling in zee.**
3. **Bestuderen van de invloed van biotransformatie op de mutageniteit van de verbindingen in een standaard assay (mutatox®).**
4. **Beleidsadvisering naar aanleiding van de behaalde resultaten in combinatie met literatuurgegevens.**

Dit rapport behandelt de resultaten welke zijn behaald met het insecticide toxafeen. Het dient gezien te worden als een aanvulling op het reeds verschenen BEON-rapport 95-4, waarin de eerste bevindingen met toxafeen al werden gerapporteerd.

Probleemstelling

Van een aantal groepen van organische microverontreinigingen wordt de kans op het optreden van biomagnificatie (bioaccumulatie via de voedselketen) van kieuwademende prooidieren naar longademende predatoren zoals vogels en zoogdieren bijzonder groot wordt geacht op grond van hun hoge vetoplosbaarheid. Hierbij wordt er impliciet vanuit gegaan, dat een stof in het lichaam niet reaktief is en dus niet van structuur verandert. De bioaccumulatie van stoffen die wel enzymatisch worden omgezet, laat zich

dus niet volgens deze regels beschrijven. Dit aspect speelt een belangrijke rol bij de doorvergiftigingsproblematiek in de normstelling (Jonkers en Everts, 1992). Het is echter nog niet mogelijk om vanuit de theorie te voorspellen of bepaalde klassen van verbindingen door verschillende diergroepen al of niet kunnen worden omgezet. Ook het verkrijgen van informatie door middel van *in-vivo* experimenten is vanwege het grote benodigde aantal dieren om ethische en logistieke redenen slechts in hoge uitzonderingsgevallen mogelijk en bovendien erg duur. Doel van het onderzoek in het kader van dit project is de ontwikkeling van een alternatief voor zulke *in-vivo* experimenten in de vorm van een *in-vitro* bioassay om de oxudatieve biotransformatiecapaciteit van verschillende soorten mariene toppredatoren voor verschillende klassen van organische microverontreinigingen te kunnen meten. Deze assay maakt gebruik van speciaal geprepareerd levermateriaal (microsomen) van één enkel dier of slechts enkele individuen per soort.

Resultaten en conclusies van het uitgevoerde onderzoek:

- In de huidige verslagperiode werd het onderzoek naar de biotransformatie van toxafeen afgerond. De *in-vitro* biotransformatiecapaciteit nam toe in de volgorde potvis < albatros, witsnuitdolfijn << gewone zeehond. In dezelfde volgorde nam het aantal pieken in het residupatroon in vetweefsel van deze soorten toe. Dit toont aan dat de in de bio-assay behaalde resultaten relevant zijn voor de veldsituatie. De resultaten bevestigen de eerdere metingen beschreven in BEON rapport 95-4, waarbij ook de zeehond veruit het meest actief was, terwijl de resultaten van een eidereend en een bruinvis overeen kwamen met die van respectievelijk de albatros en de witsnuitdolfijn. De biotransformatiecapaciteit voor toxafeen is dus sterk diergroep specifiek, waarbij de trend is dat de activiteit toeneemt in de volgorde potvis < zeevogels & dolfijn-achtigen << zeehond. Aangezien er aanwijzingen zijn dat toxafeen wordt gemetaboliseerd door iso-enzymen behorend tot de cytochroom P450 2B subfamilie, zijn biochemisch of immunochemische metingen van de activiteit van zulke enzymen wellicht goede criteria voor het onderscheiden van relatief goede en slechte metaboliseerders van toxafeen mengsels. Als dit inderdaad het geval is, zou dit betekenen dat er in het aquatisch milieu slechts een beperkt aantal soorten top-predatoren zijn die toxafeen goed kunnen omzetten, allen behorend tot de orde der carnivoren (King, 1983). Hiertoe behoren de drie families van de pinnipedia, namelijk de phocidae (échte zeehonden), de otariidae (pelsrobben en zeeleeuwen) en de odobenidae (walrussen), de mustelidae (otters, behorend tot de marter-achtigen) en de ijsbeer (Ursidae). Organismen die met kieuwen ademen bezitten vrijwel altijd geen of zeer lage en niet induceerbare concentraties van CYP 2B iso-enzymen. (zie ook de paragraaf *"Aanbevelingen voor toekomstig onderzoek"*).

- **Fysisch-chemische modellering van het gedrag van toxafeen in mariene voedselketens op grond van parameters zoals bijvoorbeeld de log $P_{octanol/water}$, de constante van Henry en het molecuulgewicht, zal in de toekomst moeten worden aangevuld met specifieke coëfficiënten voor biotransformatie activiteit. De resultaten van deze studie wijzen erop, dat voor het mariene milieu sets van aparte coëfficienten voor respectievelijk de verschillende zeehond-achtigen, zeevogels, dolfijn-achtigen en de potvis gewenst zijn.**
- **Alhoewel de met de mutatox® assay behaalde resultaten nog niet geheel eenduidig waren, kan wel gesteld worden dat technisch toxafeen direct mutageen is en dat biotransformatie niet lijkt te leiden tot een potentiëring hiervan. Dit in tegenstelling tot de situatie bij de bekende carcinogene stof benzo[a]pyreen, waarvan uitsluitend een bepaalde metaboliet zich aan DNA kan binden.**

Beleidsimplicaties

- **Doorvergiftigings problematiek: De samenstelling van toxafeen kan door biotransformatie drastisch worden gewijzigd. Dit betekent, dat in long-ademende top-predatoren zowel rekening gehouden dient te worden met een eventuele giftigheid van de ouderstoffen als de gevormde metabolieten.**
- **Normering: Van het insecticide toxafeen worden thans in de noordelijke Noordzee hogere concentraties waargenomen in de lever van kabeljauw-achtige vissen dan in de zuidelijke Noordzee (de Boer en Wester, 1993). Voor toxafeen is in Nederland nog geen norm vastgesteld. Toepassing van de in Duitsland voor dierlijke producten voorgestelde normering van 0.1 mg/g op vetbasis voor vis met een vetgehalte > 10% van het natgewicht en 0.1 mg.kg op productbasis voor vis met een vetgehalte < 10% (Bundesgesetzblatt, 1994), zou problemen kunnen opleveren voor de consumptie van sommige visserijproducten uit de noordelijke Noordzee. Een eventuele toekomstige normering dient te worden afgestemd op concentraties van toxafeen congeneren die voor de diersoort waarin wordt gemeten, persistent zijn. Voor onderzoek hiernaar kan de in dit rapport beschreven *in-vitro* assay worden gebruikt. Op grond van het huidige beeld adviseren wij monitoringsgegevens te verzamelen van de concentraties van enkele persistente en kwantificeerbare toxafeen congeneren (CHB-26, CHB-50) te bepalen met tweedimensionale gaschromatografie, aangevuld met een indicatie van het totaalgehalte aan toxafeen in het residu gebaseerd op het totaaloppervlak van de van toxafeen afkomstige pieken in de enkelkoloms chromatogrammen. Als detectie methode wordt negatieve chemische ionisatie massaspectrometrie in beide gevallen aanbevolen.**
- **Mutageniteit: Voor de normering dient vooralsnog met een directe mutagene werking van het technisch toxafeen mengsel rekening te worden gehouden.**

Overigens beoogt dit rapport niet om antwoord geven op de vraag of biotransformatie van toxafeen in algemene zin goed of slecht is; daarvoor zijn er te weinig effectgerichte aspecten bestudeerd (zie ook de rubriek *"Aanbevelingen voor toekomstig onderzoek"*). Wel blijft onverminderd van kracht, dat de kans op mogelijke nadelige effecten in het milieu toeneemt met de blootstelling. Hierdoor lopen carnivoren in de top van de voedselpyramide principieel meer gevaar dan bijvoorbeeld herbivoren.

Aanbevelingen voor toekomstig onderzoek

1. **<u>Modellering Milieugedrag van Toxische Stoffen</u>: Voor de modellering van het gedrag van contaminanten in voedselketens is het van groot belang dat het vermogen tot biotransformatie van stoffen binnen verschillende diergroepen beter kan worden voorspeld. Indien de biotransformatie van bepaalde groepen stoffen in de toekomst goed gerelateerd kan worden aan de aanwezigheid en de induceerbaarheid van bepaalde biotransformatie enzymen in alle belangrijke taxonomische groepen organismen, dan is daarmee een biochemische basis aanwezig voor een systematische classificatie van groepen organismen ten aanzien van hun biotransformatiecapaciteit. In het geval van toxafeen zouden dit wellicht de biochemisch of immunochemisch bepaalde concentraties van cytochroom P450 2B kunnen zijn.**
2. **<u>Sublethale effecten</u>: Zeer recent is gevonden, dat gehydroxyleerde PCB metabolieten in het bloed de huishouding van de geslachtshormonen en de schildklierhormonen kunnen beïnvloeden. Een gelijksoortige werking van metabolieten van toxafeen kan niet worden uitgesloten. Het belang van deze beide werkingsmechanismen zou onderzocht kunnen worden door middel van toepassing van effectgerichte bioassays speciaal ontworpen voor bestudering van deze effecten. Een uitbreiding van het huidige samenwerkingsverband met bijvoorbeeld de Vakgroep Toxicologie van de Landbouwuniversiteit Wageningen, zoals bij de oorspronkelijke indiening van dit project ook voorgesteld, zou dergelijke studies ook in logistieke zin snel uitvoerbaar maken.**

The development of an *in-vitro* Assay for the Determination of the Influence of Biotransformation on the Bioaccumulation and Mutagenicity of Lipophilic Organohalogen compounds in Marine Top Predatoren.

II. Toxaphene.

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Executive Summary

Research goals

1. **Development of an *in-vitro* bioassay to investigate the occurrence of enzyme-mediated biotransformation of lipophilic organic contaminants in marine top-predators.**
2. **Validation of the results of the *in-vitro* assays by comparison with residue patterns in environmentally exposed predators and their prey.**
3. **To study the influence of biotransformation on the mutagenicity of the compounds by means of a standard-assay (mutatox®).**
4. **To advise with respect to the development of policy measurements for new compounds on the basis of the results obtained and literature data.**

This report discusses the results obtained with the insecticide toxaphene. The results obtained are additional to those already described in BEON report 95-4.

Scientific background

A number of groups of 'new' organic contaminants are suspected to bioaccumulate through food-chains because of their structural similarity to contaminants already well-known to do so, such as certain polychlorinated biphenyl and dioxin congeners, dieldrin, p,p'-DDT. Especially at the highest levels of the food-web, i.e. from aquatic animals to lung-breathing predators, biomagnification factors can be considerable when the compounds are not reactive. Enzyme-mediated metabolism, which changes the molecular structure of a compound, can completely alter this process, since the compounds often become more polar and thus less lipophilic and more easily excretable. However, the formation of a reactive group in the molecule can make the compounds

more reactive too, which may well lead to an increased toxicity. At this moment it is not yet possible to predict the persistence to biotransformation of compounds on a theoretical basis. Since *in-vivo* experiments with live animals are not desirable for ethical, logistic and financial reasons, the development of *in-vitro* assays offers unique possibilities to study the occurrence biotransformation. In this way, a lot of information can be obtained with tissue preparations of only a few individuals. The assay described in this paper uses microsomal fractions of the liver, which is the most important organ with respect to biotransformation, of marine mammals and sea-birds.

Results and conclusions of the investigations

- **This report describes the biotransformation studies with the insecticide toxaphene. The *in*-vitro biotransformaton capacity increased in the order sperm whale < whitebeaked dolphin, albatross << harbour seal. The number of peaks in the residue of environmentally exposed animals decreased in the same order, showing that the results of the *in-vitro* assay are relevant for the bioaccumulation of toxaphene in environmentally exposed animals. The present results also confirm the first indications obtained for the biotransformation of toxaphene in another harbour seal, an eider duck and a harbour porpoise, which have already been described in BEON report 95-4. Since there are several indications that chlorinated bornanes are metabolised by isozymes of the cytochrome P450 2B subfamily, biochemical or immunochemical evidence of the activity of such enzymes might be good criteria to estimate the biotransformation capacity of species towards toxaphene. In that case only a few aquatic top predators belonging to the order of the Carnivora (King, 1983), would be able to metabolise toxaphene to a high degree. The most important species belong to the three families of the pinnipeds, i.e. the phocidae (earless seals), the otariidae (fur seals and sea lions) and the odobenidae (walrusses), otters (Mustelidae) and the polar bear (Ursidae). Gill-breathing organisms generally possess either no or very low and non-inducible levels of CYP2B isozymes (See also "*Suggestions for further research*").**
- **Bioaccumulation models based on physical-chemical descriptors such as $\log P_{octanol/water}$, Henry's constant and molecular size, will have to be expanded to include specific coefficients for biotransformation activity. The results of this study indicate that separate coefficients for all pinnipeds, sea-birds, and all dolphins might be a suitable and still practical choice to start with.**
- **Although the results of the mutatox®-assay on mutagenicity were not yet always uniform, it can be stated that the technical toxaphene mixture is directly mutagenic and that biotransformation did not lead to an apparent potentiation of mutagenicity.**

This is in contrast to the situation for the well-known mutagenic compound benzo[a]pyrene, where only a specific metabolite is able to bind covalently to DNA.

Implications for Policy

- **Bioaccumulation via the food chain: The composition of toxaphene can be drastically changed as a result of biotransformation, especially in seals. This implicates that there is a possibility for toxic effects of the metabolites formed as well as of the parent compounds.**
- **Values of maximum allowable concentrations (MAC values): At the moment higher concentrations of the insecticide toxaphene are found in the northern North Sea compared to the southern part in livers of gadoid fish (de Boer and Wester, 1993). Currently, no MAC values for toxaphene have been established in the Netherlands, but adoptation of the value of 0.1 mg/kg on a lipid normalised as proposed for animal products in Germany (Bundesgesetzblatt, 1994), could create problems for some fishery products from the northern North Sea. Future MAC values should be restricted to congeners that are persistent in the animal species under consideration. To establish the scope for biotransformation of this species, the *in-vitro* assay described in this report can be used. Based on the present results it is advised to collect monitoring data on the concentrations of selected congeners that were shown to be persistent in all species investigated so far (i.e. the congeners CHB-26 and CHB-50; to be analysed by two dimensional gas chromatography). These data should be supplemented with an indication of the total surface area of toxaphene-derived peaks in the residue as determined with single column capillary gas chromatography and detection by negative chemical ionisation mass-spectrometry.**
- **Mutagenicity: Technical toxaphene should be categorised as a directly mutagenic mixture and be treated accordingly.**

Recommendations for future research

1. **Modelling the environmental behaviour of contaminants: In the end, it will be unfeasible to investigate the scope for biotransformation of each individual species towards individual toxic compounds or mixtures of molecules with a similar basic molecular structure. The draft of maps on the presence and inducibility of different classes of biotransformation enzymes in all important taxonomic groups of biota might be good criteria to rank the biotransformation capacity of the different taxonomic groups towards different groups of compounds.**

   With respect to toxaphene, isozymes of the cytochrome P450 2B subfamily are probably the most relevant, since there are several indications that chlorinated bornanes are metabolised by these enzymes.

2. **<u>Sublethal effects</u>. Recently it was found, that hydroxylated metabolites of PCBs can have oestrogenic effects and influence the concentrations of thyroid hormones. A similar mode of action of toxaphene metabolites cannot be excluded. The relevance of both of these modes of action could be investigated by the application of specially designed bioassays for this purpose. The extension of the present research group with a group that is presently working with such assays is feasible on a reasonable short term.**

1 Organisation of the Project

This report describes part of a study of the BEON contract RKZ 165 (project NIOZ 95 M03), in which the influence of biotransformation on the bioaccumulation of lipophilic organohalogen compounds in marine top predators is studied. The project is carried out in co-operation by the Netherlands Institute for Sea Research (NIOZ), the DLO - Netherlands Institute for Fisheries Research (RIVO-DLO), and the National Institute for Coastal and Marine Management (RIKZ) of the Directorate-General Public Works and Water management (Rijkswaterstaat), Ministry of Transport, Public Works and Water Management.

2 Scientific Outline

General

Several groups of lipophilic organohalogen compounds are suspected to bioaccumulate through the food web, resulting in high levels in particularly lung-breathing top predators. Biotransformation can substantially alter the bioaccumulation and toxicity expected on the basis of the lipophilicity of the parent compounds, which is often not taken into account in modelling exercises for policy purposes. Reasons for this lack of scientific knowledge is, that experiments to study biotransformation in living organisms when seabirds, whales or seals are considered are often not desirable for ethical logistic, and financial reasons. However, the use of *in-vitro* studies would circumvents most of these problems, provided that the results are also valid for the *in-vivo* situation. In the first part of this project the potential of this microsomal assay was assessed by comparing the behaviour of a mixture of polychlorinated biphenyls (PCBs) in the assay with the *in-vivo* behaviour. It was concluded that the results of the microsomal assay carried out with microsomes of a harbour porpoise and a harbour seal indicated which CB congeners would be persistent and which congeners would be metabolisable in these species; i.e. the assay can be used in a qualitative sense (Boon *et al.*, '95).

Toxaphene concentrations were recently found to be unexpectedly high in liver of hake (*Merluccius merluccius*) and blubber of white beaked dolphin (*Lagenorhynchus albirostris*) from the northern part of the North Sea in view of the reported lack of use in countries surrounding the North Sea. The peak patterns found in both species were very similar, but the pattern differed from that of the technical mixture (de Boer and Wester, 1993). There can be many causes for this difference; biotransformation being one of them. In this study, the behaviour of toxaphene was examined in the microsomal *in-vitro* assay for harbour seal, whitebeaked dolphin, albatross, and sperm-whale, of which microsomes were available. The results of the *in*-vitro assay were compared to residue patterns in samples of environmentally

exposed animals of the same species, in some cases tissues were analysed from the same animal as used for the *in-vitro* assay.

Toxaphene: structure, analysis, toxicity

Toxaphene is a complex mixture of up to 670 polychlorinated monoterpenes with an average empirical formula of $C_{10}H_{10}Cl_8$ (Holmstead *et al.*, 1974). The product is industrially synthesised from α-pinene. From α-pinene, camphene (major product), bornylene and α-terpineol are synthesised, which are subsequently bulk chlorinated. The majority of compounds in technical toxaphene formulations consists of bornanes, but also bornenes (one C=C bond), bornadienes (2 C=C bonds) and other chlorinated and non chlorinated compounds are present (Saleh, 1991). The structure of the bornane carbon skeleton and the structural formulas of the few compounds that are commercially available as analytical standards are given in figure 1 .

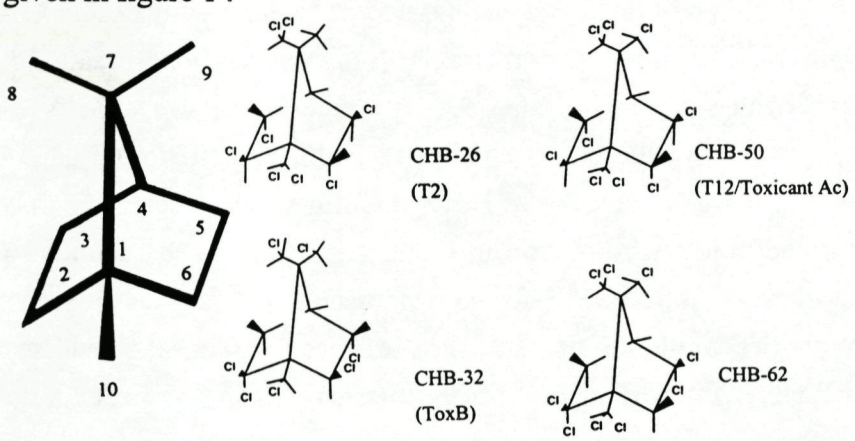


Fig. 1: Molecular structure of the bornane carbon skeleton and the structures of 4 individually synthesised toxaphene compounds. Numbers in the carbon skeleton indicate systematic numbers of positions.

Toxaphene concentrations are determined by gas chromatographic separation (GC), followed by different detection techniques, such as electron capture detection (ECD) or negative chemical ionisation mass spectrometry (NCI-MS). The components in the toxaphene mixture elute over a wide range of GC retention times, components with the lowest number of chlorine atoms having the shortest retention times. Due to the very large numbers of congeners in technical toxaphene, the vast majority of the components cannot be completely resolved by single column gas chromatography, and thus most peaks in the chromatographic pattern probably contain several compounds. Even the use of mass-spectrometry as detection method only allows the identification of compounds on the isomeric level, since difference between isomers cannot yet be made due to a lack of individual standard compounds, from which full mass-spectra can be recorded (Swackhamer, 1987; Muir and de Boer, 1993; Bruns and Birkholz, 1993).

Toxaphene is an insecticide with a broad pesticidal activity. Before its ban in 1982 it was the most heavily used pesticide in many parts of the world. Approximately 80-90% of all toxaphene production has been applied in cotton stock. It has also been used in fish eradication programs in Northern America (Saleh, 1991). Probably it is still used in regions of the former Soviet-Union, in Mexico (Voldner and Li, 1993) and in Nicaragua (de Boer and Wester, 1993).

Toxaphene is a widespread contaminant in freshwater and in the marine environment. Unexpectedly high concentration levels were found in fish from the North Sea, suggesting aerial transport from the American continent to European waters, because toxaphene was practically not used in Western Europe (de Boer and Wester, 1993).

For fish, toxaphene was found to be the second most toxic organochlorine insecticide after Endrin (Edward, 1977). Moreover, it is also highly carcinogenic to rats and mice, and mutagenic in the Ames Salmonella test (Saleh, 1991; Hooper *et al.*, 1979). In *in vivo* experiments, rats showed an increase in Aldrin epoxidase activity (yielding Dieldrin as the metabolic product) after pre treatment of the animals with toxaphene (§1.3) (Saleh, 1991), an indication that toxaphene induces cytochrome P450 2B.

Mechanisms of metabolism in animals

In every organism there is an extensive enzyme system present, which is involved in the metabolism of both endogenous and exogenous organic compounds. The order by which exogenous components are metabolised consists often of two types of consecutive reactions; called phase I and phase II metabolism. In phase I reactions a functional group is incorporated into the molecule, often oxygen, to make the component more polar but subsequently also often chemically more active. In phase II reactions an endogenous polar component is attached to the molecule, to make the complex more water-soluble, facilitating excretion.

The liver is the main organ in metabolising xenobiotics. Phase II metabolism can take place in the cytoplasm of a cell or the smooth endoplasmatic reticulum (SER), which is a phospholipid membrane into which the enzymes (proteins) are incorporated. Phase I enzymes are located in the SER of a cell.

As a result of this, phase I metabolism can be studied by metabolic activity of isolated microsomes which still show most of their catalytic activity and can thus be used for studying phase I metabolism (Gibson and Skett, 1986). Microsomes are vesiculated fragments of SER created by physical disruption, which can be achieved by grinding of the hepatocytes in a Potter tube. The microsomal fraction is isolated and purified by ultra-centrifugation and washing.

The central catalyst in phase I metabolism is the cytochrome P450 dependent monooxygenase system. It catalyses a great number of oxidative reactions, such as hydroxylation, epoxidation, O-, N- and S-dealkylation. This enzyme system is incorporated in

the membrane of the SER and consists of different components: NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase, cytochrome P450 and cytochrome b5. NADPH or NADH are required as electron donor. These electrons are passed to NADPH Cytochrome-P450 reductase or NADH-cytochrome b5 reductase. Subsequently these reducing equivalents are passed to cytochrome P450. In the case of NADH as electron donor the electrons are passed via cytochrome b5 to cytochrome P450. Together with oxygen and a suitable substrate a tri-molecular complex is formed and the substrate is oxygenated (Nebert and Gonzalez, 1987). A schematic overview of the cytochrome P450 system is given in fig. 2.

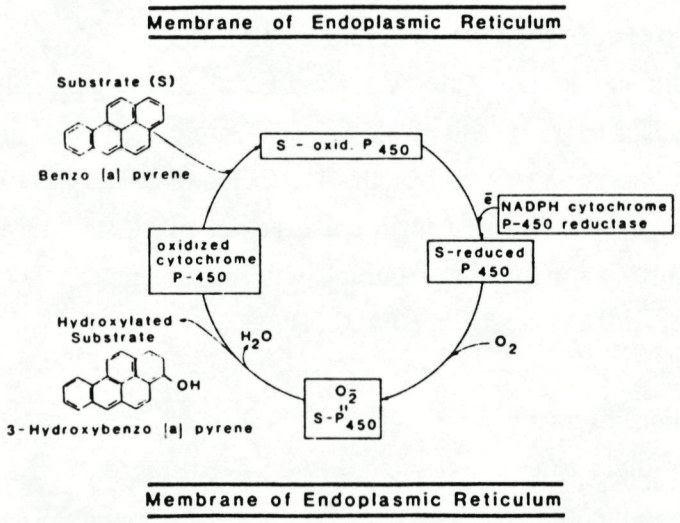


Fig. 2: Schematic representation of hydroxylation reactions of anthropogenic compounds catalysed by the cytochrome P450 system located in the smooth endoplasmatic reticulum. Source: Lee (1981).

The cytochrome P450 (CYP) system consists of a great variety of isozymes, divided into several families, mostly with a broadly overlapping substrate specificity. There are at least 10 gene families, from which eight exist in mammals. These families are subdivided into subfamilies. In this report the nomenclature according to Nebert and Gonzalez (1987) is used.

Specific activities of iso-enzymes can be induced by pre-treatment of animals with specific substrates. These animals show elevated levels of specific iso-enzymes, in this way increasing the catalytic activity of the specific enzyme. This characteristic makes the cytochrome P450 a highly flexible metabolic system. With regards to organic contaminants two subfamilies of cytochrome P450 deserve special attention: Cytochrome P450-1A (CYP1A) is induced by molecules with a planar molecule structure like 2,3,7,8-tetrachloro dibenzo-p-dioxin (TCDD), 3-methylcholanthrene (3-MC), ß-naphtoflavone (BNF) and 3,3',4,4' tetrachlorobiphenyl (CB-77). Cytochrome P450-2B (CYP2B) is induced by non planar molecules such as Phenobarbital (PB).

Substrate specificity can be used to measure the activity of specific iso-enzymes. Ethoxyresorufin O-deethylase activity (EROD) is strictly catalysed by CYP1A enzyme activity. The product of this transformation is resorufin, which is a fluorescent metabolite. The formation of this product can be followed spectrofotometrically. Aldrin epoxidase (AE) is CYP2B mediated. The product of this reaction is Dieldrin. Decrease of Aldrin and the subsequently increase of Dieldrin can be measured by gas chromatographic analysis. Both model reactions are shown in figure 3.

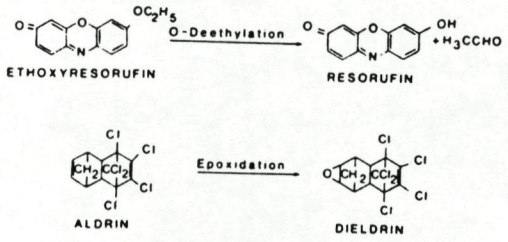


fig 3: Two model reactions catalysed by different iso-enzymes of CYP-450 in the rat. a: EROD (cytochrome P450 1A); b: Aldrin epoxidase (cytochrome P450 2B).

Substantial differences exist between concentrations and activities of the several isozymes between different animal species. Tanabe and co-workers (1988) postulated that cetaceans posses no CYP2B activity . CYP2B activity in seals was very low compared to the activity in other, mainly terrestrial species (Tanabe *et al.*, 1988).

In several species of fish-eating sea-birds no CYP2B induction was found (Ronis *et al.*, 1989a,b). In Eider Ducklings (*Somateria mollisima*) CYP1A was induced, after dosage with CB-77, but CYP2B was not induced after treatment with the technical mixture Clophen A50 (Rozemeijer *et al.*, 1995). Such a difference in metabolic capacity for PCBs illustrate that there can be large differences between marine species with regards to their metabolic capacity for toxic compounds. The present study was undertaken to investigate such differences for toxaphene congeners.

Metabolism and biomagnification of toxaphene

Several experiments have shown that there are substantial differences between chromatographic patterns of toxaphene in different organisms. This can be caused by several biochemical and physical factors, but biotransformation seems to be the most important reason (Saleh, 1991).

In blubber from Beluga whale (*Delphinapterus leucas*) two peaks were dominant, identified as CHB-26 (fig. 1; 2-exo, 3-endo, 5-exo, 6-endo,8,8,10,10-octachloro-bornane; also

known as T2) and CHB-50 (fig. 1; 2-exo,3-endo, 5-exo, 6-endo, 8,8,9,10,10,-nonachlorobornane; also called T12 or Toxicant Ac) (Stern *et al.*., 1992). In harbour porpoise (*Phocoena phocoena*) and white-beaked dolphin (*Lagenorhynchus albirostris*) biomagnification factors of respectively 100 and 40 were measured, compared with their most important food source hake, a gadoid fish. Here, also CHB-26 and CHB-50 were the dominant components in the toxaphene pattern (de Boer and Wester, 1993). In ringed seal (*Phoca hispida*) and polar bear (*Ursus maritimus*) these components were less dominant, indicating a higher rate of metabolism. For *P. hispida* no bioaccumulation of toxaphene was achieved (Zhu and Norstrom, 1993; Hargrave *et al.*, 1993). Chromatographic patterns from toxaphene in blubber from Baikal seal (*Phoca siberica*) differed substantially from patterns of their prey organisms, also indicating a greater ability to metabolise toxaphene derived compounds (Kucklick *et al.*, 1993). In general it appears that cetaceans tend to bioaccumulate at least some toxaphene congeners, where other marine mammals like seals seem to metabolise toxaphene to a great extent.

In eggs of guillemots (*Uria aalge*) the chromatographic pattern was also quite different compared to the standard mixture. There was also a downward trend of toxaphene concentrations during the period of sampling (1976-1989), but reservations were made for comparing environmental samples with standards (Wideqvist *et al.*, 1993).

Direct indications for biotransformation of toxaphene were until now only shown in experiments with rats. An *in-vitro* experiment with the 20.000*g supernatant of rat liver homogenates, toxicant B (fig. 1; 2,2, 5-endo, 6-exo, 8,9,10-heptachlorobornane; Parlar no. 32) and toxicant C (2-endo,3,3,5-exo,6-exo,8,9,10,10 nonachlorobornane) were both extensively metabolised after the addition of NADPH (Chandurkar and Matsumura, 1979). Types of metabolites formed were a dechlorination product from toxicant C (3,3,5-exo, 6-exo, 8,9,10,10 octachlorobornane), one primary alcohol from toxicant C (-OH group at the C-8, -9 or -10 position), and a number of secondary alcohols (-OH group at the C-2, -3, -5 or -6 position). No indications were found for the formation of tertiary alcohols (-OH group at the C-4 position) or diols. The fact that a primary alcohol is formed from toxicant C but not from toxicant B seems difficult to explain, since both molecules have the same configuration at the bridgehead formed by C-7, C-8 and C-9 (8b, 9c Cl-substitution). In an *in vivo* experiment, toxaphene was partly dechlorinated after excretion (Casida *et al.*, 1974).

3 Objectives of this study

Hepatic microsomes have proven to be a good qualitative indicator for the occurrence of phase I biotransformation of CB congeners in marine mammals. The purpose of the second part of the current project was to investigate biotransformation of toxaphene in the microsomal assay for different species of lung breathing marine predators. For this goal,

microsomes of a harbour seal (*Phoca vitulina*), a whitebeaked dolphin (*Lagenorhynchus albirotris*), albatross (*Diomedia imutabulis*) and a sperm whale (*Physeter macrocephalus*). Incubations were carried out with a technical toxaphene mixture and reconstituted toxaphene mixture, containing four chlorinated bornanes with a fully defined molecular structure. To validate the biochemical activity of the microsomes, EROD activity and Aldrin epoxidase assays were performed. The present results are additional to those already reported previously (Boon *et al.*, 1995) for another harbour seal, a harbour porpoise (*Phocoena phocoena*) and an eider duckling (*Sommateria mollissima*).

4 Materials and methods:

Origin of the microsomes:

Harbour seal (*Phoca vitulina*):

Female juvenile animal found dead in the ferry harbour of Texel at 5th January 1994. Animal was tagged with number N3213 of the seal rehabilitation centre at Pieterburen. Length: 1.10m Weight: 31 kg. Thickness of blubber layer: 4.0 cm, measured ventrally directly below sternum.

Cause of death: Body cavity was torn completely open (ventrally). Cause of death probably the propeller of the ferry since no indications of scavening. Also blood poured out of the heart when this was punctured. NIOZ sample code: 94PVa.

Peculiarities: Apparently good condition, time of death unknown. Samples for biochemical analyses were frozen in liquid nitrogen at 15:30.

Samples for chemical analyses were taken of blood, liver and blubber.

Whitebeaked dolphin (*Lagenorhynchus albirostris*):

Pregnant female specimen. Stranded alive at 24th January 1995; 13:45 at Texel (paal 28). Animal had died at 14:15. The animal was transferred to "Ecomare". Autopsy at 17:00. Samples were frozen in liquid nitrogen by 18:00. Thickness of blubber layer: 2.1 cm, measured ventrally at the incision from the sternum downward to obtain liver samples. Length of animal: 2.5m. Code: NIOZ sample code of animal: 95LAa.

Peculiarities: animal missed some teeth and lost milk. Autopsy at 25th January by dr. C. Smeenk (Leiden University).

Samples for chemical analyses were taken of blood, liver and blubber.

Sperm whale (*Physeter macrocephalus*):

Male specimen stranded alive at Kijkduin (near the Hague) at 12th January 1995. One of a group of three. First sighting of the animals: 9:00. Upon arrival at the beach at 19:00 the body cavity had already been opened by Ir. R. Kastelijn (Dolfinarium Harderwijk). Liver samples for biochemical purposes frozen in liquid nitrogen at 20:15. Length of animal: 14.40 m; estimated age: 15-25 years; estimated weight: 30 metric tonnes.

Samples for chemical analyses were taken of blood, liver, blubber and kidney. NIOZ internal sampling code: 95PMb.

Albatross (*Diomedia imutabulis*):

Homogenate of the livers of seven adult animals sampled in a colony at Midway (Hawaï) in April 1995. Samples were a gift of dr. Lori Ann Feyk (Michigan State University) and dr. Martin van den Berg (RITOX, University of Utrecht). NIOZ sample code: 95D6Ia

Analytical methods:

This report gives additional results on the biotransformation and bioaccumulation of toxaphene congeners to BEON report 95-4. An extensive description of the methods used has been given in Annex III of that report.

Before an *in-vitro* assay can be performed, it has to be established whether the microsomes prepared still contain a cytochrome P450 system that is generally able to catalyse any biochemical reactions. It was decided that microsomal preparations with an ethoxyresorufin-O-deethylase (EROD) activity <10 $pmol.mg^{-1}$ $protein.min^{-1}$ were unsuitable for *in-vitro* assays because post-mortem decay processes may have progressed too far (Moore and Stegeman, in press). In such cases the apparent total persistence of toxaphene might be due to a poor condition of the microsomal material only, which would be an experimental artefact.

Two types of toxaphene mixtures were used as substrates; a complex technical toxaphene mixture (TT-mix) and a mixture of five congeners with known molecular structures (T5-mix). Biotransformation of the technical mixture was quantified by GC-NCI/MS, whereas changes in the T5-mix were quantified with GC-ECD. The total number of chlorine substituents of congeners causing major peaks was established (table 1), showing that some peaks were certainly caused by at least two congeners with different chlorination levels. The other peaks containing only one mass however, may also consist of two or more isomers (de Boer and de Geus, 1995). Changes in the technical mixture due to biotransformation were expressed as a relative decrease of at least one of these peaks.

Table I: Peaks of the technical toxaphene mixture used to quantify the influence of biotransformation on the pattern. Peak numbers refer to the same peaks as in BEON report 95-4. The presence of more than one isomeric level in a peak indicates that it is caused by at least two congeners.

Peak no.	No. Cl-substituents
TP6	hepta- + octa-
TP8	hepta-
TP11	hepta-
TP13	hepta-
TP16	hepta- + octa-
TP17	hepta- + octa-
TP19	octa-
TP20	octa-
TP21	nona-
TP23	octa- + nona-

The T5-mixture consists of 5 individual chlorinated bornane congeners.

- **CHB-26**= 2-exo,3-endo, 5-exo, 6-endo, 8b,8c,10a,10b-octachlorobornane **(T2)**
- **CHB -32**= 2,2,5-endo, 6-exo, 8b,9c,10a-heptachlorobornane **(Toxicant B)**
- **CHB -50**= 2-exo,3-endo, 5-exo, 6-endo, 8b,8c,9c,10a,10b,-nonachlorobornane **(T12)**
- **CHB -62**= 2,2,5,5,8b,8c,9c,10a,10b-nonachlorobornane
- **CHB -69**= 2,2,5,5,6-exo,8b,8c,9c,10a,10b-nonachlorobornane

The latter congener showed a very low response factor on the electron capture detector and was therefore not quantified.

Mutagenicity of toxaphene

Toxaphene technical formulations were previously shown to be mutagenic in the Ames *Salmonella* test without requiring liver homogenate (S9-fraction) for activity (Hooper *et al.* 1979; Saleh, 1991). It was also shown that CHB-32 as the most easily isolated major did not have mutagenic activity, either with or without liver S9 activation in any of the standard test strains (TA1535, TA1537, TA1538, TA98 and TA100). They also showed that the more polar fraction of recrystallized toxaphene was more mutagenic.

In the present study, genotoxicity of the TT-mix was evaluated with the Mutatox assay. This bio-assay uses a dim variant of the normally photoluminescent bacterium *Vibrio fisheri*. The presence of genotoxic compounds results in mutations and consequently in a restoration of photoluminescence. A rat liver S9 homogenate is available from the supplier to test for the presence of compounds that need metabolic activation to express genotoxicity. In this project, microsomes from different marine animals were used for this purpose instead. Therefore the initial stages of the project had to be used to adapt the original test protocol.

Microsomes (Table 2) were prepared by NIOZ, and were stored at -80°C until use in the mutatox assay.

Table 2. *Microsome preparations used in this study. Protein EROD were determined by NIOZ.*

Species	Code	Protein [$mg.ml^{-1}$]	EROD [$pM.min^{-1}.mg\ protein^{-1}$]
Rat	-	54.2	-
Harbour seal	94PVa2	70.2	159
Sperm whale	95PMb1	5.9	51.8
Whitebeaked dolphin	95LAa1	11.6	49.3
Albatross	95DIb1	53.2	87.8

Protein and NADPH optimisation

The Mutatox® test kit (Microbics corp., Carlsbad, USA) is supplied with a 'direct' (**D**) and an 'indirect' (**I**) growth medium, the difference being the addition of rat liver S9 homogenate to the indirect medium. In this study we needed to prepare a customised indirect test medium (**CI**), by adding microsomes (refer to Table 1) and NADPH to medium **D** .

Optimal protein and NADPH concentrations were selected by comparing Benzo[a]pyrene (BaP) genotoxicity determined with **I** and **CI** medium.

Adaptations to the Mutatox test protocol

All dilutions were carried out under a laminar flow hood, using the standard safety procedures that apply to compounds and toxic effects under investigation. The test compounds were dissolved in a water miscible organic solvent of high purity (e.g., acetone, Mallinckrodt, Nanograde quality) at appropriate concentrations (initial concentrations: BaP: 10 $mg.ml^{-1}$, Toxaphene: 3.63-21.1 $mg.ml^{-1}$).

The Mutatox tests are normally run in dilution series according to the protocol (Microbics corp.). In summary, a 1:1 dilution series of the test compound in Mutatox S9-media (**I** and **CI**) was made; bacteria were added, and incubated for 45 minutes at 35.0 ± 0.1 °C, after which the test temperature was lowered to 27 ± 0.1 °C. Light levels were recorded at intervals of one hour between 10 and 24 hrs. after incubation. Blank controls (solvent + media) were run to determine spontaneous (i.e. not induced by the test compound) mutations. A positive genotoxic test result in the assay was scored when the test response was higher than 4 times the response of the solvent + media blank.

The original test protocol allows for qualitative results only ("+" or "-", i.e. yes or no genotoxicity). Measuring light levels from 10 to 24 hours after incubation at one hour intervals allows for a more quantitative interpretation. The test compound concentration that gives the maximum response (CMR) is calculated by quadratic regression through the three data points that construct the top of the dose-response curve. The lowest concentration that still gives a positive test result (LCR) is determined by linear interpolation between the two data points that gave responses just below and above the 4* blank value.

For the present purpose, the Mutatox S9 medium had to be replaced by microsomal preparations of the marine wildlife species. Since NADPH is normally present in the S9-fraction, it had to be added separately. A study was performed to optimise the protein and NADPH concentrations in the assay. This study was done with benzo[a]pyrene (BaP) and rat liver microsomes (a gift from drs. A.J. Murk, dept. Toxicology, Agricultural University Wageningen, the Netherlands) which were prepared in a similar way as the microsomes of the wildlife species. Rat microsomes were chosen for optimisation because the stock of microsomes of marine species was low.

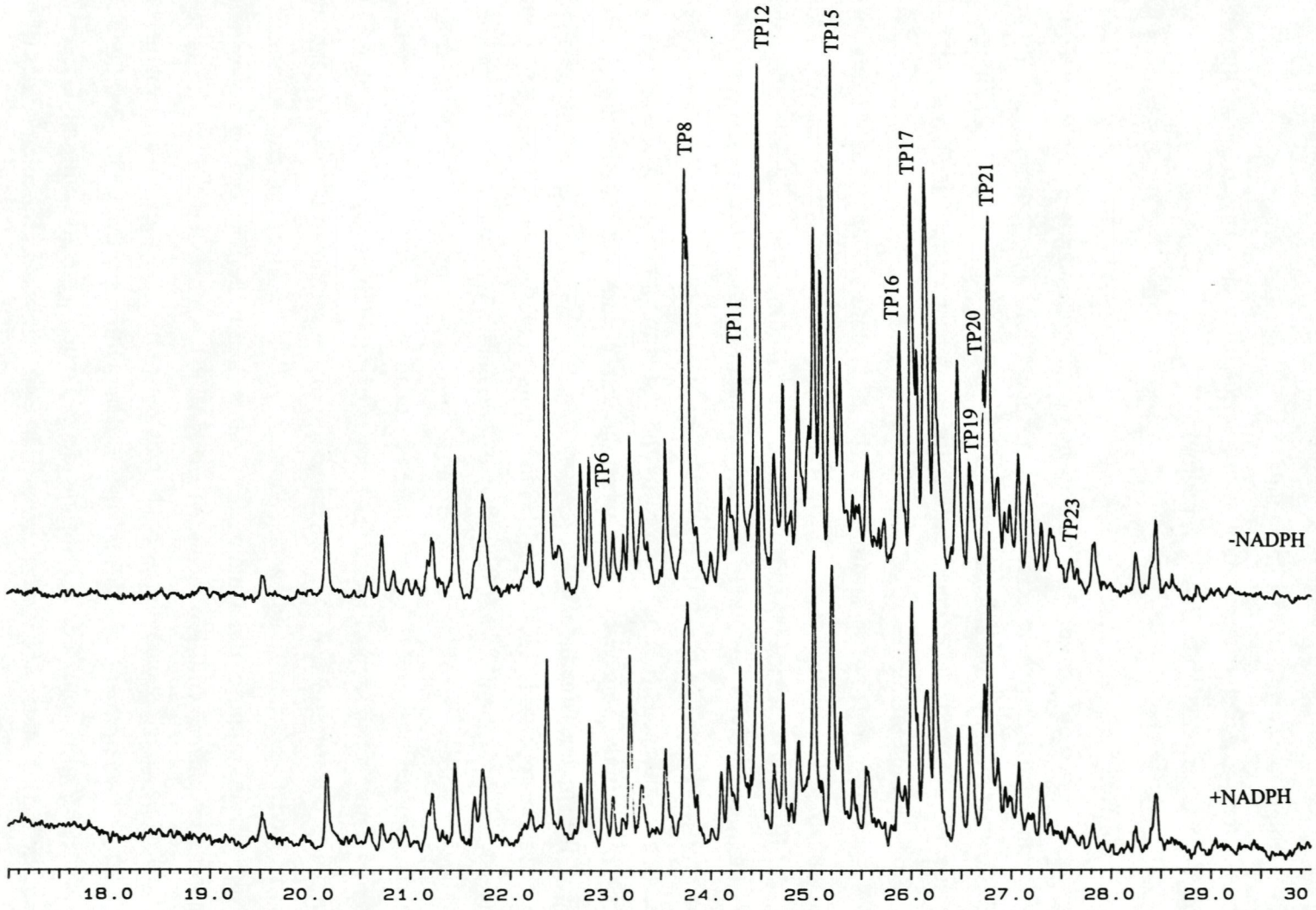


Fig.4: Total Ion Current chromatograms (as sum of the masses selected) in the absence and in the presence of NADPH as electron donor allowing biotransformation of a technical toxaphene mixture by the cytochrome P450 system in liver microsomes of a harbour seal. The peaks identified by their TP numbers were used to calculate the changes due to biotransformation.

5 Results:

Biotransformation:

Harbour seal 95PVa:

EROD activity was 159 pmol.mg^{-1} protein.min^{-1}. This value is quite high compared to the range of values reported for pinniped species, as reviewed in Boon *et al.*, 1992. It should be noted however, that theses ranges are considerable lower than those of cetaceans (data reviewed in the same paper), which is quite unexpected.

TT-mix: Qualitative changes in composition in the presence of NADPH in comparison to the reference without NADPH are illustrated in a total ion chromatogram (TIC) of the masses selected (fig. 4). Quantitative changes calculated from the comparisons of these TICs are given in a bar diagram (fig. 5). The analyses were carried out in duplicate. The influence of biotransformation resulted in decreases from less than 10 for toxaphene peak 6 (TP6) to more than 70% for TP16.

T5-mix: The assay showed a strong decrease of congeners CHB-32 and CHB-62 in contrast to the congeners CHB-26 and CHB-50 (fig.6). In view of the results with another harbour seal specimen (Boon *et al.*, 1995), the small decrease of CHBs-26 and -50 was considered an experimental error.

Tissue residue: Because of a lack of blubber tissue, the toxaphene residue was measured in liver of the same animal as used for the *in-vitro* assay. The chromatogram shows only about ten peaks higher than five times the background noise level (fig. 7). A peak with the retention time of congener CHB-50 is one of the major peaks. The estimated total toxaphene concentration was very low: only 0.6 µg.kg^{-1} extractable lipid.

Whitebeaked dolphin 95LAa:

EROD activity: 49 pmol.mg^{-1} protein.min^{-1}, which is quite low when compared to the range reported for the striped dolphin (190 ± 15 pmol.mg^{-1} protein.min^{-1}), but comparable to the values found for the short-finned pilot whale (40 ± 40 pmol.mg^{-1} protein.min^{-1}; Watanabe *et al.*, 1989) and a value of 66 ± 52 pmol.mg^{-1} protein.min^{-1} reported for unspecified whales/ dolphins by Livingstone *et al.*, 1992.

TT-mix: The bar diagram shows decreases from zero for TPs 19-21 to slightly more than 20% for TPs 11, 16 and 17 (fig. 5).

T5-mix: Only CHB-32 showed a clear decrease to about 60% (fig.6).

Tissue residue: The tissue residue in blubber of this animal shows about 20 toxaphene-derived peaks with a clear dominance of a peak with the retention time matching that of CHB-50 (fig. 7). The concentrations in a number of whitebeaked dolphins investigated cover the range of 300-12000 µg.kg^{-1} lipid.

Sperm whale 95PMb:

EROD activity: 17 pmol.mg^{-1} protein.min^{-1}. To our knowledge this is the first EROD value ever reported for a sperm whale, and it can therefore only be compared that of the whitebeaked dolphin and the values mentioned for other cetacean species in that paragraph.

TT-mix: The assay yielded no clear indications for metabolism of any peak from the technical mixture (fig. 5).

T5-mix: The results from the T5-mix confirm the absence of any indication of metabolism (fig. 6).

Tissue residue: About 50 toxaphene-derived peaks are visible in the chromatogram. The peak with the retention time of CHB-50 is only a medium peak (fig. 7). When it is assumed that this peak is caused entirely by this congener, its concentration in the three animals stranded on the Dutch coast is in the range of 3-30 μg.kg^{-1} lipid.

Albatross 95DIa:

EROD activity: 21 pmol.mg^{-1} protein.min^{-1}. This value can be compared to the value of 50 pmol.mg^{-1} protein.min^{-1} reported for birds by Livingstone *et al.*, 1992, who also noted that amongst birds, the lowest MFO activities are seen in specialised predators as fish-eaters and raptors. However, when induced under experimental conditions with relatively high doses, EROD values can increase to much higher values, as was shown by Rozemeijer and co-workers (1995) for eider ducklings.

TT-mix: The results show some biotransformation, especially of TP11 decreasing to less than 50% of its original height (fig. 5).

T5-mix: Like for the white-beaked dolphin, a clear decrease was only observed for CHB-32 (fig. 6).

Tissue residue: This chromatogram is also quite similar to that of the whitebeaked dolphin in that it contains about 20 toxaphene derived peaks and the peak with the retention time of CHB-50 dominates (fig. 7).

Mutagenicity

Mutatox test protocol optimisation

The optimisation studies with BaP an rat liver microsomes were run with protein concentrations of 0.4, 0.8, 1.6 and 3.2 mg.ml^{-1} and NADPH concentrations of 3.3 and 6.6 mM. Highest light levels were recorded with 0.4 mg protein.ml^{-1} and 3.3 mM NADPH. The response obtained with these microsomes were around 10% of that obtained with the S9-mix supplied by the manufacturer.

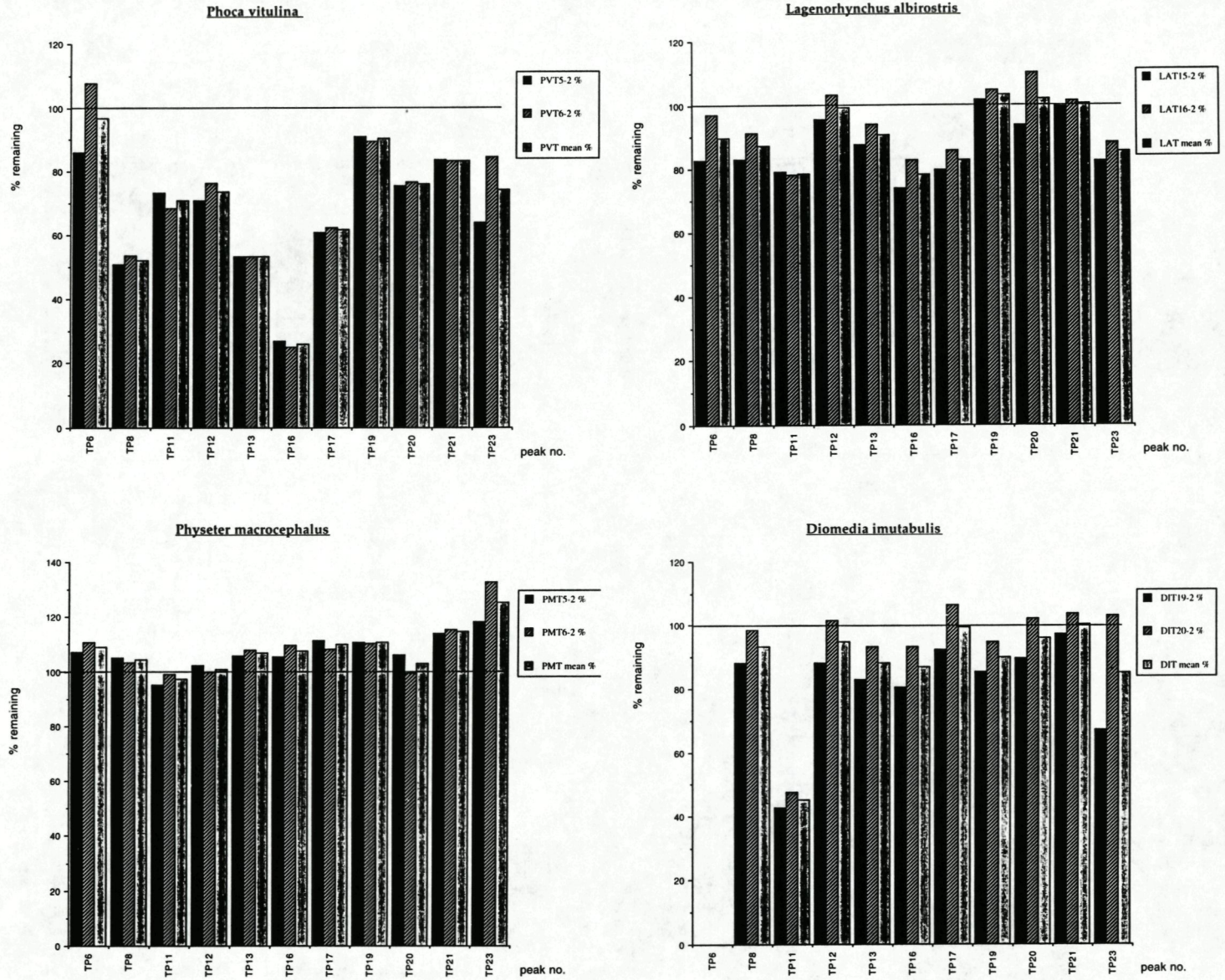


Fig. 5: Changes in major peaks of a technical toxaphene mixture due to biotransformation by hepatic microsomal preparations of different species. The TP numbers on the X-axis refer to the peaks shown in fig. 4. All analyses were carried out in duplicate, for each peak the results of both analyses are shown together with their mean.

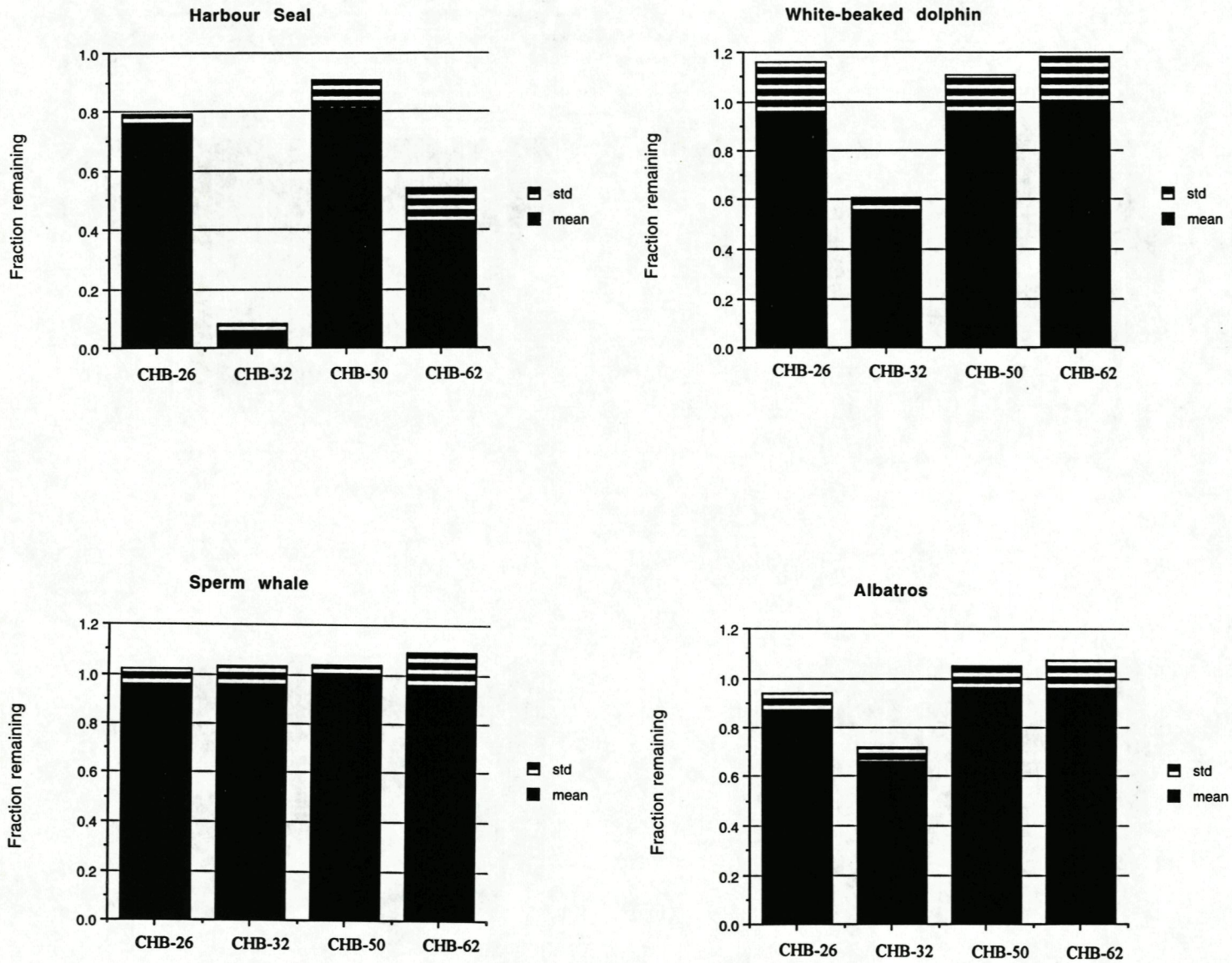


Fig. 6: Changes in the T-5 mixture after biotransformation by hepatic microsomal preparations of different species.

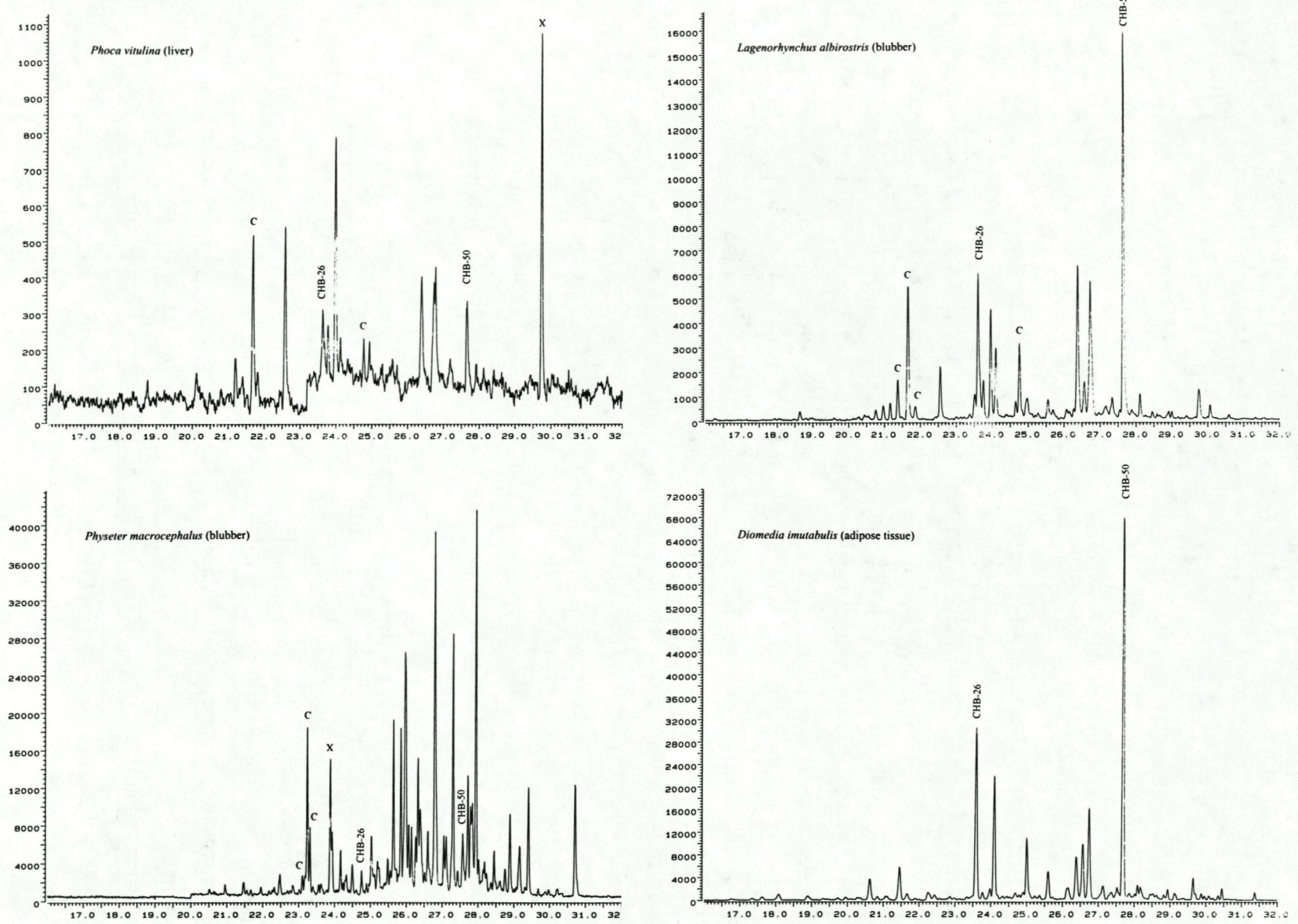


Fig. 7: TIC chromatograms (selected masses only) of the toxaphene residues in tissues of different species caused by exposure under field conditions.

Mutatox tests with Toxaphene

Table 3 summarises the results of Toxaphene genotoxicity tests with microsomes from the different organisms given in table 1. All **I** and **CI** tests were run in duplicate; ranges of LCR and CMR are given.

As is clear from Table 3, the results of the direct test and the tests with sperm whale and whitebeaked dolphin microsomes were not unambiguous. For example, of the six direct assays carried out, four gave no positive results, while two gave LCR and CMR ranges of 0.016-0.91 and 0.035-2.53 $\mu g.ml^{-1}$, respectively.

Apparently, the direct genotoxicity of the tested technical toxaphene formulation is quite low and not always detectable. In the presence of rat liver S9 supplied by Microbics® or microsomes of the harbour seal and the albatross, no indications for genotoxicity were obtained; whereas in the presence of microsomes of the whitebeaked dolphin and the sperm whale genotoxicity was retained. Thus, incubation in the presence of liver material of fast metabolisers seems to have resulted in a somewhat lower mutagenicity than observed in the direct assay or in the presence of microsomes of slower metabolisers as sperm whale or whitebeaked dolphin. Although the results were not always well reproducible, the conclusion can be drawn that biotransformation at least does not potentiate mutagenicity as in case of some well known mutagenic compounds as BaP.
The connection between the Ames *Salmonella* results (see above) is not clear.

Table 3. Toxaphene qualitative ("+", "-") and quantitative (LCR and CMR [$\mu g.ml^{-1}$]) genotoxicity results determined with the Mutatox test. n = number of replicates. npr = no positive response.

Organism	Code	n	+/-	LCR	CMR
None (direct assay)	-	6	+	npr - 0.016	npr - 2.53
Rat (supplied by Microbics)	-	2	-	npr	npr
Harbour seal	94PVa2	2	-	npr	npr
Sperm whale	95PMb1	2	+	npr - 0.092	npr - 0.65
Whitebeaked dolphin	95LAa1	2	+	npr - 1.53	npr - 2.23
Albatross	95DIb1	2	-	npr	npr

6. Discussion:

The animals investigated in the present report largely confirm the tentative conclusions of BEON report 95-4 (Boon *et al.*, 1995). In the harbour seal, a large influence of biotransformation on the pattern *in-vitro* coincides with a very low residue level and the smallest number of peaks in the chromatogram. A lower *in-vitro* capacity for biotransformation is found for the whitebeaked dolphin and the albatross. Residue patterns in the whitebeaked dolphin and the albatross contain more peaks than the harbour seal and the concentration of CHB-50 is much higher. The most complex toxaphene residue pattern in combination with an apparent absence of any *in-vitro* biotransformation capacity in spite of a well detectable EROD activity of the microsomal fractions was found for the sperm whale. For PCBs, the difference in pattern between prey and lung-breathing predators can be explained purely in terms of biotransformation (Boon *et al.*, 1987; 1992; 1994). Whether this is also true for toxaphene remains to be investigated.

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. 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 a single Cl-substituent. 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.

All chromatograms do not reveal new peaks after biotransformation. This implies that the metabolites formed are probably of a polar nature and do not elute from the alumina or silica columns used in the clean-up process before GC- analysis (*Note: In a follow-up project, the formation of hydroxylated metabolites of CHB-32 and CHB-62 by microsomal preparations of a harbour seal was confirmed by their NCI mass-spectra; see appendix I*). The formation of a dechlorination product as determined by Chandurkar and Matsumura (1979) after *in-vitro* metabolism of 2-endo,3,3,5-exo,6-exo,8,9,10,10 nonachlorobornane (ToxC) by the 20,000*g fractions of rat livers is therefore unlikely. These authors also isolated a number of polar metabolites; a primary alcohol (-OH group at the C-8, -9 or -10 position), and a number of secondary alcohols (-OH group at the C-2, -3, -5 or -6 position of the six-membered ring) were identified. The methods presently used do not allow for the detection of polar metabolites, since they are retained on the alumina column used for clean-up purposes.

The epoxidation of the pesticide aldrin to dieldrin is a classical model reaction for the activity of enzymes of the cytochrome P450 2B subfamily. On the basis of the structural resemblance of chlorinated bornanes to aldrin, we speculate that cytochrome P450 2B also fulfils a major role in the biotransformation of toxaphene congeners. Since pinnipeds are reported to posses higher activities of iso-enzymes of this subfamily than either cetaceans or fish-eating birds, this would also explain the fact that seals apparently metabolise toxaphene so much more extensively (Boon *et al.*, 1992; Rozemeijer *et al.*, 1995).

Thus, in summary, large differences exist with respect to the *in-vitro* biotransformation of toxaphene for the different species investigated, which correlates well with the complexity of the residue pattern due to *in-vivo* exposure in the field situation.

With regards to mutagenicity the results were not unambiguous, but direct mutagenicity of technical toxaphene was demonstrated. Metabolism does seem to have lead to some decrease in mutagenicity especially in the best metabolisers (rat and harbour seal) and thus certainly not to a potentiation as is the case for a number of well known mutagenic compounds such as benzo[a]pyrene.

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Appendix I

Paper presented at Dioxin96

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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.

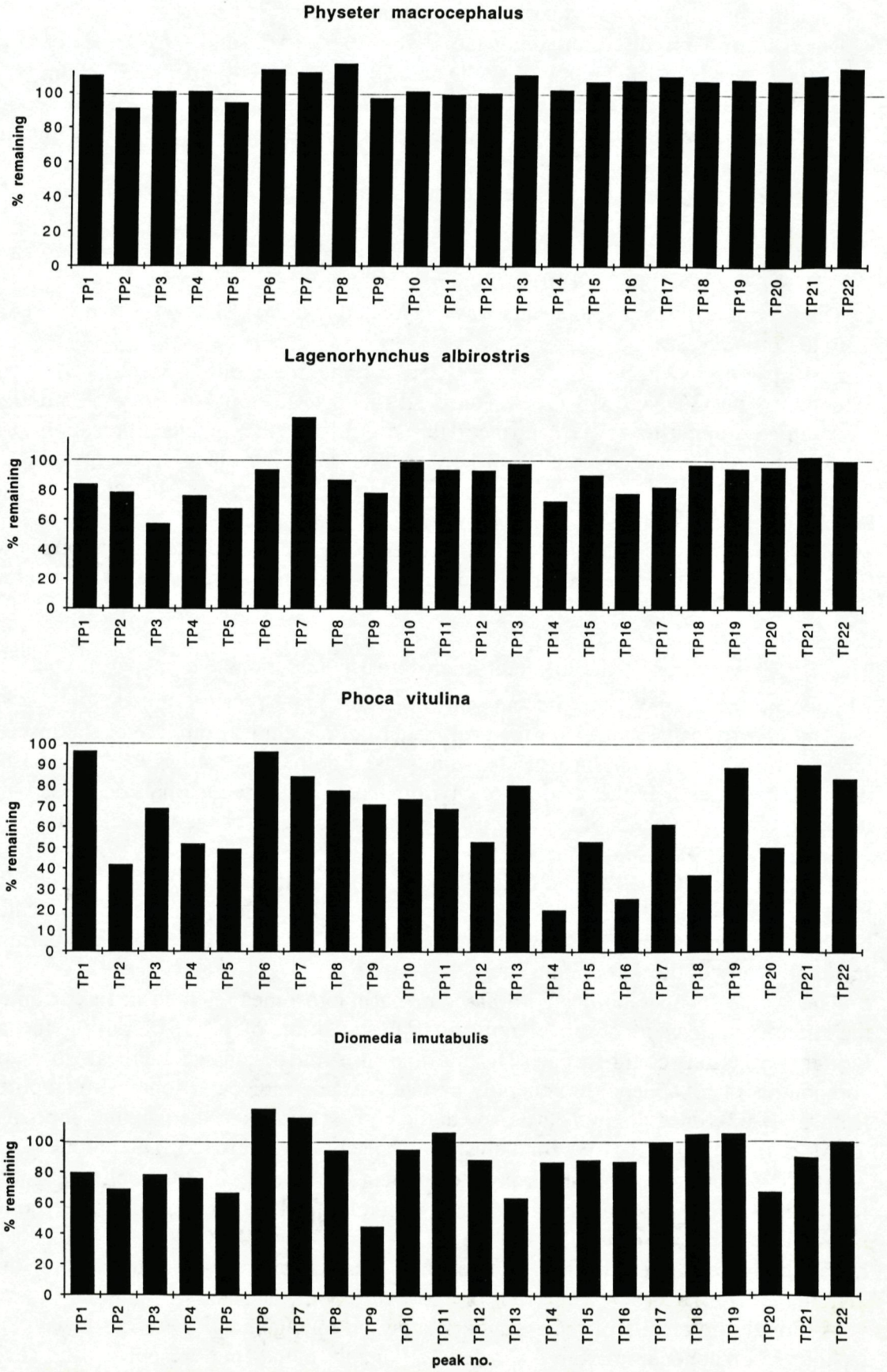


Fig.1: Differences in the NCI-SIM chromatograms of a technical toxaphene mixture between hepatic microsomal preparations that received NADPH during incubation, and reference samples that were incubated without NADPH. All results are means of duplicate sample pairs.

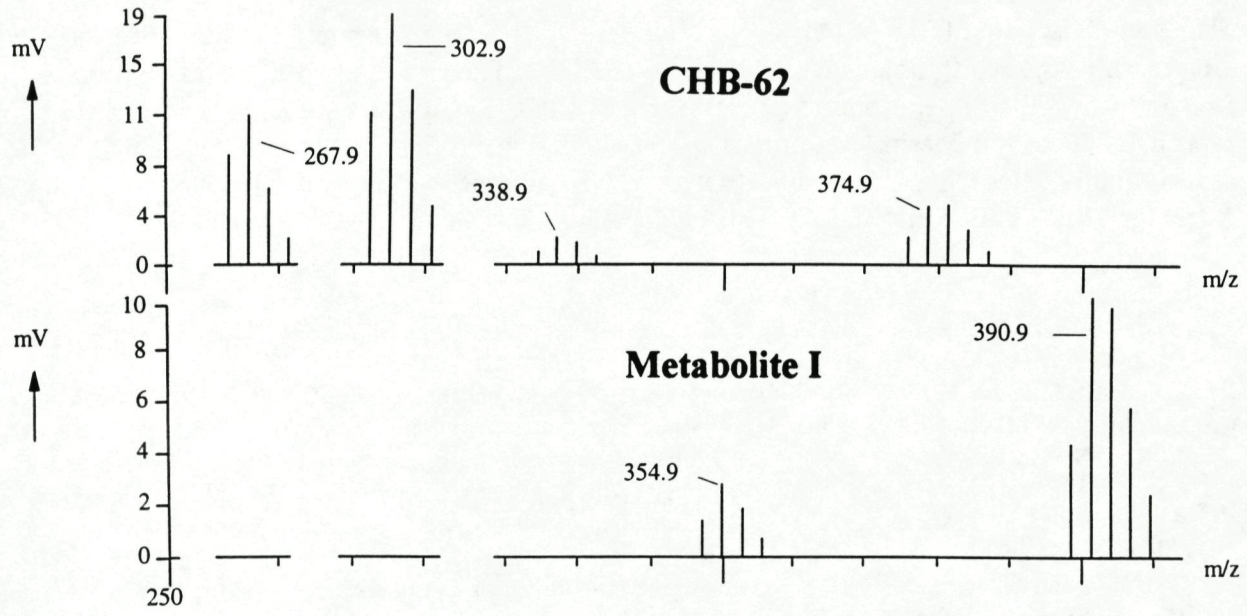


Fig. 2: NCI-mass-spectra of CHB-62 and its metabolite synthesised by harbour seal microsomes.

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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