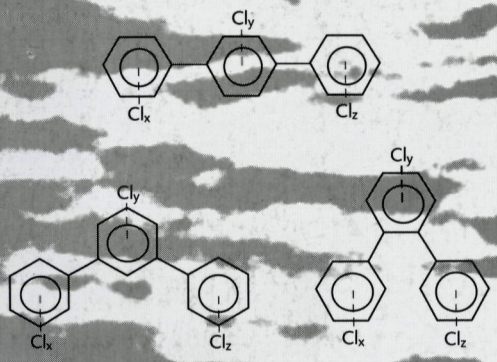

In vitro biotransformatie van organohalogeenverbindingen in zeezoogdieren en vogels. Mogelijke gevolgen voor bioaccumulatie en genotoxiciteit

IV. Polychloor terfenylen (PCT's)

Beleidsgericht
ecologisch onderzoek
van de
Noordzee/Waddenzee

BEON

NIOZ
RIVO-DLO
RWS-RIKZ

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IV. Polychloor terfenylen (PCT's)

625

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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 oxydatieve 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 met kieuwen ademende prooidieren naar longademhalende 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)). Het huidige rapport behandelt de resultaten voor de polychloor terfenylen (PCTs), een stofgroep met in essentie dezelfde toepassingen als PCBs in wassen, drukinkt, verf, lak, electrische apparatuur, hydraulische oliën, smeermiddelen, als weekmakers in plastics en als vlamvertragers. Hierna zullen de resultaten van chloordaan als laatse in deze serie rapporten van dit in totaal vier-jarige BEON project verschijnen.

Resultaten en conclusies van het uitgevoerde onderzoek met betrekking tot het te voeren beleid.

- Doorvergiftigings problematiek: In de *in vitro* assays bleek, dat de PCTs in slechts zeer geringe mate worden omgezet. Tevens werden in de onderzochte monsters van spek en lever van potvis, witsnuitdolfijn, dwergvinvis, zeehond, en eidereend PCT residuen gevonden. Op grond van de geconstateerde hoge resistentie tegen enzymatische afbraak in de *in vitro* assays en de hoge lipofiliteit van deze stoffen werd dit ook verwacht. Het voorkomen van PCTs in alle drie de in 1995 bij Kijkduin levend aangespoelde potvissen heeft hierbij een bijzondere betekenis. Potvissen bevinden zich namelijk in principe uitsluitend in de oceanen; 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 PCTs ook in deze potvissen werden gevonden, kan dus worden geconcludeerd dat PCTs ook in de diepzee voorkomen, net zoals dit voor de nauw verwante PCBs het geval is.

- Productie van PCTs: In de periode 1955-1980 is er wereldwijd ongeveer 60,000 ton PCTs geproduceerd; dit is ongeveer 5-7% van de totale PCB productie (5, 6). De PCT productie in de Verenigde Staten is in 1972 beëindigd, gevolgd door Duitsland in 1974, Italië in 1975, en Frankrijk in 1980 (7). PCTs zijn ook in Japan geproduceerd, maar daarover is geen nadere informatie beschikbaar.
- Regelgeving: PCTs worden genoemd in een aantal besluiten in nationaal en europees verband.

  1. **PCB-, PCT- en chlooretheen-besluit Wet milieugevaarlijke stoffen (WMS).**

     "Het PCB-, PCT- en chlooretheenbesluit WMS (18 april 1991, Staatsblad 232; laatstelijk gewijzigd 16 november 1993, Stb.606) omvat een verbod om polychloorbifenylen (PCB) en polychloorterfenylen (PCT) te vervaardigen of, al dan niet verwerkt in een preparaat of product, in Nederland in te voeren, toe te passen, voorhanden te hebben of aan een ander ter beschikking te stellen. Onder PCB wordt hierbij verstaan chloorbifenylen met uitzondering van mono- en dichloorbifenylen".

2. **EU-zwarte lijst**

PCTs bevinden zich op de lijst van 132 zwarte lijst stoffen die door de EU-commissie zijn geselecteerd. Lozingen van afval, dat één van de stoffen kan bevatten, moeten in principe geheel worden verboden. De stoffen zelf zijn (op een enkele uitzondering na) niet verboden. Bij productie of gebruik van deze stoffen moeten de best bestaande technieken ('best available technology') worden toegepast om de emissie van deze stoffen tegen te gaan. PCBs en PCTs zijn (destijds) als nr. 10 op 26 september 1977 op de lijst gezet.

3. **ECE-lijst**

De ECE-lijst (ECE/CEP/2; 23-6-1994) is opgenomen bij een aanbeveling van de Commissie voor Milieubeleid van de Economische Commissie van Europa (ECE) (een commissie van de Economische en Sociale Raad van de Verenigde Naties). De aanbeveling en de lijst hebben betrekking op de voorkoming, beheersing en beperking van verontreiniging van oppervlaktewater door schadelijke stoffen. De aanbeveling dient als stimulans voor de implementatie van de Convention on the Protection of Transboundary Watercourses and International Lakes. PCBs en PCTs staan op deze zogenaamde 'indicative list of priority hazardous substances'. De regeringen van de lidstaten worden aangespoord de ECE-lijst te gebruiken bij:

- de vorming van beleid (programma's en strategieën)
- het selecteren van stoffen voor monitoring en onderzoeksprogramma's
- het opstellen van lozingseisen
- het opstellen van maatregelen (bijvoorbeeld voor industriële processen)
- het uitwisselen van 'best available technologies' tussen landen.

4. **EG-verordening EEG/1492/96 (30 juli 1996)**

Deze verordening betreft de internationale procedure van kennisgeving en "voorafgaande geïnformeerde toestemming" (Prior Informed Consent; PIC) met betrekking tot de invoer vanuit en uitvoer naar 'derde' landen buiten de Gemeenschap van gevaarlijke chemische stoffen. Ook op deze lijst van stoffen staan behalve de PCTs ook de PCBs en de PBBs.

Volgens de bijlage van deze laatste lijst staan PCTs geregistreerd onder CAS-nr. 61788-33-8 en EC-nr. 262-968-2.

- Genotoxiciteit: In de mutatox assay werd voor de PCTs geen genotoxische werking vastgesteld. Dit is in overeenstemming met de meeste literatuur gegevens. Wel kunnen PCTs mogelijk de mutagene werking van andere stoffen potentiëren (promotorwerking; zie ook hieronder bij punt 3 'sublethale effecten').

Aanbevelingen voor toekomstig onderzoek

1. Voorkomen van PCTs: In de hier onderzochte monsters van top predatoren komen PCTs veelvuldig voor. De hoogste concentraties (op vetbasis) werden gevonden in de levers van eidereenden uit de Waddenzee. In tegenstelling tot de situatie voor de nauw verwante PCBs, zijn er van het voorkomen van PCTs in de Noordzee en Waddenzee nog geen gebiedsdekkende gegevens bekend. Om dit verder te onderzoeken stellen wij de volgende compartimenten voor:
   - Sediment. Gezien de te verwachten gehalten kan hierbij in eerste instantie het best worden gekozen voor metingen in een afgescheiden fijne fractie met een relatief hoog organische stof gehalte (bv. < 63 µm). Hierbij kan aansluiting worden gezocht bij het door het RIKZ reeds uitgevoerde Joint Assessment and Monitoring Programme.
   - 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 het monitoringsprogramma organochloor verbindingen in kabeljauw lever van het RIVO-DLO en ook weer bij het Joint Assessment and Monitoring Programme.
   - Zeezoogdieren. Hierbij kan worden gedacht aan 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 interne gehalten zeer sterk onder invloed van intering op de vetreserves tijdens de trekvlucht. Momenten waarop de vetreserves minimaal zijn lijken het meest kritisch omdat de interne 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.

2. Toxiciteit van PCTs: De nauw verwante PCBs beïnvloeden zowel de reproductiecapaciteit (8, 9, 10) als het imuunsysteem van zeezoogdieren (11, 12). Met behulp van speciaal voor dit doel ontwikkelde bio-assays zou nagegaan moeten worden of PCTs dit ook doen.

***In vitro* Biotransformation of Cyclic Organohalogen Compounds in Marine Mammals and Birds. Possible Consequences for Bioaccumulation and Genotoxicity. IV: Chlorinated Terphenyls (PCTs)**

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Running-title: Biotransformation of PCTs.

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

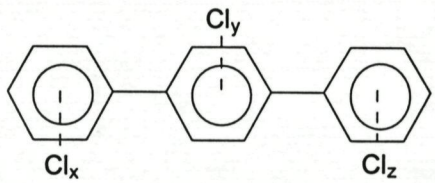
Abstract

- The *in-vitro* biotransformation capacity of hepatic microsomes of a sperm whale (*Physeter macrocephalus*), a white beaked dolphin (*Lagenorhynchus albirostris*), a harbour seal (*Phoca vitulina*), and an eider duck (*Somateria mollissima*) for the polychlorinated terphenyl (PCT) mixture Aroclor 5442 (A5442) was investigated. The biotransformation rates for PCTs were generally low. Eider duck microsomes metabolised more of the early eluting PCTs, whereas the harbour seal microsomes metabolised a wider range of PCTs. The microsomes of the whitebeaked dolphin and the sperm whale were both almost incapable of metabolising any PCTs.

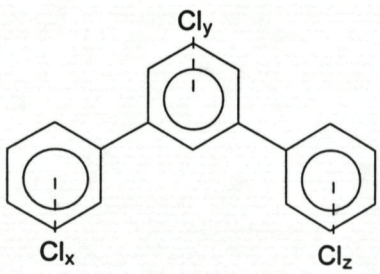
- The PCBs present as contamination in the A5442 mixture, were more readily metabolised than the PCTs. PCTs may therefore be more bioaccumulative than PCBs. On the other hand, molecular size of the PCTs may inhibit their bioaccumulation, especially for the octa- and higher chlorinated terphenyls.
- PCT residues could be detected in most of the wildlife samples. Hexa- and hepta-chlorinated congeners dominated. The highest concentrations were found in liver of eider ducks and in blubber of a whitebeaked dolphin. Relatively low, but still detectable, concentrations were found in blubber of three sperm whales. Because these animals do normally not occur in shelf seas, this strongly indicates that PCTs have also reached the deep sea.
- A 5442 was not genotoxic in the mutatox assay.

1 Introduction

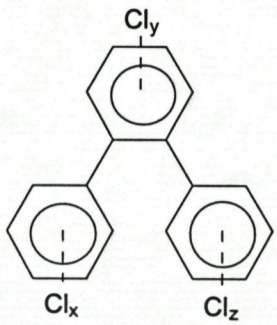
Polychlorinated terphenyls (PCTs) consist of three phenyl rings bound to each other by single C-C bonds. Structurally, they can be regarded as polychlorinated biphenyls, to which a third ring can be added in the *ortho*, *meta*, or *para* position. These three types of terphenyl 'skeletons' can be substituted with chlorine atoms at 1 to 14 positions.



Para-position



Meta-position



Ortho-position

The general formula of PCTs is $C_{18}H_{14-n}Cl_n$ (with $1 < n < 14$). Thus, the molecular weights of polychloroterphenyls span a range between 264 (nCl=1) and 713 D (nCl=14). As many as 8149 congeners are theoretically possible, which is much more than the 209 possible PCB congeners.

In 1929 the production of PCTs was started in the United States by the Monsanto Chemical Co. PCTs have also been produced in France, Italy, Germany and Japan. During the period 1955-1980, about 60,000 metric tonnes of PCTs were produced world-wide, which is about 5-7% of the total PCB production (5, 6). PCT production in the United states was terminated in 1972, followed by Germany in 1974, Italy in 1975, and France in 1980 (7). No information is available for Japan.

Like PCBs, PCTs were available as technical mixtures. The Aroclor series of the Monsanto Chemical Co. consisted of A5432, A5442, and A5460. In these names,'A' stands for Aroclor, '54' stands for the terphenyl series and the last two digits indicate the percentage of chlorine on a weight basis (13).

Because of the close structural relationship of PCTs to PCBs, chemical and physical features are also much alike. Like PCBs, PCTs were primarily used because of their heat stability. They were used in printing inks, paints, lacquers, and electronic equipment and as hydraulic fluids, lubricants, plasticizers, and fire retardants (5, 7).

The last decades much attention has been paid to the fate and bioaccumulation of polychlorinated biphenyls (PCBs). Especially marine mammals (and sea birds) turned out to be very susceptible to these compounds, since their position as top predator makes them susceptible to biomagnification (14, 15). As a group, PCBs affect reproductive processes (8, 9, 10), cause immunosuppression (11, 12), and act as promotors for cancer development. The chlorine substitution pattern of PCBs is a key factor in their biomagnification (15) and toxicity (10, 16, 17). An important factor with respect to the toxicity of the PCBs is their ability to reach a flat molecular configuration in which both aromatic rings lie in one plane (co-planar configuration).

In contrast, very little is known about the PCTs. This is caused in part by analytical problems. First, environmental concentrations can only be measured in an indicative manner, since even high resolution gas chromatography does not offer enough separation power to separate the number congeners present in technical mixtures. Moreover, only very few individual congeners have been synthesized; although the synthesis of several mono- and penta-substituted PCT congeners has been reported (18). Thus, the scarce figures are mainly based on total PCT contents expressed as equivalents of the best fitting technical PCT mixture. However, because of the multitude of mixtures that have been used, quantification of environmental residues on the basis of a single PCT mixture can at best be an approximation (6, 7). A few studies reveal a positive correlation between PCT and PCB concentrations; PCTs are present in biota and sediments at 1-10% or 10-25% of the total PCB content, respectively (7). The scarce literature information indicates that the toxic effects of PCTs are comparable to those of PCBs (7, 13). In this respect it is important to notice that in the case of the PCTs the chance that all three rings will occupy a single plane, is much smaller.

However, like PCB congeners, some PCT congeners may be enzymatically biotransformed. 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 (19). It is evident, that biotransformation can have a large impact on the behaviour of a compound in the food chain and its toxicity. Firstly, biotransformation determines whether there is accumulation from prey to predator (biomagnification). Secondly, 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 on the other hand biotransformation can also generate metabolites that are more toxic than the parent compounds (10, 20). 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 (21).

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, 22).

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. To advise with respect to the development of policy measurements for these compounds on the basis of the results obtained and literature data.

This report describes the results for the PCTs. 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). The final report, following this one, will give the results of the studies with chlordanes.

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 (23). Acetone, methanol and 2,2,4-trimethylpentane (TMP) were of nanograde quality (Promochem).

2.1.3. Organohalogen mixture

The contaminant mixture used in the *in-vitro* assays, consisted of the PCT mixture Aroclor5442 (Promochem GmbH). 2,2',4,4',5,5'-hexacholorbiphenyl (CB-153; Promochem GmbH) was added as internal standard (ISTD) because of its high resistance to biotransformation. 4,4'-dibromobiphenyl (BB-15; Promochem GmbH) and 2,3',5-trichlorobiphenyl (CB-26; Promochem GmbH) were added as positive controls to check the ability of the microsomes to carry out biotransformation of these compounds which are known to be metabolised in marine mammals and birds (2). This mixture of compounds was dissolved in acetone. The concentrations of the different congeners in the standard mixture are shown in **Table 2.1**.

The systematic numbering for the chlorobiphenyl and bromobiphenyl congeners was done according to Ballschmiter *et al.* (24).

***Table 2.1**: The contents of the standard mixture.*

Abbreviation	Compound	Concentration (ng/µl)
Aroclor 5442	Polychlorinated terphenyl mixture	4500
BB15	4,4'-dibromobiphenyl	22
CB26	2,3',5-trichlorobiphenyl	10
CB153	2,2',4,4',5,5'-hexachlorobiphenyl	16

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 there is a possibility to analyze the biotransformation ability of a unique species, this will most certainly be used. The following species were sampled for the study on PCTs:

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 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 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 (25)
NIOZ sample code	: 94SM82 and 94SM62 (two animals)

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 where 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), 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 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 (26). 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 of 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 (14), 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 (27). 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. Ethoxyresorufin-O-deethylase (EROD) activity measurements

EROD is one of the model reactions to establish the activity of the cytochrome P-4501A mediated MO-system (28, 29). 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) (30). 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} (31).

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 (32): 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, 4 assay samples with NADPH, and two blanks without either incubation mixture or NADPH. The latter was 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: Analysis of compounds

GC-ECD: The samples were analysed by gas chromatography with electron capture detection. The parameters used are given in **table 2.2**.

Table 2.2: *Parameters used for the gas chromatographic analyses of PCTs of the in vitro assays.*

Equipment	Carlo-Erba 5160 gas chromatograph
Detector	Electron capture detection
Column	25 m • 0.32 mm • 0.12 µm CP-Sil-8CB (Chrompack, NL)
Injection	1 µl splitless with a Carlo Erba A200s autosampler
Carrier gas & pressure	Hydrogen; pressure 100kPa
Linear gas velocity	107 cm/s
Injector temperature	250 °C
Detector temperature	340 °C
Oven temperature programme (controlled by a Carlo Erba MFC500 programmer)	Initial temperature: 90 °C (isothermal phase 1')/ temperature increase at 20 °C•min^{-1} to 215°C (10') / temperature increase of 5 °C•min^{-1} to 280°C (5')

A relatively short and semi wide bore capillary column was used in order to prevent that the large PCT molecules would remain on the column. An ECD chromatogram of the standard PCT mixture is shown in Figure I. Because of the great number of

possible PCT congeners, an unambiguous separation of all congeners is impossible. Consequently, pattern recognition is used instead of peak recognition (7).

2.1.12. 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:

$$Ratio^{X}_{CB-153} = \frac{H_{\text{Compound X}}}{H_{\text{CB-153}}},$$

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 PCT 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: PCT residues in wildlife

PCTs were determined in 16 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 PCT concentrations and patterns in the food of seals and dolphins.

In addition to 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.

2.2.1. Chemicals and apparatus

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

- Sodium sulphate 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
- Aroclor 5442 (technical polychlorinated terphenyl mixture), Monsanto USA
- CB-112 (internal standard)
- Waring blender
- Soxhlet apparatus
- Rotary evaporator, Rotavapor-R, Buchi
- 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 blending was not successful. 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. Extractable lipid determinations 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 to both fractions and the final volumes was adjusted to 1 ml.

2.2.3. GC/MS analysis

The samples were analysed on a HP-5988A GC-MS in the negative chemical ionisation (NCI) mode (NCI-GC-MS). A blank and a standard for recovery were also cleaned-up and analysed. A multi-level calibration curve used for the calculation of the PCT concentrations. The total-ion chromatograms used for quantification were based on all measured PCT ions, without the ions of the internal standard.
The GC/MS-parameters used are given in table 2.3.

***Table 2.3**: GC-MS parameters used for the analysis of PCT residues in samples of wildlife.*

Equipment:	Hewlett-Packard-5988A GC-MS
Column:	DB5; 20 m • 0.25 mm (internal diameter) • 0.25 µm (film thickness)
Carrier gas and pressure:	Helium; 300 kPa
Lineair gasvelocity:	~41 cm/sec t°= 48 sec
Injector temperature:	250 °C
Oven temperature programme:	90 °C (3') temperature increase of 30 °C/min to 180 °C (0')/ temp. increase of 5 °C/min to 285 °C (60'). post run: 15' at 290 °C
Injection method:	splittles, splitter open after 3.0 minutes
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:	$\sim 5 \cdot 10^{-6}$ (- reagent gas)
P source:	$\sim 4 \cdot 10^{-6}$ (- reagent gas) / $\sim 3 \cdot 10^{-4}$ (+ reagent gas)
P methane plasm:	1 torr

The m/z values used for SIM analyses are given in **table 2.4**.

Table 2.4: *Values of m/z used for SIM analyses. Tri- to nona-CTs: tri- to nona chlorinated terphenyls.*

Compound(s)	m/z values
CB-112 (ISTD)	324, 326
tri-CTs	332, 334
tetra-CTs	366, 368
penta-CTs	400, 402
hexa-CTs	436, 438
hepta-CTs	470, 472
octa-CTs	504, 506
nona-CTs	538, 540

2.3. Genotoxicity

A positive genotoxic response in the Mutatox® assay can be caused by five different modes of action (33): base substitution, frame-shift agents, DNA-damaging agents, DNA synthesis inhibitors, and DNA-intercalating agents. 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 the PCT mixture A5442 was evaluated. According to the protocol of the Mutatox® test (34), 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. .

PCT's were tested at an initial concentration of 89 µg/ml. Of the initial concentration, a series of nine serial 1:1 dilutions with growth medium was made. Thus, the minimum concentration tested was always 1/29=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 (35).

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 EROD assays

The protein concentrations and the EROD activities of the used samples are shown in **table 3.1**. Since all values were above the minimum required activity of 10 pmol• mg^{-1} protein • min^{-1}, all microsomal preparations could be used in the *in vitro* assays.

Table 3.1: *EROD activities of the microsomal preparations used for the in vitro biotransformation assays.*

Species	NIOZ code	Protein conc. (mg/ml)	EROD activity (pmol• mg^{-1} protein• min^{-1})
Harbour seal	96PVd2	56.2	233.5
	96PVd3	32.6	451.8
Whitebeaked dolphin	95LAa3	57.2	48.9
Sperm whale	95PMb3	11.3	41.9
Eider duck	94SM82	24.9	13.0
	94SM62	30.3	30.4

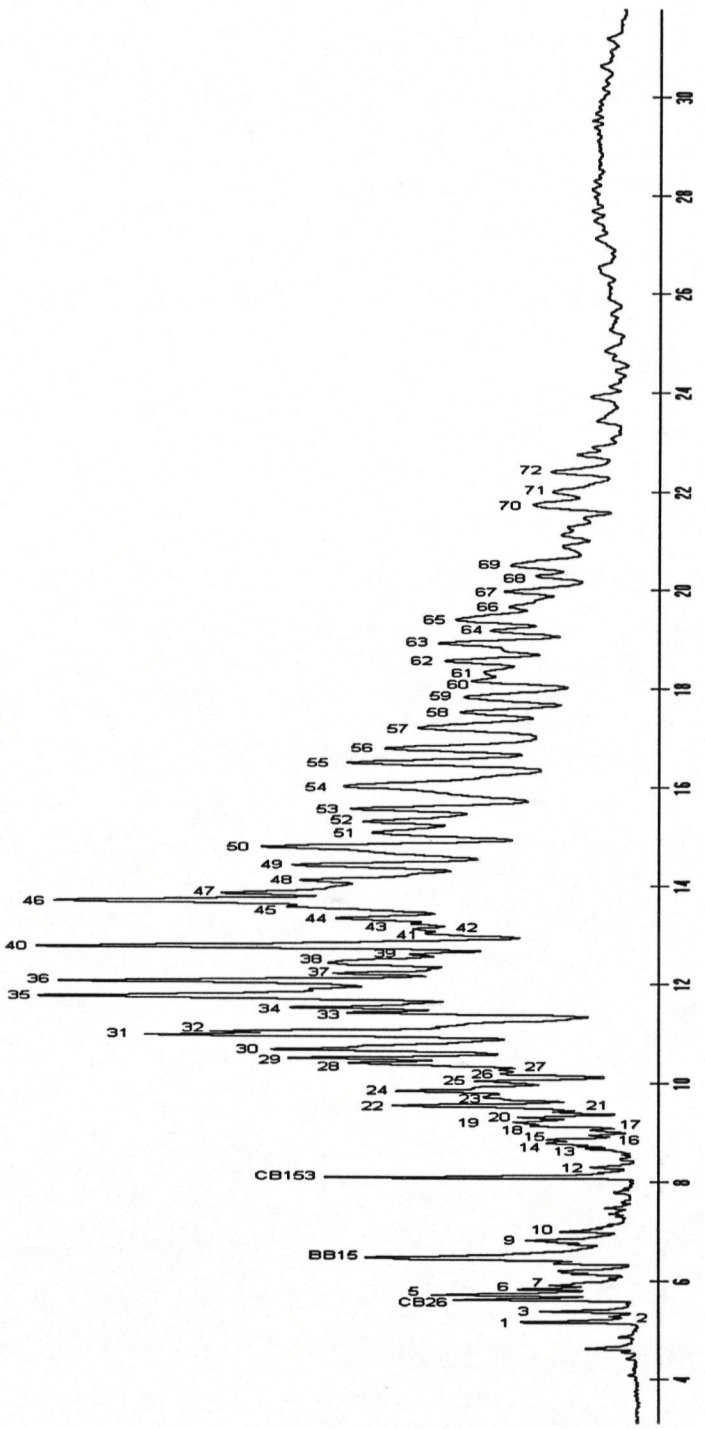
3.2. *In vitro* assays

3.2.2. PCB contamination of the A5442 mixture

The peaks with a height above 10 units in the ECD chromatograms were numbered in order of elution from 1 to 72 (**fig. I**). Peaks with a height below 10 units were discarded. Peak numbers 4, 8, and 11 were caused by the individually added compounds CB-26, BB-15, and CB-153 respectively. The ECD chromatograms of a reference and an assay sample are shown in **figure II.**

The standard PCT mixture used (Aroclor5442) is a technical mixture. Since this means that it may contain impurities, the retention times of peaks was compared with those of PCBs by GC-ECD, and the masses were examined by GC-NCI-MS. The results showed that the Aroclor5442 mixture was contaminated with PCBs. The final identification of these PCBs was based on comparison of their retention times and the masses of their molecular ions with an external standard mixture. For these

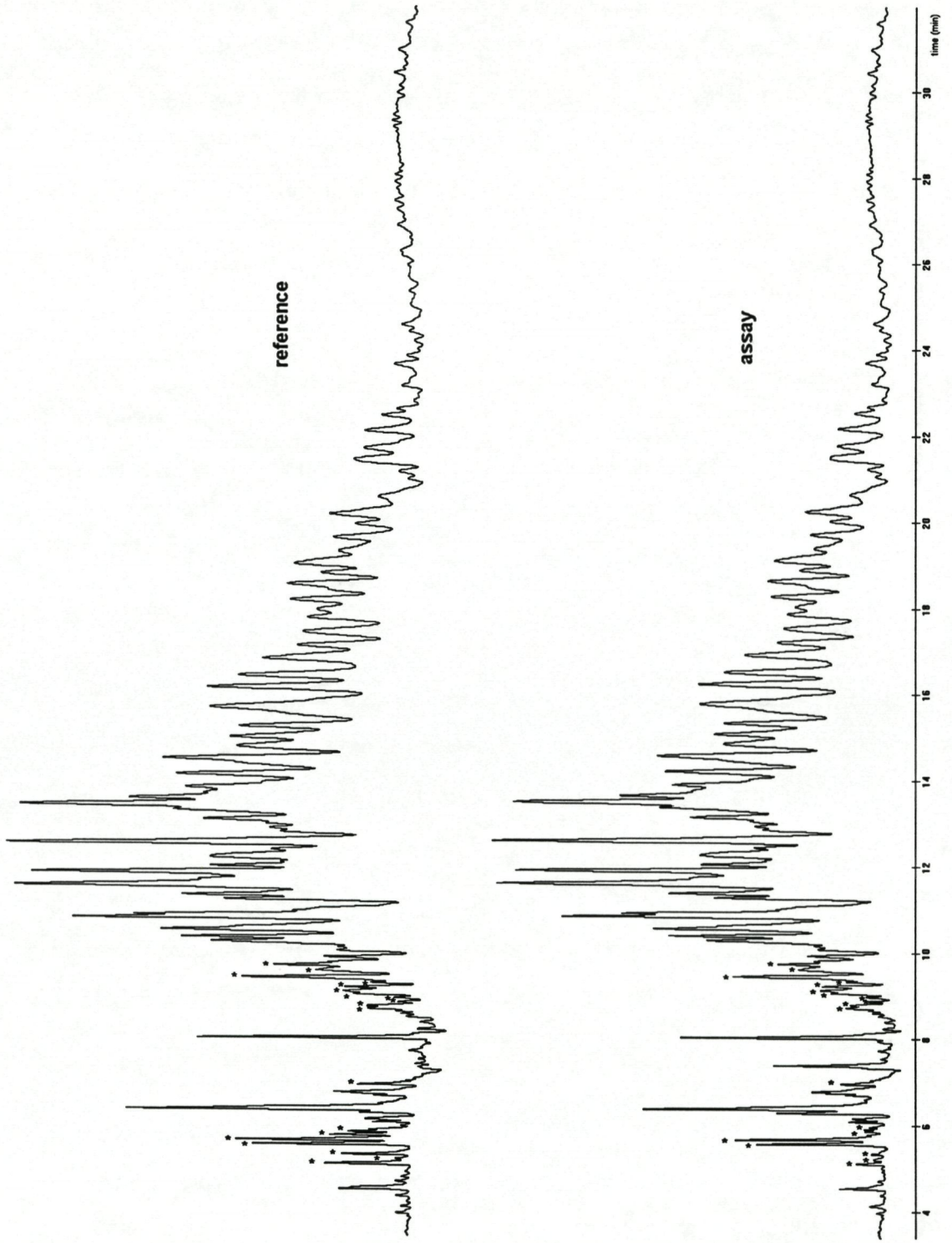
Figure I: The ECD chromatogram of the standard mixture containing the PCT mixture Aroclor 5442, CB-26 and BB-15 as positive controls, and CB-153 as internal standard.



The numbers in the chromatogram represent the peaks investigated during this assay (peaks 1-11 are PCBs and 12-72 are the actual PCTs).

Figure II: ECD chromatograms of a reference and an assay sample of the first assay of the common eider duck. (ba5973wl (ref) en ba5983wl (assay))

*The peaks tagged with a * were metabolised significantly during the in-vitro assay.*



chromatograms a CP-Sil-8 column with a smaller diameter was used (50 m • 0.25 mm • 0.25 μm). **Table 3.2** shows which peak numbers were found to correspond with particular PCB congeners. Compounds that showed low peak heights in the mass spectra, are left unassigned in **table 3.2.** In **figure III**, the ECD chromatogram of a PCB standard mixture is compared to A5442. It shows that the early eluting peaks in A5442 are caused by PCBs. This was confirmed by GC-NCI-MS analyses. Hence, peak numbers 1-11 were assigned to PCBs (except peak 8 which is BB-15), and peak numbers 12-72 were assigned to PCTs.

3.2.2 Quantification of PCTs

Because of the very large number of PCT congeners in the technical mixture, very complex chromatograms were obtained. It was impossible to obtain a complete separation of the peaks. In order to minimise the error in quantification as much as possible, the baseline was drawn manually between the valleys.

3.2.3. In vitro biotransformation.

The differences between the groups of reference and assay samples (n=4) were statistically compared with the use of a Student's t-test on the log-transformed values of $Ratio^{X}_{CB-153}$. The results for the different animals are shown in **figure IV**. The peak of which the ^{10}log of the $Ratio^{X}_{CB-153}$ were significantly lower in the assay samples than in the reference samples ($P<0.05$), are indicated by a darker pattern. Peaks that did not show significant differences between the assay and reference groups of all animals tested, have been omitted from figure IV. These peaks have the numbers 12, 13, 25, 26, 27, 29, 30, 32, 34-37, 39-43, 45-47, 49, 51-54, 57-69 and 72.

3.2.3.1. Positive controls

CB-26 and BB-15 were added as positive controls of which significant biotransformation was expected on the basis of earlier results. BB-15 was metabolised by the microsomes of all marine mammals, but not by those of the eider duck. In contrast, CB-26 was only metabolised to a significant extent by the microsomes of harbour seal and eider duck. The fact that the common eider duck could not metabolise BB-15 to a significant extent and that the whitebeaked dolphin and the

Figure III: ECD chromatograms of a standard PCB and PCT mixture. (ba6418cf (PCBs) and ba6434cf (PCTs)).

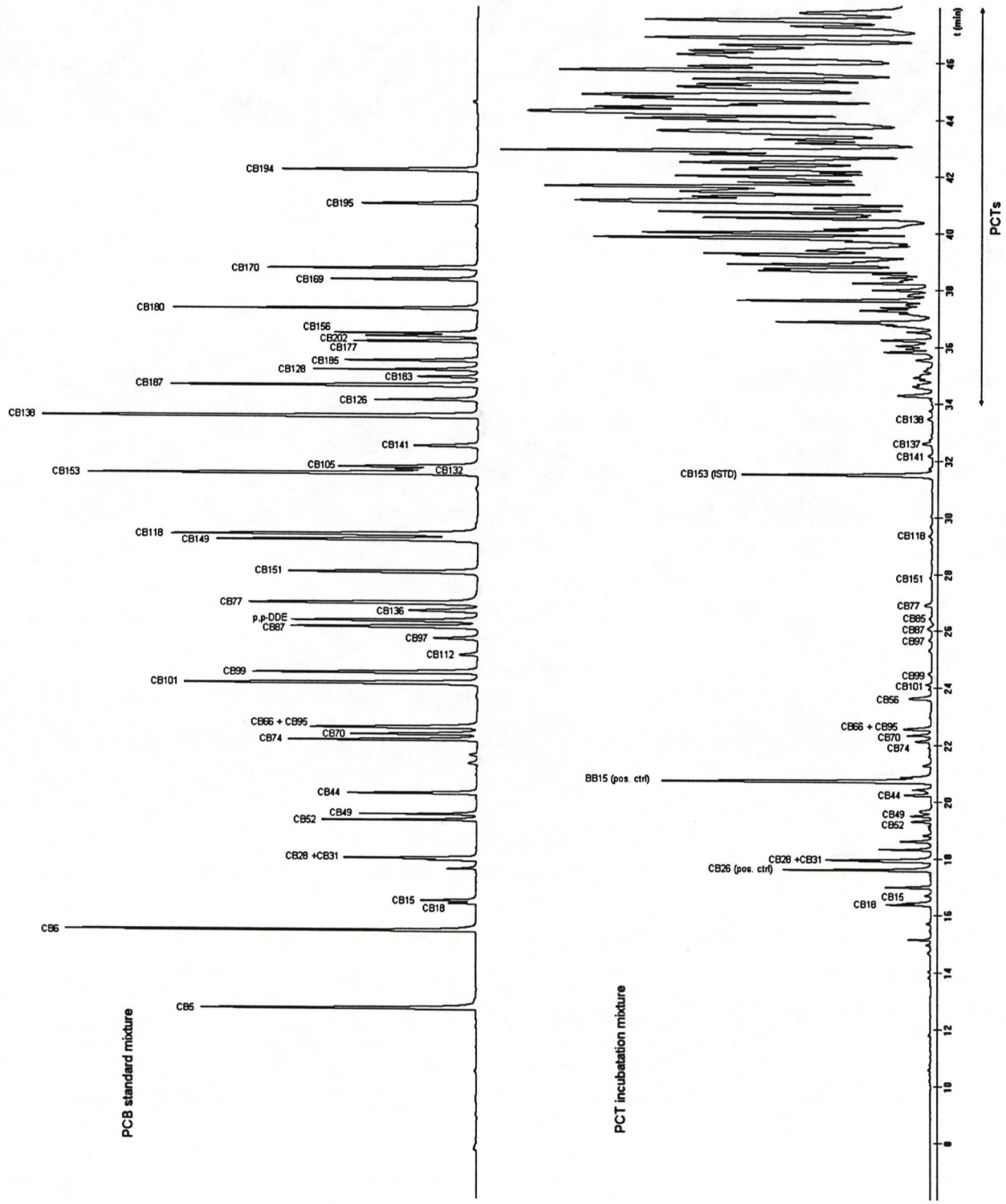
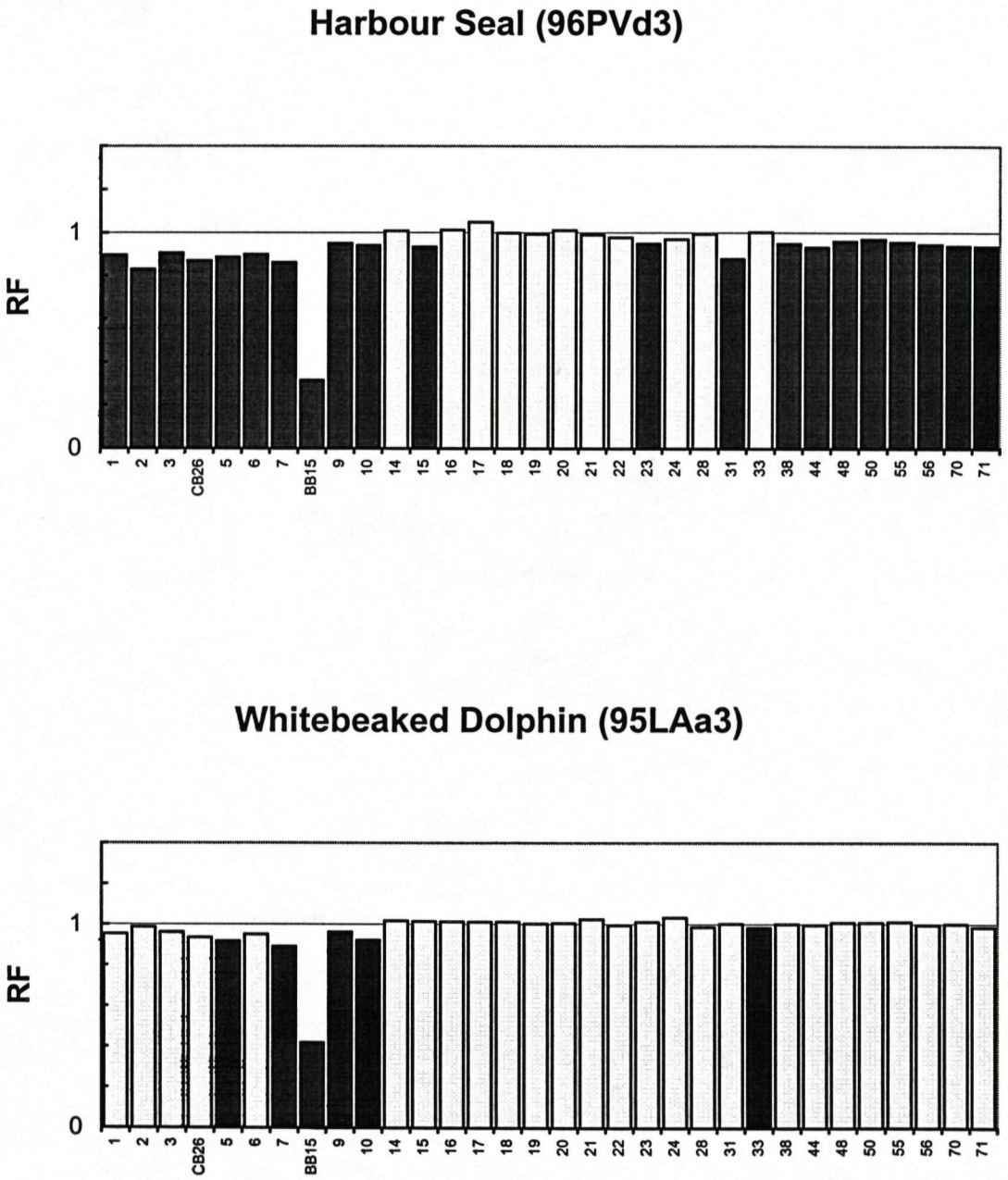
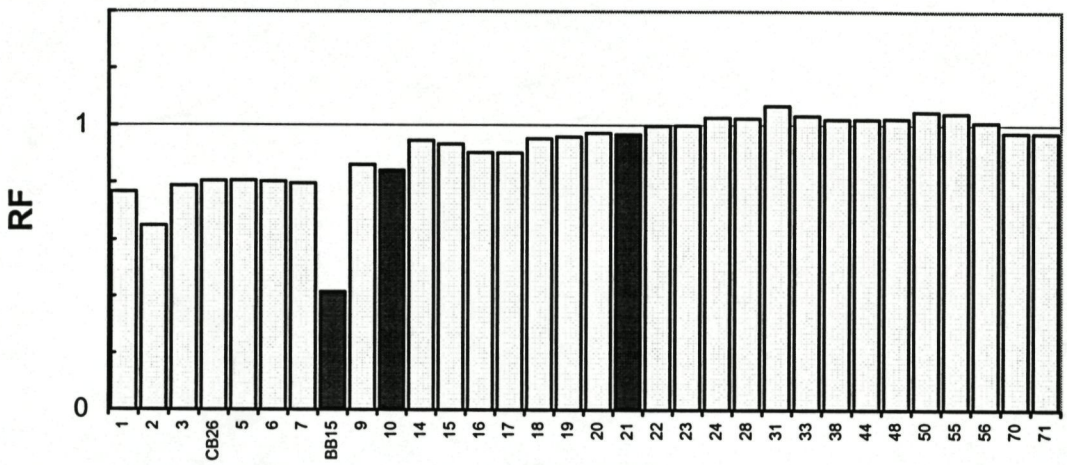
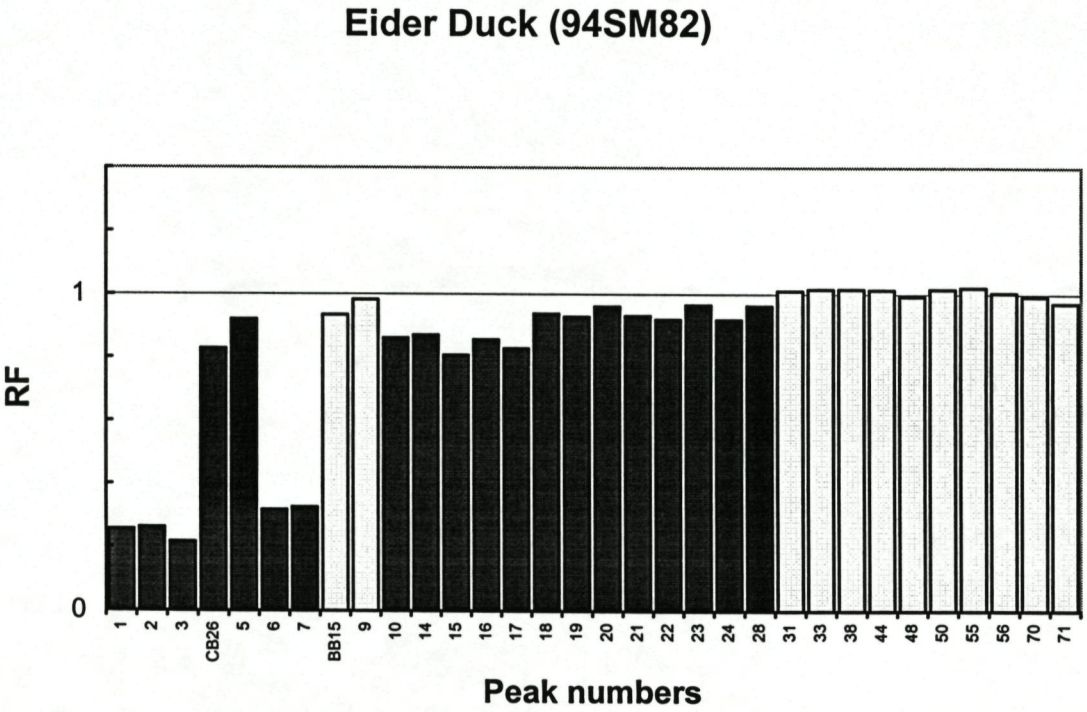


Figure IV: Histograms of the remaining fractions (RF) after allowing for biotransformation of the different peaks of the Aroclor 5442 (PCT) mixture by marine mammals and a seabird. Peaks 1-10 are PCBs (see also table 3.2); peaks 14-71 are PCTs. CB-26 and BB-15 were added as positive controls.




Sperm Whale (95PMb3)
RF
1
0
1
2
3
CB26
5
6
7
BB15
9
10
14
15
16
17
18
19
20
21
22
23
24
28
31
33
38
44
48
50
55
56
70
71


Eider Duck (94SM82)
RF
1
0
1
2
3
CB26
5
6
7
BB15
9
10
14
15
16
17
18
19
20
21
22
23
24
28
31
33
38
44
48
50
55
56
70
71
Peak numbers

sperm whale did not significantly metabolise CB-26 significantly, was also observed in earlier studies with these microsomes (4). This means that metabolism as slow as that of CB-26 will have remained undetected in the assays. However, the fact that at least one of the compounds added as positive controls was metabolised, confirmed that the cytochrome P450 complex of all preparations was still able to catalyse biotransformation reactions.

The addition of compounds as positive controls proved effective during an early assay with microsomes of harbour seal 96PVb. Because CB-26 and BB-15 were not metabolised during the assay, the results were rejected.

3.2.3.2. PCBs.

The PCB congeners are given as peaks 1-3, 5-7, 9 and 10 in **figure IV**. All PCBs were metabolised by harbour seal microsomes. All PCBs except peak 9 (CB-70), were metabolised by the eider duck microsomes. Earlier studies with PCBs have shown that harbour seal, harbour porpoise, and eider duck should be able to metabolise all these congeners, or, in case of co-eluting compounds, at least one of the congeners contributing to the peak (**Table 3.2**).

Remarkable is the inability of the sperm whale to metabolise all PCB peaks except peak no. 10 (CBs -66 + -95). This may in part be due to the inhomogeneity of the microsomes, which caused larger standard deviations. The percentages of decrease of the congeners that were significantly metabolised, are shown in **figure V**.

3.2.3.3. PCTs.

The peak numbers of the PCTs that were significantly metabolised by the microsomal preparations of the different animals, are given in **table 3.3**. In both cetaceans, the $Ratio^{X}_{CB-153}$ of only one peak was decreased significantly. Thus, the whitebeaked dolphin and the sperm whale were almost incapable of metabolising any PCTs. In harbour seal and eider duck, 11 and 12 peaks were significantly lower in the assay samples. Only three peaks were significantly metabolised by more than one species. The eider duck metabolised more of the early eluting PCTs, whereas the harbour seal metabolised a wider range of PCTs congeners (early as well as later eluting compounds).

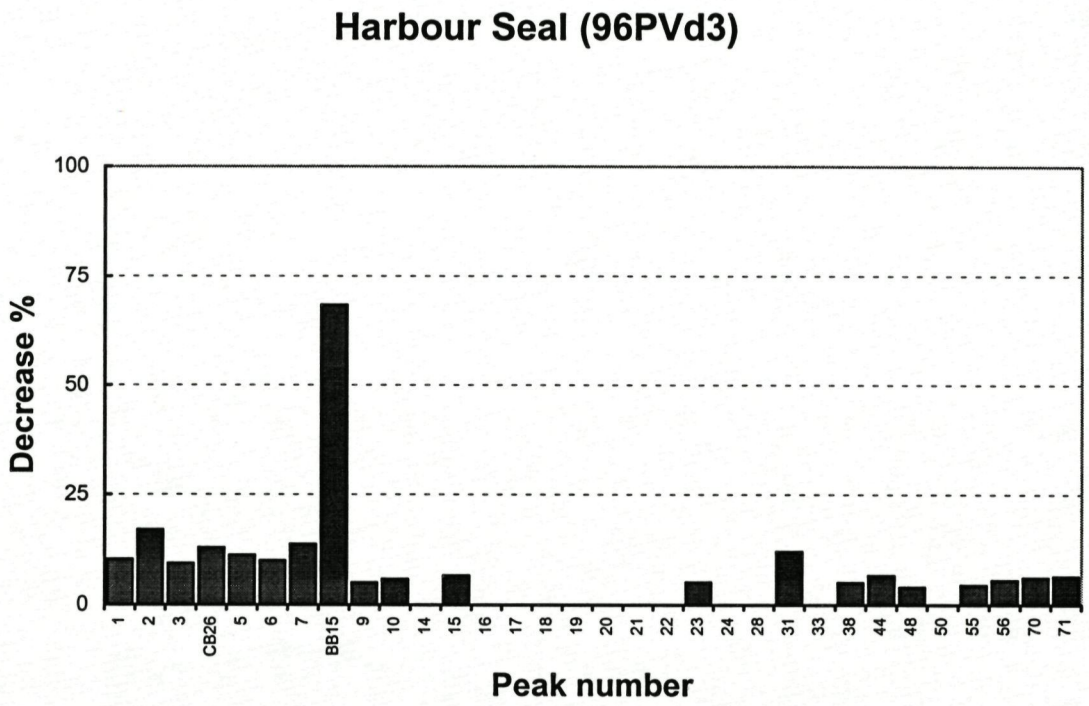
TABLE 3.2: *Identification and structural classification of halogenated biphenyls corresponding to peak numbers 1-10 in the chromatogram of A5442 with respect to phase I metabolism in harbour porpoise (HP), harbour seal (HS) and eider duck (ED). References for metabolism of PCBs: (14, 22, 36). Results on metabolism of BB-15 are taken from this study*

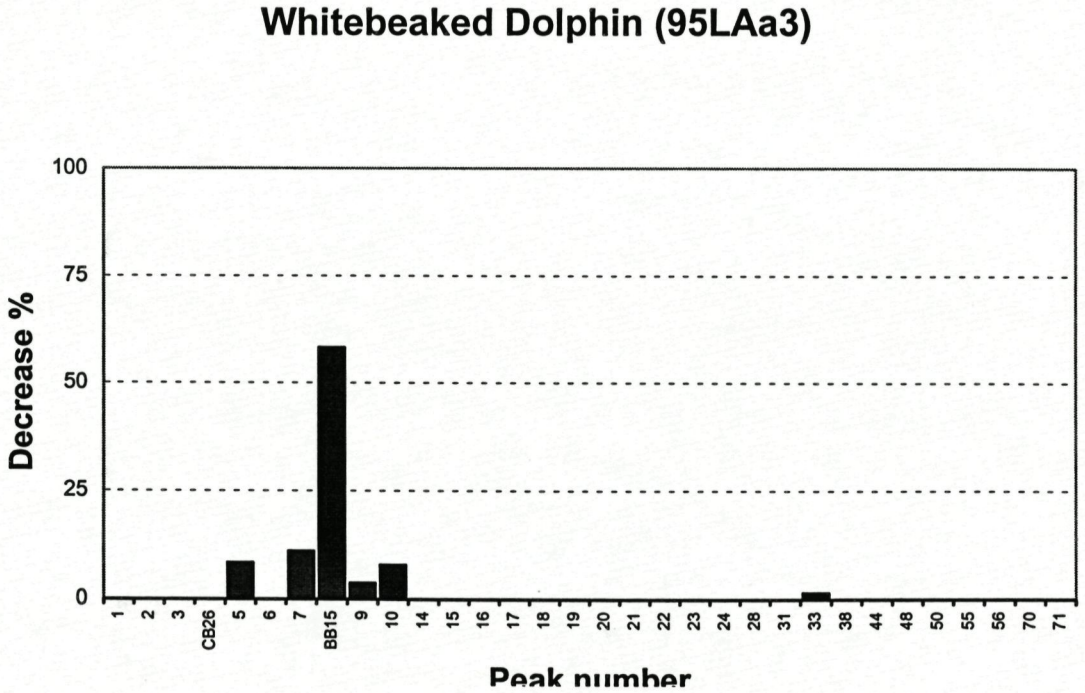
Peak no.	Syst. no.	Halogen substitution pattern	No. *ortho* Cl atoms	Position vicinal H-atoms	Metabolism by
1	CB-18	2,2',5-trichloro	2	*m,p/o,m*	*HP,HS,ED*
2	Unassigned	-	-	-	
3	Unassigned	-	-	-	
4	CB-26	2,3',5-trichloro	1	*m,p/o,m*	*HP,HS,ED*
5	CB-28	2,4,4'-trichloro	1	*o,m*	*HP,HS/*
	CB-31	2,4',5-trichloro	1	*m,p/o,m*	*HP,HS,ED*
6	Unassigned	-	-	-	
7	Unassigned	-	-	-	
8	BB-15	4,4'-dibromo	0	*o,m*	*this study*
9	CB-70	2,3',4',5-tetrachloro	1	*m,p/o,m*	*HP,HS,ED*
10	CB-66 +	2,3',4,4'-tetrachloro	1	*o,m*	*HP,HS/*
	CB-95	2,2',3,5',6-pentachloro	3	*m,p*	*HS,ED*

m,p = meta-para unsubstituted vicinal hydrogen atoms

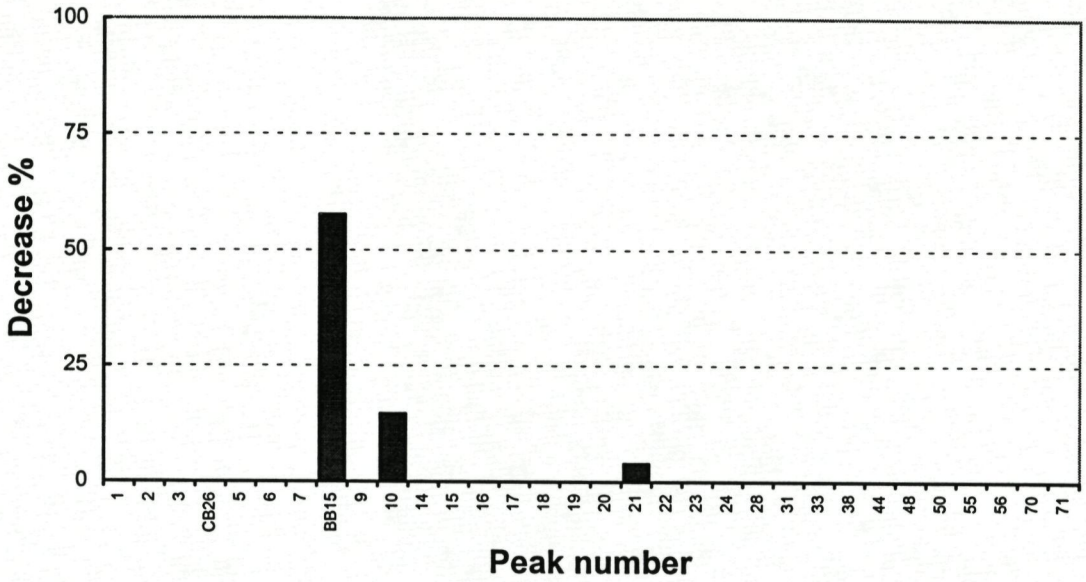
o,m = ortho-meta unsubstituted vicinal hydrogen atoms

Figure V: Histograms of the percentage decrease of the peaks of figure IV that were metabolised to a significant extent. Peaks 1-10 represent PCBs that were present in the A5442 mixture, whereas peaks14-71 represent the actual PCTs. CB-26 and BB-15 were added as positive controls.

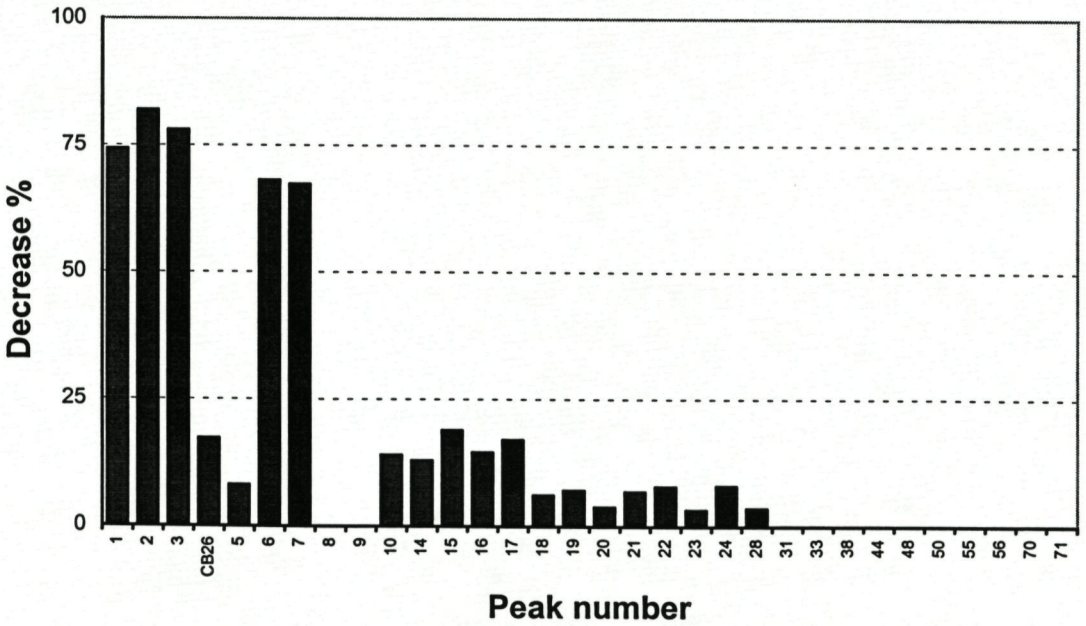




Sperm Whale (95PMb3)



Eider Duck (94SM82)



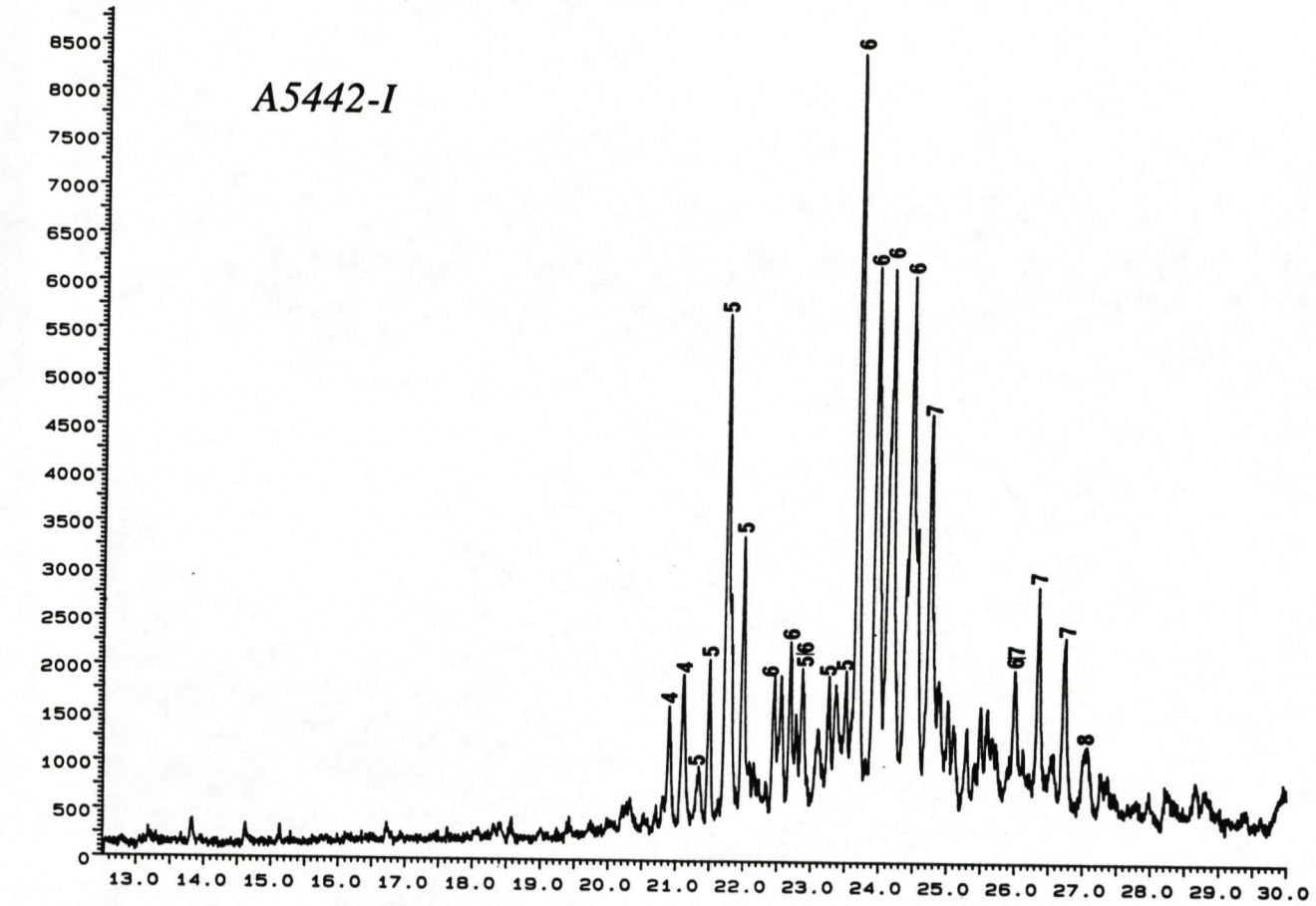
Figures IV and **V** show, that the fraction remaining after incubation in the assay samples was always more than 75% of the reference samples. In harbour seal and eider duck, the PCBs present in A 5442 were metabolised to a larger extent than the PCTs.

***Table 3.3**: The peak numbers which were metabolised significantly by the different animals. Numbers of peaks that were metabolised by more than one animal are given in a boldface font.*

	Metabolised PCT peak numbers
Sperm whale	**21**
Whitebeaked dolphin	33
Harbour seal	**15, 23**, 31, 38, 44, 48, 50, 55, 56, 70 and 71
Common eider duck	14, **15**, 16, 17, 18, 19, 20, **21**, 22, **23**, 24 and 28

3.3. Wildlife residues

Although the technical mixture A 5442 eluted in both silica fractions, the great majority eluted in the second fraction **(fig. VI)**. The contents given in **table 3.4** are based on the addition of both fractions for each sample. The NCI-MS chromatograms in **figure VII** show only the PCTs in the second silica fractions, which represented more than 80% of the total contents, The plots show, that the PCT peak pattern in wildlife residues is shifted towards higher retention times compared to A5442. Compounds with 5-7 chlorine atoms dominate in the chromatograms of wildlife. Because of this incomplete match of peak patterns, the total PCT contents expressed as A5442 equivalents in **table 3.4** should be regarded as indicative data. It was checked whether peaks with retention times below 22 min. (all PCBs eluted before this time; CB-153 eluted at 13.8 min) were perhaps caused by PCBs instead of PCTs. Remarkably high PCT contents were found in eider duck livers, which even exceeded the PCT concentrations found in cormorant livers (3.4 and 4.0 mg/kg fat, (7)). The different marine mammal species showed a wide range of PCT contents, probably due to differences in habitat, diet, and physiology. However, also within a species the range of concentrations can be quite large, as illustrated by the three samples of



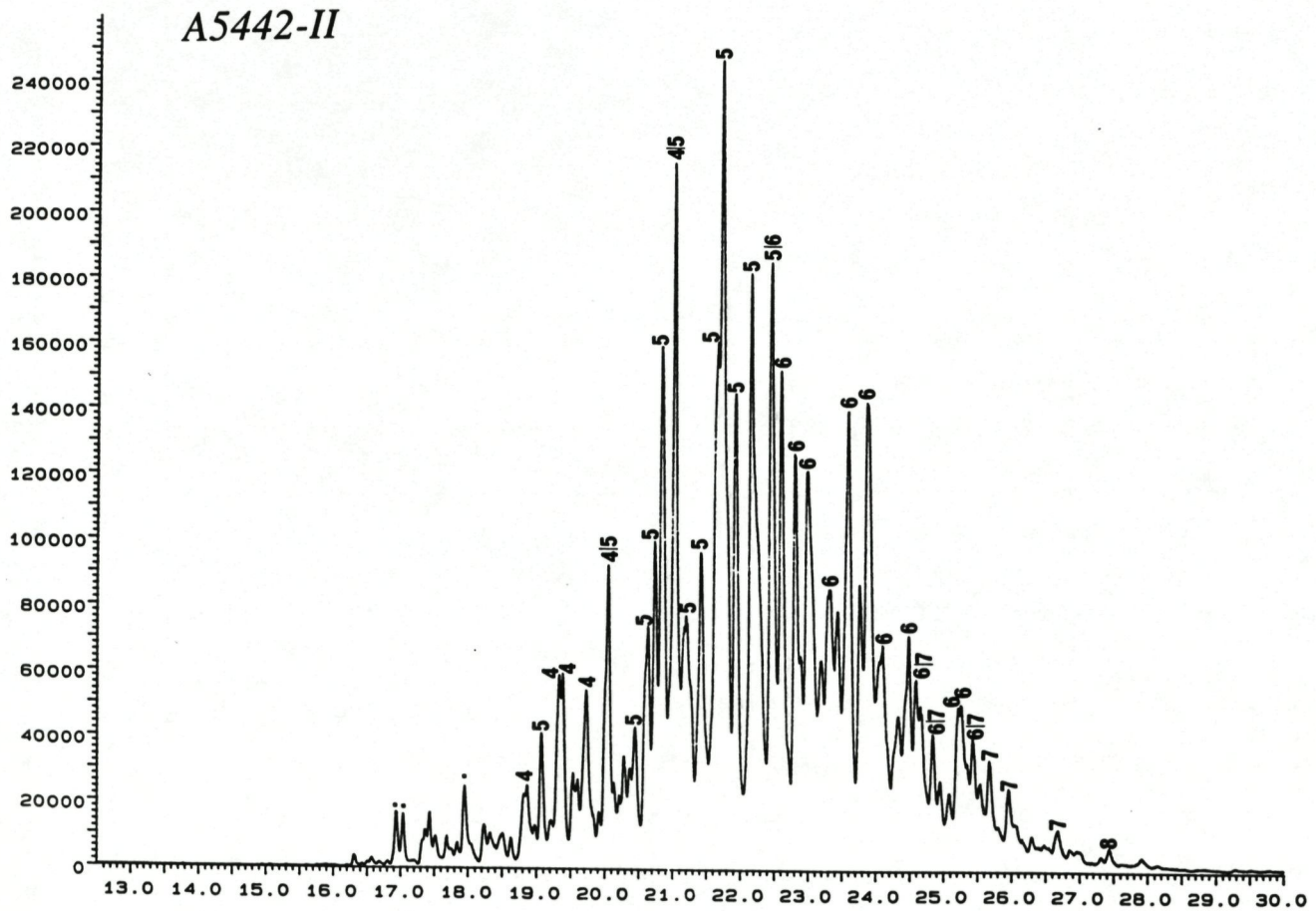


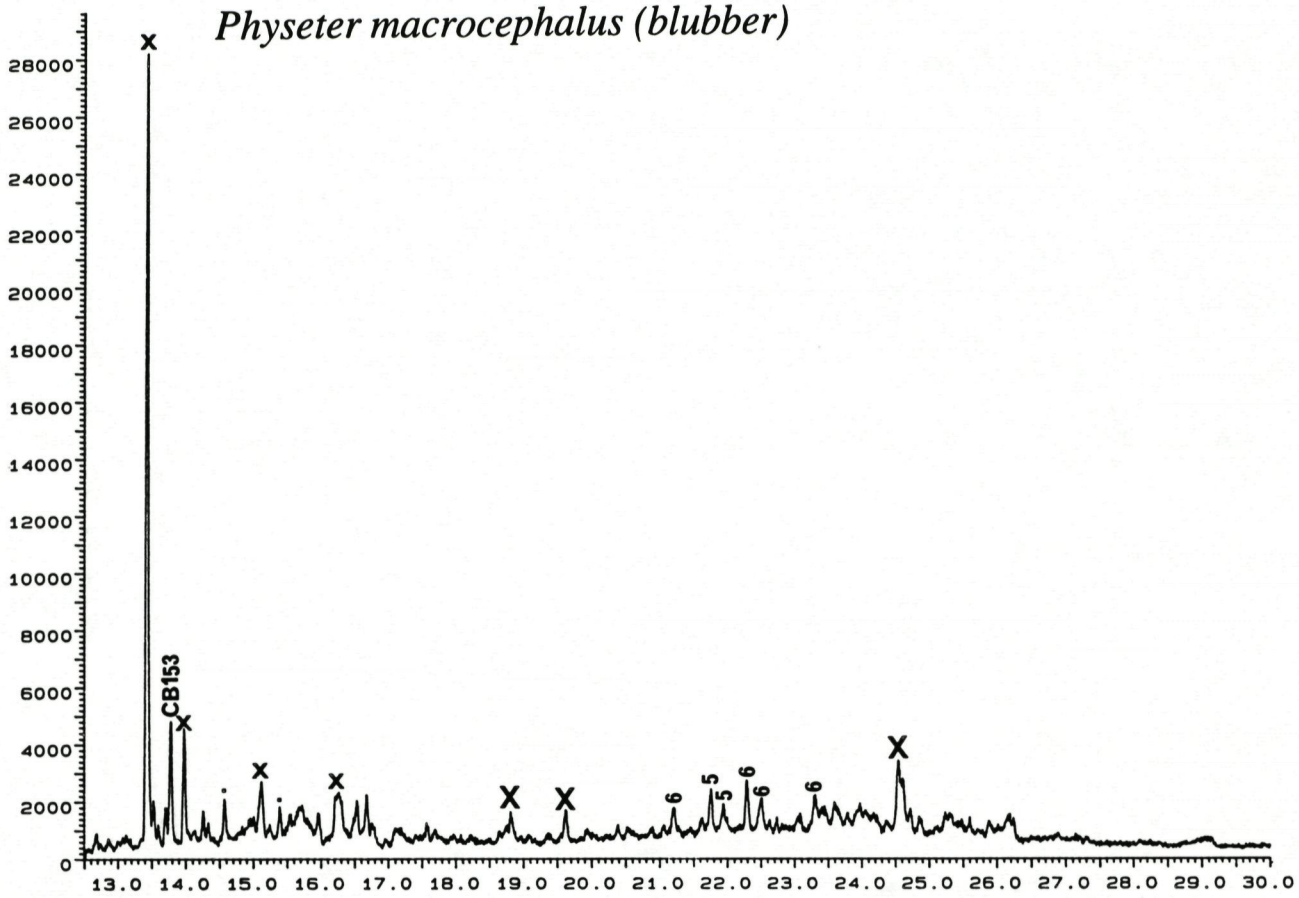
Figure VI: Chromatograms of the first (*A5442-I*) and second (*A5442-II*) silica fractions of Aroclor 5442. The chromatograms contain all peaks of compounds corresponding to the masses selected in the SIM mode of NCI mass-spectrometry. For PCTs, the overall chlorine contents are indicated. Peaks caused by PCBs are indicated with a dot at the

Table 3.4: PCT levels in tissues of marine wildlife, quantified as Aroclor 5442 equivalents.

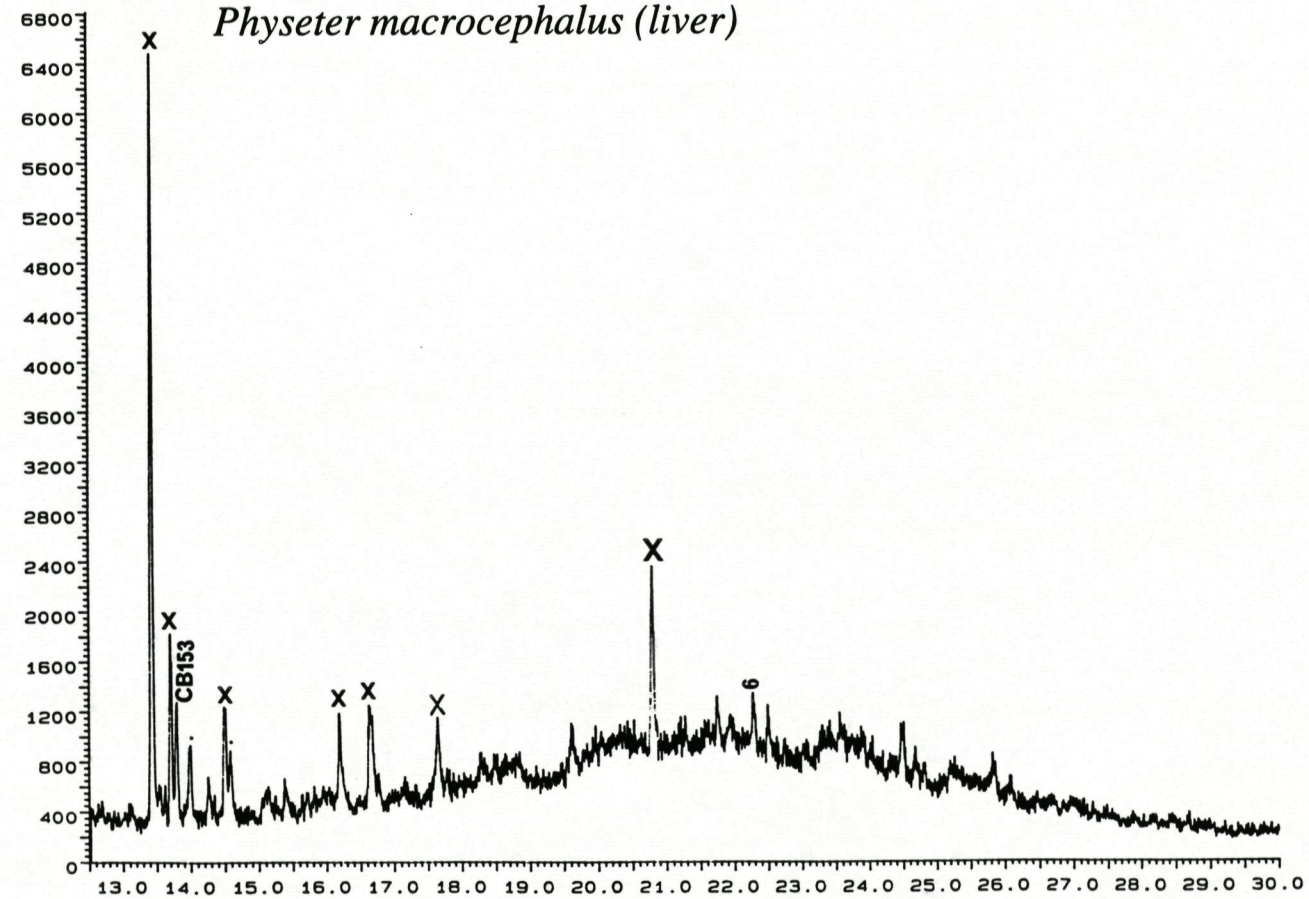
Sample	Tissue	NIOZ #	RIVO LIMS #	[A5442] (µg/kg fat)	[A5442] (µg/kg product)
Toothed whales					
Sperm whale	blubber	95PM3	15870	240	87
Sperm whale	blubber	95PM2	15871	360	92
Sperm whale	liver	95PM2	15872	<530	<21
Sperm whale	blubber	95PM1	15920	390	220
Whitebeaked dolphin	blubber	95 LA1	15926	2900	2800
Whitebeaked dolphin	liver	95 LA1	15927	1600	44
Baleen whale					
Minke whale	blubber	94BA1	15921	270	35
Seals					
Harbour seal	blubber	92PVa	15922	11000	2700
Harbour seal	liver	94PVa	15923	<440	<12
Harbour seal	blubber	96PVa	15928	<150	<130
Harbour seal	liver	96PVa	15929	780	41
Harbour seal	blubber	96PVc	19488	550	400
Harbour seal	liver	96PVc	19489	610	31
Fish					
Mackerel	muscle	-	19490-1	<100	<14
Mackerel	muscle	-	19490-2	<100	<14
Birds					
Eider duck	liver	97SMa	20331	8400	180
Eider duck	liver	96SMa	20332	10000	410
Eider duck	liver	97SMb	20333	28000	860
Eider duck	liver	97SMc	20334	29000	620
Eider duck	liver	97SMd	20335	24000	740

Figure VII: PCTs in different tissues of marine wildlife (9 plots). The chromatograms show all peaks of compounds corresponding to the masses selected in the SIM mode of NCI mass-spectrometry. The total number of chlorine atoms of the CT congeners is indicated. Peaks caused by PCBs are indicated with a dot. Peaks caused by unknown compounds are marked with a cross.

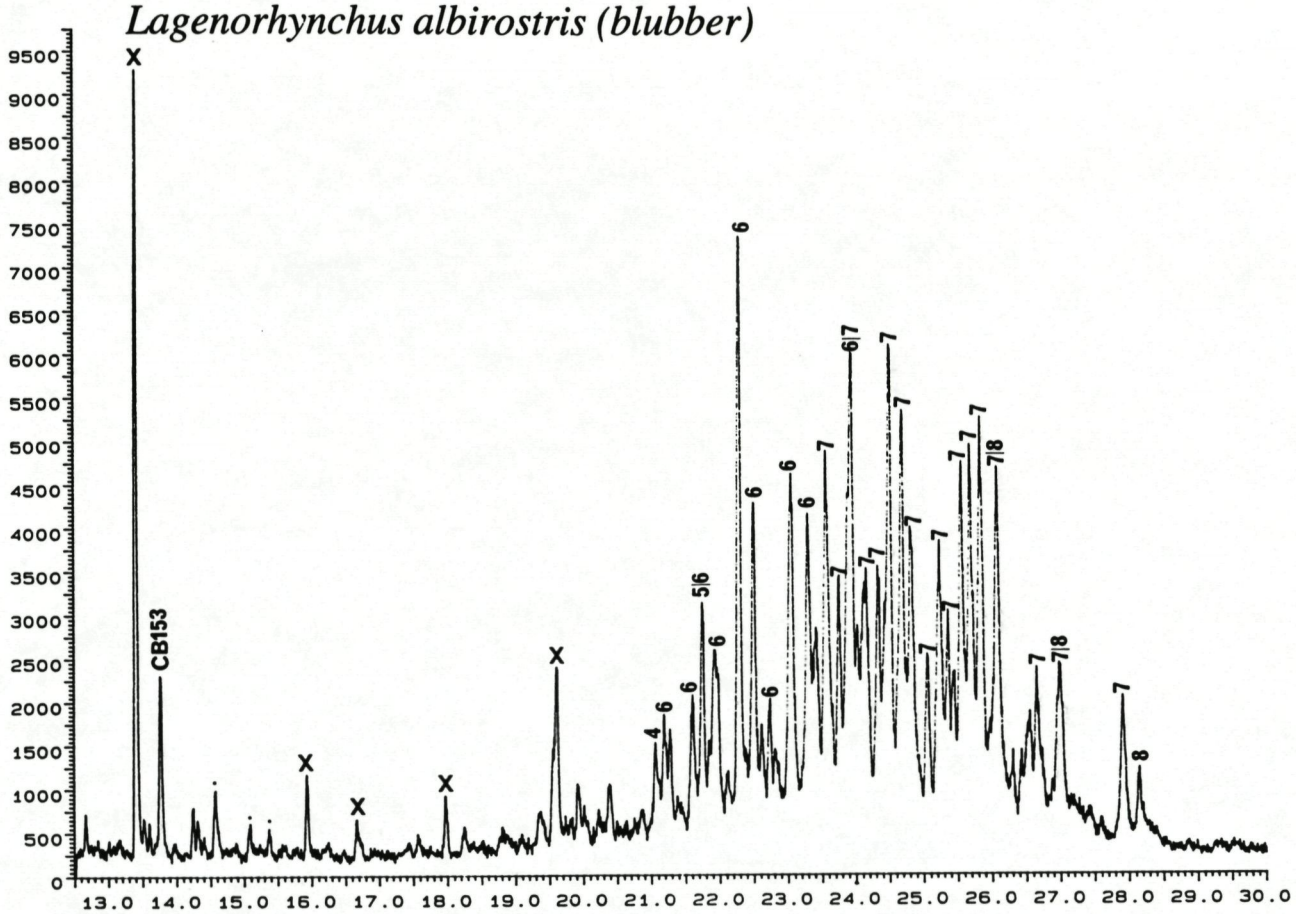
Physeter macrocephalus (blubber)



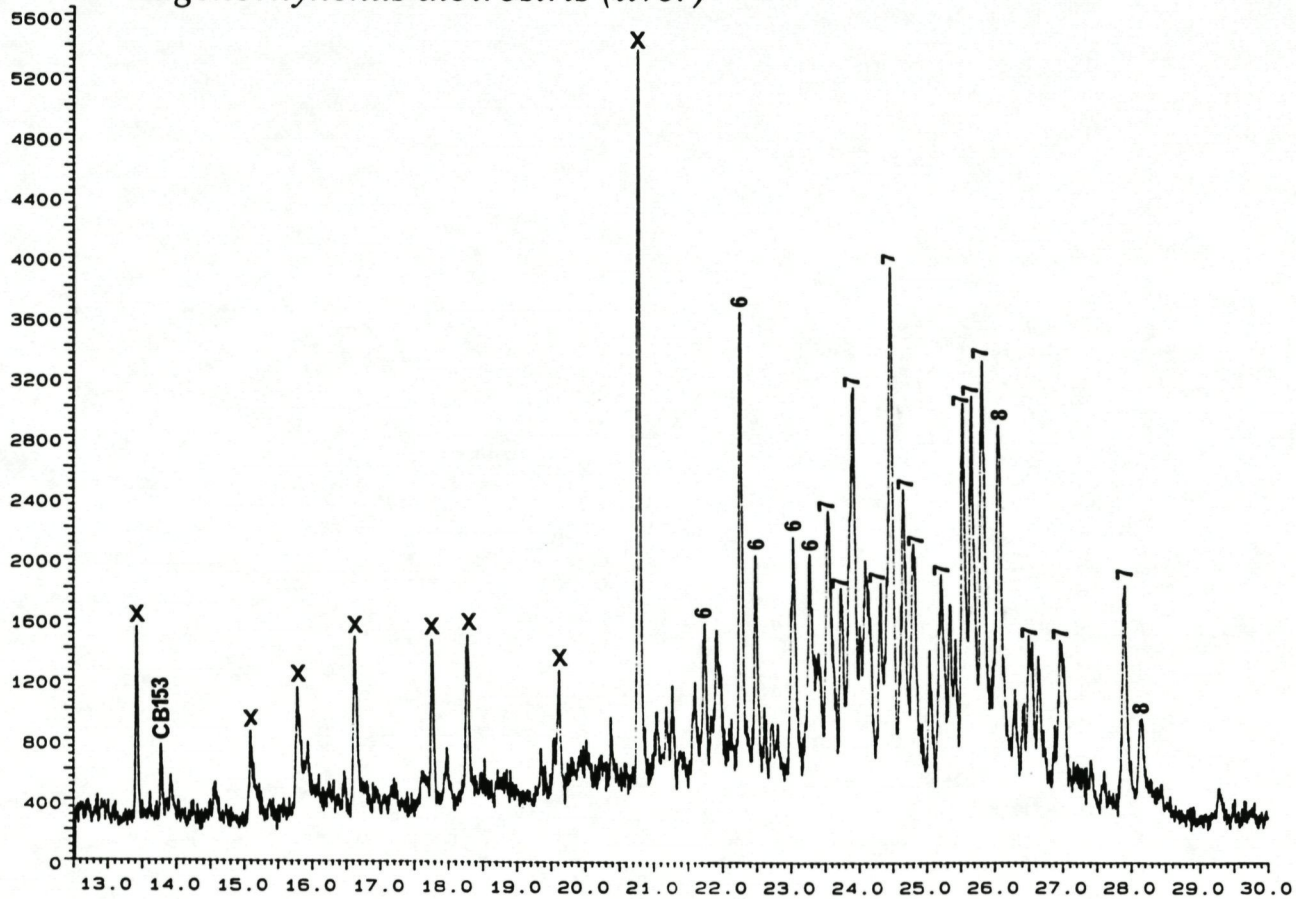
Physeter macrocephalus (liver)

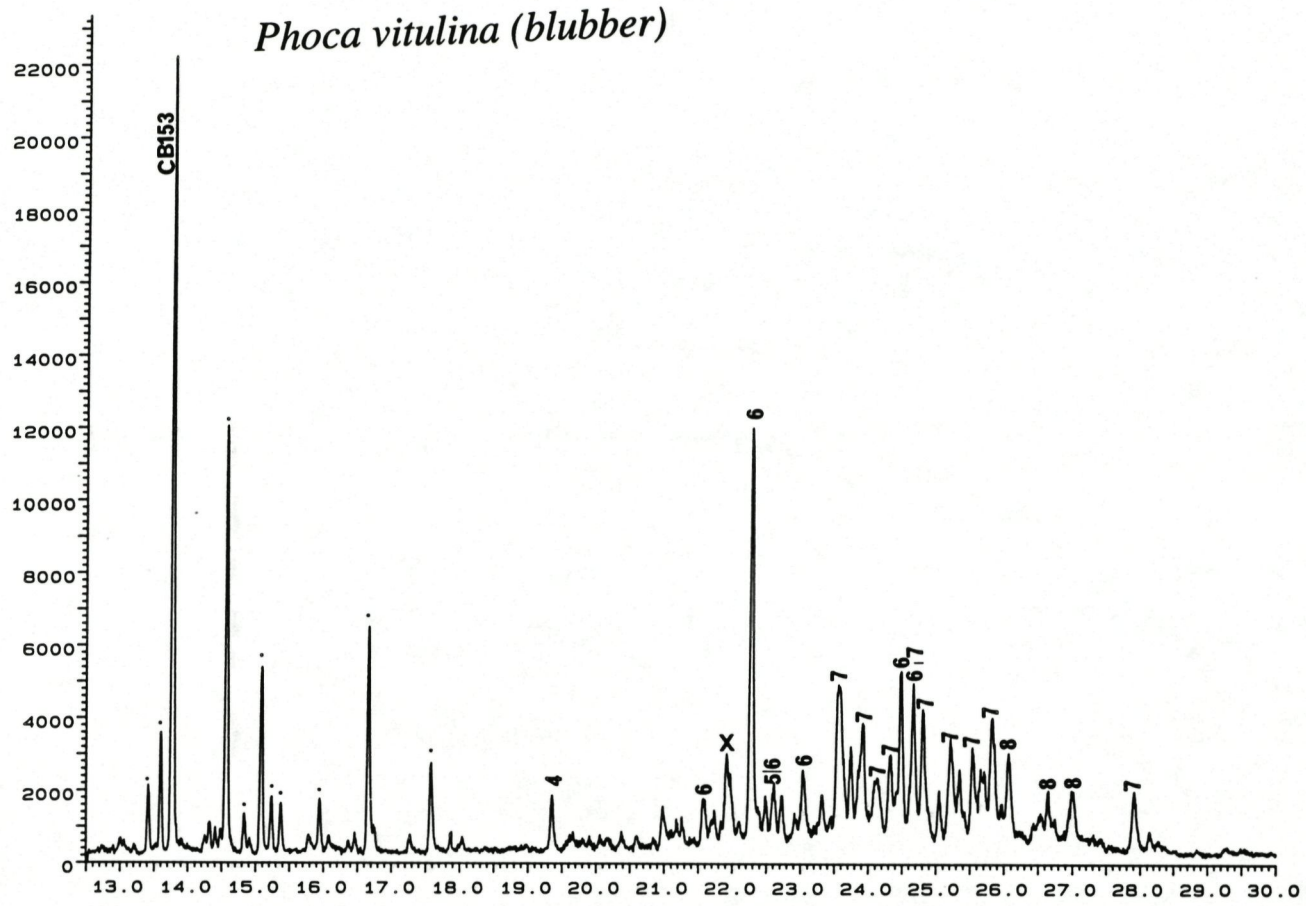


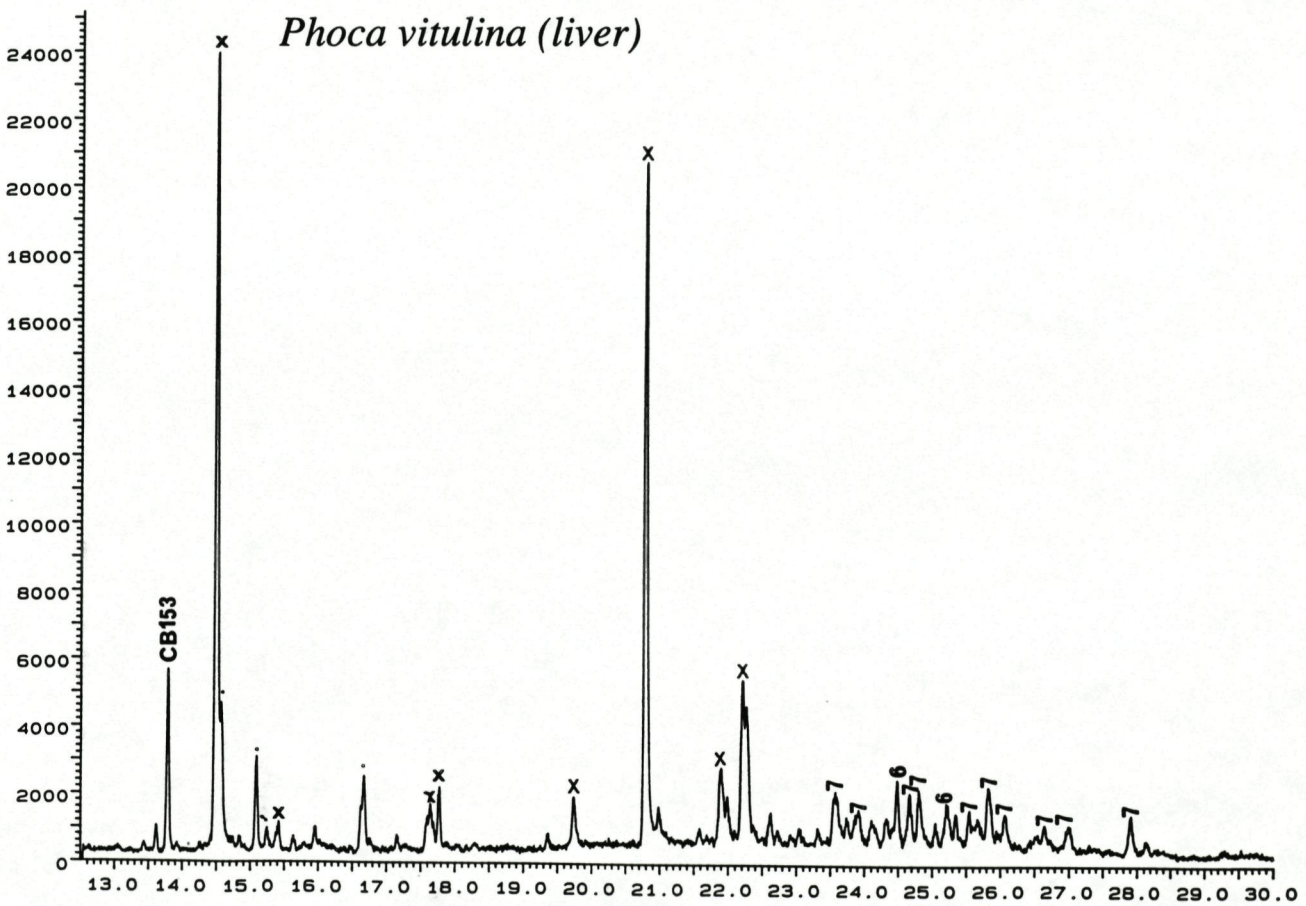
Lagenorhynchus albirostris (blubber)

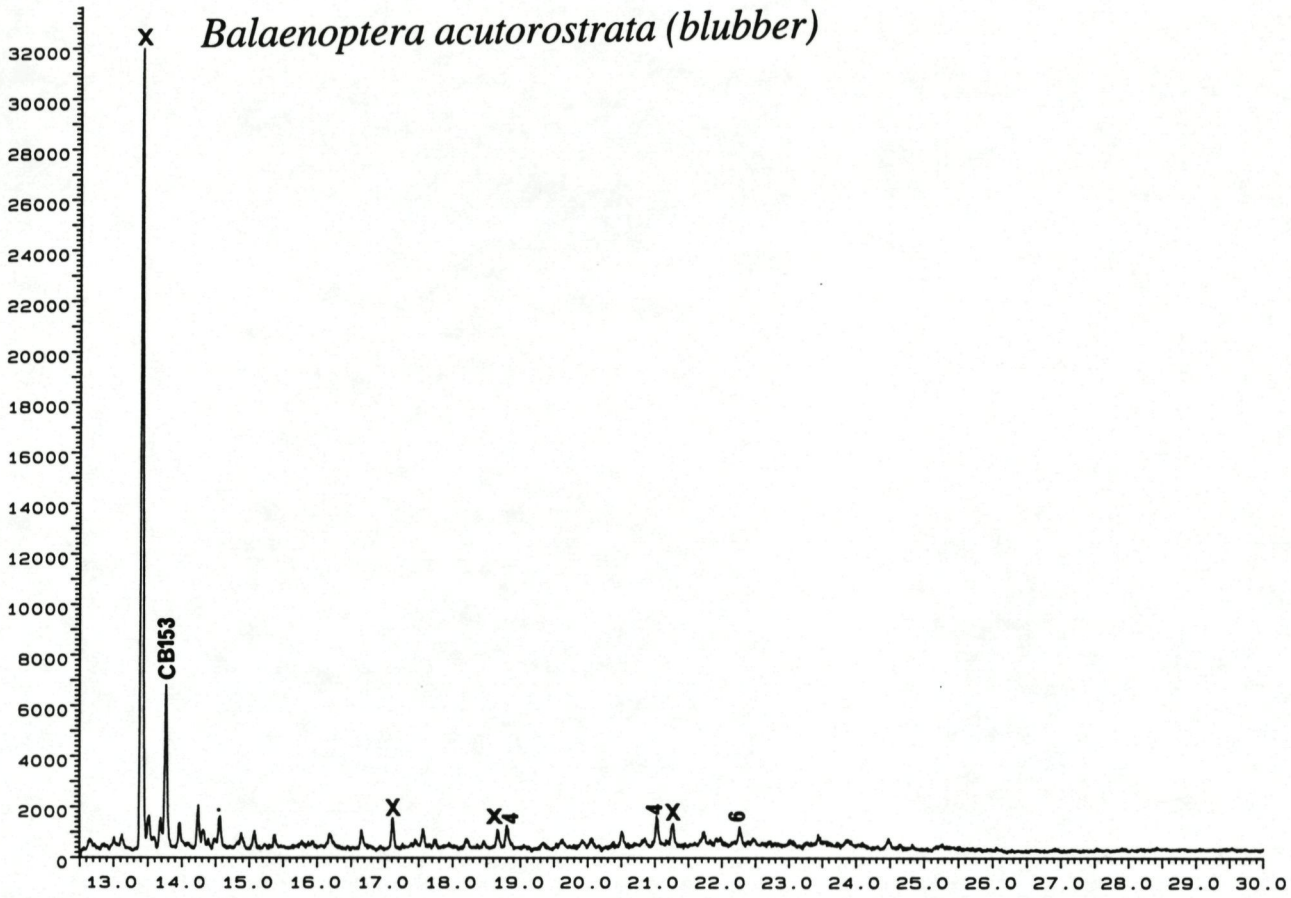


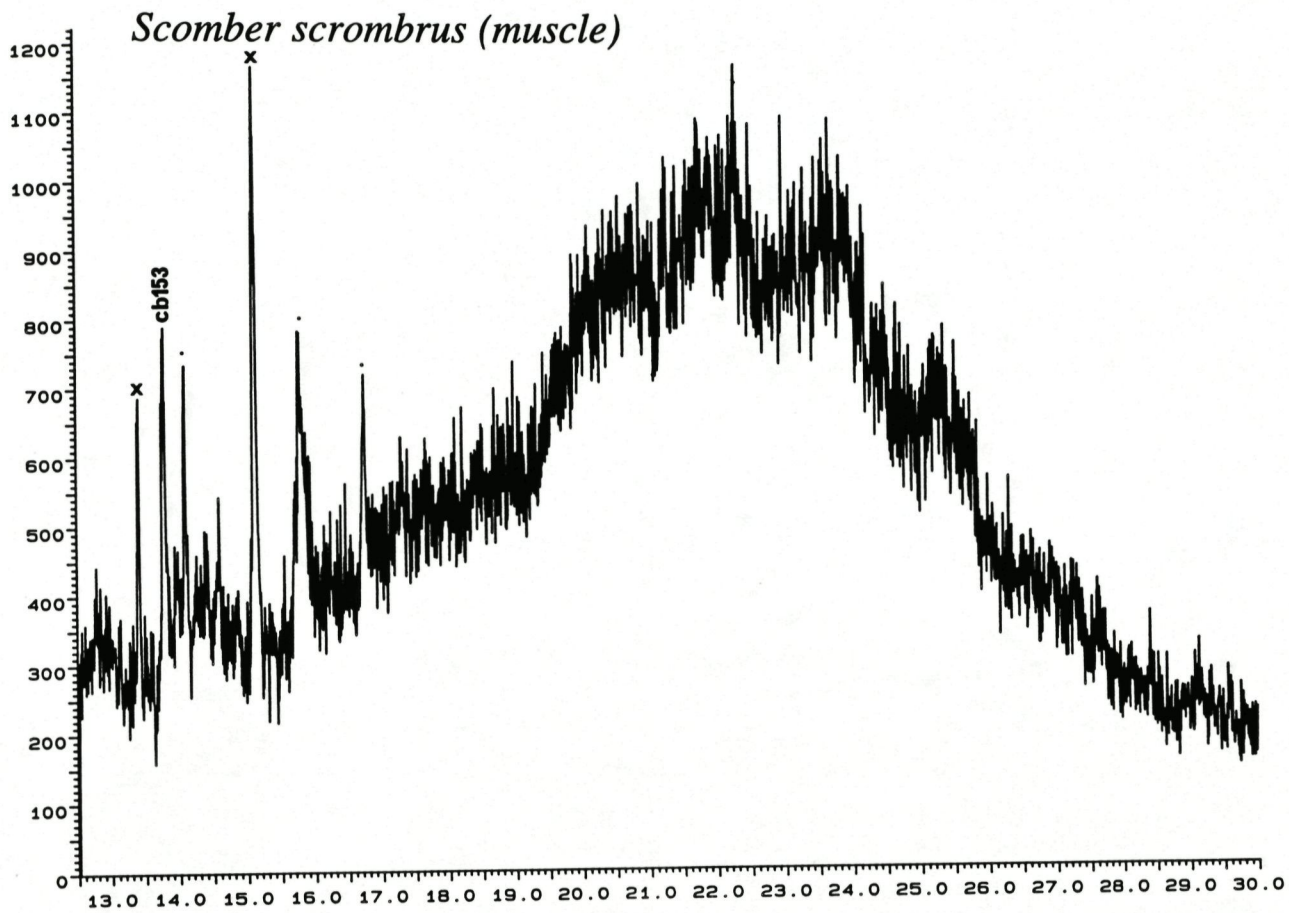
Lagenorhynchus albirostris (liver)

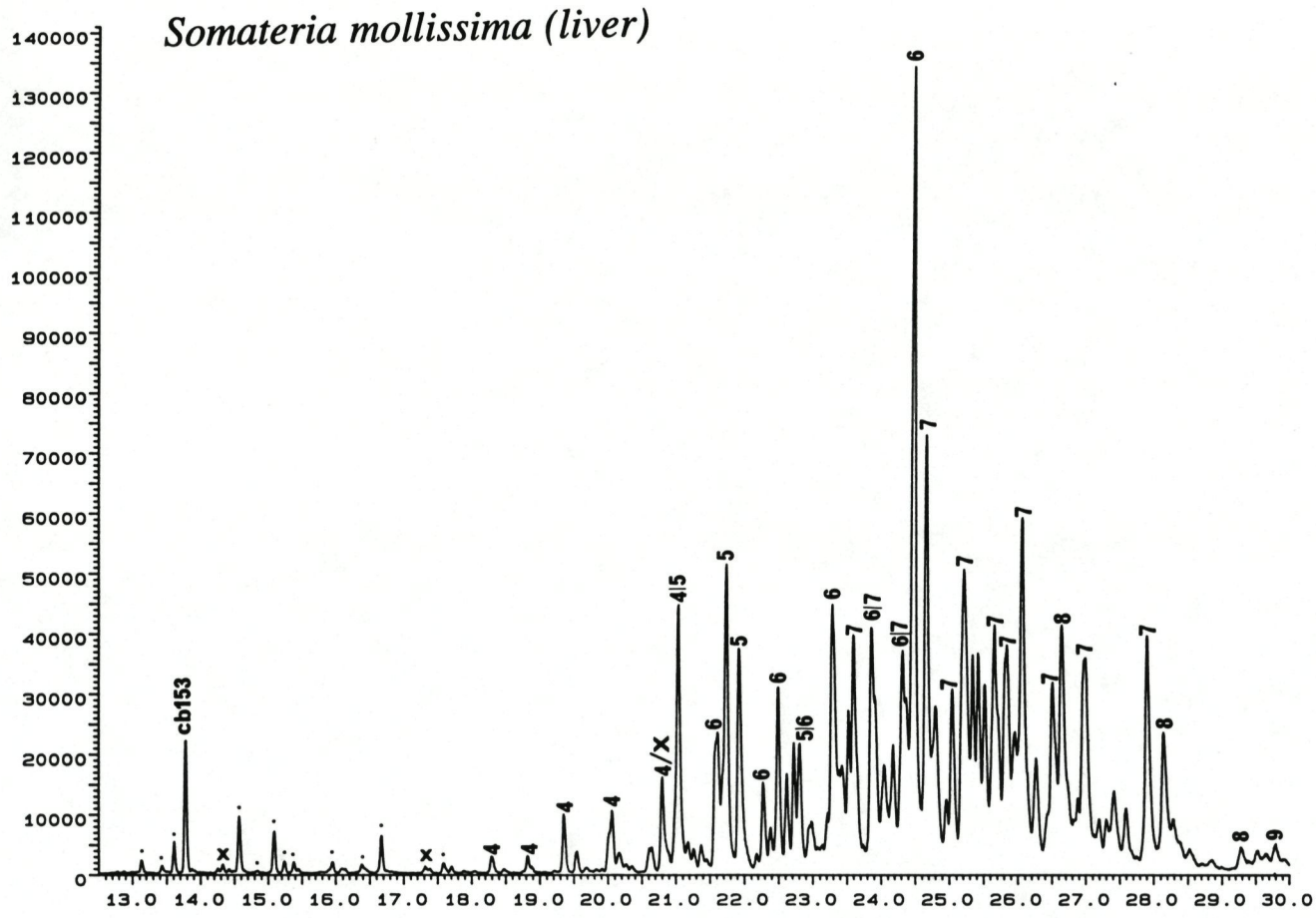



Phoca vitulina (blubber)
CB153
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
13.0 14.0 15.0 16.0 17.0 18.0 19.0 20.0 21.0 22.0 23.0 24.0 25.0 26.0 27.0 28.0 29.0 30.0


Phoca vitulina (liver)
CB153
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
13.0 14.0 15.0 16.0 17.0 18.0 19.0 20.0 21.0 22.0 23.0 24.0 25.0 26.0 27.0 28.0 29.0 30.0


Balaenoptera acutorostrata (blubber)
CB153
32000
30000
28000
26000
24000
22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
13.0 14.0 15.0 16.0 17.0 18.0 19.0 20.0 21.0 22.0 23.0 24.0 25.0 26.0 27.0 28.0 29.0 30.0


Scomber scrombrus (muscle)
cb153
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
13.0 14.0 15.0 16.0 17.0 18.0 19.0 20.0 21.0 22.0 23.0 24.0 25.0 26.0 27.0 28.0 29.0 30.0


Somateria mollissima (liver)
140000
130000
120000
110000
100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
13.0 14.0 15.0 16.0 17.0 18.0 19.0 20.0 21.0 22.0 23.0 24.0 25.0 26.0 27.0 28.0 29.0 30.0
cb153
4/X
4|5
5|6
6|7

harbour seals. The levels in sperm whale are all at the lower end of the concentration range found.

3.4 Genotoxicity

Aroclor 5442 did not give a genotoxic response in the concentration range tested (initial concentration 89 μg/ml). The results of the accompanying positive control tests, with benzo[a]pyrene and phenol, and the blanks are given in **table 3.5**.

***Table 3.5**: Results of the mutatox® assays. Since the assays with the Aroclor 5442 PCT mixture did not yield a positive genotoxic response, only the results of the positive controls, and blank values are given. Bold: Concentration showing the maximum response (CMR).*

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]	**Response**	**[Aceton]**	**Response**
(vol.%)	[a.u.]	(vol.%)	[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

4.1. Pattern recognition

A better separation of the individual peaks could have been obtained with a thinner column (0.25 mm). This can be observed by comparing the chromatograms in **figures I and III**. The advantage of a column with a smaller diameter is that the interfering PCBs are better separated from the PCTs. However, the problem is that the relatively large PCT molecules remain on the column and interfere with the next sample. Therefore, a column with a larger diameter was chosen despite the negative effects on peak separation.

4.2 The biotransformation of PCTs versus PCBs.

From Figure IV can be concluded that the early eluting compounds (the PCBs) are metabolised more than the later eluting compounds (the actual PCTs). Only small amounts of a few PCTs were metabolised. From this it can be concluded that the PCTs are less metabolised than at least some PCBs. This behaviour stimulates bioaccumulation (and biomagnification) of the PCTs. The actual bioaccumulation of PCTs also depends on the uptake efficiency of these relatively large molecules. In comparison to PCBs the location of the third phenyl group could be of great importance for the biotransformation ability; e.g. the molecular diameter of *ortho*, *meta* and *para* PCTs isomers differs considerably. This molecular size could play an important role, since large molecules may be taken up with a lower efficiency.

4.3 PCT residue patterns and levels in wildlife.

Hexa- and hepta-chlorinated terphenyls dominated in wildlife residues of cetaceans, harbour seals, and birds. Compared to Aroclor A5442, with mainly tetra-, penta-, and hexa-chlorinated terphenyls, this represents a shift to higher chlorinated congeners. However, A5442 is not the only possible source for PCTs in the environment. Hale *et al.* (13) detected Aroclor5432 (consisting of predominantly tri- to penta chlorinated terphenyls) in shellfish from an estuarine environment. In the same samples, A5460 (consisting mainly of hepta- to deca-chlorinated terphenyls) could not be identified, even though traces of this mixture could be found in soil samples. This difference in bioaccumulation was attributed to a more rapid passage of the smaller PCT molecules

through the cell membrane. The fact that the most highly chlorinated PCTs of A5460 were not detected in biota, suggests that they are too large to be bioaccumulated. Biotransformation could be another reason for the relatively low presence of tetra- and penta-chloroterphenyls in wildlife tissues. However, in view of the low metabolic rates in the *in vitro* assays, it seems unlikely that this is a major factor.

In contrast to the other species investigated, the sperm whale prefers deep water, but is also encountered near the continental slope, especially in upwelling areas to profit from the richness of food. Near Europe, the animal occurs in the Atlantic Ocean and in the Mediterranean. The animal is only seldom seen in shallow coastal waters. Female specimens do not migrate north of a latitude of 45° N (northern Spain), males occur north as far as northern Norway, Iceland and Greenland. Here, sperm whales hunt in waters with depths of 400-1200 m or more. In most areas the diet consists predominantly of squid. Most species found in sperm whale stomachs are bottom dwelling species or occur in the lower parts of the water column (=mesopelagic). The most important squid species are *Histioteuthis bonellii* and *Taningia danae* (36). It is remarkable that species which are common near the water surface in areas where sperm whales also occur, are apparently not eaten. Fish is usually a minor part of the diet, but near Iceland the animals often eat more fish than squid. Lumpsucker (*Cyclopterus lumpus*), redfish (*Sebastes* sp.), angler fish (*Lophius piscatorius*) and cod (*Gadus morhua*) were the most common species of fish eaten (37). Of these species, the angler fish is more or less confined to the bottom, whereas the other species are most often found near the bottom, but can occasionally also be pelagic. Near Iceland these species can be found down to a depth of at least 400m.

The range of PCT concentrations in marine mammals was ca. One to two orders of magnitude lower than PCB concentrations (15, 38)

4.4 Suggestions for further research

The present study leaves a number of questions, that should preferably be answered in view of the general occurrence of PCTs. For future research, the use of individually synthesized congeners is preferable to the use of technical mixtures. Differences between the three main molecular types of PCTs (*ortho-, meta-* or *para-* PCTs)

deserve first attention. Multi-dimensional GC techniques may be required for such studies (39).

- The relation between molecular configuration and bioaccumulation. In view of the difficulties to perform such experiments with marine mammals, this research could be performed first with fish.
- The relation between molecular configuration and *in-vitro* biotransformation. With the help of GC-NCI-MS, it could be determined whether the few metabolised congeners have structural similarities (position of the third phenyl ring, chlorine substitution pattern).
- Toxicity of PCTs. In contrast to PCBs, not much is known about the toxicity of interaction of PCTs with respect to hormone mediated processes and immuno-suppression. This should be done preferably with standardised assays

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