Chlorinated fatty acids in muscle lipids and blubber of harbour porpoise and harbour seals

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by

Kate Clark

Dept. Environmental Assessment
SLU
P.O.Box 7050
SE-750 07 Uppsala, Sweden

Supervisors: Peter Sundin (Dept. Environ. Assess), Helena Björn (Dept. Ecol., Lund Univ.), Clas Wesén (Dept. Techn. Anal. Chem, Lund Univ.)

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Abstract

Muscle and blubber samples from a harbour porpoise (*Phocoena phocoena*) and a blubber sample from a harbour seal (*Phoca vitulina)* were extracted and analysed to determine the presence of chlorinated fatty acids (ClFAs). Chlorinated fatty acids were detected in all samples. The highest concentrations of ClFA were found in the porpoise muscle phospholipids (about 6 mg Cl/g lipid). By GC-PICIMS, chlorohydroxy fatty acids were identified as the main ClFAs in the muscle sample. The sample also contained fatty acids which were tentatively identified as chlorinated sulphone-like derivatives. The blubber samples and the porpoise muscle triacylglycerols held ClFA concentrations below 0.1 mg Cl/g lipid.

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Introduction

Since the advent of the industrial revolution, there has been a marked increase in the load of chlorinated compounds in the environment (Walker *et al*. 1996). Organochlorine pollutants such as PCBs, DDT/DDE and chlorinated dioxins have been widely distributed throughout the environment (Rappe 1987; Beyer *et al*.1996). These compounds can be found in almost all marine biota worldwide and have been the subject of much concern due to the range of physiological disturbances they are known to cause (Sladen *et al*. 1966, Kawano *et al*. 1984, Beyer *et al*.1996, Olsson *et al*. 1992). As concentration factors of chlorinated persistent pollutants from water to higher level predators can be as high as 10^7, seals and porpoises are often chosen as indicator organisms (Mason 1996, Ofstad & Martinsen 1983, Kawano *et al*. 1995).

However recent studies have shown that this group of xenobiotic compounds usually constitutes less than 5% of the total load of extractable organically-bound chlorines (EOCl) found in fish, bivalves, lobster and sediment (Martinsen *et al*. 1988, Södergren *et al*. 1988, Håkansson *et al*. 1991, Milley *et al.* 1997). Up to 99% of the remaining proportion of EOCl can consist of esterbound, chlorinated fatty acids (Cl-FA) (Wesén *et al*. 1990).

Although few studies have been conducted into the toxicity of chlorinated fatty acids, there is growing evidence that they have an adverse effect on reproduction and membrane permeability. For instance, zebra fish fed a diet containing lipids isolated from fish with high chlorinated fatty acid concentrations experienced decreased fecundity (Håkansson *et al*. 1991) and Cherr *et al*. (1987) found that dichlorostearic acid inhibits sea urchin sperm motility. Chlorine increases the "bulkiness" of fatty acids, due to its larger van der Waals' radius in comparison to that of adjacent hydrogen atoms. The incorporation of high concentrations of bulky chlorinated stearic acid and oleic acid in mammalian membranes has been shown to increase ATP leakage (Ewald & Sundin 1993).

Despite the demonstrated toxicity of chlorinated fatty acids, organisms do not appear to differentiate and attempt to eliminate the compounds. The pathway by which chlorinated fatty acids are incorporated into lipids is unknown, however, in perch, the internal distribution of orally ingested dichlorostearic acid does not differ significantly from that of unchlorinated stearic and oleic acids (Ewald *et al*. 1996). Unlike traditional chlorinated pollutants, chlorinated fatty acids do not activate the detoxifying P-450 enzyme system (Håkansson *et al*. 1991). This suggests that chlorinated fatty acids are not recognised by organisms as xenobiotics and therefore not preferentially expelled from the body.

A study into migrating sockeye salmon (*Oncorhynchus nerka*) by Mu (1996) showed that the rate of metabolism of chlorinated fatty acids in muscle triacylglycerols was equal to that of unchlorinated fatty acids. However, in muscle phospholipids, chlorinated fatty acids were released more slowly, resulting in a proportional increase of chlorinated phospholipids within the muscle tissue. The chlorinated fatty acids present in the salmon were found to be of a different nature to the chlorinated fatty acids in grayling (*Thymallus arcticus*) that fed on salmon roe and carcasses. This suggests that chlorinated fatty acids

are incompletely catabolised within the food chain. So although regarded as chemically non-persistent, chlorinated fatty acids demonstrate a degree of "biological persistence" (Björn 1999).

Marine mammals were chosen as the study's target group because they are known to be particularly vulnerable to bioaccumulation of organochlorine pollutants (Mason 1996, Kawano *et al*. 1995). Seals and porpoises occupy a high trophic level in the environment (Tinsley & Lowry 1980) and, given their long lifetime relative to their prey species (Kawano *et al*. 1995), organochlorines increase in concentration in body lipids. Male marine mammals generally have higher concentrations of persistent organic pollutants than females who are able to excrete the compounds with their milk during lactation (Ofstad & Martinsen 1983). This paper is an introductory investigation into the presence of chlorinated fatty acids in the blubber and muscle lipids of marine mammals, with particular focus on the phospholipids.

Materials and Methods

Samples

Blubber from harbour porpoise (*Phocaena phocaena*) and harbour seal (*Phoca vitulina*) and muscle tissue from harbour porpoise were obtained from Patrik Börjesson, Dept. of Zoological Physiology, Stockholm University. Both animals originated from the west coast of Sweden and had accidentally been killed in fishing nets. The samples were stored at –80 ºC.

Chemicals and Glassware

All solvents and chemicals used in the assay were of analytical grade. All glassware was washed and soaked in chlorine-free detergent Deconex overnight and rinsed six times each in hot tap water, cold tap water and distilled water. The glassware was then heated to 400 ºC for at least 12 hours and there after stored wrapped in aluminium foil (subjected to the same heat treatment) to ensure that it was free of organic contaminants before it was used. All water used in the assays was distilled, de-ionised and stored under cyclohexane to prevent contamination.

Lipid Extraction

Lipids were extracted from three samples (seal blubber, porpoise blubber and porpoise muscle) using a method modified from Bligh and Dyer (1959). Dichloromethane was substituted for chloroform and the tissue sample was sonicated for 10 minutes and left in the dichloromethane/methanol solution overnight at room temperature to allow the lipids to be extracted. The homogenate was centrifuged rather than filtrated. After extracting the dichloromethane layer containing the lipids, more dichloromethane (1ml) was added to the homogenate and the extraction was performed again to maximise the lipid recovery. The solvent was evaporated at 30-35 °C under a gentle stream of nitrogen. The lipid residue was dissolved in cyclohexane and stored at –18 °C until further treatment.

Fractionation of lipids from porpoise muscle

To facilitate the division of fatty acids from non-polar compounds such as PCBs, DDT/DDE and HCHs, the porpoise muscle sample was separated into four fractions using activated silicic acid column chromatography modified from Håkansson *et al.* (1991). The first fraction (F1) containing the non-polar compounds was eluted with cyclohexane/diethyl ether (75:25) solution and was discarded. Fraction 2, which contained triacylglycerols (TAGs) and free fatty acids (FFA), was eluted with cyclohexane/diethyl ether (86:14) solution. The mono- and diacylglycerols of Fraction 3 were eluted with diethyl ether. Both Fractions 2 and 3 were collected in the same flask and labelled F2. The fourth fraction (F3) containing polar lipids (predominantly phospholipids) was eluted with methanol.

Esterification of lipids to FAMEs and enrichment of chlorinated FAMEs

The triacylglycerols from the porpoise and seal blubber (F2) and the lipids from the porpoise muscle (F3) were esterified (modified from Christie, 1989) to form fatty acid methyl esters (FAMEs) in order to facilitate enrichment and gas chromatographic analysis.

Approximately 100 mg of lipid was added to a 12 ml test-tube. Cyclohexane (2 ml) and 6 ml of methanol/sulphuric acid (2.0 %, v/v = 2.3 %, w/w) solution were added. The test-tube was then capped and heated to 60 °C. It was then shaken to homogenise the solution. It was then kept at 60 °C overnight (at least 12 hours). To establish a solvent vapour back-flow, the section of the test-tube above the solvent level was kept at a lower temperature than the part containing the solution.

The following day, more cyclohexane (4 ml) and water (6 ml) were added to the test-tubes. They were shaken and centrifuged. The cyclohexane layer was removed with a Pasteur pipette and the extraction was repeated with a further 6 ml of cyclohexane. The two cyclohexane phases were combined and dried with a small amount of anhydrous sodium sulphate. The solution was decanted into a weighed test-tube and evaporated at 40 °C under a gentle stream of nitrogen.

Purification of FAMEs

The dark yellow-brown colour of the FAME extracts indicated the presence of oxidised compounds and therefore further purification was performed on all the FAME extracts by rinsing them through a silica column. The 500 mg silica columns were rinsed with 3 ml of cyclohexane/diethyl ether (80/20, v/v) solution and then with 3 ml of cyclohexane. Each of the FAMEs were dissolved in a small volume of cyclohexane (1.5 ml) and transferred to the silica with a Pasteur pipette. Each column received 20-30 mg of lipid. The emptied test-tubes were flushed with 1 ml of cyclohexane and added to the appropriate silica column. FAMEs were eluted from the silica columns with 6 ml of cyclohexane/diethyl ether (90/10, v/v) and collected in weighed test-tubes (Wesén *et al*. 1995b).

Enrichment of chlorinated FAMEs

Due to the low concentration of chlorinated FAMEs, enrichment of all samples was

performed using the method of consecutive removal of silver ion and urea complexes as described by Mu *et al*. (1996a). The method was altered slightly. Half the test-tubes used for the urea complexation were fitted with stop-cocks. This was done to test the efficiency of draining the n-hexane through the urea rather than the old method which required n-hexane to be stirred into the urea and carefully removed.

Gas Chromatography (GC)

The FAMEs were studied by a Varian 3700 GC with a flame ionisation detector (FID) and an electrolytic conductivity detector (ELCD) (Tracor/Varian, model 1000) operated in parallel in the halogen selective mode (Wesén & Mu 1992). A glass jet splitter (Pressfit, Schmidlin Labor + Service) divided the column flow to the two detectors with a splitting ratio approximating 1:1. A 18 m x 0.32 mm I.D. (film thickness = 0.25 µm) HP-5 fused-silica capillary column (Hewlett Packard) was used for the gas chromatography. Helium was used as the carrier gas at a flow rate of 3 ml/min. Splitless injection (1 min) was performed at 280 °C. The column temperature was 90 °C for 3 minutes then 90 °C - 240 °C at a rate of 4 °C/min, then 20 min of isothermal conditions.

The FID and ELCD were operated at a base temperature of 280 °C and a temperature of 850 °C in the reactor tube. The ELCD used helium (99.995 %) as the make-up gas (26 ml/min) and hydrogen (99.9995 %) as the reaction gas (23 ml/min) (Wesén & Mu 1992).

Mass Spectrometry (MS)

To identify the chlorinated fatty acid methyl esters, GC-positive ion ammonia chemical ionisation (NH_3PICI) MS was used. FAMEs were separated on a fused-silica capillary column (SE-54, 25 m x 0.20 mm I.D.; film thickness=0.25 µm) fitted into a Hewlett-Packard, Model 5890 GC linked to a VG Model Trio-1S quadruple MS. The conditions were as detailed by Sundin *et al*. (1992).

Results

All samples showed the presence of chlorinated fatty acids. However the phospholipids in the porpoise muscle contained 50-250 times the concentration of chlorinated fatty acids as porpoise blubber and muscle triacylglycerols (Table 1). Given that the average chlorine concentration was found to constitute 0.6% of the phospholipids' mass and, given that the average chlorine content in chlorinated fatty acids is, by mass, about 10-20%, it can be estimated that 3-6% of the phospholipid fatty acids were chlorinated.

Table 1. Concentration of chlorine in lipids of porpoise (*Phocaena phocaena*) and seal (*Phoca vitulina*) samples

	Concentration of chlorine after esterfication (µg Cl/g)	Av. concentration of chlorine after esterfication (µg Cl/g)
Porpoise muscle phospholipids	**6 463**	**6121**
	5 790	
Porpoise muscle triacylglycerols	**114**	**77**
	40	
Seal blubber (triacylglycerols)	**41**	**34**
	26	
Porpoise blubber (triacylglycerols)	**50**	**42**
	33	

Chlorinated FAMEs within the porpoise phospholipids (Fig. 1) were initially identified by the number of carbon atoms in the acyl group, the number of double bonds (if any) and the number of chlorine atoms detected by total ion chromatography (positive ion chemical ionisation). Dichloropalmitate and dichlorostearate were detected, as was a homologous series of chlorohydroxy fatty acids with carbon chain lengths of 17,19 and 21 carbon atoms. A further two peaks were tentatively identified as chlorinated sulphones.

A few monochlorinated compounds lost a fragment of mass 64 (Fig. 2), which suggests the loss of an SO_2 group. The molecular mass related ions indicated chain lengths of 14 and 16 carbon atoms, with 3 and 4 double bonds respectively. These compounds were found by GC/MS both by using a non-polar HP5-MS and a polar DB23 column.

ELCD gas chromatograms confirmed that porpoise muscle phospholipids contained the highest concentration and diversity of chlorinated fatty acids compared to porpoise muscle and blubber triacylglycerols (Fig. 3). Prominent peaks on the ELCD (Fig. 3) supported the MS-TIC's (Fig. 1) detection of chlorinated sulphones. They have been tentatively identified as methyl chlorohexadectetraene and methyl chlorotetradecatriene sulphonic acid.

There was insufficient data to determine whether efficiency was increased by using stop-cocked test-tubes during the urea complexing phase of FAME enrichment.

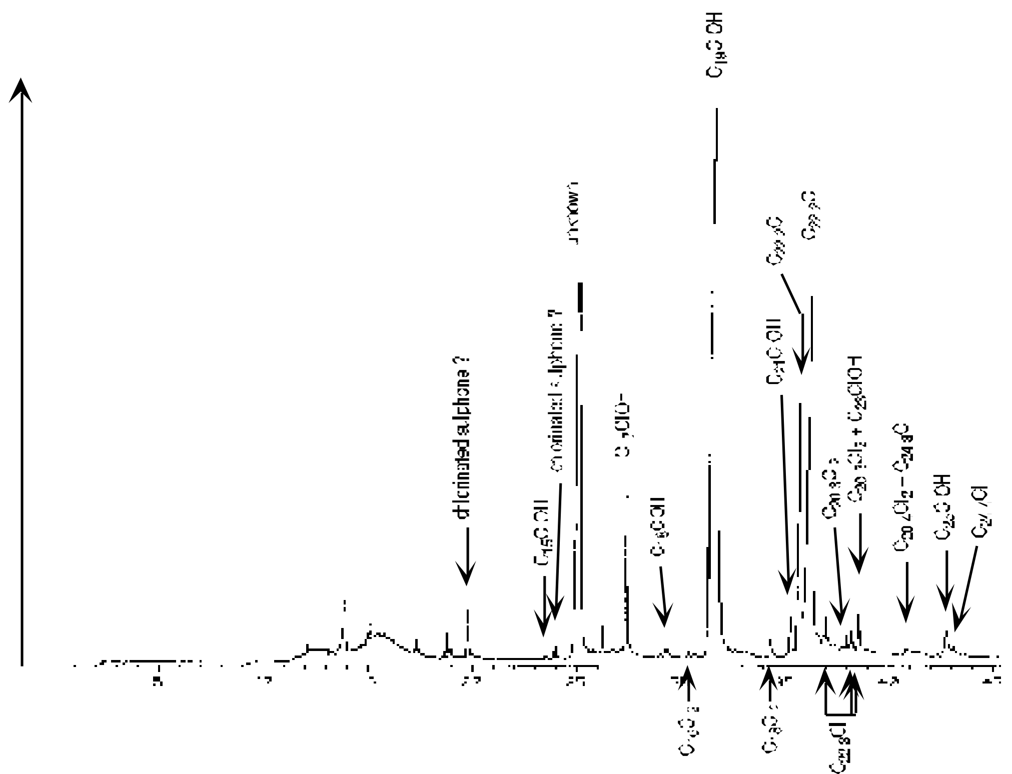

Figure 1. By total ion chromatography with positive ion chemical ionisation dichloropalmitate and dichlorostearate were detected among the FAMEs of the porpoise muscle, as was a homologous series of chlorohydroxy fatty acids with carbon chain lengths of 17, 19 and 21 carbon atoms.

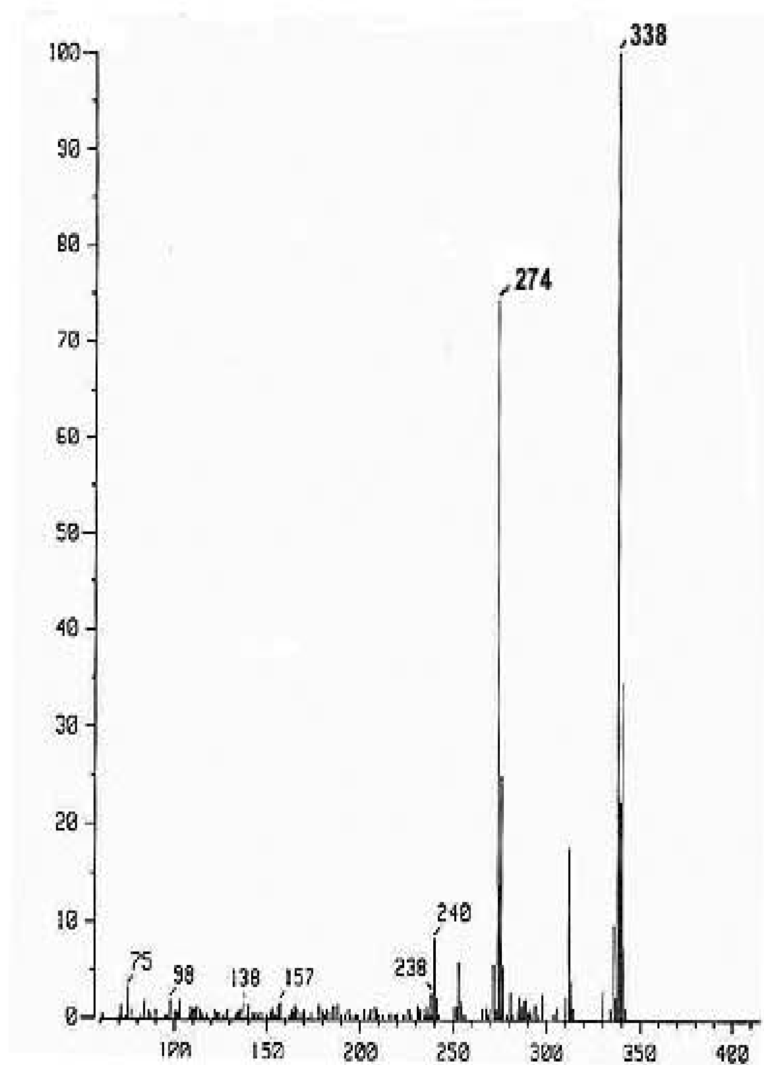

Figure 2. Ammonia PICI mass spectrum of a methyl ester obtained from porpoise phospholipids showing a loss of m/z 64, which corresponds with the mass of an SO_2 group.

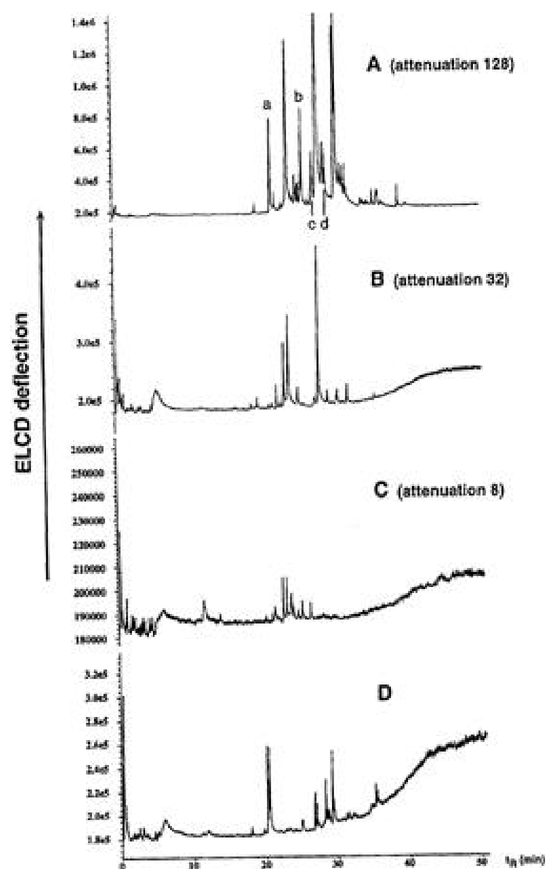

Figure 3. Electrolytic conductivity detector (ELCD) gas chromatograms of fatty acid methyl esters obtained from the lipids of : A) porpoise muscle phospholipids; B) porpoise muscle triacylglycerols; C) porpoise blubber, and D) eel phospholipids. The eel sample, where the major peaks represent dichloromyristic and dichlorostearic acids, was used to correlate retention times (Wesén *et al*. 1995a; Mu *et al*. 1996a,b). In Figure 3A, ELCD peaks have been tentatively identified as (a) methyl chlorohexadectetraene sulphonic acid and (b) methyl chlorotetradecatriene sulphonic acid. (c) and (d) mark two chlorohydroxy fatty acid methyl esters.

Discussion

This study demonstrates that the presence of chlorinated fatty acids in the storage and membrane lipids of marine species is not restricted to fish and marine invertebrates. The fatty acids of marine mammals such as seals and porpoises also seem vulnerable to chlorination which, given the dietary preferences and relatively long lifespan of marine mammals, raises concerns as to the long-term health implications of accumulation of chlorine-containing fatty acids.

While the original sources of these ClFAs remain uncertain, many possible origins have been documented. These include chlorine-bleached paper pulp production, chlorination of drinking water, disinfection of meat, and inadequate management of chlorinated pollutants (Håkansson *et al.* 1991, Björn *et al*. 1998a). In 1990, Wesén *et al*. found that fish from more remote and less polluted waters still contained chlorinated fatty acids, though in lower concentrations than fish from more polluted waters. Following Wesén's original study, ClFAs have been detected in fish and bivalves from Alaskan, Scandinavian and the Baltic waters (Wesén *et al*. 1995a, Wesén *et al*. 1995b, Mu *et al*. 1996a, Björn *et al*. 1998b). The piscivorous diet of seals and porpoises contributes to their bioaccumulation of persistent organically-bound pollutants (Kawano *et al*. 1995). Seals and porpoises incorporate chlorinated fatty acids from their fish diet into the thick layers of fatty acid-rich blubber upon which these large marine mammals rely for insulation and storage of energy. This research, which detected high concentrations of chlorinated fatty acids in porpoise phospholipids, supports the observations in Mu's (1996) study of migrating sockeye salmon in Alaska. Mu suggested that, similar to the more widely publicised POPs, chlorinated fatty acids bioaccumulate in fish and are passed to higher trophic levels through the food chain. Given the pervasiveness of chlorinated fatty acids in the marine environment, piscivorous marine mammals, irrespective of the species' distribution, are likely to be exposed to ClFAs via their diet.

The concentrations of chlorinated fatty acid found in the porpoise samples were not equal across the lipid classes. Phospholipids in the muscle contained much higher concentrations of chlorinated fatty acids than the blubber and muscle triacylglycerols. Björn's *et al*. 1999 study of membrane lipids in fish showed a similar distribution of chlorinated fatty acids. Salmon, herring and eel were all shown to have higher concentrations of chlorinated fatty acid in their phospholipids than in the triacylglycerols. Both studies are consistent with the finding by Mu (1996) that, while chlorinated and unchlorinated fatty acids in salmon and grayling triacylglycerols were released at the same rate, the total amount of chlorinated fatty acids in roe and muscle phospholipids actually increased. The turnover of unchlorinated phospholipids was higher than that of the chlorinated phospholipids. It has been suggested that the polar nature of phospholipids and their tendency to be organised as part of a membrane may slow the turnover of chlorinated fatty acids and hence cause an increase in concentration over time (Mu 1996).

The tendency of chlorinated fatty acids to bioaccumulate can also be attributed to their large size. In fish, dichlorostearic acid is the most commonly detected chlorinated fatty acid (Wesén 1995, Mu 1996, Björn *et al.* 1998b). In 9,10-dichlorostearic acid, the chlorine atoms are located in the centre of the molecule, which given the mass of chlorine atoms, increases the bulkiness of chlorinated stearic acid relative to an unchlorinated molecule (Ewald & Sundin 1993). It has been suggested that this bulkiness may obstruct the metabolic turnover of dichlorostearic acid thus conferring upon it a degree of biological stability which allows it to bioaccumulate (Ewald & Sundin 1993, Björn 1999). Dichlorostearate and dichloropalmitate were detected among the fatty acid methyl esters of the porpoise muscle in this study too but at much higher concentrations than those detected in previous studies of fish, thus indicating that seals' and porpoises' comparatively greater lifespan and fish consumption may facilitate a higher level of bioaccumulation.

The porpoise polar membrane lipids contained the highest concentration and diversity of chlorinated fatty acids, and the majority of these were chlorohydroxy fatty acids. This finding is consistent with studies of fish lipids by Björn *et al.* (1998b) and Mu (1996) which revealed that concentrations of chlorinated fatty acids were higher in polar lipids than non-polar lipids such as triacylglycerols. However in porpoises, the difference in chlorinated fatty acid concentration between polar muscle lipids and non-polar blubber lipids was far greater than the level calculated in fish. Even within an individual porpoise, the chlorinated fatty acid concentration in muscle was up to 250 times the concentration in blubber. This is in stark contrast to distribution of persistent organic pollutants (POPs) such as PCB and DDT/DDE. POPs collect in tissues which are high in non-polar lipids (Kawai *et al.* 1988).

The specific consequences of high concentrations of chlorohydroxy fatty acids in marine mammal muscle tissue have yet to be fully explored but Björn (1999) has speculated that high chlorohydroxy fatty acid concentrations may be an indication of an actively stimulated immune system. Upon infection, several types of leukocytes are known to respond by producing hypochlorous acid which in turn reacts with unsaturated fatty acids to produce chlorohydroxy fatty acids. These chlorohydroxy fatty acids are believed to play an important role in tumour cell cytotoxicity and combating micro-organisms through impairment of membrane function (Winterbourn *et al.* 1992, Carr *et al.* 1996). Therefore the high levels of chlorinated fatty acids in the sampled porpoise may suggest that the animal was physiologically distressed in the time preceding its death. However no data concerning the sampled animals' health status was collected. This would be an area of interest in future studies.

In mass spectrometry, the loss of a neutral fragment from an even-numbered ion produces an odd-numbered ion as a result of the breaking of one chemical bond. An odd-numbered ion can be transformed into an even-numbered ion through the same process. Therefore, in this study, the detection of two monochlorinated, even-numbered ions, separated by a mass of 64, indicated that the two monochlorinated compounds were either co-eluted or that a loss of a neutral fragment of mass 64 had occurred (Wesén 2002) . Given that the compounds were found in columns of different polarity, it is unlikely that the finding was

caused by the presence of pairs of unseparated, monochlorinated compounds that were co-eluted at specific retention times. Thus, given that the loss of a neutral fragment of mass 64 is an uncommon occurrence in mass spectrometric analysis, it must be considered further. It could be explained by the loss of an SO_2 group from the center of a larger substituent, such as a methyl sulphonic group, a sulphinic acid or a sulphonic acid (Wesén 2002). This would mean that two ionic bonds were broken and that an accompanying re-arrangement took place simultaneously. This process would account for the peaks in the GC-PICI MS that appear to correspond to methyl-sulphone substituted chlorinated fatty acids (Fig. 1). They have been tentatively identified as methyl chlorohexadecatetraenoate sulphone derivative and methyl chlorotetradecatrienoate sulphone derivative.

The presence of methyl sulphone substituted chlorinated fatty acids in phospholipids has not, to my knowledge, been previously reported. Chlorinated sulphones have been found in the environment but not incorporated into phospholipids. Voss (1983) sampled the biologically treated combined mill effluents from nine bleached kraft mills for chlorinated neutral organic compounds. He found chloroform, *a,a*-dichlorodimethyl sulphone*, a,-a,a'*-trichlorodimethyl sulphone and *a,a,a',a'*-tetrachlorodimethyl sulphone in concentrations between 0.3-429 µg/L. The chlorinated sulphones were far more resistant to removal by biological treatment in an aerated lagoon than the chloroform and thus likely to be relatively persistent compounds in the pulp mill receiving waters. Voss tested these compounds for their bioaccumulation potential based on the correlation between octanol-water partition coeffiecients (K*ow*) and found them to have an extremely low bioconcentration potential. Bis (4-chlorophenyl) sulphone (BCPS) has been detected in perch muscle, seal blubber and an egg from a white-tailed sea eagle (Olsson & Bergman 1995). Its detection at various trophic levels combined with its partitioning coefficient (K*ow*) suggest that BCPS is a persistent environmental pollutant but it has not yet been found bound in fatty acids. In a study by Janak *et al* (1998) methyl-sulphonyl metabolites of PCB and DDE, were found in the blubber, lung, and liver of grey seals (*Halichoerus grypus*). It was observed that in the liver there was a substantial and highly specific retention of PCB methyl sulphones but the study did not include fatty acid analysis. Thus is it possible that these chlorinated sulphonic compounds could be accumulated and incorporated into fatty acids?

This study found high concentrations of chlorinated fatty acids in a small sample of marine mammals. It is undetermined as to whether the mammals sampled were unusually exposed to chlorinated fatty acids or if they are representative of the levels of extractable, organically-bound chlorines present in the phospholipids of marine mammals. However, given these mammals dietary preferences and relatively long lifespans, there are adequate grounds for a larger study into chlorinated fatty acid concentrations in marine muscle phospholipids and the associated potential for long-term health implications of chlorine bioaccumulation. The bioaccumulation of chlorinated fatty acids has been linked with an increased risk of membrane permeability and decreased fecundity. At a time when public health professionals are advocating the benefits of a diet high in fish and their omega-3 fatty acids, research into the concentrations and physiological impact of chlorinated fatty acids is of particular relevance.

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