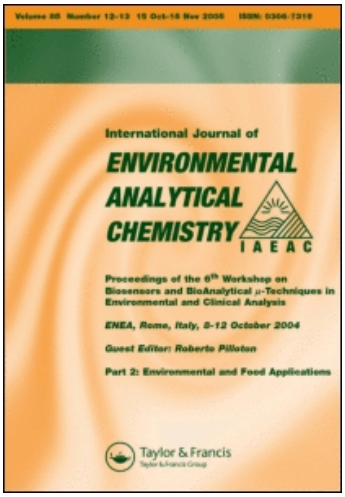

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Characterization and sources of extractable organochlorine in white sucker downstream from bleached kraft pulp mills

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Organochlorine, obtained by extraction with hexane–acetone mixture (3 : 1) of fillets of white sucker (*Catostomus commersoni*) sampled downstream of pulp mills and in a reference river, was characterized by gel-permeation chromatography, transesterification, neutron activation analysis, and gas chromatography with halogen-sensitive detection. It was found that over 78% of the extractable organochlorine (EOCl) is of relatively high molecular weight (>∼350). Chlorinated fatty acids account for 43–80% of EOCl in the high-molecular-weight portion, while chlorobenzenes, chlorinated pesticides, and polychlorinated biphenyls account for 4–55% of EOCl in the low-molecular-weight portion. Though undetectable in reference fish, three particular chlorinated fatty acids, i.e. *threo*-5,6-dichlorotetradecanoic, *threo*-7,8-dichlorohexadecanoic and *threo*-9,10-dichlorooctadecanoic acids, are characteristic of EOCl from fish collected downstream of bleached kraft pulp mills using chlorine-based bleaching, representing about 30% of total EOCl, of which *threo*-5,6-dichlorotetradecanoic acid alone accounts for 60–70%. It is thus evident that, among chlorinated compounds discharged from bleached kraft pulp mills, *threo*-9,10-dichlorooctadecanoic acid, presumably generated in chlorine-based bleaching processes, is the most bio-accumulative in fish and can be biodegraded by fish into dichlorohexadecanoic and dichlorotetradecanoic acids, presumably via β-oxidative metabolism. These three compounds were also identified in suspended solids isolated from biologically treated final effluent discharged from a bleached pulp mill using 50% ClO_2 substitution, thus confirming the effluent-related source for downstream fish. The finding also suggests that β-oxidation of dichlorooctadecanoic acid may also be operative in micro-organisms.

Keywords: EOCl; Fish lipids; Chlorinated fatty acids; Environmental analysis; Bleached kraft pulp mill effluents

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1. Introduction

Organochlorine compounds in fish are usually estimated as a group parameter, extractable organochlorine (EOCl). The detection of substantial levels of EOCl in aquatic life has caused serious public concern over agricultural use of chlorinated pesticides and industrial uses of chlorine and its derivatives. Of concern in the work is the use of chlorine as a bleaching agent in bleached kraft pulp mills [1]. Discovery of chlorinated dioxins and furans in fish downstream from bleached kraft pulp mills in 1980s prompted the majority of mills to undergo profound process changes and technology upgrades, including the switch of chlorine bleaching to chlorine dioxide bleaching, addition of oxygen delignification and implementation of biological treatment of effluents. As a result, dioxins and furans in discharged effluents decreased to levels below detection limits, and overall qualities of mill effluents were also improved dramatically [2, 3]. Dioxin levels in fish downstream from these mills consequently declined rapidly [4]. Overall, EOCl levels in these downstream fish decreased significantly but at a much slower rate, especially for male fish [5].

A significant effort has been made to analyze EOCl components in fish. Notorious chlorinated pollutants found in fish include some persistent chlorinated pesticides (OCs), polychlorinated biphenyls (PCBs), polychlorinated naphthalenes, polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, chlorinated terpenes, chlorinated paraffins, and chlorinated phenolic compounds. However, these pollutants account for only a small portion of fish EOCl, regardless of the total level of EOCl in fish [6, 7]. Although there had been some indication and speculation about the identities of the principal components of fish EOCl [8–10], they were unknown until significant work was carried out by Swedish scientists in 1990s [11, 12]. Using gas chromatography (GC) with electrolytic conductivity detection (ELCD) and GC coupled with mass spectrometry (MS) as principal tools, Wesén and coworkers identified an array of chlorinated fatty acids (FAs), including dichloroalkanoic, dichloroalkenoic, and tetrachloroalkanoic acids, in lipids of marine fish downstream from pulp mills employing chlorine bleaching [13–16]. Furthermore, in light of the work reported by Jones *et al*. [17] and Conacher *et al*. [18] on the rat metabolism of 9,10-dibromo- and 9,10-dichlorooctadecanoic acids, these researchers speculated that 9,10-dichlorooctadecanoic acid was shortened to 7,8-dichlorohexadecanoic acid, which was in turn degraded to 5,6-dichlorotetradecanoic acid in fish via peroxisomal β-oxidation [13, 15, 16]. Analytical evidence for these β-oxidative products was recently shown in our work on identification of chlorinated fatty acids in freshwater fish sampled downstream from bleached kraft pulp mills [19–21]. Thereafter, Åkesson-Nilsson and Wesén identified 5,6-dichlorotetradecanoic acid as a metabolite of 9,10-dichlorooctadecanoic acid in human cell lines, which also suggests β-oxidation [22].

There has been little reported work on the quantitative characterization of fish EOCl. In this work, EOCl from white sucker fillets was separated into relatively low- and high-molecular-weight fractions by gel-permeation chromatography (GPC) and quantitated by neutron activation analysis (NAA). Known chlorinated pesticides and polychlorinated biphenyls, and chlorinated fatty acids were quantitated by GC with electron-capture detection (ECD) and halogen-specific detection (XSD), respectively. The possible sources of major chlorinated compounds found in fish downstream of bleached kraft pull mills were also investigated.

2. Experimental

2.1 *Fish samples*

White sucker (*Catostomus commersoni* (*Lacépéde*)) samples were provided by the Canada Centre for Inland Waters (CCIW). The fish were sampled in the autumn of 1991 and 1995 in three rivers: Mattagami River downstream from a bleached kraft pulp mill at Smooth Rock Falls, Kapuskasing River downstream from a thermomechanical pulp (TMP) mill at Kapuskasing, and Groundhog River, which was a stream unpolluted by any type of pulp mill effluents [23]. Located in North Ontario, these three rivers flow northwards and converge to the Moose River. Figure 1 shows the correlation between sampling times and technology upgrading events in the mills.

2.2 *Extraction of fillets*

The frozen fillets of each fish group were ground and homogenized in a blender. After being freeze-dried, the minced fillets were extracted with hexane:acetone (3 : 1) in an accelerated solvent extractor (ASE 200, Dionex) at 55°C for 10 min and then further at 100°C for 5 min [24]. The resulting extracts in the organic solvent were washed with water (pH 3.0, H_2SO_4), dried over anhydrous Na_2SO_4, and filtered. The filtrate was concentrated in a stream of N_2 and stored at −20°C.

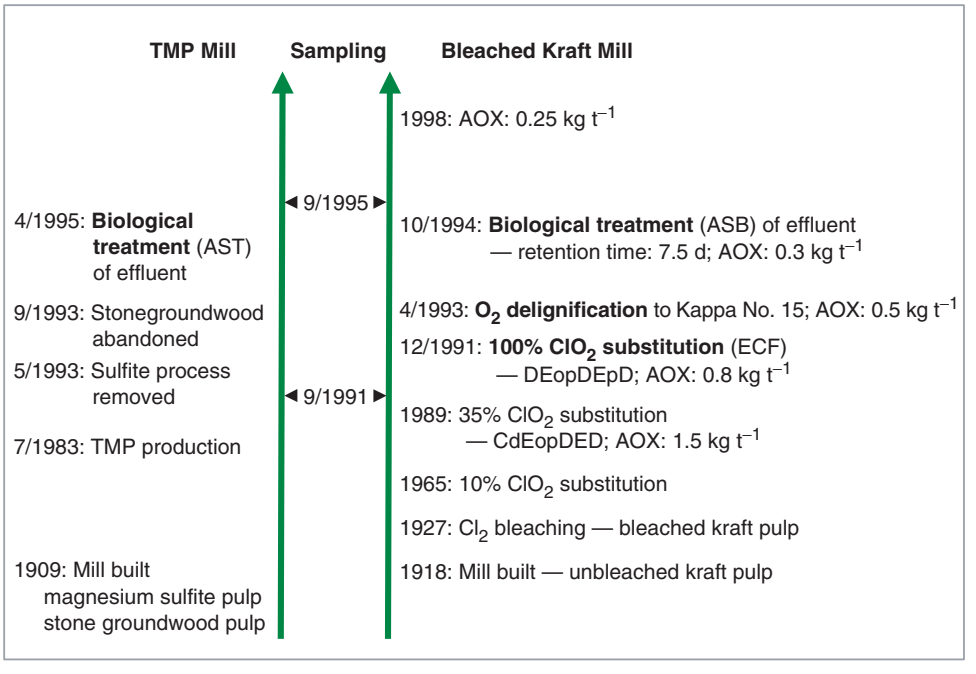

Figure 1. Fish sampling time and upgrading events in the two mills located in the Moose River basin.

2.3 *Fractionation of fish extracts by GPC*

A 0.2–1.0 g aliquot of fillet extract was loaded onto a 2.5×60 cm glass column packed with fully swelled Bio-Beads SX3 beads, and eluted with cyclohexane flowing at about 1 mL min^{-1}, driven by gravity. The eluate was collected as two fractions: high molecular weight (HMW) and low molecular weight (LMW). The elution volume at the cutoff (~350 Da) was about 170 mL, as determined from the elution profile of a mixture of triolein and biphenyl. Collected GPC fractions were washed with water, dried over anhydrous Na_2SO_4, and evaporated to appropriate concentrations. A 10 mL aliquot of each fraction was set aside for determination of its mass by gravimetry and its chlorine concentration by NAA.

2.4 *Transesterification of HMW GPC fractions of fish extracts*

About 0.6 g of the HMW fraction of fillet extract was dissolved in 1–2 mL of toluene, and 5 mL of methanol containing 2% (v/v) sulphuric acid was added. The mixture solution was left overnight in a capped tube at 50°C. The resulting fatty acid methyl esters (FAMEs) including chlorinated FAMEs (ClFAMEs) were extracted with hexane (3×10 mL) after water (10 mL) was added. The combined hexane layers were washed with water (10 mL) containing $KHCO_3$ (2%) and then with pure water (10 mL), dried over anhydrous Na_2SO_4, filtered, and concentrated to dryness in a stream of nitrogen. The yield of the transesterified extract (FAMEs/ClFAMEs) was determined by gravimetry, and the concentration of organic chlorine in it determined by NAA.

The combined aqueous phases from washings, acidified to pH 2, were extracted three times with hexane (3×20 mL). The combined hexane phases were then washed with water (20 mL), dried over anhydrous Na_2SO_4, filtered, and dried to dryness in a stream of nitrogen. The dry residue was weighed to determine non-FAME content, and the chlorine concentration in it determined by NAA. In the workup, some brown precipitates formed, which were neither dissolved in the organic phase nor dissolved in the aqueous phase. This material was dissolved in acetone and the chlorine concentration in it estimated by NAA.

2.5 *Methylation of LMW GPC fractions of fish extracts*

LMW GPC fractions were dissolved in a few drops of methanol and methylated with diazomethane dissolved in ether. Diazomethane was prepared by decomposing 1-methyl-3-nitro-1-nitrosoguanidine in a test tube containing 10% aqueous sodium hydroxide (the lower layer) and ether (the upper layer) [20].

2.6 *Extraction of suspended solids and methylation*

Suspended solids, obtained by centrifuging biologically treated effluent discharged from a mill using 50% ClO_2 substitution in 1993 [25], were provided by Dr Burnison at CCIW. After being freeze-dried, the suspended solids were extracted using a protocol described above for fish extraction. The resulting extracts were methylated with diazomethane as described above.

2.7 *Neutron activation analysis*

Chlorine concentrations in fish extracts, GPC fractions, and different portions of materials resulting from the transesterification workup were determined by NAA. A 15 min irradiation was carried out with a thermal neutron flux of 1.0×10^{12} n cm^{-2} s^{-1} in a SLOWPOKE operated at 20 kW. A 15 min counting by a Ge detector followed a 1–5 min delay. γ-Peaks of ^{38}Cl at 1643 and 2167 keV were used for quantitation using a calibration curve established with KCl.

2.8 *Identification of chlorinated fatty acids*

Major chlorinated fatty acids in the HMW GPC fraction of the fish extracts that had been transesterified to methyl esters were previously identified and confirmed by GC [20, 21, 26]. These were *threo*-5,6-dichlorotetradecanoic, *threo*-7,8-dichlorohexadecanoic, and *threo*-9,10-dichlorooctadecanoic acids found in the fish sampled downstream from a pulp mill using chlorine-based bleaching, and an isomer of dichlorotetradecanoic acid found in a control fish sample.

In this work, prominent chlorinated fatty acids in the extracts of suspended particles and in the LMW GPC fraction of fish extracts, both of which had been treated with diazomethane, were identified using GC/XSD technique described previously [20]. An HP 6890 GC equipped with an OI Analytical 5360 halogen specific detector (XSD) and either an HP-5MS or a DB-WAX column (30 m × 0.25 mm × 0.25 μm) was used under the following operation conditions: injector temperature 270°C; injection volume 1 μL (splitless); carrier gas He at 1.2 mL min^{-1}; oven temperature initially maintained at 80°C for 1 min, and then increased to 160°C at 20°C min^{-1}, to 284°C at 4°C min^{-1} and finally to 310°C at 20°C min^{-1}; detector temperature 1100°C; and detector air flow 40 mL min^{-1}. Identification was done by comparing chromatographic peaks of interest between samples before and after they were spiked with synthesized authentic standards, and then confirmed by re-matching chromatographic peaks in unspiked and spiked samples run on a second GC column which had a very different polarity from that of the first column.

2.9 *Quantification of chlorinated fatty acids*

Instruments and chromatographic conditions for quantitation were the same as those described above. A mixture of methyl *threo*-5,6-dichlorododecanoate and *threo*-10,11-dichlorononadecanoate dissolved in isooctane in known concentrations was used as an internal standard solution. Samples for quantitation were spiked with the internal standard solution prior to GC injection. Response factors of analytes relative to each of the two internal standards were determined by synthesized reference standards [27]. Concentrations of the analytes in the transesterified fish extracts were computed according to peak areas in GC/XSD chromatograms:

$$\text{Concentration of analyte } x = \frac{(A_x/A_s) \times (W_s/\text{RRF})}{W},$$

where A_x and A_s are the peak areas of analyte x and internal standard s, respectively; W and W_s are the weights of the transesterified fish extract and of the internal standard

added to it, respectively; and RRF is the response factor of the reference standard for the analyte relative to that of the internal standard.

2.10 *Analysis of chlorinated pesticides and polychlorinated biphenyls*

The LMW GPC fraction of each fish group was cleaned up using a protocol established by the National Laboratory for Environmental Testing at CCIW. The sample dissolved in 1 mL of hexane was loaded into a 2.5×60 cm glass column packed with activated silica gel and eluted with 2×2 mL and 70 mL of hexane and then 84 mL of hexane : dichloromethane (1 : 1). The first 75 mL of the eluate was collected as fraction A and the remaining 84 mL as fraction B. 1,3-Dibromobenzene and endrin ketone, 100 µL each, were added into fractions A and B, respectively, to serve as internal standards. After 2 mL of isooctane was added, each fraction was concentrated to a final volume of 1 mL. GC/ECD analysis was done on an HP 5890 GC fitted with an electron capture detector operated at 350°C with nitrogen at 60 mL min^{-1} as the makeup gas. Hydrogen at 1.2 mL min^{-1} was used as the carrier gas. The injection mode and temperature, column, and the temperature programming were the same as described above for GC/XSD. Blank and spike tests were also performed on the silica gel column. A mixture of 1 mL of CPCB standards (containing 132 PCB congeners with individual concentrations ranging from 4.925–6.055 ng mL^{-1}) and 1 mL of CBS-OCS standards (containing 26 CBs and OCs with individual concentrations of 5–50 ng mL^{-1}) was used as the spike sample.

2.11 *Synthesis of chlorinated fatty acid methyl esters*

A series of dichloro fatty acids; *threo*-5,6-dichlorododecanoic, *threo*-5,6-dichlorotetradecanoic, *threo*-9,10-dichlorohexadecanoic, *threo*-9,10-dichlorooctadecanoic, and *threo*-10,11-dichlorononadecanoic acids were synthesized from corresponding monounsaturated fatty acids; the resulting chlorinated fatty acids were then methylated in methanol under acidic conditions [20].

In summary, an overall scheme of determining EOCl in fish is illustrated in figure 2.

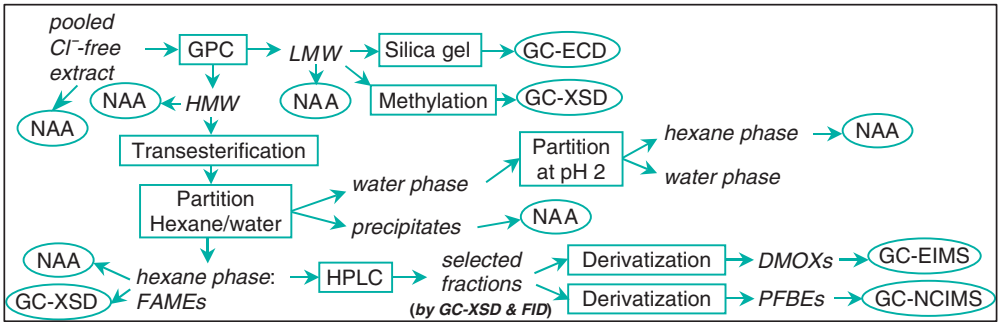

Figure 2. Overall scheme of characterizing fish EOCl. Abbreviations: DMOXs: dimethyloxazoline derivatives; EIMS: electron-impact mass spectrometry [21]; PFBEs: pentafluorobenzyl esters; NCIMS: negative ion chemical ionization mass spectrometry [26].

3. Results and discussion

3.1 *Gel-permeation chromatographic fractionation*

The recovery of the loading from GPC was evaluated using triolein and four fish extracts. The loading was about 1 g. As shown in table 1, the recovery was above 96% by weight. The % RSD calculated based on the fish extract samples was 0.5%. Thus, the absorption of the GPC column was not significant.

Fillet extracts of the same fish group (i.e. those of the same sex, sampled at the same location at the same sampling time) were pooled and separated into HMW and LMW fractions by GPC. The purpose of the GPC fractionation was to separate the EOCl compounds that are related to the source of the bleaching process from those that are unrelated. The former were collected in the HMW fraction, which is presumably composed of lipids; the latter were collected in the LMW fraction, consisting mostly of chlorobenzenes (CBs), organochlorine pesticides (OCs), and polychlorinated biphenyls (PCBs).

The mass percentages of the HMW and LMW fractions and the distribution of organic chlorine in these two fractions are listed in table 2. It can be seen from table 2 that over 94% (by weight) of each fish extract was in the HMW fraction (>~350 Da), and the majority (>78%) of EOCl in the fish extracts was bound to HMW components. In the case of the M91F, there was a particularly high proportion of EOCl in the HMW fraction (table 2). This was likely due to a high input load of the chlorinated fatty acids originating from the mill effluent derived from elemental chlorine bleaching with a

Table 1. Recovery of lipids in GPC elution.

Sample	Recovery (%)
Triolein (standard)	99.6
Fish extract #1	96.9
Fish extract #2	97.0
Fish extract #3	96.4
Fish extract #4	97.5

Table 2. Mass and Cl distributions in HMW and LMW fractions of fish extracts as separated by GPC.

	Mass (%)		Cl (%)		
Sample[a]	HMW	LMW	HMW	LMW	EOCl conc.[b] $\mu g\,g^{-1}$ extract
G91F	97	3	92	8	30
G95F	94	6	78	22	24
G95M	96	4	90	10	56
K91F	97	3	83	17	32
K95F	96	4	85	15	24
K95M	97	3	82	18	22
M91F	98	2	98	2	124
M95F	96	4	90	10	77
M95M	96	4	91	9	114

[a]Fillet extract sample labelled according to the following codes: G, K, and M: sampling locations, i.e. Groundhog (reference river), Kapuskasing (downstream of the TMP mill), and Mattagami (downstream of the bleached kraft pulp mill). 91 and 95: sampling years. F and M: fish genders.
[b]Total organic chlorine in fish extract prior to separation into HMW and LMW fractions.

low chlorine dioxide substitution. This speculation was later confirmed in the analysis of chlorinated fatty acids (*vide infra*).

3.2 *Analysis of chlorinated pesticides and PCBs in the LMW fraction*

The CBs, OCs, and PCBs are chlorinated compounds known to be persistent in the environment and highly bio-accumulative in fish. The concentrations of these chlorinated compounds in the LMW fraction were determined by GC/ECD following silica gel cleanup. The most common components were 1,2,4-trichlorobenzene, *p*,*p*′-DDE, and *p*,*p*′-DDT. Some fish groups contained particularly high concentrations of hexachlorobenzene, *p*,*p*′-DDD, γ-chlordane, mirex, PCB 105, and PCB 149. Table 3 summarizes the concentrations of CBs, OCs and PCBs as expressed in micrograms of Cl per gram of extract and the percentage of total chlorine amount in the LMW fraction of fillet extracts the chlorine amount from these compounds accounts for. Noticeably, male fish groups had higher concentrations of chlorinated pesticides and PCBs in fillets than female groups from the same sampling location. A possible explanation of this observation is that in addition to normal excretory routes such as faecal egestion, female fish can excrete the xenobiotics along with lipids via spawning. Among fish from three different rivers, those from Groundhog River had the lowest concentration, reflecting the fact that Groundhog is a pristine river. Generally speaking, fillet extracts had higher levels of PCBs than those of CBs and OCs. Sums of CBs, OCs, and PCBs accounted for a varying proportion of EOCl in the LMW fraction (4–55%), depending on the sample source and fish gender. The most prominent component of the EOCl in the LMW fraction for fish sampled downstream from the bleached kraft pulp mill was actually dichloro fatty acid (*vide infra*). However, a significant portion of the EOCl in the LMW fraction is unknown, though it represents only a small fraction of total EOCl in fish extracts.

Table 3. Chlorobenzenes (CBs), organochlorine pesticides (OCs) and polychlorinated biphenyls (PCBs) in fish extracts.

Sample[a]	CBs and Ocs $\mu g\,Cl\,g^{-1}$ extract	PCBs $\mu g\,Cl\,g^{-1}$ extract	Sum	
			$\mu g\,Cl\,g^{-1}$ extract	% of total EOCl in LMW[b]
G91F	0.1	0.1	0.2	4
G95F	0.1	0.2	0.3	5
G95M	0.4	0.5	0.9	19
K91F	0.2	1.0	1.2	21
K95F	0.2	1.3	1.4	39
K95M	0.2	1.9	2.2	55
M91F	0.1	0.9	1.0	45
M95F	0.1	0.6	0.7	9
M95M	0.2	1.9	2.0	20

[a]Fillet extract sample labelled according to the following codes: G, K, and M: sampling locations, i.e. Groundhog (reference river), Kapuskasing (downstream of the TMP mill), and Mattagami (downstream of the bleached kraft pulp mill). 91 and 95: sampling years. F and M: fish genders.
[b]Cl from dichloro C14, C16, and C18 fatty acids present in LMW fractions were excluded from the total EOCl here.

3.3 *Transesterification of HMW fractions*

Given the work by Wesén and coworkers [11–16], who identified chlorinated fatty acids in a marine fish sample of extremely high chlorine load, it is reasonable to speculate that those chlorinated substances associated with the HMW fraction obtained from GPC could also be chlorinated fatty acids, possibly present in the form of glycerolipids. Though it would be desirable to identify the specific chlorinated glycerolipids present in fish, direct analysis of the HMW fraction for chlorinated glycerolipids seems to be an unrealistic task since the number of possible glycerolipids could be so many that their individual concentrations in extract samples of a given EOCl load might be too low to be detectable. Transforming chlorinated glycerolipids into chlorinated fatty acids or their methyl esters would decrease the number of chlorinated species dramatically, thus enhancing the concentrations of analytes. This is important for the present analysis, given that the concentrations of organic chlorine are present at trace levels in fish extracts.

To transform chlorinated glycerolipids into chlorinated fatty acids, HMW fractions of fish extracts were transesterified by acidic methanolysis. The workup resulted in four portions of materials: transesterified extract dissolved in the hexane phase, which consists of fatty acid methyl esters (FAMEs) including chlorinated fatty acid methyl esters (ClFAMEs); materials that did not dissolve in either hexane or water phase; materials recovered from the diluted potassium bicarbonate aqueous phase; and materials dissolved in the water phase. NAA was employed for determining the distribution of chlorine between these portions. Detailed data regarding mass and chlorine distributions between these portions are shown in table 4. As can be seen in table 4, the amount of FAMEs is 76–98% (by weight) of the HMW extract. Consistently, the 1991 fish samples have a lower percentage of FAMEs than the 1995 fish samples at the same sampling location. This might be due to slow oxidative degradation of some lipids during their long storage. A very small amount of material (representing less than 1% of the HMW by weight) was removed by diluted $KHCO_3$ aqueous solution during the workup after transesterification (Group A). This portion of material was originally thought to be unmethylated fatty acids; it was discovered later by NAA and GC/XSD that it was composed of other unknown halogenated compounds. It was noted that some unrecovered materials were dark brown and insoluble in both hexane and water phases (Group B). As can be seen in table 4, these insoluble materials and the materials recovered from the diluted $KHCO_3$ aqueous phase represent a very small portion of the materials resulting from transesterification. Hence, the amount of organic chlorine in this portion of materials is small compared with that in the transesterfied extract portion, even though NAA indicates that the chlorine concentrations in these materials (not shown) are higher than those in the transesterified extract. Their identification was thus not pursued any further. As shown in table 4, 60–80% of the organic chlorine in the HMW samples is bound to the fatty acid components, except for G91F and K91F samples where ClFAMEs account for only 43 and 48%, respectively. It was observed that in the G91F sample, a substantial portion of organic chlorine was bound to the HMW moieties that are insoluble in both hexane and water phases. Quantification of the material designated by Group A (see note b beneath table 4) is not available in table 4 for samples G95F, G95M and K91F because this fraction had not been anticipated in the beginning of the workup.

Table 4. Characterization of HMW fish extracts subjected to transesterification.

Sample[a]	Transesterified extract[b] (g 100 g^{-1} HMW)	Material other than transesterified extract (g 100 g^{-1} HMW)				EOCl in HMW		
		Group A[c]	Group B[d]	Group C[e]	Total[f]	Total (μg g^{-1} HMW)	Contained in ClFAMEs (%)	Contained in compounds other than ClFAME[g] (%)
G91F	75.9	1.5	0.5	25.0	27.1	27.0	43	57
G95F	88.0	–	~0.3	–	15.4	20.3	63	37
G95M	96.4	–	~0.3	–	7.4	52.1	80	20
K91F	85.5	–	0.5	–	17.8	27.6	48	52
K95F	98.3	0.9	0.4	4.2	5.5	21.4	60	40
K95M	95.2	1.2	0.7	6.6	8.5	18.2	74	26
M91F	84.6	0.7	0.3	17.7	18.7	124.1	70	30
M95F	98.2	1.3	0.4	3.6	5.6	72.4	73	27
M95M	92.5	2.3	0.6	8.3	11.1	108.0	64	36

[a]Fillet extract sample labelled according to the following codes: G, K, and M: sampling locations, i.e. Groundhog (reference river), Kapuskasing (downstream of the TMP mill), and Mattagami (downstream of the bleached kraft pulp mill). 91 and 95: sampling years. F and M: fish genders.
[b]Consisting of fatty acid methyl esters, including chlorinated fatty acid methyl esters.
[c]Materials insoluble in both water and hexane phases in the transesterification workup.
[d]Materials recovered from the diluted $KHCO_3$ aqueous phase after it was acidified to pH 2.0.
[e]Deduced from total of compounds other than FAMEs–Group A–Group B; representing whatever remained in the water phase after it was acidified to pH 2.0 and extracted with hexane.
[f]Deduced from 100 – 0.961 × quantity under column heading Transesterified Extract. The coefficient of 0.961 is used to estimate the quantity of fatty acids from that of FAMEs, assuming that FAMEs have an average molecular weight of 360.
[g]Deduced from 100 – EOCl contained in C1FAMEs.

3.4 *Quantification of chlorinated fatty acids*

Methyl *threo*-5,6-dichlorotetradecanoate, *threo*-7,8-dichlorohexadecanoate and *threo*-9,10-dichlorooctadecanoate have been identified as being major EOCl components in the transesterified extracts of fish sampled downstream from bleached kraft pulp mills [20, 26]. An isomer of methyl dichlorotetradecanoate was identified in a reference sample (G95M) using GC/MS with negative ion chemical ionization [26]. These ClFAMEs were quantified by GC/XSD after known quantities of internal standards (methyl *threo*-5,6-dichlorododecanoate and 10,11-dichlorononadecanoate) were added. Methyl *threo*-5,6-dichlorododecanoate eluted before the earliest analyte and methyl *threo*-10,11-dichlorononadecanoate after the latest analyte. Table 5 lists the concentrations of the identified dichloro fatty acid methyl esters in transesterified extracts, calculated based on the two internal standards ($C_{12}Cl_2$ and $C_{19}Cl_2$).

Some fatty acids might have been liberated from lipids during the storage. If so, they would have been collected in the LMW fraction in the GPC separation. To examine if those identified dichloro fatty acids were present in the LMW fraction, the fraction was methylated by diazomethane and analyzed by GC/XSD on two different columns. Comparison was made between chromatograms obtained before and after spiking with reference standards. An example is illustrated in figure 3, which is analysis of the LMW fraction of M91F. As we see in both HP-5 and DB-WAX chromatograms, the sample peaks identified as being dichlorotetradecanoate and dichlorooctadecanoate were completely superimposed with the reference standards methyl *threo*-5,6-dichlorotetradecanoate (c in figure 3) and *threo*-9,10-dichlorooctadecanoate (e in figure 3). The sample peak identified as being dichlorohexadecanoate apparently eluted ahead of the reference standard methyl *threo*-9,10-dichlorohexadecanoate (d in figure 3). As in the case of the transesterified products of the HMW fraction [20], this sample peak can be rationalized to be a *threo*-7,8-isomer. Thus, three FAMEs identified

Table 5. Concentration of identified dichloro fatty acid methyl esters in transesterified fish extracts ($\mu g\,g^{-1}$ transesterified extract).

	Ester[b]								
	$C_{14}Cl_2$			$C_{16}Cl_2$			$C_{18}Cl_2$		
Sample[a]	$C_{12}Cl_2$	$C_{19}Cl_2$	Ave.	$C_{12}Cl_2$	$C_{19}Cl_2$	Ave.	$C_{12}Cl_2$	$C_{19}Cl_2$	Ave.
M91F	120	124	122	36	37	37	57	59	58
M95M	99	101	100	18	18	18	30	36	33
M95F	63	64	64	11	12	11	22	22	22
G95M	90	93	92		n.d.[c]			n.d.[c]	
Intestinal fat (F)	126	152	139	23	28	25	49	70	59
Carcass (F)	82	99	91	19	23	21	33	46	39
Gonad (F)	28	29	29	12	12	12	21	25	23
Gonad (M)	74	77	75	20	21	21	29	36	32

[a]Fillet extract sample labelled according to the following codes: G and M: sampling locations, i.e. Groundhog (reference river), and Mattagami (downstream of the bleached kraft pulp mill). 91 and 95: sampling years. F and M: fish genders. Extracts of other tissues were obtained from the same fish species (white sucker) sampled in 1993 downstream from a different bleached kraft pulp mill (St. Maurice R.) where 50% ClO_2 substitution was employed for bleaching [28].
[b]$C_{12}Cl_2$, $C_{14}Cl_2$, $C_{16}Cl_2$, $C_{18}Cl_2$, and $C_{19}Cl_2$ stand for methyl esters of *threo*-5,6-dichlorododecanoic, *threo*-5,6-dichlorotetradecanoic (except for G95M where the position of chlorine atoms in the molecule is unknown), *threo*-7,8-dichlorohexadecanoic, *threo*-9,10-dichlorooctadecanoic, and *threo*-10,11-dichlorononadecanoic acids, respectively.
[c]Not detected.

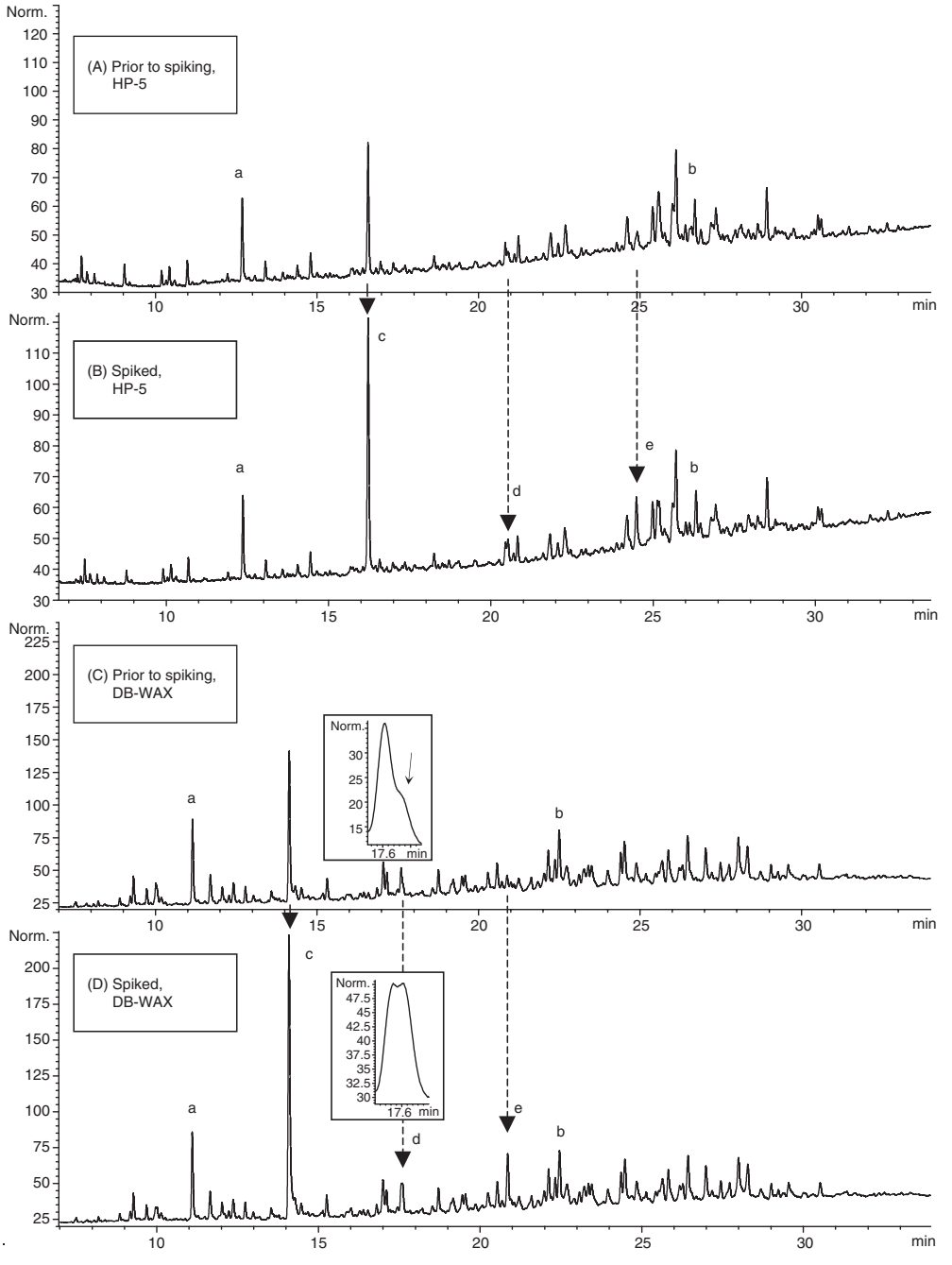

Figure 3. GC/XSD of the LMW portion of fish extracts (M91F). (A), (C) prior to spiking. (B), (D) spiked with methyl esters of *threo*-5,6-dichlorotetradecanoic, *threo*-9,10-dichlorohexadecanoic, and *threo*-9,10-dichlorooctadecanoic acids. Internal standards: methyl esters of (a) *threo*-5,6-dichlorododecanoic acid and (b) *threo* 10,11-dichlorononadecanoic acid. Standards for spiking: methyl esters of (c) *threo*-5,6-dichlorotetradecanoic acid, (d) *threo*-9,10-dichlorohexadecanoic acid, and (e) *threo*-9,10-dichlorooctadecanoic acid.

in the transesterified extracts were also present in the LMW fraction. *Threo*-5,6-dichlorotetradecanoate actually dominates in quantities in the chromatogram. The same is true for the other samples from the fish exposed to the bleached kraft pulp mill effluent (M95F and M95M). The prominent peak in the chromatogram of transesterified extracts of G95M (an isomer of methyl dichlorotetradecanoate [26]) was also found to be a predominant peak in the chromatogram of its corresponding LMW fraction that had been methylated (figure 4). Under chromatographic conditions adopted, the relative retention time of this peak to *threo*-5,6-dichlorododecanoate internal standard was 1.1, compared to 1.3 for *threo*-5,6-dichlorotetradecanoate found in the exposed fish. None of the known chlorinated fatty acids were found in the LMW fraction of the other reference fish samples.

The quantification of these chlorinated FAMEs in the methylated LMW fraction was carried out in the same way as for transesterified extracts. The quantification results are shown in table 6.

As shown in tables 5 and 6, the concentrations calculated based on these two internal standards are similar; their average is used for data interpretation. The concentrations of ClFAMEs in transesterified extracts and the methylated LMW fraction are consistent: they are all higher for the fish sampled prior to the process change to ECF (M91F) than for the fish sampled afterwards (M95M and M95F). Sample G95M shows a surprisingly high concentration of dichlorotetradecanoate. This is an unusual case for reference fish samples. Some individual fish in this group (male fish from the reference river) must have been exposed to an unknown source of this chlorinated compound. The relative concentrations among the identified dichloro fatty acids in the

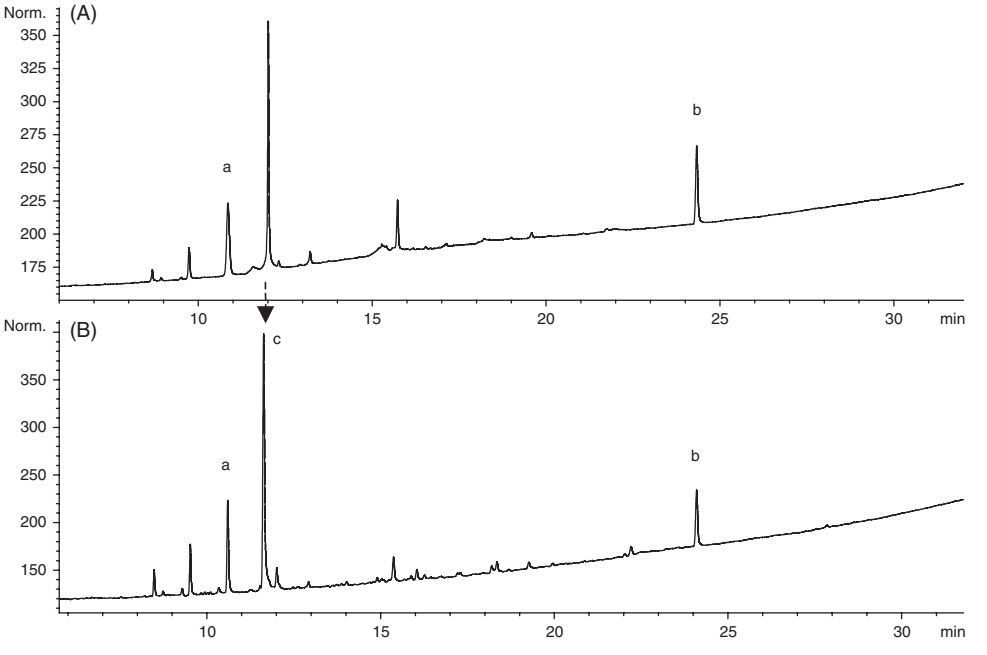

Figure 4. GC/XSD of lipid extracts from a reference fish sample (G95M). (A) HMW portion (transesterified). (B) LMW portion (methylated). Labels (a) and (b): same as those used in figure 3. Label (c): a predominant component found in this sample.

Table 6. Concentration of identified dichloro fatty acid methyl esters in LMW fraction subjected to methylation ($\mu g\,g^{-1}$ LMW).

	Ester[b]								
	$C_{14}Cl_2$			$C_{16}Cl_2$			$C_{18}Cl_2$		
Sample[a]	$C_{12}Cl_2$	$C_{19}Cl_2$	Ave.	$C_{12}Cl_2$	$C_{19}Cl_2$	Ave.	$C_{12}Cl_2$	$C_{19}Cl_2$	Ave.
M91F	111	125	118	16	18	17	19	21	20
M95M	32	33	33	6	6	6	1	1	1
M95F	11	12	12	2	2	2	1	1	1
G95M	104	98	101						

[a]Fillet extract sample labelled according to the following codes: G and M: sampling locations, i.e. Groundhog (reference river), and Mattagami (downstream of the bleached kraft pulp mill). 91 and 95: sampling years. F and M: fish genders.
[b]See Table 5 for full names.

Table 7. Percentage of each identified dichloro FAME in LMW fraction (relative to the total amount of the corresponding dichloro FAME in extracts).

Sample[a]	$C_{14}Cl_2$	$C_{16}Cl_2$	$C_{18}Cl_2$
M91F	2.5	1.2	0.9
M95M	1.3	1.3	0.1
M95F	0.8	0.7	0.2
G95M	4.2		

[a]Fillet extract sample labelled according to the following codes: G and M: sampling locations, i.e. Groundhog (reference river), and Mattagami (downstream of the bleached kraft pulp mill). 91 and 95: sampling years. F and M: fish genders.
[b]See Table 5 for full names.

LMW fraction seem to be correlated with their relative concentrations in the HMW fraction (transesterified extract). Although their concentrations in the LMW fraction are close to or just a few times less than their concentrations in the transesterified extracts, the dichloro fatty acids in the LMW actually account for a very small fraction of the total quantities in the fish extracts (table 7). Table 5 also includes the analytical data for other tissues from fish sampled in a different river downstream from a bleached kraft pulp mill using 50% ClO_2 substitution. As shown in table 5, the concentrations of the dichloro fatty acids, especially *threo*-5,6-dichlorotetradecanoic acid, vary in different tissues. The concentrations in the intestinal fat are higher than those in the carcass, which in turn are higher than those in the gonad. The male gonad extracts have higher concentrations of dichloro fatty acids than the female analogues, especially for *threo*-5,6-dichlorotetradecanoic acid.

The overall concentration of each identified dichloro fatty acid in fish extracts can be computed by converting its concentrations in transesterified fish extracts and in the LMW fraction into its concentrations in fish extracts and then summing them up (table 8). Sample M91F has higher concentrations than other samples, particularly for *threo*-7,8-dichlorohexadecanoic and 9,10-dichlorooctadecanoic acids. For a more insightful comparison, the concentrations given in table 8 are transformed into molar percentages of the total of these identified dichloro fatty acids (table 9). As shown in table 9, the proportions of *threo*-9,10-dichlorooctadecanoic acid and 7,8-dichlorohexadecanoic acid decrease in samples M95M and M95F as compared with those in M91F. In contrast, the proportion of the end product of β-oxidative metabolism, *threo*-5,6-dichlorotetradecanoic acid, increases in samples M95M and M95F as compared with

Table 8. Concentration of the identified dichloro fatty acids in total fish extracts ($\mu g\,g^{-1}$ extract).

Sample[a]	$C_{14}Cl_2$	$C_{16}Cl_2$	$C_{18}Cl_2$
M91F	99	30	47
M95M	86	16	28
M95F	58	10	20
G95M	85		

[a]Fillet extract sample labelled according to the following codes: G and M: sampling locations, i.e. Groundhog (reference river), and Mattagami (downstream of the bleached kraft pulp mill). 91 and 95: sampling years. F and M: fish genders. The analytes here are in free acid form rather than in methyl esters.

Table 9. Molar proportion of the three identified dichloro fatty acids in fish extracts (%).

Sample[a]	$C_{14}Cl_2$	$C_{16}Cl_2$	$C_{18}Cl_2$
M91F	60	16	24
M95M	69.4	11.5	19
M95F	69	11	20

[a]Fillet extract sample labelled according to the following codes: G and M: sampling locations, i.e. Groundhog (reference river), and Mattagami (downstream of the bleached kraft pulp mill). 91 and 95: sampling years. F and M: fish genders. The analytes here are in free acid form rather than in methyl esters.

that in M91F. This may indicate that the input load of *threo*-9,10-dichlorooctadecanoic acid from the bleached kraft pulp mill effluent into the downstream fish was reduced or virtually terminated as the bleaching process in the mill was upgraded from low ClO_2 substitution to ECF, and oxygen delignification and effluent biotreatment were added.

Table 10 summarizes the EOCl mass balance as determined by NAA of GPC fractions and transesterification fractions, and the GC/XSD quantification of chlorinated fatty acids. Calculated from table 10, distributions of EOCl in various compound groups are illustrated in figure 5. Total EOCl concentrations in pooled fillet extracts are presented at top of the chart in figure 5. As can be seen in table 10 and figure 5, the exposed fish sampled downstream of the bleached kraft pulp mill using chlorine-based bleaching had much higher EOCl levels than the reference fish samples. The EOCl of HMW origin accounts for the majority (over 78%) of total EOCl in fish extracts. It can be calculated from table 10 that, of the identified chlorinated fatty acids which represent 26–32% of the total EOCl in extracts from the exposed fish, *threo*-5,6-dichlorotetradecanoic acid accounts for 60–70%, *threo*-9,10-dichlorooctadecanoic acid for 19–24%, and *threo*-7,8-dichlorohexadecanoic acid for 11–16%. A chlorinated fatty acid in a reference sample, identified as an isomer of dichlorotetradecanoic acid [26], accounts for a surprisingly large portion of the total EOCl in that reference sample. This demonstrates that the discharge of any chlorinated fatty acid could be an important source of EOCl in exposed fishes.

3.5 *Sources of EOCl in fish*

It is evident from the survey of fish EOCl levels in the Moose River basin that effluent derived from the bleaching process using low ClO_2 substitution was an important

Table 10. EOCl in pooled fillet extracts[a] (g μCl contained in individual item g^{-1} extract).

	HMW								LMW[i]
	Chlorinated FAs[c]						Compounds other than FAs[g]	Total[h]	
	Known dichloro fatty acids[d]				Other[e]	Total[f]			
Sample[b]	$C_{14}Cl_2$	$C_{16}Cl_2$	$C_{18}Cl_2$	Sum					
M91F	24	6	9	39	46	86	37	122	2
M95F	14	2	4	20	31	51	19	70	8
M95M	21	3	6	30	37	67	37	104	10
K91F						13	14	27	5
K95F						13	8	21	4
K95M						13	5	18	4
G91F						11	15	26	4
G95F						12	7	19	5
G95M	20			20	21	41	10	51	5

[a]In each sample, extracts of five fish were pooled for analysis.
[b]Fillet extract sample labelled according to the following codes: G, K, and M: sampling locations, i.e. Groundhog (reference river), Kapuskasing (downstream of the TMP mill), and Mattagami (downstream of the bleached kraft pulp mill). 91 and 95: sampling years. F and M: fish genders.
[c]FAs: fatty acids.
[d]Include $C_{14}Cl_2$, $C_{16}Cl_2$, and $C_{18}Cl_2$ present in HMW and LMW fractions.
[e]Deduced from total EOCl of chlorinated FAs – sum EOCl of known dichloro fatty acids.
[f]Sum of EOCl in transesterified extract (quantified by NAA) and EOCl contained in the known dichloro fatty acids present in LMW fraction (quantified by GC/XSD).
[g]Deduced from total EOCl of HMW – total EOCl of transesterified extract.
[h]Sum of EOCl in HMW fraction (quantified by NAA) and EOCl contained in the known dichloro fatty acids present in LMW fraction (quantified by GC/XSD).
[i]Exclude the known dichloro fatty acids mentioned in footnote d.

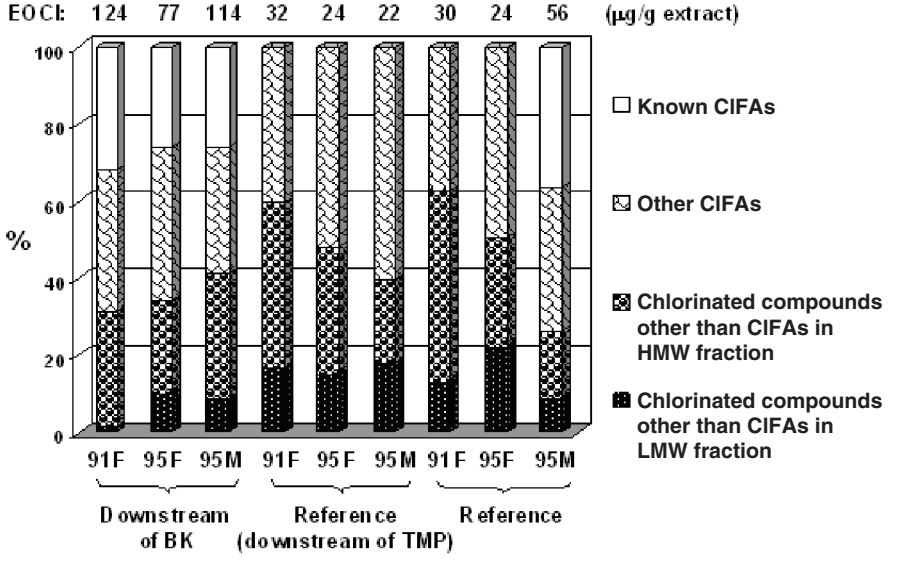

Figure 5. Concentrations and distributions of chlorine in pooled fillet extracts.

source of EOCl in fish in Mattagami River downstream from the bleached kraft pulp mill [5]. Elevated EOCl in downstream fish declined over years as the mill implemented a series of technology upgrades which were motivated by better environmental management. It appears that this effluent-related source of EOCl became insignificant after the mill switched the bleaching process to ECF (100% ClO_2), added O_2 delignification, and installed a biological treatment system to treat mill effluents prior to their discharge into the receiving river. It is believed that certain levels of EOCl that were still observed in downstream fish following these innovations were due to the following factors: (1) the background EOCl that was not bleaching effluent-related, (2) chlorinated fatty acids constituting major EOCl components, which seem to be assimilated well by fish and are thus biologically persistent, (3) physiological difference between fish genders that accounts for the fact that EOCl declined slower in male fish than in female fish, and (4) characteristics of the local aquatic environment.

Additional hints regarding sources of fish EOCl can be obtained from the results of studying the chemical nature of fish EOCl. The most prominent feature of fish EOCl is that the EOCl in extracts of fish downstream from bleached kraft pulp mills was dominated by three *threo*-dichloro fatty acids: 9,10-dichlorooctadecanoic, 7,8-dichlorohexadecanoic, and 5,6-dichlorotetradecanoic. This was true regardless of whether the extracts were from fillets, gonad, intestinal fat, or carcass. Clearly, these dichloro fatty acids were related by metabolism; dichlorooctadecanoic acid was directly formed in the bleaching process, while the other two were mainly produced from the biodegradation of dichlorooctadecanoic acid.

A deeper insight into sources of fish EOCl can be gained by comparing concentrations of EOCl (in extracts) contained in individual components or subclasses. As can be seen in table 10, decline of 50% (from years 1991 to 1995) in EOCl contained in chlorinated compounds other than fatty acids in the HMW fraction occurred for the female fish groups from all locations. Although the 1991 male fish are not available for comparison, it is noted that, for the reference sites, the 1995 male fish also have a lower concentration of this class of chlorinated materials than the 1991 female fish. It is not clear to what degrees the chlorinated materials other than fatty acids are associated with the mill effluent and how much significantly the technology upgrading in the mill has contributed to their reduction in the downstream fish. Temporal comparisons of chlorine amounts found in chlorinated fatty acids as well as in the LMW fraction are more informative: while these chlorine amounts decreased substantially from 1991 to 1995 for the fish downstream from the bleached kraft pulp mill, there was little variation for the reference fish except G95M, which represents an unusual sample compared with the other reference samples. This indicates that the changes in EOCl of these classes in the downstream fish were likely effected by the technology upgrades in the mill. The sources of chlorinated fatty acids are thus clear. Compared with reference fish groups which contain only 11–13 $\mu g\,Cl\,g^{-1}$ extract of chlorinated fatty acids (table 10), most of chlorinated fatty acids in the downstream fish (51–86 $\mu g\,Cl\,g^{-1}$ extract) must have been taken up from the mill effluent by the fish. Mill technology upgrading has caused the total EOCl of chlorinated fatty acids in the downstream female fish to decline by ~41% between 1991 and 1995, of which the three identified dichloro fatty acids decreased by ~49%, while other chlorinated fatty acids decreased only by 32%. This implies that the three identified dichloro fatty acids are strongly associated with the previous bleaching process. Decline rates are also different between the three identified dichloro fatty acids: ~56% for the dichloro C_{18} fatty acid,

~67% for C_{16}, and ~42% for C_{14}. A dramatic decline in dichloro C_{18} fatty acid should reflect a marked reduction in input of this chemical from the environment. The dichloro C_{16} fatty acid is an intermediate in the β-oxidative metabolism of the dichloro C_{18} fatty acid; its decline is expected when the concentration of its precursor has decreased. A slower decline in the dichloro C_{14} fatty acid shows that it is the end product of β-oxidation of the dichloro C_{18} fatty acid and that it is biologically persistent in fish body. It is remarkable to observe in comparing the female and male fish from the downstream site in the same sampling year (M95F *vs.* M95M) that the ratios for the concentrations of dichloro C_{18}, C_{16}, and C_{14} fatty acids are all about 0.7, while the ratios for other chlorinated materials range from 0.5 to 0.8. This may further confirm that these three dichloro fatty acids are metabolism-related. Indeed, relative concentrations of these three dichloro fatty acids are about the same in the 1995 fish regardless of the gender, 2 : 1 : 7 for dichloro C_{18} : C_{16} : C_{14}, whereas this ratio is 1.5 : 1 : 4 for the 1991 female fish, which was in a very different environment due to a very different effluent quality it received.

The increase in the LMW fraction of EOCl in the downstream fish between 1991 and 1995 (table 10) is hard to explain. It could be due to temporal variations in the background chlorine level specific to that area during that period. It could also be possible that O_2 delignification, ClO_2 bleaching, or biological transformation may have rendered some lipophilic chlorinated compounds less likely to be incorporated into HMW organics. But these explanations are merely speculative. After all, these compounds account for only 10% or less of the total EOCl in the downstream fish.

3.6 *Chlorinated fatty acids found in suspended solids from effluent derived from chlorine-based bleaching*

On the basis of the foregoing discussion, it is reasonable to assume that the majority of dichloro fatty acids present in fish downstream from bleached kraft pulp mills originate from chlorine-based bleaching. As dichloro fatty acids are practically insoluble in water, it is logical to speculate that they were adsorbed onto suspended solids in the discharged effluents. To find direct evidence, suspended solids isolated from the final effluent of a bleached kraft pulp mill using 50% CO_2 substitution and with biological treatment in place were extracted with organic solvents. After methylation, the resulting samples were analysed by GC/XSD on two different columns (HP-5 and DB-WAX). Figure 6 shows GC/XSD chromatograms obtained from these analyses with and without spiking with synthesized reference standards. As shown in figure 6, *threo*-9,10-dichlorooctadecanoic, 7,8-dichlorohexadecanoic (presumably a 7,8-isomer), and 5,6-dichlorotetradecanoic acids are all present in the suspended solids with the first being much greater in concentration than the other two. The presence of *threo*-7,8-dichlorohexadecanoic and 5,6-dichlorotetradecanoic acids demonstrates that the β-oxidative metabolism of chlorinated fatty acids may occur in the micro-organism as well. Since the ratio of the two shorter dichloro fatty acids to the dichloro C_{18} fatty acid is much smaller in suspended solid EOCl than that in fish EOCl, assuming they are bioaccumulated to the same extent, the majority of these shorter dichloro fatty acids in fish extracts must have been produced in fish as a result of β-oxidative metabolism of the dichloro C_{18} fatty acid.

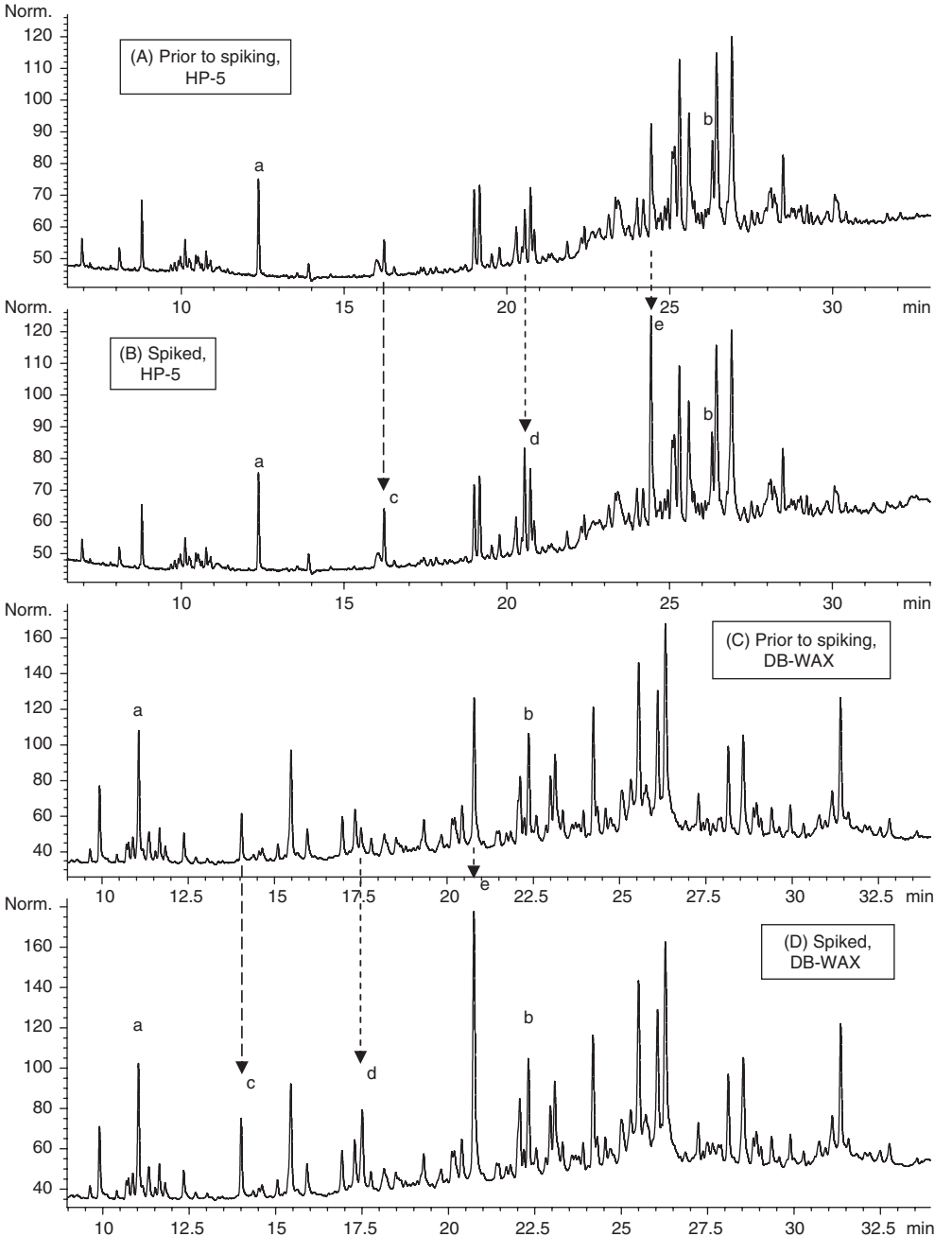

Figure 6. GC/XSD of methylated extracts of suspended particles isolated from final effluent of a bleached kraft mill using 50% CO_2 substitution and biological treatment. (A), (C) prior to spiking. (B), (D) spiked with methyl esters of *threo*-5,6-dichlorotetradecanoic, *threo*-9,10-dichlorohexadecanoic, *threo*-9,10-dichloro-octadecanoic acids. Labels (a)–(e): same as those used in figure 3.

4. Conclusions

Fish extracts have complicated compositions of organochlorine compounds. These compounds are generally present in extremely low concentrations in lipids of the fish living in unpolluted areas. However, exposure to a chlorinated fatty acid source may cause fish to accumulate high concentrations of this substance in lipids of various tissues. Fish exposed to effluents from bleached kraft pulp mills using chlorine bleaching or low ClO_2 substitution typically have high concentrations of dichlorooctadecanoic acid and its β-oxidative products (dichlorohexadecanoic and dichlorotetradecanoic acids) in lipids.

The study of EOCl obtained from white sucker fillets (hexane–acetone extraction) shows that over 78% of EOCl is of relatively HMW ($>\sim$350). The major EOCl components are chlorinated fatty acids, probably present in the form of glycerolipids. While they are undetectable in reference fish, *threo*-5,6-dichlorotetradecanoic, *threo*-7,8-dichlorohexadecanoic, and *threo*-9,10-dichlorooctadecanoic acids represent about 30% of total EOCl in extracts, of which *threo*-5,6-dichlorotetradecanoic acid accounts for 60–70%, *threo*-9,10-dichlorooctadecanoic acid for 19–24%, and *threo*-7,8-dichlorohexadecanoic acid for 11–16%. The findings support the working hypothesis that, among chlorinated compounds discharged from bleached kraft pulp mills, *threo*-9,10-dichlorooctadecanoic acid, presumably generated in chlorine-based bleaching processes, is the most bio-accumulative in fish, and that it can be biodegraded by fish into dichlorohexadecanoic and dichlorotetradecanoic acids, presumably via β-oxidative metabolism.

The identification of these three dichloro fatty acids in suspended solids isolated from biologically treated final effluent discharged from a pulp mill using 50% ClO_2 substitution confirms that effluents derived from chlorine-based bleaching are a significant source of chlorinated fatty acids in downstream fish. Furthermore, β-oxidation of dichlorooctadecanoic acid appeared to occur in micro-organisms.

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