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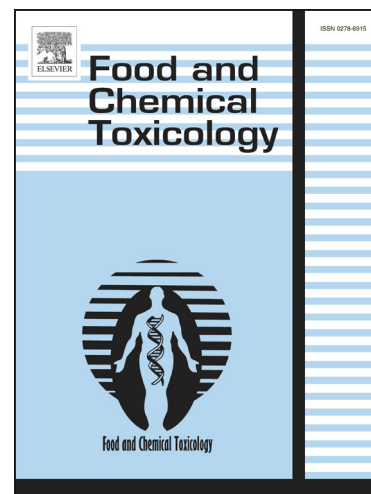
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Toxicological effect of single contaminants and contaminant mixtures associated with plant ingredients in novel salmon feeds

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Highlights

Atlantic salmon primary hepatocytes were used to screen for interaction effects caused by PAHs and pesticides.

Lipidomic and transcriptomic profiling suggested perturbation of lipid metabolism and endocrine disruption.

The pesticides gave the strongest responses, despite having less effect on cell viability than the PAHs.

The primary mixture effect was additive.

At high concentrations, the pesticides acted synergistic by decreasing cell viability and down-regulating CYP3A and FABP4.

1 **Toxicological effect of single contaminants and contaminant mixtures associated with**
2 **plant ingredients in novel salmon feeds**

3

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12

13 **Abstract**

14

15 Increasing use of plant feed ingredients may introduce contaminants not previously associated
16 with farming of salmonids, such as pesticides and PAHs from environmental sources or from
17 thermal processing of oil seeds. To screen for interaction effects of contaminants newly
18 introduced in salmon feeds, Atlantic salmon primary hepatocytes were used. The
19 xCELLigence cytotoxicity system was used to select optimal dosages of the PAHs
20 benzo(a)pyrene and phenanthrene, the pesticides chlorpyrifos and endosulfan, and
21 combinations of these. NMR and MS metabolic profiling and microarray transcriptomic
22 profiling was used to identify novel biomarkers. Lipidomic and transcriptomic profiling
23 suggested perturbation of lipid metabolism, as well as endocrine disruption. The pesticides
24 gave the strongest responses, despite having less effect on cell viability than the PAHs. Only
25 weak molecular responses were detected in PAH-exposed hepatocytes. Chlorpyrifos
26 suppressed the synthesis of unsaturated fatty acids. Endosulfan affected steroid hormone
27 synthesis, while benzo(a)pyrene disturbed vitamin D3 metabolism. The primary mixture
28 effect was additive, although at high concentrations the pesticides acted in a synergistic
29 fashion to decrease cell viability and down-regulate CYP3A and FABP4 transcription. This
30 work highlights the usefulness of ‘omics techniques and multivariate data analysis to
31 investigate interactions within mixtures of contaminants with different modes of action.

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37 **Keywords:** Atlantic salmon, PAH, pesticides, metabolomics, synergy, toxicogenomics

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39 **1. Introduction**

40

41 Marine fish oils was traditionally the main source of the persistent organic environmental
42 pollutants (POPs) in salmon feed and farmed Atlantic salmon (*Salmo salar* L.) (Berntssen et
43 al., 2010). Replacing marine ingredients with plant ingredients has reduced the levels of these
44 traditional POPs in salmon feeds, but as a consequence introduced a new cocktail of plant-oil
45 derived contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and pesticides, that
46 have not previously been associated with farming of salmonids (Berntssen et al., 2010; Glover
47 et al., 2007). The introduction of these contaminants to salmon feeds has led to concerns
48 about potential effects on fish health, including interactions with nutritional pathways. Plant
49 oils may be contaminated with PAH during the thermal processing of the oil seeds or
50 indirectly from environmental sources, such as exhaust gases from traffic or from atmospheric
51 particles deposited on the crops during growth (Fromberg et al., 2007; SCF, 2002). The
52 combustion process of oil seeds cause a predominant increase in 2-3 ring PAHs (e.g.
53 phenanthrene), while 4-5 ring PAHs (e.g. benzo(a)pyrene) are present to a lesser extent in the
54 plant crude oils (Teixeira et al., 2007; Dennis et al., 1991). In a feeding trial, a 36% increase
55 of phenanthrene was detected in the fillets of Atlantic salmon fed alternative plant feed (3.2
56 $\mu\text{g kg}^{-1}$ or 0.018 μM) compared to fish fed traditional marine fish feed (2.4 $\mu\text{g kg}^{-1}$ or 0.013
57 μM). In addition, the levels of benzo(a)pyrene increased from not being detected in the
58 traditional Atlantic salmon fillets to low concentrations being detected (0.3 $\mu\text{g kg}^{-1}$ or 0.0012
59 μM) in the plant fed fish fillets (Berntssen et al., 2010). The acute toxicity of PAH in exposed
60 rainbow trout (*Oncorhynchus mykiss*) and largemouth bass (*Micropterus salmonides*) is
61 known to increase with increasing number of aromatic rings (Black et al., 1983).
62 Phenanthrene is therefore considered to have a relatively low toxicity. Phenanthrene is a non-
63 cytochrome P450 1A (CYP1A)-inducing PAH, with aryl hydrocarbon receptor (AhR)
64 independent toxicity (Pathiratne and Hemachandra, 2010; Johnson et al., 2008), while the 4-5
65 ring PAHs have an AhR dependent mode of action. The main toxicological effects of PAHs,
66 however, are in their genotoxicity and potential endocrine disruption in teleosts (Donnelly and
67 Naufal, 2010; van der Oost et al., 2003; Johnson et al., 2008). Suppressed steroid levels and
68 steroid synthesis inhibition (Monteiro et al., 2000; Seruto et al., 2005; Yan et al., 2012) have
69 been detected in PAH-exposed teleosts as well as retinoid signalling disruption (Benisek et
70 al., 2011).

71

72 Endosulfan and chlorpyrifos are pesticides used on crops, and residue levels have been
73 reported in products from plants such as soya or maize (Jergentz et al., 2005; Marchis et al.,
74 2012) that are commonly used as ingredients in salmon feeds (Berntssen et al., 2007). In
75 2011, the concentration range measured in farmed Atlantic salmon were 0.2-5.8 µg/kg
76 (0.0005-0.014 µM) of α-endosulfan and 0.2-1.2 µg/kg (0.0005-0.003 µM) of β-endosulfan
77 (NIFES, 2014) while chlorpyrifos-methyl has recently been detected in salmon feed (Nácher-
78 Mestre et al., 2014). These pesticides act as endocrine disruptors (Krøvel et al., 2010;
79 Grünfeld and Bonefeld-Jorgensen, 2004). Disturbed steroid production and steroid
80 biosynthesis (Angelis et al., 2009; Viswanath et al., 2010) as well as histopathological
81 changes have been reported in a variety of fish species exposed to chlorpyrifos (Deb and Das,
82 2012). Adverse effects like liver metabolic perturbations (Ashad et al., 2007; Glover et al.,
83 2007; Krøvel et al., 2000) and disturbed lipid metabolism such as steatosis have been detected
84 in endosulfan exposed Atlantic salmon *in vitro* and *in vivo* (Krøvel et al., 2010; Glover et al.,
85 2007). Elevated ethoxyresorufin O-deethylase activity (EROD) has been observed in
86 endosulfan exposed Atlantic salmon *in vivo* (Glover et al., 2007).

87

88 *In vitro* models are useful supplements to animal models for the evaluation of underlying
89 mechanisms of drugs and contaminants, and for interaction studies (Bouhifd et al., 2012; Xia
90 et al., 2008; Judson et al., 2010; Walum et al., 2005; Søfteland et al., 2011). To ensure optimal
91 non-cytotoxic exposure concentrations for *in vitro* assessments, cell viability and dose-
92 response curves of well-known transcriptional markers are often evaluated (ISO, 2009;
93 Judson et al., 2010; Søfteland et al., 2011). The xCELLigence system use impedance-based,
94 continuous real-time assessment of cytotoxicity and mode of action, and is especially suitable
95 to determine when, and at which concentration, to collect cells for downstream analyses (Xia
96 et al., 2008; Atienzar et al., 2011; Judson et al., 2010; Walum et al., 2005). The xCELLigence
97 system has an equal, or even higher, cytotoxicity sensitivity than the standardised methods
98 certified by ISO (Atienzar et al., 2011; Ceriotti et al., 2007) and has been used in large-scale
99 screening of toxicants (Judson et al., 2010; Xia et al., 2008). In feed safety evaluations, a
100 contaminant-by-contaminant approach has traditionally been applied in the risk assessment.
101 This approach may however be inappropriate in animals exposed to a cocktail of
102 contaminants (Bandelet et al., 2012; Kortenkamp and Altenburger, 2011). A toxicological
103 effect of a mixture can be greater (synergistic interaction) or lesser (antagonistic interaction)
104 than expected, and these outcomes are often difficult to predict. This is especially true when
105 mixtures are composed of contaminants with differing modes of action and knowledge

106 regarding such contaminant mixtures effects is in general lacking (Kortenkamp and
107 Altenburger, 2011).

108

109 To gain toxicological knowledge about contaminants found in elevated levels in novel plant-
110 based salmon feeds, the aim of this *in vitro* study was to screen for interaction effects using
111 metabolomic, lipidomic and transcriptomic profiling. To ensure we used non-cytotoxic
112 exposure concentrations, and to find the most potent mixture concentrations, the
113 xCELLigence system was applied for cytotoxicity assessment. RT-qPCR gene expression
114 analysis of well-known and new biomarkers were used for contaminant dose-response
115 determination and interaction evaluation. Atlantic salmon primary hepatocytes were selected
116 as an experimental model.

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131 **2. Materials and Methods**

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133 *2.1 Chemicals*

134 Endosulfan (6,7,8,9,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-metano-2,4,3-benza-
135 dioxathiepin-3-oxide, $\alpha + \beta \sim 2 + 1$; PESTANAL[®], analytical standard), chlorpyrifos (O,O-
136 diethyl-O-3,5,6-trichlor-2-pyridyl phosphorothioate, PESTANAL[®], analytical standard),
137 phenanthrene ($\geq 98\%$ pure) and benzo(a)pyrene ($\geq 96\%$ pure) were all purchased from Sigma-
138 Aldrich (Oslo, Norway). Dimethyl sulfoxide (DMSO) stock solution was purchased from
139 Scientific and Chemical Supplies Ltd. (Bilston, UK), chloroform (HPLC grade) was
140 purchased from Fisher Scientific (Loughborough, UK) and ammonium acetate was purchased
141 from Sigma-Aldrich Co. Ltd (Dorset, UK).

142

143 *2.2 Isolation of primary cultures of hepatocytes*

144 Juvenile Atlantic salmon (*Salmo salar* L.) were obtained and kept at the animal holding
145 facility at the Institute of Marine Research, Bergen, Norway at Havbruksstasjonen, Matre.
146 The fish were fed once daily with a special feed produced without addition of synthetic
147 antioxidants and with low levels of contaminants, supplied by EWOS, Norway (Harmony
148 Nature Transfer 75). Feed concentrations of chlorpyrifos, endosulfan, benzo(a)pyrene and
149 phenanthrene were all under the level of quantification. All glassware, instruments and solutions
150 were autoclaved prior to liver perfusion. Hepatocytes were isolated from 8 Atlantic salmon
151 (325-515g) with a two-step perfusion method previously described in Søfteland et al. (2009).
152 The final cell pellet was resuspended in L-15 medium containing 10% fish serum (FS) from
153 salmon (Nordic BioSite, Oslo, Norway), 1% glutamax (Invitrogen, Norway) and 1%
154 penicillin-streptomycin-amphotericin (10000 units/ml potassium penicillin, 10000 $\mu\text{g}/\text{ml}$
155 streptomycin sulfate and 25 $\mu\text{g}/\text{ml}$ amphotericin B.) (Lonzo, Medprobe, Oslo, Norway). The
156 Trypan Blue exclusion method, performed in accordance with the manufacturer's protocol
157 (Lonzo), was used to determine cell viability. The different cell suspensions used in this study
158 had cell viability between 83-94%. The cell suspensions were plated on 2 $\mu\text{g}/\text{cm}^2$ laminin
159 (Sigma-Aldrich, Oslo, Norway) coated culture plates (TPP, Trasadingen, Switzerland), and
160 the hepatocytes were kept at 10°C in a sterile incubator without additional O₂/CO₂ (Sanyo,
161 CFC FREE, Etten Leur, Netherland). The following cell concentrations were used; 7.2×10^6

162 cells per well in 6-well plates (in 3 ml complete L-15 medium), 2.6×10^6 cells per well in 12-
163 well plates (in 2 ml complete L-15 medium), 0.2×10^6 cells per well in xCELLigence 96-well
164 plates (in 0.2 ml complete L-15 medium).

165

166 *2.3 Chemical exposure*

167 The primary cells were cultured for 36-40 hrs prior to chemical exposure with a change of
168 medium (containing 10% FS) after 18-20 hrs. The cells were exposed for 24 hrs to single
169 contaminants, i.e. endosulfan, phenanthrene and benzo(a)pyrene (0.01, 0.1, 1, 10, 100 μM),
170 chlorpyrifos (0.1, 1, 10, 100, 1000 μM) or to simple mixtures of endosulfan, phenanthrene,
171 benzo(a)pyrene and chlorpyrifos according to a factorial experimental design. A full factorial
172 design was used with low (1 μM) and high (100 μM) concentrations, a zero (0.4% DMSO
173 control) concentration, and one centre point (50.5 μM) in order to evaluate linearity (Table 1).
174 The concentrations used for the factorial design were determined from the cell viability and
175 RT-qPCR dose-response curves (Fig. 1-5). 1 μM was chosen as the low concentration for all
176 contaminants due to the up-regulation observed for CYP1A and CYP3A at this concentration
177 by benzo(a)pyrene, endosulfan and chlorpyrifos, in addition to that 1 μM was the lowest
178 concentration that gave a significant cell viability reduction for phenanthrene. The high (100
179 μM) concentration was chosen since all contaminants, except chlorpyrifos gave a significant
180 cell viability reduction at this concentration and since all contaminants significantly up-
181 regulated vitellogenin (VTG) and/or fatty acid binding protein 4 (FABP4) at this
182 concentration. Cells from three fish were used for cell viability and RT-qPCR dose-response
183 curves evaluation in a preliminary experiment. In a second experiment cell viability, RT-
184 qPCR, metabolomics, lipidomics and microarray analysis were used to evaluate cells
185 toxicological response when exposed to individual contaminants, accordingly to a full
186 factorial design (Table 1) or to selected contaminant mixtures from the design. Cells from five
187 additional fish were employed. The exposure medium contained 1% FS. The exposure
188 medium was substituted with new medium after 18-20 hrs and the chemical exposure was
189 sustained for another 24 hrs. The lowest concentration (0.01 μM) used in the dose-response
190 curves with endosulfan and phenanthrene corresponds to actual levels measured in Atlantic
191 salmon fillets (Berntssen et al., 2010; NIFES, 2014).

192

193 2.4 Cytotoxicity testing of chemicals

194 For the cytotoxicity assessment of the four chemical compounds, real time impedance data
195 obtained by the xCELLigence systems (Roche Diagnostics, Oslo, Norway) was used. The
196 xCELLigence system quantifies electrical impedance across electrodes in 96-well cell culture
197 E-Plates. The impedance measurement gives quantitative information regarding cells'
198 biological status including morphology, cell number and viability. Optimal plate coating
199 conditions and cell density were determined in preliminary experiments (data not shown).
200 After a background reading was measured, the appropriate number of cells was added to the
201 plate. The cells were allowed to attach at room temperature (30 min) before the plate was
202 placed on the xCELLigence plate reader in the cell incubator for continuous impedance
203 recording. The real time cell monitoring was conducted at 10°C in an incubator without
204 additional O₂/CO₂ (Sanyo, CFC FREE, Etten Leur, Netherland), using the RTCA single plate
205 xCELLigence platform. The data was collected with intervals of 2 min after contaminant
206 exposure for 12 hrs, then every 15 min for 120 h. The cell index (CI) is a parameter that is
207 derived from the measured cell-electrode impedance data that quantifies the status of the cells
208 (Abassi et al. 2009). Generally, when cells attach onto the electrodes, the CI value increases.
209 A decrease in CI correlates to cell detachment. However, changes in cell morphology will
210 affect the CI. A normalized CI (NCI) at a specific time point is calculated by dividing the CI
211 at that particular time by the CI of a reference time point which is set to 1. The last time point
212 before compound exposure was used for the normalization, allowing a more precise
213 comparison of the control versus effect of the different contaminant concentrations tested. The
214 CI values presented here were calculated from three or five replicate values. Determination of
215 cytotoxic effects was done according to the International standardised test for in vitro
216 cytotoxicity, ISO 10993-5:2009 (ISO, 2009). Contaminant will be deemed cytotoxic when
217 cells viability exceeds 30% reduction compared to the control.

218

219 2.5 Metabolomics and lipidomics

220 2.5.1 Metabolite extraction and NMR spectroscopy

221 Lyophilized samples were extracted using a 1145 µl mixture of chloroform:methanol:water
222 (2:2:1.8) and vortexed in 2 ml glass vials. The polar and non-polar phases of this bi-phasic
223 mixture were separated, and the polar phase (500 µl) was vacuum centrifuged (30 min at

224 300K), frozen and freeze dried, for Nuclear magnetic resonance spectroscopy (NMR)
225 analysis. For the non-polar phase, 300 μ l were evaporated under N_2 and stored at $-80^\circ C$
226 before shipment on dry ice for MS analysis.

227 Subsequently the dried polar metabolite fraction was resuspended in 200 μ l D_2O with 1 mM
228 TMS P and transferred to NMR tubes. All samples were maintained at 277 K and analyzed
229 within 48 hrs of resuspension. NMR was performed on a Bruker DRU 600 NMR spectrometer
230 (600.23 MHz for 1H) fitted with a 5 mm CPQCI cryogenic probe (Bruker Corporation). Three
231 mm NMR tubes were used with the Bruker Sampletrack autosampler in which the samples
232 were kept at 279 K before (and after) analysis. The spectra were recorded at 300 K with
233 suppression of the residual water resonance using the noesygprr1d pulse sequence from the
234 Bruker pulse sequence library. A pulse width of 7.91 μ s was used to collect 128 free induction
235 decays with 32K data points with a spectral window of 12,019 Hz (20 ppm). The acquisition
236 time was 2.73 s and the interscan delay was 3 s. The noesy mixing time was 10 ms. The data
237 were zero filled to 64K and exponential line broadening of 0.3 Hz applied before Fourier
238 transformation. The spectra were phased and baseline corrected.

239

240 2.5.2 FT-ICR mass spectrometry

241 All dried lipid samples were resuspended in an equal volume of 2:1 methanol:chloroform with
242 5 mM ammonium acetate. Lipidomic analyses were conducted in negative ion mode using a
243 hybrid 7-T FT-ICR mass spectrometer (LTQ FT Ultra, Thermo Fisher Scientific, Bremen,
244 Germany) with a chip-based direct infusion nanoelectrospray ionisation assembly (Triversa,
245 Advion Biosciences, Ithaca, NY). Nanoelectrospray conditions comprised of a 200 nL/min
246 flow rate, 0.4 psi backing pressure and -1.2 kV electrospray voltage controlled by ChipSoft
247 software (version 8.1.0). Mass spectrometry conditions included an automatic gain control
248 setting of 5×10^5 and a mass resolution of 100,000. Analysis time was 4.25 min (per technical
249 replicate), controlled using Xcalibur software (version 2.0, Thermo Fisher Scientific). Spectra
250 were collected using the "SIM stitching" method, i.e. acquisition of fourteen overlapping
251 selected ion monitoring (SIM) mass ranges that were subsequently fused together, ranging
252 from m/z 70 to 2000 (Southam et al., 2007, Weber et al., 2011). Each sample was analysed in
253 triplicate. A quality control (QC) sample consisting of a pooled aliquot of the samples was
254 analysed repeatedly throughout the batch of samples.

255

256 *2.6 Microarray and Quantitative real-time RT-PCR*257 *2.6.1 RNA extraction*

258 The RNeasy Plus mini kit (Qiagen, Crawley, UK) was used to extract total RNA according to
259 the manufacturer's protocol. RNA was eluted in 30 μ l RNase-free MilliQ H₂O and stored at -
260 80°C. The RNA quantity and quality were assessed with the NanoDrop® ND-1000 UV-Vis
261 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100
262 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) pursuant to the manufacturer's
263 instructions. The integrity of the RNA was evaluated with the RNA 6000 Nano LabChip® kit
264 (Agilent Technologies). The samples used in this experiment had 260/280nm absorbance
265 ratios that varied between 1.76 and 2.41, 260/230 nm ratios above 2 and RNA integrity
266 number (RIN) values above 9.5, which indicate pure RNA and intact samples (Schroeder et
267 al., 2006).

268

269 *2.6.2 Microarray; target synthesis and microarray hybridization*

270 For experimental samples, anti-sense amino-allyl RNA (aaRNA) was amplified from the
271 original individual column-cleaned total RNA samples using the Message Amp II aRNA
272 Amplification kit (Ambion, Life Technologies, Burlington, ON), following the manufacturer's
273 instructions. Only one round of amplification was necessary and it was carried out with 1 μ g
274 of total RNA input. For the common reference, an equal contribution of every sample
275 involved in the experiment was pooled and 1 μ g of this pool was used in four amplification
276 reactions. The resulting amplified aaRNA from each reaction was pooled to make a final
277 common reference. Amplified aaRNA quality and quantity was measured using UV
278 spectrophotometry and agarose gel electrophoresis, respectively. Anti-sense amino-allyl RNA
279 was labelled with either Cy3 or Cy5 (GE Healthcare, Mississauga, ON) following the
280 manufacturer's instructions with minor modifications. Twenty μ g of amplified aaRNA was
281 precipitated overnight following standard molecular biology procedures and re-suspended in
282 coupling buffer (Ambion, Life Technologies); the resulting solution was used in the labelling
283 reaction following the manufacturer's protocol. Experimental individuals were labelled with
284 Cy5 and the common reference was labelled with Cy3. Labelled aaRNA was purified using

285 the columns supplied with the kit, and labelling efficiency was measured using the
286 "microarray" function of the NanoDrop (ThermoFisher, Mississauga, ON).

287

288 Agilent 4-by-44,000 oligonucleotide probes (4x44K) custom salmonid microarrays designed
289 by the consortium for Genomic Research in All Salmonids Project (cGRASP) (Jantzen et al.,
290 2011) were used in this experiment (GEO accession # GPL11299). Hybridizations were
291 carried out following the manufacturer's instructions using 825 ng of each labelled sample
292 (i.e. one experimental sample and one reference sample) per array and the HI-RPM
293 hybridization buffer (Agilent, Mississauga, ON). Hybridizations were carried out for 16 hrs at
294 65°C with 10 rpm rotation in an Agilent hybridization oven. Following hybridizations, arrays
295 were washed following the manufacturer's instructions. Arrays were scanned using a Perkin
296 Elmer ScanArray Gx Plus at 5 µm resolution and laser power at 90%. If the average signal
297 intensity between channels was not within 300 photomultiplier tube settings (PMTs) were
298 adjusted to balance the channel in subsequent scans. Fluorescence intensity data was extracted
299 from TIFF image files using Imagen v8.5 (BioDiscovery, El Segundo, CA).

300

301 *2.6.3 Quantitative real-time RT-PCR*

302 The transcriptional levels of selected target genes were quantified with a two-step real-time
303 reverse transcription polymerase chain reaction (RT-PCR) protocol. A serial dilution curve of
304 total RNA with six points in triplicates between 1000 – 31 ng were made for PCR efficiency
305 calculations. 500 ng of total RNA was added to the reaction for each sample, and reverse
306 transcription (RT) reactions were run in duplicates using 96-well reaction plates. No-template
307 control (ntc) and no-amplification control (nac) reactions were run for quality assessment for
308 every gene assay. The 50 µl RT reactions were performed at 48°C for 60 min utilizing a
309 GeneAmp PCR 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Individual
310 RT reactions contained 1X TaqMan RT buffer (10X), 5.5 mM MgCl₂, 500 mM dNTP (of
311 each), oligo dT primers (2.5 µM), 0.4 U/µl RNase inhibitor and 1.67 U/µl Multiscribe Reverse
312 Transcriptase (Applied Biosystems) and RNase-free water.

313

314 For every gene analysed, quantitative real-time RT-PCR (real-time qPCR) was run in 10 μ l
315 reactions on a LightCycler® 480 Real-Time PCR System (Roche Applied Sciences, Basel,
316 Switzerland) containing 2.0 μ l cDNA (diluted twofold). The real-time qPCR was carried out
317 in two 384-well reaction plates using SYBR Green Master Mix (LightCycler 480 SYBR
318 Green master mix kit, Roche Applied Sciences, Basel, Switzerland) containing gene-specific
319 primers and FastStart DNA polymerase. PCR runs were performed with a 5 min activation
320 and denaturing step at 95°C, followed by 45 cycles with each cycle consisting a 10 s
321 denaturing step at 95°C, a 10 s annealing step and finally a 10 s extension step at 72°C. The
322 primer pairs had an annealing temperature of 60°C; see Table 2 for primer sequences,
323 amplicon sizes and GenBank accession numbers. Final primer concentrations of 500 nM were
324 used. For confirmation of amplification of gene-specific products, a melting curve analysis
325 was carried out and the second derivative maximum method (Tellmann, 2006) was used to
326 determine crossing point (CT) values using the Lightcycler 480 Software. To calculate the
327 mean normalized expression (MNE) of the target genes, the geNorm VBA applet for
328 Microsoft Excel version 3.4 was used to calculate a normalization factor based on three
329 reference genes. By using gene-specific efficiencies calculated from the standard curves, the
330 CT values are converted into quantities (Vandesompele et al., 2002). Elongation factor 1 AB
331 (EF1AB) and acidic ribosomal protein (ARP) and β -actin were the selected reference genes
332 for this experiment. The reference genes were stable with gene expression stability (M) values
333 of 0.38.

334

335 *2.7 Data analysis*

336 *2.7.1 Metabolomics*

337 The processed NMR spectra were imported into Matlab (The Mathworks, Inc.) using
338 Prometab v3.3 software (Viant et al. 2003). The region from 10 to 0.5 ppm was imported
339 with a resolution of 0.02 ppm which resulted in 4750 data points, and transformed using a
340 generalized log transformation. Principal Component Analysis (PCA) and Partial Least
341 Squares Discriminant Analysis (PLS-DA) was performed in PLS-toolbox v7.0.1 (Eigenvector
342 Research, Inc.) on normalized and mean centered data prior to multivariate statistical
343 analyses.

344

345 *2.7.2 Lipidomics*

346 All analyses were performed in Matlab 7.8.0 with PCA and PLS-DA analysis performed in
347 PLS-toolbox v.6.7.1. Mass spectra were processed using a three-stage filtering algorithm as
348 described in Payne et al. (2009). Samples were subsequently normalised using probabilistic
349 quotient normalisation (Dieterle et al., 2006). Processing the raw mass spectra yielded a
350 dataset that was further optimised using a QC based method as described in Kirwan and
351 Broadhurst et al. (2013). Mass features with over 20% missing values across all samples were
352 removed and the resulting intensity matrix was submitted for univariate statistical analysis as
353 described below. A k-nearest neighbour approach (Hrydziuszko et al 2012) was applied to
354 impute missing values to the same dataset and it was transformed using a generalized log
355 transformation prior to multivariate statistical analysis. The final lipidomics dataset was
356 comprised of 1603 mass features upon which statistical analyses were conducted. PCA and
357 PLSDA were performed to assess the overall effect (<http://CRAN-R-project.org>). All
358 supervised models were validated using cross validation and permutation testing to avoid
359 over-fitting. Univariate statistical analyses were conducted on the lipidomics dataset. An
360 analysis of variance (ANOVA) followed by Games-Howell (GH) post hoc testing (Games and
361 Howell 1976) was applied across the control and the highest doses of each of the four
362 contaminants using a custom adapted version of freely available Matlab scripts (Trujillo-Ortiz
363 and Hernandez-Walls 2003). ANOVA and GH post hoc testing was also applied across all
364 doses of the contaminant mixtures against control. ANOVA was also applied to compare the
365 control against the low and high doses for each individual contaminant. A Benjamini-
366 Hochberg false discovery correction of 10% was applied to all univariate statistical results
367 (Benjamini and Hochberg 1995).

368

369 Lipidomic pathway analysis was conducted according to Kanehisa (2008) utilising pathways
370 listed in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. For each mass
371 feature, KEGG and MI-Pack software were used to assign a putative empirical formula(e) and
372 identity based on accurate mass. To measure the perturbation of each pathway, the empirical
373 formulae associated with the significantly changing mass features were compared against the
374 total number of detected empirical formulae in that pathway to give a “percent of empirical
375 formulae perturbed” for each treatment.

376

377

378 *2.7.2 Microarray data pre-processing and analysis*

379 Data pre-processing and normalization were carried out in R using the mArray package. Print-
380 tip Loess normalization (i.e. per sub-grid), threshold setting and removal of low-
381 quality/flagged spots were done as described in Booman et al. (2011) and Hori et al. (2012).
382 After spot quality filtering, features absent in more than 30% of the arrays were discarded and
383 not used in the analysis, resulting in a final list of 9469 probes. Missing data for the 9469
384 probes was imputed using LSImpute (Bo et al., 2004; Celton et al., 2010) as previously
385 described (Hori et al., 2012). Differentially expressed genes between controls and exposed
386 groups were identified using the rank products algorithm (Breitling et al., 2004) as
387 implemented in the RankProd (Hong et al., 2006) R package with a percentage of false
388 positives (PFP) of 10%. A Rank Product test is demonstrated to be a robust method for
389 microarray experimental designs with few replicates (Jeffery et al, 2006).

390

391 *2.7.3 xCELLigence and RT-qPCR data*

392 GraphPad Prism 6.0 software (GraphPad Software Inc., Palo Alto, CA, USA) was used for the
393 statistical analyses of the xCELLigence and the RT-qPCR dose-response curves evaluation
394 using one-way ANOVA followed by a Dunnett's post hoc test ($p < 0.05$) to detect treatment
395 variation in contaminant-exposed hepatocytes. Mean \pm SE were calculated for three or five
396 replicates. For the statistical analyses of the VTG RT-qPCR dose-response curve, Student's t-
397 test ($p < 0.05$) was used to detect significant difference between the highest chlorpyrifos
398 concentration and the 0.4% DMSO control hepatocyte cell cultures. Regression was
399 performed with PLS (Wold et al., 1984) to correlate the design matrix to the responses of
400 different transcripts. Modde 9.0 (Umetrics, Umeå, Sweden) was used for the experimental
401 design and the PLS analysis. Before the PLS analysis the blend matrix was augmented with
402 interaction terms, the data were scaled to unit variance and mean centred. The PLS models
403 were validated with respect to explained variance and goodness of prediction (shown as Q^2),
404 obtained after cross validation (Wold, 1978). The PLS model was in addition evaluated with
405 respect to goodness of fit (R^2).

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414 3. Results

415

416 3.1 Cytotoxicity screening and RT-qPCR biomarker screening

417 3.1.1 Individual exposures

418 3.1.1.1 Cytotoxicity screening

419 Primary Atlantic salmon hepatocytes were used to establish cytotoxic dose-response curves
420 for the two individual PAHs benzo(a)pyrene and phenanthrene, and for the two individual
421 pesticides chlorpyrifos and endosulfan. With the xCELLigence cytotoxicity system,
422 phenanthrene (Fig. 1A) was the most potent compound, significantly decreased cell viability
423 of 21%, 25%, and 22% at the three highest exposure concentrations (1-100 μ M) respectively.
424 Cell viability was only significantly reduced at the highest exposure concentrations (100 μ M)
425 for benzo(a)pyrene with 25% (Fig. 1B) and endosulfan (Fig. 1C) 24% compared to the 0.4%
426 DMSO control. None of the applied chlorpyrifos concentrations (0.1-1000 μ M) induced a
427 significant cell viability reduction effects in the hepatocytes (Fig. 1D). No significant
428 difference was found between the medium control and the 0.4% DMSO control, and therefore
429 the medium control was not included in Fig. 1A-1D.

430

431 3.1.1.2 RT-qPCR biomarker screening

432 To ensure we were working in a relevant concentration range for Atlantic salmon primary
433 hepatocytes, dose-response curves were established for the four selected biomarkers CYP1A,
434 CYP3A, VTG and FABP4. Chlorpyrifos (Fig. 2) gave a bell-shaped CYP1A transcript up-
435 regulation pattern, though CYP1A was only significantly up-regulated at 10 μ M with a fold
436 change (FC) of 16.8 ($p=0.001$). The CYP3A transcript did not show a clear dose-response
437 however CYP3A was significantly elevated at 0.1 ($p=0.01$) and 10 μ M ($p=0.5$) of
438 chlorpyrifos. The FABP4 transcript was significantly expressed at 100 μ M chlorpyrifos

439 (p=0.0001) compared to the control. In similarity to FABP4, the VTG transcript was
440 according to the microarray results (data not shown) significantly induced at the highest
441 chlorpyrifos exposure concentration (100 μ M) compared to the control. The ANOVA posthoc
442 analysis of the RT-qPCR data did however not confirm this finding, even though a direct
443 comparison between the control and the 100 μ M group using the t-test suggested this
444 difference was significant (VTG, p=0.05). Endosulfan exposure (Fig. 3) gave significant up-
445 regulation of FABP4 (p=0.05) and VTG (p=0.0001) at a concentration of 100 μ M, with the
446 VTG transcript showing the highest up-regulations with a FC of 17.3. Benzo(a)pyrene (Fig. 4)
447 up-regulated CYP1A transcription (p=0.0001), most profoundly at 1 μ M (FC of 191), with a
448 significant dose-dependent reduction in the expression levels at concentrations of 10 and 100
449 μ M. As for the CYP1A transcript, CYP3A was only significantly up-regulated by
450 benzo(a)pyrene at 1 μ M (p=0.001). In addition, benzo(a)pyrene significantly up-regulated
451 VTG at 100 μ M (p=0.05). No distinct dose-dependent responses were detected in hepatocytes
452 exposed to phenanthrene (Fig. 5), however, the transcripts CYP1A (p=0.05) and VTG
453 (p=0.001) were significantly induced at the highest concentration (100 μ M) investigated.
454 VTG had the highest induction with a FC of 10.9 compared to the control.

455

456 Based on the cell viability data obtained with the xCELLigence system and transcriptional
457 dose-response curves obtained with RT-qPCR, a factorial design with non-cytotoxic
458 concentrations of the contaminants, 1 μ M (low) and 100 μ M (high), were subsequently used
459 in the cytotoxicity mixture toxicity interaction evaluation. The different contaminant mixtures
460 used in the factorial design are presented in Table1.

461

462 3.1.2. Mixture exposures

463 3.1.2.1 Cell viability screening

464 The different contaminant mixtures gave at the most a cell viability reduction of 12%
465 compared to the control, and contaminant mixture 4 and 16 showed the strongest cell viability
466 reduction effect. PLS analysis was performed on the xCELLigence normalized cell index
467 (NCI) values obtained for mixture exposures to benzo(a)pyrene, phenanthrene, chlorpyrifos
468 and endosulfan according to a factorial design. The PLS model (R^2 -value = 0.7 and the Q^2 -
469 value 0.4.) had four negative linear terms for benzo(a)pyrene, phenanthrene, chlorpyrifos and

470 endosulfan, indicating that all chemicals contributed to reduced cell viability, however only
471 two linear terms, for phenanthrene ($p=0.029$) and endosulfan ($p=0.015$), were significant (Fig.
472 6). The model also contained one negative interaction term, for chlorpyrifos and endosulfan
473 which were significant ($p=0.031$). A contour analysis of the xCELLigence cytotoxicity PLS
474 model indicated a synergistic interaction response between chlorpyrifos and endosulfan in
475 mixture-exposed hepatocytes.

476 3.2. *Metabolic profiling*

477 Cells exposed to DMSO (0.4%), 1 μM (low dose) and 100 μM (high dose) of chlorpyrifos,
478 endosulfan, phenanthrene, and benzo(a)pyrene, and contaminant mixtures 1 (1 μM of all
479 contaminants), 4 (100 μM of the PAHs and 1 μM of the pesticides) and 16 (100 μM of all
480 contaminants) were selected to be analysed with metabolomics (water-soluble metabolites)
481 and lipidomics (N=5).

482

483 3.2.1 *Lipidomics*

484 3.2.1.1 *Individual exposures*

485 Unsupervised PCA and supervised PLS-DA data analysis of the lipidomics spectra revealed
486 an apparent separation of the exposed group from the control only at the highest dose (100
487 μM) of the two concentrations analysed for chlorpyrifos ($p=0.007$; Fig. 7A and 7B),
488 endosulfan ($p=0.014$; Fig. 7C and 7D), and benzo(a)pyrene ($p=0.005$; Fig. 7E and 7F). PCA
489 also demonstrated a separation between the control samples and lowest dose of endosulfan.
490 The ANOVA followed by GH post hoc analysis of the high dose exposures of the individual
491 contaminants revealed that chlorpyrifos induced the greatest number of significantly changing
492 mass features with 92 mass features significantly changing compared to the control.
493 Endosulfan induced the second largest perturbation with 22 significant mass features,
494 followed by benzo(a)pyrene (7 significant mass features). Phenanthrene has been removed
495 from the analysis as it induced only one significant perturbation compared to the control. Fig.
496 8A characterises the overlap of the significant mass features across the individual contaminant
497 exposure. Benzo(a)pyrene shared no significant mass features with chlorpyrifos and
498 endosulfan, whereas the pesticides had 6 significant mass features in common, making the
499 pesticides more similar in their mode of action.

500

501 The KEGG pathway analysis (Table 3) of the individual contaminants revealed 10 potentially
502 perturbed pathways, five of which are linked to fatty acid and cholesterol metabolism.
503 Chlorpyrifos may have an affect on the biosynthesis of unsaturated fatty acids (suppressed
504 several desaturase pathways, e.g. $\Delta 9$ and $\Delta 11$ desaturases) (Fig. 9A), linoleic acid metabolism
505 (suppressed desaturase and elongase pathways to produce ARA, as well as increased
506 cytochrom P450 pathway eicosanoid production) (Fig. 9B) and arachidonic acids metabolism
507 (increased eicosanoid production) (Fig. 9C). Endosulfan indicates an affect on primary bile
508 acid biosynthesis and steroid biosynthesis (increased levels of cholesterol for steroid hormone
509 biosynthesis and production of VTG) (Fig. 9D) while benzo(a)pyrene mainly appears to affect
510 steroid biosynthesis (affecting vitamin D metabolism, increased levels of vitamin D
511 metabolites) (Fig. 9D).

512

513 3.2.1.2 Mixture exposures

514 The contaminant mixtures induced a higher number of significantly changing mass features
515 ($q < 0.1$ by ANOVA, $p < 0.05$ by GH), than the individual contaminants except chlorpyrifos. They
516 primarily affected mass features with putative identities that have been linked to the pathways
517 of bile acid biosynthesis and biosynthesis of unsaturated fatty acids. Contaminant mixture 16
518 induced most changes with 149 significant mass features compared to contaminant mixture 1
519 with 57 significant mass features and contaminant mixture 4, the least effective inducer, with
520 39 significant mass features (Fig. 8B). However, when modelling by PLS-DA, only
521 contaminant mixture 4 ($p = 0.031$) and contaminant mixture 16 ($p = 0.002$) could be reliably
522 distinguished from the other classes.

523

524 When comparing the overlap between the significant mass features induced by the high dose
525 of individual contaminants and those induced by the contaminant mixtures, 40% of the mass
526 features induced by the individual contaminants were found to overlap with those induced by
527 the contaminant mixtures (Fig. 8C). When comparing the different contaminant mixtures,
528 70% of significantly changing mass features were uniquely significantly changed with respect
529 to the control only in contaminant mixture 16 which contained the highest doses of all four
530 contaminants. Despite this, none of the features significant in the comparison of the control

531 class and mixture 16 were significantly different in more than two of the individual
532 contaminant classes when compared to mixture 16 suggesting that the combination of
533 contaminants caused an additive effect (Table SII). According to the KEGG pathway
534 analysis, contaminant mixture 16 may affect other pathways not perturbed by the other
535 mixtures but the coverage of these pathways was poor and thus the significance of these
536 perturbations are uncertain. According to the KEGG pathway analysis, contaminant mixtures
537 (Table 3) affected primary bile acid biosynthesis, suppressed biosynthesis of unsaturated fatty
538 acids (Fig. 9 A) and linoleic acid metabolism (Fig. 9B) and steroid biosynthesis (increased
539 levels of cholesterol for steroid hormone biosynthesis and production of VTG) (Fig. 9D).

540

541 3.2.2 Metabolomic (water-soluble metabolites)

542 The PCA and PLS-DA analyses of the polar metabolites showed no significant differences
543 between the control and exposed groups (data not presented).

544

545 3.3 Transcriptomic

546 3.3.1 Mixture exposures

547 3.3.1.1 Microarray analysis

548 In addition to the lipidomic and metabolomic screening, microarray was used for
549 identification of new biomarkers. Contaminant mixture 4 was used for the microarray
550 experiment since it showed the strongest cell viability reduction of the different contaminant
551 mixtures in the cytotoxicity screening. Top rank product lists of differentially expressed
552 features with PFP below 10% in Atlantic salmon hepatocytes exposed to contaminant mixture
553 4 (N=5), are presented in Table 4. In total 17 features were significantly regulated with PFP
554 below 10%, all significantly affected features like microtubule-associated proteins 1A/1B
555 light chain 3B precursor (MAP1LC3B), transcription factor SOX-4 (SOX4) and VTG were
556 up-regulated, and VTG (C065R146) showed the strongest response with a FC of 13.19.

557

558 3.3.1.2 RT-qPCR Contaminants interaction evaluation of mixtures

559 PLS analysis was performed on seven transcripts (Table 5) and the expression levels (MNE)
560 obtained in cells exposed to benzo(a)pyrene, phenanthrene, endosulfan and chlorpyrifos using
561 a factorial design in order to determine possible chemical interactions. VTG, MAP1LC3B,
562 SOX4 were chosen as biomarkers due to their expression levels identified with microarray
563 screening, whereas CYP1A, CYP3A, FABP4, and peroxisome proliferator-activated receptor
564 α (PPAR α) were all target genes evaluated with RT-qPCR that were not identified with the
565 microarray screening.

566 The PLS analysis of three transcripts showed no combined effect between the contaminants in
567 the mixture suggesting that only one of the contaminants was driving the observed response.
568 For example, endosulfan ($p=0.025$) was the only contaminant contributing to VTG up-
569 regulation ($R^2=0.658$ and $Q^2=0.47$), despite the fact that the PAHs and the pesticides' singly
570 induced VTG and that the PLS model contained one negative interaction term for chlorpyrifos
571 and endosulfan ($p=0.02$). The PLS analysis showed that, of the three transcripts that revealed
572 additivity, CYP1A ($R^2=0.93$ and the $Q^2=0.81$) gave the strongest response. However, of the
573 four contaminants only chlorpyrifos ($p=0.0001$) and endosulfan ($p=0.0001$) contributed to the
574 additive transcriptional reduction of CYP1A expression levels. The FABP4 ($R^2=0.95$ and
575 $Q^2=0.66$) was another transcript where additivity was identified and benzo(a)pyrene ($p=0.01$),
576 phenanthrene ($p=0.034$) and chlorpyrifos ($p=0.00007$) all contributed to this additivity.
577 Chlorpyrifos had the largest regression coefficient, and thus had a larger contribution to
578 positive regulation of FABP4. Further, the model had three significant negative interaction
579 terms. A counterplot of the interaction term between phenanthrene and endosulfan ($p=0.022$)
580 showed antagonistic interaction at low concentrations. However, the interaction term for
581 chlorpyrifos and endosulfan ($p=0.0003$) had the largest regression coefficient, and thus had a
582 larger contribution to the regulation of FABP4. The counterplot analysis of this interaction
583 term identified a synergistic interaction between chlorpyrifos and endosulfan on the up-
584 regulation of FABP4 at high concentrations, with increasing phenanthrene concentrations.
585 The PLS model for CYP3A ($R^2=0.83$ and $Q^2=0.49$), the second transcripts for which
586 synergistic interactions were identified, had only two significant terms, one negative linear
587 term for endosulfan ($p=0.0005$) and one negative interaction term for endosulfan and
588 chlorpyrifos ($p=0.042$). A counterplot analysis of the negative interaction term showed a
589 synergistic interaction between the two pesticides on the reduction of CYP3A at high
590 concentration and with increased concentration of phenanthrene.

591

592

593

594

595 **3. Discussion**

596 Cytotoxicity assays are extensively used to assess *in vitro* cell viability in fish cell cultures,
597 including to rank chemical toxicity and to evaluate chemical mixtures (Segner and Braunbeck,
598 2003; Wood et al., 2006). According to the international standard for *in vitro* cytotoxicity
599 testing (ISO, 2009), contaminants are first considered cytotoxic when cell viability are
600 reduced with more than 30%. The four compounds assessed with the xCELLigence system
601 showed only a 0-25% cell viability reduction, therefore, none of the contaminants were
602 cytotoxic in the concentration ranges used in this study. Phenanthrene was the most potent
603 compound, significantly decreasing cell viability with 21-25% at the three highest
604 concentrations (1, 10 and 100 μM). This result is in line with a study of Shirmer et al. (1998)
605 who, in exposed rainbow trout gill cells (RTgill-W1), found that five lighter PAHs with two
606 or three benzene rings, including phenanthrene, gave a stronger cell viability reduction than
607 heavier PAHs such as the five ringed PAH benzo(a)pyrene. Shirmer et al. (1998) suggested
608 that the lipid solubility of heavier PAHs prevents them from being adequately accumulated in
609 cells and membranes. In zebrafish (*Danio rerio*) larvae, phenanthrene and benzo(a)pyrene
610 exposure caused a similar toxic response (Wolinska et al., 2011), suggesting that lighter PAHs
611 can be at least as potent as heavier PAHs.

612

613 Similar to benzo(a)pyrene, the endosulfan xCELLigence screening revealed a dose-dependent
614 reduction of cell viability, which was significant only at the highest exposure concentration
615 (100 μM). This result is in line with previous findings obtained with the MTT cell viability
616 test (0.01-100 μM) in endosulfan-exposed Atlantic salmon primary hepatocytes (Krøvel et al.,
617 2010). Compared to endosulfan, none of the applied chlorpyrifos concentrations (0.1-1000
618 μM) affected cell viability in the exposed hepatocytes. In a cytotoxicity screening with the
619 rainbow trout liver (RTL-W1) and rainbow trout gonadal (RTG-2) cell lines, Babin and
620 Tarazona (2005) found chlorpyrifos (0-8.6 μM) to be the most potent compound of six
621 pesticides tested in a neutral red assay and a FRAME KB protein assay. The reason for this
622 discrepancy in sensitivity between salmonid primary hepatocytes and cell lines to chlorpyrifos
623 is not known.

624

625 Lipidomic and transcriptomics profiling were further employed to generate hypotheses about
626 the potential modes of action of the studied contaminant and contaminant mixtures. The
627 contaminant exposure showed that chlorpyrifos had the most dominant effect on the lipodome
628 despite showing no effect on cell viability. Endosulfan induced the second largest
629 perturbation, followed by benzo(a)pyrene, while phenanthrene, the most potent cell viability
630 reducing contaminant, induced no distinct perturbation according to the lipidomics data.
631 Chlorpyrifos appeared to affect pathways associated with linoleic acid metabolism as well as
632 the biosynthesis of unsaturated fatty acids, whereas endosulfan may affect steroid
633 biosynthesis and primary bile acid biosynthesis pathways. Lipids have important
634 physiological functions in fish, such as structural components in cell membranes, in cell
635 signalling, and in storage of cellular energy (Torstensen et al., 2001). Therefore, the down-
636 regulation of the biosynthesis of unsaturated fatty acids suggests that chlorpyrifos, among
637 other pathways, may disturb energy-requiring metabolic mechanisms (LeBlanc et al., 2012).
638 Inhibition of essential linolenic fatty acid metabolism has previously been seen in
639 norflurazon-exposed rat liver cells (Hagve et al., 1985). Earlier studies have shown impaired
640 fatty acid metabolism (Ortiz-Zarragoitia and Cajaraville, 2005) as well as metabolic
641 perturbation by exposure to pesticides like endosulfan and chlorpyrifos (Demur et al., 2013;
642 Wang et al., 2011) and to PAHs (Van Scoy et al., 2010; Lin et al., 2009) in exposed mammals
643 and salmonids.

644

645 Although eicosanoids can be difficult to detect since they are normally present at low
646 concentrations in biological samples, several putatively annotated eicosanoids in different
647 lipid metabolism pathways was affected by chlorpyrifos exposure in Atlantic salmon
648 hepatocytes. In the arachidonic acid metabolic pathway the putatively annotated eicosanoid
649 tromboxan (11-dehydro-TXB₂), was one of several elevated in the chlorpyrifos exposed
650 primary hepatocytes. 11-dehydro-TXB₂ is an intermediate of TXA₂, which is a
651 vasoconstrictive eicosanoid that has previously been associated with liver injury (Yokoyama
652 et al., 2005). In the linoleic acid metabolism pathway, the putatively annotated eicosanoids
653 9,12-dihydroxy-epoxyoctadecanoate, a precursor of tetrahydrofurandiols (THF-diols), and
654 TriHOME were elevated by chlorpyrifos exposure. These metabolites are involved in
655 inflammatory reactions, cellular energy metabolism and cell homeostasis (Mickalik and
656 Wahli, 2008; Penigrahy et al., 2010). THF-diols are eicosanoids that are involved in

657 inflammatory reactions and are produced by cytochrome P-450 epoxidations and epoxide
658 hydrolase (sEH) mediated hydrolysis (Konkel and Schunck, 2011; Moghaddam et al., 1996).
659 Several CYP enzymes like CYP1A1/2 and CYP3A4 take part in linoleic acid cytochrome
660 P450 eicosanoid biosynthesis in mammalian species (Konkel and Schunck, 2011). According
661 to the RT-qPCR data, both CYP1A (17-fold up-regulated) and CYP3A (1.46-fold up-
662 regulated) transcripts were induced in hepatocytes exposed to chlorpyrifos. A similar CYP-
663 induction was not seen in endosulfan-exposed hepatocytes in this study or in an earlier study
664 by Krøvel et al., (2010). CYP1A and CYP3A, via AhR- and PXR-receptor activation
665 respectively, could therefore be responsible for the biotransformation of chlorpyrifos and the
666 fatty acids and the production of lipid THF-diols (Konkel and Schunck, 2011).

667

668 The fatty acid binding proteins (FABPs) have an essential role in the regulation of the flux of
669 fatty acid in cells, and FABP4 is a well-established marker for inflammation and metabolic
670 syndrome in humans (Cabre et al., 2007; Terra et al., 2011). The putatively identified FABP
671 that may represent the adipose tissue type FABP (h6FABP or FABP11) in fish, an orthologue
672 to mammalian FABP4 (Torstensen et al., 2009), was induced by both pesticides; with
673 chlorpyrifos being the most potent inducer according to the RT-qPCR data. Endosulfan has
674 previously been shown to cause lipid metabolism disturbances such as steatosis, which is
675 triacylglycerols (TAG) accumulation in liver, in both *in vitro* [i.e. in exposed hepatocyte
676 cultures (Krøvel et al., 2010)] and *in vivo* studies (Glover et al., 2007) involving Atlantic
677 salmon. FABP4 was significantly elevated by endosulfan at 100 μ M, the same concentration
678 at which Krøvel et al., (2010) detected accumulation of TAG in endosulfan exposed primary
679 hepatocytes. In humans trophoblasts, increased lipid accumulation was found to be linked to
680 elevated transcription of FABP4 (Duttaroy, 2009; Scifres et al., 2011). FABP4 is
681 transcriptionally activated by the peroxisome proliferator activated receptor γ (PPAR γ)
682 (Michalik and Wahli, 2008), which is the key regulator of adipogenesis (Janesick and
683 Blumberg, 2011). Our results therefore suggest that PPARs may be upstream regulators of the
684 response to chlorpyrifos and endosulfan in exposed hepatocytes, explaining the observed
685 effects on eicosanoid lipids and steroids, which is in line with earlier published results
686 (Michalik and Wahli, 2008; Li and Chiang, 2009; Parkinson and Ogilvie, 2008; Peraza et al.,
687 2006).

688

689 In pesticide and contaminant mixture exposed cells the lipidomics data revealed that the levels
690 of cholesterol, the precursor of steroid hormone biosynthesis, were higher than in the other

691 exposure groups. An effect on steroid hormone biosynthesis was also suggested by the
692 observed induction of a transcript encoding VTG, the primary egg-yolk precursor protein, by
693 the contaminant mixtures and both pesticides at 100 μ M. Endosulfan was the strongest
694 inducer of VTG according to the RT-qPCR data. VTG is normally produced in female fish
695 under estrogenic stimulation of ovarian follicle development (Ekman et al., 2008; Hinton et
696 al., 2008). A number of studies have shown endocrine disruption effects caused by
697 organochlorine and organophosphate pesticides, both possessing the ability to interfere with
698 the estrogen receptor (ER) pathway (Krøvel et al., 2010; Grünfeld and Bonefeld-Jorgensen,
699 2004). The lipid metabolism perturbations induced by endosulfan and chlorpyrifos in male
700 hepatocytes can in part be linked to endocrine disruption due to an increased need for
701 cholesterol for VTG production. Comparable effects have been detected in fish exposed to
702 estrogenic compounds. Earlier fish trials have shown impaired fatty acid metabolism (Ortiz-
703 Zarragoitia and Cajaraville, 2005) and/or effects on cholesterol homeostasis (Bravo et al.
704 1999; Erickson et al. 1989) by dioxin (Fletcher et al., 2005; Moran et al., 2004) and estrogen
705 exposure (Ekman et al., 2008), as well as metabolic perturbation by exposure to pesticides
706 like endosulfan and chlorpyrifos (Demur et al., 2013; Wang et al., 2011).

707

708 The lipidomics data suggested that PAHs, similar to the pesticides and the contaminant
709 mixtures, perturbed steroid biosynthesis by elevating several cholesterol intermediates, and
710 thereby affecting steroid hormone biosynthesis. In benzo(a)pyrene and contaminant mixture
711 exposed cells, higher vitamin D3 levels (putatively identified) were detected when compared
712 to the other exposure groups. Given that fish can only acquire D3 vitamin via its feed, a time-
713 dependent reduction of vitamin D3 levels owing to cells metabolism of the vitamin, suggests
714 that vitamin D3 steroid biosynthesis has been inhibited by these contaminants (Lock et al.,
715 2010). Potent CYP1A inducers, like benzo(a)pyrene, have previously been reported to down-
716 regulate or inhibit estrogen receptor signalling (Kortenkamp, 2007; Yan et al., 2012).
717 However at the highest concentration of benzo(a)pyren (100 μ M), VTG was significantly
718 induced, as shown for chlorpyrifos, which also appears to be a potent CYP1A inducer.

719

720 Despite phenanthrene's lack of lipidomic perturbation and effect on CYP3A expression
721 levels, the RT-qPCR analysis revealed that phenanthrene significantly elevated CYP1A at the
722 highest exposure concentration (100 μ M), indicating that it is a weak CYP1A inducer.
723 Surprisingly, phenanthrene toxicity in fish therefore appears to be induced via the AhR as

724 compared to earlier findings in mammals where phenanthrene toxicity has been found to be
725 independent of the AhR induction (Pathiratne and Hemachandra, 2010; Johnson et al., 2008;
726 Wolinska et al., 2011). Weak CYP1A inducers have previously been found to act differently
727 in fish test systems possibly due to fish-specific CYP1A regulation (Søfteland et al., 2011)
728 with fish having more AhRs than mammals (Hahn and Hestermann, 2008), in addition to low
729 CYP2K/CYP2M and CYP3A induction abilities. In addition, VTG was highly elevated in
730 cells exposed to 100 μ M of phenanthrene, with a FC of 10.9. A similar VTG response was not
731 detected in corresponding studies with zebrafish or medaka (Horng et al., 2009; Wolinska et
732 al., 2011). However, hydroxylated PAHs like 2-hydroxyphenanthrene, which have structural
733 similarities to E2, have been shown to possess estrogenic activity in estrogen-sensitive
734 reporter gene assays (ER-CALUX) (Wenger et al., 2009).

735

736 According to the lipidomics data, additivity appeared to be the dominant mixture effect
737 between the PAHs and the pesticides. The high level of overlap in lipidome perturbation
738 between the individual contaminants and contaminant mixtures, and the increased number of
739 perturbations induced by contaminant mixtures with high levels of pesticides, indicate that the
740 majority of changes in the lipidome induced by the contaminant mixtures represent the sum of
741 the individual contaminants. However, some synergistic activity between the compounds
742 cannot be ruled out. In line with earlier studies with potent CYP1A inducers (van den Berg et
743 al., 2006) and EDCs (Kortenkamp, 2007), the PLS interaction evaluation of the CYP1A and
744 FABP4 transcriptional data, confirmed that additivity was the dominant mixture effect caused
745 by the PAHs and pesticides. However, at high concentrations, synergistic interaction effects
746 were detected. In the contaminant mixture cell viability screening, at high concentrations
747 chlorpyrifos synergistically interacted with and potentiated endosulfan's cytotoxic response,
748 despite having no effect on cell viability alone. A similar synergistic effect on cell viability
749 reduction has previously been shown in Ehrlich ascites tumour cells from Swiss albino mice,
750 in which Thiram, at non-cytotoxic concentration, was found to potentiate the cell viability
751 reduction of endosulfan (Rana and Shivanandappa, 2010). In line with the cell viability
752 results, at high concentrations a synergistic effect between endosulfan and chlorpyrifos was
753 detected on the transcriptional down-regulation of CYP3A and FABP4. A similar additive (at
754 low concentrations) and synergistic response (at high exposure concentration) was reported in
755 an acetylcholinesterase activity inhibition study with juvenile coho salmon (*Oncorhynchus*
756 *kisutch*) exposed to a mixture composed of organophosphate and carbamate pesticides (Laetz

757 et al., 2009). A synergistic inhibition of cholinesterase has also been detected in great
758 ramshorns (*Planorbarius corneus*) exposed to a binary mixture of chlorpyrifos and one
759 organophosphate pesticide (Cacciatore et al., 2012). The mechanism behind the pesticides'
760 synergistic effect on the down-regulation of CYP3A and FABP4 is, however, not known.
761 CYP3A is an important contaminant biotransformation enzyme in addition to having a role in
762 the metabolism of steroid hormones like testosterone (Kretschmer et al., 2005) and other
763 lipids. The observed down-regulation of this transcript may therefore affect CYP3A-
764 dependent biotransformation of contaminants and turnover of hormones and lipids in cells.
765 This illustrates that risk assessment based on toxicological data from single-contaminant
766 exposure studies can underestimate the impact of the mixture of new contaminants with
767 different modes of action, like PAHs and pesticides, which may be introduced to farmed
768 Atlantic salmon from feeds with high inclusion levels of vegetable oil.

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771 **5. Conclusions**

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773 Despite that the two PAHs benzo(a)pyrene and phenanthrene were associated with higher cell
774 viability reduction potential the two pesticides endosulfan and chlorpyrifos caused the greatest
775 lipidomics and transcriptomic perturbations. According to the lipidomics data, the two
776 metabolic pathways most strongly affected by chlorpyrifos and endosulfan, contaminants that
777 could be introduced in novel salmon feeds from plant-based ingredients, were fatty acid and
778 steroid biosynthesis. These responses were to some extent confirmed by the transcriptomic
779 data, which showed that biomarkers linked to endocrine disruption and lipid metabolism were
780 affected. According to the current observations, the interaction effects between the
781 contaminants could mostly be explained as an additive effect, however at high concentrations
782 the contaminants acted in a synergistic manner.

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1237 **Abbreviations**

- 1238 Acidic ribosomal protein (ARP)
- 1239 Analysis of variance (ANOVA)
- 1240 Aryl hydrocarbon receptor (AhR)
- 1241 Cell index (CI)
- 1242 Crossing point (CT)

- 1243 Cytochrome P450 1A (CYP1A)
- 1244 Dimethyl sulfoxide (DMSO)
- 1245 Elongation factor 1 AB (EF1AB)
- 1246 Epoxide hydrolase (sEH)
- 1247 Estrogen receptor (ER)
- 1248 Ethoxyresorufin O-deethylase activity (EROD)
- 1249 Fatty acid binding protein 4 (FABP4)
- 1250 Fish serum (FS)
- 1251 Fold change (FC)
- 1252 Games-Howell (GH)
- 1253 Genomic Research in All Salmonids Project (cGRASP)
- 1254 Goodness of fit (R^2).
- 1255 Goodness of prediction (Q^2),
- 1256 Kyoto Encyclopedia of Genes and Genomes (KEGG)
- 1257 Mean normalized expression (MNE)
- 1258 Microtubule-associated proteins 1A/1B light chain 3B precursor (MAP1LC3B)
- 1259 No-amplification control (nac)
- 1260 Normalized cell index (NCI)
- 1261 No-template control (ntc)
- 1262 Nuclear magnetic resonance spectroscopy (NMR)
- 1263 Partial Least Squares Discriminant Analysis (PLS-DA)
- 1264 Percentage of false positives (PFP)
- 1265 Peroxisome proliferator activated receptor γ (PPAR γ)
- 1266 Peroxisome proliferator-activated receptor α (PPAR α)
- 1267 Persistent organic environmental pollutants (POPs)
- 1268 Photomultiplier tube settings (PMTs)
- 1269 Polycyclic aromatic hydrocarbons (PAHs)

1270 Principal Component Analysis (PCA)
1271 Quality control (QC)
1272 Quantitative real-time RT-PCR (real-time qPCR)
1273 Rainbow trout gill cells (RTgill-W1)
1274 Rainbow trout gonadal (RTG-2)
1275 Rainbow trout liver (RTL-W1)
1276 Reverse transcription (RT)
1277 Reverse transcription (RT)
1278 Reverse transcription polymerase chain reaction (RT-PCR)
1279 Selected ion monitoring (SIM)
1280 Tetrahydrofurandiols (THF-diols)
1281 Transcription factor SOX-4 (SOX4)
1282 Triacylglycerols (TAG)
1283 Vitellogenin (VTG)

1284

1285 **Highlights**

1286 Atlantic salmon primary hepatocytes were used to screen for interaction effects caused by
1287 PAHs and pesticides.

1288 Lipidomic and transcriptomic profiling suggested perturbation of lipid metabolism and
1289 endocrine disruption.

1290 The pesticides gave the strongest responses, despite having less effect on cell viability than
1291 the PAHs.

1292 The primary mixture effect was additive.

1293 At high concentrations, the pesticides acted synergistic by decreasing cell viability and down-
1294 regulating CYP3A and FABP4.

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1297

Tables

Table 1: Overview over the different concentration (μM) combinations used for the various pesticides and PAHs used in the factorial design for microarray and RT-qPCR evaluation.

Exposure no.	Benzo(a)pyrene	Phenanthrene	Chlorpyrifos	Endosulfan
1	1	1	1	1
2	100	1	1	1
3	1	100	1	1
4	100	100	1	1
5	1	1	100	1
6	100	1	100	1
7	1	100	100	1
8	100	100	100	1
9	1	1	1	100
10	100	1	1	100
11	1	100	1	100
12	100	100	1	100
13	1	1	100	100
14	100	1	100	100
15	1	100	100	100
16	100	100	100	100
17	50.5	50.5	50.5	50.5
18	DMSO	DMSO	DMSO	DMSO

Table 2: PCR primers, GenBank accession numbers, amplicon sizes and efficiency.

Gene	Accession no.	Forward primer (5' - 3')	Reverse primer (5' - 3')	Product size (bp)	Efficiency
CYP1A	AF364076	TGGAGATCTTCCGGCACTCT	CAGGTGTCCTTGGGAATGGA	101	2.06
PPARA	DQ294237	TCTCCAGCCTGGACCTGAAC	GCCTCGTAGACGCCGTACTT	58	2.05
CYP3A	DQ361036	ACTAGAGAGGGTCGCCAAGA	TACTGAACCGCTCTGGTTTG	146	2.1
SOX4	NP_001167115	GAGCCCGATGAACGCTTTC	AGCGCTTGCCCAAGTCTCTT	110	2.1
FABP4	BT125322	CCGCCGACGACAGAAAAA	TTTTGCACAAGGTTGCCATTT	61	2.03
MAP1LC3B	NP_001239285	TGCCCCATCCTGGATAAAACC	GCCATTCAACCAGCAGGAAGA	125	1.93
VTG	C065R146	GACTTCGCCCATCAGCCCTTTC	GCCACGGTCTCCAAGAAGTCT	110	2.14
EF1AB	AF321836	TGCCCTCCAGGATGTCTAC	CACGGCCCAACAGGTACT	59	2.13
UBA52	G0050814	TCAAGGCCCAAGATCCAGGAT	CGCAGCACACAAGATGCAGAGT	139	1.9
B-ACTIN	BG933897	CCAAAGCCCAACAGGGAGAA	AGGGACAACACTGCCTGGAT	92	2.04

Table 3: Table of metabolic pathways perturbed by exposing salmon hepatocyte cells to contaminants. The extent of perturbation was measured by collating the mass features for each contaminant that had changed significantly compared to the control and mapping these as putatively annotated empirical formulae onto metabolic pathways as listed by KEGG (<http://www.genome.jp/kegg/>). The percentage perturbation of the pathway can then be estimated based on the percentage of the original pathway detected that was significantly different. Figures in red denote the greatest perturbation seen in that pathway for the individual contaminants.

KEGG Pathway*	Theoretical pathway information		Percentage of the putatively annotated empirical formula that are perturbed by the contaminant				
	Number of empirical formulae listed in KEGG pathway	Number of putatively annotated empirical formulae detected by DIMS lipids	Endosulfan	Chlorpyrifos	Benzo(a)pyrene	Phenanthrene	Mixture
Primary bile acid biosynthesis	29	7	86	71	57	14	86
Biosynthesis of unsaturated fatty acids	42	12	0	83	0	0	83
Steroid biosynthesis	19	5	80	40	60	20	80
Retinol metabolism	9	5	0	60	20	0	80
Linoleic acid metabolism	9	6	0	83	0	0	67
Vitamin digestion and absorption	29	6	33	50	17	0	67
Steroid hormone biosynthesis	45	9	56	33	22	0	44
alpha-Linolenic acid metabolism	25	6	17	50	0	0	33
Phenylalanine metabolism	47	9	0	22	0	0	33
Arachidonic acid metabolism	19	5	0	40	40	20	20

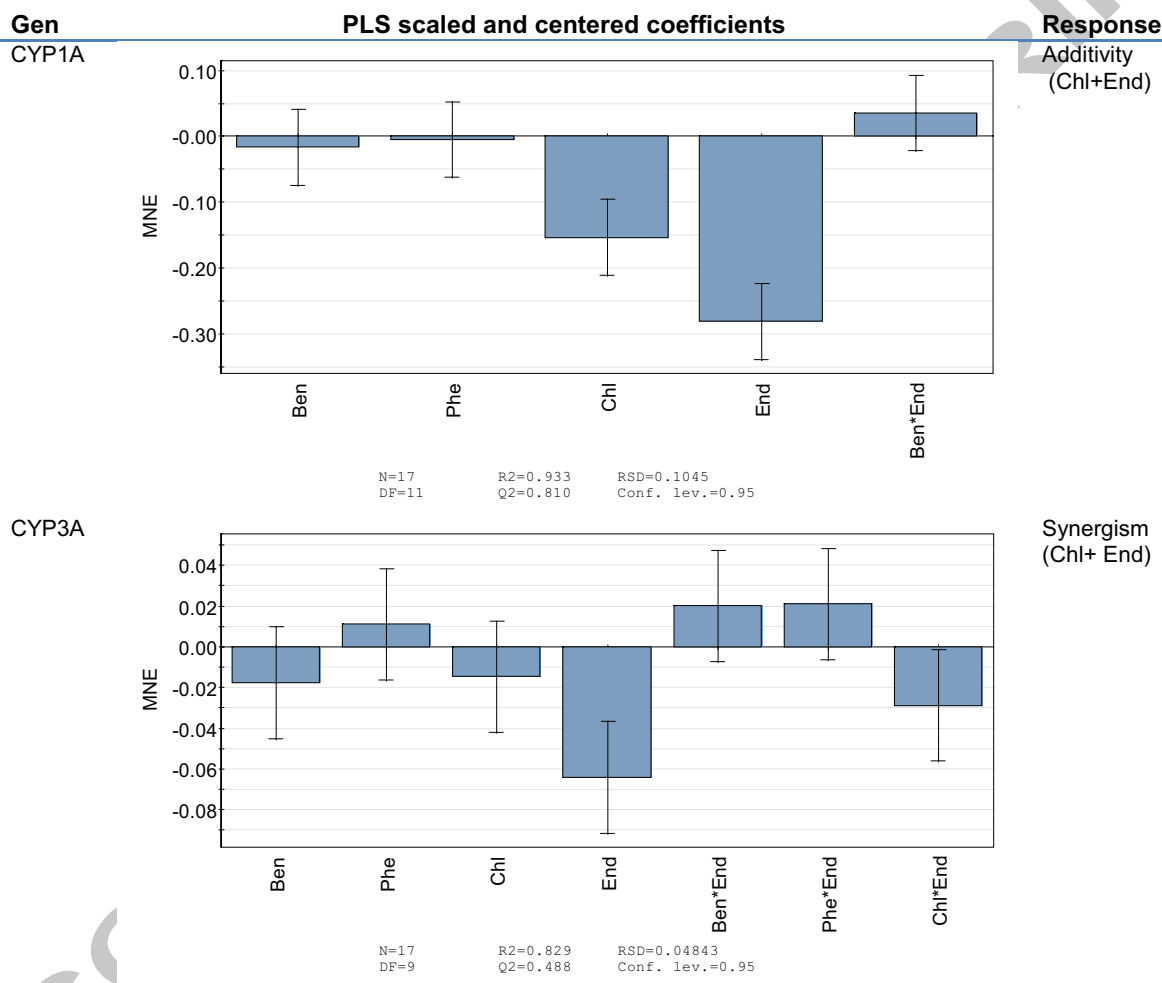
*Only pathways with 5 or more unique empirical formulae detected and 2 or more empirical formulae significantly perturbed have been included. Pathways unique to plant or microbial metabolism have been excluded.

Table 4: Top rank product list of differently expressed features with PFP below 10% in Atlantic salmon hepatocytes exposed to contaminant mixture 4^a (N=5).

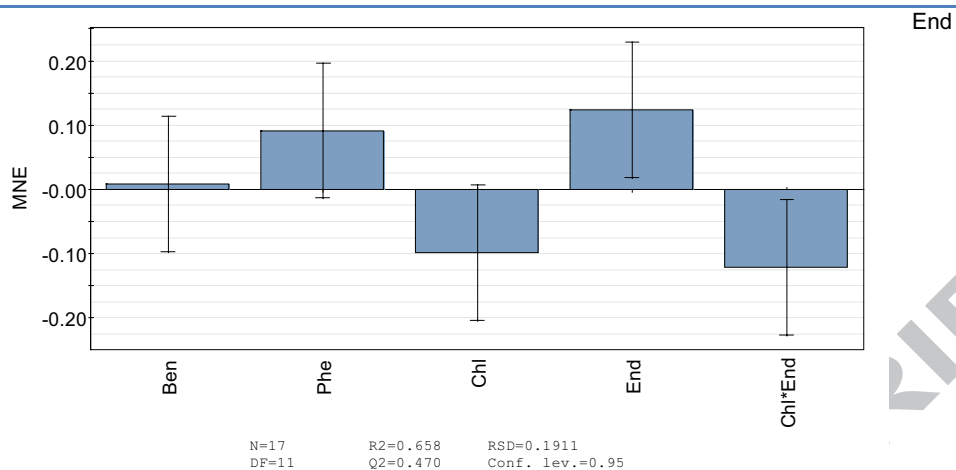
Exposure	Gene	ID	Microarray	
			Fold change	PFP ^A
Mixture 4	Vitellogenin precursor	C065R146	13.19	0.000
Mixture 4	Vitellogenin precursor	C207R010	7.72	0.005
Mixture 4	UNKNOWN	C175R143	5.59	0.013
Mixture 4	Microtubule-associated proteins 1A/1B light chain 3B precursor	C099R165	9.54	0.017
Mixture 4	Endonuclease domain-containing 1 protein precursor	C001R110	4.66	0.027
Mixture 4	Pleiotropic regulator 1	C126R053	6.88	0.028
Mixture 4	Retinoic acid receptor RXR- gamma-B	C159R064	7.71	0.028
Mixture 4	Transcription factor SOX-4	C084R080	4.05	0.029
Mixture 4	Actin-related protein 2/3 complex subunit 1A	C080R051	4.59	0.033
Mixture 4	Vitellogenin precursor	C088R103	2.87	0.045
Mixture 4	UNKNOWN	C033R065	1.16	0.047
Mixture 4	Ferritin, middle subunit	C023R056	3.97	0.048
Mixture 4	Zinc finger FYVE domain- containing protein 1	C131R039	4.41	0.051
Mixture 4	Pre-mRNA cleavage complex II protein Clp1	C124R050	5.46	0.064
Mixture 4	UNKNOWN	C072R085	3.05	0.081
Mixture 4	Neuropeptide B precursor	C080R062	3.05	0.081
Mixture 4	UNKNOWN	C105R067	2.85	0.095

^aContaminant mixture 4 is composed of benzo(a)pyrene and phenanthrene (100 μ M), endosulfan and chlorpyrifos (1 μ M).

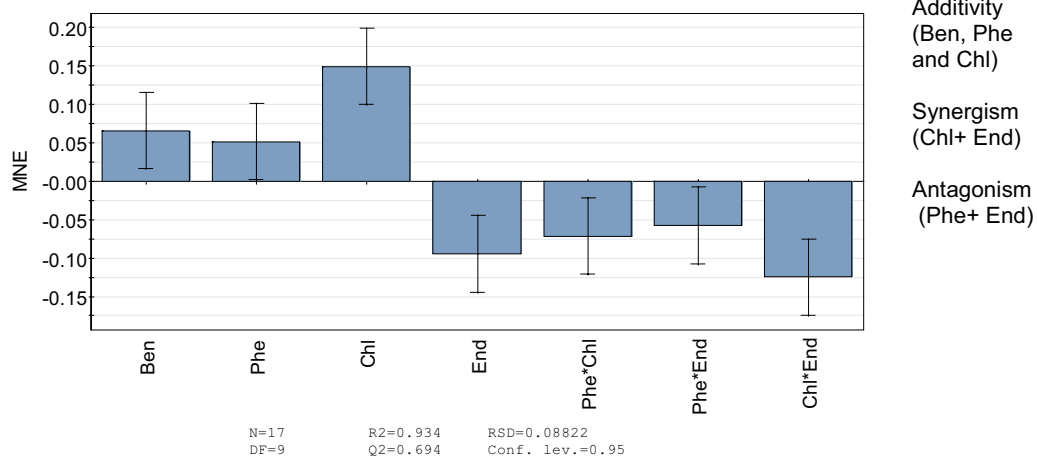
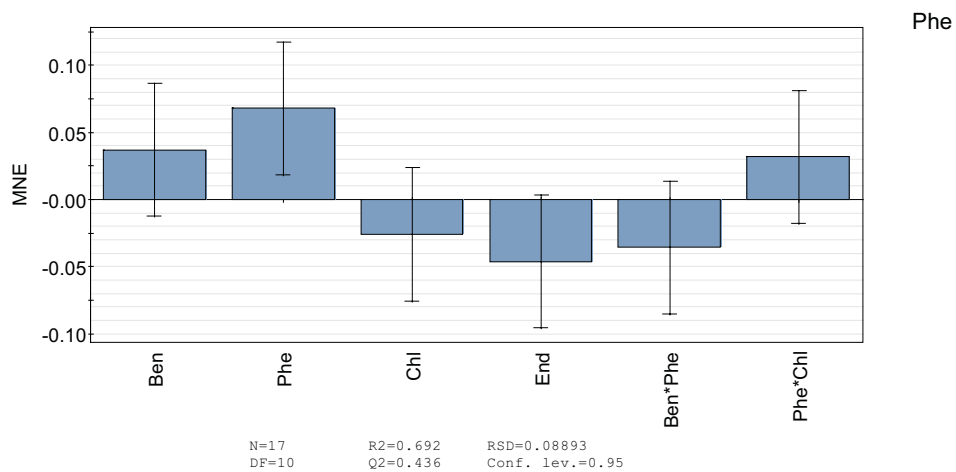
Table 5: Scaled and centered PLS regression coefficient models for different genes measured in primary Atlantic salmon hepatocytes exposed to chlorpyrifos (Chl), endosulfan (End), benzo(a)pyrene (Ben) and phenanthrene (Phe) using mean normalized expression (MNE) and factorial design. The combined effects identified with contour plot analysis for the different models are presented in the response column. The specific contaminants that contributed to a particular combined effect like additivity, synergism or antagonism are specified in parentheses behind the identified combined response in the response column. PLS plots of genes where only one contaminant was responsible for the observed response is indicated in the response column by naming the contaminant responsible for the effect.

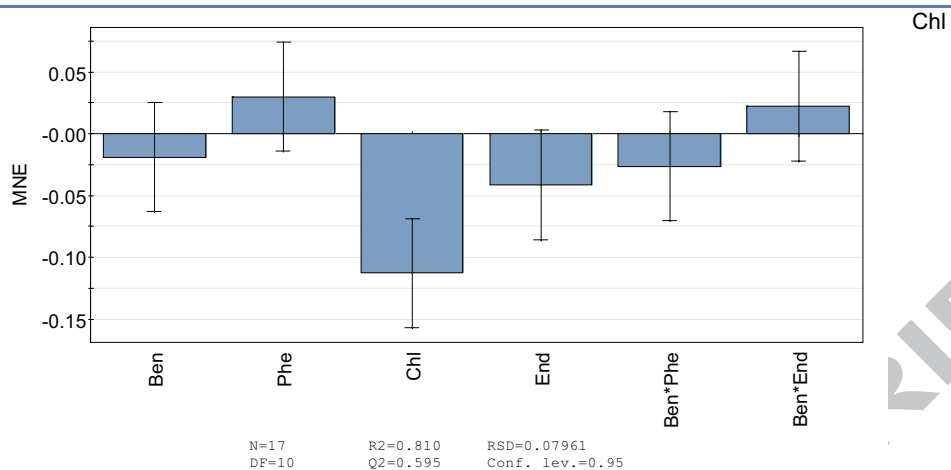


VTG

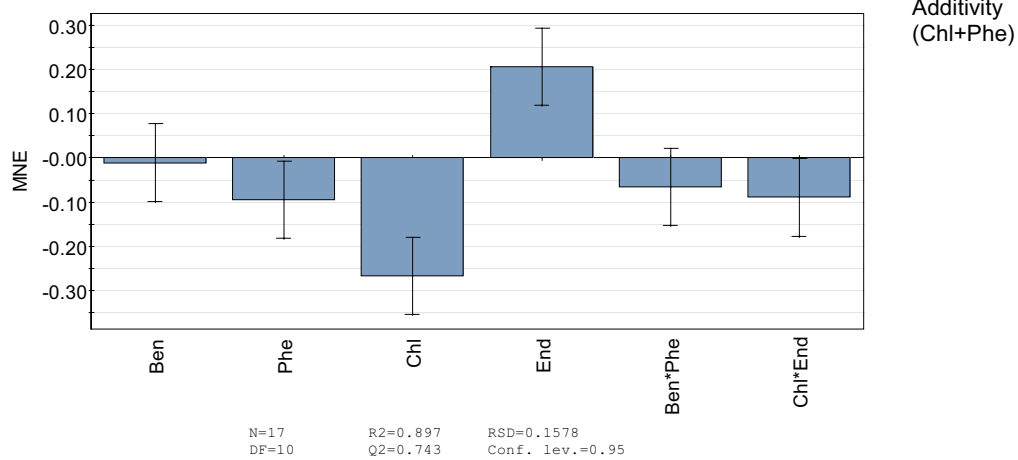


FBP4

PPAR α 

MAP1L
C3B

SOX4



Figures

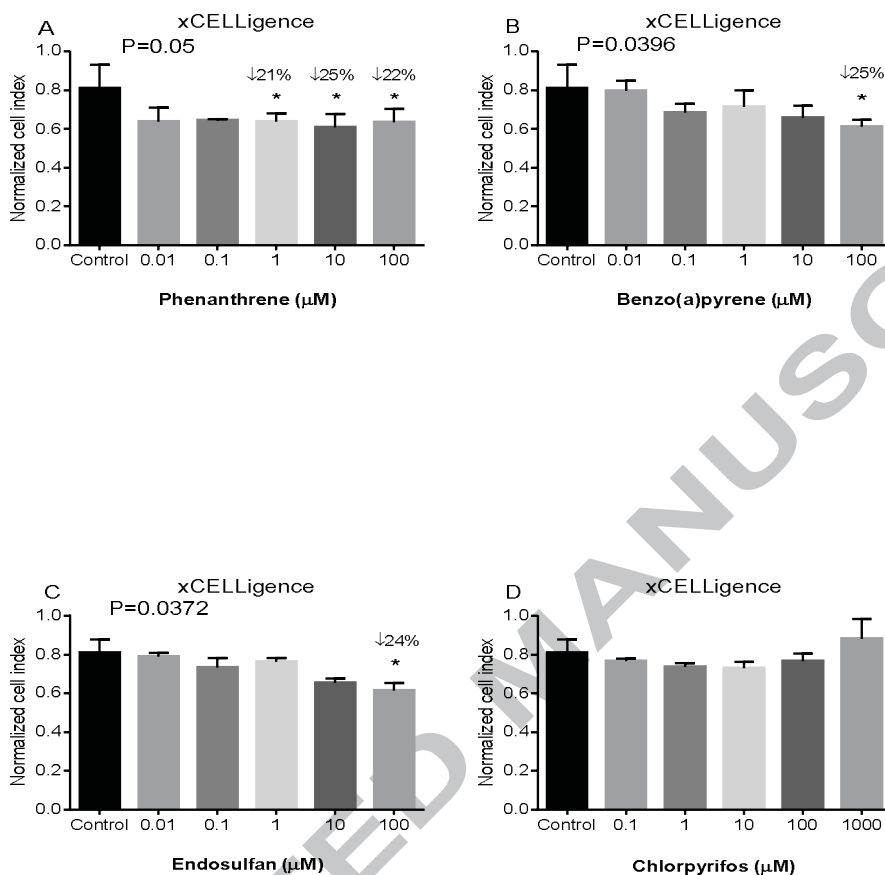


Fig. 1: Dose-response curves for Normalized cell index (NCI) values obtained for primary Atlantic salmon hepatocytes (N=3) exposed to (A) phenanthrene (B) benzo(a)pyrene, (C) endosulfan, (D) and chlorpyrifos. The values represent the mean \pm SE of three replicates (N=3). The analyses showed significant difference between the control (DMSO 0.4%) and the exposed group indicated by * (p=0.05).

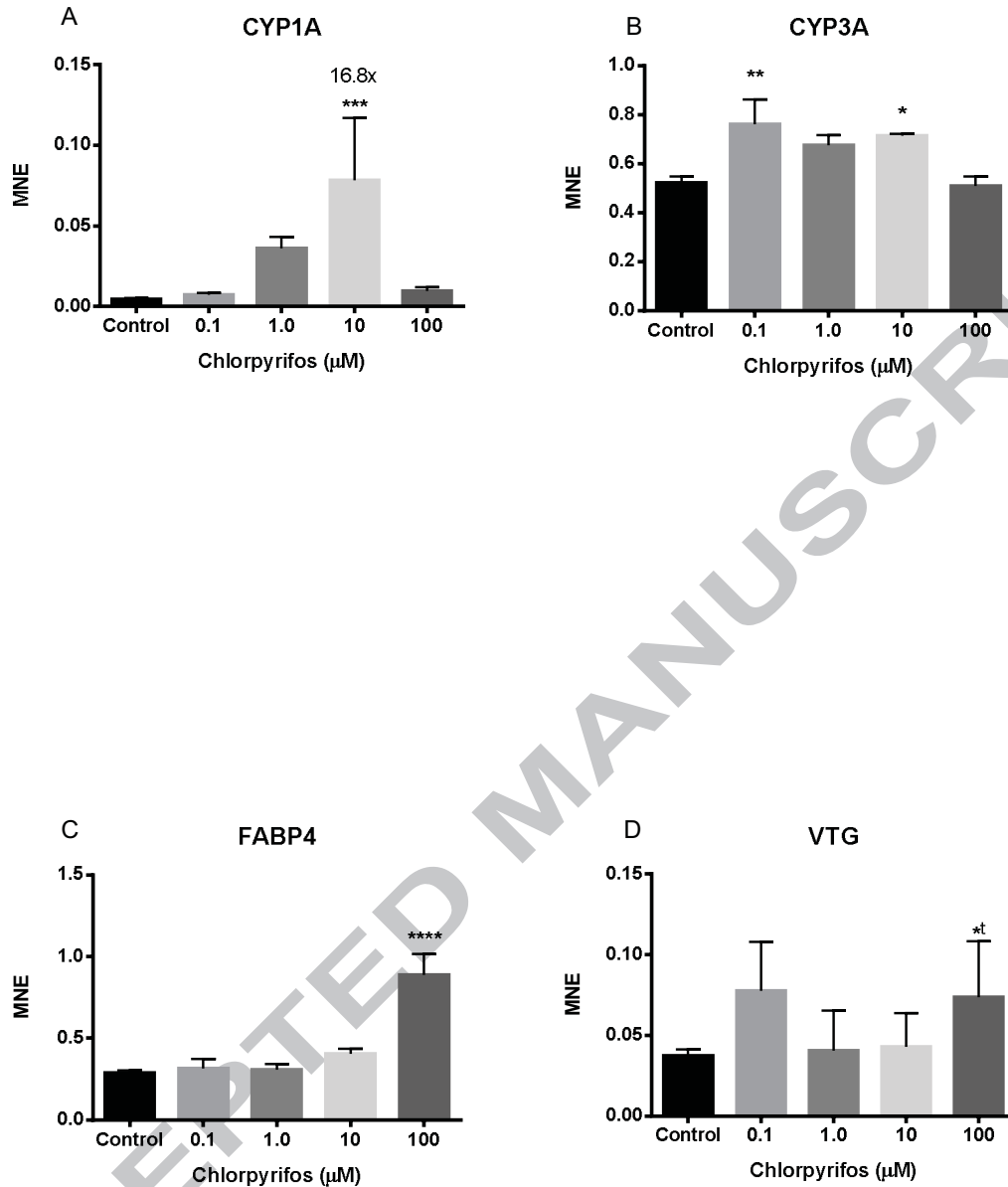


Fig. 2: Dose-response curves for A) CYP1A, B) CYP3A, C) FABP4, D) VTG, using normalized expression (MNE) values, obtained for primary Atlantic salmon hepatocytes exposed to chlorpyrifos and the DMSO control (0.4%). The values represent the mean±SE of three replicates (N=3). The ANOVA analyses showed significant difference between the control and the exposed group indicated by **** (P=0.0001), *** (p=0.001), ** (p=0.01) and * (p=0.05). The Students't-test analyses showed significant difference between the control (DMSO 0.4%) and the exposed group indicated by *t (p=0.05).

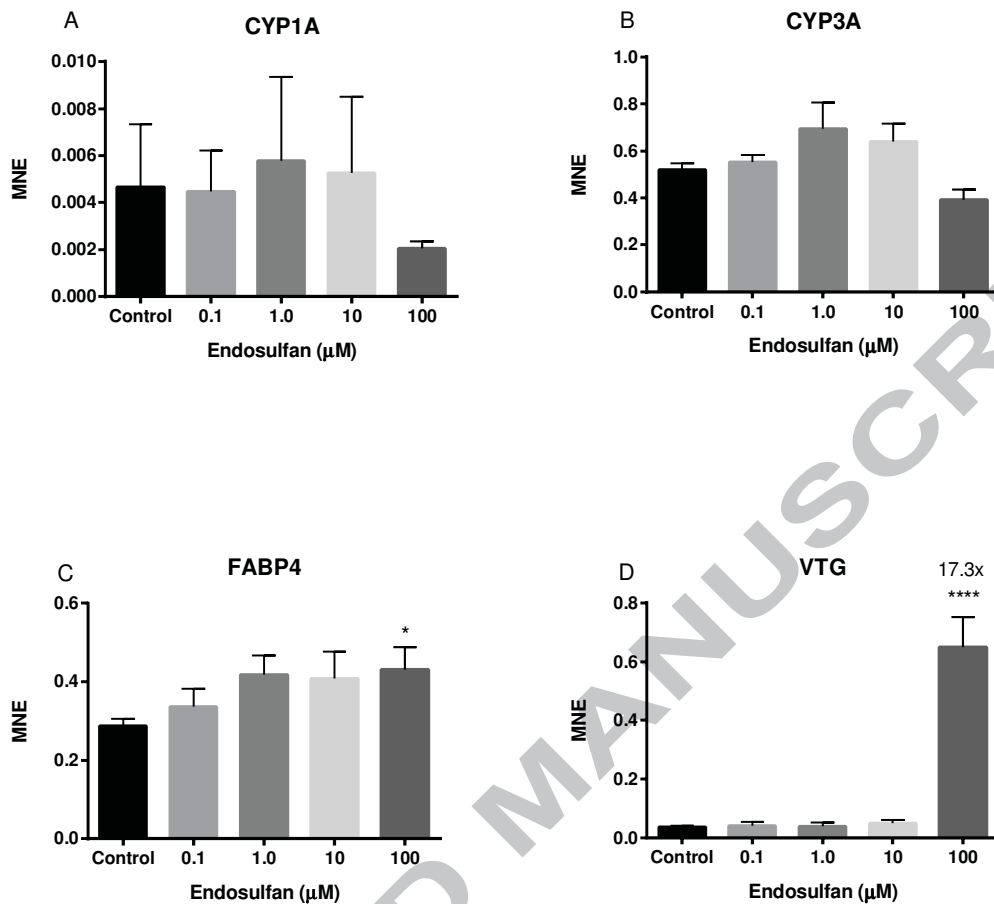


Fig. 3: Dose-response curves for A) CYP1A, B) CYP3A, C) FABP4, D) VTG, using normalized expression (MNE) values, obtained for primary Atlantic salmon hepatocytes exposed to endosulfan and the DMSO control (0.4%). The values represent the mean \pm SE of three replicates (N=3). The ANOVA analyses showed significant difference between the control and the exposed group indicated by **** (P=0.0001) and * (p=0.05).

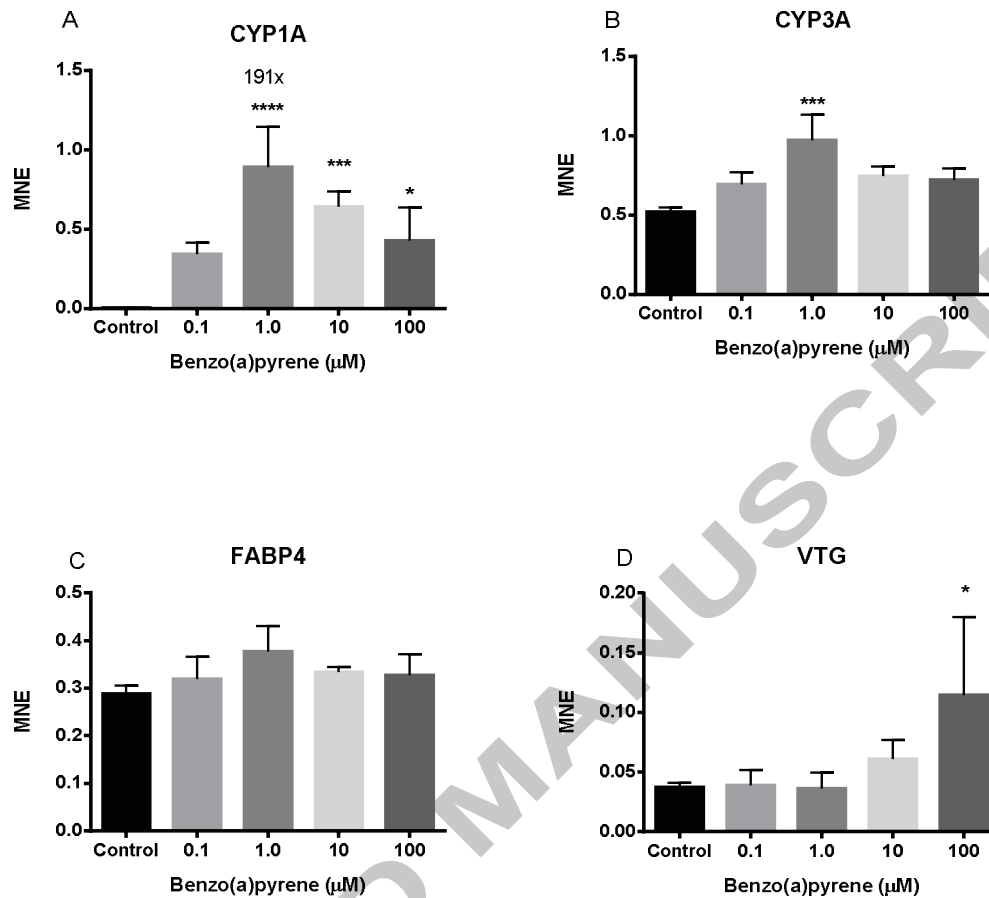


Fig. 4: Dose-response curves for A)CYP1A, B)CYP3A, C)FABP4, D) VTG, using normalized expression (MNE) values, obtained for primary Atlantic salmon hepatocytes exposed to benzo(a)pyrene and the DMSO control (0.4%). The values represent the mean \pm SE of three replicates (N=3). The ANOVA analyses showed significant difference between the control and the exposed group indicated by **** (P=0.0001), *** (p=0.001) and * (p=0.05).

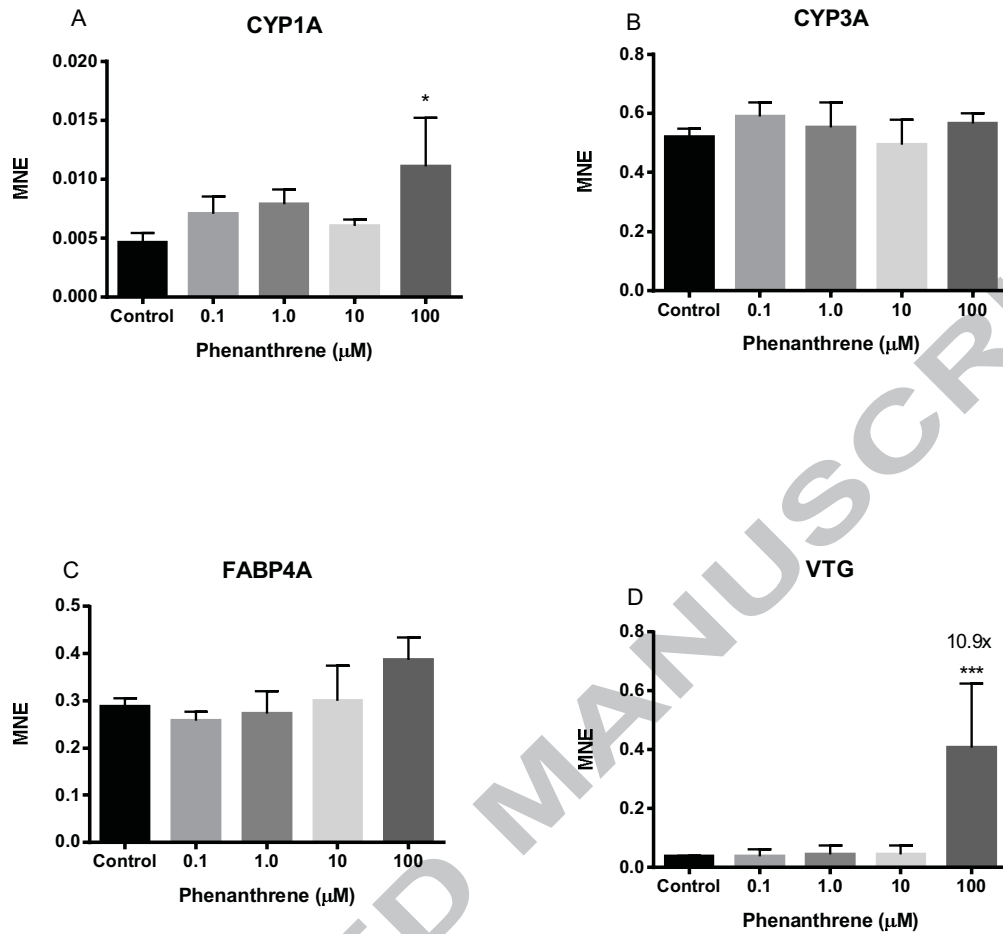


Fig. 5: Dose-response curves for A) CYP1A, B) CYP3A, C) FABP4 D) VTG, using normalized expression (MNE) values, obtained for primary Atlantic salmon hepatocytes exposed to phenanthrene and the DMSO control (0.4%). The values represent the mean \pm SE of three replicates (N=3). The ANOVA analyses showed significant difference between the control and the exposed group indicated by *** (p=0.001) and * (p=0.05).

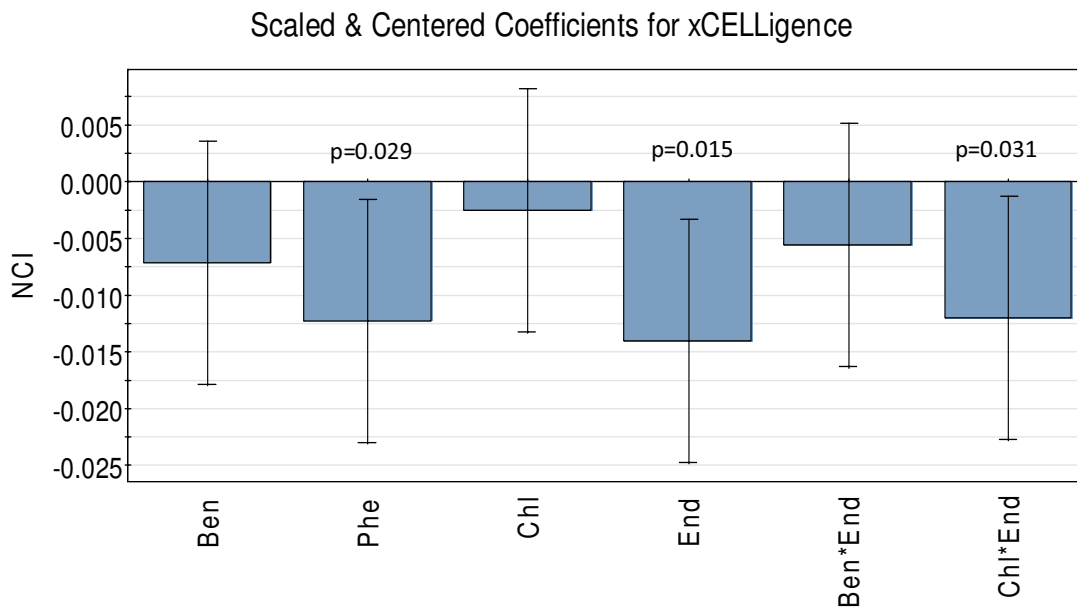


Fig. 6: Scaled and centered PLS regression coefficients with 95% confidence intervals for Normalized cell index (NCI) levels measured in primary Atlantic salmon hepatocytes exposed to benzo(a)pyrene, phenanthrene, chlorpyrifos and endosulfan accordingly to the factorial design (N=5). The model is based on 17 experimental objects, and had one PLS component. The model was good ($R^2=0.7$ and $Q^2=0.4$), containing four linear terms and two interaction terms.

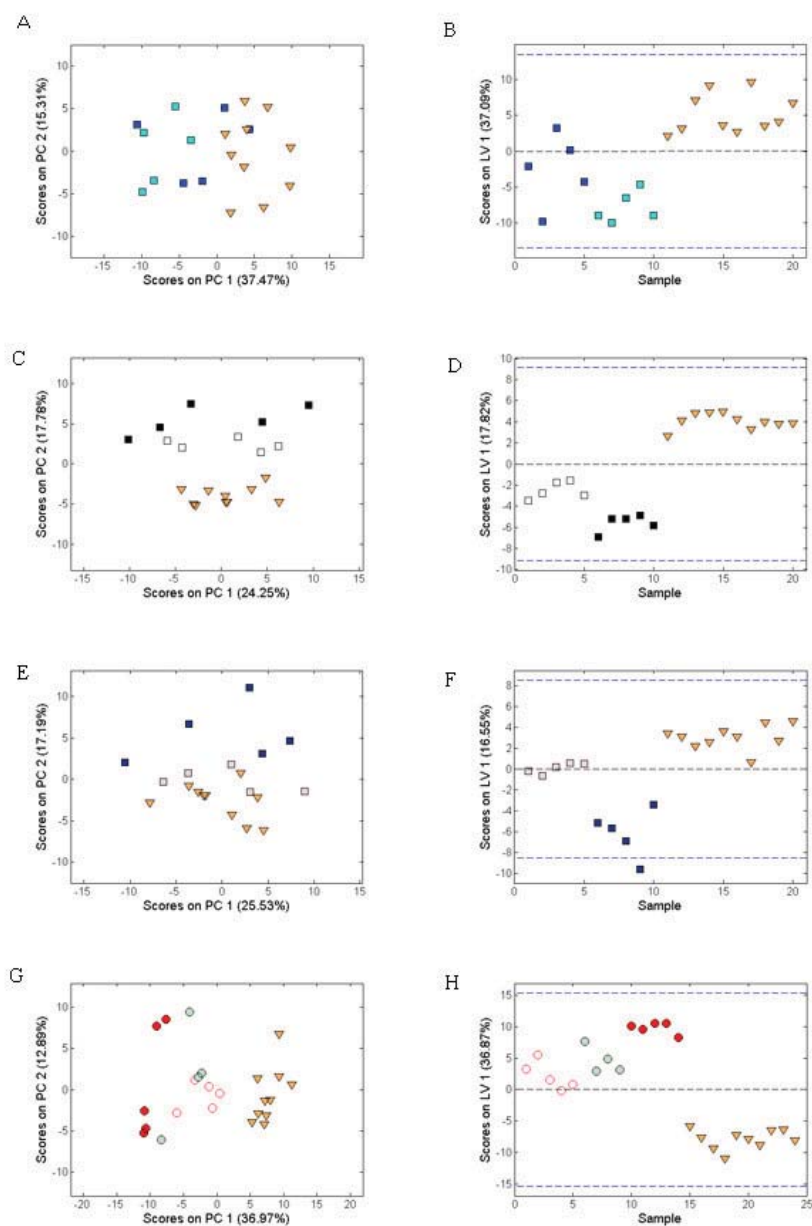


Fig. 7: PCA and PLSDA scores plots for lipidomics. PCA (left hand column) and PLSDA (right hand column) scores plots data from salmon hepatocyte cell cultures treated with chlorpyrifos (A and B), endosulfan (C and D), benzo(a)pyrene (E and F), or a mixture (I and J) of four contaminants (benzo(a)pyrene, chlorpyrifos, endosulfan and phenanthrene) at one of three different doses or DMSO control. One sample from the contaminant mixture 4 class has been removed as an outlier in the DIMS lipidomics data. Key to treatments: control (∇) low dose (1 μ M) chlorpyrifos, (\blacksquare) high dose (100 μ M) chlorpyrifos (\blacksquare), low dose endosulfan (\square), high dose endosulfan (\blacksquare), low dose benzo(a)pyrene

(\square), high dose benzo(a)pyrene (\blacksquare) or mixtures 1 (\circ), 4 (\odot) and 16 (\bullet) of the four contaminants.

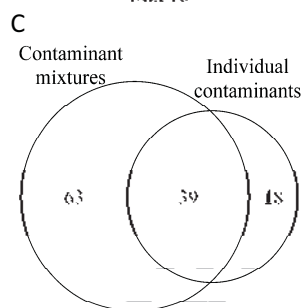
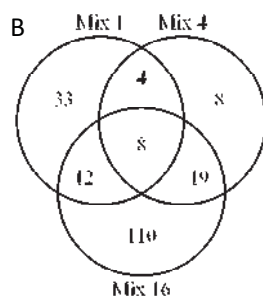
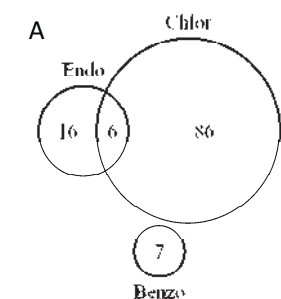
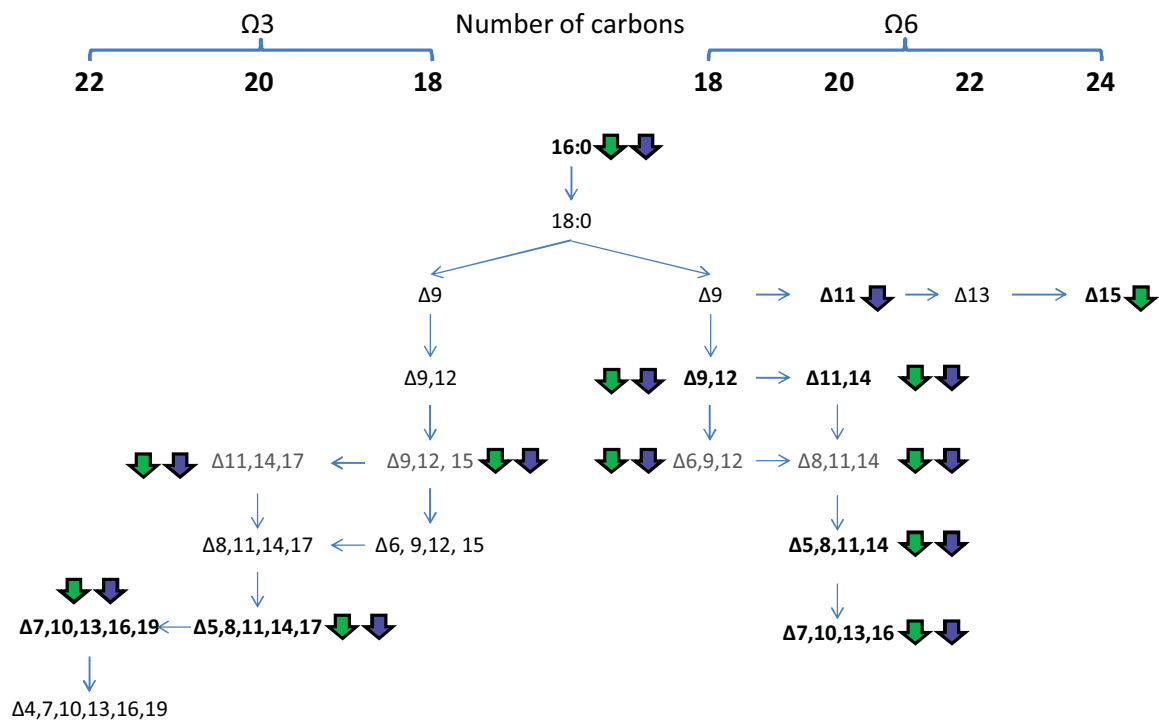
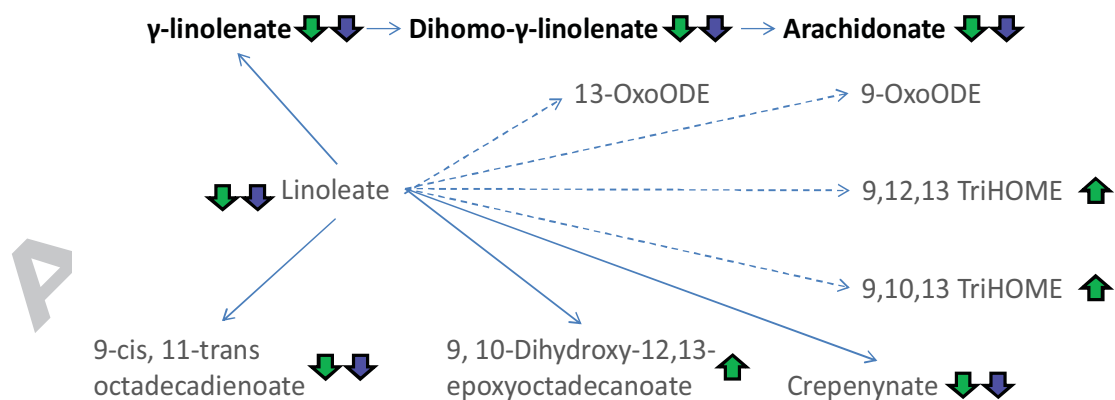


Fig. 8: Venn diagrams to summarise the significant mass features by ANOVA with Games-Howell post-hoc testing. The significant mass features between high dose individual contaminants versus control do not show much overlap (A). Phenanthrene was excluded from this analysis since statistical tests revealed there was only one mass feature significantly different from the control after FDR correction. This mass feature was also significantly different in endosulfan compared to the control. (B) The Venn diagram displays the difference in significant mass features between different contaminant mixes and the control, and (C) displays the overlapping mass features that were significant in any individual contaminant and the contaminant mixes.

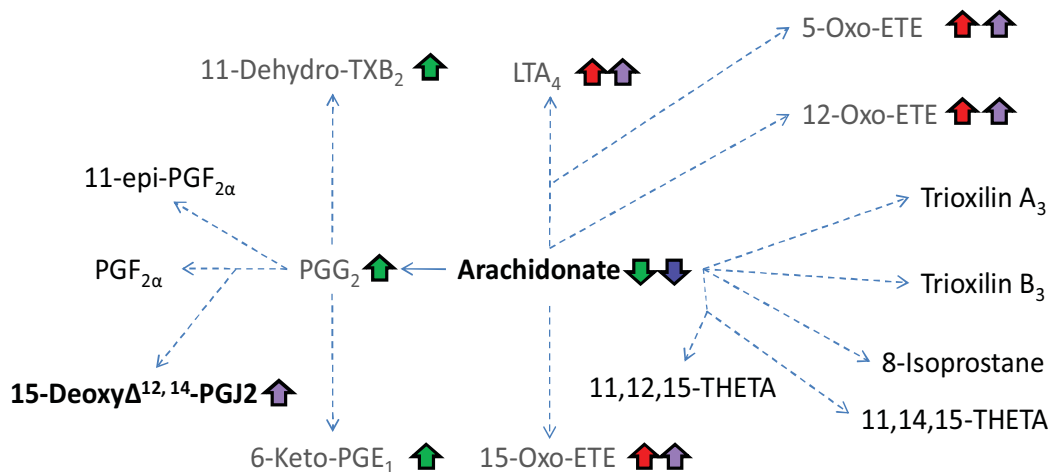
A



B



C



D

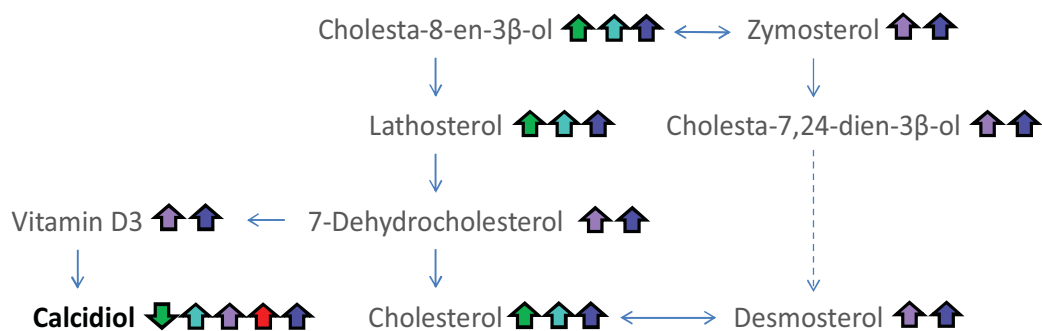


Fig. 9: Examples of metabolic pathways with significantly changing putatively annotated empirical formulae mapped onto them for each contaminant. All pathways have been reduced to show only the metabolites detected by direct infusion lipidomics. Block arrows demonstrate a significant fold increase or decrease following exposure to the contaminants in relation to the control group. Key (↑) chlorpyrifos (↑) endosulfan (↑) phenanthrene (↑) benzo(a)pyrene (↑) contaminant mixture. Dotted arrows represent two or more reactions between the metabolites of interest and metabolites in grey font indicate that potentially multiple metabolites of the same m/z exist in this pathway. Biosynthesis of unsaturated fatty acids pathway (A) and the linoleic pathway (B) shows perturbations only when chlorpyrifos or a contaminant mixture is used. By contrast, the arachidonic acid metabolism pathway (C) and the steroid pathway (D) (reduced to show only the section of interest) appear disrupted by a much greater range of contaminants.