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RESEARCH ARTICLE

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Environmentally realistic concentrations of chlorinated, brominated, and fluorinated persistent organic pollutants induce the unfolded protein response as a shared stress pathway in the liver of Atlantic cod (*Gadus morhua*)

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Abstract

In the North Sea and North Atlantic coastal areas, fish experience relatively high background levels of persistent organic pollutants. This study aimed to compare the mode of action of environmentally relevant concentrations of mixtures of halogenated compounds in Atlantic cod. Juvenile male cod with mean weight of 840 g were exposed by gavage to dietary mixtures of chlorinated (PCBs, DDT analogs, chlordane, lindane, and toxaphene), brominated (PBDEs), and fluorinated (PFOS) compounds for 4 weeks. One group received a combined mixture of all three compound groups. The results showed that the accumulated levels of chemicals in cod liver after 4 weeks of exposure reflected concentrations found in wild fish in this region. Pathway analysis revealed that the treatment effects by each of the three groups of chemicals (chlorinated, brominated, and fluorinated) converged on activation of the unfolded protein response (UPR). Upstream regulator analysis predicted that almost all the key transcription factors (XBP1, ERN1, ATF4, EIF2AK3, and NFE2L2) regulating the UPR were significantly activated. No additive effect was observed in cod co-treated with all three compound groups. In conclusion, the genome-wide transcriptomic study suggests that the UPR pathway is a sensitive common target of halogenated organic environmental pollutants in fish.

KEYWORDS
Atlantic cod liver, pathway analysis, persistent organic pollutants, RNA-seq, unfolded protein response

1 | INTRODUCTION

Chemical pollution is ubiquitous in marine ecosystems and is causing global concern (Landrigan et al., 2020). With its extensive gas and oil exploration, the North Sea has been subjected to pollution for decades. Substantial efforts have been made to understand the impact of petroleum hydrocarbons released from offshore anthropogenic activity on fish and other wildlife. However, the North Sea and adjacent coastal areas are also heavily impacted by other industrial activities (Barbosa et al., 2021; Deschutter et al., 2017; Green & Knutzen, 2003; Paasche et al., 2015). River runoff, urban wastewater, and atmospheric deposition, as well as coastal industries, all contribute to marine pollution.

High concentrations of persistent organic pollutants have been documented in liver tissue of many fish species from the North Sea. Gadoid fish with fatty liver tend to accumulate particularly high levels of lipid-soluble pollutants (Falandysz et al., 1994; Green & Knutzen, 2003; Kallenborn et al., 2004). Seafood safety baseline studies of undesirables have documented worrying high levels of pollutants such as dioxins (PCDD/PCDF) and dioxin-like PCBs, noncoplanar PCBs, brominated flame retardants, and some legacy pesticides in liver of fish from this region (Frantzen et al., 2020; Julshamn et al., 2013; Nilsen et al., 2013). Of the PCBs, highest levels in liver are usually found for the congeners PCB-138 and PCB-153. Among the brominated flame retardants, PBDEs and alpha-HBCD are the dominant chemicals found in fish liver. For PBDEs, PBDE-47 and PBDE-100 are the dominant congeners (Nøstbakken et al., 2018). Thus, organisms living in this ecosystem are exposed to and impacted by numerous environmental contaminants.

Chemicals never appear alone in the environment. Multiple chemicals are present in most ecosystems, and their potential harmful effects can often be hard to predict. Chemicals acting on the same systems, for example, receptors, are often considered to act additive, while chemicals acting on different systems may have synergistic or antagonistic effects (Celander, 2011; Kortenkamp et al., 2009). Adverse effects of chemical mixtures can occur at low exposure concentrations, even when individual chemicals in the mixture are present at concentrations below their no-effect levels (Kortenkamp et al., 2007). Recently, high-throughput technologies have generated knowledge for a large array of chemicals, compiled in projects such as ToxCast (Dix et al., 2007), CompTox (Williams et al., 2017), and Tox21 (Richard et al., 2021). Adverse outcome pathways and biological process and function targets offer an opportunity to predict and compare the hazards of chemicals, not only after exposure to single contaminants but potentially also after exposure to chemical mixtures. Prioritizing contaminants using toxicity data from the ToxCast project (Dix et al., 2007), Barbosa et al. (2021) recently ranked 158 chemicals of concern in the North Sea. Based on knowledge of biological process targets reported in the ToxCast database, they identified major knowledge gaps on mixture toxicity especially for PCBs and PBDEs.

Atlantic cod (*Gadus morhua*) has for a long time been used as an environmental monitoring species in the North Atlantic (Hylland et al., 2009). Declining stocks due to of overfishing and pollution have raised concerns about the future sustainability of the North Sea Atlantic cod population. Recently, complex responses to chemicals have been studied with omics methods in Atlantic cod exposed to various environmental contaminants such as benzo[a]pyrene, estradiol, chlorpyrifos-methyl, and perfluoroalkyl substances (Dale et al., 2020; Olsvik et al., 2019; Yadetie et al., 2018). However, there is a general lack of knowledge of mixture toxicity of environmental contaminants for this species.

The aim of this study was, therefore, to compare molecular responses in liver of Atlantic cod exposed to mixtures of chlorinated, brominated, and fluorinated compounds and their combined effect to improve risk characterization under natural conditions. Juvenile male cod was fed diets enriched with chemical mixtures of chlorinated (PCBs, pesticides), brominated (polybrominated biphenyl ethers, PBDEs), and fluorinated (PFOS) compounds, as well as a combined mixture of all these chemicals, for 4 weeks. Exposure concentrations were designed to reflect high but environmentally relevant concentrations documented in wild fish from the North Sea area. Gas chromatography (GC) was applied to quantify the accumulated levels of chemicals, and transcriptional responses in liver were examined with RNA-seq technology. We hypothesized that environmentally relevant concentrations of the studied chemicals would have an impact on hepatic transcription.

2 | MATERIALS AND METHODS

2.1 | Experimental fish

This study is analyzing new samples from an exposure experiment with Atlantic cod (*G. morhua*) previously described in detail by Bratberg et al. (2013). In the present study, we are analyzing transcriptomic data from exposure groups of (1) chlorinated (PCBs, DDT analogs, chlordane, lindane, and toxaphene), (2) brominated (PBDEs), (3) fluorinated (PFOS) compounds, and (4) a group that received a mixture of all three compound groups. Those groups were not included in the study of Bratberg et al. (2013). The study was approved by the governmental Norwegian Animal Research Authority (NARA, reference number FOTS ID 04/04).

The transcriptomic data presented in this paper were obtained from a strain of Norwegian coastal Atlantic cod (see Bratberg et al., 2013). Farmed cod with mean weight 840 ± 180 g were transferred to 20 m^3 indoor tanks and fitted with internal Trovan electronic tags. Cod were divided into five experimental groups (control, plus four exposed groups) with 20 fish in each tank. The fish tanks were supplied with well-oxygenated seawater from a depth of 100 m and water temperature remained stable at 8.8–9.5°C throughout the experiment. To avoid variation from sex differences, only male fish were selected for this study.

2.2 | Exposure chemicals

Three technical mixtures of polychlorinated biphenyls (PCB), Aroclor 1242, 1254, and 1260, were purchased from LGC Standards AB (Borås, Sweden) and mixed (10% A1242, 60% A1254, and 30% A1260) to give a congener profile resembling the PCB profile in the marine environment (Hellou et al., 1993; Sather et al., 2003). Pesticides *p,p*-DDT, *p,p*-DDE, *p,p*-DDD, gamma-lindane (HCH), technical toxaphene (DE-TOX 483), technical chlordane (CHL), and brominated flame retardant (technical pentabromodiphenyl ether [PBDE], DE-71 mixture) were obtained from LGC Standards AB (Borås, Sweden). DDT and metabolites were mixed (20% DDT, 65% DDE, and 15% DDD) to reflect the pattern documented in Atlantic cod from the North Sea (Green & Knutzen, 2003). Perfluorooctanesulfonate (PFOS) was obtained from Chiron AS (Trondheim, Norway).

2.3 | Feeding experiment

The results presented in this study were obtained from five treatment groups: control ($n = 10$), chlorinated compounds ($n = 8$), brominated

TABLE 1 Treatment dose (μg/kg) at each exposure.

Treatment	Dose (μg/kg)
Chlorinated compounds	
PCB (mixture of Aroclor 1242, 1254, 1260)	228
4,4′DDT	5
4,4′DDE	17
4,4′DDD	4
Chlordane (technical)	20
Lindane (gamma-HCH)	2
Toxaphene (DE-TOX 483 [technical])	26
Brominated compounds	
PBDE mixture (DE-71)	20
Fluorinated compounds	
PFOS (perfluorooctane sulphonic acid)	13

Note: The fish was exposed four times, once a week for 1 month. The combined mixture group received all three groups of contaminants.

compounds ($n = 10$), fluorinated compounds ($n = 10$), and a mixture group ($n = 9$) exposed to a cocktail of all chemicals. Exposure concentrations were designed to reflect the environmental levels found in Atlantic cod from the most contaminated areas of the North Sea, the Norwegian Coast, and the Baltic Sea (see Table S1) (chlordane, Falandysz et al., 1994; PCB, PBDE, and pesticides, Green & Knutzen, 2003; PFOS, Kallenborn et al., 2004). Total treatment doses (μg/kg) used in the study are shown in Table 1.

The experimental diets were prepared by mixing feed pellets with vehicle (control), chlorinated compounds (PCBs, DDTs chlordane, lindane, and toxaphene), brominated compounds (PBDEs), fluorinated compounds (PFOS), and a combined mixture of the chlorinated, brominated, and fluorinated compounds, as detailed in Bratberg et al. (2013). The feed was administered by tube-feeding except for the vehicle control group that was fed with uncontaminated commercial feed pellets (Amber Neptun 300, Skretting AS, Stavanger, Norway). Prior to tube-feeding, the fish were anaesthetized with metomidate hydrochloride (5 mg/L, Syndell International, Vancouver, Canada), and the paste was administered directly into the stomach using a syringe fitted with a sterile Stomach Tube (Uno Plast, Hundested, Denmark). To ensure that the fish received the correct dose, both the fish and the paste were weighed immediately prior to feeding. Fish were individually tube fed once each week for 4 weeks (from November 2008 until December 2008). The tube-fed fish were also fed with commercial fish pellets by hand once between each tube feeding. The control group was fed with pellets twice each week. The total amount of food corresponded to 0.5% of the body weight per feeding.

2.4 | Tissue sampling

Tissue sampling was conducted 1 week after the administration of the final treatment dose. The cod were anaesthetized with metomidate hydrochloride and killed by a blow to the head. Liver samples were rapidly excised with a scalpel, snap frozen in liquid nitrogen, and stored at −80°C before further processing. The duration between the fish that were killed and to the liver samples that were frozen was less than 5 min.

2.5 | Chemical analysis of chlorinated, brominated, and fluorinated compounds

Pooled liver tissue (200 mg from each fish or approximately 2 g liver sample in total) was used for quantification of accumulated levels of the studied chemicals. A detailed description of the methods used to quantify the levels of the studied compounds in Atlantic cod liver is described by Abihssira-Garcia et al. (2022). Briefly, eight mono-ortho PCBs (105, 114, 118, 123, 156, 157, 167, and 189) and seven non-ortho PCBs (28, 52, 101, 118, 138, 153, and 180) were analyzed with gas chromatography/high resolution mass spectrometry (GC-MS/MS) (Agilent, Wilmington, DE, United States). Seven PBDEs (28, 47, 99, 100, 153, 154, and 183) were analyzed with GC-MS/MS (Agilent, Wilmington, United States). The pesticides (DDT and metabolites, lindane, chlordane, and toxaphene) and PFOS were analyzed using GC-MS/MS positive EI modus (Agilent).

2.6 | RNA-seq analysis

Liver tissue (50 mg from each fish) was homogenized with a Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA was extracted using the BioRobot EZ1 and RNA Tissue Mini Kit (Qiagen, Hilden, Germany) and treated with DNase. The RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA, United States) was used to evaluate the RNA integrity of the samples. The RNA integrity number (RIN) was 9.9 ± 0.2 (mean ± SD, $n = 47$).

RNA-seq was performed as previously described by Yadetie et al. (2018) using the Illumina HiSeq 4000 system (Illumina, San Diego, CA, United States). About 50 million 2 × 75 bp paired-end reads were generated per sample/individual fish (47 samples in total, $n = 47$). The RNA-seq data were analyzed using the RASflow pipeline (Zhang & Jonassen, 2020). Read mapping and counting were performed as described previously (Yadetie et al., 2018) using *G. morhua* reference assembly (gadMor1) (Star et al., 2011). Differential expression analysis (with TMM normalization) was performed using edgeR (Robinson et al., 2010). Lists of differentially expressed genes (DEGs) were generated using a 1.5-fold change and a false discovery rate (FDR) <0.05 cutoff.

Pathway and network analysis was performed as described by Olsvik et al. (2021). Briefly, pathway analysis was performed using human orthologs of the Atlantic cod DEGs, retrieved from the Ensembl database (Ensembl.org). Pathway enrichment analysis was performed using Metascape (Zhou et al., 2019) and Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA). Gene set enrichment analysis (GSEA) was performed using the GSEA software applying default parameters (Subramanian et al., 2005).

Hierarchical clustering analysis was performed in Qlucore Omics Explorer 3.4 (Qlucore AB, Lund, Sweden). Causal network and upstream regulator analyses were performed in IPA (Kramer et al., 2014). Upstream regulator analysis determines likely molecules or chemicals that are connected to dataset genes through a set of direct or indirect relationships. Predicted activation or inhibition are calculated by using the direction of gene regulation (up- or down-regulation).

2.7 | Statistical analyses

One-way ANOVA with Tukey's post hoc test was used to compare fish weight and chemical concentrations. In case the SDs differed significantly (Brown–Forsythe test), or the data did not have a normal distribution (Shapiro–Wilk test), the data were log-transformed before ANOVA. For hierarchical clustering of RNA-seq data, log2-transformed expression values (normalized RNA-seq cpm) were analyzed in Qlucore Omics Explorer (Qlucore, Lund, Sweden), using multi-group comparison test (ANOVA) with Benjamini-Hochberg correction for multiple hypotheses testing, to identify top discriminating genes. All tests were considered significant at adjusted $p < 0.05$ (except for the GSEA $p < 0.25$).

3 | RESULTS

3.1 | Growth and mortality during the experiment

Mean weight of the fish was 848 ± 154 g before the experiment started and 895 ± 167 g (mean ± SD, $n = 47$) at the end of the experiment. Fish mortality was observed at approximately 3%, which is considered low in all treatment groups during the experiment. There were no statistically significant differences between the treated groups and the controls for end weight, length, hepatosomatic index, gonadosomatic index, or Fulton condition factor (Table S2).

3.2 | Bioaccumulation of exposure chemicals in liver tissue

The levels of the different chemicals were calculated in one pooled liver sample per treatment group. The levels of PCBs, PBDEs, *o,p* and *p,p* DDT isomers, lindane, chlordane, toxaphene, and PFOS in cod liver are shown in Table 2. Except for toxaphene and PFOS, background concentrations above the detection levels were documented for all studied chemicals in liver of the control fish. The concentrations of these chemicals in liver of cod exposed to chlorinated, brominated, and fluorinated compounds confirmed that the treated fish bioaccumulated the respective contaminants. A substantial amount of the dose given to the cod was found accumulated in the liver. Of the studied chemicals, PFOS showed the lowest degree of liver bioaccumulation in all four treatment groups. The accumulated levels of chlorinated chemicals (PCBs and pesticides) in liver of cod from the combined mixture group were only about half of the nominal levels in the chlorinated group. The reason for the discrepancy observed between the chlorinated and combined mixture groups for accumulation of chlorinated compounds is unknown.

3.3 | Gene expression and pathway analysis

There were 597, 658, 2,491, and 239 significant DEGs (FDR < 0.05) in the chlorinated, brominated, fluorinated, and mixture groups, respectively. Exposure to fluorinated compounds gave the strongest response, followed by brominated and chlorinated compounds. Thirty-five DEGs were common for all four treatment groups, including the mixture of all contaminants. Functional annotation identified

TABLE 2 Concentrations of exposure chemicals in Atlantic cod liver (ng/g wet weight).

	Concentration liver (ng/g wet weight)				
Compound/treatment	**Control**	**Chlorinated**	**Brominated**	**Fluorinated**	**Mixture of all**
∑PCB (9 congeners)	173	4,180	159	146	2,110
∑mono-ortho-PCB (8 congeners)	1	30	1	1	15
∑DDT (p,p isomers)	109	1,677	120	126	833
Lindane (gamma-HCH)	1.8	108	4.7	0.7	42
Chlordane (trans-nonachlor)	60	443	63.9	69.7	231
Toxaphene (DE-TOX 483 technical)	n.d.	133	n.d.	81	101
∑PBDE (18 congeners)	20	18	647	21	21
PFOS[a]	<LOD	<LOD	<LOD	21	21

Note: Data are from pooled liver samples. Control: pool of 10 liver, chlorinated: pool of 8 livers, brominated: pool of 10 livers, fluorinated: pool of 10 livers, mixture: pool of 8 livers. Seven non-ortho PCB congeners were analyzed: PCB-28, PCB-52, PCB-101, PCB-118, PCB-138, PCB-153, and PCB-180. Eight mono-ortho-PCB congeners were analyzed: PCB-105, PCB-114, PCB-118, PCB-123, PCB-156, PCB-157, PCB-167, and PCB-189. Seven PBDE congeners were analyzed: BDE-28, BDE-47, BDE-99, BDE-100, BDE-153, BDE-154, and BDE-183.
Abbreviation: LOD, level of detection; n.d., not detected.
[a]PFOS = 3 ng/g wet weight.

28 of these DEGs, that is, *taf6l*, *rnf216*, *sf3b1*, *rbm5*, *sgsm3*, *srrm1*, *fam184a*, *rasip1*, *anks3*, *u2surp*, *tk2*, *znf341*, *clk1*, *srsf11*, *rsrc2*, *rcor1*, *gramd1c*, *fnbp4*, *ogt*, *gpatch8*, *rbm25*, *inpp5b*, *sult1c3*, *gpbp1l1*, *ints3*, *txndc5*, *depdc5*, and *brf1*. The number of overlapping DEGs in the four treatment groups is shown in Figure 1A,B. A list of all significant DEGs including human gene names is shown in File S1. Applying an additional fold-change cutoff (at least 1.5 for both down- and up-regulated genes) in addition to FDR < 0.05 (edgeR analysis), there were 246, 189, 685, and 83 significant DEGs (in the chlorinated, brominated, fluorinated, and mixture groups, respectively) that could be annotated with human identifiers and used in downstream pathway analyses (such as KEGG, Reactome, and Wikipathway) and Gene Ontology enrichment analyses using Metascape. For IPA, all significant DEGs with human identifiers and FDR < 0.05, independent of fold change cutoff, were included. Independent of fold change, there were unexpectedly few significant DEGs in liver of cod exposed to the combined mixture of chlorinated, brominated, and fluorinated compounds. Co-exposure of fluorinated compounds with chlorinated and brominated compounds reduced the effect on transcription in cod liver as suggested by lower number of DEGs in the mixture group. The lower amount of significant DEGs in the combined mixture group could in part be due to the lower concentration of chlorinated compounds in this treatment group. However, since the fluorinated compounds gave a stronger response than the chlorinated compounds alone, the lower amount of significant DEGs in the combined mixture group suggests an interaction effect of all the studied chemicals. Whether this observed response on transcription in the combined mixture group reflects a true antagonistic effect due to competition at target sites, or is due to some other mechanism, needs further study.

Hierarchical clustering analysis of the samples and the normalized expression values of the top discriminating genes shows relatively large overlap between the treatment groups (Figure 2). Based on expression patterns, the heatmap shows that samples obtained from cod exposed to the fluorinated compounds to a large degree clustered together. Samples obtained from cod exposed to a mixture of halogenated compounds clustered most closely with the controls, further underlining the limited response in liver of cod exposed to the combined chlorinated, brominated, and fluorinated mixture.

Gene Ontology and pathway enrichment analysis performed in Metascape is summarized in Figure S1A–E (File S2). Pathways related to the unfolded protein response (UPR) such as response to topologically incorrect protein, protein processing in the endoplasmic reticulum (ER), and protein glycosylation were enriched in the brominated, chlorinated, and fluorinated groups (Figure S1A–C,E). Other pathways enriched are largely related to RNA processing, metabolism, and transport (Figure S1A–E). Only two general cellular pathways related to RNA and protein metabolism were significantly enriched in the combined mixture group (Figure S1D). On the biological processes level, Gene Ontology analysis suggested a negative impact on growth in cod exposed to brominated and fluorinated compounds but no significant effect of chlorinated compounds or of the combined mixture (Figure S2).

Gene set enrichment analysis (GSEA), using normalized counts as input, identified the UPR as the most significant pathway (FDR *q* values < 0.25) for all three mixture groups (Figure S3A–C). By comparing all three groups exposed to halogenated compounds with the control, GSEA also identified the UPR pathway as the most significant (Figure S3D), followed by the MYC targets V2 and protein secretion pathways (not shown). GSEA did not identify any significantly affected pathway in the combined mixture group.

According to the IPA comparison analysis, the UPR was the most significantly activated pathway in liver of cod exposed to chlorinated and brominated compounds and one of the most significant pathways in the fluorinated group (Figure 3A). However, the UPR pathway was

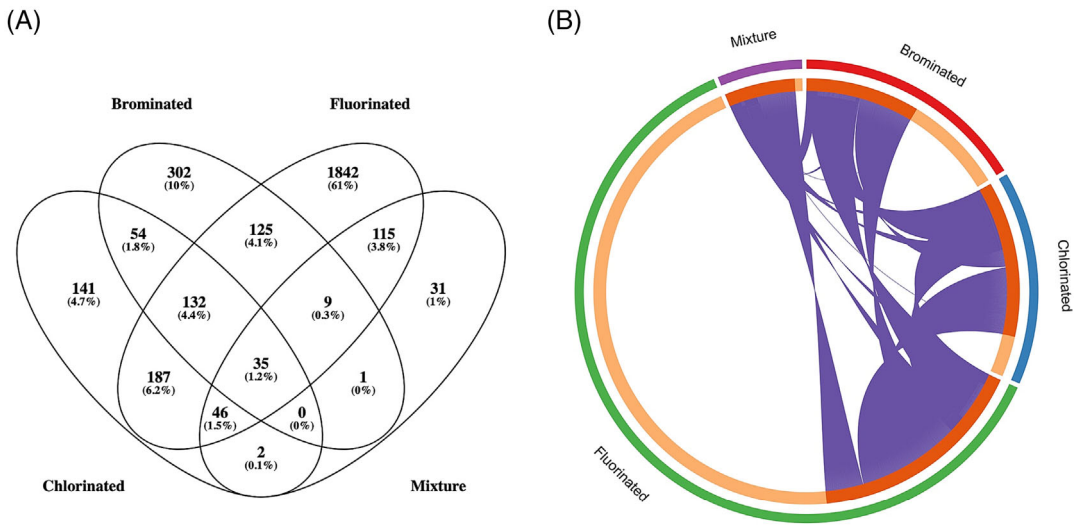

FIGURE 1 Overlapping DEGs in liver of the four treatment groups of Atlantic cod based on RNA-seq analysis. (A) Venn diagram. (B) Circos plot (Metascape): color coded external arc indicates individual list of DEGs by the indicated compounds; light orange and dark orange internal arcs indicate unique and shared DEGs, respectively; shared DEGs are linked by purple lines. Significant DEGs: chlorinated compounds: 597, brominated compounds: 658, fluorinated compounds: 2491 and mixture: 239. DEGs with FDR < 0.05 are shown.

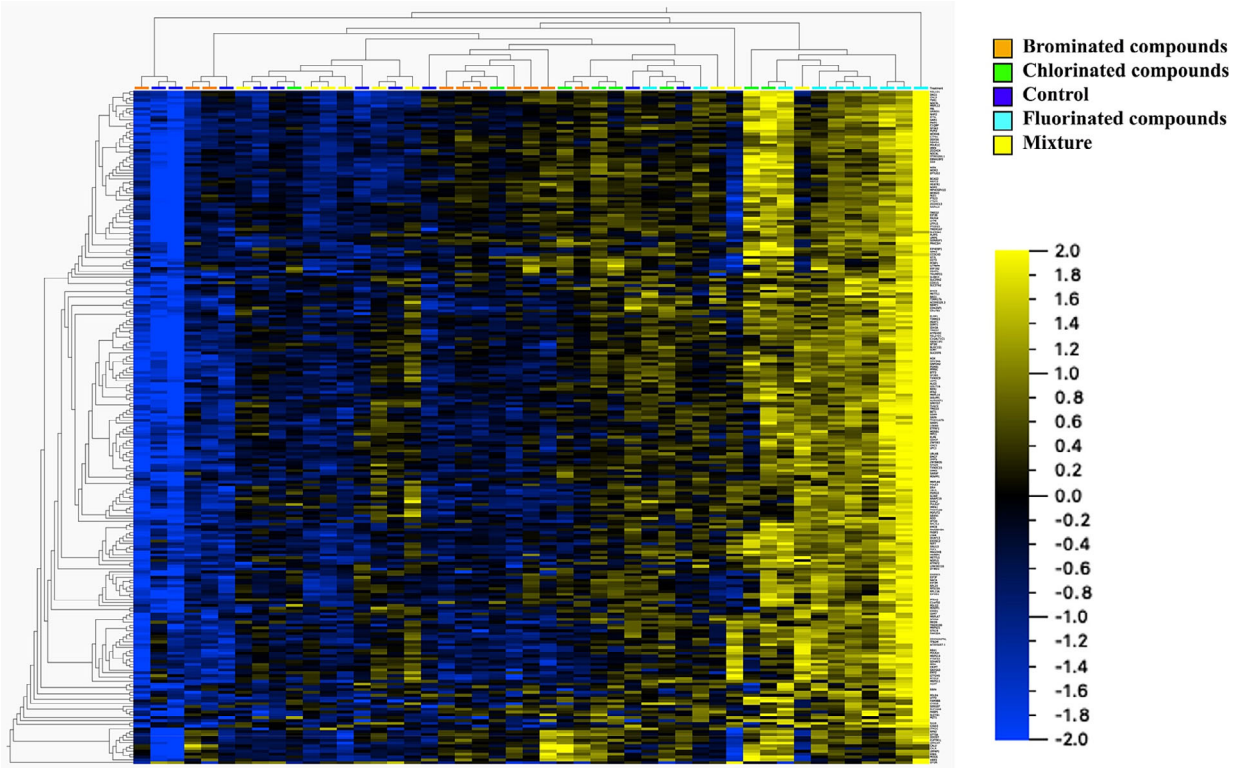

FIGURE 2 Heatmap of hierarchical clustering analysis of samples and the top discriminating genes in cod liver after exposure to halogenated compounds. Analysis was performed with log2-transformed normalized gene expression values and the top discriminating genes are presented (ANOVA, Qlucore Omics Explorer).

not significantly affected in liver of cod exposed to the combined mixture of all halogenated compounds. Still, independent of analytical tools, pathway analyses point to UPR as the main target of all studied toxicants in liver of cod. IPA analysis using DEGs as input and GSEA using normalized counts as inputs both showed that the transcriptional responses were weaker in cod exposed to a combination of all three types of halogenated compounds. Furthermore, IL-15 production, IL-6 signaling, and ERK/MAPK signaling were predicted as inhibited in cod exposed to all three compounds singly but not in cod exposed to a combined mixture of all halogenated compounds. Autophagy, one of the outcomes of the UPR pathway, was predicted as activated in liver of cod exposed to chlorinated and fluorinated compounds and inhibited by brominated compound treatment. Oxidative phosphorylation was predicted as inhibited by brominated compounds, while exposure to fluorinated compounds leads to a predicted activated state of this pathway.

For the chlorinated group, the UPR pathway significant enrichment (FDR < 0.05) was based on differential expression of 12 DEGs (*hsp90b1*, *hspa5*, *pdia6*, *calr*, *canx*, *hspa4*, *edem1*, *sel1l*, *ero1b*, *hspa9*, *dnajc11*, and *pparg*) (Table S3). For the brominated group, 13 DEGs in the UPR pathway were significantly differentially expressed (*hsp90b1*, *hspa5*, *pdia6*, *calr*, *canx*, *edem1*, *sel1l*, *ero1b*, *cebpz*, *p4hb*, *syvn1*, *scap*, and *mbtps1*), while in the fluorinated group, 17 DEGs were significantly differentially expressed in this pathway (*pdia6*, *hspa9*, *cebpz*, *cd82*, *dnajc11*, *dnaja1*, *dnajc19*, *dnaja2*, *hspa8*, *dnaja3*, *dnajc7*, *dnaja4*, *dnajb14*, *dnajb12*, *dnajc13*, *dnajc10*, and *map 3 k5*) (Table S3). *Hsp90b1*, the most strongly regulated DEG in the chlorinated and brominated groups, was 4.3- and 4.6-fold higher expressed in these groups than in the control, respectively (Table S3). *Hspa5*, which showed the second strongest regulation, was 4.3-fold higher expressed in these two treatment groups compared to the control. *Cd82* was the most strongly regulated gene in liver of cod exposed to fluorinated compounds (2.1-fold upregulated). Combined treatment of all three halogenated compounds only resulted in significance for one DEG in this pathway, *map 2 k7*. Significance levels are shown in Table S3.

Furthermore, according to the upstream regulator analysis, many of the key genes associated with the UPR pathway were predicted to be upregulated by the chlorinated, brominated, and fluorinated compounds in cod liver. XBP1, ERN1, ATF4, EIF2AK3, and NFE2L2, key regulators in the UPR pathway, were all predicted activated with a *z* score of >2 in the treatment groups (except NFE2L2, *z* score 1.7 in the brominated group) (Figures 3B and 4). In addition, ATF6 was predicted to be activated in liver of cod exposed to chlorinated and brominated compounds (with activation *z* scores of 1.5 and 2.2, respectively). None of these regulators were predicted affected in cod exposed to the combined mixture. Furthermore, comparison analysis showed that there were 134 common annotated DEGs in the chlorinated, brominated, and fluorinated treatment groups (shown in File S1, "Common DEGs"). By using these DEGs as input, XBP1, ERN1,

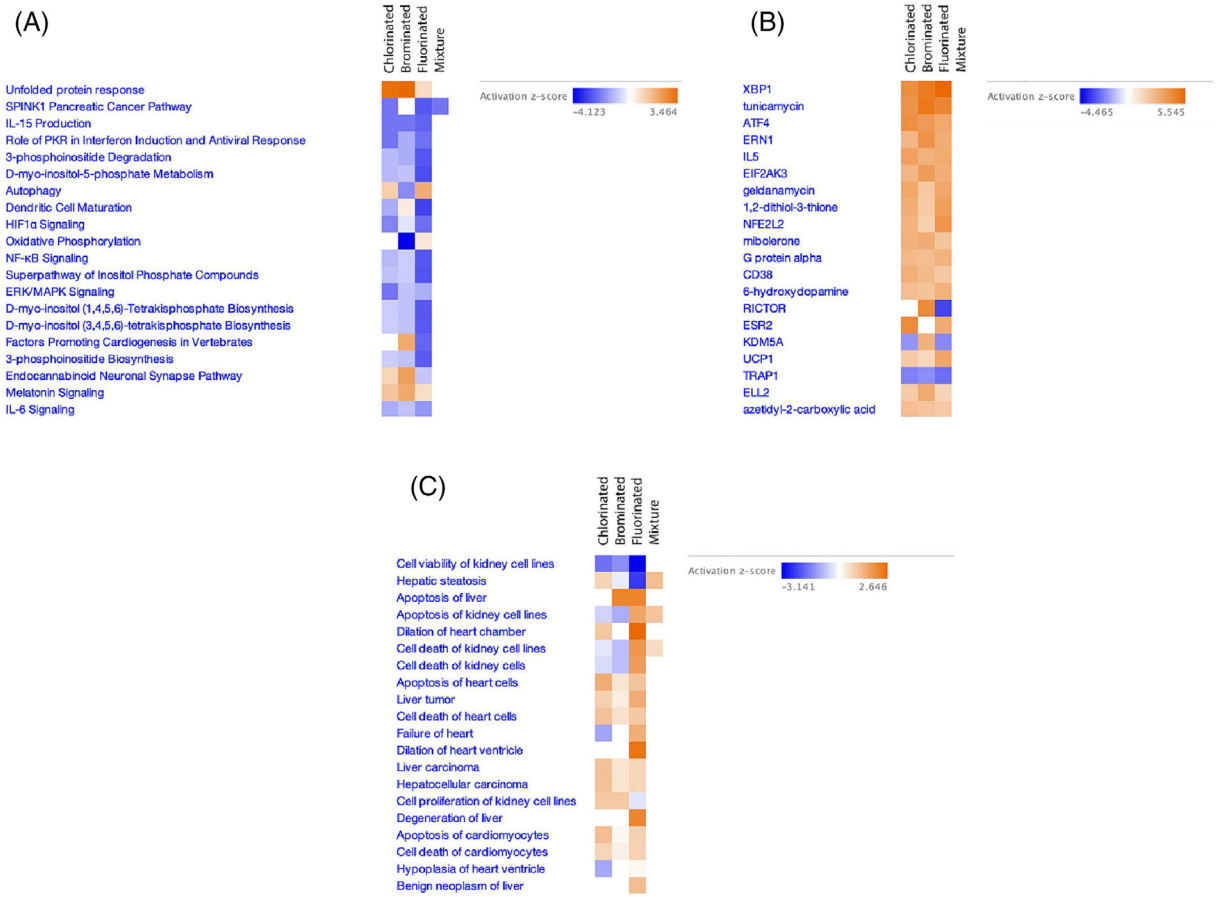

FIGURE 3 Comparison analyses of gene expression patterns in liver of Atlantic cod exposed to chlorinated, brominated, fluorinated, or a mixture of these compounds. (A) Heatmap of canonical pathways. (B) Heatmap of upstream regulators. (C) Heatmap of top tox function. Blue color: activation *z* score lower than zero. Orange color: activation *z* score higher than zero. Data from IPA core analysis showing the top 20 entities. Based on DEGs with FDR < 0.05

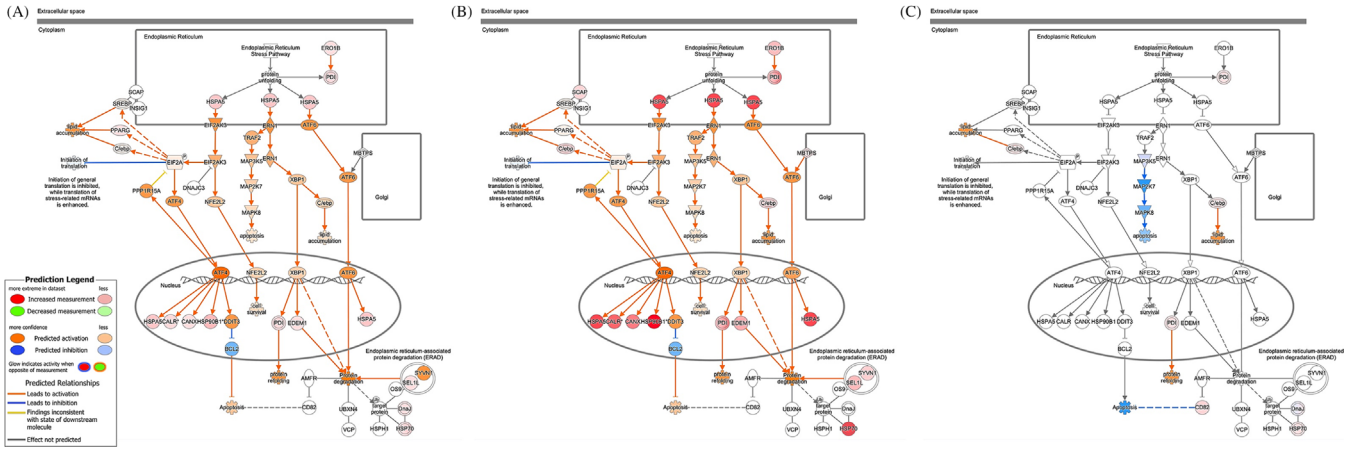

FIGURE 4 Unfolded protein response in liver of Atlantic cod exposed to halogenated compounds. (A) Chlorinated compounds, (B) brominated compounds, and (C) fluorinated compounds. Highlighted in red and green color are significantly affected transcripts.

EIF2AK3, MMP3, and imatinib were the five most significant predicted upstream regulators in the combined data set. With both approaches, XBP1 was the upstream regulator with the highest *z* score. Figure S4 shows the significant DEGs used to predict the possible outcome of exposure in the chlorinated, brominated, and fluorinated treatment groups. Pathway analysis with the 134 common DEGs predicted liver damage and hyperplasia as a possible outcome of exposure (IPA Core Analysis–hepatotoxicity, degeneration of liver,

P value 1.02E03, liver hyperplasia/hyperproliferation, *P* value 4.86E01–2.28E03).

The top tox function in IPA generates hypotheses to understand mechanism of action and mechanism of toxicity. Based on the significant DEGs in the dataset, hepatic steatosis was predicted to be activated in the liver of cod exposed to chlorinated compounds and by the combined mixture of all contaminants (Figure 3C). In contrast, hepatic steatosis was predicted inhibited in liver of cod exposed to brominated and fluorinated compounds. Apoptosis was predicted stimulated in liver of cod exposed to brominated and fluorinated compounds. Furthermore, the degeneration of liver pathway was predicted to be stimulated in the liver of cod exposed to fluorinated compounds but not in the other treatment groups. Key toxicological responses appear to be differentially affected. Exposure to chlorinated compounds was predicted to have a stronger impact on lipid accumulation than the other two contaminants.

4 | DISCUSSION

By using environmentally relevant exposure concentrations, this study demonstrates how Atlantic cod could be negatively impacted by POPs and their mixtures commonly found in coastal waters in the North Atlantic Ocean. After 1 month of dietary exposure, the accumulated levels of halogenated contaminants in cod liver reflected levels earlier documented in wild fish. We have previously documented even higher concentrations of PCBs in fish from coastal waters in Norway. Accumulated levels of PCB7 were up to 7,900 ng/g in liver of cod collected from Western Norway (Olsvik et al., 2009) or about 10 times higher than the values reported here. Concentrations as high as 272 ng/μg ∑PBDE have been documented in cod liver collected in Norway (Green & Knutzen, 2003). Later surveillance has documented levels of 143 ng/μg ∑PBDE in liver of cod collected in the North Atlantic (Nøstbakken et al., 2018). These levels are about half the concentrations quantified in cod liver in this experiment. In the Baltic Sea, PFOS levels as high as 62 ng/g have been documented in cod liver (Kallenborn et al., 2004), while liver of cod collected in Southern Norway contained up to 9 ng/g PFOS (Valdersnes et al., 2017). The accumulated concentrations of PFOS in our experimental fish, at 21 ng/g, is thus in range with levels previously documented in wild cod in the North Atlantic. According to the functional analyses, growth was predicted to be negatively impacted by brominated and fluorinated compounds. Exposure to the studied contaminants most significantly impacted molecular mechanisms associated with protein folding and function in cod liver. Induction of the UPR by these compounds might result in adverse outcome in fish that may be used in development of AOPs to facilitate risk assessment (Andersen & Krewski, 2010; Ankley et al., 2010). Further studies aimed at strengthening weight of evidence linking induction of UPR by chlorinated, brominated, and fluorinated compounds to adverse outcome in an AOP framework will be needed.

At the cellular level, oxidative stress is one of the main effects of chemical exposure. Toxicant-induced oxidative stress can cause modifications in cysteine, an amino acid that forms disulfide bonds and stabilizes the three-dimensional structure of proteins. Impaired protein folding in the ER due to inhibition of disulfide bond formation may lead to accumulation of unfolded proteins. In this study, all three compound groups induced the UPR, most strongly in liver of cod exposed to chlorinated and brominated compounds. Interestingly, this response was not evident in cod that were exposed to the combined mixture of all compounds. The weaker response in the combined mixture group could in part be due to the lower levels of chlorinated compounds in that exposure group. Induction of the UPR is a sign of ER stress. ER stress occurs when the ER fails to perform proper folding of proteins, and this could be related to factors such as impaired protein glycosylation or disulfide bond formation, by overexpression of proteins, or by mutation in the amino acid sequence of proteins (Walter & Ron, 2011). When the protein folding machinery is impaired, the cells will try to prevent accumulation of unfolded or misfolded proteins by reducing global protein synthesis and inducing the production of chaperones (Pesonen & Vahakangas, 2019). Effects on the UPR have been reported in several studies of wild fish exposed to high levels of chemical mixtures in nature. For example, enrichment of the GO term protein folding (GO:0006457) was shown in liver of European flounders (*Platichthys flesus*) from contaminated sites in UK waters (Williams et al., 2011). Similarly, the ER stress response and the UPR were affected in liver of the climbing perch (*Anabas testudineus*) collected from sites contaminated with electronic waste enriched with metals and PBDEs in China (Zhang et al., 2019). In a fjord in Western Norway, the UPR was enriched in liver of Hg-exposed wild tusk (*Brosme brosme*) (Olsvik et al., 2021). It is, therefore, plausible that environmental relevant concentrations of ubiquitous contaminants affect the UPR in wild fish.

In mammals, the protein kinase R-like ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol requiring enzyme-1 (IRE1) regulate three different branches of the UPR (Szegezdi et al., 2006). Initiation of the UPR is primarily by non-transcriptional events. One of the most abundant proteins within the ER that effect the efficiency of protein folding is the HSP70-type chaperone BIP (GRP78). Under unstressful ER conditions, BIP binds to these three receptors and suppress the UPR. Upon ER stress, accumulation of unfolded or misfolded proteins competes for BIP binding, and this leads to activation of the UPR (Szegezdi et al., 2006). Each branch of the UPR starts a signaling cascade that ends with the production of a transcriptional activator (Arensdorf et al., 2013). The cellular outcome of the UPR will thus depend on which branch is activated. In this study, the three transmembrane proteins PERK, IRE1, and ATF6 were predicted to be activated in liver of cod exposed to chlorinated and brominated compounds but not in cod exposed to fluorinated compounds or the combined mixture of all compounds. PERK, IRE1, and ATF6 are encoded by the *eif2ak3*, *ern1*, and *atf6* genes, respectively. Nonetheless, in this study, none of these genes were differentially expressed in the exposed cod.

Activation of the PERK branch affects the phosphorylation of EIF2A and the translation of ATF4 (CREB2), one of the major effectors of the UPR (Jennings et al., 2013). PERK activation can induce

upregulation of ATF4, which promotes an antioxidant response that contributes to greater ER folding capacity (Adams et al., 2019). ATF4 in turn can induce the expression of pro-apoptotic proteins and instigate apoptosis in the cells (Read & Schröder, 2021). Activation of the PERK branch may also affect cell survival via the transcription factor NRF2, which controls the expression of many antioxidant response element-dependent genes (Ma, 2013), and lipid accumulation, which may lead to hepatic steatosis (Lee & Glimcher, 2009). In this study, chlorinated and brominated compounds were predicted to have an impact on NRF2-mediated cell survival, while all three compounds were predicted to induce lipid accumulation in the liver cells. Accumulation of lipids in the liver is a common response to pollutant exposure in fish and other organisms (Foulds et al., 2017; Maradonna et al., 2015; Olsvik & Søfteland, 2018). Taken together, pathway analysis predicted that chlorinated and brominated compounds affect the PERK branch of the UPR in cod, potentially resulting in lipid accumulation.

Apoptosis, protein refolding, protein degradation, and lipid accumulation are potential outcomes of the IRE1 branch of the UPR. All these responses were predicted to be activated in liver of cod exposed to chlorinated and brominated compounds in the current study. The chlorinated compound PCB-153 has been shown to upregulate lipid accumulation promoting genes in Atlantic cod (Yadetie et al., 2014). Upstream regulator analysis predicted that differential expression of the XBP1 could explain this outcome. Upon accumulation of unfolded proteins in the ER, IRE1 mediates a splicing mechanism resulting in XBP1 activation (Yoshida et al., 2001). XBP1 is a transcription factor that drives the transcription of genes such as *atf6*, *hspa5*, *hsp90b1*, *edem1*, *serp1*, *pdia6*, *hif1a*, and several *DnaJ* (HSP40) genes (Jennings et al., 2013). Based on activation *z* score, XBP1 was the transcription regulator that best could explain the pattern of gene expression in liver of cod exposed to chlorinated and fluorinated compounds and the second most significant regulator in the brominated group. The most significantly activated upstream regulator in the brominated group was tunicamycin, an antibiotic that causes cell cycle arrest in G1 phase and causes apoptosis. The current study suggests that both chlorinated and brominated compounds at environmentally relevant concentrations impact the XBP1-mediated IRE1 branch of the UPR.

The third activator of the UPR, ATF6, controls a response that promotes protein folding and ER-associated protein degradation (ERAD) (Yoshida et al., 2001). Downstream genes controlled by the ATF6 branch of the UPR includes *hspa5*, *xbp1*, *hsp90b1*, and *ddit3*. Activation of XBP1 is thus controlled by both IRE1 and ATF6 (Yoshida et al., 2001). The gene encoding BIP, *hspa5*, as well as *hsp90b1*, were significantly overexpressed in liver of cod exposed to chlorinated and brominated compounds in this study. *Edem1*, encoding a protein that targets misfolded glycoproteins for degradation, was differentially regulated in liver off cod exposed to chlorinated and brominated compounds, while several protein disulfide isomerase chaperone genes were significantly affected by the treatments (chlorinated: *pdia3*, *pdia4*, and *pdia6*, brominated: *pdia3*, *pdia4*, *pdia5*, and *pdia6*; fluorinated: *pdia6*). PDIA6 affects UPR signaling by binding to IRE1 (Eletto et al., 2014). The proteins encoded by the protein disulfide isomerase genes have multiple functions, including assisting in protein folding in the ER. However, of all genes upregulated by ER stress, only relatively few can be linked directly to ATF4, XBP1, and ATF6 (Adams et al., 2019), further highlighting the need for more research on how chemical stress affects the UPR response in fish.

It is well established that activation of the UPR is one of the major responses to toxicological insult (Jennings et al., 2013). In line with our findings, Williams et al. (2008) exposed European flounder to Aroclor 1254 (PCB mix) (50 mg/kg) and PFOA (100 mg/kg) and observed impacts on chaperones and heat shock proteins, suggesting an impact on protein folding and the UPR pathway. Oxidative stress responses were among the pathways activated in Atlantic cod liver slices exposed to PFAS mixtures (51.7 μg/kg) (Dale et al., 2020). Enrichment of the UPR pathway has also been documented in human and mammalian cells after PCB exposure (Espin-Perez et al., 2019; Xu et al., 2015; Yang et al., 2020). Several of the other chemicals included in the chlorinated mixture (DDT and its metabolites, and gamma-HCH [lindane]) can also impact the UPR or essential pathway genes (Burgos-Aceves et al., 2021; Liu et al., 2013). PBDE exposure activates XBP1 and the UPR via IRE1 in human endothelial cells (100 mM PBDE-209) (Hou et al., 2019) and in human H295R adrenocortical carcinoma cells (10 μM OH-PBDE) (Song et al., 2009). In mouse liver, PBDE-49 exposure (5 ml/kg/day) strongly activated the UPR (Zhang et al., 2015). Furthermore, it has been shown that PBDE quinone metabolites (10 μM PBDEQ) cause oxidative stress and DNA damage and subsequently trigger apoptosis in human cells (Wang et al., 2021). In mouse pancreatic cells, PFOA (50 μg/ml) activates all branches of the UPR (Hocevar et al., 2020). Unique to the fluorinated group was the strong impact on *DnaJ* genes, with 11 *DnaJ* genes significantly affected (Table S3). These genes encode proteins that act as co-chaperones regulating HSP70 and facilitate protein folding (Qiu et al., 2006). Many of the above reported studies were conducted with unrealistically high exposure concentrations. Better documentation of how the UPR response is affected in fish exposed to complex mixtures of contaminants found in the aquatic environment is, therefore, still needed.

Terminally misfolded proteins can be discarded either through the ubiquitin-proteasome pathway or the lysosomal pathway (autophagy) (Bravo et al., 2013). In this study, autophagy was predicted to be activated by chlorinated and fluorinated compounds but inhibited by brominated compounds. Fluorinated compounds had the strongest effect on the autophagy pathway, with 33 DEGs being significantly affected (Figure S5). *Igf1*, encoding an autophagy pathway protein associated with growth and development, was significantly downregulated by chlorinated and fluorinated compounds, but not by brominated compounds. One gene that was differentially regulated by all chemical compounds (but not by the combined mixture) was *atg14*, encoding a protein required for both basal and inducible autophagy (Yorimitsu et al., 2006). These findings suggest that the accumulated levels of the studied compounds were high enough in cod exposed to chlorinated and fluorinated compounds to interfere with mechanisms associated with protein misfolding and autophagy.

Many of the 28 genes common for all four treatments have been associated with liver injury, necrosis, and endocrine disruption. However, none of these common genes have been directly linked to the UPR pathway. Nevertheless, many of the common genes for the chlorinated, brominated, and fluorinated groups (by excluding the combined mixture group) are associated with the UPR response.

Of other interesting pathway responses observed in this study, ERK/MAPK signaling was predicted inhibited by all three halogenated compounds. The MAPK signaling pathway regulates a wide variety of cellular processes such as proliferation, differentiation, apoptosis, and stress responses (Yue & Lopez, 2020). HIF1A signaling was also predicted to be inhibited by all three compounds. HIF1A signaling controls the effect of hypoxia in the cells. Impaired protein folding in the ER might occur as a result hypoxia-induced inhibition of disulfide bond formation (Rozpedek et al., 2016). Furthermore, oxidative phosphorylation was predicted stimulated by fluorinated compounds and inhibited by brominated compounds while chlorinated compounds had no impact on this pathway.

5 | CONCLUSION

In conclusion, this study suggests that exposure to chlorinated, brominated, and fluorinated compounds at levels designed to reflect environmentally relevant exposure concentrations can negatively impact Atlantic cod at the molecular level. Remarkably, pathway analysis suggested that the effects of exposure to the three group of different compounds converged on ER stress and the UPR as the main cellular outcome in liver of exposed fish. Thus, when evaluating the hazards of chemical mixtures of halogenated compounds in wild fish, the UPR pathway appears to be an essential pathway-based biomarker.

AUTHOR CONTRIBUTIONS

Conception and design of the research: Sonnich Meier, Fekadu Yadetie, Pål A. Olsvik, and Anders Goksøyr. *Obtaining financing*: Anders Goksøyr. *Analysis and interpretation of the data*: Pål A. Olsvik, Fekadu Yadetie, and Xiaokang Zhang. *Writing of the manuscript*: Pål A. Olsvik, Fekadu Yadetie, and Sonnich Meier. *Critical revision of the manuscript for intellectual content*: Fekadu Yadetie, Sonnich Meier, Xiaokang Zhang, Odd Andre Karlsen, and Anders Goksøyr. All authors read and approved the final draft.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The RNA-seq dataset discussed in this publication has been deposited in the EBI ArrayExpress database with accession E-MTAB-12338.

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SUPPORTING INFORMATION

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