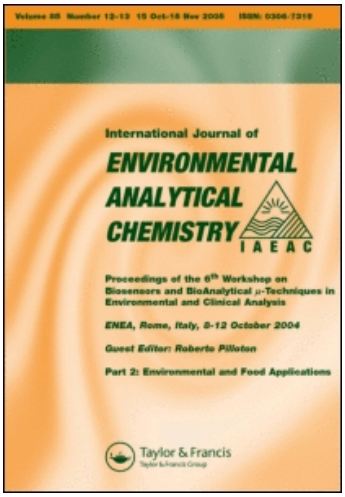

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Determination of Individual Chlorobiphenyls (CBs), Including Non-Ortho, and Mono-Ortho Chloro Substituted CBs in Marine Mammals from Scottish Waters

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DETERMINATION OF INDIVIDUAL CHLOROBIPHENYLS (CBs), INCLUDING NON-ORTHO, AND MONO-ORTHO CHLORO SUBSTITUTED CBs IN MARINE MAMMALS FROM SCOTTISH WATERS

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Twenty individual chlorobiphenyls (CBs) have been determined in marine mammals. The more toxic non-*ortho* and mono-*ortho* CBs have been separated from di and tri-*ortho* CBs using a pyrenyl silica HPLC column prior to the final determination by capillary GC. The comparative metabolism of the biologically active CBs in whale, porpoise, dolphin and seal tissue is described. An estimate of the TCDD toxic equivalence concentration (TEC) for the non ortho and mono ortho CBs is made.

KEY WORDS: Non *ortho*-chlorobiphenyl, mono *ortho*-chlorobiphenyl, marine mammals, pyrenyl silica HPLC, capillary gas chromatography, toxic equivalence factor.

INTRODUCTION

The determination of chlorobiphenyls (CBs) in marine mammal tissue species has developed over the past decade from a measurement of the 'total' PCB value, based on the industrial formulations, to the estimation of the individual CB congeners[1–5]. This change has been brought about primarily by the need to separate, identify and determine those congeners in marine biota which (i) are measured for monitoring purposes[6,7], and (ii) are known to be toxic and exhibit specific biological effects, particularly the induction of liver microsomal enzyme activities[8,9]. The CBs which are the most toxic and induce both aryl hydrocarbon hydroxylase (AHH) and ethoxy resorufin-O-deethylase (EROD) and bind with high affinity to the cytosolic receptor protein are approximately iso-electronic with 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD). Both the toxic mechanism and the enzyme induction involve an initial binding of the CB to the same arylhydrocarbon, Ah, receptor[10], and one of the common spatial features between the TCDD and these toxic CB molecules is the

possible planar configuration for those congeners having unsubstituted ortho positions on the biphenyl molecule. A high correlation has been found between affinity to the receptor and AHH/EROD induction, and between induction and toxicity (weight loss, thymic atrophy)[11,12]. However, any similar correlation between biological activity and the concentration of these toxic, planar molecules in marine mammals is considerably more tenuous[5]. This gap in our knowledge is partly due to a combination of poor or limited sampling, poor measurement of the co-factors, e.g. seasonality, sex, lipid/blubber thickness, which affect contaminant concentrations, and the lack of good analytical methods to separate and measure these toxic CBs.

In 1982 the Community Bureau of Reference (BCR) working group on CB analysis identified seven CBs which could be used in environmental monitoring programmes (Table 1). This list has currently been extended to include three mono ortho-(*o*) chloro CBs: CB 118, CB 105, CB 156 as well as CB 128 and CB 170. Although there have been considerable improvements in the determination of the CBs used for monitoring purposes[13,14,15] there are still some questions related to the identity and measurement of the mono-*o* and non-*o* CBs. De Boer[16,17,18] has addressed the problem of separation and identification using multi-dimensional gas chromatography (MDGC) and Hagland[19] has reported an improved separation of these toxic CBs prior to GC

Table 1 Chlorobiphenyls determined in marine mammals.

Chlorobiphenyls Determined		*CBs which may interfere on 5% phenyl methyl without further separation*	*P450 induction potential of the CBs determined*
IUPAC No.[1]	*Substitution pattern*		
28*	2,4,4′	31	None
52*	2,5,2′,5′	None	Non/weak
44	2,5,2′,3′	None	Non/weak
70	3,4,2′,5′		Non/weak
101*	2,4,5,2′,4′	90, 84	P450 IIB
138*	2,3,4,2′,4′,5′	163	P450 IA/IIB
149	2,4,5,2′,3′,6′	118	None
153*	2,4,5,2′,4′,5′	132, 105	P450 IIB
180*	2,3,4,5,2′,4′,5′	None	P450 IIB
105	2,3,4,3′,4′	132, 153	P450 IA/IIB
114	2,3,4,5,4′		P450 IA/IIB
118*	2,4,5,3′,4′	149	P450 IA/IIB
128	2,3,4,2′,3′,4′	167, 162	P450 IA/IIB
156	2,3,4,5,3′,4′	171, 202	P450 IA/IIB
170	2,3,4,5,2′,3′,4′	190	P450 IA/IIB
Non-orthochloro CBs			
77	3,4,3′,4′	110	P450 IA
126	3,4,5,3′,4′	129, 178, 166	P450 IA
169	3,4,5,3′,4′,5′	170, 190	P450 IA

* Original "seven" CBs selected for certification and are the basis of most marine enviromental moniroring programmes.
1. Ballschmiter ref. 28.

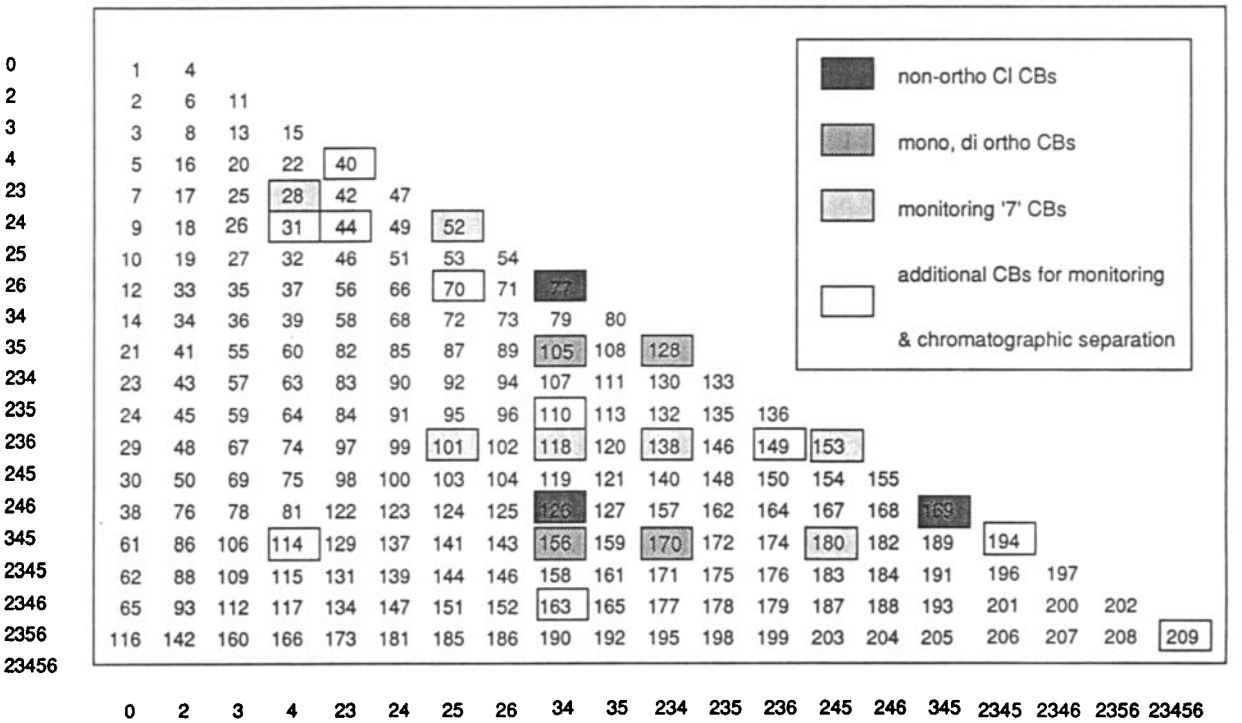

Figure 1 The substitution pattern on each aryl ring of the chlorobiphenyl structure and the IUPAC Nos for each CB, after Ballschmiter (ref. 28). The CBs selected for analysis in the marine mammal tissue shown in the shaded rectangles.

using a pyrenyl (*PYE*) bonded silica high performance liquid chromatographic (HPLC) column.

The chlorobiphenyls studied in the SOAFD Marine Mammals Programme are given in Figure 1 which shows the selection of the CBs in relation to their substitution patterns. The choice was based on abundance, persistence and toxicity, and includes the non-*o* CBs which exhibit pure 3-methylcholanthrene (3-MC) type induction of the P450-IA sub-family, along with the mono-*o* and di-*o* CBs, which exhibit a mixed function induction (P450 IA/IIB)[8,20,21] (Table 1). These specific CBs were included in addition to the original seven CBs selected for monitoring purposes[13].

This paper reports on the development of the separation of CBs in marine mammal tissue using a pyrenyl-silica (PYE) HLPC column to separate the more toxic, trace CBs prior to analysis by capillary GC. Particular attention has been given to the separation of key groups of CBs which are normally unresolved on a 5% phenyl-methyl silicone GC stationary phase. The concentration of the CBs and the TCDD toxic equivalence factors (TEFs) in the Scottish marine mammals are determined and are compared with data from animals taken from other regions. The relative pattern of these CBs is compared in relation to the metabolism of each CB and its TEF for each of the four marine mammal species selected.

EXPERIMENTAL

Samples

Blubber samples from four marine mammal tissues were taken from the laboratory's specimen bank for this study and were originally obtained in co-operation with the Veterinary Investigation Service of the Scottish Agricultural College, Inverness.

Sperm whale A male sperm whale, ***Physeter macrocephalus*** was stranded at Garbh Ailean on the west coast of the Isle of Mull on 31-05-90 and samples of blubber and visceral tissue were taken for pathological and chemical investigation. This whale attracted considerable media attention since a stranding of an animal of this size and species is a relatively rare occurrence. It also followed in the wake of the phocine distemper virus (PDV) epizootic amongst the common seal population in the UK during 1988–89.

Bottle-nosed dolphin A male bottle-nosed dolphin, ***Tursiops truncatus***, length 1.97 m was stranded at Spittle Point, Berwick and had died from meningitis and parasitism.

Harbour porpoise A male harbour porpoise, ***Phocoena phocoena***, length 1.47 m beached at Kingston (NGR NJ 334658) on 19-10-90 had drowned suffering from pneumonia.

Common seal A female common seal pup, ***Phoca vitulina***, length 0.97 m, weighing 15 kg was found ill at Torrin on the Isle of Skye (NGR NG 576199) and died from

an upper respiratory tract infection, probably associated with the 1988 PDV infection.

Each blubber tissue from these animals was originally analysed for organic contaminants and heavy metals as part of the laboratory's surveillance programme on marine mammal strandings in Scotland and were now re-examined to determine the toxic CB content of the tissue.

Chemicals

All solvents used were of the highest purity and were obtained as *'glass distilled grade'* from Rathburn Chemicals (Walkerburn, Scotland). The individual pure, solid CBs were obtained from the Community Bureau of Reference (CB 28, 52, 101, 105, 118, 128, 138, 149, 153, 156, 163, 170, and 180) as either certified or well characterized materials, from Promochem GmbH (CB 44, 70, 77, 114, 126, 158, 169, 194) and the CLB mixture of 51 CBs in *iso*-octane from the National Research Council of Canada (NRCC) Halifax, Nova Scotia.

The internal standards (2,4 dichlorobenzyl aklyl ethers) were prepared and characterized in this laboratory[33].

All other materials used in the sample preparation have been reported elsewhere[22,23].

Extraction and clean-up

Samples of blubber tissue (20 g) were taken and macerated with anhydrous sodium sulphate. The resultant powder was extracted in a Soxhlet for 4 h with dichloromethane (DCM) (100 ml). The bulk of the DCM was reduced in a rotary evaporator and then completely removed by adding hexane and evaporating in a clean, dry stream of air. A portion of the extract was taken to determine the amount of extractable lipid residue by removing the solvent in a weighed dish.

The lipid in the remaining portion of the sample was hydrolysed by saponification with 20% potassium hydroxide in ethanol (300 ml) at 60°C for 1 hr. A 1 ml portion of CB 209 (50 mg ml^{-1}) was added to the extraction mixture to determine the recovery of the CBs. The reaction mixture was cooled and diluted with distilled water (200 ml) and extracted with hexane (2 × 50 ml). The hexane extract was evaporated to ca 1 ml in a clean, dry stream of air and cleaned-up by passing the sample sequentially through an alumina and then a silica column to remove any traces of remaining lipid and co-extracted material and to separate the CB fraction from other unhydrolysed pesticides[22,23].

An alternative or additional method of clean-up using a sulphuric acid column was also used. The sample extract is eluted with hexane through a 3 g silica column which has been impregnated with concentrated sulphuric acid. The scheme of the sample preparation is given in Figure 2.

Liquid chromatography

The CBs were separated using a Cosmosil 5-PYE HPLC column (2-(1-pyrenyl) ethyldimethylsilylated silica gel), particle size 5 μm, Nacalai Tesque (Promochem

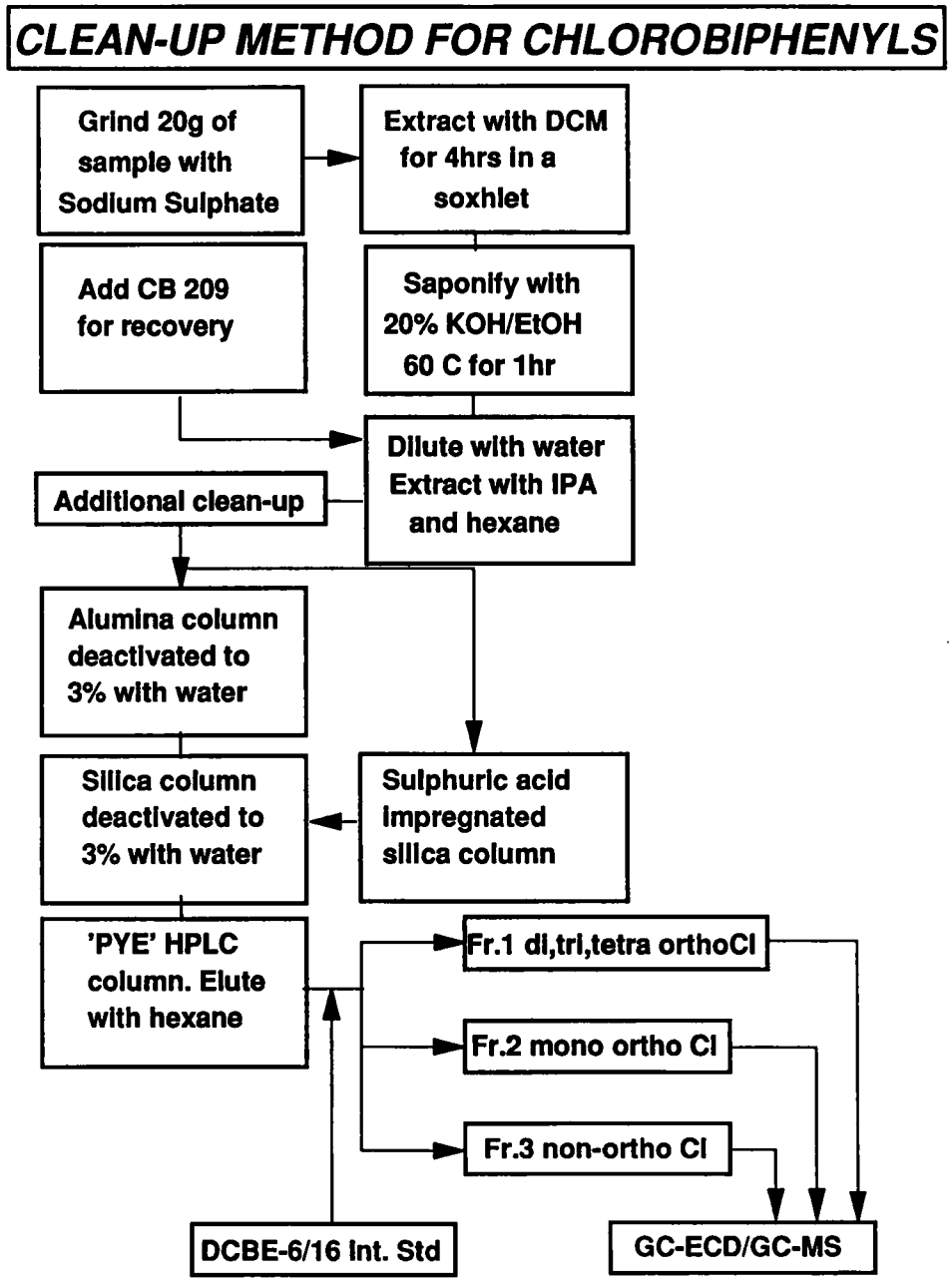

Figure 2 Schematic diagram for the extraction, clean-up and separation of non-*o*, mono-*o*, and di, tri, tetra CBs.

GmbH). The HLPC system consisted of Gilson 321 autosampler fitted with a 50 μl loop, a Gilson 302 pump and 401 diluter to dispense the sample into the loop. The fractions from the column were monitored with a Philips PU 4020 uv detector set at 254 nm and collected with a Gilson 202 fraction collector. The injection and fraction cycle was controlled by the Gilson autosampler and the data collection was controlled by an Apple IIe microcomputer running Chromatochart software.

The cleaned-up samples from the alumina and silica were concentrated to ca 80 μl in Chromcol tapered vials and eluted with hexane at a flow rate of 0.5 ml min^{-1}. Two internal standards, 2,4 dichlorobenzyl hexyl (D_6) and dodecahexyl (D_{16}) ether[33] were added (1 ml of 1 $\mu g\ ml^{-1}$) to each fraction, the sample was reconstituted in iso-octane and the CB content determined by capillary GC.

Gas chromatography

The Philips 302 GC and autosampler was fitted with a 50 m × 0.22 mm i.d. CPSil 8 CB fused silica column and a ^{63}Ni electron capture detector (ECD). The samples were injected (1 μl) into a splitless injector at 270°C and chromatographed at 80°C for 1 min and at 3°C min^{-1} to 280°C using hydrogen as carrier gas at a linear velocity of 40 cm s^{-1}. The chromatograph and data collection was controlled by a microcomputer operating Minichrom (VG Data Systems).

RESULTS AND DISCUSSION

Sample preparation

Kannan *et al*[24,25] reported the levels of the planar CBs in the major commercial formulations and from these data we determined the most appropriate sample mass required for the determination of the toxic CBs at the ultra trace level in the different compartments in the aquatic environment[4]. The total CB loading, based on a wet tissue mass, for fatty fish species with a high lipid content and for marine mammals is between 1–500 mg kg^{-1} and for the non-*o* CBs 77, 126, and 169 (Table 1) the concentration would most likely fall between 0.01 and 50 $\mu g\ kg^{-1}$. Since these planar CBs are present at a substantially lower concentration than the most abundant congeners it is necessary to increase the intake mass accordingly. This amount should be between 0.2 and 10 g as a minimum sample size and between 10 to 500 g to produce an ideal response on the ECD.

Since the marine mammals selected for this study contain between 1000 and 20,000 $\mu g\ kg^{-1}$ *"total"* CB the sample mass taken was 20 g. It is possible to increase this mass to ca 30 g before increasing the size of the Soxhlet apparatus or changing the extraction method to a column percolation system. In the percolation system the tissue is ground with an anhydrous sodium sulphate as before and placed in a large glass column. Solvent, such as dichloromethane (DCM) is passed through the column under gravity or with a small positive pressure. Although this column system can hold a greater mass of tissue, the volume of solvent used is considerably larger (500–1000 ml) than in the conventional Soxhlet (100 ml). This increase in solvent

volume is costly. It raises the level of impurities in the sample and blank and, unlike the Soxhlet, does not have the advantage of being left unattended.

Saponification

The initial clean-up requirement following the extraction of a large mass of marine mammal tissue was to remove or destroy the bulk of the co-extracted lipid, which may be as high as 90% of the original tissue mass. In this study the lipid levels ranged from 13% for the whale blubber, which is relatively low, to 84% for the dolphin (Table 2). There are four main ways which can be used to achieve this: (i) adsorption

Table 2 Concentration of individual chlorobiphenyls in marine mammal taken from Scottish water.

IUPAC No.	*PYE HPLC fraction*	*Whale*	*Dolphin*	*Porpoise*	*Seal n = 9*	*CV%*[1]
			Concentration in μg kg⁻¹			
Di (and Tri-)-*ortho* chloro CBs						
52	I	15	243(228)	1262	108	7.4
44	I	22	44	58	48	8.8
101	I	48	309(281)	670	159	4.9
149	I	42	925(925)	2255	222	4.4
114	II	ND	ND	ND	ND	
153	I	117	2491(2243)	4901	2773	2.8
138	I	46	1133(1814)	2402	962	6.7
163	II	58	708	2218	660	
158	II	4	18	89	15	17.7
128	II	ND	180(299)	416	126	15.7
170	II	19	306(340)	512	267	9.2
180	I + II	56	924(1076)	1259	699	
194	II	15	123	187	137	16.7
Mono-*ortho* chloro CBs						
70	I	3	25	13	ND	
105	II	7	117(167)	129	33	11.1
118	II	48	283(285)	408	71	9.0
156	II	6	21(130)	5	37	7.7
Non-*ortho* chloro CBs						
77	III	0.42	2.4	1.3	0.22	
126	III	0.41	1.2	0.35	1.6	
169	III	0.12	0.54	0.17	1.6	
Lipid content		13%	88%	79%	85%	
"Total PCB" based on wet weight		705	14,520 (14,800)	26,300	11,750	
"Total PCB" based on lipid		5500	16,500 (16,830)	33,200	14,000	

Figures in parenthesis for the dolphin are the values obtained in a previous analysis without the separation of the *PYE* column.
"Total PCB" is a sum of the seven congeners CB 28, 52, 101, 118, 138(+163), 153 and 180 multiplied by 2.5 to give an "Aroclor 1254 equivalent" comparable to the early existing data obtained on packed columns.
Limit of detection CB 52–CB 156 0.5 μg kg⁻¹
CB 77–CB 169 0.05 μg kg⁻¹
1. CV% = Coefficient of variation.

column chromatography, (ii) gel permeation chromatography (GPC), (iii) concentrated sulphuric acid digest, and (iv) saponification with alcoholic potassium hydroxide. The advantages of methods (i) and (ii) are that they do not destroy other determinands which are less robust than the CBs, but they do require large quantities of adsorbent or GP material to cope with 15 g of lipid. The adsorption column can remove between 50 mg (for a small 3 g alumina column) to 1 g with a 60 g alumina column[23,26]. A large GPC column packed with biobeads SX-2 or SX-3 can remove up to 500 mg of lipid, but will also use up to 300 ml of solvent. Concentrated sulphuric acid will effectively destroy most organic compounds containing elements other than C, H, X, but the exothermic oxidation associated with such a large organic mass can be an unnecessary hazardous laboratory operation. The more favoured method is alkaline hydrolysis. Van de Valk and Dao[27] has studied the conditions for the removal of fish lipid, by saponification, for the determination of CB and they recommend a maximum of 60°C for 1 hr to maximize the destruction of the lipid without degrading any of the more highly chlorinated congeners. Ethanol was preferred to methanol since the latter tended to form emulsions more readily during the subsequent extraction into hexane. Any emulsion that formed was destroyed by the addition of *iso*-propyl alcohol (30 ml) prior to the addition of the distilled water (200 ml) and back extraction into hexane. The efficiency of the saponification was checked by measuring the lipid content after the saponification. More than 99% of the solvent extractable lipids are destroyed by this method. However, even with an efficiency of 99%, a sample mass intake of 20 g would still have a residue of up to 200 mg of lipid.

Further clean-up to remove the traces of lipid and group separation[23,26] of the contaminants was undertaken using standard alumina and silica adsorption columns.

Liquid chromatography

The separation techniques to isolate planar CBs from other contaminants has been fully reviewed by de Voogt *et al*[1]. The initial methods used carbon or granular charcoal in various grades and particle sizes. The carbon was used in mg quantities as a free powder or adsorbed onto foam to offer a greater surface area. More recently, silica has been coated with graphite as the basis of the commercially available HPLC *Hypercarb* columns. The separation is based upon the retention of the planar, or near planar molecules by the graphitic surface of the adsorbent. Non-planar molecules are either unretained or have a limited retention, whilst molecules with a 'flat' structure similar to TCDD interlock with the graphite surface and are only removed by backflushing with a more polar solvent, like dichloromethane or toluene. Whilst most of these columns were able to separate the planar molecules from the other contaminants they also retained some of the planar compounds, reducing the recovery. The resolution of these columns, even the *Hypercarb* type, are insufficient to obtain a more refined separation of the planar and semi-planar molecules with slightly differing structures.

The development of the pyrenyl silica 'PYE' HPLC column[19] has made it possible to separate structurally similar molecules with different π-electron densities resulting

from the spatial configuration of the aryl rings. This stationary phase gives sufficient resolution between non-*o*, mono-*o* and the other ortho substituted CBs. Initially this type of column was developed to separate the toxic non-*o* CBs (CB 77, 126, and 169), which the carbon columns achieve, and the mono-*o* CBs, which the carbon columns could not achieve. However, in addition to the separation of these toxic CBs it is also possible to improve the separation between other key CBs which can co-elute on the 5% phenyl methyl GC column. This technique, therefore, has the potential to remove a number of ambiguities that can exist in the final determination of CBs using GC.

The retention profile of the toxic non-*o*, and mono-*o* CBs and the CBs determined in routine monitoring on the PYE column is given in Figure 3. The lower chlorinated CBs and the di- and tri-*o* CBs (CB 28, 52, 101, 149, 153 and 138) have a limited retention on the column and are eluted in fraction I. The mono-*o* CBs (CB 118, 105 and 156 are eluted in fraction II, along with the other chlorinated, di-*o* CBs (CB 163, 128 and 170). The non-*o* CBs (CB 77, 126 and 169 elute in the final third fraction.

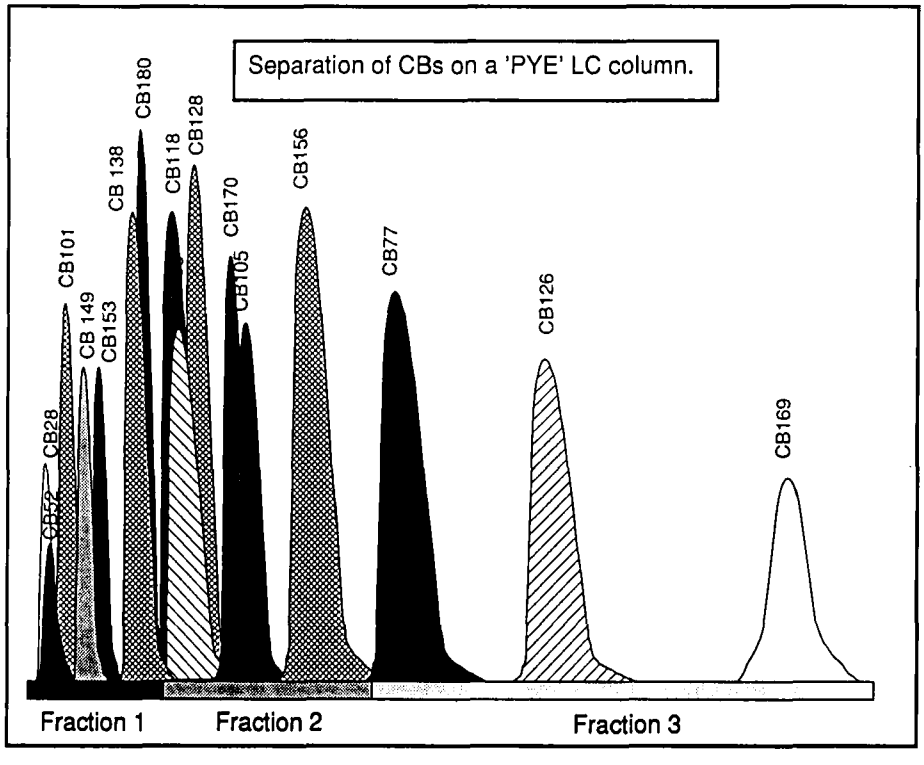

Figure 3 The reconstructed chromatogram of the toxic and monitoring CBs determined in marine mammals. Each CB was injected via a 50 μl loop into the 150 mm × 4.6 mm i.d. *PYE* column with hexane as solvent at a flow rate of 0.5 ml min^{-1}.

Table 3 Separation of the NRCC CLB and SOAFD mixture of chlorobiphenyls using the PYE HPLC column.

IUPAC No.	*Ring substitution*	*"PYE" fraction*	*IUPAC No.*	*Ring substitution*	*"PYE" Fraction*
15 4	4′	I	138 2 4 5	2′3′4′	I + II
18 2	2′5′	I	163 2 3 5 6	3′4′	II
54 2 6	2′6′	I	129 2 3 4 5	2′3′	I
31 2 5	4′	I	158 2 3 4 6	3′4′	I
28 2 4	4′	I	126 3 4 5	3′4′	III
52 2 5	2′5′	I	187 2 3 5 6	2′4′5	I
49 2 5	2′4′	I	182 2 3 4 5	2′4′6′	I
44 2 5	2′3′	I	159 2 3 4 5	3′5′	II
40 2 3	2′3′	I	183 2 3 4 6	2′4′5′	I
103 2 4 6	2′5′	I	128 2 3 4	2′3′4′	II
121 2 4 6	3′5′	I	185 2 3 4 5 6	2′5′	I
60 2 3 4	4′	I	202 2 3 5 6	2′3′5′6′	I
101 2 4 5	2′5′	I	156 2 3 4 5	3′4′	II
86 2 3 4 5	2′	I	200 2 3 5 6	2′3′4′6′	I
87 2 3 4	2′5′	I	173 2 3 4 5 6	2′3′	I
110 2 3 6	3′4′		180 2 3 4 5	2′4′5′	I + II
77 3 4	3′4′	III	169 3 4 5	3′4′5′	III
154 2 4 6	2′4′5	I	170 2 3 4 5	2′3′4′	II
151 2 3 5 6	2′5′	I	201 2 3 5 6	2′3′4′5′	I
149 2 4 5	2′3′6′	I	196 2 3 4 6	2′3′4′5′	I
118 2 4 5	3′4′	II	203 2 3 4 5 6	2′4′5′	I
143 2 3 4 5	2′6′	I	189 2 3 4 5	3′4′5′	III
114 2 3 4 5	4′	II	208 2 3 4 5 6	2′3′5′6′	I
153 2 4 5	2′4′5′	I	207 2 3 4 5 6	2′3′4′6′	I
132 2 3 6	2′3′4′	I	194 2 3 4 5	2′3′4′5′	II
105 2 3 4	3′4′	II	205 2 3 4 5 6	3′4′5′	III
141 2 3 4 5	2′5′	I	206 2 3 4 5 6	2′3′4′5′	I + II
137 2 3 4 5	2′4′	I	209 2 3 4 5 6	2′3′4′5′6′	I

Hagland *et al*[19] have determined the retention times of 150 CBs and we have added a further 18 congeners to that list (Table 3).

The elution order of the CBs is not dependent upon the degree of *ortho*-chloro substitution alone. A number of the more highly chlorinated CBs occur in the second and third fractions (Table 3). These data were obtained by chromatographing a composite mixture of the four *NRCC CLB* solutions on the *PYE* column into three fractions prior to analysis by GC. The effect on retention of the *ortho* substitution with the more highly chlorinated CBs can be seen by comparing CB 205, 206 and 209, where one aryl ring is fully substituted. The second ring has a 3′4′5′ substitution pattern for CB 205, which elutes in fraction III. By adding one further *o*-chloro atom to the ring the elution of CB 206 is now split between fractions I and II, and the fully substituted CB 209 elutes completely in first fraction I.

Hagland *et al*[19] implied that all of the CBs in their study were separated into one of three fractions, with little or no overlap. However it was clear that this was not so when we tested the *PYE* column with the *NRCC CLB* mixture of 51 CBs. A complete separation of even the main CBs that occur in environmental samples into

just three fractions would be difficult and it is likely that there will always be some degree of overlap even with a highly efficient column like the *PYE*.

Despite this overlap between fractions for some CBs, it is possible to make the essential separations between (i) CBs that co-elute or elute closely on the CPSil 8 or SE 54 type column and (ii) between the toxic mono-*o* and non-*o* CBs and other known interferences, to obtain an unambiguous measurement of these compounds in marine biota (Tables 2 and 3).

The GC retention indices of all 209 congeners has only been measured on a column of one polarity type, namely a 5% phenyl methyl (SE 54, CPSil 8)[29] and although information on the retention times for a number of the CBs on other columns is available[21,17], the data set is not yet complete. MDGC has been applied[17,18] to obtain a confirmative identification where the GC separation is difficult and where mass spectrometry has been of limited confirmatory use, due to the similarity of the mass spectra. This multidimensional technique has considerable potential but, has not yet been fully refined to *'heart-cut'* the chromatographic peaks and to fully quantitate each peak with the internal standard. The cost of the MDGC, relative to a single oven GC, may also restrict the technique to a small number of laboratories for the present time. However, the two techniques (MDGC and PYE HPLC) are beginning to play an important complementary role in the separation of CBs.

The key CB separations that are required for the monitoring and ecotoxicological programmes are given in Table 1.

CB 28 and 31 Both CB 28 and 31 elute in fraction I from the *PYE* column, but it is possible to separate these CBs on a 50 m narrow bore (0.22 mm i.d.) CPSil 8 CB column. This GC separation can be used to confirm the quality of the column and the optimization of the GC. The measurement of these lower chlorinated CBs is less important in marine mammals since they are metabolized by small cetaceans and seals. They are relatively less toxic than other congeners and contribute little to the overall CB body burden of the animal.

CB 52 CB 52 elutes in fraction I on the *PYE* column and there is no apparent interference from any other congener[21] with CB52 on the CPSil 8 column. This has also been confirmed by a number of other workers.[13,14,16]

CB 101, 90 and 84 De Boer *et al*[30] has reported that CB 84 may interfere with the determination of CB 101. However, Schulz *et al*[21] have shown that it is possible to resolve the CB 84 from CB 101 on the 5% phenyl methyl column. CB 90 has an even closer retention time to CB 101 than CB 84, but it is only present at trace level in environmental samples. Our own investigations indicate that the CB 101 peak on the CPSil 8 is homogeneous for these marine mammal samples.

CB 118 and 149 Both of these CBs are well resolved from each other when the GC is optimized and the capillary column is of reasonable quality. This separation can also be used to test the efficiency of the column. However, the *PYE* column separates CB 149 into fraction I, and the mono-*o* chloro CB 118 into fraction II.

CB 138 and 163 Until recently the GC peak assigned CB 138 was considered to be homogeneous. Roos *et al*[31] identified a second compound in the MS peak profile with negative ion chemical ionization MS. They tentatively suggested that this compound was CB 163. Larsen *et al*[32] synthesized the CB 163 and confirmed its presence in a number of environmental samples. Both CB 138 and 163 co-elute on the CPSil 8, SE 54 and the CPSil 19, OV 210 polarity columns.

Schulz *et al*[21] also did not find CB 163 in the Arochlor mixtures, even though they used MDGC to determine each congener. However, they were unfortunate to select OV 210 as the second GC column, which also does not resolve this pair of congeners.

By using the *PYE* column we have found that it is possible to separate CB 138, which elutes in fraction I, from CB 163 which elutes in fraction II (Figure 3). The HPLC split between fraction I and II is critical for the separation of these two congeners, and has been used as the dividing line between the two fractions. By monitoring the separation it is possible to isolate >95% of CB 138 with no interference from CB 163 in fraction I. This HPLC separation divides some CBs, such as CB 180, between fractions I and II. However, this is not critical to the measurement of CB 180 since it elutes as a single peak on the GC column and can be determined without any separation by the *PYE* column.

CB 153, 132 and 105 Both CB 153 and 105 are determined in all samples, but all three compounds have relatively similar retention times on the CPSil 8 column. This potential problem has been overcome with an initial separation of CB 153 and CB 132 eluting in fraction I on the HPLC column and the mono-*o* CB 105 eluting later in fraction II. This HPLC separation improves the identification and reduces any bias in the quantification of these congeners by GC due to unresolved peaks.

CB 128, 167 and 162 CB 128 has a di-*o* chloro substitution pattern but still elutes in fraction II because the 3,4,3′,4′ configuration on both rings retains some degree of planar nature. CB 128 can be separated from CB 167 on the 5% phenyl methyl GC phase and CB 162 is <0.05% in three main technical mixtures[21].

CB 156, 171 and 202 CB 156 elutes in fraction II from the *PYE* column and can be separated from both CB 171 and CB 202, which occur in fraction I and co-elute on the CPSil 8 column. This separation is critical since CB 156 can make a significant contribution to the overall toxicity associated with the induction of the P450 IA sub-family. It is important not to overestimate the concentration of CB 156 by inadvertently including CB 171 and/or 202 in any measurement that will subsequently be used to correlate, for example, with any EROD measurement.

CB 170 and 190 CB 170 has a 2,3,4,5,2′,3′,4′ substitution pattern and elutes in fraction II, suggesting that it retains some degree of a planar configuration. CB 190 (2,3,4,5,6,3′,4′) has both *o*-chloro atoms on the same ring. This may be compared to CB 205 (2,3,4,5,6,3′,4′,5′) which elutes in fraction III, partially due to the planar angle between the rings and the degree of chlorination on the molecule. It is therefore most likely that CB 190 also elutes in fraction III giving a separation between CB 170 and

CB 190. However, this is yet to be confirmed in our laboratory. CB 190 may be present in these samples up to 25% of the value of CB 170[21].

QC and repeatability of the method The overall quality control for the determination of CBs in marine biota in this laboratory have been reported previously[34]. The short term, within laboratory, repeatability is $<10\%$ and the long term reproducibility is ca 15% or less (Table 2). Control is maintained with charts for the analysis of a Laboratory Reference Material (LRM), in this case a cod liver oil LRM 110 and the bias of the method checked with Certified Reference Materials, CRM 349, and CRM 350, both of which are fish oils.

The percentage of the sample in the final fraction was between 79–89%, which is not a 'recovery' value, but a correction factor for the percentage of the total taken from the HPLC microvial. CB 209 is added at the commencement of the analysis to correct for any recovery in the method.

Metabolic pattern of CBs in marine mammals

The sperm whale was selected as an example of a large cetacean which is relatively rare in Scottish coastal waters. The measurement in the organic contaminant in this mammal was made following the PVD epidemic and gave an opportunity to obtain some chemical data on a deep ocean animal.

The porpoise and dolphin were chosen from samples taken from an existing study on the distribution of organic contaminants in small cetaceans in the Moray Firth, North East Scotland. These tissues contained above average levels of the seven 'monitoring' CBs found in the dolphin and porpoise in that area, and were, therefore, likely to have measurable levels of the toxic 'planar' CBs present in the lipid.

The juvenile seal also contained relatively high levels of CBs in the blubber tissue for these animals in the West of Scotland. The samples were brought to the laboratory in 1988 as a result of the PDV epidemic which mortally affected large numbers of the common seal population around the Scottish coast. One indirect biological effect of elevated levels of CBs could be to suppress the immune system and so increase the susceptibility of an animal to circum to such a virus. Although anthropogenic sources of environmental contaminants, and PCBs, in particular, were implicated in the outbreak of this disease, there is no firm evidence, at present, to substantiate these claims[5].

The results of the determination of the CBs in the blubber taken from the four marine mammals for this study are given in Table 2. Thirteen di- and tri-*o* chloro CBs were determined in fraction I from the *PYE* column, four mono-*o* chloro CBs were determined in fraction II and the three non-*o* chloro CBs were determined in fraction III. It has been possible to compare the distribution CBs with very different levels of chlorination, substitution pattern and metabolism in marine mammal tissue.

Unlike the fish, which absorbs the majority of organic contaminants from the surrounding water via the gill membranes, the body burden of contaminants in air breathing marine mammals is obtained either at birth via the placenta or from the food chain. The relative concentration and congener distribution pattern of the CBs in marine mammal tissue will reflect the changes in the composition of the CBs in the

food source compared with the original industrial formulations and the degree of metabolism of the congeners by the mammals themselves. Ballschmiter *et al*[28] found that the CBs which have a 4,4′ or a 3,5,4′ substitution pattern were not readily metabolized by biota and were consequently bioaccumulated through the food chain. Equally, the distribution pattern of CBs in different tissue of the same animal were very similar.

Boon *et al*[35] has reported that the relative pattern of CBs in different organs of harbour seals is virtually identical. The *actual* concentrations in each organ are different and depend primarily on the lipid levels in that tissue, but the ratio of the CBs remains approximately constant, even for the brain which contains a significantly higher percentage of phospholipids and sterols.

The body burden of the organochlorine contaminants in any tissue or body fluid does not remain in static equilibrium. Although the lipid-rich blubber contains high concentrations of CBs, these compounds are not bound irreversibly in the tissue, but are highly mobile through the blood[35] or into the mother-milk during lactation[36]. So, although the actual concentration of each CB in each animal and tissue is dependent on the exposure, the relative patterns of the congeners will be reasonably constant for the same species and depend primarily on the CBs in the food chain. Once the relationship of the concentration of CBs in different tissues for the same species has been established it may only be necessary to determine the levels of CB in one tissue, e.g. blubber, to describe the distribution in the other organs. A number of the more persistent CBs have been used to normalize the CB pattern, but the most abundant, stable and more easily measured congeners is CB 153[2]. This congener is biomagnified through the food chain and can be used to normalize the measurements of the other CBs in marine mammal blubbler.

The concentrations of each congener in each of the four marine mammals relative to the value for CB 153 has been plotted for each CB (Figure 4). In addition, the same CBs, relative to CB 153, in fish (cod) from the northern north sea, and in the industrial formulations of Aroclor 1254 and 1260[21] are also plotted for comparison. The distribution of CBs in each of the industrial formulations is different. However, a number of the formulations have a relatively similar congener distribution in mixtures containing approximately the same degree of chlorination. The most widely used industrial mixtures contain between 50% and 60% chlorination, and a theoretical composition containing 70% Aroclor 1254 (54% chlorine) and 30% Aroclor 1260 (60% chlorine) has been used to represent the type of mixture which is released to the open marine environment, either from atmospheric deposition or from discharge from rivers or coastal zone.

The bar graph in Figure 4 for the industrial formulation is *only* an indicative series of values based on the analysis of one batch of material. Although each CB will be metabolized to some degree through the food chain some congeners are considerably more biologically active and are, as a consequence modified by hydroxylation, or sulphonation. The extent of the metabolism of any one congener is dependent on the substitution pattern of the CB and the enzyme activity in each animal. The congeners which have been determined in the marine mammal blubber and the cod liver oil in this study may be divided structurally into the following classes:

1) Congeners with adjacent hydrogen atoms (H) at the meta-(*m*) and para-(*p*) positions only. These are CB 52, 44, 101, and 149.

2) Congeners with adjacent H at the *o* and *m* positions and one *o*-chloro atom only. These are CB 105, 118, and 156.

3) Congeners with adjacent H at the *o* and *m* positions and two *o*-chlorines. These are CB 128, 158, 138 and 170.

4) Congeners with no *o*-chlorine and substituted in the 3,4 or 3,4,5 position. These are CB 77, 126, and 169.

5) None of the above (1–4). These are CB 153, 180 and 194.

The congeners in groups 3 and 5 (Figure 4A), are least likely to be metabolized[28,35] as they have neither adjacent *o–m* or *m–p* H atom nor do they contain less than two *o*-chloro atoms. In general, the second *o*-chloro, one on each ring, prevents the biphenyl rings from achieving sufficient planarity of the ring surfaces to aid the metabolism.

There appear to be two exceptions to these rules amongst the CBs studied. CB 128 has two *o*-chloro atoms and is extensively metabolized by all four marine mammals (Figure 4A). The metabolic process is primarily aided by the adjacent *o–m* H atoms on *both* rings. CB 158 also has two *o*-chlorines, but in this molecule they are on the *same* aryl ring, with a 3,4 chloro substitution and adjacent *o–m* H atoms on the second ring. This configuration also appears to be metabolized by each of the four marine mammals, regardless of the second *o*-chloro atom.

CBs 153, 138, 170, 180 and 194 have relatively unreactive substitution patterns and remain as the major congeners in the blubber of each species. However, even these recalcitrant molecules undergo some degradation. The small losses in each animal are, generally, in the order: whale < dolphin < porpoise < seal. A similar order of species is maintained for the CB 128 and 158 which are metabolized.

The congeners in group 1 (Figure 4B) are all metabolized to some degree relative to the industrial formulation. The tri-chloro CB 52 is removed in all species including the fish. The penta chloro CB 101 is also present at lower concentrations in all marine mammals and in the fish, but the hexachloro CB 149 is relatively unmetabolized, except in the seal. CB 149 is more resilient to degradation primarily because of the third *o*-chloro atom, which appears to block the metabolism of this congener in the whale, dolphin and porpoise, whereas the seal is able to remove a significant proportion of this compound. The metabolism of this group of CBs is not dependent on the planarity of the CBs, since they are all weak or P450 IIB inducers, and the reactivity may simply be related as much to the number (bulk) of chlorine atoms present on the rings as to the spatial configuration of the molecule.

The group of CBs containing 1 *o*-chlorine and adjacent *o–m* H atoms (CB 105, 118 and 156) are all metabolized by each of the four marine mammals, but to a lesser extent by the fish (Figure 4C). In each case the extent of the metabolism of the CBs is in the order: fish ≤ whale < dolphin < porpoise < seal. The differences are more pronounced for CB 118, but this may be primarily a function of the relative concentrations of each of the three CBs.

The final three CBs (CB 77, 126, and 169) (Figure 4D) contain no *o*-chloro atoms

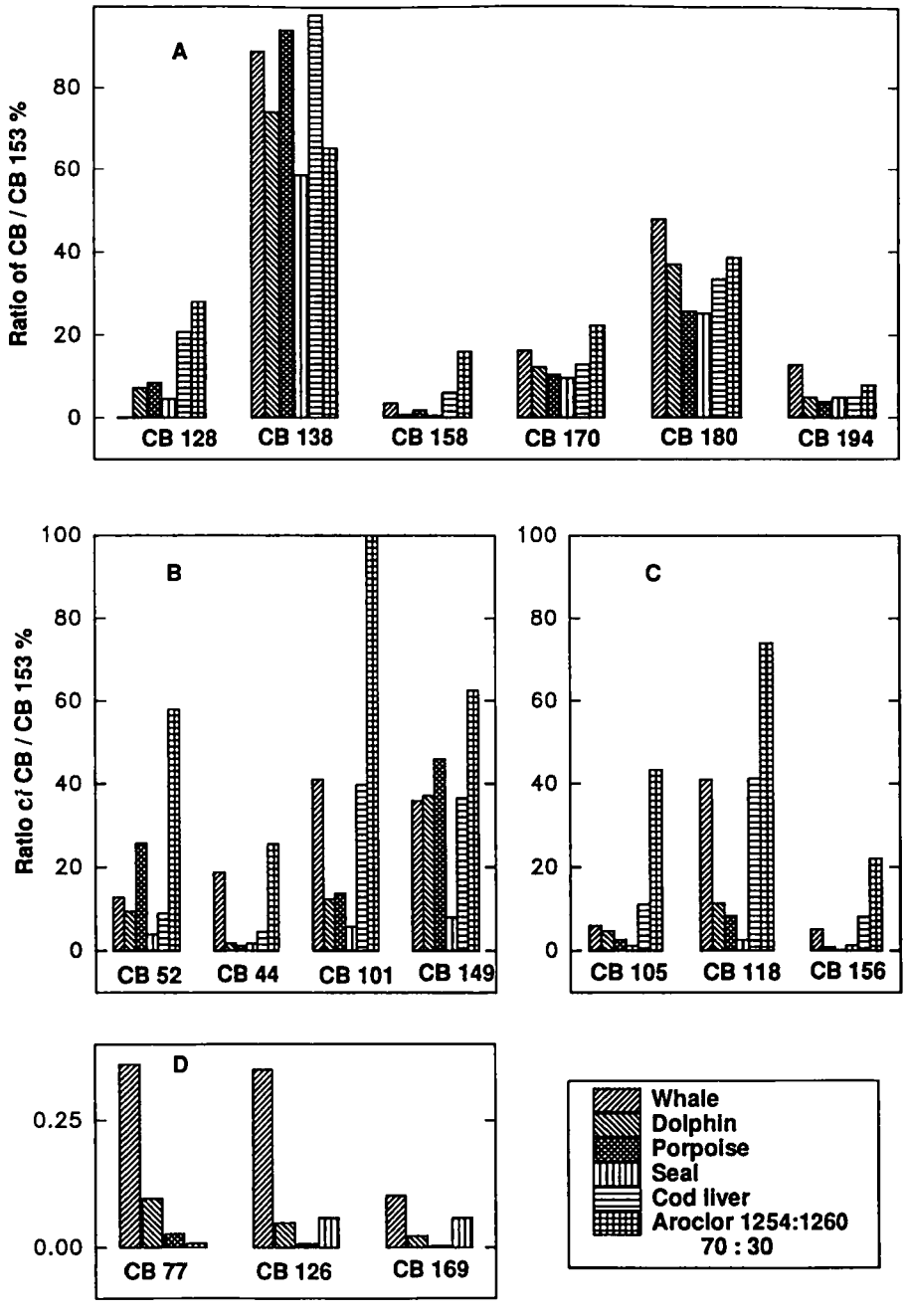

Figure 4 Bar graph giving the concentration of each CB determined in each of the four sea mammals, cod liver oil and a hyperthetical mixture of Aroclor 1254 and 1260, relative to CB 153. Graph A: CBs with more than 1 *o*-chloro atom and no adjacent *o–m* H atoms. Graph B: CBs containing *o–m* H atoms. Graph C: CBs containing 1 *o*-chloro atom and *m–p* H atoms. Graph D: Non *o*-chloro CBs. Each of the series of bars for each CB is in the order whale, dolphin, porpoise, seal, fish oil, and formulation.

and are also metabolized by each animal. The relative extent to which these mammals metabolize these planar CBs is in the order: whale < dolphin < porpoise < seal. Currently, there is no data on the amount of these planar congeners relative to CB 153 in the industrial formulation. Kannan *et al*[25] determined the concentration of the planar and mono-*o* CBs in Kaneclors and Aroclors, but not the major constituents, and Schultz *et al*[21] determined all 209 congeners in the formulations, but did not quantify those CBs at concentrations of <0.5%! However, from the data reported by Kannan *et al*[25] the metabolism of these planar CBs is in the order CB 77 (2 adjacent *o–m* H atoms) > CB 126 (1 adjacent *o–m* H atoms > CB 169 (no *o–m* H atoms). Again, there is an enrichment of the higher chlorinated congeners.

Concentration of CBs in the marine mammals

The concentration of the lower chlorinated CBs containing less than four chlorine atoms in marine mammal tissue are either below the detection limit or are present at significantly lower concentration compared to the more chlorinated CBs. CB 28 was below the limit of quantification (LOQ) (10× limit of detection (LOD)) which was $\simeq 0.05\ \mu g\ kg^{-1}$.

The predominant CBs in each blubber tissue were CB 138, 153, and 180, with CB 149, 118, 170 and 194 present at relatively lower concentrations.

The data given in parenthesis in Table 3 for the CB concentrations in the bottle-nosed dolphin relates to the values obtained without any separation on the *PYE* HPLC column. There is no significant difference between the two sets of data for CB 52, 101, 149, 118, 153, and 180. However, the 'CB 138' value in parentheses is actually for CB 138 + 163, which can be separated by HPLC to give the individual congener values. CB 128, 105 and 156 are also corrected, after separation by HPLC, for the interference caused by other CBs. This correction is likely to have the greatest effect on the measurement of the metabolizable CBs in marine mammal tissue where the determinands (e.g. CB 156) are present at the low concentrations similar to the potentially interfering congeners, e.g. CB 171/202. In other matrices (e.g. fish tissue) where the determinands are not metabolized to the same extent as in marine mammals, the relative concentration of determinand to interference will be much greater and the need for this type of separation may be less critical. However, the *PYE* column (or equivalent) will always be required where there are large differences in concentration between congeners, and where any interference with the measurement would preclude the main 'monitoring' CBs and the trace 'toxic' CBs from being determined in the same extract.

The 'total' PCB value for each marine mammal was calculated to give an approximate comparison with previous work of the laboratory and with measurements reported by other workers. However, the term 'total' PCB can be misleading since the method of estimation is made in different ways by different workers. The value in some reports is the sum of all the CB measured, which may range from 7 to over 30 CBs. In other cases the total is multiplied by a factor equivalent to a complete summation of all the CB peaks in the chromatogram. In this paper the total PCB value is equivalent to Aroclor 1254 based on the original measurement

by packed column GC to obtain the closest comparison with earlier data. The total PCB value is obtained by the summation of the value for the seven CBs (28, 52, 101, 118, 138 + 163, 153 and 180) with a multiplication factor of 2.5 (Table 2).

The level of total PCBs in the sperm whale is 705 $\mu g\,kg^{-1}$, which is the lowest value for the four mammals. However, this is not surprising since this species spends most of its time in the open ocean feeding at depths of 400 m or more primarily, or in many cases exclusively on cephalopods (small squid and octopi). The pattern and concentration of CBs in the whale reflect the different diet by comparison with the porpoise, dolphin and seal.

The concentration of total PCB in the dolphin was 12,750 $\mu g\,kg^{-1}$ which is close to the mean PCB value (range 3,200 to 30,000 $\mu g\,kg^{-1}$) obtained in blubber taken from the small cetacean population in the Moray Firth, Scotland. The porpoise taken from the same area contained 26,300 $\mu g\,kg^{-1}$ in the blubber. The range of CBs in these animals from the Moray Coast is between 1,800 and 32,400 $\mu g\,kg^{-1}$ with a mean of around 14,000 $\mu g\,kg^{-1}$ (SOAFD data in preparation).

Most of these animals are only obtained as a result of stranding or netting operations and so chemical data is only available for individual or small numbers of animals. These values also vary from animal to animal depending on the feeding habits in any one location, and the condition of the individual, e.g. blubber thickness, sex and age. Morris *et al*[4] found 310,000 $\mu g\,kg^{-1}$ of total PCB in the blubber of a juvenile bottlenosed dolphin and 93,000 ug kg^{-1} in harbour porpoise from Cardigan Bay, West Wales and Tanabe *et al*[37] reported values of 53,000 $\mu g\,kg^{-1}$ in a Pacific whitesided dolphin, and 12,000 $\mu g\,kg^{-1}$ in Dall's porpoise taken from Japanese coastal waters.

The concentration of the total PCB in the common seal from the west coast of Scotland was 11,750 $\mu g\,kg^{-1}$ and was one of a number of animals analysed during the PDV epizootic in 1988. The range of concentrations of PCB in the common seal taken from that area was between 8,000 and 24,000 $\mu g\,kg^{-1}$ with a mean value around 10,000 $\mu g\,kg^{-1}$. These data from Scotland along with similar information on the levels of organochlorines in seals from England, Wales and Northern Ireland have been reported by Hall *et al*[5].

Toxic equivalent factors for CBs in marine mammals

Safe *et al*[9] applied the *in vitro* AHH/EROD-induced bioassay to an *in vivo* animal model using the Wistar rat. The high correlation obtained between loss in body weight, thymic atrophy, and the *in vitro* enzyme induction for fifteen polychlorodibenzofurans (PCDFs), five polychlorodibenzo-*p* dioxins (PCDDs) and eight CBs has been used as a basis for developing the *Toxic Equivalence Concentration* (*TEC*) for compounds that show AHH or EROD activity. The TEC is calculated from the enzyme activity of the compound of interest, its concentration in the tissue and the activity of TCDD;

$$\mathrm{TEC(CB)} = \frac{\mathrm{EC}_{50\,\mathrm{CB}}}{\mathrm{EC}_{50\,\mathrm{TCDD}}} \times [\mathrm{CB}]$$

where the EC_{50} values relates to the estimated dose of the CB to produce 50% of the maximum enzyme induction.

Using this approach proposed by Tanabe[37], it is possible to obtain a ranking of the potential biological effect of each of the toxic CBs based on their activity and environmental concentrations.

The TEC values from the analysis of the marine mammals taken from Scottish waters are compared with the values obtained by Tanabe *et al*[37] in Japan in Table 4. Although the seal was more effective at metabolizing the CBs, particularly the mono *o*-chloro CBs, it also has the highest TEC of the Scottish samples analysed in this study, which ranged from 0.3 to 1.3 $\mu g\,kg^{-1}$ TCDD equivalent concentration. We reported our calculated values for the TECs for non *o*-chloro and mono *o*-chloro CBs in Pacific marine mammals obtained by Tanabe, and other species in a recent review[1]. These values of 4.02 $\mu g\,kg^{-1}$ for killer whale, 0.28 $\mu g\,kg^{-1}$ for Bairds beaked whale and 0.41 ug kg^{-1} for Dalls porpoise covers a similar range of values obtained in this study. Of the most toxic non *o*-chloro CBs only CB 126 contributes significantly to the overall TEC for the Scottish samples, with an additional contribution from the mono *o*-chloro CBs 105, 118 and 156. The order of contribution of the six "toxic" CBs to the TEF was as follows:

Sperm whale CB 126 > CB 105 > CB 156 > CB 77 ~ CB 118 ~ CB 169
B.N. Dolphin CB 126 > CB 105 ≫ CB 118 ~ CB 156 ~ CB 77 ~ CB 169
H. Porpoise CB 126 ~ CB 105 > CB 118 > CB 77 ~ CB 156 ~ CB 169
Common seal CB 126 > CB 105 > CB 156 > CB 118 ~ CB 169 ~ CB 77

Clearly, the relative contribution of each of these CBs will be dependent on the overall contamination of the environment from which the sample is taken, but also on the relative degree of metabolism for each CB. Although the overall body burden of CBs in marine mammals is greater than for other marine species, due to the high lipid content of the tissue and the position at the top of the food chain, a measure of the intrinsic toxicity of these CBs on marine mammals may be underestimated if it is based on the amount of CB in the blubber alone. Since these toxic CBs are metabolized as part of the de-toxifying process it would be more appropriate to determine the total uptake of toxic CBs and relate this value to any biological effect rather than to the measurement of the CB residue(s) in the body.

SUMMARY

Many environmental studies on marine species are attempting to relate biological effect both at the physiological level and at the cellular level to causative agents, for example, the induction of EROD/ECOD/AHH activity and chlorobiphenyl concentrations in the sediment, target species and their food chain.

The specific CBs which are toxic and induce well characterized biological effects are known. It is essential to concentrate on the determination of these toxic CBs in any biological effects programme and to have the specific methods available for the unequivocal measurement of these congeners at the ultra trace level.

Table 4 Toxic equivalence concentrations (TEC) for CBs in marine mammals.

CB IUPAC No.	*EROD relative induction potential (EROD-RIP)*[1]	*Toxic equivalent concentration for each CB*						
		Conc. of CB × EROD RIP (μg kg⁻¹ of TCDD)						
		Sperm whale[2]	*Bottlenosed dolphin*[2]	*Harbour porpoise*[2]	*Common seal*[2]	*Bairds beaked whale*[3]	*Killer whale*[3]	*Dalls porpoise*[3]
114	3.1×10^{-4}							
105	1.5×10^{-3}	1.5×10^{-2}	1.8×10^{-1}	1.9×10^{-1}	5.0×10^{-2}	7.0×10^{-4}	4.5×10^{-1}	1.5×10^{-2}
118	2.0×10^{-5}	9.6×10^{-4}	5.7×10^{-3}	8.2×10^{-3}	1.4×10^{-3}	1.0×10^{-4}	2.3×10^{-2}	6.0×10^{-4}
156	2.0×10^{-4}	1.2×10^{-3}	4.2×10^{-3}	1.0×10^{-3}	7.4×10^{-3}	4.7×10^{-4}	3.9×10^{-2}	2.2×10^{-4}
77	2.0×10^{-3}	8.4×10^{-4}	4.8×10^{-3}	2.6×10^{-3}	4.4×10^{-4}	2.7×10^{-3}	1.0×10^{-1}	4.8×10^{-3}
126	7.2×10^{-1}	3.0×10^{-1}	8.6×10^{-1}	2.5×10^{-1}	1.2	2.8×10^{-1}	2.8	1.2×10^{-1}
169	7.5×10^{-3}	9.0×10^{-4}	4.1×10^{-3}	1.3×10^{-3}	1.2×10^{-3}	1.9×10^{-3}	6.1×10^{-1}	8.7×10^{-4}
Total TCDD TEC of CBs		3.09×10^{-1}	1.05	4.6×10^{-1}	1.3	2.86×10^{-1}	4.02	1.41×10^{-1}

[1] Parkinson and Safe, Goldstein and Safe.
[2] This study.
[3] Tanabe *et al.*

In this paper we have shown that it is possible to make the necessary key chromatographic separations to identify and quantify both the major CBs, selected for monitoring purposes, and those CBs essential for the estimation of the TCDD toxic equivalence concentrations at the fg g^{-1} (LOD 50×10^{-15} g g^{-1}).

Each CB, which has a known EROD/AHH activity, has been separated using pyrenyl silica HPLC followed by capillary GC and a measure of the TEC attributable to these CBs has been made in the four marine mammal species studied.

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