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Dietary pesticide chlorpyrifos-methyl affects arachidonic acid metabolism including phospholipid remodeling in Atlantic salmon (*Salmo salar* L.)

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ABSTRACT

The pesticide chlorpyrifos-methyl (CLP-m) has been identified in plant ingredients intended for aquaculture feed production. To investigate systemic effects of CLP-m with emphasis on lipid metabolism, post-smolt Atlantic salmon were fed in duplicate (n = 2) either diets with no CLP-m (Control) or CLP-m at different concentrations (0.1, 1.0 or 8.0 mg kg^{-1}) for a total of 67 days (Low, Medium, High). Fish in all groups almost doubled their weight during the feeding trial from 262 ± 26 g (mean ± SD) to 465 ± 64 g (overall mean), with no significant effects on any growth parameters. There was a significant dose-dependent inhibition of plasma cholinesterase activity (BuChE) after 67 days. The CLP-m biotransformation metabolite, TCP was detected in liver and bile, with low levels of the parent compound in the organs. Spleen somatic index decreased significantly with increasing dietary CLP-m intake. Hematocrit (%) decreased linearly with increasing dietary exposure to CLP-m after 30 days of exposure, but this decrease was less at 67 days of exposure. A significantly reduced content of arachidonic acid (ARA 20:4n − 6), accompanied by a significantly increased content of the saturated fatty acid, palmitic acid (PA 16:0), was observed in liver phospholipids (PLs) with increasing dietary content of CLP-m. Major effects were seen on the PL classes in liver which showed a significantly decreased absolute content, possibly indicating inhibition of PL remodeling pathways or other membrane perturbation effects from CLP-m exposure. In conclusion, this study shows that the pesticide CLP-m is a relatively potent toxicant in Atlantic salmon, especially affecting liver PLs and ARA metabolism.

1. Introduction

Mainly due to the fish oil (FO) replacement, hazardous environmental contaminants such as dioxins and PCBs have decreased in fish feed. The shift from marine to plant feed ingredients, has introduced new undesirable substances, such as non-organochlorine pesticides, used on crop intended as fish feed ingredients. Chlorpyrifos-methyl (CLP-m) has been identified in feed ingredients by the ARRAINA EU-project and in fish feed by Norwegian feed surveillance as one of the new potential undesirables (Nacher-Mestre et al., 2014). Recent studies with Atlantic salmon hepatocytes have reported perturbation of lipid metabolism as one of the main target effects of CLP exposure (Softeland et al., 2014; Softeland et al., 2016; Olsvik et al., 2015). At the same time, the European Union (EU) pesticide maximum residue limit (MRL) for animal feeds do not include the non-persistent organophosphate (OP) pesticides (EC, 2002) such as CLP-m. EU MRL legislation comprises most food commodities (EC, 2005), but for feed (crops exclusively used for animal feed purposes) and fish, harmonized EU MRLs are not yet established. In 2013, crops used as feed ingredient and fish were added as commodity categories with no set MRL yet (EC, 2013), emphasizing the need for feed borne exposure studies on marine farmed fish such as Atlantic salmon. The levels of CLP-m reported in commercially available Norwegian fish feed ranged from 10 to 14 μg kg^{-1} in 2015 (Sanden et al., 2016a). At present, there is no specific MRL for CLP-m in fish feed. The potential adverse effects of this pesticide are well researched in fish, but only for waterborne exposures (e.g. Maryoung et al., 2015; Mhadhbi and Beiras, 2012; Xing et al., 2015; Topal et al., 2016). The route of uptake is of great importance for the

Abbreviations: AChE, acetylcholinesterase; ARA, arachidonic acid; BuChE, butyrylcholinesterase; CLP-m, chlorpyrifos-methyl; EROD, ethoxyresorufin-*O*-deethylase; K, condition factor; MRI, magnetic resonance imaging; MRL, maximum residue limit; OCPs, organochlorine pesticides; OP, organophosphate; PC, phosphatidylcholine; PLs, phospholipids; RBC, red blood cell; SSI, spleen somatic index

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potential adverse effect on fish. Studies on dietary CLP-m exposure in fish are lacking.

The main target toxic effects of Chlorpyrifos (CLP) is an irreversible inhibition of acetylcholinesterase (AChE) (Boone and Chambers, 1997) which has also been suggested as a biomarker for CLP exposure in fish (Topal et al., 2016). CLP is activated to the oxon metabolite by cytochrome P450 systems (Racke, 1993). The activated oxon metabolite inhibits AChE by phosphorylating a serine hydroxyl group in the active site of the enzyme (Murphy, 1966; Tang et al., 2001). AChE is responsible for cholinesterase activity in the nervous system of vertebrates and where inhibition of AChE in red blood cells is recognized as an early biomarker of effect (Garabrant et al., 2009). Butyrylcholinesterase (BuChe) is the main B-esterase in human plasma and has been regarded as an early biological response in the category of biomarkers of exposure to OP pesticides (Garabrant et al., 2009). BuChe activity has been found in freshwater teleosts belonging to the family Cyprinidae (Chuiko et al., 2003) and marine teleosts (Sturm et al., 1999). Dose-response models have predicted that no inhibition of RBC AChE activity will be observed until exposure levels sufficient to produce substantial inhibition of plasma BuChe activity (> 50% inhibition) are attained (Garabrant et al., 2009). In addition to CLP oxon metabolite formation, cytochrome P450 systems cause CLP to undergo dearylation forming diethyl thiophosphate and 3,5,6-trichloro-2-pyridinol (TCP) metabolite (Kamataki et al., 1976; Smith et al., 2012), which can be conjugated with endogenous molecules to facilitate its excretion (Smith et al., 2012; Nolan et al., 1984). TCP is considered as the main chlorpyrifos (methyl) metabolite in risk assessment of livestock and crop (EFSA, 2012). In the liver of common carp exposed to waterborne CLP, a general increasing trend for the activity of the cytochrome P450 biotransformation enzymes (EROD and PROD) and the gene transcript level *cyp1A,* were observed (Xing et al., 2014). Similarly, a 17-fold upregulation of *cyp1A* gene transcript was shown in salmon hepatocytes exposed to CLP (Softeland et al., 2014).

Non-target general toxic effects of CLP-m include immunotoxic responses (Xing et al., 2015; Li et al., 2013) and oxidative stress (Chen et al., 2015; Ma et al., 2015; Ural, 2013). CLP induced changes in physiopathological alterations, and hematological characteristics have also been reported in *Tilapia guineensis* (Chindah et al., 2004) and *Cyprinus carpio carpio* (Ural, 2013). On a biochemical level, recent *in vitro* studies with Atlantic salmon hepatocyte showed perturbation of lipid metabolism as one of the main non-AChE target effects of CLP exposure (Softeland et al., 2014; Softeland et al., 2016; Olsvik et al., 2015). Primary bile acid, linoleic acid and unsaturated fatty acid biosynthesis were reported as the three main metabolic pathways affected in salmon hepatocytes exposed to CLP (Softeland et al., 2014). Furthermore, recent *in vivo* lipidomic studies in Japanese medaka (*Oryzias latipes*) exposed to waterborne CLP, showed strong decrease in the levels of phosphatidylcholine (PC) in the liver (Jeon et al., 2016). In addition, several putative annotated eicosanoids belonging to the arachidonic acid (ARA, 20:4n − 6) metabolic pathway was affected by CLP exposure (Softeland et al., 2014). ARA, the physiologically most important n − 6 polyunsaturated fatty acid (PUFA), is required as a constituent of membrane phospholipids (PLs) and is the precursor of the n − 6 family of eicosanoids. Likewise, Medina-Cleghorn et al. (2014) examined the metabolic alterations caused by OPs in mice, and found that OPs induced disruption in lipid metabolism by inhibiting several serine hydrolases.

Disturbed lipid metabolism such as steatosis has been detected in salmon exposed to OP pesticides (Krovel et al., 2010; Glover et al., 2007). Likewise, a recurrent negative effect in salmon fed plant-based diets, is increased lipid accumulation (Torstensen et al., 2011; Liland et al., 2013; Ruyter et al., 2006; Sissener et al., 2014). The exact causative factor(s) related to increased lipid accumulation in salmon is still being investigated, but suboptimal levels of nutrients in plant-based salmon feed have been suggested (Sanden et al., 2016b; Hamre et al., 2016; Hemre et al., 2016; Alvheim et al., 2013). Salmon fed on a diet high in soybean oil showed liver lipid accumulation, increased levels of endocannabinoids (Alvheim et al., 2013) and pro-inflammatory eicosanoids (Araujo et al., 2014; Bell et al., 1996). Likewise, the amount and balance of eicosanoids was altered in salmon hepatocytes exposed to CLP (Softeland et al., 2014). Current Norwegian salmon feed use plant oil as the main lipid source, predominantly rapeseed oil. We hypothesized that the presence of CLP-m residues in salmon feed would aggravate or modify the recurrent negative effect of replacing fish oil with plant oils, possibly leading to perturbation of lipid metabolism and increased lipid accumulation. Therefore, the current study aimed at determining systemic effects of dietary CLP-m exposure in Atlantic salmon (fed on a high soybean oil background diet) with emphasis on possible perturbation effects on hematology and lipid metabolism.

2. Material and methods

2.1. Feeding trial

The trial was initiated September 21st, and ended November 26th, 2015. Locally bred post-smolt Atlantic salmon (*Salmo salar* L.) of the SalmoBreed strain were distributed among eight fiberglass tanks (450 L; 0.95 m × 0.95 m × 0.5 m; 32 fish per tank) at Industrilaboratoriet (ILAB), Bergen, Western Norway (60°N′5°E). Weight, length (fork-tail) and condition factor (K) of fish were 262 ± 26 g, 27 ± 1 cm and 1.3 ± 0.1 (mean ± standard deviation; $n = 256$), while hepatosomatic index (HSI) and spleen somatic index (SSI) were 1.1 ± 0.1 and 0.08 ± 0.01, respectively (mean ± standard deviation; $n = 15$) at the beginning of the experiment. During a three-week acclimatization period to holding facilities, all fish were fed the control diet (without CLP-m). The control diet (produced by Skretting ARC) was composed of: Soyprotein concentrate (32%), wheat gluten (17%), fish meal (10%), wheat (4%), sunflower meal (3%), faba beans (5%), soybean oil (20%), fish oil (5%, South American and Northern hemisphere fish oil 70:30) and premixes (4%) including crystalline DL-methionine, lysine, vitamins and minerals, the latter supplemented to cover requirements according to NRC (2011). No CLP-m was detected (LOQ 0.01 mg kg^{-1}) in the control diet. Thereafter for 67 days, duplicate tanks (n 2) received one of the four experimental diets: either no CLP-m (Control) or CLP-m at three different concentrations (0.1, 1.0 and 8.0 mg kg^{-1}) for a total of 67 days (Low, Medium, High). Fish were fed to apparent satiety by automatic feeders once a day for 6 h. Feed intake per tank was measured by collecting feed waste once daily after the feeding, permitting assessment of the average daily dose of CLP-m consumed (ng kg^{-1} fish day^{-1} Table 1). The fish were reared in sea water (34 g L^{-1}, 12 °C) using a 12 h light, 12 h dark photoperiod regime. The O_2 saturation of the outlet water was always above 80%. Mortality was recorded on a daily basis. The experiment complied with the guidelines of the Norwegian Regulation on Animal Experimentation and EC Directive 86/609/EEC. The National Animal Research Authority approved the protocol (ID 7583).

Table 1
Analyzed concentration (mg kg^{-1}) of CLP-m in experimental feeds, and estimated daily dose (μg kg^{-1} fish day^{-1}) for each feeding period.

Nominal feed level mg kg^{-1}	Measured feed level mg kg^{-1} [a]	Estimated CLP-m dose μg kg^{-1} fish day^{-1} Feeding days 1–30	Estimated CLP-m dose μg kg^{-1} fish day^{-1} Feeding days 31–67
Control	n.d.	–	–
0.1, Low	0.095	0.97	0.66
1.0, Medium	0.92	9.04	5.93
8.0, High	7.80	76.26	51.26

[a] Analyzed by Eurofins Scientific, Hamburg Germany. Limit of Quantification (LOQ) of method 0.01 mg kg^{-1}.