Mixture effects of environmental contaminants in Atlantic cod *(Gadus morhua)*

Combining experimental approaches to study contaminant-induced biological responses

Karina Dale

Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
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Scientific environment

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Abstract

Release of chemicals from anthropogenic activities is considered a substantial threat to the global environment. Marine ecosystems are sinks for accumulation of contaminants, therefore marine organisms are highly susceptible to contaminant exposure and possible adverse effects. Importantly, organisms are continuously exposed to complex mixtures of chemicals and interactions among these chemicals may occur. Thus, acquiring knowledge of which components that provoke certain effects can be challenging and needs to be addressed in detail.

The main objective of this dissertation was to assess the biological responses of Atlantic cod (*Gadus morhua*) exposed to contaminant mixtures, focusing primarily on the liver as the target organ for effects. An additional aspect was to investigate mixture effects of contaminants using different experimental approaches, including a field/caging study (**Paper I**), an *in vivo* exposure study (**Paper II**), and an *ex vivo* exposure study with precision-cut liver slices (PCLS) (**Paper III**). The caging and *in vivo* studies (**Paper I** and **II**) assessed effects of environmentally relevant concentrations of contaminants, whereas a mechanistic approach with a wider range of concentrations was applied in the PCLS study (**Paper III**). While **Paper I** included several groups of environmental contaminants, the contaminants investigated in the studies comprising this dissertation were the polycyclic aromatic hydrocarbons (PAHs) (**Paper II**) and the per- and polyfluoroalkyl substances (PFAS) (**Paper II** and **III**). The distribution and possible adverse effects of these contaminants have previously been studied in isolation in marine species, however, limited research exists on their combined effects.

In **Paper I**, an *in situ* approach was applied to assess whether Atlantic cod would be affected by contaminants leaking from a capped waste disposal site in the bay of Kollevåg in Western Norway. To this end, farmed juveniles of Atlantic cod were caged for six weeks at different locations within Kollevåg bay, with three cages at, or close to the capped disposal site, and a reference cage at a nearby, unpolluted location.

Chemical measurements and analyses of biological responses were combined to investigate the possible impact of the waste disposal site on caged cod. We observed higher concentrations of certain contaminant groups at the Kollevåg stations both in sediment, cod liver, and cod bile compared to the reference station. Contaminant concentrations in cod liver were greatest in cod caged at station 1, which was situated closest to the waste disposal site. In contrast, station 2 had the highest concentrations of PAHs and polychlorinated biphenyls (PCBs) in sediments, in addition to the highest levels of PAH metabolites in cod bile. Several biological responses were measured in cod tissues. Specifically, condition factor, hepatosomatic index, and hepatic antioxidant enzyme activities were reduced in cod caged at station 1. Station-dependent increases in expression of genes involved in lipid metabolism and steroidogenesis were also observed. Effects on reproductive parameters, including induction of steroid hormone synthesis and genes encoding proteins involved in steroidogenesis were most prominent among the biological responses assessed, and had the highest correlation with contaminant concentrations in the liver. These findings suggest that reproduction of cod caged in Kollevåg may be disrupted by contaminants leaking from the waste disposal site, which may also apply for local fish inhabiting the area.

In **Paper II**, an *in vivo* approach was applied by exposing Atlantic cod for two weeks to mixtures of PAHs and PFAS through two intraperitoneal injections (day 0 and day 7), using environmentally relevant (1x, L) and higher (20x, H) concentrations. PAH metabolite and PFAS concentrations were determined in cod bile and liver, respectively, and confirmed a dose-dependent accumulation of contaminants. Chemical analyses revealed trends of potential chemical interactions between PAHs and PFAS, with regards to accumulation and/or metabolization. Biological responses were assessed by applying both single biomarker assays and toxicogenomic approaches (proteomics and lipidomics). Observed changes in biological responses were mostly related to PFAS exposure. Proteomic and lipidomic analyses of liver

samples revealed an increase in the levels of proteins involved in lipid degradation pathways and a decrease in levels of triacylglycerols in cod exposed to H-PFAS, respectively. In addition, hepatic antioxidant enzyme activities were significantly induced following PFAS exposure. For several other biomarker assays, including plasma vitellogenin concentrations, cytochrome P450 1a (Cyp1a) response (both at mRNA and protein levels/activity) and DNA fragmentation, no clear effects were observed following contaminant exposure. Nonetheless, our findings demonstrated that two weeks of exposure to environmentally relevant concentrations of PFAS were sufficient to affect the lipid composition and homeostasis in cod liver.

In **Paper III**, PCLS from Atlantic cod were applied as an *ex vivo* approach to expand on PFAS mixture toxicity, in addition to comparing mixture effects to effects of single PFAS. Cod PCLS were exposed for 48 h to perfluorooctanesulfonic acid (PFOS), perfluorooctanoic acid (PFOA), and perfluorononanoic acid (PFNA) at three different concentrations (10, 50 and 100 µM), and ternary mixtures of these PFAS (10, 50 and 100 µM of each compound). Changes in the transcriptome of exposed PCLS were assessed by RNA sequencing. When comparing single compounds, PFOS exposure generated a larger number of differentially expressed genes (DEGs) compared to PFOA and PFNA exposure. Affected pathways following PFOS exposure included sterol metabolism, nuclear receptor pathways and oxidative stress-related responses. Compared to single compound exposure, mixture exposure generated a 10-fold higher number of DEGs compared to PFOS exposure alone. PFOS clustered closer to the PFAS mixture in hierarchical clustering and principal component analyses compared PFOA and PFNA, and the majority of DEGs following PFOS exposure was shared with the DEGs following mixture exposure. The PFAS mixture caused significant enrichment of several stress-related pathways, including antioxidant responses, ferroptosis, cancer-related responses, and nuclear receptor pathways. Mixture effect analysis showed that approximately 10% of the DEGs from the mixture exposure displayed non-additive effects compared to the prediction from the single compound exposures. The majority

of these genes showed synergistic effects. To summarize, PFAS exposure promoted effects on nuclear receptors, sterol metabolism and oxidative stress-related pathways in cod PCLS. Although some genes had synergistic or antagonistic expression patterns in the PFAS mixture, the majority of DEGs showed additive expression patterns, suggesting additivity to be the main mixture effect.

List of publications

The dissertation is based on the following publications and manuscript, which will be referred to in the text by their roman numerals (**Paper I-III**).

Paper I

Dale, K., Müller, M. B., Tairova, Z., Khan, E. A., Hatlen, K., Grung, M., Yadetie, F., Lille-Langøy, R., Blaser, N., Skaug, H. J., Lyche, J. L., Arukwe, A., Hylland, K., Karlsen, O. A., and Goksøyr, A. (2019). **Contaminant accumulation and biological responses in Atlantic cod (*Gadus morhua*) caged at a capped waste disposal site in Kollevåg, Western Norway**. *Marine Environmental Research*, *145, 39-51.* https://doi.org/10.1016/j.marenvres.2019.02.003

Paper II

Dale, K., Yadetie, F., Müller, M.B., Pampanin, D.M., Gilabert, A., Zhang, X., Tairova, Z., Haarr, A., Lille-Langøy, R., Lyche, J.L., Porte, C., Karlsen, O.A. and Goksøyr, A. (2020). **Proteomics and lipidomics analyses reveal modulation of lipid metabolism by perfluoroalkyl substances in liver of Atlantic cod (*Gadus morhua*).** *Aquatic Toxicology, 227, 105590.* https://doi.org/10.1016/j.aquatox.2020.105590

Paper III

Dale, K., Yadetie, F., Horvli, T., Zhang, X., Frøysa, H.G., Karlsen, O.A., and Goksøyr, A. (2021). **Single PFAS and PFAS mixtures affect nuclear receptor-and oxidative stress-related pathways in precision-cut liver slices of Atlantic cod (*Gadus morhua*).** *Manuscript.*

Abbreviations

Table 1 – Abbreviations included in the dissertation*

Acly	ATP-citrate lyase
Acox	Acyl-coenzyme A oxidase
Acsl4	Acyl-coenzyme A synthetase long-chain family member 4
Ahr	Aryl hydrocarbon receptor
AO	Adverse outcome
AOP	Adverse outcome pathway
Arnt	Aryl hydrocarbon receptor nuclear translocator
BaP	Benzo[a]pyrene
BCF	Bioconcentration factor
Cat	Catalase
CEC	Contaminant of emerging concern
CF	Condition factor
Cpt	Carnitine palmitoyltransferase
Crat	Carnitine O-acetyltransferase
Cyp	Cytochrome P450
DDT	Dichlorodiphenyltrichloroethane
Decr2	2,4-Dienoyl-CoA Reductase 2
DEG	Differentially expressed gene
dpf	Days post fertilization
EC	European Commission
EDC	Endocrine disrupting compound
EFSA	European Food Safety Authority
EROD	7-ethoxyresorufin-O-deethylase
Esr	Estrogen receptor
E2	Estradiol
EE2	Ethynylestradiol
Fasn	Fatty acid synthase
Fgf	Fibroblast growth factor
Gclc	Glutathione cysteine ligase catalytic unit
Gclm	Glutathione cysteine ligase modifier unit
GEM	Genome-scale metabolic model
Gpx	Glutathione peroxidase
Gst	Glutathione S-transferase
hpf	Hours post fertilization
HPT	Hypothalamic-pituitary-thyroid
Hsd	Hydroxysteroid dehydrogenase
HSI	Hepatosomatic index
i.p.	Intraperitoneal
IVIVE	In vitro-In vivo extrapolation
KE	Key event
K_{ow}	Octanol-water partition coefficient
MHC	Major histocompatibility complex
MIE	Molecular initiating event
MoA	Mode of action
mTORC1	Mechanistic target of rapamycin complex 1
Nrf2	Nuclear factor erythroid 2-related factor 2
OECD	The Organisation for Economic Cooperation and Development
OSPAR	Oslo and Paris Commision
PAH	Polycyclic aromatic hydrocarbons
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PCLS	Precision-cut liver slices
PerFAS	Perfluoroalkyl substance
PFAS	Per-and polyfluoroalkyl substances
PFCA	Perfluoroalkyl carboxylates
PFDoA	Perfluorododecanoic acid
PFHxA	Perfluorohexanoic acid
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonic acid
PFNA	Perfluorononanoic acid
PFSA	Perfluoroalkyl sulfonates
PFTeA	Perfluorotetradecaonic acid
PFTrDA	Perfluorotridecanoic acid

PLHC	Poeciliopsis lucida hepatocellular carcinoma
PNEC	Predicted no effect concentration
PolyFAS	Polyfluoroalkyl substances
POP	Persistent organic pollutant
PP	Peroxisome proliferator
Ppar	Peroxisome proliferator-activated receptor
Pxr	Pregnane X receptor
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	Reactive oxygen species
SDG	Sustainable development goals
Slc	Solute carrier
Sod	Superoxide dismutase
Star	Steroidogenic acute regulatory
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
Vtg	Vitellogenin
WHO	World Health Organization
WOA	World Ocean Assessment
XRE	Xenobiotic response element

*Abbreviations for fish proteins are used.

Contents

1. Introduction

1.1 Marine environmental pollution

The health of the global environment is affected by several processes directly or indirectly related to human activities, including overfishing, climate change, deforestation and environmental pollution (WWF, 2021). The human imprints on earth have become so pronounced that it has been suggested to name a new epoch of geological time as the "Anthropocene", underlining the global environmental challenges we are currently facing (Steffen et al., 2011). In particular, marine ecosystems, covering approximately 70% of the planet, are essential to global health for a number of reasons. These ecosystems contribute to primary and food production, oxygen synthesis, carbon sequestration, and are important for balancing climate change (Brierley & Kingsford, 2009; Friedman et al., 2020). Therefore, sustaining marine ecosystems is of crucial importance.

The World Ocean Assessment (WOA), reporting the health of our oceans, made the following statement in 2016: "*Over the coming decades, a changing climate, growing global population and multiple environmental stressors will have significant impacts*" (UNEP & Grid-Arendal, 2016). The issues of climate change and environmental pollution are complicated and often tightly intertwined, and several review papers have focused on how a warming climate accelerates the spread and effects of contaminants (Manciocco et al., 2014; Noyes et al., 2009). Furthermore, environmental contaminants may affect marine ecosystems by reducing biodiversity and species distribution (Johnston & Roberts, 2009). Worldwide, substantial efforts are made to understand and protect the diverse aspects of marine environments. In 2017, the United Nations declared 2021-2030 as the "Ocean Decade", or more specifically, "*a decade of ocean science for sustainable development*". One of the main objectives with the Ocean Decade initiative is to obtain "*a clean ocean where sources of pollution are identified and removed*" (Ryabinin et al., 2019).

Landrigan and colleagues recently published a review on ocean pollution and human health (Landrigan et al., 2020). Ocean pollution is here described as "*complex ever-changing mixtures of chemicals and biological materials that includes plastic waste, petroleum-based pollutants, toxic metals, manufactured chemicals, pharmaceuticals, pesticides, and a noxious stew of nitrogen, phosphorus, fertilizer, and sewage"* (Fig. 1). Release of pollutants from anthropogenic activities is an important threat: the global chemical production is rapidly increasing, and the majority of these chemicals have not been tested for toxicity or safety (ibid). Even less is known about effects of exposures to chemical mixtures.

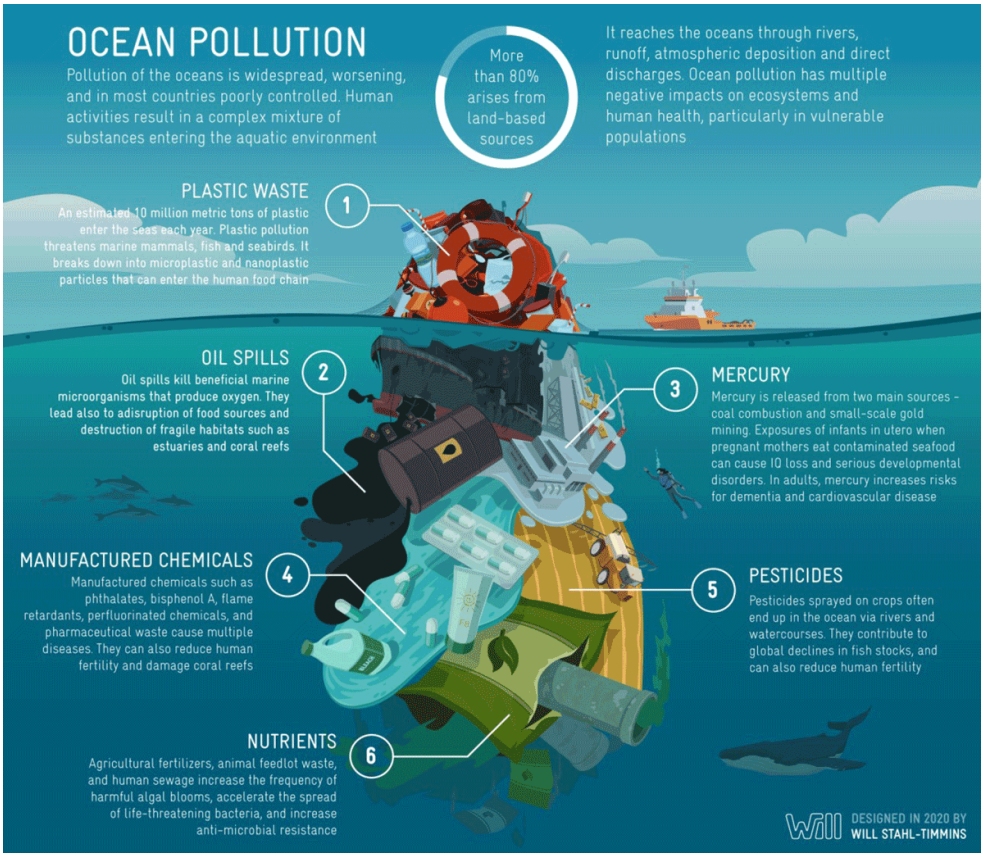


Figure 1 – Complex mixtures of ocean pollution (Landrigan et al., 2020). Examples of man-made contributions to ocean pollution include oil spills, plastic waste and a large amount of manufactured chemicals, metals, and pesticides. License link: https://creativecommons.org/licenses/by/4.0/

1.2 Mixture toxicity

"Despite the fact that human exposure to chemicals rarely is limited to only a single chemical, an overwhelmingly large portion of the toxicology studies to date deals with single, pure chemicals".

This is the first sentence of the book "Toxicology of Chemical Mixtures" by Raymond S.H. Yang (Yang, 1994). Even though this book was published nearly 30 years ago, the problem is still highly relevant. In the environment, organisms are constantly exposed to complex mixtures of natural and anthropogenic compounds through air, water, and food, usually in low doses. However, in terms of risk assessment of compounds being introduced to the market, it relies heavily on toxicity studies of single compounds, not reflecting real-life exposure scenarios (Bopp et al., 2019). Certainly, studying effects of single compounds is essential in toxicology, and obtaining knowledge on compound mixtures is complicated without knowing how the individual constituents act separately. To fully understand underlying mechanisms of toxicity, it is important to reduce and control confounding factors, which is easier in single compound studies (Yang, 1994).

1.2.1 Challenges of investigating effects of contaminant mixtures

There are several reasons for the previous lack of focus on contaminant mixture effects. The complexity of studying mixtures is evident; in the environment, factors such as exposure routes, timing of exposure, mixture composition, and non-chemical factors (climate change, temperature, oxygen levels) should (preferably) all be considered (Bopp et al., 2019). Moreover, there are large differences in species sensitivities towards single chemicals, and thus towards complex mixtures. Another major challenge of understanding mixture toxicity is that chemicals, when combined, might interact with each other. Without accounting for interactions, the toxicity of mixtures is usually assessed by summarizing the effects of the single compounds within the mixture, which is known as additive effects (Cedergreen, 2014). However,

interactions between chemicals could cause effects that are stronger (synergism) or weaker (antagonism) than the additive effects (Celander, 2011). In some cases, a compound that alone does not induce adverse effects, may enhance the toxicity of a co-exposed compound, a phenomenon known as potentiation (Burcham, 2014). Regulated chemicals have defined threshold limits below which they are regarded as "safe". Thus, if environmental contaminants are regulated on a single-compound basis, additive, potentiating, or synergistic interactions of chemicals may exceed these limits of safety.

1.2.2 Current status of mixture toxicity assessment

Chemical mixtures have gained increased attention throughout the past two decades, underlined by the large increase in the number of publications related to mixture toxicity (Fig.2). Effects of combined exposures to environmental contaminants were acknowledged by the European Commission (EC) in 2012, where the commission in communication to the Council wrote: "*In recent years there has been an increasing focus on the effects on human health and on the environment arising from exposure to many different chemicals. These effects are variously referred to as* ***combination effects, mixture effects, or cocktail effects****. The European Parliament has consistently drawn attention to* ***the need to take account****, in the context of EU chemicals' legislation, of the combined effects of different chemicals on human health and the environment*" (European Commission, 2012). In 2018, Bopp and colleagues published a review of ongoing European research activities looking into mixture toxicity exposures (Bopp et al., 2018). One example is the large Horizon 2020 "EuroMix" project, which aims to develop methodology and tools for risk assessment of mixtures, consistent with and expanding on recent guidelines on mixture risk assessment from The Organisation for Economic Cooperation and Development (OECD) and the European Food Safety Authority (EFSA) (Beronius et al., 2020; More et al., 2019; OECD, 2018). Another project, named "EU-ToxRisk", aims to promote toxicology that assess safety of chemicals with a mechanism-based approach without using animals (Bopp et

al., 2018). Hopefully, the efforts put into understanding chemical mixtures will provide increased knowledge on use and regulation of environmental contaminants.

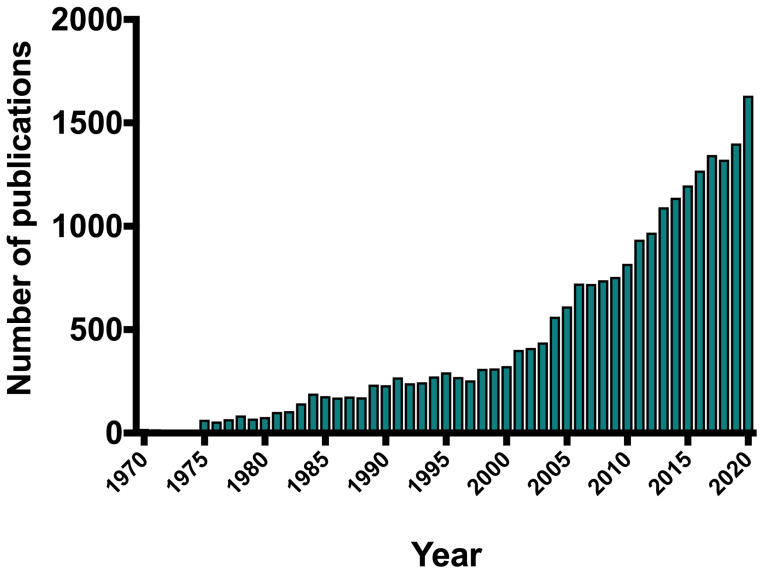


Figure 2 – Number of publications in PubMed including the keyword "Mixture toxicity" from 1970 to 2020.

One of the more recent strategies of assessing risks of environmental contaminants, both single chemicals and mixtures, are adverse outcome pathways (AOPs). The aim of the AOP framework is to organize existing knowledge on biological responses and adverse effects, and link initial cellular responses (molecular initiating events, MIEs) to downstream biological responses (key events, KEs), and finally to an observable adverse outcome (AO) at the individual or the population level (Ankley et al., 2010; Villeneuve et al., 2014). By linking different parts of AOPs together, one can form networks that can combine endpoints and multiple chemicals, thereby supporting an assessment of effects of chemical mixtures. Furthermore, these networks can facilitate a grouping of chemicals based on their mode of action (MoA), which is regarded as essential for understanding mixture toxicity (Bopp et al., 2019).

1.3 Environmental contaminants

1.3.1 Persistent organic pollutants (POPs)

One of the main issues of environmental contaminants is their persistence in nature. The term persistent organic pollutants (POPs) includes a wide array of contaminants sharing certain chemical properties. POPs were highlighted through the Stockholm Convention, which originally (in 2004) recognized 12 chemicals ("the dirty dozen") as causing adverse effects in humans and global ecosystems (Stockholm Convention, 2021). Since then, more chemicals have been added. The World Health Organization (WHO) defines POPs as "*chemicals of global concern due to their potential for long-range transport, persistence in the environment, ability to biomagnify and bioaccumulate in ecosystems, as well as their significant negative effects on human health and the environment*" (WHO, 2014). The term bioaccumulation describes the enrichment of contaminants in organisms relative to the environment (Borgå, 2008). Whereas bioaccumulation refers to uptake of contaminants via all exposure routes, another term, bioconcentration, refers to exposure routes via water only for aquatic organisms. Accumulation of chemicals via water is often described through the bioconcentration factor (BCF), which equals the concentration ratio of a chemical in the tissue of an aquatic organism compared to the water (Erickson et al., 2008). The accumulation processes are further linked to biomagnification, which describes the increase in chemical concentration in animals at higher trophic levels, meaning that the concentration of a contaminant is elevated in an organism compared to the concentration in its diet (Drouillard, 2008). The chemical properties of environmental contaminants such as POPs play crucial roles to how these compounds are distributed in aquatic systems, and how they bioaccumulate in organisms. An important concept to consider with regards to contaminant exposure is bioavailability, which may be defined as the relative facility of which a chemical is transferred from the environment to a specific location in an organism of interest (Erickson et al., 2008). The bioavailability of chemicals depends on several factors, such as environmental influences (interactions with biotic and abiotic ligands) and the properties of the

chemicals and the organism itself, e.g. the surface tissue of the organism (gills, intestine, olfactory epithelium etc.) (Anderson & Hillwalker, 2008; Erickson et al., 2008). One important property of a chemical dictating its fate in aquatic systems is its hydrophobicity, estimated through the octanol-water partition coefficient (K_{ow}). Contaminants with log $K_{ow} > 5$ are considered hydrophobic, and these compounds tend to associate with organic material such as particles and sediments in aquatic systems. At the same time, hydrophobic contaminants will diffuse through membranes and hence be more bioavailable if they are in the water phase. The hydrophobicity and the following distribution of contaminants in the environment is important to consider, as it dictates the routes by which organisms will be exposed to these compounds (Anderson & Hillwalker, 2008). Finally, due to their lipophilic nature, several POPs may accumulate in adipose tissue, rendering such tissues vulnerable for adverse effects of contaminant exposure following release due to e.g., starvation or reproductive requirements (Barouki & Clément, 2013; Jørgensen et al., 2006).

Not all contaminants are persistent or bioaccumulate in marine organisms. With some exceptions, several groups of environmental contaminants, including bisphenols, phthalates and polycyclic aromatic hydrocarbons (PAHs), are taken up in organisms, but may be rapidly metabolized (Casals-Casas et al., 2008; Chen et al., 2016; Hylland, 2006). There are however species differences involved in the ability to metabolize these contaminants, and e.g., aquatic invertebrates have lower metabolic capacities of PAHs compared to fish species (Honda & Suzuki, 2020). Despite rapid metabolization, these contaminants may however still be hazardous to organisms, due to extensive and widespread use, but also through adverse effects caused by the original substance or by reactive metabolites.

The presence and effects of some contaminants on marine environments have been well-described and monitored for decades by international agreements including the Stockholm Convention and Oslo and Paris Commission (OSPAR) (Hutchinson et al.,

2013). Such compounds are commonly known as legacy contaminants, and include e.g., PAHs, polychlorinated biphenyls (PCBs), cadmium, and mercury. In contrast, emerging contaminants or contaminants of emerging concern (CECs), are terms utilized for (1) new contaminants appearing recently in the environment and in scientific literature, (2) contaminants being detected for some time in the environment, but where concerns have been raised recently, and (3) new issues related to legacy contaminants (Sauvé & Desrosiers, 2014). Some contaminants, such as personal care products and UV-filters, have been documented fairly recently in the marine environment. Others, e.g. the per-and polyfluoroalkyl substances (PFAS) have been synthesized and used for decades, but are categorized as CECs due to more recent awareness of their bioaccumulating properties and adverse effects (Aminot et al., 2019; Giesy & Kannan, 2001).

1.3.2 Per- and polyfluoroalkyl substances (PFAS)

PFAS are synthetic, organic compounds that have been produced since the 1950s and are utilized in a wide array of consumer products (Xiao, 2017). They consist of a hydrophobic fluorinated alkyl chain and a hydrophilic functional group (Fig. 3). This composition makes PFAS excellent surfactants that can repel both water and oil (Kissa, 2001). Due to their unique thermal and chemical resistance, partly due to the great strength of the C-F bond, they are used in non-stick cookware, as waterproof and stain-resistant material in clothing, in fire-fighting foams, and in paint. It was not until the early 2000s that the adverse effects of PFAS were documented, and thus, they are currently categorized as CECs (Aminot et al., 2019). Their beneficial properties in consumer products are the same properties that make PFAS potent environmental contaminants. They persist in the environment, are spread by ocean currents or by long-range atmospheric transport, and bioaccumulate in organisms (Ahrens, 2011).

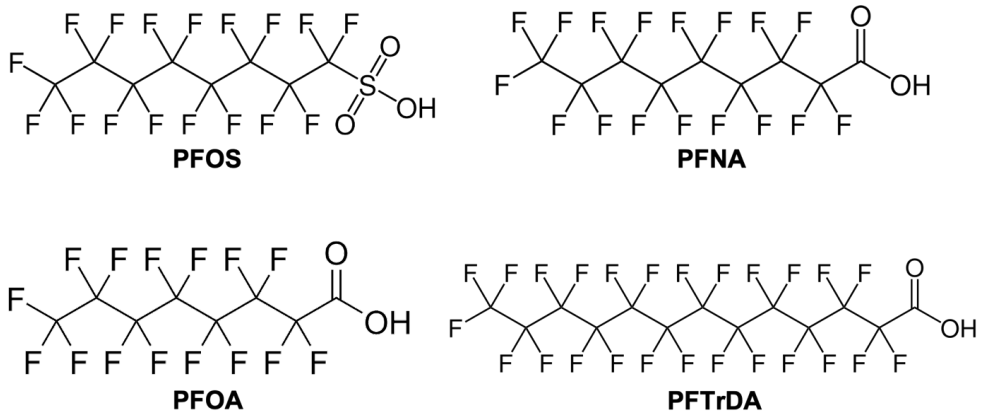


Figure 3 – Structures of PFAS relevant for this dissertation. Structures from PubChem.

PFAS are usually divided into three main classes: perfluoroalkyl substances (PerFAS), polyfluoroalkyl substances (PolyFAS), and fluorinated polymers (Ahrens & Bundschuh, 2014). The PerFAS refers to the compounds with fully fluorinated carbon chains, i.e., where all hydrogen atoms (except for in functional groups) are replaced by fluorine atoms. In this dissertation, the PerFAS are in focus, but will be referred to as PFAS. Within this class, the two largest subclasses of compounds are the perfluoroalkyl carboxylates (PFCAs) and perfluoroalkyl sulfonates (PFSAs) (Buck et al., 2011). In these subclasses, we find some of the most studied PFAS worldwide, such as perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA). PFOS and its precursor and salts were added to Annex B of the Stockholm Convention in 2009, and PFOA with its related compounds have been reviewed by the POP Review Committee since 2015. In 2020, five European countries (Norway, Sweden, Denmark, Germany and the Netherlands) proposed a total ban of usage of all PFAS (Norwegian Environment Agency, 2020).

PFAS are usually distinguished as short-chain or long-chain variants, but in the literature, there are some inconsistencies in the distinction between these terms. OECD defines long-chain PFAS in the following manner: (1) PFCAs with eight carbons and greater (i.e. with seven or more perfluorinated carbons in addition to the

functional group) and (2) PFSAs with six carbons and greater (i.e. with six or more perfluorinated carbons) (OECD, 2020). The reason for the differences regarding carbon length between these two definitions is that PFCAs with a given carbon number bioaccumulate to a lesser extent compared to PFSAs with the same carbon number (Buck et al., 2011). In general, long-chain PFAS are believed to be more persistent and bioaccumulative, and thus may be more harmful for the environment, compared to short-chain variants. Hence, both chain length and type of functional group dictate the fate of PFAS in the environment. In biota, the bioaccumulation potential of PFAS is related to their high affinity for binding to fatty acid binding proteins and serum albumin (Ahrens & Bundschuh, 2014). PFOS is known to predominate among the PFAS in biota (Sturm & Ahrens, 2010). Once PFAS have accumulated in organisms, they may cause a variety of hazardous effects, including endocrine and metabolic disruption, oxidative stress, developmental toxicity and more (summarized in Lee et al., 2020).

1.3.3 Polycyclic aromatic hydrocarbons (PAHs)

The toxic and carcinogenic potentials of PAHs have been known for decades. As important components in crude oil, their toxic impacts have gained significant attention due to numerous oil spills, e.g. the Exxon Valdez and the Deepwater Horizon accidents (Incardona et al., 2013). The environmental input of PAHs can occur from natural sources such as forest fires and underwater seeps, but the majority are released due to anthropogenic activities including oil spills, incomplete combustion of wood and fossil fuels, sewage sludge, and ship discharges (Santana et al., 2018). Four categories of PAHs exist in the marine environment: pyrogenic, petrogenic, biogenic and diagenetic, derived from incomplete combustion processes, fuels, organic metabolism and sediment transformation, respectively (Hylland, 2006). Of these four types, pyrogenic and petrogenic PAHs are the main contributors to environmental PAH pollution (Honda & Suzuki, 2020).

PAHs are composed of aromatic rings combined in different ways (Fig. 4). The structures can be fairly simple, such as the two-ringed naphthalene, but can also be larger, more complex 10-ring structures (Hylland, 2006). In general, pyrogenic PAHs are composed of a larger number of rings compared to petrogenic PAHs (Pampanin & Sydnes, 2013). PAHs are hydrophobic compounds and tend to associate with organic matter (sediment/soil) in aquatic systems. Their water-solubility decreases with increasing number of aromatic rings; thus, the lower-ring structures are more bioavailable for uptake in marine organisms (Burgess et al., 2003). PAHs are taken up either through water or diet and may, due to their structural differences, exert varying effects on biological systems (Hylland, 2006). Many marine organisms, including fish, have well-established biotransformation capacities, enabling rapid metabolism of PAHs into toxic or excretable metabolites. Therefore, in contrast to POPs, PAHs do not generally biomagnify in vertebrate aquatic food chains. The problem, however, is that many of the metabolites created through biotransformation are electrophilic, highly reactive, and thus toxic to the organism (Santana et al., 2018). PAHs are well-known for their carcinogenic effects through DNA adduct formation, but have also been linked to developmental toxicity (e.g. cardiac dysfunction), immunotoxicity, oxidative stress and endocrine disruption (Honda & Suzuki, 2020).

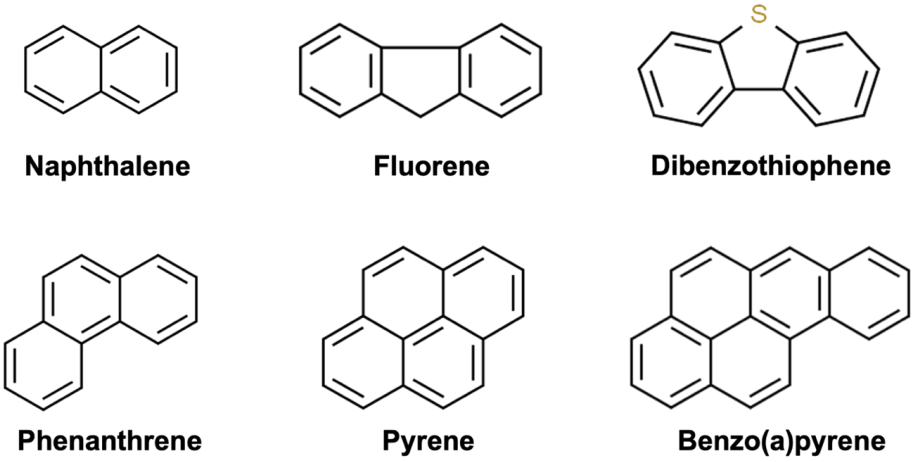


Figure 4 – PAH structures relevant for this dissertation. Structures from PubChem.

1.4 Biological responses of contaminant exposure: from molecular biomarkers to systems toxicology

Understanding how organisms are affected by exposure to environmental contaminants requires a set of tools. These tools range from studying responses with single or a few molecular biomarkers, which has been the focus of classical toxicology, to using omics methodologies that provide a deeper and more comprehensive understanding to contaminant-induced toxicity, and further to *in silico* approaches where computational and other non-animal approaches are used to assess adverse effects of chemicals (Brooks et al., 2020; Garcia-Reyero & Perkins, 2011). The following sections will introduce relevant approaches in more detail.

1.4.1 Molecular biomarkers

In environmental research, adverse effects of contaminants on organisms, populations and ecosystems are difficult to detect at an early stage, as such effects often only appear following longer periods of exposure. Thus, it is important to identify and develop methods for determining early warning signals of effects, and to establish links between these warning signals, contaminant exposure and tissue accumulation (van der Oost et al., 2003). Interactions of contaminants with biota start at the molecular and cellular level; therefore, molecular and cellular responses are both early manifestations of toxicity, and potentially suitable tools for sensitive detection of the effects of chemical exposure (Fent, 2001). Hence, the use of biomarkers has been essential in environmental toxicology. A common definition of a biomarker is a change in a biological response (ranging from molecular/cellular and physiological responses to behavioral change) which can be related to exposure to or toxic effects of environmental contaminants (Peakall, 1994; van der Oost et al., 2003). Biomarkers have traditionally been divided into three subclasses, which are biomarkers of exposure, effects and susceptibility (Schlenk et al., 2008).

Studying biomarker responses using various fish species as model organisms is a common way of investigating effects of aquatic pollution. In early phases of aquatic toxicology, the main focus was to quantify contaminant concentrations leading to fish mortality. Later, this focus shifted to consider sublethal concentrations that do not cause immediate mortality, but affect phenotypes and cause subtle adverse effects without being directly observable in an individual. Detection of sublethal changes is linked to the use of biomarkers (van der Oost et al., 2003). When an organism is exposed to harmful compounds, there are biochemical defense systems that initiate protective responses by self-regulation of signal transduction mechanisms (Schlenk et al., 2008). The defense systems are usually inhabited by proteins with various functions, and these proteins are commonly used as biomarkers as contaminant exposure may cause their levels and/or activities to deviate from normal conditions. Such biomarkers are usually regulated at the transcriptional level and can be measured both as transcript and protein abundances, in addition to catalytic activity, if the protein in question is an enzyme (ibid). It is however important to know the presence and activity of these components during normal conditions to know how they respond to chemicals, and thus avoid inappropriate interpretation. Below, biomarkers relevant for this dissertation are described in more detail.

PAH metabolites in bile as biomarker for PAH exposure

As mentioned above, fish have well-established biotransformation capacities for metabolizing and excreting PAHs. Therefore, compared to many other contaminants, a direct measurement of parent compounds in fish is not regarded as a reliable method for determining PAH exposure (Beyer et al., 2010). In contrast, as PAHs are metabolized by the liver, their metabolites are temporarily stored in fish bile, and biliary concentrations of PAH metabolites is regarded as a biomarker of recent (days) PAH exposure (Pampanin & Sydnes, 2013). Several methods exist for measuring PAH metabolites in bile, e.g. fixed wavelength fluorescence and various chromatography- and spectrometry-based approaches (Beyer et al., 2010).

Cytochrome P450 1a (Cyp1a)

Cytochrome P450 monooxygenases (Cyps[1]) are a large multi-gene family of enzymes occurring in nearly all plants and animals (Schlenk et al., 2008). Within this family of enzymes, Cyp1a is a classic example of a well-established biomarker in fish, with measurement of gene induction, protein synthesis and/or enzyme activity (ethoxyresorufin-O-deethylase, EROD) (A. Goksøyr, 1995). Cyp1a is involved in phase I detoxification, contributing to conversion of environmental contaminants to more hydrophilic, excretable metabolites (van der Oost et al., 2003). The basal expression level of Cyp1a is low in most species and tissues. However, many environmental contaminants, including dioxins, dioxin-like PCBs, and several PAHs (e.g., benzo[a]pyrene, BaP), are known to induce Cyp1a through binding to the aryl hydrocarbon receptor (Ahr). Ahr is a ligand-activated transcription factor with an important role in mediating toxicity of halogenated and polycyclic aromatic hydrocarbons in vertebrates (Aranguren-Abadía et al., 2020). Ligand binding causes heterodimerization of Ahr with the aryl hydrocarbon receptor nuclear translocator (Arnt), which further binds to xenobiotic response elements (XREs) upstream of target genes, such as *cyp1a*. Therefore, Cyp1a can be used as a biomarker of exposure for Ahr-activating contaminants (Schlenk et al., 2008), and have been applied in a range of studies with different fish species (Beyer et al., 1996; Brammell et al., 2010; A. Goksøyr et al., 1994; Holth et al., 2014; Sturve et al., 2006, 2014; Sundt et al., 2012; Wolińska et al., 2013). Finally, Cyp1a induction indicates a molecular response, thus it may also act as a biomarker of effect for potentially (but not definite) harmful responses of contaminant exposure (van der Oost et al., 2003).

Antioxidant responses and oxidative stress

In all aerobic organisms, metabolism of oxygen produces harmful oxyradicals named reactive oxygen species (ROS) (Winston & Di Giulio, 1991). Cells have well-established defense mechanisms for neutralizing ROS, such as antioxidant enzymes and low-

[1] In this dissertation, fish nomenclature is used as default. In the text, nomenclature proposed by zfin.org is followed, i.e., fish: *gene*/Protein, human: *GENE*/PROTEIN, mouse: *Gene*/PROTEIN.

molecular-weight compounds that directly scavenge ROS, both endogenously produced (glutathione) or acquired through the diet (vitamin C, E) (Di Giulio & Meyer, 2008). Numerous environmental contaminants have been shown to induce the antioxidant defense systems in fish, in some cases overwhelming the defense mechanisms and causing cellular damage through a process known as oxidative stress (Lushchak, 2016). Examples of teleost antioxidant enzymes whose expression and activities may be used as biomarkers of ROS induction, are the superoxide-neutralizing superoxide dismutases (Sod), and the hydrogen peroxide-metabolizing catalases (Cat) and glutathione peroxidases (Gpx). Additionally, the enzyme glutathione S-transferase (Gst), responsible for conjugating electrophilic phase I metabolites with glutathione, also protects against oxidative damage and may be used as a biomarker of oxidative stress for some fish species (van der Oost et al., 2003).

Endocrine and metabolic disruption

To sustain ecosystem health, it is vital for organisms within a given population to maintain functional reproductive and metabolic capacities. Many environmental contaminants, commonly categorized as endocrine-disrupting compounds (EDCs), are known to affect reproduction-related processes through mechanisms such as interference with hormone-related receptors, disruption of critical components of the hypothalamic-pituitary-thyroid (HPT) axis, and morphological damage of gonads (Kime, 1998). Several biomarkers acting as warning signals for reproductive toxicity in fish have been established. Biomarkers commonly used to assess endocrine-disrupting effects are induction of vitellogenin (Vtg), changes in expression of genes and levels of hormones in steroidogenesis, and gonad histology (Hutchinson et al., 2006). Vtg is an egg yolk precursor protein, synthesized in the liver of sexually maturing female fish in response to endogenous estrogen. Vtg induction in male fish is a well-established biomarker for endocrine disruption by exogenous estrogens (Arukwe & Goksøyr, 2003). Regarding components of steroidogenesis, both changes in transcript levels and direct hormone concentrations are used to assess reproductive impacts of EDCs. Sex

steroids (estrogens and androgens) function both during sexual differentiation and maturation in fish, and their synthesis and clearance may be disrupted by contaminant exposure (Thibaut & Porte, 2004). Examples of enzymes involved in the stepwise synthesis of sex hormones, that can be used as molecular biomarkers for EDCs, are the steroidogenic acute regulatory (Star) protein, hydroxysteroid dehydrogenases (Hsd) and Cyp19 aromatases, among others (Arukwe, 2008; Arukwe et al., 2008). Although linkage between EDC exposure and a fish population collapse has been shown previously (Kidd et al., 2007), establishing links between EDC exposure and reproductive endpoints (e.g. decreased fecundity) may be challenging. Thus, the above-mentioned biomarkers are valuable for detecting environmental exposure to EDCs.

Environmental contaminants including EDCs may also disrupt metabolic processes, such as lipid metabolism (Heindel et al., 2017). Lipids play essential roles in growth and reproduction and in fish, as for other species, they are vital for energy storage, which is mediated through metabolism of fatty acids. Components of lipid metabolism have been suggested as biomarkers for monitoring of environmental contaminants in fish species (Olivares-Rubio & Vega-López, 2016). Similar to mammals, lipid metabolism in fish is regulated through peroxisome proliferator-activated receptors (Ppars). Ppars are ligand-dependent transcription factors whose natural ligands are fatty acids, but they can also bind certain pharmaceutical ligands, including fibrate drugs (Hahn & Hestermann, 2008) and environmental contaminants such as phthalates, organotins and PFAS (Arukwe & Mortensen, 2011; Cajaraville et al., 2003; Olivares-Rubio & Vega-López, 2016). Thus, Ppars may be targets for metabolic disruptors, and Ppar target genes may be potential biomarkers for modulation of lipid homeostasis (Casals-Casas et al., 2008). In mammals, PPARs exist in three forms; PPARα, PPARβ/δ, and PPARγ, and these vary in their ligand-binding affinities, target genes and tissue distribution. Homologs of all three isoforms have been identified in teleosts, but some fish species may possess additional variants (Hinton et al., 2008). Among the Ppars, Ppara regulates transcription of genes involved in e.g., fatty acid β-oxidation. Ppar-induced liver

responses, linked to changes in components involved both in mitochondrial and peroxisomal β-oxidation, may thus be used as biomarkers of metabolic-disrupting chemicals in aquatic organisms (Cajaraville et al., 2003). Examples of possible biomarkers in these processes are the carnitine palmitoyltransferases (Cpt) and the acyl-coenzyme A oxidases (Acox) for mitochondrial and peroxisomal β-oxidation, respectively (Schulz, 2008).

1.4.2 Systems toxicology – from single biomarkers to toxicogenomics

The use of biomarker assays to study effects of contaminant exposure is part of the bottom-up approach that has been utilized in toxicology for decades (Garcia-Reyero & Perkins, 2011). Although biomarkers are valuable for understanding contaminant effects, performing multiple assays is time-consuming and not compatible with assessing the large number of chemicals being introduced to the market and released into the environment. For the past two decades, there has been an increase in the application of a more systems biology-oriented approach, where the aim is to integrate organism responses from genotype to phenotype, facilitated by the development of omics technologies such as transcriptomics, proteomics and metabolomics (Simmons et al., 2015; Sturla et al., 2014). Systems biology is based on a holistic fundament: that all parts of a given system cannot be explained by combining its separate components. Therefore, the goal is to combine responses from distinct biological parameters to integrated networks within an organism, and to characterize the links between these elements to an emergent biological process (Garcia-Reyero & Perkins, 2011). Systems toxicology is a branch of systems biology, where the overall aim is to describe effects on biological systems in response to hazardous contaminant exposures, and to relate any potential alterations of biological processes to impacts at a populational level (Hartung et al., 2017). The AOP approach is one of the strategies aimed to aid the development of systems toxicology and chemical risk assessment.

An important part of systems toxicology is the application of computational (*in silico*) approaches to predict the outcome of chemical exposure (Hartung et al., 2017). The utilization of *in silico* methodology to evaluate the large number of commercial chemicals will be essential in future predictive toxicology (Brooks et al., 2020). Developing robust *in silico* methods for assessing contaminant effects relies on a large input of experimental data, which is utilized to construct computational network models, such as organ reconstructions or genome-scale metabolic models (GEMs) (Gu et al., 2019). Although experimental data from simple bioassays may be included in constructing such network models, the network development benefits from the application of toxicogenomic approaches to establish mechanisms of toxicity following contaminant exposure (Spurgeon et al., 2010).

Toxicogenomic approaches

Toxicogenomics is a collective term for different technologies (e.g., transcriptomics, proteomics, metabolomics) applied to investigate adverse effects of environmental contaminants (NRC, 2007). The transition from the classical bottom-up approach using only single biomarkers to a more integrative systems toxicology approach would not have been achieved without the rapid development of such toxicogenomic or omics technologies (Fig. 5). The omics methods facilitate high-throughput screening by enabling simultaneous assessment of thousands of molecular responses that may have resulted from contaminant exposure, providing high-content datasets of molecular changes of gene transcripts (transcriptomics), proteins (proteomics) and/or metabolites (metabolomics, e.g. lipidomics) (Brockmeier et al., 2017; Brooks et al., 2020; Garcia-Reyero & Perkins, 2011; Martins et al., 2019; Simmons et al., 2015). Utilizing these technologies in a systems toxicology approach can therefore enhance our understanding of contaminant-induced toxicity. Furthermore, omics may be utilized to generate exposure-specific patterns that can be useful for discovery of novel biomarkers, which may be especially important for gaining increased knowledge on effects of contaminant mixtures.

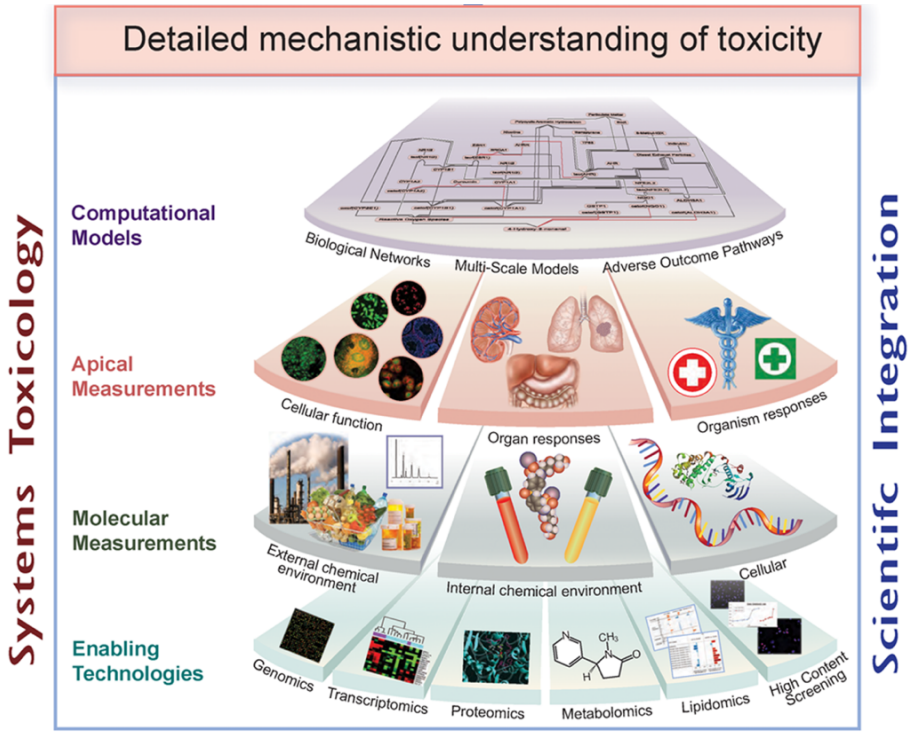


Figure 5 – The systems toxicology approach. Different toxicogenomic approaches are combined with molecular and apical measurements and computational models to improve understanding of underlying toxicity mechanisms. Figure from Sturla et al. (2014). Further permission to use this material should be directed to the American Chemical Society (ACS).

1.5 Experimental approaches

Understanding the effects of environmental contaminants on aquatic organisms is a complex undertaking. The "perfect" test conditions do not exist. In field studies, it is possible to obtain environmentally realistic exposure scenarios, but controlling important parameters such as contaminant concentrations, exact exposure routes etc. is difficult. In the lab, however, these parameters can be controlled, but at the expense of appropriate environmental conditions. The overall aim when selecting which experimental system to use in a research study is to balance ethical considerations with obtaining valuable and expedient results.

1.5.1 Fish as model species

Model species are required to investigate the impacts of contaminants on aquatic systems. As essential organisms with central positions in marine ecosystems, numerous fish species play a prominent role as model organisms in environmental toxicology (Di Giulio & Hinton, 2008). Advantages of using fish as model organisms include: (1) being vertebrates, they are genetically closely related to mammals, and may also be applied as model systems for humans; (2) their bodies are immersed in water; thus, they have a close physiological relationship with their surrounding environment, and are therefore sensitive models for environmental pollution; and (3) the enormous species diversity offers a large tool-box for testing adaptations and variety of responses to environmental contaminants (Cossins & Crawford, 2005). Fish are frequently chosen as vertebrate test species in regulatory ecotoxicology, e.g., based on guidelines from OECD and the EC Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) (Rehberger et al., 2018). Examples of fish species commonly used as model organisms in toxicology through *in vitro* and *in vivo* studies include, but are not limited to, zebrafish (*Danio rerio*), rainbow trout (*Oncorhynchus mykiss*), fathead minnow (*Pimephales promelas*), medaka (*Oryzias latipes*), three-spined stickleback (*Gasterosteus aculeatus*), Atlantic killifish (*Fundulus heteroclitus*), and Atlantic cod (*Gadus morhua*) (Carvan III et al., 2007; Di Giulio & Hinton, 2008; Eide et al., 2021). Below, more details on the use of fish model systems in toxicology are described.

1.5.2 In vitro – *seeking knowledge in accordance with the 3R principles*

With the large numbers of new chemicals continuously being synthesized and released into the environment, there is a need for cost-efficient and rapid, high-throughput methodologies for chemical testing. *In vitro* is Latin for “within the glass” and refers to studies being performed outside of the living body, in an artificial environment (Klaassen & Watkins, 2001). Considering the 3R concept (replace, reduce, refine) (Russell & Burch, 1959), *in vitro* studies are highly applicable for minimizing and

refining the use of experimental animals in science. Currently, fish primary cell cultures and cell lines are established approaches for use in assessment of the MoA and biotransformation of chemicals (Rehberger et al., 2018). Although *in vitro* assays have proven valuable for understanding toxic mechanisms of chemicals, there has been a certain lack of predictability when extrapolating results to whole organisms *in vivo*. This is partly due to cultivation events such as cell differentiation and mutagenesis (Segner, 1998), but most importantly the absence of processes involved in chemical toxicokinetics (absorption, distribution, metabolism and excretion) (Yoon et al., 2012). In chemical risk assessment, where parameters such as the Predicted No Effect Concentrations (PNEC) are used, sensitivity is an important issue to consider. If PNEC values are underestimated through *in vitro* studies, non-protective concentrations will be used in chemical regulations (Rehberger et al., 2018). Therefore, regulatory toxicology still relies heavily on animal studies. There are recent advances in "*in vitro-in vivo* extrapolation" (IVIVE) methods for increasing the predictive capabilities of *in vitro* assays (Brinkmann et al., 2016), however, these will not be discussed further in this dissertation.

1.5.3 In vivo – *whole animal exposures*

In vivo is Latin for "within the living", and in toxicology, it refers to studies of toxic effects of chemicals performed within an intact, living organism (Klaassen & Watkins, 2001). Although *in vivo* exposures are less suitable in relation to the 3R principles, a major benefit of such studies is the opportunity to combine whole-animal exposure with control of important parameters such as exposure route, temperature, feeding, light-regime etc. Controlled experiments like these are valuable for investigating cellular and organismal responses to environmental contaminants. In fish, *in vivo* exposures can be performed using various methods, including water exposures, injections and feeding trials, each of these methods with associated advantages and disadvantages. Exposures through water and food reflect natural conditions to a higher degree compared with injections. However, many contaminants are poorly dissolved

in water, and force-feeding or injections may better control and balance the delivery of compounds into the model species. Even though *in vivo* exposure studies are more realistic compared to the *in vitro* alternatives, they cannot reflect a true field exposure situation where natural background conditions are involved. Nonetheless, such studies provide valuable information on adverse effects of environmental contaminants.

1.5.4 Precision-cut liver slices (PCLS) – an ex vivo *approach*

Extensive use of *in vivo* exposure studies does not agree well with the 3R concept, whereas *in vitro* methods may have limited prediction of *in vivo* toxicity. An alternative to these approaches is *ex vivo* techniques, such as precision-cut liver slices (PCLS). *Ex vivo* means "out of the living" and relates to the study of tissues or cells of an organism outside of the body, without changing its natural environment to a great extent. The use of PCLS from fish to study general hepatic responses, or hepatic effects of environmental pollutants, has increased during the past decades (Aranguren-Abadía et al., 2020; Bizarro et al., 2016; Eide, Karlsen, et al., 2014; Harvey et al., 2019; Lemaire et al., 2011; Singh et al., 1996; Tapper et al., 2018; Yadetie et al., 2018). With the PCLS method, the tissue integrity is maintained by retaining not only the hepatocytes, but also other cell types of the liver, thus reflecting *in vivo* exposure situations to a greater extent compared to primary hepatocyte cultures. Furthermore, a great number of liver slices can be obtained from a few individual fish, offering an opportunity of a more rapid testing of environmental contaminants using a low number of subjects. The use of PCLS combined with transcriptomics has proved valuable for assessing effects of legacy POPs (Yadetie et al., 2018, 2021) and effects on lipid metabolism (Harvey et al., 2019).

1.5.5 In situ *field and caging studies*

One of the greatest disadvantages of laboratory studies is the lack of a natural environment. In the lab, it is challenging to include all environmental factors such as temperature, oxygen saturation, heterogeneity of pollution, food availability, and

species interactions (van der Oost et al., 2003). Therefore, conducting field studies is the most optimal way of assessing exposures with natural background influences. Field measurements can integrate several exposure routes and processes that may affect bioaccumulation of chemicals, and account for ecosystem variables such as spatial and temporal variations in chemical concentrations. However, due to such variations, field studies may only represent a snapshot in time. Furthermore, one cannot know the exposure history of the species sampled, which is especially true for migratory animals. Finally, in comparison to laboratory test organisms, field populations are often more genetically heterogeneous, thereby increasing variability in individual responses (Weisbrod et al., 2009).

To overcome some of the problems related to field studies, researchers have implemented the use of *in situ* ("on site") techniques, including caging. Caging enables control of animal placement and exposure duration, while simultaneously reflecting exposure with natural background influences in a more realistic manner compared to laboratory experiments. It is possible to use both hatcheries species and wild fish caught from local, clean areas, and many different species have been used successfully in caging studies (Oikari, 2006). A wide range of research questions regarding effects of environmental contaminants, industrial effluents, and seasonal changes have been assessed using caged fish (Berge et al., 2011; Beyer et al., 1996; Brammell et al., 2010; Chesman et al., 2007; Goksøyr et al., 1994; Hylland et al., 2006; Vincze et al., 2015). It is however important to ensure that cages are exposed to comparable physical conditions, thus being placed in areas with similar temperatures, salinity and sediment characteristics (A. Goksøyr et al., 1994).

1.6 Atlantic cod (*Gadus morhua*) as a model organism in environmental toxicology

Atlantic cod (*Gadus morhua*) is a benthopelagic teleost species in the *Gadidae* family of the Gadiformes order. It is a major fishery species in the North Atlantic and has

greatly impacted Norwegian economy and lifestyle for centuries. Due to being a high trophic level species and that its habitat overlaps with areas with certain anthropogenic impact, Atlantic cod is used as an indicator species in marine environmental monitoring programs such as OSPAR, and in water column monitoring of offshore petroleum activities in Norway (Norwegian Environment Agency, 2011; Sundt et al., 2012; Vethaak et al., 2017). For example, long-term monitoring studies of various POPs including PFAS have been performed using Atlantic cod as a monitoring species (Boitsov et al., 2019; Schultes et al., 2020). Along the Norwegian coastline, two main ecotypes of Atlantic cod exist: the oceanic and the coastal cod. Whereas the oceanic cod is spread and migrates across large distances during the spawning season, the coastal cod is more stationary, and several site-specific stocks exist along the Norwegian coast (Ono et al., 2019; Vøllestad, 2020). However, coastal cod populations have been declining for the past decades and have now collapsed in several areas, including the Oslofjord. Already in 2014, no cod recruits were found in Inner Oslofjord during the beach seine survey (Espeland & Knutsen, 2014). This decline has been linked to climate change; warmer oceans have changed the plankton populations along the Norwegian coastline, thus making necessary food unavailable for cod recruits. Also, a report on fish communities in the inner Oslofjord suggests that an increase in the whiting (*Merlangius merlangus*) population, with a subsequent increase in predation on cod recruits, contributes to maintaining a reduced cod population (Hylland & Holth, 2021). Another study investigating various coastal cod depopulations in Norway has linked alterations in reproductive potential and subsequent changes in cod populations to contaminant exposure (Ono et al., 2019). Cod populations along the southern coast of Norway are currently protected through strict regulations of fishery activities.

Contaminant exposure studies involving both single and mixtures of contaminants, such as PAHs, PCBs and other EDCs have been performed using cod as a model species (Bizarro et al., 2016; A. Goksøyr et al., 1986; Hasselberg et al., 2004; Holth et al., 2014; Ruus et al., 2012; Sydnes et al., 2016; Yadetie et al., 2014, 2017). The popularity of

using Atlantic cod as a model species in environmental toxicology increased when its genome sequence was published in 2011, with the annotated version ("gadMor1") available in Ensembl (Star et al., 2011). The cod genome has later been re-sequenced and updated to "gadMor2" (Tørresen et al., 2017), and "gadMor3" (GenBank assembly accession: GCA_902167405). Furthermore, an available annotated genome facilitates the use of Atlantic cod as a model organism with omics technologies (Khan et al., 2021; Yadetie et al., 2017, 2018). Finally, it allows the mining of the genome for the set of genes and proteins that constitute the intrinsic defense against hazardous contaminants, known as the chemical defensome, as was recently published for Atlantic cod and four other teleost species (Eide et al., 2021). Knowing which components that are involved in the chemical defense is valuable for investigating effects of environmental contaminants, which adds to the advantages of using Atlantic cod as a model species in toxicological studies.

1.6.1 Cod liver as target organ for environmental contaminants

The liver has three essential functions in organisms; (1) uptake, storage, metabolism, and redistribution of nutrients and other endogenous and exogenous molecules, (2) metabolism of lipophilic compounds, including contaminants, and (3) synthesis and excretion of bile (Hinton et al., 2008). Due to its role in first pass xenobiotic metabolism, the liver is a target for accumulation of lipophilic contaminants, and therefore a well-suited organ for studying contaminant-induced effects. This is especially true for Atlantic cod, whose lipid contents reside mostly in the liver, with a total lipid content of 40-80% (A. Goksøyr et al., 1994). Cod hepatocytes are quite large (50-70 µm), with large lipid droplets (5-15 µm) that occupy more than two-thirds of the cellular volume (Fujita et al., 1986). Isolation of primary hepatocytes from cod have been performed previously (Ellesat et al., 2011; Søfteland et al., 2010), but due to their unique cellular structure and high lipid content, isolation techniques for obtaining primary hepatocyte cell cultures are less efficient in cod compared to other species (Eide, Karlsen, et al., 2014). Thus, in contrast to common *in vitro* hepatocyte

techniques, the use of PCLS is one of several useful methods for using Atlantic cod as a model species to investigate effects of contaminants. Furthermore, based on the recent cod genome assemblies, researchers in our dCod 1.0 project have drafted a metabolic reconstruction of the cod liver, with detoxification as a primary focus (Hanna et al., 2020). This reconstruction may aid future studies on biological effects in Atlantic cod liver following exposure to environmental contaminants.

2. Dissertation objectives

Molecular biomarkers have a central position as early warning signals of contaminant exposure in environmental toxicology. Several biomarkers are well-established in fish species and have been widely applied in studies with complex mixtures, including field and monitoring studies. Still, increased understanding on the assessment of biomarker responses following mixture exposures is needed. Furthermore, the suitability of applying omics methodologies to investigate mixture effects compared to biomarker assays must be addressed. Finally, as effects following contaminant mixture exposures are often studied using a variety of experimental approaches, differences among these methods may affect the endpoints in question, an issue that must be accounted for.

Therefore, the objectives of this dissertation were to:

1) Quantify biological responses in caged Atlantic cod exposed to a complex chemical cocktail originating from an old waste disposal site, thus assessing the suitability of applying a range of established biomarkers in field studies.

2) Assess the effects of *in vivo* exposure of Atlantic cod to environmentally relevant mixtures of PAHs and PFAS with regards to biological responses, using both biomarker assays and omics approaches. PAHs and PFAS were chosen as these contaminants are important contributors to global pollution and more knowledge of their mixture toxicities is needed.

3) Combine *ex vivo* PCLS exposures with RNA-seq analyses to improve the mechanistic understanding of effects following PFAS exposure, including differences in transcriptome responses between single compound and mixture toxicity of PFAS, and as a comparison with the similar *in vivo* exposure experiment.

Overall, combining these objectives will increase our knowledge regarding the use of conventional biomarkers and omics approaches to evaluate biological responses of Atlantic cod following contaminant mixture exposures, and how the application of different experimental approaches, including *in situ* by caging, *in vivo* aquaria studies, and *ex vivo* PCLS exposures, may affect the results.

3. Summary of results

3.1 Leakage of environmental contaminants from a capped waste disposal site promotes biological effects in caged Atlantic cod (Paper I)

Caged Atlantic cod were utilized as a proxy to assess whether possible leakage of environmental contaminants from a capped waste disposal site in Kollevåg (Western Norway) could induce biological effects in fish inhabiting this area.

In this field study, cod were caged at three different locations in Kollevåg, from inner to outer parts of the bay, with one station at the disposal site and two stations further out in the bay. In addition, one cage was placed at a nearby reference location. We demonstrated station-dependent differences in contaminant burden in sediments and in cod tissues. Elevated concentrations of PCBs and PAHs in sediment, in addition to significantly higher concentrations of PAH metabolites in bile of caged cod, were observed for all Kollevåg stations compared to the reference station. Significantly higher concentrations of PCBs, polybrominated diphenyl ethers (PBDEs), and dichlorodiphenyltrichloroethanes (DDTs) were observed in the liver of cod caged at the station closest to the capped disposal site (station 1). Growth performance parameters, the condition factor (CF) and hepatosomatic index (HSI), were reduced for cod caged at station 1. Station-specific patterns in hepatic and ovarian gene expressions were also observed, with significant increases in lipid pathway-related genes in liver and steroidogenic genes in ovaries of caged cod at several of the Kollevåg stations. Furthermore, site-specific reductions in lipid peroxidation and antioxidant activities (Gst and Cat), with a gradient from inner to outer parts of Kollevåg, were observed. By combining chemical and biological data with multivariate statistics (principal component analysis (PCA)), we demonstrated that responses clustered in a station-dependent manner, with a site-specific gradient from inner to outer parts of Kollevåg bay, compared to the responses of the reference cod. Finally, correlation

analysis showed that contaminant levels in cod liver correlated well with reproductive responses (Pearson´s $r \geq 0.70$). Based on our findings, we concluded that contaminants leaking from the capped disposal site accumulated and caused biological responses in caged cod, with reproductive endpoints as the most prominent response.

3.2 Lipid metabolism in liver of Atlantic cod is modulated by perfluoroalkyl substances (Paper II)

The aim of this study was to assess the effects of PAH and PFAS mixtures in Atlantic cod. Through a repeated intraperitoneal (i.p.) injection of the contaminants (at day 0 and day 7), Atlantic cod were exposed to environmentally relevant mixtures (1x, L-PAH and L-PFAS), and mixtures with higher concentrations (20x, H-PAH and H-PFAS). The cod were sampled at day 14 and biological parameters, mainly in the liver, were assessed through biomarker assays and omics approaches.

The results indicated a possible interacting effect of PAHs and PFAS regarding chemical uptake and metabolism. Trends of increasing PAH metabolite concentrations with combined PAH/PFAS exposure compared to PAH exposure alone were observed. Concentrations of PFOS and perfluorotridecanoic acid (PFTrDA) were trending higher following combined PAH/PFAS exposure compared to H-PFAS exposure alone, whereas the opposite trend was observed for PFOA and perfluorononanoic acid (PFNA). Changes in biological responses were most prominent in cod exposed to PFAS. Importantly, proteomics and lipidomics analyses showed that lipid metabolism was affected in cod liver. Proteomic analyses showed that PFAS exposure caused an up-regulation of enzymes involved in fatty acid degradation pathways, including Cpt2, carnitine O-acetyltransferase (Crat), and 2,4-dienoyl-CoA reductase 2 (Decr2). In accordance with an increase in lipid degradation, lipidomic analysis showed a decrease in levels of triacylglycerols in livers of cod exposed to H-PFAS. In addition, induction of antioxidant enzyme activities was more strongly related to PFAS exposure compared to PAH exposure. The lack of responses for several important biological parameters,

such as *cyp1a* expression, changes in vitellogenin concentrations and effects on DNA fragmentation were most likely related to the low (but environmentally relevant) concentrations utilized in the study, combined with the one-week duration between final injection and sampling. Nevertheless, the observed effects on lipid-related processes in liver suggests that PFAS can cause adverse effects on lipid metabolism in Atlantic cod at low concentrations.

3.3 PFAS exposure induces stress-related responses in precision-cut liver slices of Atlantic cod (Paper III)

In this study, a mechanistic approach was applied to investigate transcriptome responses of Atlantic cod PCLS following 48h of PFAS exposure. Exposures included both single compounds (PFOS, PFOA and PFNA) and a ternary mixture of these with equimolar concentrations. Three different concentrations (10, 50 and 100 μM) of each compound were included. Transcriptome responses of cod PCLS following PFAS exposure were assessed using high-throughput RNA sequencing.

We demonstrated differences between single PFAS in the number of differentially expressed genes (DEGs) generated following exposure. PFOS generated a higher number of DEGs (86) compared to PFOA (25) and PFNA (31). The PFAS mixture generated a larger number of DEGs (841) compared to single PFAS. Hierarchical clustering analysis showed that PFOS clustered closer to the mixture compared to PFOA and PFNA. Furthermore, 40 of 52 annotated DEGs following PFOS exposure were common with DEGs following mixture exposure. PFOS exposure caused significant enrichment of pathways related to nuclear receptor activation, oxidative stress, and cholesterol metabolism. Exposure to PFAS mixtures caused a dose-dependent difference on significantly enriched pathways, with enrichment of fatty acid biosynthesis and cancer-related pathways following exposure to Mix50, and an increased effect on stress-related responses including oxidative stress, ferroptosis and nuclear-receptor pathways following Mix100 exposure. Compared to the *in vivo* study

(**Paper II**), less effects were observed on β-oxidation and Ppara-related pathways in PFAS-exposed PCLS. Analysis of interactive effects among the PFAS in the mixture showed non-additive effects among 71 genes of the DEGs from the Mix100 exposure, which accounted for 10% of the total number of DEGs analyzed. 52 of these genes had synergistic expression patterns compared to the prediction from the single compounds and included genes encoding antioxidant and membrane-related proteins. To summarize, PFAS exposure caused effects on pathways involved in oxidative stress, nuclear receptors, and sterol metabolism. The mixture exposure generated a higher number of DEGs compared to single PFAS exposure, which could partly be explained by synergistic effects among the PFAS. However, as 90% of the DEGs in the mixture showed additive expression patterns, additivity was suggested as the main mixture effect.

4. Discussion

Mixture toxicity is the common denominator in this dissertation. In the environment, organisms are rarely, if at all, exposed to single contaminants. Thus, increasing our understanding of mixture effects is a challenging, but nevertheless an important task. The studies included in this dissertation have scratched the surface of this challenge by applying both conventional biomarker assays and toxicogenomic approaches to assess biological responses following exposure to contaminant mixtures. The suitability of investigating mixture toxicity using various bioassays, and different experimental approaches for exposure (caging, *in vivo*, PCLS), will be discussed. The majority of responses studied in this dissertation follow exposure to PFAS and PAHs; hence, effects of these contaminants will receive a considerable amount of attention.

4.1 Contaminant accumulation and chemical interactions

It is important to determine contaminant concentrations within the model species to identify the contaminants present in field studies, and/or determine accumulation from the exposure media in experimental situations. We applied chemical analyses to determine contaminant load in sediment (**Paper I**) and in cod liver following exposure (**Paper I** and **II**). In addition, we determined concentrations of PAH metabolites in bile as biomarkers of exposure to PAHs. In cod liver, we could verify accumulation of PCBs, DDTs, and other contaminants from the caging areas (**Paper I**), and uptake of PFAS following i.p. injections (**Paper II**). In cod bile, we observed significantly higher concentrations of all PAH metabolites in cod caged at Kollevåg stations compared to cod caged at the reference station (**Paper I**), and for some metabolites in cod exposed to H-PAH alone or combined with PFAS in the *in vivo* study (**Paper II**). After determining contaminant accumulation, we investigated the response of biomarkers related to chemical exposure. However, detecting a certain concentration of contaminants or their metabolites does not necessarily imply induction of biomarkers or contaminant-induced adverse effects in cod, an issue which is further discussed below.

Interactions between chemicals are a well-known phenomenon in toxicology (Celander, 2011). It is suggested that severe interactions are more likely to occur when few chemicals dominate the toxicity (Cedergreen, 2014). Furthermore, the presence of contaminants with surfactant properties in environmental mixtures, such as PFAS, may increase dispersion of co-pollutants, which limits sorption to soil/sediments. Such chemical interactions may lead to increased bioavailability of contaminants (Spurgeon et al., 2010). For the caging study (**Paper I**), it is difficult to interpret whether or not chemical interactions have taken place, due to the complexity of the contaminant mixture and other factors (biotic or abiotic) that might have played a part in the observed results. We did, however, observe some interesting features of contaminant concentrations in **Paper II**. Trends of increased concentrations of biliary PAH metabolites (1-OH pyrene and 1-OH phenanthrene) in cod co-exposed to H-PAH and L- or H-PFAS, compared to H-PAH alone were observed. This finding suggests that PFAS may increase PAH accumulation and/or biotransformation, which in turn may be observed as higher concentrations of metabolites in bile. Surfactants may modulate cell membrane permeability, and it is suggested that PFAS can enhance uptake of other chemicals through this mechanism (Lee et al., 2020; Liu et al., 2009). However, Liu et al. (2009) observed that PFAS, in this case PFOS, exhibited opposing effects on cellular uptake of different co-exposed pesticides in green alga. For example, uptake of pentachlorophenol increased, whereas uptake of atrazine and diuron decreased, in the presence of PFOS. The authors attributed these differences to physicochemical properties of the co-exposed contaminants; PFOS increased the uptake of the more hydrophobic pentachlorophenol (log K_{ow} = 5.01), while it decreased the uptake of the less hydrophobic atrazine and diuron (log K_{ow} 2.7 and 2.6, respectively) (Liu et al., 2009). These findings are in line with another study, where pre-exposure to PFOS or PFOA modified membrane integrity and the toxicity of different herbicides in an aquatic microorganism (cyanobacterium *Anabaena* CPB4337), and specifically, pre-exposure to PFOS or PFOA reduced atrazine toxicity, but increased toxicity of other herbicides (Rodea-Palomares et al., 2015). To our knowledge, no studies exist on PFAS-

mediated effects on the cellular uptake of PAHs in fish. PAHs are hydrophobic compounds, and their hydrophobicity increases with their molecular weight. The six PAHs in our study (**Paper II**) have log K_{ow} of 3.33, 4.18, 4.38, 4.57, 5.18 and 6.04 for naphthalene, fluorene, dibenzothiophene, phenanthrene, pyrene and BaP, respectively (Hylland, 2006). Therefore, it is possible that PFOS and the other PFAS may have enhanced uptake of several of the co-exposed PAHs. Differences in carbon length and functional group among PFAS could influence their ability to affect membrane permeability. In freshwater alga, both PFOS, PFOA and the longer-chained perfluorododecanoic acid (PFDoA, C12) and perfluorotetradecaonic acid (PFTeA, C14) increased cell membrane permeability (Liu et al., 2008), suggesting that the PFAS used in our studies (C8-C13) may cause similar effects on cellular membranes in Atlantic cod. Finally, it should be noted that also PFAS concentrations in liver varied in our *in vivo* study. Concentrations of PFOA and PFNA were trending higher following exposure to H-PFAS alone compared with combined PAH/PFAS exposure, whereas the opposite was observed for PFOS and PFTrDA. Therefore, we cannot exclude that PAHs also may affect uptake and/or metabolization of PFAS. More targeted studies are needed to investigate the underlying mechanisms of these potential interactions.

Aspects of chemical interactions and the ability of PFAS to affect membrane properties were also highlighted in our PCLS study (**Paper III**). No chemical analysis was performed in this study; therefore, the discussion of possible interactions among individual PFAS is based on mixture effect analysis of RNA-seq data. The mixture exposure generated a larger number of DEGs compared to single PFAS exposure, and we wanted to test whether this observation could be related to interacting effects among the individual PFAS within the mixture. A simple mixture effect analysis was performed by calculating predicted expression values of genes in the mixture based on single PFAS expression values and comparing these with the actual expression values in the mixture. We observed non-additive interactive effects in approximately 10% (71 genes) of the DEGs from the Mix100 exposure, indicating that 90% of the genes displayed an additive

response pattern. 52 of the non-additive responding genes had synergistic expression patterns in the mixture and included genes involved in cellular processes such as antioxidant responses and membrane-related transport. The effects of PFAS on oxidative stress-related responses are discussed in detail in section 4.2.2. The effects on membrane-related components, including several genes encoding solute carrier (Slc) proteins, is interesting in light of the ability of PFAS to affect membrane permeability. Membrane transporters play important roles in the toxicological response following xenobiotic exposure. For example, hepatic transporters are crucial in first pass metabolism for uptake and clearance of contaminants absorbed by the intestine (Klaassen & Lu, 2008). Several studies in zebrafish have observed interactions of PFAS with a subfamily of Slcs, the organic anion transporting polypeptides (Oatp or Slco) (Jantzen, Annunziato, & Cooper, 2016; Jantzen, Annunziato, Bugel, et al., 2016; Popovic et al., 2014). In humans and rats, OATPs and organic anion transporters (OATs, SLC22) are suggested to contribute to renal reabsorption of PFAS, which may lead to an extended biological half-life (Yang et al., 2010; Zhao et al., 2017). No genes within the Slco and Slc22 subfamilies were among the significantly affected genes in our mixture analysis, partly because only liver-related transcriptome responses were assessed. However, effects on other membrane-related components may add to the implications of PFAS-mediated membrane effects. Finally, the fact that membrane-related genes showed synergistic expression patterns in the PFAS mixture suggests that combined PFAS exposure may provoke interactive effects on membrane properties and/or PFAS uptake and clearance, which in turn is crucial for biological responses following PFAS exposure.

4.2 Biomarker responses following mixture exposures

4.2.1 Cyp1a as a biomarker for exposure to contaminant mixtures

Biomarkers used for indicating exposure to single compounds may not be equally effective to indicate mixture exposure. One example from our studies is the detoxification enzyme Cyp1a. Dioxins, dioxin-like PCBs and some PAHs, e.g. BaP, are

Ahr agonists and therefore, induction of Cyp1a is a well-established biomarker of exposure to these contaminants (Schlenk et al., 2008), also in Atlantic cod (Aranguren-Abadía et al., 2020; A. Goksøyr, 1985, 1995). Despite having detected several of these contaminants in either cod liver (PCBs, **Paper I** only) or bile (PAH metabolites), we did not observe hepatic *cyp1a* induction in our caging study (**Paper I**) or *in vivo* exposure study (**Paper II**). In comparison, in cod PCLS exposed to the same PAH mixture as in the *in vivo* study, we observed *cyp1a* induction following 48h of continuous exposure (Fig. 6). There might be several reasons for the lack of Cyp1a response in our caging and *in vivo* studies, such as the dose reaching the target organ, exposure duration (linked to metabolism and excretion of compounds), and antagonistic effects of chemicals within the mixtures. The mere presence of Ahr agonists is not enough to provoke a Cyp1a response, a certain concentration level of these compounds is also needed (Aranguren-Abadía et al., 2020). However, another study investigating effects of crude oil exposure in Atlantic cod observed similar biliary PAH metabolite concentrations compared to our studies, with an associated induction of Cyp1a (EROD) activity, following three weeks of continuous exposure (Holth et al., 2014). This finding, combined with the observed *cyp1a* induction by the PAH mixture in cod PCLS, suggest that the contaminant burden in our studies should be sufficient to induce *cyp1a* in Atlantic cod. For the caging study (**Paper I**), the potential antagonistic effects between compounds may be an explanation for lack of Cyp1a response. Within similar contaminant groups, different compounds may cause confounding effects on a given biomarker. Whereas some PAHs and PCBs are Ahr agonists, others, including non-dioxin like PCBs and the PAH fluoranthene, may inhibit Cyp1a activity/induction, and there are also species differences involved (Besselink et al., 1998; Brenerová et al., 2016; Brown et al., 2016; Hylland, 2006). Our findings are supported by another study, where sole (*Solea solea*) were exposed to a mixture of three PAHs (BaP, fluoranthene and pyrene), and despite significantly higher concentrations of biliary PAH metabolites, there was no significant increase in EROD activity in exposed groups compared to control (Wessel et al., 2010). In addition, a recent study assessing EROD activity in PLHC liver cell cultures exposed

to extracts from contaminated sediments, observed the lowest EROD activity following exposure to the most PAH-contaminated sediment extract (S. Ø. Goksøyr et al., 2021). Reduced sensitivity to the AhR agonists PCB-126 and BaP, determined through reduced responsiveness of *cyp1a* induction, has been observed in Atlantic killifish native to highly contaminated areas (Nacci et al., 2002, 2010). However, this type of reduced responsiveness to Ahr activation is acquired through generations and is therefore an unlikely explanation for the lack of *cyp1a* induction in our six-week caging study.

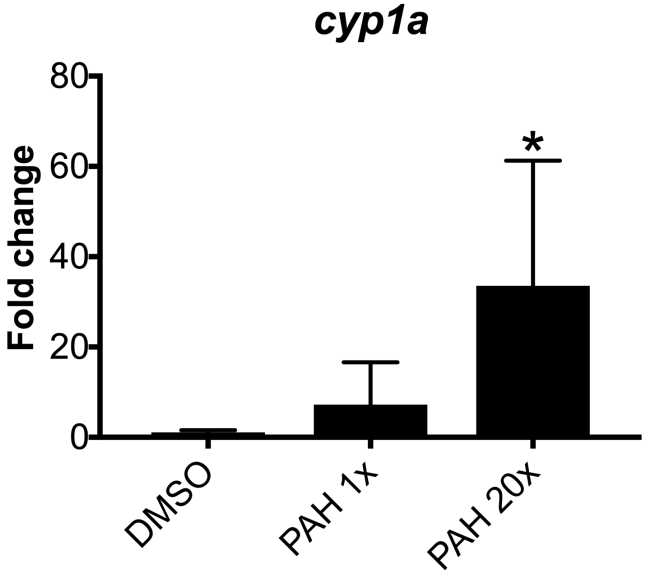


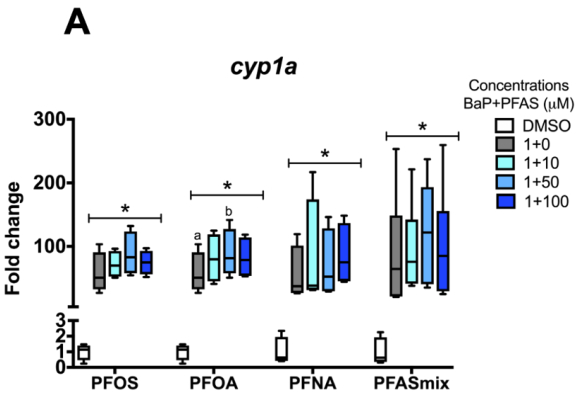
Figure 6 – Transcript levels of *cyp1a* determined in PCLS of Atlantic cod after exposure to PAH mixtures. Reverse transcriptase quantitative PCR was performed in PCLS exposed to a PAH mixture at low (1x – 40 µg/kg) and high (20x – 800 µg/kg) concentrations. DMSO (0.1%) was used as control. n=4, and data are presented with mean values + SD. Asterisk (*) indicated statistical significance ($p<0.05$) when comparing exposed groups to DMSO control (RM-ANOVA). Data are from unpublished results.

Although opposing effects of chemicals could have been a possible reason for the lack of Cyp1a response also in the *in vivo* study (**Paper II**), this is less likely as the same PAH mixture induced *cyp1a* expression following 48h of continuous exposure in cod PCLS (Fig.6). This finding underline that the aspect of exposure duration is important to

consider. In the *in vivo* study, there was one week between the last i.p. injection and sampling. As PAHs are rapidly metabolized in fish (Hylland, 2006), any Cyp1a response that may have been present at an earlier time point during the exposure may have ended. The results from a previous study support this statement, where an initial induction of *cyp1a* expression was reversed following a one-week recovery period in liver of PFAS-exposed Atlantic salmon (*Salmo salar*) (Mortensen et al., 2011). Based on our findings, applying Cyp1a as a biomarker of exposure to complex mixtures may be suitable for some mixtures, depending on their composition, but may be less applicable compared to single compound exposures. That said, other studies have successfully applied Cyp1a as a biomarker for complex mixtures (Beyer et al., 1996; Bratberg et al., 2013; Costa et al., 2009). Ultimately, as indicated by our studies, the type of compounds and their concentrations and ratios, combined with other potential contributors such as exposure situation, duration, and routes, are all factors that need to be addressed in order to fully understand the Cyp1a response in exposure studies.

The PCLS methodology has been shown to be suitable for assessing induction of *cyp1a* expression (Eide, Karlsen, et al., 2014). We have measured c*yp1a* expression in cod PCLS co-exposed to PAHs and PFAS to further investigate possible impacts of PFAS on uptake and/or metabolism of PAHs (discussed in section 4.1). Enhancement of PAH metabolization could be observed through stronger induction of *cyp1a*. We tested this hypothesis by exposing PCLS to PFAS in combination with BaP (a well-known Ahr agonist) to see if *cyp1a* expression was stronger with the PFAS+BaP combination compared to BaP alone. Gene expression was assessed using both reverse transcriptase quantitative PCR and RNA-seq analysis. These results were not included in **Paper III** but are presented here to continue the discussion on combined effects of PAH and PFAS. All exposures produced a significant induction of *cyp1a* compared to the DMSO control (Fig. 7). Mostly trends, but not significant increases (except for the combination BaP+50 µM PFOA, Fig. 7A) of stronger *cyp1a* expression following exposure of BaP combined with PFAS, compared to BaP alone, were observed. For the

mixture exposures, the trends were similar among the two methods: The highest *cyp1a* expression was observed in the BaP+Mix50 group, whereas the BaP+Mix100 group had expression levels similar to BaP alone. These findings indicate that PFAS-induced effects on *cyp1a* expression are concentration-dependent. In the literature, there are conflicting results as to whether PFAS alone may bind Ahr and induce the Cyp1a response. PFOS significantly induced *cyp1a* expression after 24h exposure in Atlantic salmon hepatocytes (Krøvel et al., 2008), whereas both PFOS and PFOA increased EROD activity in tilapia (*Oreochromis niloticus*) following 72h exposure (Han et al., 2012). In our DEGs lists from single PFAS exposure in **Paper III**, *cyp1a* was among the top DEGs following PFOA100 exposure. In contrast, PFOS did not induce *cyp1a* expression or EROD activity following exposure for two or 16 days in thicklip grey mullet (*Chelon labrosus*) (Bilbao et al., 2010). Also, there may be time-or development-specific differences in Cyp1a response following PFAS exposure. Expression of *cyp1a* was significantly increased and then decreased at 4 and 10 days post fertilization (dpf), respectively, in PFOS-exposed medaka (Fang et al., 2012). In combination with the Ahr agonist PCB126, PFOS and perfluorohexanoic acid (PFHxA) caused a significant increase, followed by a significant decrease, in *ahr2* expression in zebrafish embryos at 48 and 72 hours post fertilization (hpf), respectively (Blanc et al., 2017). Furthermore, a previous study observed that PFOS may increase cell membrane permeability and enhance uptake of another Ahr agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and further increase *cyp1a* expression compared to TCDD alone (Hu et al., 2003). In summary, these findings suggest that effects on both cellular uptake and increased PAH metabolization may occur following co-exposure of PFAS and PAHs. Further studies are necessary to elucidate the mechanisms behind these potential interaction effects.



B

Exposure	Fold change *cyp1a* RNA-seq
BaP	91 ± 49*
BaP + Mix10	110 ± 58*
BaP + Mix50	118 ± 54*
BaP + Mix100	91 ± 46*

Figure 7 – Expression levels of *cyp1a* in cod PCLS co-exposed to PFAS and BaP. Expression of *cyp1a* in cod PCLS exposed to per-and polyfluoroalkyl substances (PFAS) and benzo[a]pyrene (BaP) was analyzed using A) Reverse transcriptase quantitative PCR for BaP, binary (single PFAS+BaP) and quaternary (three PFAS+BaP) mixtures, or B) RNA sequencing for BaP and quaternary mixtures. Concentrations applied of single PFAS in binary or quaternary mixtures was 10, 50 and 100 µM, whereas the BaP concentration was constant at 1 µM. DMSO (0.1%) was used as control. n=6, and data are presented with median values and 25-75% quartiles, whiskers are calculated using Tukey (A) or as mean values ± SD (B). Asterisk (*) and letters (a, b) indicate statistical significance ($p<0.05$) when comparing exposed groups to DMSO control and PFAS+BaP exposed groups to the BaP group, respectively (RM-ANOVA). Data are from unpublished results.

4.2.2 Mixture effects on oxidative stress biomarkers

Effects related to oxidative stress, such as changes in expression and/or activity of antioxidant enzymes, are common responses following exposure to environmental contaminants (Lushchak, 2016). In this dissertation, oxidative stress-related responses were assessed mainly through measurement of antioxidant enzyme activities (**Paper I** and **II**) and pathway analyses of omics data (**Paper II** and **III**). The use of antioxidant enzymes as biomarkers is complex, as environmental contaminants may either decrease or increase their activities (van der Oost et al., 2003). Such opposing effects on enzyme activities were also observed in our studies. In the Kollevåg study (**Paper I**), a significant reduction of Cat and Gst activities were observed in liver of cod caged closest to the waste disposal site. In contrast, activities of the same enzymes were

increased by PFAS mixtures alone or combined with PAHs in the *in vivo* study (**Paper II**). In line with the *in vivo* findings, exposure of PCLS to a similar PFAS mixture, in addition to PFOS alone (**Paper III**) induced genes encoding several components of the antioxidant systems, including *sod*, *cat*, and glutathione cysteine ligase modifier and catalytic units (*gclm* and *gclc*, respectively). The findings from our studies suggest that changes in expression and/or activity of antioxidant enzymes may act as valuable biomarkers of oxidative stress for mixture exposures. However, as both up- or down-regulation of these components may indicate contaminant exposure and cellular stress, combining them with additional biomarkers is desirable.

In addition to effects on antioxidant enzymes, other oxidative stress-related pathways were significantly affected following PFAS exposure in cod PCLS (**Paper III**). Although some effects related to ROS pathways were affected by single PFOS exposure, most of the oxidative stress-related responses were observed following exposure to the PFAS mixture. Interestingly, several of these pathways were interlinked through the ferroptosis pathway. Ferroptosis is caused by lipid peroxidation and is a non-apoptotic, iron-dependent cell death (Tarangelo & Dixon, 2019). Two pathways involved in preventing ferroptosis, the mechanistic target of rapamycin complex 1 (mTORC1) and glutathione synthesis (controlled by the nuclear factor erythroid 2-related factor 2 (Nrf2)), where *gclm* and *gclc* play an essential role, were both induced, suggesting ferroptosis and other stress-related responses as possible outcomes of PFAS exposure. It must be mentioned that since oxidative stress responses in the PCLS study were more related to mixture exposure compared to single PFAS exposure, this may be attributed to the total exposure load in the mixture. Oxidative stress-related responses are shown to be correlated with the concentration of the inducer (Lushchak, 2014, 2016), suggesting that the PFAS mixture, with its higher exposure load, had a greater capability to induce such responses compared to the single exposure. Nevertheless, our findings suggest that oxidative stress-related responses are valuable for assessing

effects of contaminant mixtures, bearing in mind that the chosen exposure concentrations could have an impact on the outcome.

4.2.3 Mixture effects on biomarkers of metabolic disruption

Environmental contaminants may cause adverse effects through disruption of metabolic processes (Casals-Casas & Desvergne, 2011; Heindel et al., 2017). In recent years, effects on lipid metabolism have gained a great deal of attention. Of Ppar-regulated lipid metabolism pathways, fatty acid or β-oxidation is an essential energy-providing pathway in fish, and involves a set of enzymes located in mitochondrial and peroxisomal membranes (Olivares-Rubio & Vega-López, 2016; Weil et al., 2013). Through our proteomic analyses (**Paper II**), we could highlight an up-regulation of enzymes involved in mitochondrial and peroxisomal β-oxidation, including Cpt2, Crat, and Decr2, following PFAS mixture exposure. We also observed a decrease in triacylglycerols which agrees well with their hydrolysis into fatty acids that are oxidized in β-oxidation. These interesting findings led us to further investigate PFAS-induced effects on lipid metabolism in our PCLS study (**Paper III**). Here, however, no effects on the β-oxidation pathways were observed. Compounds that may affect lipid metabolism by activating Ppars are termed peroxisome proliferators (PPs) and e.g., fibrate drugs are well-known PPs due to their structural similarities to fatty acids, the natural ligands of Ppars (Olivares-Rubio & Vega-López, 2016). In mammalian species, e.g., rodents, it has been documented that PFAS toxicity is linked to peroxisomal proliferation through activation of PPARA. In fish, however, there are conflicting findings regarding the ability of PFAS to activate Ppara (summarized in Olivares-Rubio & Vega-López, 2016). Although many PFAS compounds are structurally similar to fatty acids (Fig. 8), PFAS are shown to have low affinity to PPARA in humans compared to e.g. fibrates (Lau et al., 2007). Furthermore, there may be structural differences involved in the ability of single PFAS to bind and activate Ppara. In Atlantic cod, it has been shown that the carboxylated PFOA and PFNA, but not the sulfonated PFOS, are able to activate Ppara1 *in vitro* (Søderstrøm et al., 2021). Moreover, these PFAS differ in their ability to

accumulate in cod tissues, where sulfonated PFAS are shown to accumulate to a higher extent compared to carboxylated PFAS (Conder et al., 2008; Liu et al., 2019). If this is the case, the time aspect of 48h versus two weeks of exposure for the PCLS and *in vivo* studies, respectively, may have provoked the differences regarding induction of the β-oxidation pathway, as it is likely that more PFAS accumulated over time in the *in vivo* study. It is also suggested that some PFAS may perturb lipid metabolism downstream of PPARA in rats, by binding directly to fatty acid binding proteins and displacing their endogenous ligands from these proteins, leading to peroxisome proliferation (Luebker et al., 2002). To summarize, effects on Ppara-related pathways such as β-oxidation may to some extent indicate PFAS toxicity in various species but should not be accepted as reliable biomarkers of PFAS exposure. Several parameters of β-oxidation were significantly affected following mixture exposure in the *in vivo* study (**Paper II**), but not in the PCLS study (**Paper III**). Mixture exposure complicates the application of such parameters as potential PFAS biomarkers when single PFAS differentiate in their ability to affect these components. In addition, factors including exposure duration may cause differences among the studied endpoints within the different experimental approaches, which is discussed in section 4.4.

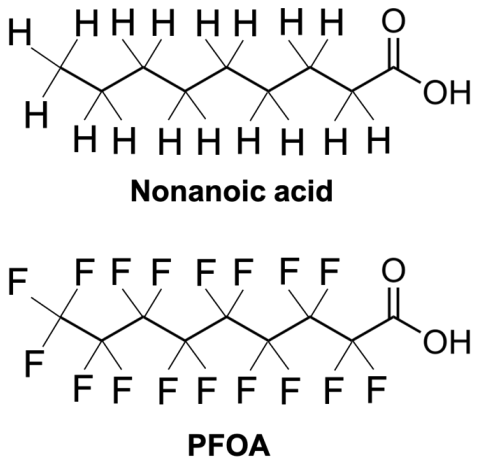


Figure 8 – Structural similarities of fatty acids (illustrated by nonanoic acid) and perfluoroalkyl substances (illustrated by PFOA). Structures from PubChem.

In addition to effects related to lipid degradation, we observed some effects of contaminant exposure on lipid biosynthesis. Gene expression of ATP-citrate lyase (*acly*) and fatty acid synthase (*fasn*) were significantly up-regulated at station 1 in our caging study (**Paper I**) and following exposure to the 50 µM PFAS mixture in our PCLS study (**Paper III**). These enzymes are essential in lipogenesis (Mashima et al., 2009). In addition, several genes involved in pathways related to cholesterol synthesis were significantly affected by exposure to PFOS and PFAS mixtures (50 and 100 µM). The expression of *cyp7a1*, the gene that controls the expression of the rate-limiting enzyme in cholesterol catabolism and bile acid synthesis, was down-regulated by PFAS exposure. Previous studies have shown that environmental contaminants including PFAS may disrupt lipid homeostasis through interaction with these components in fish and rodents (Arukwe et al., 2013; Fuller et al., 2021; Lai et al., 2017). Combined with the lipid degradation effects discussed above, these findings underline the possible effects environmental contaminants and contaminant mixtures may provoke on lipid homeostasis.

4.2.4 Mixture effects on biomarkers of endocrine disruption

Many contaminants are categorized as EDCs and may exert adverse effects on biological processes related to reproductive parameters, which might eventually affect populations (Kidd et al., 2007). Mixtures of EDCs may cause significant effects on organisms, even at low doses (Hamid et al., 2021). Reproductive responses from the experiments in this dissertation have been published and presented in detail in another doctoral thesis from the dCod 1.0 project (Khan, 2021) and will therefore receive less attention here. Of the studies included in the present dissertation, effects on reproductive endpoints were mostly assessed in the caging study (**Paper I**). Here, we observed effects on several reproduction-related parameters, including induction of ovarian steroidogenic genes and an increase in plasma concentrations of estradiol (E2), with a similar trend of increase of testosterone. Despite the lack of increased Vtg concentrations in caged cod, correlation analysis performed with chemical and

biological data highlighted the greatest correlation between the reproductive responses and contaminant levels in cod liver. Ovarian responses were further assessed by applying toxicogenomic approaches (transcriptomics and lipidomics) with ovaries of female cod caged in Kollevåg (Khan et al., 2020). A correlation between contaminant concentrations in cod and the number of DEGs in ovarian tissues for the different stations was observed, with a gradient from inner to outer parts of Kollevåg. In addition, compliance between contaminant burdens and responses important for maintaining a healthy reproduction, such as effects on ovarian immunomodulation, epigenetic regulation of gene expression, and lipid metabolism, was observed.

Although most effects following exposure of PCLS to PFAS in our *ex vivo* study (**Paper III**) were related to oxidative stress, some indications of reproductive effects were observed. The estrogen receptor pathway was among the top-enriched pathways following PFOS and PFAS mixture exposures, partly due to induction of the gene encoding estrogen receptor 1 (*esr1*). Furthermore, effects on metabolism of sterols such as cholesterol were also observed following PFAS exposure, which is related to reproduction due to cholesterol being a precursor in ovarian steroidogenesis (Gwynne & Strauss, 1982). The observed effects on reproductive endpoints from our studies suggest that biomarker responses such as steroid hormone concentrations, ovarian steroidogenic transcripts, and induction of reproductive-related nuclear receptors may be suitable for indicating exposure to contaminant mixtures. However, the ability of omics approaches to generate more detailed information regarding reproductive and other biological responses following mixture exposure is important to highlight, a topic which is further discussed below.

4.3 Assessing mixture toxicity using toxicogenomic approaches

To fully understand the effects of contaminant mixtures, it is essential to investigate the underlying MoA that provokes effects at higher biological levels. Toxicogenomic approaches have proven valuable in studying pathways related to a chemical´s MoA,

and to investigate mechanisms of interactions within a mixture (Bopp et al., 2019; Martins et al., 2019). Such information may facilitate linkage between exposure and biological responses of contaminant mixtures (Hamid et al., 2021).

In our studies, we applied omics methodologies to investigate transcriptomic (**Paper III**), proteomic and lipidomic (**Paper II**) changes caused by PAH and/or PFAS exposure. Except for a significant induction of antioxidant enzyme activities following PFAS exposure, few effects were observed using single biomarker assays in the *in vivo* study (**Paper II**). In contrast, although less pronounced changes in proteome and lipidome responses were observed for PAH and the combined PAH/PFAS mixtures, we detected effects on pathways related to lipid metabolism in livers of cod exposed to H-PFAS, with a similar trend for L-PFAS exposure. These findings suggest that toxicogenomic approaches may be more sensitive in unveiling effects of contaminant exposure compared to conventional biomarker assays. This sensitivity may be of greater importance when studying environmentally relevant concentrations. Omics approaches may also be valuable for discovering novel biomarkers. For example, during research on transcriptomic responses in PCLS following exposure to the synthetic estrogen ethynylestradiol (EE2), the induction of *fgf* (fibroblast growth factor) *3* and *4* transcripts were suggested as novel biomarkers of estrogen exposure in fish liver (Yadetie et al., 2018). An example from **Paper II** is the assessment of genes and proteins involved in β-oxidation. Whereas expression of *acox1*, a possible biomarker for indicating Ppara activation and subsequent induction of β-oxidation, was not induced following PFAS exposure, a battery of other components of β-oxidation, including Cpt2, Crat and Decr2, was found significantly increased with proteomics analysis. In PCLS exposed to a similar PFAS mixture (**Paper III**), the oxidative stress-related pathways, such as ferroptosis, were prominent among the observed effects (discussed in section 4.2.2). Components of ferroptosis-related pathways, such as the acyl coenzyme-A synthetase long-chain family member 4 (*acsl4*) involved in promoting ferroptosis, and *gclc* and *gclm* involved in the anti-ferroptotic glutathione synthesis

pathway, were all differentially expressed following PFAS mixture exposure. Although we do not suggest that the components discussed above should be established as biomarkers of PFAS exposure, these observations underline the ability of omics approaches to assess batteries of potential biomarkers within the same pathway, in addition to the discovery of novel biomarkers.

When toxicogenomic approaches are applied simultaneously on a given mixture together with the single chemicals within this mixture, these methods may be valuable in elucidating what, if any, interactive effects are induced by the chemicals in question. In our PCLS study (**Paper III**), the PFAS mixture exposure produced a relatively large number of DEGs compared to single PFAS exposures. To further investigate this observation, we performed a simple mixture effect analysis using the DEGs from the mixture exposure as a basis. This was done by comparing the expression values of the genes in the mixture with a calculated prediction from the expression values for the single PFAS exposures. We found non-additive effects on gene expression for 71 genes, accounting for approximately 10% of the DEGs in the mixture, and 52 of these genes had synergistic expression values in the mixture compared to the prediction from the single PFAS. This finding suggests that additivity is the main mixture effect in our study, but that a certain degree of interaction among the PFAS occurs. Other studies have also applied omics methodologies for investigating patterns of mixture exposures. Mixture effects of EE2 and BaP in PCLS from both Atlantic cod and Arctic cod (*Boreogadus saida*) have been studied previously by Yadetie et al. (2018, 2021). In these studies, the transcriptomic approach proved valuable in investigating unique expression patterns of the binary mixture in comparison to the effects of the individual compounds. Søfteland and colleagues studied effects on Atlantic salmon hepatocytes following exposure to contaminant mixtures by combining several omics methodologies, and could highlight additivity as a dominant mixture effect in their study, based on comparisons between mixtures and single contaminant effects (Søfteland et al., 2014). To summarize, the expression patterns and affected pathways

generated from the omics analyses gave valuable insights in the effects of both single PFAS and the PFAS mixture in our PCLS study, underlining the superior capability of toxicogenomic approaches to assess effects of contaminant exposure. However, to get a deeper understanding of possible interactions among PFAS, other types of mixture effect analysis should be explored. Such investigations may include the combination of multiple bioassays with mixture modelling analysis (Altenburger et al., 2018), or through exploring bioinformatic tools for discovering interacting effects within chemical mixtures (Tung et al., 2018).

4.4 Implications of applying different experimental approaches for investigating effects of contaminant exposure

In this dissertation, three different experimental approaches were applied to study contaminant-induced biological responses in Atlantic cod: Caging (*in situ*, **Paper I**), i.p. injections (*in vivo*, **Paper II**) and PCLS (*ex vivo*, **Paper III**). These approaches were different in terms of exposure route, contaminant concentrations, and duration of exposure, which are factors that may affect downstream analyses and cannot be neglected.

4.4.1 Exposure situation

Our Kollevåg caging study (**Paper I**) differs from the other two studies, as the composition of the contaminant exposure was determined by the cocktail of contaminants present at the caging sites. This study was the most "realistic" exposure scenario in terms of including natural biotic and abiotic factors, such as temperature, salinity, presence of other organisms etc., which is an important experimental advantage of this approach compared to the laboratory studies. The fact that natural influences are involved when studying environmental contaminants is advantageous for understanding their biological effects in a realistic setting but may also complicate the interpretation of results in a given study. Chemicals may interact with abiotic components such as sediment particles, soil and other substances, which might affect

both degree of exposure and bioavailability of a given chemical to an organism (Spurgeon et al., 2010).

Another factor that may affect biological responses in field studies is food availability. Although using cages was necessary in our study to monitor the exposure situation at the desired location, a disadvantage of caging compared to a genuine field study is the restriction of movement within the cages. Caged fish may only obtain limited amounts of food, and the uptake of contaminants via food will likely differ from free-living organisms in a given area (Oikari, 2006). In our studies (caging and *in vivo*) we have used Fulton's condition factor (CF) and the hepatosomatic index (HSI) to evaluate general health of the cod. In the caging study (**Paper I**), both parameters were significantly decreased for cod caged at the station closest to the waste disposal site. The cod were caged for a period of six weeks and could only feed from the sediments at the cage bottoms or from food floating by. Therefore, even though environmental contaminants are known to affect these health parameters, we could not conclude whether the observed effects were caused by contaminant exposure, food shortage or a combination of both.

Studies performed in the laboratory are obviously less realistic in terms of "true" environmental conditions compared to field/caging studies. Despite the lack of influence by biotic and abiotic factors, laboratory studies may still provide valuable information on biological responses following contaminant exposure. Also, for the lab studies within this dissertation (**Paper II** and **III**), we could better control the exposure conditions and importantly, choose which contaminants to study. In this way, it is more likely that any observed changes in biological responses compared to a control group is caused by contaminant exposures.

4.4.2 Exposure concentrations

The choice of exposure concentrations is an important parameter to consider in toxicology. In the studies within this dissertation, we have utilized both environmentally relevant concentrations (**Paper I** and **II**) and higher concentrations for obtaining a mechanistic understanding (**Paper III**) of contaminant effects. The whole range of concentrations is important for different reasons: using higher concentrations in the lab is important for mechanistic studies of effects linked to contaminant exposure, whereas observing effects at lower concentrations is more relevant for risk analyses (Hook, 2010). Environmentally relevant concentrations may occasionally be too low to affect biomarker responses. Biomarkers need a certain concentration of an inducer/inhibitor in order to respond to chemical exposure (Lam & Gray, 2003), which may require some time to achieve through bioaccumulation alone. However, despite using environmentally relevant concentrations both in our caging (**Paper I**) and *in vivo* (**Paper II**) studies, we observed a variety of effects on biological responses in Atlantic cod. These findings underline the importance of including a large range of concentrations to improve the understanding of effects following contaminant exposure.

For mixture exposure studies, the choice of concentration is even more important, as biological responses may be affected by concentration ratios of the components within the mixture (Hamid et al., 2021). We applied different strategies for our compound ratios in the *in vivo* and PCLS studies. Since we focused on environmentally relevant concentrations in our *in vivo* study (**Paper II**), the ratios among the compounds within the PAH and PFAS mixtures were similar to the ratios of the same compounds measured in the liver of wild Atlantic cod captured through monitoring studies (Grøsvik et al., 2012; Kallenborn et al., 2004; Norwegian Environment Agency, 2013). For the PCLS study (**Paper III**), however, we chose equimolar concentrations of each PFAS within the mixture. In zebrafish embryos, Ding and colleagues saw differences in interacting effects between PFOS and PFOA by combining these compounds with varying concentration ratios (Ding et al., 2013), whereas changes in gene expression

patterns among mixtures containing different PFAS ratios were observed in primary hepatocytes of rare minnow (*Gobiocypris rarus*) (Wei et al., 2009). It is therefore likely that choosing a different combination of contaminant concentrations would provoke some changes in the observed biological effects in our studies.

4.4.3 Exposure route

Waterborne exposure is usually the preferred method for *in vivo* exposures in aquatic organisms (Rand, 2008). For the *in vivo* study, however, cod were exposed to contaminants through i.p. injections (**Paper II**), a method which will simulate oral exposure. Lipophilic contaminants dissolve poorly in water and injections and feeding exposures may therefore be better at ensuring delivery of such contaminants to the fish body. Furthermore, unless force-feeding is used, injections may also compensate for differences in food intake, thereby balancing and providing a more similar exposure dose to each individual test species. The choice of exposure route does not seem to affect biomarker responses of PAHs and PFAS. For both contaminant groups, most of the biomarker responses assessed have shown similar effects regardless of exposure routes in aquatic organisms (Lee et al., 2020; Santana et al., 2018). Even so, there are differences in the exposure routes between the *in vivo* (**Paper II**) and *ex vivo* (**Paper III**) studies that need to be addressed. For the *in vivo* study, the contaminant mixtures were injected into the intraperitoneal cavity of the fish, whereas the PCLS were kept in exposure media containing the contaminants. Although clear effects following PFAS exposure in cod PCLS were observed through a certain number of generated DEGs, we cannot exclude possible differences in uptake of contaminants into hepatocytes between the *in vivo* and PCLS studies. A reduced uptake of contaminants from the exposure media has earlier been proposed as a limitation of liver slices as a model system, which may depend on the physicochemical properties of the exposure compounds as well as the liver slice thickness (De Graaf et al., 2010).

In contrast to our laboratory studies, we could not control the contaminant exposure

routes in the Kollevåg study (**Paper I**). Here, caged cod may have been exposed through different routes: through water (gills, intestine) or through ingestion of sediment particles or feeding on epibenthic organisms. It is likely that the bioavailability of the contaminants differs in this study compared to the laboratory studies. Although biomarker responses following PAH and PFAS exposure are shown to be similar independent of exposure route (Lee et al., 2020; Santana et al., 2018), we cannot exclude an impact of exposure route on biological responses in our caging study compared to our laboratory studies.

4.4.4 Exposure duration

Another aspect to consider when comparing the experimental approaches is the exposure duration, which differs among our three approaches. In the caging study (**Paper I**), a six-week exposure duration was chosen to provide sufficient time for accumulation of contaminants from the waste disposal site. In our *in vivo* study (**Paper II**), where the aim was to provoke changes in protein and lipid responses, the cod were exposed for 14 days with one week between the final i.p. injection and sampling. Using PCLS (**Paper III**), however, there are restrictions to exposure length due to reduced viability of the liver slices over time. We chose an exposure duration of 48 hours, as a slightly reduced viability (measured by ATP content) after 72 hours of exposure has been observed (Eide et al., 2014). The differences in exposure duration between the studies provided an opportunity to assess how biological responses may be affected by these variations. One of the advantages of biomarker responses is their rapid induction, which is the reason for their use as “early warning signals”. However, biomarker induction is a dynamic process balancing between injury and repair, thus choosing an optimal timing for assessing biomarker responses is critical (Hook et al., 2014). Two examples of this aspect have been highlighted in this dissertation. The first example was timing of assessment of the Cyp1a response. A PAH mixture caused induction of *cyp1a* following 48 h of exposure in PCLS (Fig. 6), but no induction was observed after 14 days of exposure in the *in vivo* study, with one week after the final

injection (**Paper II**). In addition, effects on components within the β-oxidation pathway were prominent among the biological responses in the *in vivo* study, but not in cod PCLS exposed to a similar PFAS mixture (**Paper III**). The latter observation may be linked to a reduced time window for the PFAS to accumulate in the PCLS compared to the *in vivo* study. Although other differences among the exposure conditions may contribute to these observations, the findings underline the importance of choosing the correct timing for determining biomarker responses following contaminant exposure.

4.5 Atlantic cod as a model species for studying effects of environmental contaminants

The suitability of using Atlantic cod as a model species in various experimental approaches has been underlined in this dissertation. Several previous caging and *in vivo* studies have used Atlantic cod as model species to assess different research questions (Beyer et al., 1996; Bratberg et al., 2013; A. Goksøyr et al., 1987, 1994; Olsvik et al., 2013; Ruus et al., 2012; Sundt et al., 2012; Sydnes et al., 2016; Yadetie et al., 2016). Further, the PCLS technique has successfully been applied in several exposure studies with downstream analyses including biomarker assays such as western blots and qPCRs, and more complex analyses such as RNA-seq (Aranguren-Abadía et al., 2020; Bizarro et al., 2016; Eide, Karlsen, et al., 2014; Yadetie et al., 2018).

Although only farmed Atlantic cod were utilized for the exposure studies in this dissertation, the results obtained might predict how cod, and potentially other fish and marine organisms, are affected by exposure to environmental contaminants. However, it is well established that there are species differences involved in the response to contaminant exposure. Previous studies have observed lower sensitivity in hepatic biomarker responses of cod compared to other fish species, using both cod primary hepatocytes exposed 24 h to copper and statins (Ellesat et al., 2011), and in liver of cod caged for three months in a polluted area (Beyer et al., 1996). The high lipid content of the cod liver could contribute to these differences. Lipophilic compounds may strongly

associate with the large lipid droplets and thereby reduce the total burden on molecular targets, compared to the responses in other fish species with less fatty livers (Beyer, 1996; A. Goksøyr, 1984). Furthermore, sequencing of the cod genome has revealed unique properties, e.g. a lack of several genes involved in the immune system, including class II major histocompatibility complex (MHC II), CD4 and Ii (invariant chain), which have important roles in the immune system of other vertebrates (Star et al., 2011). Despite this finding, the cod appears to be equally robust towards disease under normal conditions compared to other vertebrates, indicating that compensatory mechanisms have evolved (Malmstrøm et al., 2013). In addition, members of our research project have characterized the full complement of the genes involved in the defense against chemical stressors (denoted the chemical defensome) of the Atlantic cod and four other teleost species (Eide et al., 2021). Interestingly, Atlantic cod and most of the other teleosts belonging to the Gadiformes order lack the nuclear receptor pregnane X receptor (Pxr, Nr1i2) (Eide et al., 2018), which is important for regulating several biotransformation enzymes in other vertebrates. Hence, Atlantic cod must regulate these enzymes through other mechanisms, which may cause differences in biological responses following contaminant exposures, compared to other species. Due to the importance of Pxr in relation to biotransformation of contaminants in many other fish species, its absence in Atlantic cod may be a disadvantage for using cod as a model organism for fish species carrying this gene.

The life cycle of fish species is important to consider when choosing test species in toxicology. Atlantic cod reach sexual maturity after 2-10 years, depending on ecotypes, habitats and growth performance (Food and Agriculture Organization of the United Nations, 2021). Therefore, assessing research questions related to long-term effects on several generations, such as epigenetics, is less convenient using cod compared to other species with faster generation time, such as zebrafish, medaka and stickleback (Lawrence et al., 2012).

It is shown that local populations of Atlantic cod are genetically different from one another. Some fjords along the Norwegian coast (Western Skagerrak) possess locally differentiated Atlantic cod, which is characterized by an inherited fitness in their native environment compared to non-native populations (Barth et al., 2017). These adaptations are linked to chromosomal rearrangements, where important, functional genes are passed on through generations (ibid). Moreover, these chromosomal rearrangements have led to the development of 'supergenes' in the Atlantic cod genome, which are coadapted genes that are inherited as a single Mendelian locus (Matschiner et al., 2021). It is possible that such local adaptations and variations within cod populations can lead to differences in the ability to respond to contaminant exposures, which is important to be aware of when assessing biological responses in wild Atlantic cod.

5. Conclusions

In this dissertation, the aim was to increase our understanding of biological responses of Atlantic cod following exposure to contaminant mixtures, using different experimental approaches. Reproductive parameters of Atlantic cod were affected following six weeks of caging near a capped waste disposal site. In addition, environmentally relevant concentrations of a PFAS mixture induced antioxidant responses and disrupted lipid homeostasis in cod liver following *in vivo* exposure. Both these studies underline the ability of contaminant mixtures to induce adverse effects in marine fish species, even at low concentrations. Further, higher concentrations of PFAS induce significant changes in transcriptomic responses in cod PCLS, with pathways related to oxidative stress, nuclear receptors, and sterol metabolism as most prominent. In addition, we identified a set of genes whose expression patterns were shown to be synergistic or antagonistic in the mixture compared to a prediction from the single PFAS exposures.

Through the studies included in this dissertation, we have assessed well-known biomarkers of contaminant exposure and discussed how suitable they are for indicating exposure to contaminant mixtures. Some of the biomarkers, including the antioxidant enzymes, proved appropriate for indicating mixture exposure whereas others, such as the well-established Cyp1a biomarker, were less responsive in our mixture exposure studies. These observations underline the complexity of assessing mixture toxicity compared to single compound toxicity. Conventional biomarkers are still valuable for rapid evaluation of initial responses following contaminant exposure; however, caution is needed when evaluating their responses in mixture toxicity studies. In comparison to biomarker assays, toxicogenomic approaches proved their ability to uncover contaminant-induced effects on several important cellular pathways, even following exposure to moderate contaminant concentrations. Such approaches may be especially valuable for assessing batteries of pathway-related biomarkers following exposure to contaminant mixtures. In addition, these methods are useful for

large-scale analysis of possible interacting effects among single compounds within a mixture.

Studying biological responses of contaminant mixtures is a complex task and several factors may affect the final result. We applied three diverse experimental approaches for assessing mixture effects and important differences among these approaches have been highlighted. Specifically, concentrations and ratios of exposure compounds, in addition to the duration and route of exposure are factors whose impacts must be addressed in studies on biological responses following contaminant exposure.

Our studies underline that Atlantic cod can successfully be used as a model species in several experimental approaches (caging, *in vivo*, PCLS), combined with both conventional biomarker assays and toxicogenomic approaches. Limitations of using Atlantic cod as a model species, such as a potential reduced sensitivity of hepatic biological responses following chemical exposure, or the unique properties of the cod immune system and chemical defensome, underline the importance of carefully choosing the model species in toxicological studies, depending on the objectives of the study in question.

6. Perspectives and future studies

6.1 Continuing the development of cod model systems

The PCLS methodology is a useful system for a rapid evaluation of contaminant toxicity. However, currently the cod liver slices are only viable for 48-72 h, an exposure duration that may not be sufficient to fully induce protein and lipid responses. For future studies, we wish to further develop the PCLS methodology in Atlantic cod by improving the slice viability, which in turn can provide longer exposure durations in PCLS studies. Other researchers have obtained an increased length of viability (9-10 days) in Atlantic salmon and rainbow trout liver slices (Fisher et al., 1996; Harvey et al., 2019). With the possibility to perform longer experiments with cod PCLS, it would be interesting to follow up on the time-dependent Cyp1a response, by exposing liver slices to PAHs and analyze *cyp1a* expression at different time points, e.g., 24 h, 48 h, 72 h and one week after exposure. Such an experiment could also be executed through a new *in vivo* exposure study; however, this is less suitable in a 3R perspective. Further, finding a suitable way of combining chemical analyses with biological bioassays in PCLS would provide an opportunity to link chemical accumulation in PCLS with cellular endpoints. Moreover, as the combination of PCLS with transcriptomic analysis has proven valuable, it would be interesting to investigate whether other toxicogenomic approaches, such as proteomics and metabolomics, possibly also as an integrated multi-omics approach, could be applied using cod PCLS. Finally, the cod liver is heterogenous and individual cell types may respond differently to contaminant exposure. Therefore, integrating cod PCLS with application of the rapidly growing single-cell sequencing technologies (Zhang et al., 2017) would be interesting to investigate mechanisms of contaminant effects in cod liver in more detail.

Due to the high lipid content of the cod hepatocytes, obtaining primary hepatocytes is a challenging task and requires, similar to PCLS, the sacrifice of a certain number of cod. The alternative to these methods is cell lines. Although cell lines may cause poorer prediction of chemical toxicity compared to primary hepatocytes, *ex vivo* and *in vivo*

approaches, they are still convenient to use in terms of the 3R perspective, easy maintenance, and the ability of rapid screening of a large number of chemicals (Eide, Rusten, et al., 2014; Rehberger et al., 2018). Furthermore, there have been advances in development of three-dimensional (3D) cell culture systems that may provide closer prediction of *in vivo* toxicity compared to the 2D systems (Ravi et al., 2015). 3D hepatocyte spheroid cultures have been established and applied in assessment of chemical biotransformation in rainbow trout (Hultman et al., 2019; Lammel et al., 2019; Uchea et al., 2015), and it would be interesting to explore the possibility of establishing a similar system with Atlantic cod hepatocytes.

In silico approaches are deemed essential for developing the systems toxicology concept. Members of our research project dCod 1.0 are currently working on a metabolic reconstruction of the cod liver, with a focus on lipid and xenobiotic metabolism pathways (Hanna et al., 2020). Establishing such reconstructions could greatly enhance studies of biological effects following contaminant exposure in Atlantic cod and may also aid in development of AOPs.

6.2 Chemical interactions: Effects of PFAS on membrane permeability and accumulation of other contaminants

The indication of chemical interactions between PAHs and PFAS, where PFAS may affect membrane permeability and enhance PAH uptake and/or metabolization, is an interesting observation that may be further assessed. Membrane permeability can be determined using various methods, e.g., flow cytometry, a method that has been successfully applied using mouse PCLS (Bartucci et al., 2020) and may also be tested using cod PCLS. We recently performed an exposure study where cod PCLS were exposed to binary and quaternary mixtures of PFAS and BaP, part of which was presented through *cyp1a* expression in Fig.7. Hopefully, a full analysis of these data may provide increased insights into potential interactions between PFAS and PAHs.

6.3 Perspectives

Through this dissertation, a detailed evaluation of how Atlantic cod has responded to contaminant exposure using different experimental approaches has been presented. Our findings may be discussed in relation to important topics within environmental toxicology that are also of societal interests. One example is the Kollevåg study, where the area containing household- and industrial waste after 45 years of waste dumping has been repaired and recapped on several occasions from the termination of the disposal in 1975 until today. The caging study in Kollevåg was executed after discovering new damage on the capping in 2014, to investigate possible effects of the "old sins" beneath the ocean surface. The fact that effects on biological responses, especially those related to reproduction, were observed in caged Atlantic cod following only six weeks of exposure, is alarming. The findings in this study underline several important questions: If we can find biological effects after six weeks of exposure, how are the local organisms responding to the presence of the waste disposal site? How many other disposal sites in Norway, or across the world, are present where this is an equal or much larger problem? The disposal site in Kollevåg was recapped after our caging study, and it would be possible to repeat the study to investigate whether recapping has improved the conditions in the area. However, an important question remains: Is capping/recapping of areas such as Kollevåg the best option, or is it just an "out of sight, out of mind"-strategy? Our findings suggest that the alternative approach of removing the waste should also be considered.

The observation that environmentally relevant concentrations of contaminant mixtures may affect biological responses in Atlantic cod following short exposure periods (both in the caging and the *in vivo* studies) has other societal implications. As the number of chemicals introduced to the market has exploded, the importance of a proper examination of the chemicals in question should not be underestimated. Despite the demand of several EU regulations to include consideration of chemical mixtures, risk assessments are mostly based on single chemicals (Bopp et al., 2019).

Through our studies, we have underlined the complexity of investigating effects of contaminant mixtures, with several aspects that may affect the final observations. We have studied environmentally relevant contaminant mixtures in our caging (**Paper I**) and *in vivo* (**Paper II**) studies, and our results contribute to improve the understanding of potential effects following exposure to contaminants such as PFAS. Despite our findings, the mixtures applied in our experiments are quite specific in terms of their compositions and concentrations. Therefore, they cannot be directly applied in risk assessment of contaminant mixtures. Although the attention to effects of contaminant mixtures has increased greatly over the past decades, the problem with several of these studies, including the present studies, is that they are often designed to address specific research questions or assess mixtures of local polluted areas with a unique chemical composition. The results from such studies may be less appropriate for implementation in risk assessment analysis, underlining the importance of developing more standardized study designs for investigating biological responses following mixture exposure.

In light of the Ocean Decade and the Sustainable Development Goals (SDGs), where SDG14 states "*Conserve and sustainably use the oceans, seas and marine resources for sustainable development*", the health of our marine ecosystems has never received more attention. Mixture toxicity is highlighted as one of the main topics to focus on during the Ocean Decade (European Marine Board, 2019), underlining the relevance of the topic within this dissertation. Increasing our understanding of the impact of environmental contaminants is crucial to contribute to these initiatives, and above all, to secure a healthy environment where all organisms may thrive.

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Paper I

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Contaminant accumulation and biological responses in Atlantic cod (*Gadus morhua*) caged at a capped waste disposal site in Kollevåg, Western Norway

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ABSTRACT

The aim of this study was to assess whether fish in Kollevåg, a sheltered bay on the western coast of Norway, previously utilized as a waste disposal site, could be affected by environmental contaminants leaking from the waste. Farmed, juvenile Atlantic cod (*Gadus morhua*) were caged for six weeks at three different locations in Kollevåg bay and at one reference location. Sediments and cod samples (bile and liver) were analyzed for polychlorinated biphenyls (PCBs), organochlorine pesticides (OCPs), brominated flame retardants (BFRs), per- and polyfluoroalkyl substances (PFASs) and polycyclic aromatic hydrocarbon (PAH) metabolites, revealing a contamination gradient at the four stations. Furthermore, hepatosomatic index (HSI) and Fulton's condition factor (CF) were significantly lower in cod caged closest to the disposal site. Levels and activities of biomarker proteins, such as vitellogenin (Vtg), metallothionein (Mt), and biotransformation and oxidative stress enzymes, including cytochrome P450 1a and 3a (Cyp1a, Cyp3a), glutathione s-transferase (Gst) and catalase (Cat), were quantified in blood plasma and liver tissue. Hepatic Cat and Gst activities were significantly reduced in cod caged at the innermost stations in Kollevåg, indicating modulation of oxidative stress responses. However, these results contrasted with reduced hepatic lipid peroxidation. Significant increases in transcript levels were observed for genes involved in lipid metabolism (*fasn* and *acly*) in cod liver, while transcript levels of ovarian steroidogenic enzyme genes such as *p450scc*, *cyp19*, *3β-hsd* and *20β-hsd* showed significant station-dependent increases. Cyp1a and Vtg protein levels were however not significantly altered in cod caged in Kollevåg. Plasma levels of estradiol (E2) and testosterone (T) were determined by enzyme immunoassay (EIA) and showed elevated E2 levels, but only at the innermost station. We conclude that the bay of Kollevåg did not fullfill adequate environmental condition based on environmental quality standards (EQSs) for chemicals in coastal waters. Following a six weeks caging period, environmental contaminants accumulated in cod tissues and effects were observed on biomarker responses, especially those involved in reproductive processes in cod ovary.

1. Introduction

During the past century, oceans and coastlines were utilized deliberately for dumping of wastes (Goldberg, 1985). The bay of Kollevåg outside the City of Bergen (Norway) was used as a waste disposal site from 1930 to 1975, with dumping of in total 450,000 m^3 of industrial and household wastes. About 90% of this waste was deposited on the seabed (Vassenden and Johannessen, 2009). After terminating the

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Abbreviations

BaP	Benzo[a]pyrene
BNF	β-naphthoflavone
BFR	Brominated flame retardants
Cat	Catalase
CHL	Chlordanes
CF	Condition factor
CTD	Conductivity Temperature Depth
Cyp	Cytochrome P450
DDT	Dichlorodiphenyltrichloroethane
EDC	Endocrine disrupting compounds
EIA	Enzyme immunoassay
EQS	Environmental quality standard
EROD	7-ethoxyresorufin-O-deethylase
E2	Estradiol
Gst	Glutathione S-transferase
Hsd	Hydroxysteroid dehydrogenase
HSI	Hepatosomatic index
HCH	Hexachlorocyclohexane
HPLC	High-performance liquid chromatography
HRGC	High-resolution gas chromatography
LOD	Limit of detection
LOQ	Limit of quantification
MNE	Mean normalized expression
Mt	Metallothionein
OCP	Organochlorine pesticides
PFAS	Per- and polyfluoroalkyl substances
POP	Persistent organic pollutant
PBDE	Polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PAH	Polycyclic aromatic hydrocarbons
Ssc	Side-chain cleavage
Star	Steroidogenic acute regulatory protein
T	Testosterone
TBARS	Thiobarbituric acid reactive substances
Vtg	Vitellogenin

dumping in 1975, the site was capped by sand and stone. Kollevåg was reopened as a recreational area in 1983 and has since been routinely monitored. In 2004, comparatively high levels of environmental contaminants were observed both in the sediments (e.g. polychlorinated biphenyls (PCBs): 271 µg/kg dw of PCB-7) and in liver (PCB-7: 8679 µg/kg ww) of cod captured in the area. Following this discovery, the sediment was covered with geotextile (Vassenden and Johannessen, 2009). After a few years of decreasing contaminant concentrations, increasing levels of PCBs were nonetheless observed in the sediments after 2009, both within and outside the capped area (Hatlen and

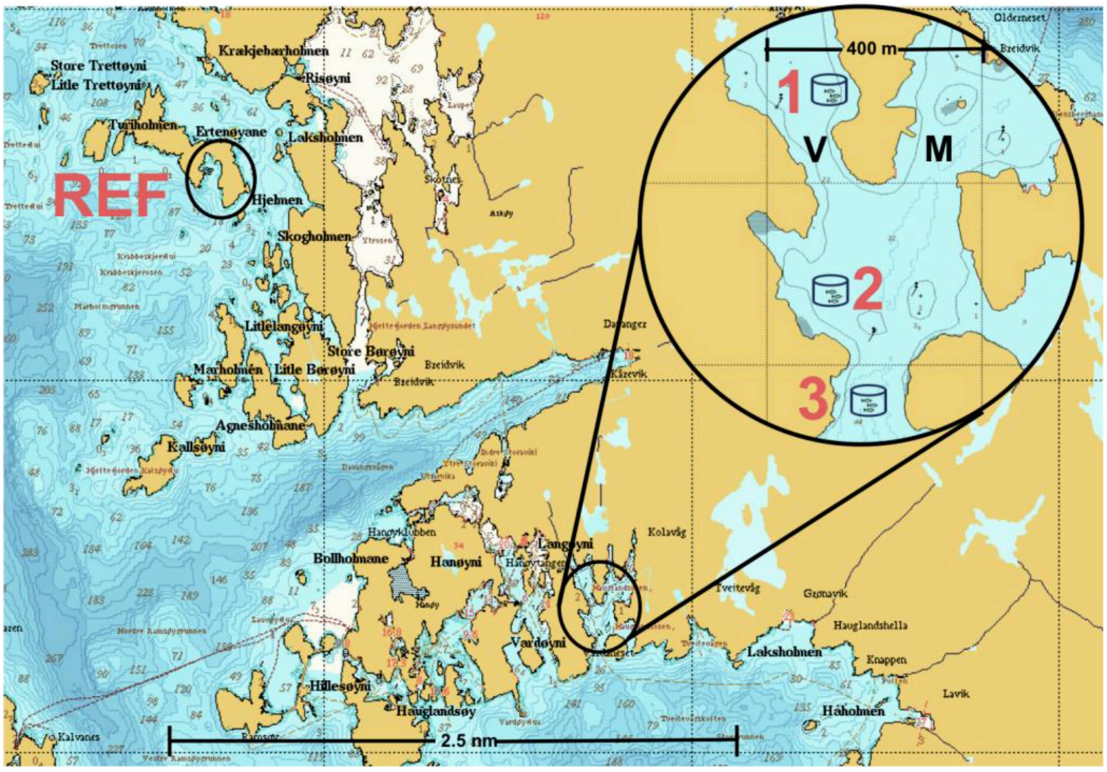

Fig. 1. Location of fish cages. Cages with 22 cod per cage were placed at four different locations: Three cages were placed in or near the capped waste disposal site in Kollevåg (enlarged in black circle), with one station (1) in the capped area in Vestrevågen (V), one station (2) at the borderline between Vestrevågen and Medavågen (M) and one station (3) at the outer parts of Kollevåg. In addition, a reference cage (REF) was placed near Ertenøyane. Nm = nautical miles. Map adapted from Olex AS.

Johansen, 2016). In 2014, extensive damage to the capping was observed and waste was visible on the surface (Hatlen et al., 2017). The area is still a popular beach and site for recreational fishing; it is therefore important to monitor environmental contaminant concentrations and biological effects on biota in the area.

PCBs, polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides (OCPs), and heavy metals, together with contaminants of emerging concern, such as endocrine disrupting compounds (EDCs), have been shown to affect the detoxification systems, lipid metabolism, antioxidant pathways and the endocrine system in fish (Arukwe and Goksøyr, 2003; El haimeur et al., 2017; Enerstvedt et al., 2018; Vincze et al., 2015; Yadetie et al., 2014). For example, EDCs are known for their potential to induce masculinization and feminization of female and male fish, respectively (Bergman et al., 2013). Furthermore, through disturbance of components involved in steroidogenesis, EDCs can cause effects on the whole organism level, potentially affecting sexual differentiation, growth, reproduction, and population structure (Arukwe, 2008; Kidd et al., 2007). In environmental toxicology, biomarkers such as cytochrome P450s (Cyps), vitellogenin (Vtg) and metallothionein (Mt) are used for monitoring purposes to indicate exposure to pollutants (Arukwe and Goksøyr, 2003; Hook et al., 2014; van der Oost et al., 2003).

The Atlantic cod (*Gadus morhua*) is a major fisheries species in the North Atlantic and is an important component of coastal and continental shelf ecosystems. It is commonly used as an indicator species in marine environmental monitoring programs, including the OSPAR convention and water column monitoring of offshore petroleum activities in Norway (Hylland et al., 2006; Norwegian Environment Agency, 2011; Vethaak et al., 2017). Cod has previously been used as a target species in caging studies to investigate pollution effects (Hylland et al., 2006). Caged fish have been utilized to study a wide variety of research questions including effects of wastewaters, environmental contaminants and seasonal changes (Berge et al., 2011; Beyer et al., 1996; Brammell et al., 2010; Chesman et al., 2007; Goksøyr et al., 1994; Vincze et al., 2015). Caging studies enable control of both animal placement and duration of exposure and most importantly, reflect exposure with natural background influences in a more realistic manner compared to laboratory experiments (Oikari, 2006).

Therefore, the aim of this study was to investigate whether environmental contaminants leaking from the waste disposal site in Kollevåg could accumulate and cause adverse biological responses in fish inhabiting the area, using caged cod as a proxy. Atlantic cod were caged at the site for a period of six weeks, and contaminant concentrations were determined in cod liver and in sediments, in addition to PAH metabolites in bile as a biomarker of PAH exposure. Biomarkers of effect were determined at transcript and protein levels in liver, ovaries and plasma to assess possible impacts on oxidative and biotransformation stress responses, and reproductive processes.

2. Methods

2.1. Fish husbandry, caging and sampling

The experimental animals were juvenile, farmed Atlantic cod (mean weight 289.8 ± 73.2 g for all groups) that originated from Austevoll Research Station, Institute of Marine Research (IMR). Fish husbandry was performed in compliance to Norwegian animal welfare act and national regulations on the use of animal in research. The Norwegian Food Safety Authorities approved the experimental setup (Application number FOTS #9195) and the experiments were performed accordingly. Cod were given feed pellets up until two weeks prior to caging, after which pellets were gradually replaced with crustaceans (*Mysis* sp.). Cages (1.22 m^3) with 22 cod per cage were placed on the seabed at four different locations. Three stations were located in or near the capped waste disposal site in Kollevåg, Askøy, while one cage was placed at a reference location close to Ertenøyane (Fig. 1; Table S1). Conductivity, temperature and depth (CTD) profiles taken at deployment and retrieval of the cages are found in Figs. S1–S4. The cod were caged for six weeks from early September to mid-October 2016. The cod were transferred to tanks on board R/V Hans Brattstrøm, and euthanized with a blow to the head. Tissue samples were collected and frozen in liquid nitrogen before being transferred to −80 °C for downstream analyses. Regarding gonads, only female ovaries were collected due to the small size of male testis. For chemical analysis, liver tissue was stored at −20 °C until analysis. Plasma samples were obtained from whole blood by centrifugation for 10 min at 1000 g × 4 °C. Mucus was sampled using the TECO® Mucus collection set according to manufacturer's instructions (Product no. TE1034, Pathway diagnostics ltd, Dorking, UK).

2.2. Growth and condition parameters

Hepatosomatic index (HSI = liver mass (g)•100/body mass (g)) and Fulton's condition factor (CF = body mass (g)/body length (mm) •100,000) were used to evaluate the post-caging condition of the cod.

2.3. Chemical analyses

2.3.1. Sediment sampling and analyses

Sediment was collected from four spots at each station using a van Veen grab (KC Denmark, Silkeborg, Denmark). Sediment from the different spots were pooled to one sample, consisting of the upper 1 cm of the sediment layer, and was transferred to rilsan plastic bags (Tub-Ex ApS, Taars, Denmark). In order to not disturb the fish, sediments were sampled after recovery of the cages. The samples were frozen at −20 °C for four months until elemental analyses with standardized methods at Eurofins Environment Testing Norway AS (accredited by the Norwegian Accreditation, NS-EN ISO/IEC 17025). These analyses included arsenic, lead, cadmium, copper, chromium, nickel and zinc according to NS EN ISO 17294-2; Mercury was analyzed with a modified version of EN ISO17852; Loss of ignition and dry matter by NS 4764; PCB-7 according to EN 16167; PAH-16 by a modified version of ISO 18287 and grain size distribution by gravimetrics. The concentrations of metals, PCBs and PAHs were compared to the environmental quality standards (EQS) for organic contaminants in sediment according to EU legislation, summarized in the Norwegian water framework directive (Norwegian Environment Agency, 2018).

2.3.2. Cod liver analyses

2.3.2.1. OCPs, PCBs, BFRs and PFASs. The chemical analyses of persistent organic pollutants (POPs) and PFASs in cod livers were performed at the Laboratory of Environmental Toxicology, Norwegian University of Life Sciences (NMBU), Oslo, Norway. The laboratory is accredited by the Norwegian Accreditation for chemical analysis of OCPs, PCBs and BFRs in biota according to the requirements of the NS-EN ISO/IEC 17025 (TEST 137). PFAS analysis is not included in this accreditation, but it is validated according to the same procedures. POPs and PFASs were analyzed in eight pooled samples from each cage by assembling 2 g of cod liver from two fish of same sex in each pool (four pooled samples of each sex per cage). The analytical method for OCPs, PCBs and BFRs is based on Brevik (1978) and Polder et al. (2014), whereas the analytical procedure for PFASs is described by Grønnestad et al. (2017). The procedures are described in detail in supplementary material (S2).

2.3.2.2. Metals. Metals were analyzed in the pooled liver samples, i.e. the samples included in the chemical analyses described above, at the Faculty of Environmental Sciences and Natural Resource Management (MINA), Norwegian University of Life Sciences (NMBU). The liver samples were weighed, with approximately 500 mg, in ultrapure teflon tubes pre-rinsed in 7 M HNO_3 and MQ water, before 5 mL HNO_3 (Ultrapure, subboiled) was added. As internal standards, Sc, Ge, Rh,

In, Bi and Au were added. Further, the samples were decomposed at 260 °C for 20 min in an UltraClave (Milestone Inc, Shelton, CT, USA). After digestion, the samples were diluted to 50.0 mL using distilled water in centrifuge tubes (Sarstedt, Nümbrecht, Germany). The elements were quantified with an Agilent 8900 ICP-MS-MS in oxygen reaction mode using standards for each element.

The analytical quality was approved by satisfactory quality control measures, and is further described in supplementary material (S2).

2.3.3. PAH metabolites in bile

Analysis of PAH metabolites in cod bile was performed at the Norwegian Institute for Water Research (NIVA). The method is previously described by Krahn et al. (1992) and in more detail in Kammann et al. (2013) (lab code 7). In brief, PAH metabolites were hydrolyzed with β-glucuronidase/arylsulphatase, separated with high performance liquid chromatography (HPLC) and detected by a fluorescence detector. Fluorescence was measured at the following wavelengths (nm, excitation/emissions): 1-OH-phenanthrene 256/380; 1-OH-pyrene 346/384; triphenylamine 300/360; 3-OH-benzo[*a*]pyrene 380/430).

2.4. RNA extraction and real-time-qPCR

2.4.1. Liver tissue

Liver tissue (100 mg) from Atlantic cod was homogenized with the Precellys 24 homogenizer by using ceramic beads CK28 (Bertin Technologies, Montigny-le-Bretonneux, France). Total RNA was extracted using Agencourt® RNAdvance™ Tissue Kit (Product no. A32645) on a Biomek NX^{P} workstation (Beckman Coulter Life Sciences, Indianapolis, IN, USA). The samples were treated with DNase according to manufacturer's instructions. RNA concentration, quality and integrity were checked using NanoDrop ND-1000 ultraviolet–visible (UV–Vis) spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). The RNA integrity number (RIN) of all samples were > 7. Transcript levels of target genes (*cyp1a*, *cat*, *gst3ab*, *fasn*, *acly*, *scd*, *hsp70*) and two reference genes (*uba52*, *ef1a*) (primer information in Table S3) were quantified using quantitative PCR (qPCR) as described by Olsvik et al. (2013), conducted according to the MIQE guidelines (Bustin et al., 2009). Mean normalized expression (MNE) of the target genes was determined using a normalization factor calculated by the geNorm software (Vandesompele et al., 2002). The geNorm stability index M for the reference genes was 0.73.

2.4.2. Gonad tissue

RNA was extracted from cod ovaries using Direct-zol™ RNA extraction kit (Zymo Research Corporation Irvine, CA, USA), following the manufacturer's protocol. RNA quality was evaluated using NanoDrop ND-1000 ultraviolet–visible spectrophotometric analysis (Nanodrop Technologies) and formaldehyde agarose gel electrophoresis. cDNA was generated by following the instructions of iScript cDNA synthesis kit (Bio-Rad Laboratories Hercules, CA, USA). Primers specific for Atlantic cod *star*, *cyp17*, *p*450scc, *cyp19*, *3β-hsd*, *11β-hsd*, *17β-hsd*, and *20β-hsd* genes were amplified using iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories) and Mx3000P real-time PCR machine (Stratagene, La Jolla, CA, USA). A detailed qPCR procedure and primer sequences are presented in the supplementary material (S3.2 and Table S4).

2.5. Liver protein analyses

Liver S12 fractions for various protein analyses were prepared as follows: Liver samples were kept on dry ice while cut and homogenized with a Precellys 24 homogenizer using 2.8 mm bulk zirconium oxide beads (Ref no. KT03961-1-102.BK, Bertin Technologies) in homogenization buffer as previously described (Nilsen et al., 1998). Post-mitochondrial supernatant (S12) fractions were obtained by centrifugation for 20 min at 12,000 g × 4 °C. Protein concentration of individual aliquots were determined using Pierce™ 660 nm Protein Assay Reagent (Catalog no. 22660, TermoFischer Scientific, Waltham, MA, USA). For the enzyme and TBARS assays, the results were normalized to the protein concentration of individual S12 fractions. All spectrophotometric and fluorometric measurements for the following liver analyses were performed using the 2300 EnSpire™ Multilabel Reader (Perkin Elmer, Turku, Finland). Liver S12 fractions to be used as positive controls for Cyp1a induction were prepared by injecting Atlantic cod intraperitoneally with 50 mg/kg β-naphthoflavone (BNF), and euthanizing the cod one week after injection. However, the liver tissue preparation (homogenization and centrifugation) to obtain the S12 fractions were identical for positive controls and Kollevåg samples.

2.5.1. Western blotting

Liver S12 fractions were subjected to SDS-PAGE and western blotting according to Laemmli (1970) and Towbin et al. (1979), respectively. 15% polyacrylamide gels were utilized, and 7 μg protein was loaded into each well. Primary antibodies utilized were anti-Cyp1a (1:800), anti-Cyp3a (1:800), anti-Mt (1:1000) and anti-actin (1:1000), and HRP-conjugated anti-IgG was used as secondary antibody (Table S5). Blots were developed with SuperSignal™ West Femto Maximum Sensitivity Substrate (Item no. 34095, ThermoFischer Scientific) and visualized using the ChemiDoc™ XRS + System (Bio-Rad Laboratories). For protein quantification, individual band intensities were divided by the intensity of the corresponding actin band using the Image Lab™ Software (Bio-Rad Laboratories).

2.5.2. Antioxidant enzyme activity

The enzyme activities of Cat and Gst were analyzed using Catalase Assay Kit and Glutathione-S-transferase Assay Kit (Item no.707002 and item no. 703302, Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The liver S12 fractions were diluted in the kit's separate sample buffers 1:2–3 and 1:80 for the Gst and Cat assays, respectively.

2.5.3. EROD activity

The 7-ethoxyresorufin *O*-deethylase (EROD) activity was determined in gills and in liver tissue. The EROD activity in liver was measured in S12 fractions as described in Burgeot et al. (1996), using the method of Burke and Mayer (1974), modified for plate readers by Galgani and Bocquene (1991). The EROD activity in gill filaments was performed essentially as described in Holth et al. (2014).

2.5.4. Lipid peroxidation (TBARS)

Lipid peroxidation was assessed in liver S12 fractions using the TBARS (TCA method) assay kit according to manufacturer's instructions (Item no. 700870, Cayman Chemical). The S12 fractions were diluted 1:2–10 in deionized H_2O prior to analyses. Samples were heated at 99 °C using a heating block (Eppendorf® Thermomixer® R, Sigma-Aldrich, St.Louis, MO, USA).

2.6. Quantification of plasma and mucus Vtg by ELISA

Levels of Vtg in blood plasma were determined both in male and female cod, using the Cod (*Gadus morhua*) Vitellogenin ELISA kit (Item no. V01006401-480, Biosense Laboratories AS, Bergen, Norway) according to manufacturer's instructions. The plasma samples were diluted 1:50 in dilution buffer before being analyzed. Levels of Vtg were also measured in mucus using the TECO™ Multi-species Vitellogenin ELISA kit (Product No. TE1042, Pathway diagnostics ltd, Dorking, UK).

2.7. Steroid hormone analysis

Enzyme immunoassay (EIA) was used to determine the

concentration of 17β-estradiol (E2) and testosterone (T) in plasma from female cod by using respective EIA kits (Item no. 582271 and Item no. 582701, Cayman Chemical). A detailed description of hormone extraction and quantification is presented in supplementary material (S5).

2.8. Statistics

Detection rate is defined as percentage of samples with a detectable value, i.e. above limit of detection (LOD). For POPs, PFASs and metals in liver, the compounds with detection rate below 50% within each cage are not included in further statistical analyses and only reported with range in Tables S8–S11. Compounds with detection rate above 50% within each cage are reported with descriptive statistics in Fig. 2 and Tables S8–11, the actual determined levels for samples below LOD are used, and missing values are replaced with the lowest determined level of the compound. For metals with detection rate above 50%, levels below LOD are replaced with ½ LOD and levels between LOD and limit of quantification (LOQ) are replaced with ½ LOQ.

For all results presented, Graphpad Prism 7 (Graphpad Software, San Diego, CA, USA) and R version 3.4.3 (R Core Team, 2017) were used for statistical analyses. Normality of the data was tested using Shapiro-Wilk. If one of the stations failed the Shapiro-Wilk test, the responses of all stations were log-transformed. Responses following normal distribution were analyzed for station-dependent (Kollevåg stations vs reference station) statistical differences ($p < 0.05$) using a two-sample *t*-test with Welch Correction. Nonparametric tests (Mann-Whitney) were used to analyze responses failing Shapiro-Wilk after being log-transformed. In both tests, a two-sided alternative was used. To account for multiple testing, a Bonferroni corrected significance level 0.05/(3*34) = 0.00049 was applied in addition to the ordinary 0.05 significance level. Sediment results could not be analyzed statistically (n = 1).

To visualize the results, the investigated parameters were displayed in a spider plot (only reproduction parameters are shown). All values were rescaled to [0, 1] before calculating mean values by station. In addition, all determined parameters were combined in a principle component analysis (PCA). For the PCA, all missing data was imputed using the median values by station. The first two principle components and their eigenvectors are reported. In addition to a PCA, the relationship between all different parameters was visualized in a correlation plot. Pearson correlation was calculated and the parameters were plotted in a graph where parameters with a positive or negative correlation above 0.7 were connected. All parameters without any connections above this correlation cut-off were removed from the plot. The nodes were colored by parameter family (PCBs, OCPs, reproduction parameters, BFRs, liver transcripts, metals, PAHs, or PFASs).

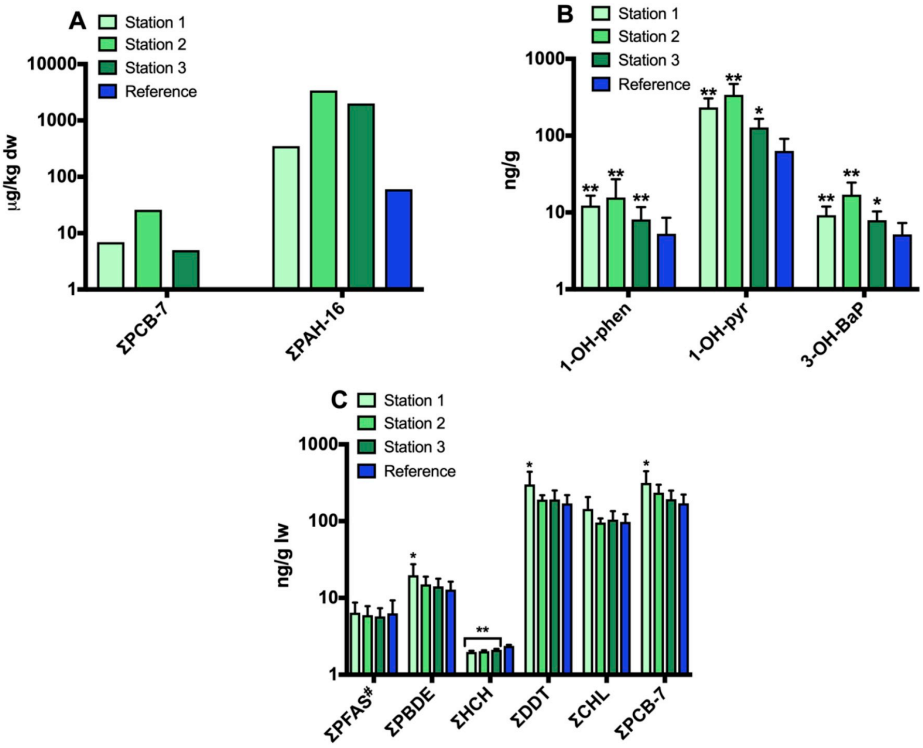

Fig. 2. Contaminant concentrations in sediments from caging areas and in liver and bile of Atlantic cod caged in Kollevåg. Sediment concentrations are presented as µg/kg dry weight (dw) (A) and OH-metabolites of phenanthrene (1-OH-phen), pyrene (1-OH-pyr) and benzo(a)pyrene (3-OH-BaP) in bile (B) are presented as ng/g bile. In liver (C), contaminant concentrations are presented as ng/g lipid weight (lw), except ΣPFAS which is expressed as ng/g wet weight[#]. The y-axis is presented in log scale for all figures. For sediment analyses, four samples from each caging area were pooled prior to analyses (A). n = 8 for all stations in liver (C) measurements (two cod of the same sex were combined per n measurement) and n = 19, 22, 16 and 21 for stations 1, 2,3 and reference stations in bile (B) measurements, respectively, data are presented as mean ± s.d. ΣPCB-7 in sediments for the reference station were non-detectable. Asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. Double asterisk (**) indicates p-values below the Bonferroni corrected significance level ($p < 0.00049$).

3. Results

3.1. PCBs, PAHs and other contaminants were detected in sediments and cod tissues

The station-dependent differences in contaminant burdens in sediments are shown in Fig. 2A and Table S6. All Kollevåg stations had higher sediment concentrations of PCBs and PAHs, compared to the reference station. ΣPCB-7 exceeded EQS (4.1 μg/kg dw) for all three Kollevåg stations (6.9, 26 and 5.0 μg/kg dw for station 1, 2 and 3, respectively). No EQS exists for ΣPAH-16 in the EU water framework directive, but several of the individual PAHs observed in sediments (anthracene, fluoranthene, pyrene, benzo[a]anthracene, benzo[b] fluoranthene, benzo[a]pyrene, indeno[1,2,3cd]pyrene, dibenzo[a,h] anthracene and benzo[ghj]perylene) exceeded their respective EQSs in one or several Kollevåg stations (Table S6). All Kollevåg stations had higher concentrations of all determined metals, except Cd, compared to the reference station, but the concentrations did not exceed the corresponding EQS (Table S6). The organic content within the sediments, measured as loss on ignition (LOI), were similar for the Kollevåg stations, but higher compared to the reference station (Table S7).

Selected hydroxylated PAH metabolites were determined in bile and the concentrations of selected contaminants including PCBs, DDTs, CHLs, PBDEs, PFASs and metals were determined in liver (detailed overview in Tables S8–S11). Significantly higher concentrations ($p < 0.05$) of the PAH metabolites 1-OH-phenanthrene, 1-OH-pyrene and 3-OH-benzo[a]pyrene were observed in cod bile from all Kollevåg stations compared to cod from the reference station (Fig. 2B). Station 2 had the highest concentrations for all three metabolites, followed by station 1 and station 3. In cod livers, ΣPCB-7, ΣDDTs and ΣPBDEs were significantly higher in cod from station 1, compared to the reference station (Fig. 2C). Cod from all Kollevåg stations had significantly lower levels of ΣHCHs compared to the reference ($p < 0.05$). No significant differences were found between stations for ΣPFASs, ΣCHLs and metals (Fig. 2C; Tables S8, S9 and S11).

3.2. CF and HSI were reduced in cod caged in the inner parts of Kollevåg

The parameters indicating growth performance, i.e. CF and HSI, are presented in Fig. 3. Compared to cod from the reference station, both CF and HSI were significantly reduced ($p < 0.05$) for cod from station 1, but not for the other Kollevåg stations (Fig. 3A and B).

3.3. Station-dependent differences in abundance of liver and ovarian gene transcripts

The transcript levels of selected genes were studied to elucidate any station-dependent differences in gene expression levels. Only a few significant differences ($p < 0.05$) in expression levels were observed among the eight genes investigated in cod liver (Fig. 4A). Station 1 had a significantly higher expression level for *acly*, *fasn* and *gsta3b*. *Gsta3b* was significantly higher also for station 3, compared to the reference station. No significant changes were observed between the Kollevåg stations and the reference station for *cyp1a*, *cat*, *hsp70*, *fabp7* and *scd*. Among ovarian steroidogenic genes, significant increases ($p < 0.05$) were observed for *p450scc* and *20β-hsd* in an apparent station-dependent manner (Fig. 4B). Station 1 and 2 had significantly higher expression level for *cyp17*, *cyp19* and *3β-hsd*, while station 1 and 3 were highest for *17β-hsd*. Compared to the reference site, *star* and *11β-hsd* were significantly increased in station 2.

3.4. Cyp3a and Mt, but not Cyp1a, were detected in cod liver samples using protein immunoblotting

Western blots were performed to study levels of Cyp1a, Cyp3a and Mt in cod liver from the different stations. Cyp3a and Mt were detected in livers from all stations (Fig. 5) whereas Cyp1a was only detected in the positive control (Fig. 5A). When normalizing the band intensities of Cyp3a and Mt to the loading control (Actin), no significant differences in relative protein levels were seen for Cyp3a (Fig. 5B), however cod from station 1 and 2 had significant lower liver levels ($p < 0.05$) of Mt compared to the reference station (Fig. 5C).

3.5. No difference in Cyp1a (EROD) activity in cod caged at the different locations

EROD activities were assessed in both cod gill filaments and liver tissues, in order to reveal any changes in Cyp1a activity between the stations. In both tissues, very low activities (< 10 pmol/min/mg in liver, and close to, or below, the detection limit in gills), and no significant differences were observed when comparing the Kollevåg stations to the reference station (data not shown).

3.6. Reduced lipid peroxidation and antioxidant enzyme activities in Kollevåg stations

The enzymes Cat and Gst were studied to assess possible station-dependent differences in oxidative stress levels. The activities of both enzymes were significantly lower ($p < 0.05$) in cod from station 1 and 2, compared to cod from the reference station (Fig. 6A and B). Furthermore, the TBARS assay was performed to study the degree of lipid peroxidation and thereby possible oxidative stress in the cod livers. Compared to the reference station, cod from all Kollevåg stations had significantly lower hepatic concentrations ($p < 0.05$) of TBARS (Fig. 6).

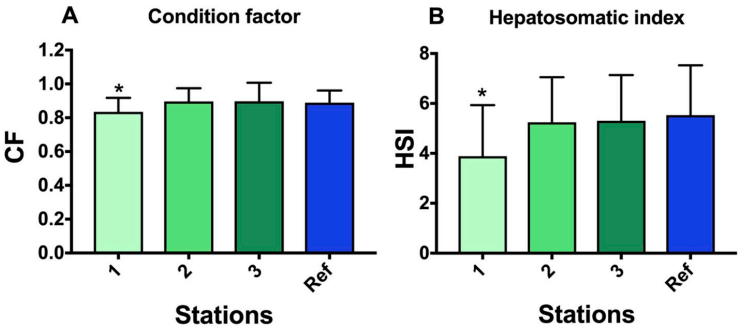

Fig. 3. Growth performance of caged Atlantic cod. Post-caging condition was assessed by calculating CF (A) and HSI (B). Data are presented as mean ± s.d. n = 21, 22, 16 and 23 for stations 1,2,3 and Ref, respectively, asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. None of the responses had p-values below the Bonferroni corrected significance level ($p < 0.00049$).

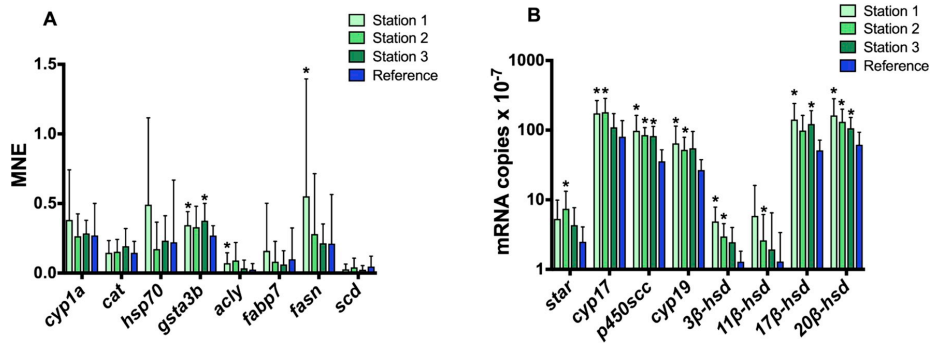

Fig. 4. Transcript levels of selected genes determined in liver and ovary samples from caged Atlantic cod. Quantitative PCR was performed on liver and ovary tissues from caged Atlantic cod. The expression levels are presented as mean normalized expression (MNE) for liver samples (A) and as transcript copies x 10^{-7} for ovary samples (B). n = 12–15, 17–20, 13–15 and 15–19 for stations 1, 2, 3 and Ref, respectively (A). n = 10, 7, 8 and 9 for stations 1, 2, 3 and Ref, respectively (B). Data are presented as mean values ± s.d. Asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. None of the responses had p-values below the Bonferroni corrected significance level ($p < 0.00049$).

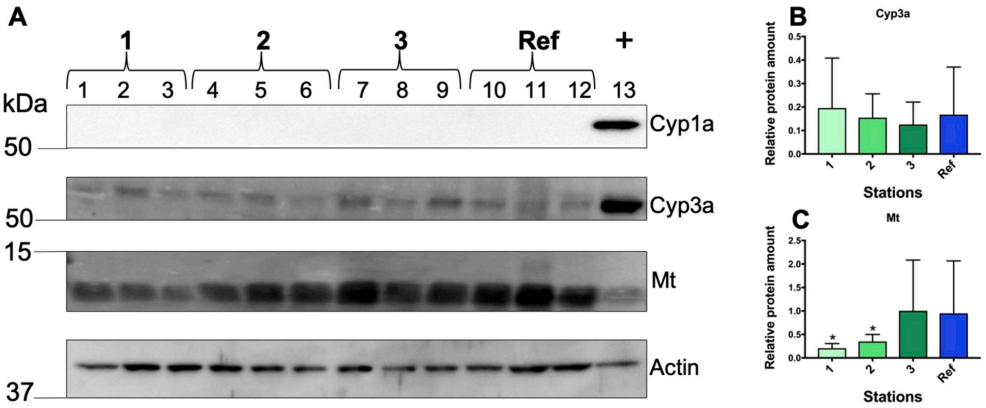

Fig. 5. Levels of Cyp1a, Cyp3a and Mt in liver S12 fractions of caged Atlantic cod. All samples were analyzed with western blotting and representative blots are shown in A and include the proteins Cyp1a, Cyp3a, Mt and Actin. The protein sizes are indicated on the left. Three samples representing individual fish from each station (1, 2, 3 and Ref in bold) are shown in A, 1–13 indicate well numbers. The positive control (+) in well no.13 is liver S12 fraction of cod exposed to 50 mg/kg BNF. For the protein quantification (B-C), individual band intensities were divided by the intensity of the corresponding actin band. n = 18, 18, 12 and 13 for stations 1, 2, 3 and Ref, respectively, and mean values ± s.d. are presented in B-C. Asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. None of the responses had p-values below the Bonferroni corrected significance level ($p < 0.00049$).

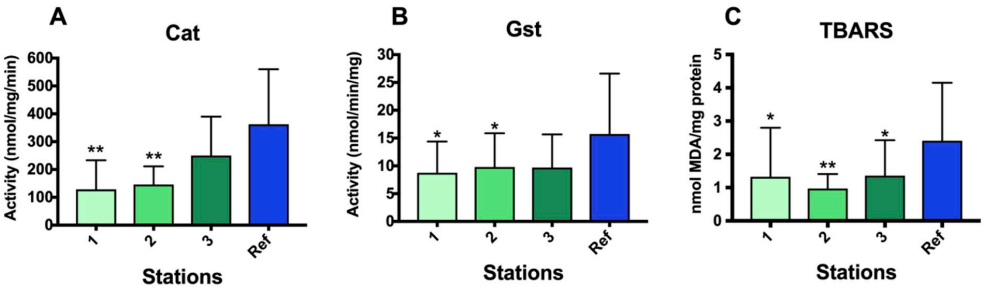

Fig. 6. Enzyme activities of Cat and Gst, and TBARS levels in liver S12 fractions of caged Atlantic cod. The enzyme activities of Cat (A) and Gst (B) and TBARS levels (C) were determined in cod liver S12 fractions. n = 18, 20, 13 and 17 (A), n = 18, 21, 15 and 20 (B) and n = 21, 22, 16 and 23 (C) for stations 1, 2, 3 and Ref, respectively. Asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. Double asterisk (**) indicates p-values below the Bonferroni corrected significance level ($p < 0.00049$).

3.7. Limited changes were observed in steroid hormones and Vtg levels in cod plasma

The plasma concentrations of E2 and T in female cod were determined using EIA. Cod from station 1 showed a significant increase ($p < 0.05$) in E2 concentrations compared to cod from the reference station (Fig. 7A), whereas no significant differences were found for T concentrations (Fig. 7B). Concentrations of Vtg were determined both in blood plasma and in mucus of male and female cod. No significant differences in plasma (Fig. 7C) or mucus (data not shown) Vtg concentrations were observed in comparisons among the stations or between males and females.

3.8. Combination of parameters show station-specific patterns and link biological responses to contaminant accumulation

The measured parameters involved in reproductive and steroidogenic pathways were combined in a spider plot to better visualize station-specific patterns (Fig. 8). The plot shows that the reference station is clearly separated from the Kollevåg stations regarding expression of steroidogenic enzyme genes. For steroid hormones and Vtg, this pattern is less clear, and only station 1 is markedly different from the reference station for E2 and T.

All chemical (individual compounds) and biological results except sediment data (due to n = 1) were combined in a principal component analysis (PCA) to clarify whether the contaminant levels and biological responses grouped in a station-dependent manner. In this analysis, the innermost stations of Kollevåg, stations 1 and 2, separated from the reference station, while station 3 was closer to and intersected with the 95% confidence ellipse of the reference station (Fig. 9A). Approximately 49% of the total variance was explained by the first two principal components (PCs). Using a Kruskal-Wallis-test, both PCs showed a statistically significant relationship to the stations ($p < 0.0001$) and Freeman's theta was higher for PC2 (0.757) than PC1 (0.643), indicating that PC2 captured more of the environmental differences between the stations. When studying the corresponding eigenvalues (Table S12), it was found that ovarian transcripts, bile PAHs, oxidative stress parameters, steroid hormones and some liver contaminants, explained most of the variability in PC2. The same chemical and biological data were subjected to a correlation analysis (Fig. 9B), revealing that among the biological data, the reproductive responses showed the highest correlations ($r \geq 0.70$) with liver contaminant levels. Using a lower correlation cut-off (< 0.70), the oxidative stress and condition parameters also correlated with the liver contaminant levels, whereas responses of liver transcripts, sex and Cyp3a seemed less correlated to other parameters (data not shown).

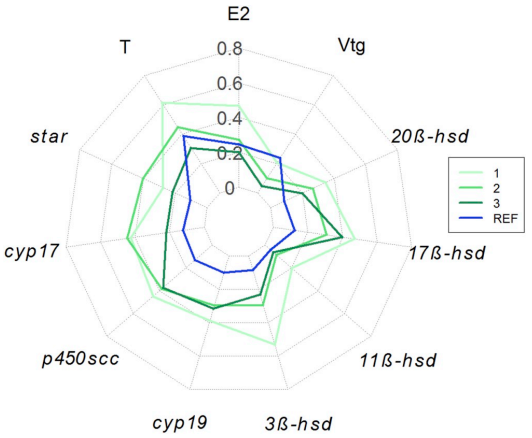

Fig. 8. Spider plot combining investigated reproduction parameters. The plot shows combined reproductive gene expression (italics) in ovaries and EIA (T, E2) and Vtg measurements in plasma of female cod. The values for all female fish were rescaled to [0, 1] and the mean values by station are shown.

4. Discussion

4.1. Contaminant concentrations in cod tissues reflect environmental concentrations

The present study investigated contaminant accumulation and biological responses of Atlantic cod caged for six weeks at a formerly capped waste disposal site in Kollevåg in Western Norway. We found that the sediment levels of contaminants, such as PAHs and PCBs, were higher in Kollevåg compared to the reference station (Fig. 2A). The main dumping site was located in Vestrevågen, close to station 1 in our study, whereas stations 2 and 3 were located further away from the original dump site (approx. 400–700 m), in an area that had not been capped. The results showed, however, that the sediments from station 2 contained the highest levels of PCBs and PAHs, and several of the compounds (e.g. BaP and pyrene) exceeded their respective EQSs (Norwegian Environment Agency, 2018). These findings are consistent with previous monitoring surveys in Kollevåg (Hatlen et al., 2013; Hatlen and Johansen, 2016), and might be explained by the location of station 2 at the borderline between two bays: Vestrevågen (where the capped area resides) and Medavågen (Fig. 1). Previously, elevated concentrations of both PCBs and PAHs have been detected in Medavågen, possibly from former local dumping activities. Thus, station 2

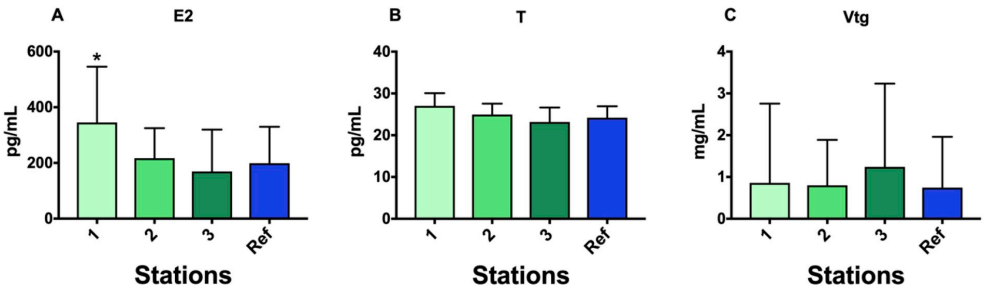

Fig. 7. Concentrations of estradiol, testosterone and vitellogenin in plasma of caged Atlantic cod. Estradiol (E2), testosterone (T), and vitellogenin (Vtg) concentrations (A, B and C, respectively) were determined in plasma samples. n = 7, 6, 7, 9 (A and B) and 19, 21, 16, 21 (C) for stations 1, 2, 3, Ref, respectively. Data are presented as mean ± s.d. Asterisk (*) indicates statistical significance ($p < 0.05$) between Kollevåg stations (1–3) and reference station. None of the responses had p-values below the Bonferroni corrected significance level ($p < 0.00049$).

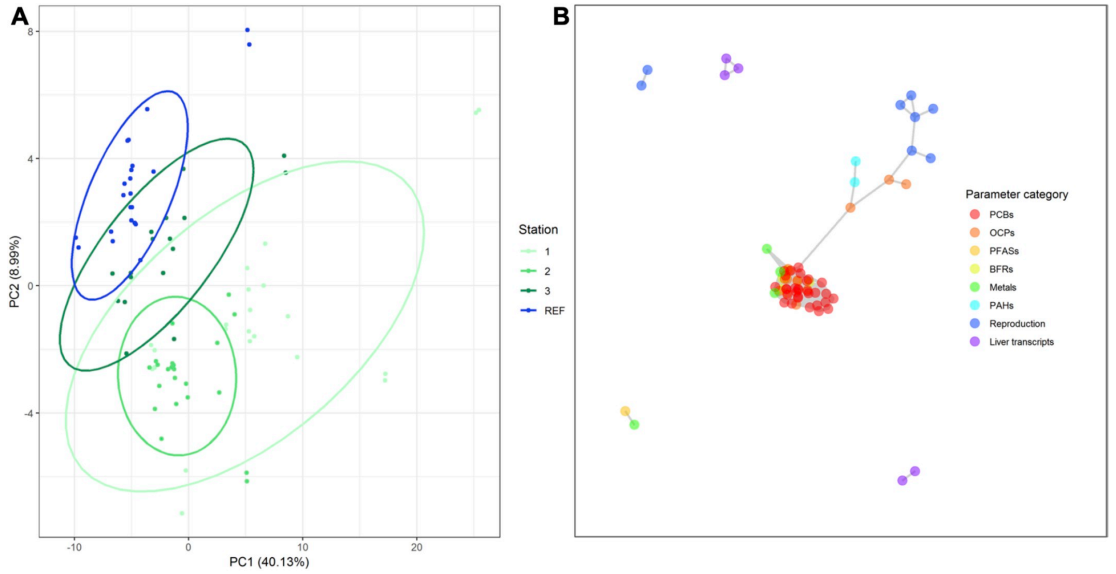

Fig. 9. Combination of measured parameters in principal component analysis (PCA) and correlation analysis. The data included is liver contaminant levels (individual compounds) and biliary PAH metabolites, condition parameters, qPCR data, oxidative stress analyses and parameters involved in steroidogenic and reproductive pathways. For the PCA (A), individual scores of the first and second principal component (PC) are plotted on an x-y scatter, giving a two-dimensional visualization of 49.12% of the variance. Each dot represents individual fish (n = 84). The ellipses are station-wise 95% confidence ellipses calculated from the first two principal components. The right panel (B) shows a correlation network where parameters which are correlated with a Pearson correlation above 0.7 or below −0.7 are connected. Each dot represents one parameter and parameters without connections were removed. The liver contaminants are grouped by PCBs, OCPs (including HCHs, CHLs and DDTs), BFRs (including PBDEs, HBB and HBCD), PFASs and metals, while reproduction includes ovarian genes, steroid hormones and Vtg.

may receive contaminant inputs from both bays (Hatlen and Johansen, 2016). Furthermore, drainage from Medavågen is also channeled into areas close to station 3, which may explain the similar and higher levels of PCBs and PAHs in sediments from station 3 compared to station 1. The present study shows that despite the capping of the main dump site in 2004, sediments within and outside the capped area in Kollevåg are still contaminated with PAHs and PCBs at concentrations of environmental health concern, with specific regards to long-term exposure and chronic toxic effects in the local fauna.

Bile PAH metabolites (in ng/g bile) from all Kollevåg stations and liver Σ7PCBs, ΣDDTs and Σ7PBDEs (in ng/g lipid weight) in cod from station 1, were significantly higher compared to cod from the reference station (Fig. 2). This demonstrates that caging of cod on the seabed for a six-week period resulted in accumulation of contaminants from the sediment, through water (gills and intestine) and/or through feeding on epibenthic organisms. The highest levels of bile PAH metabolites were observed in cod from station 2, which agrees with the pollution burdens observed in the sediments. Background and environmental assessment criteria (BAC/EAC) for phenanthrene and pyrene metabolites are 2.7/21 ng/g bile and 518/483 ng/g bile, respectively (Hylland et al., 2012). Three of the cod caged at station 2 exceeded EAC, indicating exposure of environmental concern. No assessment criteria have been developed for 3-OH-BaP, but the concentrations of this metabolite suggest elevated toxicological risk, since BaP is known to possess higher carcinogenic potential than many other PAHs (Delistraty, 1998). Liver PCB concentrations, however, were highest in station 1, while the sediment PCB concentrations were highest in station 2. This finding suggests that other factors, in addition to the sediment concentrations, can influence the exposure and contaminant accumulation in the caged cod, including water contaminant concentrations, the bioavailability of sediment-bound contaminants and accumulation in the local food web etc.

4.2. Condition parameters indicate health implications in cod caged at inner parts of Kollevåg

The condition parameters CF and HSI were significantly reduced for cod from station 1, compared to the reference station (Fig. 3). CF and HSI indicate energy status of the fish and a reduction can be caused by either food shortage, contaminant exposure and/or other factors (e.g. parasite infections) (Chellappa et al., 1995; Rätz and Lloret, 2003). Although Adeogun et al. (2016b) and Scarcia et al. (2014) attributed increased HSI and CF to contaminant exposure, PCB and other contaminant exposures are also reported to reduce condition parameters (Adeogun et al., 2016a; Ozmen et al., 2006; Scarcia et al., 2014). It is therefore uncertain whether the observed reductions in condition indices were caused by contaminant exposure or food shortage, or a combination of these.

4.3. Conflicting results among biomarkers of stress response, detoxification and oxidative stress responses in cod liver

We quantified the expression of genes involved in various biological pathways in cod liver (Fig. 4A). Increased expression of the lipogenic marker enzyme genes, *acly* and *fasn* might be related to the higher levels of PCBs detected in station 1, compared to the reference station. Indeed, increased mRNA of *acly* and *fasn* transcripts, and Acly protein levels have been observed in cod liver exposed to PCB153 (Yadetie et al., 2017, 2014). These results are in accordance with other studies showing that PAHs and POPs, such as PCBs, are able to interfere with lipid metabolism (Grün and Blumberg, 2007; Ruzzin et al., 2010; Vieweg et al., 2018).

Cyp1a is an established biomarker for PAHs and dioxin-like PCBs (Brammell et al., 2010; Goksøyr, 1994; van der Oost et al., 2003; Whyte

et al., 2000). Surprisingly, despite our findings showing elevated levels of PAH metabolites in bile and dioxin-like PCBs in liver in cod from Kollevåg stations, Cyp1a was not significantly induced at neither the gene (Fig. 4A) nor protein level (Fig. 5), including enzymatic activity. Lack of Cyp1a induction may be explained by factors such as low concentrations of the inducers, or possible inhibitory effects of some components (e.g. fluoranthene) in the mixture (Billiard et al., 2004; Goksøyr et al., 1986; Wolińska et al., 2013; Brown et al., 2016; Hylland, 2006). Low inducibility and in some cases inhibition of Cyp1a activity in flounder (*Platichthys flesus*) exposed to selected PCB congeners has been reported (Besselink et al., 1998). Chronic contaminant exposure has also been shown to cause reduced Cyp1a inducibility in some fish species (Brammell et al., 2013).

In addition to Cyp1a, levels of Cyp3a and Mt were also assessed by immunoblotting. Whereas no significant differences in levels of Cyp3a were observed, a significant decrease in the levels of Mt were detected at station 1 and 2. The Mt decrease might be attributed to exposure to BaP and PCBs, contaminants previously shown to inhibit Mt synthesis and activity (Hurk et al., 2000; Maria and Bebianno, 2011; Sandvik et al., 1997).

Environmental contaminants can both induce and inhibit antioxidant enzyme activities (Benedetti et al., 2015; Scarcia et al., 2014) and reviewed in van der Oost et al. (2003). In the present study, hepatic activities of the antioxidant enzymes Gst and Cat were significantly reduced in the innermost stations of Kollevåg (Fig. 6). Benedetti et al. (2015) suggested that oxidative stress biomarkers can be induced in the initial phase of a response, followed by a depletion after longer periods. Other studies have indicated that an excessive ROS production can overwhelm the antioxidant system through direct interaction with enzymes, depletion of essential cofactors or substrates, or downregulation of corresponding transcripts (Dale et al., 2017; Shao et al., 2012). The reduction in enzyme activities could not be related to increased lipid peroxidation, as significant decreases in TBARS levels were observed in cod from stations 1 and 2. In accordance with our results, other studies suggested that there is no obvious link between antioxidant enzyme activities and lipid peroxidation (Oruç, 2010; Talcott et al., 1979).

4.4. Station-specific differences in biomarkers involved in reproductive pathways

To investigate possible endocrine disrupting effects, we analyzed the expression of a set of ovarian genes involved in the steroidogenic pathways (Fig. 4B). The gene encoding cytochrome P450 aromatase (*cyp19*), responsible for catalyzing the conversion of androgens to estrogens (Cheshenko et al., 2008), showed significantly higher expression levels at Kollevåg stations 1 and 2, compared to the reference station. Other genes including steroidogenic acute regulatory protein (*star*), cytochrome P450 side-chain cleavage (*p*450scc), 3β-hydroxysteroid dehydrogenase *(3β-hsd), cyp17, 11β-hsd, 17β-hsd* and *20β-hsd,* which are also involved in the stepwise synthesis of sex hormones (progesterone, testosterone and estrogens) (Abbaszade et al., 1997; Adamski and Jakob, 2001; Kime and Ebrahimi, 1997; Miller et al., 1997) followed similar expression patterns as *cyp19.* In addition to the transcript results, it was shown that female cod from station 1 had significantly higher levels of circulatory E2 compared to the reference (Fig. 7). Albeit non-significant, a decreasing trend in the T level was observed across the stations. E2 and T play important roles in the development of female and male reproductive organs and control reproductive functions in adults. In accordance with our results, previous studies have shown that some PCBs, PBDEs and DDTs induce steroidogenesis, however, other congeners decrease the components of this pathway (Gregoraszczuk et al., 2008b, 2008a; Kraugerud et al., 2010; Murugesan et al., 2007; Song et al., 2008). Further, E2 binds to the estrogen receptor (ER) and induces the synthesis of Vtg, a precursor of yolk protein important for reproduction (Arukwe and Goksøyr, 2003; Tyler et al., 1996). Despite the higher E2 levels in cod at station 1, no significant differences in Vtg levels were found between stations or sex. Previously, decrease or no change in the levels of Vtg was reported in female fish exposed to PCBs and PAHs in the wild (Casillas et al., 1991). Additionally, it is known that contaminants that bind and activate the ER can antagonize AhR pathway effects (Marques et al., 2013), while AhR agonists can antagonize estrogenic effects (Boverhof et al., 2008), resulting in ER-AhR cross-talk (Göttel et al., 2014; Mortensen and Arukwe, 2007). Complex contaminant interactions are important in understanding their effects on aquatic organisms as these may be synergistic, antagonistic or agonistic in nature (Celander, 2011; Silva et al., 2002). Considering the higher levels of PCBs, DDTs and PBDEs in cod liver from station 1 compared to the reference, the possible endocrine disrupting effects may be related to the complex mixture of environmental contaminants at the Kollevåg sites. Hence, these results may indicate possible effects on reproductive processes in feral fish inhabiting the area.

4.5. Combined chemical and biological parameters created a station-specific pattern

In order to investigate the pattern of all measured parameters in our study, we collected liver chemistry and bile PAH metabolite data of individual compounds and all biological responses in a PCA. Combining the contaminant concentrations and biomarker responses revealed a separation from inner to outer parts of Kollevåg, with station 3 grouping closest to the reference station, while station 1 and 2 were separated from the reference, using 95% confidence ellipses (Fig. 9A). To further identify any relationships between the contaminant levels and biological responses, all parameters were subjected to correlation analysis (Fig. 9B). Among the biological responses, parameters involved in steroidogenic pathways were found to strongly correlate to liver contaminant levels, suggesting a potential impact on reproductive pathways in the cod caged near the old waste disposal site in Kollevåg.

In addition to contaminant exposure, there are several factors that can contribute to the observed station-dependent differences. The complexity within field studies is evident – non-pollution-related confounding factors including individual biological variance (health, sex, metabolic activity, developmental and reproductive status) and environmental contributions (temperature, oxygen saturation, heterogeneity of environmental pollution) can contribute to observed effects (reviewed in van der Oost et al. (2003)). However, our analyses indicate strong correlation between contaminant levels and certain biological responses, suggesting that site-specific contaminant load is the main reason for the observed biomarker effects pointing to possible endocrine disruption.

4.6. Conclusion

In this study, we have shown that levels of PCBs and PAHs in sediments in Kollevåg exceeded the EQSs set in the EU water framework directive, and that these contaminants accumulated in cod tissues during the six-week caging period. Several biological responses were included in cod caged in Kollevåg compared to cod from the reference location, where reproductive endpoints were most prominent. Our results indicate that the area near the capped waste disposal site did not fulfill the requirements for the established environmental quality standards during our caging study, with a potential to affect reproductive processes in local aquatic organisms. The area surrounding station 1 was recapped during summer 2018, with an aim not only to improve conditions at station 1, but also the general environmental quality in the area. The caging strategy may be useful in follow-up studies to investigate the effect of the recapping of the Kollevåg waste disposal site.

Declarations of interests

AG is a major shareholder in Biosense Laboratories AS, supplier of Vtg ELISA kits.

Author contributions

MM, ZT, KHa, FY, RLL, JLL, AA, KHy, OAK and AG were involved in experimental design and sampling planning. KD, MM, ZT, KHa, FY, RLL, JLL, KHy, OAK, and AG contributed to sampling. KD performed liver qPCRs, enzyme activity assays (Gst, Cat, liver EROD), immunoblotting, vitellogenin and TBARS assays, in addition to contributing to statistical analyses. MM contributed to liver chemical analyses, and ZT performed laboratory analyses (EROD in gill filaments, biliary PAH metabolite extraction and data analysis). EK performed ovarian qPCRs and EIA measurements, and MG performed PAH metabolite analyses. HS and NB contributed with statistical and combination analyses. KD was the lead author, MM, ZT, EK, MG, NB, KHa, RLL, and FY wrote parts of the manuscript, and all authors contributed to interpretation of the results and revision of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2019.02.003.

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Supplementary information Paper I

Contaminant accumulation and biological responses in Atlantic cod (*Gadus morhua*) caged at a capped waste disposal site in Kollevåg, Western Norway

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Materials and methods

S1. Caging areas

Table S1 – Position and depth of different stations.

Station	Location / Coordinates	Depth (m)
1	Kollevåg, 060°26.896N; 005°06.922E	14
2	Kollevåg, 060°26.675N; 005°06.926E	19
3	Kollevåg, 060°26.555N; 005°06.991E	38
REF	Ertenøyane, 060°29.123N; 005°02.736E	25

S2. Chemical analyses

S2.1 Liver analyses

S.2.1.1 Chemicals

Cyclohexane, acetone and methanol of HPLC quality were supplied from VWR Chemicals, VWR International S.A.S, Radnor, Pennsylvania. Purified water was obtained from a Milli-Q Gradient A10 water system (Millipore, Bedford, MA, USA). Primary standards were supplied from Ultra Scientific, N. Kingstown, RI, USA (PCB-29, -112 and -207) and Cambridge Isotope Laboratories, Inc., MA, USA (BDE-77, -119 and -181 and 13C12-BDE-209). H_2SO_4 (≥ 97.5%) was supplied from Sigma-Aldrich, Saint-Louis, Missouri, USA. Certified Reference Materials (CRM) (CRM 2525, 350) were supplied by Cerilliant Corporation, Round Rock, TX, USA. Interlaboratory tests (human serum) were supplied by Arctic Monitoring and Assessment Program (AMAP), Québec, Canada. According to the accreditation requirements, all chemicals are routinely quality tested.

S2.1.2 Pooling

For chemical analyses, four pooled samples of each gender were prepared from each cage (in total eight pooled samples from each cage). Liver from two fish of same gender were pooled together by assembling 2 grams of cod liver from each fish in a 50 mL Falcon tube. The pooled sample was homogenized using a T25 Ika Ultra-Turrax®.

S2.1.3 Analysis of OCPs, PCBs and BFRs

The analytical method is based on Brevik (1978) and Polder et al. (2014), with further modifications described herein. During the analytical procedure the samples were protected from daylight to avoid degradation of the BFRs. One gram of homogenized liver tissue from each pool was weighed in centrifuge tubes. The following internal standards were added: PCB-29, -112 and -207 (Ultra Scientific, RI, USA); and BDE-77, -119 and -181 and 13C12-BDE-209 (Cambridge Isotope Laboratories, Inc., MA, USA). After adding solvents and distilled water, lipids were extracted twice with cyclohexane and acetone (3:2) using an ultrasonic homogenizer followed by centrifugation and separation. The lipid determination was done gravimetrically using 1 mL aliquot of the lipid extract. Removal of lipids was performed using ≥97.5% H_2SO_4 (Sigma Aldrich, Missouri, USA). All extracts were concentrated approximately with a factor 10 by careful evaporation under N2, before GC analyses.

Detection of the OCPs (hexachlorobenzene (HCB), β- and γ-hexachlorocyclohexanes (ΣHCHs), oxychlordane and trans-nonachlor (ΣCHLs), bis-2,2-(4-chlorophenyl)-1,1,1-trichloroethane (p,p′-DDT) and its metabolites p,p′-DDE, o,p′-DDD, p,p′-DDD, and o,p′-DDT (ΣDDTs), and mirex) and PCBs (IUPAC nos.: PCB-28, -47, -52, -56, -66, -74, -87, -99, -101, -105, -110, -114, -118, -128, -136, -137, -138, -141, -149, -151, -153, -156, -157, -170, -180, -183, -187, -189, -194, -196, -199, -206 and -209) was performed on a high resolution (HR) GC (Hewlett Packard HP 6890 Series, USA) with NPD and ECD detectors (Agilent Technologies, 5975c inert XL EI/CI MSD triple axis detector, USA). The separation and identification of the compounds were performed on a DB-5 MS column (60 m, 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific). The temperature program was: 90 °C (2 min hold); 25 °C/min increase to 180 °C (2 min hold); 1.5 °C/min increase to 220 °C (2 min hold); and 3 °C/min increase to 275 °C (12 min hold) and 25 °C/min increase to 300 °C (4 min hold). The total run time was 71.6 min. The target ions are given in Table S2.

Table S2. Overview of the analyzed organochlorine pesticides (OCPs) and PCBs and the target ions.

Organochlorine	Target ion	Organochlorine	Target ion	Organochlorine	Target ion
α-HCH	71	PCB-28	291.9	PCB-151	359.9
β-HCH	71	PCB-47	291.9	PCB-153	359.9
γ-HCH	71	PCB-52	291.9	PCB-156	359.9
		PCB-56	291.9	PCB-157	359.9
p,p'-DDE	317.9	PCB-66	291.9	PCB-170	393.8
o,p'-DDD	248	PCB-74	291.9	PCB-180	395.8
p,p'-DDD	248	PCB-87	325.9	PCB-183	393.8
o,p'-DDT	248	PCB-99	325.9	PCB-187	393.8
p,p'-DDT	71	PCB-101	325.9	PCB-189	393.8
		PCB-105	325.9	PCB-194	429.8
HCB	283.8	PCB-110	325.9	PCB-196	429.8
		PCB-114	325.9	PCB-199	429.8
Oxychlordane	351.8	PCB-118	325.9	PCB-206	463.8
trans-Chlordane	409.8	PCB-128	359.9	PCB-209	497.7
cis-Chlordane	409.8	PCB-136	359.9		
trans-Nonachlor	443.8	PCB-137	359.9		
cis-Nonachlor	443.8	PCB-138	359.9		
		PCB-141	359.9		
Mirex	438.7	PCB-149	359.9		

For detection of BFRs (PBDEs: BDE-28, -47, -99, -100, -153, -154, -183, -196, -202, -206, -207, -208, -209; hexabromocyclododecane (HBCD), 2-bis(2,4,6-tribromophenoxy) ethane (BTBPE); Hexabromobenzene (HBB); (2,3-Dibromopropyl) (2,4,6-tribromophenyl) ether (DPTE); pentabromoethylbenzene (PBEB) and 2,3,4,5,6-pentabromotoluene (PBT)) were performed on a HRGC (Agilent 6890 Series GC system, USA) coupled with a low resolution (LR) MS (Agilent 5973 Network Mass Selective Detector, USA) configured with a split/splitless injector (Agilent Technologies, Santa Clara, US). The separation and identification of the compounds were performed on a DB-5 MS column (30 m, 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific). The temperature program was: 90 °C (1 min hold); 25 °C/min increase to 180 °C; 2.5 °C/min increase to 220 °C (1 min hold); and 20 °C/min increase to 320 °C (10 min hold). The total run time was 36.60 min. The BFRs were monitored using ECNI in SIM mode at m/z 79/81 for PBDEs, HBCD, BTBPE, PBEB and PBT, at m/z 79/551 for HBB and at m/z 160/79 for DPTE.

For detection of BDE-197, -206, -207, -208 and -209, extracts (10 µL) were injected on a HRGC–MS (Agilent 6890 Series/5973Network) configured with a programmable temperature vaporization (PTV) injector (Agilent Technologies). The separation and identification of BDE -197, -206, -207, -208 and -209 were performed on a DB-5-MS column (10 m, 0.25 mm i.d., 0.10 µm film thickness; J&W Scientific, Agilent Technologies). The temperature program was: 80 °C (2 min hold); 30 °C/min increase to 315 °C (6 min hold). The total run time was 15.83 min. BDE-197, -206, -207, -208 and -209 was monitored at m/z 484/486 and 13C12-BDE-209 at m/z 495/497.

S2.1.4 Analyses of PFASs

The analytical procedure is described by Grønnestad et al. (2017). In brief, 0.25 grams of homogenized liver tissue from each pool were weighed in Falcon centrifuge tubes (VWR International, LLC Radnor, USA) and spiked with internal standards containing a ^{13}C-labeled PFAS mix (Wellington Laboratories). The samples were extracted twice with methanol (MeOH). For lipid removal, active carbon (EnviCarb) was used.

Separation and detection of PFASs (perfluorohexane sulfonate (PFHxS), perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoid acid (PFDA), perfluoroundecanoic acid (PDUdA), perfluorododecanoic acid (PFDoA) and perfluorotridecanoic acid (PFTrDA), perfluoro-n-hexanoic acid (PFHxA), perfluoro-n-heptanoic acid (PFHpA), perfluoro-n-tetradecanoic acid (PFTeDA), potassium perfluorobutane sulfonate (PFBS), perfluorooctanesulfonamide (FOSA), metylperfluorooctanesulfonamide (N-MeFOSA), methylperfluorooctanesulfonamidoethanol (N-MeFOSE), ethylperfluorooctanesulfonamid (N-EtFOSA) and ethylperfluorooctanesulfonamidoethanol (N-EtFOSE)) were performed using a high-performance liquid chromatography (HPLC) with a Discovery C18 column, connected to a C18 pre-column (Supelco, Sigma-Aldrich, Oslo, Norway) and a liquid chromatography (LC) tandem mass spectrometry (MS-MS) (API 3000, LC/MS/MS System).

S2.1.5 QA/QC OCPs, PCBs, BFRs and PFASs

For analyses of OCPs, PCBs and BFRs, the analytical batches included one non-spiked and two spiked samples of commercial cod oil, three blanks (solvents), one harp seal blubber (*Pagophilus groendlandicus*) as internal reference material (IRM). For analyses of PFASs, the

analytical series included one non-spiked (blind) and two spiked salmon liver samples and three blanks (solvents).

For OCPs, PCBs, BFRs, PFASs, the lowest level of detection (LOD) for individual compounds was defined as three times the noise level for each compound. The LOD (ng/g wet weight (ww)) (Table S8-S10) ranged for OCPs: 0.002-0.041, PCBs: 0.007-1.297, BDE -28-183: 0.006-0.027, BDE -196-209: 0.007-0.11, HBCD: 0.066, and PFASs: 0.05-2.64. The means of the relative recoveries ranged for OCPs: 82-105%, PCBs: 84-104%, BDE -28-183: 89-114%, BDE -196-209: 81-115%, HBCD: 99-116% and PFASs: 76-123%. Positive procedural blanks were found for some compounds. If the blank concentrations were consistent for all blank samples, results were corrected for blanks. The analytical quality was approved by satisfactory quality control measures, and results were within the accepted ranges for the analysed Certified Reference Materials (CRM 2525, 350) (Cerilliant Corporation, Round Rock, TX, USA) and interlaboratory tests (Arctic Monitoring and Assessment Program (AMAP), Québec, Canada).

S2.1.6 QA/QC metals

LOD and LOQ (0.001 and 0.004) were calculated as three times SD and ten times SD of blank samples (n=5), respectively. The detection limits (mg/kg ww) were Li: 0.001, Mg: 0.03, Al: 0.01, V: 0.0003, Cr: 0.001, Fe: 0.01, Co 0.00004, Ni: 0.002, Cu: 0.001, Zn: 0.005, As: 0.0003, Se: 0.001, Mo: 0.00004, Ag: 0.0001, Cd 0.0004, Hg: 0.002, Pb: 0.0003. One Dolt-4 (Dogfish Liver Certified Reference Material for Trace Metals) sample and two Dolt-5 (Dogfish Liver Certified Reference Material for Trace Metals and other Constituents) samples from National Research Council, Canada, were digested and analysed at the same time. The concentrations of Mg, V, Cr, Fe, Co, Ni Cu, Zn, As, Se, Mo, Hg, and Pb were within the limits of uncertainty for Dolt 5. Concentrations of Al were within the range of two times the uncertainty, and Ag and Cd were within three times. The concentrations of Ni, Cu, Zn, Hg and Pb were within the limits of uncertainty of Dolt-4. The measured concentrations of Fe, As, Ag and Cd were within the ranges of two times the uncertainty.

S3. Quantitative PCR

S3.1 Liver qPCR

Table S3 – Sequences of cod primers for reference (*) and target genes used in liver qPCR

Gene name	Gene product	Gene ID/accession no.	Forward sequence	Reverse sequence	Amplicon size (bp)
acly	ATP citrate lyase	ENSGMOG00000000077	CTGCGGTGGATTTACACGAGATGA	CTTCTGGTCCAGGTAGTGTCCGATGA	115
cat	Catalase	ENSGMOG00000005683	GCCAAGTTGTTTGAGCACGTT	CTGGGATCACGCACCGTATC	101
cyp1a	Cytochrome P450 1a	ENSGMOG00000000318	CACCAGGAGATCAAGGACAAG	GCAGGAAGGAGGAGTGACGGAA	101
*ef1a**	Translation elongation factor 1a	ENSGMOG00000012005	CGGTATCCTCAAGCCCAACA	GTCAGAGACTCGTGGTGCATCT	93
fabp7	Fatty acid binding protein 7	ENSGMOG00000011090	ACAAGGCAAGTTGGCAATGTGA	CCTCGGTGTTCTTGAAGGTGCT	99
fasn	Fatty acid synthase	ENSGMOG00000005697	GGAGGTCACCGCACGACTT	TTCCACCTCGCCATGGTATT	132
gsta3b	Glutathione S-transferase	JW196096	CGGGCAATGGTCAACATGTA	TAGCGGCCTGTTGCTTTTGT	131
hsp70	Heat shock protein 70	JW198668	AGAGCACGGGCAAACAGAAC	GTTGGTCTGCTTCCTGAACCA	96
scd	Stearoyl-CoA desaturase	ENSGMOG00000000395	GCACCCCGACGTCATAGAAA	CACCATGGGCAGCATGAAG	130
*uba52**	Ubiquitin A-52 residue ribosomal protein fusion	ENSGMOG00000007530	GGCCGCAAAGATGCAGAT	CTGGGCTCGACCTCAAGAGT	69

S.3.2. Ovary qPCR

The primer pairs (Table S4) were tested by analysing single amplified product of expected size. A parallel running control lacking cDNA template was used to validate the specificity and target sequence amplification. The PCR program included an enzyme activation step at 95 °C (4 min) followed by 40 cycles of 95 °C (15 s), 60 °C (30 s) and 72 °C (15 s) and last step temperature profile include 95 °C (60 s), 65 °C (30 s) and 95 °C (30 s). Expression of each gene was determined by following the well-validated procedure of absolute quantification in our laboratory (Mortensen and Arukwe, 2007). Known amount of plasmid cloned with an amplicon of interest generated a standard curve of log copy number versus cycle threshold (Ct) to quantify the expression of target genes.

Table S4 – Sequences of cod primers used in ovary qPCR

Gene name	Gene product	Accession no.	Forward sequence	Reverse sequence	Amplicon size (bp)
cyp17	Cytochrome P450 17	ENSGMOG00000011243	CTTTGACCCAGGTCGTTTCC	CCACGACAGGAAGAGGAAGA	142
cyp19	Cytochrome P450 19	ENSGMOG00000017406	GAGGAGACGCTCATCCTCAG	TAGCTGCGTGTCTTCTTCCA	166
p450ssc	P450 side-chain cleavage	ENSGMOG00000008338	AACAACTACTTCCGCAGCCT	CGGTAGAACAATGAGCTGGA	171
star	Steroidogenic Acute Regulatory Protein	ENSGMOG00000016690	CAACGTCAAGCAGGTCAAGA	GCAGCATCGGGCTTCAACACTAT	249
3β-hsd	3β-hydroxysteroid dehydrogenase	ENSGMOG00000002557	CGTCTACTACGTCTCCGATGA	CCTCCAGCAGGAAGCAAA	143
11β-hsd	11β-hydroxysteroid dehydrogenase	ENSGMOG00000011762	CCAGTTGGGAGGACTGGATTA	TTCCAGGCCCAGTGACAA	132
17β-hsd	17β-hydroxysteroid dehydrogenase	ENSGMOG00000014848	CACTTCAACATCCACATGAGC	GCAGGTACTGCCCGTAAA	142
20β-hsd	20β-hydroxysteroid dehydrogenase	ENSGMOG00000018824	ATCACTGTGCAGTGTGTGG	GCAGCCGTTGGTGTAGTT	144

S4. Western Blotting

Table S5 – List of antibodies and dilutions utilized in western blotting

Type	Antibody	Species	Producer	Product no.	Dilution
Primary	Anti-cod Cyp1a	Mouse	Biosense Laboratories AS	C02406101	1:800
Primary	Anti-MT	Rabbit	Biosense Laboratories AS	M04406201	1:1000
Primary	Anti-Cyp3a	Rabbit	Isolated as described in Celander et al. (1996), Goksøyr (1985)	-	1:800
Primary	Anti beta-actin	Mouse	Abcam	ab8224	1:1000
Secondary	Anti-mouse IgG/HRP Linked Whole Ab	Sheep	GE Healthcare UK	NA931	1:3000
Secondary	Polyclonal anti-rabbit IgG/HRP	Goat	Dako Denmark AS	P0448	1:2000

S5. Enzyme immunoassay (EIA) measurement

Plasma samples (100 µL) were mixed thoroughly with diethyl ether (1:4) and the two phases were allowed to separate. The organic phase containing steroid hormone was transferred into a new glass tube and the combined extract was allowed to evaporate at 30 °C. The dry extract was reconstituted in 100 µL EIA buffer and analysed, following the manufacturer's guideline. The plate was read with Bio-Tek Synergy HT microplate reader (Bio-Tek instruments,

Winooski, VT, USA) at 410 nm. Hormone levels were quantified against a standard curve, made by a 4-parameter logistic fit between log concentration and logit transformation of B/Bo (Bound sample/maximum bound).

Results

Table S6 – Concentrations of metals, PCB-7 and PAH-16 in sediment from reference and Kollevåg stations. Values exceeding EQS sediment (Norwegian Environment Agency, 2018) are presented in bold. Values in italics are below the detection limit. Concentrations are measured in mg/kg dry weight (DW) for metals and µg/kg DW for PCBs/PAHs. nd = not detected.

		Station 1	Station 2	Station 3	Reference	EQS sediment
METALS						
Arsenic (As)	mg/kg DW	3.1	6.1	15	1.8	18
Lead (Pb)	mg/kg DW	17	39	72	7.2	150
Cadmium (Cd)	mg/kg DW	0.13	0.11	0.055	0.11	2.5
Mercury (Hg)	mg/kg DW	0.076	0.04	0.134	0.005	0.52
Copper (Cu)	mg/kg DW	29	27	19	2.4	84
Chrom (Cr)	mg/kg DW	18	16	10	4.1	660
Nickel (Ni)	mg/kg DW	13	5	3.8	1.4	42
Zink (Zn)	mg/kg DW	80	57	90	11	139
Dry weight (DW)	%	53.1	57	63.7	62.9	
PCBs						
PCB 28	µg/kg DW	*<0.5*	*<0.5*	*<0.5*	*<0.5*	
PCB 52	µg/kg DW	*<0.5*	0.93	*<0.5*	*<0.5*	
PCB 101	µg/kg DW	0.7	2.7	0.51	*<0.5*	
PCB 118	µg/kg DW	0.75	2.7	*<0.5*	*<0.5*	
PCB 153	µg/kg DW	2	8	1.5	*<0.5*	
PCB 138	µg/kg DW	2.2	8	2	*<0.5*	
PCB 180	µg/kg DW	1.2	4	1	*<0.5*	
Sum 7 PCB	µg/kg DW	**6.9**	**26**	**5**	nd	4.1
PAHs						
Naphthalene	µg/kg DW	*<10*	25	11	*<10*	27
Acenaphthylene	µg/kg DW	*<10*	*<10*	*<10*	*<10*	33
Acenaphthene	µg/kg DW	*<10*	42	*<10*	*<10*	100
Fluorene	µg/kg DW	*<10*	41	10	*<10*	150
Phenanthrene	µg/kg DW	17	360	110	*<10*	780
Anthracene	µg/kg DW	*<10*	**92**	**30**	*<10*	4.6
Fluoranthene	µg/kg DW	50	**540**	280	*<10*	400
Pyrene	µg/kg DW	44	**500**	**220**	*<10*	84
Benzo[a]anthracene	µg/kg DW	26	**270**	**190**	*<10*	60
Chrysene	µg/kg DW	22	210	150	*<10*	280
Benzo[b]fluoranthene	µg/kg DW	58	**370**	**300**	14	140
Benzo[k]fluoranthene	µg/kg DW	15	99	91	*<10*	140
Benzo[a]pyrene	µg/kg DW	35	**330**	**210**	*<10*	180
Indeno[1,2,3-cd]pyrene	µg/kg DW	43	**220**	**150**	23	63
Dibenzo[a,h]anthracene	µg/kg DW	*<10*	**63**	**54**	*<10*	27
Benzo[ghi]perylene	µg/kg DW	39	**220**	**160**	23	84
Sum PAH(16) EPA	µg/kg DW	350	3400	2000	60	

Table S7. Sediment particle composition and loss on ignition (LOI) at different stations

		Weight (%)			
Definition	**Grain size (mm)**	**Station 1**	**Station 2**	**Station 3**	**REF station**
Gravel	>4.0-2.0	3.8	70.8	26.1	30.8
Coarse sand	0.5-2.0	9.4	9.8	63.6	37.8
Medium sand	0.25-0.50	8.6	5.4	5.3	15.3
Fine sand	0.063-0.25	24.5	11.9	3.5	13.9
Clay/silt	<0.063	53.8	2.0	1.5	2.3
Loss on ignition (LOI)	%	4.10	4.89	4.71	2.92

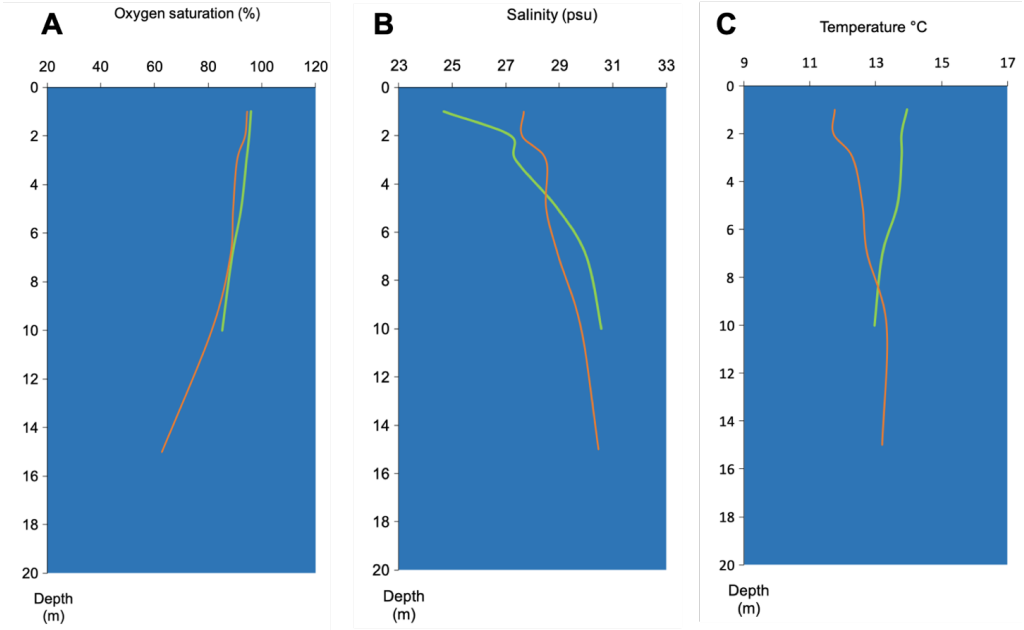

Figure S1 – CTD profile at Kollevåg station 1. Oxygen saturation (A), salinity (B) and temperature (C) were measured at deployment of the cage the 2nd of September 2016 (green line) and at retrieval of the cage the 16th of October 2016 (orange line).

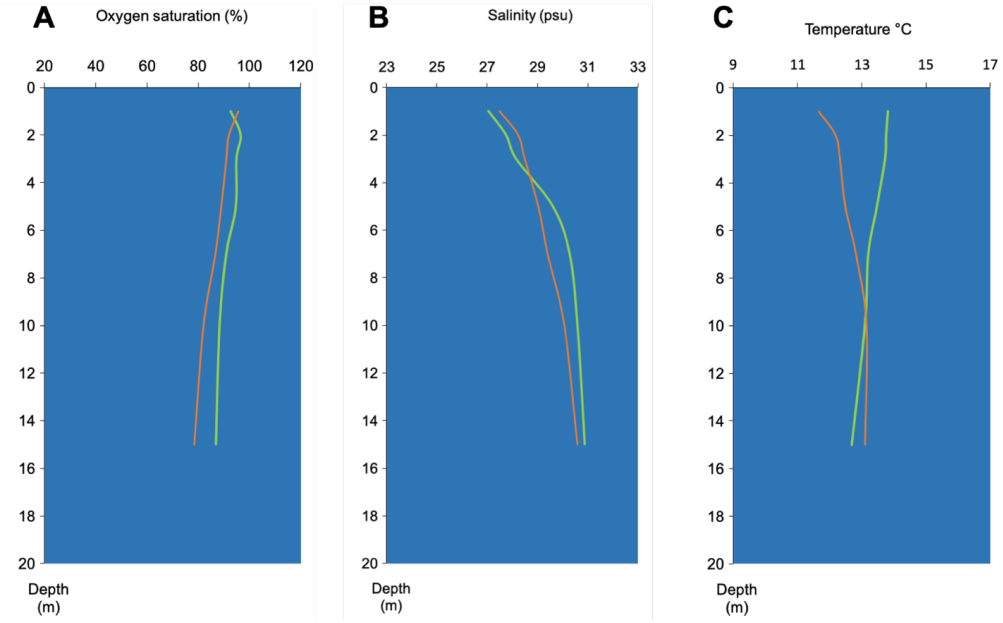

Figure S2 – CTD profile at Kollevåg station 2. Oxygen saturation (A), salinity (B) and temperature (C) were measured at deployment of the cage the 2nd of September 2016 (green line) and at retrieval of the cage the 16th of October 2016 (orange line).

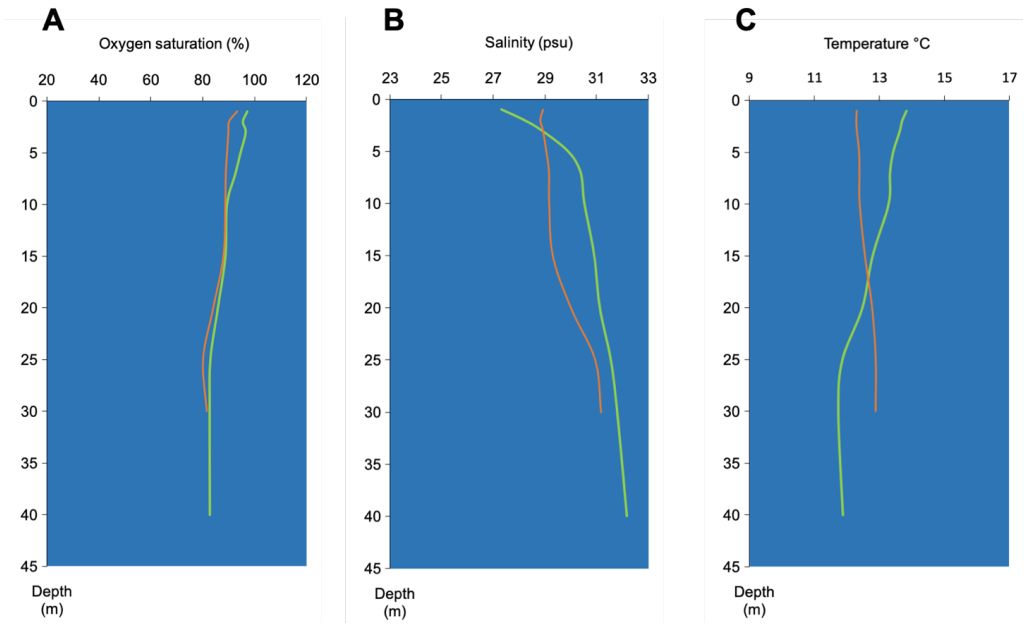

Figure S3 – CTD profile at Kollevåg station 3. Oxygen saturation (A), salinity (B) and temperature (C) were measured at deployment of the cage the 2nd of September 2016 (green line) and at retrieval of the cage the 17th of October 2016 (orange line).

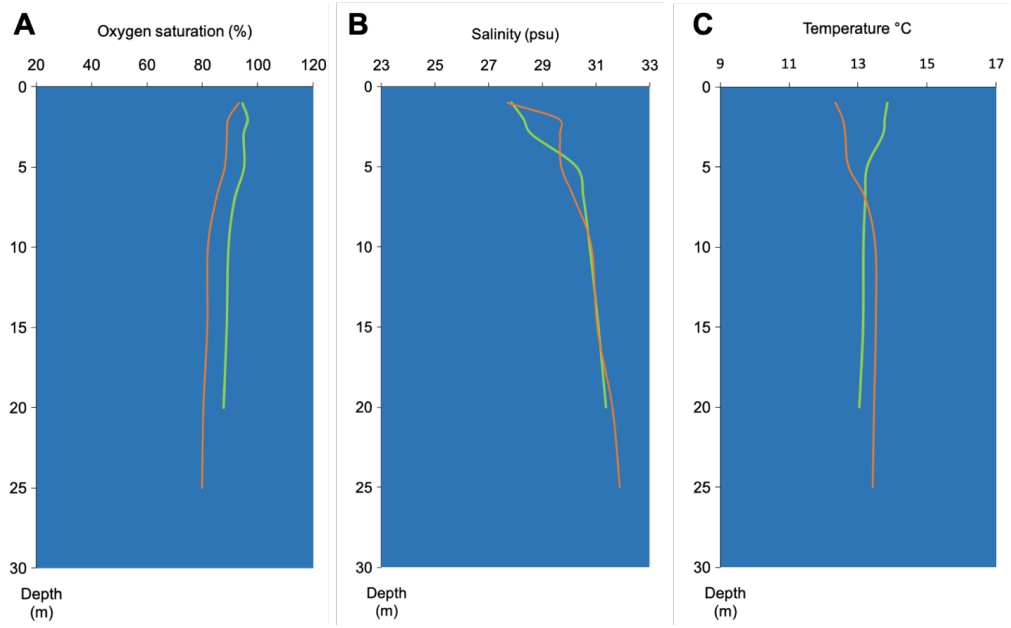

Figure S4 – CTD profile at reference station. Oxygen saturation (A), salinity (B) and temperature (C) were measured at deployment of the cage the 2nd of September 2016 (green line) and at retrieval of the cage the 17th of October 2016 (orange line).

Table S.8. Lipid% and levels of OCs in ng/g wet weight (ww) in liver of caged Atlantic cod.

		Station 1					Station 2					Station 3					Reference				
	LOD	% > LOD	Mean	Median	Min	Max	% > LOD	Mean	Median	Min	Max	% > LOD	Mean	Median	Min	Max	% > LOD	Mean	Median	Min	Max
Lipid %		100	67.3	68.7	54.0	75.5	100	74.4	73.3	68.6	79.3	100	71.0	71.7	62.8	77.8	100	68.8	68.8	58.7	77.7
α-HCH	*0.012*	100	0.443	0.458	0.360	0.498	100	0.510	0.506	0.480	0.561	100	0.553	0.554	0.482	0.602	100	0.552	0.551	0.468	0.635
β-HCH	*0.025*	100	0.526	0.522	0.437	0.606	100	0.591	0.592	0.540	0.640	100	0.549	0.551	0.467	0.607	100	0.619	0.613	0.517	0.715
γ-HCH	*0.014*	100	0.364	0.375	0.301	0.417	100	0.407	0.410	0.366	0.448	100	0.401	0.405	0.348	0.443	100	0.460	0.461	0.375	0.543
∑HCH			**1.33***	**1.36**	**1.10**	**1.52**		**1.51***	**1.51**	**1.40**	**1.65**		**1.50***	**1.51**	**1.30**	**1.65**		**1.63**	**1.63**	**1.36**	**1.85**
p,p'-DDE	*0.093*	100	134	130	78.9	215	100	97.8	95.5	86.2	127	100	97.2	90.7	71.9	146	100	84.7	80.3	53.2	121
o,p'-DDD	*0.084*	100	3.64	3.57	2.70	4.75	100	3.26	3.28	2.87	3.70	100	2.88	2.84	2.12	3.35	100	2.51	2.61	1.78	2.96
p,p'-DDD	*0.602*	100	38.2	39.3	23.8	54.5	100	29.6	29.7	25.3	36.1	100	23.1	20.9	17.2	36.4	100	20.0	19.3	16.6	28.8
o,p'-DDT	*0.286*	100	7.75	0.924	0.458	36.9	100	2.94	0.972	0.517	17.2	62.5	2.58	0.642	*<LOD*	16.8	75	0.911	1.10	*<LOD*	1.57
p,p'-DDT	*0.071*	100	11.2	10.6	7.06	18.4	100	7.96	7.85	6.92	10.1	100	8.36	8.07	5.65	11.7	100	7.32	7.19	4.49	9.13
∑DDTs			**194***	**185**	**113**	**293**		**142**	**141**	**124**	**178**		**134**	**124**	**100**	**197**		**116**	**111**	**76.2**	**161**
HCB	*0.007*	100	23.9	23.6	19.6	27.7	100	22.7	22.5	21.4	24.7	100	22.9	23.1	19.8	26.9	100	21.5	21.9	17.1	24.5
Oxychlordane	*0.032*	100	2.08	2.04	1.48	2.70	100	2.04	1.99	1.80	2.39	100	2.96	2.79	2.41	3.86	100	2.48	2.47	1.90	3.25
trans-Chlordane	*0.015*	100	9.58	9.21	7.07	13.0	100	7.55	7.47	6.66	8.67	100	7.54	7.39	5.86	10.0	100	6.85	6.74	4.43	8.75
cis-Chlordane	*0.014*	100	26.4	26.7	17.3	37.8	100	19.9	20.4	12.9	24.1	100	20.7	19.9	15.6	29.0	100	18.7	18.0	12.5	24.2
trans-Nonachlor	*0.011*	100	38.8	38.0	23.0	59.6	100	29.2	28.9	25.6	36.4	100	29.7	27.7	21.9	44.0	100	26.8	25.8	17.0	35.7
cis-Nonachlor	*0.007*	100	16.7	16.2	10.1	26.0	100	12.5	12.4	11.1	15.5	100	12.8	12.0	9.48	18.8	100	11.7	11.3	7.37	15.5
∑Chlordanes			**93.5**	**92.9**	**59.0**	**139**		**71.2**	**71.2**	**58.1**	**87.0**		**73.6**	**70.2**	**55.3**	**106**		**66.5**	**63.9**	**43.2**	**87.3**
Mirex	*0.061*	100	1.94	1.88	1.02	3.09	100	1.40	1.35	1.17	1.63	100	1.23	1.19	0.860	1.84	100	1.05	1.03	0.750	1.38
PCB-28	*n.a.*																				
PCB-47	*1.110*	87.5	3.71	3.61	*<LOD*	6.32	100	3.80	3.79	2.36	5.05	100	2.88	2.42	1.96	5.97	87.5	2.66	2.86	*<LOD*	3.36
PCB-52	*1.024*	100	12.6	12.9	7.86	19.1	100	9.77	9.28	8.86	12.6	100	8.42	8.03	5.99	13.0	100	7.44	7.03	4.97	9.84
PCB-56	*0.167*	100	1.38	1.46	0.726	1.93	100	0.869	0.741	0.554	1.41	100	0.708	0.691	0.527	0.939	100	0.450	0.389	0.265	0.712
PCB-66	*0.115*	100	7.92	7.86	5.61	11.7	100	5.92	5.75	4.76	7.51	100	5.00	4.83	3.75	7.00	100	4.34	4.13	2.84	6.42
PCB-74	*0.172*	100	7.13	7.22	4.71	10.2	100	5.34	5.30	4.46	6.83	100	4.60	4.30	3.40	6.39	100	3.98	3.80	2.88	5.38
PCB-87	*0.120*	100	22.4	20.6	13.4	41.7	100	15.8	15.5	13.4	19.8	100	14.1	13.9	10.8	20.1	100	12.2	11.6	7.62	17.0
PCB-99	*0.147*	100	22.6	22.6	13.9	35.2	100	16.7	15.8	13.1	21.0	87.5	12.8	12.5	*<LOD*	22.1	100	12.6	11.9	8.06	18.7

PCB-101	*0.054*	100	30.7	30.5	19.1	46.1	100	24.0	23.9	20.3	29.6	100	20.7	20.2	15.2	29.5	100	17.1	16.2	10.9	23.7
PCB-105 [DL]	*0.012*	100	7.63	7.66	4.88	11.7	100	6.02	5.74	4.88	7.18	100	5.20	5.05	3.90	7.58	100	4.46	4.15	2.83	6.73
PCB-110	*0.133*	100	35.6	36.0	23.4	51.4	100	27.9	27.2	23.3	36.4	100	25.8	25.6	17.6	35.3	100	20.5	20.5	13.7	24.8
PCB-114 [DL]	*0.009*	100	0.427	0.447	0.271	0.695	100	0.342	0.337	0.276	0.450	100	0.304	0.279	0.209	0.511	100	0.286	0.263	0.167	0.434
PCB-118 [DL]	*0.014*	100	25.5	25.5	15.7	40.0	100	20.0	19.0	16.2	24.4	100	17.0	16.5	12.4	25.4	100	14.5	13.5	9.09	21.8
PCB-128	*0.014*	100	6.12	6.13	3.75	9.68	100	4.73	4.58	3.86	5.85	100	4.21	4.08	3.11	6.27	100	3.61	3.39	2.28	5.51
PCB-136	*1.297*	0					12.5			*<LOD*	0.683	12.5			*<LOD*	1.22	37.5			*<LOD*	1.07
PCB-137	*0.012*	100	1.38	1.37	0.848	2.16	100	1.12	1.05	0.906	1.55	100	0.973	0.919	0.731	1.44	100	0.859	0.813	0.549	1.23
PCB-138	*0.012*	100	45.8	45.3	27.5	72.4	100	35.5	34.4	27.9	45.5	100	30.8	29.9	21.4	48.2	100	26.5	25.2	17.0	40.3
PCB-141	*0.009*	100	2.85	2.80	1.77	4.48	100	2.33	2.12	1.81	3.80	100	1.94	1.88	1.45	2.88	100	1.66	1.59	1.07	2.36
PCB-149	*0.029*	100	14.1	14.0	9.44	19.4	100	12.7	12.6	10.6	15.1	100	11.3	11.3	8.77	14.1	100	9.48	9.53	6.50	10.8
PCB-151	*0.011*	100	10.4	10.3	6.51	15.7	100	8.91	8.17	7.07	14.4	100	7.39	7.24	5.50	10.6	100	6.22	6.11	4.04	8.15
PCB-153	*0.014*	100	72.9	72.9	43.8	116	100	65.5	54.0	45.4	126	100	48.6	46.8	35.6	73.7	100	41.6	38.9	25.8	63.9
PCB-156 [DL]	*0.007*	100	3.19	3.12	1.95	5.08	100	2.96	2.27	1.90	6.96	100	2.18	2.13	1.59	3.23	100	1.85	1.72	1.15	2.82
PCB-157 [DL]	*0.010*	100	1.11	1.11	0.673	1.74	100	1.06	0.834	0.699	2.42	100	0.783	0.768	0.558	1.16	100	0.661	0.620	0.426	1.00
PCB-170	*0.009*	100	6.95	6.80	4.20	11.0	100	7.39	4.86	4.04	23.0	100	4.27	4.12	3.10	6.38	100	3.54	3.32	2.22	5.48
PCB-180 [DL]	*0.007*	100	16.3	16.0	9.58	25.9	100	17.8	11.4	9.58	57.6	100	10.1	9.49	7.37	15.1	100	8.38	7.82	5.18	12.9
PCB-183	*0.009*	100	4.15	4.07	2.49	6.73	100	3.93	3.06	2.59	8.75	100	2.78	2.66	2.04	4.22	100	2.39	2.23	1.47	3.66
PCB-187	*0.007*	100	10.2	10.1	6.39	16.1	100	10.6	8.00	7.01	28.5	100	7.54	7.34	5.61	10.9	100	6.34	6.26	4.06	8.07
PCB-189 [DL]	*0.007*	100	0.209	0.209	0.126	0.330	100	0.226	0.149	0.118	0.689	100	0.132	0.128	0.0939	0.199	100	0.108	0.100	0.0637	0.171
PCB-194	*0.007*	100	1.87	1.80	1.09	3.12	100	2.60	1.33	1.08	11.0	100	1.21	1.14	0.924	1.82	100	0.995	0.914	0.588	1.62
PCB-196	*0.007*	100	1.70	1.65	1.02	2.72	100	2.06	1.23	1.02	7.50	100	1.09	1.03	0.830	1.63	100	0.925	0.868	0.564	1.43
PCB-199	*0.007*	100	0.0772	0.0745	0.0484	0.126	100	0.0888	0.0597	0.0492	0.295	100	0.0504	0.0493	0.0389	0.0745	100	0.0436	0.0415	0.0294	0.0634
PCB-206	*0.007*	100	0.551	0.514	0.325	0.899	100	0.903	0.405	0.330	4.25	100	0.377	0.366	0.287	0.556	100	0.305	0.282	0.176	0.502
PCB-209	*0.007*	100	0.700	0.732	0.429	1.11	100	0.695	0.556	0.381	1.94	100	0.505	0.495	0.365	0.682	100	0.398	0.337	0.208	0.820
∑7PCBs[1]			**203***	**197**	**138**	**310**		**173**	**158**	**131**	**242**		**136**	**135**	**101**	**198**		**116**	**109**	**75.5**	**167**
∑33PCBs			**378***	**375**	**236**	**579**		**318**	**285**	**244**	**485**		**254**	**242**	**188**	**377**		**217**	**206**	**140**	**308**

[1]PCB 28,52,101,118,138,153,180

DL = Dioxin-like

n.a.= not analyzed

* Statistically different ($p<0.05$) between means in Kollevåg stations and reference station (only presented for sums).

Table S9. Levels of BFRs in ng/g wet weight (ww) in liver of caged Atlantic cod.

		Station 1					Station 2					Station 3					Reference				
	LOD	**% > LOD**	**Mean**	**Median**	**Min**	**Max**	**% > LOD**	**Mean**	**Median**	**Min**	**Max**	**% > LOD**	**Mean**	**Median**	**Min**	**Max**	**% > LOD**	**Mean**	**Median**	**Min**	**Max**
Lipid %		100	67.3	68.7	54.0	75.5	100	74.4	73.3	68.6	79.3	100	71.0	71.7	62.8	77.8	100	68.8	68.8	58.7	77.7
BDE-28	*0.006*	100	0.326	0.325	0.233	0.447	100	0.270	0.255	0.236	0.337	87.5	0.236	0.251	*<LOD*	0.372	100	0.238	0.230	0.167	0.346
BDE-47	*0.017*	100	7.27	7.35	5.34	9.78	100	6.07	5.85	5.24	7.41	100	5.91	6.09	4.70	7.71	100	5.21	5.05	3.71	7.22
BDE-99	*0.015*	100	0.244	0.251	0.152	0.337	100	0.250	0.256	0.185	0.297	100	0.284	0.289	0.252	0.316	100	0.266	0.249	0.241	0.320
BDE-100	*0.027*	100	3.18	3.11	1.82	5.43	100	3.40	2.16	1.85	11.7	100	2.15	2.04	1.61	3.38	100	1.82	1.72	1.12	2.78
BDE-153	*0.025*	87.5	0.0531	0.0522	*<LOD*	0.0921	100	0.0470	0.0446	0.0347	0.0699	100	0.0584	0.0561	0.0389	0.0849	100	0.0467	0.0440	0.0385	0.0581
BDE-154	*0.018*	100	1.65	1.61	0.964	2.58	100	1.13	1.08	0.969	1.43	100	1.20	1.22	0.850	1.77	100	1.01	1.00	0.667	1.30
BDE-183	*0.023*	100	0.0821	0.0764	0.0292	0.145	100	0.0532	0.0467	0.0300	0.0837	87.5	0.0801	0.0842	*<LOD*	0.153	100	0.106	0.0978	0.0727	0.146
∑7PBDEs			**12.8***	**12.8**	**8.80**	**18.6**		**11.2**	**10.1**	**8.73**	**19.2**		**9.92**	**9.90**	**7.83**	**13.7**		**8.69**	**8.42**	**6.06**	**12.0**
HBCD	*0.066*	100	8.87	7.87	6.10	13.8	100	6.14	5.98	4.57	7.15	100	6.75	6.40	5.19	9.73	100	6.04	5.71	3.49	8.65
HBB	*0.010*	100	0.410	0.419	0.266	0.578	100	0.314	0.314	0.279	0.361	100	0.311	0.302	0.231	0.451	100	0.283	0.279	0.185	0.379

* Statistically different ($p<0.05$) between means in Kollevåg stations and reference station (only presented for sums).

Table S10. Levels of PFASs in ng/g wet weight (ww) in liver of caged Atlantic cod.

		Station 1					Station 2					Station 3					Reference				
	LOD	% > LOD	Mean	Median	Min	Max	% > LOD	Mean	Median	Min	Max	% > LOD	Mean	Median	Min	Max	% > LOD	Mean	Median	Min	Max
PFHxA	*0.682*	87.5	2.16	1.78	0.744	4.52	100	2.38	1.86	1.06	4.17	100	1.50	1.32	0.744	2.81	100	2.07	1.76	0.956	3.69
PFHpA	*0.218*	87.5	0.564	0.508	*<LOD*	1.40	100	0.627	0.545	0.293	1.31	62.5	0.302	0.268	*<LOD*	0.862	100	0.785	0.779	0.304	1.19
PFOA	*0.485*	25			*<LOD*	1.36	12.5			*<LOD*	1.50	62.5	0.587	0.518	*<LOD*	1.75	62.5	0.743	0.842	*<LOD*	2.07
PFNA	*0.187*	75	0.377	0.288	*<LOD*	0.887	50	0.298	*0.183*	*<LOD*	0.988	50	0.381	0.236	*<LOD*	0.964	62.5	0.462	0.343	*<LOD*	1.39
PFDA	*0.236*	62.5	0.306	0.250	*<LOD*	0.689	50	*0.224*	*0.139*	*<LOD*	0.599	50	0.245	0.200	*<LOD*	0.566	12.5			*<LOD*	0.636
PFUdA	*0.283*	62.5	0.422	0.456	*<LOD*	1.10	87.5	0.436	0.413	*<LOD*	0.647	75	0.572	0.415	*<LOD*	1.87	62.5	0.420	0.388	*<LOD*	0.909
PFDoDA	*0.122*	37.5			*<LOD*	0.485	0			*<LOD*	*<LOD*	62.5	0.260	0.176	*<LOD*	0.579	25			*<LOD*	0.140
PFTrDA	*0.119*	75	0.298	0.319	*<LOD*	0.531	75	0.281	0.278	*<LOD*	0.628	62.5	0.202	0.154	*<LOD*	0.493	25			*<LOD*	0.348
PFTeDA	*0.663*	0			*<LOD*	*<LOD*	12.5			*<LOD*	1.14	0			*<LOD*	0.532	0			*<LOD*	*<LOD*
PFHxS	*0.230*	25			*<LOD*	0.484	0			*<LOD*	*<LOD*	12.5			*<LOD*	0.424	37.5			*<LOD*	0.520
PFOS	*0.122*	100	1.41	1.26	0.677	2.88	100	1.09	0.929	0.502	2.30	100	1.17	1.11	0.500	2.08	100	1.07	0.943	0.723	2.44
FOSA	*0.047*	100	0.394	0.344	0.198	0.848	100	0.321	0.332	0.231	0.414	100	0.522	0.449	0.302	0.860	100	0.434	0.269	0.224	1.33
N-MeFOSA	*0.246*	12.5			*<LOD*	0.903	12.5			*<LOD*	0.327	12.5			*<LOD*	1.72	37.5			*<LOD*	1.18
N-EtFOSA	*0.744*	0			*<LOD*	*<LOD*	0			*<LOD*	*<LOD*	0			*<LOD*	0.241	37.5			*<LOD*	2.78
N-EtFOSE	*2.64*	0			*<LOD*	*<LOD*	0			*<LOD*	*<LOD*	25			*<LOD*	4.50	37.5			*<LOD*	3.16
∑10PFAS			**6.43**	**5.93**	**4.05**	**10.9**		**5.97**	**5.20**	**4.55**	**10.6**		**5.74**	**5.94**	**3.65**	**8.04**		**6.30**	**5.04**	**4.01**	**12.9**

Table S11. Levels of metals in ng/g dry weight (dw) in liver of caged Atlantic cod.

		Station 1					Station 2					Station 3					Reference				
	LOQ	% > LOQ	Mean	Median	Min	Max	% > LOQ	Mean	Median	Min	Max	% > LOQ	Mean	Median	Min	Max	% > LOQ	Mean	Median	Min	Max
Li	0.002	100	0.006	0.005	0.004	0.010	100	0.005	0.005	0.004	0.006	100	0.005	0.005	0.004	0.008	100	0.004	0.004	0.003	0.006
Mg	0.09	100	59	55.5	42	86	100	47	49	34	59	100	43.5	38.5	37.0	60.0	100	46.8	42.5	37.0	58.0
Al	0.044	100	1.34	1.07	0.636	0.473	100	0.845	1.10	0.660	0.335	100	0.610	0.230	0.270	0.230	100	3.20	1.60	1.00	1.30
V	0.001	100	0.016	0.013	0.007	0.035	100	0.012	0.010	0.006	0.030	100	0.011	0.009	0.007	0.025	100	0.009	0.009	0.007	0.012
Cr	0.003	75	0.009	0.009	<LOQ	0.016	50	0.007	0.004	<LOQ	0.028	75	0.005	0.004	<LOQ	0.012	50	0.007	0.006	<LOQ	0.020
Fe	0.04	100	19	19	12	28	100	15.0	13.5	8.30	31.0	100	16.3	14.5	12.0	31.0	100	12.2	9.10	6.10	30.0
Co	0.0001	100	0.009	0.007	0.002	0.018	100	0.005	0.004	0.003	0.010	100	0.008	0.007	0.005	0.017	100	0.004	0.004	0.003	0.006
Ni	0.007	87.5	0.009	0.010	<LOQ	0.012	12.5			<LOQ	0.014	87.5	0.017	0.017	<LOQ	0.039	37.5			<LOQ	0.014
Cu	0.004	100	1.40	1.55	0.83	1.90	100	1.59	1.65	0.560	2.60	100	1.75	1.85	0.770	2.30	100	1.57	1.55	0.970	2.20
Zn	0.016	100	14.4	14	10	21	100	12.6	12.0	9.80	16.0	100	11.7	12.0	8.60	13.0	100	11.7	11.0	9.60	15.0
As	0.001	100	4.24	4.20	3.60	5.40	100	4.04	4.05	3.60	4.50	100	3.75	3.80	2.90	4.10	100	3.75	3.65	3.30	4.70
Se	0.003	100	0.388	0.370	0.250	0.560	100	0.298	0.280	0.230	0.420	100	0.308	0.265	0.260	0.450	100	0.269	0.240	0.210	0.360
Mo	0.0002	100	0.077	0.069	0.049	0.140	100	0.062	0.056	0.046	0.097	100	0.056	0.052	0.048	0.091	100	0.045	0.043	0.034	0.077
Ag	0.0004	100	0.017	0.013	0.007	0.036	100	0.012	0.013	0.004	0.027	100	0.021	0.018	0.009	0.039	100	0.011	0.011	0.005	0.025
Cd	0.001	100	0.024	0.026	0.011	0.039	100	0.018	0.016	0.010	0.027	100	0.018	0.015	0.013	0.035	100	0.015	0.014	0.008	0.024
Hg	0.008	75	0.011	0.012	<LOQ	0.016	75	0.011	0.011	<LOQ	0.018	75	0.010	0.009	<LOQ	0.020	87.5	0.008	0.008	<LOQ	0.011
Pb	0.001	100	0.003	0.003	0.002	0.009	100	0.008	0.003	0.002	0.039	100	0.004	0.003	0.002	0.008	100	0.003	0.002	0.001	0.011

Table S12 – Eigenvalues of PCA in Figure 3.8. The eigenvalues are sorted with decreasing absolute eigenvalues in PC2.

Parameter	PC1 (40.13%)	PC2 (8.99%)	Type of parameter/process
p450scc	0,039	-0,237	Ovary gene
Oxyklordan	0,056	0,230	Liver chemistry
α-HCH	-0,091	0,211	Liver chemistry
3β-HSD	0,064	-0,210	Ovary gene
γ-HCH	-0,078	0,206	Liver chemistry
Cyp17	0,061	-0,205	Ovary gene
1-OH-pyr	0,051	-0,200	Bile PAH
Cat	-0,040	0,191	Oxidative stress
Testosterone	0,029	-0,184	Steroid hormone
20β-HSD	0,054	-0,180	Ovary gene
StAR	0,024	-0,179	Ovary gene
3-OH-BaP	0,017	-0,174	Bile PAH
17β-HSD	0,032	-0,169	Ovary gene
Estradiol	0,027	-0,165	Steroid hormone
PFOA	-0,034	0,163	Liver chemistry
TBARS	-0,002	0,158	Oxidative stress
cyp19	0,058	-0,154	Ovary gene
BDE-99	0,029	0,153	Liver chemistry
FOSA	0,061	0,150	Liver chemistry
β-HCH	-0,062	0,146	Liver chemistry
Mt	-0,013	0,131	Mt
PFHxS	-0,005	0,126	Liver chemistry
BDE-183	-0,043	0,120	Liver chemistry
HCB	0,133	0,119	Liver chemistry
Gst	-0,029	0,117	Oxidative stress
1-OH-phen	0,037	-0,113	Bile PAH
N-EtFOSE	-0,047	0,107	Liver chemistry
PFOS	0,098	0,099	Liver chemistry
acly	0,023	-0,093	Liver gene
PFUdA	0,043	0,092	Liver chemistry
cis-Nonaklor	0,141	0,092	Liver chemistry
N-MeFOSA	0,022	0,091	Liver chemistry
cis-Klordan	0,138	0,089	Liver chemistry
HBCDD	0,137	0,087	Liver chemistry
trans-Nonaklor	0,142	0,086	Liver chemistry
PFNA	-0,001	0,083	Liver chemistry
PCB-206	0,064	-0,082	Liver chemistry
PCB-136	-0,025	0,080	Liver chemistry
p,p'-DDT	0,140	0,080	Liver chemistry
PCB-56	0,121	-0,076	Liver chemistry
PCB-194	0,079	-0,076	Liver chemistry
trans-Klordan	0,142	0,073	Liver chemistry
p,p'-DDE	0,145	0,073	Liver chemistry
PCB-196	0,094	-0,070	Liver chemistry
BDE-47	0,143	0,069	Liver chemistry
PCB-114	0,144	0,067	Liver chemistry
BDE-154	0,143	0,066	Liver chemistry
PCB-180	0,109	-0,066	Liver chemistry
HBB	0,143	0,065	Liver chemistry
PCB-170	0,113	-0,064	Liver chemistry
PCB-189	0,113	-0,063	Liver chemistry
PCB-199	0,101	-0,062	Liver chemistry
BDE-28	0,131	0,060	Liver chemistry
fasn	0,022	-0,057	Liver gene
PFHpA	0,006	0,056	Liver chemistry
Pb	-0,009	-0,055	Liver chemistry

11β-HSD	0,046	-0,054	Ovary gene
cyp1a	0,010	-0,053	Liver gene
Gender	0,029	0,049	Sex
PFDA	0,075	0,049	Liver chemistry
o,p'-DDT	0,049	-0,048	Liver chemistry
PFHxA	0,066	0,048	Liver chemistry
cat	0,014	0,047	Liver gene
hsp70	0,017	-0,047	Liver gene
PFTrDA	0,084	-0,046	Liver chemistry
PFTeDA	-0,018	0,045	Liver chemistry
Hg	0,090	0,042	Liver chemistry
As	0,105	-0,042	Liver chemistry
PCB-128	0,148	0,041	Liver chemistry
N-EtFOSA	-0,030	0,041	Liver chemistry
PCB-138	0,148	0,037	Liver chemistry
PCB-187	0,120	-0,035	Liver chemistry
CF	-0,070	-0,034	Condition parameter
PCB-137	0,150	0,034	Liver chemistry
PCB-47	0,097	0,033	Liver chemistry
PCB-99	0,139	0,032	Liver chemistry
gsta3b	0,033	-0,030	Liver gene
PCB-209	0,121	-0,030	Liver chemistry
Mirex	0,145	0,027	Liver chemistry
HSI	-0,077	-0,026	Condition parameter
PCB-105	0,150	0,026	Liver chemistry
PCB-52	0,147	0,025	Liver chemistry
PCB-118	0,150	0,025	Liver chemistry
PCB-110	0,146	0,022	Liver chemistry
Cd	0,110	0,022	Liver chemistry
Ni	0,007	0,020	Liver chemistry
PCB-183	0,138	-0,019	Liver chemistry
PCB-66	0,146	0,019	Liver chemistry
PCB-157	0,136	-0,018	Liver chemistry
PCB-156	0,136	-0,018	Liver chemistry
PCB-74	0,146	0,017	Liver chemistry
Cu	-0,012	-0,017	Liver chemistry
PCB-141	0,149	0,014	Liver chemistry
PCB-149	0,143	0,013	Liver chemistry
PCB-101	0,149	0,013	Liver chemistry
PCB-87	0,142	0,012	Liver chemistry
p,p'-DDD	0,145	-0,012	Liver chemistry
Cyp3a	0,048	0,011	Cyp3a
scd5	-0,026	0,011	Liver gene
Cr	0,019	-0,011	Liver chemistry
Zn	0,115	-0,009	Liver chemistry
PCB-153	0,145	-0,009	Liver chemistry
fabp7	-0,005	-0,008	Liver gene
o,p'-DDD	0,136	-0,005	Liver chemistry
PCB-151	0,148	0,004	Liver chemistry
Vtg	-0,015	-0,003	Vitellogenin
PFDoDA	-0,017	-0,002	Liver chemistry
BDE-153	-0,011	0,002	Liver chemistry
BDE-100	0,086	-0,002	Liver chemistry

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Paper II

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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 $[M\text{-}H^+]$, 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.

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

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

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).

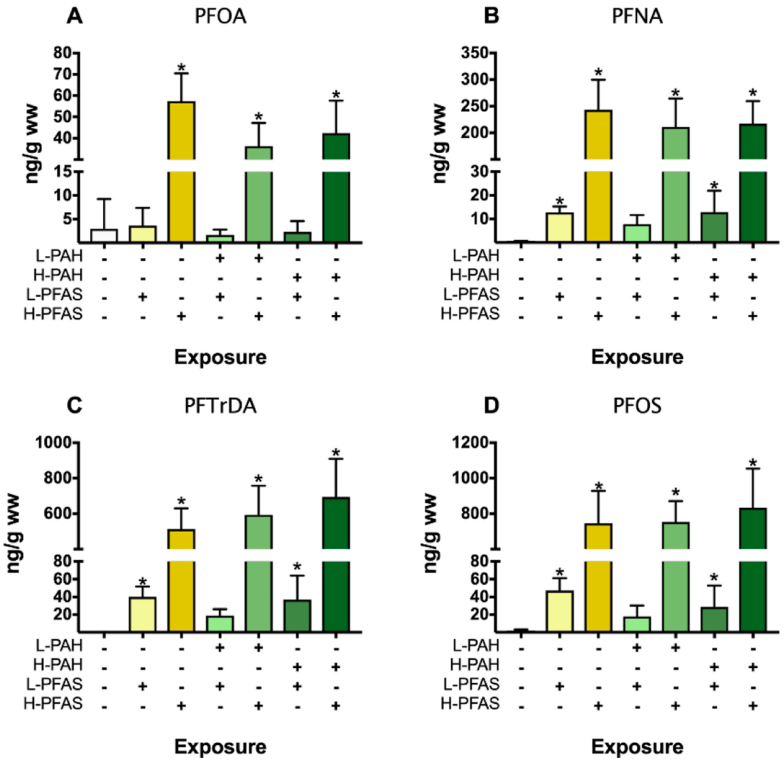


Fig. 1. PFAS concentrations in liver of Atlantic cod exposed to PAHs and PFASs. Atlantic cod were exposed to low (L = 1×) or high (H = 20×) doses of PAHs and PFASs either separately (PFAS, yellow), or combined (green). PFAS concentrations were determined in cod liver and are presented as ng/g wet weight 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). Distinct 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

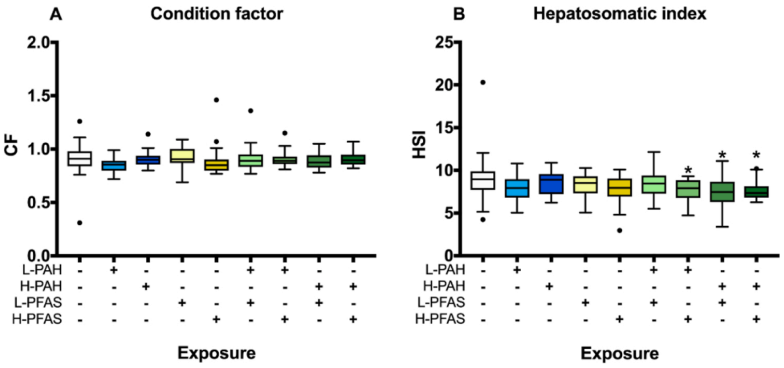


Fig. 2. Growth performance of Atlantic cod exposed to PAHs and PFASs. Post-exposure condition was assessed by calculating CF (A) and HSI (B) of Atlantic cod exposed to low (L = 1×) or high (H = 20×) doses of PAHs and PFASs either separately (blue and yellow, respectively) or combined (green). Data are 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).

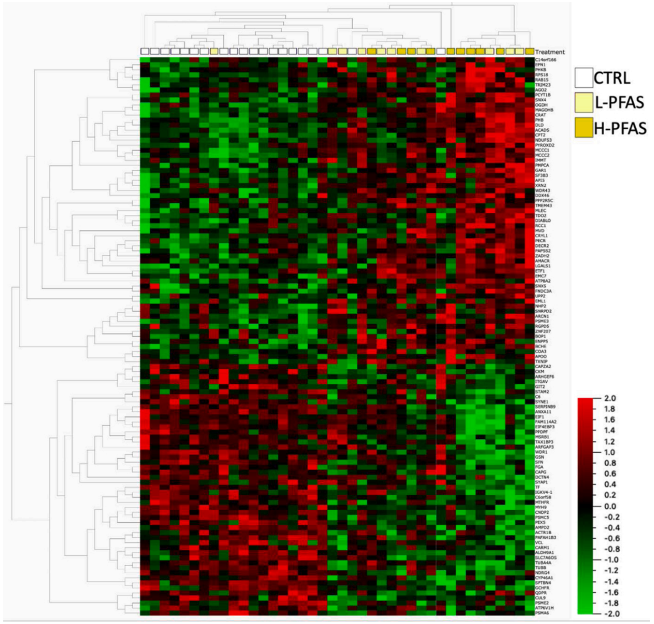


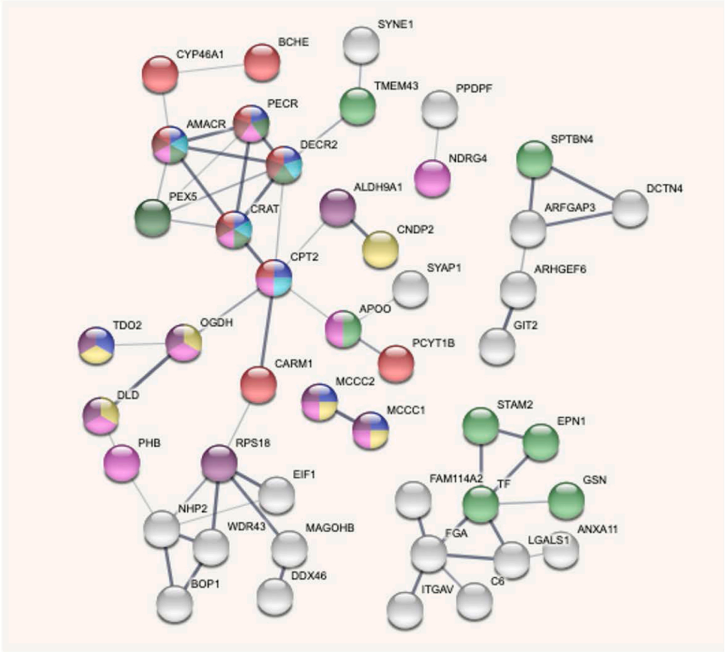
Fig. 3. Two-way hierarchical clustering of the top differentially expressed proteins in livers of cod exposed to L-PFAS and H-PFAS versus control. Data represent expression values (log-transformed) of 111 top differentially expressed proteins ($p = 0.005$, q-value < 0.125). The heatmap shows relative 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

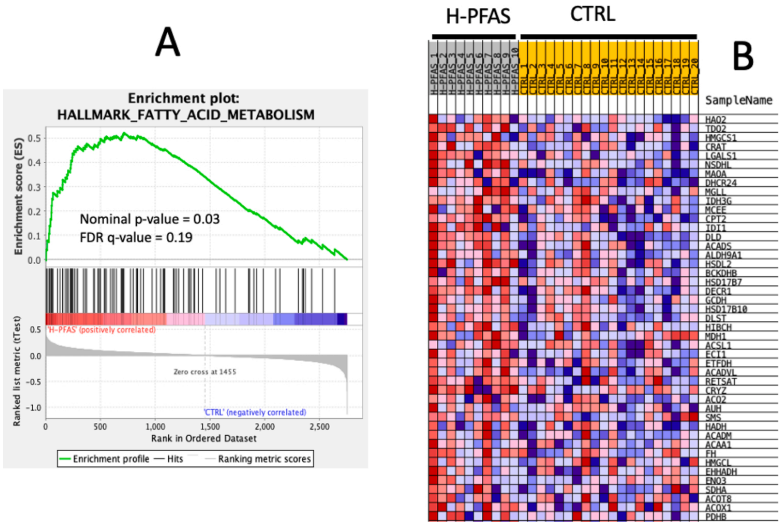


Fig. 5. Enrichment plot of the FATTY_ACID_METABOLISM pathway (A) and the corresponding core enrichment proteins (B) in livers of cod exposed to H-PFAS versus control. Data represent log2-transformed liver protein expression values in control cod (CTRL) and cod exposed high dose of PFAS (H-PFAS). The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

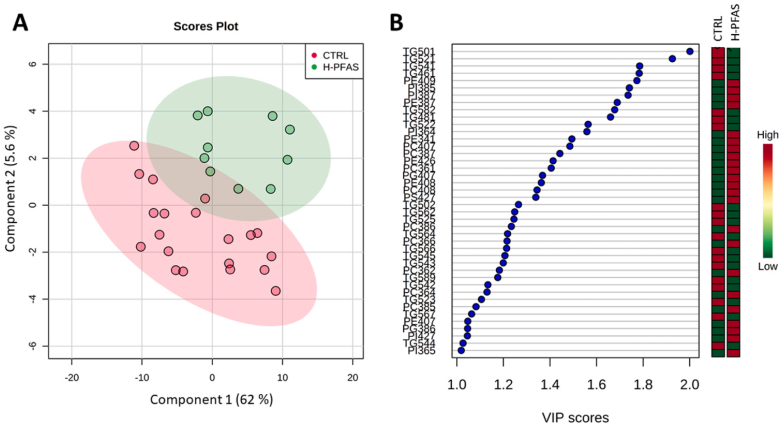


Fig. 6. Scores plot and Variable Importance in Projection (VIP) scores from PLS-DA multivariate analysis of liver microsomal lipidome in cod exposed to H-PFAS. Comparison in scores plot (A) between CTRL and H-PFAS. 42 VIP > 1 were considered relevant to explain the differences between groups (B). CTRL: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

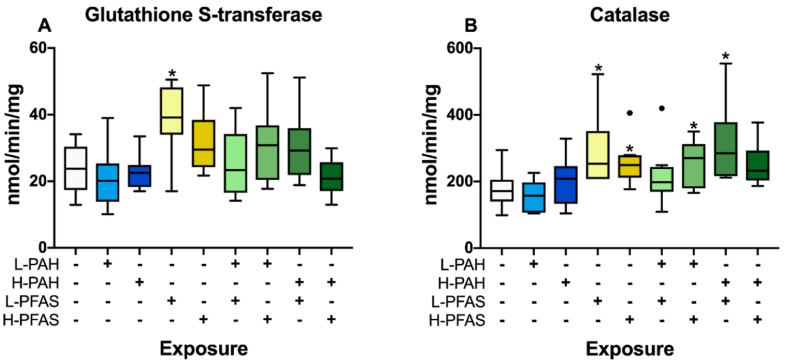


Fig. 7. Gst and Cat activities in liver of Atlantic cod exposed to PAHs and PFASs. The enzyme activities of (A) glutathione S-transferase (Gst) and (B) catalase (Cat) were determined in liver of Atlantic cod exposed to low (L = 1×) or high (H = 20×) doses of PAHs (blue) and PFASs (yellow) either separately 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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Supplementary information Paper II

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

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Materials and methods

S1. Chemical information

Table S1 – Overview of exposure chemicals.

Compound group	Full name	Abbreviation	CAS no.	Item no.*
PAH	Benzo[a]pyrene	BaP	50-32-8	48564
	Dibenzothiophene	DBT	132-65-0	45776
	Fluorene	Fluo	86-73-7	48568
	Naphthalene	Naph	91-20-3	84679
	Phenanthrene	Phen	85-01-8	48569
	Pyrene	Pyr	129-00-0	48570
PFAS	Heptadecafluorooctanesulfonic acid	PFOS	2795-39-3	77282
	Perfluorononanoic acid	PFNA	375-95-1	394459
	Perfluorooctanoic acid	PFOA	335-67-1	171468
	Perfluorotridecanoic acid	PFTrA	72629-94-8	654973

* All chemicals were purchased from Sigma Aldrich (St.Louis, MO, USA).

S2. PFAS chemical analysis

The analytical series included one non-spiked (blind) and two spiked salmon liver samples and three blanks (solvents). The lowest level of detection (LOD) for individual compounds was defined as three times the noise level for each compound. The LOD (ng/g wet weight (ww)) ranged between 0.032 and 0.554. Means of the relative recoveries ranged between 90 and 109%. Positive procedural blanks were found for some compounds. If the blank concentrations were consistent for all blank samples, results were corrected for blanks.

S3. Comet Assay

White blood cells were isolated from freshly sampled whole blood on a Percoll® gradient. The isolated cells were diluted in PBS EDTA (ethylenediaminetetraacetic acid) to ensure an optimal cell density for scoring. Cells were mixed with agarose gel (1:9), cast onto Gelbond ®films, and stored in cold lysis buffer (4 °C) prior to electrophoresis. For unwinding and relaxation of supercoiled DNA, films were rinsed in electrophoresis buffer at alkaline conditions (pH 13-14). Electrophoresis was run for 25 minutes at 4 °C. Films were treated with a neutralising buffer,

dehydrated with 96% ethanol and stored dry and without direct light exposure. The DNA staining was done using SYBR® Gold and the cell scoring was conducted using the Comet Assay IV software (Perspective Instruments, version 4.2). Cells around the edge of the gel, overlapping cells, cells with irregular shape and cells close to foreign objects were not scored. A tail intensity around 10% was considered acceptable for baseline measurements and indicates that cells were in good conditions for scoring (Collins, 2004).

S4. Proteomics analysis

Proteomics analysis was performed as described previously (Yadetie et al., 2016). Sample preparation: 20-100 mg of cod liver tissue were homogenized by sonication in a lysis buffer consisting of 8 M UREA, 0.1 M Tris-HCl, pH 8.5, 20 mM methylamine (10 µL added per mg tissue). After homogenization, samples were incubated at 37 °C for 5 min, centrifuged at 13000 rpm for 10 min, before protein concentrations in the lysates (supernatants) were determined with the Pierce BCA-protein assay kit (Thermo Fischer Scientific). 30 µg of protein from each sample was transferred to a LB Eppendorf tube, and volumes adjusted to 20 µL. 20 µL of 50 mM Tris-HCl, pH 7.9 was added, and samples incubated at room temperature (RT) for 5 min. Reduction and subsequent alkylation of the samples were performed by addition of 4 µL 100 mM DTT and 5 µL of iodoacetamide, respectively, with 1 hour incubation at RT for each reaction. 0.8 µL 100 mM DTT was thereafter added to quench unreacted iodoacetamide and avoid unwanted protease alkylation. Protein samples were then digested with trypsin (PROMEGA, Sequencing Grade Modified Trypsin) for 16 hours at 37 °C by adding 110 µL 50 mM Tric-HCl, pH 7.9, and 0.6 µg trypsin. Trypsin digestion was stopped by adding 10% TFA to a 1% final concentration, and resulting peptide samples were desalted with OASIS C18 filtration (Waters) and dried in a freezevac.

NanoLC-ESI-LTQ Orbitrap Elite mass spectrometry: About 0.5-1 µg tryptic peptides dissolved in 2% acetonitrile (ACN), 0.5% formic acid (FA), were injected into an Ultimate 3000 RSLC system (Thermo Scientific, Sunnyvale, California, USA) connected online to a linear quadrupole ion trap-orbitrap (LTQ-Orbitrap Elite) mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nanospray Flex ion source (Thermo Scientific).

The sample was loaded and desalted on a pre-column (Acclaim PepMap 100, 2cm × 75µm i.d. nanoViper column, packed with 3µm C18 beads) at a flow rate of 7 µl/min for 5 min with 0.1% TFA (trifluoroacetic acid, vol/vol).

Peptides were separated with a biphasic ACN gradient from two nanoflow UPLC pumps (flow rate of 200 nl /min) on a 50 cm analytical column (Acclaim PepMap 100, 50cm × 75µm ID nanoViper column, packed with 2 µm C18 beads). Solvent A and B was 0.1% FA (vol/vol) in water and 100% ACN respectively. The gradient composition was 5% B during trapping (5 min) followed by 5-7% B over 1 min, 7–21% B for the next 134 min, 21-34% B over 45 min, and 34–80% B over 10min. Elution of very hydrophobic peptides and conditioning of the column were performed during 20 minutes isocratic elution with 80% B and 20 minutes isocratic elution with 5% B respectively.

The eluting peptides from the LC-column were ionized in the electrospray and analyzed by the LTQ-Orbitrap Elite. The mass spectrometer was operated in the DDA-mode (data-dependent-acquisition) to automatically switch between full scan MS and MS/MS acquisition. Instrument control was through Tune 2.7.0 and Xcalibur 2.2. Survey full scan MS spectra (from m/z 300 to 2000) were acquired in the Orbitrap with resolution R = 240,000 at m/z 400 (after accumulation to a target value of $1e^6$ in the linear ion trap with maximum allowed ion

accumulation time of 300ms). The 12 most intense eluting peptides above a ion threshold value of 3000 counts, and charge states 2 or higher, were sequentially isolated to a target value of $1e^4$ and fragmented in the high-pressure linear ion trap by low-energy CID (collision-induced-dissociation) with normalized collision energy of 35% and wideband-activation enabled. The maximum allowed accumulation time for CID was 150 ms, the isolation with maintained at 2 Da, activation q = 0.25, and activation time of 10 ms. The resulting fragment ions were scanned out in the low-pressure ion trap at normal scan rate, and recorded with the secondary electron multipliers. One MS/MS spectrum of a precursor mass was allowed before dynamic exclusion for 40s. Lock-mass internal calibration was not enabled. The spray and ion-source parameters were as follows. Ion spray voltage = 1800V, no sheath and auxiliary gas flow, and capillary temperature = 260 °C.

Protein identifications were obtained by searching with the MS/MS data using the MaxQuant software and the Ensembl *Gadus morhua* protein database.

Protein quantification data (named Table S4 in the publication) with the file names “Paper II_Proteomics_Pre-processed data”, “Paper II_Proteomics_Protein Groups_1” and “Paper II_Proteomics_Protein Groups_2” can be found here:

https://github.com/kda036/PhD-thesis

S5. Lipidomics analysis

Reconstituted lipid extracts were analyzed by direct injection in an Orbitrap-Exactive (Thermo Fisher Scientific) equipped with an electrospray source (H- ESI II). Methanol/dichlorometane 80:20 at 50 µL/min was used as mobile phase. Mass spectra were acquired in full scan (ESI+/-), with an acquisition mass range (*m/z* 200-2000) and resolving power of R=100,000 (m/z 200,

FWHM). Data from LIPID MAPS®, exact mass, isotopic distributions, charge, adducts formed, number of ring plus double bond (RDB=0.5-15) and elements in formula, were used for the identification of the lipid molecules with a maximum permitted mass error fixed at < 5 ppm. Lipid species were quantified with a mixture of internal standards that consisted of 1,2,3-17:0 triacylglycerol (TG) (200 pmol), 15:0-18:1D7 diacylglycerol (DG) (100 pmol), 16:0D31-18:1 phosphatidylcholine (PC) (200 pmol), 17:0 lysophosphatidylcholine (LPC) (200 pmol), C18(Plasm)-18:1D9 phosphatidylcholine plasmalogen (PC-P) (200 pmol), 16:0D31-18:1 phosphatidylethanolamine (PE) (200 pmol), 16:0D31-18:1 phosphatidylinositol (PI) (100 pmol), 16:0D31-18:1 phosphatidylserine (PS) (200 pmol), 16:0D31 sphingomyelin (SM) (200 pmol), 16:0-D31 ceramide (Cer) (200 pmol), 17:0 cholesteryl ester (CE) (800 pmol). Repeatability, expressed as intra-day relative external standard deviation calculated for all lipid standards, was satisfactory (TG: 28%, DG: 16%, PC: 11%, LPC: 17%, PC-P: 17%, PE: 24%, PI: 22%, PS: 49%, SM: 16%, Cer: 19%, CE: 37%).

S6. Quantitative PCR

Table S2 – Sequences of cod primers for reference (*) and target genes used in liver qPCR

Gene name	Gene product	Accession no.	Forward sequence	Reverse sequence	Amplicon size (bp)
acox1	Acyl-coenzyme A oxidase 1				
ahrrb	Aryl hydrocarbon receptor repressor b		GTGTCCCCCACA ACACAAGG	GAGTGGAAGAG ATTGCTCACCA	
cyp1a	Cytochrome p450 1a		CACCAGGAGAT CAAGGACAAG	GCAGGAAGGAG GAGTGACGGAA	101
*ef1a**	Translation elongation factor 1a	ENSGMOG00 000012005	CGGTATCCTCAA GCCCAACA	GTCAGAGACTC GTGGTGCATCT	93
hsd17b7	Hydroxysteroid 17-beta dehydrogenase 7		ATTCGCGACCA ACCTATTTG	ATGGAGGAGGT CCAGATCAC	118
hsf2	Heat shock factor protein 2		ACGACCTGCTG GAGAACATC	GTTTCTTGTTTG CCGTGGAC	124
hmgcr	3-hydroxy-3-methylglutary-coenzyme A reductase		GGACATGCTGTC CTCCAGAC	GCATGTAGCCA ATGACGTTC	116
*uba52**	Ubiquitin A-52 residue ribosomal protein fusion	ENSGMOG00 000007530	GGCCGCAAAGA TGCAGAT	CTGGGCTCGACC TCAAGAGT	69

S7. ELISA

Table S3 – List of antibodies and dilutions utilized in ELISA

Type	Antibody	Species	Producer	Product no.	Dilution
Primary	Anti-cod Cyp1a	Mouse	Biosense Laboratories AS	C02406101	1:800
Secondary	Anti-mouse IgG/HRP Linked Whole Ab	Sheep	GE Healthcare UK	NA931	1:3000

Results

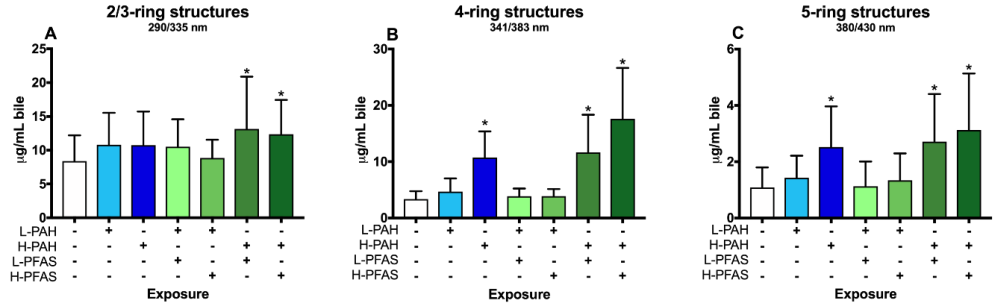

Figure S1 – Concentrations of PAH metabolites measured by fix wavelength fluorescence, in Atlantic cod exposed to PAH and PFAS. Atlantic cod were exposed to low (L=1×) or high (H=20×) doses of PAH and PFAS, either separately (PAH in blue) or combined (green). Data are presented as ug metabolite per mL bile for 2-3 ring structures (A), 4-ring structures (B) and 5-ring structures (C). n = 42 for CTRL and 19-21 for exposed groups, and asterisk indicates statistical significance ($p < 0.05$) between control and exposed groups (Kruskal-Wallis).

Table S5 – Concentrations of single PFAS compounds in cod liver exposed to PFASs alone or combined with PAHs. The table reports mean, median, minimum (min) and maximum (max) values in ng/g wet weight. Levels below LOD replaced with LOD to calculate the SUM. Detection rate below 60% for each compound within one group, only given range.

	PFOA				PFNA				PFTrDA				PFOS				∑PFAS			
	Mean	Median	Min	Max	Mean	Median	Min	Max	Mean	Median	Min	Max	Mean	Median	Min	Max	Mean	Median	Min	Max
CTRL			< LOD	25.1			< LOD	< LOD			< LOD	0.563			< LOD	6.64	5.14	1.85	1.82	32.4
L-PFAS	3.63	2.54	1.45	13.8	12.8	12.9	9.14	16.4	40.1	35.3	26.2	60.1	47.1	41.1	33.8	72.3	104	94.9	82.2	150
H-PFAS	57.4	54.8	42.0	86.1	243	230	183	365	513	512	398	787	746	707	457	1070	1560	1480	1110	2240
L-PAH/L-PFAS	1.64	1.16	0.420	3.71	7.84	6.54	3.97	14.4	18.8	15.8	10.2	29.4	18.0	11.1	6.88	37.6	46.3	34.3	22.1	84.4
L-PAH/H-PFAS	36.3	34.2	22.4	62.0	211	207	134	333	593	640	380	866	754	738	660	1060	1600	1570	1280	2170
H-PAH/L-PFAS	2.31	1.30	<LOD	7.45	12.9	8.16	5.79	33.9	37.0	25.2	17.4	88.9	28.6	13.3	9.58	79.7	80.8	47.2	33.6	210
H-PAH/H-PFAS	42.4	43.9	23.5	70.4	217	224	132	266	694	765	281	977	833	779	466	1280	1790	1800	920	2500

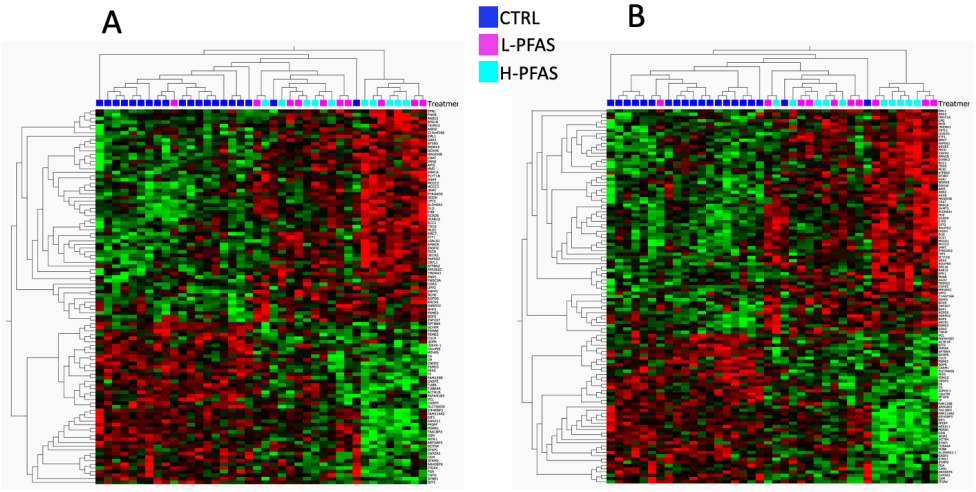

Figure S2 – Hierarchical clusters of top differentially expressed (p = 0.005, q-value < 0.125) proteins (after statistical analysis without correction for gender effect (A) and with correction for gender effect (B). For detailed figure legend Fig 3.

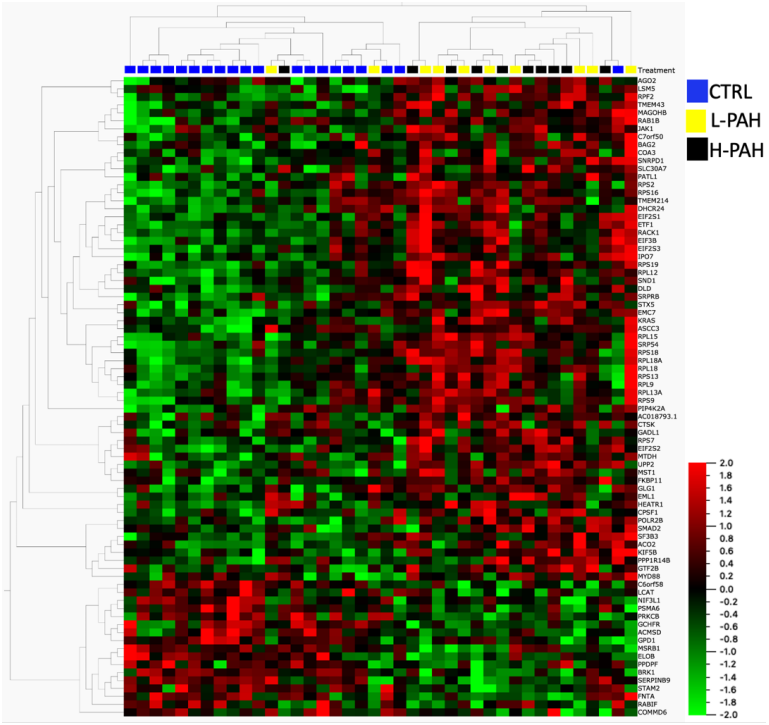

Figure S3 –A Two-way hierarchical clustering of the top differentially expressed proteins in control (CTRL), L-PAH and H-PAH groups. Data represent expression values (log-transformed) of top differentially expressed proteins (though not significantly, FDR qVal = 0.3). The heatmap shows relative expression 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. The control and treated groups are indicated by the color legend (top right).

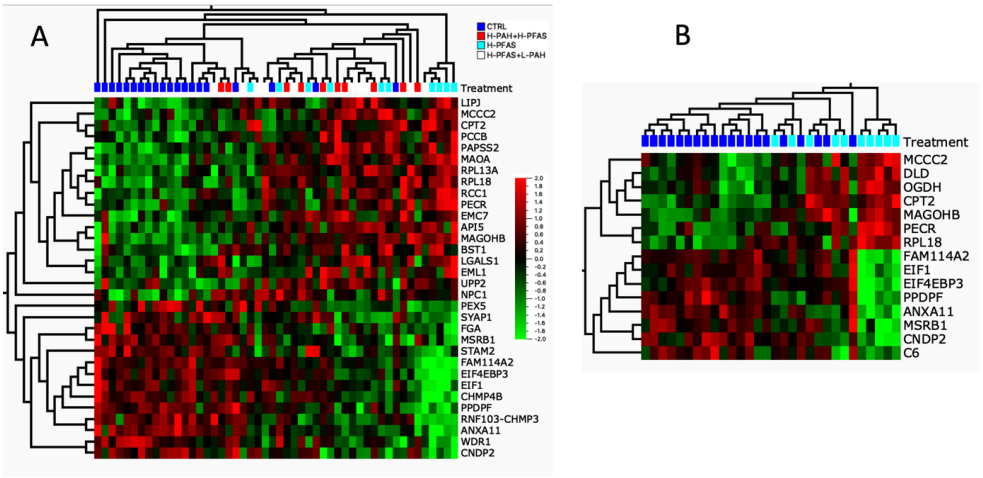

Figure S4 – Hierarchical cluster analysis of top differentially expressed (p = 0.0006, q-value < 0.05) proteins in high dose PFAS groups (H-PFAS, H-PAH/H-PFAS and L-PAH/H-PFAS) compared to control (CTRL) group (A) and H-PFAS alone compared to CTRL group (p = 0.0003, q-value < 0.05) (B).

Figure S5 Full network and pathways for proteomics data L-PFAS and H-PFAS group can be found with the file name "Paper II Figure S5 full network" here:

https://github.com/kda036/PhD-thesis

Table S6 – Significantly enriched gene sets in combined H-PFAS (H-PFAS, H-PAH/H-PFAS and L-PAH/H-PFAS) groups.

NAME	NES	NOM p-val	FDR q-val
HALLMARK_OXIDATIVE_PHOSPHORYLATION	1.7	0.03	0.13
HALLMARK_FATTY_ACID_METABOLISM	1.6	0.03	0.17
HALLMARK_ADIPOGENESIS	1.5	0.059	0.24
HALLMARK_CHOLESTEROL_HOMEOSTASIS	1.5	0.073	0.24

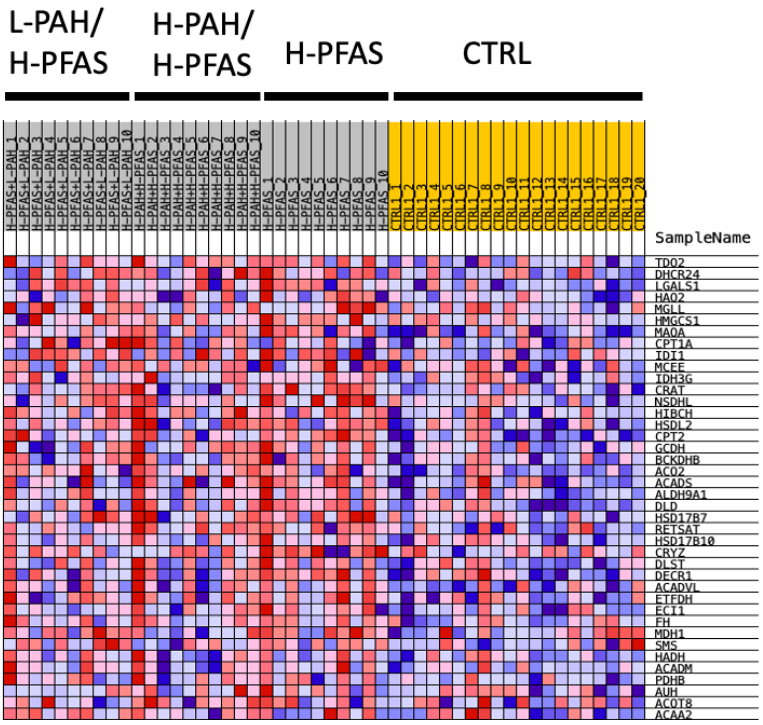

Figure S6 – Core enrichment proteins in FATTY_ACID_METABOLISM gene set enriched in combined H-PFAS (H-PFAS, H-PAH/H-PFAS and L-PAH/H-PFAS) groups. The heatmap represents protein expression levels, with red and blue representing high and low relative expression, respectively.

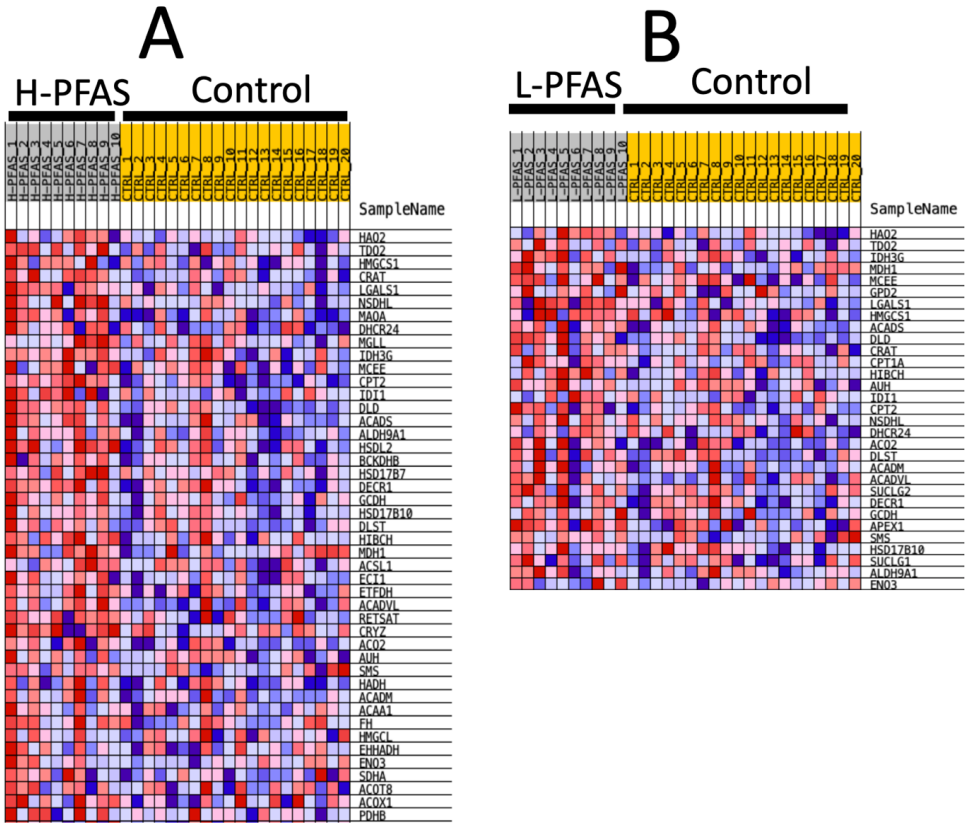

Figure S7 – Core enrichment proteins in FATTY_ACID_METABOLISM gene set from H-PFAS vs control (A) L-PFAS vs control (B) groups. The heatmap represents protein expression levels, with red and blue representing high and low relative expression, respectively. Note that the H-PFAS representation in (A) is identical to Figure 5 in the main manuscript, and is included here for comparison to the L-PFAS group in (B).

In agreement with proteomics data, PLS-DA analysis did not allow to differentiate the lipidome of the liver samples treated with L- or H-PAH from control (Fig. S8A,B) (-72% of the covariance explained for the latter; accuracy: 0.63; Q2: -0.13). Regarding the lipidome of fish exposed to mixtures of PAHs and PFASs, no differences were observed between samples exposed to L-PAH/L-PFAS and control (Fig. S8D). Almost no discrimination was observed either between control and H-PAH/H-PFAS exposed fish (71 % of the covariance explained; accuracy: 0.6, Q2: -0.09) (Fig. S8G). Nonetheless, it is worth mentioning that mainly PLs were selected as VIPs, all of them were up-regulated in exposed samples (Fig. S9). Interestingly, VIPs with higher values are PLs composed by highly unsaturated long-chain fatty acids (PE40:7, PE38:7, PI38:7, PE42:6). TGs were found down-regulated in the treated samples. This pattern is in agreement with the one detected in H-PFAS exposed group.

For the L-PAH/H-PFAS and H-PAH/L-PFAS mixture, the PLS-DA analysis did not allow discrimination between control and exposed cod (Fig. S8E, F).

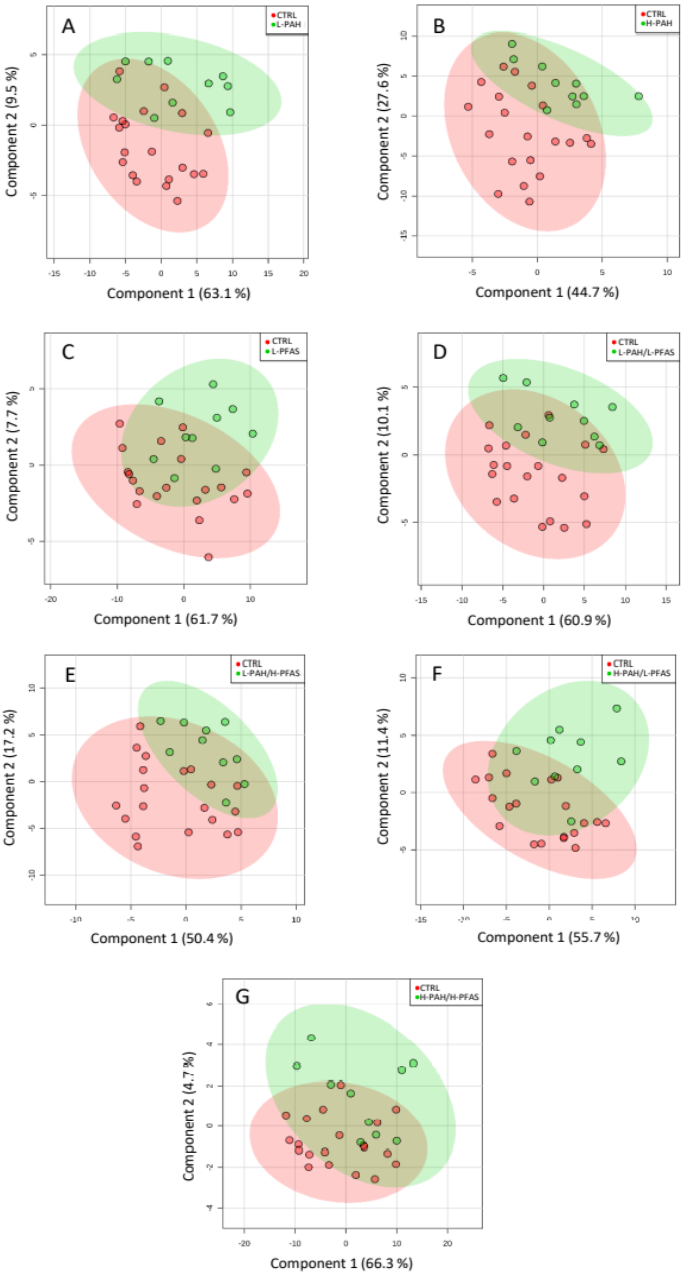

Figure S8 - Scores plots from PLS-DA multivariate analysis of liver lipidome. Comparison between control (CTRL) and L-PAH (A), H-PAH (B), L-PFAS (C), L-PAH/L-PFAS (D), L-PAH/H-PFAS (E), H-PAH/L-PFAS (F) and H-PAH/H-PFAS (G). Only the lipidome of fish exposed to a high concentration of PFAS (H-PFAS) showed difference from control samples (D).

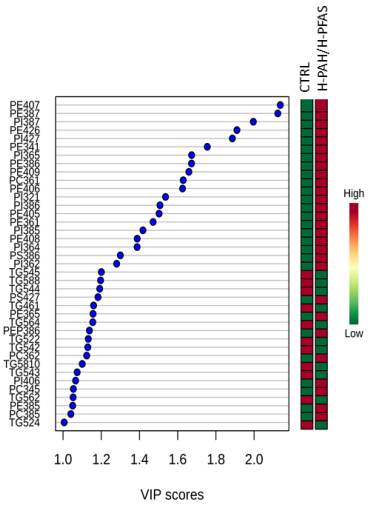

Figure S9 - Variable Importance in Projection (VIP) scores from PLS-DA multivariate analysis of liver lipidome in cod exposed to H-PAH/H-PFAS. 39 VIP > 1 were considered relevant to explain the differences between groups. CTRL: control cod; H-PAH/H-PFAS: cod exposed to a mixture of PAHs and PFASs, high concentration (20×).

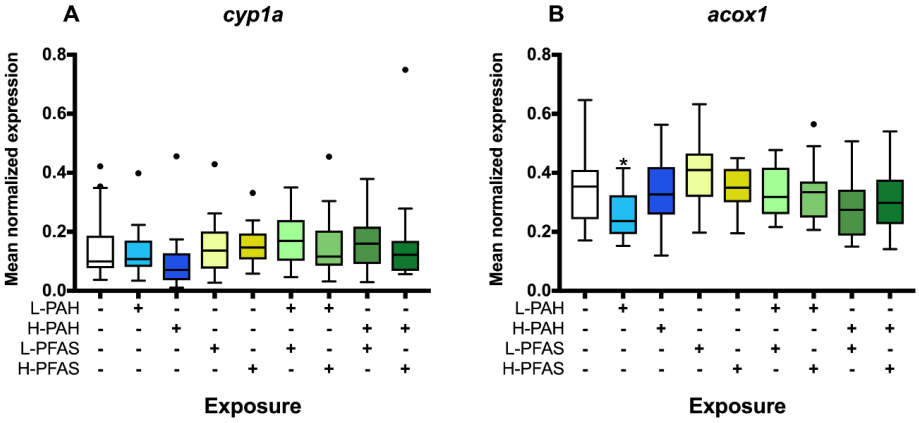

Figure S10 – Hepatic *cyp1a* and *acox1* expression of Atlantic cod exposed to PAHs and PFASs. Atlantic cod were exposed to low (L=1×) or high (H=20×) doses of PAHs and PFASs either separately (blue and yellow, respectively), or combined (green). Data are presented as mean normalized expression levels of *cyp1a* (A) and

acox1 (B), with median values and 25-75% quartiles. Whiskers are calculated using Tukey. n = 42-43 and 18-22 for control and exposed groups, respectively, and asterisk indicates statistical significance ($p < 0.05$) between control and exposed groups (Kruskal-Wallis).

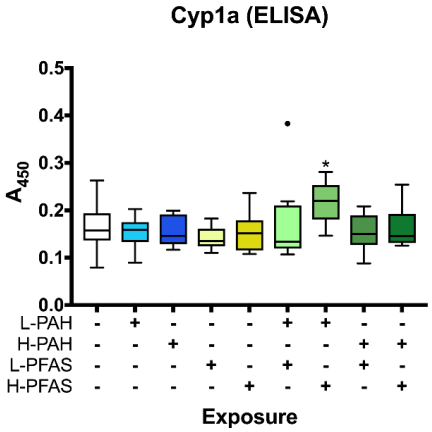

Figure S11 – Determination of Cyp1a protein amount in liver S12 fractions of Atlantic cod exposed to PAHs and PFASs. Atlantic cod were exposed to low (L = 1×) or high (H = 20×) doses of PAHs (blue) and PFASs (yellow) either singly, or combined (green). Protein amount presented as absorbance was determined in cod liver using ELISA. Data are presented as mean values and 25-75% quartiles, whiskers are calculated using Tukey. n = 20 and 10 for control and exposed groups, respectively, and asterisk indicates statistical significance ($p < 0.05$) when comparing exposed groups to control (ANOVA).

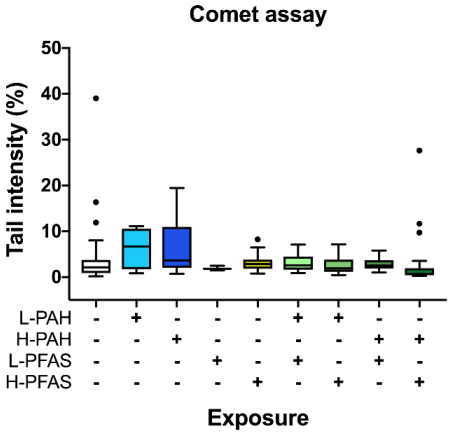

Figure S12 – DNA fragmentation measured by Comet assay in blood of Atlantic cod exposed to PAHs and PFASs. Atlantic cod were exposed to low (L = 1×) or high (H = 20×) doses of PAHs (blue) and PFASs (yellow) either separately, or combined (green). Data are presented as tail intensity, with median values and 25-75% quartiles. Whiskers indicate data range. N = 44 (CTRL) and 18-22 (exposed groups), with the exception of L-PAH (n = 4) and L-PFAS (n = 3). Statistical significance was tested by comparing exposure groups to control (Kruskal-Wallis).

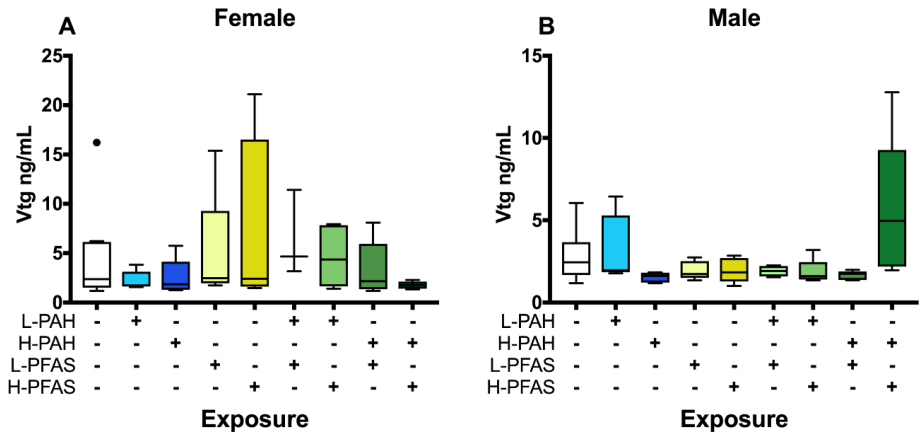

Figure S13 – Concentrations of vitellogenin in plasma of Atlantic cod exposed to PAHs and PFASs. The protein concentrations of vitellogenin (Vtg) were measured in plasma of both female (A) and male (B) Atlantic cod exposed to low (L = 1×) or high (H = 20×) doses of PAHs (blue) and PFASs (yellow) either separately or combined (green). n = 10 for control and n = 5 for other exposure groups per gender. Data are presented with median values and 25-75% quartiles, whiskers indicate data range. Statistical significance was tested by comparing exposed groups to control (ANOVA).

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