Mass Spectrometric Screening for Organohalogen Substances (OHS) in Blood Plasma from Baltic Salmon (*Salmo salar*)

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Abstract

Wild living salmon (*Salmo salar*) from the Baltic suffer from a disease complex characterised by high fry mortality at the yolk-sac stage. The syndrome is known as M74 and so far there is no explanation for the occurrence of the disease. Several hypothesis have been put forward to explain M74, including the presence of environmental contaminants in the salmon. The aim of the present project is to verify if any correlation can be made between the syndrome and anthropogenic substances in the salmon muscle, blood and/or in their eggs. Samples from salmons producing healthy fryes and fryes suffering from M74 were analysed for exogenous neutral and phenolic substances. Hitherto unknown hydroxylated polybrominated diphenyl ethers (OH-PBDE) has been detected in the salmon plasma together with hydroxylated polychlorinated biphenyls (OH-PCB) and halogenated phenols, including pentachlorophenol (PCP) in high concentrations. All traditional neutral environmental contaminants were detected in plasma and in muscle in addition to several polybrominated diphenyl ethers (PBDEs) and methoxylated PBDE (MeO-PBDE).

Introduction

Baltic salmon suffer from a syndrome characterised by salmon fry showing a very high mortality as they reach the yolk-sac stage. The syndrome was first observed in the early 1970-ties and has thereafter occurred with different intensities over the years[1] . With the causal connection behind the M74 being unknown, different hypotheses for the syndrome have been suggested. Thus, genetic factors in the salmon populations, large scale ecological changes in the environment, virus infections and environmental pollutants have been suggested as possible causes of the syndrome. It has been shown that salmon eggs that have been washed in dilute thiamine (vitamin B_1) water solutions have significantly improved hatching success[1]. The potential influence for the development of M74 by genetic factors and microbial infections are under investigation.

Since the concentrations of the dominating environmental contaminants PCB and DDT, have been decreasing for many years in the Baltic Sea and their concentrations in e.g. herring is today 10% and 20-25%, respectively of the levels 20 years ago[3]. It thus seems unlikely that these substances play a role in the development of M74. On the other hand, there are other substances present, in the environment, e.g. a number of different flame retardants including PBDEs and phenolic type compounds used in technical applications or as metabolites of xenobiotics. PBDEs have shown increasing concentrations in the Baltic Sea ecosystems[3, 4]. While no corresponding studies have been performed for any of the phenolic compounds.

Major effects observed in wildlife have often been due to reduced fertility or other reproductive problems as observed for birds of prey, alligators and seals from the Baltic Sea[5]. The reproductive impairment may be due to endocrine disrupting substances such as PCB and DDT via disturbances of the thyroid or sex hormone homeostasis. Several halogenated phenolic compounds have been shown to bind to the thyroxin (T_4) transporting protein, transthyretine (TTR)[6]. Several hydroxylated metabolites of PCB have been shown to have a 5-15 times higher affinity for TTR than the natural ligand T_4 itself[7,8]. Also pentachlorphenol and other chlorinated phenols compete with T_4 for the binding site on TTR [9]. Presence of phenolic compounds in blood has been verified for environmentally exposed species, such as mammals, humans and birds [10,11].

The specific aims of this study was to investigate the presence of phenolic substances and phenolic metabolites of OHS in Baltic salmon blood by mass spectrometry and to investigate if a correlation exists between the levels of the substances of interest and the appearance of M74 in the salmon fry.

Material and Methods

Chemicals: All solvents were of p.a.quality unless otherwise stated. The brominated diphenyl ether standards: 2,2′,4, 4′-tetraBDE (BDE-47), 2,2′4,4′,5-pentaBDE (BDE-99) and 2,2′,3,4,4′-pentaBDE (BDE-100) were synthesized as described elsewhere[12,13,14]. Diazomethane was synthesised as described by Fieser and Fieser (1968).

Instruments: The HPLC system consisted of a Hitachi L-6200 pump, a Rheodyne 7125 injector equipped with a 500 µl loop, and a variable UV detector. Separation was performed on two PL gel (5µm, 50Å, 7.5 mm i.d..) columns coupled in series (Polymer Laboratories, Shropshire, UK9). n-Hexane/dichlormetane (70:30, v/v) was used as the mobile phase at a flow of 1.0 ml/min.

Gas chromatography (GC) was performed on a Varian 3400 GC with a Varian 8200 autosampler and an electron capture detector (ECD), and split-splitless injector operate in the splitless mode. The column used was a DB-5 (J&W, Scientific, Folson, CA, USA) 30m, 0.25 mm i.d., and 0.25um film thickness. Hydrogen was used as carrier.

The gas chromatography-mass spectrometry (GC-MS) analysis was performed on a Finnigan TSQ 700, provide with a Varian 3400 gas chromatograph and a fused-silica capillary column (DB-5, 30m, 0.25, µm, 0.25 mm i.d. J&W, Scientific, Folson, CA, USA). The carrier gas was helium.

Samples: In Sweden smolts are reared in hatcheries to compensate for the losses of river production of salmons through damming for power production. Fishes were cached to be stripped on eggs for breeding smolts in the hatcheries. The samples in this investigations (blood, muscle and eggs) were taken from salmon cached in a hatchery, located in the lower part of river Dalälven 150 km north of Stockholm. The salmon spanning in this river lives and feed in the Baltic Sea. Samples were taken from 30 females fishes for OHS analysis (Table 1).

Extraction and clean-up and analysis

Blood samples: A new method for extraction and clean-up of neutral and phenolic compounds in blood plasma has been developed. The project is a collaborative effort between several projects and a detailed description of the extraction method are given elsewhere[15]. The method can briefly be decried as; Plasma samples (5g) were denatured by HCl and 2-propanol, followed by extractions of the orgnohalogens with hexane:methyl *tert*-butyl ether. The lipid amount were determined gravimetrically and the sample were resolve in hexane and partitioned with KOH (0.5M in 50% ethanol). The phenolic compounds were extracted after acidification (HCl, 2M) and derivatised with diazomthane. The residual lipids were removed in both neutral and phenolic fractions by high-performance gel permeation chromatography (HR-GPC) followed by a silica gel column.

Muscle samples; The samples were extracted according to Jensen et al. (1983). Lipids were removed by gel permeation chromatography (GPC), followed by partition with KOH, for isolation of the phenolic compounds. The neutral fraction was further cleaned-up on a silica gel column (0.5g). Followed by a separation by high performance liquid chromatography using Nuclesil-NO_2, (unpublished).

Results and Discussion

Mean concentrations, SD and range of the OHS analysed in muscle from individual salmons, with (n=15) and without (n=12) symptoms of M74 are given in Table 1. p,p´-DDE is still the dominating contaminant in the salmon, with a concentration 3 times the most abundant PCB congeners (CB-138, CB-153). Also, it is interesting to note that BDE-47 is present in the salmon muscle in the same range as HCB. BDE-47 is present in approximately 3-4 times higher concentration than BDE-99 and BDE-100. Even though PCP is not likely to accumulate in lipids this compound is also found in the muscle samples but only at low concentrations (c.f. Table 1). No difference in the PCB, p,p-DDE and PBDE concentrations determined can be seen between salmons suffering from M74 and healthy fish.

Table 1. Concentrations of OHS in Salmon muscle tissue:

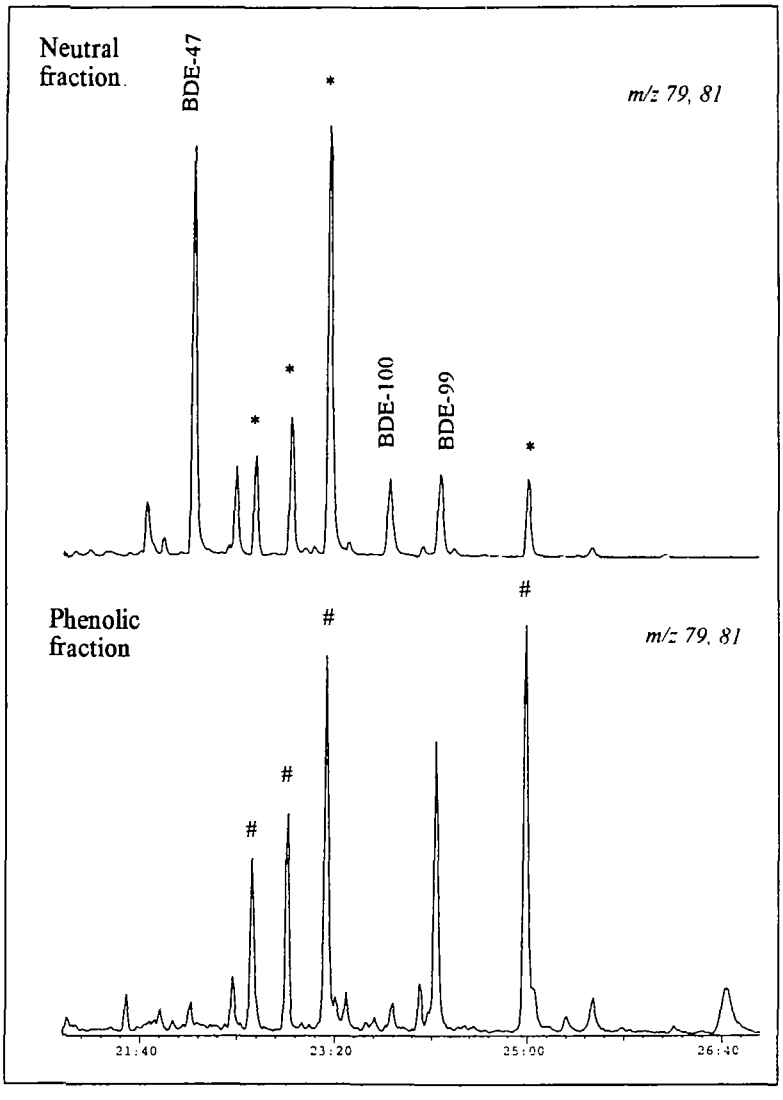
	Legnth	Weight	Lipid	PCP	HCB	p,p-DDE	sPCB*	CB-153	CB-138	BDE-47	BDE-100	BDE-99
Healthy	(cm)	(kg)	(%)	ng/g lip	ng/g lip	ng/g lip	ng/g lip	ng/g lip	ng/g lip	ng/g lip	ng/g lip	ng/g lip
Average	89	6,4	5%	33	97	3300	4100	1200	1200	200	48	56
Std dev	10	2,4	1%	15	22	730	830	270	240	98	13	16
MIN	76	3,5	2%	16	62	2400	2700	710	770	100	34	26
MAX	106	10,3	6%	64	130	4700	5400	1600	1500	410	83	73
M-74												
Average	86	5,3	5%	38	88	3100	3700	1000	1000	190	46	50
Std dev	8	1,6	1%	12	21	600	690	220	200	65	9	14
MIN	71	2,9	2%	21	67	2300	2500	650	700	110	30	22
MAX	100	8,5	7%	55	130	4100	4800	1400	1400	300	63	78

* sPCB: Sum of seven.

In addition, the salmon muscle samples were found to contain compounds with NICI mass spectra (M^- at *m/z* 512 with a isotope cluster corresponding to 4 bromine atoms, and an abundant fragment corresponding to a loss of Br^-) indicating the presence of methoxylated PBDEs (MeO-PBDEs) in salmon muscle. According to MS data three MeO-tetraBDE and one MeO-pentaBDE was present in the muscle tissue. The corresponding compounds were recently unambiguously identified in several biological samples, including salmon, by Haglund and co-workers[16].

The same OHS detected in salmon muscle were detected in their plasma, including also the MeO-PBDEs (Figure 1, upper chromatogram). However, plasma is shown to contain a large number of phenolic compounds, dominated by high concentrations of PCP. Several hydroxylated PCB congeners are also present in the plasma. This is the first time that OH-PCBs have been detected in a fish species while it is well-known that OH-PCBs are retained in mammalian, including human and bird plasma[10,11]. To our knowledge, there are no previous reports on the occurrence of hydroxylated PBDEs (OH-PBDEs) in fish. In fact, three OH-tetraBDEs and one OH-pentaBDE were detected in the salmon plasma (Figure 1, lower chromatogram), that after methylation correspond (GC, and GC/MS) to the MeO-PBDEs present in the same sample and in muscle tissue. The presence of the OH-PBDEs in the plasma was confirmed by analysis of the underivatized compound (GC/MS).

Figure 1: Mass fragmentograms (NICI, CH_4)
Bromide ions (*m/z* 79, 81) detected in Salmon blood plasma; neutral fraction (upper chromatogram) and phenolic fraction (lower chromatogram). The peaks indicating MeO-PBDEs are marked in the chromatogram with an asterisk (*) and with (#) for OH-PBDEs after methylation.



The origin of the OH-PBDEs and MeO-PBDEs is at present not clarified. It is according to our study it is not likely that MeO-PBDEs are demethylated during the clean-up of the plasma samples, but this issue may need to be further addressed. The strong retention of other phenolic compounds in the plasma may be taken as a support that also OH-PBDEs may be retained in the plasma.

The origin of OH- and MeO-PBDEs may be due to the metabolism of PBDE, a hypothesis that is supported by the fact that three OH- and MeO-substituted tetraBDEs and one pentaBDE were detected. Their precursors may be BDE-47, a tetraBDE, and BDE-99 and BDE-100, two pentaBDEs, are present in the salmon, available for metabolism in the fish. It can not be ruled out that MeO-PBDE and OH-PBDE are formed through microbial methylation/demethylation, respectively. It cannot be excluded however that the OH-PBDEs and/or the MeO-PBDEs are produced commercially, even though it seems to be less likely.

It must be pointed out that the presence of phenolic type compounds, some of which in high concentrations, in the salmon plasma must be considered as a potential risk for the salmon. Several of the compounds detected in the salmon plasma (OH-PCB, PCP, OH-PBDE) are known to compete with T_4 for the transport protein TTR[5] but very little is known about the situation in fish. Further, even though indications of health related effects may be seen for phenolic anthropogenic

compounds no conclusive evidence of thyroidogenic effects have yet been published. Further work in relation to sex hormone effects must be initiated.

In conclusion, so far no correlation's between salmon producing M74 fryes and traditional neutral OHS, have been determined. Several, hitherto unknown OHS have been detected in salmon. The toxicological implications of these new OHS are still unknown.

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