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Omega-3 and alpha-tocopherol provide more protection against contaminants in novel feeds for Atlantic salmon (*Salmo salar* L.) than omega-6 and gamma tocopherol

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ABSTRACT

Extended use of plant ingredients in Atlantic salmon farming has increased the need for knowledge on the effects of new nutrients and contaminants in plant based feeds on fish health and nutrient-contaminant interactions. Primary Atlantic salmon hepatocytes were exposed to a mixture of PAHs and pesticides alone or in combination with the nutrients ARA, EPA, α-tocopherol, and γ-tocopherol according to a factorial design. Cells were screened for effects using xCELLigence cytotoxicity screening, NMR spectroscopy metabolomics, mass spectrometry lipidomics and RT-qPCR transcriptomics. The cytotoxicity results suggest that adverse effects of the contaminants can be counteracted by the nutrients. The lipidomics suggested effects on cell membrane stability and vitamin D metabolism after contaminant and fatty acid exposure. Co-exposure of the contaminants with EPA or α-tocopherol contributed to an antagonistic effect in exposed cells, with reduced effects on the VTG and FABP4 transcripts. ARA and γ-tocopherol strengthened the contaminant-induced response, ARA by contributing to an additive and synergistic induction of CYP1A, CYP3A and CPT2, and γ-tocopherol by synergistically increasing ACOX1. Individually EPA and α-tocopherol seemed more beneficial than ARA and γ-tocopherol in preventing the adverse effects induced by the contaminant mixture, though a combination of all nutrients showed the greatest ameliorating effect.

1. Introduction

Increased use of plant feed ingredients has introduced a new cocktail of plant-oil derived contaminants, such as polycyclic aromatic hydrocarbons (PAHs) and pesticides, formerly not related with farming of salmonids [5,23]. Plant oils intended for animal feed production can be contaminated with PAH like phenanthrene and benzo(a) pyrene [81,12] due to atmospheric deposition of particles on crops before harvesting or later during the thermal processing of the oil seeds [20,64]. Residue levels of pesticides like endosulfan and chlorpyrifos have been reported in products from plants such as soya or maize [33,49] which are commonly used as ingredients in salmon feeds [4]. Both PAH and pesticides have been shown in several *in vitro* and *in vivo* experiments to cause lipid and endocrine disturbances and to induce cytochrome P450 enzymes in teleost fish [43,53,68,79,100,102]. PAHs are genotoxic [16,86,35] and exposure has been suggested to cause vitamin D signalling disruption [79] as well as an effect on cell membrane integrity [65,70]. The pesticides endosulfan and chlorpyrifos have been shown to induce lipid accumulation in Atlantic salmon liver cells both *in vitro* and *in vivo* [43,23,79].

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In addition to an altered contaminant profile, the plant-based feed ingredients also change the nutrient profile of the fish. Marine oils contain high levels of the n-3 polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) [36] while common plant oils can contain high levels of n-6 PUFA like linoleic acid (LA, 18:2n-6) [92]. Since the fatty acid composition of oily fish reflects the fatty acid composition of their feed [85], replacing fish oil with vegetable oil in fish feed typically reduces the concentration in Atlantic salmon fillet of the n-3 PUFAs EPA and DHA and increases concentrations of the n-6 PUFAs LA and arachidonic acid (ARA, 20:4n-6) [25,62,84,85]. The liver is a central organ for lipid metabolism [7] and the synthesis of cholesterol and bile [42]. Immediately after uptake in the liver, the PUFA can be converted to energy by β-oxidation [74], stored in adipocytes and in intracellular lipid droplets in different tissues [55] or incorporated into phospholipid membranes [92]. The n-3 and n-6 PUFAs can be converted to their respective group of eicosanoids or lipid mediators by the lipoxygenase (LOX), cyclooxygenase (COX) and cytochrome P450 (CYP) enzyme pathways [92]. The n-6 eicosanoids are a general group of pro-inflammatory eicosanoids [92]. By contrast, the n-3 PUFA can be converted to n-3 eicosanoids that have anti-inflammatory abilities [92]. Several studies have reported increase in liver lipid when fish oil was replaced with plant oils indicating that nutrients in fish oil such as EPA and DHA, n-6, saturated fatty acids, cholesterol and phytosterols play a role (Torstensen et al., 2011) [45,47,62]. High levels of the n-3 PUFAs DHA and EPA in fish feed can protect against induction of liver steatosis in Atlantic salmon [45,47]. α-tocopherol is an essential nutrient for fish [28] and is also the main form of vitamin E in fish fillet [76]. Dietary α-tocopherol is taken up more rapidly than γ-tocopherol [93], which is the main vitamin E congener in most plant seeds [34] and maize, rapeseed and soya oils [77]. γ-tocopherol seems to be a more effective trap for lipophilic electrophiles than α-tocopherol [34] and in contrast to α-tocopherol, γ-tocopherol possesses anti-inflammatory properties [34]. Similar to the n-3 PUFAs, high levels of tocopherol can inhibit induction of liver steatosis [91] and protect organisms against lipid oxidation [28].

The aim of this study was to examine how relevant nutrients can modulate the toxicological outcome of a contaminant mixture associated with plant feed ingredients using metabolomic, lipidomic and transcriptomic methods to search for novel biomarkers and possible interaction effects. The study utilised Atlantic salmon primary hepatocytes as a biological model system.

2. Materials and methods

2.1. Chemicals

Endosulfan (6,7,8,9,10-hexachloro-1,5,5a,6,9,9,a-hexahydro-6,9-metano-2,4,3-benza-dioxathiepin-3-oxide, α + β–2 + 1; PESTANAL®, analytical standard), chlorpyrifos (*O*,*O*-diethyl-*O*-3,5,6-trichlor-2-pyridyl phosphorothioate, PESTANAL®, analytical standard), phenanthrene (≥98% pure), benzo(a) pyrene (≥96% pure), arachidonic acid (ARA ≥99%), eicosapentaenoic acid (EPA, ≥99% pure), potassium hydroxide, α-tocopherol (αT, >95.5% pure) and γ-tocopherol (γT, ≥96% pure) were all purchased from Sigma–Aldrich (Oslo, Norway). Dimethyl stock solution was purchased from Scientific and Chemical Supplies Ltd. (Bilston, UK), chloroform (HPLC grade) was purchased from Fisher Scientific (Loughborough, UK) and ammonium acetate was purchased from Sigma–Aldrich Co., Ltd. (Dorset, UK). The fatty acid free-BSA (FAF–BSA) was purchased from PAA (Parching, Austria).

2.1.1. Bovine Serum Albumin (BSA) coupling of fatty acids

Binding of fatty acids (FA) to fatty acid free-BSA (FAF–BSA) was performed as per Ghioni et al. [22]. To summarize, FA dissolved in 0.04 ml chloroform per mg FA was added to a glass sample tube and N_2 was used to evaporate the chloroform. Potassium hydroxide (KOH) was applied to the FA in a 1:3 ratio and the solution was shaken for 10 min using a vortex mixer. FAF–BSA was employed in a 2.5:1 relationship to the FA and the solution was mixed for 45 min before it was sterile-filtered and preserved at −80 °C in anoxic conditions.

2.2. Isolation of primary cultures of hepatocytes

Juvenile Atlantic salmon were obtained and kept at the animal holding facility at Ilab, University of Bergen (UiB), Bergen, Norway. The fish were fed once daily with a special feed produced without addition of synthetic antioxidants and with low levels of contaminants, supplied by EWOS, Norway (Harmony Nature Transfer 75). All glassware, instruments and solutions were autoclaved prior to liver perfusion. Hepatocytes were isolated from eight Atlantic salmon (278–381 g) with a two-step perfusion method previously described in Ref. [78]. The final cell pellet was resuspended in L-15 medium containing 10% fish serum (FS) from salmon (Nordic BioSite, Oslo, Norway), 1% glutamax (Invitrogen, Norway) and 1% penicillin–streptomycin–amphotericin (10,000 units/ml potassium penicillin, 10000 μg/ml streptomycin sulphate and 25 μg/ml amphotericin B) (Lonzo, Medprobe, Oslo, Norway). The Trypan Blue exclusion method, performed in accordance with the manufacturer's protocol (Lonzo, Medprobe, Oslo, Norway), was used to determine cell viability. The different cell suspensions used in this study had cell viability between 85–90%. The cell suspensions were plated on 5 μg/cm^2 laminin (Sigma–Aldrich, Oslo, Norway) coated culture plates (TPP, Trasadingen, Switzerland), and the hepatocytes were kept at 10 °C in a sterile incubator without additional O_2/CO_2 (Sanyo, CFC FREE, Etten Leur, The Netherlands). The following cell concentrations were used; 7.2×10^6 cells per well in 6-well plates (in 3 ml complete L-15 medium), 2.6×10^6 cells per well in 12-well plates (in 2 ml complete L-15 medium), 0.2×10^6 cells per well in xCELLigence 96-well plates (in 0.2 ml complete L-15 medium).

2.3. Chemical and nutrient exposure

The primary cells were cultured for 36–40 h prior to chemical exposure with one change of medium (containing 10% FS) after 18–20 h. The cells were exposed for 48 h to single nutrients to establish cytotoxic dose-response curves and to nutrients and a contaminant mixture according to a factorial experimental design for interaction evaluation. Cytotoxicity dose-response curves were established for α-tocopherol (1, 10, 100, 1000 and 10,000 μM) and EPA (100, 200, 4000 and 600 μM) and cells from three fish ($n = 3$) were used to make the dose-response curves. Based on individual cytotoxicity dose-response curves for the nutrients, non-cytotoxic concentrations of the α-tocopherol (100 μM) and the fatty acid EPA (200 μM) were used (data not shown). To be able to assess the effect of both marine and plant derived nutrients on the toxicity of a mixture of PAHs and pesticides, comparable concentrations were used for the plant derived nutrients γ-tocopherol (100 μM) and ARA (200 μM) in a factorial design. The contaminant mixture used here was selected based on a previous study by Søfteland et al. [79] and was composed of 100 μM of the PAHs benzo(a) pyrene and phenanthrene and 1 μM of the two pesticides chlorpyrifos and endosulfan. These concentrations were chosen after earlier individual assessment of the four contaminants aiming for levels just below the onset of cytotoxicity, and the contaminant mixture used in the present study was a potent combination [79]. A full factorial design was used with two levels (low and high concentrations), a zero

Table 1
Overview of the different concentration (μM) combinations used for the various nutrients (eicosapentaenoic acid (EPA), arachidonic acid (ARA), α-tocopherol (αT) and γ-tocopherol (γT)) and contaminant mixture (CM) used in the factorial design for lipidomic and RT-qPCR evaluation. CM contained 100 μM of benzo(a) pyrene and phenanthrene and 1 μM of chlorpyrifos and endosulfan.

Exp. no.	EPA	ARA	αT	γT	CM
1	0	0	0	0	0
2	200	0	0	0	0
3	0	200	0	0	0
4	200	200	0	0	0
5	0	0	100	0	0
6	200	0	100	0	0
7	0	200	100	0	0
8	200	200	100	0	0
9	0	0	0	100	0
10	200	0	0	100	0
11	0	200	0	100	0
12	200	200	0	100	0
13	0	0	100	100	0
14	200	0	100	100	0
15	0	200	100	100	0
16	200	200	100	100	0
17	0	0	0	0	100
18	200	0	0	0	100
19	0	200	0	0	100
20	200	200	0	0	100
21	0	0	100	0	100
22	200	0	100	0	100
23	0	200	100	0	100
24	200	200	100	0	100
25	0	0	0	100	100
26	200	0	0	100	100
27	0	200	0	100	100
28	200	200	0	100	100
29	0	0	100	100	100
30	200	0	100	100	100
31	0	200	100	100	100
32	200	200	100	100	100
33	100	100	50	50	50

point (control, 0.4% DMSO), and one centre point in order to evaluate linearity (Table 1). In addition a BSA control was included. Cells from five fish were employed for the factorial design experiment. The exposure medium contained 1% FS. The chemical exposure medium was substituted with new medium after 18–20 h.

2.4. Cytotoxicity testing of chemicals

For the cytotoxicity assessment of the nutrients and contaminant mixtures, real time impedance data obtained by the xCELLigence systems (ACEA Biosciences, Inc. (ACEA), Aarhus, Denmark) was used. The xCELLigence system quantifies electrical impedance across electrodes in 96-well cell culture E-Plates. The impedance measurement gives quantitative information about the cells' health status including morphology, cell number and viability and is indicated with the parameter Cell index (CI) or the normalized CI (NCI). The xCELLigence instrument was used to establish dose-response relationship for EPA and α-tocopherol exposed singly to the primary hepatocytes and interaction evaluation using cells exposed according to a factorial design, as mentioned above. The real time cell monitoring was conducted at 10 °C in an incubator without additional O_2/CO_2 (Sanyo, CFC FREE, Etten Leur, Netherland), using the RTCA single plate xCELLigence platform. The data was collected according to Søfteland et al. [79]. Briefly, the data was collected with intervals of 2 min after contaminant exposure for 12 h, and then every 15 min for 120 h. The last time point before compound exposure was used for the normalization, allowing a more precise comparison of the effect of the different contaminant concentrations tested. The CI values presented here were calculated from three to five replicate values. Determination of cytotoxic effects was done according to the International standardised test for *in vitro* cytotoxicity, ISO 10993-5:2009 [32]. Contaminants will be deemed cytotoxic when cells viability exceeds 30% reduction compared to the control.

2.5. Metabolomics and lipidomics

Based on the factorial design cytotoxicity screening, a selection of mixtures were analysed with lipidomic and metabolomic methods to determine how nutrients modify the toxic effect of the contaminant mixtures. The exposure groups analysed were EPA, ARA, αT, γT, CM, C-EPA, C-ARA, C-αT, C-γT, C-All high, and C-All low, and these groups corresponded to following mixtures 2, 3, 5, 9, 17, 18, 19, 21, 25, 32 and 33, respectively, in Table 1.

2.5.1. Metabolite extraction

Lyophilized samples were extracted using a 1145 μl mixture of chloroform:methanol:water (2:2:1.8) and vortexed in 2 ml glass vials. The polar and non-polar phases of this bi-phasic mixture were separated, and the polar phase (500 μl) was vacuum centrifuged (30 min at 300 K), frozen and freeze dried for nuclear magnetic resonance spectroscopy (NMR) analysis. For the non-polar phase, 300 μl were evaporated under N_2 and stored at −80 °C before MS analysis.

2.5.2. FT-ICR mass spectrometry based lipidomics

All dried lipid samples were resuspended in an equal volume of 2:1 methanol:chloroform with 5 mM ammonium acetate. Lipidomic analyses were conducted in negative ion mode using a hybrid 7-T FT-ICR mass spectrometer (LTQ FT Ultra, Thermo Fisher Scientific, Bremen, Germany) with a chip-based direct infusion nanoelectrospray ionisation assembly (Triversa, Advion Biosciences, Ithaca, NY). Nanoelectrospray conditions comprised of a 200 nl/min flow rate, 0.4 psi backing pressure and -1.2 kV electrospray voltage controlled by ChipSoft software (version 8.1.0). Mass spectrometry conditions included an automatic gain control setting of 5×10^5 and a mass resolution of 100,000. Analysis time was 4.25 min (per technical replicate), controlled using Xcalibur software (version 2.0, Thermo Fisher Scientific). Spectra were collected using the "SIM stitching" method, *i.e.*, fourteen overlapping selected ion monitoring (SIM) mass ranges, ranging from *m/z* 70 to 2000, were acquired and subsequently fused together, [72,95]. Duplicate analyses were averaged for each sample. A quality control (QC) sample consisting of a pooled aliquot of the samples was analysed before during and after the analysis.

2.5.3. NMR spectroscopy based metabolomics

Subsequently the dried polar metabolite fraction was resuspended in 200 μl D_2O with 1 mM TMSP and transferred to NMR tubes. All samples were maintained at 277 K and analysed within 48 h of resuspension. NMR was performed on a Bruker DRU 600 NMR spectrometer (600.23 MHz for ^1H) fitted with a 5 mm CPQCI cryogenic probe (Bruker Corporation). Three mm NMR tubes were used with the Bruker Sampletrack autosampler in which the samples were kept at 279 K before (and after) analysis. The spectra were recorded at 300 K with suppression of the residual water resonance using the noesygppr1d pulse sequence from the Bruker pulse sequence library. A pulse width of 7.91 μs was used to collect 128 free induction decays with 32 K data points with a spectral window of 12,019 Hz (20 ppm). The acquisition time was 2.73 s and the interscan delay was 3 s. The noesy mixing time was 10 ms, the data were zero filled to 64 K and exponential line broadening of 0.3 Hz applied before Fourier transformation and the spectra were phased and baseline corrected.

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	1.93
PPARA	NM001123560	TCTCCAGCCTGGACCTGAAC	GCCTCGTAGACGCCGTACTT	58	2.00
CYP3A	DQ361036	ACTAGAGAGGGTCGCCAAGA	TACTGAACCGCTCTGGTTTG	146	1.90
ACOX1	DY733173	CACTGCCAGGTGTGGTGGTA	GGAATTCGTACGTTCTCCAATTTCA	94	2.04
FBP4	BT125322	CCGCCGACGACAGAAAAA	TTTTGCACAAGGTTGCCATTT	61	1.99
CPT2	BG934647	TGCTCAGCTAGCGTTCCATATG	AGTGCTGCAGGACTCGTATGTG	49	2.07
VTG	AY049952	GACTTCGCCATCAGCCTTTC	GCCACGGTCTCCAAGAAGTCT	110	2.11
EF1AB	AF321836	TGCCCCTCCAGGATGTCTAC	CACGGCCCACAGGTACT	59	2.04
UBA52	GO050814	TCAAGGCCAAGATCCAGGAT	CGCAGCACAAGATGCAGAGT	139	2.01
B-ACTIN	BG933897	CCAAAGCCAACAGGGAGAA	AGGGACAACACTGCCTGGAT	92	1.96

2.6. Quantitative real-time RT-PCR

2.6.1. RNA extraction

The RNeasy Plus mini kit (Qiagen, Crawley, UK) was used to extract total RNA according to the manufacturer's protocol. RNA was eluted in 30 μl RNase-free MilliQ H_2O and stored at −80 °C. The RNA quantity and quality were assessed with the NanoDrop® ND-1000 UV–vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) pursuant to the manufacturer's instructions. The integrity of the RNA was evaluated with the RNA 6000 Nano LabChip® kit (Agilent Technologies). The samples used in this experiment had 260/280 nm absorbance ratios and a 260/230 nm ratios above 2 and RNA integrity number (RIN) values above 9, which indicate pure RNA and intact samples [66].

2.6.2. Quantitative real-time RT-PCR

The transcriptional levels of selected target genes were quantified with a two-step real-time RT-PCR protocol. A serial dilution curve of total RNA with six points in triplicates between 1000–31 ng were made for PCR efficiency calculations. 500 ng of total RNA was added to the reaction for each sample, and reverse transcription (RT) reactions were run in duplicates using 96-well reaction plates. No-template control (ntc) and no-amplification control (nac) reactions were run for quality assessment for every gene assay. The 50 μl RT reactions were performed at 48 °C for 60 min utilizing a GeneAmp PCR 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Individual RT reactions contained 1X TaqMan RT buffer (10×), 5.5 mM $MgCl_2$, 500 mM dNTP (of each), oligo dT primers (2.5 μM), 0.4 U/μl RNase inhibitor and 1.67 U/μl Multiscribe Reverse Transcriptase (Applied Biosystems) and RNase-free water.

For every gene analysed, real-time qPCR was run in 10 μl reactions on a LightCycler® 480 Real-Time PCR System (Roche Applied Sciences, Basel, Switzerland) containing 2.0 μl cDNA (diluted twofold). The real-time qPCR was carried out in two 384-well reaction plates using SYBR Green Master Mix (LightCycler 480 SYBR Green master mix kit, Roche Applied Sciences, Basel, Switzerland) containing gene-specific primers and FastStart DNA polymerase. PCR runs were performed with a 5 min activation and denaturing step at 95 °C, followed by 45 cycles with each cycle consisting of a 10 s denaturing step at 95 °C, a 10 s annealing step (60 °C) and finally a 10 s extension step at 72 °C. The primer pairs had an annealing temperature of 60 °C; see Table 2 for primer sequences, amplicon sizes and GenBank accession numbers. Final primer concentrations of 500 nM were used. For confirmation of amplification of gene-specific products, a melting curve analysis was carried out and the second derivative maximum method [82] was used to determine crossing point (CT) values using the Lightcycler 480 Software. To calculate the mean normalized expression (MNE) of the target genes, the geNorm VBA applet for Microsoft Excel version 3.4 was used to calculate a normalization factor based on three reference genes. By using gene-specific efficiencies calculated from the standard curves, the CT values are converted into quantities [89]. Elongation factor 1 AB (EF1AB), acidic ribosomal protein (ARP) and β-actin were the selected reference genes for this experiment. The reference genes were stable with gene expression stability (M) values of 0.304.

2.7. Data analysis

2.7.1. xCELLigence

GraphPad Prism 6.0 software (GraphPad Software Inc., Palo Alto, CA, USA) was used for the statistical analyses of the xCELLigence dose-response evaluation using one-way ANOVA followed by a Dunnett's post hoc test ($p < 0.05$) to detect treatment variation in nutrient exposed hepatocytes. Mean ± SE were calculated for three replicates.

2.7.2. Metabolomics

The processed NMR spectra were imported into Matlab (The Mathworks, Inc.) using ProMetab v3.3 software [90]. The region from 10 to 0.5 ppm was imported with a resolution of 0.02 ppm which resulted in 4750 data points, and transformed using a generalized log transformation. PCA and PLS-DA analysis was performed in PLS-toolbox v7.0.1 (Eigenvector Research, Inc.) on normalized and mean centred data prior to multivariate statistical analyses. The quantitative data from NMR were first subjected to the Shapiro–Wilk test for normality and the metabolites were subjected to the Kruskal–Wallis analysis of variance followed by Games–Howell post hoc testing [21] in order to assess the comparisons of statistically significant changes between groups compared to the control.

2.7.3. Lipidomics

All analyses were performed in Matlab 7.8.0 with PCA and PLS-DA analysis performed in PLS-toolbox v.6.7.1. Mass spectra were processed using a three-stage filtering algorithm as described in Ref. [59]. Samples were subsequently normalised using probabilistic quotient normalization [14]. Processing the raw mass spectra yielded a dataset that was further optimised using a QC based method as described in Ref. [39]. Mass features with over 20% missing values across all samples were removed and the resulting intensity matrix was submitted for univariate statistical analysis as described below. A k-nearest neighbor approach [31] was applied to impute missing values to the same dataset and it was transformed using a generalized log transformation (glog) prior to multivariate statistical analysis [14]. The final lipidomics dataset was comprised of 901 mass features upon which statistical analyses were conducted. PCA and PLSDA were performed to assess the overall effect (three outliers which were outside the Hotellings t2 plot were first removed). All supervised models were validated using cross validation and permutation testing to avoid overfitting. Univariate statistical analyses were also conducted on the

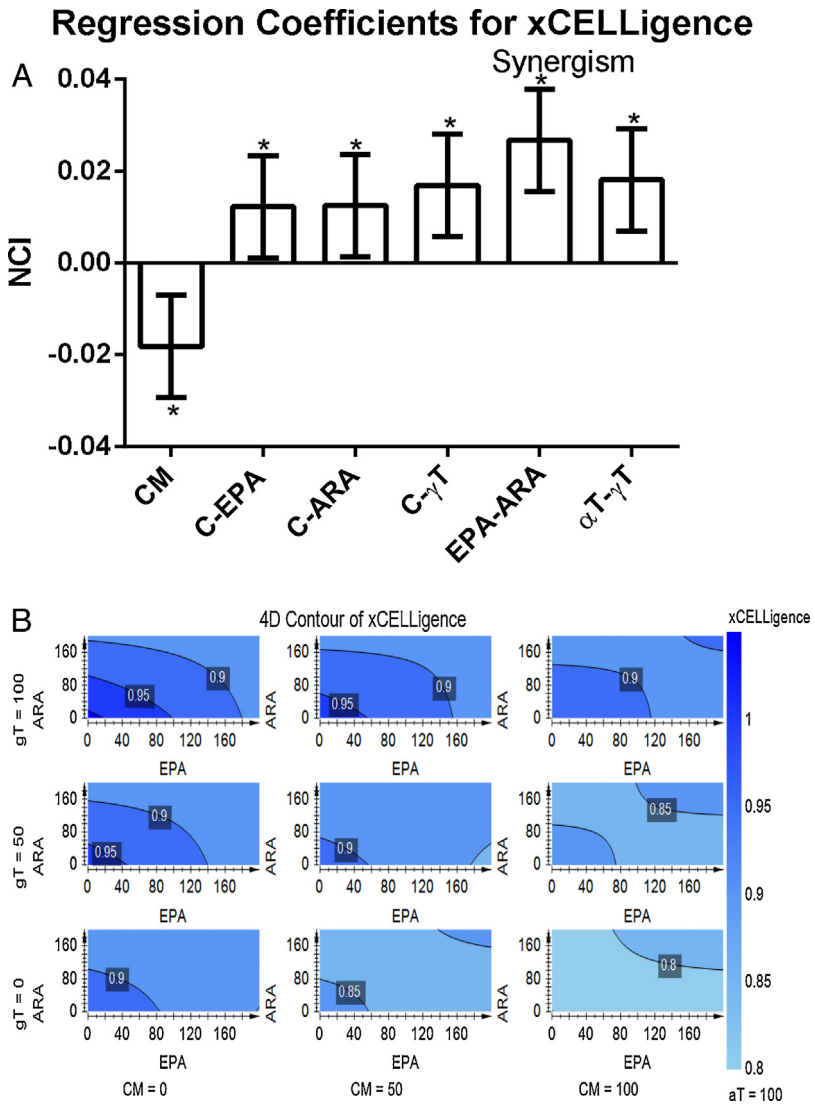

Fig. 1. A Simplified scaled and centered PLS regression coefficients with 95% confidence intervals for Normalized cell index (NCI) levels measured in primary Atlantic salmon hepatocytes exposed to eicosapentaenoic acid (EPA), arachidonic acid (ARA) and α-tocopherol (αT), γ-tocopherol (γT) and contaminant mixture (CM) accordingly to the factorial design ($N = 5$). The CM was composed of 100 μM of benzo(a) pyrene and phenanthrene and 1 μM of chlorpyrifos and endosulfan. The model is based on 33 experimental objects, and had one PLS component. The model containing five linear terms and eight interaction terms ($R^2 = 0.85$ and $Q^2 = 0.55$). Only linear (CM) and interaction terms representing contaminant-nutrient interactions (C-EPA, C-ARA, C-γT) and important nutrient-nutrient interaction (EPA-ARA, αT-γT) were included in the figure (confidence level = 0.95). Significant PLS regression coefficients are indicated with a *($p < 0.05$). The complete PLS regression model equation is described in the supplementary A1. The regression coefficients reflecting the impact of the factors on the PLS model. B 4D contour plot of xCELLigence cytotoxicity NCI levels as a function of EPA and ARA with increasing levels of CM and γT on the *X*- and *Y*-axis, respectively, keeping αT constant at 100 μM. The highlighted values in the plot represent NCI levels for the different stratification beddings (isoboles).

lipidomics dataset. A Kruskal–Wallis analysis of variance followed by Games–Howell post hoc testing [21] was conducted in order to assess the comparisons of statistically significant changes between groups compared to the control. A Benjamini–Hochberg correction of 10% was applied to the KW results to control for false discovery [3]. The eigenvalues for the different groups in the different PCA plots are presented in the Supplementary Table A3.1-A3.5.

For putative annotation of the detected mass features, the KEGG database and MI-Pack software ('single peak search' approach) were first used to assign one or more empirical formula(e), *e.g.*, $C_cH_hN_nO_oP_pS_s$, as well as putative metabolite names to each mass feature [94], assuming a maximum mass error of ±2 ppm. This assignment equates to MSI level 2 [75].

2.7.4. RT-qPCR

Regression was performed with PLS [98] to correlate the design matrix to the responses of different transcripts. MODDE 9.0 (Umetrics, Umeå, Sweden) was used for the experimental design and the PLS analysis. Before the PLS analysis the blend matrix was augmented with interaction terms, the data were scaled to unit variance and mean centred. The PLS models were validated with respect to explained variance and goodness of prediction (shown as Q^2), obtained after cross validation [97]. In addition, the PLS models were evaluated with respect to goodness of fit (R^2).

3. Results

3.1. xCELLigence cytotoxicity screening

A multivariate model was used to analyse the cytotoxicity data. The PLS model, based on the xCELLigence normalized cell index (NCI), contained three significant positive nutrient–contaminant interactions. Of the nutrient–contaminant interactions, the interaction of the contaminant mixture (CM) and γT, the C-γT ($p = 0.005$),

displayed increased cell viability (Fig. 1A). In addition, the PLS plot contained two significant nutrient–nutrient interactions of the lipids (EPA–ARA) and the tocopherols (αT–γT) with the EPA–ARA ($p = 0.000078$) interaction showing the strongest positive effect on the response, increasing the cell viability. The contour plot analysis of all the nutrients and the CM showed that the cell viability reduction caused by the CM was almost completely counteracted when cells were exposed to the CM in combination with all the nutrients due to a synergistic effect (Fig. 1B). The individual nutrients only moderately reduced cell viability. Only co-exposure to all nutrients provided sufficient protection against CM induced cytotoxicity.

3.2. Metabolomics and lipidomics

3.2.1. FT-ICR mass spectrometry lipidomics

A selection of the different mixtures were analysed with lipidomics and metabolomics to examine the mechanism of how nutrients affect the toxic effects of the CM. The exposure groups analysed were; cells exposed to a single nutrient (EPA, ARA and αT and γT) or CM, one nutrient in combination with the CM (C-EPA, C-ARA, C-αT and C-γT) and either a low or high concentration of the CM and all nutrients (C-All low and C-All high). Initially each of the four nutrients along with the CM was investigated with PCA and PLSDA to reveal the individual effects of the treatments. In the lipidomics PCA plots, the cells treated with the CM, ARA and EPA showed clear separation from each other and the control along PC1 (Fig. 2A and B). ARA was the nutrient that showed the greatest global effect on the lipidome. The PLSDA analysis displayed similar effects with $p \leq 0.05$ (from permutation testing) and classification error rate ≤5% for these particular compounds (data not shown). Cells treated with the other nutrients (tocopherols) could not be reliably separated from the control cells (Fig. 2C ($p = 0.104$, Table A4.4) and 2D ($p = 0.004$, Table A4.5)). In contrast to αT, the γT was significant different from the other groups, however, due to small sample size and large number of latent variables, γT were therefore together with αT excluded from further analysis.

PCA scores plots for cells treated with either control DMSO, CM or a fatty acid plus CM showed that the addition of the fatty acids to the CM (Fig. 2A and B) shifted the lipidome back towards the normal (untreated) state. A similar but dose related effect on the lipidome was observed when all nutrients were combined together and administered alongside the CM at either a high or a low dose (Fig. 2E).

To assess if the fatty acids had an ameliorating effect on the CM toxicity, mass features that had been shown to be significantly changing between CM treated cells and the control DMSO treated cells were used as a proxy indicator of toxicity. The list of significantly different mass features was checked to see whether these features remained significantly different from the control class in the combined nutrient and CM treatment group. Analysis was conducted using Kruskal–Wallis analysis of variance (level of significance set at $q < 0.1$) followed by Games Howell post hoc testing (level of significance defined as $p < 0.05$). In total, 88 mass features were deemed significant across the different treatment groups (Tables A2.1 and A2.2 in Supplementary), however, only 13 metabolites were annotated with individual putative identities and are presented in Table 3. The C-EPA group had the largest number of 41 affected metabolites, *e.g.*, four different adducts of dihydroxy norvitamin D3 (hydroxypropyl) with a mean fold change of between 6.84 and 11.34 in addition to xeniasterol, phosphatidylinositol (PI 42:5) and cholesterol sulphate with mean fold changes of 2.26, 1.96, and −1.52, respectively. The CM was the second most affected group with 31 individual metabolites that changed significantly compared to the control. Four features, all putatively annotated as different adduct forms of dihydroxy norvitamin D3, had a mean fold change of between 10.11 and 15.39 and the phosphatidylglycerol (P-33:2) and phosphatidylserine (P-32:1) levels were both significantly reduced in the CM. In the ARA exposed group, 27 metabolites were affected overall, and of the individual putatively annotated metabolites, three metabolites were elevated compared to the control; two phosphatidylglycerols (PG 22:4) with fold changes of 13.44 and 13.42, and phosphatidylethanolamine (PE 42:10) with a fold change of 2.60. Eighteen metabolites were affected in C-ARA group, *e.g.*, phosphatidylethanolamine (PE 42:10) and phosphatidylethanolamine (PE 42:9) were both elevated compared to the control with a fold change of 2.43 and 1.52, respectively. In the EPA group, 6 metabolites changed significantly compared to the control, however no putative identifications could be ascribed to these. Both EPA and ARA effected the CM lipidomic response in cells, however cells exposed to all nutrients showed globally a stronger protective effect against the lipidomic disturbances caused by the CM.

3.2.2. NMR spectroscopy metabolomics

The PCA and PLSDA analyses of the water-soluble metabolites showed a larger effect globally than at the individual metabolite level. The variations in the entire metabolic fingerprints from NMR represented by PCA and PLS scores plots showed shifts in some of the groups compared to the control. The CM group was separated from the control along PC2 (Fig. 3A), thus indicating metabolic shifts due to the exposure. The addition of the two fatty acids in combination with the CM treatment (C-EPA and C-ARA) shifted the metabolic status observed by NMR towards the control samples compared to the CM group alone (Fig. 3B and C). As can be observed, the direction of change in the scores plots was the same for the C-EPA and C-ARA, indicating similar shifts in the NMR observable metabolome, but with different magnitudes. The alleviating effect was more expressed for ARA where the C-ARA group was better separated from the CM group (Fig. 3C). For the EPA and C-EPA groups, both clustered between the control and the CM group (Fig. 3B). No apparent clustering was observed to support any alleviating effect from the C-αT and C-γT groups (Fig. 3D and E). The control and both C-All groups were separated from the CM group along PC2, but there was no observable differences between C-All high and C-All low groups (Fig. 3F). The low and high levels of the CM in combination with all nutrients reduced the toxicity more than individual fatty acids exposed in combination with CM, by shifting the metabolic response to almost overlapping the metabolic response observed in the control treatment. At the individual metabolite level, statistical analysis with a Kruskal–Wallis test followed by the Games Howell post hoc test resulted in no significant metabolites (data not presented) which may be reflective of the small sample size combined with the lower statistical power inherent in all non-parametric tests. Cells exposed to all nutrients in combination with the CM showed a stronger ameliorating effect than individual fatty acids exposed in combination with CM.

3.3. RT-qPCR

PLS interaction evaluation was performed on candidate biomarkers that belong to well-known toxicological pathways; cytochrome P450 1A (CYP1A, Fig. 4A), CYP3A (Fig. 4B), vitellogenin (VTG, Fig. 4G), and four lipid metabolism candidate markers; fatty acid-binding protein 4 (FABP4, Fig. 4F); peroxisome proliferator-activated receptors (PPARa, Fig. 4C), carnitine palmitoyltransferase 2 (CPT2, Fig. 4D) and peroxisomal acyl-coenzyme A oxidase 1 (ACOX1, Fig. 4E). PLS models were based on the mean normalized expression levels (MNE) obtained from cells exposed to nutrients and contaminant mixture using a factorial design in order to determine possible contaminant- nutrient interactions.

In all PLS models, except for ACOX1, the CM had the strongest effect on the transcriptional levels compared to the other treat-

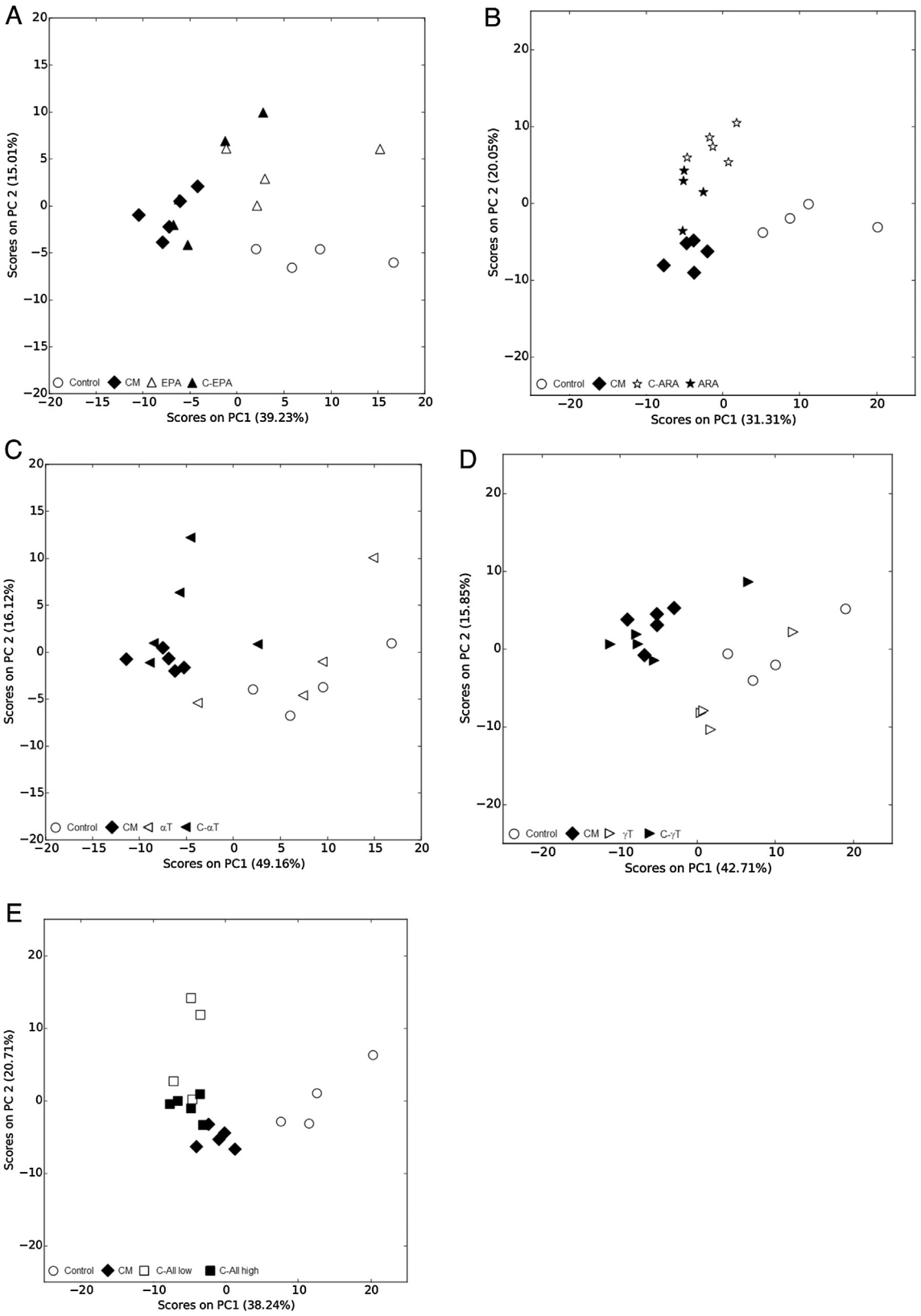

Fig. 2. PCA scores plots from lipidomics data of salmon hepatocytes treated with different nutrients combined with a contaminant mixture (CM) versus control DMSO and the CM alone. A Effect of eicosapentaenoic acid (EPA) compared to the C-EPA, B arachidonic acid (ARA) compared to C-ARA, C α-tocopherol (αT) compared to C-αT, D γ-tocopherol (γT) compared to C-γT. E The dose related effect on the lipidomic profile when a low dose (C-All low) or a high dose (C-All high) of combination of CM and all nutrients were used.

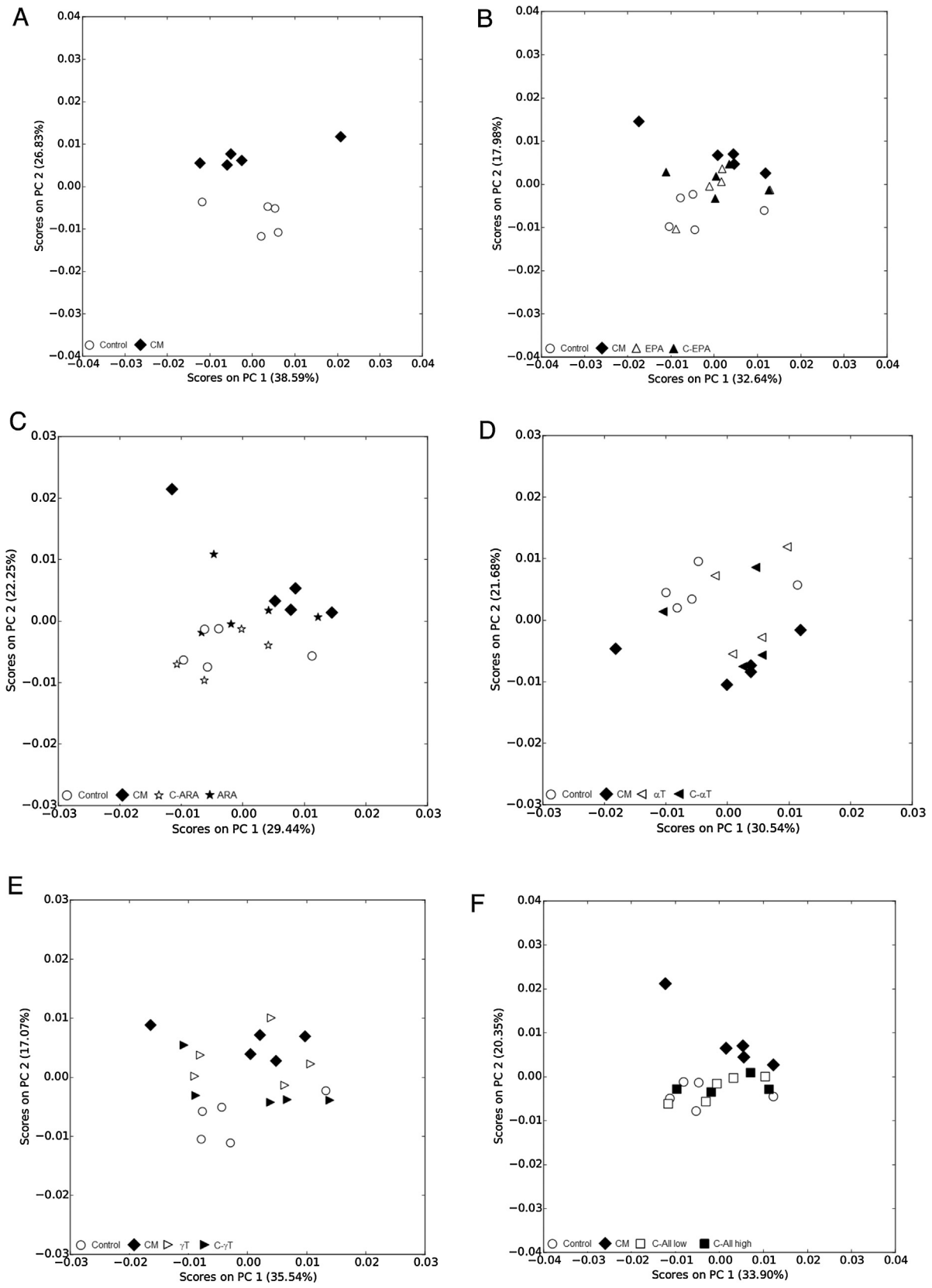

Fig. 3. Multivariate analyses of NMR metabolic fingerprints for Atlantic salmon hepatocyte responses to exposure shown by A PCA analysis of the response of the contaminant mixture (CM) exposure versus control (DMSO). B The effects of eicosapentaenoic acid (EPA) and C-EPA, C arachidonic acid (ARA) and C-ARA, D α-tocopherol (αT) and C-αT, E γ-tocopherol (γT) and C-γT, and F C-All low and C-All high (low or high concentration of the CM and all nutrients) compared to the effect of CM.

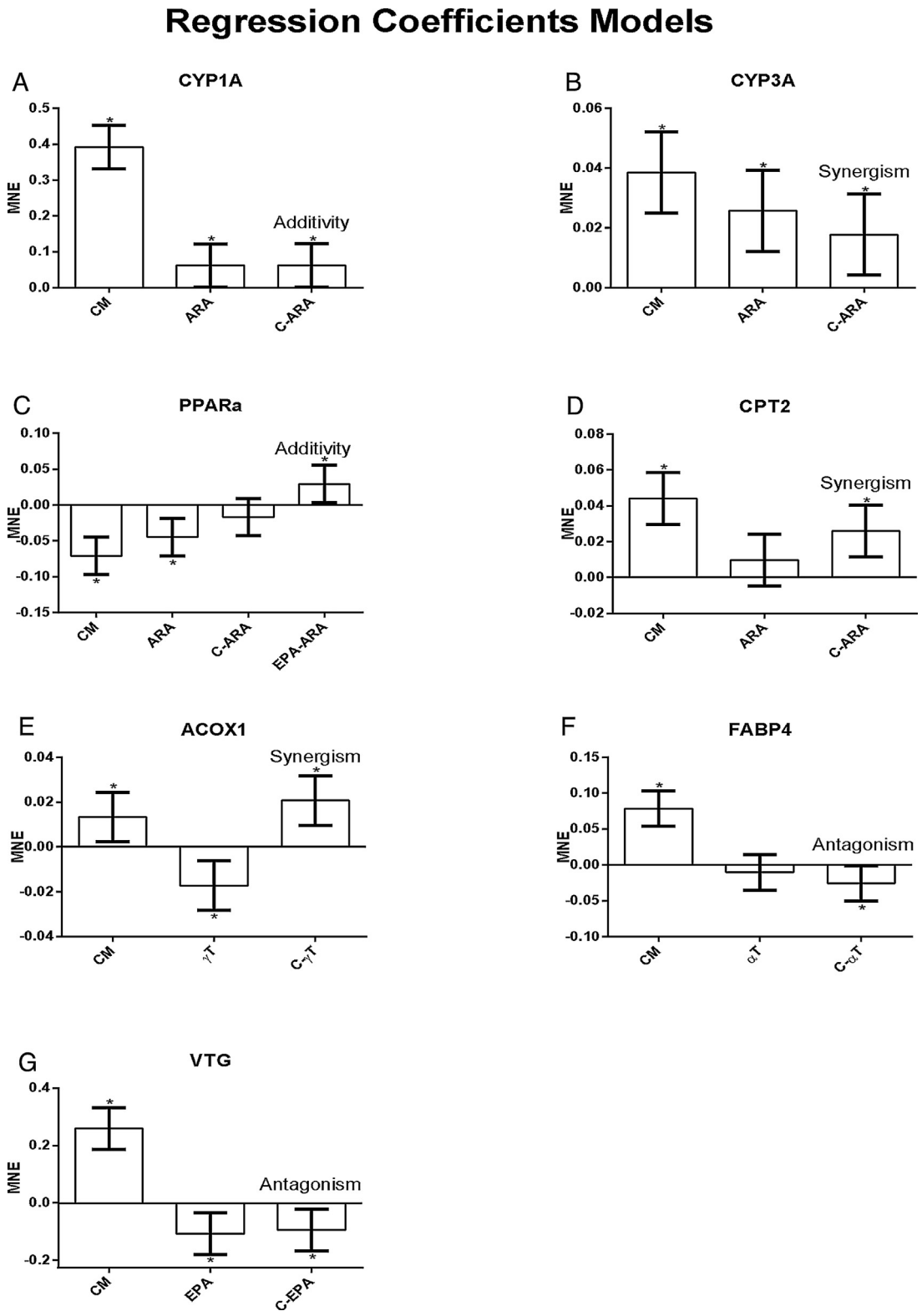

Fig. 4. Simplified scaled and centered PLS regression coefficient models for different transcripts measured in primary Atlantic salmon hepatocytes exposed to eicosapentaenoic acid (EPA), arachidonic acid (ARA), α-tocopherol (αT), γ-tocopherol (γT) and contaminant mixture (CM) using mean normalized expression (MNE) and a factorial design ($N = 5$). CM contained 100 μM of benzo(a) pyrene and phenanthrene and 1 μM of chlorpyrifos and endosulfan. The combined effects identified with contour plot analysis like additivity, synergism or antagonism are presented in the different PLS regression coefficient models (confidence level = 0.95). Significant PLS regression coefficients are indicated with a *($p < 0.05$). A Cytochrome P450 1A (CYP1A), $R^2 = 0.90$, $Q^2 = 0.76$. B Cytochrome P450 3A (CYP3A), $R^2 = 0.84$, $Q^2 = 0.62$. C Peroxisome proliferator-activated receptors (PPARα), $R^2 = 0.76$, $Q^2 = 0.53$. D Carnitine palmitoyltransferase 2 (CPT2), $R^2 = 0.78$, $Q^2 = 0.52$. E Peroxisomal acyl-coenzyme A oxidase 1 (ACOX1), $R^2 = 0.79$, $Q^2 = 0.55$. F Fatty acid-binding protein 4 (FABP4), $R^2 = 0.74$, $Q^2 = 0.47$. G Vitellogenin (VTG), $R^2 = 0.77$, $Q^2 = 0.58$. The complete PLS regression model equations are described in the supplementary A1. Only important liner and interaction terms representing contaminant-nutrient and nutrient–nutrient interactions were presented in the figures. The regression coefficients reflecting the impact of the factors on the PLS model.

Table 3
Table of putatively annotated mass features significantly different between hepatocytes treated with control DMSO and those treated with either a contaminant mixture (CM), eicosapentaenoic acid (EPA), arachidonic acid (ARA), or CM and ARA (C-ARA) or CM and EPA (C-EPA). Fold changes in bold indicate results that were significantly different with respect to the control. Mass features are represented more than once if they were significantly different with respect to the control for more than one class. Analysis was conducted using Kruskal Wallis analysis of variance (level of significance set at $q < 0.1$) followed by Games Howell post hoc testing (level of significance defined as $p < 0.05$).

Mean fold change (compared to the control)								
m/z	EPA	C-EPA	CM	ARA	C-ARA	Empirical formula	Putative Annotation	Adducts
461.3636	1.39	**6.91**	**10.11**	1.26	8.95	C29H50O4	(Hydroxypropyl) Dihydroxy norvitamin D3	[M – H]-
462.3671	1.38	**6.84**	**10.12**	1.29	8.86	C29H50O4	(Hydroxypropyl) Dihydroxy norvitamin D3	[M – H]- C13
521.3846	1.51	**11.24**	**15.26**	1.08	12.35	C29H50O4	(Hydroxypropyl) Dihydroxy norvitamin D3	[M + Hac-H]-
522.3882	1.51	**11.34**	**15.39**	1.04	12.40	C29H50O4	(Hydroxypropyl) dihydroxy norvitamin D3	[M + Hac-H]- C13
559.3044	**–**	**–**	-1.89	**13.42**	7.40	C28H49O9P	PG(22:4)	[M – H]-
560.3077	**–**	**–**	-1.61	**13.44**	7.68	C28H49O9P	PG(22:4)	[M – H]-
810.5082	**–**	**–**	1.01	**2.60**	**2.43**	C47H74NO8P	PE(42:10)	[M – H]-
812.5241	–	–	1.02	1.66	**1.52**	C47H76NO8P	PE(42:9)	[M – H]-
501.28166	−1.53	−**1.52**	1.2	–	**–**	C27H46O4S	Cholesterol sulphate	[M + Cl]-
551.39556	1.32	**2.26**	2.36	–	**–**	C30H52O5	Xeniasterol B	[M + Hac-H]-
775.51214	−1.04	−1.18	−**1.27**	–	**–**	C39H73O9P	PG(P-33:2)	[M + Hac-H]-
776.50978	−1.05	−1.18	−**1.26**	–	**–**	C38H72NO9P	PS(P-32:1)	[M + Hac-H]-
939.59786	2.25	**1.96**	1.20	–	**–**	C51H89O13P	PI(42:5)	[M – H]-

ments. The PLS analysis of the transcripts CYP1A and PPARα showed that ARA was the only nutrient that had a significant contribution to the transcription levels; having an additive combined effect together with the CM. For CYP1A (Fig. 4A), the additive response ($p = 0.044$) caused increased transcription while the PPARα additivity ($p = 0.002$) gave a reduced transcription (Fig. 4C). The other nutrients, EPA, αT and γT did not have any significant contribution on CYP1A and PPARa transcription.

A synergistic interaction effect between ARA and the CM was predicted by the PLS models for CYP3A (Fig. 4B, $p = 0.013$) and CPT2 (Fig. 4D, $p = 0.001$), resulting in increased transcription. The PLS model for ACOX1 (Fig. 4E) showed a similar synergistic interaction effect between yT and the CM ($p = 0.0008$). For FABP4 (Fig. 4F), the negative interaction term for C-αT predicted an antagonistic effect between αT and the CM ($p = 0.044$), reducing the transcription levels. Lastly, the VTG PLS model (Fig. 4G) revealed an antagonistic effect between EPA and the CM ($p = 0.014$). Based on the effects on toxicological important markers, EPA and α-tocopherol seemed more beneficial than ARA and γ-T in preventing the adverse effects induced by the CM.

4. Discussion

Today's Atlantic salmon feed with high inclusion levels of plant ingredients contain a reduced amount of EPA, αT and contaminants such as persistent organic pollutants (PCBs, dioxins etc.) and heavy metals (Cd, Hg). At the same time, higher levels of plant-derived contaminants are found in plant based feeds like PAH and pesticides, and nutrients such as γT and ARA derived from linoleic acid (LA). The levels of feed nutrients and contaminants will vary with which type of feed ingredients used and actual feed composition of the feed ingredients [5,25,28,34,62,77,84,85]. To assure that the feed contain sufficient levels of tocopherol, synthetic αT is supplemented as a-tocopheryl acetate to Atlantic salmon feeds [28]. These tocopherols and fatty acids EPA and ARA have different kinetics and biological roles in cultured salmon [2,28,60], with the possibility to affect the toxicity of contaminants differently. This study show that the nutrients EPA, ARA and αT, γT affect, individually and together, the toxicity of a CM composed of two PAHs and two pesticides. The addition of nutrients to the contaminant mix (CM) significantly increased the viability of Atlantic salmon hepatocyte cells compared to exposure to CM alone. PAHs and particularly lighter PAHs, like phenanthrene, are able to reduce cell viability [65,79] in teleost *in vitro* systems, used at the same concentration range as in this study [79]. Of the pesticides, only endosulfan has previously been shown to negatively affect cell viability of Atlantic salmon hepatocytes, however the reduction was observed at higher exposure concentration [79] than used in the present study. Therefore, it is most likely that it is the PAHs that are driving the negative effect observed in cell viability. The cytotoxicity of PAHs has been suggested to be caused by their ability to embed in and disrupt cellular membranes [65] by increasing their fluidity [46]. The cytotoxicity data obtained from the current contaminant-nutrient interaction evaluation suggest that the negative effects of the CM might be counteracted by the nutrients. Of the individual nutrients, γT had the strongest positive effect on the cell viability. Evaluation of the C-γT interaction term showed that γT seems to ameliorate the toxicity of the CM. αT is known to be the most important antioxidant that resides within cell membranes, protecting the lipids from peroxidation [1], and maintaining the integrity of membranes by preventing the initiation of cell lysis [28]. Mammalian endothelial cells enriched with the n-6 PUFA LA showed a weakened endothelial barrier for PCB owing to a discrepancy in cellular status of antioxidant/oxidative stress [101]. In addition, PCB caused an increased uptake of LA. However, the dysfunction of the endothelial barrier caused by the PCB was completely restored by αT owing to reduced oxidative stress and production of inflammatory cytokines [101]. In a study where rats were exposed to iron-dextran in combination with αT or γT, it was shown that γT similar to αT is able to inhibit lipid oxidation [29]. This suggests that γTs, by preventing lipid oxidation, are able to ameliorate the effect on the cell viability caused by the CM.

To generate hypotheses about the potential modes of action of the studied CM, nutrients and nutrient–contaminant combinations, lipidomic and metabolomic profiling were employed. ARA was the nutrient that showed the greatest global effect on the lipidome, having a stronger effect than the CM and EPA. Both forms of vitamin E had the least effect on the lipidomic and metabolomic responses, even though previous research suggest vitamin E has low effect on hepatocytes cell viability [50]. In total, 13 putative annotated metabolites were significantly affected. The C-EPA combination induced the most significant perturbed mass feature changes followed by the CM, ARA and C-ARA. EPA did not induce any significant mass feature changes. Both C-EPA and the CM significantly altered four mass features, identified as the vitamin D analogue 19 nor-2α-(3-hydroxypropyl)-1α,25(OH) 2D3 (dihydroxy norvitamin D3). The lipidomic data showed that 19 nor-2α-(3-hydroxypropyl)-1α,25(OH) 2D3 was significant increased 10.11–15.39 fold in the CM group and 6.84–11.34 fold in the C-EPA group compared to the control. 19 nor-2α-(3-hydroxypropyl)-

1α,25(OH) 2D3 is an analog of the active vitamin D form 1α,25(OH) 2D3 [9]. This confirms previous results where benzo(a) pyrene and a similar contaminant mixture of PAH and pesticides induced an effect on the steroid synthesis pathway and vitamin D3 metabolism in primary Atlantic salmon hepatocytes [79]. The 19 nor-2α-(3-hydroxypropyl)-1α,25(OH) 2D3 has been shown to be effective in inhibiting cell proliferation in HepG2 cells [9] and in preventing cell growth in MCF-7 cells, and BxPC-3 tumor development in mouse [8]. The increased synthesis of the 19 nor-2α-(3-hydroxypropyl)-1α,25(OH) 2D3 in exposed Atlantic salmon hepatocytes might therefore prevent potential genotoxic effects induced by PAHs, like benzo(a) pyren [16,86,35].

Perturbed lipid and cholesterol homeostasis by contaminants has been demonstrated in numerous mammalian [19] and teleostean [19,79] studies. Contaminants affecting the lipid composition of membranes can significantly influence membrane transport, bioenergetics and cell signalling, as well as membrane function and integrity [19]. ARA and C-ARA treatments gave the strongest effect on cell membrane lipids in exposed cells; phosphatidylglycerol (PG 22:4) was 13-fold upregulated in ARA exposed cell and phosphatidylethanolamine, (PE 42:10) was 2.6- and 2.43-fold upregulated in the ARA and C-ARA exposed cells, respectively. The lipid phosphatidylglycerol is synthesized in the mitochondria and is used for production of cardiolipin, a mitochondrial inner membrane lipid stabilizing the electron transfer complex [51,88,87]. Phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol, key structural lipids in eukaryotic membranes [88] and particularly enriched in the inner cytoplasmic leaflet [38], were all found to be affected in this study. The physical properties of the membrane depends on the n-3 and n-6 PUFA acyl chains which affect the lateral organization, curvature etc. [69]. Bilayers becomes more deformable and disordered with ARA than those containing more double bonds like DHA. In PAH and PCB contaminated mussels, an increase in the non-polar/polar lipid ratio in the mussels has been observed, suggesting that the contaminants affected the lipid homoestasis by reducing the conversion of storage lipid to membrane lipids [19]. A similar reasoning can be used to explain the downregulation of the membrane lipids phosphatidylglycerol and phosphatidylserine by the CM treatment in the present study. Both excessive and deficient amount of fatty acids can induce mitochondrial and ER-stress responses, production of free radicals and steatosis in hepatocytes [11,24,99]. Only hepatocytes treated with C-EPA showed an effect on the cholesterol analogue cholesterol sulphate that was −1.52-fold down regulated. The homeostasis of cholesterol is tightly regulated in animals, *i.e.*, the amount absorbed via the diet, produced via de novo cholesterol synthesis, and excreted as bile salts or biliary cholesterol [10,37,48]. In mammals, cholesterol sulphate is an important component of cell membranes where it function as a stabilizing agent in a similar way as cholesterol [73]. This suggests that the C-EPA treatment reduced the stability of the cell membrane, though this effect was minor.

To further identify nutrient–contaminant interactions, genes encoding selected targeted biotransformation enzymes, lipid metabolism and endocrine effect markers were analysed with qPCR and PLS. Similar to the lipidomics data, ARA gave the strongest effect on gene transcription. Cells exposed to the CM in combination with ARA showed an additive and synergistic induction of the CYP1A and CYP3A transcripts, respectively. The other nutrients, EPA, αT and γT did not significantly alter the CM-induced response. Several CYP transcripts like CYP1A and CYP3A are not only involved in biotransformation of contaminants like benzo(a) pyrene and chlorpyrifos [79] but also play a critical role in degradation and synthesis of endogenous compounds like lipids [6]. Several CYP enzymes like CYP1A and CYP3A have previously been found to be involved in the CYP-dependent metabolising of ARA or LA into eicosanoids in mammals both *in vivo* and *in vitro* [6,13,41] CYP1A, which has been seen as the primarily biotransformation enzyme and activator of toxic and carcinogenic contaminants [56], is important in the bioactivation of benzo(a) pyrene, into the ultimate carcinogen, the diol epoxide [40]. In mammals, CYP1A and CYP3A have been shown to bioactivate chlorpyrifos to a chlorpyrifos–oxon, which is a potent anticholinesterase [80], and CYP3A to convert β-endosulfan to endoulfan sulphate which is as toxic as the mother compound [44]. CYP1A biotransformation of poorly metabolised inducers can cause production of ROS, lipid peroxidation and ultimately can change the membrane function [19]. Thus, the additive and synergistic increased expression of CYP1A and CYP3A by ARA can increase the toxicity of the CM when exposed in combination.

Peroxisome proliferator-activated receptor α (PPARα) regulates the expression of several target genes linked to mitochondrial and peroxisomal β-oxidation of lipids [61]. ARA exposure gave an additive effect on the CM induced downregulation of PPARα. Though, despite the downregulation of PPARα, the C-ARA caused a weak synergistic increase of carnitine palmitoyltransferase 2 (CPT2). The CPT2 transcript is an inner mitochondrial enzyme that takes part in the oxidising of long-chain fatty acids [21], and is used as a marker for mitochondrial β-oxidation. γT contributed synergisticly to the CM induced increase of the transcription of peroxisomal β-oxidation enzyme peroxisomal acyl-coenzyme A oxidase 1 (ACOX1). ACOX1 is the first enzyme in peroxisomal β-oxidation and is in charge of the desaturation of acyl-CoAs to 2-*trans*-enoyl-CoAs [21]. This finding is in contrast to a study with rats where the mitochondria were found to have the main role in β-oxidation of αT [54]. The synergistic effect of the plant nutrients ARA and γT on the CM effect on mitochondrial and peroxisomal β-oxidation might cause the Atlantic salmon to lose weight. Pesticides like endosulfan have previously been shown to cause lipid metabolism disturbances such as steatosis in Atlantic salmon, both in exposed hepatocytes [43] and in *in vivo* studies [23]. However, an unbalanced diet can produce a similar effect in Atlantic salmon fed diets with high inclusion levels of plant ingredients in addition to low levels of EPA and DHA [45]. The fatty acid binding proteins (FABPs) are a family of proteins involved in lipid flux in cells [71]. In human hepatocytes and trophoblasts, increased lipid accumulation has been linked to elevated transcription of FABP4 [17,67,96]. An orthologue to mammalian FABP4 that may represent the adipose tissue type FABP (h6FABP or FABP11) in fish [83], was induced by both chlorpyrifos [58,79] and endosulfan [79] in previous Atlantic salmon hepatocytes studies. In the present study, FABP4 was significantly upregulated in cells exposed to the CM. In cells co-treated with αT, however, the FABP4 transcript level was downregulated, possibly due to an antagonistic interaction between the CM and the antioxidant. A similar downregulation has been observed in Atlantic salmon hepatocytes co-treated with chlorpyrifos and αT [58]. The present finding thus confirms that αT can reduce FABP4 transcription and possible protect against chemical-induced steatosis in Atlantic salmon.

Further, an antagonistic effect was also detected for the VTG transcript where EPA in combination with the CM induced a weak antagonistic effect on the VTG expression level. The egg yolk precursor protein VTG is generally produced in the female liver cells by estrogenic stimulation, originating from the developing ovarian follicles [18,30]. All four contaminants in the CM have the ability to interfere with the estrogen receptor (ER) pathway in fish [43,27,79]. In accordance with Søfteland et al. [79], exposure to the CM significantly increased VTG transcription, however, in combination with EPA, VTG upregulation was substantially reduced due to an antagonistic interaction. These results suggest that EPA can ameliorate the negative effect induced by the CM on the endocrine system. The mechanism behind EPA's ameliorating effect is however not known.

The PCA analysis of both the lipidomics and metabolomics data showed that all nutrients, in combination with the CM, gave a shifted response closer to the control compared to the CM alone, and the lipidomics data indicated that the fatty acids had the strongest ameliorating effect. The cytotoxicity analysis showed a similar effect. From all the individual CM-nutrient combinations, only γT showed ameliorating effect on the CM treatment. However, the cell viability reduction caused by the contaminants was only completely restored when the hepatocytes were co-exposed with all the nutrients. It is not surprising that the lipids EPA and ARA can affect the PAHs impact on cell viability because both lipids are dietary essentials for marine fish due to their limited ability to elongate and/or desaturate 18-carbon PUFA [63]. In teleosts, the composition of the neutral lipids (*e.g.*, storage lipids (TAG) and cholesterol) reflects the diet, and the polar lipids (*e.g.*, membrane phospholipids) the requirements of the membranes [63]. Fish membrane phospholipids hold a large surplus of EPA relative to ARA [2]. Whereas ARA has a more even distribution between the neutral and polar lipids, as seen in haddock larvae [60]. Lipophilic, non-polar contaminants like PCBs are more closely linked with neutral lipids, than the polar membrane phospholipids [19]. Non-polar contaminants with non-specific mode of action, like narcotics compounds and PAHs, are able to embed into membranes and disturb their function. Sequestration of the contaminants into neutral storage lipids will therefore protect the cells by preventing the contaminants to interact with the target, the polar membrane lipids [19]. Further, co-exposure of EPA and ARA might have reduced the uptake of contaminants into the primary hepatocytes. In a previous study it was found that the bioavailability of PCB in the intestine of Channel catfish (*Ictalurus punctatus*) was reduced when micelle carriers composed of different lipids like myristic, palmitic, stearic acids and LA were used while the bioavailability was increased when micelles were comprised of only LA [15]. This suggests that lipids and tocopherols in combination can have ameliorating effects on the contaminants ability to disrupt the integrity of cell membranes; the tocopherols are likely to prevent lipid peroxidation and the lipids prevent sequestration of the contaminants into polar lipids and possibly by reducing the bioavailability of contaminants.

5. Conclusion

The cytotoxicity results suggest that the negative effects of contaminants that can be found in plant-based salmon feed might be counteracted by nutrients. Of the nutrients, the fatty acids ARA and EPA showed the strongest perturbation effect on the lipidome, in contrast to the tocopherols that showed no effect. Responses most affected were cell membrane stability and vitamin D metabolism. The transcriptomic analysis identified nutrient-contaminant interaction effects on biotransformation, lipid metabolism and endocrine effects. Co-exposed with the contaminants, the n-3 PUFA EPA and αT showed ameliorating effects by antagonising the contaminants negative effect while the n-6 PUFA ARA and γT the toxicity of the contaminants due to an additive and synergistic interaction. Individually, at the selected concentrations the nutrients EPA and αT seem more beneficial than ARA and γT, nevertheless, a combination of all nutrients gave the strongest ameliorating effect on the toxicity of the contaminant mixture composed of PAH and pesticides.

Transparency document

The http://dx.doi.org/10.1016/j.toxrep.2016.01.008 associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxrep.2016.01.008.

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