

In vitro biotransformatie van organohalogeenverbindingen in zeezoogdieren en vogels. Mogelijke gevolgen voor bioaccumulatie en genotoxiciteit

V. Chloordanen

Beleidsgericht
ecologisch onderzoek
van de
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Uitgebreide Samenvatting:

Doel van het onderzoek:

Het onderzoek binnen dit BEON project beoogt een viertal doelen:

1. Ontwikkeling van een *in-vitro* bioassay voor de oxidatieve biotransformatie van verschillende groepen van lipofiele organische microverontreinigingen in mariene toppredatoren.
2. Validatie van de met deze assay behaalde resultaten door vergelijking met residu patronen in prooi en predator als gevolg van natuurlijke blootstelling in zee.
3. Bestuderen van de invloed van biotransformatie op de genotoxiciteit van de verbindingen in een bacteriële standaard bio-assay (Mutatox®).
4. Beleidsadvisering op grond van de behaalde resultaten en de verzamelde literatuurgegevens.

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 bijzonder groot geacht op grond van hun moleculaire eigenschappen, bijvoorbeeld een 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 wèl enzymatisch worden omgezet, laat zich dus niet volgens

deze regels beschrijven. Dit aspect speelt een belangrijke rol bij de doorvergiftigingsproblematiek in de normstelling (1). Het is echter nog niet mogelijk om vanuit de theorie te voorspellen of bepaalde klassen van verbindingen al of niet kunnen worden omgezet in verschillende diergroepen. In het geval van zeezoogdieren en zeevogels is ook het verkrijgen van informatie door middel van experimenten met dieren in gevangenschap (*in-vivo* experimenten) slechts in hoge uitzonderingsgevallen mogelijk vanwege ethische en logistieke redenen. Bovendien zijn zulke experimenten erg duur.

Doel van het huidige BEON project is dan ook de ontwikkeling van een alternatief voor zulke *in-vivo* experimenten in de vorm van een *in-vitro* bioassay om de oxidatieve 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 dieren die kort na het moment van sterven kunnen worden bemonsterd.

Voordat er met 'nieuwe' stoffen werd begonnen, is de gebruikte *in vitro* assay eerst gevalideerd voor de polychloorbiphenylen (PCBs), omdat het bioaccumulatie- en biotransformatiegedrag hiervan in zeezoogdieren en zeevogels al goed bekend is. De resultaten hiervan zijn beschreven in BEON rapport 95-4 (2).

Nadat uit het onderzoek aan de PCBs de waarde van de *in vitro* assay was gebleken, zijn vervolgens een aantal stofgroepen onderzocht, waarvan op grond van hun molecuulstructuur ophoping in de voedselketen kan worden verwacht, maar waarover nog weinig gegevens bekend zijn omtrent hun bioaccumulatie in top-predatoren uit de Noordzee en Waddenzee. De reeds verschenen rapporten in de serie behandelen de insecticiden toxafeen (resultaten verschenen in de BEON rapporten 95-4 (2) en 96-1 (3)), de als vlamvertragers gebruikte polybroom biphenylen (PBBs) en polybroom difenylethers (PBDEs) (BEON rapport 97-6 (4), en de polychloor terfenylen (PCTs; een stofgroep met in essentie dezelfde toepassingen als PCBs) (5). In het huidige rapport (het laatste in deze serie) wordt het onderzoek naar invloed van biotransformatie van de voornaamste componenten van technisch chloordaan op de bioaccumulatie en genotoxiciteit beschreven.

Resultaten en conclusies van het uitgevoerde onderzoek met betrekking tot de beleidsvoering ten aanzien van chloordanen.

- Doorvergiftigings problematiek: Uit de *in vitro* assays bleek, dat biotransformatie veelvuldig voorkwam en dus zeer belangrijk is voor de beschrijving van het bioaccumulatieproces en de giftigheid van chloordaan componenten in mariene voedselketens. Wel waren er duidelijke verschillen tussen de microsomen van verschillende soorten: Bij de eidereend en de zeehond was de biotransformatiecapaciteit duidelijk groter dan bij de beide geteste tandwalvis soorten, de witsnuitdolfijn en de potvis.

  Deze uitkomst van de *in vitro assays* is duidelijk terug te vinden in de residuen van dieren, die op natuurlijke wijze via de mariene voedselketen zijn blootgesteld: Bij zeehond en eidereend waren de geoxideerde metabolieten in de meerderheid, terwijl bij tandwalvissen de concentraties van de ouderstoffen hoger waren. Het voorkomen van chloordaan componenten in alle drie de bij Kijkduin levend aangespoelde potvissen heeft hierbij een bijzondere betekenis; gezonde potvissen bevinden zich namelijk in principe uitsluitend in de diepzee; op onze breedte zijn dit de Noord Atlantische Oceaan en de Middellandse Zee. Zij leven daar voornamelijk van inktvis, maar ook diep levende vissoorten worden gegeten. Zowel de prooidieren als de predator komen dus slechts bij hoge uitzondering in kustgebieden voor. Uit het feit, dat de chloordaan componenten ook in deze potvissen werden gevonden, kan dus worden geconcludeerd dat we hier te maken hebben met wereldwijd verspreide groepen van stoffen.
- Genotoxiciteit: Zowel een mengsel van ouderstoffen als een mengsel van metabolieten waren genotoxisch in de Mutatox® assay. Deze toxiciteit verdween bij beide mengsels na toevoeging van rat S9 fractie, die net als microsomen van mariene organismen biotransformatie mogelijk maakt. Er zijn dan ook verschillende indicaties dat de in deze studie gemeten metabolieten niet de eindstadia van biotransformatie zijn. Dit aspect behoeft nader onderzoek, aangezien de genotoxiciteit van metabolieten blijkbaar ook erg stofafhankelijk is.
- Normering: De wereldwijde productie van technisch chloordaan sinds 1946 wordt geschat op 70.000 ton (6, 7). Net als aldrin en dieldrin behoren chloordanen tot de gechloreerde cyclodiëen insecticiden, welke tot de meest giftige en persistente verbindingen van alle pesticiden worden gerekend (8). Aangezien vertegenwoordigers van de cyclodiënen zeer lipofiel, zeer toxisch en zeer stabiel zijn in het milieu, kunnen zij een bedreiging vormen

voor organismen. Voor technisch chloordaan is een productie piek in de 70-er jaren gerapporteerd en een halfwaardetijd van 10-20 jaar (9). Op grond hiervan is berekend, dat 25-50 % van al het geproduceerde technisch chloordaan nog onveranderd in het milieu aanwezig is (10). Chloordaan componenten komen voor op de volgende stoffenlijsten en in de volgende kaders:

Internationaal:

1. De 'list of Potential Endocrine Disrupters - Part B' van de Oslo and Paris Commision (OSPAR). Deze bevat de namen van stoffen waarvan in de wetenschappelijke literatuur is is gerapporteerd dat ze veranderingen in hormonale processen teweeg kunnen brengen in tenminste *in vitro* tests.
2. Het UNEP Persistent Organic Pollutants (POP-) Programme: de 'list of substances for international action, including a global legally binding instrument'.
3. Het UNECE POP-protocol: De 'list of substances requiring international action'. Dit protocol is onlangs uitonderhandeld, en zal (samen met een protocol over zware metalen) op 25-26 juni 1998 tijdens de Pan-Europese Ministersconferentie in Århus worden ondertekend. In het POP-protocol is er voor 11 in Annex I genoemde stoffen, waaronder chloordaan en heptachloor, overeengekomen tot wereldwijde eliminatie van productie en gebruik.
4. Medio 1998 zullen ook de onderhandelingen starten voor een wereldwijd POP-protocol, waarvoor het UNECE-POP protocol één van de belangrijkste uitgangspunten is.
5. De EU-lijst van 132 geselecteerde stoffen, die in aanmerking komen voor plaatsing op de zwarte lijst . Chlordaan werd hiervoor reeds in 1977 geselecteerd.
6. Noordzee Actieprogramma: De internationale lijst van 18 pesticiden die beperkt of verboden dienen te worden (lijst 1B).
7. Technisch chloordaan en alle hoofdcomponenten staan vermeld op de "hazardous substance list" van de Environmental Protection Agency in de VS. Vanwege de carcinogeniteit van chloordaan is al het gebruik in de VS vanaf 1988 verboden.

Nationaal:

Ook in Nederland is de toepassing van chloordanen reeds lang verboden. In nationaal verband komen chloordaan componenten bovendien voor in de volgende kaders:

1. Milieukwaliteitsnormen van het programma 'Integrale Normstelling Stoffen'.
2. Voor het compartiment water ook het regeringsvoornemen beschreven in de 4e Nota Waterhuishouding (NW4).

Gezien de in deze studie geconstateerde wijde verspreiding van chloordaan componenten in het mariene milieu, in combinatie met hun carcinogene en hormoon verstorende eigenschappen, ondersteunen wij de internationale en nationale beleidsmaatregelen om de huidige nivo's verder terug te dringen. Wel achten wij het van groot belang om chloordaan componenten op te nemen in bestaande monitoringsprogramma's om het succes van dit beleid vast te kunnen stellen. Als componenten stellen wij voor *cis*- en *trans*-chloordaan, *cis*- en *trans*-nonachloor, heptachloor en de metabolieten oxychloordaan en heptachloorepoxide B.

Aanbevelingen voor toekomstig onderzoek

1. Voorkomen van chloordanen: In de hier onderzochte monsters van toppredatoren komen chloordanen veelvuldig voor. De hoogste concentraties (op vetbasis) werden gevonden in de levers van eidereenden uit de Waddenzee. In tegenstelling tot de situatie voor een aantal andere organochloor pesticiden, zoals de DDT groep, de verschillende hexachloor cyclohexaan (HCH-) isomeren, hexachloorbenzeen (HCB) en een aantal andere cyclodiënen als dieldrin en endrin, zijn er van het voorkomen van de verschilllende componenten van technisch chloordaan in de Noordzee en Waddenzee nog geen gebiedsdekkende gegevens bekend. Om dit verder te onderzoeken stellen wij de volgende compartimenten voor:
   - Sterk plaatsgebonden ongewervelde dieren. Bij het NIOZ zijn hierbij goede ervaringen opgedaan met het gebruik van de worm *Nephtys spp.* voor dit doel.
   - Vis. Hierbij kan aansluiting worden gezocht bij een door het RIVO-DLO jaarlijks reeds uitgevoerd monitoringsprogramma, waarin organochloor verbindingen in levers van kabeljauw uit de Noordzee worden gemeten.
   - Zeezoogdieren. Hierbij kan worden gedacht aan een een actief monitoring programma aan zeehonden, waarbij gegevens op een non-invasieve manier worden verkregen door

bemonstering van bloed en het nemen van vetbiopsies. Aanvullend zouden er meer monsters van dolfijn-achtigen uit de Noordzee dienen te worden onderzocht.

- Vogels: Vooral bij trekvogels worden de gehalten zeer sterk beïnvloed door intering op de vetreserves tijdens de trekvlucht. Momenten waarop de vetreserves minimaal zijn lijken het meest kritisch omdat de contaminant gehalten dan wellicht zeer hoog zijn. Hiervoor zijn laboratoriumstudies noodzakelijk. Op het NIOZ zijn hiervoor goede faciliteiten en biologische achtergrondkennis aanwezig met betrekking tot kanoetstrandlopers, welke trekken tussen hun overwinteringsgebieden in Mauretanië en hun broedgebieden in Noord-Siberië. Hierbij dient de Waddenzee als een tussenstop om weer reserves op te bouwen voor de tweede helft van de route.

Dankwoord.

Wij willen graag de beleidsmentor van dit BEON project, drs. D.A. Jonkers van het Directoraat Generaal Milieuhygiëne van het Ministerie van Volkshuisvesting, Ruimtelijke Ordening, en Milieuhygiëne (VROM) hartelijk danken voor het verstrekken van het overzicht van de internationale en nationale programma's en kaders waarin componenten van technisch chloordaan worden genoemd. Dr. R.W.P.M. Laane heeft, als voorzitter van de Begeleidingsgroep 'Microverontreinigingen' van BEON, ook bij alle rapporten in deze serie de moeite genomen om waardevolle opmerkingen te geven. De medewerkers van het programmaburo BEON, drs. J. Visser, drs. M.B.A.M. Scheffers en de heer M.-J. Heesen willen wij tenslotte hartelijk bedanken voor de plezierige wijze waarom zij dit vier-jarige project logistiek hebben ondersteund.

***In vitro* Biotransformation of Cyclic Organohalogen Compounds in Marine Mammals and Birds. Possible Consequences for Bioaccumulation and Genotoxicity. V: Chlordanes.**

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Running-title: Biotransformation of Chlordanes.

Key words: Chlordanes, *in vitro* Biotransformation, Residue Patterns, Genotoxicity, Wildlife.

Abstract

- The *in-vitro* capacity for biotransformation of the major constituents of technical chlordane was investigated with hepatic microsomal preparations of a sperm whale (*Physeter macrocephalus*), a white beaked dolphin (*Lagenorhynchus albirostris*), a harbour seal (*Phoca vitulina*), and an eider duck (*Somateria mollissima*).
- The ability to metabolise chlordanes increased in the order sperm whale < whitebeaked dolphin < eider duck < harbour seal. Harbour seal microsomal preparations were able to metabolise all chlordane congeners except *trans*-chlordene. In contrast, the microsomes of sperm whale microsomes (*Physeter macrocephalus*) could metabolise *trans*-chlordene only to a significant extent. The whitebeaked dolphin was able to metabolise *trans*-

chlordene, heptachlor and *trans*-chlordane. Finally, the common eider duck was able to metabolise *trans*-chlordene, heptachlor, *trans*-chlordane and *cis*-chlordane significantly.

- Three metabolites were formed in the *in-vitro* assays. Two could be identified as heptachlor epoxide B and oxychlordane. However, the relative amounts of oxychlordane formed in the *in-vitro* assays were very low in comparison to residue levels. A further metabolism to diols by epoxide hydrolase enzymes would account for the low yield of oxychlordane. The heptachlorepoxide A isomer was not formed in the *in vitro* assays, which is probably due to the stereoselectivity of the MFO system. The third metabolite, with a relative retention time of 0.53 * the retention time of CB-153, remained unknown.
- Chlordane residues could be detected in all wildlife samples. From the *in-vitro* assay as well as biota residue levels it can be concluded that *cis*- and *trans*-nonachlor are the most persistent of all chlordane components. However, a slow metabolism of these compounds occurred in the harbour seal microsomes. Of the chlordanes, *trans*-chlordane is more readily metabolised than *cis*-chlordane, which is therefore more bioaccumulative.
- Relatively high concentrations were also found in blubber of three sperm whales. Because these animals do normally not occur in shelf seas, this shows that chlordanes have also reached the deep sea. The metabolic capacity of sperm whales seems to be low even among other cetacean species.
- Both a mixture containing the main parent compounds of technical chlordane and a mixture containing three oxygenated metabolites were genotoxic in the direct Mutatox® assay. This genotoxic response disappeared after the addition of rat S9 fraction, allowing for biotransformation. This means that the primary metabolites formed were still genotoxic and ongoing metabolism must have taken place to detoxify both mixtures.

1. Introduction

Chlordane was first registered in 1945 as an organochlorine insecticide. Structurally it is part of the chlorinated cyclodiene family (aldrin and dieldrin are also part of this family). Technical chlordane is a mixture of more than 140 compounds. The major constituents are *cis*- and *trans*-chlordane, heptachlor, *cis*- and *trans*-nonachlor and chlordene (10). The composition of the technical chlordane mixture with respect to these major constituents is given in **table 1.1**.

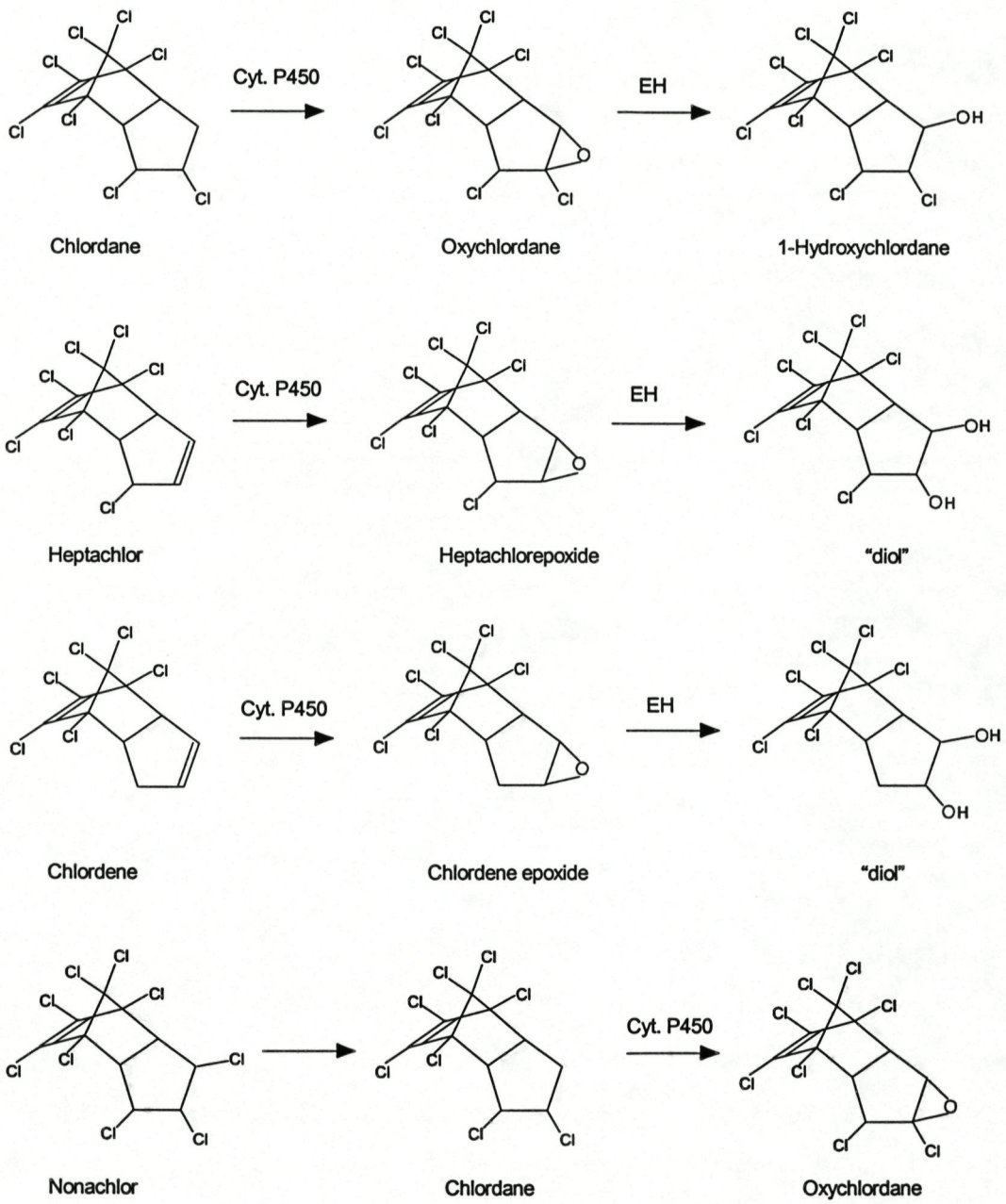
***Table 1.1**: Major constituents of technical chlordane (11).*

Compound	% in technical chlordane
Trans-chlordane	25
Cis-chlordane	20
Heptachlor	10
Trans-nonachlor	7
Cis-nonachlor	<5

The world wide production of technical chlordane is estimated as more than 70,000 tons since 1946 (6, 7). Since chlordanes are highly lipophilic, the log K_{ow} of technical chlordane is 5.58 (12), and very stable in the environment, they can pose a threat to aquatic organisms. A half-life of 10-20 years and a production peak in the 1970s is reported for technical chlordane (9), suggesting that 25-50 % of the technical chlordane ever produced still is present unaltered in the environment (10). Between 1983 and 1988, the only permitted use for chlordane was for control of subterranean termites. Technical chlordane and all major individual components are on the hazardous substance list of the EPA. Because of concern about the risk of cancer, all use of chlordane was cancelled in 1988 in the U.S. Chlorinated cyclodiene insecticides are among the most toxic and persistent of all pesticides and their use for most agricultural purposes is banned or restricted in most countries. The only commercial use still permitted is for fire ant control in power transformers (1993) (8).

Several chlordanes are known to be metabolised enzymatically in biota. The major constituents of technical chlordane are investigated in this research project. Their structure is shown in **figure I**. They are discussed in detail below.

FIGURE I: The structures of the chlordanes and their most probable metabolites



Cyt. P450= cytochrome P450
EH = epoxide hydrolase

***Cis*- and *trans*-chlordane.**

Pure chlordane (also known as octachlor) consists of the 2 isomers; *cis*- and *trans*- chlordane. The *cis*- isomer is by far the more toxic of the 2 geometric isomers. Both isomers can be epoxidised to the more toxic metabolite oxychlordane, which is relatively stable and can be found in environmental residues The reaction is shown in **figure I.** However, a slow metabolism to a variety of secondary metabolites (for example diols) occurs. Thus, the biotransformation of chlordane will do an animal, at least initially, more harm than good (8, 13, 14).

Heptachlor.

Heptachlor was first isolated from technical chlordane in 1946. It was also used separately as a pesticide. The main and most persistent metabolite of heptachlor is heptachlor epoxide. Its acute toxicity is greater than the parent compound. For example, the intravenous lethal dose for heptachlor is 40 mg/kg body weight and for heptachlor epoxide 10 mg/kg body weight. So, at least the initial biotransformation of heptachlor will do the animal more harm than good. Heptachlor epoxide, like oxychlordane, appears to be fairly stable. Heptachlor epoxide occurs in 2 geometric isomers: heptachlor epoxide A (*endo*-epoxide) and heptachlor epoxide B (*exo*- epoxide) (13).

***Cis*- and *trans*-nonachlor.**

Cis- and *trans*-nonachlor represent a small portion of the technical chlordane mixture. However, due to their persistent nature, these nonachlors often represent large fractions of the Σ-chlordane residues in wildlife. Nevertheless, there is a very slow dechlorination to *trans*-chlordane and subsequently biotransformation to oxychlordane (13)(15).

***Trans*-chlordene.**

Trans-chlordene is the intermediate product during the production of all the other chlordanes. The reaction is shown in **figure 1.1.**

Trans-chlordene can be metabolised to chlordene epoxide and the subsequent hydroxy compound. This reaction is shown in **figure I.**

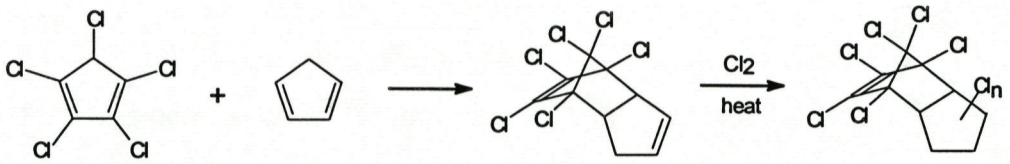


***Figure 1.1**: The formation reaction of the chlordanes.*

As stated above, enzyme mediated biotransformation plays an important role in the environmental fate of chlordane components. The initial oxidative step in the biotransformation pathway of polyaromatic halogenated hydrocarbons (PHAHs) is mediated by the cytochrome P-450 dependent monooxygenase (HMO) system. This first step is called phase I metabolism. During the subsequent phase II metabolism, the products of phase I metabolism are conjugated to endogenous substrates, which further increases the water solubility and polarity of the metabolites. Since in this research only the phase I metabolism is investigated, phase II metabolism is not discussed any further. The cytochrome P450 complex metabolises endogenous and exogenous compounds in order to facilitate the excretion of these compounds (16). It is evident, that biotransformation can have a large impact on the behaviour of a compound in the food chain and its toxicity. First, biotransformation determines whether there is accumulation from prey to predator (biomagnification). Second, the change in molecular structure strongly affects toxicity. On the one hand, the formation of water soluble metabolites that are more easily excreted is advantageous, but biotransformation can also generate metabolites that are more toxic than the parent compounds (17, 18). Cytochrome P450 consists of a family of closely related iso-enzymes (a super gene family). Each iso-enzyme (subfamily) is specialised in the transformation of a group of closely related compounds. Not all iso-enzymes are present in every species. Therefore, difference in biotransformation abilities between different species can be expected (19).

It is obvious that if one wishes to investigate hepatic xenobiotic metabolism then the best model is the liver itself. In the case of the marine mammals and birds, the examined animals are relatively rare. Therefore, *in-vivo* experiments with these species are often not feasible due to the ethical and logistic factors, and an alternative must be pursued. The cytochrome P450 complex is incorporated in the smooth endoplasmatic reticulum, which can be isolated from liver homogenate by (ultra-)centrifugation. The isolated membrane parts spontaneously close themselves to vesicles, which are called microsomes. These microsomal preparations can be used in *in vitro* experiments where the substrate is added to the microsomal suspension in a

buffer. Upon the addition of NADPH as electron donor, the phase I oxidation reaction can, in principle, occur when a compound is a suitable substrate for a particular iso-enzyme. Microsomal preparations are easy to work with, but of course they represent a strong simplification compared to a living organism. In order to determine the validity of the results obtained with such a strongly simplified system, they have to be compared to the *in-vivo* situation. The results for the well-studied PCBs have shown that the results of the *in-vitro* assay indicated very well which PCBs were biomagnified from fish to seals and birds as predators, and which did not biomagnify due to biotransformation (2, 20).

This research project is part of a larger BEON project called 'the *in-vitro* biotransformation of organic contaminants by the cytochrome P450 system. Possible consequences for bioaccumulation and genotoxic effects'. The purpose of this project is to:

1. Investigate the ability of hepatic microsomal preparations of different species of marine mammals and birds to metabolise halogenated organohalogen compounds in an *in vitro* bioassay.
2. Obtain information on residue patterns of the same classes of compounds in environmentally exposed marine mammals and birds, in relation to the results of the *in vitro* assays.
3. Obtain information on the genotoxicity of the compounds in a standard microbial assay (Mutatox®), both of the parent compounds and after allowing for biotransformation to occur.
4. Recommend (changes in) policy measurements for these compounds on the basis of the results obtained and literature data.

This report describes the results for chlordanes; it is the last report in the series of this BEON project. Other compound classes that have been investigated in the course of this project are the polychlorinated biphenyls (PCBs) (2), toxaphene (2, 3), polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs) (4), and the polychlorinated terphenyls (PCTs) (5).

2. Materials and methods

2.1. In vitro assays

2.1.1. Glassware

All glassware was thoroughly washed with soap, rinsed with demineralised water, heated overnight at 350°C and rinsed with hexane prior to use.

2.1.2. Organic Solvents

Hexane (Chemproha) was purified as described previously (21). Acetone, methanol and 2,2,4-trimethylpentane (TMP) were of nanograde quality (Promochem).

2.1.3. Organohalogen mixture

The (absolute) amount of the compound that is metabolised is usually quite small. Therefore the concentration of the standard chlordane mixture must be as low as possible to measure a significant decrease of a peak. On the other hand, the concentrations in the contaminant mixture must be well above the noise signal. The final detection of a significant biotransformation activity with respect to a particular compound, depends on a combination of the true difference between reference and assay samples and the standard deviations of both sets. Since the average standard deviation was about 2.5% of the absolute peak height in each set (the peak heights of the congeners range from 200-900 units), biotransformation has to cause a decrease of peak height of at least 10% to be significant.

The standard chlordane mixture used in the *in-vitro* assay, consisted of the compounds shown in **table 2.1**. This mixture of compounds was dissolved in acetone. CB 153 (2,2',4,4',5,5'-hexachlorobiphenyl) was added as an internal standard (because of its high resistance to biotransformation). CB26 (2,3',5-trichlorobiphenyl) was added as a positive control to check the ability of the microsomes to carry out biotransformation reactions (2, 4). This mixture was designated as chlordane mixture-I.

The systematic numbering for the chlorobiphenyl congeners was done according to Ballschmiter *et al.* (22).

***Table 2.1**: The contents of chlordane mixture-I.*

Compound	Concentration (μg/ml)
Trans-chlordene	*
CB 26 (2,3',5-trichlorobiphenyl)	7
Heptachlor	36
Trans-chlordane	13
Cis-chlordane	16
Trans-nonachlor	27
CB-153 (2,2',4,4',5,5'-hexachlorobiphenyl)	8 (ISTD)
Cis-nonachlor	13

** The concentration of trans-chlordene was unknown due to impurities in the standards of the other compounds, which contributed to this peak.*

The standard metabolite mixture used in the determination of the metabolites consisted of the compounds shown in **table 2.2**. This mixture of compounds was also dissolved in acetone. It was designated as chlordane mixture-II.

***Table 2.2**: The contents of the standard chlordane metabolite mixture (chlordane mixture-II).*

Compound	Concentration (μg/ml)
Oxychlordane	33
Heptachlor epoxide B	77
Heptachlor epoxide A	30
CB153 (2,2',4,4',5,5'-hexachlorobiphenyl)	5.4 (ISTD)

The chlordanes and metabolites were obtained from the Institute of Organic Industrial Chemistry (Warsaw, Poland) and Riedel-de-Haën a.g. (Seelze, Germany).

2.1.4. Origin of samples

The type of research described in this report depends largely upon the availability of animals of which the liver can be sampled as soon as possible after the moment of dying, because the

enzymes rapidly start to degrade after the moment of death. Since the availability of the studied species almost always is coincidental, it has been attempted to study the most important animal groups. When the possibility arose to analyse the biotransformation ability of unique species such as a sperm whale, it was also done. For the study of chlordanes, samples were taken from the following four species:

Harbour seal (*Phoca vitulina*).

Sex	: female.
Age	: approximately 4 years.
Location of stranding	: Camperduin.
Sampling date	: 10 October 1996.
Weight	: 49.5 kg.
Length	: 1.35 m.
Blubber layer	: < 0.5 cm thick (ventrally below sternum).
Sampled	: 4 hours after arrival at Ecomare (signs indicated that the animal had recently died).
Condition	: moulting, many inflammations on the skin.
Cause of death	: illness.
NIOZ sample code	: 96PVd.

Whitebeaked dolphin (*Lagenorhynchus albirostris*).

Sex	: female.
Age	: adult.
Location of stranding	: pole 28 the beach of Texel.
Date of sampling	: 24 January 1995.
Length	: 2.5 m.
Blubber layer	: 2.1 cm thick (ventrally below sternum).
Sampled	: 3:45 hours after demise at 'Ecomare'.
Condition	: pregnant, lost milk, missed some teeth.
Cause of death	: illness.
NIOZ sample code	: 95LAa.

Sperm whale (*Physeter macrocephalus*).

Sex	: male.
Age	: approximately 15-25 years.
Location of stranding	: Beach of Kijkduin (near The Hague).
Date of sampling	: 12 January 1995.
Weight	: 30 metric tons.
Length	: 14.40 m.
Sampled	: within 11 hours after demise by Ir. R. Kastelijn from 'Marine Mammal Centre' in Harderwijk.
NIOZ sample code	: 95PMb.

Eider duck (*Somateria mollissima*).

Age	: approximately 38 days.
Catching Area	: 'Kroonpolders' at the island of Vlieland, further raised at NIOZ.
Experimental treatment	: After an acclimatisation period of 27 days, the cytochrome P-450 system was induced by treatment with CB77 at 50 mg/kg body weight. (incubation during 10 days) This treatment had induced CYP1A significantly (23)
NIOZ sample code	: 94SM82, and 94SM5 (two animals)

The protein concentrations and the EROD activities of the used samples are shown in **table 2.3**.

***Table 2.3**: Microsomes used for this study, clean-up method, total protein concentrations and EROD activity.*

Species	NIOZ sample code	Protein conc. ($mg \cdot ml^{-1}$)	EROD activity ($pmol \cdot mg^{-1}$ protein$\cdot min^{-1}$)
Harbour seal	96PVd4	26.8	404.3
Whitebeaked dolphin	95LAa3	57.2	48.9
Sperm whale	95PMb3	11.3	41.9
Eider duck	94SM82	24.9	13
Eider duck	94SM5	18.4	14.4

Besides the animals of which microsomes were prepared for *in vitro* analyses, samples from a number of other animals were used for residue analyses only:

Minke whale (*Balaenoptera acutorostrata*).

Sex : male

Age : juvenile

Location of stranding : Zoutelande in the southwest of the Netherlands (province of Zeeland).

Sampling : The animal could be sampled within a day after its demise by Ir. R.A Kastelein of the Harderwijk Marine Mammal Park on 25th November 1994

Weight : approximately 1100 kg

Length : 4.53 m

Width of tail : 1.18 m

Condition : Animal was lean (thickness of blubber layer at the neck *dorso-laterally*: 3 cm; halfway between the dorsal fin and the tail: 2 cm) and dried-out; most probably it was an infant that had lost its mother.

NIOZ sample code : 94BAa

Mackerel (*Scomber scombrus*).

Homogenate of fish caught in the Northern Irish Sea / Eastern Atlantic Ocean (obtained from the educational centre for North Sea and Wadden Sea 'Ecomare' at Texel.

2.1.5. Preparation of liver homogenates

All procedures with liver tissue were conducted at 0-5°C to maintain the integrity of the enzymes. Liver lobes were cut to small pieces on site and immediately homogenised with an Ultra Turrax TP 18/10 after the addition of half a volume of ice-cold glycerol. The beaker containing the liver material was cooled by placing it into a larger beaker filled with crushed ice during homogenisation. The homogenate was divided over a number of 10 ml nalgene tubes, directly frozen on site in liquid nitrogen, and subsequently transported to the laboratory were it was stored in a -80°C freezer for the preparation of the microsomal fraction at a later date.

2.1.6. Preparation of the microsomes

The thawed liver homogenate was mixed with three times its volume of a 0.1 M sodium phosphate buffer (pH 7.6) with 10% glycerol, 0.15M KCl, 1mM EDTA, and 1mM dithiothreitol (DTT) and subsequently homogenized with first an Ultra-turrax TP 18/10 and finally in a Potter-Elvehjem tube. The homogenate was first centrifuged at 12,500*g for 15 min. Subsequently, the supernatant was centrifuged at 100,000*g for 1 h. The pellet containing the microsomes, was redissolved in 0.1 M sodium phosphate resuspension buffer (pH 7.6) containing 1mM EDTA, 1mM DTT and 20% glycerol and homogenized in a Potter-Elvejhem tube. The homogenate was again centrifuged at 100,000*g for 1 h to wash out excess blood and the pellet was homogenized in the resuspension buffer with a Potter-Elvejhem tube. The homogenate was divided into a number of Nalgene cryovials containing 0.5ml portions which were frozen in liquid nitrogen and stored at -80°C for total protein analysis, EROD activity measurements, or use in the *in vitro* assays.

2.1.7. Quality criteria for the microsomal preparations

Liver samples were taken from the bodies of dead animals, but only when samples could be taken within a short time span (2-6 hours) after the moment of death. This precaution was taken to reduce the danger that *post-mortem* decay processes would have adversely affected the condition of the tissues (24). As a further precaution it was established whether the microsomal preparations still contained a viable cytochrome P450 system by measurement of

the ethoxyresorufin-O-deethylase (EROD) activity. Although this is strictly speaking a measurement for the activity of the iso-enzyme cytochrome P450 1A1 only, it was used as a general quality index for the activity of the microsomes. In view of the range of EROD activities published in the literature for marine mammals (25), it was decided that the EROD activity of the microsomal preparations for use in the *in-vitro* assays, should be at least 10 pmol • mg-1 protein • min-1.

2.1.8. Total Protein Analysis

Total protein concentrations were determined with the Biorad assay based on the method developed by Bradford (26). The analysis was conducted on a Biorad model 3550 microplate reader at a wavelength of 595 nm. Biorad protein assay dye reagent was used to stain the proteins. Bovine serum albumin was used as an external standard.

2.1.9. EROD activity measurements

Ethoxyresorufin-*O*-deethylase (EROD) is one of the model reactions to establish the activity of the cytochrome P-4501A mediated MO-system (27, 28).. In the context of this paper, EROD activity was used as a general index of the viability of the microsomes used next to the behaviour of the chlorobiphenyl congeners added as positive controls in the assays.

The EROD activities were determined fluorimetrically with a microplate reading fluorimeter (Titertek fluoroskan II; Flow Laboratories) (29). Measurements were carried out at 37°C. The pH was 7.6 for marine mammals and 7.4 for eiderduck microsomes. Assays were performed in a 0.1 M NaH_2PO_4*H_2O buffer containing 0.9 µM ethoxyresorufin and 150 µM NADPH. The resorufin concentration in the external standard was calculated with the extinction coefficient 73 mM-1 • cm-1 (30).

2.1.10. In vitro bioassay

With the bioassay, the phase-I biotransformation of the compounds investigated by the microsomal fractions of the liver is monitored. The basic methodology has been described previously (31): 0.1 ml of a microsomal suspension containing 10 mg protein/ml was added to 1 ml of a 0.08 M NaH_2PO_4*H_2O buffer with 1 mM EDTA in a 25 ml Erlenmeyer flask (pH 7.6 for marine mammals, pH7.4 for sea birds). 3 µl of an acetone solution of the test compound mixture was added with a 10 µl Hamilton syringe to the erlenmeyer flasks for incubation. Substrate concentrations are mentioned above (refer to "organohalogen mixture").

Assays were carried out in a water bath at 37°C. After 3 min pre-incubation, 0.1 ml of a 11 mM NADPH solution was added every 10 min to four assay samples; four other samples that did not get NADPH served as a set of reference samples where no reaction could occur. The reaction was terminated after 90 min. by the addition of 1 ml ice-cold methanol and 1 ml of milli-Q water to the samples. In total, each assay consisted of 9 samples: 4 reference samples without NADPH, and 4 assay samples with NADPH, and two blanks without either incubation mixture or NADPH. These blanks were used to check for the presence of any compounds in the microsomes due to bioaccumulation during the lifetime of the animals. All samples were analysed simultaneously.

2.1.11. Clean-up methods

A clean-up step was included to remove the co-extracted lipids from the sample. Two methods were used. Initially, a clean-up with concentrated sulphuric acid was pursued. After the clean-up with the sulphuric acid, the samples were neutralised with bicarbonate buffer until a pH of 6 was reached. After the discovery of a few minor new peaks in the assay samples another clean-up method was sought in order to try enlarge these peaks, which were presumably due to biotransformation.

It was tested whether a clean-up by normal phase HPLC would be more suitable for this purpose. A Kontron 325 HPLC equipped with a normal phase radial compression silica column (100 mm • 8 mm, particle size 5 μm) was used for the clean-up. The chlordane components were eluted from the column with a hexane/dichloromethane gradient; flow $1ml \bullet min^{-1}$. 100 μl injections were made by a Kontron 360 autosampler and the 2.1 - 17.1 ml fraction was collected with a Gilson 206 fraction collector. After the clean-up the samples were evaporated and dissolved in 0.5 ml trimethylpentane.

The recovery of the compounds in chlordane mixture-I (see **table 2.1** for composition) was calculated by dividing the values of the $Ratio^{X}_{CB-153}$ in samples that were incubated in the *in-vitro* assay (no NADPH added) and subsequently subjected to one of the clean-up methods, by the values of the $Ratio^{X}_{CB-153}$ in mixture-I when it was directly analysed by GC-ECD.

2.1.12. Analysis of compounds

GC-ECD: The samples were analysed by gas chromatography with electron capture detection. The parameters used are given in **table 2.4**. The samples were compared to the standard chlordane mixture and the standard metabolite mixture.

Table 2.4: *Parameters used for the gas chromatographic analyses of chlordanes of the in vitro assays.*

Equipment	Hewlett Packard 5880A gas chromatograph
Detector	Electron capture detection
Column	50 m • 0.15 mm • 0.20 µm CP-Sil-19 CB column (Chrompack, NL)
Injection	1 µl splitless with a Hewlett Packard 7673A autosampler
Carrier gas & pressure	Hydrogen; pressure 350 kPa
Linear gas velocity	107 cm/s
Injector temperature	250 °C
Detector temperature	350 °C
Oven temperature programme	Initial temperature: 90 °C (isothermal phase (IP) 3')/ temperature increase at 30 °C•min^{-1} to 215°C (IP 40') / temperature increase of 5 °C•min^{-1} to 270°C (IP 5')

2.1.13. GC-NCI-MS of metabolites formed

An attempt was made to identify the peaks that emerged only in the samples to which NADPH was added in the *in-vitro* assay. To this end, all samples of the assay group were combined and the solvent was evaporated to approximately 100 µl. The same was done with all samples of the reference groups. Full scan mass-spectrometry was performed as well as selective ion monitoring (SIM). Peaks that can be attributed to metabolites of chlordane components should (i) appear only in the assay samples and (ii) show the typical chlorine clusters in full scan mass-spectra The masses for SIM analyses were selected on the expectation that biotransformation would result in the formation of an epoxide. Thus, the m/z values selected were the masses of the parent compounds +14 for chlordanes and nonachlors and +16 for heptachlor and chlordenes. Since the parent compounds possess 6-9 chlorine atoms, their masses are not fixed but show the typical chlorine distribution clusters caused by

the occurrence of ^{35}Cl and ^{37}Cl in a ratio of 3:1. Details of the GC-NCI-MS analyses are given below in paragraph 2.2.3.

2.1.14. Statistical treatment

With the analytical results, calculations were made to determine a difference in peak heights between the reference samples and the assay samples. The following calculations were made: first the ratio of the peakheight (H) of each compound X to the internal standard (CB-153) was calculated ($Ratio^{X}_{CB-153}$) according to the following formula.

$$Ratio^{X}_{CB-153} = \frac{H_{CompoundX}}{H_{CB-153}}$$

The $Ratio^{X}_{CB-153}$ values of the reference samples were then divided by the $Ratio^{X}_{CB-153}$ values of the organohalogen mixture to check the recovery. It was stated that there should be a minimal recovery of 75 % of each individual peak in the reference sample. The significance of the difference between assay and reference samples in $Ratio^{X}_{CB-153}$ of both positive controls and the peaks of the chlordane mixture was tested with analysis a student's t-tests on the log-transformed data (to obtain homogeneity of variance), using the SYSTAT 5.0.4 programme.

For a graphical display in bar diagrams, the remaining fraction of each compound in the assay was determined by dividing its $Ratio^{X}_{CB-153}$ value in a randomly chosen assay sample by the value in a randomly chosen reference sample according to the equation:

$$Rf = \frac{Ratio^{X}_{CB-153}.assay}{Ratio^{X}_{CB-153}.reference}$$

R*f* is the remaining fraction of compound X after biotransformation. Means were calculated for the four combinations of assay and reference samples.

2.2. Chlordane residues in wildlife

Chlordane components were determined in 15 wildlife samples of lung-breathing animals feeding on invertebrates or fish: whitebeaked dolphin (*Lagenorhynchus albirostris*; main food

source fish), sperm whale (*Physeter macrocephalus*; main food source squid and fish from deep water), minke whale (*Balaenoptera acutorostrata*), harbour seal (*Phoca vitulina*; main food source fish) and eider duck (*Somateria mollissima*; main food source invertebrates). A mackerel sample from the southern North Sea was also analysed to get a first impression of chlordane concentrations and patterns in the food of seals and dolphins.

2.2.1 Chemicals and equipment

For this study the following materials were used for the analyses of the wild-life samples:

- sodiumsulphate anhydrous, Merck cat. no. 6649
- pentane, Promochem, nanograde, cat. no. 6145
- hexane, Promochem, für die HPLC, cat. No. UN 1208
- 2,2,4-trimethylpentane (iso-octane), Promochem, nanograde, cat. no.6051
- diethylether, Promochem, nanograde, cat. no.3434
- acetone, Promochem, zur Rückstandanalyse, cat. no. UN 1090
- sulphuric acid 95-97%, Baker cat. no. 6057
- SiO_2 silicagel 60 63-200µm, Merck cat. no. 7754
- Chlordane standards
- CB-112 (internal standard)
- Waring blender
- Soxhlet apparatus
- Rotary evaporator, Rotavapor-R, Buchli
- HP5988A gas chromatograph/mass spectrometer

2.2.2. Extraction and clean-up:

The wildlife samples were homogenized with a Waring Blender. Blubber samples were cut into very fine pieces if homogenizing by blender succeed. Subsequently, the samples were ground with anhydrous sodium sulphate to obtain a dry powder for Soxhlet extraction. The samples were extracted at 70 °C for six hours with an acetone: hexane 1:3 (v/v) mixture. The fat extracts were evaporated with a rotary evaporator and transferred to 50 ml volumetric flasks and adjusted to a volume of 50 ml with pentane. Free fat analyses were performed gravimetrically with 5 ml fat extract and approximately 500 mg, if available, of free fat was taken for analyses. Co-extracted fat was removed by the addition of 25 ml of concentrated sulphuric acid (95-97%) to the fat extracts and letting it stand overnight. The organic layers

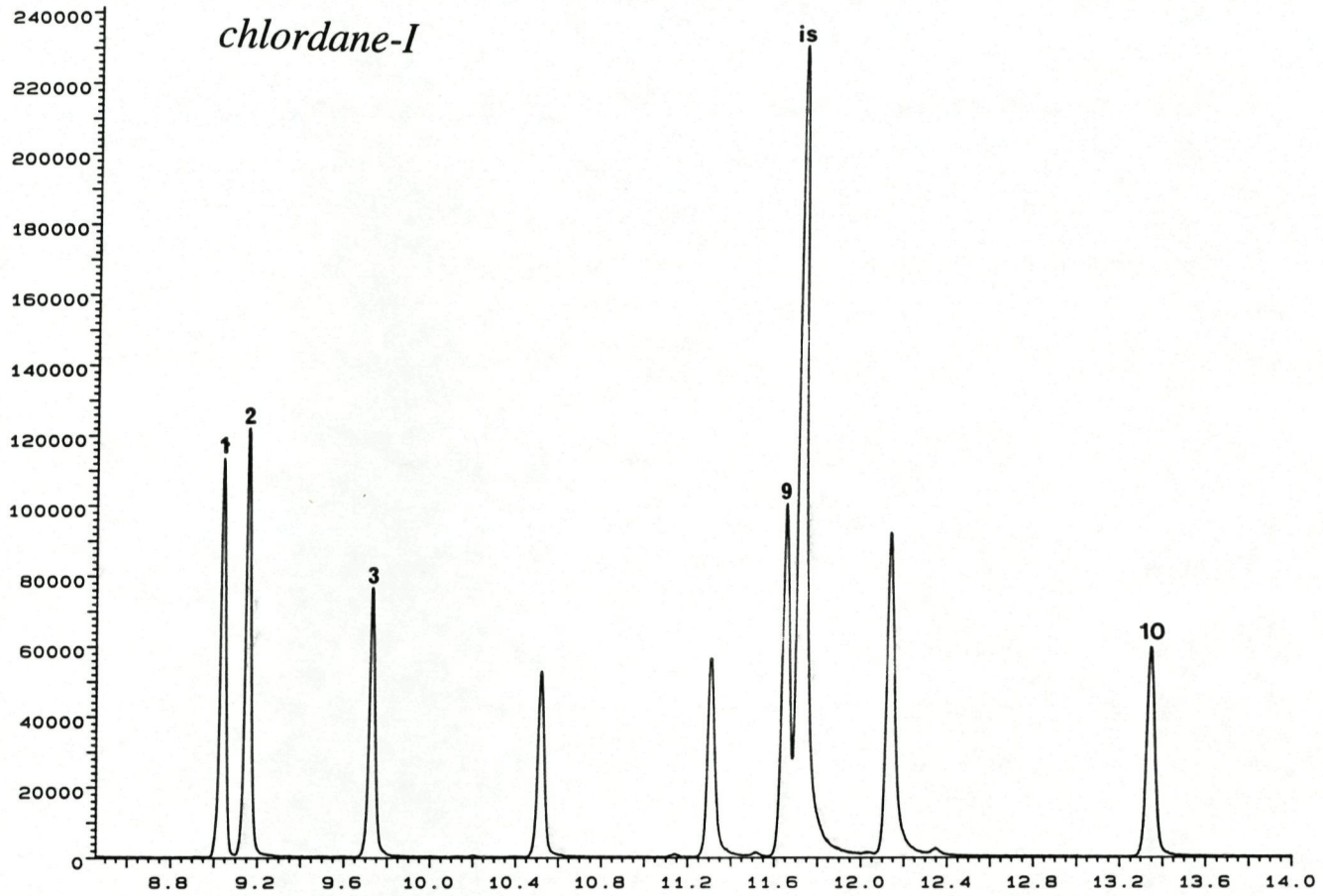
were transferred quantitatively and washed with bi-distilled water. The cleaned-up extracts were evaporated under a gentle nitrogen stream to approximately 2 ml in volumetric cylinders. These were brought on top of a column filled with 1.6 g SiO_2 deactivated with 1.5% H_2O for fractionation. The volumetric cylinders were rinsed with another 1 ml which was also transferred to the silica columns. The first fraction consisted of 11 ml TMP, the second fraction of 10 ml 20% di-ethylether in TMP. 1 ml of 0.04 µg/ml CB-112 was added as internal standard (IS) to both fractions and the final volumes was adjusted to 1 ml. The compounds present in the external standard mixture (ES) eluted into both silica fractions (**fig. II**). Heptachlor, and *cis-* / *trans*-chlordene eluted mainly in the first silica fraction. *Cis*-nonachlor eluted in about equal portions in both silica fractions, whereas *trans*-nonachlor eluted mainly in the second silica fraction. Most of the compounds in the ES however, eluted >95% in the second fraction (*Cis-* and *trans*-chlordane and the oxygenated compounds heptachlorepoxide A and B, and oxychlordane).

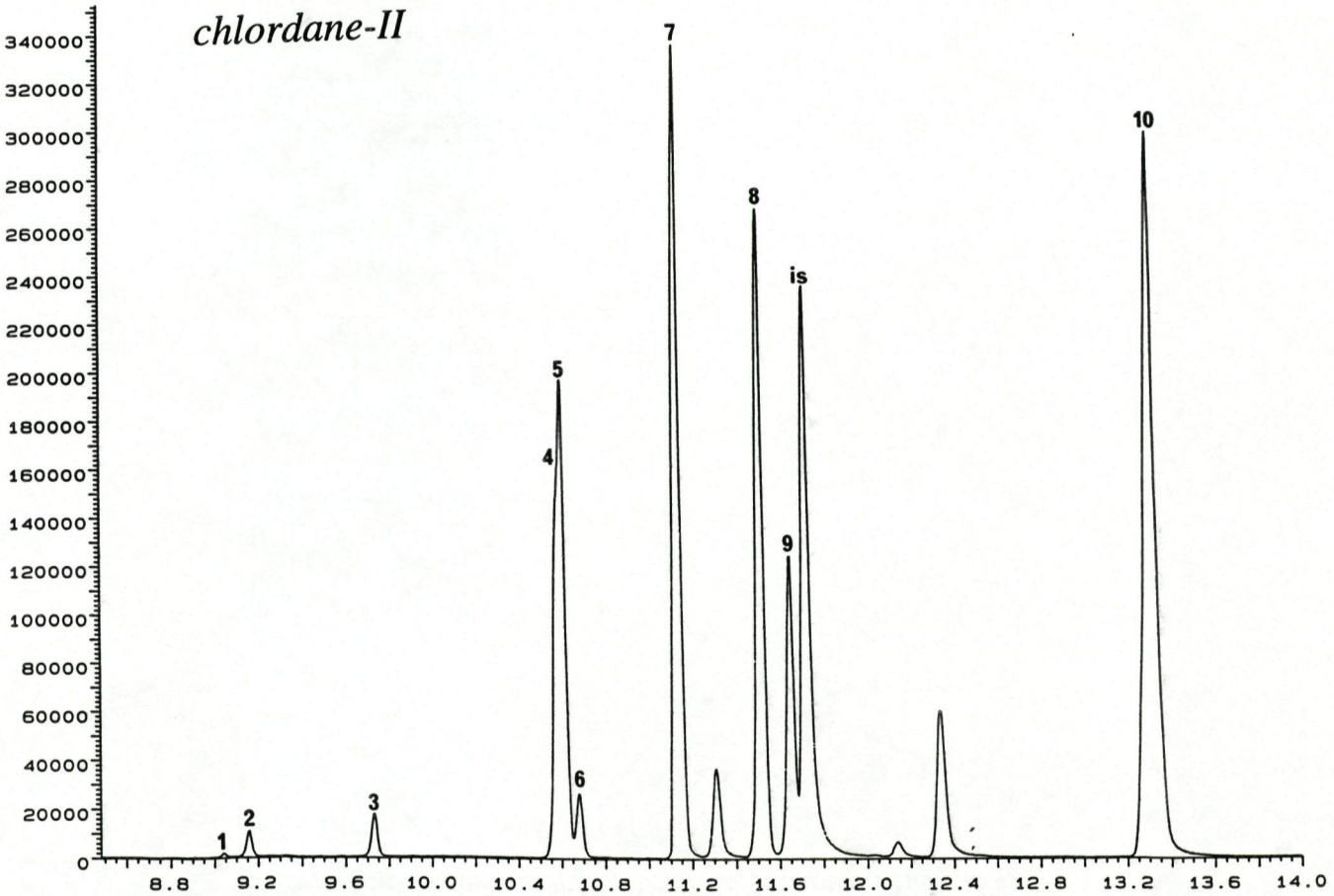
Table 2.4: *GC-MS Parameters used for the analyses of chlordane residues in wildlife tissues.*

Equipment	Hewlett Packard -5988A GC-MS
Column	20 m • 0.25 mm • 0.20 µm DB-5 column
Injection	1 µl splitless, splitter open after 3.0 minutes
Carrier gas & pressure	Helium; pressure 300 kPa
Linear gas velocity	41 cm/s; t°= 48 sec
Injector temperature	250 °C
Oven temperature programme	Initial temperature: 90 °C (isothermal phase (IP) 3')/ temperature increase at 30 °C•min^{-1} to 180°C (IP 0') / temperature increase of 5 °C•min^{-1} to 285°C (IP 60'). Post run: 15' at 290 °C
Ionisation method:	negative chemical ionisation (NCI)
Reagent gas:	methane
Source temperature:	100 °C
T analysator	120 °C in front and 75 °C at the rear
T transferline	280 °C
P analysator:	~5 • 10^{-6} (- reagent gas)
P source:	~4 • 10^{-6} (- reagent gas) / ~3 • 10^{-4} (+ reagent gas)
P methane plasm:	1 torr

Figure II: *Chromatograms of the first (chlordane-I) and second (chlordane-II) silica fractions of the external standard mixture of chlordane compounds. The chromatograms are total ion chromatograms which contain the peaks of all compounds with the masses selected in the SIM mode of GC-NCI-MS.*

Peak number	Compound	Retention time (min.)
IS	CB-112(internal standard)	11.73
1	heptachlor	9.04
2	*cis*-chlordene	9.16
3	*trans*-chlordene	9.73
4	heptachlorepoxide B	10.58
5	oxychlordane	10.61
6	heptachlorepoxide A	10.68
7	*trans*-chlordane	11.14
8	*cis*-chlordane	11.51
9	*trans*-nonachlor	11.65
10	*cis*-nonachlor	13.31


chlordane-I
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
1
2
3
9
is
10
8.8
9.2
9.6
10.0
10.4
10.8
11.2
11.6
12.0
12.4
12.8
13.2
13.6
14.0


chlordane-II
340000
320000
300000
280000
260000
240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
1
2
3
4
5
6
7
8
9
is
10
8.8
9.2
9.6
10.0
10.4
10.8
11.2
11.6
12.0
12.4
12.8
13.2
13.6
14.0

2.2.3. GC-NCI-MS analysis

Both silica fractions of the samples were analysed by gas chromatography-mass spectrometry in the negative chemical ionization mode (GC-NCI-MS). A blank and a standard for recovery were also cleaned-up and analyzed. A multi level calibration curve was constructed and the samples' contents were calculated on it. The GC-MS-parameters are given in **table 2.4**:

The m/z values used for SIM analyses are given in **table 2.5**.

Table 2.5: *m/z values used for GC-NCI-MS analyses in the SIM mode.*

Compound(s)	m/z values
CB-112 (ISTD)	324, 326
heptachlor, *cis*- and *trans*-chlordene	35, 37
heptachloro epoxides -a and -b	388, 390
cis- and *trans*-chlordane	408, 410
oxychlordane	422, 424
cis- and *trans*-nonachlor	442, 444

2.3 Genotoxicity

A positive genotoxic response of a compound in the Mutatox® assay can be caused by five different modes of action (32): base substitution, frame-shift, DNA-damage, DNA synthesis inhibition, and DNA-intercalation. Only the first two processes result in genetically stable revertants. This bio-assay uses a dim variant of the normally photoluminescent bacterium Vibrio fisheri. The presence of genotoxic compounds may result in mutations and consequently in a restoration of photoluminescence (direct genotoxicity). A rat liver S9 homogenate is available from the supplier to test for the presence of compounds that need biotransformation to express genotoxicity (indirect or induced genotoxicity).

2.3.1. Procedure:

In the present study, the genotoxicity of both chlordane mixtures described in **tables 2.1** and **2.2** was evaluated. According to the protocol of the Mutatox® test (33), all compounds or mixtures should be tested over a range of concentrations. Stock solutions of contaminants or

mixtures thereof were made in acetone. The highest concentration tested (initial concentration) was made by adding 10 µl of a stock solution to 500 µl of growth medium. .

Both chlordane mixtures were tested at an initial concentration which were diluted by a factor of five compared to the values given in **table 2.1** for the mixture of parent compounds (mix I) and **table 2.2** for the mixture of the oxygenated metabolites (mix II). Of the initial concentration, a series of nine serial 1:1 dilutions with growth medium was made. Thus, the minimum concentration tested was always $1/2^9=1/512$ of the initial concentration. Cuvettes with S9 were pre-incubated for 45 minutes at 35.0 ± 0.1 °C to activate the biotransformation enzymes. All cuvettes, both with and without S9 were incubated at 27.0 ± 0.1 °C for 22 hours, recording the light levels at intervals of one hour. Blank controls (acetone + media) were run to determine spontaneous mutations (i.e. not induced by the test compound). Positive controls with phenol and benzo[a]pyrene dissolved in acetone were run according to the protocol. 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 (34).

2.3.2. Quantification

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 (LOEC) is determined by linear interpolation between the two data points that gave responses just below and above the 4 times the blank value.

3 Results

3.1. Sulphuric acid versus normal phase HPLC clean-up

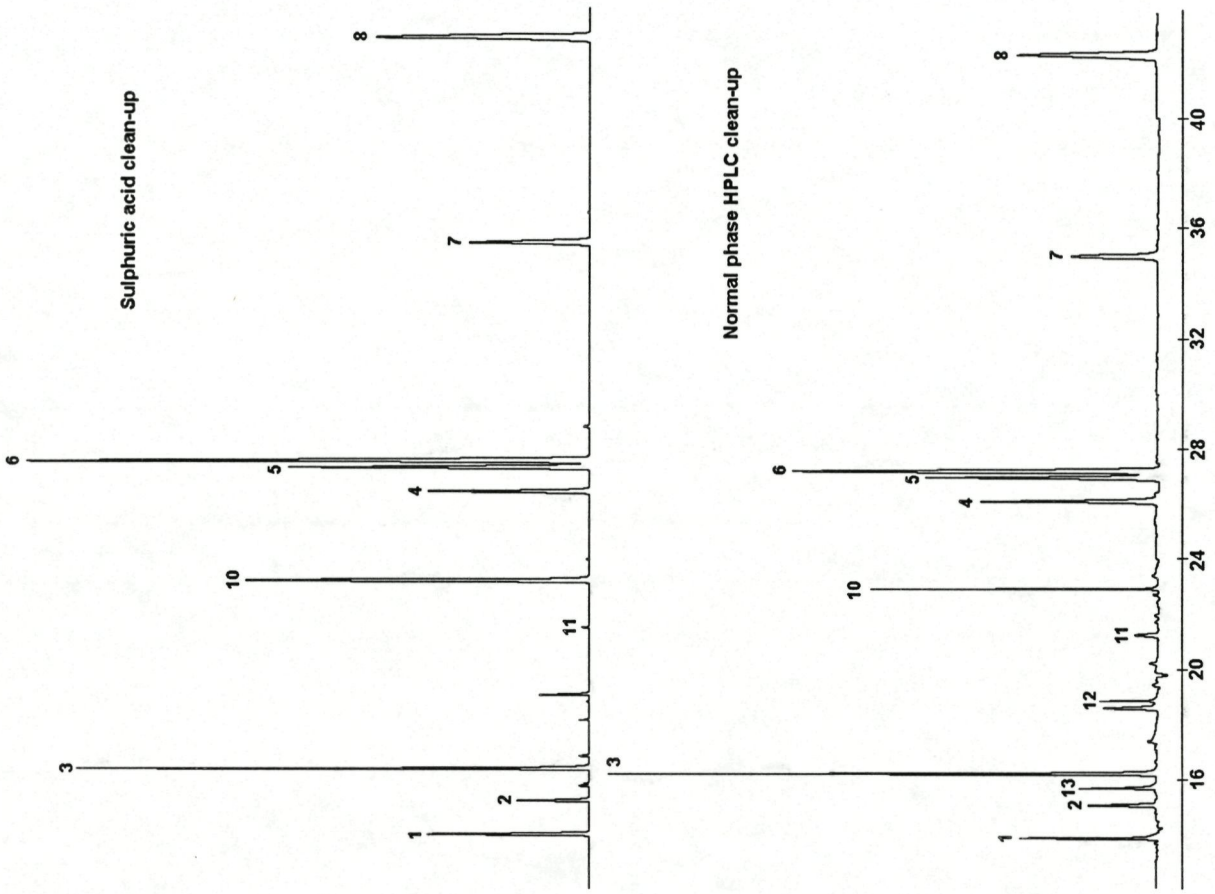
From **table 3.1**, it can be concluded that the use of a normal phase HPLC clean-up had a favourable effect on the recovery of a number of compounds after extraction and clean-up. Moreover, the standard deviation of several compounds improved considerably.

Table 3.1: *Comparison of the recovery of the compounds in the solution added to the in vitro assays (see table 2.1 for composition) after clean-up with sulphuric acid or normal phase HPLC.*

	Recovery with H_2SO_4 clean-up		Recovery with HPLC clean-up	
Compound	Average	Std. dev.	Average	Std. Dev.
Trans-chlordene	0.629	0.080	0.881	0.009
CB-26	0.541	0.014	0.831	0.015
Heptachlor	0.627	0.039	0.888	0.002
Trans-chlordane	0.537	0.012	0.824	0.029
Cis-chlordane	0.802	0.025	0.963	0.006
Trans-nonachlor	0.990	0.026	1.048	0.008
Cis-nonachlor	1.010	0.044	1.069	0.062

Furthermore, it can be concluded from **figure III** that the peak height of the unknown metabolite increased significantly with the change of clean-up to normal phase HPLC. In the case of the whitebeaked dolphin microsomes of the same batch were used during the assay with the sulphuric acid clean-up and the assay with the HPLC clean-up. Comparable results were obtained, except for the peak height of the unknown metabolite, which increased significantly after HPLC clean-up. In the case of the common eider duck, different samples were used (with different EROD activities).

Figure III: GC-ECD chromatograms of *in vitro* assays with a sulphuric acid clean-up (hp2156ds), and with a normal phase HPLC clean-up (hp2241ds).



Parent compounds:

1. *trans*-chlordene
2. CB-26 (positive control)
3. heptachlor
4. *trans*-chlordane
5. *cis*-chlordane
6. *trans*-nonachlor
7. CB-153 (internal reference)
8. *cis*-nonachlor

Metabolites:

9. oxychlordane
10. heptachlor epoxide B
11. heptachlor epoxide A
12. unknown metabolite

13. Extra peak also present in reference samples after HPLC clean-up

3.2. I*n vitro* biotransformation

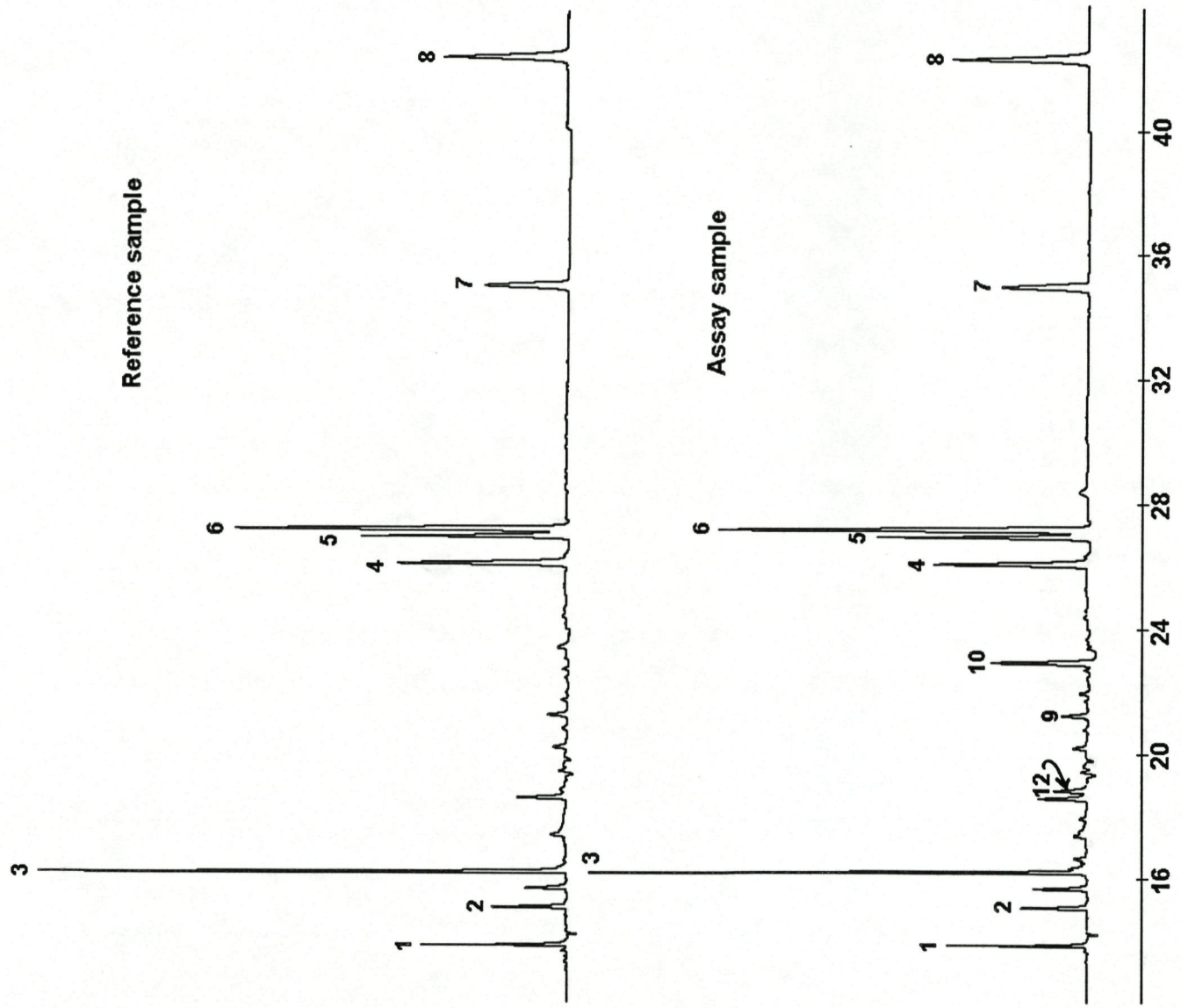
3.2.1 Decrease of the parent compounds

Chlorobiphenyl -26, that was added to the microsomal preparations as a positive control for metabolic activity, was metabolised to a significant extent in all animals, proving the viability of the microsomes. An example of a chromatogram of a reference and an assay sample of the assays carried out with eider duck microsomes, is shown in **figure IV**.

The results of the *in vitro* assays are summarised in **figure V**. The harbour seal metabolised all compounds except *trans*-chlordene to a significant extent ($P<0.05$), which is indicated by the darkened bars. *Trans*-chlordane was metabolised at the highest rate; faster than the *cis*-isomer and heptachlor. The harbour seal microsomes were even able to significantly metabolise minor fractions of *cis*- and *trans*- nonachlor. Microsomes of the eider duck and whitebeaked dolphin metabolised *trans*-chlordene, *trans*-chlordane and heptachlor. *Cis*-chlordane and both nonachlors were not metabolised to a significant extent, although a probability value of 0.067 for *cis*-chlordane in the assay with microsomes of the whitebeaked dolphin indicates that this compound might be metabolisable by this species. In an earlier assay, where sulphuric acid was used as a clean-up, the common eider duck was also capable of significantly metabolising *cis*-chlordane. The fact that the present sample could not metabolise this congener significantly, is probably due a lower EROD-activity rather than a real inability to metabolise this compound. Therefore, it can be assumed that the eider duck is able to metabolise *cis*-chlordane too.

Trans-chlordene was the only chlordane component that was metabolized to a significant extent by sperm whale microsomes. The results have been summarized in **table 3.2**. It can be concluded that the metabolising ability of chlordanes decreased in the order: harbour seal (*Phoca vitulina*) > common eider duck (*Somateria mollissima*) ≈ whitebeaked dolphin (*Lagenorhynchus albirostris*) > sperm whale (*Physeter macrocephalus*). The common eider duck and the harbour seal metabolised *trans*-chlordane faster than the *cis*-isomer. For the whitebeaked dolphin this difference was somewhat less apparent, but still present. If this would be the general situation, it would make the *cis*-chlordane more bioaccumulative.

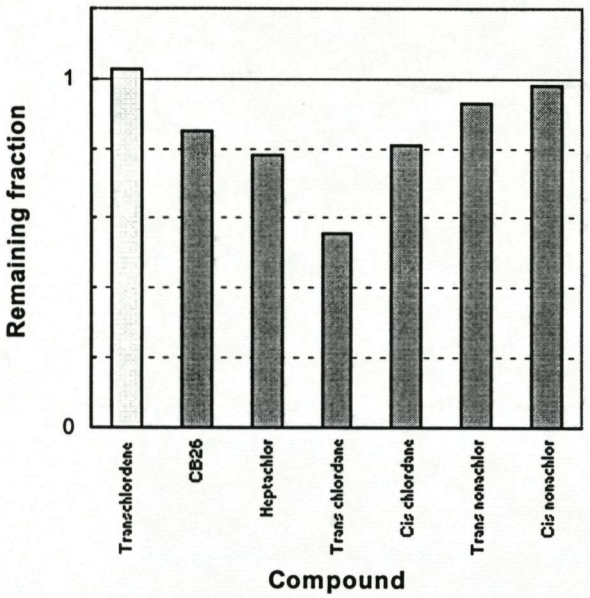
Figure IV: ECD chromatograms of a reference and an assay of an *in-vitro* assay with the common eider duck. Reference sample: no NADPH added; Assay sample: + NADPH.



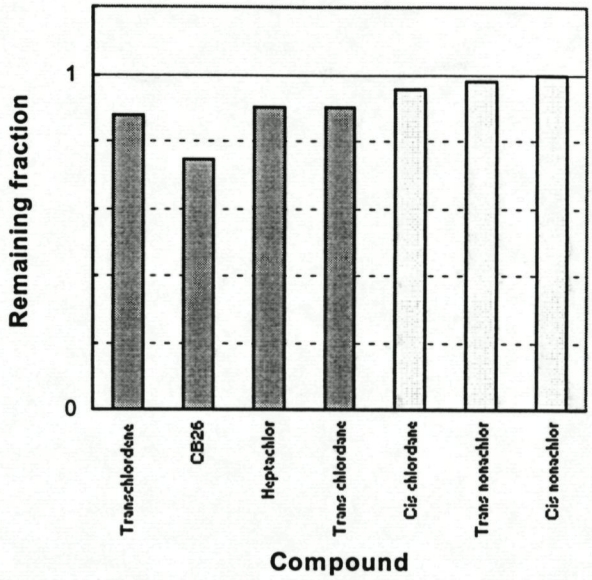
1. Transchlordene.
2. CB26.
3. Heptachlor.
4. Trans chlordane.
5. Cis chlordane.
6. Trans nonachlor.
7. CB153
8. Cis nonachlor
9. Oxychlordane
10. Heptachlor epoxide B
11. Heptachlor epoxide A.
12. Unknown metabolite.

Figure V: Charts of the remaining fraction of the biotransformation of the chlordanes by different marine mammals and an eider duck (HPLC clean-up). Compounds which were significantly lowered ($P < 0.05$) in the assay after the addition of NADPH are indicated by a dark grey colour.

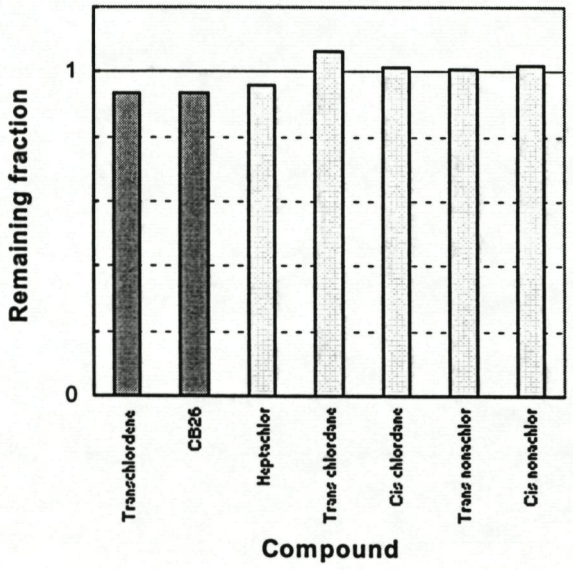
***Phoca vitulina* (96PVd4)**



***Lagenorhynchus albirostris* (95LAa3)**



***Physeter macrocephalus* (95PMb3)**



***Somateria mollissima* (94SM5)**

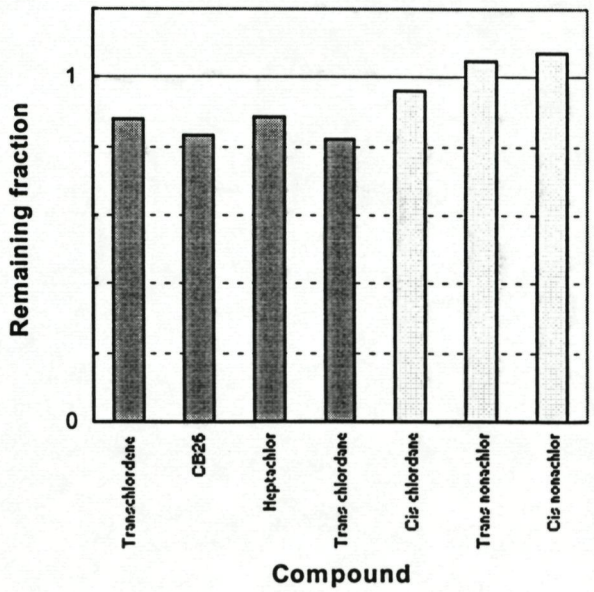


Table 3.2: *Chlordane metabolism by the different microsomal preparations.*
+: metabolisable; -: persistent.

Compound	Sperm whale	Whitebeaked dolphin	Harbour seal	Eider duck
Trans-chlordene	+	+	-	+
Heptachlor	-	+	+	+
Trans-chlordane	-	+	+	+
Cis-chlordane	-	±	+	±
Trans-nonachlor	-	-	+	-
Cis-nonachlor	-	-	+	-

3.2.2. Metabolites formed

When the parent chlordanes are metabolised, metabolites must be formed. T*rans*-chlordene can be metabolised by the MFO system to *trans*-chlordene epoxide (35). Heptachlor is metabolised in mice to heptachlor epoxide, *cis*- and *trans*-chlordane to oxychlordane and nonachlors are slowly dechlorinated to chlordane and subsequently metabolised to oxychlordane (11).

3.2.2.1 GC-ECD

Three new peaks were formed in the assay samples, that were not present in the reference samples (**Fig. IV**). Their $Ratio^{X}_{CB-153}$ values and relative proportions are shown in **table 3.3**.

Table 3.3: *The* $Ratio^{X}_{CB-153}$ *and relative proportions of the metabolites (given between brackets), formed in the* in-vitro *assays with microsomes of the different species.*

	$Ratio^{X}_{CB-153}$ (relative proportion as %)			
Species	Sperm whale	Whitebeaked dolphin	Harbour seal	Eider duck
NIOZ sample code	95PMb3	95LAa3	96PVd4	94SM5
Oxychlordane	-	-	0.04 (3)	0.01 (1)
Heptachlor epoxide B	-	0.22 (64)	0.96 (70)	1.38 (76)
Unknown metabolite	0.11 (100)	0.13 (36)	0.37 (27)	0.44(23)

The retention times of two of these peaks agreed with those of the metabolites oxychlordane and heptachlorepoxide B in the external standard mixture of the metabolites (mix. II, **table 2.2**). The retention time of the third peak did not match with that of any compound in the ESTD mixture. The metabolite heptachlor epoxide A was never formed in quantities above its detection limit in the *in vitro* assays.

3.2.2.2. GC-NCI-MS

All animals were able to produce the unknown metabolite. An effort was made with GC-NCI-MS to determine structural properties of this compound. However, only the identity of heptachlor epoxide B could be confirmed by full scan GC-NCI-MS of the combined assay samples. For the other potential metabolites, full scan GC-NCI-MS was unsuccessful because the amounts of metabolites formed were below the detection limit. Therefore, masses for selective ion monitoring had to be selected. When it is assumed that all chlorine is present as ^{35}Cl, the masses of the molecular ions are 336 for both chlordenes isomers, 370 for heptachlor, 406 for both chlordanes and 440 for the nonachlors. Due to the existence of ^{35}Cl and ^{37}Cl isotopes in a ratio of 3:1, the typical chlorine clusters show up in the mass-spectra of chlorinated compounds. The ratios of the different masses within a cluster of a particular ion are indicative for its overall chlorination level. The masses of the different ions of the anticipated metabolites were assumed to be 14 units (in case of the formation of an epoxide) or 16 units (in case of the replacement of an H atom by a hydroxy group) higher than those of the parent compounds. The masses selected for selective ion monitoring are summarised in **table 3.4.**

Table 3.4: *The masses which were measured by SIM to determine whether mono-oxidised metabolites where formed during the in-vitro assay.*

	Mass of parent compound		Anticipated mass of metabolite	
Chlordene (Cl_6)	336	338	352	354
Heptachlor (Cl_7)	372	374	388	390
Chlordane (Cl_8)	408	410	422	424
Nonachlor (Cl_9)	442	444	456	458

The reconstructed TIC-chromatogram of all masses of possible oxygenated metabolites given in **table 3.4**, is shown in **figure VI**. Considering the relative retention times in GC-NCI-MS compared to GC-ECD, the retention time of the unknown metabolite should be around 24 minutes. The unknown peak could be an oxygenated-metabolite of heptachlor or a chlordane. In order to be sure, the anticipated $[M]^-$ and $[M\text{-}Cl+H]^-$ / $[M\text{-}HCl]^-$ clusters of this compound were measured again. The masses selected are given in **table 3.5**, and the resulting spectra are shown in **figure VII**.

Table 3.5: *Masses of ions measured in the SIM mode for the identification of the unknown metabolite in the* in vitro *assays.*

O-metabolite of	$[M\text{-}HCl+H]^-$ / $[M\text{-}HCl]^-$					$[M]^-$				
Heptachlor	350	352	354	356	358	**386**	**388**	**390**	392	394
Chlordane	384	**386**	**388**	**390**	392	420	422	424	426	428

However, the resulting spectra were also inconclusive, since the dominant masses 386, 388, and 390 can be attributed to the $[M]^-$ ions of oxygenated metabolites of heptachlor as well as to $[M\text{-}(H)Cl]^-$ ions of the chlordanes. Thus, future *in vitro* assays with single compounds are necessary to establish the origin of this metabolite.

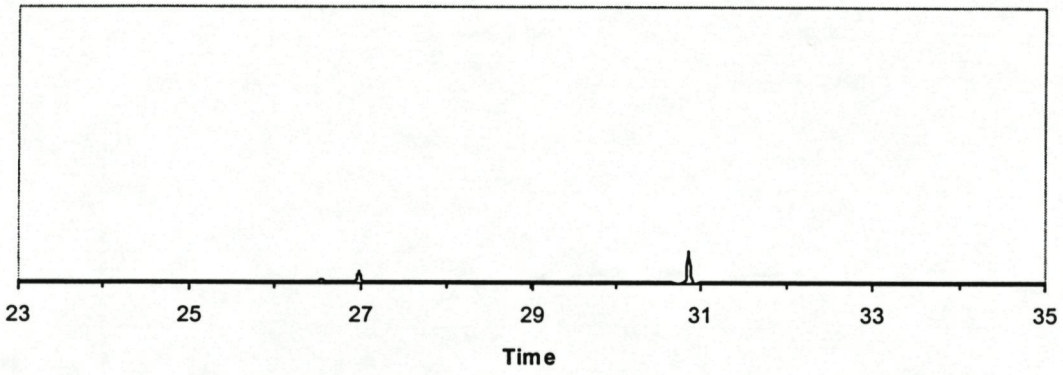
3.3. Wildlife residues

Table 3.6 gives the concentrations of the individual chlordane components in $\mu g \bullet kg^{-1}$ wet weight. The concentrations given are based on the values in both silica fractions. The two analyses of the mackerel sample showed a large difference for *cis*-chlordenepresumably due to analytical difficulties. Illustrations of the reconstructed TIC chromatogrammes of all masses selected in the SIM mode of GC-NCI-MS are given in **figure VIII.** The plots only show the results of the second silica fractions, which contained the majority of the compounds. Exceptions are heptachlor, and *cis-* / *trans*-chlordene which elute mainly in the first silica fractions, and *cis*-nonachlor which eluted in about equal portions in both silica fractions. A minor fraction of *trans*-nonachlor also eluted in the first silica fraction.

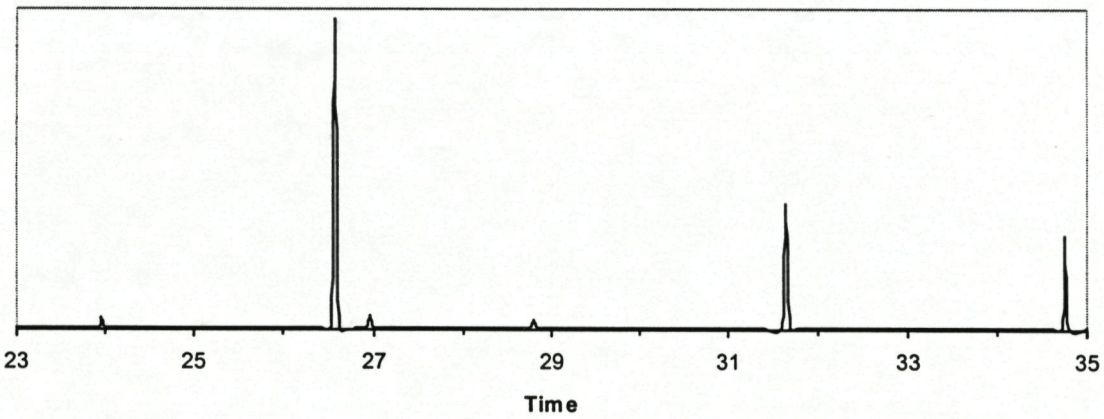
To compare the levels between the different animals, or between liver and blubber of the same animal, the concentrations of all individual components were added and normalised to lipid content. **Table 3.7** shows, that liver concentrations on a lipid basis were about one third to

Figure VI: The TIC chromatograms of the possible O-metabolites of the chlordanes.

TIC chromatogram of the possible O-metabolites of chlordene



TIC chromatogram of the possible O-metabolites of heptachlor.



TIC chromatograms of the possible O-metabolites of chlordane.

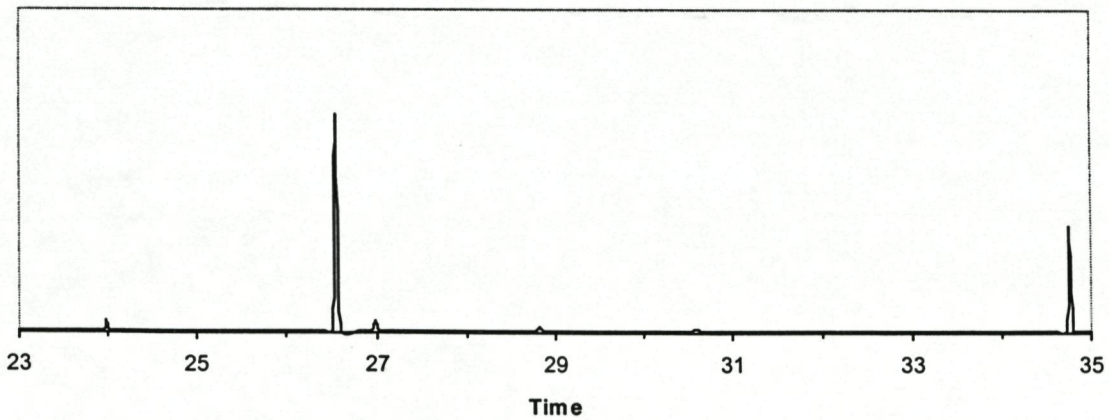
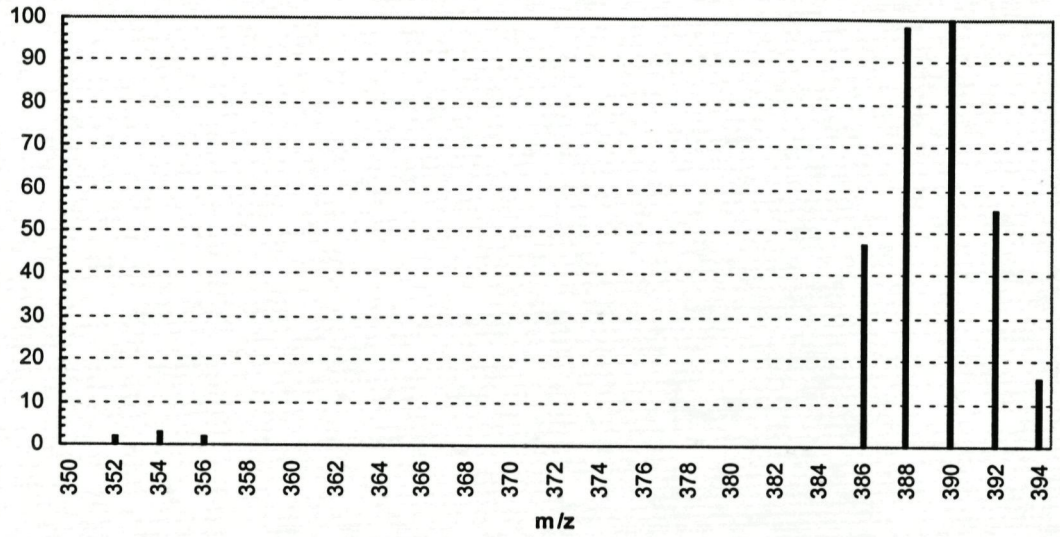


Figure VII: The SIM spectra of the possible identification of the unknown metabolite in the *in-vitro* assay.

SIM spectrum of possible heptachlor metabolite (23.96 min).



SIM spectrum of possible chlordane metabolite (23.96 min).

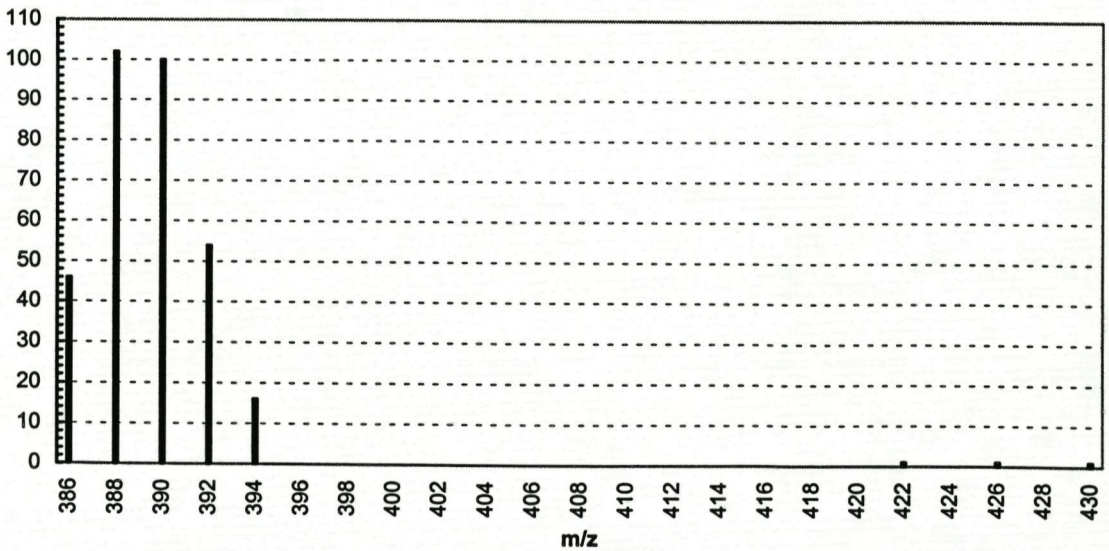


Table 3.6: *Concentrations of chlordane components in marine wildlife (in $\mu g \bullet kg^{-1}$ wet weight).*

Species	Tissue	NIOZ-code	RIVO LIMS#	*cis*-chlordene	*trans*-chlordene	*cis*-chlordane	*trans*-chloordane
Cetaceans							
Physeter macrocephalus	blubber	95PC1	15920	4.6	<0.1	210	87
Physeter macrocephalus	blubber	95PC3	15870	11	5.4	250	78
Physeter macrocephalus	blubber	95PC2	15871	15	11	130	60
Physeter macrocephalus	liver	95PC2	15872	16	0.22	7.8	3.7
Lagenorhynchus albirostris	blubber	95 LA1	15926	1.7	7.1	130	16
Lagenorhynchus albirostris	liver	95 LA1	15927	3.8	<0.03	0.55	0.05
Mysticete							
Balaenoptera acutorostrata	blubber	94BA1	15921	6.2	2.4	75	1
Pinnipeds							
Phoca vitulina	blubber	92PVa	15922	11	14	1.5	0.43
Phoca vitulina	liver	94PVa	15923	3.8	<0.03	0.05	0.02
Phoca vitulina	blubber	96PVa	15928	3	4.6	3.8	0.67
Phoca vitulina	liver	96PVa	15929	2.7	<0.03	0.14	0.06
Phoca vitulina	blubber	96PVc	19488	2.6	11	2	0.22
Phoca vitulina	liver	96PVc	19489	1.9	0.46	0.03	0.01
Pisces							
Scomber scombrus	muscle	-	19490-1	2.8	<0.04	0.69	0.12
Scomber scombrus	muscle	-	19490-2	17	<0.03	0.65	0.42
Aves							
Somateria mollissima	liver	97SMa	20331	0.54	0.24	0.01	0.004
Somateria mollissima	liver	96SMa	20332	0.71	0.66	0.02	0.005
Somateria mollissima	liver	97SMb	20333	0.38	0.13	0.04	0.02
Somateria mollissima	liver	97SMc	20334	0.3	0.13	0.02	0.01
Somateria mollissima	liver	97SMd	20335	0.79	<0.01	0.03	0.01

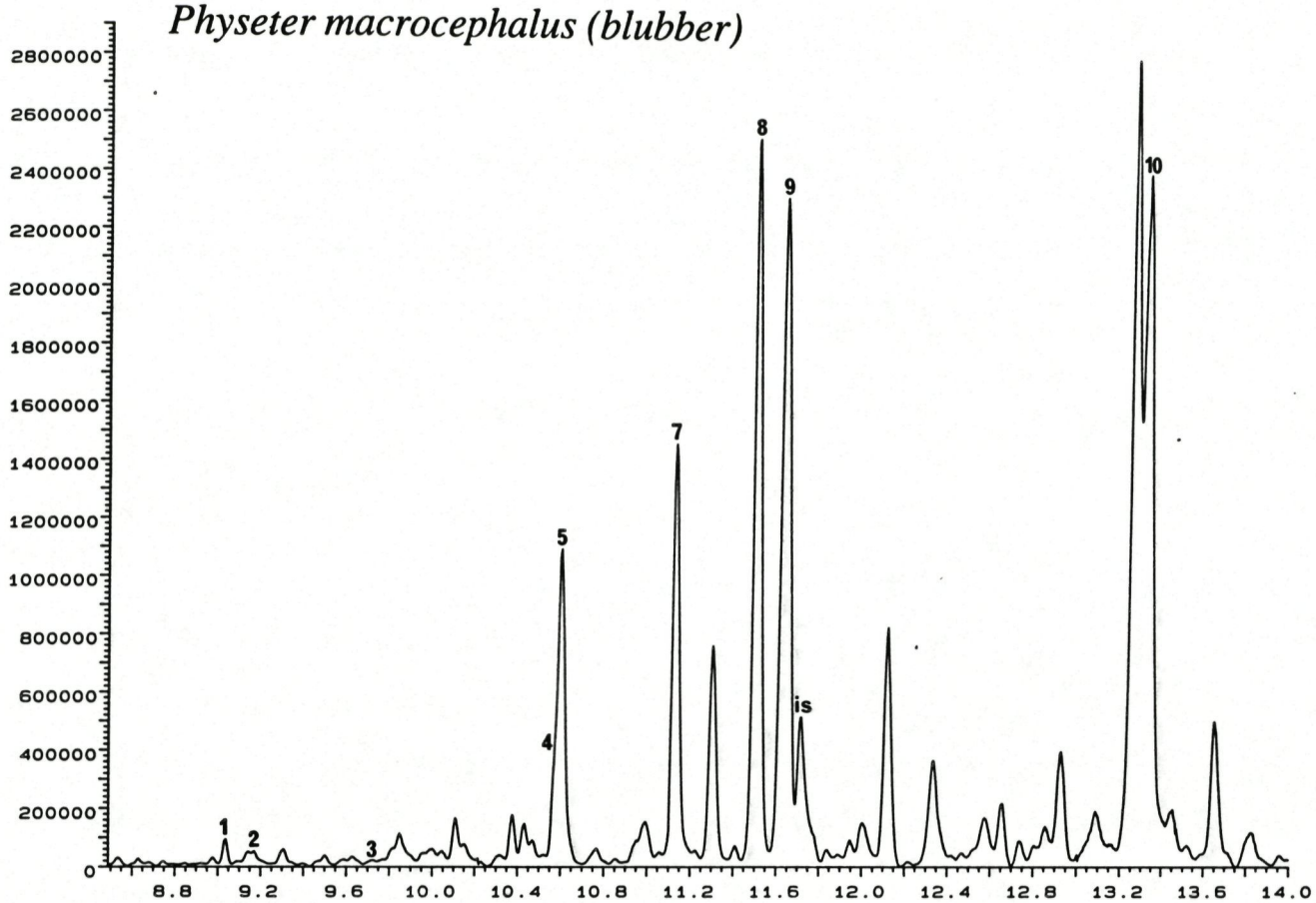
***Table 3.6** (continued)*

Species	Tissue	NIOZ-code	*cis*-nonachlor	*trans*-nonachlor	heptachlor	oxychlordane	hepox-a	hepox-b
Cetaceans								
Physeter macrocephalus	blubber	95PC1	130	310	3.2	94	<0.1	19
Physeter macrocephalus	blubber	95PC3	95	390	3.9	81	<0.1	17
Physeter macrocephalus	blubber	95PC2	77	310	7.7	66	<0.1	14
Physeter macrocephalus	liver	95PC2	5.1	17	0.31	3.4	<0.05	1.3
Lagenorhynchus albirostris	blubber	95 LA1	180	600	3.2	110	<0.85	63
Lagenorhynchus albirostris	liver	95 LA1	0.7	2	0.19	0.55	<0.02	0.94
Mysticete								
Balaenoptera acutorostrata	blubber	94BA1	110	370	5.4	64	<0.05	55
Pinnipeds								
Phoca vitulina	blubber	92PVa	9.1	510	6.9	610	<0.3	110
Phoca vitulina	liver	94PVa	0.05	0.44	0.13	0.61	<0.03	1.6
Phoca vitulina	blubber	96PVa	15	130	0.86	150	<0.3	45
Phoca vitulina	liver	96PVa	0.24	1.2	0.69	3.1	<0.03	3.6
Phoca vitulina	blubber	96PVc	4.8	190	0.92	310	<0.2	60
Phoca vitulina	liver	96PVc	<0.01	2.7	0.27	12	<0.02	4.2
Pisces								
Scomber scombrus	muscle	-	0.28	0.77	0.07	0.32	<0.04	0.73
Scomber scombrus	muscle	-	0.28	0.82	0.04	0.3	<0.03	0.75
Aves								
Somateria mollissima	liver	97SMa	0.36	1.3	0.47	9.7	<0.01	12
Somateria mollissima	liver	96SMa	0.31	1.7	0.15	31	<0.01	45
Somateria mollissima	liver	97SMb	0.54	3.4	0.06	7.8	<0.01	16
Somateria mollissima	liver	97SMc	0.12	0.61	0.04	6.7	<0.01	9.3
Somateria mollissima	liver	97SMd	0.62	2.4	0.39	16	<0.01	16

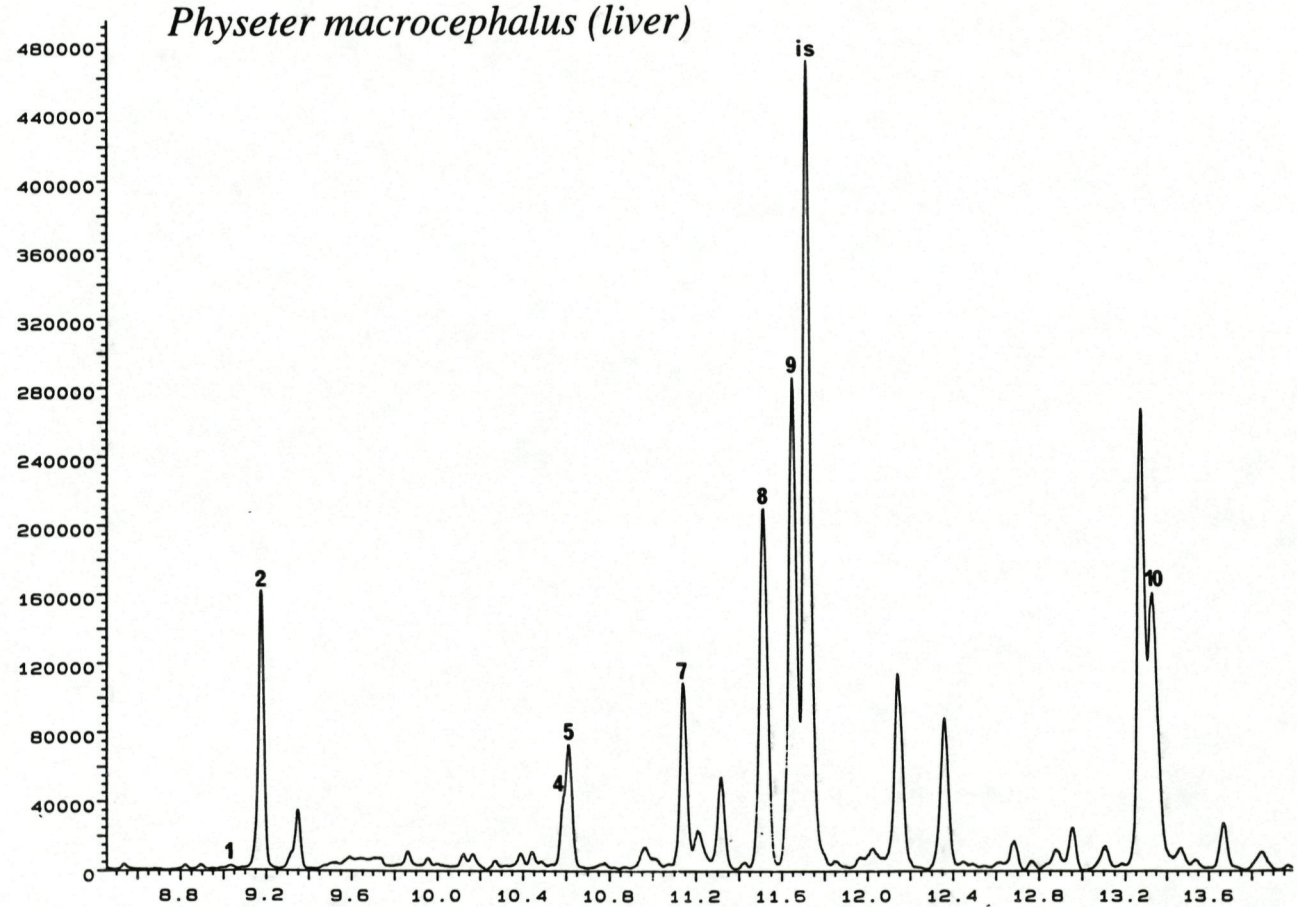
Figure VIII: *Chlordane residues in the second silica fractions of liver, blubber and adipose tissue extracts of marine wildlife. The chromatograms are total ion chromatograms which contain the peaks of all compounds with the masses selected in the SIM mode of GC-NCI-MS.*

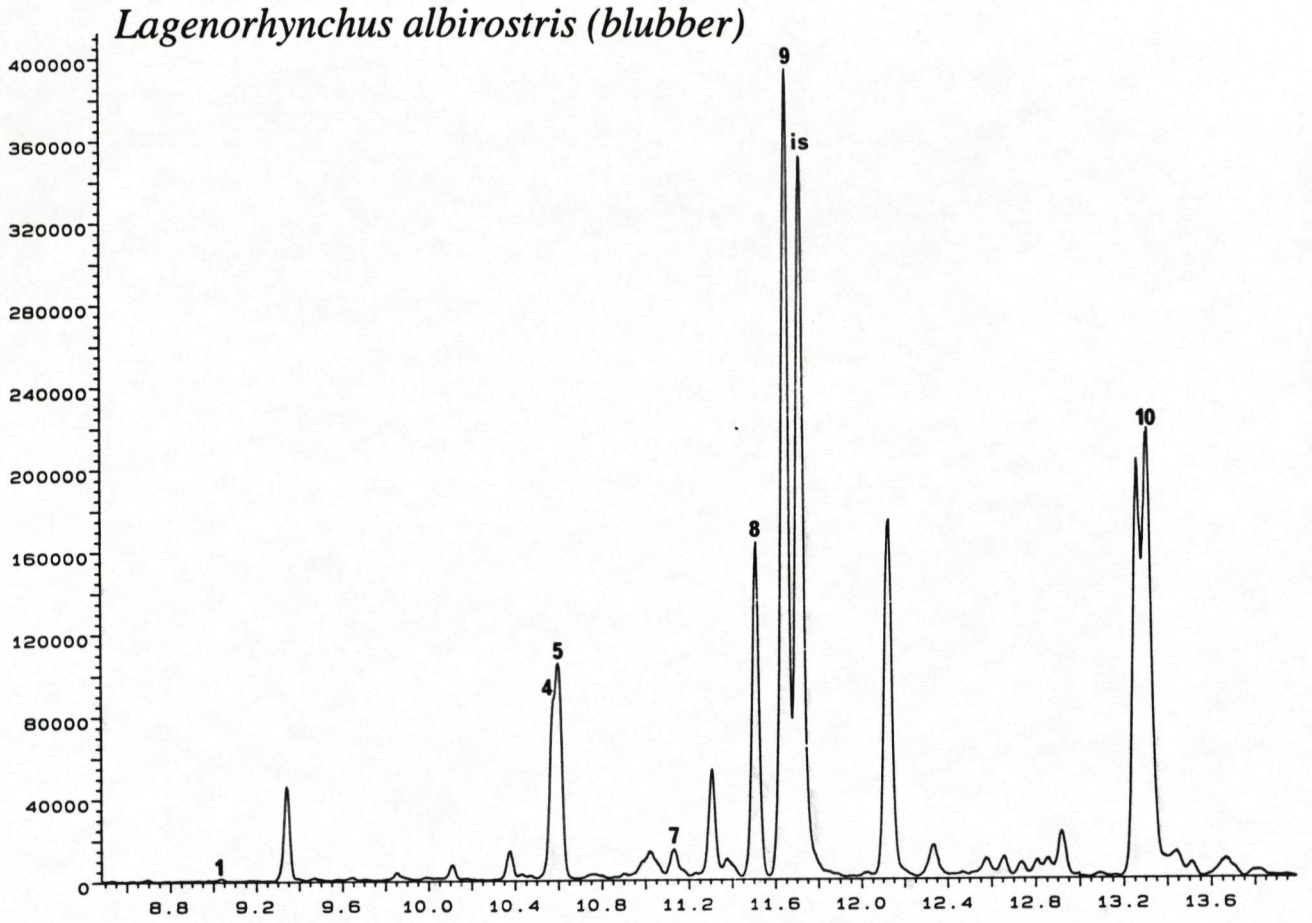
Peak number	Compound	Retention time (min.)
IS	CB-112(internal standard)	11.73
1	heptachlor	9.04
2	*cis*-chlordene	9.16
3	*trans*-chlordene	9.73
4	heptachlorepoxide B	10.58
5	oxychlordane	10.61
6	heptachlorepoxide A	10.68
7	*trans*-chlordane	11.14
8	*cis*-chlordane	11.51
9	*trans*-nonachlor	11.65
10	*cis*-nonachlor	13.31

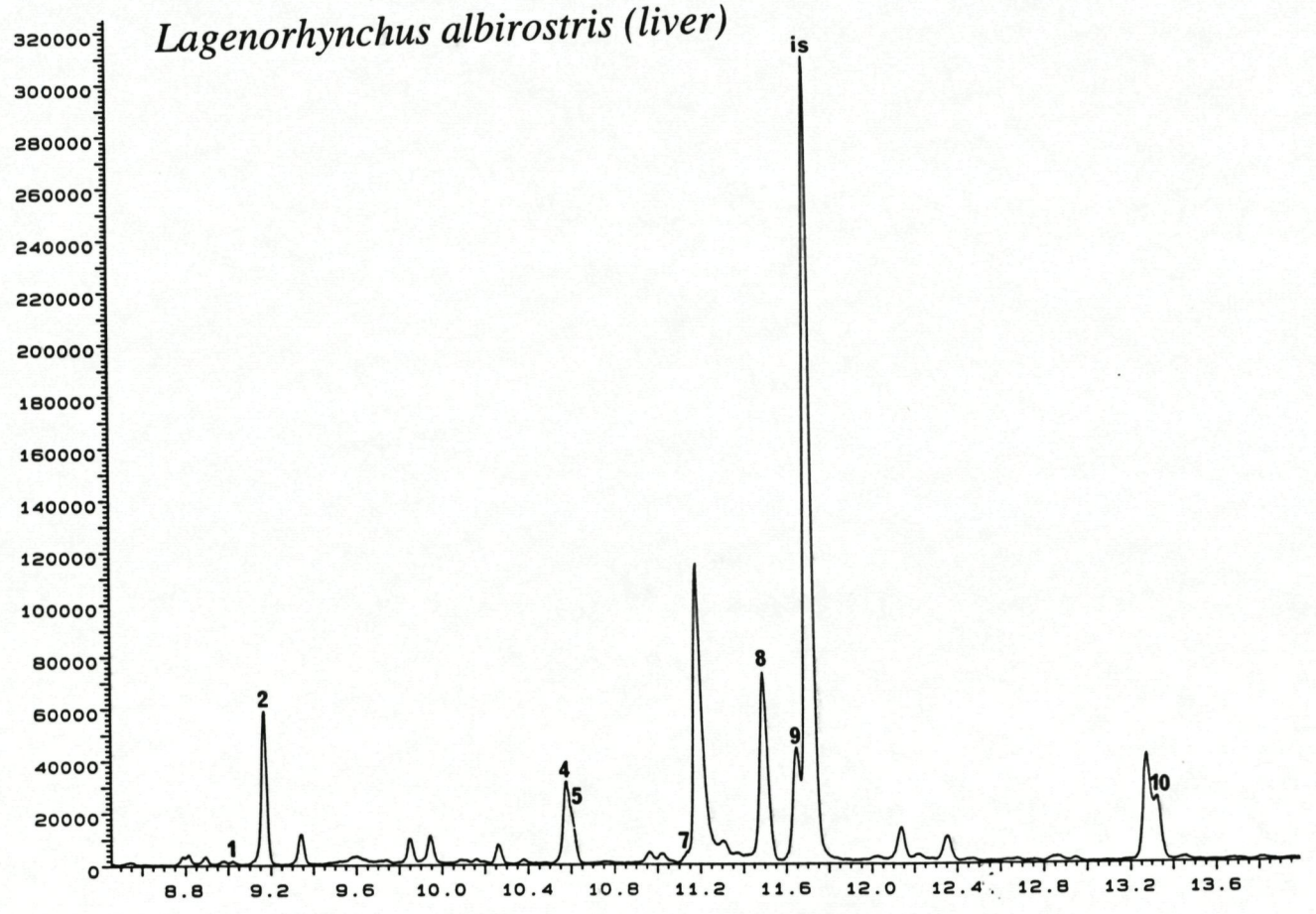
Physeter macrocephalus (blubber)



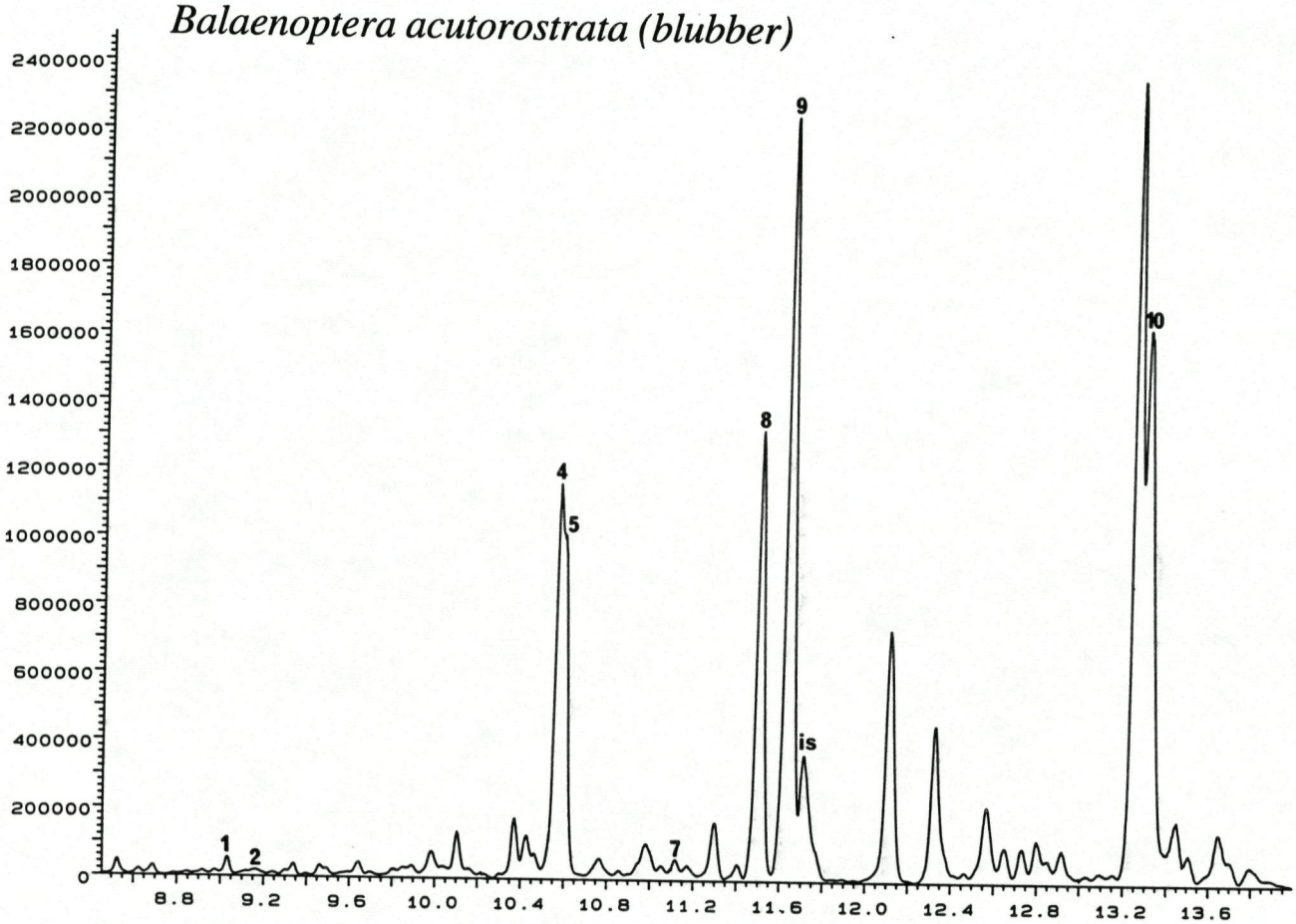
Physeter macrocephalus (liver)

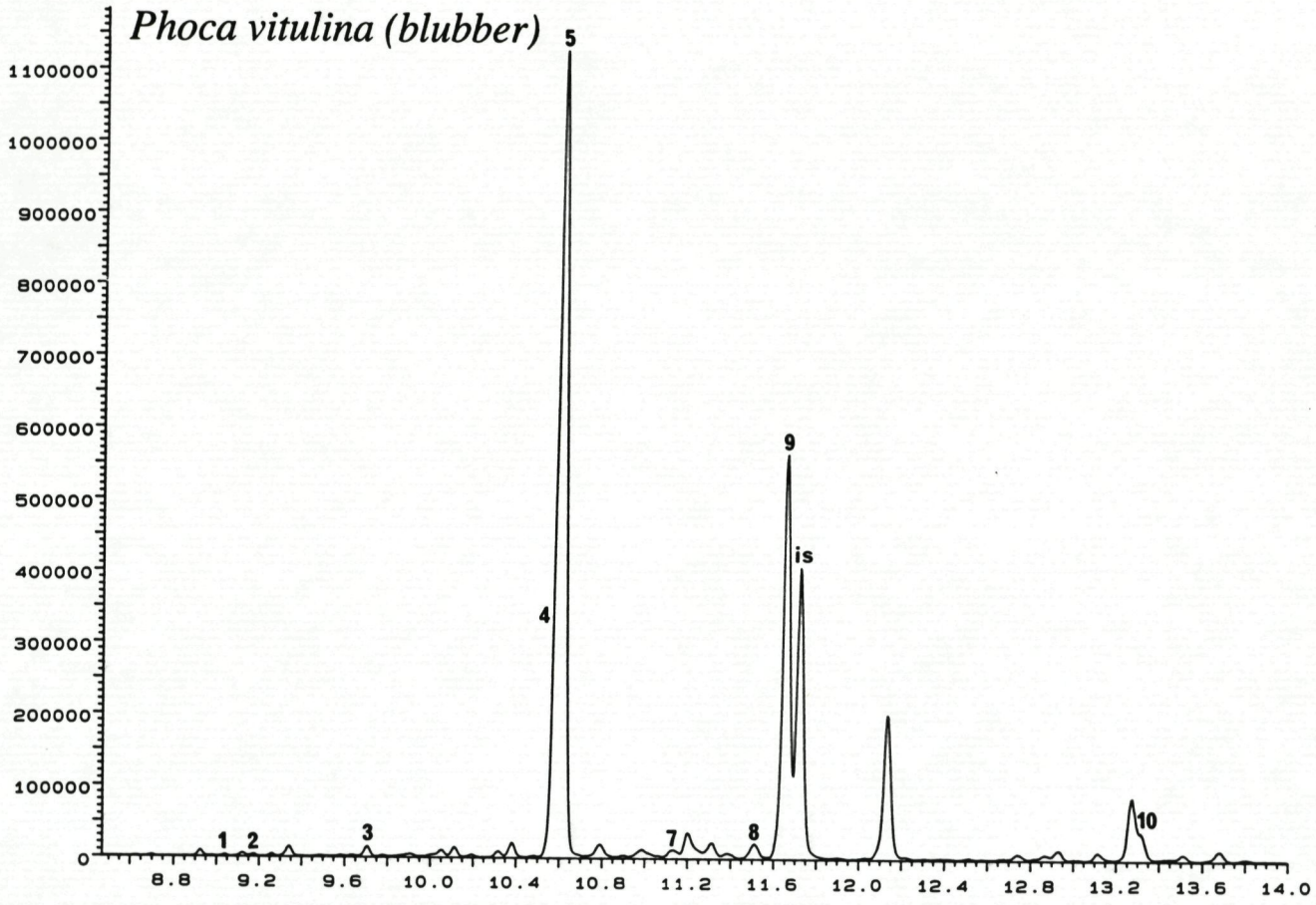


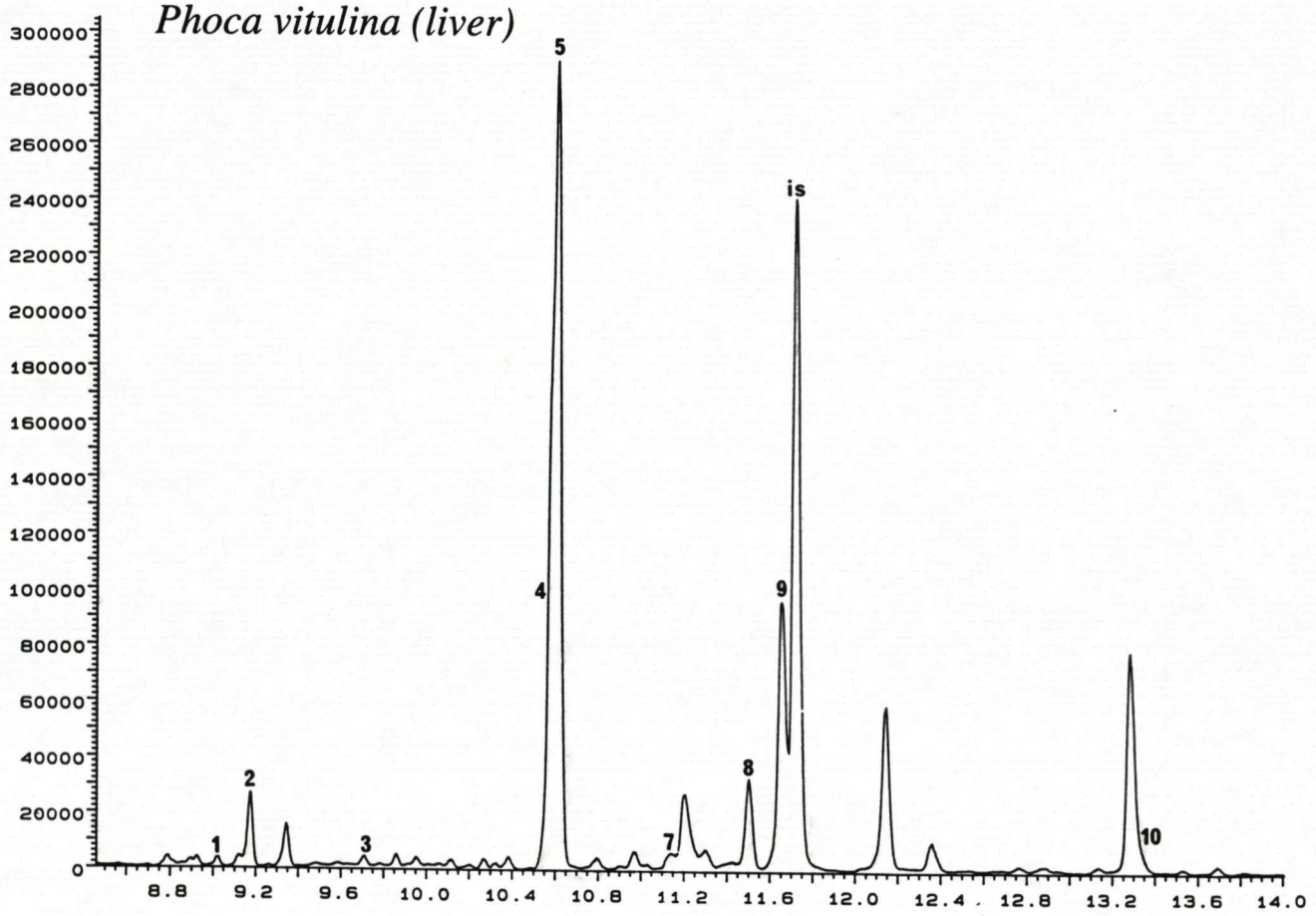

Lagenorhynchus albirostris (blubber)
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200000
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120000
80000
40000
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9
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9.2
9.6
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11.6
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12.8
13.2
13.6

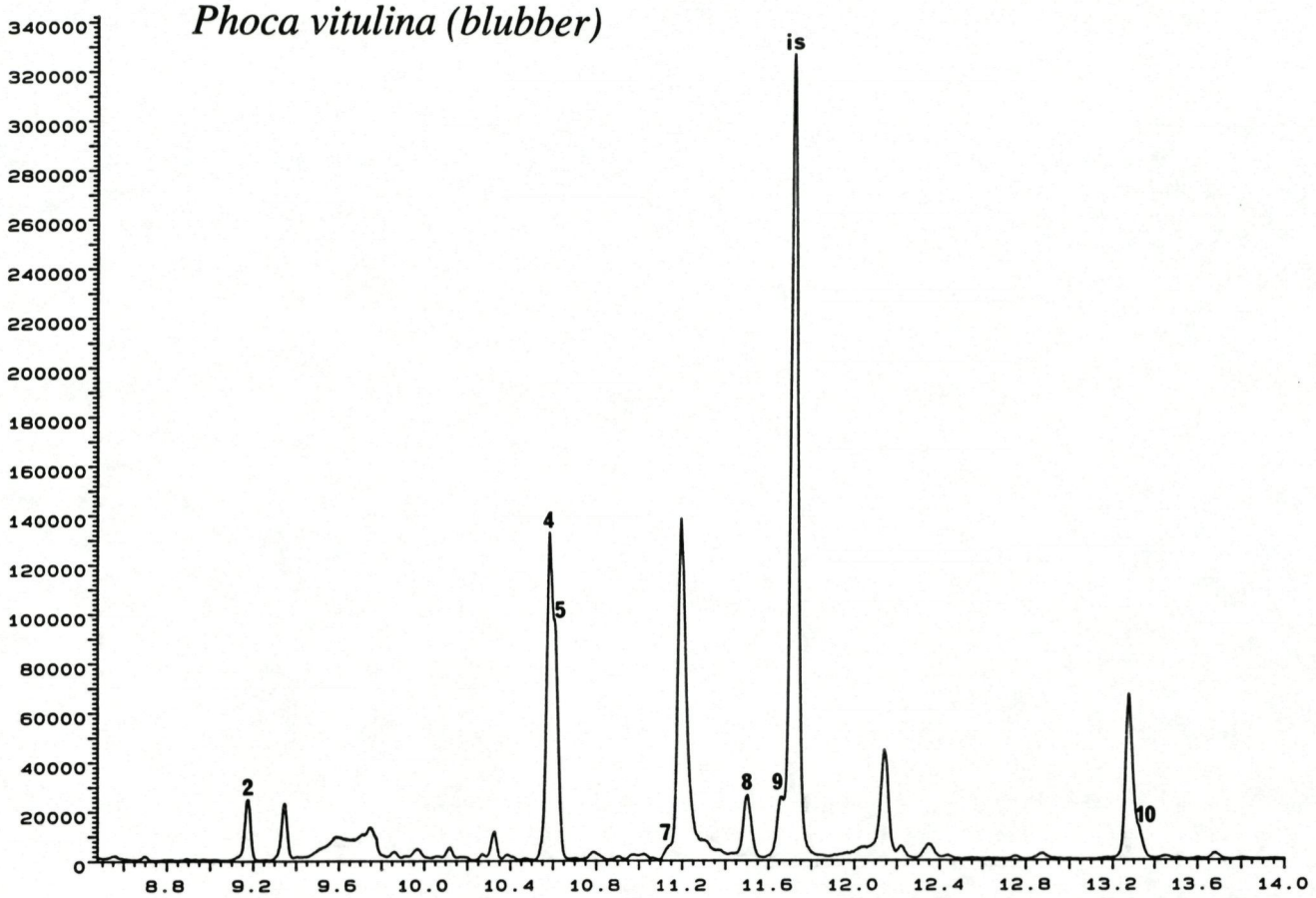

Lagenorhynchus albirostris (liver)
320000
300000
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260000
240000
220000
200000
180000
160000
140000
120000
100000
80000
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40000
20000
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1
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10
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12.0
12.4
12.8
13.2
13.6

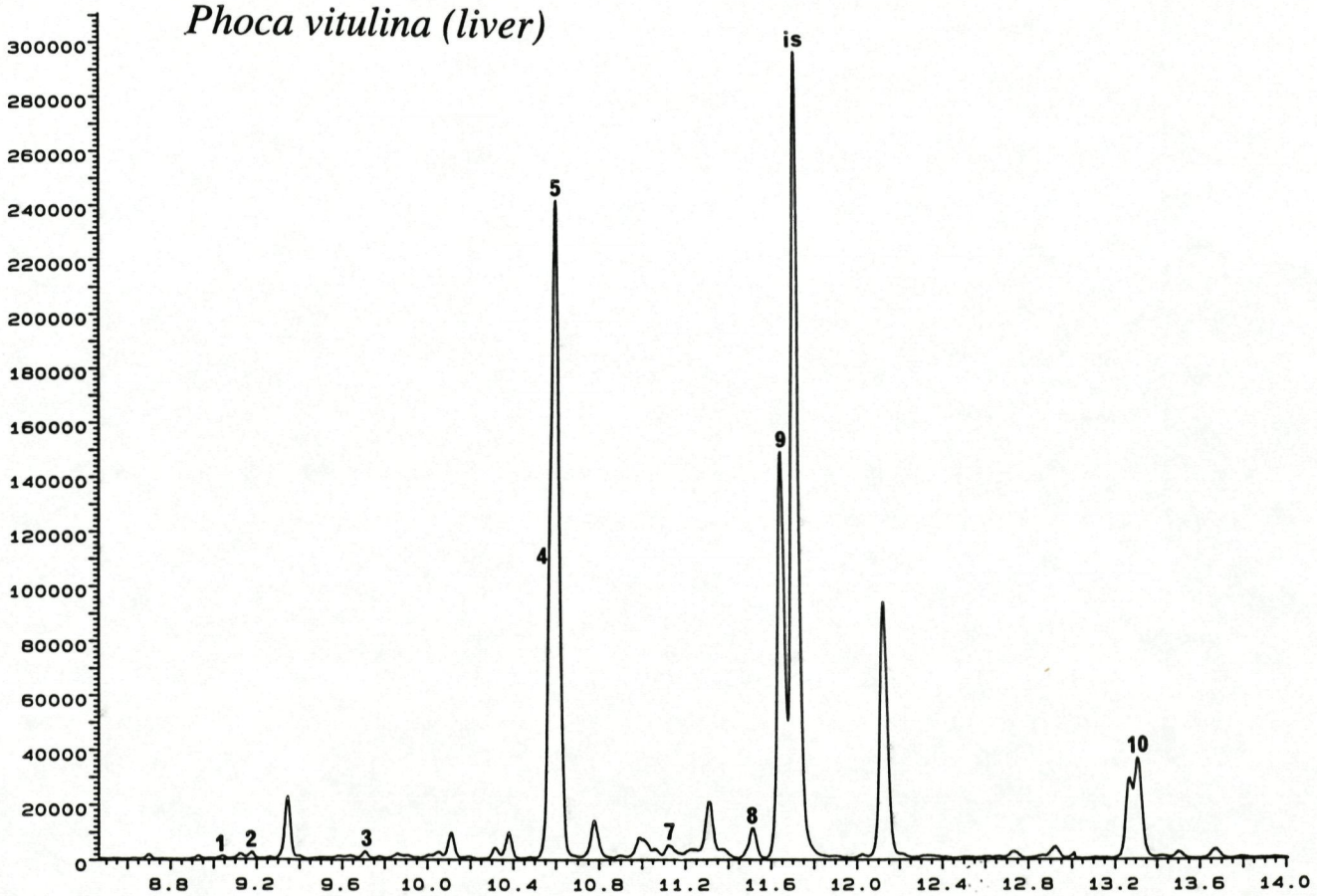
Balaenoptera acutorostrata (blubber)



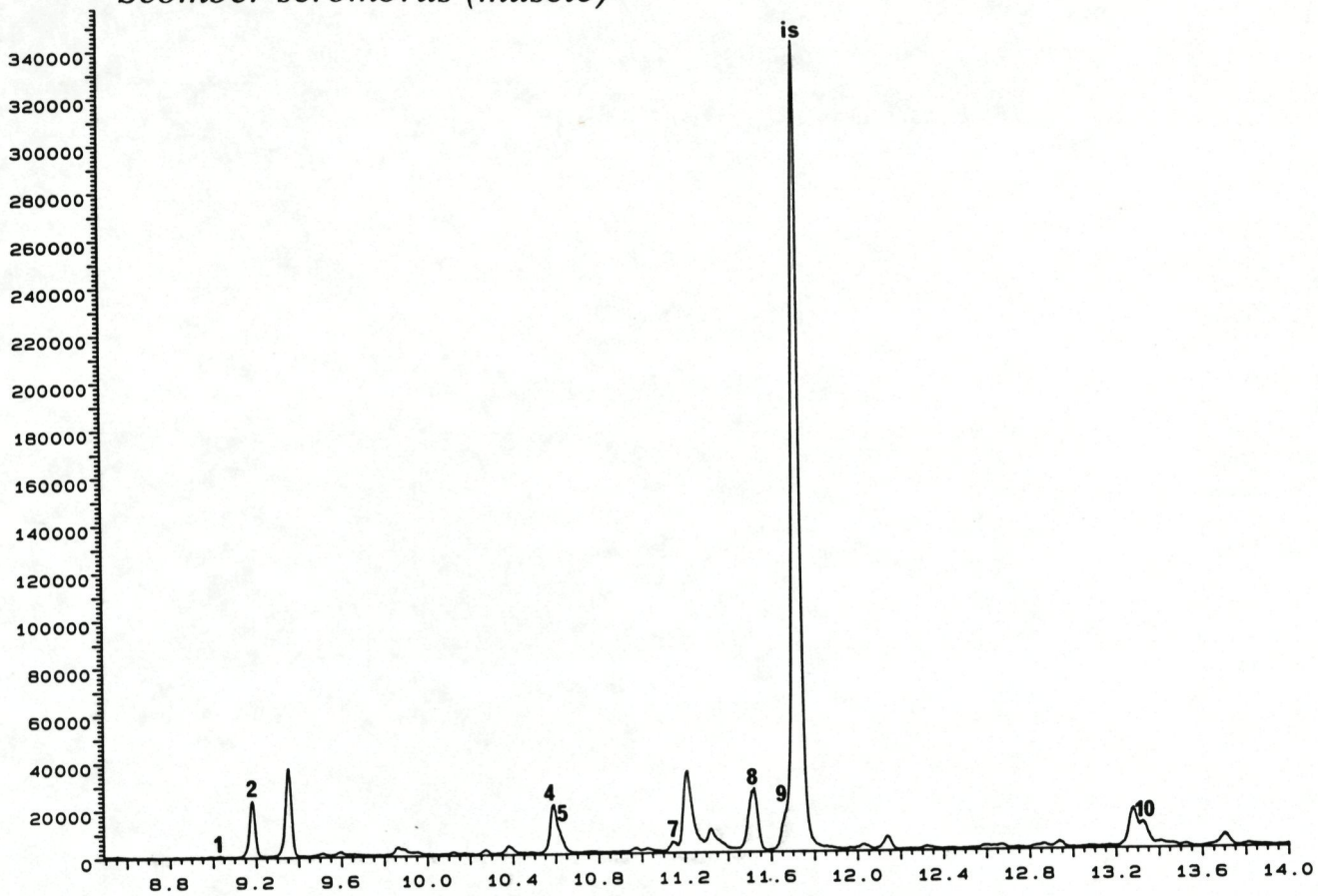

Phoca vitulina (blubber)
1100000
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
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9.6
10.0
10.4
10.8
11.2
11.6
12.0
12.4
12.8
13.2
13.6
14.0


Phoca vitulina (liver)
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240000
220000
200000
180000
160000
140000
120000
100000
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40000
20000
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Phoca vitulina (blubber)
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Phoca vitulina (liver)
300000
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240000
220000
200000
180000
160000
140000
120000
100000
80000
60000
40000
20000
0
8.8
9.2
9.6
10.0
10.4
10.8
11.2
11.6
12.0
12.4
12.8
13.2
13.6
14.0
is
5
9
4
10
1
2
3
7
8

Scomber scrombrus (muscle)



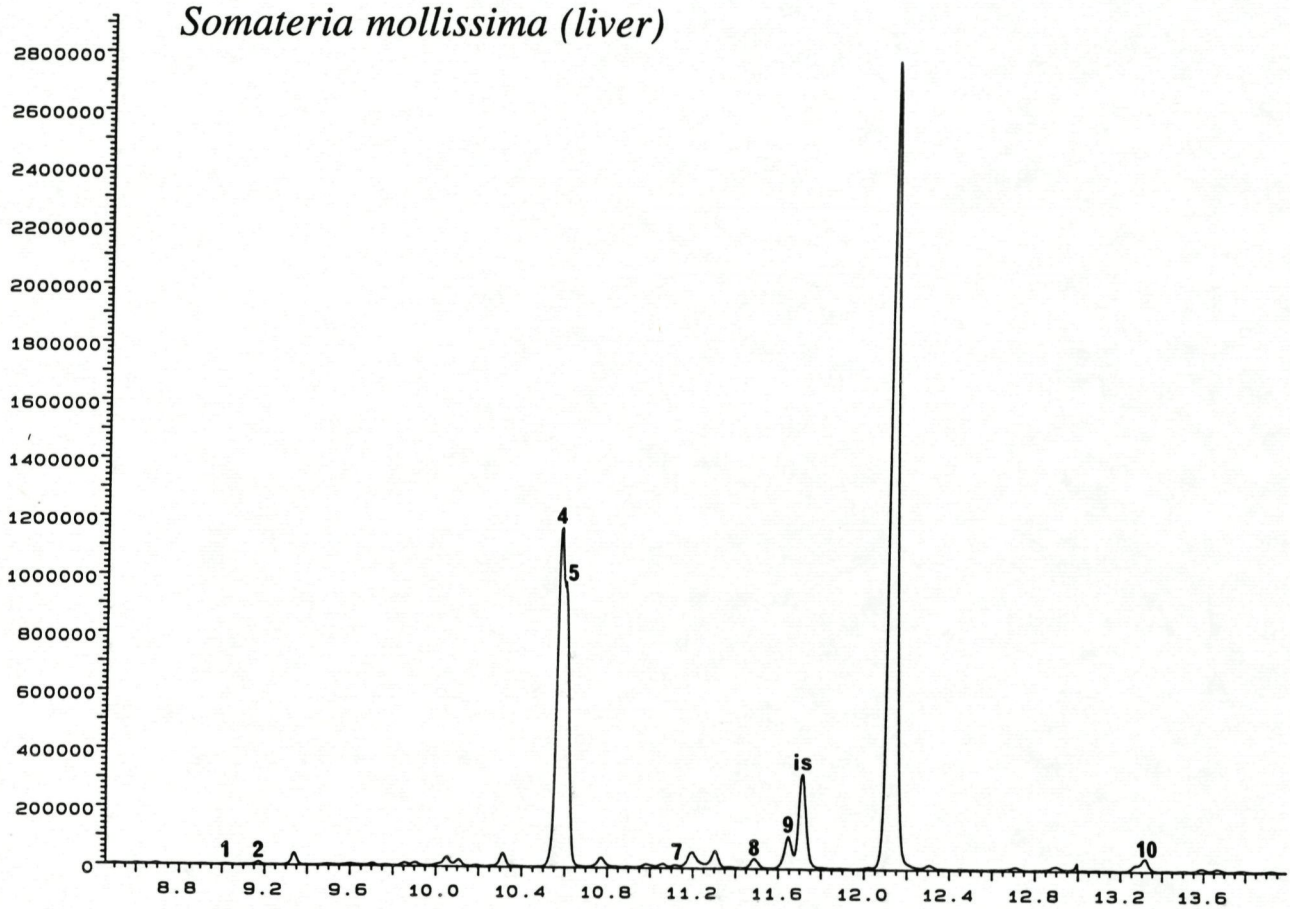

Somateria mollissima (liver)
2800000
2600000
2400000
2200000
2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
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0
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8.8
9.2
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10.8
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11.6
12.0
12.4
12.8
13.2
13.6

Table 3.7: *Total concentrations (in μg•kg^{-1}) of all chlordane components on a wet weight (WW) and on a lipid weight basis.*

Species	Tissue	NIOZ-code	RIVO LIMS#	Σ-chlordanes (WW)	Lipid Content (%)	Σ-chlordanes (lipid)
Cetaceans						
Physeter macrocephalus	blubber	95PC1	15920	858	56.4	1521
Physeter macrocephalus	blubber	95PC3	15870	931	35.8	2601
Physeter macrocephalus	blubber	95PC2	15871	691	25.4	2719
Physeter macrocephalus	liver	95PC2	15872	55	4.0	1367
Lagenorhynchus albirostris	blubber	95 LA1	15926	1111	96.1	1156
Lagenorhynchus albirostris	liver	95 LA1	15927	9	2.8	314
Mysticete						
Balaenoptera acutorostrata	blubber	94BA1	15921	689	12.9	5341
Pinnipeds						
Phoca vitulina	blubber	92PVa	15922	1273	24.4	5217
Phoca vitulina	liver	94PVa	15923	7	2.7	248
Phoca vitulina	blubber	96PVa	15928	353	82.6	427
Phoca vitulina	liver	96PVa	15929	12	5.2	226
Phoca vitulina	blubber	96PVc	19488	582	72.2	805
Phoca vitulina	liver	96PVc	19489	21	5.1	414
Pisces						
Scomber scombrus	muscle	-	19490-1	6	15.2	38
Scomber scombrus	muscle	-	19490-2	20	14.0	145
Aves						
Somateria mollisssima	liver	97SMa	20331	25	2.1	1173
Somateria mollisssima	liver	96SMa	20332	80	4.0	1989
Somateria mollisssima	liver	97SMb	20333	28	3.1	915
Somateria mollisssima	liver	97SMc	20334	17	2.1	820
Somateria mollisssima	liver	97SMd	20335	36	3.1	1169

half of the concentrations in the blubber of the same animal. The concentrations ranged between 95 and 5300 $\mu g \bullet kg^{-1}$ lipid. The lowest concentrations were found in samples of mackerel and knot. The highest concentrations were surprisingly found in the sample of minke whale. The concentrations in sperm whale were also certainly not at the low end of the range observed, suggesting a wide geographical spread of chlordane in the environment and the occurrence of chlordanes in deep ocean food webs.

Table 3.8 gives the sums of all parent compounds (i.e. the sum of *cis-* & *trans*-chlordene, *cis-* & *trans*-chlordane, *cis-* & *trans*-nonachlor, and heptachlor) and all metabolites (only heptachlorepoxide-b and oxychlordane, since heptachlorepoxide-a was always below the detection limit), as well as their ratios, indicated as 'Ratio Σ-PC/Σ-met'. These ratios are above 4 for all whale samples and mackerel, but < 1 for all eider duck samples and all samples except one of harbour seal. This supports the results of the *in vitro* assays where harbour seal and eider duck metabolised chlordanes to a higher extent than cetaceans.

3.4 Genotoxicity

The results are given in **table 3.9**. The chlordane parent compound mixture (mix I) and the metabolite mixture (mix II) gave a significant genotoxic response in the *direct* test mode. However, the response disappeared for both mixtures in the *indirect* test, i.e. after the addition of rat S9-fraction, allowing for biotransformation. This indicates that the mixture of metabolites generated by the rat liver microsomal preparation as supplied by the manufacturer, are no longer genotoxic, in contrast to the metabolites present in mix II (oxychlordane, heptachlorepoxides A and B).

Table 3.8: *Total concentrations (in μg•kg^{-1} wet weight) of all parent chlordane components (Σ-PC) and all oxygenated metabolites (Σ-met.). The ratio of Σ-PC/ Σ-met. is used as an indicator for the role of metabolism in the bioaccumulation process.*

Species	Tissue	RIVO LIMS #	Σ-PC	Σ-met.	Ratio Σ-PC /Σ-met.
Cetaceans					
Physeter macrocephalus	blubber	15920	744.8	113.0	6.59
Physeter macrocephalus	blubber	15870	833.3	98.0	8.50
Physeter macrocephalus	blubber	15871	610.7	80.0	7.63
Physeter macrocephalus	liver	15872	50.1	4.7	10.67
Lagenorhynchus albirostris	blubber	15926	938.0	173.0	5.42
Lagenorhynchus albirostris	liver	15927	7.3	1.5	4.89
Mysticete					
Balaenoptera acutorostrata	blubber	15921	570.0	119.0	4.79
Pinnipeds					
Phoca vitulina	blubber	15922	552.9	720.0	0.77
Phoca vitulina	liver	15923	4.5	2.2	2.03
Phoca vitulina	blubber	15928	157.9	195.0	0.81
Phoca vitulina	liver	15929	5.0	6.7	0.75
Phoca vitulina	blubber	19488	211.5	370.0	0.57
Phoca vitulina	liver	19489	4.9	16.2	0.30
Pisces					
Scomber scombrus	muscle	19490-1	4.7	1.1	4.50
Scomber scombrus	muscle	19490-2	19.2	1.1	18.30
Aves					
Somateria mollisssima	liver	20331	2.9	21.7	0.13
Somateria mollisssima	liver	20332	3.6	76.0	0.05
Somateria mollisssima	liver	20333	4.6	23.8	0.19
Somateria mollisssima	liver	20334	1.2	16.0	0.08
Somateria mollisssima	liver	20335	4.2	32.0	0.13

Table 3.9: *Results of the mutatox® assays with mixtures of chlordane compounds, positive controls, and blank values. Chlordane mix. I: parent compounds; mix. II: oxidised metabolites. Test concentrations are given as dilution factors, which refer to concentrations of individual compounds given in table 2.1 for mix I and table 2.2 for mix. II. Only results of the direct assays with are given, since the positive responses disappeared after the addition of rat S9 fraction.* ***Bold****: Concentration showing the maximum response (CMR).*

Direct Assay			Direct Assay		
Chlordane mix. I dilution factor	**Response (1)** [a.u.]	**Response (2)** [a.u.]	**Chlordane mix. II** dilution factor	**Response (1)** [a.u.]	**Response (2)** [a.u.]
2560	6	6	2560	7	6
1280	6	7	1280	7	5
640	6	6	640	4	5
320	5	6	320	7	6
160	7	6	160	8	6
80	5	6	80	8	6
40	6	5	40	10	6
20	9	9	20	13	9
10	22	43	10	65	16
5	**1288**	**590**	5	**231**	**905**

Positive Controls			
Direct Assay		**S9- activated**	
Phenol [µg/ml]	**Response** [a.u.]	**BaP** [µg/ml]	**Response** [a.u.]
0.78	5	0.02	18
1.56	5	0.04	19
3.13	6	0.08	18
6.25	6	0.16	13
12.5	7	0.31	15
25	9	0.63	34
50	27	1.25	188
100	418	2.50	**3307**
200	**9363**	5	172
400	5	10	8

Blank (medium + solvent)			
Direct Assay		**S9- activated**	
[Aceton] (vol.%)	**Response** [a.u.]	**[Aceton]** (vol.%)	**Response** [a.u.]
0.625	24	0.625	14
1.25	22	1.25	24
2.5	17	2.5	30
5	11	5	16
10	3	10	7

4. Discussion

From **figure V** it can be concluded that the biotransformation capacity decreased in the order harbour seal > whitebeaked dolphin ≈ eider duck > sperm whale. Thus, there is a substantial difference in biotransformation capacity between the different species. Differences in activity of different iso-enzymes of cytochrome P-450 iso-enzymes may rationalise the observed differences in chlordane metabolism. Besides genetic inter-species differences, several other *intra*-species factors can also contribute to the differences in metabolism: age, sex, nutritional status and inter-individual genetic variability (16). Obviously it is difficult to draw general conclusions from experiments with only one or two animals per species. However, the results clearly show that the *in-vitro* assays yield useful results to answer this type of questions in the end.

The order of metabolic ability for chlordanes was similar to the results obtained with toxaphene (2), which is also a group of cyclic aliphatic compounds (chlorobornanes; a group of cyclic mono-terpenoids derived from α-pinene). Two representatives of this group appear to be metabolised by a cytochrome P450 3A-like iso-enzyme in the harbour seal. (36).

When a compound is metabolised *in-vitro*, it can be assumed that it will also be metabolised *in vivo* because the iso-enzymes responsible for its metabolism are present in the animal. Of course chlordanes will not be available as a substrate in excess to the enzyme and the enzyme will not be as readily reachable in the *in vivo* situation. In the end, a multitude of factors (like tissue levels and the effective local concentration at the active site) will determine which substrates will be metabolised first (16) by the P450 system. Consequently the metabolism of the chlordanes could be even less in *in-vivo* situations.

In view of this, the amounts of oxychlordane produced in the *in vitro* assays were unexpectedly low when compared to the often quite large peaks of this compound in environmental residues. Oxychlordane can originate from both chlordane isomers (**fig. I**). Since especially *trans*-chlordane was significantly metabolised in most assays (except for the sperm whale), oxychlordane was expected as the product. Several explanations can be given to explain this behaviour:

- Oxychlordane is further metabolised by epoxide hydrolase (EH) to a dihydrodiol. This diol could have escaped from our attention because it is either destroyed by sulphuric

acid treatment, and did not separate from the lipids in HPLC clean-up. Alternatively, it may not have eluted from the GC-column, because of its high polarity.

- Another reason is based on the fact be that EH also plays a role in the transformation of endogenous substrates (epoxides). Often endogenous substrates are preferred over exogenous epoxides. This could explain why oxychlordane is found *in-vivo*, when an abundance of endogenous compounds is present, and not *in-vitro,* when there is only an abundance of exogenous compounds present (16).

With respect to the biotransformation of heptachlor to heptachlorepoxide, Buser and Müller (37) also reported the formation of only the heptachlor epoxide B isomer, which they attributed to the stereoselectivity of the MFO system.

Large differences were found in the values of the ratios of the Σ-parent compounds: Σ-metabolites, which decreased in the order sperm whale>whitebeaked dolphin>harbour seal> eider duck. This is consistent with the results of the *in-vitro* assay where harbour seal and eider duck showed the highest rates of biotransformation. The fact that no heptachlor epoxide A was found in these biota samples is also consistent with the results of the *in-vitro* assay.
The results in **figure VIII** also illustrate that the cetaceans contained proportionally more parent compounds than the harbour seal and eider duck. The sperm whale was the only species that contained substantial amounts of *trans*-chlordane. This agrees with the results of the *in-vitro* assay, where the sperm whale was the only species incapable metabolising *trans*-chlordane to a significant extent. The harbour seal contained proportionally more heptachlor than the other animals. This also agrees with the results of the *in-vitro* assay, where *cis*- and *trans*-chlordane were metabolised faster than heptachlor in comparison to other animals. Remarkable is that *trans*-chlordene, the only chlordane congener that could not be metabolised by the harbour seal in the *in-vitro* assay, does not account for a large proportion of the parent compounds in the *in-vivo* situation. Perhaps there are other biogeochemical processes in the environment that cause the disappearance of this compound before it can reach animals representing high trophic levels.
Table 4.1 shows the residue levels reported for some other cetacean species. Like in the cetacean samples investigated in this study, the ratios of parent compounds:metabolites are all > 3. The biota residue levels of the different samples shown in **table 4.1** were taken from a number of publications, so the analytical methods were divers. From a comparison with the

composition of technical chlordane (**table 1.1**), it appears that both nonachlors, but especially the *trans*-isomer, become strongly enriched in cetaceans compared to both chlordanes. Of the latter, *cis*-chlordane seems to be more bioaccumulative than *trans*-chlordane. When the sperm whale is compared to the other species listed, it seems that the enrichment factor of *trans*-nonachlor to chlordanes is considerably less. In general, these conclusions agree well with the results obtained in the *in-vitro* assay of this research project.

***Table 4.2**: Concentrations of chlordanes in blubber of different cetaceans (μg/kg wet weight).*

Compound	Sperm whale	Killer whale	Risso's dolphin	Dall's porpoise	Harbour porpoise	Beluga
Individual parent compounds						
Trans-chlordane	139	110	11	42	32	28
Cis-chlordane	271	640	41	110	340	191
Trans-nonachlor	687	5400	310	900	2500	1720
Cis-nonachlor		400	19	130	300	356
Σ Parent compounds	1097	6550	381	1182	3172	2295
Metabolites						
Oxychlordane		280	26	110	250	460
Heptachlor epoxide		110	9	190	180	151
Σ metabolites	0	390	35	300	430	611
Ratio ΣPC/ΣMet	-	16.8	10.9	3.94	7.38	3.76

Sources: 1: (38); 2-5: (39); 6: (40)

The results with respect to the genotoxicity in the Mutatox® test clearly indicate that chlordanes are genotoxic. This is in agreement with the literature (7). Addition of a rat S9 fraction to the Mutatox® assay resulted in a clear decrease of genotoxicity. However, this cannot be due to the metabolites as present in our incubation mixture II (oxychlordane, heptachlorepoxides A and B), which still showed a positive genotoxic response. So, a different mixture of metabolites must have been formed by these rat microsomes.

5. Suggestions for further research

After this study, a number of questions remain with respect to the route of metabolism of chlordane components:

- The metabolites that were measured in the present study can probably be metabolised further, thereby losing their genotoxicity. This route of biotransformation should be investigated in more detail. By performing *in-vitro assays* with single components, it should be determined whether the unknown metabolite is an oxidised metabolite of chlordane or heptachlor.
- With the help of specific inhibitors for epoxide hydrolase could be determined whether oxychlordane is formed and metabolised further during the *in-vitro* assay.
- By use of specific inhibitors, it could be determined which subfamily of the cytochrome P450 complex is responsible for the biotransformation of individual chlordane components.

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