DEBROMINATED AND HYDROXYLATED METABOLITES OF INDIVIDUAL POLYBROMINATED DIPHENYL ETHERS (PBDEs) IN JUVENILE COMMON SOLE (*SOLEA SOLEA*)

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Abstract
The uptake, elimination and transformation of six PBDE congeners (BDE-28, -47, -99, -100, -153, -209) were studied in juvenile common sole (*Solea solea*) exposed to spiked contaminated food during three months followed by a five-month depuration period. Fish contamination by individual PBDEs was followed in muscle and liver, while methoxylated (MeO-) and hydroxylated (HO-) PBDEs were determined in plasma.
Several PBDE congeners that were not present in the spiked food, nor in control fish, were identified in the tissues of fish fed contaminated food, revealing transformation of PBDEs; mainly via debromination. The main debrominated congeners were BDE-49, BDE-154, and a penta-BDE tentatively identified as BDE-119 and reported here for the first time. MeO- and HO- congeners were also identified in fish plasma. While all MeO- and also some HO- congeners probably originate from natural sources, two HO- congeners, i.e. 4'-OH-BDE-49 and 4'-OH-BDE-101, were thought to derive from metabolism. 4'-OH-BDE-49 has been previously reported as BDE-47 metabolite. 4'-OH-BDE-101, identified here for the first time, may be the result of BDE-99 metabolic transformation. The results show that PBDEs undergo metabolisation in juvenile soles, either via debromination and formation of HO- metabolites.

Introduction
PBDE behaviour in fish has been the focus of several studies because these compounds may exert toxic effects such as endocrine disruption and alteration of thyroid hormone homeostasis, and neurotoxicity[1]. Studies dealing with fish artificially exposed to PBDEs via food have shown that the fate of individual congeners is highly dependant upon the studied species. For example, major differences in assimilation and transformation abilities have been reported for different fish species: while juvenile carp (*Cyprinus carpio*) did not show any accumulation of BDE-99 nor any formation of hydroxylated (HO-) metabolites following dietary exposure[2], juvenile trout (*Salvelinus namaycush*)[3] and pike (*Esox lucius*)[4] accumulated this congener. HO-PBDEs are likely to accumulate in organisms because of their high log Kow, in the 4.5 – 10.7 range[5]. Data about the occurrence of HO-PBDEs and methoxylated-PBDEs (MeO-PBDEs) exist in the literature, although data about the unequivocal identification of HO-PBDEs from metabolism in fish are still scarce. Their origins may result from natural sources (MeO-PBDEs, and some HO-PBDEs) or from in vivo metabolism (HO-PBDEs)[6,7,8]. Owing to their similarity to the thyroxine (T4), HO-PBDE effects on thyroid hormone homeostasis has been previously reported[5].
The objectives of this study were to determine accumulation and elimination kinetics, and the transformation of selected PBDEs in *Solea solea* exposed to spiked food under experimental conditions. Emphasis was placed upon the formation of the debrominated, MeO- and HO- congeners. This research is part of a larger project on the fate and effects of selected organic contaminants (PCBs, PBDEs, PAHs) in juvenile soles.

Materials and methods
Experimental design
Juvenile soles (*Solea solea*) (G0, n = 470), obtained from a hatchery (Solea BV, Ijmuiden, Netherlands), were exposed to artificially contaminated food (congeners BDE-28, BDE-47, BDE-99 and BDE-100 at 100 ng g^{-1} dw, and BDE-153 and BDE-209 at 200 ng g^{-1} dw) for 84 days, followed by 150 days of non-spiked food. In parallel, control fish (n = 474) were maintained in a separate tank and fed non spiked food over the whole period. PBDE concentrations in non-spiked food were between 16 +/- 1 pg g^{-1} dw (BDE-153) and 394 +/- 17 pg g^{-1} dw (BDE-47). Food lipid content was determined on replicate samples at a level of 13.1 +/- 0.15 %.
Fish were contained in separate 400 L tanks (initial fish density: 120.sqm^{-1}) receiving a continuous flow of sea water. A photoperiod of 12 h light / 12 h dark, optimum water temperature (20°C +/- 1°C) and oxygen rate (>

80% saturation) were maintained. During the experiment, fish mortality was < 2% of the initial population, and food utilisation and growth rates were similar in both the PBDE and control tanks.
Fish were sampled from the tank receiving contaminated food on days 0, 8, 14, 28, 56, 84 (contamination period), and on days 91, 98, 112, 140, 168 and 233 (decontamination period). Fish from the control tank were sampled on days 0, 14, 84, 91, 140, 168 and 233. Between 12 and 33 fish were sampled at each time in each tank and anaesthetized with phenoxy-ethanol before further handling. Individual fish size and weight was recorded. Fish blood was collected from cardiac muscle, placed into tubes containing heparin as the anticoagulant, immediately centrifugated at high speed (12000 rpm) during 5 minutes, with the resultant plasma stored at -80°C until further treatment. Fish muscle and liver were dissected in a clean laboratory maintained under positive pressure. Samples were made from pooled individuals of n = 8 to 20 individuals depending on the fish size, and replicate pools were generally processed (n = 2 to 3). Animal care guidelines were applied throughout the experiment by well-trained-staff.

Analysis
Analyses of PBDEs in fish tissues were conducted on freeze-dried samples. The detailed analytical protocols for extraction and cleanup have been described previously[9]. PBDEs were analysed using a Gas Chromatograph (Agilent 6890) coupled to a Mass Spectrometer (5973N) operated in negative chemical ionisation mode using methane as the reagent gas. Results were systematically calculated on two capillary columns: a DB-5-MS (40 m x 0.18 mm x 0.18 µm) and a DB-1 (15 m x 0.25 mm x 0.10 µm), both fitted with a 1–2 m deactivated non-polar retention gap column. BDE-209 was quantified on the DB-1 column only. BDE-139 and ^{13}C BDE-209 were used as recovery surrogates. BDE-190 was used as a syringe standard for all PBDEs.
Fish plasma samples were analysed for MeO-PBDEs and HO-PBDEs using a protocol adapted from [10]. Plasma samples, mixed with acidified sodium sulphate, were extracted using cyclohexane:acetone 3:1 v/v, purified by Gel Permeation Chromatography, and fractionated on Florisil columns. The HO- fraction was derivatized using diazomethane in ether. Both the MeO and HO- fractions were purified on acidified silica column and analysed by High Resolution Gas Chromatography - High Resolution Mass Spectrometry with an AutoSpec Ultima (Micromass, Manchester, UK) operated in electronic impact ionisation mode at a minimum resolution of 10,000 in the selected ion monitoring mode. The GC was equipped with a DB-1MS capillary column (30 m x 0.25 mm x 0.1 µm), which allowed good separation of MeO- derivatives within 20 minute runs. This column was used to quantify the samples, while a longer column (Rtx 5 – MS, 60 m x 0.25 mm x 0.1 µm) was also used to confirm identification of the compounds. The calibration standards (Wellington Laboratories, Canada) used to identify and quantify the MeO-PBDEs contained the eight native 5-MeO-BDE-47, 6-MeO-BDE-47, 4'-MeO-BDE-49, 2'-MeO-BDE-68, 5'-MeO-BDE-99, 5'-MeO-BDE-100, 4'-MeO-BDE-101, 4'-MeO-BDE-103, and the two ^{13}C labelled 6-MeO-BDE-47 and 6'-MeO-BDE-100 used as internal standards. The native HO- congeners used for HO-PBDEs quantification were obtained from individual solutions (AccuStandard Inc., USA), i.e. 4'-OH-BDE-17, 4-OH-BDE-42, 3-OH-BDE-47, 5-OH-BDE-47, 6-OH-BDE-47, 4'-OH-BDE-49, 2'-OH-BDE-68, 6'-OH-BDE-85, 4'-OH-BDE-90, 6'-OH-BDE-99, and the two ^{13}C labelled 6-OH-BDE-47 and 6'-OH-BDE-100 (Wellington Laboratories, Canada) were used as internal standards.

Results and discussion
PBDEs
Linear accumulation kinetics were observed for PBDEs in fish tissues during the contamination phase followed by an exponential decrease during the depuration phase. Some PBDE congeners, not given in food nor present in control fish, were identified in fish exposed to contaminated food during the whole studied period, with similar increasing and decreasing profiles over time as the congeners given in food. Among them, BDE-49 (2,5,2',4') and BDE-154 (2,4,6,2',4',5'); potentially originating from BDE-99 (2,4,5,2',4') and BDE-209 respectively. BDE-119 (2,4,6,3',4') was also identified, and may result from BDE-154 debromination[11]. While BDE-49 was present at the same levels as BDE-99 in fish during the contamination phase, BDE-119 levels were about 10 times higher than those of BDE-154 (figure 1). These results indicate different transformation kinetics depending on the congeners. To our knowledge, BDE-119 is identified here for the fist time as a debrominated product of BDE-154 in fish maintained under experimental exposure.

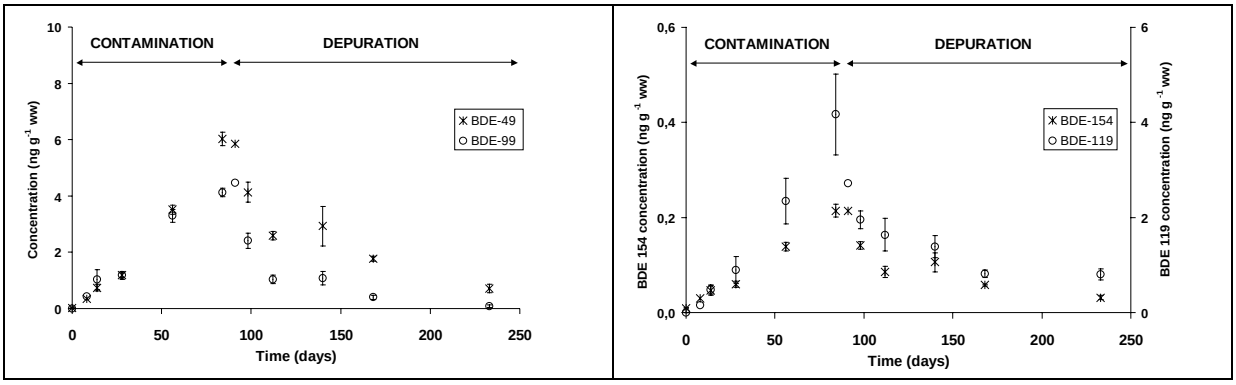

Figure 1: Accumulation and depuration kinetics of PBDEs in juvenile soles exposed to contaminated food during a 3-month contamination period followed by a 5-month depuration time. Concentrations are expressed in ng g^{-1} ww in whole fish body and corrected for fish growth

MeO-PBDEs

Among the eight MeO-PBDEs which were looked for, three congeners were identified in the fish plasma samples. The most abundant one was 6-MeO-BDE-47, followed by 2'-MeO-BDE-68 and 5'-MeO- BDE-100. Although the first two compounds have already been identified in marine biota such as sponge, green alga and fish[12,13], to our knowledge, 5'-MeO-BDE-100 has not been reported before. This latter congener has the MeO-group in the *meta* position, which contrasts with the general findings about the substitution of MeO-PBDEs, with the MeO- group commonly found in the *ortho* position. The three congeners were quantified at levels between 116-270 pg g^{-1} ww, 58-158 pg g^{-1} ww and 5-12 pg g^{-1} ww respectively (average values calculated on replicate fish pools). The levels showed relatively high variation in between pools (rsd between 1 % and 39 %), and did not show any tendency over the studied period. The three congeners were also present in plasma of the control fish, at the same concentration ranges as in fish exposed to contaminated food. This supports the idea that the three congeners originate from natural sources and not from PBDE metabolism. Indeed, although maintained in a controlled environment, fish were directly exposed to natural sea water throughout the whole experiment, the tanks being supplied by a continuous flow of sea water. Thus, MeO-PBDEs in fish could result from accumulation from the water. However, some authors have suggested that certain MeO-PBDEs could originate from their HO-PBDE homologues[8].

HO-PBDEs

Several HO-PBDEs were identified in the fish plasma samples: 6-OH-BDE-47, 4'-OH-BDE-49, 4'-OH-BDE-101 and 4'-OH-BDE-103. Two additional penta-OH-BDEs were also identified, but could not be compared to any of the standards which were looked for. While 6-OH-BDE-47 and 4'-OH-BDE-103 were detected in the plasma of control fish at levels in the same range as of those determined in exposed fish, the other HO-congeners were much higher in exposed fish (as illustrated for 4'-OH-BDE 49 in figure 2). The two main HO-congeners, 6-OH-BDE-47 and 4'-OH-BDE-49 were detected at concentrations between 6 and 70 pg g^{-1} ww and 3 to 30 pg g^{-1} ww respectively. The other congeners were in the 0.3-1.8 pg g^{-1} ww range.

4'-OH-BDE-49 and 4'-OH-BDE-101 present their HO- groups in the *para* position, which suggests that they originate from PBDE metabolism[7,8]. 4'-OH-BDE-49 may be derived from BDE-47 via CYP enzyme-mediated metabolism[8], and could also be derived from BDE-49[7], which was present in both fish tissues and blood. The shift in the bromine position between BDE-47 (2,4,2',4') and 4'-OH-BDE-49 (2,4,2',5') is explained by the 1,2 shift of the *para*-bromine atom during the formation of HO-metabolites[14]. The same process could explain the formation of 4'-OH-BDE-101 (2,4,5,2',5') from BDE-99 (2,4,5,2',4'). The two other penta-OH congeners are also thought to be from metabolic origin, as they were not identified in the control fish. Considering that both BDE-47 and BDE-49 are metabolised into 4'-OH-BDE-49, ratios 4'-OH-BDE-49 / (BDE-47 + BDE-49) calculated in fish plasma were between 0.07 % and 0.34 %. These values are consistent with other data available in the literature[8] and show that HO-PBDEs do not represent major residues in fish exposed to PBDEs.

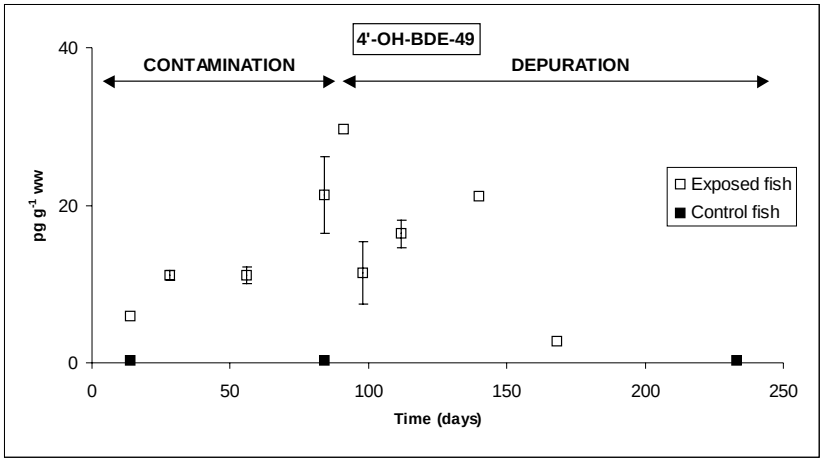

Figure 2: 4'-OH-BDE-49 accumulation and elimination in fish plasma during a 3-month dietary exposure by PBDEs followed by a 5-month depuration period. Concentrations are expressed in pg g^{-1} ww.

Conversely, 6-OH-BDE-47 and 4'-OH-BDE-103 were detected at similar levels in exposed fish and in control fish, ruling out their main origin from metabolic processes. 6-OH-BDE-47 has been found in red algae from the Baltic as well as in different marine species[13], supporting its natural origin. This HO- BDE also had its corresponding MeO- analogue in fish plasma samples. 4'-OH-BDE-103 has not been reported before. Its HO- substitution in the *para* position is not common, as all OH-PBDEs from natural origin reported before have their HO- group in the *ortho* position[13]. *Para* HO- metabolites like those found in fish plasma are reported to be the most potent in terms of thyroxine-like activity and/or estrogen-like activities[14]. However, their concentrations in the fish plasma were very low, in the pg g^{-1} ww range.

Acknowledgements
This research was funded by the "Agence Nationale pour la Recherche" under the "Vulnérabilité Milieux et Climat" frame. The authors thank the technical staff for assistance in experimental work and fish maintenance. Mehran Alaee, Grazina Pacepavicius and Colin Darling from Environment Canada are gratefully acknowledged for guidance in the development of PBDE metabolite analysis in fish plasma.

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