Effects of decontaminated fish oil or a fish and vegetable oil blend on persistent organic pollutant and fatty acid compositions in diet and flesh of Atlantic salmon (*Salmo salar*).

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Abbreviations: ASE™, accelerated solvent extractor; cNFO, control northern fish oil; deNFO, decontaminated northern fish oil; DL-PCBs, dioxin-like polychlorinated biphenyls; FCR, feed conversion ratio; HUFA, highly unsaturated fatty acid; PBDEs, polybrominated diphenyl ethers; POPs, persistent organic pollutants; toxic equivalency factors, TEF; SFO/RO/SO, southern fish oil, rapeseed oil, soybean oil; SGR, specific growth rate; toxic equivalents, TEQ; TGC, thermal growth coefficient; World Health Organisation, WHO;

Abstract

The health benefits of seafood are well documented and based on the unique supply of n-3 highly unsaturated fatty acids (HUFA). Aquaculture now contributes ~50% of food-grade seafood globally and Atlantic salmon is a rich source of *n*-3 HUFA. However, salmon and other oily fish can accumulate lipophilic persistent organic pollutants (POPs), including dioxins (PCDD/Fs), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs), derived largely from feed. In this study, triplicate groups of salmon, of initial weight 0.78 kg were fed one of three experimental diets for 11 weeks. The diets were coated with either a northern fish oil (FO) with a high POPs content (cNFO), the same oil that had been decontaminated (deNFO) or a blend of southern fish oil, rapeseed and soybean oils (SFO/RO/SO). Dietary PCDD/F + dioxin-like PCB (DL-PCB) concentrations were 17.36, 0.45 and 0.53 ng TEQ/kg, respectively. After 11 weeks, the flesh concentrations in fish fed the cNFO, deNFO and SFO/RO/SO diets were 6.42, 0.34 and 0.41 ng TEQ/kg, respectively. There were no differences in flesh eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) between fish fed the cNFO or deNFO diets although EPA and DHA were reduced by 50 and 30%, respectively, in fish fed the SFO/RO/SO diet. Thus, decontaminated FO can be used to produce salmon high in n-3 HUFA and low in POPs. Salmon produced using deNFO would be of high nutritional value and very low in POPs and would utilise valuable fish oils that would otherwise be destroyed due to their high pollutant concentrations.

Keywords: fatty acid composition, Atlantic salmon, dioxins, PCBs, decontaminated fish oil

In the 1970s, studies on Greenland Inuit populations established that, despite consuming high lipid diets, cardiac disease was virtually absent in these populations and that this was due to their high fish intake[1]. Subsequently, the beneficial effects of *n*-3 highly unsaturated fatty acids (HUFA), especially eicosapentaenoic (20:5*n*-3; EPA) and docosahexaenoic acids (22:6*n*-3; DHA) have been shown to have cardioprotective activity[2,3]. More recently, the benefits of increased EPA and DHA intake have been shown for a wide range of lifestyle disorders with an associated inflammatory pathology[4,5] as well as improving the symptoms of certain neurological disorders[6]. Due to the proven benefits of *n*-3 HUFA, numerous governmental and non-governmental organisations across the world currently advise increased fish intake as a means of improving the health of their citizens[7,8].

Atlantic salmon can store large quantities of oil in their flesh and this makes them a rich source of *n*-3 HUFA[9,10]. However, the oil-rich flesh can also accumulate lipophilic undesirable compounds, including polychlorinated dibenzo-*p*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDF) (collectively known as dioxins or PCDD/F), dioxin-like non-*ortho* and mono-*ortho* polychlorinated biphenyls (PCBs), known collectively as dioxin-like (DL) PCBs and polybrominated diphenyl ethers (PBDEs), among others[11,12,13,14,15]. For these reasons there is currently considerable interest to investigate the transfer of persistent organic pollutants (POPs) from feed to farmed fish as well as assessing the potential for transfer to humans and any consequences for human health[16,17].

There are 210 dioxin (75 PCDD & 135 PCDF congeners) and 209 PCB congeners of which 17 PCDD/PCDF congeners and 12 DL-PCBs have been assigned toxic equivalency factors (TEFs) by the World Health Organisation (WHO), according to their relative toxicity to 2,3,7,8 tetra chlorinated dibenzo dioxin (TCDD). The concentration of PCDD/F and DL-PCB is expressed as toxic equivalents (TEQ), where a toxic equivalency factor (TEF) is applied to the concentration of the individual congeners and summed to generate the total TEQ in a mixture of the 29 PCDD/F and DL-PCB congeners. The TEFs established in 1997 by the World Health Organisation have been most widely used for human risk assessment by government bodies[18], these TEFs were re-evaluated in 2005[19]. Application of these factors to individual PCDD/F and DL-PCB congener concentrations in a feed or fish can be used to determine the level of toxic equivalents (TEQs) present. PCDD/Fs and PCBs have originated either by natural means, such as forest fires and incomplete combustion (dioxins), or by industrial activity (PCDD/F and PCBs) and both have half-lives of several decades and are highly persistent in the marine biota[20].

In addition to PCDD/Fs and PCBs, the PBDEs are of more recent origin, having been introduced as flame retardants in household furnishings, computers and electrical circuits since the 1970s[12]. As with PCDD/Fs and PCBs, PBDEs are lipophilic and accumulate in the aquatic biota and humans[12,21] although at the present time no TEQs have been assigned for any PBDE congeners. Although there are 209 possible PBDE congeners, only a few are present in commercial mixtures and those are represented in tissues by 7 principal congeners namely PBDE 28, 47, 99, 100, 153, 154 and 183[21].

Within the European Union, limit values for PCDD/Fs were established in 2001, which were revised in 2006 to include concentrations of all 29 PCDD/Fs and DL-PCB congeners assigned WHO TEFs[22,23]. These limit values cover raw materials, feeds and fish products. The current EU limit values are 24 ng, 4.5 ng, 7 ng and 8 ng TEQ/kg, for fish oil, fish meal, fish feed and fish products, respectively. The main contributor to POPs in fish feeds and fish is fish oil (FO) derived from pelagic marine fish[24]. In recent years a number of studies have been conducted where FO was replaced by terrestrial vegetable oils (VO) in salmon feeds[9,10,14,15]. The fish produced using diets with a high VO content contained significantly lower levels of POPs compared to fish grown on diets with a high marine FO content[14,25]. However, the reduction in POPs was accompanied by reductions in EPA and DHA of 50-65%, which would obviously reduce the health benefits of this product to the consumer[14,15]. With the introduction of the revised EU limits for PCDD/Fs and DL-PCBs in 2006, some of the FOs previously used in aquafeeds would no longer comply with these new limits and so would have to be removed from the food chain. The loss of valuable *n*-3 HUFA in this way, at a time when FO have reached their sustainable production limits and prices are rising rapidly, is a major dilemma and has increased the desire to develop technology to clean FO of POPs so that they might be used in aquafeeds[26,27].

The aim of the present study was to investigate the effects on tissue POPs concentrations of replacing northern FO, containing high levels of POPs, with either the same oil which had been cleaned using a decontamination protocol or a blend of southern FO, soya and rapeseed oils (4:3:3 by volume). The 3 diets were fed to triplicate groups of Atlantic salmon in sea cages for a period of 11 weeks. The effects of these 3 treatments on PCDD/F, DL-PCB, PBDE and *n*-3 HUFA concentrations in fish feed and flesh are presented.

Materials and methods

Fish, husbandry and diets

Three 9 mm extruded diets were prepared with the same basal composition, but were top coated with 3 different oils and were prepared at the BioMar Tech Centre, Brande, Denmark (Table 1). The diets were formulated to satisfy the nutritional requirements of salmonid fish,[28] and contained 33% protein and 34% lipid. The three diets contained either a) 100% northern FO (cNFO) as control, b) 100% decontaminated northern FO (deNFO), and c) a blend of southern FO and rapeseed and soya oil in a ratio of 40/30/30; (SFO/RO/SO). The deNFO was the same product and batch as the cNFO following the decontamination process. The cNFO was selected to contain high levels of POPs and this oil was subjected to a two-stage decontamination process (FF Skagen, Denmark) that involved an initial adsorption using activated carbon that was designed to remove ~90% of the PCDD/Fs. The second step was a thin-film deodourisation step that should remove up to 95% of PCBs as well as pesticides and other contaminants, free fatty acids and peroxides. The deNFO oil was representative of decontaminated oil currently commercially available in Europe. Atlantic salmon of initial mean weight 0.78 ± 0.01 kg were fed one of the three experimental diets for 11 weeks in 9 x 5 m^3 net pens containing 120 fish/pen at the Fjord Research Station, Dønna, Norway. The experiment was conducted between July and October 2006 under ambient photoperiod and the mean seawater temperature was 11.5 ± 2.7 ^oC and salinity 31.9 ± 0.8 ppt. Feed was supplied manually to apparent satiation with waste feed collection provided by an uplift system. Experimental procedures complied with the Norwegian code of practice for the care and use of animals for scientific purposes and there are no aspects of this trial that would cause aggravated or unnecessary harm or stress to the fish involved.

Sample collection

Samples of the three feeds were collected at the start of the trial and wrapped in foil before placing in sealable polythene bags and storing at -20^oC. Samples of fish were collected at the start and end of the trial with 3 fish per pen (9 per dietary treatment) anaesthetised by metacaine (50 mg/L) and then killed by a blow to the head. Samples of flesh from the Norwegian Quality Cut (NQC) region were collected, wrapped in foil and immediately frozen at -20^oC and transported to the laboratory where they were stored at -70^oC until analysed. The samples were selected from fish that were close to the average fish weight in each pen. The three NQC samples from each pen were then pooled, to provide three samples per treatment group. Before analysis, the pooled steaks were homogenised in a food processor after removal of skin and bones. Samples of these homogenates were used for POPs and fatty acid analyses.

Analysis of PCDD/Fs, DL-PCBs and PBDEs

Homogenised samples of wet flesh (~50g) were freeze dried for minimum 12 h before extraction. Samples of diet (~10g) were weighed as above and ground in a coffee grinder prior to extraction. The entire flesh and diet samples were extracted using isohexane in an accelerated solvent extractor (ASE™, Dionex, Camberley, UK). Each sample was accurately weighed, mixed with hydromatrix (Varian Inc., USA) before addition of 1ml of a 2 $ng.ml^{-1}$ ^{13}C-labelled PCDD/F and PCB internal standard (EPA-1613-LCS; Wellington Laboratories, Guelph, Cananda). Samples were extracted by ASE™ for 20 minutes x 2 cycles with isohexane under pressure (1500 psi) and temperature (125^{o}C). Fat and organic matter were removed from the 120 ml ASE™ extract by sulphuric acid treatment. Fifty ml of 95-97% sulphuric acid was added to the lipid extract in a separation funnel and left for 72h. The sulphuric acid was drained off and the organic phase washed with 50 ml of nanopure water (Millipore UK Ltd., UK) and left for 3 h.

Clean-up of the extracted lipid samples was then performed using the automated multi-column Power-Prep™ System (Fluid Management Systems, Waltham, MA, USA) using a series of disposable teflon columns of multi-layered silica (4 g acid, 2 g base and 1.5 g neutral), basic alumina (8 g) and carbon (2 g). For high lipid samples, high capacity silica columns (28 g acidic, 16 g basic and 6 g neutral) were used. The total run time was 150 min followed by a 40 min preventative decontamination programme. The mono-ortho PCBs were collected in 120 ml isohexane-dichloromethane (1:1 v/v) and the PCDD/F and non-ortho PCBs eluted in 120 ml toluene. The purified fractions were evaporated to ~5 ml by rotary evaporation (Rotavapor® R-200; Büchi Ltd, Oldham, UK) in a heated water bath set at 25^{o}C and 320 mbar pressure for mono-ortho PCBs and 40^{o}C and 70 mbar pressure for PCDD/F and non-ortho PCBs. The mono-ortho PCB fraction was evaporated to dryness under nitrogen (N-Evap™111; Organomation Associates Inc., Massachusetts, USA) at room temperature before 5 ml isohexane and 5 ml concentrated sulphuric acid was added, vortex mixed, and centrifuged (5 min, 478 *g*). The organic phase was transferred to a clean conical tube and evaporated to approximately 1 ml under nitrogen before addition of 1 ml isooctane and further evaporation to 500 µl. The sample was finally transferred to a conical GC vial containing 150 µl of nonane as the keeper and evaporated to 100 µl prior to analysis by GC/MS/MS.

The PCDD/F and non-ortho PCB fraction was evaporated to 500 µl under nitrogen and transferred to a conical GC vial containing 60 µl of nonane before further evaporation to 10 µl where 400 µl of isohexane and 10 µl of recovery standard (EPA-1613-ISS for PCDD/F and

68A-IS for PCBs; Wellington Laboratories, Guelph, Canada) was added and vortex mixed. Thirty-five µl of sulphuric acid was added to the vial, vortex mixed and left for 1 hour before centrifugation (5 min, 1328 *g*). The organic phase was transferred to a clean conical GC autosampler vial containing 10 µl of nonane as keeper and evaporated to 10 µl or 50 µl level prior to analysis by GCMS for PCDD/F or non-ortho PCB analysis respectively.

Analysis of the 29 PCDD/F and DL-PCB congeners with WHO TEFs was conducted using GC/MS/MS on a Trace GC 2000 coupled to a Polaris Q ion trap MS/MS (Thermo Finnegan, Hemel Hempsted, England). The chromatographic separations were conducted on a Rxi-5ms (5% phenyl/95% dimethyl polysiloxane) fused silica column (Thames Restek Ltd., Saunderton, England) 30 m x 0.25 mm i.d. x 0.25 mm film thickness with helium as carrier gas at 0.8 ml/min. Injector temperature was kept at 250^{o}C and samples and standards were injected in the splitless mode. MS conditions were in positive electron ionisation mode (EI+) using automatic gain control with electron energy of 70eV and emission current of 250 µA. The transfer line and ion source were kept at 305 and 250^{o}C respectively. Xcalibar version 1.3 software was used for data acquisition and processing of results. The limit of quantification (LOQ), which was determined by using 9 times the background level (3 x limit of detection; LOD in the blanks of each congener. Procedural blanks were run for each batch of up to ten samples. Each batch of samples were analysed with one procedural blank, one external QC sample and one internal QC sample. The external QC was cod liver oil (Food Analysis Performance Assessment Scheme®, CSL, York, England; T0623) and the internal QC was internally validated salmon flesh. Percentage recoveries were in the range 78-114%. Quantification of each congener is based on the isotope dilution method of the USEPA methods 1613 and 1668[29,30]. The range of LOQs for whole fish sample were 0.03-0.18 pg g^{-1} wet weight for PCDD/Fs, 0.03-0.06 pg g^{-1} wet weight for non-ortho PCBs and 0.03-0.06 pg g^{-1} wet weight for mono-ortho PCBs. The congeners analysed included the 17 PCDD/Fs and 12 DL- PCBs for which the World Health Organization has established toxicity equivalent factors, TEFs from 1997[18] and recently re-evaluated TEFs[19] (Table 2).

Analysis of PBDEs

For PBDE analysis, 25 g of homogenised wet flesh were freeze dried as described above. The dried flesh sample or 10 g of diet were ground by mortar and pestle or coffee grinder, respectively, and 2.5 g of flesh or diet was extracted using the ASE™ extraction cell containing 19 g silica gel-sulphuric acid mixture (1:1 wt/wt) and hydromatrix before addition of 20 µl of a 500 ng.ml^{-1} PBDE-119 internal standard (Wellington Laboratories, Guelph,

236 Canada). Samples were extracted by ASE for 20 minutes x 2 cycles with dichloromethane-
237 isohexane (4:1 v/v) under pressure (1500 psi) and temperature (40^{o}C). The extracts from the
238 ASE™ was reduced to ~5ml by rotary evaporation at 30^{o}C and 320 mbar and then reduced to
239 1ml under nitrogen at room temperature. Five ml of isohexane and 2ml of concentrated
240 sulphuric acid were added, vortex mixed and centrifuged (15 min, 1328*g*). The organic phase
241 was transferred to a clean tube and the procedure repeated. The combined organic phases
242 were reduced to 1ml under nitrogen and 100µl of concentrated sulphuric acid added, mixed
243 and centrifuged (4 min, 212*g*). The isohexane layer was then transferred to a silanized GC
244 autosampler vial containing 500 µl of nonane as the keeper and the solvent evaporated under
245 nitrogen to the nonane mark prior to analysis by GC/MS.
246 Analysis of the seven PBDE congeners (28, 47, 99, 100, 153, 154 & 183) was conducted
247 using a Thermo Finnegan Trace GC Ultra equipped with a ZB5-MS column (30m x 0.25 mm
248 i.d. x 0.25 micron phase; Phenomenex, Macclesfield, England) using helium as carrier gas
249 coupled to a Trace DSQ MS, (Bremen, Germany) in negative chemical ion (CI-) mode and
250 methane as reagent gas at a flow rate of 2.0 ml/min. The mass spectrometer was operated in
251 the selective ion monitoring mode at the *m/z* = 79 and 81. Procedural blanks were the same as
252 described for PCDD/F + DL-PCBs above. The external QC was supplied by the Norwegian
253 Institute of Public Health and the internal QC was internally validated salmon flesh. Xcalibar
254 version 1.4 software was used for data acquisition and processing of results. The range of
255 limits of quantification for whole fish sample were 2-63 pg g^{-1} wet weight for PBDEs.
256
257 *Lipid content and fatty acid compositions*
258 Total lipid was extracted from 1 g of diet or flesh homogenates by homogenising in 20
259 volumes of ice-cold chloroform/methanol (2:1, v/v) using an Ultra-Turrax tissue disrupter
260 (Fisher Scientific, Loughborough, U.K.). The total lipid fraction was prepared according to
261 the method of Folch *et al*[31] with non-lipid impurities removed by washing with 0.88% (w/v)
262 KCl. The lipid weight was determined gravimetrically after evaporation of solvent under
263 nitrogen and desiccation *in vacuo* for at least 16h.
264 Fatty acid methyl esters (FAME) were prepared from diet and flesh total lipid by acid-
265 catalysed transesterification according to the method of Christie[32]. Extraction and
266 purification of FAME was performed as described by Tocher and Harvie[33]. FAME were
267 separated and quantified by GC (Carlo Erba Vega 8160, Milan, Italy) using a 30 m x 0.32 mm
268 i.d. x 0.25 mm film thickness capillary column (CP Wax 52CB, Chrompak, London, U.K.)
269 and cold on-column injection. Hydrogen was used as carrier gas and temperature

programming was from 50°C to 150°C at 40°C min^{-1} and then to 230°C at 2.0°C min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data[33,34]. Data were collected and processed using the Chromcard for Windows (version 2.01) computer package (Thermoquest Italia S.p.A., Milan, Italy).

Statistical analysis

Significance of differences ($P < 0.05$) between dietary treatments was determined by one-way ANOVA. Differences between means were determined by Tukey's test. Data identified as non-homogeneous, using Bartlett's test, were subjected to log or arcsin transformation before applying the ANOVA. ANOVA was performed using a GraphPad Prism (version 4.0) statistical package (GraphPad Software, San Diego, CA, USA.

Results

The fish grew from an initial weight of 0.78 kg to 2.19 kg over the 11 week trial and there were no significant differences in final weights between the three treatments. Specific growth rate (SGR; %/day) values were in the range 1.34-1.35 and Thermal Growth Coefficient (TGC) between 3.86 and 3.92 while the Feed Conversion Ratio (FCR) values were in the range 0.96-0.98. There were no differences between treatments and all these values were comparable with normal commercial practice for salmon of this size and culture conditions.

The cNFO diet contained 17.4 ng TEQ/kg of the 29 WHO PCDD/F and DL-PCB congeners with assigned TEFs (Table 2). This value is significantly higher than the EU limit for fish feeds of 7 ng TEQ/kg[22]. However, following the two-step decontamination of the cNFO oil, this value was reduced to 0.45 ng TEQ/kg in the deNFO diet that was comparable to the value of 0.53 ng TEQ/kg in the SFO/RO/SO blend diet (Table 2). These values represent 6.3 and 7.6% of the EU limit, respectively[22], and indicate that the decontamination process was successful. When comparing the POPs concentrations in the 3 diets, a reduction of PCDD/Fs of 97% was observed for both the deNFO and SFO/RO/SO diets compared to the cNFO diet. Similarly, an overall 98% reduction of both mono-*ortho* and non-*ortho* PCBs was noted compared to the cNFO diet with individual congeners being reduced by 71-99% and 93-99%, respectively (Table 2).

The sum concentrations of the 7 PBDE congeners in diets were 5.9, 1.9 and 0.31 ng/g wet weight, for the cNFO, deNFO and SFO/RO/SO diets, respectively (Table 3). The sum PBDEs were reduced by 68% for the deNFO compared to the cNFO diet, and 95% for the SFO/RO/SO, compared to the cNFO diet. The individual PBDE congeners were reduced by

35-94% in the deNFO diet and by 93-100% in the SFO/RO/SO diet compared to the cNFO diet (Table 3).

In the initial fish at the start of the trial, the sum of flesh PCDD/Fs + DL-PCBs was 1.06 ng TEQ/kg (Table 4). In fish fed the cNFO diet for 11 weeks this value increased significantly to 6.42 while in fish fed the deNFO and SFO/RO/SO diets the concentrations were significantly reduced to 0.34 and 0.41, respectively, compared to the initial fish (Table 4). The flesh concentration for the fish fed cNFO was however below the EU limit value of 8 ng TEQ/kg while the deNFO and SFO/RO/SO flesh represented 4.3 and 5.1% of the EU limit value, respectively[23].

In the initial fish flesh at the start of the trial, the sum concentration of the 7 PBDE congeners was 0.26 ng/g wet weight. After feeding the experimental diets for 11 weeks the flesh value in fish fed the cNFO diet increased significantly to 0.94 ng/g, while fish fed the deNFO diet had similar levels to the initial fish of 0.25 ng/g and fish fed the SFO/RO/SO diet had a significantly reduced concentration of 0.095 ng/g, compared to the initial flesh (Table 5). The sum PBDEs were reduced by 73% for the deNFO compared to the cNFO diet and 90% for the SFO/RO/SO compared to the cNFO diet.

Flesh total lipid content increased from 9.0 ± 0.9% in initial fish to 17.0 ± 1.6% in the final fish. There were no differences between treatments. Flesh fatty acid levels were closely correlated to dietary values and DHA values were similar for fish fed the cNFO and deNFO diets although EPA was significantly higher in the latter (Figure 1). By contrast, the DHA and EPA values in the flesh of fish fed the SFO/RO/SO diet were significantly lower than in the two FO groups, being reduced by 50 and 30%, respectively (Figure 1). At the same time fish fed the SFO/RO/SO diet had increased levels of linoleic (18:2*n*-6 and linolenic acids (18:3n-3) of 3.6 and 2-fold, respectively (Figure 1).

Data presented from this study shows that consumption of 2 x 140g portions of cNFO salmon would represent 171% of the weekly limit for PCDD/Fs + DL-PCBs for girls and women of reproductive age[35] (UK Food Standards Agency (FSA); www.food.gov.uk) (Figure 2) 140g portions are the standardised portion sizes used by the FSA. However, this value assumes that salmon is the only dietary input of PCDD/Fs and DL-PCBs while in practice the 2 x 140g portions would contribute to more than 171% of the weekly limit due to consumption of other food products that also contain POPs, albeit at a lower level than that seen in the cNFO salmon. In the UK, as in most countries in the developed world the largest contribution to weekly POPs intake is derived from dairy products[4]. Consuming 4 x 140g portions would represent 86% of the FSA weekly limit value for boys, men and women over

reproductive age. By comparison, consuming 2 x 140g portions of salmon fed the deNFO or SFO/RO/SO diets represents 9.0 and 10.8%, respectively, of the weekly limit for girls and women of reproductive age while consuming 4 x 140g portions represents 4.5 and 5.4%, respectively of the weekly limit for PCDD/Fs + DL-PCBs for boys, men and women over reproductive age (Figure 2). Over the years many advisories have been issued on guideline intakes for EPA and DHA in humans[4,7,8]. The intake advice of the International Society for the Study of Fatty Acids and Lipids (ISSFAL) is for a daily intake of EPA + DHA of 500 mg or 3.5g/week[8]. Thus, 2 x 140 g portions of salmon fed the cNFO, deNFO and SFO/RO/SO diets provides 4.81 5.14 and 2.83 g EPA + DHA, respectively, while 4 x 140 g portions provides 9.62, 10.29 and 5.67 g EPA + DHA, respectively (Figure 3).

Discussion

The cNFO diet used a sprat oil from the Baltic Sea that was selected to be high in POPs and this same oil was subjected to the two step decontamination process at Skagen FF to produce the deNFO oil. Previously, removal of > 90% of PCDD/F from FO could be achieved using activated carbon treatment alone. However, removal of DL-PCBs was less effective and required the use of high temperature deodourisation that could cause oxidation of HUFA[26,36]. More effective removal of both PCDD/Fs and DL-PCBs can be achieved using activated carbon coupled with short path distillation as has been used in the present study[27]. Other methods for removal of POPs from FO are being developed, including supercritical CO_2 extraction along with activated carbon[37]. However, this methodology is equally efficient but has only been used in relatively small scale extractions at the present time.

The cNFO diet in the present study contained 17.4 ng TEQ/kg of PCDD/Fs + DL-PCBs and, as such, exceeds the EU maximum permitted value of 7.0 ng TEQ/kg for fish feeds[22]. Fish oils are generally considered to be the major source of PCDD/Fs and DL-PCBs in high energy fish diets although a smaller contribution is derived from the fish meal component[14,15,24,39]. Despite there being considerable variation in contaminant concentrations in northern FO, due to season and location of capture, they are generally higher in PCDD/Fs and DL-PCBs compared to those of Pacific origin[40,41]. As a result of high POPs levels, a significant tonnage of FO from the North Atlantic, North and Baltic Seas would currently exceed EU permissible limits for PCDD/Fs + DL-PCBs. Currently, oils such as the cNFO used in the present study could not be used in animal feeds and would be destroyed by high temperature incineration. Given the nutritional value of *n*-3 HUFA as a beneficial nutrients to

attenuate a range of inflammatory disorders in humans[6,42], and the fact that FO prices exceeded $1700/tonne in January 2008, it seems wasteful to dispose of valuable nutrients in this way. The decontamination of the cNFO was very successful with the dietary concentration of PCDD/Fs + DL-PCBs falling by 97% in the deNFO diet, which was similar to the concentration of 0.53 ng TEQ/kg in the SFO/RO/SO diet. This reduction in PCDD/Fs and DL-PCBs, indicates that the efficiency of the decontamination process was consistent with reports in the literature[26,43].

Hites et al.[11] reported PCDD/Fs + DL-PCBs in Scottish farmed salmon feeds in the range 2.5-7 ng TEQ/kg with lowest values ~1 ng TEQ/kg in feed from Canada. Both the deNFO and SFO/RO/SO diet in the present study had lower levels of PCDD/Fs + DL-PCBs compared to the Canadian feeds sampled in 2003/04. Easton et al., (2002) sampled five feeds from North America and found concentrations for DL-PCBs alone in the range 1.73-10.9 ng TEQ/kg. The highest concentration found by Easton et al.,[38] was similar to the DL-PCB concentration in the cNFO diet (8.71) while the concentrations in the deNFO and SFO/RO/SO diets were 83% lower than the lowest diets sampled from North America. This clearly demonstrates the value of using either a decontaminated FO or a FO/VO blend as a means of reducing feed contaminant levels. The contaminant concentrations found in the present study, in the deNFO and SFO/RO/SO diets, are broadly similar to those seen in previous studies where FO has been substituted, partially or completely, with VO[14,15,25]. In a study where salmon were fed diets containing either 17 or 34% oil, which was a 100% replacement of FO with a blend of rapeseed and linseed oils, the PCDD/F + DL-PCB concentrations in the diet were 0.95 and 0.78 ng TEQ/kg, respectively[25]. These values are about double those in the deNFO and SFO/RO/SO diets used in the present study. These differences probably reflect the use of northern fish meal in the study of Bell *et al*.,[25] compared to southern fish meal in the present study. Two similar studies using different levels of FO substitution were conducted by Berntssen *et al*.[13,14] In the first trial, fish were fed 100% northern FO or 100% of a VO blend comprising RO, linseed and palm oils for the whole production cycle. As FO content increased with pellet size the PCDD/F + DL-PCB in ng TEQ/kg feed increased from 2.43 in the 0.3 mm pellet to 4.74 in the 9 mm pellet[14]. In the VO diet, as the VO inclusion increased with pellet size, the PCDD/F + DL-PCB in ng TEQ/kg reduced from 1.07 in the 0.3 mm pellet to 0.61 and 0.33 in the 6 and 9 mm pellets. Thus, the values for PCDD/Fs + DL-PCBs recorded in the present study, for the deNFO and SFO/RO/SO diets of 0.447 and 0.533 ng TEQ/kg, respectively, were broadly similar to the 100% VO diets used by Berntssen *et al*[14]. In a second study where 100% southern FO was replaced with 30% or 60% RO, the diet

406 concentrations of PCDD/Fs + DL-PCBs were 1.18, 0.93 and 0.86 ng TEQ/kg, respectively[13].
407 The value of 0.86 is slightly higher than for the SFO/RO/SO diet of 0.53 ng TEQ/kg, which
408 was also a 60% replacement with VO.
409 The reduction of PBDEs in the deNFO diet, compared to the cNFO diet, was 73%, while
410 the SFO/RO/SO diet had a 90% reduction compared to the cNFO diet, which suggested less
411 efficient removal of PBDEs compared to dioxins and PCBs. However, use of activated carbon
412 alone did not reduce PBDE levels in FO[43] and so the addition of the short-path distillation
413 step resulted in a significant reduction in PBDEs in the deNFO diet. However, the
414 decontamination method used in the present study is less efficient for PBDEs than for
415 PCDD/Fs and DL-PCBs. In four samples of feed from Scotland, Hites *et al*.,[12] reported total
416 PBDE concentrations in the range 2.44-10.9 ng/g wet weight while in 6 samples from Canada
417 the range was 0.49-9.3. By comparison Easton *et al*.,[38] measured two North American feeds
418 which contained 1.87 and 1.90 ng/g. It is noteworthy that the sum PBDEs for 3 of the Scottish
419 diets and 2 of the Canadian diets[12] were higher than the value of 5.94 ng/g observed in the
420 cNFO diet. By comparison, the PBDE concentration in the deNFO diet of 1.93 ng/g is lower
421 than 9 of the feeds from Scotland, Canada and Chile analysed by Hites *et al*.,[12] and similar to
422 the two feeds analysed by Easton *et al*[38]. The sum PBDE in the SFO/RO/SO diet was 0.31
423 ng/g, and was lower than any value reported by either Hites *et al*.,[12] or Easton *et al*[38].
424 In the present study, the PCDD/F and DL-PCB concentrations in flesh were closely
425 correlated with diet concentrations with highest values in fish fed the cNFO diet (6.4 ng
426 TEQ/kg). This value was increased compared to the initial flesh (1.1 ng TEQ/kg) but was
427 much lower than the diet concentration. This was also observed in previous studies with
428 salmon where the accumulation efficiency of PCDD/Fs was shown to be lower than for DL-
429 PCBs[13,14]. In comparison to the fish fed the cNFO diet, the PCDD/F + DL-PCB
430 concentrations in flesh of fish fed the deNFO and SFO/RO/SO diets were decreased
431 compared to the initial values to 0.34 and 0.41, respectively. Although there are no data in the
432 literature on effects of feeding decontaminated oils, values are reported for fish fed varying
433 levels of VO, as well as different sources of FO. In the study by Bell *et al*.,[25] flesh PCDD/F
434 + DL-PCB concentration in salmon fed a similar oil level to the present study, but where the
435 VO was 50% RO and 50% linseed oil, was 0.73 ng TEQ/kg, which was more than double the
436 values seen in the fish fed deNFO and SFO/RO/SO in the present study. Despite having a
437 higher level of VO, this higher value is probably due to the use of northern fish meal in the
438 2005 study. The concentrations in the study by Bell *et al*.,[25] are similar to those found by
439 Berntssen *et al*.,[13] who reported values of 0.95 and 0.86 ng TEQ/kg for fish fed either 30%

or 60% RO, respectively. The sum ng TEQ/kg for fish fed the deNFO and SFO/RO/SO diets, in the present study, were 0.34 and 0.41, respectively. These values are similar to the results reported by Berntssen *et al*.,[14] where salmon were fed a 100% VO oil inclusion for 97 weeks. Although the fish in the present study were harvested at a smaller size than those produced by Berntssen *et al*.,[14] it is likely that the values would decrease even more with continued culture on the deNFO and SFO/RO/SO diets. The present study lasted for only 11 weeks, while the half-life of PCDD/Fs and DL-PCBs is estimated at approximately 5 months[13], and an additional growth dilution would cause a continued lowering of POP loads. Thus, a further decrease in PCB levels, at least, would be expected with continued feeding on deNFO and SFO/RO/SO diets.

In the study of Hites *et al*.,[11] PCDD/F + DL-PCB concentrations for Scottish salmon were ~2.9 ng TEQ/kg (n = 30) while the concentrations in fish fed the deNFO and SFO/RO/SO diets were around 13% of this value. In a more recent study Ikonomou *et al.,*[44] recorded a maximum value of 1.85 ± 0.27 ng TEQ/kg in Canadian salmon. By comparison the fish fed the deNFO and SFO/RO/SO diets were ~20% of this value. The mean feed PCDD/F + DL-PCB value in the Canadian diets was 3.74 ng TEQ/kg[44] which is 7.5 times higher than the deNFO and SFO/RO/SO diets in the present study. The PCDD/F + DL-PCB concentration for wild Pacific Chinook salmon was ~0.13 ng TEQ/kg[11] while the DL-PCB values alone for a different source of Chinook salmon were ~0.45 ng TEQ/kg[38]. Thus, the PCCD/F + DL-PCB concentrations in fish fed the deNFO and SFO/RO/SO diets are only slightly higher than those quoted by Hites *et al*.,[11] and considerably lower than those quoted by Easton *et al*.,[38] for wild Pacific salmon.

The flesh PBDE concentrations for the fish fed the cNFO, deNFO and SFO/RO/SO diets were 0.94, 0.25 and 0.10 ng/g, respectively. These values are all significantly lower than the values recorded for wild Canadian Chinook (4.2) Scottish farmed (3.9) or wild Oregon Chinook (2.2) by Hites *et al*.[12] These values were also considerably lower than the average PBDE concentration of 5 ng/g reported by Jacobs *et al*.,[45] in 13 samples of European salmon. Excluding the 9 wild Chinook salmon, the average value for the 36 wild Pacific salmon, including Pink, Coho, Sockeye and Chum was 0.130 ± 0.020 ng/g[12]. The PBDE concentrations in salmon fed the deNFO and SFO/RO/SO diets were slightly higher and lower than the wild Pacific salmon, respectively.

There is currently considerable interest in fish, and especially oily fish, as a means of increasing n-3 HUFA intake in populations that currently have low fish intake. However, while the benefits of consuming n-3 HUFA are widely known, there is also concern about the

relative risks and benefits due to the presence of POPs in oily fish[4,14,16,47]. The current guideline of the UK Food Standards Agency (www.food.gov.uk) suggests that we should consume up to four 140g portions of oily fish per week. However, the FSA add the caveat that girls and women of child bearing age should only consume two 140g portions of oily fish due to concerns that POPs present in the fish might pose a risk to the developing foetus. Thus, for girls or women of child bearing age the FSA recommendation is in a agreement with the EU Scientific Committee on Food that the maximum intake of PCDD/Fs + DL-PCBs should be 2pg/kg body weight/day[35]. However, the FSA guideline suggests that boys, men and women over reproductive age can consume up to 8pg/kg body weight/day. The salmon fed the cNFO diet in this study would give 171% of the weekly limit for girls and women of reproductive age or 86% of the weekly limit value for boys, men and women over reproductive age, from 2 or 4 x 140g portions of cNFO salmon, respectively. However, salmon with these levels of POPs would not be available for public consumption due to current EU limits on PCDD/Fs and DL-PCBs in fish feeds[22]. In a sample of standard production Scottish salmon analysed in our laboratory, the average PCDD/F + DL-PCB concentration in 2 x 140g portions was 493 pg TEQ. This represents 47% of the value for girls and women of child bearing age or 24% of the limit for boys, men and women over reproductive age. By comparison, for the deNFO and SFO/RO/SO fish, consuming 2 or 4 x 140g portions represents only 9.0 and 10.8% of the weekly limit for girls and women of reproductive age or 4.5 and 5.4% respectively, of the weekly limit for PCDD/Fs + DL-PCBs for boys, men and women over reproductive age. While the POPs concentrations are very low for both the deNFO and SFO/RO/SO salmon, the same 2 and 4 x 140 g portions provide 147% and 81% and 294% and 162%, respectively, of the ISSFAL recommended weekly intake of EPA + DHA[8].

In summary, this study has confirmed that FOs with high POP concentrations can be successfully decontaminated with removal of more than 97% of PCDD/Fs and DL-PCBs using two stage activated carbon and thin-film deodourisation. Removal of PBDEs was less successful with concentrations being reduced by around 70%. Feeding the deNFO and SFO/RO/SO diets to 0.8kg Atlantic salmon for 11 weeks resulted in excellent growth rates and feed conversion. Flesh PCDD/F and DL-PCB concentrations were reduced to very low levels in fish fed the deNFO and SFO/RO/SO diets with values being similar to those seen in wild Pacific salmon while PBDE concentrations were comparable or lower than Pacific salmon. Feeding the deNFO diet did not significantly alter the n-3 HUFA content of the fillet as was observed in fish fed the SFO/RO/SO diet. This suggests that decontaminated fish oils can be used successfully to produce salmon that are both high in n-3 HUFA and low in POPs

508 although further longer term trials should be conducted to ensure that no detrimental effects
509 on fish performance and health occur. These results could be of significant benefit in
510 producing highly nutritious farmed salmon that are very low in undesirables as well as
511 utilising valuable FOs that would otherwise be destroyed by incineration.
512
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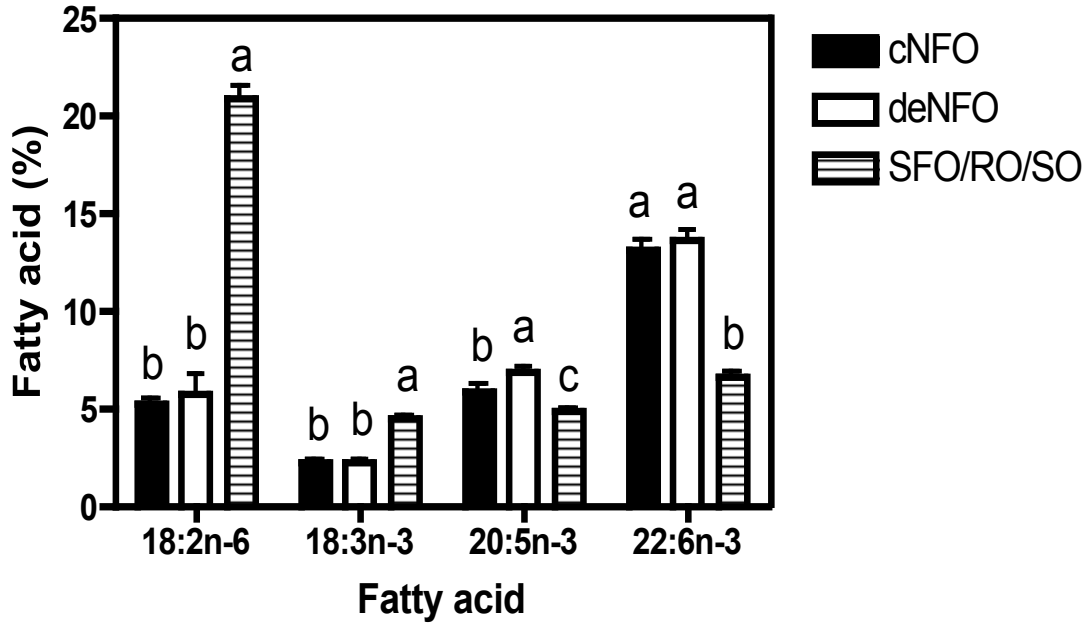
Figure legends

Figure 1. Concentrations (% of total fatty acids) of 18:2n-6, 18:3n-3, 20:5n-3 and 22:6n-3 in flesh of salmon fed either northern fish oil (cNFO), decontaminated northern fish oil (deNFO) or a blend of southern fish oil, rapeseed and soybean oil (SFO/RO/SO) for 11 weeks. Columns assigned a different letter are significantly different ($P < 0.05$).

Figure 2. Amount of dioxin + DL-PCBs present in 2 or 4 x 140g portions of salmon, produced using cNFO, deNFO or SFO/RO/SO diets. Upper dotted line indicates UK FSA maximum dioxin/PCB intake of 56 pg/kg/week for a 75kg person while the lower dashed line is the UK FSA maximum intake of 14 pg/kg/week for a 75kg girl or woman of reproductive age.

Figure 3. Amount of EPA + DHA provided by 2 or 4 x 140g portions of salmon, produced using cNFO, deNFO or SFO/RO/SO diets. Dotted line indicates ISSFAL recommended EPA + DHA intake of 3.5g week.

687 **Fig. 1**.



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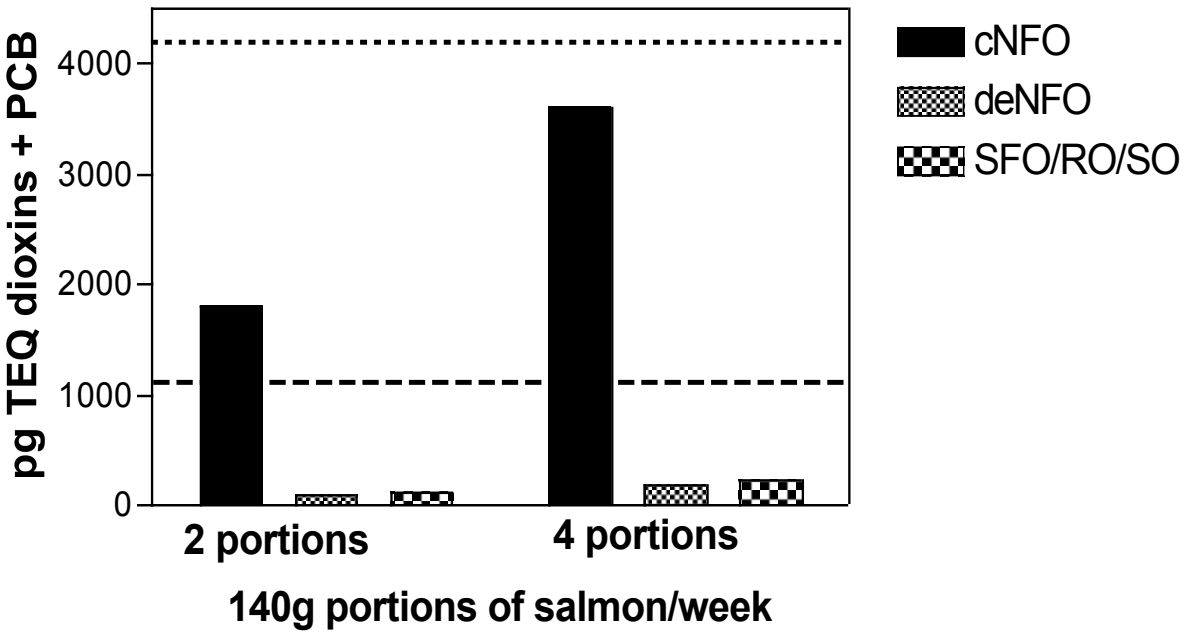
689 **Fig. 2**



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696 **Fig. 3.**

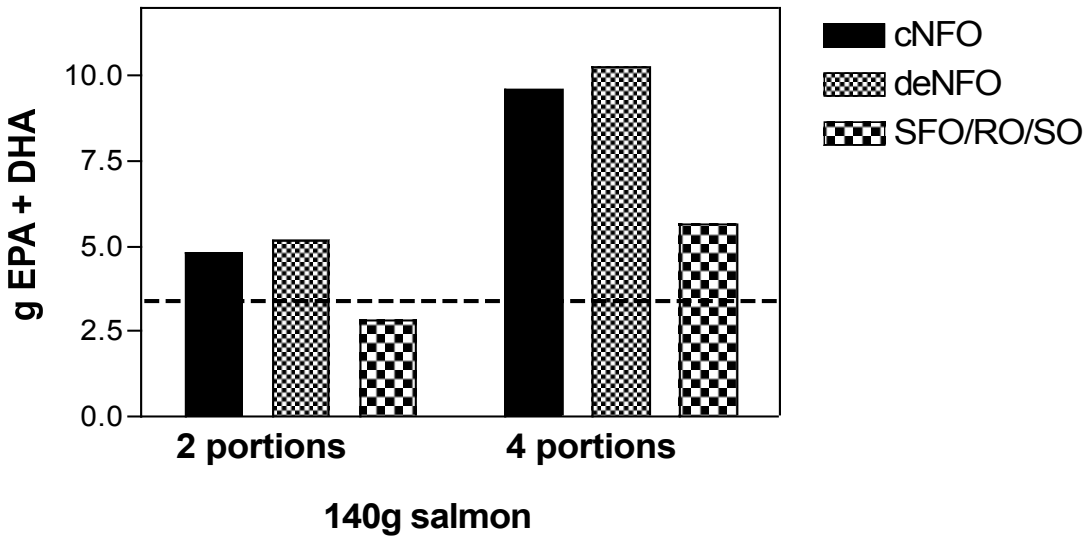

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Table 1. Diet formulations, proximate compositions (g/kg), energy (kJ/g) and major fatty acid compositions (% of total fatty acids) of the three experimental diets fed to Atlantic salmon for 11 weeks.

Component/Diet	cNFO	deNFO	SFO/RO/SO
Fish meals	378	378	378
Legume meals	149	149	149
Northern fish oil (NFO)	325	-	-
Decontaminated NFO	-	325	-
Southern fish oil	-	-	130
Rapeseed oil	-	-	98
Soybean oil	-	-	98
Binder	140	140	140
Premixes	9.2	9.2	9.2
Composition (g/kg)			
Protein	326	322	328
Oil	336	343	342
Moisture	60	55	55
Ash	78	77	78
Fibre	21	22	20
Digestible energy (kJ/g)	25.3	25.3	25.2
Fatty acid			
16:0	19.2	18.8	13.5
Total saturates[1]	27.8	29.3	23.1
18:1n-9	25.9	21.6	29.5
Total monounsaturates[2]	42.0	40.0	36.5
18:2n-6	3.8	4.3	23.1
Total n-6[3]	5.6	5.9	23.9
18:3n-3	2.7	2.3	5.1
20:5n-3	6.7	7.2	5.2
22:6n-3	11.6	11.4	4.3
Total n-3[4]	24.7	24.7	16.5

cNFO, northern fish oil; deNFO, decontaminated northern fish oil; SFO/RO/SO, southern fish oil, rapeseed oil and soybean oil. [1]Includes 14:0, 15:0, 18:0, 20:0 and 22:0. [2]Includes 16:1n-7, 16:1n-9, 20:1n-11, 20:1n-7, 18:1n-7, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n-9. [3] Includes 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6 and 22:5n-6. [4]Includes 18:4n-3, 20:4n-3 and 22:5n-3.

Table 2. Dietary concentrations (ng TEQ/kg wet weight) of dioxin and DL-PCB congeners in the northern fish oil, decontaminated northern fish oil and southern fish oil, rapeseed oil and soybean oil diets.

Congener/Diet	cNFO	deNFO	SFO/RO/SO
1234678-HpCDD	0.005	0.001	0.001
123478-HxCDD	0.028	0.009	0.007
123678-HxCDD	0.113	0.013	0.012
12378-PeCDD	1.386	0.081	0.038
123789-HxCDD	0.028	0.007	0.010
2378-TCDD	0.609	0.040	0.082
Sum PCDD	**2.169**	**0.153**	**0.151**
1234678-HpCDF	0.002	nd	nd
123478-HxCDF	0.084	0.013	0.005
1234789-HxCDF	0.001	nd	0.001
123678-HxCDF	0.102	0.006	0.005
12378-PeCDF	0.147	0.006	0.002
123789-HxCDF	0.018	0.012	0.009
234678-HxCDF	0.125	0.008	0.008
23478-PeCDF	4.775	0.058	0.050
2378-TCDF	1.223	0.011	0.015
Sum PCDF	**6.477**	**0.114**	**0.094**
PCB 105	0.355	0.004	0.007
PCB 114	0.081	0.002	0.001
PCB 118	1.043	0.010	0.014
PCB 123	0.010	0.001	nd

PCB 156	0.612	0.029	0.012
PCB 157	0.153	0.008	0.002
PCB 167	0.005	nd	nd
PCB 189	0.010	0.003	nd
Sum mono-ortho PCB	**2.269**	**0.057**	**0.036**
PCB 77	0.001	0.001	0.001
PCB 81	0.020	nd	nd
PCB 126	6.4	0.12	0.25
PCB 169	0.070	0.007	0.003
Sum non-ortho PCB	**6.4**	**0.12**	**0.25**
Sum dioxins + PCBs	**17.4**	**0.45**	**0.53**

cNFO, northern fish oil; deNFO, decontaminated northern fish oil; SFO/RO/SO, southern fish oil, rapeseed oil and soybean oil. nd = not detected.

Table 3. Dietary concentrations (ng/g wet weight) of seven PBDE congeners in the northern fish oil, decontaminated northern fish oil and southern fish oil, rapeseed oil and soybean oil diets.

PBDE	cNFO	deNFO	SFO/RO/SO
PBDE 28	0.22	0.013	nd
PBDE 47	3.6	0.87	0.23
PBDE 99	0.84	0.34	0.027
PBDE 100	0.89	0.45	0.059
PBDE 153	0.27	0.17	nd
PBDE 154	0.15	0.095	nd
PBDE 183	nd	nd	nd
Sum PBDE 7	**5.94**	**1.90**	**0.31**

cNFO, northern fish oil; deNFO, decontaminated northern fish oil; SFO/RO/SO, southern fish oil, rapeseed oil and soybean oil. nd = not detected.