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Proteomics and lipidomics analyses reveal modulation of lipid metabolism by perfluoroalkyl substances in liver of Atlantic cod (*Gadus morhua*)

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ABSTRACT

The aim of the present study was to investigate effects of defined mixtures of polycyclic aromatic hydrocarbons (PAHs) and perfluoroalkyl substances (PFASs), at low, environmentally relevant (1× = L), or high (20× = H) doses, on biological responses in Atlantic cod (*Gadus morhua*). To this end, farmed juvenile cod were exposed at day 0 and day 7 via intraperitoneal (i.p.) injections, in a two-week *in vivo* experiment. In total, there were 10 groups of fish (n = 21-22): two control groups, four separate exposure groups of PAH and PFAS mixtures (L, H), and four groups combining PAH and PFAS mixtures (L/L, H/L, L/H, H/H). Body burden analyses confirmed a dose-dependent accumulation of PFASs in cod liver and PAH metabolites in bile. The hepatosomatic index (HSI) was significantly reduced for three of the combined PAH/PFAS exposure groups (L-PAH/H-PFAS, H-PAH/L-PFAS, H-PAH/H-PFAS). Analysis of the hepatic proteome identified that pathways related to lipid degradation were significantly affected by PFAS exposure, including upregulation of enzymes in fatty acid degradation pathways, such as fatty acid β-oxidation. The increased abundances of enzymes in lipid catabolic pathways paralleled with decreasing levels of triacylglycerols (TGs) in the H-PFAS exposure group, suggest that PFAS increase lipid catabolism in Atlantic cod. Markers of oxidative stress, including catalase and glutathione S-transferase activities were also induced by PFAS exposure. Only minor and non-significant differences between exposure groups and control were found for *cyp1a* and *acox1* gene expressions, vitellogenin concentrations in plasma, Cyp1a protein synthesis and DNA fragmentation. In summary, our combined proteomics and lipidomics analyses indicate that PFAS may disrupt lipid homeostasis in Atlantic cod.

Abbreviations: Acox1, Acyl coenzyme-A oxidase 1; Ahr, Aryl hydrocarbon receptor; BaP, Benzo[a]pyrene; BNF, Beta-naphthoflavone; Cat, Catalase; CF, Condition factor; Cyp, Cytochrome P450; DG, Diacylglycerol; EDC, Endocrine disrupting compounds; ER, Endoplasmic reticulum; EROD, 7-ethoxyresorufin-O-deethylase; FA, Free fatty acid; Gst, Glutathione S-transferase; HSI, Hepatosomatic index; HPLC, High-performance liquid chromatography; HRGC, High-resolution gas chromatography; LOD, Limit of detection; LOQ, Limit of quantification; PFAS, Perfluoroalkyl substances; PFNA, Perfluorononanoic acid; PFOS, Perfluorooctane sulfonate; PFOA, Perfluorooctanoic acid; PFTrDA, Perfluorotridecanoic acid; PP, Peroxisome proliferator; Ppar, Peroxisome proliferator-activated receptor; POP, Persistent organic pollutant; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; PG, Phosphatidylglycerol; PI, Phosphatidylinositol; PS, Phosphatidylserine; PL, Phospholipids; PCB, Polychlorinated biphenyls; PAH, Polycyclic aromatic hydrocarbons; TG, Triacylglycerol; Vtg, Vitellogenin.

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1. Introduction

The marine environment is under constant pollution pressure, and in addition to offshore activities such as oil and gas operations, a considerable amount of pollutants originates from land-based sources, including industrial and agriculture activities, as well as sewage discharges (Windom, 1992). Two abundant groups of environmental contaminants are polycyclic aromatic hydrocarbons (PAHs), and perfluoroalkyl substances (PFASs). Depending on their origin, PAHs are categorized as pyrogenic or petrogenic. Petrogenic PAHs are naturally occurring in crude oil and coal, while pyrogenic PAHs are formed by incomplete combustion of organic matter, such as forest fires, fossil fuels and tobacco smoke. Pyrogenic PAHs are generally composed of a higher number of aromatic rings compared to petrogenic variants (Pampanin & Sydnes, 2013). In contrast to PAHs, PFASs are utilized in a wide array of consumer products, including cosmetics, non-stick cookware, as stain- and water repellents, and in fire-fighting foams (Collí-Dulá et al., 2016). PAHs and PFASs have been detected in marine organisms, including several fish species and marine mammals (Grøsvik et al., 2012; Kallenborn et al., 2004; Law, 2014). Exposure of several teleost species to PAHs and PFASs have been linked to deleterious effects, such as developmental toxicity, behavioral abnormalities and disruption of the reproductive system (Cherr et al., 2017; Dale et al., 2019; Jantzen et al., 2016). However, limited number of studies exists on how PAHs and PFASs exert their toxicities when combined. Furthermore, the majority of toxicological research concerning effects of environmental contaminants are using concentration ranges exceeding environmentally relevant concentrations. Thus, studying effects of combined exposures at environmentally relevant concentrations is important for expanding our knowledge on their potential toxic effects in the marine environment.

In environmental toxicology, biomarkers are used as sensitive and early warning signals of chemical exposure. Common biomarkers for PAH exposure are induction of cytochrome P450 1A (Cyp1a) through activation of the aryl hydrocarbon receptor (Ahr), formation of DNA adducts and DNA fragmentation, and accumulation of PAH metabolites in bile (Frenzilli et al., 2004; Goksøyr, 1995; Pampanin & Sydnes, 2013; van der Oost et al., 2003). Specific biomarkers of PFAS exposure are less established. However, previous studies suggest that the liver is an important target organ for PFAS toxicity, causing oxidative stress, endocrine disruption and altered lipid metabolism (Han & Fang, 2010; Wei et al., 2009; Wielsøe et al., 2015). For example, exposure to PFASs induced vitellogenin (Vtg) levels in cultured tilapia hepatocytes (Liu et al., 2007) and in juvenile rainbow trout (Benninghoff et al., 2011), and caused DNA damage and oxidative stress in a human hepatoma cell line (Wielsøe et al., 2015). Additionally, it has been shown that several PFASs, including perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS), can activate the peroxisome proliferator-activated protein alpha (PPARA) in humans and rodents (Behr et al., 2020; Berthiaume & Wallace, 2002). PPARA is a transcription factor controlling the expression of genes involved in energy metabolism, including the acyl coenzyme-A oxidase 1 (*acox1*). Therefore, assessing alterations in antioxidant enzyme activities, energy metabolism, and DNA homeostasis in liver tissue could provide valuable information regarding the toxicity of PFASs. In addition, high-throughput methods such as proteomics and lipidomics may help understand toxicity mechanisms and provide potential biomarkers of PFAS exposure.

Atlantic cod (*Gadus morhua*) is a major fisheries species in the North Atlantic, and is also used as an indicator species in marine environmental monitoring programs, including the Oslo and Paris Convention for the Protection of the Marine Environment of the North-East Atlantic (OSPAR convention) and water column monitoring of offshore petroleum activities in Norway (Norwegian Environment Agency, 2011; Sundt et al., 2012; Vethaak et al., 2017). Furthermore, Atlantic cod is increasingly used as a model organism in environmental toxicology, facilitated by the availability of the annotated cod genome (Star et al., 2011; Tørresen et al., 2017). Exposure studies with cod as a model organism have involved both single and mixtures of contaminants, such as PAHs, polychlorinated biphenyls (PCBs) and other endocrine-disrupting compounds (EDCs) (Bizarro et al., 2016; Goksøyr et al., 1986; Hasselberg et al., 2004; Holth et al., 2014; Pampanin et al., 2016; Ruus et al., 2012; Yadetie et al., 2014, 2017). However, limited knowledge exists on the effects of PFASs in fish, and in particular on combined effects of PAH and PFAS exposure. Recently, we have shown that exposure to environmentally relevant mixtures of PAHs and PFASs can modulate the neuro-dopamine homeostasis in female Atlantic cod (Khan et al., 2019), where PAHs and PFASs promoted both separate and interactive effects. Here, we report other biological effects measured in liver and plasma of cod from the same exposure experiment.

The aim of this study was to investigate biological responses in Atlantic cod after exposure to environmentally relevant mixtures of PAHs and PFASs, either separately or combined. The low dose mixtures were based on reported concentrations of PAHs and PFASs in liver of wild Atlantic cod, and the high dose mixtures were twenty times (20×) the low dose concentration. To confirm the exposure, accumulated PFASs were measured in cod liver, while PAH metabolites were measured in bile. Proteomics analysis was performed in liver samples to study changes in the hepatic proteome, and lipidomics analysis of liver microsomal fractions was carried out to investigate alterations in lipid metabolism. In addition, biological responses such as DNA fragmentation, oxidative stress and endocrine disruption were assessed in white blood cells, and at transcript and protein levels in liver and plasma, respectively.

2. Methods

2.1. Fish husbandry

Information regarding fish husbandry is available in Khan et al. (2019). At the start of the exposure, fish were 18 months with an average body weight of 172 ± 34 g.

2.2. Exposures

Details of exposures can be found in Khan et al. (2019). Briefly, chemicals were purchased from Merck (Darmstadt, Germany) (Table S1) and stock solutions were dissolved in 1:1 (v/v) mixture of 100% refined rapeseed oil (Eldorado, Unil AS, Oslo, Norway) and PBS and injected at 1 mL/100 g fish. The 1:1 (v/v) mixture of rapeseed oil and PBS was used as a solvent control (Control in Table 2). Atlantic cod were injected intraperitoneally twice (day 0 and day 7), using two different doses: 1× (low = L) and 20× (high = H) dose of PAH and PFAS mixtures (Table 1), individually and in various combinations with a total of 10 groups (Table 2). The concentrations of the low dose mixtures (L-PAH, L-PFAS) were based on reported levels and proportions of these compounds in liver samples obtained from wild Atlantic cod (Grøsvik et al., 2012; Kallenborn et al., 2004; Norwegian Environment Agency, 2013). In the Nordic Environment, median PFAS concentrations less than 12 ng/L and up to 250-300 ng/g wet weight (ww) were found in seawater and sediment, respectively (Kallenborn et al., 2004). Median PFAS concentrations of up to 80 ng/g ww were reported in marine fish liver (Kallenborn et al., 2004). Intraperitoneal injection was chosen as exposure method to ensure delivery of correct doses and proportions of the complex mixtures to the fish. We aimed at 14 days exposure and chose to inject the doses twice at seven days interval, based on the half-lives of the compounds and expected response times at both proteomics and lipidomic levels. Fish were euthanized with a blow to the head 14 days after the first injection. The exposure experiment was conducted according to national regulations and was approved by the Norwegian Food Safety Authorities (FOTS ID: 11730).

Table 1
Detailed overview of chemical compounds and concentrations in polycyclic aromatic hydrocarbon (PAH) and perfluoroalkyl substance (PFAS) mixtures (adapted from Khan et al. (2019)).

Compound type	Compound	Low dose (1×) µg/kg	High dose (20×) µg/kg	% of total
PAH mixture	Naphthalene	12.64	252.8	31.6
	Phenanthrene	8.38	167.6	21.0
	Dibenzothiophene	0.58	11.6	1.4
	Pyrene	1.45	29.0	3.6
	Benzo[a]pyrene	1.93	38.5	4.8
	Fluorene	15.03	300.5	37.6
	Total concentration	**40**	**800**	**100**
PFAS mixture	PFOS	25	500	48.3
	PFTrDA	16.95	339	32.8
	PFNA	5.925	118.5	11.5
	PFOA	3.825	76.5	7.4
	Total concentration	**51.7**	**1034**	**100**

Table 2
Exposure setup of low (L = 1×) and high (H = 20×) doses of polycyclic aromatic hydrocarbon (PAH) and perfluoroalkyl substance (PFAS) mixtures. +/- indicate presence/absence of the mixtures, respectively.

	Control	Single exposures				Combined exposures			
L-PAH	-	+	-	-	-	+	+	-	-
H-PAH	-	-	+	-	-	-	-	+	+
L-PFAS	-	-	-	+	-	+	-	+	-
H-PFAS	-	-	-	-	+	-	+	-	+

2.3. Growth and condition parameters

Hepatosomatic index (HSI = liver weight (g) × 100/total weight (g)) and Fulton's condition factor (CF = 100000 × W/L^3, where W is body weight (g), and L is body length (mm)) were used to evaluate the general physiological condition.

2.4. Chemical analyses

2.4.1. PFAS in liver

The analytical procedure is described by Grønnestad et al. (2017), and further details are found in section S2. In brief, 0.25 grams of homogenized liver tissue were weighed and spiked with internal standards containing a ^{13}C-labeled PFAS mix (Wellington Laboratories). Samples were extracted twice with methanol. Active carbon (EnviCarb) was used for removal of lipids. Separation and detection of PFASs (PFOS, PFOA, perfluorononanoic acid (PFNA) and perfluorotridecanoic acid (PFTrDA)) were performed using a high-performance liquid chromatography (HPLC) with a Phenomenex Kinetex 2.6U C18 column with a C18 pre-column (Phenomenex, Torrance, USA) and a Agilent 1200 liquid chromatography (LC) system coupled to an Agilent 6460 tandem mass spectrometry (MS/MS) instrument. The analytical quality was approved by satisfactory quality control measures, and results were within the accepted ranges for the interlaboratory tests (Arctic Monitoring and Assessment Program, Québec, Canada).

2.4.2. PAH metabolites in bile

2.4.2.1. Fixed wavelength fluorescence. PAH metabolites were analyzed using the fix wavelength fluorescence (FF) screening method (Aas et al., 1998, 2000). Bile samples were diluted 1:1600 in 50% methanol. Slit widths were set at 2.5 nm for both excitation and emission wavelengths, and samples were analyzed in a quartz cuvette using a Lumina Fluorescence spectrometer (ThermoFisher Scientific, Waltham, MA, USA). All bile samples were measured by FF at the excitation/emission wavelength pairs 290/335, 341/383 and 380/430 nm, optimized for the detection of 2-3 ring, 4-ring and 5-ring PAH metabolites, respectively. The fluorescence signal was transformed into 1-hydroxypyrene fluorescence equivalents (PFE) using pyrene external standard curve (Merck). The concentration of PAH metabolites in bile samples was expressed as µg PFE/ mL bile.

2.4.2.2. GC-MS. The quantification of the PAH metabolites in Atlantic cod bile samples was carried out using a gas chromatography-mass spectrometry approach (GC-MS). Details are reported in previous studies (Jonsson et al. 2003; Jonsson et al., 2004). The GC-MS system consisted of a HP5890 series II Gas chromatograph, Shimadzu QP2010 GCMS. Helium was used as the carrier gas and the applied column was a CP-Sil 8 CB-MS, 50 m ×0.25 mm and 0.25 µm film thickness (Varian). Molecular ions were selected for determining alkylated and non-alkylated trimethylsilyl ethers of OH-PAHs (Jonsson et al., 2003). Results were expressed as ng/g bile.

2.5. Comet assay

The comet assay was performed as described by Haarr et al. (2018), with minor adjustments, and is described in section S3.

2.6. RNA extraction and Real-Time qPCR

RNA was extracted and transcript levels of target genes (*cyp1a*, *acox1*) and reference genes (*uba52*, *ef1a*) (Table S2) were quantified using quantitative PCR (qPCR), as described in Dale et al. (2019), with some modifications. According to manufacturer's instructions, total RNA was extracted using QIAzol Lysis Reagent (Cat.no 79306, Qiagen, Hilden, Germany) and the BioRobot EZ1 (Qiagen), and eluted in 100 µL RNase-free deionized H_2O. All tested samples had RNA integrity numbers (RIN) > 9. For the reference genes, the geNorm stability index M was < 0.91.

2.7. Liver protein analyses

Liver S12 fractions from experiment samples and for positive controls (BNF samples) were prepared and utilized for enzymatic analyses (Gst, Cat and EROD) as described in Dale et al. (2019). 10 samples (five per gender) were analysed per exposure group. The enzyme-linked immunosorbent assay (ELISA) were performed as described in Nilsen et al. (1998). A concentration of 100 µg/mL protein in coating buffer was chosen for each sample. Primary and secondary antibodies utilized were anti-cod Cyp1a (1:800) and HRP-conjugated anti-IgG (1:3000), respectively (Table S3). The ELISA was developed with 100 µL 3,3',5,5'-tetramethylbenzidine (TMB) for 30 min and the reaction was stopped by adding 100 µL 0.3 M H_2SO_4.

2.8. ELISA quantification of plasma Vtg levels

Levels of Vtg in blood plasma were determined for 10 cod from each exposure group (five per gender) as described in Dale et al. (2019).

2.9. Proteomics

2.9.1. Sample preparation, quantitation of proteins and preprocessing of data

Quantitative proteomics of liver samples (100 samples in total) were conducted at the Proteomics Unit at the University of Bergen (PROBE). A label-free LC-MS/MS approach was used with an LTQ Orbitrap Elite mass spectrometer. Details about sample preparation, running conditions, and instrumentation, are provided in section S4.

2.9.2. Differential expression analysis

Differential expression analysis and hierarchical clustering were performed using the Qlucore Omics Explorer (version 3.4) software (Qlucore AB, Lund Sweden) using pre-processed protein expression data from 100 samples (n = 20 for control group, and n = 10 for each of the other exposure groups) (Table S4).

2.9.3. Pathway and Gene Set Enrichment Analysis (GSEA)

Pathway analysis and GSEA were performed on human orthologs of the cod genes, extracted from ENSEMBL database as previously described (Yadetie et al., 2018). The pathway analysis tool Enrichr (Kuleshov et al., 2016) and the STRING protein-protein interaction network analysis tool (Szklarczyk et al., 2019) were used in pathway and network analyses, with FDR < 0.05 for significant enrichment. For GSEA, the Hallmark gene sets database (Liberzon et al., 2015) was used to perform gene set enrichment analysis using GSEA software (https://www.gsea-msigdb.org), with the tTest metric for ranking and the default FDR < 0.25 for significant enrichment (Subramanian et al., 2005).

2.10. Lipidomics

2.10.1. Sample fractionation

Liver samples were homogenized as described in Blanco et al. (2019), with some modifications. Homogenates were centrifuged at 1000 × g for 5 min at 4 °C. The supernatant was collected and further centrifuged at 12,000 × g for 45 min and 20,000 × g for 30 min. The obtained supernatant was centrifuged at 100,000 × g for 90 min. Microsomal pellets were then resuspended in a small volume of 100 mM phosphate buffer pH 7.4, 1 mM EDTA, 0.1 mM DTT, 0.1 mM PMSF and 20% w/v glycerol. Protein concentrations were determined by the method of Bradford, using bovine serum albumin as a standard (Bradford, 1976).

2.10.2. Lipid extraction

Microsomal lipids were extracted by a modification of Folch et al. (1957). 200 µg protein were homogenized in ice-cold chloroform: methanol (2:1 v/v) containing 0.01% butylated hydroxytoluene (BHT). Samples were incubated for 30 min at room temperature and soft shaking. 0.88% KCl was added to the supernatant (1:4 v/v), thoroughly mixed and further incubated for 10 min. After centrifugation (2500 rpm, 10 min, 10 °C), the organic layer was collected, the extraction was repeated, and the solvent was evaporated under nitrogen.

2.10.3. Instrumental analysis

The analysis of lipids was performed as described in Vichi et al. (2012), with some modifications. Reconstituted lipid extracts were directly injected in an Orbitrap-Exactive (Thermo Fisher Scientific). Mass peaks considered were single positive charged sodium molecular ions, [M + Na^+], including triacylglycerols (TG), diacylglycerols (DG), phosphatidylcholines (PC); and single negative charge [M-H^+], including phosphatidylethanolamines (PE), their plasmanyl/plasmenyl forms (PE-O/PE-P), phosphatidylserines (PS), phosphatidylinositols (PI), phosphatidylglycerols (PG), and free fatty acids (FA). Mass spectra were processed with Xcalibur (v2.1, Thermo Fisher Scientific, Bremen, Germany) and lipid species were quantified with internal standards (see Section S5).

2.11. Statistics

To account for any possible effects of sampling day, all biological parameters were treated with the Eq. (1) and (2), before combining the two control groups to one group, in order to compare all exposure groups to one control.

$$x_i^{'} = \frac{x_i}{\bar{x}} \times \frac{\bar{x}+\bar{y}}{2} \quad (1)$$

$$y_i^{'} = \frac{y_i}{\bar{y}} \times \frac{\bar{x}+\bar{y}}{2} \quad (2)$$

where $i \in \{1, 2, \ldots 110\}$, $\bar{x}$ is the mean abundance of one parameter measured on the samples from the first day, and $\bar{y}$ is that of the second day.

For all results presented, Graphpad Prism 7 (Graphpad Software, San Diego, CA, USA) was used for statistical analyses. Normality of the data distribution was tested using Shapiro-Wilk test. If one of the exposure groups failed the Shapiro-Wilk test, the responses of all groups were log-transformed. Responses following normal distribution were analyzed for exposure-dependent (exposed groups vs control) statistical differences ($p < 0.05$) using one-way ANOVA, followed by Dunnett's multiple comparisons test. Nonparametric tests (Kruskal-Wallis) were used to analyze responses failing Shapiro-Wilk test after being log-transformed.

The preprocessing of the proteomics data was performed by first removing the proteins where more than half of the values were missing in any group, followed by log2-transformation of the data. Remaining missing values were imputed using a maximum likelihood estimation (Schafer, 1997) and finally, the values were standardized using Eqs. (1) and (2) (Table S4).

Lipid profiles were analyzed by univariate and multivariate approaches using Metaboanalyst software (version 3.0; Xia et al. 2015). Missing values were replaced by the half of the minimum value in the original data. Data was normalized to the sum, scaled by mean-centering and dividing by standard deviation of each variable (Xia & Wishart, 2016). Partial least square - discriminant analyses (PLS-DA) was performed to identify lipids that differed in control and exposed fish. The goodness of the model was validated by a leave-one-out cross validation test. The predictive capacity (accuracy) and the explained Y variance (Q2) were calculated in order to assess the quality of the multivariate approach. Volcano plots based on fold-change values (> 1.5) and a significance threshold of $p < 0.05$ (Student's t-test) were used to visualize the significance and magnitude of the changes.

3. Results

3.1. PFASs and PAH metabolites were detected in cod liver and bile

Concentrations of the four compounds in the PFAS mixture (PFOS, PFNA, PFOA and PFTrDA) were determined in liver (Fig. 1, Table S5). Groups exposed to L- or H-PFAS alone or in combination with PAHs had significantly ($p < 0.05$) higher liver PFAS concentrations compared to the control, except for the L-PAH/L-PFAS group for all four compounds, and all L-PFAS groups for PFOA. The levels of PFASs were not analyzed in the two groups exposed only to PAHs. Furthermore, the PFAS congeners showed different accumulation patterns. Trends of higher PFOA and PFNA concentrations (Fig. 1A and B) were observed for exposure to H-PFAS alone compared to combined exposure with PAH, while PFTrDA and PFOS concentrations (Fig. 1C and D) were higher in combination with PAH than with H-PFAS alone. Such differences in accumulation patterns were not evident in the L-PFAS groups.

Concentrations of selected PAH metabolites were measured in bile (Table 3). Hydroxylated metabolites of naphthalene (1-OH-Naph and 2-OH-Naph) were below LOQ for all exposure groups. Hydroxylated metabolites of phenanthrene (1-OH-Phen) and pyrene (1-OH-Pyr) were significantly increased in the H-PAH, L-PAH/H-PFAS, H-PAH/L-PFAS and H-PAH/H-PFAS groups compared to control. Interestingly, concentrations of 1-OH-Phen and 1-OH-Pyr were higher in groups where H-PAH were co-exposed with either L-or H-PFAS, compared with H-PAH alone. These results were also in line with the screening of PAH metabolites with the FF method, where the clearest differences among exposure groups were observed for 4 and 5 ring PAHs (Fig. S1).

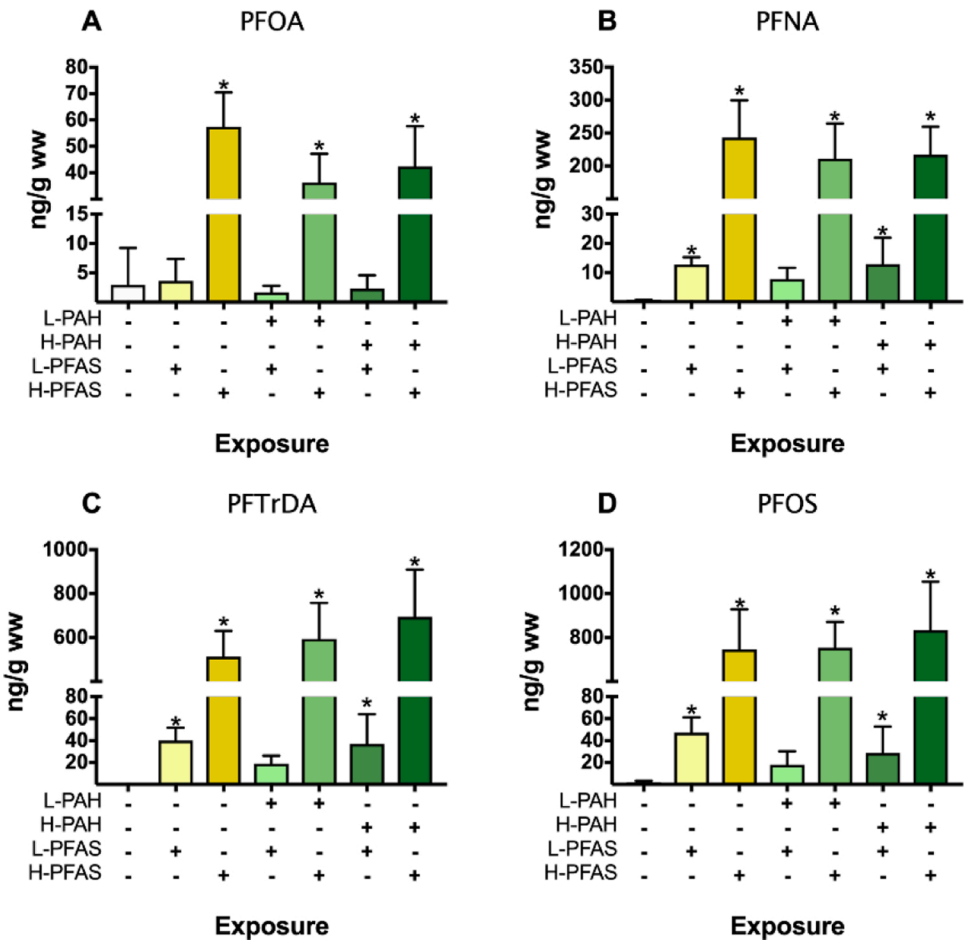


Fig. 1. PFAS concentrations in liver of Atlantic cod exposed to PAHs and PFASs. Atlantic cod were exposed to low (L = 1×) or high (H = 20×) doses of PAHs and PFASs either separately (PFAS, yellow), or combined (green). PFAS concentrations were determined in cod liver and are presented as ng/g wet weight liver tissue for PFOA (A), PFNA (B), PFTrDA (C), and PFOS (D). Data are presented as mean + s.d. and n = 20 and 10 for control and exposure groups, respectively. Asterisk indicates statistical significance ($p < 0.05$) between the exposed groups and control (Kruskal-Wallis). Disticnt color code is used for each exposure group based on presence or absence of PAH and PFAS and doses as indicated in the figure (A-D).

Table 3
PAH metabolites in bile (ng/g) of Atlantic cod exposed to PAHs and PFASs. Asterisks indicate $p < 0.05$ compared to control (CTRL) (Kruskal-Wallis). Values are presented as mean + s.d. (n = 43 for CTRL, n = 20-22 for exposure groups). Values below limit of quantification (LOQ, > 30 ng/g) were replaced with ½ LOQ.

Metabolite	CTRL	L-PAH	H-PAH	L-PAH/ L-PFAS	L-PAH/ H-PFAS	H-PAH/ L-PFAS	H-PAH/ H-PFAS
1-OH-Naph	15.0 + 0.0	15.0 + 0.0	15.0 + 0.0	15.0 + 0.0	15.0 + 0.0	15.0 + 0.0	15.0 + 0.0
2-OH-Naph	15.4 + 2.5	14.8 + 0.0	19.2 + 18.5	15.2 + 0.0	14.8.0 + 0.0	15.2 + 0.0	21.3 + 19.9
1-OH-Phen	23.5 + 17.8	20.0 + 17.2	63.0 + 32.7*	23.3 + 22.3	42.2 + 30.1*	87.8 + 45.3*	117 + 95.6*
1-OH-Pyr	47.0 + 43.7	107 + 92.7	579 + 391*	82.9 + 69.2	145 + 119*	700 + 515*	1162 + 963*

3.2. Combined PAH/PFAS exposure caused reduction in hepatosomatic index

Parameters indicating growth performance, i.e. effects of exposure on Fulton's condition factor (CF) and hepatosomatic index (HSI), are presented in Fig. 2. No significant differences were found when comparing control to exposed groups for CF (Fig. 2A). However, there was a significant reduction of HSI ($p < 0.05$) for the L-PAH/H-PFAS, H-PAH/L-PFAS and H-PAH/H-PFAS groups compared to the control group (Fig. 2B).

3.3. Liver proteome analysis revealed modulation of lipid homeostasis in Atlantic cod exposed to PFAS

3.3.1. Differential expression of proteins

When analyzing the proteomics data, we first studied PAH and PFAS exposure groups separately and performed cluster analyses to get an overview of proteins separating the exposure groups from the control group. For PFAS groups, the top 111 differentially expressed proteins in the comparison between control *versus* exposure groups (L-PFAS and H-PFAS, $p = 0.005$, q-value < 0.125) were used in hierarchical clustering (Fig. 3). The L-PFAS and H-PFAS groups had many top proteins showing similar expression profile, suggesting a dose-response trend in the data. A possible gender effect was tested by performing the differential expression for the PFAS groups with and without eliminating the gender factor, with approximately the same number of proteins being detected in both cases (Fig. S2). Thus, correction for gender effect was not performed in subsequent analyses.

None of the PAH treatments (L-PAH and H-PAH) resulted in statistically significant differential expression (Fig. S3). The effect of the PAH treatment was considered minimal, and therefore the separate PAH exposure groups (without PFAS) were excluded from further analysis. Mixture groups containing H-PFAS (H-PAH/H-PFAS and L-PAH/H-PFAS) were combined with the H-PFAS group and compared with the control group in further analysis of H-PFAS effects (Fig. S4). We found 32 significantly differentially expressed ($p = 0.0006$, q-value < 0.05) proteins (Fig. S4A), similar to the separate L-PFAS and H-PFAS groups (Fig. 3). Comparison of the H-PFAS group alone with the control resulted

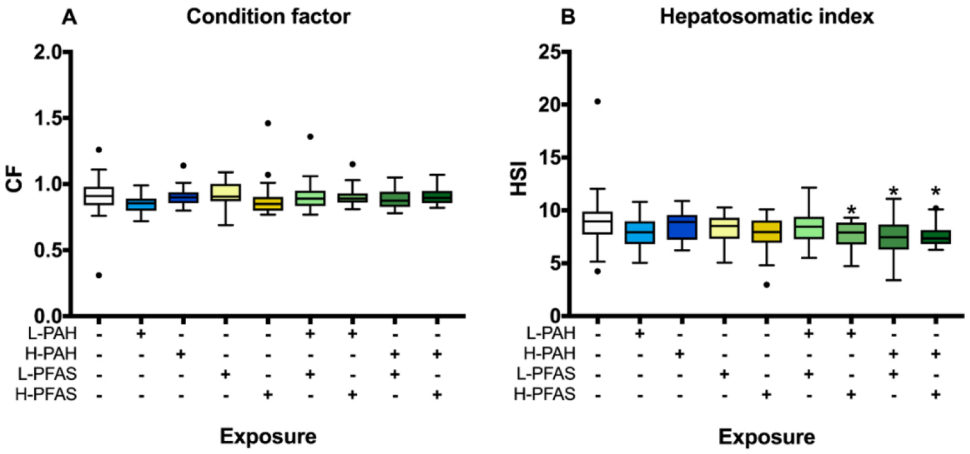


Fig. 2. Growth performance of Atlantic cod exposed to PAHs and PFASs. Post-exposure condition was assessed by calculating CF (A) and HSI (B) of Atlantic cod exposed to low (L = 1×) or high (H = 20×) doses of PAHs and PFASs either separately (blue and yellow, respectively) or combined (green). Data are presented with median values and 25-75% quartiles, whiskers are calculated using Tukey. n = 43 and 20-22 for control and exposed groups, respectively. Asterisk indicates statistical significance ($p < 0.05$) when comparing exposed groups to control (ANOVA).

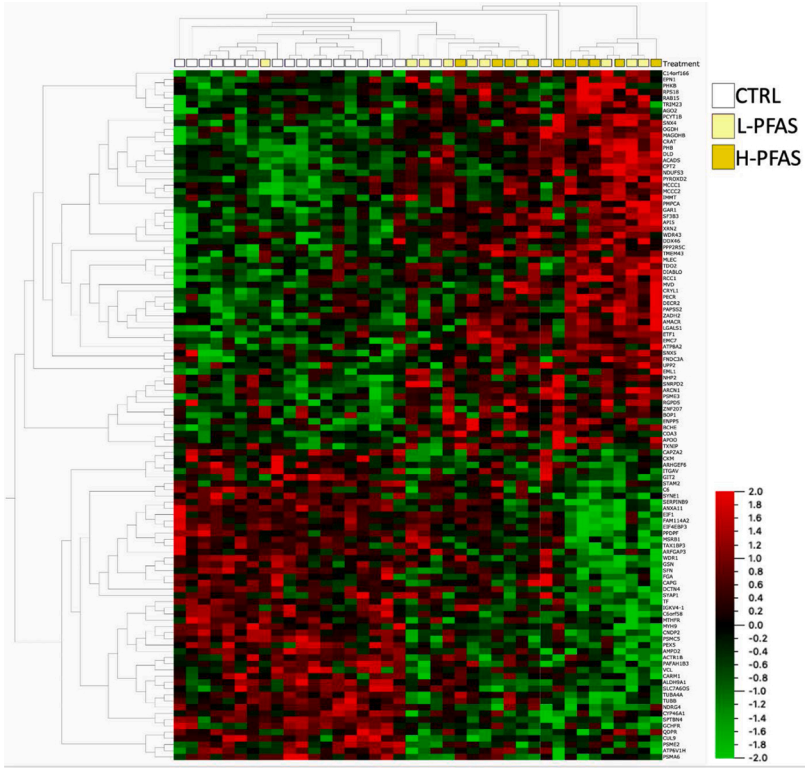


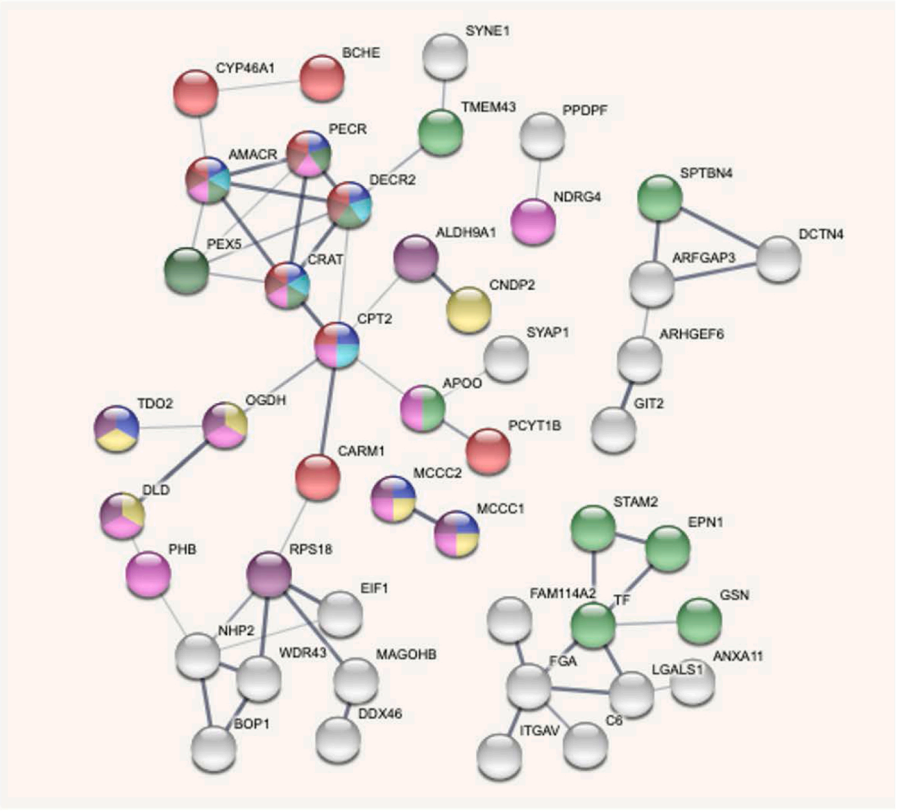
Fig. 3. Two-way hierarchical clustering of the top differentially expressed proteins in livers of cod exposed to L-PFAS and H-PFAS versus control. Data represent expression values (log-transformed) of 111 top differentially expressed proteins ($p = 0.005$, q-value < 0.125). The heatmap shows relative expression levels as shown by the color scale (bottom right), with red and green ends representing highest and lowest relative expression, respectively. Rows represent proteins and columns represent samples. Control (CTRL) and exposure groups (L-PFAS, H-PFAS) are indicated by the color legend (top right).

in 15 significantly differentially expressed ($p = 0.0003$, q-value < 0.05) proteins (Fig. S4B), where 12 of these are a subset of the 32 proteins (Fig. S4A).

3.3.2. *Effects related to lipid metabolism in liver proteomes of cod exposed to H-PFAS*

3.3.2.1. Pathway and network analysis. Pathway and network analyses were performed with 76 of the top 111 differentially expressed ($p = 0.005$, q-value < 0.125) proteins (Fig. 3), with fold-changes of at least 1.2 in the L-PFAS or H-PFAS groups, using the STRING protein interaction database to visualize enriched networks and pathways (Fig. 4). The complete network and pathway lists can be found in the supplementary material (Fig. S5). This analysis revealed that many proteins involved in mitochondrial and peroxisomal lipid metabolism were affected by PFAS exposure. Some of the up-regulated proteins (Fig. 3), including Cpt2, Amacr, Pecr and Decr2, are known to be Ppara target genes and important enzymes in β-oxidation. Overall, the significantly enriched pathways were largely related to energy metabolism, especially peroxisomal and mitochondrial lipid catabolism pathways, but also amino acid metabolism pathways and processes (Figs. 3, 4, and S5).

3.3.2.2. Gene Set Enrichment Analysis (GSEA). In general, moderate expression changes between exposure groups and control were observed for the proteomics data presented here. Therefore, GSEA, that may help in identification of gene sets enriched from modest changes in expression levels (Subramanian et al., 2005), was also performed. For the H-PFAS group, GSEA identified one significantly enriched pathway, FATTY_ACID_METABOLISM, that contains several up-regulated enzymes in fatty acid degradation pathway such as fatty acid β-oxidation (Fig. 5A and B), including those identified in pathway enrichment and network analysis (Figs. 3, 4, and S5). Similar analysis was performed using GSEA for all three groups with high PFAS (H-PFAS, H-PAH/H-PFAS and L-PAH/H-PFAS) combined (n = 30) compared with the control



GO-term/pathway	Description	count in gene set	FDR
GO:0046395	carboxylic acid catabolic process	8 of 237	0.0049
GO:0006635	fatty acid beta-oxidation	4 of 56	0.0066
GO:0051186	cofactor metabolic process	9 of 467	0.0067
GO:0061024	membrane organization	9 of 729	0.0473
GO:0005777	peroxisome	6 of 127	0.00076
GO:0005739	mitochondrion	15 of 1531	0.0122
HSA-390918	Peroxisomal lipid metabolism	4 of 23	0.00057
HSA-71291	Metabolism of amino acids and derivatives	9 of 354	0.00092
HSA-556833	Metabolism of lipids	10 of 721	0.0280

Fig. 4. STRING interaction network of 76 proteins differentially expressed in L-PFAS and H-PFAS groups in cod liver. $p = 0.005$, q-value < 0.125, with a fold change > 1.2. For clarity, disconnected nodes were removed, and only some of the significantly enriched pathways were highlighted. For the whole network and enriched pathway list, see Fig. S5 in supplementary materials.

group (n = 20). This analysis resulted in four significantly enriched ($q <$ 0.25) pathways related to energy metabolism (Table S6). Among those, the FATTY_ACID_METABOLISM pathway is populated by enzymes involved in lipid degradation similar to the list identified in H-PFAS group (Figs. 3, 5, and S6).

Although no significantly enriched pathways were found using GSEA in the L-PFAS group, the top enriched pathways were similar to the H-PFAS enriched pathways and many were related to lipid metabolism. For example, the FATTY_ACID_METABOLISM pathway was among the top pathways in the L-PFAS group, and it was populated by many proteins similar to the list for the H-PFAS group (Figs. 5 and S7). A similar list of proteins was upregulated in the H-PAH/L-PFAS (data not shown). Thus, L-PFAS treatment appears to elicit similar effects as the H-PFAS, being consistent with the dose-response trend in expression profiles of the top differentially expressed proteins (Fig. 3).

The GSEA performed with the other exposure groups, including the H-PAH groups, did not result in a significant enrichment of pathways (data not shown), compared to the control group.

3.4. Significant changes in the liver microsomal lipidome of Atlantic cod exposed to H-PFAS

In the liver microsomal fraction, 130 lipid species were identified belonging to nine different lipid subclasses. TGs (49%) represented the most abundant subclass of the ER fraction of the control fish, followed by PCs (31%), PGs (9%), PEs (6%), PIs (3%), PS (1%) and PE-P/PE-O (0.2%). When comparing lipid profiles in the liver of the different exposure groups to the control (Fig. S8), only the lipidome of the H-PFAS group was significantly different from the control group (Fig. 6A). The multivariate analysis demonstrated significant differences between H-PFAS and control (68% of the covariance explained in the PLS-DA; accuracy: 0.83; Q2: 0.44). The 42 VIPs > 1 generated demonstrated a relative decrease of some TGs and a concomitant increase of some phospholipids, which accounted for the discrimination between H-PFAS and control. Mono-unsaturated TGs (TG46:1, TG50:1, TG52:1, TG54:1, TG58:2), and highly unsaturated phospholipids, mainly PEs (PE34:1, PE38:7, PE40:7, PE40:8, PE40:9), PCs (PC36:1, PC36:2, PC36:6, PC38:6, PC38:7, PC40:7) and PIs (PI36:4, PI36:5, PI38:5, PI38:7, PI42:7) were highlighted as VIPs (Fig. 6B).

For the groups exposed to a mixture of PAHs and combinations of

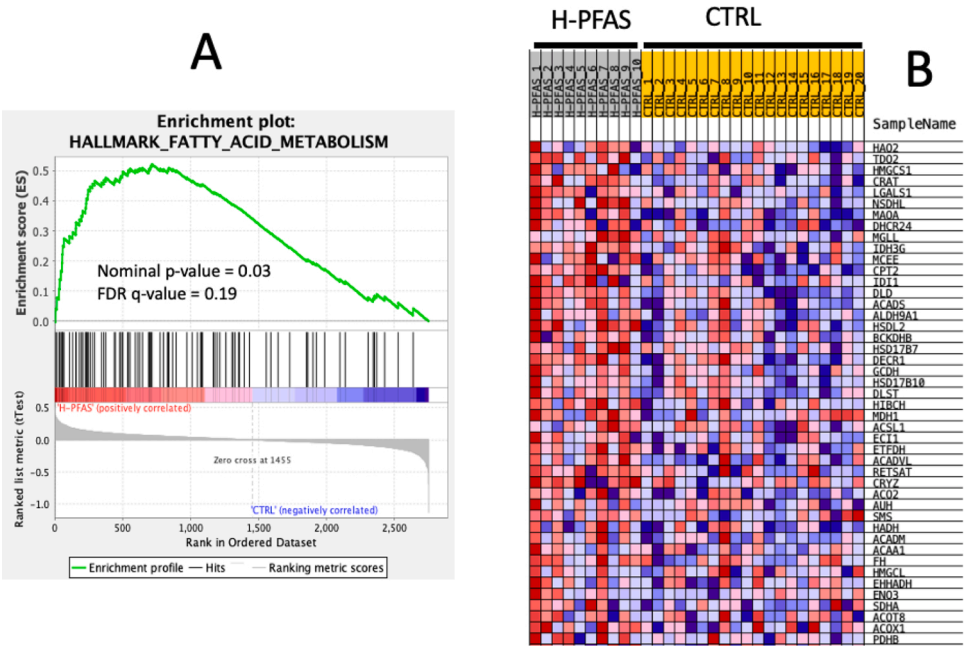


Fig. 5. Enrichment plot of the FATTY_ACID_METABOLISM pathway (A) and the corresponding core enrichment proteins (B) in livers of cod exposed to H-PFAS versus control. Data represent log2-transformed liver protein expression values in control cod (CTRL) and cod exposed high dose of PFAS (H-PFAS). The heatmap (B) represents protein expression levels, with red and blue representing high and low relative expression, respectively.

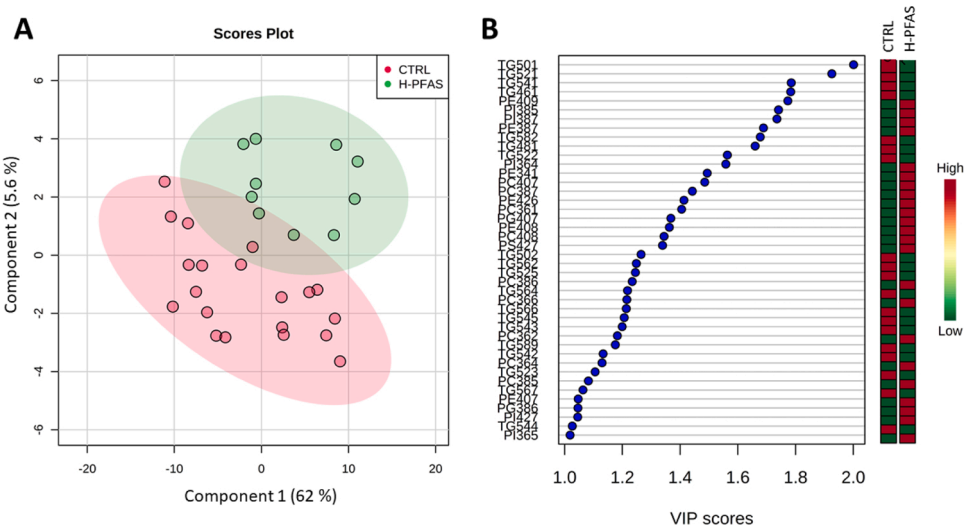


Fig. 6. Scores plot and Variable Importance in Projection (VIP) scores from PLS-DA multivariate analysis of liver microsomal lipidome in cod exposed to H-PFAS. Comparison in scores plot (A) between CTRL and H-PFAS. 42 VIP > 1 were considered relevant to explain the differences between groups (B). CTRL: control cod; H-PFAS: cod exposed to a mixture of PFAS, high concentration (20×).

PAH and PFASs, the PLS-DA analysis did not allow the discrimination between exposure groups and control (Figs. S8 and S9), however some trends were observed and are commented in supplements.

3.5. Induction of antioxidant enzyme activities

Cat and Gst enzyme activities were studied to assess possible exposure-dependent differences in oxidative stress levels (Fig. 7). PAH exposure alone did not affect the Cat and Gst activity, whereas the L-PFAS group caused significant ($p < 0.05$) induction in activity of both enzymes. The H-PFAS group alone, and in combination with PAHs (L-PAH/H-PFAS and H-PAH/L-PFAS) significantly induced Cat, but not Gst activity.

3.6. No or few significant effects on classical biomarker responses in PAH/PFAS exposure groups

For several of the measured biomarkers, no or few significant effects were observed. No significant changes in gene or protein expression were observed between exposure groups and control for Cyp1a (Figs. S10A and S11, respectively), except for a significant increase ($p < 0.05$) in Cyp1a protein in the L-PAH/H-PFAS exposure group. The EROD activity was too low (< 10 pmol/min/mg) for a reasonable comparison of groups (data not shown). For *acox1* expression, the only significant ($p < 0.05$) change was a reduction of expression levels in the L-PAH exposure group (Fig. S10B). No significant differences in DNA fragmentation or vitellogenin concentrations were observed when comparing exposure groups to control (Figs. S12 and S13, respectively).

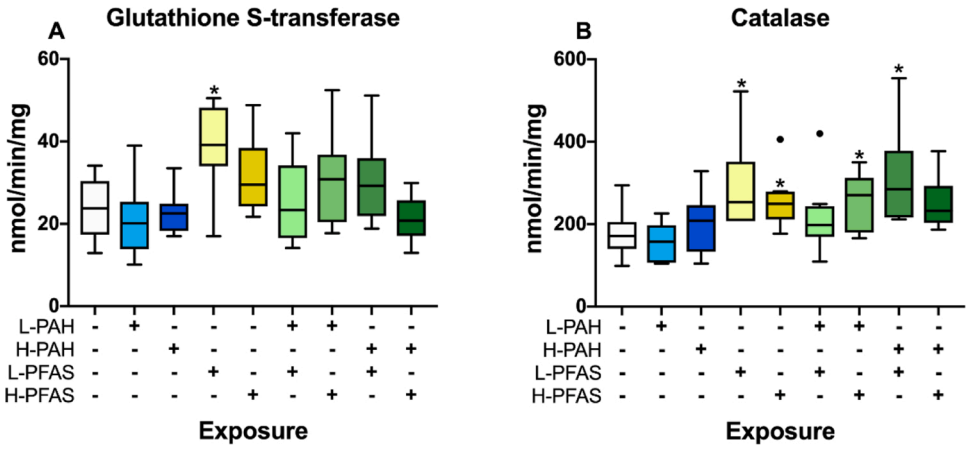


Fig. 7. Gst and Cat activities in liver of Atlantic cod exposed to PAHs and PFASs. The enzyme activities of (A) glutathione S-transferase (Gst) and (B) catalase (Cat) were determined in liver of Atlantic cod exposed to low (L = 1×) or high (H = 20×) doses of PAHs (blue) and PFASs (yellow) either separately or combined (green). Enzyme activities are presented as nmol/min/mg protein, with median values and 25-75% quartiles, whiskers are calculated using Tukey. n = 20 and 10 for control and exposure groups, respectively, and asterisk indicates statistical significance ($p < 0.05$) when comparing exposure groups to control (ANOVA).

4. Discussion

The present study investigated biological responses of Atlantic cod exposed to mixtures of PAHs and/or PFASs in two different doses: a low dose reflecting concentrations detected in wild Atlantic cod liver (Grøsvik et al., 2012; Kallenborn et al., 2004; Norwegian Environment Agency, 2013), and a 20 times higher dose. Previously published findings from the same experiment indicated that combined PAH/PFAS exposure affected dopaminergic pathway in cod brain (Khan et al., 2019). Here, we present results related to biological responses in liver and blood/plasma, in addition to chemical analyses.

4.1. Accumulation of PFASs and PAH metabolites confirms contaminant exposure

PFASs and PAH metabolites were measured in liver and bile, respectively. Except for naphthalene metabolites, PFASs and other PAH metabolites were detected in higher levels in all exposure groups compared to the control group, confirming the absorption and accumulation from the exposure (Fig. 1, Table 3). PFAS concentrations in liver of cod exposed to L-PFAS were within the same range as PFAS compounds (e.g. PFOS 6-62 ng/g and PFNA 0.5-18 ng/g) detected in Atlantic cod liver in the Nordic marine environment (Kallenborn et al., 2004; Miljødirektoratet., 2009; Valdersnes et al., 2017). As intended, the PFAS levels detected in cod livers indicated that the H-PFAS group accumulated more than 15 times higher levels than the L-PFAS groups (Table S5). PAH metabolite analyses were used to confirm the exposure to PAHs, as in previous studies (Pampanin et al., 2016; Sundt et al., 2012). PAH metabolites represent a good indication of the actual exposure to this class of compounds, showing a dose related response in most cases (Beyer et al., 2010). The results highlight the exposure of cod to the selected PAH compounds. Significantly higher levels of PAH metabolites of higher molecular weight (1-OH-phen and 1-OH-pyr) were found in the H-PAH groups compared to both control and L-PAH groups. The metabolite concentrations in bile of cod exposed to L-PAH reflect PAH metabolite levels in cod bile from the North Sea (Sundt et al., 2012). Thus, the exposure resulted in environmentally relevant levels of PAHs and PFAS in cod tissues in the low dose groups, and in the desired ratio between the two different exposure doses.

It is however important to take into account that the levels of PFASs and PAH metabolites measured reflect the different half-lives of these compounds. Goeritz et al. (2013) observed a half-life of 16, 12 and 7 days for PFOS, PFNA and PFOA, respectively, in juvenile rainbow trout (*Oncorhynchus mykiss*). In comparison, Djomo et al. (1996) observed a half-life of 5.8, 4.8 and 4.0 days for benzo[a]pyrene (BaP), pyrene, and phenanthrene, respectively, in zebrafish (*Danio rerio*). Mortensen et al. (2011) also showed that bioaccumulation and elimination of PFASs vary between congeners in Atlantic salmon (*Salmo salar*) exposed to PFOS and PFOA for six days, where PFOA was more rapidly excreted from the liver compared to PFOS in the recovery period. In the present study, PFASs showed different accumulation patterns in the group exposed to H-PFAS, compared to the combined exposure to H-PFAS and PAHs, suggesting an interaction with PAHs on accumulation and/or elimination. PFOA and PFNA accumulated at higher levels when exposed to H-PFAS alone compared to combined exposure with PAHs, while PFTrDA and PFOS accumulated at higher levels when exposed to the combination than when exposed to H-PFAS alone. Similar observations were made for the PAH metabolites, where the concentrations of 1-OH-phen and 1-OH-pyr were higher for H-PAH in combination with L-or H-PFAS, compared to H-PAH exposure alone. Mixture-specific induction of the P450 system has been observed in zebrafish exposed to PFASs and the Ahr agonist PCB-126 (Blanc et al., 2017). Thus, PAHs and PFASs may have interfered with each other's toxicokinetics. The mechanisms behind the different contaminant patterns in the present study cannot be elucidated and warrants further research.

4.2. – PFAS exposure disrupts lipid homeostasis in cod liver

The proteomics analysis showed significant effects of the PFAS mixtures on lipid degradation, with up-regulation of enzymes in peroxisomal and mitochondrial fatty acid catabolic pathways (Figs. 3–5). Although we detected statistically significant induction only with H-PFAS exposure, L-PFAS exposure showed a similar trend, indicating that effects may occur at environmentally relevant levels. Earlier, it has been shown that PFASs such as PFOS and PFOA may act as peroxisome proliferators (PPs) in rodents (Berthiaume & Wallace, 2002) and in humans (Behr et al., 2020). PPs are known to activate PPARA, which is responsible for regulation of fatty acid oxidation (Jiang et al., 2015; Latruffe & Vamecq, 1997). PFASs can activate PPARA in cell culture reporter assays (Wolf et al., 2008), and are thought to have similar mode of action as PPs. However, there are species differences in response to PPs, and rodents appear to be more sensitive than humans and fish (Scarano et al., 1994). The lower sensitivity in fish may have led to conflicting reports in the literature, with both activation (Arukwe & Mortensen, 2011) and no effects (Ren et al., 2009) of PFAS on Ppara pathway genes. Atlantic cod Ppara variants have been shown to be activated *in vitro* by the Ppara agonist Wy-14643 and some PFASs, including PFOA and PFNA (Söderström et al., in preparation), which is consistent with the results shown here. Species differences may also exist in sensitivities of fish Ppara activation by PFASs. Our large-scale proteomics and lipidomics data show a strong evidence that PFASs have the potential to perturb lipid metabolism in Atlantic cod. The consequence of lipid degradation for fish by environmental levels of PFAS needs to be further studied.

We measured gene expression of *acox1* as a possible biomarker for PFAS-induced effects on lipid metabolism through Ppara activation. However, no change *acox1* mRNA levels was observed (Fig. S2B), although there was a slight increase of the protein (Fig. 5B). In the proteomics analysis, many other enzymes involved in fatty acid β-oxidation pathways, including Cpt2, Crat, Decr2, Pecr and Amacr were up-regulated by H-PFAS exposure, similar to observations from other studies (Guruge et al., 2006; Krøvel et al., 2008). These findings illustrate the superior performance of OMICs based expression signature as biomarkers. Such transcriptomics and/or proteomics-based expression signatures of a set of fatty acid β-oxidation related transcripts or proteins, may be used as biomarkers of exposure to PFAS and other Ppara activating environmental pollutants in fish. Some of these proteins have previously been shown to be induced by other PPs in rodents (Willumsen et al., 1997) and/or by PFOA in a human liver cell line (Peng et al., 2013).

The endoplasmic reticulum (ER), together with the Golgi apparatus, are major sites of de novo synthesis of phospholipids (PL), and neutral lipids (TGs). In our study, a decrease of mono-unsaturated TGs and a concomitant increase of highly unsaturated PLs was observed in liver microsomes from cod exposed to H-PFAS (Fig. 6). In agreement with the proteomics data, these results may indicate a preferential hydrolysis of mono-unsaturated TGs and their hydrolysis to FAs that are oxidized in β-oxidation. Furthermore, in two groups involving H-PFAS (L-PAH/H-PFAS and H-PAH/H-PFAS), we observed a significant reduction of HSI, with a similar decreasing trend for the H-PFAS group (Fig. 2B). Other studies have observed HSI reduction paralleled with an increase in lipid-metabolizing enzymes. Chen et al. (2017) found an upregulation for *cpt1* and *ppara* mRNA levels in combination with reduced HSI and lipid content in livers of javelin goby (*Synechogobius hasta*) exposed to iron for 21 days. Zheng et al. (2013) found reduced HSI combined with increased CptI activity in livers of yellow catfish (*Pelteobagrus fulvidraco*) exposed for 96 h to zinc. PFAS exposure has also been linked to reduced HSI in other fish species (Hagenaars et al., 2008; Schultes et al., 2019). Thus, our results suggest that decrease in HSI might be caused by PFAS-induced lipid degradation in cod liver.

In parallel, we observed an enrichment of polyunsaturated acyl chains in PLs, which will confer membranes more flexibility in the z direction compared to saturated and monounsaturated acyl chains. In other words, they will create a third dimension of fluidity in a structure that is essentially fluid in the x–y plane (Barelli & Antonny, 2016). The vertical movements of the polyunsaturated acyl chain will enable density adjustments across the bilayer to compensate for an acute membrane deformation, possibly caused by PFAS exposure.

Regarding the increase of PIs, these are minor phospholipids with a characteristic fatty acid profile; they are highly enriched in stearic acid at the sn-1 position and arachidonic acid at the sn-2 position. Although PI constitutes < 5 % of ER lipids, in eukaryotic cells, they play a major role generating phosphorylated derivatives of PI (Zhao et al., 2018). Phosphorylated PIs have many functions in the cell including regulation of the actin cytoskeleton, endocytosis, autophagy, and cell signaling (Blunsom & Cockcroft, 2019). Interestingly, in a previous study where the digestive gland microsomal fraction of *Mytilus sp*, was incubated in the presence of ATP and CoA to investigate lipid synthesis, increased synthesis of PIs in the presence of PFOS/PFOA was observed (Gilabert, unpublished results).

4.3. – Possible increase in ROS levels induced by PFAS exposure

Generation of reactive oxygen species (ROS) and oxidative stress are often linked to an induction of antioxidant enzyme activities. In the present study, the observed increases in antioxidant enzymes activities (Fig. 7) seemed to be more strongly related to PFAS exposure compared to PAH alone, indicating possible oxidative stress in PFAS-exposed individuals. PFASs have previously been suggested to induce oxidative stress in fish. Kim et al. (2010) found significant increase in Cat activity in common carp (*Cyprinus carpio*) after four days of high PFOA exposure (55 mg/L), but PFOS did not induce the activity at similar concentrations. In contrast, Arukwe & Mortensen (2011) found that both PFOA and PFOS (0.2 ug/kg fish) could significantly increase *cat* gene expression in Atlantic salmon (*Salmo salar*) after two and five days of exposure, in addition to an increase in TBARS, both findings indicating oxidative stress.

4.4. – No or little change in several biological responses

Previous research has linked PAH and PFAS exposure to several biomarker responses, such as Cyp1a induction and DNA damage for PAHs (Pampanin & Sydnes, 2013; Yazdani, 2018), and oxidative stress and endocrine disruption for PFASs (Arukwe & Mortensen, 2011; Fang et al., 2012; Kim et al., 2010). In our study, however, several of the measured biomarkers, including gene expression (*cyp1a*, *acox1*), DNA fragmentation (Comet assay), Cyp1a protein expression (ELISA) and vitellogenin concentrations showed only minor changes in PAH/PFAS-exposed cod compared to control (Fig. S2, S10, S11 and S12, respectively). This lack of responses is likely due to a combination of multiple factors. One possible reason is that concentrations used in our experiment are similar to environmentally relevant levels, while the majority of toxicological studies focusing in these contaminants utilize higher concentration ranges, e.g. 4-16 mg/L PFOS (Fang et al., 2012) or 55 mg/L PFOA/PFOS (Kim et al., 2010). In comparison, we used concentrations below 1 mg/L in our study. Also, we chose to have one week between final injection and sampling/experiment end, to hopefully induce protein and lipid responses. If there were initial inductions of e.g *cyp1a* or *acox1* mRNA, it is likely that these changes would have returned to background levels by the time we ended our experiment. Previous studies in Atlantic salmon (*Salmo salar*) found that the expression of both *acox1* and *cyp1a* returned to non-induced levels after a one-week recovery period following PFAS exposure (Arukwe & Mortensen, 2011; Mortensen et al., 2011). Furthermore, as we have used mixtures in our exposures, the compounds within these mixtures might cause opposing effects, giving a total response similar to control. For example, even though Cyp1a is an established biomarker for certain PAHs (e.g. BaP), other PAHs including DBT can inhibit Cyp1a activity (Wassenberg et al., 2005).

4.5. – Strength and limitations

In the present study, fish were exposed through i.p. injections with the exposure compounds dissolved in vehicle solutions. An advantage of i.p. injections is that the exposure dose may be precisely calculated based on the fish weight. However, the absorption of the exposure compounds from the exposure vehicle to the circulation within the animal is not possible to control. Nevertheless, determination of PFAS levels in liver and PAH metabolites in bile confirm the exposure load, although different toxicokinetic patterns were observed among individual compounds. Thus, the exposure route and the desired exposure load in the present study were shown to be successful. Noteworthy is the environmental relevance of the low doses used in this experiment. In the environment, wild organisms are likely to be chronically exposed to these contaminants, possibly leading to stronger/other effects on biological responses than observed in the present study. Investigating effects of long-term exposure would have gained increased knowledge on the effects of these contaminants. However, studying effects of short-term exposure is valuable to detect early warning signals.

4.6. - Conclusion

The main finding of the present study is that PFAS exposure affects lipid composition and lipid-related pathways in liver of Atlantic cod. Chemical analyses revealed a possible interaction between contaminant groups on chemical metabolism. For several biological parameters,

however, no changes in responses were observed from exposures to PAH and/or PFAS. The combination of relatively low concentrations (environmentally relevant) and one week between the final i.p. injection and termination might be the reason for observed lack of responses for certain biological parameters. Nevertheless, as the aim of the study was to investigate biological responses related to PAH and PFAS mixture exposure, the observed effects on HSI, antioxidant enzyme activities and liver lipidome and proteome suggest that environmentally relevant concentrations may adversely impact Atlantic cod after only two weeks of exposure. Observed effects were to a larger extent associated with groups exposed to higher dose of PFAS alone or combined with PAHs, rather than with PAH exposure alone. However, in the proteomics analysis the L-PFAS treatment appeared to elicit similar effects on lipid degradation enzymes as the high dose treatment, suggesting that PFAS exposure at environmentally relevant levels may lead to modulation of lipid homeostasis in Atlantic cod.

Author contributions

KD, FY, MM, DP, ZT, RLL, JLL, OAK and AGo were involved in experimental design and sampling planning. KD, FY ZT, AH, RLL, JLL, OAK and AGo contributed to sampling. KD performed liver qPCRs, enzyme activity assays (Gst, Cat, EROD), vitellogenin and ELISA assays, in addition to contributing to statistical analyses. MM contributed to liver chemical analyses, and ZT and AH performed comet assay and analysis. DP contributed to PAH metabolite analyses, while AGi and CP performed and analyzed lipidomics data. FY analyzed proteomics data. XZ contributed with data standardization and proteomics analysis. KD and FY are the lead authors. MM, DP, AGi, AH and OAK wrote parts of the manuscript, and all authors contributed to interpretation of the results and revision of the manuscript.

Declarations of Competing Interest

AGo is a major shareholder in Biosense Laboratories AS, supplier of Vtg ELISA kits. Anders Goksøyr is a major shareholder in Biosense Laboratories AS, supplier of Vtg ELISA kits.

Research data

All data in this study including the assays and associated files can be accessed at the FairdomHub repository: 10.15490/fairdomhub.1.study.753.2

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105590.

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