# PREPARATION OF A CONTAMINATED FEED FOR A PCB, PCDD, AND PCDF CHEMOBIOKINETIC STUDY IN THE TROUT IN A PILOT LEVEL FARMING PLANT

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## Introduction

Farmed fish, such as rainbow trout, can be heavily exposed to the alimentary intake of *persistent toxic substances* (PTS) because fed on a diet containing up to 25 % fish oil and 40 % fish meal. A progressive and extensive accumulation of polychlorinated biphenyls (PCBs), dibenzodioxins (PCDDs), and dibenzofurans (PCDFs) can be enhanced by two additional risk factors: the 14-month long life of the commercial trout and the less efficient detoxification mechanisms in fish, when compared with warm-blooded animals. Recently, the European Union (EU) has pragmatically fixed the maximum limits of PCDD+PCDF contamination in feed and food of animal origin.[1,2] Nevertheless, poor documentation is available about the correlation between such levels in feed and food, in particular in intensive aquaculture. Within the above framework, a presently ongoing chemobiokinetic study was organized at a farming pilot level with the goal of providing the Italian Ministry of Health with practicable risk management options to reduce the PCDD+PCDF and PCB content in fish edible tissues. In this progress report, the plan of the study, how the feed was prepared to have as low contamination as possible,[3] the technique used to add contamination to the basic feed, and the analytical characterization of the produce are described.

*The chemobiokinetic study layout*

Four 3-$m^3$ flow-through freshwater pools, kept at 14 °C, were arranged to rear as many groups of 200 70-g-*bw* rainbow trout each. All trout groups were fed with an all-vegetal feed during the first rearing month. After such a conditioning period, a first 10-specimen sample was drawn from each pool (Day 0). Thereafter, three groups were exposed to feeds fortified at different levels with PCBs (Aroclor 1254) and six selected PCDD+PCDF congeners (see below) for one more month, with samples drawn on Day 7, 14, and 30; the low, medium, and high fortification levels (approximate ratios, 1:3:9) roughly reflected the low, medium, and high fish feed contamination concentrations, according to an EU inventory (Table 1). Exposure was stopped; during the following 120-day clearance period, the unfortified feed was re-introduced in all groups and samples were drawn every 15 days. Trout were continuously monitored for feed intake, welfare, and health. Fæces were removed by sedimentation and filtration, and properly disposed of.

*Preparation of contaminated fish feed*

The all-vegetal fish feed formula devised for trout rearing meant to replace all animal proteins and fat/oil components with those of vegetal origin, for a final composition made respectively of 70 % legumes, 17 % vegetal oil, and 10 % cereals. Relevant ingredients were assayed in advance for PCB and PCDD+PCDF contents and cleared for use only if background-compatible.

**Table 1.** Prediction of low, medium, and high PCDD+PCDF contamination in animal-based and vegetable-based trout feeds as per the reported contamination of each component. Values expressed as pgWHO-TE/g dry matter, 12% moisture.

| Feed ingredient | Animal-based feeds | | | | Vegetable-based feeds | | | |
|---|---|---|---|---|---|---|---|---|
| | % | Low | Medium | High | % | Low | Medium | High |
| Cereals | 15 | 0.001 | 0.015 | 0.060 | 10 | 0.001 | 0.010 | 0.040 |
| Fish oil * | 15 | 0.006 | 0.091 | 0.390 | — | — | — | — |
| *Fish oil* ** | | *0.105* | *0.720* | *3.000* | — | — | — | — |
| Vegetal oil | — | — | — | — | 17 | 0.001 | 0.017 | 0.068 |
| Fishmeal * | 52 | 0.010 | 0.070 | 0.125 | — | — | — | — |
| *Fishmeal* ** | | *0.020* | *0.600* | *2.800* | — | — | — | — |
| Legumes | 15 | 0.001 | 0.015 | 0.060 | 70 | 0.007 | 0.070 | 0.280 |
| Other | 3 | 0.001 | 0.003 | 0.019 | 3 | 0.005 | 0.003 | 0.019 |
| Total* | 100 | 0.019 | 0.194 | 0.654 | 100 | 0.014 | 0.100 | 0.407 |
| Total** | 100 | *0.128* | *1.353* | *5.939* | — | — | — | — |

(*) Fish oil and meals from South Pacific. (**) Fish oil and meals from Northern Europe.

Known amounts of two standard mother solutions in *n*-nonane — one containing PCBs, the other the six selected PCDD+PCDF congeners (see below) — were added to some 0.5–1-L amounts of vegetal oil and vigorously mixed on a magnetic stirrer for more than 24 hours.

All ingredients but the vegetal oil were extensively minced, thoroughly mixed, and made into small hard cylindrical pellets (approximately: Ø, 5 mm; length, 10 mm). The four finished feeds were obtained by letting a proper amount of vegetal oil — with or without added contaminants — slowly trickle down (1 L/hour) on a 50-kg feed pellet batch, gently stirred in a small-scale rotary mixer. Feed batches were produced in the following order of contamination levels: blank, low, medium, and high. They were stocked at room temperature in plastic-lined paper bags, and allow to rest 15 days. Aliquots (10 g) were then analyzed to test the homogeneity/stability of each batch.

*Chemicals*

Solvents (acetone, *n*-hexane, *n*-pentane), chromatographic materials (alumina, Extrelut™, anhydrous $Na_2SO_4$, silica gel), and reagents (96 % $H_2SO_4$, $NaHCO_3$) were high quality grade, suitable for residue analysis, as assayed in the laboratory. The following PCB, PCDD, and PCDF standards were provided as toluene solutions by Ultra Scientific Italia (Bologna, Italy): 2,3,7,8-$T_4$CDD, 20 mg/mL (´ 2); 1,2,3,7,8-$P_5$CDD, 50 mg/mL; $O_8$CDD, 50 mg/mL; 2,3,7,8-$T_4$CDF, 50 mg/mL; 1,2,3,7,8-$P_5$CDF, 50 mg/mL; $O_8$CDF, 50 mg/mL; 1-g and 50-mg Aroclor 1254, 99 % pure. Natural decachlorobiphenyl ($D_{10}$CB) internal/external standard and natural PCB external standards were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Fully $^{13}$C-labelled PCB, PCDD, and PCDF internal standards were purchased from Lab Service Analytica (Bologna, Italy).

*Analysis*

Five grams of anhydrous $Na_2SO_4$ were thoroughly mixed with 10-g finely ground feed specimen; $D_{10}$CB was canonically added as an internal standard. When fish was assayed, a 20-g muscle amount was extensively homogenized and then combined with excess anhydrous $Na_2SO_4$ (some 20 g); during homogenization, $D_{10}$CB was again added to the matrix as an internal standard. When quantitation was

**Table 2.** Background and fortified (Aroclor 1254) PCB levels in trout feeds used for the chemobiokinetic study described. Concentrations expressed in ng/g, whole weight.

| Feed contamination | Assessed analytes | Nominal concentrations | Measured concentrations * | | |
|---|---|---|---|---|---|
| | | | *HRGC-LRMS* | *HRGC(ECD)* | |
| Blank | Σ $PCB_7$ | — | < 4 | — | — |
| | Σ $PCB_{18}$ | — | < 7 | — | — |
| | Σ $PCB_{TOT}$ | — | < 10 | » 7 | — |
| Low | Σ $PCB_7$ | — | 52.9 | — | — |
| | Σ $PCB_{18}$ | — | 93.5 | — | — |
| | Σ $PCB_{TOT}$ | 132.0 | 134 | 144.0 ± 7.0 | 4.7 % ** |
| Medium | Σ $PCB_7$ | — | 145 | — | — |
| | Σ $PCB_{18}$ | — | 257 | — | — |
| | Σ $PCB_{TOT}$ | 396.8 | 370 | 378 ± 14 | 3.6 % |
| High | Σ $PCB_7$ | — | 453 | — | — |
| | Σ $PCB_{18}$ | — | 799 | — | — |
| | Σ $PCB_{TOT}$ | 1191 | 1150 | 1180 ± 57 | 4.9 % |

(*) In five-sample pools for HRGC-LRMS; from five replicates for HRGC(ECD). (**) CV% (s ´ $m^{-1}$ ´ 100).

by high resolution gas chromatography (HRGC) in tandem with mass spectrometry (MS), the spiked original matrix was divided into two portions for parallel independent assessments of PCBs and PCDDs+PCDFs; in this case, several fully $^{13}C$-labelled PCB, PCDD, and PCDF congeners were added as internal standards to the portions, as required.

Extraction was performed with a Soxhlet apparatus, using a mixture of *n*-hexane-acetone (1:1, v/v) and a number of cycles greater than 300. The extract was evaporated under reduced pressure, taken up with 100-mL *n*-hexane, and divided into two aliquots for determination of PCBs and PCDDs+PCDFs, respectively.

The cleanup procedure consisted in two different steps. The first step included a column of Extrelut impregnated with 96% sulphuric acid. The column was pre-washed with 100-mL *n*-pentane; then, an extract aliquot was applied and eluted with 150-mL *n*-pentane. The eluate was reduced to 200 mL under a gentle nitrogen flow, transferred to an alumina column pre-washed with *n*-hexane, and eluted with 5-mL *n*-hexane (*Fraction 0*), 18-mL *n*-pentane-$CCl_4$ (1:1, v/v) (*Fraction 1*, containing PCBs), and 9-mL $CH_2Cl_2$ (*Fraction 2*, containing PCDDs and PCDFs). The *F1* and *F2* eluates were reduced to appropriate volumes for instrumental determination based on HRGC.

Measurements of PCBs in feed were carried out with a Varian Mod. 3300 gas chromatograph equipped with a SPI injector, a $^{63}Ni$ electron capture detector (ECD), and an Ultra-2 capillary column (30 m × 0.32 mm × 0.17 µm). PCBs were assessed in fish — and on occasions in feed — by HRGC combined with low resolution mass spectrometry utilized in the single ion monitoring mode (LRMS(SIM)), the GC unit being equipped with a SGE HT-5 capillary column (25 m × 0.22 mm × 0.10 µm). PCDD+PCDF contents in feed were determined by HRGC-HRMS(SIM) with a VG Mod. AutoSpec mass spectrometer in tandem with a Carlo Erba Mod. 8000 gas chromatograph equipped with a SGE BPX-5 capillary column (50 m × 0.32 mm × 0.25 µm).

When HRGC(ECD) was used, the analytes were identified by their retention time relative to $D_{10}$CB; when the assessment was by combined GC-MS, the isotopic ratios of the two most intense masses of the parent ion clusters were used in addition to relative retention times. All final results, estimated according to the medium bound approach, were corrected for recovery rates.[4–7]

**Results and discussion**

The results reported in Table 2 showed that the contamination procedure was effective in yielding reasonably homogeneous feed products, suitable to carry out the chemobiokinetic study. The vegetal fish feed formula gave satisfactory daily weight gains in the trout, with no altered taste and fat composition of the fish muscle (data unreported).

The unfortified (base) feed had very low PCB and PCDD+PCDF contents: <10 ng/g, whole weight (*ww*), and <0.2 pgTE/g *ww* (I-TEQs or WHO-TEQs),[8,9] respectively. Such figures are in agreement with those recurring in vegetal feeds within the EU (Table 1). The PCB and PCDD+PCDF levels — <70 ng/g, lipid base (*lb*) for PCBs and <4 pgTE/g *lb* (I-TEQs or WHO-TEQs) — detected in trout fed on the base feed were also very low and well below the average contamination levels found in the wild or conventionally farmed fish.[10]

The above results are in agreement with the goals of our study, indicating both the efficacy and the practicability of the feed formula adopted to lower the contaminant levels in trout, thus giving new toxicological and commercial perspectives to farmed fish in human diets.

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