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

I. Validatie van de assay met PCBs en de eerste testresultaten met toxafeen

Beleidsgericht ecologisch onderzoek van de Noordzee/Waddenzee

BEON

NIOZ
RIVO-DLO

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I. Validatie van de assay met PCBs en de eerste resultaten met Toxafeen.

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Samenvatting van het onderzoek:

I. Inleiding

Van een aantal groepen van organische microverontreinigingen wordt de kans op het optreden van biomagnificatie (bioaccumulatie via de voedselketen) van kieuwademende prooidieren naar longademende predatoren zoals vogels en zoogdieren bijzonder groot wordt geacht op grond van hun vetoplosbare karakter. Hierdoor worden hoge concentraties van dergelijke verbindingen verondersteld in genoemde toppredatoren (Reijnders, 1986; Tanabe *et al.*, 1988), waardoor dit trofische nivo een sterk verhoogd risico loopt op toxische effecten van deze stoffen. Deze denkwijze speelt een belangrijke rol bij de normstelling (Jonkers en Everts, 1992). Als voorspeller van de te verwachten mate van biomagnificatie van prooi naar longademende toppredator wordt hierbij gebruik gemaakt van fysisch-chemische karakteristieken van de moleculen, waarbij de logaritme van de verdelingscoëfficient tussen 1-octanol en water, afgekort log P_{OW}, een vooraanstaande plaats inneemt.

Dergelijke berekeningen veronderstellen echter, dat een stof in het lichaam niet van structuur verandert; de bioaccumulatie van stoffen die in het lichaam enzymatisch omgezet worden laat zich dus niet volgens deze regels beschrijven. Echter, juist bij zeevogels en zeezoogdieren treden veelvuldig enzymatische omzettingen van organische microverontreinigingen op; dit proces wordt metabolisme of biotransformatie genoemd. Men onderscheidt fase I en fase II metabolisme; fase I betreft de werkelijke veranderingen in de moleculen van giftige stoffen, fase II betreft de koppeling (conjugatie) van stoffen of hun omzettingsproducten aan biologische moleculen. Veel fase I biotransformatiereacties worden gekatalyseerd door het cytochroom P450 systeem, of voluit het cytochroom P450 afhankelijke mono-oxygenase systeem (Nebert & Gonzales, 1987). In vele gevallen leidt biotransformatie tot een hogere wateroplosbaarheid van de stoffen, waardoor de uitscheiding wordt versneld. en er dus juist relatief veel lagere gehalten van deze stoffen voorkomen in zeevogels en-

zoogdieren ten opzichte van hun met kieuwen ademhalende prooidieren. De gevormde metabolieten kunnen echter zelf ook bioaccumuleren en/of een ander mechanisme van toxiciteit vertonen dan de oorspronkelijke stof (Guengerich & Liebler, 1985); bekende voorbeelden hiervan zijn de omzetting van p,p'-DDT in p,p'-DDE, de omzetting van chloorbiphenyl congeneren in gehydroxyleerde en zwavelbevattende metabolieten en de omzetting van benzo[a]pyreen in een carcinogene metaboliet. Het vermogen tot biotransformatie van organische stoffen kan echter zelfs tussen vrij nauw-verwante diersoorten, zoals zeehonden en walvissen, nog verschillen, waardoor grote verschillen in toxiciteit kunnen ontstaan. Het al of niet optreden van biotransformatie kan dus een grote invloed hebben op het gedrag van een stof in de voedselketen en de gevoeligheid van soorten voor bepaalde stoffen.

Het verkrijgen van informatie omtrent de metaboliseerbaarheid van verschillende (groepen) organische verbindingen door middel van experimenten met levende dieren (*in-vivo* experimenten) is echter erg duur en vanwege het grote aantal dieren dat hiervoor nodig is om ethische en logistieke redenen slechts in hoge uitzonderingsgevallen mogelijk.

Het onderzoek in het kader van dit project heeft tot doel om het inzicht in de bioaccumulatie van mengsels van lipofiele organische verbindingen in longademhalende toppredatoren te verkrijgen door een bioassay te ontwikkelen die gebruik maakt van speciaal geprepareerd levermateriaal (microsomen) van één enkel dier of slechts enkele dieren per soort. Een assay waarbij gebruik wordt gemaakt van slechts een deel van een organisme noemt men een *in-vitro*-assay.

II. Validatie van de* in-vitro *assay met behulp van PCBs:

In de eerste periode (tot 1 juli 1994) werd de *in-vitro* assay gevalideerd. Hiertoe werd gebruik gemaakt van de stofgroep de polychloorbiphenylen (PCBs) omdat voor de PCBs het waargenomen gedrag in de bio-assay goed kan worden vergeleken met reeds opgestelde Structuur Activiteits Relaties (QSARs) voor biotransformatie aan de hand van PCB patroon vergelijkingen tussen prooi (schelpdieren, vis) en predator (vogels, zeezoogdieren) (Boon *et al.*, 1987, 1989; 1992, 1994; Borlakoglu *et al.*, 1988; Tanabe *et al.*, 1988; Duinker *et al.*, 1989). Naar aanleiding van deze studies werden de verschillende chloorbiphenyl congeneren op grond van hun moleculaire structuur ingedeeld in de volgende zes groepen:

I: congeneren zonder vicinale H-atomen (vicinale H-atomen zijn waterstof atomen gebonden aan naburige koolstofatomen van de aromatische ringen).
II: congeneren met *o,m* vicinale H-atomen and ≥ twee *ortho* Cl atomen.
III: congeneren met *o,m* vicinale H-atomen en één *ortho* Cl atoom
IV: congeneren met *m,p* vicinale H-atomen en ≤ twee *ortho* Cl atomen
V: congeneren met *m,p* vicinale H-atomen en ≥ drie *ortho* Cl atomen

VI: congeneren met karakteristieken van zowel groep III als groep IV.

Op grond van patroonvergelijkingen tussen prooi en predator zijn de volgende biotransformatieregels voor PCBs opgesteld: Voor zowel zeevogels als zeehonden en dolfijnachtigen geldt, dat alle congeneren behorend tot de groepen I en II niet enzymatisch kunnen worden omgezet; deze congeneren zijn dus persistent.
Zeevogels zijn in staat om congeneren behorend tot de groepen IV, V, en VI om te zetten. Zeehonden kunnen dit ook en zijn bovendien in staat om de tot groep III behorende congeneren om te zetten. Dolfijnachtigen en bruinvissen zijn in staat om congeneren behorend tot de groepen III, IV en VI om te zetten. Congeneren behorend tot groep V zijn hier dus persistent.

Uitgangspunt voor de *in-vitro* assay vormde een technisch PCB mengsel (Clophen A40), waarin het gedrag van een 15-tal individuele stoffen, PCB congeneren genoemd, werd gemeten. In het Clophen A40 mengsel bevinden zich vertegenwoordigers van de verschillende boven beschreven structurele groepen. De resultaten van de *in-vitro* assay zijn op hoofdlijnen goed in overeenstemming met de veldwaarnemingen in de zin dat congeneren behorend tot de groepen I en II inderdaad altijd persistent waren en dat verschillende vertegenwoordigers van de andere groepen verlaagde gehalten vertoonden na incubatie in aanwezigheid van NADPH als elektronendonor ten opzichte van een set referentiemonsters die zonder toevoeging van NADPH werden geïncubeerd, zodat de enzymatische omzetting niet kon verlopen. Wel is het zo, dat binnen een structurele groep de biotransformatiesnelheid soms enigszins lijkt terug te lopen met een toenemend totaal chloreringsgraad van het molecuul. Dit verschil tussen assay veldwaarneming kan worden verklaard doordat in de *in-vitro* assay het substraat voor de enzymatische reaktie (=het PCB mengsel) zich in overmaat in de reageerbuis bevindt. Het enzym kan dus zijn favoriete substraat uit het mengsel uitkiezen, waardoor de relatieve omzettingssnelheden van de verschillende substraten een functie worden van hun relatieve affiniteit. Bij de veldsituatie wordt daarentegen continu aan een dusdanig lage dosis blootgesteld, dat waarschijnlijk de kans dat een PCB molecuul dat potentiëel omgezet kan worden het enzym in een levercel ook werkelijk bereikt, een belangrijke, mogelijk zelfs de snelheidsbepalende stap is. Als een molecuul de bindingsplaats van het enzym werkelijk bereikt en het molecuul kan in principe worden omgezet, dan zal dit in deze situatie ook daadwerkelijk gebeuren. Hierbij speelt de **relatieve** omzettingssnelheid van moleculen die allemaal in potentie kunnen worden omgezet dus geen rol.

Samenvattend kan worden gesteld, dat de assay geschikt is gebleken om vast te stellen of bepaalde structurele hoofdgroepen van PCB congeneren in mengsels gemetaboliseerd kunnen worden in verschillende diersoorten.

Een meer uitgebreide rapportage van de uitgevoerde assays met PCBs gaat hierbij als Annex I. Annex II is een publicatie waaruit blijkt dat er in deze assay ook werkelijk fase I metabolieten worden gevormd (Murk *et al.*, 1994). De in deze publicatie verwerkte resultaten voor de zeehond en de bruinvis zijn behaald met lever microsomen van dezelfde dieren als gebruikt voor de hier gerapporteerde studie.

III. Gebruik van de in-vitro *assay voor onderzoek naar de biotransformatie van Toxafeen*

Van het insecticide toxafeen worden thans in de noordelijke Noordzee hogere concentraties waargenomen in de lever van kabeljauw-achtige vissen dan in de zuidelijke Noordzee (de Boer en Wester, 1993). Voor toxafeen is in Nederland nog geen norm vast gesteld, maar toepassing van de Duitse normen (0.1 mg/g op vetbasis) zou problemen kunnen opleveren voor sommige (vetrijke) visserijproducten uit de noordelijke Noordzee. Net als PCBs bestaat toxafeen uit een groot aantal afzonderlijke stoffen met dezelfde basisstructuur (congeneren). In dit geval zijn het echter geen aromatische stoffen maar norbornanen (verzadigde ringstructuur), norbornenen (1 dubbele binding in het molecuul aanwezig) en norbordiënen (2 dubbele bindingen aanwezig).

In de huidige verslagperiode werd de biotransformatie van toxafeen onderzocht in microsomale fracties afkomstig van de lever van een zeehond, een eidereend en een bruinvis. De *in-vitro* resultaten werden vergeleken met monsters van dezelfde organismen afkomstig uit de Noordzee of de Waddenzee. Hoewel geen mariene soort, werd ook de rat meegenomen in het onderzoek omdat van dit klassieke laboratoriumdier relatief veel meer bioaccumulatie gegevens bekend zijn dan van mariene organismen.

Bij het gebruik van kunstmatig verkregen preparaten uit weefsels van dode organismen dient allereerst de algemene conditie te worden vastgesteld. Immers, een negatief resultaat voor de biotransformatie van een milieugevaarlijke stof heeft geen enkele waarde als het preparaat in slechte conditie verkeert, bijvoorbeeld door afbraak van eiwitten door verrotting. Een indruk van de conditie van de microsomen werd verkregen door een tweetal modelreakties uit te voeren welke elk karakteristiek zijn voor de activiteit van een bepaald iso-enzym van cytochroom P450. Pas als tenminste één van deze reakties goed wordt uitgevoerd, heeft het zin om de microsomen te gebruiken voor biotransformatie assays van stoffen.
In de *in-vitro* assay werden de biotransformatiesnelheden afgeleid uit de verdwijning van de ouderstoffen uit het chromatogram van toxafeen; moleculaire structuren van de gevormde metabolieten konden dus niet worden vastgesteld. Microsomen van rat en zeehond vertoonden de hoogste omzettingssnelheden voor componenten uit toxafeen. Er werden grote verschillen gevonden in de verdwijningssnelheid van 23 geselecteerde pieken van het technische mengsel. Er was in het algemeen een goede overeenkomst tussen de met electronen invangst detectie (ECD) en de met massaspectrometrie behaalde resultaten, hoewel de intensiteiten van de verschillende pieken sterk kon wisselen tussen deze beide detectiemethoden.. Een voorbeeld

van de veranderingen in het chromatogram na incubatie in aanwezigheid van NADPH als electronendonor is gegeven in figuur 1. Van de rat werden zowel microsomen van een dier gebruikt dat van te voren met phenobarbital was geïnduceerd als van een ongeïnduceerd dier. Phenobarbital is een modelstof welke bij de rat met name het enzym cytochroom P4502B induceert. Aangezien deze rat het toxafeen mengsel nog weer aanzienlijk sneller omzette dan het controledier, is dit een sterke aanwijzing dat toxafeen hier wordt omgezet door het iso-enzym cytochroom P450 2B. Microsomen van de zeehond en de bruinvis werden ook gebruikt om de omzetting van een mengsel bestaande uit 5 individueel gesynthetiseerde stoffen uit toxafeen waarvan de molecuulstructuurer van 4 stoffen volledig bekend is. Het gebruik van dit mengsel in plaats van het veel complexere technische mengsel heeft als voordeel, dat het waargenomen gedrag voor 4 stoffen verklaard kan worden vanuit bekende molecuulstructuren. Van deze 5 componenten werden er door de zeehond twee bijna volledig omgezet (toxicant B en Parlar no. 69) en een derde gedeeltelijk (Parlar no. 62). De congeneren T2 en T12 waren persistent. Een verschil tussen de gemetaboliseerde componenten en de persistente is, dat laatstgenoemde stoffen aan alle 4 laterale posities van de (alifatische) zesring van koolstof atomen een chlooratoom bezitten, terwijl er bij twee van de drie gemetaboliseerde stoffen er tenminste één laterale positie geen chlooratomen bevat. De metabolische capaciteit van de microsomen van een eidereend en een bruinvis vertoonden veel lagere omzettingssnelheden. Voor de eidereend lijkt dit een relevant resultaat, aangezien de omzettingssnelheden van de biochemische testassays voldoende hoog waren om een goede conditie van de microsomen te mogen aannemen. Bij de bruinvis waren deze activiteiten echter laag en dus blijft het onzeker of de lage omzettingssnelheden het gevolg waren van een lage intrinsieke capaciteit van het cytochroom P450 systeem in de bruinvis of van een slechte conditie van de microsomen. Literatuur gegevens duiden echter wel op een lage CYP2B activiteit in zowel zeevogels als cetaceën (walvis-achtigen).
In het geval van de zeehond was er in het ECD chromatogram na biotransformatie consequent één piek aanwezig welke afwezig was in het technische mengsel. Geprobeerd zal worden om met GC/MS vast te stellen of deze piek door een gechloreerde norbornaan wordt veroorzaakt.

De resultaten van de *in-vitro* assay werden tevens vergeleken met extracten van milieumonsters van de zeehond, de bruinvis (zelfde dieren als gebruikt in de assay), een ei van een eidereend, spek van een witsnuitdolfijn en lever van heek (een kabeljauw-achtige vis). Ook hier vertoonde het chromatogram van de zeehond slechts minieme toxafeen pieken; zelfs T12 was afwezig. Het chromatogram van het ei van de eidereend vertoonde daarentegen een flink aantal pieken. In het chromatogram van de witsnuitdolfijn was de congeneer T12 dominant; dit was ook het geval in de bruinvis, zij het in mindere mate. In deze beide dieren werd de piek ook uitsluitend veroorzaakt door deze stof; bij het technische mengsel echter bleken er zich op de plaats van T12 tenminste twee stoffen te bevinden.

Samenvattend kan worden geconcludeerd, dat de resultaten van de gebruikte *in-vitro* assay ook voor toxafeen goed bruikbaar zijn om in kwalitatieve zin te voorspellen hoe toxafeen bioaccumuleert in verschillende mariene top predatoren, waartussen net als voor PCBs aanzienlijke verschillen lijken te bestaan.

Gevolgen voor het Beleid

Modellering van de bioaccumulatie

De resultaten van deze studie laten zien, dat de **bioaccumulatie** van toxafeen niet kan worden voorspeld door het modelleren van uitsluitend fysisch-chemische processen, zoals wel het geval is voor de meer klassieke organochloorverbindingen als p,p'-DDE, PCBs zonder vicinale waterstof atomen (CB-153, CB-180) en volledig lateraal (2,3,7,8) chloorgesubstitueerde dibenzodioxinen en -dibenzofuranen. **Fysisch-chemische modellering op grond van parameters zoals bijvoorbeeld de log $P_{octanol/water}$, de constante van Henry en het molecuulgewicht zullen moeten worden aangevuld met stofspecifieke coëfficiënten voor biotransformatie activiteit.** Op grond van de grote waargenomen verschillen tussen soorten, dienen deze coëfficiënten tevens diergroepspecifiek, of zelfs soortspecifiek te zijn. Groepen van relatief goede en relatief slechte metaboliseerders van toxafeen kunnen wellicht worden onderscheiden op grond van de aanwezigheid van iso-enzymen van cytochroom P450 2B.

Eventuele toekomstige normering van toxafeen

Momenteel gelden in Nederland nog geen **normen voor toxafeen. Dit in tegenstelling tot de situatie in Duitsland, waar reeds een norm van 0,1 mg/kg op vetbasis geldt (Bundesgesetzblatt, 1994). Een eventuele toekomstige normering dient te worden afgestemd op concentraties van toxafeen congeneren die voor de diersoort waarin wordt gemeten, persistent zijn. Voor onderzoek hiernaar kan de in dit rapport beschreven *in-vitro* assay worden gebruikt.** Aangezien van technische toxafeen mengsels de structuur van de overgrote meerderheid van congeneren zeer moeilijk is vast te stellen, adviseren wij het gebruik van zowel het uit vijf bekende componenten bestaande mengsel (tox-5 mengsel) als een technisch toxafeen mengsel in dergelijke tests. Alleen zo wordt een zo goed mogelijke inschatting verkregen van de persistentie van toxafeen componenten in de voor monitoringsdoeleinden te kiezen diersoort. Daarnaast dienen de te selecteren congeneren goed te kwantificeerbaar te zijn in milieumonsters; als analysetechniek geniet hier meerdimensionale gaschromatografie/ massaspectrometrie duidelijk de voorkeur (de Boer en de Geus, 1995). **Op grond van het huidige beeld adviseren wij monitoringsgegevens van de concentratie van enkele persistente en kwantificeerbare componenten (T2, T12, te bepalen met MDGC) aangevuld met een indicatie van het totaalgehalte aan toxafeen gebaseerd op het totaaloppervlak van de van toxafeen afkomstige pieken in de enkelkoloms**

chromatogrammen, bepaald met behulp van negatieve chemische ionisatie massaspectrometrie.

Een meer uitgebreide rapportage van het uitgevoerde werk gaat hierbij als Annex III.

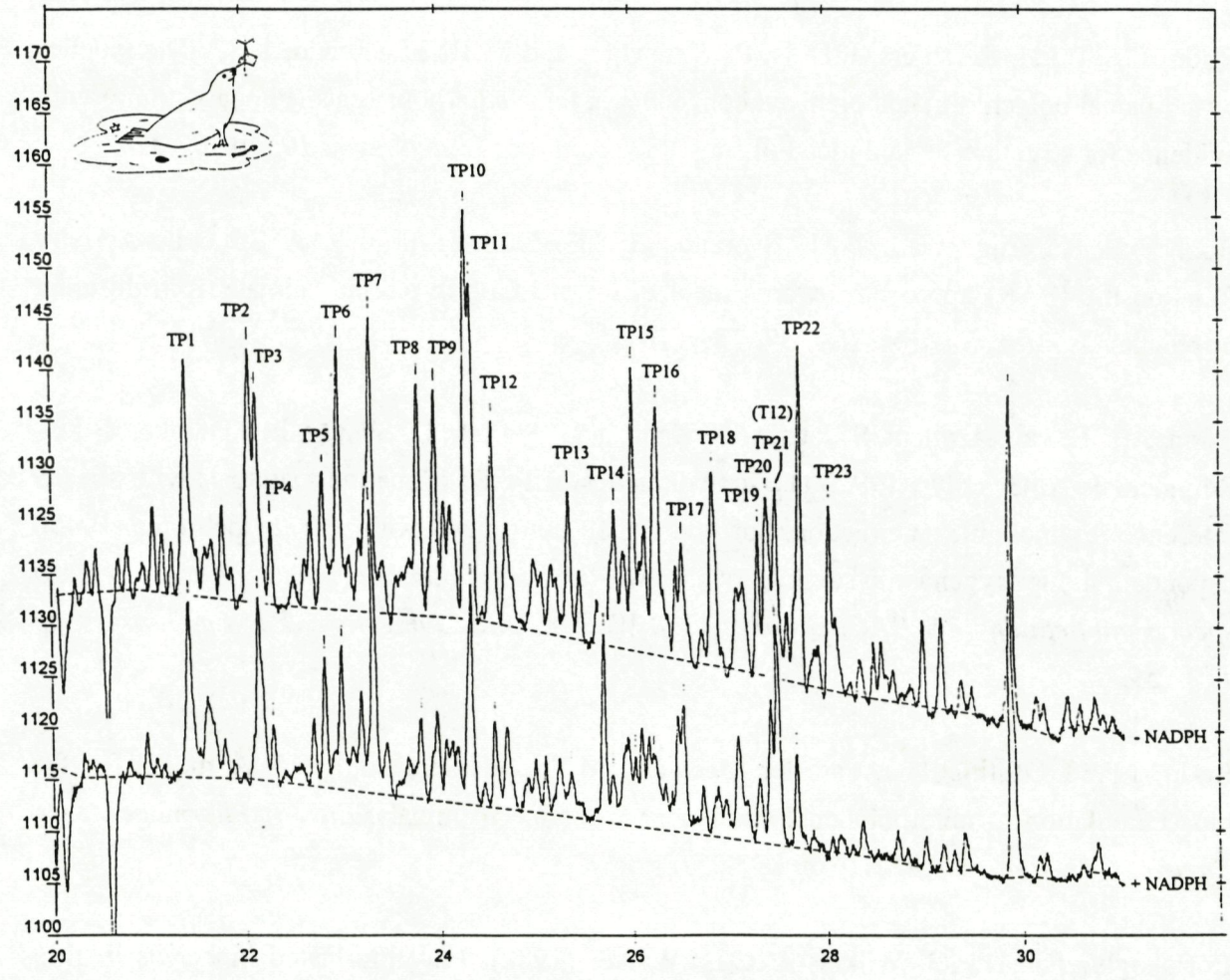


Fig.1: GC-ECD chromatogrammen van de *in-vitro* biotransformatie van een technisch toxafeen mengsel door een microsomaal leverpreparaat van een gewone zeehond (*Phoca vitulina*). Het bovenste chromatogram toont de uitgangssituatie, het onderste chromatogram de situatie na enzymatische omzetting.

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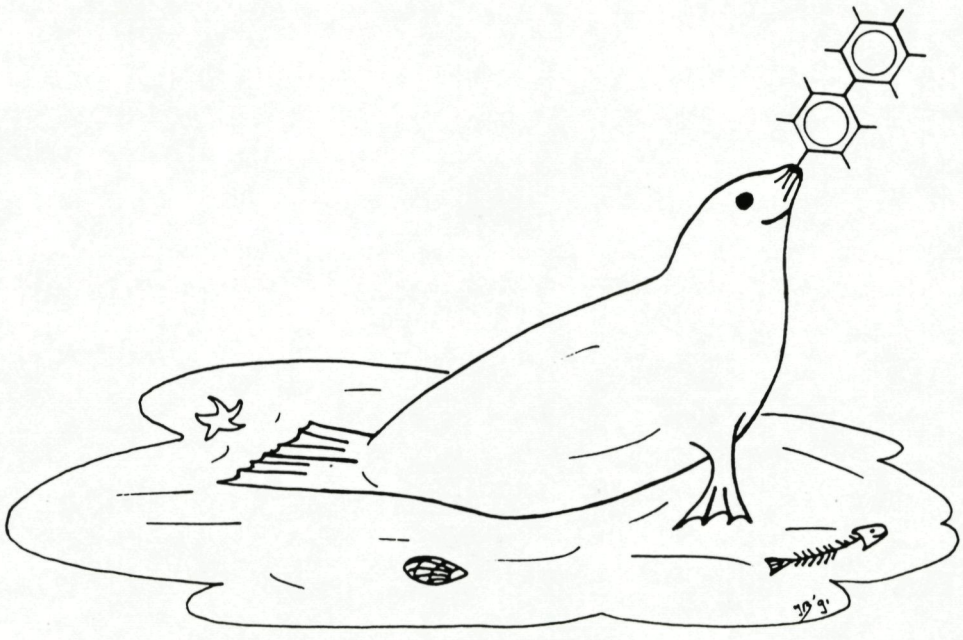
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Annex I



Metabolism of chlorinated biphenyl congeners in microsomal preparations of harbour porpoise and harbour seal and rat.

34011

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1 Objectives of this study

A simple standardised tool to screen phase I metabolism in diverse species can increase our understanding of the factors underlying biomagnification of organic contaminants. Microsomes have shown to be a valuable model for metabolism in the entire organism, though reservations have to be made for the influence of additional phase II metabolism. Investigations were started with rat microsomes because data of metabolism in rat are abundantly available in literature, allowing an easy validation of experimental results.

Secondly incubations with microsomes of a harbour seal and a harbour porpoise were carried out and compared with *in vivo* data. The experiment with the porpoise microsomes was carried out in triplicate to prove the significance of the results. Because of the expected correlation of PCB metabolism with the activity of enzymes of the cytochrome P450 1A (CYP1A) and the cytochrome P4502B (CYP2B) subfamily, the catalytic activities of these enzymes in the microsomes were also determined by measurement of the CYP1A associated ethoxyreorufin O-deethylase (EROD) and the CYP2B associated pentoxyresorufin O-deethylase (PROD) activities (Burke *et al.*, 1985).

This report presents an assay that provides the possibility to estimate the capacity of a species to biotransform selected chemicals, without having to carry out time-consuming and expensive experiments with whole animals.

2 Materials and methods

2.1 Glassware, chemicals and animals

Glassware: all glassware was thoroughly washed with soap, rinsed with demineralised water, heated overnight at 350°C and rinsed with hexane prior to use.
Organic Solvents: Hexane, pentane and 2,2,4-trimethylpentane (TMP) (Chemproha) were purified as described in Boon *et al.* (1994). All other solvents used were nanograde quality (Mallinckrodt), except methanol (BAR-grade, Baker).
Other Chemicals: Clophen A40 (Bayer), CB standards (Promochem), NADPH (98% pure), bovine serum albumin (BSA, Sigma), ethoxy- and pentoxyresorufin (Boehringer Mannheim), resorufin (Aldrich), NaOH, NaH_2PO_3 and ethyleneglycol (Baker), basic alumina super 1 (Al_2O_3, ICN) and silica (SiO_2, Merck). Al_2O_3 and SiO_2 were pretreated as described in Boon *et al.* (1994).
Microsomes: Livers were taken from a harbour porpoise (*Phocoena phocoena*) and a harbour seal (*Phoca vitulina*) within two hours after their death at the seal rehabilitation centre 'Ecomare' at Texel.
Of the rat, the microsomal fractions of two animals were used. One animal had been previously induced by three injections of 30 mg per kg body weight of β naphtaflavone (BNF), a model compound causing induction of the CYP1A subfamily (i.e. the proteins CYP1A1 and 1A2; Nebert & Gonzales, 1987). The second animal had been previously induced by oral administration of a 0.1% Phenobarbital (PB) solution in water. PB acts a model compound for the induction of the CYP2B subsystem (i.e. the proteins CYP2B1 and 2B2; Nebert & Gonzales, 1987). Livers were taken the day after the last treatment. Microsomes were prepared as described in Gibson and Skett (1986) and stored at -80°C until used. Protein was determined according to Bradford (1976).

2.2 EROD and PROD activity measurements

The EROD and PROD activities were determined fluorimetrically according to the method of Eggens and Galgani (1992) using a Flow Laboratories Titertek fluoroskan II plate reader. The temperature was 37°C. The pH was 7.6 for porpoise and seal and 7.8 for rat microsomes (the pH optimum for seal microsomes was determined in a pilot study). Assays were performed in a 0.1 M phosphate buffer containing 0.9 µM ethoxy-/pentoxyresorufin and NADPH (EROD: 150 µM, PROD: 185 µM). For the PROD assay 440 mg/l BSA was added to increase the fluorescent intensity of resorufin and by that the sensitivity of the assay (Rutten *et al.*, 1992). Before PROD measurements were started great care was taken to remove all air bubbles resulting from the addition of BSA. Final volumes for EROD and PROD assays were

335 and 270 µl respectively. Resorufin concentration in the external standard was calculated with the extinction coefficient 73 $mM^{-1} * cm^{-1}$ (Klotz *et al.*, 1984). Protein concentrations were determined spectrophotometrically according to Bradford (1976) at a wavelength of 595 nm using a Biorad model 3550 microplate reader. BSA was used as an external standard.

2.3 Biotransformation assay

The biotransformation capacities of the animals mentioned in 2.1 were tested in a microsomal assay. In a pilot study several experimental conditions were optimised: number of extractions, the incubation time and the added amount of PCBs.

To 25 ml Erlenmeyer flasks 1 ml 0.08 M phosphate buffer, 0.1 ml microsome suspension (containing 10 mg/ml protein) and 3 µl Clophen A40 solution (100 mg/l in acetone) were added. After two minutes of pre incubation in a 37°C water bath, 0.1 ml 11 mM NADPH was added. After 90 minutes incubation the reaction was terminated by transferring the microsomal suspension to 1 ml of ice-cold methanol in a test tube. Subsequently 1 ml of milli-Q water was added and the test tube was vortexed for two minutes. Two blanks were treated in the same way: one without NADPH, one without both Clophen and NADPH. A single experiment was conducted with seal and both kinds of rat microsomes. The experiment was carried out in triplicate with porpoise microsomes.

2.4 Sample preparation

To the incubation mixtures 100 µl of internal standard (CB 112, 18 µg/l in TMP) was added, then they were extracted four times with 2 ml hexane. Hexane was collected and evaporated to approximately 0.5 ml using a micro-Snyder column. The samples were cleaned from fat and pesticide residues by subsequent chromatography over alumina and silica columns as described by Boon *et al.* (1994). The eluates were evaporated to approximately 0.1 ml and transferred quantitatively to GC-vials for automated injection.

2.5 Gas chromatographic analysis

Analyses were performed on a Carlo Erba 5160 and a Hewlett Packard 5880 gas chromatograph, both equipped with an electron capture detector and a narrow bore capillary column (Chrompack, Middelburg, the Netherlands, properties: length, I.D., film thickness stationary phase): respectively a CPSil-8 (50 m * 0.15 mm * 0.30 µm), and a CPSil-19 (60m * 0.15 mm * 0.20 µm). The carrier gas was hydrogen; pressure 350 kPa, linear gas velocity 30 cm/sec. 1 µl injections were made using an autosampler. Other conditions were as mentioned in Boon *et al.* (1994). Throughout this paper, the PCB congeners are numbered according to Ballschmiter & Zell (1980).

Analyses were quantified by comparing peak heights of individual compounds with reference compounds in a dilution series of an external reference mixture containing 40 PCB congeners (multi-point log peak height-log concentration calibration) using the PANDORA® quantification programme developed at NIOZ. The results presented here are based on CBs No. 26, 31, 70, 132, 136, 44, 49, 87, 97, 101, 141, 28, 74, 105, 118, 138, 170, 177, 195, 180, 183, 187, 194 and 202. The other reference compounds were either not present in Clophen A40 or microsome samples (CB No. 15, 169, 185 and 209, Schulz *et al.*, 1989), hindered by co-eluting with other CBs on one or both columns (CBs No. 18, 66, 77, 95, 99, 126, 128, 149, 151 and 156, (Larsen *et al.*, 1991)), or could not be detected reliably (CB No. 52). The concentrations of the individual congeners in the samples were corrected for the recovery of the internal standard, but only samples with more than 80% internal standard recovery were considered reliable.

The laboratory has also participated in an intercalibration exercise organized by ICES for the determination of chlorobiphenyl congeners in marine sediments and biota and is currently participating in the EC-based QUASIMEME programme as part of its quality assurance procedure. Samples of standard reference materials are analysed at regular intervals to check the performance of the analytical procedure.

2.6 Comparison of PCB patterns between incubations

From all peak heights the ratio to CB 153 (R_{153}) was calculated. The rationale for this transformation is described by Boon *et al.* (1992). For each congener the R_{153} in an incubation with NADPH was devided by the R_{153} in an incubation without NADPH. Congeners were devided into six groups based on earlier studies with seals (Boon *et al.*, 1987; 1994), cetaceans (Duinker *et al..*, 1989; Tanabe *et al.*, 1988) and seabirds (Borlakoglu *et al.*, 1988; Everaarts *et al.*, 1991).

I: congeners without vicinal H-atoms
II: congeners with *o,m* vicinal H-atoms and two or more *ortho* Cl
III: congeners with *o,m* vicinal H-atoms and one *ortho* Cl
IV: congeners with *m,p* vicinal H-atoms and two or less *ortho* Cl
V: congeners with *m,p* vicinal H-atoms and three or more *ortho* Cl
VI: congeners showing characteristics of both group III and IV

The number of chlorine atoms in the *ortho*-positions especially influences the activation energy required to obtain a planar configuration of the molecule (i.e. both aromatic rings lying in the same plane) (Cullen & Kaiser, 1984).

3 Results and discussion

3.1 EROD and PROD activity measurements

The EROD and PROD activities were determined in microsomal fractions of livers of rat, seal and porpoise as shown in table 1.

Table 1. Protein content, EROD and PROD activity in porpoise and seal liver microsomes.

Species	Protein content	EROD activity	PROD activity
BNF induced Rat	20	$3.2 * 10^2$	n.m.
PB induced Rat	10	n.m.	83
Harbour Porpoise	10	$4.7 * 10^2$	28
Harbour Seal	10	$2.5*10^3$	16

Protein content in mg/ml

EROD and PROD activity in pmol . mg protein^{-1} . min^{-1}

n.m. = activity not measured

EROD activity in harbour seal is much higher then the value of 28 pmol*mg p^{-1}*min^{-1} reported by Addison *et al.* (1986). This difference could have several causes: in the first place the experimental animals are so hard to obtain that researchers only have microsomes originating from one animal. It is obvious that great differences exist in induction states between animals of different age and origin (Gibson and Skett, 1986). Second in this investigation microsomal activities were determined at the body temperature of marine mammals (37°C) and an optimal pH (7.6), while other researchers carried out their experiments at room temperature and pH 7.8.

3.2 Biotransformation assay

The results of the biotransformation assay are presented in figures 2 to 5 and summarised in table 2.

None of the animals tested was able to metabolise PCBs of groups I and II. For the preliminary studies with rat microsomes, a smaller set of congeners is reported than for the assays with microsomes of harbour seal and porpoise. The relative metabolic capacity differed between the different animals and is summarised in table 2.

Table 2: Summary of the results from the biotransformation assay, relative rates of metabolism for the different samples. The data were interpreted as follows: "-", mean value for the entire class between 1.05 and 0.95; "+/-": mean values between 0.95 and 0.85; "+" : between 0.85 and 0.75 , "++": between 0.75 and 0.65 and "+++": below 0.65. n.d.: not detected.

Congener class	I	II	III	IV	V	VI
PB induced rat	-	-	+	+++	n.d.	+++
BNF induced rat	-	-	+	+++	n.d.	+++
Harbour Seal	-	-	+++	+++	++	+++
Harbour Porpoise	-	-	++	+	+/-	+++

When vicinal hydrogen atoms are present in the *meta-* and *para-* positions the presence of three or four *ortho* Cl atoms (group V) seems to prevent oxidation totally in the harbour porpoise, but not in the harbour seal.

Only quantitative differences between the PB and BNF induced rat microsomes show up in the metabolism of CB 28 (faster in the BNF-treated rat) and all congeners of group IV (faster in the PB-induced animal); in principle both animals metabolised congeners belonging to groups III and IV; group V congeners were not determined for this species. Mills *et al.*, (1985) found increased metabolism of polybromobiphenyl (PBB) congeners with vicinal H-atoms in the *ortho-* and *meta-* positions in rat microsomes of animals induced with 3-methylcholanthrene, like BNF an inducer of CYP1A, but an enhanced turnover of PBB congeners with vicinal H-atoms in the *meta-* and *para-* positions in rat microsomes of PB induced animals. Thus, the differences observed between microsomes of the BNF and PB-induced rat are in line with this observation. The conclusion of Mills *et al.* (1985) that *penta-* and higher brominated biphenyls cannot be metabolised appears not valid for the chlorinated biphenyls, since reduced levels of CBs -118 and -141 were found in the assay as well as in field data.

Compared with both rat samples, the harbour seal and porpoise microsomes appear to be better metabolisers of group III PCBs. Both the seal and porpoise microsomes revealed the same relationship between molecular structure and the ability to undergo metabolism as found in earlier *in vivo* studies (Boon *et al.*, 1987, Boon *et al.*, 1994, Wells and Echarri, 1992), including an appearingly better ability of the harbour seal to metabolize congeners of group V.

4 Conclusions

- From the comparison between the *in vitro* results obtained with harbour seal and harbour porpoise microsomes and *in vivo* studies with these animals, it is concluded that the assay can serve as a qualitative tool to establish the ability of an animal to metabolise PCBs congeners. If a compound is metabolised in the assay, it will certainly also be metabolised *in-vivo*. The influence of the total chlorination level within a structural group as observed in some of the *in-vitro* assays may not exist in the same way in the *in-vivo*situation. Reservations should also be made for the influence of subsequent phase II metabolism and biotransformation by the intestinal micro flora in the *in-vivo* situation.
- The ability to metabolise PCBs with *m,p* vicinal H-atoms *in-vitro* as well as the observed PROD activity of the microsomes from a harbour porpoise are indications for the presence of enzymes of the CYP2B subfamily in this animal.

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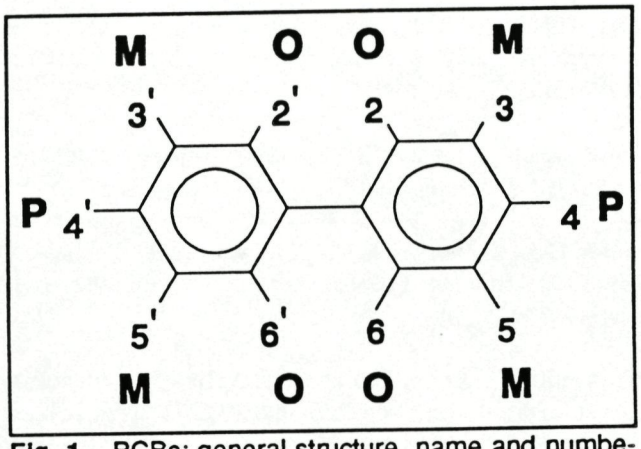


Fig. 1. PCBs: general structure, name and numbering of the positions (O =ortho, M =meta, P =para)

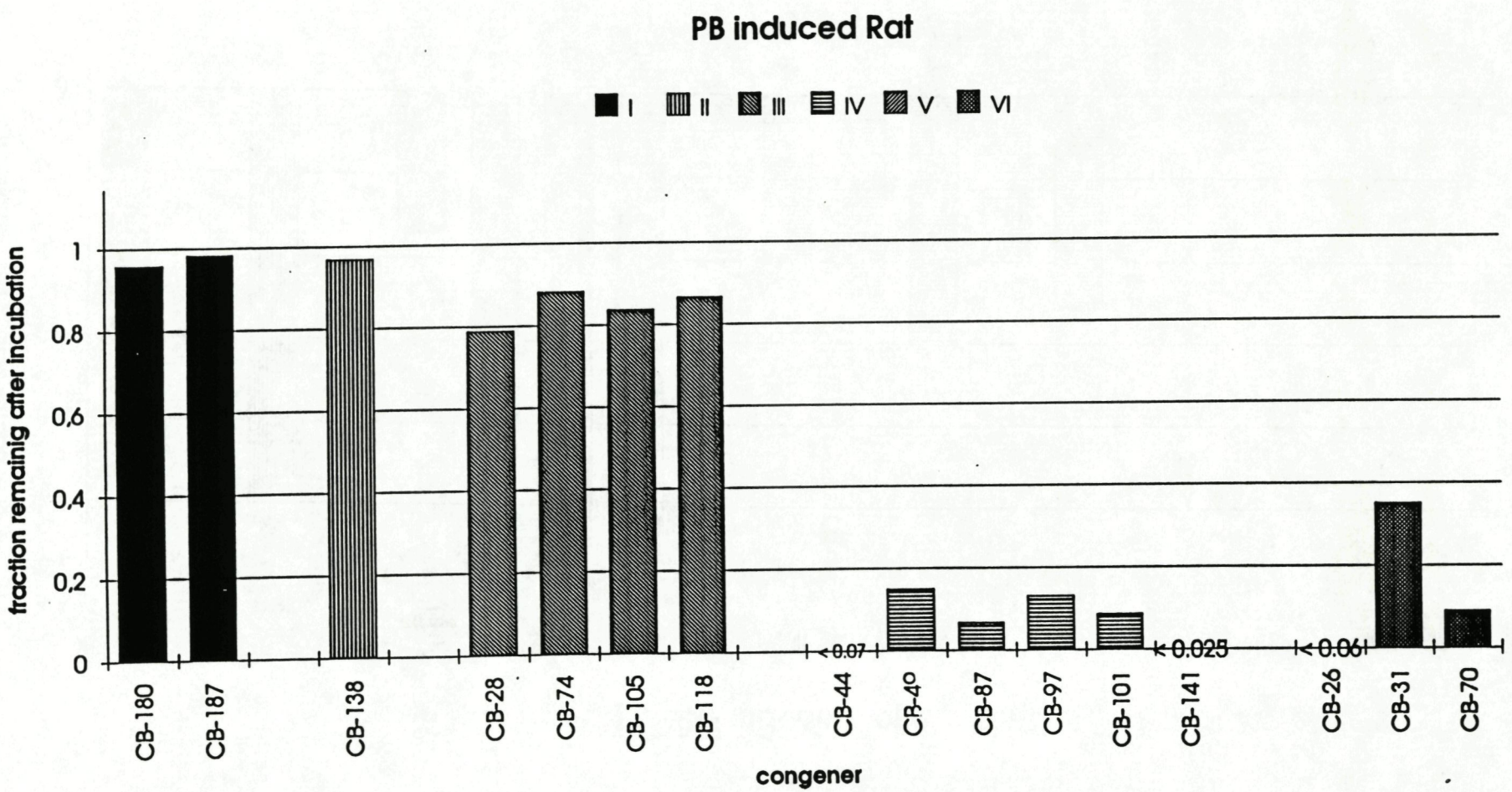


Figure 2: PCB metabolism in PB induced rat microsomes

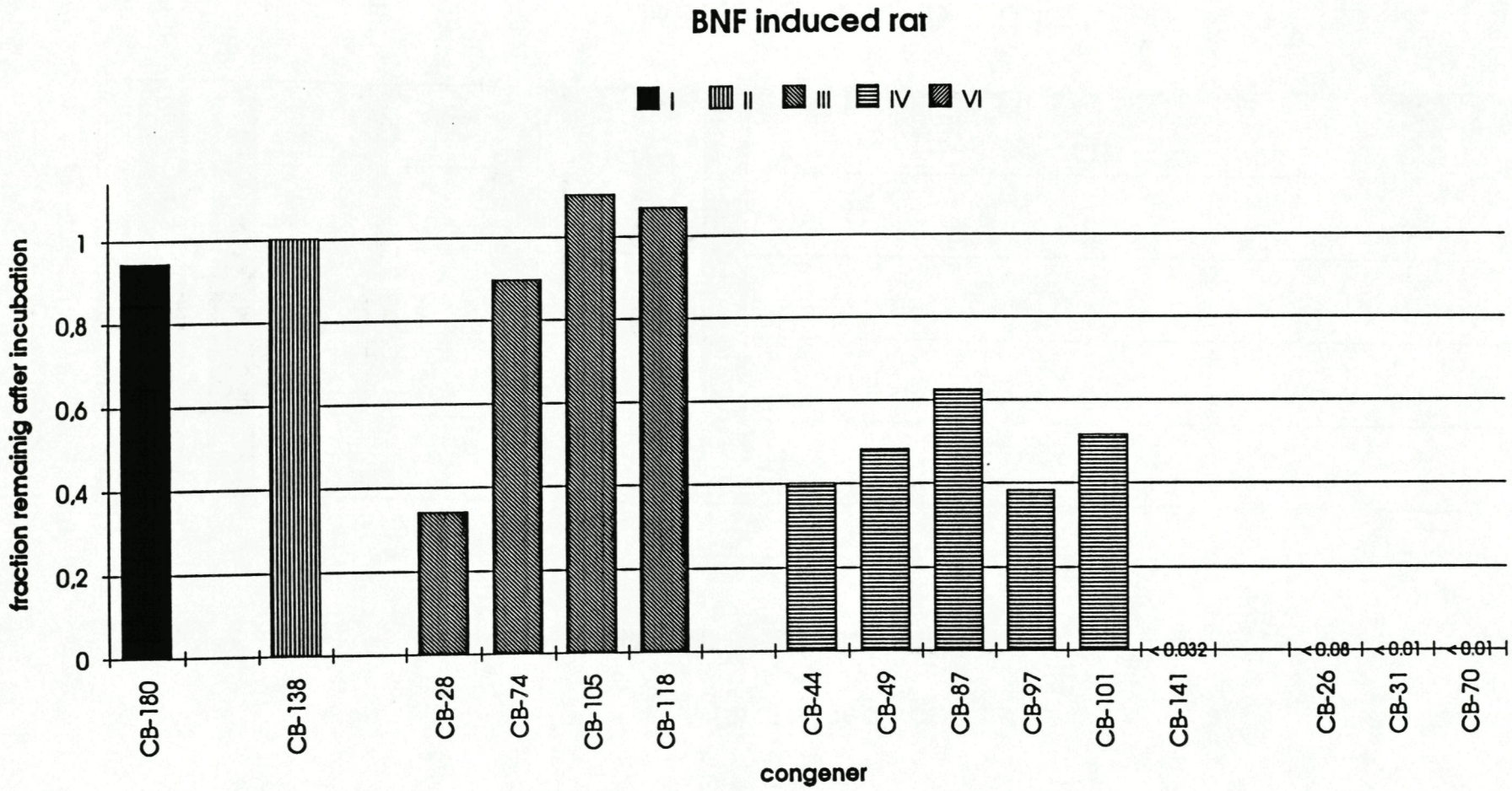


Figure .3 PCB metabolism in BNF induced rat microsomes

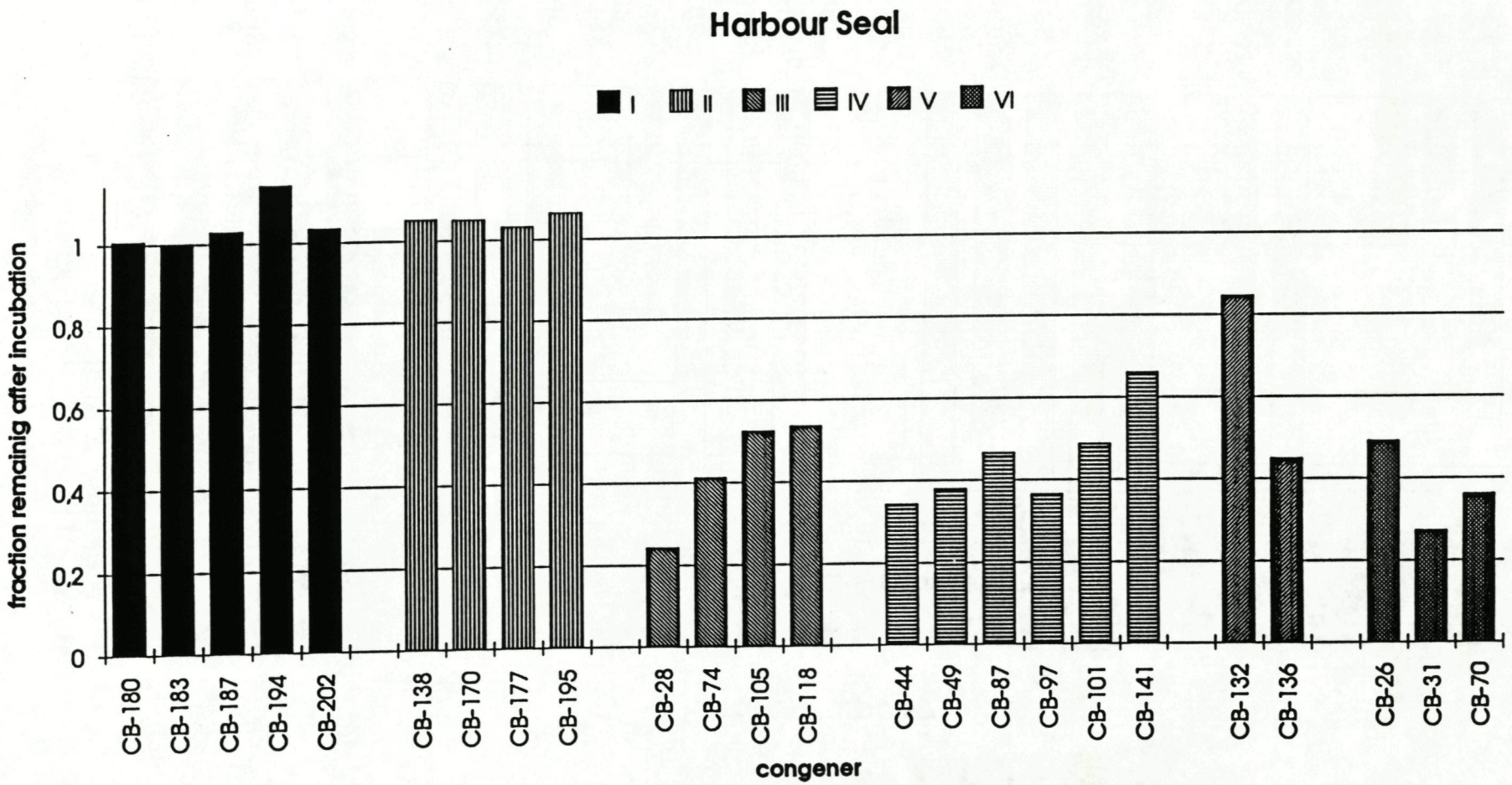


Figure 4 PCB metabolism in Harbour Seal microsomes

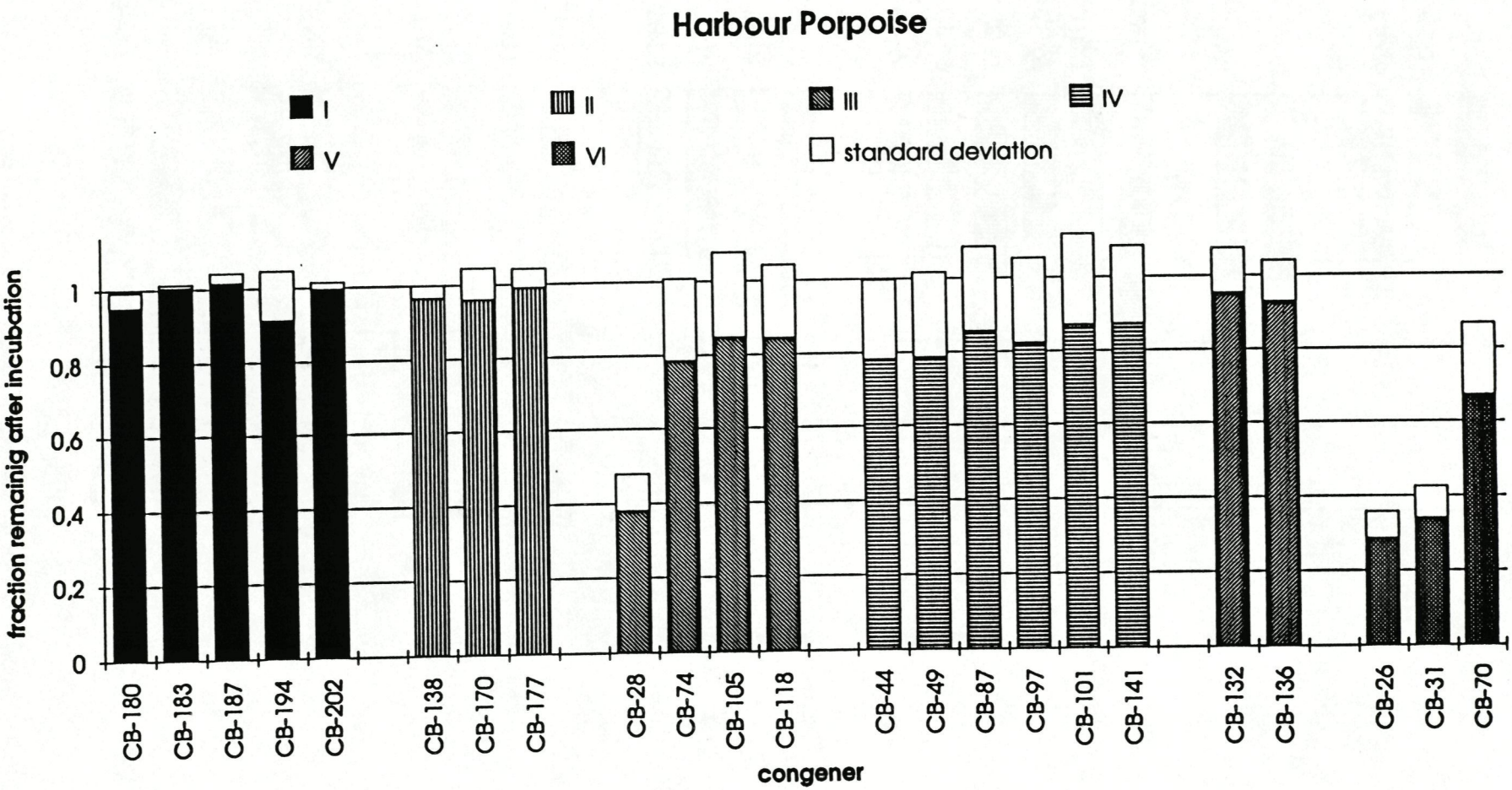


Figure 5 PCB metabolism in Harbour Porpoise microsomes

Annex II

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In vitro metabolism of 3,3′,4,4′-tetrachlorobiphenyl in relation to ethoxyresorufin-*O*-deethylase activity in liver microsomes of some wildlife species and rat

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In vitro metabolism of 3,3′,4,4′-tetrachlorobiphenyl in relation to ethoxyresorufin-*O*-deethylase activity in liver microsomes of some wildlife species and rat

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Abstract

A qualitative study was performed of the capacity of hepatic microsomes of several wildlife species to metabolize 3,3′,4,4′-tetrachlorobiphenyl (TCB). Hepatic microsomes of species environmentally exposed to polychlorinated biphenyls (PCBs): harbour porpoise (*Phocoena phocoena*), harbour seal (*Phoca vitulina*), common tern (*Sterna hirundo*), and hepatic microsomes from species experimentally exposed to PCBs: eider duck (*Somateria mollissima*), rainbow trout (*Salmo gairdneri*), flounder (*Platichthys flesus*), and Wistar rat, were incubated with ^{14}C-labelled TCB ([^{14}C]TCB). The mammals and birds were able to metabolize TCB at a rate that correlated with their ethoxyresorufin-*O*-deethylase (EROD) activity. No [^{14}C]TCB metabolism was observed in the fish, despite elevated EROD activity in the trout. HPLC analysis of diisopropylether extracts of the microsomal incubations indicated the presence of 4-OH-, 5-OH-, and 6-OH-tetrachlorobiphenyl metabolites and a yet unidentified metabolite. The ratio of the different hydroxy metabolites formed varied for the various species.

Key words: TCB (3,3′,4,4′-tetrachlorobiphenyl); PCB (polychlorinated biphenyl) metabolism; Ethoxyresorufin-*O*-deethylase (EROD); (Bird); (Mammal); (Fish)

1. Introduction

Polychlorinated biphenyls (PCBs) are ubiquitous environmental pollutants that accumulate in the environment due to their great lipophilicity and resistance to biodegradation. Due to additional biomagnification, greatest concentrations of PCBs have been found in body lipids of lung breathing top predators of especially the aquatic food chain, like fish eating birds and mammals. For example, in cormorant eggs collected in the period 1988–1990 in the Biesbosch area (the delta of the rivers Rhine and Meuse) in the Netherlands, concentrations as great as 350 mg/kg yolk lipid were observed for 2,2′,4,4′,5,5′-hexachlorobiphenyl (CB-153) (Van den Berg et al., 1994). A similar concentration of CB-153 was observed in blood of a starved harbour seal (399 mg/kg lipid (Boon et al., 1994)).

Although internal PCB concentrations increase with the trophic level, the pattern of individual PCB congeners is considerably different in sediments and in species of different trophic levels (Muir et al., 1988; Boon et al., 1989). In general, lesser chlorinated PCB congeners (tetra and less chlorine substitutions) disappear progressively from the congener patterns in, e.g., body lipids, with increasing trophic level, due to biotransformation. As a general rule, PCB congeners with unsubstituted adjacent *meta* and *para* positions, and congeners with unsubstituted adjacent *ortho* and *meta* positions and less than one *ortho*-chlorine are most susceptible to oxidative metabolism, catalysed predominantly by cytochrome P450-dependent monooxygenases (Walker, 1992; Boon et al., 1992).

Cytochrome P450 IA1 (P450IA1) can be induced by several PCB congeners, mediated by the arylhydrocar-

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bon (Ah) receptor. Non-*ortho*-chlorine substituted PCB congeners such as TCB (CB-77), 3,4,5,3′,4′-pentaCB (CB-126) and 3,4,5,3′,4′,5′-hexaCB (CB-169), are the most potent PCB congeners with respect to induction of P450IA1 and IA2 and its associated ethoxyresorufin-*O*-deethylase (EROD) activity (Goldstein, 1989; Safe, 1990). In addition, the induction of P450IA and EROD activity correlates well with several toxicity parameters like reduced growth, thymic atrophy and hepatomegaly (Goldstein and Safe, 1989).

P450IA1 is an efficient enzyme involved in the oxidative biotransformation of non-*ortho*-PCBs (Ishida et al., 1991). Several phenolic PCB metabolites and mercapturic acid pathway metabolites, such as methyl sulphonyl PCBs, have been identified in various laboratory species. For example, Darnerud et al. (1986) identified a number of hydroxy metabolites and a methylsulphone metabolite of TCB in C57/Bl mice and their fetuses. Bergman et al. (1992) showed that methylsulphonyl PCBs are relatively persistent metabolites in mink. Yoshimura et al. (1987) reported several hydroxylated metabolites of TCB in rats.

Several studies over the past 8 years have indicated that phenolic metabolites of PCBs are not just inert intermediate metabolites of PCBs, but do possess metabolite-specific toxic potencies. Hydroxylated PCB metabolites have been found to competitively displace thyroxine from its binding site on the plasma carrier protein transthyretin (TTR), thus enhancing the elimination of thyroxine from the circulation (Brouwer, 1987; Lans et al., 1993). Hydroxylated PCB metabolites have also been shown to inhibit hepatic thyroxine 5′-deiodinase (Rickenbacher et al., 1989; Adams et al., 1990), interact with oestrogen receptors (Korach et al., 1988), and to uncouple oxidative phosphorylation in mitochondria (Ebner and Braselton, 1987) whereas TCB itself has no such effects (Lans et al., 1990).

The ability of a species to metabolize specific PCB congeners, has mainly been estimated from differences in congener composition of food with that of the predators (Borlakoglu et al., 1989; Tanabe et al., 1988; Boon et al., 1992). A limitation of this approach is that it is indirect, not all congeners can easily be separated from each other by gas-chromatography analysis and it is not known what metabolites are being formed. The relatively toxic planar TCB for example could not be quantified in most experiments because it co-elutes with the more abundant 2,3,3′,4′,6-pentaCB (CB-110). No data have been reported on in vitro biotransformation of TCB in hepatic microsomes of marine mammals, fish eating birds, and fish. Study of the metabolism of a toxic CB congener in addition to analysing the model substrate EROD, has the additional advantage of being relevant to the possible occurrence of the above-mentioned metabolite specific toxic effects in certain species.

Here we report on a qualitative comparative study for in vitro metabolism of ^{14}C-labelled 3,4,3′,4′-tetraCB ([^{14}C]TCB) by hepatic microsomes of several wildlife species. Some of these species, such as harbour seal (*Phoca vitulina*), harbour porpoise (*Phocoena phocoena*) and common tern (*Sterna hirundo*), were environmentally exposed to PCBs and other substances. Also hepatic microsomes from experimentally PCB-exposed eider duck (*Somateria mollissima*), rainbow trout (*Salmo gairdneri*) and flounder (*Platichthys flesus*) were used in this study. The amount and type of hydroxy metabolites formed were quantified with high perfor-

Table 1
Per sample, the EROD activity, total amount of metabolites formed, relative amount of individual metabolites, and ratio amount of metabolites formed/ethoxyresorufin metabolized

Animal	Exposure (see also section 2.1)	EROD activity pmol/mg · min	−ΔTCB [a] pmol/mg · min	4-OH-TCB % [b]	5-OH-TC3 % [b]	6-OH-TCB % [b]	Unknown % [b]	Ratio (%) ΔTCB/EROD [c]
Porpoise	Environment	471	39.6	20.1	32.9	29.25	17.75	8.4
Seal	Environment	2460	39.7	18	53.8	19.3	8.9	1.6
Tern 1	Environment	130	1.37	10	73.9	14.6	–	1.1
Tern 2	Environment	45	0.94	–	–	–	–	2.1
Tern 3	Environment	11	0.62	–	–	–	–	5.6
Eider 1	CloA50	616	20.7	9.9	76.1	6.9	7.1	3.4
Eider 2	TCB	1050	46.9	9.8	74.4	6.3	9.5	4.5
Trout 1	CloA50 (215; 10 d) [d]	336	0	–	–	–	–	0.0
Trout 2	CloA50 (54; 10 d) [d]	328	0	–	–	–	–	0.0
Trout 3	CloA50 (215; 20 d) [d]	252	0	–	–	–	–	0.0
Trout 4	CloA50 (54; 20 d) [d]	259	0	–	–	–	–	0.0
Flounder	CloA50 (500; 10 d) [d]	2	0	–	–	–	–	0.0
Rat	β-Naphthoflavone	2290	91.4	27.9	23.5	31.3	19.6	4.0

[a] Amount of TCB metabolized. [b] Relative amount of specific hydroxylated metabolites. [c] Ratio between metabolism of TCB over EROD activity. [d] Clophen A50 (dose in mg/kg; number of days of exposure).

mance liquid chromatography (HPLC) and compared with the EROD activities measured in the same microsomes.

2. Materials and methods

2.1. Origin of microsomes

The liver microsomes used in this experiment originated from environmentally and experimentally exposed species.

Environmentally exposed species

Harbour seal and harbour porpoise were washed ashore alive at the beach of the island of Texel, Netherlands. The adult male harbour seal was extremely lean, with the blubber layer completely depleted. The liver contained 130 mg CB-153/mg lipid, the blubber 128 and the blood 399 mg CB-153/kg lipid. The adult female harbour porpoise had concentrations of 48.9 mg CB-153/kg lipid in the liver, 19.2 in the blubber and 87.8 mg/kg lipid in the blood. More information on these animals (respectively referred to as HS2 and HP1) is given in Boon et al. (1994). The animals died at the mammal rehabilitation centre "Ecomare", where the livers were removed within 2 h after death.

Common tern microsomes were obtained from 1-day-old chicks, artificially bred from eggs collected in colonies with different degrees of PCB contamination. As only 100 μl of microsomes was avaible per individual for this metabolism study, microsomes were pooled to get two portions of 1 mg microsomal protein each. Three pools of microsomes were created, from either five animals with a relatively great (around 147 mg CB-153/kg yolk sac lipid, tern 1), four with an intermediate (around 86 mg CB-153/kg yolk sac lipid, tern 2), and four with a lesser mean PCB concentration (around 65 mg CB-153/kg lipid, tern 3) (Table 1). PCB, PCDF and TCDD concentrations and EROD activities are described by Bosveld et al. (1993), the biochemical effects found are described by Murk et al. (1993).

Experimentally exposed species

Eider duck microsomes were prepared from livers of 38-day-old animals killed 10 days after i.p. exposure to 200 mg of the commercial PCB mixture Clophen A50 per kg bodyweight (bw) or to 50 mg TCB/kg bw (Murk et al., 1994).

Trout microsomes originated from an experiment where trout were orally dosed with 54 or 215 mg Clophen A50/kg bw. The animals were killed 10 or 20 days after exposure. Samples of four different animals were used: 215 mg/kg at 10 days (trout 1), 54 mg/kg at 10 days (trout 2), 215 mg/kg at 20 days (trout 3), 54 mg/kg at 20 days (trout 4).

A 1-year-old *flounder* was killed 10 days after i.p. exposure to 500 mg Clophen A50/kg bw in a laboratory experiment. Microsomes with the relatively greatest EROD activity were used for this study.

Rat microsomes were obtained from a 16-week-old Wistar rat that had been given three daily i.p. administrations of 30 mg β-naphthoflavone/kg bw and had been killed 24 h after the last treatment. These microsomes were used as a positive control, since [^{14}C]TCB metabolism has already been characterized in this species (Morse et al., in preparation).

2.2. Preparation of microsomes, and measurement of microsomal protein concentrations and EROD activity

Microsomes were prepared according to the method of Gibson and Skett (1986), and immediately stored at −80°C until further analysis.

Protein concentrations were measured according to the method of Bradford (1976) using the Bio-Rad protein assay reagent. Measurement of EROD activity was performed as described by Prough et al. (1978) at a temperature of 37°C.

2.3. In vitro [^{14}C]TCB metabolism and metabolite analysis

[^{14}C]TCB obtained from Sigma Chemical, with a specific activity of 37.1 mCi/mmol was purified according to the method described by Morse et al. (in preparation). Four hydroxylated metabolites, 2-hydroxy-3,4,3′,4′-TCB (2-OH-TCB), 4-hydroxy-3,5,3′,4′-tetraCB (4-OH-tetraCB) 5-hydroxy-3,4,3′,4′-TCB (5-OH-TCB) and 6-hydroxy-3,4,3′,4′-TCB (6-OH-TCB), kindly donated by Dr. E. Klasson Wehler (Wallenberg Laboratory, Stockholm, Sweden) were used as internal standards for HPLC analysis.

Microsomal incubations and HPLC analysis of TCB metabolites were performed according to the method described by Morse et al. (in preparation). Duplicate portions of 1 mg hepatic microsomal suspension with 10 μM [^{14}C]TCB (added in 25 μl acetone) and 50 nM Tris-HCl buffer (pH = 7.5) with a total volume of 900 μl were pre-incubated in glass tubes for 2 min at 37°C. The reaction was started by adding 100 μl of a 10 mM NADPH solution. After 5 min 1 ml ice-cold methanol was added to the incubation mixture. TCB and metabolites were extracted four times with 2 ml of diisopropylether. The ether extract was pooled and dried under nitrogen gas and resuspended in 50 μl of methanol. The samples were stored at −20°C until further analysis. The recovery of mono-hydroxy-TCB (OH-TCB) metabolites using this extraction method was on average 94 ± 1% (Morse et al., in preparation).

Aliquots of 20 μl methanol extract enriched with the four standard metabolites were injected into a HPLC reversed phase column (Perkin Elmer 30 × 3 mm coupled to a 83 × 3 C-18 3 μm column). Separation of metabolites and parent TCB was performed with 78% methanol and 22% water for 15 min followed by 100% methanol for 16 min. Flow rate was 0.8 ml/min and 0.4 min fractions were collected with a Pharmacia fraction collector (LKB.RediFrac). Scintillation fluid (4.5 ml, Ultima-Gold, Packard) was added to the collected fractions and they were counted in a Packard liquid scintillation counter (1600 TR) for a period of 5 min. The pattern of radioactive peaks was compared to the position of the standard metabolites on the UV-absorbance chromatogram (265 nm).

3. Results

3.1. EROD activity

Hepatic microsomal EROD activities differed greatly among the species tested (Table 1). EROD activity was greatest in microsomes from the rat (2290 pmol/mg · min) and the environmentally exposed harbour seal (2460 pmol/mg · min). The EROD activity of the TCB exposed eider was 1050 pmol/mg · min. Microsomes from the environmentally exposed harbour porpoise and Clophen A50 exposed trout showed intermediate EROD activity (250–450 pmol/mg · min). Low EROD activities (2–130 pmol/mg · min) were found in the microsomes of the Clophen A50 exposed flounder and in the environmentally exposed tern chicks.

3.2. Total amount of hydroxy-TCB metabolites formed

The rates of formation of hydroxy-TCB metabolites are given in Table 1. Of the species tested, rat microsomes showed the greatest rate of OH-TCB formation, followed by the environmentally exposed porpoise and seal and the experimentally exposed eiders. Although the EROD activity was more than five times greater in the seal than in porpoise microsomes, the rate of metabolite formation was the same for both species. The microsomes of common tern exhibited only limited conversion rates of TCB (between 0.62 and 1.37 pmol/mg · min). The TCB exposed eider showed the greatest production of metabolites (46.9 pmol/mg · min), about half of the production of the BNF induced rat microsomes (91.4 pmol TCB/mg · min). A striking result was the complete absence of metabolite formation in the trout samples, which showed intermediate EROD activity. The EROD activity in the flounder microsomes was very low, and no metabolite formation was observed.

The ratio (×100) of OH-TCB metabolites formed (pmol/mg · min) to EROD activity (pmol resorufin/mg · min) (OH-TCB/EROD) of the rat was the same as the mean ratio of both eiders (Table 1). The OH-TCB/EROD ratio was greatest in the porpoise (8.4) and least in the tern (1.1–5.6). The OH-TCB/EROD ratio of the tern microsomes increased with decreasing EROD activity. However, the total amount of metabolites was just at the level of detection in terns 2 and 3. Fig. 1 shows the correlation of the rate of formation of OH-TCB metabolites with the EROD activity of the microsomes of all species tested.

3.3. Relative amount of individual hydroxy-TCB metabolites

The standard OH-TCB metabolites 2-OH-TCB, 4-OH-tetraCB, 5-OH-TCB and 6-OH-TCB were well separated from each other and from the parent compound under the chromatographic conditions used. The sequence of eluted compounds was: 4-OH-tetraCB, 2-OH-TCB, 5-OH-TCB, 6-OH-TCB and finally the parent compound. Fig. 2A–F gives the OH-te-

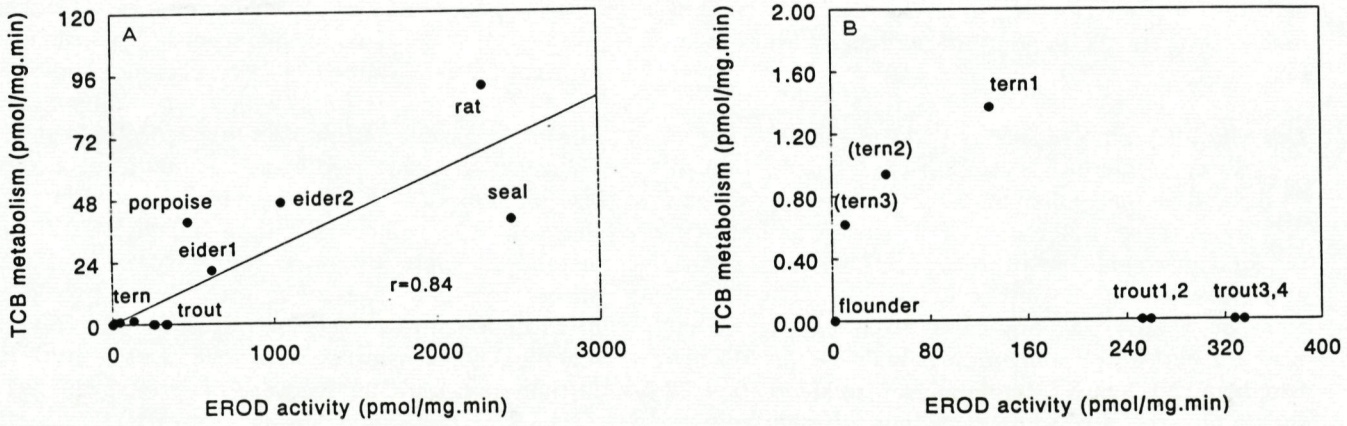


Fig. 1. Relationship between ethoxyresorufin-*O*-deethylase (EROD) activity and amount of metabolites formed by microsomes of various species. A: all species. B: enlargement of the lower left corner of A. The TCB metabolism of tern 2 and tern 3 is not very precise.

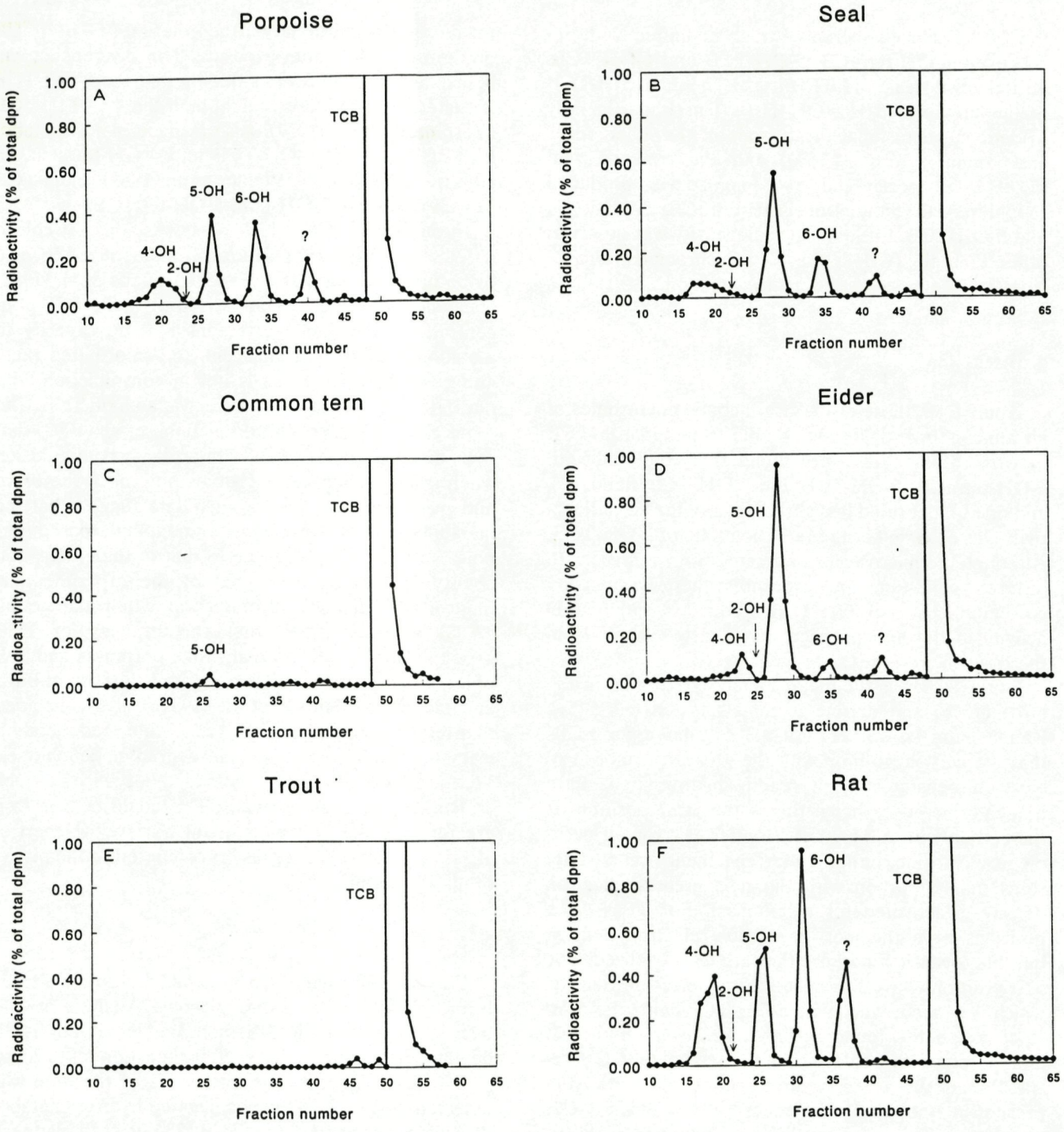


Fig. 2. The characteristic pattern of TCB and its metabolites per species: porpoise (A), seal (B), tern no. 1 (C), eider no. 2 (D), trout (E), rat (F). The codes 4-OH, 5-OH and 6-OH indicate the positions of the standard hydroxy-TCB metabolites on the corresponding chromatogram. The arrow indicates the position of the 2-OH-TCB standard.

traCB metabolites extracted from microsomal incubations of different species. No radioactivity was observed at the retention time of 2-OH-TCB (arrows in Fig. 2). In most chromatograms also a yet unidentified metabolite is visible. Although the total amount of metabolites could just be detected for terns 2 and 3, this amount was too small to determine the formation of individual metabolites.

The relative contribution of the individual metabolites to the total amount of metabolites is given in Table 1. In the microsomal incubations of ^{14}C-TCB in most species five peaks of radioactivity were visible in

the HPLC chromatograms, which co-eluted with respectively 4-OH-tetraCB, 5-OH-TCB and 6-OH-TCB, an unknown peak and TCB itself. The main OH-TCB metabolite formed is 5-OH-TCB. This is particularly evident in microsomal incubations of the eider ducks and common tern (±75%) and the common seal (53.8%). Porpoise and rat microsomes produced roughly equal amounts of 4-OH-tetraCB, 5-OH-TCB, and 6-OH-TCB. Little or no radioactivity was observed other than to TCB in the HPLC chromatograms of trout and flounder microsomal incubations.

4. Discussion

Apart from those of the fish, hepatic microsomes of all animals investigated were able to metabolize TCB in vitro. Metabolites were found that co-eluted with 4-OH-tetraCB, 5-OH-TCB and 6-OH-TCB standards, and one unidentified less polar metabolite. No radioactivity was observed at the retention time of 2-OH-TCB. Although the microsomes originated from animals with different conditions and exposures, there was a good correlation between OH-TCB production and EROD activity in the hepatic microsomes tested, apart from the trout microsomes.

5-OH-TCB was the major metabolite formed in the birds (75%) and marine mammals tested (33–54%). Kannan and co-workers (1989a) calculated for PCBs that the carbon position with the greatest frontier (π) electron density is most readily hydroxylated. In a non-chlorinated biphenyl this is the *para* position. If the carbon with the greatest frontier electrons is occupied by chlorine, either a rearrangement occurs (NIH shift) or the carbon with the next greatest electron density is activated for metabolism. For TCB *ortho* positions have the most favourable electron density, but due to steric hindrance they are least oxidized. The 5,5′ positions have the next greatest electron density, which is in accordance with 5-OH-TCB being the principal metabolite found in birds. However, in the rat and porpoise the chlorine shift metabolite 4-OH-tetraCB and the *ortho* hydroxylated 6-OH-TCB were present in amounts almost equal to those of the 5-OH metabolite. This suggests different substrate specificities for the cytochrome P450IA-isoenzyme homologues between the species investigated.

4.1. Mammals

Most information on TCB metabolite formation by mammals is from studies with laboratory rodents. Ishida et al. (1991) showed that 4-OH-tetraCB and 5-OH-TCB were produced at a ratio of 2.2:1 in vitro in rat liver microsomes. In our experiment this ratio was 1.2:1. In an in vivo experiment, the amount of the 5-OH-TCB in rat faeces was a little larger than that of 4-OH-tetraCB on the first 5 days after exposure. This discrepancy can be explained by a previous finding that the 5-OH-TCB is excreted much more rapidly than the 4-OH-tetraCB (Yoshimura et al., 1987). Also in mice major metabolites of TCB were 5-OH-TCB and 4-OH-tetraCB. In adipose tissue Klasson Wehler et al. (1989) found TCB as well as 4-OH-, 5-OH- and 6-OH-tetraCB.

In our study TCB, which possesses adjacent chlorinated *meta* and *para* positions, is metabolized in vitro by both the porpoise and the seal. Tanabe et al. (1988) concluded, based on a comparison of tissue PCB concentrations, that small cetaceans had no capacity to metabolize PCBs with adjacent non-chlorinated *meta* and *para* positions. This is not in contradiction with our data, although no data were available on TCB. The same goes for the conclusion of Boon et al. (1992) that seals can metabolize PCBs with a planar configuration when there are adjacent H atoms present in the *ortho* and *meta* positions. Our in vitro data suggest that the metabolic capacity of the environmentally exposed porpoise and seal is comparable to that of the eiders, and about twice as small as that of the experimentally induced rat. This is in contradiction with Tanabe et al. (1988) who suggested that the capacity of PCB metabolism of small cetaceans (like porpoises and dolphins) is extremely small as compared to those of birds and mammals. Kannan et al. (1989b) also concluded that terrestrial organisms are better equipped metabolically than marine mammals in degrading xenobiotics. We found that the rat metabolizes about twice as much TCB as the marine mammals. This could be due to a greater exposure to "animal-plant warfare" (Nebert et al., 1989) of the omnivorous rat compared to the piscivorous marine mammals.

4.2. Birds

The only information on TCB metabolism by birds is from chicken embryos and pigeons. Analysis of bile from chicken embryos (Klasson Wehler et al., 1990) demonstrated that 6 days after injection of 0.3 mg [^{14}C]TCB/kg egg into the airsac, 4% of the dose was detected in the bile. Three conjugated hydroxy metabolites were identified: 5-OH-TCB (234 ng/gall bladder), 4-OH-tetraCB (45 ng/gall bladder) and 2-OH-TCB (3 ng/gall bladder). The presence of a dihydroxy-tetrachlorobiphenyl and a dihydroxy-trichlorobiphenyl was also indicated. Borlakoglu et al. (1991) also found di-hydroxylated PCB metabolites in Aroclor 1254 treated pigeons and rats. They showed that induction of isoforms of P450 by PCBs results in an enhanced secondary hydroxylation of the mono-hydroxylated metabolites. In our incubations however only mono-hydroxylated metabolites were formed. The unknown peak is not polar enough to be a di-hydroxylated PCB

metabolite. This difference could be due to the differences in incubation time: 60 min against 5 min in our experiment.

On the basis of comparison of PCB congeners in Aroclor and in fish eating sea birds, Boon et al. (1989), Borlakoglu et al. (1990), Roozemeijer et al. (1991) and Walker (1992) concluded that only PCB congeners with unsubstituted adjacent *meta* and *para* positions can be metabolized by birds. Thus, congeners with only unsubstituted adjacent *ortho* and *meta* positions appeared to be metabolizable in marine mammals (when no more than one *ortho*-Cl atom is present) but not in birds. These conclusions in regard to birds, however, were only based on measurements of mono-*ortho* substituted congeners with *ortho-meta* vicinal H atoms (2,3,3',4,4'-PCB (CB-105), 2,3',4,4',5-PCB (CB-118) and 2,3,3',4,4',5-HCB (CB-156)), because congeners with *ortho-meta* vicinal H atoms without *ortho*-Cl substitution (such as 3,3',4,4'-TCB and 3,3',4,4',5-PCB (CB-126)) were not investigated due to GC separation problems and their relatively low environmental concentrations. The results of the present study show, however, that TCB is rapidly metabolized by the eider hepatic microsomes. Thus, the differences observed in PCB congener patterns in vivo may only involve the mono-*ortho* substituted congeners with *ortho-meta* vicinal H atoms.

Although predatory birds were expected to have lesser P450 activities than mammals (Walker, 1980), the eider microsomes in this experiment showed great EROD activities and were very well capable of metabolizing TCB. The rate of metabolism was comparable to that of the microsomes of the marine mammals tested.

4.3. Fish

We observed that exposure of trout to PCBs induced EROD activity. This is in accordance with the results of Melancon and Lech (1983) who found that TCB does induce EROD activity in trout in a dose dependent way. However we found that the induced trout were not able to metabolize TCB. Apparently the substrate specificity of P450IA is different between fish and birds and mammals. Two main factors are responsible for substrate specificity towards the cytochromes P450. First, the molecular dimensions which determine whether a chemical fits into the binding site. Second, the enzyme-substrate complex has to be activated to permit the oxidative metabolism of the substrate (Parke et al., 1990). The last factor involves the position of the frontier molecular orbitals in relation to the shape of the active site of the P450 enzyme. Parke et al. (1990) show in a plot of the molecular shape parameter (area/depth^2) against electronic parameter (ΔE) for a number of chemicals metabolized by P450I, IIB, IIE and IV, that clusters can be observed of chemicals metabolized by certain P450 iso-enzymes. It is conceivable that due to the evolutionary distances the active sites of fish P450IA and mammal/bird P450IA have slightly different requirements for those parameters. As a result ethoxyresorufin could answer the demands of fish P450IA but TCB could not. Additionally it has been demonstrated that contrary to birds and mammals, fish do not have two iso-enzymes of P450IA (IA1 and IA2) but only one (IA1) that can be induced by planar PCBs (Goksøyr and Förlin, 1992; Stegeman and Kloepper-Sams, 1987). Possibly, ethoxyresorufin is metabolized by IA1 and TCB by IA2.

For fish no data on TCB metabolism are available. Of some other PCB congeners, e.g., 2,5,2',5'-tetraCB, it was observed that 4-OH-glucuronides were excreted by trout into the bile (Melancon and Lech, 1976). Recent experiments in our laboratory suggest that flounders have little or no Ah receptor and are hardly inducible by PCBs (Besselink et al., 1993). However, in other fish species an Ah receptor has been demonstrated (Hahn et al., 1992).

Our findings of the inability of trout and flounders to metabolize TCB are in accordance with the findings of De Boer et al. (1994). They analysed the concentrations of some non-*ortho* and mono-*ortho* substituted chlorobiphenyls and chlorinated dibenzo-*p*-dioxins and dibenzofurans in 14 marine and freshwater fish species and four shellfish species from The Netherlands. In all fish and shellfish but the yellow eel (*Anquilla anquilla*), TCB concentrations were present in two-fold greater relative concentrations than in the technical PCB mixture Aroclor 1254, while the pattern of the other non-*ortho* substituted PCBs was rather similar to that of Aroclor 1254. This indicates that apart from the eel none of the tested (shell)fish species, including the flounder, can metabolize TCB.

4.4. Possible consequences of TCB metabolism

The ability of animals to metabolize PCBs can lead to an increased excretion of these congeners and accordingly to decreased toxicity associated with the parent compound. However, hydroxylated PCB metabolites have been shown to exhibit biochemical effects of their own. With in vivo (Brouwer et al., 1990) and in vitro experiments (Lans et al., 1994) it has been demonstrated that various hydroxy-polychlorobiphenyls (and -dibenzo-*p*-dioxins and -dibenzofurans) show a competitive interaction with transthyretin (TTR), a plasma transport protein of thyroxine. The greatest competitive binding potency was observed for hydroxylated PCB congeners with the hydroxy group substituted on *meta* or *para* positions and one or more chlorine atoms substituted adjacent to the hydroxy group on either aromatic ring or both rings. The metabolites that were formed in our study all had a relative potency greater than that of the physiological

ligand thyroxine (Lans et al., 1994). All hydroxylated PCBs identified in this study have also been shown to uncouple mitochondrial oxidative phosphorylation (Lans et al., 1990). Additionally the 5-OH-TCB, 4-OH-tetraCB and 2-OH-TCB metabolites were shown to bind to the Ah receptor, although the affinity was significantly less than that of the parent compound (Klasson Wehler et al., 1990).

4.5. Conclusions

From our experiments we conclude that although EROD activity is a measure of the exposure of animals to PCBs and related compounds, it is not a direct measure of the ability of a species to metabolize TCB. The structural requirements for PCB metabolism can be quite different between different animal species. Within the same species (apart from fishes) and when exposed under the same circumstances, there is a relationship between biotransformation of ethoxyresorufin and TCB.

The observed species differences in P450IA1 induction and amount and composition of metabolites formed from TCB may be a consequence of evolutionary differences in which the divergence of the IA family into the isoforms IA1 and IA2 may play an important role. Also food specialization is expected to be of influence (Nebert et al., 1989). The accompanying differences in detoxification capacity and potential toxicity of metabolites formed pose serious problems for extrapolation of toxicity data from one type of species to another. Identification of the characteristics that make a specific species vulnerable for the toxic actions of PCBs will help to find the species at risk.

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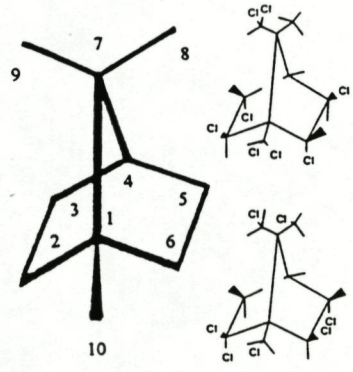
Annex III

In-vitro metabolism of toxaphene in microsomes of harbour seal, eider duck, harbour porpoise, and rat.

Comparison to toxaphene patterns in marine wildlife

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Summary and possible consequences for policy.

This paper is the result of a study carried out jointly by the Netherlands Institute for Sea Research (NIOZ) and the Netherlands Institute for Fisheries Research (RIVO-DLO). Subject is the biotransformation of the pesticide toxaphene in a microsomal assay that was validated for PCBs in the first part of this project (Annexes I and II) Biotransformation rates were derived from the disappearance of the parent compounds from the gas chromatograms; thus structures of polar metabolites could not be revealed at the present stage. The microsomes used originated from several species. Differences in metabolic capacities between animal species caused by different patterns of (iso-)enzymes that are able to metabolise anthropogenic compounds, can cause inter-species differences in the bioaccumulation of individual compounds.

In the present study, the condition of the microsomes used was checked by the measurement of standard biochemical assays for the activity of different iso-enzyme subfamilies of cytochrome P450; the ethoxyresorufin-O-deethylase assay was used as a model reaction for the activity of the cytochrome P450 1A subfamily (CYP1A), whilst the aldrin epoxidase activity was used as a model reaction for the activity of the cytochrome P450 2B (CYP2B) subfamily.

Microsomes from rat and a harbour seal showed the highest metabolic activities towards toxaphene in this study. Large differences were observed between the rates of biotransformation for the different peaks present in the technical toxaphene mixture. Rat microsomes from non-induced rats and rats induced with phenobarbital (PB) were used. Phenobarbital is a model inducer of CYP2B in rat. Since the PB-induced rats showed even higher rates of biotransformation, the CYP2B subfamily appears to be involved in the biotransformation of chlorinated norbornanes and norbornenes. Microsomes of the harbour seal were also used to test the biotransformation rates of 5 individually synthesised toxaphene congeners with a known environmental relevance. Of these toxicant B and Parlar no.69 were almost completely metabolised, whereas the compounds T2 and T12 were completely persistent. Parlar no. 62 was partially metabolised. Compared to the persistent compounds T2 and T12, toxicant B and Parlar no. 62 lack a chlorine substituent at one of the lateral positions of the ring formed by the carbon atoms C1-C6.

The metabolic capacity of a harbour porpoise and a juvenile eider duck showed much lower metabolic activities towards the technical toxaphene mixture, indicating low levels of CYP2B activity. For the eider duck this seems to be a valid result, since EROD and Aldrin epoxidase activities were detected. In the case of the harbour porpoise these activities were very low, and thus it remains uncertain whether the persistence of toxaphene in this animal is characteristic for the species or due to a bad condition of the microsomes. However, lower

activities of CYP2B in these species are in accordance with the published literature, reviewed by Boon *et al.* (1992).

Since no peak in the chromatograms obtained with either electron capture detection (ECD) or negative chemical ionisation mass spectrometry (NCI-MS) detection were higher after metabolism, it can be concluded that the metabolites formed were not detected with the analytical methods used. thus formation of products that are only dechlorinated seems unlikely. Oxygen containing metabolites that might have been formed are likely to have been incompletely recovered in one or more stages of the analytical procedure.

Consequences for policy towards toxaphene

In the Netherlands, no maximum allowable concentrations of toxaphene in fishery products have been established yet. This in contrast to the situation in Germany, where a MAC value of 0.1 mg/kg extractable lipid has been established (Bundesgesetzblatt, 1994). With regard to measurements of the technical toxaphene mixture, the results clearly show that toxaphene cannot be treated in the same physical chemical ways as many 'classical' persistent organochlorines such as p,p'-DDE, PCBs without vicinal hydrogen atoms and 2,3,7,8-substituted dibenzodioxins and dibenzofurans, of which the unaltered molecular structure exerts the toxic effects in biota. Thus, physical-chemical parameters such as log $P_{octanol/water}$ do not predict the bioaccumulation and expected effects of toxaphene in marine food-chains. Moreover, large differences in the oxidative metabolic capacity may exist between species, e.g. between eider duck and harbour seal. The inducibility of enzymes belonging to the cytochrome P450 2B subfamily may play a key role in the case of toxaphene.

As a consequence of the occurrence of extensive metabolism in some marine species, persistent toxaphene congeners should be carefully selected for the establishment of maximum allowable concentrations in marine products Congeners T2 and T12 are at present the most likely candidates. MDGC seems the analytical method of choice for such measurements, since single column GC yields insufficient separation of toxaphene congeners. Since the ratio of persistent toxaphene congeners to the total of toxaphene derived peaks differs enormously between species, a complementary single column GC-MS analyses should be performed to get an impression of total toxaphene present (de Boer and de Geus, 1995). *In-vitro* assays should be performed for monitoring purposes to check the persistence of potential combinations of candidate species and toxaphene compounds. Based on current knowledge we would suggest to test the persistence of the reconstituted mixture of five individual chlorobornanes in species already regularly used for monitoring, e.g. mussel, or the fish species dab, cod or hake.

Acknowledgement

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1. Introduction

1.1 Goal and Organisation of the Project

This study is the second part of the BEON contract RKZ 95-4 (project NIOZ 94 M01), in which the influence of biotransformation on the bioaccumulation of lipophilic organohalogen compounds in marine top predators is studied. The project is carried out in cooperation with the Netherlands Institute for Fisheries Research (RIVO-DLO).

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

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

Toxaphene: structure, analysis, toxicity

Toxaphene is a complex mixture of up to 670 polychlorinated monoterpenes with an average empirical formula of $C_{10}H_{10}Cl_8$ (Holmstead *et al.*, 1974). The product is industrially

synthesised from α-pinene. From α-pinene, camphene (major product), bornylene and α-terpineol are synthesised, which are subsequently bulk chlorinated. The majority of compounds in technical toxaphene formulations consists of bornanes, but also bornenes (one C=C bond), bornadienes (2 C=C bonds) and other chlorinated and non chlorinated compounds are present (Saleh, 1991). The structure of the bornane carbon skeleton and the structural formulas of the few compounds that are commercially available as analytical standards are given in figure 1.1 .

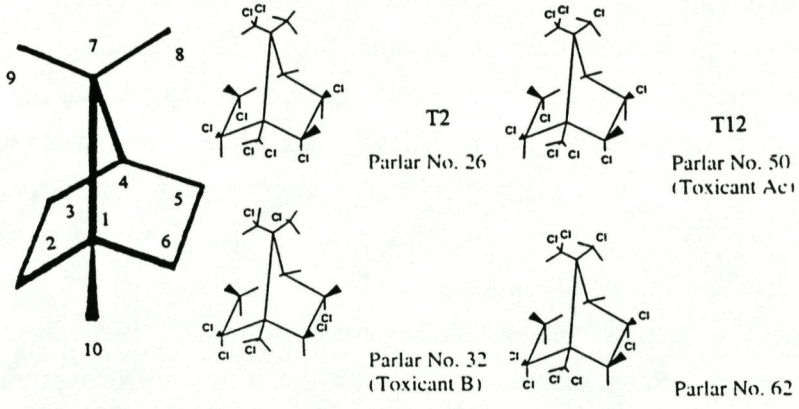


Fig. 1.1: Molecular structure of the bornane carbon skeleton and the structures of 4 individually synthesised toxaphene compounds. Numbers in the carbon skeleton indicate systematic numbers of positions.

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

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

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

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

1.3 Mechanisms of metabolism in animals

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

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

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

The central catalyst in phase I metabolism is the cytochrome P450 dependent monooxygenase system. It catalyses a great number of oxidative reactions, such as hydroxylation, epoxidation, O-, N- and S-dealkylation. This enzyme system is incorporated in the membrane of the SER and consists of different components: NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase, cytochrome P450 and cytochrome b5. NADPH or NADH are required as electron donor. These electrons are passed to NADPH Cytochrome-P450 reductase or NADH-cytochrome b5 reductase. Subsequently these reducing equivalents

are passed to cytochrome P450. In the case of NADH as electron donor the electrons are passed via cytochrome b5 to cytochrome P450. Together with oxygen and a suitable substrate a trimolecular complex is formed and the substrate is oxygenated (Nebert and Gonzalez, 1987). A schematic overview of the cytochrome P450 system is given in fig. 1.2.

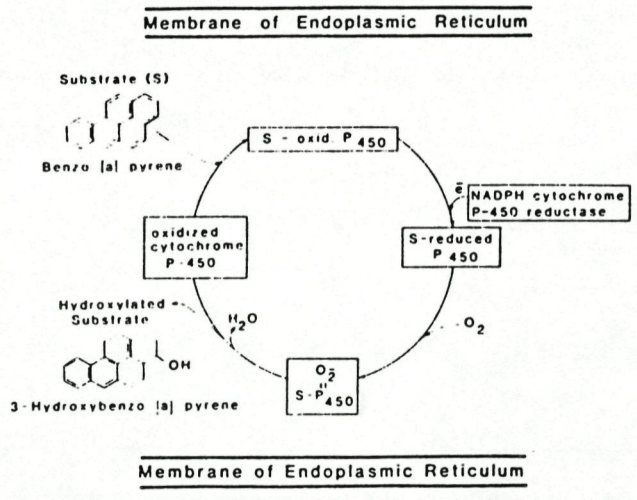


Fig. 1.2: Schematic representation of hydroxylation reactions of anthropogenic compounds catalysed by the cytochrome P450 system located in the smooth endoplasmatic reticulum. Source: Lee (1981).

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

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

Substrate specificity can be used to measure the activity of specific iso-enzymes. Ethoxyresorufin O-deethylase activity (EROD) is strictly catalysed by CYP1A enzyme activity. The product of this transformation is resorufin, which is a fluorescent metabolite. The formation of this product can be followed spectrofotometrically. Aldrin epoxidase (AE) is CYP2B mediated. The product of this reaction is Dieldrin. Decrease of Aldrin and the

subsequently increase of Dieldrin can be measured by gas chromatographic analysis. Both model reactions are shown in figure 1.3.

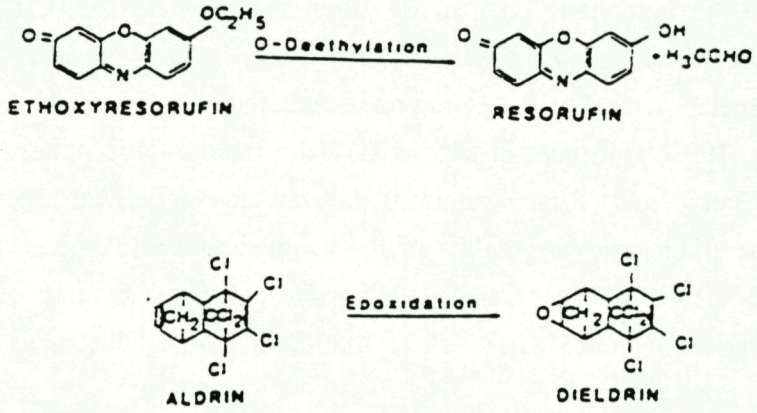


fig1.3: Two model reactions catalysed by different iso-enzymes of CYP-450 in the rat. a: EROD (cytochrome P450 1A); b: Aldrin epoxidase (cytochrome P450 2B).

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

In several species of fish-eating sea-birds no CYP2B induction was found (Ronis *et al.*, 1989a). In Eider Ducklings (*Somateria mollisima*) CYP1A was induced, after dosage with CB-77, but CYP2B was not induced after treatment with the technical mixture Clophen A50 (Rozemeijer *et al.*, 1992, Rozemeijer *et al.*, 1994). Such a difference in metabolic capacity for PCBs illustrate that there can be large differences between marine species with regards to their metabolic capacity for toxic compounds. The present study was undertaken to investigate such differences for toxaphene congeners.

1.4 Metabolism and biomagnification of toxaphene

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

In blubber from Beluga whale (*Delphinapterus leucas*) two peaks were dominant, identified as T2 (fig. 1.1; 2-exo, 3-endo, 5-exo, 6-endo,8,8,10,10-octachloro-bornane; also known as Parlar no. 26) and T12 (fig. 1.1; 2-exo,3-endo, 5-exo, 6-endo, 8,8,9,10,10,-nonachlorobornane; also called Toxicant Ac or Parlar no. 50) (Stern *et al..*, 1992). In harbour porpoise (*Phocoena phocoena*) and white-beaked dolphin (*Lagenorynchus albirostris*)

biomagnification factors of respectively 100 and 40 were measured, compared with their most important food source hake, a gadoid fish. Here, also T2 and T12 were the dominant components in the toxaphene pattern (de Boer and Wester, 1993). In ringed seal (*Phoca hispida*) and polar bear (*Ursus maritimus*) these components were less dominant, indicating a higher rate of metabolism. For *P. hispida* no bioaccumulation of toxaphene was achieved (Zhu and Norstrom, 1993; Hargrave *et al.*, 1993). Chromatographic patterns from toxaphene in blubber from Baikal seal (*Phoca siberica*) differed substantially from patterns of their prey organisms, also indicating a greater ability to metabolise toxaphene derived compounds (Kucklick *et al.*, 1993). In general it appears that cetaceans tend to bioaccumulate at least some toxaphene congeners, where other marine mammals like seals seem to metabolise toxaphene to a great extent.

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

Direct indications for biotransformation of toxaphene were until now only shown in experiments with rats. An *in-vitro* experiment with the 20.000*g supernatant of rat liver homogenates, toxicant B (fig. 1.1; 2,2, 5-endo, 6-exo, 8,9,10-heptachlorobornane; Parlar no. 32) and toxicant C (2-endo,3,3,5-exo,6-exo,8,9,10,10 nonachlorobornane) were both extensively metabolised after the addition of NADPH (Chandurkar and Matsumura, 1979). Types of metabolites formed were a dechlorination product from toxicant C (3,3,5-exo, 6-exo, 8,9,10,10 octachlorobornane), one primary alcohol from toxicant C (-OH group at the C-8, -9 or -10 position), and a number of secondary alcohols (-OH group at the C-2, -3, -5 or -6 position). No indications were found for the formation of tertiary alcohols (-OH group at the C-4 position) or diols. The fact that a primary alcohol is formed from toxicant C but not from toxicant B seems difficult to explain, since both molecules have the same configuration at the bridgehead formed by C-7, C-8 and C-9 (8b, 9c Cl-substitution). In an *in vivo* experiment, toxaphene was partly dechlorinated after excretion (Casida *et al.*, 1974).

1.5 Objectives of this study

Hepatic microsomes have proven to be a good qualitative indicator for the behaviour of PCBs in living organisms, though reservations were made for additional phase II metabolism (Annexes I and II of this report). The purpose of the second part of this project was to investigate toxaphene in the microsomal assay for different species of lung breathing marine predators. For this goal, microsomes of a harbour seal (*Phoca vitulina*), a harbour porpoise (*Phocoena phocoena*) and eider ducklings (*Sommateria mollissima*) were available.

The experiments were started with microsomes of rat, because these have proven to be a good indicator for Phase I metabolism. For the harbour porpoise and the harbour seal incubations were carried out with a reconstituted toxaphene mixture, containing five chlorinated norbornanes, of which four are fully identified. To validate the activity of the microsomes, EROD activity and Aldrin epoxidase activity were measured.

2. MATERIALS AND METHODS

2.1 Glassware, chemicals and animals

Glassware: all glassware was thoroughly washed with soap, rinsed with de mineralised water, heated overnight at 350 °C and rinsed with hexane prior to use.

Organic solvents: Hexane and pentane were purified as described in Boon *et al.* (1994). 2,2,4-Trimethylpentane (TMP), acetone and dichloromethane were nanograde quality (Mallinckrodt). Methanol was Baker Analysed Reagent-grade quality.

Other chemicals used:

- NADPH (98% pure, Boehringer)
- Silica (SiO_2, Merck).
- Alumina (Al_2O_3, ICN)
- Silica and alumina were pre-treated as described in Boon *et al.* (1994).
- Toxaphene, technical mixture (Quant grade, Polyscience Corp.)
- Toxaphene, reconstituted standard mixture containing the following five components (Dr. Ehrenstorfer GmbH):
  - **Parlar no. 26=T2** (2-exo,3-endo, 5-exo, 6-endo, 8b,8c,10a,10b-octachlorobornane;)
  - **Parlar no. 32=Toxicant B** (2,2,5-endo, 6-exo, 8b,9c,10a-heptachlorobornane)
  - **Parlar no. 50=T12** (2-exo,3-endo, 5-exo, 6-endo, 8b,8c,9c,10a,10b,-nonachlorobornane;)
  - **Parlar no.62** (2,2,5,5,8b,8c,9c,10a,10b-nonachlorobornane)
  - **Parlar no.69** (2,2,5,5,6-exo,8b,8c,9c,10a,10b-nonachlorobornane).

The first four congeners are also present in the toxaphene technical mixture. T2, T12 and Parlar 62 tend to bioaccumulate in many types of environmental samples, whereas Toxicant B and Parlar no. 69 are reported to be more easily degradable under environmental conditions (Stern *et al.*, 1993).

- Dieldrin (Fluka AC)
- Aldrin (Quant grade, Polyscience corp.)

Origin of the microsomes:

Male Wistar rats were treated seven days with 0.1 % Phenobarbital (PB) in their drinking water. The animals were sacrificed under ether anaesthesia 24 hours after the last treatment and livers were collected.

28-day-old Eider ducklings (*Somateria mollisima*) were dosed once by intraperitoneal injection with 5 (code: EE47) or 50 (code: EE17) mg/kg body weight of 3,3',4,4'-tetrachlorobiphenyl (CB-77). After ten days livers were collected.

Also livers were taken from a 'wild' harbour porpoise (*Phocoena phocoena*) and a harbour seal (*Phoca vitulina*) within two hours after their death at the seal rehabilitation centre 'Ecomare' at Texel.

All hepatic microsomes were prepared as described in Gibson and Skett (1986), frozen in liquid nitrogen and stored at -80°C until used.

2.2 EROD activity measurements

EROD activities were measured fluorimetrically, using the microplate fluorometer Titertek Fluoroskan II (Flow laboratories), according to the method of Eggens and Galgani (1992).

The assays were performed in a 0.1 M phosphate buffer containing 1 µl ethoxyresorufin and 330 µM NADPH. The temperature of the assay was 37°C. The pH was 7.6 for harbour porpoise and harbour seal and 7.8 for rat microsomes, according to van Schanke (1993). For eider duck microsomes the pH was 7.4 according to Murk *et al.* (1994).

Protein concentrations of the microsomes were determined spectrophotometrically according to Bradford (1976) at a wavelength of 595 nm using a microplate reader (Biorad model 3550). A dilution range of Bovine Serum Albumin was used as an external standard. Protocol for protein determination and EROD measurement are included in appendix 1 and 2.

2.3 Biotransformation assay

The biotransformation capacities of the animals mentioned in 2.1 for toxaphene were tested in a *in vitro* assay.

1 ml of a a 0.08 M phosphate buffer, containing 1 mM EDTA and 0.1 ml of a microsomal suspension (containing 10 mg/ml protein) was added to a number of 25 ml erlenmeyer flasks . Either 3 µl toxaphene solution (65,98 µg/ml technical mix in acetone, or 7 µg/ml standard mix in acetone) or 3 µl aldrin-solution (7 µg/ml in acetone) were added. The Aldrin epoxidase-assay was used as a positive control, testing the activity of the microsomes.

After three minutes of pre-incubation at 37°C in a waterbath, every 10 minutes 0.1 ml 11 mM NADPH was added. After 90 minutes the reaction was stopped by transferring the mixture to a test tube containing 1 ml ice-cold methanol and 100 µl of an internal standard compound to determine the recovery of the entire analytical procedure (CB 112, 13.97 ng/ml in TMP), were added. 1 ml MilliQ water was added and the test tube was vortexed for two minutes. Two blanks were treated in the same way: one without NADPH and one without both toxaphene and NADPH. All experiments were carried out in duplicate.

2.4 Aldrin epoxidase assay

For harbour seal, harbour porpoise and eider duck, Aldrin epoxidase activity was quantified in a separate assay. This assay was carried according to the same procedure as for toxaphene, only with a shorter incubation period to maintain zero-order reaction kinetics, i.e. metabolism of aldrin to dieldrin at the maximum velocity (V_{max}).

To 25 ml erlenmeyer flasks 1 ml 0.08 M phosphate buffer, containing 1 mM EDTA. Four incubations were carried out; 2, 5, 8 and 10 minutes. All experiments were carried out in duplicate.

2.5 Sample preparation

All samples were extracted four times with 2 ml hexane. All hexane phases were collected and 0.4 ml TMP was added. The solvent was evaporated to approximately 0.5 ml, using a micro-Snyder column. The samples were cleaned from fat by chromatography over an alumina column, as described by Boon *et al.* (1994). After evaporation, samples were separated from other pesticides and PCB's over a silica column. The second elution fraction contained 96% of the toxaphene. A complete separation of PCBs from toxaphene is essential for analysis by GC-ECD and GC-NCI/MS to avoid interference problems (Muir and de Boer, 1993, 1995). The eluates were evaporated to approximately 1 ml and transferred quantitatively to GC-vials for automated injection.

2.6 Gas chromatographic analysis

All analyses were performed on a Carlo Erba 5160 and a Hewlett Packard 5880 gas chromatograph, both with electron capture detector (ECD). Both GCs were equipped with a narrow bore capillary column (Chrompack, Middelburg, the Netherlands), respectively a CPSil-8 (50m * 0.15 mm * 0.30 μm) and a CPSil-19 (60m * 0.15 mm *0.20 μm). The carrier gas was hydrogen; the pressure for the HP was 350 kPa and for the CE 170 kPa), the linear gas velocity was 30 cm/sec. Aliquots of 1 ul were injected automatically. Other conditions were mentioned as in Boon *et al.* (1994). All peak heights were corrected for the recovery of the internal standard (CB-112), but only measurements with a recovery of ≥ 80 % were taken in account.

2.7 Comparison of toxaphene patterns between incubations by GC-ECD

From each chromatogram of the technical toxaphene mixture 23 peaks were selected, each peak presenting one or more toxaphene components. The peak heights between an incubation without NADPH and an incubation with NADPH were compared, thus giving an indication for the rate of metabolism for the selected peaks by that specific animal. Because most peaks are suspected to contain more than one toxaphene congener (de Boer and de Geus, 1995), GC-ECD has its restrictions in determining the metabolic rate of individual toxaphene congeners, since it cannot make a distinction between different toxaphene congeners contributing to a peak, but only measures the total amount of electrons which are captured at a certain retention time. It is possible that in a peak consisting of several toxaphene congeners, only one is metabolisable, while others remain as they are. In this way an incorrect metabolic rate is determined. In this respect GC-ECD is only suitable for qualitative analysis of the total toxaphene pattern. GC-MS gives more information, because it is possible to determine whether peaks consist more than one molecular mass. However, also GC-MS cannot distinguish different isomers with the same retention time. Because of the multitude of toxaphene congeners present, this is an actual problem. It was found that GC-ECD was only suitable for well defined peaks at high concentrations, whereas the detection limit of GC-MS was much lower when the negative chemical ionisation mode is chosen (Muir and de Boer, 1993).

2.8 Comparison of toxaphene patterns between incubations by GC-MS

After GC-ECD analysis the samples were sent to RIVO-DLO for GC-MS analyses. Identification and quantification were routinely carried out in the selective ion monitoring

(SIM) mode to obtain a high sensitivity, but in some cases full mass-spectra were recorded of the mass-range m/z 200-500 amu.

The following GC/MS parameters were used:

equipment : HP-5988A GC/MS
injection method : splittles, splitter open after 3.0 minutes
ionisation method: NCI, 200 eV
ionisation gas : methane
T source : 100 °C
T injector : 285 °C
T analysator : 120 °C front / 75 °C at the detector side (manifold)
T transferline : 280 °C
P kolom : 200 kPa
P analysator : ~3 * 10^{-6} (- reagent gas)
P source : ~1 * 10^{-6} (- reagent gas)
: ~5 * 10^{-5} (+ reagent gas)
P methane plasma: 1 torr
carrier gas GC : helium
linear gas velocity: ~18.6 cm/sec t_i= 263 sec
GC column : CP Sil-8; length * internal diameter * thickness of stationary phase film = 49 m * 0.21 mm * 0.20 µm (RIVO nr. 52).
method file : MNIOS2::SC
tuning file : MTNCI1
multiplier voltage : 2600 Volt
emission current : 300 µA

- temperature programme:
  injection temperature 90 °C, 3 min
  increase with 30 °C/min to 180 °C, kept constant for 10 min
  increase with 5 °C/min to 280 °C, kept constant for 25 min
  290 °C, kept constant for a period of 8 min post run

- m/z ratios used in SIM mode:

m/z plus dwell:	group 1: R_t 25-30 minutes		group 2: R_t 30-55 minutes	
	264	50 msec	305	50 msec
	266	50 msec	306	50 msec
	305	50 msec	307	50 msec
	306	50 msec	308	50 msec

307	50 msec	340	50 msec
308	50 msec	341	50 msec
340	50 msec	342	50 msec
341	50 msec	343	50 msec
342	50 msec	375	50 msec
343	50 msec	377	50 msec
375	50 msec	411	50 msec
377	50 msec	413	50 msec
411	50 msec	447	50 msec
413	50 msec	449	50 msec

3 RESULTS

3.1 Condition of the microsomes

The EROD activities in harbour porpoise, harbour seal and eider duck are shown in table 3.1

Table 3.1: EROD, Aldrin epoxidase (AE) activity and protein contents in the microsomal preparations used.

Animal	EROD	AE	protein contents
Rat	n.d	n.d	31.2
PB-induced rat	n.d.	n.d.	54.2
Harbour Seal	700	9.2	9.34
Porpoise	20	0.0	15.5
Eider ducks: EE17	130	n.d	44.9
EE47	64	4.8	42.5

EROD and Aldrin epoxidase activity in pmol/mg protein/min.

Protein content in mg/g

n.d: not determined

EROD activity was very low in the harbour porpoise. All the Aldrin epoxidase activities were quite low, compared to other activities found in comparable animals (Boon *et al.*, 1992, Tanabe and Tatsukuwa, 1992, Renzoni, 1992).

In the assays of the rats, Aldrin epoxidase activity was not measured quantitatively, but after 90 minutes the entire amount of Aldrin administered was transformed into Dieldrin, a clear indication for the presence of a CYP2B type activity.

3.2 Biotransformation assay

3.2.1 GC-ECD analysis of toxaphene

Figure 3.1 gives an example for the GC-ECD chromatograms obtained in the presence of NADPH as electron donor for the cytochrome P450 system and in the absence of NADPH (no biotransformation possible). The fractions of the 23 selected peaks that remained in the animals tested after incubation for 90 minutes in the presence of NADPH are shown in figs. 3.2 - 3.6. Both samples of rat extensively metabolised the toxaphene mixture; the PB induced rat clearly showing the highest activity. Large differences were observed for the metabolic

Figure 3.1: GC-ECD chromatograms of harbour seal microsomes in the presence (lower trace) and in the absence of NADPH. TP numbers indicate the positions of the 23 peaks that were used to estimate the metabolic capacity of the microsomes from different animal species.

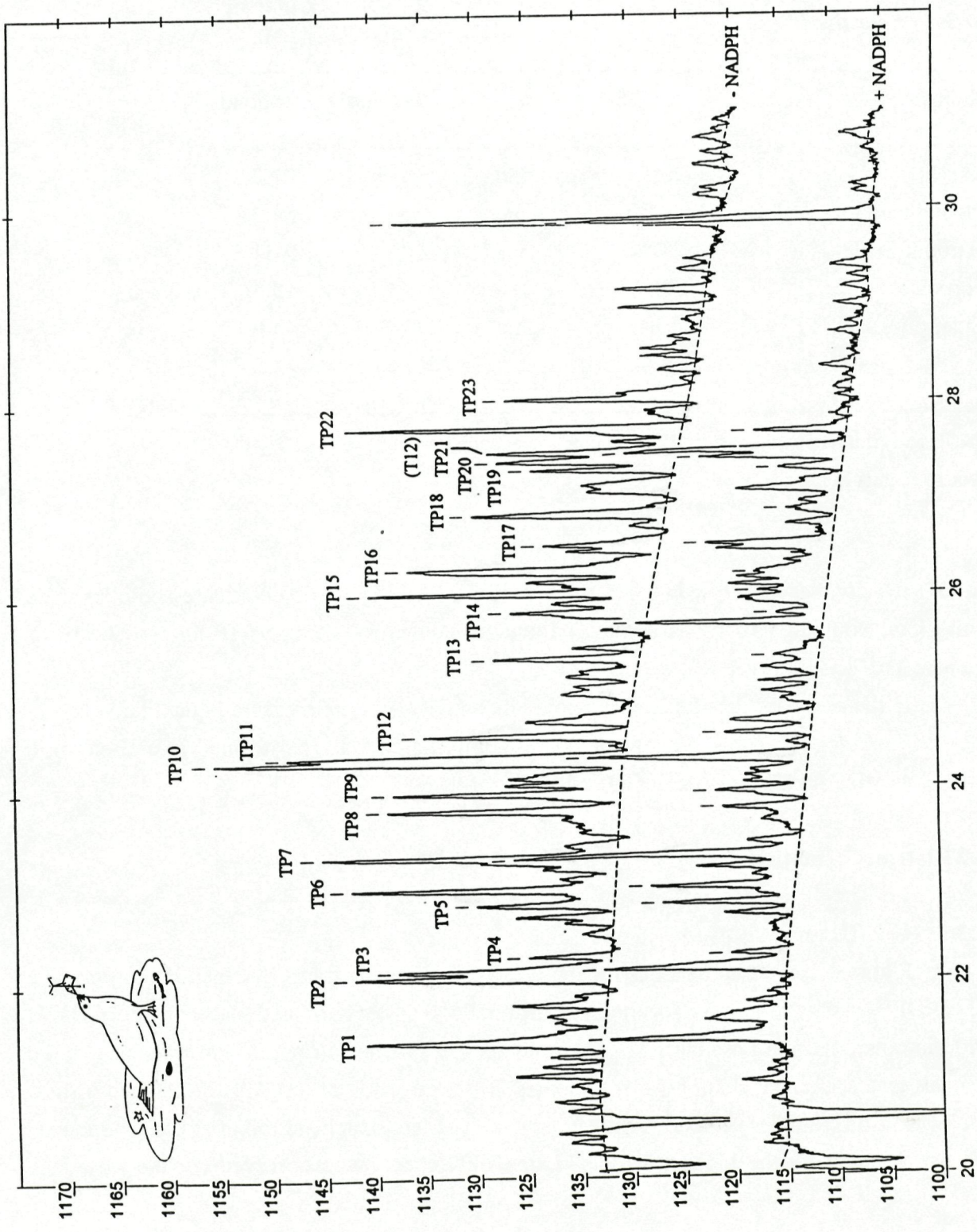


Figure 3.2: Toxaphene metabolism in microsomes of an uninduced rat.

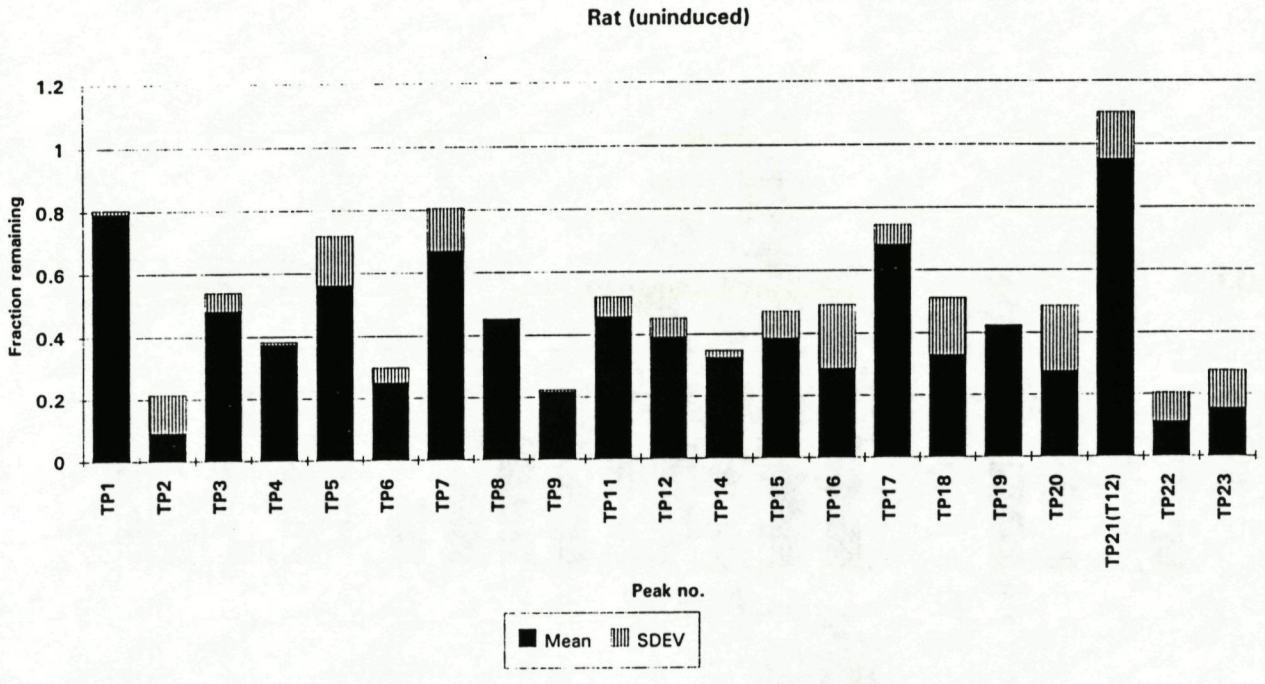


Figure 3.3: Toxaphene metabolism in microsomes of a PB induced rat.

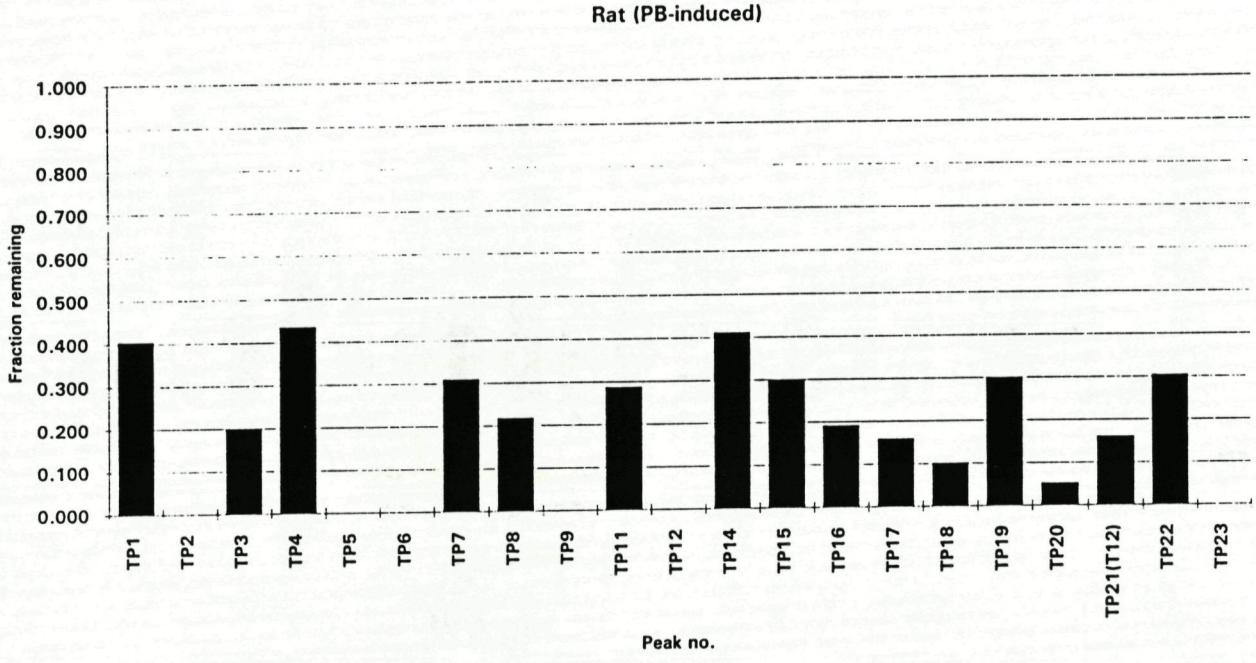


Figure 3.4 Toxaphene metabolism in harbour seal microsomes.

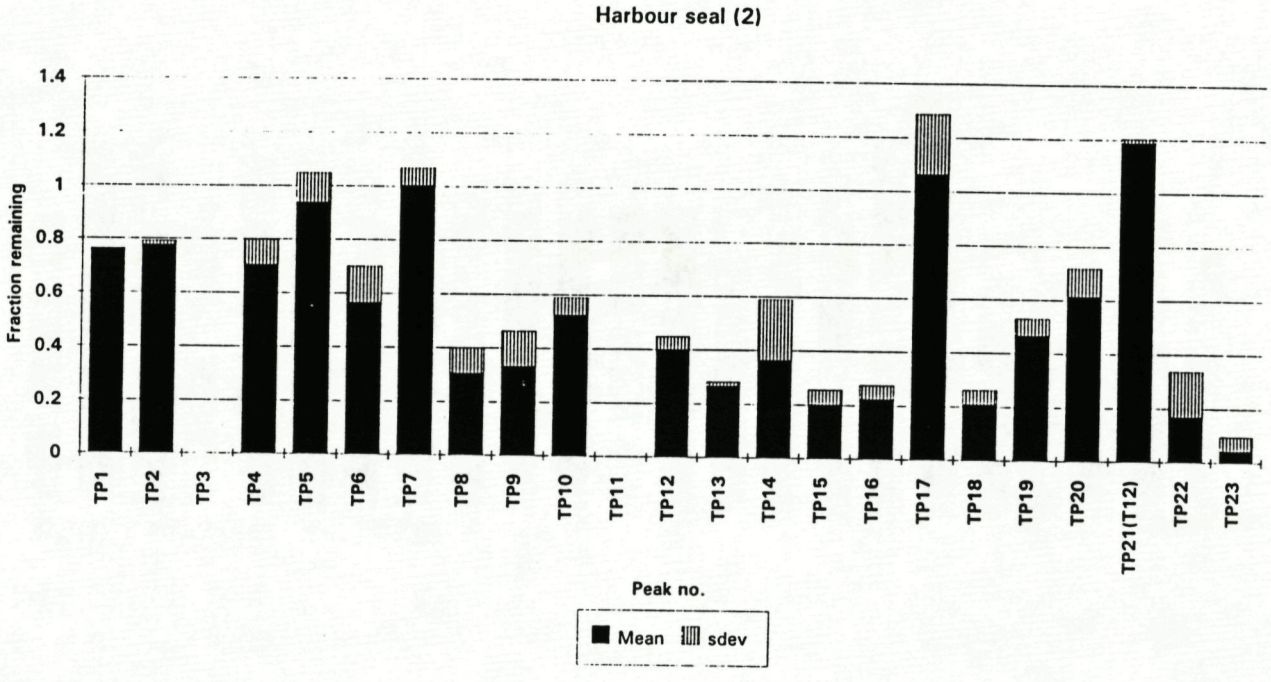


Figure 3.5 Toxaphene metabolism in eider duck microsomes.

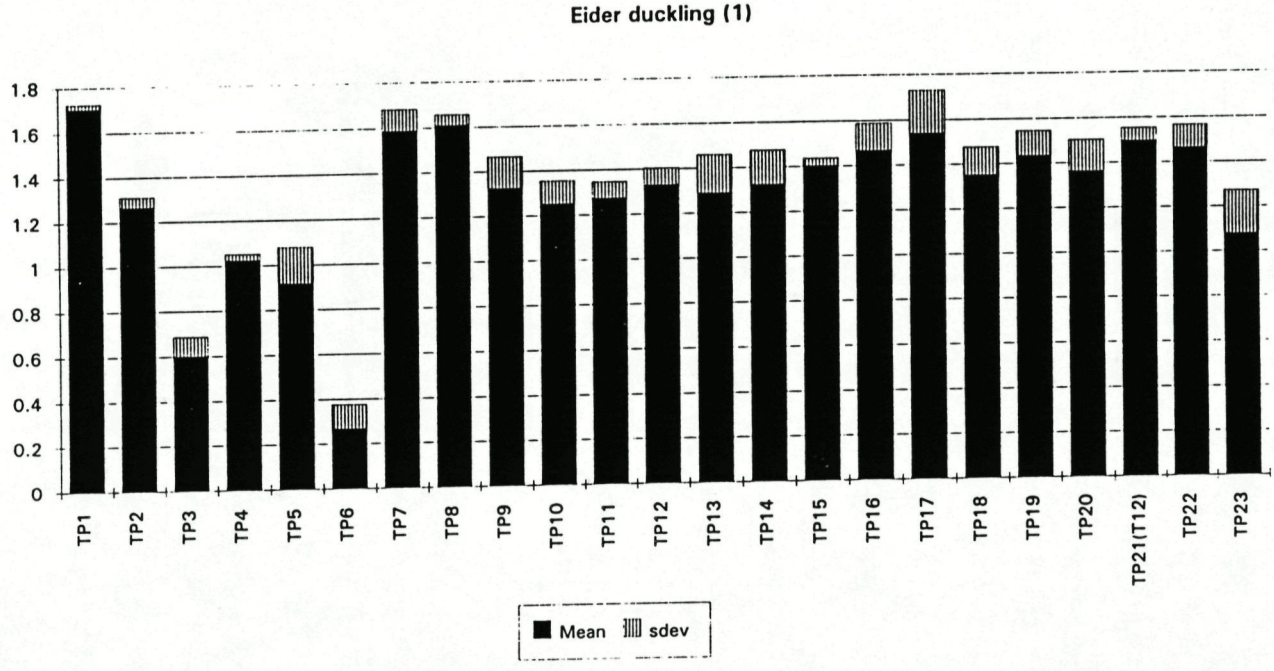
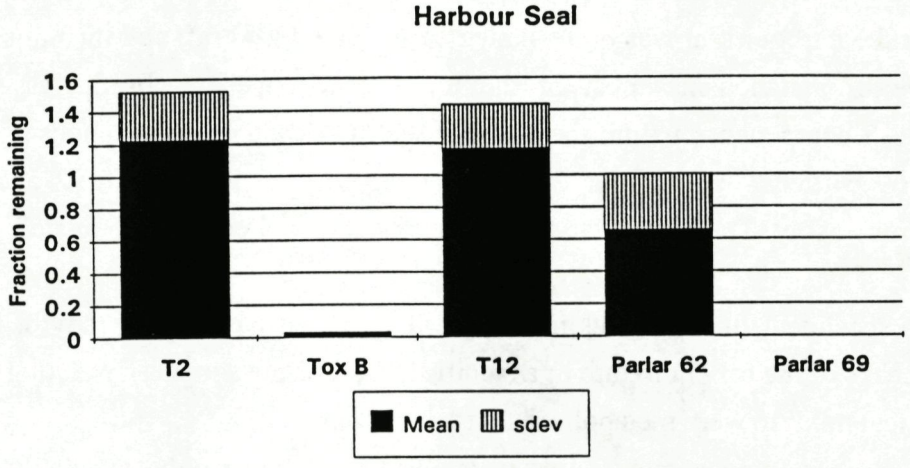
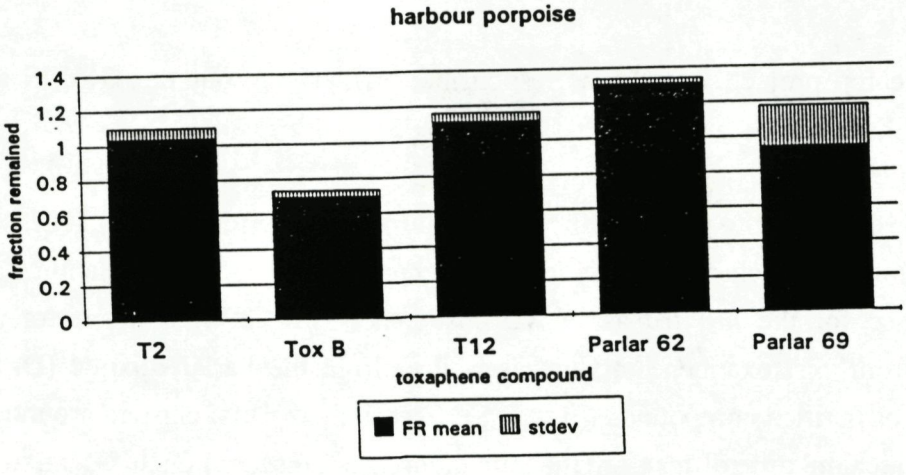


Figure 3.6 Metabolism of five chloronorbornanes by harbour seal and harbour porpoise microsomes. Molecular structures of 4 of these compounds are given in fig. 1.1.





capacities of the animals with respect to the selected peaks. A few peaks could not be metabolised at all, while other peaks disappeared completely. Only the PB induced rat was able to metabolise peak TP 21, having the same retention time as the pure compound T12 (fig. 1.1). Since this identification is based only on retention time, it is still uncertain whether this peak is really caused entirely by compound T12. The mass-spectrometric determination at RIVO at least confirmed that this peak was caused entirely by a nonachlorobornane. The situation for the harbour seal was quite similar to the uninduced rat, and thus this animal was also capable of metabolising most of the toxaphene congeners. In the assay with the reconstituted 5 component mixture the harbour seal was capable of metabolising toxicant B and Parlar 69 entirely, while Parlar 62 was partially metabolised. Both T2 and T12 were persistent in the assay. The molecular structures of 4 of the 5 compounds tested are given in fig. 1.1.

Two specimens of eider ducklings were incapable of metabolising any of the selected toxaphene peaks of the tox-5 mixture in a significant way. Only some early eluting compounds of the technical mixture were metabolised to a minor amount.

The harbour porpoise microsomes were able to metabolise only toxicant B of the tox-5 mixture to a minor extent, while Aldrin epoxidation activity could not be detected and EROD activity was also low. Since this indicates a low overall activity of the harbour microsomes, the results of the *in-vitro* assay of this animal have to be regarded with some suspicion and should in the future be confirmed with microsomes of another animal before a more definite conclusion with regards to toxaphene metabolism by the harbour porpoise can be reached.

3.2.2 GC/MS analyses

In this research project, RIVO was responsible for carrying out the GC/MS analyses. The results are discussed below.

Characterisation of the peaks in the technical toxaphene standard by GC/MS.

A reconstituted ion chromatogram of the technical toxaphene formulation and the tox-5 mixture used for the *in-vitro* assay was also made (Fig. 3.7). Full spectra of T12 were recorded from this toxaphene mixture as well as from the Parlar mixture (Dr.Ehrenstorfer) consisting of purified compounds (Figs. 3.8). Especially the m/z clusters around 340 and 378 of the technical mixture differ from the pure compound present in the tox-5 mixture, indicating that the T12 peak in the technical mixture is contaminated by at least one other compound. Thus, the decrease of compound TP21 in the technical mixture in case of the PB-induced rat does not prove that compound T12 is actually metabolised. This should be confirmed by using the tox-5 mixture.

Comparison of GC/MS and GC/ECD peak patterns

The chromatograms obtained by GC-NCI/MS were compared with those obtained by GC/ECD (NIOZ). The patterns were obviously different due to the different response factors of the individual compounds on GC/NCI-MS and GC/ECD. Strong peaks present in GC/ECD chromatograms can therefore seemingly be absent in GC/MS chromatograms, whereas the opposite is true as well. Peaks obtained by both identification techniques were identified by their TP numbers on the basis of their relative retention times vs. the peak of compound T12 (TP21). This is possible since the GC columns used with both techniques had the same stationary phases (CP Sil 8). Eleven of the 23 peaks that were identified in GC/ECD chromatograms could be found also in the GC/MS chromatograms, Nos.: TP6, 8, 11, 12, 13, 16, 17, 19, 20, 21 (T12) and 23. The other peaks may be present, but, as mentioned above, may have a lower response with GC/MS. On the other hand a number of relatively large peaks were found at the end of the GC/MS chromatograms which could not be found in the GC/ECD chromatograms.

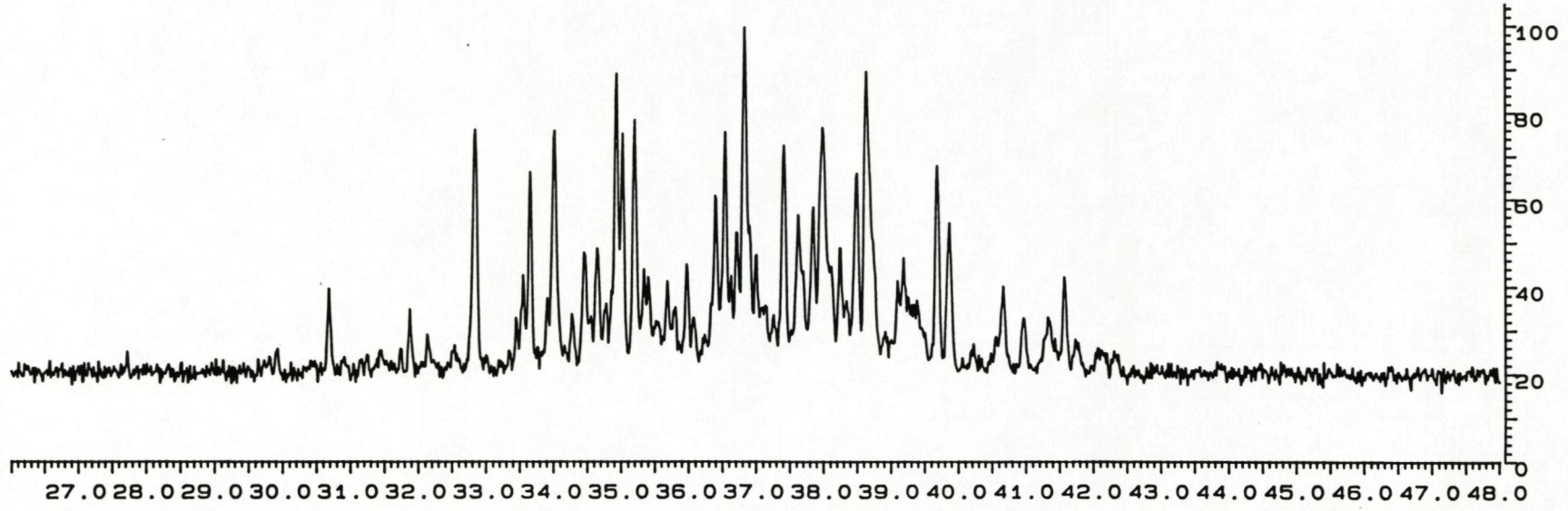
The eleven peaks identified on the basis of their relative retention times merely showed a similar behaviour to the peaks identified in GC/ECD chromatograms. The main deviation was found for TP6, which was present in high quantities in the GC/MS chromatograms. This is presumably due to an interference at that position. The comparisons were carried out for uninduced (fig. 3.9) and PB-induced rat (fig. 3.10), harbour seal (fig. 3.11) and eider duckling (fig. 3.12). Just as for the GC/ECD results, GC/MS analyses show that the harbour seal and rat have a large metabolic capacity,whereas only minor changes are found for the eider duckling.

Analysis of toxaphene in *in-vivo* samples

Toxaphene was determined by GC/MS in tissue samples of several animals from the North Sea or Wadden Sea. The chromatograms of harbour seal and harbour porpoise liver originated from the same animals of which the microsomes were used for the *in-vitro* assays. The chromatogram of the harbour seal clearly shows that toxaphene derived compounds are virtually lacking (fig. 3.13). In harbour porpoise liver, more peaks (a.o. of T12) are present, but the chromatogram is still much more simple than that of the technical mixture. The peak of T12 shows the highest peak of the chlorinated bornanes (fig. 3.14). A blubber sample of another harbour porpoise specimen was also analysed (fig. 3.15). Here, the same peaks were present as in the liver sample, but the relative height show some minor differences. Also here, T12 dominates in the chromatogram. This was even more strongly the case in a blubber sample of a whitebeaked dolphin (*Lagenorhynchus albirostris*)(fig. 3.16). The full scan mass spectrum of the T12 peak in the dolphin strongly resembles that of pure T12 in the Parlar mixture, which points to a pure T12 peak in the dolphin chromatogram (fig. 3.17). This was

confirmed by MDGC-analysis (de Boer and de Geus, 1995). Since gadoid fish is a major food source for these marine mammals, a chromatogram of hake liver (*Merluccius merluccius*) is given in fig.3.18. It shows a more complex pattern than found in all marine mammals investigated, which is an indication for metabolic changes in all marine mammal species. Yet, also the pattern in hake is less complex than that of the technical mixture. A highly different chromatogram was found in the eider duck egg. Here, T12 is present but other toxaphene-derived peaks are much higher (fig. 3.19). The number of peaks is similar to the chromatogram of hake liver, but the relative heights of the individual peaks can differ considerably.

Fig. 3.7: Reconstructed ion GC-ECNIMS chromatograms of the technical toxaphene formulation (a) and the reconstituted mixture of five chloronorbornanes (tox-5 mix) (b) used for the *in-vitro* assay.



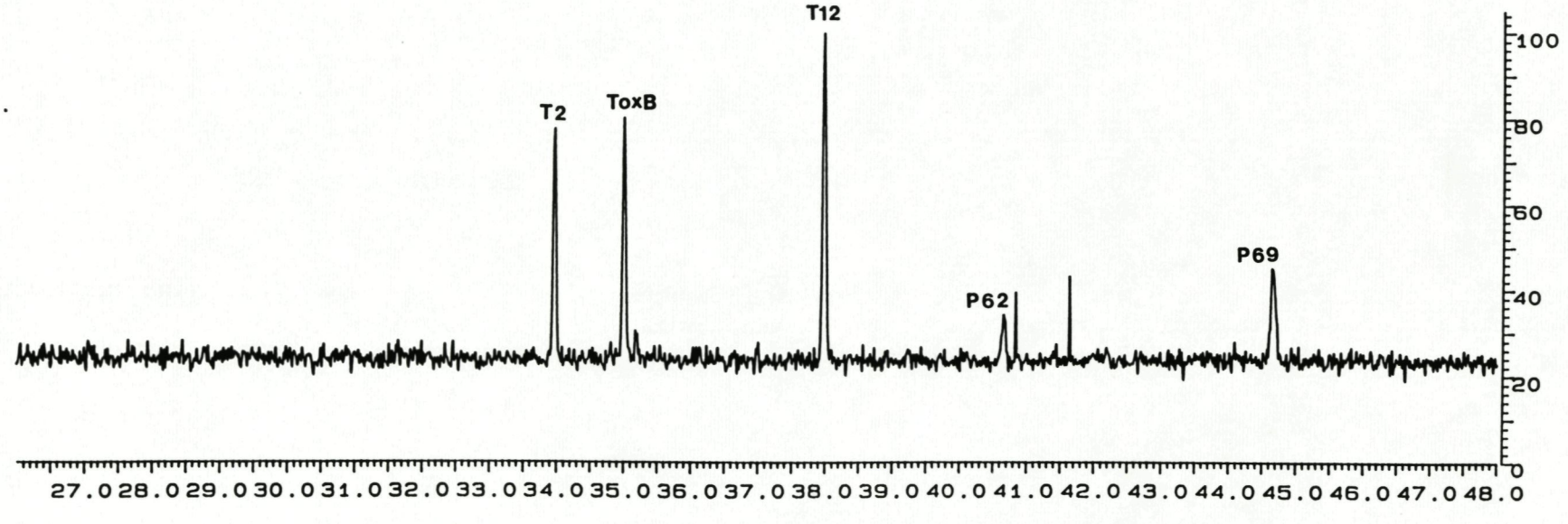


Fig. 3.8: Full mass spectra of the peak identified as T12 in the technical toxaphene formulation used (a) and of pure T12 as present in the tox-5 mixture (Parlar mix).

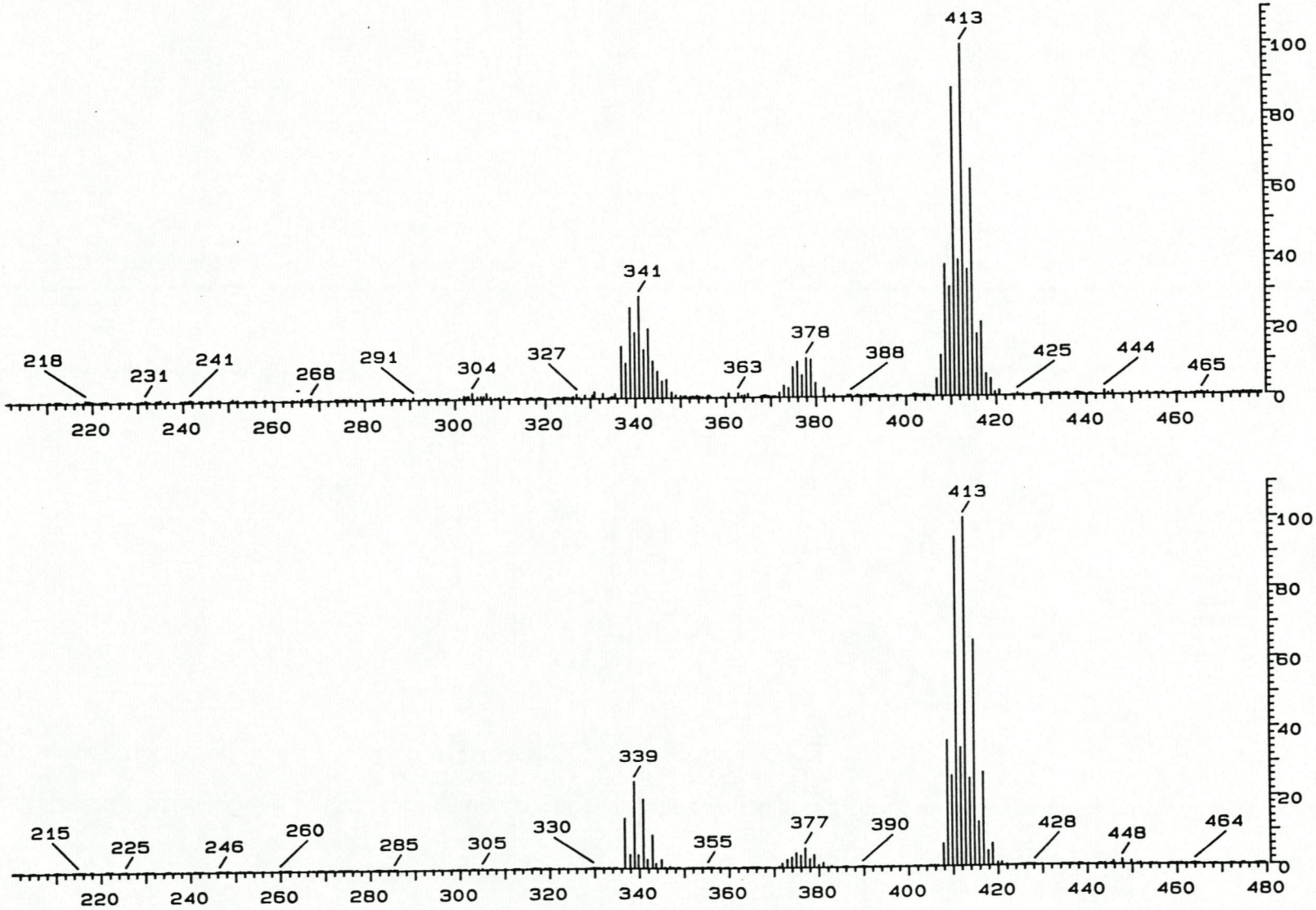


Fig. 3.9: Fraction of peaks remaining after *in-vitro* biotransformation by microsomes of an uninduced rat as measured by GC-MS. Identification of peaks was based on comparison of relative retention times (RR_t) compared to peak TP21 (T12) using GC-ECD or GC-MS. A close match of the RR_t's was only found for peaks for which bars are given.

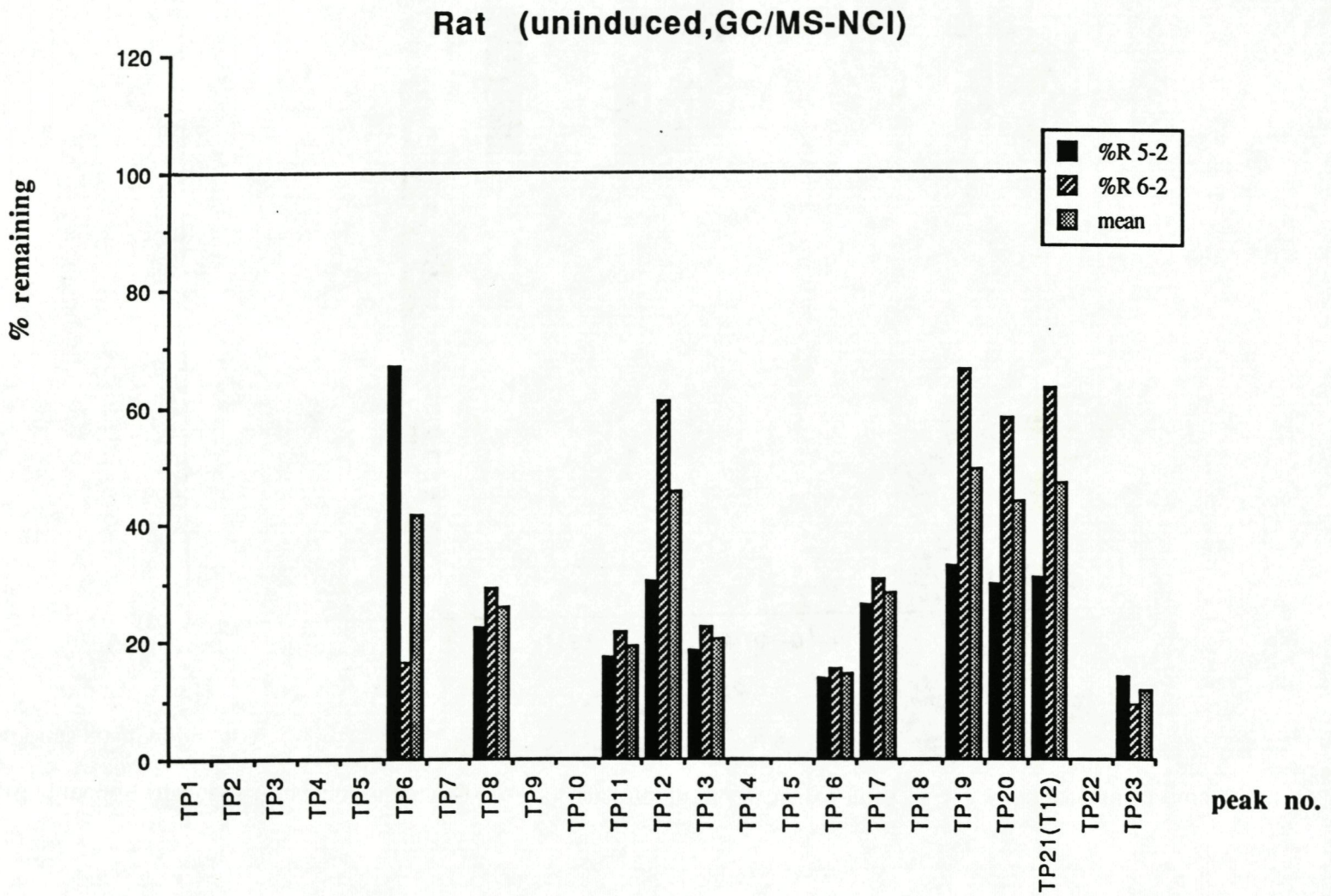


Fig. 3.10: Fraction of peaks remaining after *in-vitro* biotransformation by microsomes of a PB-induced rat as measured by GC-MS. Further explanation is given in fig. 3.9.

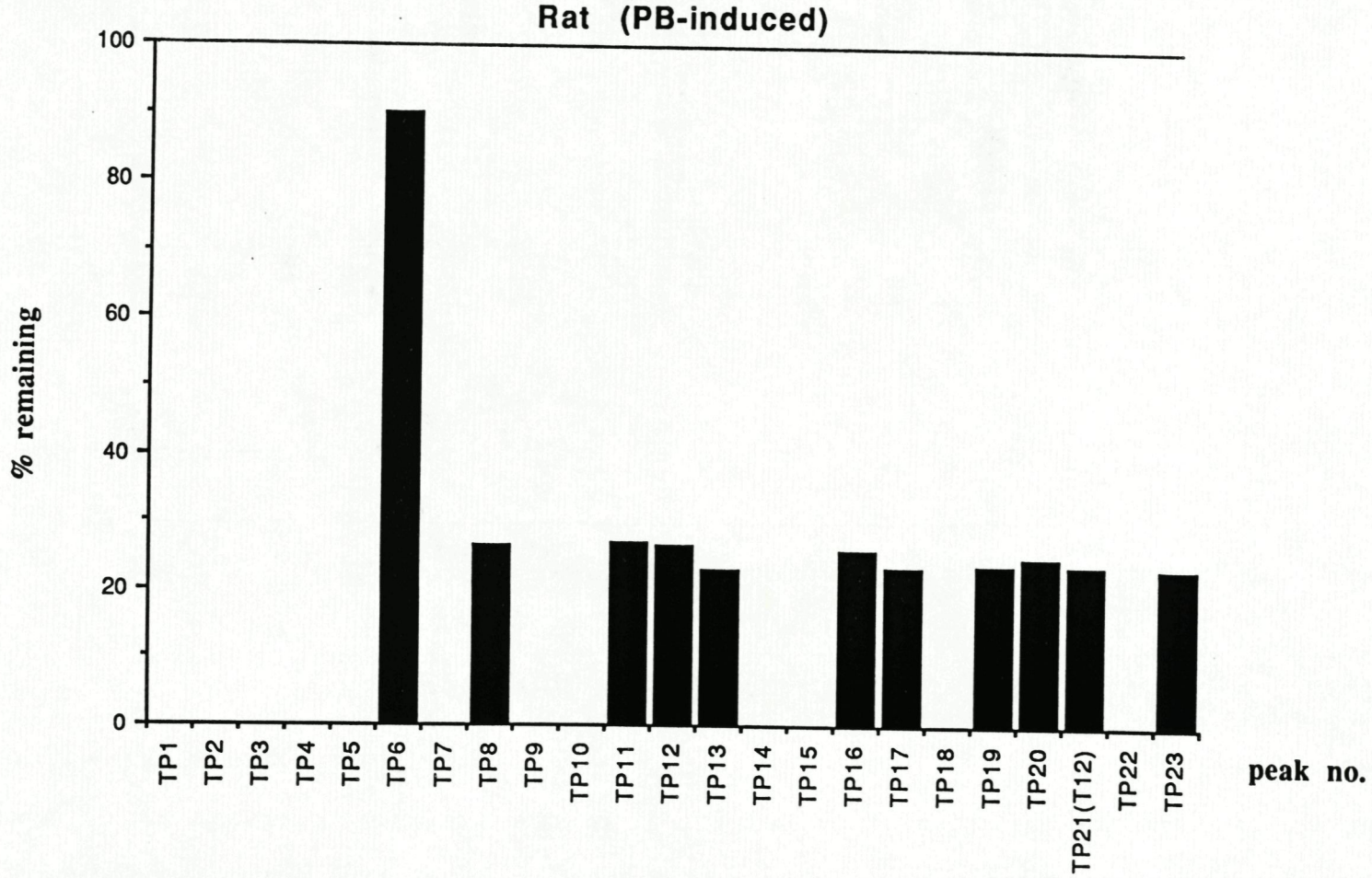


Fig. 3.11: Fraction of peaks remaining after *in-vitro* biotransformation by microsomes of a harbour seal as measured by GC-MS. Further explanation is given in fig. 3.9.

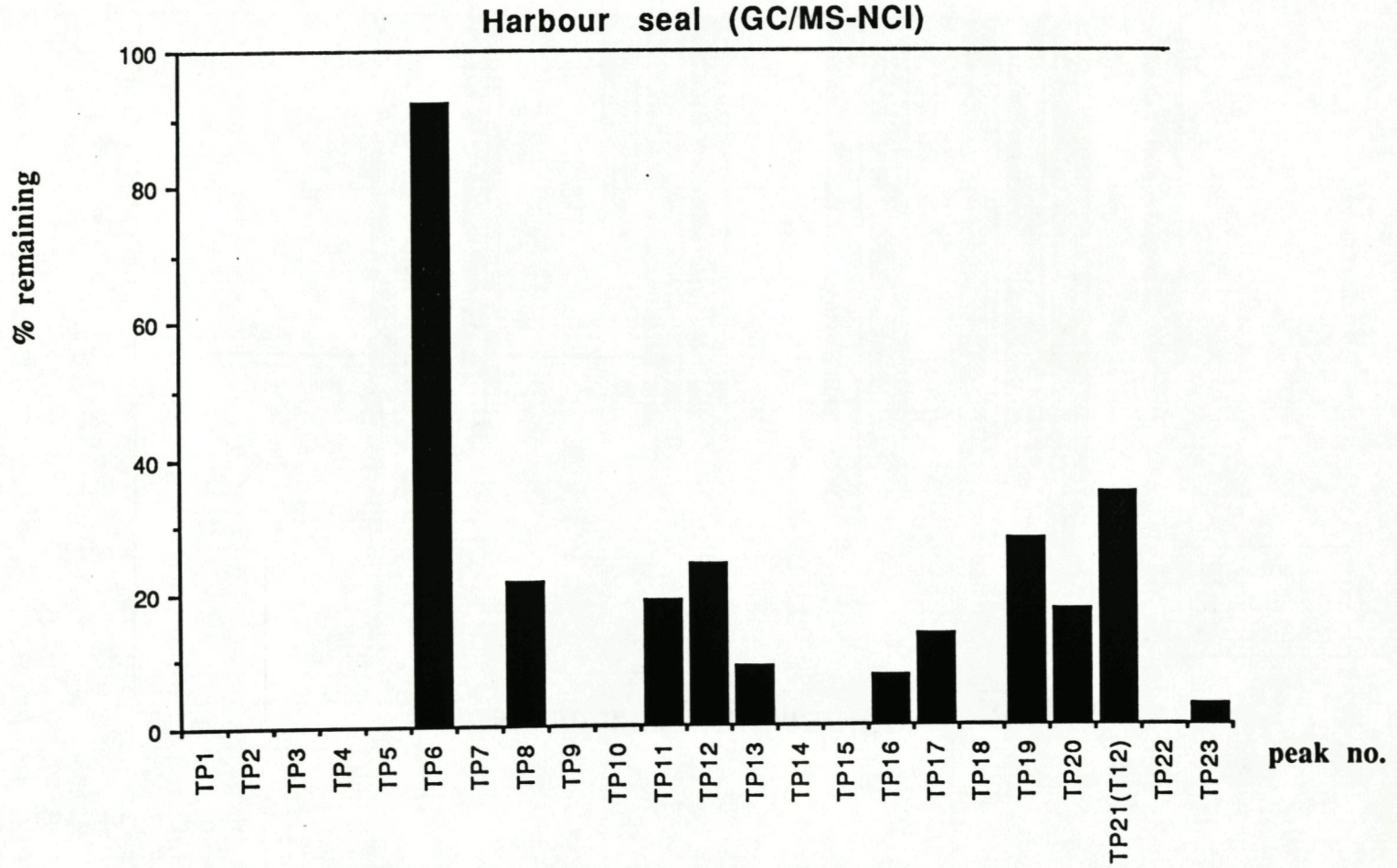


Fig. 3.12: Fraction of peaks remaining after *in-vitro* biotransformation by microsomes of an eider duck as measured by GC-MS. Further explanation is given in fig. 3.9.

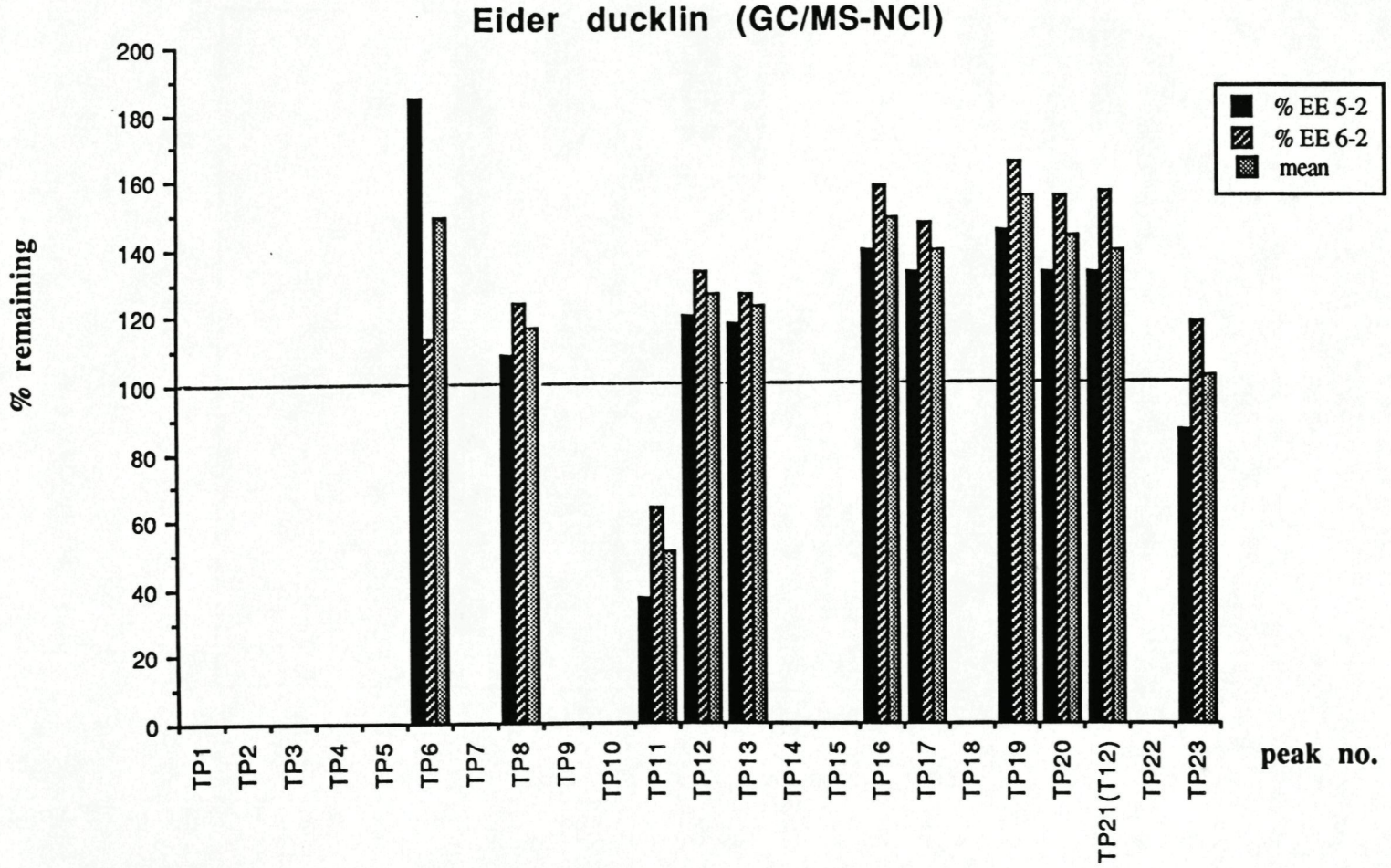


fig. 3.13: GC-MS chromatogram of liver of a harbour seal. TCN: tetrachloronaphtalene; ?= unknown compound; X= unknown compound, but no chloronorbornane. C= chlordane derived compound.

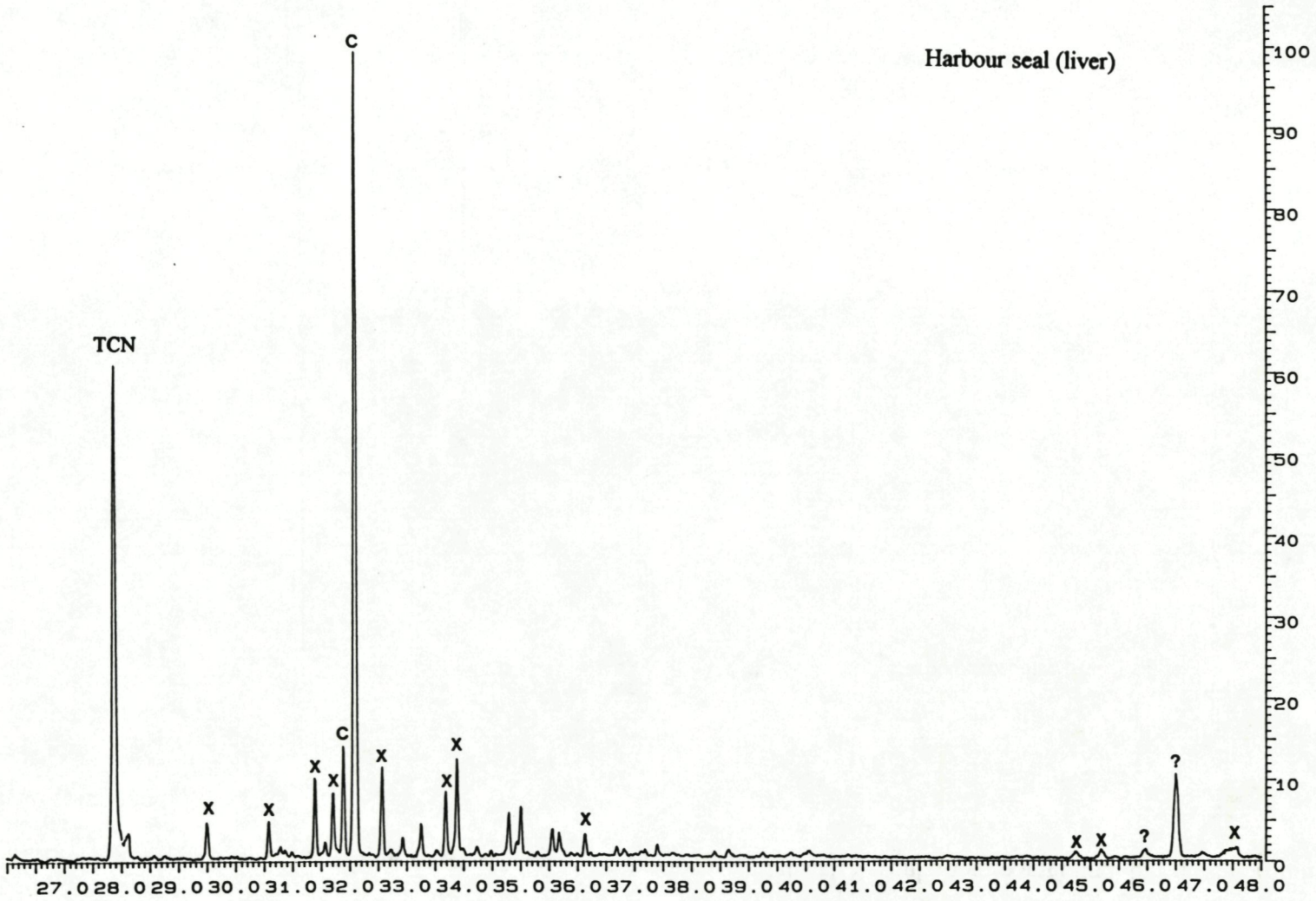


fig. 3.14: GC-MS chromatogram of liver of a harbour porpoise. TCN: tetrachloronaphtalene; X= unknown compound, but no chloronorbornane. C= chlordane derived compound.

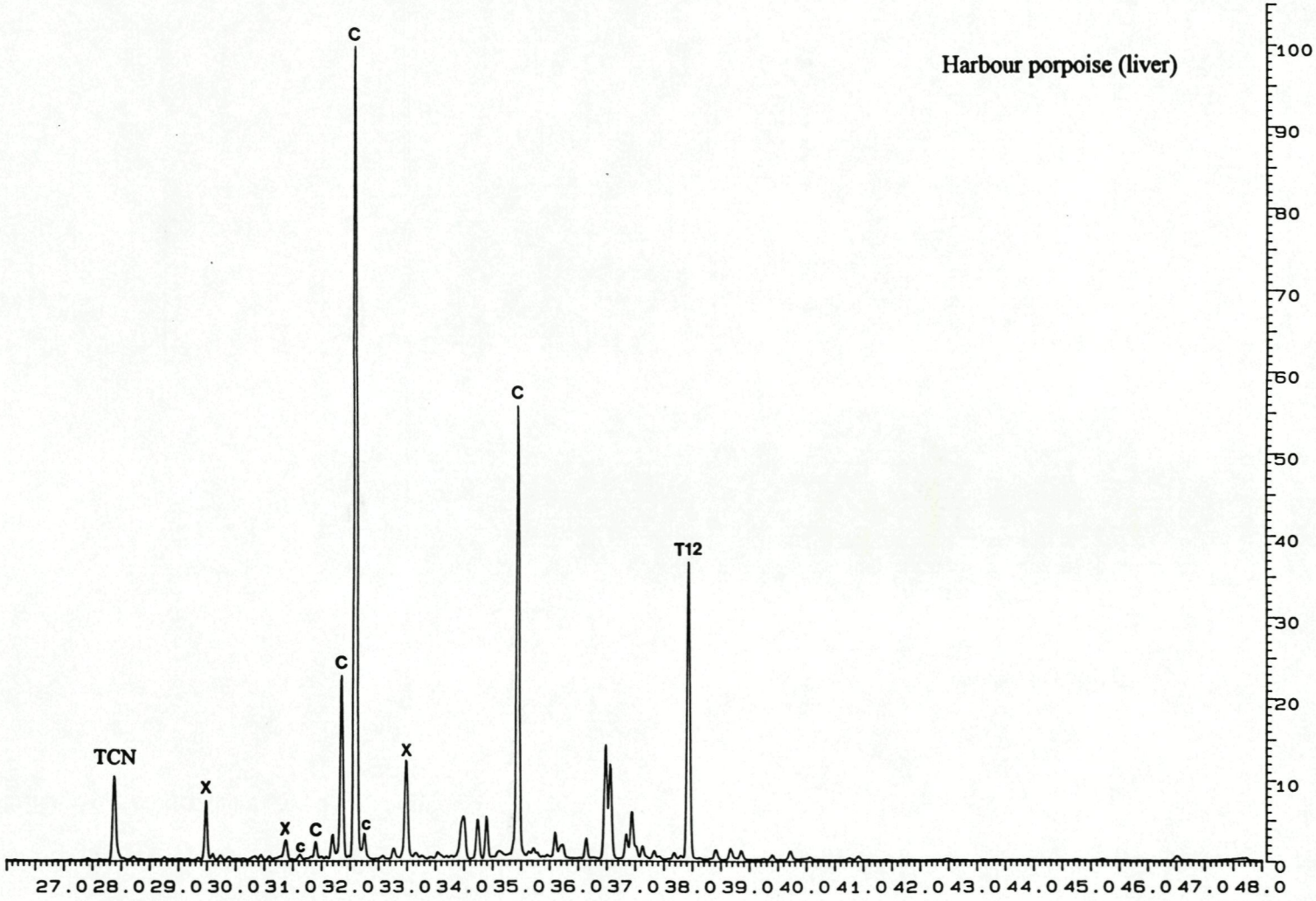


fig. 3.15: GC-MS chromatogram of blubber of a harbour porpoise (not the same animal as fig. 3.14). T12?: Peak is at least in part caused by T12 but it is not certain whether any co-eluting compounds were present; ?= unknown compound; X= unknown compound, but no chloronorbornane. C= chlordane derived compound.

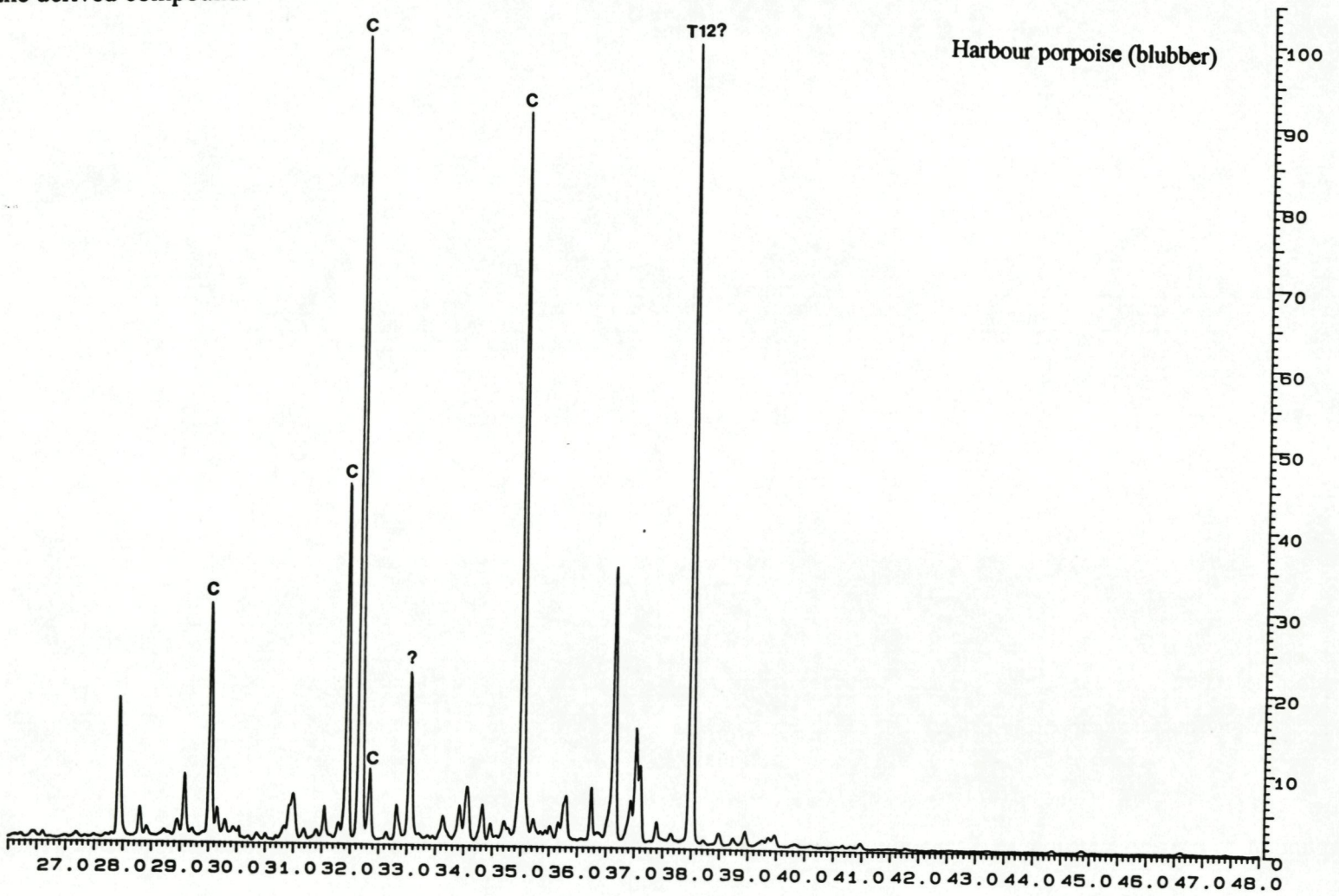


fig. 3.16: GC-MS chromatogram of blubber of a whitebeaked dolphin. TCN: tetrachloronaphtalene; C= chlordane derived compound.

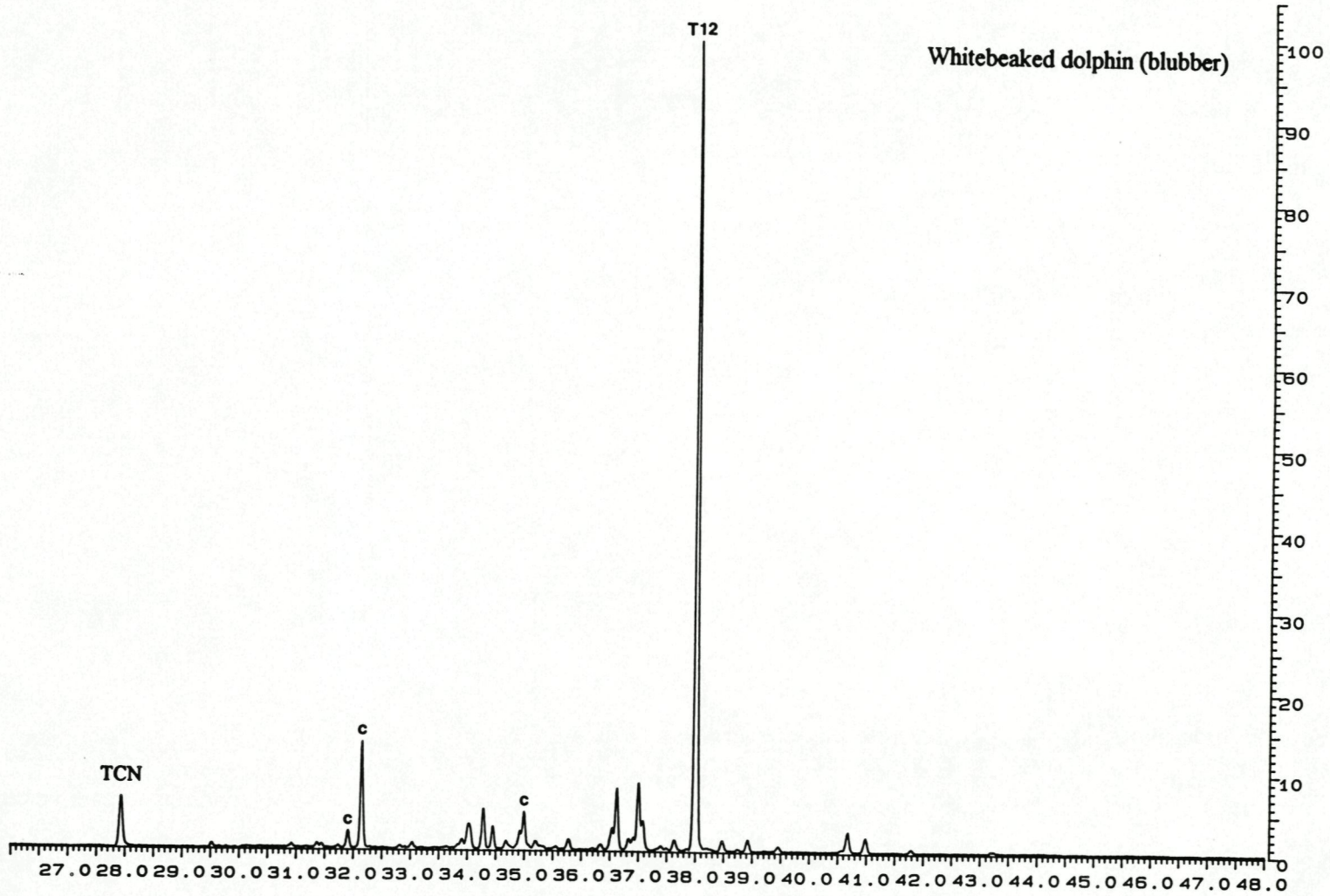


Fig. 3.17: Mass-spectra of pure T12 from the tox-5 mixture (a) and of the peak indicated as T12 in the GC-MS chromatogram of whitebeaked dolphin blubber in fig. 3.16 (b).

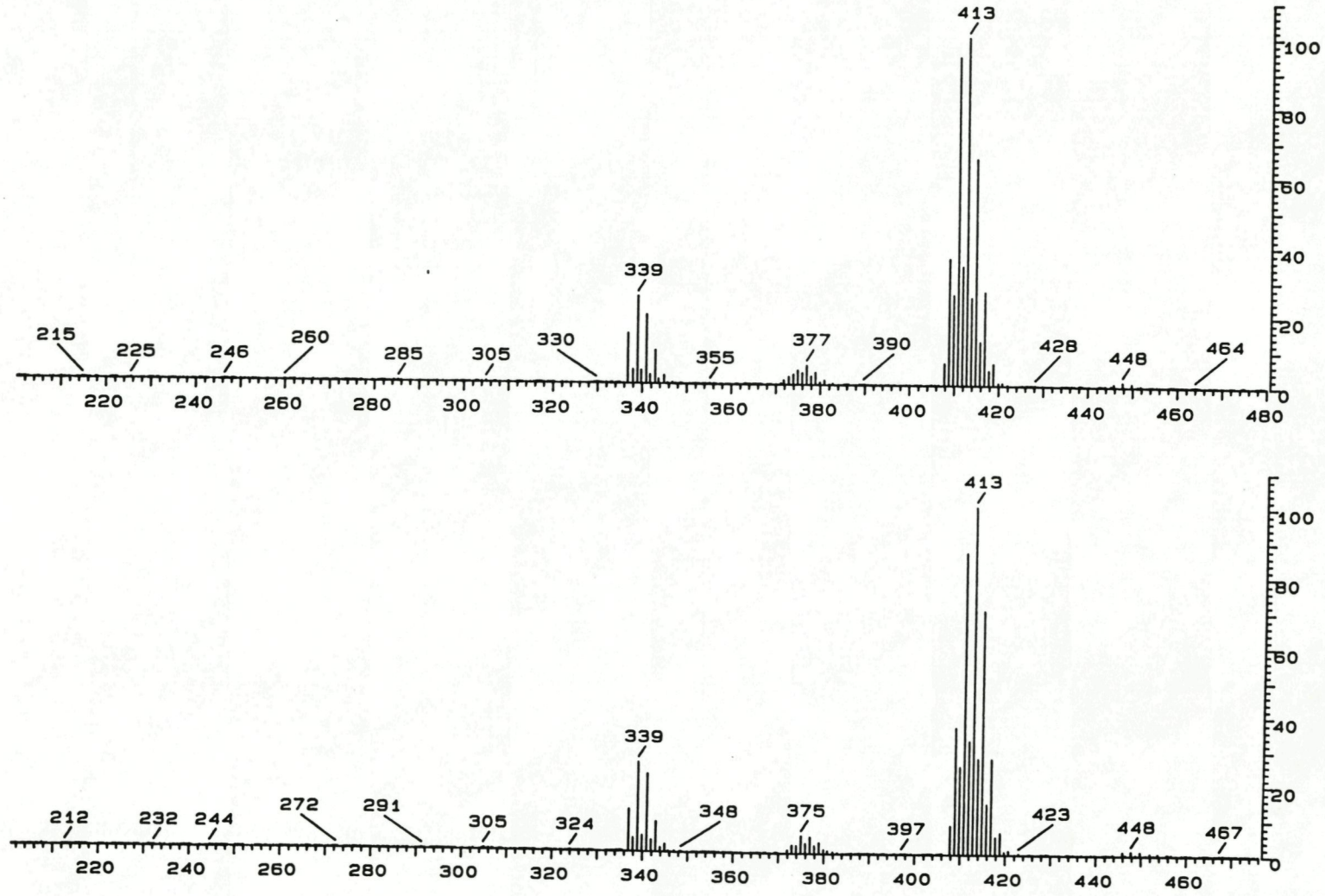


fig. 3.18: GC-MS chromatogram of hake liver. TCN: tetrachloronaphtalene; C= chlordane derived compound.

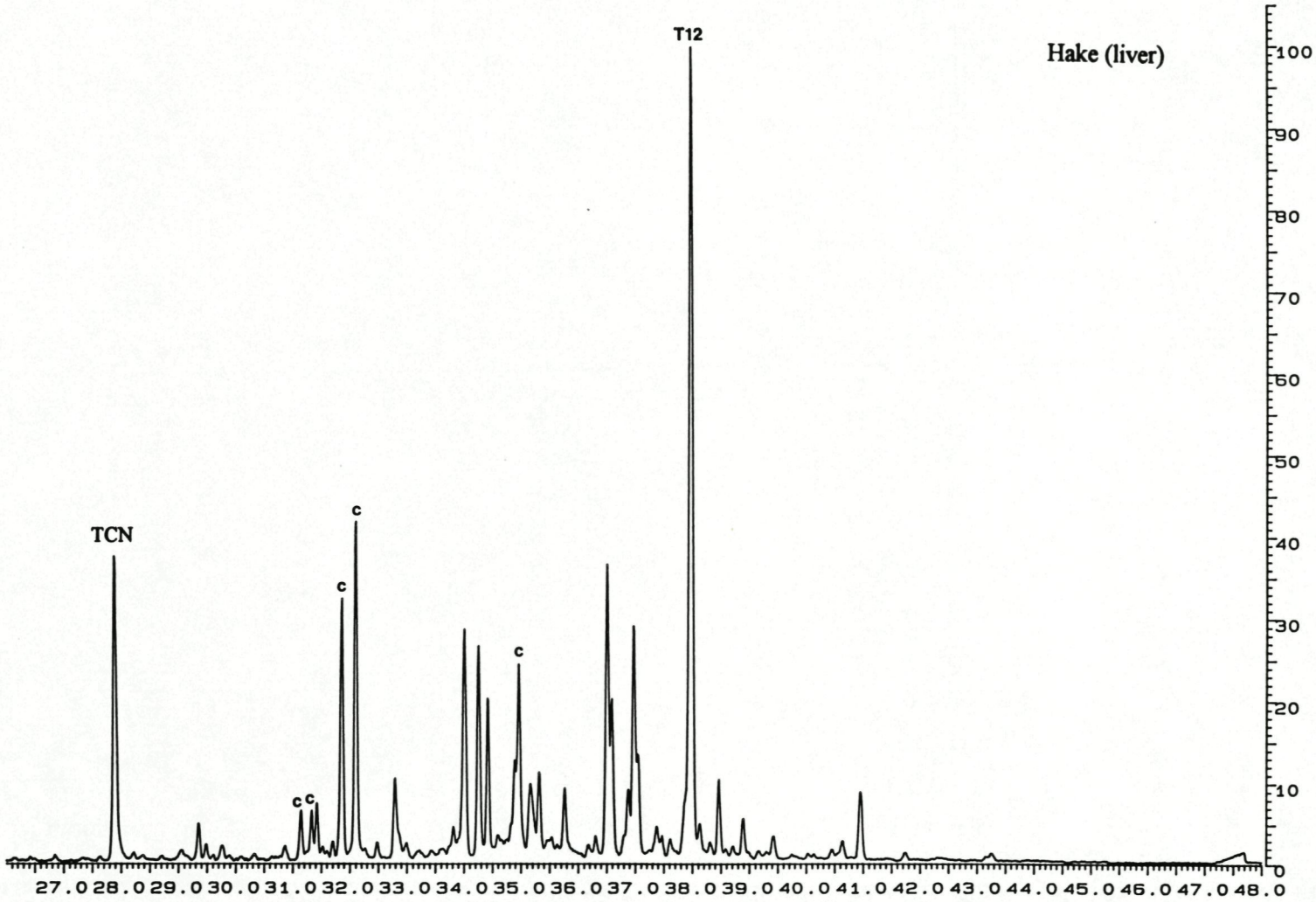
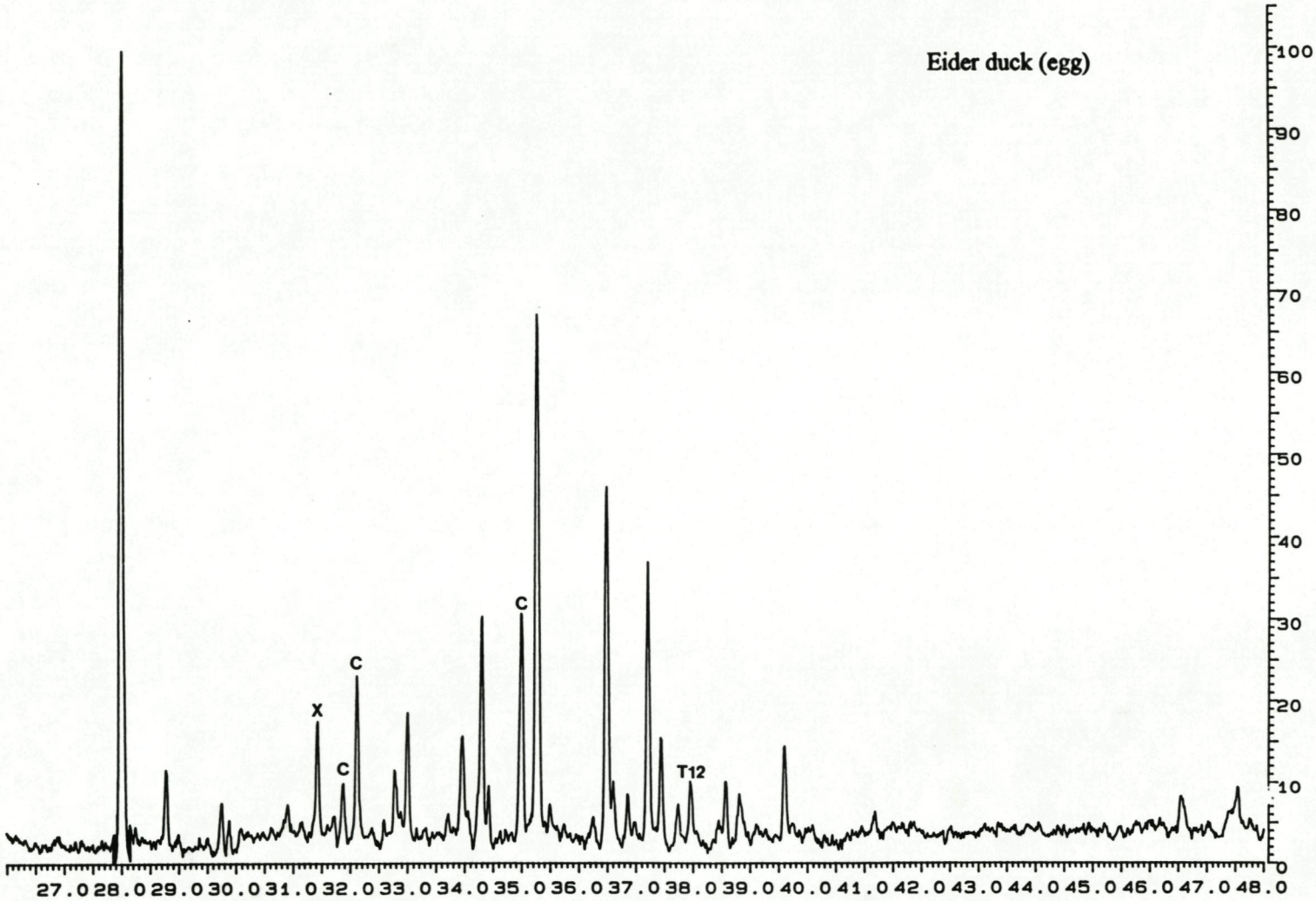


fig. 3.19: GC-MS chromatogram of an eider duck egg. X= unknown compound, but no chloronorbornane. C= chlordane derived compound.



4 Discussion and conclusions

Condition of microsomes

Rat and harbour seal microsomes showed relatively high aldrin epoxidase activities, indicating a good condition of the microsomes. Also the eider duck microsomes showed Aldrin epoxidase activity, though it was lower. Here the condition of the microsomes was also confirmed by the EROD activity, which was in the expected range for an eider duck.

In contrast to the rat, harbour seal and eider duck, the harbour porpoise microsomes showed no Aldrin epoxidase activity at all. Also the EROD activity was very low compared with the other microsomes used. These results indicate that the overall metabolic activity of the microsomes of the harbour porpoise was very low, and that the condition of the microsomes was very doubtful.

Toxaphene metabolism

Rat and harbour seal showed good metabolic activity towards the technical toxaphene mixture. There were minor differences between the chromatographic patterns of de different assays. The peak with the same retention time as component T12 was bioaccumulated by the control rat and the harbour seal, but was metabolised by the PB-induced rat. GC/MS analysis showed however, that this peak was caused by at least two compounds, one of them being T12. In the chromatogram of the harbour seal, one peak consistently emerged only in the presence of NADPH in the reaction mixture. Since oxygen containing metabolites were probably all lost in the clean-up procedure, this peak may be caused by a dechlorination product. Confirmation awaits further GC/MS analysis. Future research should provide more insight into the nature of the metabolites formed. The use of the reconstituted mixture with 5 compounds seems to be preferable to the technical mixture here, because of the overwhelming numbers of compounds that can be expected from the technical mixture.

In contrast, the microsomes of the eider ducks showed a low ability to metabolise the toxaphene mixture. The microsomes of the harbour porpoise were only able to metabolise toxicant B to a minor extent, but these results have to be regarded with some suspicion because of the low activity of these microsomes in the EROD and the Aldrin-epoxidase assay.

In the case of PCBs and chlorinated dibenzodioxins, it is well known that the presence of chlorine substituents at certain positions prevents biotransformation. Especially the substitution pattern at the outer positions of the rings are important in these molecules. In this respect it deserves attention that the persistent T2 and T12 molecules possess one chlorine substituent at all lateral (2,3,5,6) positions of the ring formed by the carbon atoms C1-C6,

whereas toxicant B and Parlar 69 have no chlorine substituents at the 3 position and Parlar no. 62 even lacks chlorine substitution at both the 3 and the 6 position. However, Chandurkar and Matsumura (1979) reported the formation of a number of secondary alcohols from toxicant C (2-endo, 3,3, 5-exo, 6-exo, 8b,9c,10a,c nonachlorobornane) in an *in-vitro* assay with the 20000*g fraction of rat liver homogenates. Similar to T2 and T12, toxicant C also carries at least one Cl substituent at all lateral positions. Thus, the structural requirements for biotransformation of toxaphene remain at the moment largely unknown.

The result from the microsomal assay show that the harbour seal extensively metabolises toxaphene compounds whereas only minor biotransformation activity was found for the eider duck. This was confirmed by comparison of GC/MS analyses of wildlife samples in the sense that a more complex toxaphene pattern was found in the eider duck egg. In case of the seal, liver of the same animal as used for the *in-vitro* assay could be used. For the eider duck, this was not possible; in this case an eider duck egg from the Wadden Sea population was taken. The virtual absence of toxaphene peaks in the sample harbour seal liver is quite spectacular, since this animal contained very high concentrations of persistent PCB congeners, but relatively low concentrations of metabolisable CB congeners due to prolonged starvation while the cytochrome P450 enzyme system had remained intact until the moment of death (Boon *et al.*, 1994). In summary, the results of the residues in wildlife confirm the results obtained in the *in-vitro* studies in the sense that the most complex toxaphene patterns were found in the species with the lowest biotransformation activities in the *in-vitro* assay.

The similarity between the results of the rat and the harbour seal indicates similar metabolic pathways in both species. The microsomes of the PB-induced rat metabolised technical toxaphene more rapid than microsomes of the uninduced rat. This supports the hypothesis that the metabolism of toxaphene congeners may be catalysed by iso-enzymes belonging to the CYP2B subfamily, because of the expected higher levels of CYP2B in microsomes of a PB-induced animal. Also, from the low ability of eider duck to metabolise the technical mixture, it can be concluded that the pathway for metabolism of toxaphene supposed to be present in harbour seal and rat is lacking in eider duck . This is confirmed by the hypothesis that other fish-eating seabirds seem to lack CYP2B. Probably the aldrin epoxidase reaction is catalysed by another iso-enzyme incapable of metabolising toxaphene, related to CYP2B in this species (Ronis *et al.*, 1989a, Ronis *et al.* 1989b).

In summary, it can be concluded that the *in-vitro* results are supported by the *in-vivo* data on residues in environmentally exposed animals. Thus, this *in-vitro* bio-assay can serve as a valuable, rapid and relatively very cheap instrument to measure the metabolic capacity of phase I enzymes in different marine species towards mixtures of lipophilic organic contaminants.

Recommendations for further research

Because certain peaks of the chromatogram of the technical mixture of toxaphene consist of several unresolved congeners it is difficult to quantify the biotransformation of these congeners. Analysis by multidimensional GC (MDGC) may be helpful in this respect, but a suitable MDGC method for toxaphene has only recently been developed (de Boer and de Geus, 1995). The reconstituted mixture of five components seems a promising alternative in this respect. The difference between the PB-induced rat on the one hand and the control rat and harbour seal on the other hand in the metabolic activity of peak TP21 of the technical mixture consisting in part of component T12, might also become more clear when the reconstituted mixture is applied.

The fact that only a few components of the toxaphene mixture are identified, makes it difficult to predict a relationship between structure and metabolism of certain congeners. Therefore more analytical standards of individual congeners are needed.

The results obtained so far only concern two marine species, since the results for the harbour porpoise remained inconclusive due to the low ability of the microsomes to perform the standard biochemical assays for cytochrome P450 activity (EROD and Aldrin epoxidase). However, very recently viable microsomes could be obtained from another harbour seal, a sperm whale (*Physeter macrocephalus*) and a whitebeaked dolphin. These will also be used in the continuation of this project in 1995. Also, the effect of biotransformation on the mutagenic effects of toxaphene will be studied using the standard mutatox assay (contribution of RIKZ-RWS).

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