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

Sanden, M.¹*, Olsvik P.A.¹, Søfteland L.¹, Rasinger J. D.¹, Rosenlund G.², Garlito B.³, Ibáñez M.³ and M.H.G. Berntssen¹.

¹ Natl Inst Nutr & Seafood Res, POB 2029, N-5817 Bergen, Norway
² Skretting ARC, PB 48, N-4001, Stavanger, Norway.
³ Research institute for Pesticides and Water (IUPA), University Jaume I, Av. Sos Baynat S/N, 12071, Castellón, Spain

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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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 ± 26g (mean ± SD) to 465 ± 64g (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., 2015b, Topal et al., 2016). The route of uptake is of great importance for the 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 (greater than 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., 2015a, 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 have 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 21\textsuperscript{st}, and ended November 26\textsuperscript{th}, 2015. Locally bred post-smolt Atlantic salmon (\textit{Salmo salar} L.) of the SalmoBreed strain were distributed among eight fiberglass tanks (450L; 0.95m x 0.95m x 0.5m; 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 ± 26g, 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 six hours. 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).

2.2 Preparation of CLP-m fortified diets
The CLP-m fortified diets were prepared by dissolving CLP-m (Sigma-Aldrich AS, Norway) directly into the feed oil (mixture of soybean oil and fish oil, 1:6) and further vacuum coated on the basal pellet (3 mm diameter) at a level of 6% oil inclusion. Control diets were vacuum coated with 6% of CLP-m free feed oil. Immediately after production, samples were taken from each feed batch and analysed for supplemented levels and were: < limit of quantification (LOQ) 0.01 mg kg$^{-1}$ (Control), 0.095 (Low), 0.92 (Medium), and 7.80 mg kg$^{-1}$ (High), respectively (Table 1). Pellets were stored at −20 °C until fed to the fish. Feed samples were taken at the end of the trial, and after 16 months of storage, analysis showed no significant CLP-m degradation during the frozen storage. CLP-m feed analyses were performed by EUROFINS by gas chromatography with flame photometric detection (GC-FPD), based on the multi residue method DFG S19 (CEN EN12393).
2.3 Sampling

Tricaine methanesulfonate (FINQUEL MS-222 ~7 g L\(^{-1}\)) was used for immobilization and sedation of the fish before euthanization by a blow to the head. At days 30 and 67, six fish from each tank were sampled, postprandial time was 24 h. Weight, length (fork-tail) and organ somatic indices (liver and spleen) were recorded for all fish (six fish per tank at day 30 and, 16 fish per tank at day 67). Blood samples were drawn from the caudal vein using vacutainers coated with lithium heparin. Only hematocrit and plasma samples were collected at day 30, while an extended blood analysis package was investigated at day 67 (see below). The left fillet was sampled from each fish for fatty acid (FA) composition determination. For comparative purposes, sections of the liver were excised from the same anatomical position and collected for ethoxyresorufin-O-deethylase (EROD) analysis, lipid class composition and FA composition determinations, before being flash frozen in liquid nitrogen. For analysis of CLP-m metabolites including parent compound, liver and bile were collected at day 67 and flash frozen in liquid nitrogen. Additionally, the remaining fish in the Control and High CLP-m group was sampled at day 67 for chemical analysis of total lipid in whole fish and for quantification of mass fat fractions using magnetic resonance imaging (MRI).

2.4 Hematology and plasma parameters

Whole blood analyses (aliquot 1 and 2) for hematocrit (Hct), number of red blood cells (RBCs) and hemoglobin (Hb) were measured as described in Sissener et al. (2009). Mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) were calculated as described in Sandnes et al. (1988). Osmotic resistance of erythrocytes (from whole blood aliquot 1) was measured based on the method described previously (Dacie and Lewis, 1975). Briefly, 20 µL of heparinized blood was added to a series of 12 concentrations of 5-mL saline solution (0.9 g L\(^{-1}\)) in phosphate buffer. After gentle mixing and 1 h incubation at 4°C, the suspensions were centrifuged at 3000 rpm for 10 min and the absorbance of the supernatant recorded at 540 nm. Results are given as the salinity causing 50% lysis of the erythrocytes. Blood smears (from aliquot 2) were prepared on glass microscope slides, air dried and fixed in absolute methanol for 5 min, then stained with May-Grünwald (Merck) for 5 min and Giemsa (Merck) for 15 min. The samples were blinded and examined with an Olympus light microscope (BX 51) at 400×. Hundred white blood cells (granulocytes, monocytes and lymphocytes) from each smear
were assessed. Morphological parameters such as cell size, shape, nuclear lobulation, presence of granules, and general cellular irregularities were evaluated. The final aliquot was centrifuged at 3000 g for 10 min to obtain the plasma fraction, which was frozen on dry ice and stored at –80°C until analysis. Analysis of chloride, creatinine, cholinesterase and total protein in plasma were performed on Maxmat Biomedical Analyser (SM1167, Maxmat S.A., France) using Maxmat reagents and the appropriate calibrators, standards and controls for the different methods. Plasma osmolality was measured using Fiske One Ten Osmometer (Bergman Instrumentering AS, Norway).

2.5 Liver EROD activity

The preparation of microsomal fractions for EROD (7-ethoxyresorufin-0-deethylase) activity assay was performed according to Nilsen et al. (1998) with minor modifications. Briefly, microsomal fractions were prepared from 250-300 mg hepatic tissue that was homogenised (0.1 M Na$_3$PO$_4$, pH 7.4, 0.15 M KCl, 1 mM EDTA, 1 mM DTT, without glycerol, pH 7.6) using a metal bead (Retsch, Anders Pihl AS MM301, Dale, Norway) homogeniser. The homogenates were centrifuged sequentially at respectively 12000 x g for 20 min and 100,000 x g for 60 min at 4°C. The final cell pellet was dissolved in a resuspension buffer (0.1 M Na$_3$PO$_4$, pH 7.6, 0.15 M KCl, 1 mM EDTA, 1 mM DTT) containing 20% (v/v) glycerol. 10 μL microsomal fraction per well was added and incubated in 0.98 mL EROD buffer (0.1 M Na$_3$PO$_4$, pH 7.6) with 5 μL 7-ethoxyresorufin (ER) solution (0.4 mM ER Sigma–Aldrich, in DMSO), and 5 μL NADPH solution (10 mM NADPH Sigma–Aldrich, in distilled water) for 12 min. For background subtraction, two wells were prepared by adding the following solutions: 0.99 mL EROD buffer, 5 μL ER solution and 5 μL NADPH solution (in duplicate). The resorufin production was measured as fluorescence change over time (Victor ™X5 2030 Multilabel Plate Reader, Perkin Elmer, Upplands Väsby, Sweden, with cw lamp energy: 8904 and counting time: 0.2s, and equipped with 530 excitation and 595 nm emission filters). The calculation of the enzyme activity (μmol min$^{-1}$ mg protein$^{-1}$) was conducted according to Ganassin et al. (2000). The total protein concentration in the microsomal fraction was measured using a Pierce 660 nm protein assay, according to the manufacture’s specification (Life Technologies, Oslo, Norway).
2.6 Residues of pesticide and metabolite in liver and bile

For the analysis of CLP-m and TCP content, approximately 0.2 mL of bile or 0.5 g (wet weight) of liver tissue were transferred to an Eppendorf tube. After adding 0.8 mL (1 mL for liver analysis) of acetonitrile:acetone (80:20, v/v) containing 1% formic acid, the tube was stirred in a vortex for 1 min. The extract was then passed through a hybridSPE-phospholipid cartridge (Sigma-Aldrich) (in the case of liver, 0.4 g magnesium sulphate were previously added to the mix and stirred in a vortex for 30 s). The eluate was diluted with HPLC water (two- and five-fold for bile and liver, respectively). After adding the internal-labelled internal standard mix (composed by CLP-m-D₆ and TCP-13C₃), 2 µL were finally injected in the liquid chromatography-tandem mass spectrometry (LC-MS/MS) system, equipped with a triple quadrupole analyser (TQS, Waters). For further details, please see supplementary material (Appendix B).

2.7 Lipid class and fatty acid analysis

Lipids from liver were extracted in a mixture of chloroform–methanol 2:1 (Merck) with 1% 2,6-di-tert-butyl-4-methylphenol (Sigma-Aldrich) as described by Torstensen et al. (2004). Briefly, chloroform:methanol (at approximately twenty times the weight of the sample) was added to the samples and lipids extracted overnight at -20 °C. After the extraction of lipids as described above, the samples were filtered and the quantification of lipid class composition was carried out by HPTLC as described by Torstensen et al. (2011). Lipid classes were quantified by scanning densitometry using a CAMAG TLC Scanner 3 and calculated using an integrator (WinCATS-Planar Chromatography, version 1.2.0; CAMAG, Berlin, Germany). Quantitative determination (mg lipid class g⁻¹ tissue) of lipid classes was performed by establishing standard equations for each lipid class within a linear area, in addition to including a standard mixture of all the lipid classes at each high-performance TLC plate for corrections between plate variations. After the extraction of lipids as described above, neutral lipids (NLs) and polar lipids (PLs) were separated and an aliquot of 10 mg lipids (solved in 200 µL chloroform) was applied to a solid-phase extraction column (Isolute; Biotage). NLs were eluted with 10 mL chloroform–methanol (98:2, v/v) and PLs were eluted with 20 mL methanol. For analysis of FAs, the two lipid extracts were filtered and the remaining samples were saponified and methylated using
12% boron trifluoride (BF₃) in methanol. To determine FA composition the methyl esters were separated using a Trace gas chromatograph 2000 (Fison, Elmer, USA) equipped with a 50-m CP-sil 88 (Chrompack) fused silica capillary column (id: 0.32 mm) (Lie and Lambertsen, 1991, Torstensen et al., 2004). The FAs were identified by retention time using standard mixtures of methylesters (Nu-Chek, Elyian, USA), and the FA composition (area %) was determined. All samples were integrated using the software Chromeleon® version 6.8 connected to the Gas liquid chromatography (GLC). Amount of FA per gram sample was calculated using 19:0 methyl-ester as internal standard. FA composition was analyzed in fillet before the trial, after 30 and 67 days of exposure, as described above. Data on fillet FA composition are presented as percentage of total FAs, to facilitate comparison between samplings and exposure groups when the fish differ in total lipid content.

2.8 Fat fraction quantification using magnetic resonance imaging (MRI)

Water-fat separation MRI was used to quantify and localize dense lipid in whole fish. We acquired 20 transverse slices of 1 mm thickness and with 1 mm gap between each slice on a whole salmon in order to evaluate the volume of dense lipid in one predefined area covering 40 mm. To rule out possible confounding factors linked to the position of the whole fish in the coil and to standardize the acquired images for each fish, a working zone of 50 mm anterior to the dorsal fin was defined. After scanning, each individual image was verified according to the anatomical positions of internal organ such as the orientation of the liver and swim bladder. MR images were acquired using a 7T horizontal bore magnet (Pharmascan 70/16, Bruker BioSpin, Ettlingen, Germany) operating at 300 MHz. A 60 mm diameter linear volume resonator was used for transmission and reception of RF radiation. After a TriPilot scan, two T₁-weighted multi-spin multi-echo images were acquired with the following geometry: 6.5 x 6.5 cm² field-of-view, 512 x 512 matrix size, in plane resolution of 127x127 μm², 20 slices of 1 mm thickness and with 1 mm gap between each slice, covering 40 mm. Imaging parameters were: TE/TR=11.3ms/1180ms, 10 averages and total scan time of 18 min 53 sec. Images were acquired with and without fat suppression, to generate “fat_water” and “water_only” images. Eppendorf tubes with different amount of dense lipid from salmon was used as a lipid standard to set the MR parameters for each scan. Each scan was processed using ImageJ Software, Fiji version
First, a fat subtraction was performed using the image calculator before each image was adjusted using common threshold windowing, the triangle algorithm (Zack et al., 1977), for all fish in order to remove the background noise and standardize pixel signal intensities between scans. All images were converted to a binary 8-bit scale with background dark (0) and lipid signal white (255) before drawing tools were used to define regions of interest (ROI). Only the visceral/abdominal region is reported, as the other measured ROI showed similar fat distribution patterns. Total lipid in the same fish were analyzed gravimetrically after ethyl acetate and isopropanol extraction as described by Lie 1991 (Lie, 1991).

2.9 Calculations

Condition factor (K) = (bw/fl^3) * 100 (bw=body weight, fl=fork length). Organsomatic index = (ow/bw)*100 (ow=organ weight). MCV = (Hct/rbc)*10. MCH = (Hb/rbc)*10. MCHC = (Hb/Hct)*100.

2.10 Statistics

Statistica 13.1 (StatSoft Inc, Tulsa, OK, USA) and GraphPad Prism version 7.0 (GraphPad Software Inc.) were used in the statistical work and for the graphics. Data were tested for normality using a Kolmogorov–Smirnov test and homogeneity of variance using Levene’s test. Nested ANOVA with the different tanks nested within the diet treatments was used for parameters measured on individual fish (n=12). Nested ANOVA maintains both the between-tank and within-tank variabilities in the analysis and can be more powerful in resolving treatment differences (Ruohonen 1998). A Tukey’s honest significant difference post hoc test was used to detect differences between diet groups. Correlations in the data were tested using Spearman’s rank order correlation. For all the tests a p-values less than 0.05 were set as the significant level. Heat maps were created in GraphPad using the negative logarithm of the models p-values from nested design ANOVA. Data, which passed significance testing in nested ANOVAs were also submitted to multivariate regression analysis using log (x+1) CLP-m intake (µg kg⁻¹ fish day⁻¹) as the abscissa and any given parameter as the ordinate. Multilevel linear modelling was performed in the statistical programming language R (version 3.2.3) (R Development Core Team, 2013) as described.
earlier (de Gelder et al., 2016). In brief, fixed and random intercept models were created for each parameter of interest. The models produced were compared to each other using log likelihood tests and the best fitting and most parsimonious model was chosen for further analyses in which the concentration of CLP-m was added as predictor. Statistical significance was accepted at p < 0.05. All figures are given with the mean values ± 95% confidence intervals. A comprehensive overview of all models created and all parameters determined is presented in the supplementary material (Appendix A, Table 3 and appendix C).

3. Results

3.1 Growth performance and organ indices

There were no mortalities in any of the experimental treatments. Fish in all groups almost doubled their weight during the 67 - day feeding trial in which they grew from 262 ± 26g (n=256) at the start sampling, to 359 ± 36g (n=48) after 30 days of exposure (overall mean) and to 465 ± 64g (n=128) at the final sampling (overall mean). There were no significant differences in weight, condition factor (K) or liver somatic index (HSI) amongst the dietary groups after 30 days or 67 days of feeding and all details are found in supplementary material (Appendix A, Table 1). There was a significant (p=0.006) dose-response effect of CLP-m on spleen somatic index (SSI) (Fig. 1A) after 67 days of feeding, where the model follows a linear trend y = a + (-0.008 * Dose).

3.2 Hematology and plasma biochemistry

There were no significant effects of CLP-m on plasma BuChE levels after 30 days of exposure. After 67 days of feeding the model shows that there was a significant dose-dependent effect of CLP-m on plasma BuChE (Fig. 1B), where plasma BuChE levels decreased linearly with increasing dietary CLP-m intake (y = a + (-73.02 * Dose, p=0.03)). After 30 days of feeding, hematocrit (%) decreased linearly with increasing dietary CLP-
m intake \( (y = a + (-2.14 \times \text{Dose}, p<0.0001) \) (Fig. 1C). For the other plasma parameters measured (total protein, creatinine, osmolality or chloride) no significant effects were observed at any of the sampling points. The hematological parameters hemoglobin (Hb), red blood cell count, mean cell volume (MCV), mean cell hemoglobin concentration (MCHC) and mean cell hemoglobin (MCH) were only analyzed at the final sampling, but these parameters did not reveal any significant differences among the groups (results not shown). No significant differences were observed in fragility of erythrocytes at the final sampling; the concentration where hemolysis occured in 50% of the erythrocytes (IC50) were 7.39 (control), 7.15 (0.1 mg kg\(^{-1}\)), 7.28 (0.1 mg kg\(^{-1}\)) and 7.38 (8 mg kg\(^{-1}\)) with overlapping confidence intervals and no significant differences. Analysis of blood smears illustrated that the differential counts of leukocyte types did not differ significantly with increasing dietary CLP-m intake (Table 2).

3.3 Biotransformation of pesticide
The biotransformation enzyme EROD was assessed in liver samples after 67 days of exposure. EROD activity increased linearly with increasing dietary CLP-m intake \( (y = a + (422.5 \times \text{Dose}, p=0.002) \) from 480 μmol/min/mg protein (± SD 266) in salmon fed the control diet to 1223 (± SD 444) in salmon fed the High diet (Fig. 2). The concentrations of CLP-m and its metabolite 3,5,6-trichloro-2-pyridinol (TCP) in fish liver and bile are summarized in Table 3. The metabolite TCP showed significantly \( (p<0.05, \text{t-test}) \) higher concentrations in bile compared with the liver of fish fed the High and Medium diet. The TCP concentrations in fish fed the High diet ranged from 42.5-65.8 ng g\(^{-1}\) in the bile and from 29.2-36.9 ng g\(^{-1}\) in the liver. CLP-m was only detected in fish fed the High diet and ranged from 3.9 – 7.4 ng g\(^{-1}\) in bile and from 2.6 – 6.7 ng g\(^{-1}\) in the liver, with no significant differences between the organs. TCP and CLP-m were not detected in bile and liver of salmon fed the Low or the Control diet.

3.4 Liver lipid classes
As several liver lipid classes were affected by the experimental treatment, heat maps are shown as an overall aggregated view of all the lipid classes (Fig. 3F), while details on absolute values (mg g\(^{-1}\)) are found in the supplementary material (Appendix A, Table 2AB). The most abundant lipid classes in the liver were phosphatidylcholine (PC), phosphatidylethanolamine (PE) and triacylglycerol (TAG). After 30 days of exposure, PC
content in the liver (Fig. 3A) followed a second order polynomial trend where PC increased in salmon fed the Low and Medium diet and decreased in salmon fed the High CLP-m diet; \[ y = a + (12.5 \times \text{Dose}) + (-6.2 \times \text{Dose}^2). \] After 67 days of exposure PC content in liver followed a third order polynomial trend where PC decreased in salmon fed the Low and Medium diet before flattening out in salmon fed the High CLP-m diet. The content of cardiolipin (CL) followed a second order polynomial trend at both samplings where CL liver content significantly decreased linearly in the liver of salmon fed the Low and Medium diet, before flattening out in salmon exposed to the High CLP-m dose (Fig. 3B). Liver phosphatidylinositol (PI) content significantly decreased with increasing CLP-m dose at the first sampling \[ y = a + (-0.46 \times \text{Dose}, p<0.001), \] while the decrease in liver PI content followed at third order polynomial trend with increasing CLP-m dose at the second sampling (Fig. 3C). Sphingomyelin (SM) and cholesterol (CH) followed the same trend as PI with a linear decrease after 30 days of exposure followed by a third order polynomial trend at the second sampling (Fig. 3DE). After 67 days of exposure to CLP-m, even fish fed the Low diet showed a significantly decreased content of SM and CH in the liver compared to the Control (also seen for other lipid classes; PC, PI, PE, PS).

The liver TAG concentrations were similar for all the dietary groups, but with large individual variations, especially in fish exposed to the High CLP-m diet at the final sampling. A significant negative correlation was seen for PC and lysoPC \((r=-0.5659)\) after 30 days of exposure. While lysoPC decreased (significantly between fish fed the Medium diet and control), the content of free FAs increased significantly in all fish exposed to CLP-m, compared to control \((p=0.01)\) (supplementary material). The graphical presentation of the lipid classes are summarized in Fig. 3F, where the individual p-values (negative log transformed) are contained in a matrix and represented as colors (from white (not significant) to black \((p<0.001)\).

3.5 Liver FA composition in phospholipids (PLs) and neutral lipids (NLs)

As several FAs were affected by the experimental treatment, heat maps are shown as an overall aggregated view of all the FAs (Fig. 4C). FAs mostly affected are given in Fig. 4AB and details in the supplementary material (Appendix A, Table 4AB). Despite being fed the same background diet with the same FA composition, only differing in the content of CLP-m, fish fed all CLP-m dietary levels, showed a significantly lower content of ARA \((20:4n-\))
6) in liver PLs after 30 days (p<0.05) and after 67 days of exposure (p=0.001) compared to control (Fig. 4A). At both time samplings the model followed a second order polynomial trend with the most significant effects observed at the second sampling; \( y = a + (-2.1 * \text{Dose}) + 0.9 * \text{Dose}^2 \)). In addition, fish fed the High CLP-m diet showed a significantly increased content of PA (16:0) in liver PLs compared to control (30 days of feeding; p<0.05) and Low CLP-m diet (67 days of feeding; p<0.05). After 30 days of feeding (Time 1) the model followed a linear dose response; \( y=a + (0.4 * \text{Dose}, p=0.01) \) (Fig 4B). At the first sampling, SA (18:0) increased significantly, but was not affected by the dietary treatments at the second sampling. The total sum of saturated fats in liver PLs was not affected by the experimental diets at any samplings. Overall, the absolute content of sum FAs (mg g\(^{-1}\)) in liver PLs decreased significantly with increasing CLP-m dose from 19.0 mg g\(^{-1}\) to 15.3 mg g\(^{-1}\) at the final sampling (Appendix A, Table 4AB). Other than that, no significant dietary effects on FA composition in liver PLs were found. Generally, DHA (22:6n-3) was the predominant FA in liver PLs, while the most abundant FAs in the NL fraction of the liver was oleic acid (OA 18:1n-9) and linoleic acid (LA 18:2n-6) accounting for more than 50% of FAs. Only minor effects were seen on the FAs in the liver NL fraction, except for OA (p=0.01) which decreased at the final sampling (Appendix A, Table 3A and 4C) following a linear dose response; \( y = a + (-1.3 * \text{Dose}, p=0.01) \). A graphical representation of the FAs are summarized in Fig. 4C, where the individual p-values (negative log transformed) are contained in a matrix and represented as colors (from white (not significant) to grey (p<0.05) to black (p<0.001).

3.6 Fat fraction distribution quantification using magnetic resonance imaging (MRI)

On a whole-body level, no significant differences were observed in the quantified dense lipid volume using MRI in a standardized predefined transverse section (Fig. 5A). Neither did it reveal any differences in dense lipid volume of the abdominal region (Fig. 5B) which accounted for 52% and 54% of the total dense lipid volume in the control group and salmon fed 8 mg kg\(^{-1}\) CLP-m, respectively (Fig. 5C). The total whole fish lipid determined gravimetrically, correlated significantly with the dense abdominal lipid volume determined by MRI (r=0.81, p<0.05) (Fig. 5D).
4.0 Discussion

This is the first study investigating dietary CLP-m exposure in farmed fish and results show that the pesticide is a relatively potent toxicant in Atlantic salmon. Although it is known that fish will rapidly metabolize and excrete CLP-m, continuous exposure through the feed may have detrimental effects. Summary of the findings indicate that CLP-m affects liver membrane PLs remodeling and ARA metabolism with significant impact even at the lower CLP-m dose. This is in accordance with a recent study in salmon hepatocytes exposed to CLP which also reported effects on ARA metabolism (Olsvik et al., 2015). The low CLP-m dose used in the present work is only slightly higher compared with the concentrations reported for commercial salmon feed (Sanden et al., 2016a). In addition, CLP-m inhibited the main target of CLP, namely plasma BuChe activity and affected several non-target systemic pathways in Atlantic salmon.

There were no effects on growth performance within the time frame of the feeding trial. The main toxic effect of CLP is an irreversible inhibition of AChe, but it is suggested that there will be no inhibition of red blood cell AChe activity before the exposure levels are sufficiently high to produce substantial inhibition of plasma BuChe activity (greater than 50% inhibition) (Garabrant et al., 2009). Therefore, we measured plasma BuChe, regarded as an early response biomarker of exposure (Garabrant et al., 2009), and found a significant inhibition in Atlantic salmon plasma with increasing exposure to dietary CLP-m. Serum BuChe and brain AChe inhibition was also observed in several fresh water fish species following exposure to DDVP, another OP pesticide (Chuiko, 2000). We also measured plasma AChe (results not shown), but found no effects of CLP-m on AChe activity, possibly indicating that the dietary levels were not high enough to affect AChe activity in the present study. CLP is twelve time more toxic compared with CLP-m in mice (Sultatos et al., 1982), which may also explain the lack of AChe inhibition in plasma.

Non-target systemic physiopathological alterations from CLP exposure such as decreased hematocrit has been reported in *Tilapia guineensis* (Chindah et al., 2004) and *Cyprinus carpio carpio* (Ural, 2013). In the present study we observed a decreased hematocrit indicating that the red blood cells (RBCs) were destroyed or the production affected. As
we did not measure other hematological characteristics at the first sampling (30 days of exposure), we do not know the mechanisms responsible for the effects on hematocrit. However, at the final sampling the fish may seem to have compensated with increased production of RBCs, as we observed no significant effects on any hematological parameters. This transient effect on hematocrit may be linked to the reduced relative size of the spleen observed after 67 days of exposure, as this organ plays important roles in RBC homeostasis including erythropoiesis and the immune system. Further examination of white blood cells at 67 days of exposure did not indicate a reaction of the immune system. The reduced relative size of the spleen could also be linked to histopathological changes, as a recent study with CLP exposed rainbow trout (*Oncorhynchus mykiss*), found liver hyperaemia and degeneration, which was accompanied with a strong inhibition of the enzyme carbonic anhydrase (Topal et al., 2014).

The main detoxification system of CLP is via the cytochrome P450 (CYP) enzyme system (Lavado and Schlenk, 2011, Choi et al., 2006). In the mammalian liver CLP is completely metabolized to CLP-oxon and then to 3,5,6-trichloro-2-pyridinol (TCP) by the cytochrome P450 system (Hodgson and Rose, 2008). In the present work ethoxyresorufin-O-deethylase (EROD) enzyme activity was measured as a marker of enzymatic biotransformation and we observed that EROD activity increased significantly with increasing CLP-m dose. A general increasing trend for the activity of EROD and the gene transcript levels of cyp1a, was observed in the liver of common carp exposed to waterborn CLP (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). Earlier trials with waterborne CLP exposed common carp, showed a dose response induction of biotransformation enzymes with an accumulation of CLP and CLP-oxon metabolite the liver (Xing et al. 2014). In the present study, dietary exposures also caused a dose response induction of EROD activity, with minor accumulation of CLP-m at the highest exposure level and a higher accumulation of hepatic TCP at the medium and high exposure levels. Highest TCP levels were observed in bile with increasing exposure levels, indicating an effective biotransformation and biliary excretion of dietary CLP-m exposed Atlantic salmon.

In our study, the level of the saturated fatty acid PA (16:0) increased and polyunsaturated fatty acid ARA (20:4n-6) decreased in the membrane PLs, (Fig. 6A). Generally, ARA
released from membranes by the action of phospholipase A (PLA2) are further metabolized through different enzymatic pathways to generate lipid signaling messengers such as prostaglandins from the cyclooxygenase (COX) pathway, leukotrienes from the lipoxygenase (LOX) pathway or epoxyeicosatrienoic acids (EETs) from the cytochrome P450 pathway (CYP) (Rowley et al., 1995). As, we did not measure any of the lipid signaling molecules, the fate of released ARA in the present work is not known. But increased content of the eicosanoid tromboxan and THF-diols with decreased content of ARA, was reported in salmon hepatocytes exposed to CLP (Softeland et al., 2014). THF-diols are eicosanoids involved in inflammatory reactions and produced by the cytochrome P450 pathway (Moghaddam et al., 1996). Another study with salmon hepatocytes exposed to CLP also reported diminished content of ARA and suggested that decreased levels of this metabolite in the hepatocytes could indicate conversion to lipid peroxidation products such as 13-HODE and 9-HODE that are peroxisome proliferator activated receptor (PPAR) ligands associated with inflammation (Olsvik et al., 2015).

CLP-m is a relatively small molecule with a lipophilic nature (logKow 4.3) and may interfere with lipid metabolic pathways in membranes. Incorporation of CLP-m could change the membrane fluidity and the membrane structure. Fat soluble molecules such as benzo[a]pyrene and phenanthrene were shown to affect the membrane fluidity in model membranes (Liland et al., 2014). Adaption to incorporation of lipid soluble molecules into PL membranes is through homoviscous adaption (Sinensky, 1974). The observed change in membrane PL could be a mechanism of homoviscous adaptation alike more fat soluble compounds. We know that membrane integrity must be maintained for membranes to operate as a selective barrier and cholesterol is a key molecule for membrane structure.

The decreased content of membrane cholesterol observed in the present work may further influence membrane structure and possibly affect biosynthesis of steroid hormones, bile acid and vitamin D. Perturbed cholesterol homeostasis by contaminants has been demonstrated in numerous mammalian and teleostean studies (Elkskus et al., 2005). A recent study reported that cell membrane stability and vitamin D metabolism were the main responses affected in salmon hepatocytes exposed to a contaminant mixture containing CLP (Softeland et al., 2016). Another recent study revealed effects on
cholesterol metabolism and lysophospholipids in salmon hepatocytes exposed to pirimiphos-methyl (Olsvik et al., 2017b).

As hepatic steatosis has been associated with exposure to pesticides such as endosulfan resulting in dyslipidemia in Atlantic salmon (Glover et al., 2007, Krovel et al., 2010), we hypothesized that the exposure diets would result in lipid accumulation in Atlantic salmon. However, within the time frame of the present study, we observed no significant changes in the total neutral lipid fraction of the liver nor any increase in dense lipid in the abdominal region of the fish. There was a tendency though, of increased liver levels of TAG at the final sampling in fish fed the high exposure diet, but there were large individual variations at this sampling. Long-term feeding studies are needed to confirm if this was only a random effect or if it could be linked to lipid metabolic perturbations.

Only minor effects were seen on the FAs in the liver neutral lipid fraction, except for OA; (18:1n-9) which decreased at the final sampling, possibly indicating increased energy expenditure with increasing CLP-m dose. Generally, lipids are secreted by the liver mainly as TAG in very-low-density lipoprotein (VLDL). In a study with salmon hepatocytes incubated with a range of seven 14C-labelled FAs, the highest recovery of radioactivity in exported lipids was PA (16:0) and OA (18:1n-9) (Stubhaug et al., 2005), indicating that these FAs are readily used for energy in salmon hepatocytes. In general EROD detoxification of organic pollutant in fish is associated with altered energy metabolism (Gourley and Kennedy, 2009, Lindberg et al., 2017), and also for CLP exposed bird (Coturnix coturnix japonica) increased energy expenditure was associated with detoxification processes (Narvaez et al., 2016). Altered FA turn-over due to increased energy expenditure to cope with sub chronic CLP exposures might explain the decrease in OA in the liver NL fraction in the present study.

Rapid turnover of FAs from the membrane PLs is attributed to the concerted and coordinated activities of PLA2 and lysophospholipid acyltransferases (LPLATs) (Lands, 1958). Although the ultimate toxicity of OP pesticides is the inhibition of acetylcholinesterase (AChE) at nerve synapses, they may additionally have detrimental effects through inhibition of other serine hydrolases, such as phospholipases (PLA),
lysoPLA, monoacylglycerol lipases and triacylglycerol hydrolases (Casida and Quistad, 2005). Inhibition of one of the enzyme hydrolases in the PL remodeling pathway would result in an imbalance between free FAs and lysophospholipids and finally membrane perturbation effects such as direct damage or effects on lipoprotein transport. A rat study investigating the toxicity of chronic exposure to a mixture of four OP pesticides, including CLP, reported that the intensities of metabolites significantly changed in the OP mixture-treated group compared with the control group for the metabolites lysoPE (16:0/0:0), lysoPC (17:0/0:0), lysoPC (15:0/0:0) and ARA (Cao et al., 2016). Similarly, pirimiphos-methyl exposure in Atlantic salmon hepatocytes significantly down regulated several lysophospholipids in the cells and it was suggested that this could be due to enzyme inhibition of PLA2 (Olsvik et al., 2017a). Another study by our research team showed that Atlantic salmon hepatocytes exposed to CLP exhibited increased levels of several lysolipids such as 1-palmitoyl-GPC (16:0) and 1-oleoyl-GPC (18:1) (not published data). Perturbation of PL metabolism was also reported in crayfish exposed to CLP (Gradwell et al., 1998). Taken together our results supports previous findings and it is suggested that CLP-m affects the PL remodeling pathway in Atlantic salmon by affecting one or more lipid pathways (Fig. 6B).

Specifically, we quantified and found significant effects of CLP-m exposure on several of the classes of PLs which generally decreased with increasing exposure doses. The most abundant PL class in the liver, PC, exhibited a bell shaped response at the first sampling and a linear response which decreased with CLP-m exposure, at the second sampling, possibly indicating compensation mechanisms (increased de novo synthesis of PC) after short term exposure before non-compensatory adverse effects (membrane damage) after long term exposure. This is in accordance with recent work on Japanese medaka (Oryzias latipes) which reported a dramatically decreased content of PC and proposed it as a lipid biomarker for CLP exposure (Jeon et al., 2016).

For some of the PL classes such as phosphatidylinositol (PI), there was a significant decrease even at the low CLP-m dose. Interestingly, PI is the membrane PL with the highest relative content of ARA in liver and heart (Bell et al., 1991). Gradwell et al. (1998) suggested that the PL metabolism perturbation observed in crayfish could also be linked to impairment of mitochondrial energy metabolism. In mammals, it is suggested that the mitochondrial cardiolipin level decrease during apoptosis and increase when the
abundance of mitochondria increase (Nomura et al., 2000). The significant decreased content of cardiolipin observed after short term feeding at the lower CLP-m dose may suggest implications on the energy metabolism as CL is a key PL of the inner mitochondrial membrane. Impairment of mitochondrial energy metabolism would have detrimental long term effects in Atlantic salmon. It is also reported that CLP can activate sphingomyelinases (SMases), the catabolic enzymes that convert sphingomyelin (SM) to ceramide (Lima et al., 2014). In our study we found a significant reduced content of SM in Atlantic salmon exposed to CLP-m at 67 days of exposure.

4. Conclusions

In summary, our work show that the pesticide CLP-m is a relatively potent toxicant in Atlantic salmon. The known main toxic effect of the pesticide, which is inhibition of plasma cholinesterase activity (BuChE), was also evident in this trial as we found inhibition of the enzyme with increasing CLP-m dose in Atlantic salmon plasma. In addition CLP-m biotransformation metabolite TCP was detected in liver and bile, with low levels of the parent compound in the organs, suggesting a fast biotransformation. Although this short term dietary exposure study found no effects on growth performance, our findings indicate that CLP-m affects liver membrane PLs remodelling and ARA metabolism with impact even at the lower CLP-m dose. In addition to the above mentioned effects on lipid metabolism pathways, CLP-m exposure had an overall affect as both the relative spleen size and hematocrit (%) decreased with increasing pesticide dose, while BuChE was inhibited at increased doses. The low CLP-m dose used in the present work is only slightly higher compared with the concentrations reported for commercial Norwegian salmon feed and at present there are no EU MRL legislation for fish feed. We do not know the long term effects of pesticide exposure in real farming conditions. The observed effects on cell membrane stability and FA metabolism which could implicate effects on the immune system and barrier functions, needs further attention.
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Appendix A, B and C. Supplementary data
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Tables and Figure texts:

**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\(^{-1}\) | 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                                                               |

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

**Table 2** Atlantic salmon differential counts of white blood cells in response to dietary CLP-m (mean ± SD) after 67 days of exposure. Nested ANOVA with the different tanks nested within the diet treatments was used for parameters measured on individual fish (n=12).

| Granulocytes (%) | 0.1 mg kg\(^{-1}\) | 1.0 mg kg\(^{-1}\) | 8.0 mg kg\(^{-1}\) | Anova |
|------------------|--------------------|--------------------|--------------------|-------|
| Control          | 9.3 ± 5.6          | 11.8 ± 4.6         | 11.9 ± 5.3         | 8.7 ± 5.0 | ns    |
| Low              | 1.3 ± 1.0          | 1.4 ± 1.3          | 1.5 ± 1.5          | 2.5 ± 4.0 | ns    |
| Medium           | 89.5 ± 5.2         | 86.8 ± 4.9         | 86.6 ± 6.2         | 88.8 ± 7.7 | ns    |
| High             |                    |                    |                    |       |
**Table 3** Levels of CLP-m and its metabolite TCP in liver (ng g\(^{-1}\)) and bile (ng mL\(^{-1}\)) of Atlantic salmon fed the four experimental diets for 67 days (minimum–maximum (number of positive samples)). Not detected (n.d). T-tests were run to check differences between organs (liver and bile) at p<0.05. Two fish from each dietary group was pooled to six samples per dietary group (n 5-6).

|                | 0.1 mg kg\(^{-1}\) | 1.0 mg kg\(^{-1}\) | 8.0 mg kg\(^{-1}\) |
|----------------|---------------------|---------------------|---------------------|
| **Liver ng g\(^{-1}\)** | Control Low Medium High |
| CLP-m          | n.d                 | n.d                 | 5.1 ± 1.6 (6)      |
| TCP            | n.d                 | 3.2 ±0.5 (6)\(^{a}\) | 32.1 ± 3.1 (6)\(^{A}\) |
| **Bile ng mL\(^{-1}\)** | Control Low Medium High |
| CLP-m          | n.d                 | nd                  | 5.1 ± 1.4 (5)      |
| TCP            | n.d                 | 9.7 ± 2.6 (6)\(^{b}\) | 53.4 ± 9.3 (6)\(^{B}\) |

Concentration range: 0.05 – 25 ng mL\(^{-1}\) (CLP-M), 0.25 – 25 ng mL\(^{-1}\) (TCP)
Figure 1ABC Dose response curves showing significant (p<0.05) multivariate regression model relationships between dietary CLP-m intake using log (x+1) of CLP-m intake (mg CLP-m kg⁻¹ body weight per day⁻¹) as the abscissa and A) Spleen somatic index (SSI) (only after 67 days), B) Plasma cholinesterase activity (BuChE) and C) Hematocrit (%) as the ordinates after 30 days (T1) and 67 days (T2) of feeding four diets with increasing levels of the pesticide CLP-m to Atlantic salmon postsmolt. Whole lines (T1) and dotted lines (T2). Each value represented the mean ± 95% confidence interval (CI) of 12 individuals (except SSI, 32 individuals). Model outputs are found in the supplementary material (Appendix A, Table 3 and appendix C). Small letters for T1 (a, b) or capital letters for T2 (A, B) indicate significant (p<0.05) differences between the groups.

Figure 2 Dose response curve showing significant (p<0.05) multivariate regression model relationship between dietary CLP-m intake using log (x+1) of CLP-m intake (mg CLP-m kg⁻¹ body weight per day⁻¹) as the abscissa and EROD activity as the ordinates after 67 days (T2) of feeding four diets with increasing levels of the pesticide CLP-m to Atlantic salmon postsmolt. Each value represented the mean ± confidence interval (CI) of 12 individuals. Model outputs are found in supplementary (Appendix A, Table 3 and appendix C). Capital letters (A, B) indicate significant (p<0.05) differences between the diet groups.

Figure 3ABC Dose response curves showing significant multivariate regression model relationships between dietary CLP-m intake using log (x+1) of CLP-m intake (mg CLP-m kg⁻¹ body weight per day⁻¹) as the abscissa and A) Phosphatidylcholine (PC) mg g⁻¹ B) Cardiolipin (CL) mg g⁻¹ C) Phosphatidylinositol (PI) mg g⁻¹ as the ordinates after 30 days (T1) and 67 days (T2) of feeding four diets with increasing levels of the pesticide CLP-m to Atlantic salmon postsmolt. Whole lines (T1) and dotted lines (T2). Each value represents the mean ± confidence interval (CI) of 12 individuals. Model outputs are found in the supplementary material (Appendix A, Table 3 and appendix C). Small letters for T1 (a, b) or capital letters for T2 (A, B, C) indicate significant (p<0.05) differences between the groups.
Figure 3D Đose response curves showing significant multivariate regression model relationships between dietary CLP-m intake using log \((x+1)\) of CLP-m intake (mg CLP-m kg\(^{-1}\) body weight per day\(^{-1}\)) as the abscissa and D) Sphingomyelin (SM) mg g\(^{-1}\) E) Cholesterol (CH) mg g\(^{-1}\) as the ordinates after 30 days (T1) and 67 days (T2) of feeding four diets with increasing levels of the pesticide CLP-m to Atlantic salmon postsmolt. Whole lines (T1) and dotted lines (T2). Each value represents the mean ± confidence interval (CI) of 12 individuals. Model outputs are found in the supplementary material (Appendix A, Table 3 and appendix C). Small letters for T1 (a, b, c) or capital letters for T2 (A, B) indicate significant (p<0.05) differences between the groups. F) Heat map of all lipid classes using the negative logarithm of the models p-values (from nested ANOVA model) spanning from white (not significant) to greyscale (p=0.05 on scale 1.3) to black (p<0.001). SM; Sphingomyelin, PC; Phosphatidylcholine, PI; Phosphatidylinositol, PS; Phosphatidylserine, CL; Cardiolipin, PE; Phosphatidyl-ethanolamine, DAG; Diacylglycerol, CH; Cholesterol, FAs; fatty acids; TAG; Triacylglycerol.

Figure 4ABC Đose response curves showing significant multivariate regression model relationships between dietary CLP-m intake using log \((x+1)\) of CLP-m intake (mg CLP-m kg\(^{-1}\) body weight per day\(^{-1}\)) as the abscissa and A) Arachidonic acid (ARA 20:4n-6) in liver phospholipids (PLs) B) Palmitic acid (PA 16:0) in liver PLs as the ordinates after 30 days (T1) and 67 days (T2) of feeding four diets with increasing levels of the pesticide CLP-m to Atlantic salmon postsmolt. Whole lines (T1) and dotted lines (T2). Each value represents the mean ± confidence interval (CI) of 12 individuals. Model outputs are found in the supplementary material (Appendix A, Table 3 and appendix C). Small letters for T1 (a, b) or capital letters for T2 (A, B) indicate significant (p<0.05) differences between the groups. C) Heat map of FAs using the negative logarithm of the models p-values (from nested ANOVA model) spanning from white (not significant) to greyscale (p=0.05 on scale 1.3) to black (p<0.001). PLs; Phospholipids, NLs, Neutral lipids.

Figure 5 A) Shows the standardized predefined area of Atlantic salmon after fat subtraction. The orientation of the liver, size of the swim bladder and quantity of pyloric sacs were used to standardize the images for each fish. B) Shows the standardized abdominal region of interest in Atlantic salmon after converting the image to a binary 8-bit scale with background dark and lipid signal white. C) The total dense lipid in whole fish expressed as megapixels with the abdominal dense lipid D) correlation between MRI quantification of abdominal dense lipid and chemically quantification of whole fish lipid.
Figure 6 Proposed mechanisms in Atlantic salmon liver PL membrane exposed to dietary CHL-m. A) **Mechanisms of homoviscous adaption** (Sinensky, 1974). The incorporation of lipophilic insecticide CHL-m into phospholipid (PL) bilayer would change the membrane fluidity. An increase in saturated fatty acid (FA), palmitic acid (PA 16:0) and decrease in polyunsaturated FA, arachidonic acid (ARA 20:4n-6) iFign the PL membrane might be a mechanism of homoviscous adaption to the higher membrane fluidity caused by CHL-m incorporation. Free ARA acts as a substrate for eicosanoid production. Would ultimately result in membrane damage and inflammation. B) **Mechanism of PL remodeling** (Lands, 1958). Lipophilic insecticide CHL-m could directly change the phospholipase A (PLA2) and lysophospholipid acyltransferases (LPLATs) activity. Increased PLA2 and decreased LPLATs activity would affect the concentration of membrane PLs, lysolipids and free FAs. Free ARA acts as a substrate for eicosanoid production. Would ultimately result in membrane damage and inflammation.