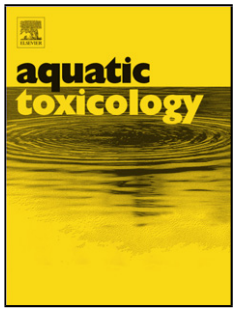

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Transcriptional effects of phospholipid fatty acid profile on rainbow trout liver cells exposed to methylmercury

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Highlights

- Fatty acid composition of fish liver cell phospholipids can be manipulated
- Methylmercury (MeHg) has few impacts on fatty acid composition of cell phospholipids
- n-3 and n-6 polyunsaturated fatty acids (PUFAs) have no impact on MeHg accumulation
- Fatty acid metabolism and stress response genes are influenced by PUFAs and MeHg

Abstract

Lipids, and their constitutive fatty acids, are key nutrients for fish health as they provide energy, maintain cell structure, are precursors of signalling molecules and act as nuclear receptor ligands. These specific roles may be of crucial importance in a context of exposure to pollutants. We recently showed that the fatty acid profile of rainbow trout liver cell phospholipids modulates sensitivity to an acute methylmercury challenge. In order to investigate mechanisms of effects, we herein tested whether specific polyunsaturated fatty acids may protect cells from methylmercury through decreasing intracellular mercury accumulation and/or enhancing cellular defences (e.g. via modulation of gene expression patterns). We also investigated the inverse relationship and assessed the impact of methylmercury on cellular fatty acid metabolism. To do so, the fatty acid composition of rainbow trout liver cell phospholipids was first modified by incubating them in a medium enriched in a specific polyunsaturated fatty acid (PUFA) from either the n-3 family (alpha-linolenic acid, ALA; eicosapentaenoic acid, EPA) or the n-6 family (linoleic acid, LA; arachidonic acid, AA). Cells were then exposed to methylmercury (0.15 or 0.50 µM) for 24 h and sampled thereafter for assessing phospholipid fatty acid profile, intracellular total mercury burden, and expression pattern of genes involved in fatty acid metabolism, synthesis of polyunsaturated fatty acid-derived

signalling molecules and stress response. We observed that cells incorporated the given polyunsaturated fatty acid and some biotransformation products in their phospholipids. MeHg had few impacts on this cellular phospholipid composition. None of the PUFA enrichments affected the cellular mercury burden, suggesting that the previously observed cytoprotection conferred by ALA and EPA was not linked to a global decrease in cellular accumulation of mercury. Fatty acid enrichments and methylmercury exposure both modulated gene expression patterns. Genes involved in the synthesis of polyunsaturated fatty acid-derived signalling molecules, in stress response and the orphan cytochrome P450 20A1 were identified as possible sites of interaction between fatty acids and methylmercury in rainbow trout liver cells.

KEY WORDS: methylmercury, polyunsaturated fatty acids, fish, gene expression, RTL-W1 cells

1. Introduction

Methylmercury (MeHg) is an ubiquitous persistent pollutant resulting from methylation of mercury by sulphate-reducing bacteria at the oxic-anoxic interface of sediments (Selin, 2009). Total mercury (THg) concentrations in the freshwater ecosystems range from 0.3 ng/L to 450 µg/L, with higher levels found downstream of industrial discharges or mines (Kidd and Batchelar, 2011; Rytuba, 2003). MeHg is prone to bioaccumulation and biomagnification throughout the aquatic food webs (Kidd and Batchelar, 2011; Lavoie et al., 2013), so that high contamination levels can be expected in carnivorous fish, such as salmonids (Schwindt et al., 2008).

MeHg impairs survival, growth and reproduction in wildlife and humans. This organo-metal acts as a neurotoxicant in mammals and fish, but is also toxic to other organs such as kidney and liver (Kidd and Batchelar, 2011). Cytotoxicity is mainly linked to MeHg ability to (i) alter homeostasis of some ions, such as calcium (Farina et al., 2011; Kidd and Batchelar, 2011); (ii) bind sulfhydryl and selenohydryl groups (thereby interfering with the activity of a wide variety of cellular proteins) (Farina et al., 2011; Kanda et al., 2014; Kidd and Batchelar, 2011); and (iii) generate oxidative stress (by both enhancing the direct production of reactive oxygen species and reducing antioxidant defences) (Farina et al., 2011; Kidd and Batchelar, 2011). Importantly, oxidative attacks to membrane phospholipids may lead to uncontrolled chain reactions of hydrogenation that can propagate throughout the membrane and alter membrane functionality (Catalá, 2012). This is of particular concern in fish as they are rich in long chain polyunsaturated fatty acids (LC-PUFAs) that are very sensitive to oxidation.

Lipids, and their constitutive fatty acids, are important nutrients for fish health. They can markedly exceed protein contents in fish body, and thus are crucial energetic resources (Tocher, 2003). They also play a key role in cell structure maintenance, are precursors of signalling molecules (e.g. eicosanoids) and act as nuclear receptor ligands (Tocher, 2003). It therefore appears plausible that lipids could play key roles in organismal, tissue and cellular response to pollutants such as MeHg in fish. This topic is of importance to both fundamental (mechanistic toxicology) and applied (aquaculture) research fields. In line with this view, a change in dietary fatty acid composition,

obtained by substituting fish oil (rich in n-3 LC-PUFAs) with vegetable oils (deprived of n-3 LC-PUFAs) in diets was found to modulate the transcriptional response to MeHg in liver, white muscle and brain of Atlantic salmon (Olsvik et al., 2011). Nevertheless, the use of complex fatty acid mixtures (like oils) precluded a detailed mechanistic understanding of the effect of individual fatty acids in the elicited responses. Other studies, however, have investigated the role of individual LC-PUFAs on molecular response to MeHg in mammalian and fish cells. Data showed that specific cellular PUFAs can modulate metal uptake, ion homeostasis, redox status, energy metabolism, cell signalling and survival under MeHg stress (Ferain et al., 2016; Kaur et al., 2008; Nøstbakken et al., 2012a; Nøstbakken et al., 2012b).

In particular, we recently demonstrated that the phospholipid composition of rainbow trout liver cells (RTL-W1 cell line) was an important parameter influencing cell sensitivity to MeHg (Ferain et al., 2016). Indeed, RTL-W1-cells with phospholipids artificially enriched in alpha-linolenic acid (ALA, 18:3n-3) or eicosapentaenoic acid (EPA, 20:5n-3) were significantly more tolerant to an acute MeHg stress while an enrichment in their n-6 counterparts, i.e. linoleic acid (LA, 18:2n-6) and arachidonic acid (AA, 20:4n-6), had no impact (Ferain et al., 2016). The mechanisms involved remain to be elucidated. It could be that individual n-3 PUFAs increase tolerance to MeHg by decreasing MeHg burden in cells, through a reduced uptake and/or an increased efflux of the organo-metal. It could also be that individual PUFAs act to improve the capacity of RTL-W1 cells to cope with the toxic effects of MeHg, among others through the expression of defensive genes. On the other hand, it is increasingly recognized that pollutants, such as MeHg can affect cellular fatty acid homeostasis (i.e., uptake, synthesis and use for signalling, structural or energetic purposes) (Dimastrogiovanni et al., 2015; Yadetie et al., 2016).

The present study thus aimed at investigating interactions between PUFAs and MeHg at the molecular level. RTL-W1 cells enriched or not (control) in ALA, EPA, LA or AA were exposed or not (blank) to two levels of MeHg (0.15 or 0.50 µM). After 24 h of metal challenge, the intracellular content of THg, the fatty acid profile of the cell phospholipids and the expression of specific genes involved in fatty acid metabolism, PUFA-derived signalling and stress response were determined.

2. Materials and methods

2.1. *Experimental design*

The fatty acid profile of RTL-W1 cells (rainbow trout liver - waterloo 1; (Lee et al., 1993)) was first modified by incubating cells for one week in growth medium supplemented with one of the four selected PUFAs (50 µM ALA, EPA, LA or AA) (Fig. 1). Control cells were grown in the same medium but without any PUFA supplementation. Subsequently, cells were exposed to MeHg (0, 0.15 or 0.50 µM CH_3HgCl) for 24 h in a minimal medium deprived of fatty acids before being harvested (independent repetitions were performed) (Fig. 1). Intracellular quantification of THg levels (n=3), cell phospholipid fatty acid profiles (n=3) and gene expression levels (n=4) were then assayed (Fig. 1).

2.2. Routine RTL-W1 cell culture and PUFA enrichments

The RTL-W1 cell line was kindly provided by Dr. S. Bony (UMR 5023 LEHNA, INRA, ENTPE, France). For routine cell culture, cells were grown in 175 cm² flasks (CELLSTAR, Greiner Bio-One, Belgium) with Leibovitz's L15 medium supplemented with 5% foetal bovine serum (FBS; PAA Laboratories, France) and antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin) at 19°C, as previously described (Ferain et al., 2016). Cells were regularly checked and confirmed the absence of mycoplasma (MycoAlert kit, Promega, The Netherlands).

Individual PUFA enrichments were performed as described by (Ferain et al., 2016). Briefly, RTL-W1 cells were grown in 175 cm^2 flasks for one week, at 19°C. Growth medium contained Leibovitz's L15 medium supplemented with 2% FBS, antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin) and one of the PUFA of interest (50 µM of ALA, EPA, LA or AA; Larodan, Sweden) complexed with fatty acid free-bovine serum albumin (FAF-BSA) in the molar proportion of 4:1 (FAF-BSA:PUFA of interest) (Best et al., 2006). This resulted in a final concentration of 12.5 µM FAF-BSA in the growth media. Control cells were grown in Leibovitz's L15 medium supplemented with 2% FBS, antibiotics (100 IU/mL penicillin, 100 µg/mL streptomycin) and FAF-BSA (12.5 µM) only. During the PUFA enrichment period, growth medium was renewed twice. Experiments were conducted with RTL-W1 cells from passage 56 to 66.

2.3. MeHg contamination

Stock solutions of CH_3HgCl (15 µM and 50 µM; CAS 115-09-3, Sigma-Aldrich, USA) were prepared in cell culture grade water and kept at 4°C in the dark until use. Contaminated medium was prepared fresh daily by diluting 100 times the stock solutions in L15/ex medium, a restrictive medium containing concentrations of salts, galactose and pyruvate similar to Leibovitz's L15 medium (Dayeh et al., 2002). After one week of PUFA enrichments, growth medium was replaced by L15/ex medium containing 0, 0.15 or 0.50 µM CH_3HgCl (nominal concentrations). Exact contamination levels were measured by atomic absorption spectroscopy (see 2.6). After 24 h of MeHg exposure, RTL-W1 cells were sampled for downstream analyses. Besides, 24 h cytotoxicity induced by the two doses of MeHg tested was assessed using both the CellTiter-Blue (CTB) (Promega, The Netherlands) and the 5-carboxyfluorescein diacetate, acetoxymethyl ester (5-CFDA,AM) (Life Technology, USA) assays, as previously described (n=6) (Ferain et al., 2016).

2.4. Cell sampling

RTL-W1 cells were washed with 10 mL of phosphate-buffered saline supplemented with 0.03 % ethylenediamine tetraacetic acid (PBS-EDTA), then detached from the flasks with trypsin (0.0625% in PBS-EDTA). Each cell suspension was washed once with ice-cold FAF-BSA (10 g/L in Leibovitz's L15 medium), then once with L15/ex medium. After centrifugation of the cell suspensions (1,500 g, 6 min, 19°C), cells were resuspended in L15/ex medium and counted using hemocytometers (C-chip Neubauer improved, NanoEnTek, USA). Aliquots of $4x10^6$ cells were used for phospholipid fatty acid profile determination while remaining cells were subsampled in aliquots of $1x10^6$ cells each.

The latter aliquots were centrifuged (400 g, 5 min, 19°C), the cell pellets were flash-frozen in liquid N_2 and kept at -80°C for downstream analyses (intracellular THg and gene expression levels).

2.5. Phospholipid fatty acid profile determination

For each sample, $4x10^6$ freshly collected cells were resuspended in 2 mL of ice-cold L15/ex medium. Total lipids were extracted from 2 x 0.8 mL of the cell suspension (technical duplicates), with methanol:chloroform:water (2:2:1.8, v:v:v, (Bligh and Dyer, 1959)). Lipid extracts were then separated in three fractions (i.e. neutral lipids, free fatty acids and phospholipids) by solid phase extraction (Schneider et al., 2012). Each fraction was then methylated and extracted with 1 mL hexane (Ferain et al., 2016). The resulting fatty acid methyl esters (FAMEs) of the phospholipid fraction were separated by gas chromatography following Ferain et al. (2016) with minor adaptations. The gas chromatograph (GC Trace 1310, Thermo Fisher Scientific, Italy) was equipped with an autosampler TriPlus TP100 (Thermo Fisher Scientific, Italy). Separation was carried out on a RT2560 capillary column (biscyanopropyl polysiloxane, 100 m x 0.25 mm internal diameter, 0.2 µm film thickness; Restek, USA) with H_2 as carrier gas (200 kPa, 2 mL/min). The temperature program started with an increase from 80°C to 175°C at a rate of 25°C/min. After 25 min at 175°C, the temperature increased to 200°C at a rate of 10°C/min. After 20 min at 200°C, the temperature increased to 220°C at 10°C/min. After 5 min at 220°C, the temperature increased to 235 at 10°C/min. After 15 min at 235°C, the temperature decreased to 80°C at 20°C/min. FAME detection was performed using a Flame Ionization Detector (Thermo Quest, Italy) flowed by H_2 (35 mL/min), air (350 mL/min) and N_2 (40 mL/min), at a constant temperature of 255°C. The ChromQuest software (version 5.0, ThermoFinnigan, Italy) was used to process spectra. FAMEs were identified and quantified by comparing both retention times and peak areas with an external standard made from known concentrations of 44 FAMEs (Larodan, Sweden). Internal standards were added during the extraction step (1,2-dipentadecanoyl-sn-glycero-3-phosphatydylcholine, triheptadecanoin and tridecanoic acid; Larodan, Sweden) and before the injection (methyl-undecanoate; Larodan, Sweden) to control for whole-process quality. Fatty acid profiles were expressed as % of total fatty acids identified in the phospholipid fraction. For each biological replicate, data from the technical duplicates were averaged. Concentrations that were below the limit of quantification were set at 0.

2.6. THg quantification

THg concentrations were determined by atomic absorption spectroscopy with a Direct Mercury Analyzer 80 (Milestone, Italy) (Habran et al., 2013). Frozen cell pellets, containing $1x10^6$ cells, were lysed at room temperature with 1 mL ultrapure water and homogenized with a Vortex. Four aliquots of 100-150 µL of each cell lysate were then analysed using the EPA method 7473. Data from the technical quadruplicates were averaged. Procedural blanks, Hg standards and positive control material (DOLT 3 from the National Institute of Standards and Technology, USA) were always included. THg level of contaminated media (400 µL aliquots) was dosed using the same method in technical duplicates. Exposure levels were 32.8 ± 2.8 and 108.8 ± 19.3 µg THg/L (corresponding

respectively to 0.16 ± 0.01 and 0.54 ± 0.10 µM CH_3HgCl). These levels are within the concentration ranges observed in salmonids (Schwindt et al., 2008).

2.7. Gene expression

Total RNA was extracted from $1x10^6$ cells with the Aurum total RNA mini kit (Bio-Rad Laboratories, USA), following manufacturer's guidelines. The DNAse I treatment was always included. The concentration and purity of RNA eluates were assessed on a Nanodrop ND-1000 spectrophotometer (Isogen Life Sciences, The Netherlands) with the following optical densities OD_{260}, $OD_{260/280}$ and $OD_{260/230}$. Aliquots of 1 µg total RNA were reversed transcribed with the iScript cDNA synthesis kit (Bio-Rad Laboratories, USA), following manufacturer's guidelines. cDNA samples were diluted 3 times with nuclease-free water, and stored at -20°C until gene expression analyses. Quantitative polymerase chain reactions (qPCR) were run on a Step One Plus thermocycler (Applied Biosystems, Life Technologies, USA), using SYBR Select Master Mix I (Applied Biosystems, Life Technologies, USA) in a final reaction volume of 20 µL per well. For each gene, samples were run in technical duplicates. The thermal profile was: 95°C for 2 min, then (95°C for 15 s followed by 1 min at the annealing temperature (60 or 58°C, cf. Table 1) for 40 cycles. A melt curve analysis was always included. The $E^{-\Delta\Delta Ct}$ method was applied for relative gene expression analysis (Livak and Schmittgen, 2001). For each biological replicate, Ct values of technical duplicates were averaged prior to relative expression calculation. The geometric mean of *actin* and *18S* Ct values was used as housekeeping value (Vandesompele et al., 2002). Note that other genes and gene combinations were also tested as housekeepers but proved unreliable (i.e., substantially higher variation of Ct values across treatments; data not shown). Control cells that were not challenged with MeHg were used as reference condition, for relative expression of data. An arbitrary expression value of 1 was given to each biological replicate of this condition. Table 1 provides a list of genes used in qPCR studies along with primer characteristics. These genes are involved in fatty acid uptake (*fatty acid transport protein 1 (FATP1)*), fatty acid *de novo* synthesis (*fatty acid synthase (FASN)*), fatty acid bioconversion (*fatty acid desaturase 2 (FADS2), fatty acid elongases 2 and 5 (ELOVL2 and ELOVL5)*), β-oxidation (*carnitine palmitoyl transferase 1 (CPT1)*), synthesis of PUFA-derived signalling molecules (*cytosolic phospholipase A2 (cPLA2), cyclooxygenase 2 (COX2), arachidonate 5-lipoxygenase (ALOX5)*) and stress response (*metallothioneins a* and *b* (*MTa* and *MTb*), *heat shock proteins 70a* and *70b* (*HSP70a* and *HSP70b*), apoptosis regulator *BCL2-Like1* (*BCLx*)) (Fig. 2). The orphan *cytochrome P450 20A1* gene (*CYP20A1*) was also studied. Primer pairs (Table 1) were either gleaned from the literature or designed based on transcript sequences available at the European Nucleotide Archive (for identifiers, see (Marancik et al., 2014)). Optimal primer pair concentrations were determined experimentally and randomly selected amplicons were sent to Beckman Coulter Genomics for confirmation of sequence identity.

2.8. Statistical analyses

Statistical analyses were performed using R (R Core Team 2016) and JMP PRO 12.0 softwares.

For all statistical tests, results were considered significant at the level α = 0.05. A Bonferroni-Sidak correction was applied to correct the p values in any case of multiple testing (Sidak, 1967). For all parametric tests used, homoscedasticity and normality of the residuals were previously checked with appropriate tests (*i.e.* Fligner-Killeen test; (Conover et al., 1981) and Shapiro-Wilk test; (Royston, 1982)).

Metabolic activity and membrane integrity of the non-exposed cells as well as gene expression levels of non-exposed control cells had no variance, which restricted the use of classical statistical tests. To overcome this limitation, Student's t-tests (Dagnelie, 2006) were performed to determine (i) whether metabolic activity and membrane integrity of cells exposed to MeHg were significantly different from 100% and (ii) whether gene expression levels of control cells exposed to MeHg and non-exposed PUFA-enriched cells were different from 1. In any other case, two 1-way ANOVAs or Kruskal-Wallis tests (if non-parametric tests were required (Hollander and Wolfe, 1973)) were performed to assess (i) the impact of the PUFA enrichment strategy at each MeHg concentration (with Dunnett's test (Bretz et al., 2016) or Dunn's test as post-hoc analyses for parametric and non-parametric tests respectively) and (ii) the impact of MeHg exposure level for each PUFA enrichment strategy (with Tukey test (Yandell, 1997). or Dunn's test as post-hoc analyses for parametric and non-parametric tests respectively). Note that THg intracellular concentration data were Log transformed prior statistical analyses.

To summarize the genetic dataset and highlight possible interactions between gene expression patterns, intracellular THg levels and phospholipid composition, a principal component analysis (PCA) was performed. The relative expressions of the 12 selected genes were used as quantitative variables and both the THg and the phospholipid level of selected fatty acids were used as illustrative variables. The latter do not participate in the generation of principal components but give important clues for interpreting results from the PCA. The PCA was based on correlations between standardized variables. In addition, linear regressions between relevant couples of variables from the PCA were performed. The confidence interval for those regressions was set at 95%.

3. Results

In vitro manipulation of the phospholipid fatty acid profile of RTL-W1 cells

One week of growth in a medium supplemented with a specific PUFA significantly changed the fatty acid composition of RTL-W1 cell phospholipids (Table 2). ALA-, EPA-, LA- and AA-enriched cells contained significantly higher amounts of the specific PUFA added to the growth medium and higher amounts of its biotransformation products than control cells (see Fig. 2 for the biotransformation pathway). Indeed, ALA, 18:4n-3, 20:3n-3 and 20:4n-3 were found in phospholipids of ALA-enriched cells and not in those of control cells. In addition, ALA-enriched cells contained significantly more EPA than control cells (about 3 times). The phospholipids of EPA-enriched cells contained 13 fold higher amounts of EPA and were significantly richer in 20:4n-3 and 22:5n-3 than control cells. A significantly higher amount of LA (11 to 15 times), 18:3n-6, 20:2n-6 and 20:3n-6 was found in the

phospholipids of LA-enriched cells than in control cells. Phospholipids of AA-enriched cells were significantly richer in AA (about 8-9 times) and 22:4n-6 than those of control cells. As a result of these changes in fatty acid composition, the ratio between n-3 and n-6 PUFA (n-3/n-6) was significantly higher in n-3 PUFA-enriched cells and significantly lower in n-6 PUFA-enriched cells than in control cells. Also the total PUFA contents in cellular phospholipids were significantly higher (about 3 to 4 times) in PUFA-enriched cells than in control cells.

Few impacts of MeHg on the phospholipid fatty acid profile

MeHg had few significant impacts on the fatty acid composition of RTL-W1 cell phospholipids. In ALA-enriched cells, 24 h of exposure to MeHg slightly decreased the level of 18:1n-9 (- 6.5%), the total MUFA content (- 7.5%) as well as the level of 18:4n-3 (- 12 to 15%) (Table 3). In AA-cells, MeHg slightly decreased the phospholipid content in 20:3n-6 (- 12 to 17%) (Table 3). MeHg had no impact on fatty acid profile of phospholipids in control cells and cells enriched in either LA or EPA (not shown).

Slight reduction of cell viability by MeHg exposure

Slight reduction of both membrane integrity and metabolic activity was observed in control, ALA- and LA-enriched cells exposed to MeHg (0.15 or 0.5 µM; Fig. A1). However, due to a higher variability of the data, metabolic activity of the control, ALA- and LA-enriched cells challenged at the highest MeHg dose was not significantly different from that of the unchallenged cells. MeHg had no impact on the metabolic activity in EPA- and AA-enriched cells. The same phenomenon was observed for membrane integrity at the low MeHg concentration. On the other hand, membrane integrity of EPA-enriched cells was significantly lowered while it remained stable in AA-enriched cells. Within each MeHg concentration, PUFA enrichments had no significant impact on either metabolic activity or membrane integrity of RTL-W1 cells. Still, a trend for higher metabolic activity was observed in EPA-enriched cells challenged with the low MeHg concentration as compared to control cells challenged with the same MeHg concentration. Altogether, these results confirm the low toxicity of the MeHg concentrations selected in this experiment and therefore the possibility to observe mostly sub-lethal effects.

No impact of PUFA on MeHg accumulation

After 24 h of MeHg exposure, RTL-W1 cells significantly accumulated mercury, in a dose-dependent way (Fig. 3). THg accumulated in cells represented 33-47% of the mercury added to the exposure medium. PUFA enrichments had no significant impact on intracellular THg levels.

Impacts of PUFA and MeHg on gene expression patterns

Fatty acid metabolism

In all cases, *ELOVL2* expression level in RTL-W1 cells was below quantification limit (not shown). In absence of MeHg, PUFA enrichments increased the expression levels of *CPT1* and *FATP1*, and reduced the expression levels of *FADS2* and *ELOVL5* (Table 4), while having no significant impact

on *FASN* expression. In particular, ALA enrichment significantly reduced *FADS2* and *ELOVL5* expressions while it significantly stimulated *FATP1* expression. The expressions of *FADS2* and *ELOVL5* were also significantly inhibited by EPA-enrichment, while LA enrichment significantly reduced *ELOVL5* expression only.

MeHg modulated the expression level of *FATP1* in control cells (Table 4). The expression level of *FATP1* was significantly lower in control cells exposed to 0.50 µM MeHg than in control cells exposed to 0.15 µM MeHg. Whatever the PUFA-treatment, MeHg did not influence significantly the expression of other genes involved in fatty acid metabolism (i.e., fatty acid *de novo* synthesis, bioconversion and β-oxidation).

PUFA-derived signalling

In absence of MeHg, PUFA enrichments led to increases of the expression level of *cPLA2* (significant for ALA- and AA-enriched cells). Decreases of the expression level of *COX2* were detected in ALA-, EPA- and AA-enriched cells (significant for EPA-enriched cells).

MeHg exposure increased the expression levels of *COX2* (significant for ALA-enriched cells) and *ALOX5* (Table 4). Besides, MeHg seemed to upregulate *cPLA2* expression in control cells, LA- and AA-enriched cells. However, these effects were not significant (Table 4)

Stress response

In all cases, *MTa* and *HSP70b* expression levels in RTL-W1 cells were below quantification limits (not shown). In the absence of MeHg, PUFA enrichment tended to increase both *MTb* and *HSP70a* gene expression. Nevertheless, these impacts were not significant (Table 4).

MeHg modulated the expressions of *BCLx, MTb* and *HSP70a*. In ALA-enriched cells, *BCLx* expression was significantly lower after exposure to 0.50 µM MeHg than after exposure to 0.15 µM MeHg. In addition, exposure to 0.50 µM MeHg significantly increased, by about 3.5 times, *MTb* expression level. After exposure to 0.15 µM MeHg, *MTb* expression level was higher in PUFA-enriched cells than in control cells (significant for EPA- and AA-enriched cells). *HSP70a* expression was also strongly induced by MeHg (significant for ALA- and EPA-enriched cells).

The case of CYP20A1

In absence of MeHg, PUFA enrichments increased *CYP20A1* expression level. These variations were, however, not significant (Table 4).

MeHg exposure (0.50 µM) decreased the expression level of *CYP20A1* gene (significant in control cells and LA-enriched cells). In addition, at both MeHg concentrations tested, *CYP20A1* expression level was significantly higher in EPA- and AA-enriched cells than in control cells. This trend was only significant at the lowest MeHg concentration tested (i.e., 0.15 µM) in ALA-enriched cells.

Multivariate analysis

The PCA was performed using the relative expressions of the 12 selected genes as quantitative variables and both the intracellular concentration in THg and the phospholipid level in selected fatty

acids as illustrative variables. Fig. 4 shows the score plot (A) and loading plots (B) resulting from the principal component analysis. The two first principal components (PC) explained 50.5% of the total variance. PC1 and PC2 explained 31.1% and 19.4% of the total variance, respectively (Fig. 4). Loadings, that represent the correlations between the variables and the principal components, are listed in Table 4 for PC1 and PC2.

The PC1/PC2 biplot separates control cells from cells enriched in a specific PUFA (Fig. 4A). The loadings of these components (Table 5, Fig. 4B) suggest that control cells are characterized by a higher expression level of *ELOVL5, FADS2* and *COX2*, a higher phospholipid content in MUFAs, a lower expression level of *FATP1, CPT1* and *CYP20A1* and a lower phospholipid content in PUFA than cells enriched in a specific PUFA, irrespective of both the nature of the supplemented PUFA and the intensity of the MeHg challenge.

The PC1/PC2 biplot gradually separates the RTL-W1 cells exposed to MeHg from the non-exposed ones (Fig. 4A). The direction of the observed gradient is materialized by an arrow on the score plot (Fig. 4A). The loadings of the two first components (Table 5, Fig. 4B) suggest that cells challenged with increasing MeHg concentrations are characterized by an increasing intracellular THg level, an increasing expression level of *HSP70a, ALOX5* and *MTb*, irrespective of the PUFA enrichment condition. Linear regressions of the expression level of *MTb, HSP70a* and *ALOX5* genes as a function of the intracellular THg level, highlight the linear correlation between these parameters (Fig. 5).

Linear regression analyses also highlight several correlations of gene expression in the dataset that could suggest co-regulation (Fig. A.2). Positive correlations with a regression coefficient above 0.5 were found for *FATP1-CPT1, ALOX5-MTb, ALOX5-HSP70a* and *FADS2-ELOVL5*. Negative correlations of comparable strength were notably found for *ELOVL5-FATP1* and *COX2-CYP20A1*.

4. Discussion

We previously highlighted the importance of the fatty acid profile of rainbow trout liver cell phospholipids in a context of MeHg acute (24 h) toxicity (Ferain et al., 2016). RTL-W1 cells with phospholipids rich in ALA or EPA were significantly more tolerant to MeHg (4-4.5 times higher 24 h median effect concentration (EC50) value than non-enriched cells) while enrichment in LA or AA, their respective n-6 counterparts, had no impact on MeHg acute toxicity (Ferain et al., 2016). In the present study, we investigated plausible mechanisms by which the fatty acid profile of RTL-W1 cell phospholipids could improve the cellular resistance to MeHg exposure by applying sub-lethal scenarios of short term exposure. In addition, we studied the molecular impact of MeHg on cellular fatty acid metabolism, as this organo-metal was recently shown to affect cellular fatty acid homeostasis (Dimastrogiovanni et al., 2015; Yadetie et al., 2016). A summary of the main results observed after one week of PUFA enrichment and a subsequent exposure to MeHg for 24 h is presented in Table 6.

PUFA supplementations modify the phospholipid fatty acid profile of RTL-W1 cells

The phospholipid composition of RTL-W1 cells was modified by adding individual PUFAs to the culture media, as previously shown (Ferain et al., 2016). Within one week, cells had absorbed individual PUFAs at high levels and incorporated them with some biotransformation products in their phospholipids. These observations are in line with our previous results with the same cell line (Ferain et al., 2016) and emphasize, once more, the importance of the fatty acid composition of growth media in defining the fatty acid profile of fish cells in culture (Buzzi et al., 1996; Gregory et al., 2011; Tocher, 1990). Noteworthy, the range of n3/n6 ratio resulting from the different PUFA enrichment strategies (at fixed PUFA concentrations and incubation periods), encompassed a 200-fold range. The RTL-W1 cell line therefore appears a valuable model for toxicological investigations focusing on the combined effects of dietary PUFAs and xenobiotics in fish liver.

Salmonids, such as rainbow trout, have a naturally high ability for fatty acid bioconversion (Tocher, 2003). In the present study, RTL-W1 cells were also able to bioconvert the given PUFAs, although to a lower extent than observed *in vivo*. Indeed, while docosahexaenoic acid (DHA, 22:6n-3) and AA, two major biotransformation products of the n-3 and n-6 PUFA pathway respectively, are efficiently synthetized in rainbow trout liver *in vivo*, RTL-W1 cells showed no production of these from their respective precursors ALA (and EPA) and LA. Instead, an accumulation of intermediary metabolites resulting from elongation and/or desaturation (Δ5 and Δ6) of the given precursors was observed, as previously described (Ferain et al., 2016). These observed elongation and desaturation abilities of RTL-W1 cells were confirmed in the present experiment, by the quantifiable expression of the genes coding for ELOVL5 and FADS2, respectively. The inability of RTL-W1 cells to produce DHA from 22:5n-3 could result from either a non-functional *ELOVL2* gene or an inactivation of the gene product (enzyme) catalysing the elongation of 22:5n-3 into 24:5n-3 (Gregory and James, 2014), i.e. the first and limiting step for DHA production from EPA (Tocher, 2003). Of note, gene expression of *ELOVL 2* in RTL-W1 cells was always below the limit of quantification in the present study (not shown).

MeHg only slightly modulates the phospholipid fatty acid profile in RTL-W1 cells

While numerous studies reported increased lipid peroxide levels in fish under MeHg stress (e.g. (Berntssen et al., 2003), (Mozhdeganloo et al., 2015)), only a few assessed the impact of MeHg on the global or specific phospholipid fatty acid profile of cells and on the integrity of biological membrane composition. Data so far suggest that MeHg has few impacts on the relative amounts of PUFAs in liver, brain and muscle of Atlantic salmon (Amlund et al., 2012; Olsvik et al., 2011) and in mouse liver phospholipids (Zeng et al., 2016). Nevertheless, MeHg induced AA release from membrane phospholipids in rat astrocytes (Shanker et al., 2002), rat neurons (Shanker et al., 2004) and bovine pulmonary artery endothelial cells (Mazerik et al., 2007). Similarly, chronic MeHg intoxication (5 mg Hg/kg in the diet during 3 months) decreased the relative amounts of AA in brain phospholipids of Atlantic salmon (Amlund et al., 2012). In the present study, MeHg did not substantially impact phospholipid fatty acid profiles in RTL-W1 cells, whatever the PUFA enrichment strategy applied before the metal challenge. Only a subtle decrease of 18:4n-3 level, the desaturation

product of ALA, in ALA-enriched cells, and of 20:3n-6 level, the precursor of AA through desaturation, in AA-enriched cells could be observed. The biological relevance of these changes remains unclear. Note that the duration of the metal challenge (24 h) combined to the timing of cell sampling (right after the 24h challenge) were maybe too short for enabling pronounced impact of MeHg on cellular fatty acid composition.

PUFA-enrichments influence the expression of genes involved in fatty acid metabolism

In the present study, the expression of genes involved in cellular fatty acid uptake (*FATP1*), β-oxidation (*CPT1*), desaturation (*FADS2*) and elongation (*ELOVL5*) was influenced by the PUFA enrichment strategies. The supplementation of the growth media with any of the PUFA increased (or tended to do so) RTL-W1 cell expression levels of *FATP1* and *CPT1* while decreasing that of *ELOVL5* and *FADS2*. Besides, the relative expression of these four genes was highly correlated, suggesting a common mechanism of regulation (Fig. A.2).

FATP1 is a transmembrane protein responsible for the fatty acid uptake through the plasma membrane while CPT1 is an enzyme with transmembrane domains that catalyses the generation of acyl-carnitine (from acyl-CoA and carnitine), necessary to allow its translocation to the mitochondrial matrix (Fig. 2). This step is the limiting one for fatty acid uptake through the mitochondrial outer membrane for β-oxidation purpose. The higher expression level of *FATP1* and *CPT1* in cells enriched in fatty acids observed in the present experiment, highlights the high predisposition of fish cells to absorb fatty acids and use them as energy source (Tocher, 2003).

In contrast to our observations, EPA, DHA and AA supplementation (300 µM, 72 h) had no impact on either *FATP* or *CPT1* expression level in the Atlantic salmon kidney (ASK) cell line (Nøstbakken et al., 2012a). This might reflect that salmonid kidney cells, contrary to liver cells, are not a prime site for fatty acid metabolism in fish. While the impact of specific PUFA supplementation on salmonid hepatic *FATP1* expression pattern has, to our knowledge, never been investigated, effects of ALA, EPA, LA and AA supplementation on *CPT1* gene expression have previously been studied in rainbow trout primary hepatocytes (Coccia et al., 2014). As in the present experiment, authors observed that ALA and AA (1 mM, 24 h) increased the cellular expression level of *CPT1β*, a *CPT1* isoform (Coccia et al., 2014), while LA and EPA enrichment decreased it (Coccia et al., 2014). The differences observed with our study may be due to the duration of exposure (1 week instead of 24 h) and/or to the concentration used (50 µM instead of 1 mM), and/or to the isoform of the gene targeted (α instead of β). Indeed, up to 5 different isoforms of the *CPT1* gene have been identified in rainbow trout liver (Morash et al., 2010). These isoforms showed specifications in their regulation and function. However, the impact of specific PUFA on the expression pattern of these 5 isoforms has, to our knowledge, not been investigated yet. Additional experiments assessing the kinetic expression pattern of *CPT1* isoforms under specific PUFA supplementation would be needed to better understand to role of individual lipids in cellular catabolism.

The lower expression level of *FADS2* and *ELOVL5* genes in PUFA-enriched cells may reflect a negative feedback exerted by PUFA on LC-PUFA biosynthesis (see Fig. 2 for the biotransformation

pathway). In line with this view, EPA supplementation (50 µM, 24 h) decreased *Δ6FADSb* and *ELOVL5b* gene expression patterns in Atlantic salmon primary hepatocytes (Kjær et al., 2016). Also, a lower expression level of FADS2 genes was observed in liver of rainbow trout fed for 3 weeks on a diet supplemented with ALA (linseed oil), EPA (EPA-enriched fish oil), DHA (DHA-enriched fish oil) or combinations of those n-3 PUFAs, as compared to a low n-3 PUFA diet (Gregory et al., 2016). However, in contrast with the present experiment, EPA supplementation (50 µM, 24 h) was also found to upregulate *Δ5FAD* gene expression without affecting *Δ6FADa,c* and *ELOVL5a* gene expression in Atlantic salmon primary hepatocytes (Kjær et al., 2016). These examples illustrate how various experimental parameters (PUFA concentration, exposure duration or isoform of the genes targeted) can influence both the intensity and the direction of gene expression variations, emphasising the difficulty to interpret and compare genetic data from salmonid experiments (Kjær et al., 2016). Moreover, in the present *in vitro* study, in absence of MeHg challenge, n-3 PUFAs (ALA or EPA) significantly downregulated (by 27 and 25 %, respectively) the expression of *FADS2* genes while n-6 PUFAs (LA or AA) only slightly lowered it (by 20 and 13 %, respectively, not significant). This could suggest that n-3 PUFAs may exert a higher negative feedback on *FADS2* gene than n-6 PUFAs. Additional experiments are needed to validate this hypothesis, as the present experiment is, to our knowledge, the first one testing the impact of specific n-6 PUFA supplementation on *FADS2* gene expression in rainbow trout liver cells.

MeHg has few impacts on the expression of genes involved in fatty acid metabolism

The present study could not identify interactive effects of PUFAs and MeHg on the expression levels of targeted genes involved in fatty acid metabolism. This is in accordance with an *in vivo* study by Nøstbakken et al. (2012c) on Atlantic salmon exposed to MeHg (3.8 mg/kg fresh weight diet during 8 weeks). Nevertheless, other studies (*in vitro* and *in vivo*) have highlighted an influence of MeHg on the expression of genes involved in fatty acid uptake (Nøstbakken et al., 2012a), β-oxidation (Liu et al., 2013; Nøstbakken et al., 2012a; Olsvik et al., 2011)*, de novo* synthesis (Klaper et al., 2008) and bioconversion (Ho et al., 2013; Liu et al., 2013; Olsvik et al., 2011) in fish cell lines or in fish tissues. Noteworthy, the results from these studies were sometimes contradictory. For instance Olsvik et al. (2011) and Liu et al. (2013) found opposite pattern of MeHg impact on *CPT1* expression in Atlantic salmon and zebrafish, respectively. Overall, the present results and the available literature underline the complex and indirect influence that MeHg might exert on fatty acid metabolism in fish.

PUFA-enrichment does not influence THg burden

Both passive and (specific or non-specific) active mechanisms are suggested to participate in MeHg uptake (Kidd & Batchelar 2011). The relative importance of active and passive mechanisms may depend on the MeHg concentration, with possibly an increasing preponderance of passive mechanisms at higher MeHg concentrations. Alterations of the fatty acid composition of RTL-W1 cell phospholipids could modulate the physicochemical properties of the bilayer and embedded proteins, potentially disrupting passive and active uptake-efflux of MeHg, respectively.

In the present study, THg intracellular level was found to be independent of the cellular fatty acid composition. This has previously been shown in lipid membranes (Bienvenue et al., 1984) and in both ASK and Human embryonic kidney (HEK293) cell lines (Nostbakken et al 2012). The higher tolerance to MeHg of ALA and EPA-enriched RTL-W1 cells observed previously (Ferain et al., 2016) is thus unlikely due to a lower uptake of MeHg. However, experiment testing higher MeHg concentrations would be needed to validate this hypothesis as uptake mechanisms may be different at low and high MeHg concentration. Besides, one cannot exclude that MeHg intracellular distribution into membranes and organelles, the possible conversion into other less toxic Hg species such as inorganic Hg, or the amount of unbound Hg species could be different in PUFA-enriched cells.

PUFA-enrichments and MeHg slightly modulated cell viability

MeHg slightly decreased metabolic activity and membrane activity of RTL-W1 cells. EPA and AA enrichments seemed to prevent RTL-W1 cells from MeHg toxicity. EPA-derived cytoprotection against MeHg challenge was previously observed on the same cell line (increased 24h-EC50, metabolic activity) (Ferain et al., 2016) and on the ASK cell line (reduction of MeHg-induced apoptosis) (Nøstbakken et al., 2012a). In contrast to the present experiment, AA enrichment did not significantly modulate MeHg toxicity in both RTL-W1 and ASK cell lines (Ferain et al., 2016; Nøstbakken et al., 2012a). Of note, ALA enrichment conferred no significant protection in the present study as was the case in Ferain et al. (2016). This probably reflects that two discrete MeHg concentrations were tested herein, while EC50 data measured before are based on modelling data (i.e., concentration response analyses and curve fitting implying extrapolation). The two discrete MeHg concentrations were at the lower limit of the concentration range for which protection became significant in the previous study. The concentration-dependent increase of THg burden in RTL-W1, in light of the above observations, suggests that a fast sequestration of MeHg occurs in RTL-W1 cells under the experimental conditions tested herein.

PUFA enrichments and MeHg both modulate the expression of key genes of PUFA-derived signalling

PUFA-derived mediators, such as the eicosanoids and the resolvins, are important signalling molecules playing roles in biological processes such as inflammation (Kremmyda et al., 2011). Eicosanoid production starts with the liberation of 20 carbons fatty acid precursors (mainly AA but also EPA and, to a lower extent, 20:3n-6) from the phospholipid membranes, while resolvin production starts with the liberation of either 20 or 22 carbon fatty acids (EPA, 22:5n-3, DHA) (Kremmyda et al., 2011). These first steps are catalysed by phospholipases (mainly cPLA2) (Fig. 2). The released precursors will then be oxidized to generate prostanoids, leukotrienes or resolvins. These oxidation steps are catalysed by cyclooxygenases, such as COX2, and lipoxygenases, such as ALOX5 (Fig. 2) (Kremmyda et al., 2011). Several studies highlighted the ability of MeHg to trigger inflammation, increase eicosanoid content and rise *cPLA2* and *COX2* transcription and/or activity of

the protein gene products (Mela et al., 2007; Nøstbakken et al., 2012a; Shanker et al., 2004; Shanker et al., 2002; Sherwani et al., 2013). In line with those studies, MeHg (0.50 µM, 24 h) tended to increase *cPLA2*, *COX2* and *ALOX5* gene expression in the present study. Besides, MeHg impact on *cPLA2* transcription seemed to be influenced by the type of PUFA supplementation (n-6 or n-3). Indeed, MeHg (0.50 µM, 24 h) tended to upregulate *cPLA2* expression in control cells and in cells enriched in either LA or AA (2-3.5 fold increase) while cPLA2 expression remained constant under MeHg exposure in ALA- and EPA-enriched cells. The effect needs to be confirmed (as it was not significant) but it would suggest an implication of PUFA-derived mediators in the protection against MeHg toxicity provided by ALA and EPA supplementation as previously demonstrated in the same cell line (Ferain et al., 2016). MeHg also slightly (but significantly) decreased the level of 20:3n-6 in AA-enriched cell phospholipids, which could suggest mobilisation of this PUFA from the biological membranes for eicosanoid production under MeHg challenge in AA-enriched cells. The presence of 20:3n-6 in phospholipids of RTL-W1 cells was correlated to a higher sensitivity to MeHg (Ferain et al., 2016). The latter effect was suggested to be linked to the apoptotic properties of 20:3n-6 (Ferain et al., 2016; Watts and Browse, 2006). Altogether, it appears that the eicosanoid/resolvin signalling pathway could well constitute a site of PUFA and MeHg interaction in RTL-W1 cells.

The expression of stress responsive genes is affected by both MeHg and PUFA enrichments

In line with previous literature (Morcillo et al., 2016; Nøstbakken et al., 2012a; Yadetie et al., 2013), MeHg upregulated (or tended to do so) *HSP70a* and *MTb* genes in RTL-W1 cells in a concentration-dependent manner. Besides, the expression of these two genes increased linearly with the intracellular THg burden. In addition, PCA and linear regression analyses revealed a tight correlation between the expression of these two stress-responsive genes and that of *ALOX5*, suggesting these genes to be possible sites of interaction between PUFAs and MeHg in RTL-W1 cells. Interestingly, even though not significant, there were trends to a higher basal level of *HSP70a* expression in PUFA-enriched RTL-W1 cells, compared to control cells. Such an effect has rarely been investigated. Yet, data so far support the view that PUFA enrichments can increase expression levels of *HSP* genes (Blondeau et al., 2002; Pérez et al., 2016). This might be a way through which some PUFAs could exert protective effects against metals in RTL-W1 cells (i.e., through transcriptional induction of defensive genes).

Opposite effects of PUFA enrichments and MeHg on CYP20A1 expression

CYP enzymes catalyse oxidation of a vast variety of endogenous and exogenous compounds, including PUFAs (Morrison et al., 2014; Schlezinger et al., 1998). Some vertebrate CYP isoforms have unidentified functions and are termed orphans. CYP20A1 is one of those orphans (Stark et al., 2008). A recent study revealed that *CYP20A1* expression level was increased in response to selected PUFAs (suggesting possible involvement in the production of PUFA-derived signalling molecules) and decreased in response to MeHg (10 or 50 µg/L, 24 h) in developing zebrafish (Lemaire et al., 2016). Accordingly, in the present experiment, PUFA enrichments tended to

upregulate while MeHg tended to downregulate *CYP20A1* expression level. Also, a negative correlation was found between *COX2* and *CYP20A1* expression across samples. It is therefore plausible that the orphan CYP20A1 represents another site of interaction between PUFAs and MeHg effects in RTL-W1 cells.

5. Conclusion

The present study highlights that the phospholipid fatty acid composition of fish liver cells can modulate the expression pattern of genes involved in fatty acid metabolism, PUFA-derived signalling and stress response, both in the absence and in the presence of a MeHg challenge. Still, the phospholipid fatty acid composition does not influence cellular THg burden. While MeHg exposure and PUFA enrichments had little or no interactive effects on cellular PUFA profile, on intracellular THg level or on genes related to PUFA metabolism, both were found to modulate PUFA-derived signalling, stress response and *CYP20A1* expression. Modulation of cell sensitivity to MeHg by PUFA therefore seems to be driven by complex molecular interactions, possibly including PUFA-derived lipid mediators and metal stress responsive genes. Further investigations are required to confirm or infirm this view. Focus should be put in future studies on proteomic and metabolomic analyses to provide a deeper understanding of the molecular action of individual PUFA enrichments towards MeHg stress.

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Figure captions

Figure 1: Experimental design. The illustration has been done using the "general items" package from Servier Medical Art. Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid, LA, linoleic acid; MeHg, methylmercury; PUFA, polyunsaturated fatty acid; THg, total mercury.

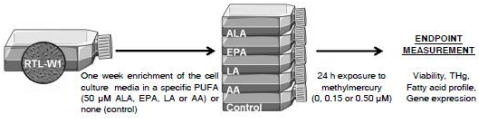

Figure 2: Roles of the proteins encoded by the genes investigated. The illustration has been done using the "cell biology" package from Servier Medical Art. Abbreviations used: Δ5, Δ5 desaturation; Δ6, Δ6 desaturation; ALOX5, arachidonate 5-lipoxygenase; BCLx, apoptosis regulator BCL2-Like1; COX2, cyclooxygenase 2, cPLA2, cytosolic phospholipase A2; CPT1, carnitine palmitoyl transferase 1; cs, peroxisomal chain shortening; E, elongation; ELOVL2, fatty acid elongase 2; ELOVL5, fatty acid elongase 5; FA, fatty acid; FADS2, fatty acid desaturase 2; FASN, fatty acid synthase; FATP1, fatty acid transport protein 1; HSP, heat shock protein; MT, metallothionein

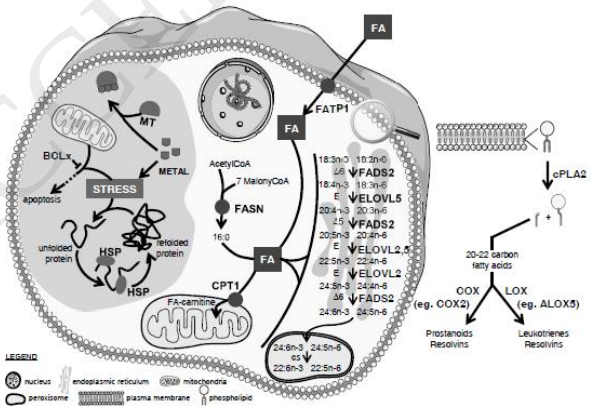

Figure 3: Intracellular concentrations of THg (ng/million cells) (means ± standard errors) *in RTL-W1 cells enriched or not (control) with a specific PUFA (ALA, EPA, LA or AA) and challenged with MeHg (0, 0.15 or 0.50 µM) during 24 h (n=3). For each PUFA enrichment strategy, levels with different letters are significantly different from each other (1-way ANOVA with a post-hoc Tukey test, p<0.05). PUFA enrichments had no significant impact on the intracellular THg concentration. Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid, LA, linoleic acid; MeHg, methylmercury; PUFA, polyunsaturated fatty acid; THg, total mercury.*

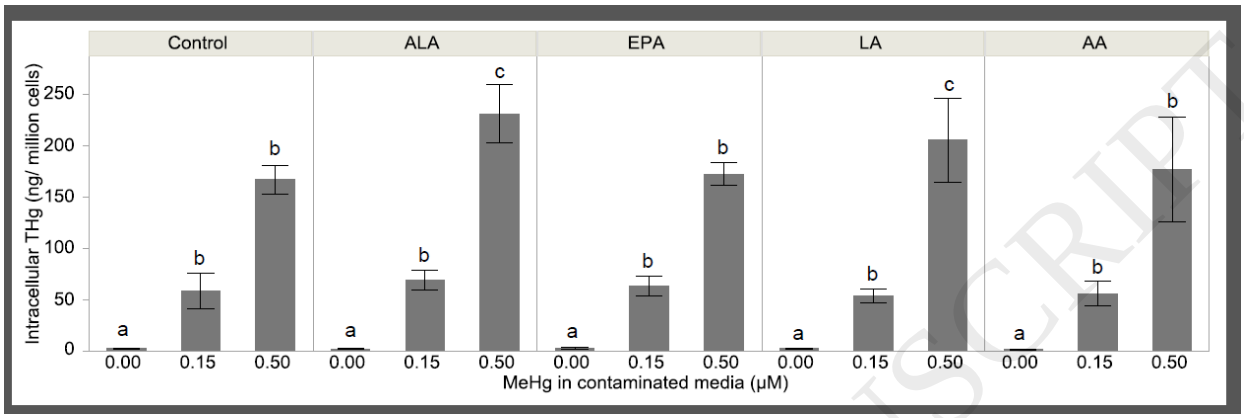

Figure 4: Principal component analysis score plot (A) and loading plot (B). The principal component analysis was performed using the relative expressions of the 12 selected genes as quantitative variables and both the intracellular concentration in THg and the phospholipid level in selected fatty acids as illustrative variables. The arrow on the score plot materializes the observed gradient separating RTL-W1 cells challenged with MeHg (0.15 µM in orange and 0.50 µM in red) from the unchallenged ones (in grey). The ellipse on the score plot groups the non-enriched RTL-W1 cells (control cells, stars) that were separated from the RTL-W1 cells enriched with a specific PUFA (either ALA (solid triangles), EPA (solid diamonds), LA (hollow triangles), or AA (hollow diamonds)) by PC1 and PC2. Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; ALOX5, arachidonate 5-lipoxygenase; BCLx, apoptosis regulator BCL2-Like1; COX2, cyclooxygenase 2; cPLA2, cytosolic phospholipase A2; CPT1, carnitine palmitoyl transferase 1; CYP20A1, cytochrome P450 20A1; ELOVL5, fatty acid elongase 5; EPA, eicosapentaenoic acid; FADS2, fatty acid desaturase 2; FASN, fatty acid synthase; FATP1, fatty acid transport protein 1; HSP70a, heat shock protein 70a; LA, linoleic acid; MeHg, methylmercury; MUFA, total monounsaturated fatty acids in cellular phospholipids; MTb, metallothionein b; PC, principal component; PUFA, total polyunsaturated fatty acids in cellular phospholipids; THg, intracellular total mercury level.

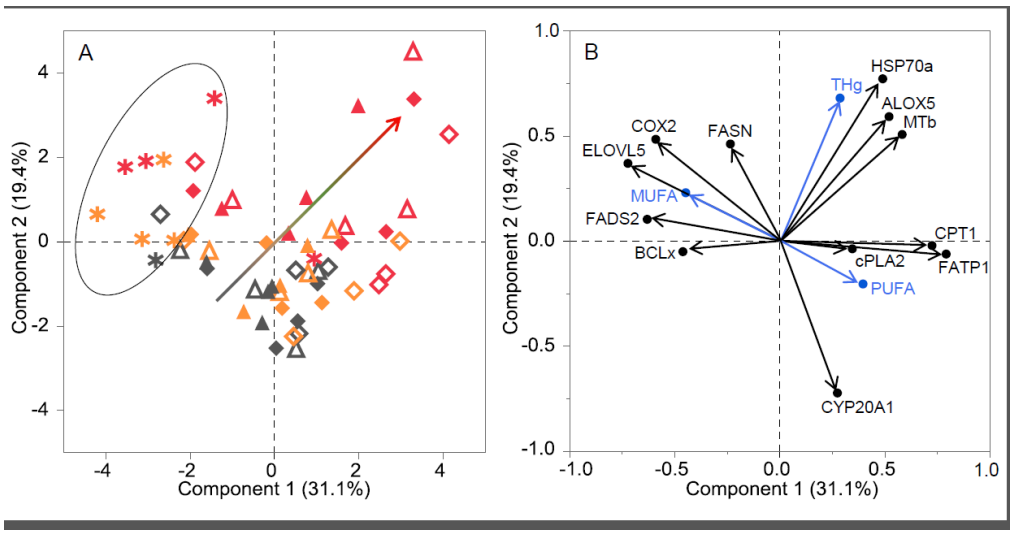

Figure 5: Linear regressions of the relative expression level of MTb (A), HSP70a (B) and ALOX5 (C) genes as a function of the intracellular THg level. Plain lines represent the linear regressions; shaded areas represent the confidence intervals at 95% for the regressions (n=3). RTL-W1 cells enriched with either ALA, EPA, LA, AA or no PUFA (control cells) are respectively represented by solid triangles, solid diamonds, hollow triangles, hollow diamonds and stars. RTL-W1 cells challenged with 0, 0.15 or 0.50 µM MeHg are respectively coloured in grey, orange and red. Abbreviation used: AA, arachidonic acid; ALA, alpha-linolenic acid; ALOX5, arachidonate 5-lipoxygenase; EPA, eicosapentaenoic acid; HSP70a, heat shock protein 70a; LA, linoleic acid; MeHg; methylmercury; MTb, metallothionein b; r, Pearson's correlation, THg, intracellular total mercury level.

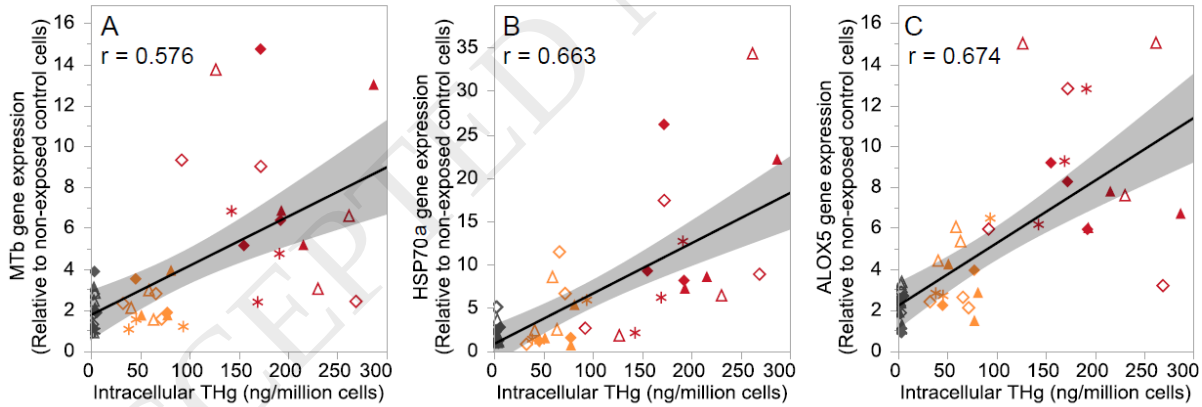

Table captions

Table 1: Sequence, amplicon size, annealing temperature and optimal concentration in master mix for the primers of the different genes investigated.

Gene	Sequence 5'→3' Forward	Reverse	Size (bp)	Annealing T (°C)	Concentration (nM)	Reference
Actin *Housekeeping gene 1*	AAGACAGCTACGTGGGAGAC	CAGCTCGTTGTAGAAGGTGTG	135	60	200	*Present study*
18S *Housekeeping gene 2*	CACGCGAGATGGAGCAATAA	CGCAGAGTAGACACACGCTGAT	96	60	200	*Gagné et al. 2012*
FASN *fatty acid synthase*	TGATCTGAAGGCCCGTGTCA	GGGTGACGTTGCCGTGGTAT	162	60	200	*Kamalam et al. 2013*
ELOVL2 *fatty acid elongase 2*	CCAGATCACCTTCCTGCAC	GACAGGCCATAGTAGGAGTAC	151	60	200	*Present study*
ELOVL5 *fatty acid elongase 5*	CCAAGTACATGAGACACAGAC	CACACAGCAGACACCATCTC	115	60	100	*Present study*
FADS2 *fatty acid desaturase 2*	AGGGTGCCTCTGCTAACTGG	TGGTGTTGGTGATGGTAGGG	175	60	200	*Kamalam et al. 2013*
FATP1 *fatty acid transport protein 1*	CGAATGCAACTGTAGCATCG	CTCYGTGGTCTCCTCATCC	122	60	300	*Present study*
CPT1 *carnitine palmitoyl transferase 1*	CTCCTGGAAGAAGAGGTTCATACG	ACTCACCACCACCACCAAAAC	95	60	100	*Present study*
cPLA2 *cytosolic phospholipase A2*	GAGATGTCGTTAGAGGTGTG	GTCTCTGCGTGTCTGTCTGA	94	60	200	*Present study*
COX2 *cyclooxygenase 2*	CATCGGACAAGAACCCTTGA	CTTCATTGGAGAGGCTGGTGTGC	73	60	100	*Chettri et al. 2011*
ALOX5 *arachidonate 5-lipoxygenase*	CGGCAGAGCACGGTTGCCAAAG	CCACTCACTCCATCTGTAGGTT	104	58	200	*Baron et al. 2005*
MTa *metallothionein a*	CATGCACCAGTTGTAAGAAAGCA	GCAGCCTGAGGCACACTTG	75	60	300	*Ceyhun et al. 2011*
MTb *metallothionein b*	ATCCTGCAAGTGCTCAAACT	ATGACTGCATTGTCACGGTA	139	60	300	*Ceyhun et al. 2011*
HSP70a *heat shock protein 70a*	CAGCATTGAGATTGACTCC	GTCAGAACACATCTCCTC	86	60	300	*Present study*
HSP70b *heat shock protein 70b*	CAGCATTGAGATTGACTCT	GTCGGAACACATTTCCTC	71	60	200	*Present study*
BCLx *apoptosis regulator BCL2-Like1*	GGACTCAGTGGATGAGTTTGAGC	GAACACTTCGTCCATCACACTCTC	122	60	200	*Present study*
CYP20A1 *cytochrome P450 20A1*	GCTGTCACTCAACTCGCTCTG	CTGATGGAGCTCTTCTCCATG	137	60	300	*Present study*

Transcript sequences used for primer design were gleaned from the literature or designed (*"Present study"*) based on transcript sequences annotated by *Marancik et al. 2014.* (see *"Reference"* column). Abbreviations used: bp, base pairs ; T, temperature

Table 2: Fatty acid composition of RTL-W1 cell phospholipids after one week culture in a growth medium supplemented or not (control) with a specific PUFA (50 µM ALA, EPA, LA or AA) (% of total fatty acids identified in cellular phospholipids).

Fatty acid	Control	ALA	EPA	LA	AA
14:0	1.8 ± 0.0	0.8 ± 0.1 *	1.4 ± 0.0 *	1.0 ± 0.0 *	1.4 ± 0.0 *
16:0	28.0 ± 0.9	21.5 ± 1.1 *	26.6 ± 1.3	21.9 ± 0.6 (*)	24.3 ± 0.6
18:0	19.5 ± 1.0	18.5 ± 1.0	16.7 ± 1.1	16.8 ± 0.7	15.8 ± 0.5 (*)
Total SFA	**49.3 ± 1.9**	**40.8 ± 2.2 ***	**44.6 ± 2.3**	**39.7 ± 1.1 ***	**41.4 ± 1.0 (*)**
16:1n-7	1.4 ± 0.1	0.0 ± 0.0 *	0.8 ± 0.0 *	0.0 ± 0.0 *	0.7 ± 0.0 *
18:1n-7	3.7 ± 0.2	1.8 ± 0.1 *	2.6 ± 0.1 *	1.7 ± 0.2 *	2.4 ± 0.1 *
18:1n-9	34.4 ± 1.3	11.5 ± 0.2 *	13.7 ± 0.4 *	8.6 ± 0.4 *	12.6 ± 0.4 *
Total MUFA	**39.5 ± 1.5**	**13.3 ± 0.0 ***	**17.0 ± 0.5 ***	**10.3 ± 0.5 ***	**15.7 ± 0.6 ***
ALA 18:3n-3	0.0 ± 0.0	**25.0 ± 2.0 ***	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:4n-3	0.0 ± 0.0	7.0 ± 0.2 *	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3n-3	0.0 ± 0.0	1.9 ± 0.1 *	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:4n-3	0.0 ± 0.0	2.3 ± 0.2 *	1.6 ± 0.1 *	0.0 ± 0.0	0.0 ± 0.0
EPA 20:5n-3	1.6 ± 0.1	4.7 ± 0.4 *	**19.9 ± 0.7 ***	0.7 ± 0.1 *	0.0 ± 0.0 *
22:5n-3	1.5 ± 0.1	1.2 ± 0.1	11.8 ± 0.8 *	0.9 ± 0.0 (*)	1.1 ± 0.0
22:6n-3	2.2 ± 0.1	1.5 ± 0.0 *	1.6 ± 0.1 *	1.3 ± 0.0 *	1.0 ± 0.0 *
Total n-3 PUFA	**5.3 ± 0.2**	**43.5 ± 2.0 ***	**34.9 ± 1.6 ***	**3.0 ± 0.1 ***	**2.1 ± 0.1 ***
LA 18:2n-6	2.8 ± 0.1	1.7 ± 0.2 *	1.6 ± 0.1 *	**34.1 ± 0.3 ***	1.4 ± 0.1 *
18:3n-6	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	6.6 ± 0.1 *	0.8 ± 0.0 *
20:2n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.1 *	0.0 ± 0.0
20:3n-6	2.0 ± 0.1	0.6 ± 0.0 *	0.8 ± 0.1 *	3.6 ± 0.2 *	2.0 ± 0.1
AA 20:4n-6	3.2 ± 0.1	1.6 ± 0.1 *	2.1 ± 0.1 *	2.6 ± 0.2 (*)	**27.5 ± 0.6 ***
22:4n-6	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.1 *	0.0 ± 0.0	10.3 ± 0.6 *
Total n-6 PUFA	**8.2 ± 0.3**	**3.9 ± 0.2 ***	**5.1 ± 0.2 ***	**48.3 ± 0.8 ***	**41.9 ± 0.6 ***
Total PUFA	**13.5 ± 0.5**	**47.4 ± 2.2 ***	**40.0 ± 1.8 ***	**51.3 ± 0.9 ***	**43.9 ± 0.7 ***
n-3/n-6 ratio	**0.66 ± 0.01**	**11.11 ± 0.21 ***	**6.84 ± 0.03 ***	**0.06 ± 0.00 ***	**0.05 ± 0.00 ***

Data are expressed as means ± standard error of mean (n=3). Stars indicate significant differences as compared to the control condition (1-way ANOVA with a post-hoc Dunnett's test, $p<0.05$) while stars into brackets indicate similar trends but without significance ($0.05<p<0.1$). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid

Table 3: Fatty acid composition of RTL-W1 cell phospholipids after one-week culture in a growth medium supplemented in ALA or AA (50 µM) and a subsequent challenge with MeHg (0, 0.15 or 0.50 µM during 24 h) in a medium deprived of fatty acid (% of total fatty acids identified in cellular phospholipids).

Fatty acid	ALA			AA		
	0 µM MeHg	0.15 µM MeHg	0.50 µM MeHg	0 µM MeHg	0.15 µM MeHg	0.50 µM MeHg
14:0	0.8 ± 0.1	0.8 ± 0.0	0.9 ± 0.1	1.4 ± 0.0	1.3 ± 0.1	1.4 ± 0.1
16:0	21.5 ± 1.1	23.5 ± 1.1	24.5 ± 1.2	24.3 ± 0.6	28.1 ± 0.6	27.1 ± 1.5
18:0	18.5 ± 1.0	21.0 ± 0.8	21.3 ± 1.1	15.8 ± 0.5	19.5 ± 1.1	18.3 ± 2.0
Total SFA	**40.8 ± 2.2**	**45.3 ± 2.0**	**46.7 ± 2.3**	**41.4 ± 1.0**	**48.9 ± 1.6**	**46.7 ± 3.3**
16:1n-7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.1
18:1n-7	1.8 ± 0.1	1.6 ± 0.1	1.5 ± 0.0	2.4 ± 0.1	2.0 ± 0.1	2.2 ± 0.1
18:1n-9	11.5 ± 0.2 (a)	10.7 ± 0.2 (b)	10.8 ± 0.11 (ab)	12.6 ± 0.4	11.2 ± 0.3	12.1 ± 0.8
Total MUFA	**13.3 ± 0.0 a**	**12.3 ± 0.2 b**	**12.3 ± 0.1 b**	**15.7 ± 0.6**	**13.9 ± 0.3**	**15.0 ± 1.0**
ALA 18:3n-3	**25.0 ± 2.0**	**23.5 ± 1.7**	**22.6 ± 1.5**	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
18:4n-3	7.0 ± 0.2 (a)	6.1 ± 0.0 (ab)	5.9 ± 0.3 (b)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3n-3	1.9 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:4n-3	2.3 ± 0.2	2.3 ± 0.1	2.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
EPA 20:5n-3	4.7 ± 0.4	4.3 ± 0.3	4.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:5n-3	1.2 ± 0.1	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	0.9 ± 0.1	1.0 ± 0.1
22:6n-3	1.5 ± 0.0	1.4 ± 0.0	1.4 ± 0.0	1.0 ± 0.0	0.9 ± 0.0	1.2 ± 0.3
Total n-3 PUFA	**43.5 ± 2.0**	**40.3 ± 1.6**	**39.1 ± 2.0**	**2.1 ± 0.1**	**1.8 ± 0.1**	**2.2 ± 0.3**
LA 18:2n-6	1.7 ± 0.2	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1	1.2 ± 0.1
18:3n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.0	0.6 ± 0.1	0.8 ± 0.1
20:2n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
20:3n-6	0.6 ± 0.0	0.4 ± 0.2	0.4 ± 0.2	2.0 ± 0.1 a	1.6 ± 0.0 b	1.7 ± 0.1 ab (b)
AA 20:4n-6	1.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.0	**27.5 ± 0.6**	**24.1 ± 0.6**	**24.7 ± 1.7**
22:4n-6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	10.3 ± 0.6	8.6 ± 0.8	9.0 ± 1.0
Total n-6 PUFA	**3.9 ± 0.2**	**3.4 ± 0.3**	**3.3 ± 0.3**	**41.9 ± 0.6**	**36.3 ± 1.3**	**37.3 ± 2.6**
Total PUFA	**47.4 ± 2.2**	**43.8 ± 1.9**	**42.4 ± 2.2**	**43.9 ± 0.7**	**38.1 ± 1.4**	**39.5 ± 2.6**
n-3/n-6 ratio	**11.11 ± 0.21**	**11.93 ± 0.61**	**12.00 ± 0.67**	**0.05 ± 0.00**	**0.05 ± 0.00**	**0.06 ± 0.01**

Data are expressed as means ± standard error of mean (n=3). For each fatty acid enrichment, letters indicate significant impact of the MeHg challenge, with levels not connected to the same letter being significantly different from each other (1-way ANOVA with a post-hoc Tukey test, $p<0.05$) while letters into brackets indicate similar trends but without significance ($0.05<p<0.1$). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; MeHg, methylmercury; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid

Table 4: Gene expression levels in RTL-W1 cells after one week culture in a growth medium supplemented or not (control) with a specific PUFA (50 µM ALA, EPA, LA or AA) and a subsequent challenge with MeHg (0, 0.15 or 0.50 µM during 24 h) in a medium deprived of fatty acid.

	Control			ALA			EPA			LA			AA		
Gene	0 µM MeHg	0.15 µM MeHg	0.50 µM MeHg	0 µM MeHg	0.15 µM MeHg	0.50 µM MeHg	0 µM MeHg	0.15 µM MeHg	0.50 µM MeHg	0 µM MeHg	0.15 µM MeHg	0.50 µM MeHg	0 µM MeHg	0.15 µM MeHg	0.50 µM MeHg
Lipid metabolism															
FAS	1.00 ± 0.00	1.92 ± 0.54	1.28 ± 0.49	1.36 ± 0.30	1.35 ± 0.33	1.51 ± 0.23	1.55 ± 0.32	1.69 ± 0.68	1.63 ± 0.38	1.66 ± 0.35	1.70 ± 0.31	1.69 ± 0.61	2.98 ± 0.73	2.35 ± 0.41	1.90 ± 0.87
FAT P1	1.00 ± 0.00 ab	1.35 ± 0.19 a	0.83 ± 0.06 b	5.59 ± 0.88 *	8.53 ± 1.90 (*)	6.89 ± 1.27 (*)	7.34 ± 2.02	8.86 ± 3.38 *	9.42 ± 2.86 *	7.36 ± 1.67 (*)	10.04 ± 2.04 *	10.86 ± 2.30 *	8.44 ± 1.79 (*)	11.21 ± 3.40 *	10.20 ± 3.02 *
CPT 1	1.00 ± 0.00	1.61 ± 0.36	1.74 ± 0.55	13.90 ± 4.06 (*)	13.04 ± 4.27 (*)	14.05 ± 4.84 (*)	26.86 ± 9.95	25.03 ± 9.27 *	27.10 ± 7.06 *	22.42 ± 6.58 (*)	20.83 ± 8.19 *	20.18 ± 6.91 *	41.09 ± 12.78	42.04 ± 17.93 *	40.89 ± 22.37 *
FAD S2	1.00 ± 0.00	1.21 ± 0.28	1.07 ± 0.40	0.73 ± 0.06 *	0.70 ± 0.08 *	0.65 ± 0.05	0.75 ± 0.05 *(ab)	0.79 ± 0.08 (a)	0.58 ± 0.07 (b)	0.80 ± 0.15	0.70 ± 0.04 *	0.53 ± 0.06	0.87 ± 0.08 (a)	0.75 ± 0.09 (*)(ab)	0.60 ± 0.11 (b)
ELO VL5	1.00 ± 0.00	0.99 ± 0.13	0.80 ± 0.17	0.54 ± 0.05 *	0.53 ± 0.05 (*)	0.54 ± 0.03	0.45 ± 0.04 *	0.51 ± 0.05 *	0.39 ± 0.05 *	0.47 ± 0.09 *	0.45 ± 0.07 *	0.40 ± 0.06 (*)	0.61 ± 0.12 (*)	0.47 ± 0.10 *	0.40 ± 0.09
PUFA-derived signalling															
cPL A2	1.00 ± 0.00	1.51 ± 0.22	3.74 ± 2.42	1.97 ± 0.07 *	2.19 ± 0.11 *	2.02 ± 0.25	1.81 ± 0.27	2.37 ± 0.32 *	1.81 ± 0.21	1.71 ± 0.33	1.76 ± 0.18	5.91 ± 3.80 (*)	2.09 ± 0.22 *	1.89 ± 0.25	4.54 ± 2.86
COX 2	1.00 ± 0.00	1.66 ± 0.33	2.22 ± 0.83	0.79 ± 0.08 a	1.45 ± 0.46 ab	1.87 ± 0.51 b	0.61 ± 0.06 *(a)	1.19 ± 0.40 (b)	1.52 ± 0.80 (ab)	1.11 ± 0.13	1.21 ± 0.19	1.67 ± 0.57	0.50 ± 0.15 (*)	0.59 ± 0.26 *	1.05 ± 0.71
ALO X5	1.00 ± 0.00	3.49 ± 1.03	7.70 ± 2.21	1.67 ± 0.28 (a)	2.55 ± 0.66 (ab)	5.45 ± 1.46 (b)	1.71 ± 0.55 (a)	2.65 ± 0.68 (ab)	6.20 ± 1.77 (b)	2.48 ± 0.58 (a)	4.16 ± 1.18 (ab)	9.88 ± 3.21 (b)	1.95 ± 0.45	1.99 ± 0.43	5.80 ± 2.55
Stress response															
BCL x	1.00 ± 0.00	1.03 ± 0.22	0.92 ± 0.11	0.78 ± 0.12 ab	0.88 ± 0.07 a	0.62 ± 0.08 b	0.89 ± 0.09	0.93 ± 0.12	0.73 ± 0.06	0.83 ± 0.11	0.77 ± 0.05	0.77 ± 0.21	0.83 ± 0.15	0.66 ± 0.17	0.61 ± 0.11 (*)
HSP 70a	1.00 ± 0.00 (ab)	2.56 ± 1.13 (a)	6.54 ± 2.23 (b)	1.78 ± 0.20 (*)a	2.36 ± 1.03 a	10.87 ± 3.83 b	2.10 ± 0.33 (*)ab(a)	1.37 ± 0.12 a	12.71 ± 4.53 b	1.72 ± 0.64 (a)	3.61 ± 1.69 (ab)	12.45 ± 7.40 (b)	3.63 ± 0.94	5.11 ± 2.51	9.18 ± 3.06
MTb	1.00 ± 0.00 ab(a)	1.22 ± 0.13 a	4.36 ± 0.96 b	2.05 ± 0.41 a	2.24 ± 0.57 (*)a	6.93 ± 2.21 b	2.04 ± 0.65 a	2.45 ± 0.55 *ab	7.32 ± 2.59 b	1.80 ± 0.51 a	1.97 ± 0.38 (*)a	6.66 ± 2.50 b	1.95 ± 0.31 a	2.08 ± 0.31 *a	6.39 ± 1.69 b
Cytochrome P450															
CYP 20A 1	1.00 ± 0.00 a	0.73 ± 0.08 ab(b)	0.47 ± 0.08 b(c)	1.76 ± 0.50	1.42 ± 0.22 *	1.00 ± 0.41	2.18 ± 0.62	1.85 ± 0.40 *	1.15 ± 0.21 *	2.01 ± 0.64 a	1.44 ± 0.29 (*)ab	0.91 ± 0.19 b	2.08 ± 0.38 (a)	1.94 ± 0.52 *(ab)	1.20 ± 0.28 *(b)

The $E^{-\Delta\Delta Ct}$ method was applied for relative gene expression analysis (Livak and Schmittgen, 2001), using the geometric mean of actin and 18S Ct values as housekeeper, and control cells non-exposed to MeHg as reference condition, for normalization. Data are finally expressed as means ± standard error of mean (n=4). For each MeHg dose, stars indicate significant difference as compared to the control condition (Kruskal-Wallis test with a post-hoc Dunn's test, $p<0.05$), while stars into brackets indicate similar trends but without significance ($0.05<p<0.1$). For each fatty acid enrichment strategy, letters indicate significant impact of the MeHg challenge, with levels not connected to the same letter being significantly different from each other (Kruskal-Wallis test with a post-hoc Dunn's test, $p<0.05$), while letters into brackets indicate similar trends but without significance ($0.05<p<0.1$). Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; ALOX5, arachidonate 5-lipoxygenase; BCLx, apoptosis regulator BCL2-Like1; cPLA2, cytosolic phospholipase A2; COX2, cyclooxygenase 2, CPT1, carnitine palmitoyl transferase 1; CYP20A1, cytochrome P450 20A1; ELOVL5, fatty acid elongase 5; EPA, eicosapentaenoic acid; FADS2, fatty acid desaturase 2; FASN, fatty acid synthase; FATP1, fatty acid transport protein 1; HSP70a, heat shock protein 70a; LA, linoleic acid; MeHg, methylmercury; MTb, metallothionein b; PUFA, polyunsaturated fatty acid.

Table 5: Principal component analysis loadings.

	PC1	PC2
Quantitative variables		
FASN - fatty acid synthase	-0.24	0.46
FADS2 - fatty acid desaturase 2	-0.63	0.10
ELOVL5 - fatty acid elongase 5	-0.72	0.37
CPT1 - carnitine palmitoyl transferase 1	0.72	-0.02
FATP1 - fatty acid transport protein 1	0.79	-0.06
cPLA2 - cytosolic phospholipase A2	0.34	-0.04
ALOX5 - arachidonate 5-lipoxygenase	0.52	0.59
COX2 - cyclooxygenase 2	-0.59	0.48
BCLx - apoptosis regulator BCL2-Like1	-0.46	-0.05
HSP70a - heat shock protein 70a	0.49	0.77
MTb - metallothionein b	0.58	0.51
CYP20A1 - cytochrome P450 20A1	0.27	-0.72
Qualitative variables		
MUFA	-0.45	0.23
PUFA	0.39	-0.20
THg	0.29	0.68
Part of the total variance explained (%)	31.1	19.4
Cumulative part of the variance explained (%)	31.1	50.5

The principal component analysis was performed using the relative expressions of the 12 selected genes as quantitative variables and both the THg and the phospholipid level in selected fatty acids as illustrative variables. A loading is the correlation between one variable and one principal component. Abbreviation used: PC, principal component; MUFA, total monounsaturated fatty acids in cellular phospholipids; PUFA, total polyunsaturated fatty acids in cellular phospholipids; THg, intracellular total mercury level

Table 6: Summary of the main results observed after PUFA enrichment and a subsequent exposure to MeHg for 24 h.

Parameter	Control	ALA		EPA		LA		AA		Multivariate analysis	
	MeHg impact	PUFA impact	MeHg impact	PUFA impact	MeHg impact	PUFA impact	MeHg impact	PUFA impact	MeHg impact	PUFA impact	MeHg impact
Intracellular THg [1]	↑		↑		↑		↑		↑		↑
Fatty acid profile of cellular phospholipids [2]											
Supplemented PUFA		↑		↑		↑		↑		na	
Direct biotransformation products		↑		↑		↑		↑		na	
Total PUFAs		↑		↑		↑		↑		↑	
Total MUFAs		↓		↓		↓		↓		↓	
n-3/n-6 ratio		↑		↑		↓		↓		na	
Genes involved in fatty acid metabolism [3]											
FASN											
FATP1		↑				(↑)		(↑)		↑	
CPT1		(↑)				(↑)				↑	
FADS2		↓		↓					(↓)	↓	
ELOVL5		↓		↓		↓		(↓)		↓	
Genes involved in PUFA-derived signalling [3]											
cPLA2		↑						↑			
COX2			↑	↓				(↓)		↓	
ALOX5			(↑)		(↑)		(↑)				↑
Genes involved in stress response [3]											
BCLx											
HSP70a		(↑)	↑	(↑)	(↑)		(↑)				↑
MTb	(↑)		↑		↑		↑		↑		↑
Cytochrome P450 gene [3]											
CYP20A1	↓						↓		(↓)	↑	

[1]: ng/million cells; [2] % of total identified fatty acids in cellular phospholipids; [3] relative gene expression; "↑" and "↓" stand respectively for significant ($p<0.05$) up- and down-regulation by either MeHg or PUFA, depending on the column in which the symbol in found (grey for PUFA impact and white for MeHg impact) while these symbols into brackets stand for similar trends but without significance ($0.05<p<0.1$). Note that "MeHg impact" evaluates the effect of the highest dose of MeHg tested only (0.5 µM), except for the multivariate analysis. The "multivariate analysis" columns summarize the main results that came from the principal component analysis, which was performed using the relative expressions of the 12 selected genes as quantitative variables and both the intracellular concentration in THg and the phospholipid level in selected fatty acids as illustrative variables. Abbreviations used: AA, arachidonic acid; ALA, alpha-linolenic acid; ALOX5, arachidonate 5-lipoxygenase; BCLx, apoptosis regulator BCL2-Like1; COX2, cyclooxygenase 2, cPLA2, cytosolic phospholipase A2; CPT1, carnitine palmitoyl transferase 1; CYP20A1, cytochrome P450 20A1; ELOVL5, fatty acid elongase 5; EPA, eicosapentaenoic acid; FADS2, fatty acid desaturase 2; FASN, fatty acid synthase; FATP1, fatty acid transport protein 1; HSP70a, heat shock protein 70a; LA, linoleic acid; MeHg, methylmercury; MTb, metallothionein b; MUFA(s), monounsaturated fatty acid(s); na, not-applicable; PUFA(s), polyunsaturated fatty acid(s); THg, total mercury