Mixture effects of benzo(a)pyrene and perfluoroalkyl substances on the aryl hydrocarbon receptor signalling pathway and energy metabolism of Atlantic cod (*Gadus morhua*)

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ABSTRACT

Most contaminants of emerging concern are man-made, and when introducing these compounds to consumers and our environment, it comes with a responsibility of obtaining the necessary knowledge of their toxicities. Polycyclic aromatic hydrocarbons (PAHs) are organic, lipophilic compounds that are carcinogenic and also ubiquitous within our environment. Per- and polyfluoroalkyl substances (PFAS) are organic, fluorinated compounds used both in industry and consumer products. They are stable and extremely persistent and can cause reproductive toxicity and affect metabolism. Toxicity testing has traditionally been performed on one compound at a time, but the exposure to wildlife and humans is complex. Mixtures might give rise to so-called "cocktail effects", where the combined response could be synergistic, potentiating, or antagonistic. It is important to gain insights into what the effects of these compounds might be, and if different combinations of compounds can make an exposure scenario more severe for biota. In this thesis Atlantic cod was used as a model organism, and precision cut liver slices were exposed *ex vivo* to three relevant PFAS compounds and benzo(a)pyrene. Effects on the Ahr and Ppara-signalling pathways were assessed by gene expression analyses of *cyp1a*, *acox1*, and *acly* using qPCR. An Ahr/Arnt/XRE based system was also used to study transactivation of the Ahr2a receptor *in vitro*. All three PFAS congeners were able to induce activation of *cyp1a* on their own, and produced apparent synergistic effects in combination with B(a)P. Trends of dose-dependent induction was shown for the expression of *acly* after exposure to PFOA and PFNA, which also induced the expression of *acox1*. For these two Pparα target genes, weak trends of further induction were present after co-exposures between all PFAS congeners and B(a)P. As expected, B(a)P transactivated the Ah-receptor in COS7-cells, but no further activation was shown in co-exposure to B(a)P and PFAS, presumably due to cytotoxic concentrations. The experiments performed in this master's thesis have accentuated that mixture effects is a field that must be explored further, as there are indications that combinations of B(a)P and PFAS could indeed give rise to responses beyond additive effects. The results from this thesis provide additional information that enhances the importance of integrating mixture effects into the toxicity testing and risk assessment of chemicals.

ABBREVIATIONS

Abcb4	ATP-binding cassette B4 (multidrug resistance protein)
AhR	Aryl hydrocarbon receptor
acly	APT Citrate Lyase
acox1	Acyl-CoA oxidase 1
ANOVA	Analysis of variance
Arnt	Aryl Hydrocarbon Receptor Nuclear Translocator
B(a)P	Benzo(a)pyrene
BNF	Beta-naphthoflavone
cDNA	Complementary DNA
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
CYP	Cytochrome P450
cyp1a	Cytochrome P450 1A
DDT	Dichlorodiphenyltrichloroethane
DEHP	Bis(2-ethylhexyl)-phthalate
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
EC_{50}	50% of maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EE2	Ethynylestradiol
EFSA	European Food Safety Authority
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
FICZ	6-formylindolo[3,2-*b*]carbazole
LRA	Luciferase Reporter Gene Assay
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NRT	No reverse transcriptase
NTC	No template control
OSPAR	Oslo/Paris convention (For the Protection of the Marine Environment of the North-East Atlantic)

PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate-Buffered Saline
PCB126	Polychlorinated Biphenyl 126 (3,3',4,4',5-pentachlorobiphenyl)
PCLS	Precision cut liver slices
PCR	Polymerase chain reaction
POP	Persistent organic pollutant
PFAS	Perfluoroalkyl substances
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctane sulfonic acid
PFNA	Perfluorononanoic acid
PMSF	Phenylmethylsulfonyl fluoride
Ppara	Peroxisome proliferator-activated receptor alpha
qPCR	Quantitative real time polymerase chain reaction
REACH	The European Regulation on Registration, Evaluation, Authorisaion and Restriction of Chemicals
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT	Room temperature
SEM	Standard error of mean
TCDD	2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
TBE	Tris-Borate-EDTA
TWI	Tolerable weekly intake
XRE	Xenobiotic response element

In this thesis, proteins are given in capital letters, gene names are written in italics, in accordance with HGNC Guidelines.

1 INTRODUCTION

1.1 BACKGROUND

Environmental contaminants are chemicals that are released into the environment either deliberately or unintended, and usually as a result of human activities. Examples are pesticides, industrial biproducts, waste materials from production and refinement of oil, and effluents from waste discharges. Once released into the environment, these compounds can spread via air, rivers, and ocean currents. An important group of environmental contaminants is referred to as persistent organic pollutants (POPs). These compounds originate from anthropogenic activities and are characterized by their stability and their persistence in the environment. Thus, a central property these compounds share is the ability to bioaccumulate within an organism, and further biomagnify up the food chain. To be defined as a POP, the compound must be persistent, bioaccumulative, toxic, and be subjected to long-range transport (*WHO | Persistent Organic Pollutants (POPs)*, n.d.). Examples of POPs are chlordane, dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs). These compounds are all listed in the Stockholm Convention on POPs. This is a convention that was put forth for signatories to identify and manage this class of contaminants, and the parties of the convention have agreed to reduce or, if possible, eliminate the manufacture, use, and import of these POPs to limit the release into the environment (Landis et al., 2018)

1.2 POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) comprise a group of organic, lipophilic compounds, consisting of aromatic carbon ring structures. They occur naturally in crude oil, and are central elements in creosote, tar, and asphalt. They were identified as carcinogens early on, and experiments performed already in 1963 showed that exposure to PAHs lead to formation of cancer cells (Aune, 2007). PAHs are released to the environment mainly through incomplete combustion of organic materials, and there are both natural and anthropogenic sources. Generation of power using fossil fuel and burning of waste are two examples of man-made emissions, while volcanic activity and forest fires are natural sources. Because of this, PAHs are ubiquitous within the environment. In most organisms, these compounds are metabolised through P450-enzymes and epoxide hydrolases into metabolites that can interact with the

genetic material and form DNA-adducts, and the biological half-life varies among different organisms (e.g. around 24 hours for humans, and 2-3 weeks in blue mussels) (Aune, 2007). The most studied PAH is benzo(a)pyrene (B(a)P), and one of the reasons is the broad toxicological properties that this compound inhabits. It is known to induce Cyp1a activity through activation of the aryl hydrocarbon receptor (Ahr), and Ahr-activation is therefore a commonly used biomarker for exposure. In this thesis, B(a)P will be used in co-exposures as a known agonist of Ahr. B(a)P itself is one of the xenobiotics that are transformed by P450-enzymes and will therefore be able to affect its own metabolism through activation of the Ah-receptor (Mao et al., 2018) .

1.3 PERFLUOROALKYL SUBSTANCES (PFAS)

Per- and polyfluoroalkyl substances (PFAS) are a group of organic, fluorinated compounds that are used both in industry and consumer products. There are over 5000 PFAS congeners on the commercial market (Miljødirektoratet, 2019) that are used in various applications, including water-resistant fabrics, food packaging, paint, firefighting foams, as non-stick surfactants in cookware, and more. Perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA) are two of the PFAS congeners that have been most widely used. PFAS compounds are stable and extremely persistent in nature due to the high energies of the carbon-fluorine covalent bonds. Because of the slow decay, they will remain in the environment for long periods and have therefore been referred to as “forever chemicals” (Allen, 2018; Gibbens, 2020). This property, along with the widespread use, gives these chemicals extended potential for exposure, and therefore, PFAS represent one of the more contemporary groups of chemicals of environmental concern.

PFASs are able to bind to blood proteins in vertebrates and accumulate mainly in the liver and kidney (Galatius et al., 2013). There are several reported effects of PFAS, and some of them are related to immunotoxicity (Grandjean et al., 2012), reproductive toxicity (Lau et al., 2003; Luebker et al., 2005), and effects on energy metabolism (Berthiaume & Wallace, 2002). As they are amphiphilic, the PFAS tend to accumulate within living organisms and high levels have been found in marine mammals and predators on top of the food chain, such as seals and dolphins (Galatius et al., 2013; Kannan et al., 2001). The PFAS compounds can be transported over long distances, through air via long range atmospheric transport or via rivers and ocean currents

(Landis et al., 2018). Accordingly, PFAS have been found in arctic waters and species, such as polar bear (*Ursus maritimus*) and polar cod (*Boreogadus saida*) (Kannan et al., 2001; *Perfluorinated Alkylated Substances, Brominated Flame Retardants and Chlorinated Paraffins in the Norwegian Environment - Screening 2013*, n.d.). Several monitoring studies along the Norwegian coastline have shown that several PFAS congeners are present in sediments, runoff water, as well as in fish tissues, including cod liver samples (*Kartlegging av utvalgte nye organiske miljøgifter 2004*, 2005; *Perfluorinated Alkylated Substances, Brominated Flame Retardants and Chlorinated Paraffins in the Norwegian Environment - Screening 2013*, n.d.; Valdersnes et al., 2017). PFOS and PFOA are shown to be the most ubiquitous congeners in biota, and they are also the most studied PFAS molecules. In addition, PFOS is one of the chemicals classified as a POP in the Stockholm Convention on Persistent Organic Pollutants, and all production, import, export and sale of merchandise containing PFOS and PFOS-related compounds were banned in 2007 (*Begrensningsdirektivet - PFOS*, 2006).

1.3.1 PFAS TODAY

Since the production of PFAS compounds started over half a century ago, there has been a lack of regulation regarding manufacture and use of these substances. One example of industrial release of PFAS into the environment is linked to a paper-factory's 50-year long history of discharge of such compounds, which were used in the production of various paper wrappings. The factory was located along the river Randselva that empties into Tyrifjorden, Norway. Tyrifjorden serves as a source of drinking water, and a screening of 53 different PFAS congeners performed by the Norwegian Environment Agency showed elevated levels of PFAS in sediments throughout the river and fjord (*Tyrifjorden*, 2019). The skiing industry has also been a contributor of PFAS-exposure and release of PFASs into the environment. Through the article series "Glidens pris", the Norwegian magazine Dagbladet shed light on poor working conditions, high mortality-, and cancer rates amongst workers in an Italian factory producing ski wax for the Norwegian company Swix. A cohort study of workers that most likely had been exposed to PFAS and exhibited elevated serum concentrations of PFOA, showed a higher risk of overall mortality, diabetes, liver cancer, and liver cirrhosis (Girardi & Merler, 2017). As a part of the article series, Norwegians applying wax to skis on an amateur level also got the PFAS-levels in their blood tested, and PFOA-levels varied between 8 and 36 ng/ml compared to an average of 2.4 ng/ml in the general population (Folkehelseinstituttet, 2017). Elevated levels of

PFAS have also been found in soil-samples, bank vole (*Myodes glareolus*) and earthworm (*Eisenia fetida*) in a skiing area in Granåsen, Norway, and the PFAS patterns in the soil resembled the patterns found in commonly used ski wax samples, indicating that this is indeed an important source of environmental contamination (Grønnestad et al., 2019).

As food is also a source of PFAS exposure for humans, the European Food Safety Authority (EFSA) set a new safety threshold for the main PFAS that accumulate in humans in September 2020. Tolerably weekly intake (TWI) of PFAS congeners as a group is now lowered to 4.4 ng per kilogram bodyweight (EFSA, 2020). From July 2020, EU banned the production and sale of chemicals and products containing more PFOA than 0.025 ng/kg product, and this also includes around 100 other compounds that could be precursors of PFOA (*Skadelig fluorstoff forbudt - Miljødirektoratet*, n.d.). From the season 2021/2022, the International Ski Federation (FIS) has also decided to ban the use of fluorinated compounds in competitions. (Aftenposten, 2020; *Decisions of the FIS Council Meeting in Constance (GER) Autumn 2019*, 2019). It is important that one regulated PFAS compound is not simply replaced by another congener with similar or more harmful effects. Since there are many similarities between different PFAS molecules, it is suggested that these compounds should be managed as a class, such as for example organophosphate pesticides already are, to ensure an efficient approach to reduce adverse effects on humans and environmental health (Kwiatkowski et al., 2020).

1.4 MIXTURE EFFECTS

Toxicity testing has traditionally been performed on one compound at a time, but the scenarios in which organisms are exposed are more complex than that. Different xenobiotics are present in mixtures, and seldom one by one. These mixtures might give rise to so-called “cocktail effects”, where the combined response is not simply the sum of the effects induced by each toxicant alone. The combined effects may be higher (synergistic or potentiating), or they may be less than the effects produced by the compounds separately (antagonistic) (Bizarro et al., 2016; Silkworth et al., 1993). As mentioned, benzo(a)pyrene is a well-studied compound, and so are some of the most used PFAS congeners as well, but the combinations of these compounds and the mixture effects they could give rise to is a less explored field. Previous research has suggested that PFAS can modulate the uptake and toxicity of other chemicals, and PFOS has shown to inhabit the ability to act as a chemosensitizer for other chemicals through

interfering with cellular efflux transporter ATP-binding cassette B4 (Abcb4) proteins (Keiter et al., 2016). Notably, mixture-specific induction of the P450 system has been shown in zebrafish (*Danio rerio*) after exposure to polychlorinated biphenyl 126 (PCB126) and PFAS (Blanc et al., 2017), and combinations of PAHs and PFAS have shown to increase lipid catabolism in Atlantic cod by affecting lipid degradation pathways in liver (Dale et al., 2020). It is therefore important to assess how different compounds interact in relevant mixtures, in addition to the traditional toxicity analyses of single substances.

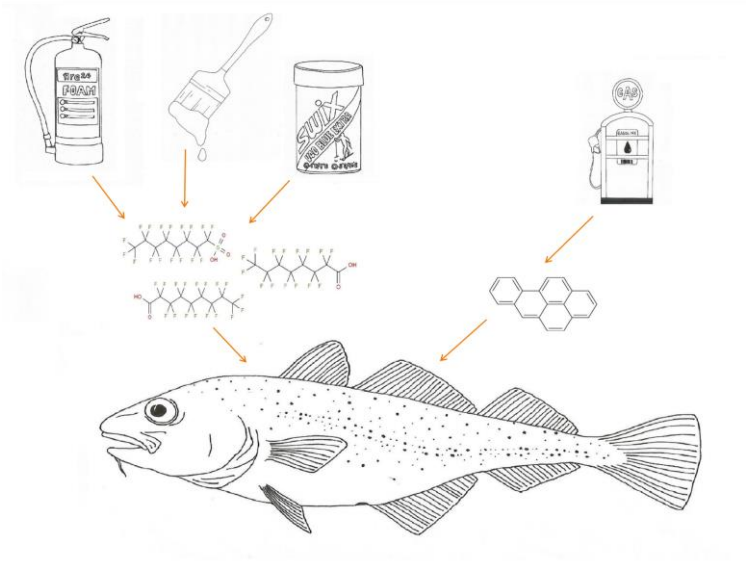


Figure 1: ***Complex exposure scenarios to environmental contaminants might give rise to so-called cocktail effects.***

1.4.1 THE ARYL HYDROCARBON RECEPTOR

The Aryl hydrocarbon receptor (Ahr) is a xenosensor that mediates cellular responses to environmental pollutants. It belongs to the helix-loop-helix family of dimeric transcription factors, and unliganded, the receptor is located in the cytoplasm of the cell in a protein complex with two HSP90 proteins, a cochaperone protein (p23), and Ahr-interacting protein (AIP) (Avilla et al., 2020). The binding of a ligand allows the ligand-receptor-complex to dissociate from the chaperones and translocate into the nucleus of the cell (Larigot et al., 2018). Inside the nucleus, the ligand-receptor complex heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (Arnt), and Ahr-Arnt is a competent DNA-binding heterodimer. The dimer binds to xenobiotic response elements (XREs) upstream of Ahr-target genes and induces the transcription of genes encoding enzymes that are involved in the biotransformation of xenobiotics, e.g. cytochrome P450 1a (CYP1a) (Figure 2). Transcription of Ahr repressor (AhRR)

is also induced by Ahr, and AhRR will bind to Arnt, competing with the dimerization to Ahr and forming a negative feedback regulation of Ahr transcriptional activity. The CYP1 metabolism of ligands and the interaction of AhRR with Arnt will prohibit further Ahr-Arnt dimerization (Avilla et al., 2020).

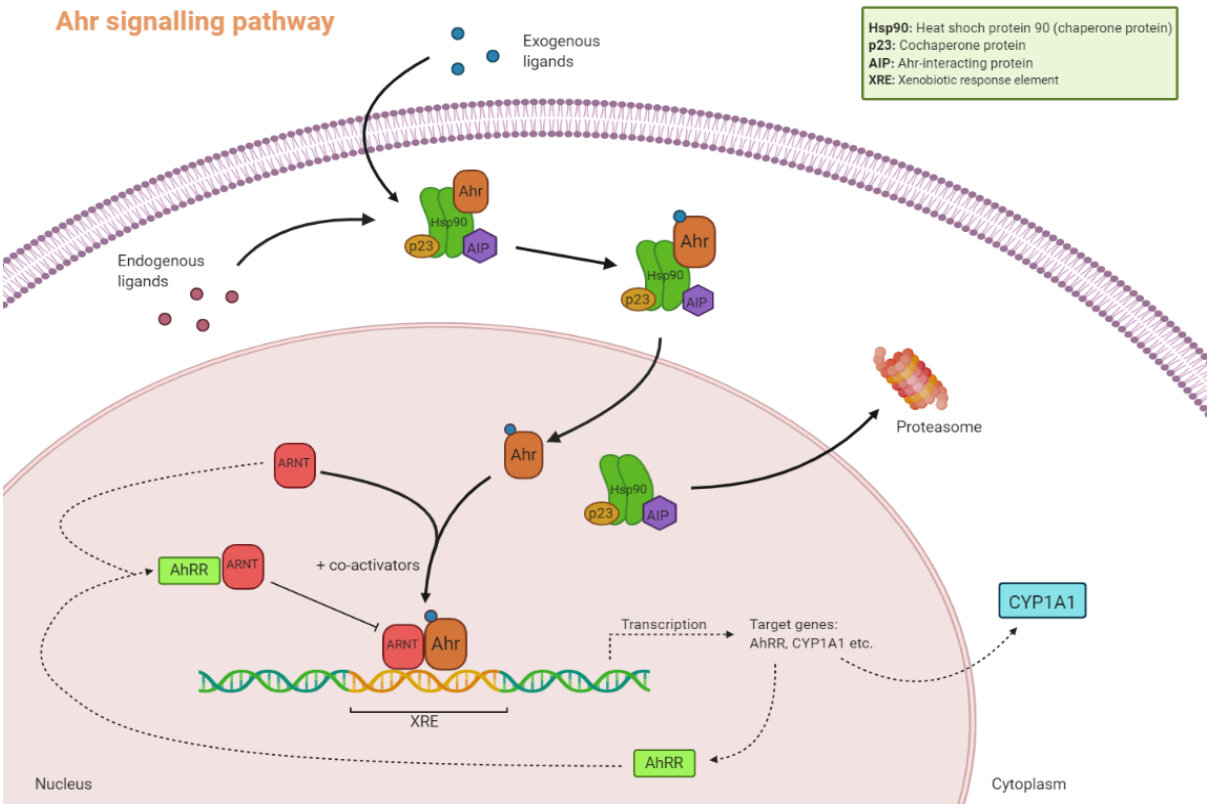


*Figure 2: **Ahr signalling pathway and regulatory functions**. Created in Biorender.com, redrawn after* (Larigot et al., 2018)

Some well-known Ahr agonists are B(a)P, beta-naphthoflavone (BNF), PCB126, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), where the latter is the most potent exogenous ligand. Experiments performed on mice have shown that B(a)P-exposure induced cancer tumours in Ahr-positive mice, but that no tumours were formed in Ahr-deficient mice. This indicates that the biotransformation caused by activation of the Ah-receptor and mediated through Cyp1a is central in giving B(a)P its cancer forming potential (Shimizu et al., 2000).

1.4.2 PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR (PPAR)

A similar family of xenobiotic-responsive transcription factors are the peroxisome proliferator-activated receptors (Ppar). As the name indicates, they were first described as cellular receptors

for peroxisome proliferators, which are xenobiotics that can increase the size and number of peroxisomes within liver cells (Issemann & Green, 1990). PPAR alpha (Pparα) is one of the PPARs that regulates fatty acid oxidation through induction of the gene Acyl-coA oxidase 1 (*acox1*), which is a key enzyme in the first stage of β-oxidation. ATP citrate lyase (*acly*) is also an important PPARα target gene as it codes for the enzyme that function as a cross-link between the glycolysis and the fatty acid synthesis pathway. PPARα has shown to play a role in the cancer development in rodents exposed to the phthalate bis(2-ethylhexyl)-phthalate (DEHP) as PPARα knockout mice are resistant to DEHP-induced tumours (Burcham, 2014). PFOS, PFOA and perfluorononanoic acid (PFNA) have previously shown to modulate the activity of mouse, human and cod PPARα and (Behr et al., 2020; Söderström, 2017; Takacs & Abbott, 2007).

1.5 ATLANTIC COD

The term "In cod we trust" might have occurred as a misprint on American coins but made perfect sense to our Norwegian ancestors. For thousands of years, the cod has been a central part of living and frankly the basis of existence in Norway. It has been of great importance to the Norwegian economy and identity, and was used to trade for other goods, and as a currency in payment for tax or rent (Jenssen, 2012). The cod is still the "white gold" of the North East Atlantic, and the most valuable species within the fisheries sector in Norway. In 2019, amounts worth a total of 10.1 billion NOK were exported. This was a 7% increase in value since 2018 despite a 8% decrease in volume (*Sjømateksport for 107,3 milliarder kroner i 2019*, 2020). In addition to being an important food source, the cod's widespread distribution makes it vulnerable to effluents of anthropogenic sources, and hence it is also used as an indicator species for monitoring the presence of pollutants in the environment. Cod is included in the Convention for the Protection of the Marine Environment of the North-East Atlantic (the OSPAR convention) List of threatened and/or declining species or habitats. The Atlantic cod belongs to the family Gadidae and is widely spread throughout the North Atlantic and the Baltic ocean (Figure 3).

Cod resides both in the shoreline and the continental shelf, and although it is considered a demersal fish it inhabits the pelagic ocean at different conditions and phases of its life cycle (*FAO Fisheries & Aquaculture - Species Fact Sheets - Gadus morhua (Linnaeus, 1758)*, n.d.).

Along the coast of Norway, we mainly separate between three main types of Atlantic cod: the coastal cod, the fjord cod and the "skrei", which migrates from the Barents Sea and spawns along the Norwegian coast.

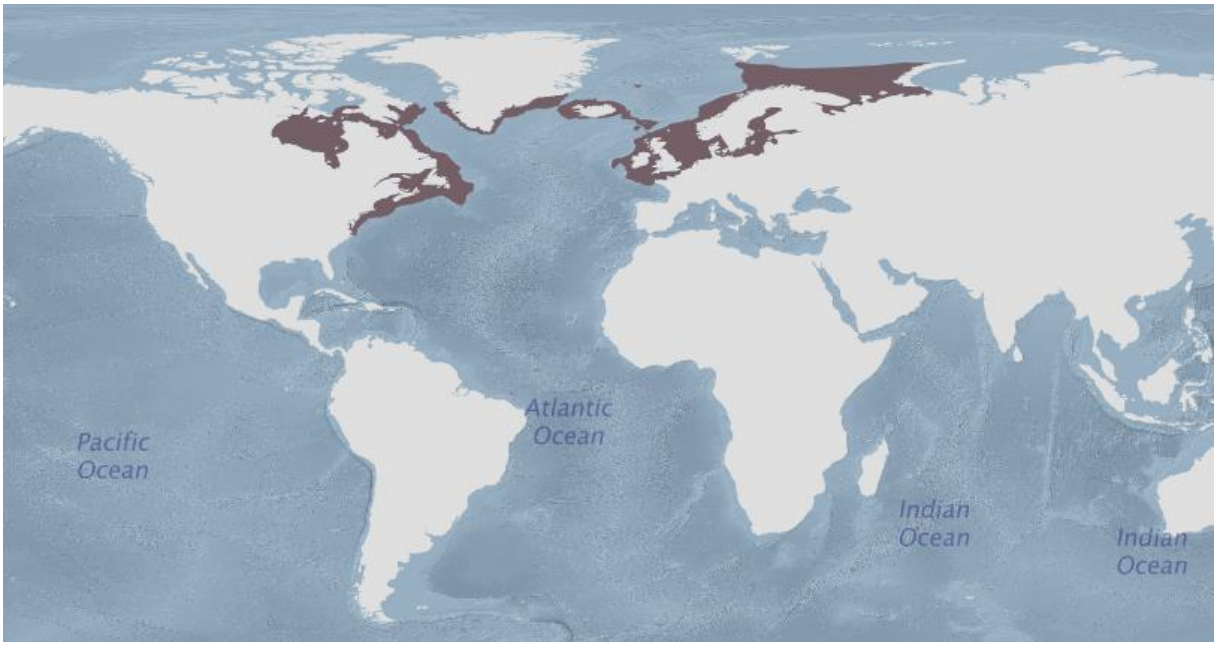


Figure 3: ***Distribution of Atlantic cod (Gadus morhua).*** *Map:* (*FAO Fisheries & Aquaculture - Species Fact Sheets - Gadus Morhua (Linnaeus, 1758)*, n.d.)

The genome of the Atlantic cod was recently sequenced and annotated (Star et al., 2011), and this has made it possible to perform large scale toxicogenomic studies on this species. It has been frequently used as a model organism (Bizarro et al., 2016; Dale et al., 2020; Meier et al., 2007; Sturve et al., 2006). The cod has two gmAhr-encoding genes, Ahr1a and Ahr2a, which demonstrate tissue-specific expression profiles and differences in ligand binding affinities (Aranguren-Abadía et al., 2020). The liver is a central target organ for contaminants as it plays a major role in detoxification of xenobiotics, and it may have a higher susceptibility to accumulation of lipophilic contaminants as the cod has a very fatty liver where the hepatocytes contain several large lipid droplets (Fujita et al., 1986).

1.6 PCLS AS AN EX VIVO MODELLING SYSTEM

Cell culture, such as cell lines or primary cells, is an *in vitro* model system that is widely used, and in line with the 3R principles (Tannenbaum & Bennett, 2015) by considerably reducing the use of animals in scientific experiments. It is a convenient methodology to use as most cell cultures can be stored in liquid nitrogen for long periods before use, and the cells can be easily

quantified. However, some limitations exist. For instance, due to cell differentiation and mutagenic events as a result of cultivation, isolated cell systems have shown limited ability to predict toxicity *in vivo* (Segner, 1998). Also, the unique hepatocyte morphology in Atlantic cod makes the isolation techniques of primary cells less efficient than with other species. Precision cut liver slices (PCLS) is another model system that also has been applied in several toxicological studies (Bizarro et al., 2016; Du et al., 2016; Eide et al., 2014). As with cell cultures, this method creates the opportunity to conduct experiments in a lab environment reducing the amounts of test individuals needed, and at the same time provide highly relevant results, as it is a versatile *ex vivo* model where the distribution of the cells maintain the 3-dimensional tissue structure as found *in vivo*. PCLS have shown to give a closer prediction of *in vivo* toxicity than cell cultures (Elferink et al., 2008). Furthermore, in this system the biotransformation of compounds could occur, and through activation of the P450 system, the compounds could affect their own metabolism. In 1985 Smith *et al.* published the first paper on isolation and maintenance of PCLS and use in toxicology (Davies, 2012). In the later years, different variations of the technique have been presented, and applied to numerous other species, including Atlantic cod (*Gadus morhua*) (Eide et al., 2014). As in most other vertebrates, the detoxification of xenobiotics occurs mainly in the liver of Atlantic cod. Therefore, cod liver slices were used to assess the toxicity of the compounds and mixtures used in this thesis. When preparing precision cut liver slices, the central part of the cod liver was cut into thin slices using a vibratome and kept in culture medium. The slices must be thin enough to make sufficient diffusion of oxygen and nutrients possible, and given the right conditions, Atlantic cod PCLS are viable in culture for at least 72 hours (Eide et al., 2014).

1.7 THE LUCIFERASE REPORTER GENE ASSAY

The Luciferase Reporter Gene Assay (LRA) is a commonly used *in vitro* system for characterization of ligand activation of transcription factors, such as Ahr and nuclear receptors, and is used to study regulation of gene expression at a transcriptional level. It is a quick and sensitive assay that provides quantitative measurements of potencies and efficacies, and it has been used to characterize Ahr in various fish species, such as zebrafish , Atlantic cod and rainbow trout (*Oncorhynchus mykiss*) (Abnet et al., 1999; Aranguren-Abadía et al., 2020; Hansson & Hahn, 2008). Different variations of the method exist, but in general, a cell line is transfected with plasmids encoding the receptor of interest and the reporter gene.

In this thesis, an Ahr/Arnt/XRE based system was used, where COS-7 cells were initially transfected with the receptor plasmids (pcDNA3.1_gmAhr2a; pcDNA3.1_gmArnt), a luciferase reporter plasmid (pGudLuc6.1), a normalization plasmid (pCMV-β-galactosidase) and empty pcDNA3.1/Zeo. Through ligand binding, Ahr will dimerize with Arnt and bind to xenobiotic response elements (XRE) that are located in the promoter region upstream of the luciferase reporter gene. Binding of the Ahr/Arnt dimer induces the transcription of the reporter gene, and luciferase is produced. Figure 4 illustrates schematically how this happens.

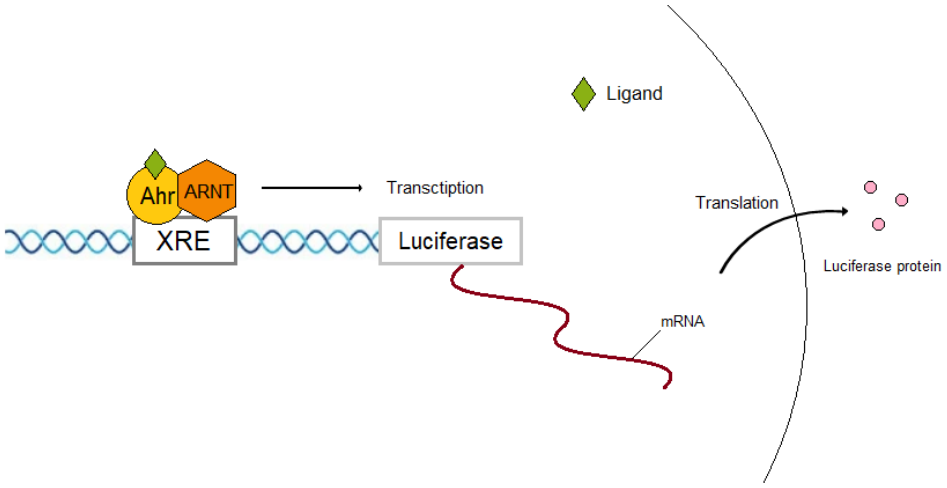


Figure 4: ***In vitro reporter gene system for ligand binding and transactivation.***

After lysis of the cells, D-luciferin is added to the wells and the luciferase enzyme will catalyse the reaction illustrated in Figure 5. Oxyluciferin is a luminescent compound, and nearly all the energy put into the reaction is rapidly converted to light as oxyluciferin falls back to its ground state. The measured luminescence will reflect the receptor activation.

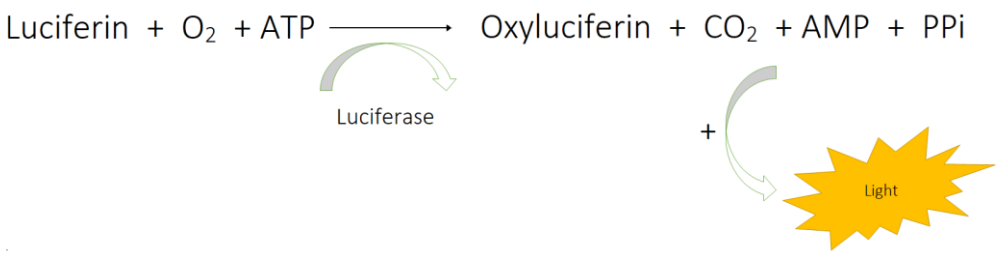


Figure 5: ***Chemical reaction catalysed by luciferase and producing light.***

1.8 AIM

This study is considered a continuation of previous work done in our group by (Dale et al., 2020) where Atlantic cod was exposed *in vivo* to mixtures of 6 different PAHs and 4 different PFASs in either a low dose considered environmentally relevant (1x), or a high dose (20x). Fish were exposed to low or high doses of PAHs and PFAS either separately or combined to study potential mixture effects between the two classes of compounds. Few mixture effects were revealed in this experiment, but the results showed that the high dose exposure to PFAS congeners influenced peroxisomal and mitochondrial fatty acid catabolic pathways, as several PPARα target genes and important enzymes in β-oxidation were upregulated.

The aim of this master's thesis is to characterize toxicological effects in Atlantic cod after co-exposure to three relevant PFAS compounds and benzo(a)pyrene, focusing on changes in gene expression levels and Ahr activation. Two model systems are used, precision cut liver slices and a luciferase reporter gene system using COS-7 cells. By studying alterations in gene expression, modulation of the energy metabolism and the biotransformation of xenobiotics will be especially targeted. Three of the PFAS compounds used by (Dale et al., 2020) in addition to a PFAS mixture was selected, and exposure to the PFAS compounds was done both separately and in combination with benzo(a)pyrene to characterize potential mixture effects between PFAS and PAHs. The following questions were asked:

- Could PFASs alter the energy metabolism and the biotransformation of xenobiotics in Atlantic cod?
- Could PFASs modulate the toxicity of B(a)P in Atlantic cod?
- Could B(a)P modulate any potential effects demonstrated by PFASs in Atlantic cod?

2 MATERIALS

2.1 ATLANTIC COD (*GADUS MORHUA*)

The Atlantic cod used in this work were hatched during the spring 2018 at Havbruksstasjonen i Tromsø AS (Tromsø, Norway) and transported to Bergen in August the same year. The cod were kept at the Industrial and Aquatic Laboratory (ILAB, Bergen, Norway) in 500L tanks in natural seawater at 8-10°C at a 12/12 h light cycle regime, and fed *ad libitum* with commercial feed, Amber Neptun (Skretting, Stavanger, Norway).

2.2 CHEMICALS AND REAGENTS

Table 1: List of chemicals and reagents used in the thesis

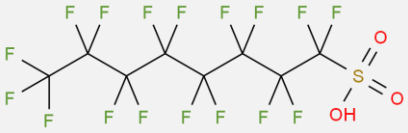
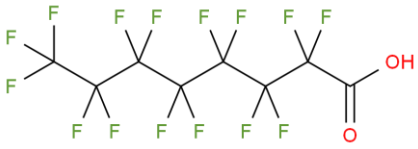
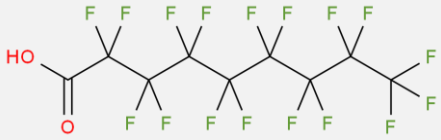
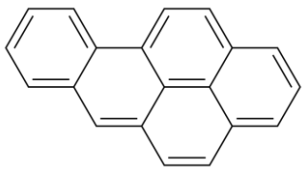
Chemical/reagent	*Supplier / Product number*
2-log ladder (0.1 – 10 kb)	New England Biolabs / N3200s
5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM)	Thermo Fisher Scientific / C1354
ß-mercaptoethanol	Aldrich / M6250
Adenosine triphosphate (ATP)	Sigma / A2383
Agarose	Sigma / 2A9538
Ampicillin sodium salt	Sigma-Aldrich / 69-52-3
BlueJuice™Gel loading buffer (10 X)	ThermoFisher / 10816-015
Boric acid	Merck / A9647
Bovine serum albumin (BSA)	Sigma / A4503
Calcium chloride ($CaCl_2$)	Merck

CHAPS	Appli-Chem / A1099
Chloroform	Sigma-Aldrich / 67-66-3
Coenzyme A	Fisher Scientific / 18439-24-2
D-Luciferin (Firefly)	Bio-Synth / L-8200
Dimethyl sulfoxide (DMSO)	Sigma / D8418
Disodium hydrogen phosphate Na_2HPO_4	Sigma / 30435
DL-Dithiothreitol (DTT)	Sigma / D0632
Dulbecco's Modified Eagle's Medium – high glucose (w/ phenol red)	Sigma / D5671
Dulbecco's Modified Eagle's Medium - high glucose (w/o phenol red)	Sigma / D1145
Erythrosine B	Sigma-Aldrich
Ethanol	Sigma / 32221
Ethylene diamine tetraacetic acid (EDTA)	Merck / 108418
Ethylene glycol tetraacetic acid (EGTA)	Sigma / E3889
Foetal bovine serum (FBS)	Sigma / F7524
Formamide	Sigma / F9037
Galactose	Sigma-Aldrich / G0625
GelRed	Biotium / 41003
Glycerol	Sigma / G5516
Isopropanol	Kemetyl / 603117000

L-α-phosphatidylcholine	Sigma / P3644
Leibowitz-15 medium (-phenol red + L-glutamine)	TermoFisher / 21083- 027
L-glutamine	Sigma / G7513
Magnesium carbonate hydroxide pentahydrate $Mg(CO_3)4Mg(OH)·5H2O$	Sigma-Aldrich / 56378-72-4
Magnesium chloride $MgCl_2$	Merck
Magnesium chloride hexahydrate $MgCl_2·6H2O$	Sigma-Aldrich / M9272
Magnesium sulfate heptahydrate $MgSO_4·7H2O$	Sigma-Aldrich / 63140
OptiMEM w/glutamax	Life Technologies / 31985- 062
Ortho-Nitrophenyl-β-galactoside (ONPG)	Sigma / N1127
Penicilin-streptomycin-amphotericin (1%)	Sigma-Aldrich
Penicillin-streptomycin (1000U)	Sigma / P4458
Phenylmethylsulfonyl fluoride (PMSF)	Sigma / P7626
Phosphate buffered saline (PBS) 10X	Sigma / P5493
Potassium chloride KCl	Sigma / P9541
Potassium dihydrogen phosphate KH_2PO_4	Merck
Resazurin	Sigma-Aldrich
Sodium bicarbonate $NaHCO_3$	Merck
Sodium chloride NaCl	Merck / 106404

Sodium phosphate dibasic dihydrate $Na_2HPO_4 \cdot 2H_2O$	Sigma-Aldrich
Sodium pyruvate	Sigma / S8636
TransIT® LT1	Mirus / MIR 2300
Tricine	Sigma / T0377
TriReagent	Sigma / T9424
Tris HCl (pH 7.8)	Merck
Triton® X100	Sigma-Aldrich / T4258
Trypsin-EDTA 1X (0,05% trypsin, 0,02% EDTA)	Sigma / 59417c

Table 2: Ligands used in PCLS- and COS-7 cell exposure

Compound	*Structure*	*Supplier*
Perfluorooctane sulfonic acid (PFOS)		Sigma Aldrich
Perfluoroctanoic acid (PFOA)		Sigma Aldrich
Perfluorononanoic acid (PFNA)		Sigma Aldrich
Benzo(a)pyrene		Sigma Aldrich

2.3 KITS

Table 3: Commercial kits used in PCLS

Kit	*Description*	*Supplier/product number*
Cytotoxicity Detection kit (LDH)	Cytotoxicity testing (PCLS)	Roche / 11644793001
iScript™ cDNA Synthesis kit	Reverse transcription	BioRad/1708891
Lightcycler® 480 SYBR Green	Quantitative real-time PCR	Roche / 04887352001

Table 4: Commercial kits used in Luciferase reporter gene assay

Kit	*Description*	*Supplier/product number*
NucleoBond® Xtra Midi plasmid purification kit	Plasmid purification	Macherey-Nagel / 740410.100

2.4 SOLUTIONS

2.4.1 PRECISION CUT LIVER SLICES

Table 5: PCLS-buffer

Component	*Mass (g)*
NaCl	7.13
KCl	0.35
$Na_2HPO_4 \cdot 2H_2O$	1.95
$MgSO_4 \cdot 7H_2O$	0.29
$NaHCO_3$	0.31

pH adjusted to 8.4, volume brought to 1 L with deionized water, solution sterile filtered and stored at 4°C

Table 6: Culture medium used for PCLS

Component	*Volume (mL)*
Leibowitz-15 medium (÷ phenol red, + L-glutamine)	44.5
Charcoal-stripped + heat-inactivated FBS	5.0
penicillin-streptomycin-amphotericin (1%)	0.5

Table 7: Tris-borate-EDTA (TBE) buffer (5X)

Component	*Concentration (M)*
Tris HCl	0.45
Boric acid	0.45
Ethylenediamine tetraacetic acid (EDTA)	0.01
Deionized water	-

Table 8: Preparation of 0.75% agarose gel

Component	*Amount*
Agarose	1.125 g
0.5 X Tris borate EDTA (TBE) buffer	150 mL
GelRed	0.5 µL

2.4.2 LUCIFERASE REPORTER GENE ASSAY

Table 9: Culture medium used for cultivation of COS-7 cells (DMEM w/10% FBS)

Component	*Volume (mL)*
Dulbecco's modified Eagle medium (DMEM)	500
Foetal Bovine Serum (FBS)	50
L-glutamine	10
Sodium pyruvate	5.0
Penicillin-streptomycin (10 000U)	5.0

During ligand exposure, DMEM w/o phenol red and super stripped FBS was used.

Table 10: Composition of DMEM-transfection medium

Component	*Volume per well (µl)*
Plasmid mixture	0.1
OptiMEM w/glutamax	9.0
TransIT LT1	0.2
DMEM w/10% FBS (Table 9)	92.1
Total	101.4

Table11: Cell lysis base buffer (1.05X) for luciferase reporter gene assay

Component	*Concentration*
Tris HCl (pH 7.8)	25 mM
Glycerol	15%
CHAPS	2.0%
L-α-phosphatidylcholine	1.0%
BSA	1.0%

Table 12: β-galactosidase base buffer (10 X, pH 7.2) for luciferase reporter gene assay

Component	*Concentration (mM)*
Na_2HPO_4	60
$NaH_2PO_4 \cdot H_2O$	40
KCl	10
$MgCl_2 \cdot 6H_2O$	1.0

Table 13: Luciferase base buffer (4 X, pH 7.8) for luciferase reporter gene assay

Component	*Concentration (mM)*
Tricine	80
$Mg(CO_3)4 \cdot Mg(OH) \cdot 5H_2O$	4.28
EDTA	0.40
$MgSO_4 \cdot 7H_2O$	10.68

Table 14: Lysis buffer

Component	*Concentration*
Cell lysis base buffer (1.05 X) (Table 11)	1 X
EGTA (100 mM)	4.0 Mm
DTT (1000 mM)	1.0 mM
$MgCl_2$ (500 mM)	8.0 mM
PMSF (250 mM)	0.4 mM

Table 15: β-galactosidase reaction buffer

Component	*Concentration*
β-gal base buffer (1 X) (Table 12)	1.0 X
β-mercaptoethanol (14.2 M)	52.9 M
ONPG	8.6 mM

Table 16: Luciferase reaction buffer

Component	*Concentration*
Luciferase base buffer (4 X, pH 7.8) (Table 13)	1 X
Deionized H_2O	-
DTT (1000 mM)	5 mM
ATP (100 mM)	0.5 mM
*Coenzyme A **	0.2 mM
*D-luciferin (10 mM) **	0.5 mM

* Added just before use

Table 17: L15/ex medium

Component	*Volume (mL)*
Solution A (Table 18)	34
Solution B (Table 19)	6.0
Solution C (Table 20)	17
Galactose (90 mg/l)	5.0
Sodium Pyruvate (110 mM)	2.5
Water, sterile filtered (0.2µm)	500

Table 18: L15-solution A

Component	*Mass (g)*
NaCl	80
KCl	4.0
$MgSO_4 \cdot 7H_2O$	2.0
$MgCl_2 \cdot 6H_2O$	2.0

Volume brought to 600mL with deionized water, solution autoclaved and stored in RT.

Table 19: L15-solution B

Component	*Mass (g)*
$CaCl_2$	1.4

Volume brought to 100mL with deionized water, solution autoclaved and stored in RT.

Table 20: L15-solution C

Component	*Mass (g)*
Na_2HPO_4	1.9
KH_2PO_4	0.6

Volume brought to 300mL with deionized water, solution autoclaved and stored in RT.

Table 21: Resazurin-CFDA-AM solution

Component	*Volume (µL)*
Resazurin (0.15 mg/ml)	20
CFDA-AM (4 Mm)	0.1
L15/ex medium (Table 17)	79.9

2.5 PRIMERS, PLASMIDS & CELL LINES

Table 22: Primers used in PCLS

Primer	*Direction*	*Nucleotide sequence 5' - 3'*	*Supplier*
acly	F	CTGCGGTGGATTTACACGAGATGA	Sigma Aldrich
	R	CTTCTGGTCCAGGTAGTGTCCGATGA	
acox1	F	ACTGGAGCATGTGCGAATC	Sigma Aldrich
	R	ACCATGGTGCCGTAGGTTAG	
arp	F	TGATCCTCCACGACGATGAG	Sigma Aldrich
	R	CAGGGCCTTGGCGAAGA	
cyp1a	F	CACCAGGAGATCAAGGACAAG	Sigma Aldrich
	R	GCAGGAAGGAGGAGTGACGGAA	

Table 23: Plasmids used in luciferase reporter gene assay

Plasmid	*Description*
pcDNA3.1_gmAhr2a	Eukaryote expression plasmid encoding cod Ahr2, receptor plasmid)
pcDNA3.1_gmArnt	Eukaryote expression plasmid encoding cod Arnt
pCMV-ß-galactosidase	Eukaryote expression plasmid encoding β-galactosidase, LRA transfection control plasmid
pGudLuc6.1	Eukaryote expression plasmid encoding firefly luciferase, LRA reporter plasmid
pcDNA3.1/Zeo	Eukaryote expression vector plasmid, empty

Table 24: Cell lines used in luciferase reporter gene assay

Cell line	*Description*	*Supplier / product number*
StrataClone Solo Pack competent cells	Prokaryote cloning (*E. coli*)	Agilent / 240205
COS-7 cells	Eukaryote kidney cell (African green monkey)	

2.6 INSTRUMENTS

Table 25: Instruments used

Instrument	*Application*	*Supplier*
CFX96™ RealTime PCR System C1000™ Thermal Cycler	qPCR	BioRad
ChemiDocTM XRS+ System	Gel scan	BioRad
DOPPIO Thermal Cycler	PCR Thermo Cycler	VWR
EnSpire 2300 Multilabel Reader	Plate reader	PerkinElmer
Heraeus Multifuge X3R Centrifuge	Centrifuge	Thermo Fisher Scientific
Homogenization tool	Homogenization of PCLS-samples	Xenox / D-54343
HS 501 Digital	Shaker	IKA-Werke
Leica DMBL Leica Microsystems	Microscope	LEICA
Leica VT1200 S vibrating blade vibratome	Vibratome	LEICA
Mini Vortex 230V	Mixer	VWR
Nanodrop One	Spectrophotometer	Thermo Scientific
New BrunswickTM Galaxy® 170 R	CO2-incubator	Eppendorf
PowerPac™ HC	High-current power supply	BioRad
Ultrospec 10	Cell density meter	Amersham Biosciences
Z 216 MK microliter centrifuge	Centrifuge	Hermle

2.7 SOFTWARE

Table 26: Software used

Software	*Application*	*Supplier*
BioRad CFX Manager 3.1	Analysis of qPCR data	BioRad
Biorender.com	Figure	
EnSpire Manager	Operation of plate reader	PerkinElmer
Excel (2009)	Data treatment	Microsoft
ImageLab	Agarose gel visualization	BIO RAD
Prism V.7.05	Statistics and figures	Graphpad

3 METHODS

3.1 PRECISION CUT LIVER SLICES

3.1.1 EXPERIMENTAL DESIGN

In the first part of the thesis, precision cut liver slices (PCLS) were prepared and exposed to the compounds of interest for 48 hours. The slices were then harvested, and extraction of RNA was done to prepare complementary DNA (cDNA). The cDNA was used in quantitative real-time PCR (qPCR) to measure the expression of the genes of interest. To assess the cell viability, a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was also conducted (Figure 6).

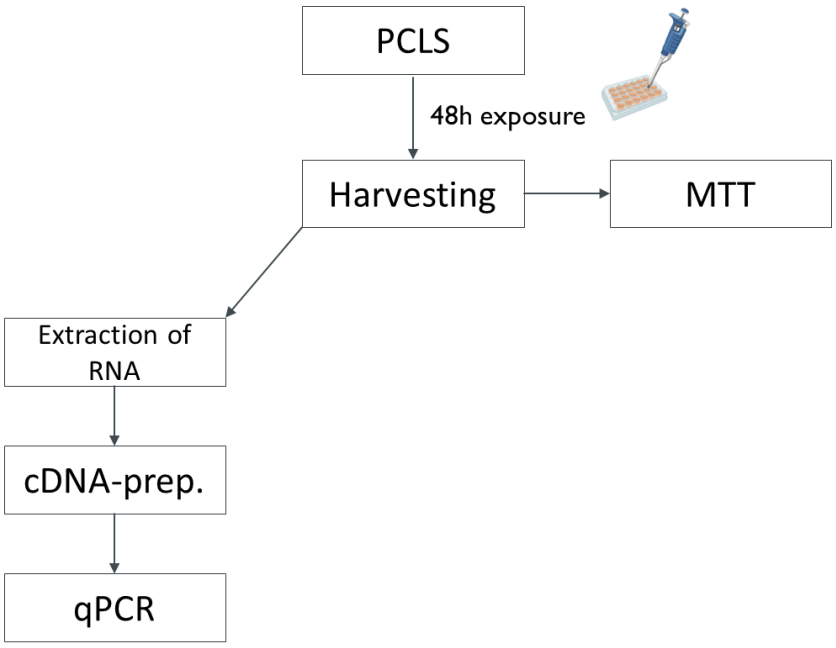


*Figure 6: **Flow chart of the process from collecting the cod liver to running the qPCR analyses and cell viability assay (MTT)***

3.1.2 EXPERIMENTAL DESIGN

The compounds studied in this thesis were the perfluoroalkyl substances (PFAS) PFOS, PFOA and PFNA, in addition to the PAH compound B(a)P. All compounds were dissolved in 100% DMSO, and 1000x stock solutions were prepared at 10, 50 and 100 mM. As 2 µL of these stock

solutions were added to 2 mL of culture medium, all exposures would have a 0.1% final DMSO concentration, and 0.1% DMSO was therefore used as solvent control. Exposure to B(a)P alone was also done to demonstrate the compound's capability of activating *cyp1a* gene expression. Three concentrations of B(a)P were used, 0.1, 1.0 and 10 µM, and four replicate exposures of each concentration were carried out (n=4 fish). An LDH assay was conducted to assess the cytotoxicity of B(a)P before choosing the fixed concentration for the co-exposures. This work is not described here.

Two experiments were performed using PCLS. In Experiment 1, the liver slices were exposed to PFOS, PFOA, PFNA, or a PFAS-mix, in increasing concentrations in addition to a solvent control. In Experiment 2, the PCLS were exposed to the same PFAS-compounds, but in combination with B(a)P at a fixed concentration. The concentrations given for the PFAS-mix represent ΣPFAS, which means that the 10 µM exposure consists of 3.33 µM PFOS, 3.33 µM PFOA and 3.33 µM PFNA. This also applies to the 50 µM and 100 µM PFAS-mixtures.

Experiment 1

The PCLS were exposed to three different concentrations (10, 50 and 100 µM) of each PFAS congeners, as well as the mix containing all three PFASs as shown in Table 27. Four replicates of each exposure were carried out using n=4 fish (n=6 for B(a)P + PFAS-mixture).

Table 27: Overview of test compounds and concentrations used in the single compound and PFAS-mixture exposures of the PCLS (Experiment 1).

Compound (µM)	*Exposures*				
	Solvent control	PFOS	PFOA	PFNA	PFAS-mix
DMSO (0.1%)	x	X	x	X	X
PFOS (10, 50, 100)		X			X
PFOA (10, 50, 100)			x		X
PFNA (10, 50, 100)				X	X

Experiment 2:

In Experiment 2, the liver slices were exposed to the same PFAS-concentrations as used in Experiment 1, and in addition in combination with a fixed B(a)P-concentration of 1 µM (Table 28).

Table 28: Overview of test compounds and concentrations used in combined exposures of the PCLS (Experiment 2).

Compound (µM)	*Exposures*					
	Solvent control	B(a)P	B(a)P + PFOS	B(a)P + PFOA	B(a)P + PFNA	B(a)P + PFAS-mix
DMSO (0.1%)	x	x	x	x	x	x
BaP (1.0)		x	x	x	x	x
PFOS (10, 50, 100)			x			x
PFOA (10, 50, 100)				x		x
PFNA (10, 50, 100)					x	x

3.1.3 PREPARATION OF PRECISION CUT LIVER SLICES

Juvenile fish of both sexes were used for the experiments, in total 11 male and 9 female fish. To obtain a semi-sterile work environment, work benches and all equipment were washed using 70% ethanol before the experiments began. The PCLS buffer was sterile filtered (0.2 µm) into an autoclaved glass flask and kept at 4°C (for up to two weeks). Each individual cod was euthanized with a blow to the head before length and weight were registered, --and the fish opened on the ventral side, from the gills to the anus using a scalpel. The sex of the fish was recorded, before the liver was dissected free, weighed, and placed in PCLS-buffer. The liver was kept on ice throughout the procedure to reduce degradation of liver cells. Cubes measuring 3x2x1-2 cm were prepared from the central part of the liver using a Feather® cutting blade and transferred to culture medium (Table 6). The cubes were then glued onto a directional specimen plate that was lowered into the vibratome chamber filled with PCLS-buffer and

surrounded with ice. A vibratome (Leica VT1200 S vibrating blade vibratome, LEICA biosystems) was used to cut 250 µm thick tissue slices, which were further sliced into squares measuring approximately 5x5 mm. Prepared slices and squares were kept in culture medium until sufficient material needed for conducting the exposure experiments was obtained. Four squares were transferred to each well of a 12-well plate containing 2 ml culture medium, and equilibrated for 2 hours on 50 revolutions per minute (rpm) shaking at 10°C.

3.1.4 EXPOSURE AND HARVESTING OF PCLS

After the equilibration period, the liver slices were exposed to the compounds of interest by adding the compounds (2 µl of 1000x exposure concentrations) directly into the wells. To avoid direct exposure of the stock solutions to the hepatic cells, the plate was tipped, and the liver slices moved to one side of the well as the test compound was added to the opposite side. The compounds were mixed thoroughly into the medium using a pipette before the plate was levelled and the slices moved away from the wall of the well. Exposures were performed for 48 hours at 50 rpm shaking at 10°C. After 48 hours of exposure, the cells were harvested. Using tweezers, the liver slices were transferred to 1.5 mL microcentrifuge tubes, weighed and snap-frozen by being placed directly in liquid nitrogen, and subsequently stored at -80°C.

3.1.5 RNA EXTRACTION

Precision cut liver slices were collected from the -80°C-freezer and placed in liquid nitrogen. 500 µl trizol (TriReagent Sigma) was added to the frozen tissue in each tube and the samples were homogenized using a homogenization tool (Xenox). Additional 500 µl trizol was added, and the tubes were flipped a few times and placed on ice. The homogenization tool was rinsed with dH_2O between samples and washed thoroughly when changing between PCLS exposed to different compounds. When all samples were homogenized, they were incubated in room temperature (RT) for 5 minutes before 200 µl chloroform was added to each tube. The tubes were closed and shaken for approx. 30 seconds before another 5 minutes of incubation in RT. They were then centrifuged using a Z216MK microliter centrifuge (Hermle) at 12000 x g for 15 minutes at 4°C for separating the material into the organic and aqueous phases. From the upper aqueous phase, 300 µl was transferred to a new tube using a pipette, and an equal amount (300 µl) of 100% isopropanol was added for the RNA to precipitate. The samples were again left in RT for 5 minutes, and then centrifuged at 12000 x g for 10 minutes at 4°C. The

supernatant was removed with a pipette, leaving the RNA-pellet in the tube. To wash the RNA, 1 ml of 75% ethanol was added, and the tubes were vortexed to make sure the pellets were no longer attached to the tube wall. The tubes were centrifuged at 7500 x g at 4°C for 5 minutes, the supernatant was removed, and the washing step with 1 ml of 75% ethanol was repeated. The supernatant was again removed before the samples were centrifuged for 5 seconds and the residues of ethanol removed by pipetting. The samples were left to dry with the lids open for approximately 20 minutes in RT, until all the ethanol had evaporated, and the pellets had changed colour from white to transparent. The RNA was then dissolved in 50 µl RNase-free dH_2O and incubated at 60°C for 20 minutes. The tubes were then vortexed and centrifuged before the concentration and purity of the RNA was measured using a Nanodrop One (Nanodrop-One Thermo Scientific) instrument.

3.1.6 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis was used to assess the integrity of the RNA. First, a 0.75% agarose gel solution was prepared as described in Table 7. GelRed was used to visualise the nucleic acids, and 0.5 X Tris-borate-EDTA (TBE) buffer (Table 6) was used both in the preparation of the gel and as running buffer in the electrophoresis. The samples were prepared by adding 5 µL formamide, 1 µL 10x loading buffer (BlueJuice™Gel, ThermoFisher) and 3.5 µL RNA-free H_2O to 0.5 µL RNA-sample. Formamide was added to remove any secondary structures, and the samples were further denatured by incubating them at 60°C for 10 minutes before they were centrifuged and 10 µl of each sample was loaded into the wells of the gel. A 2log ladder (New England Biolabs) was used as molecular weight marker, and the electrophoresis was run at 100V for 1 hour and 5 minutes. A picture of the gel was obtained using ChemiDocTM XRS+ System (BioRad).

3.1.7 PREPARATION OF cDNA

The RNA isolated from the PCLS was used to synthesize cDNA for further use as templates in polymerase chain reactions. The RNA samples were thawed and diluted in 8-tube PCR-strips to give a final concentration of 50 ng/µl. The RNA was then denatured to avoid any secondary structures, using a thermocycler (DOPPIO Thermal Cycler, VWR) at 70°C for 5 minutes. Using the iScript™ cDNA Synthesis Kit (Bio-Rad), a master-mixture (Table 29) of 5x iScript buffer, iScript Reverse Transcriptase and nuclease-free dH_2O was added to each RNA sample and

mixed by pipetting before it was incubated in the thermocycler following the program described in Table 30 to produce cDNA.

Table 29: Components used to synthesise cDNA using iScript™ cDNA Synthesis Kit (BioRad).

Component	*Amount*
5x iScript buffer	4.0 µL
iScript Reverse Transcriptase	1.0 µL
RNA	1000 ng
Nuclease free dH_2O	-
Total	20 µL

Table 30: Reaction protocol for synthesis of cDNA using iScript™ cDNA Synthesis Kit (BioRad)

Component	*Temperature (°C)*	*Time (min.)*
Priming	25	5
Reverse transcription	46	20
RT inactivation	95	1
Hold (optional)	4	Forever

From a mixture of RNA-samples, a "no reverse transcriptase" (NRT) sample was prepared to monitor potential DNA contamination. The same procedure as for the RNA samples was followed, but with no reverse transcriptase added. A "no template control" (NTC) where the RNA-sample was replaced with nuclease-free H_2O was also prepared, to monitor background signal and probe stability. All cDNA samples were stored at -20°C.

3.1.8 GENE EXPRESSION ANALYSES

To assess the expression of *cyp1a*, *acox1* and *acly*, qPCR analyses were performed. This is a sensitive assay with high accuracy and throughput. The prepared cDNA was collected from the freezer and thawed before it was used in qPCR analyses. The cDNA was diluted 1:10 before 5.0 µl (125ng) was added to the wells of a white qPCR-plate as it is optimized for signal detection during real time qPCR. Forward and reverse primers were mixed and diluted to 2 µM before a master mix of primers and SYBR Green was made as described in table 31 and added to the cDNA in the qPCR-plate. Each sample was added to the plate in triplicates concerning the target genes analysed, and duplicates for the reference gene, *arp*. This gene was used as a reference gene in all experiments to normalise differences in gene expression that could arise because of the varying cell distribution in PCLS. The components were mixed before the plate was sealed with Microseal® 'B' Seal (Bio-Rad) and centrifuged at 1200 x g for 2 minutes. The qPCR was run in CFX96™ RealTime System C1000™ Thermal Cycler (BioRad) following the protocol described in Table 32.

Table 31: Components used in preparation for qPCR.

Component	*Volume (µl)*
cDNA	5.0
Primer mix (2µM)	5.0
SYBR Green Master Mix	10.0
Total	20.0

Table 32: Reaction protocol qPCR analyses using Lightcycler® 480 SYBR Green.

Component	*Temperature (°C)*	*Time*
Enzyme activation	95	10 min
Amplification (45 rounds)	95	10 sec
	55	20 sec
	72	40 sec
Melting curve	95	10 sec
	72	5 sec
Hold (optional)	4	Forever

3.1.9 MTT

To assess if the concentrations used were cytotoxic to the liver slices, an MTT assay was carried out on three replicates per exposure. The MTT assay is a colorimetric assay used to assess cell metabolic activity as the water soluble MTT will be transformed into a coloured insoluble formazan compound by healthy, living cells. The amount of colour (formazan) produced is directly proportional to the number of viable cells. During exposure, a 12-well plate identical to the exposure plate was prepared, using cut-offs from the liver slices (~15mg). These liver slices were exposed to the same compounds as the other slices for 48 hours. Triton (1%) was used as positive control of cytotoxicity. After 48 hours of exposure, each slice was weighed and transferred to a new well containing 1 ml cold phosphate buffered saline (PBS) and incubated on a shaker at 50 rpm at RT for 5-10 minutes. The PBS was then replaced by 2 ml MTT solution (2 mg/ml dissolved in L-15 medium), and the plate was covered in aluminium foil and incubated in RT at 50 rpm for 90 minutes.

After incubation, the MTT solution was replaced by 1 ml cold PBS and incubated at 50 rpm in RT for 5-10 minutes. The PBS was then replaced by 1 ml DMSO (100%) and again incubated in

RT at 50 rpm for 20 minutes. Triplicates of 100 µl per sample were transferred to a transparent 96 well-plate, and the absorbance of the formazan solution was measured at 590 nm using a Perkin Elmer Enspire 2300 Multilabel Plate Reader.

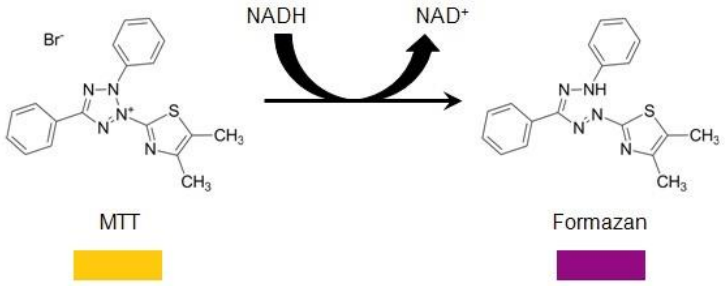


Figure 7: ***The reaction happening when MTT solution is added to viable cells.*** *Modified version from* ***(Riss et al., 2016).***

3.1.10 DATA TREATMENT AND STATISTICS

The cq-values retrieved from the CFX96™ RealTime System C1000™ Thermal Cycler were processed using Microsoft Excel (2007) before GraphPad Prism V.7.05 (Graphpad Software, San Diego, CA, USA) was used for statistical analyses and to produce graphical illustrations of the data. To calculate relative gene expression, relative quantification (RelQ = 2^-cq) was calculated from the average cq of all three replicates before the values were normalised to the reference gene (*arp*). Change in target gene expression was calculated as a fold change relative to the solvent control (DMSO 0.1%). The data was then transformed (y=log2(y)) before a one-way ANOVA with Greenhouse-Geisser corrections and Dunnet's multiple comparisons test was performed in order to calculate significant fold induction in the means of the test concentrations over the mean of the solvent control.

For the MTT-data collected from the EnSpire 2300 Multilabel Reader (PerkinElmer), the mean blank values were subtracted from the mean of the test concentrations and then divided on the weight of the slice to get abs/mg slice. Fold change per mg slice relative to the solvent control (DMSO 0.1%) averages was calculated and normality was tested using a Shapiro-Wilk

test. Transformation (y=log2(y)) and repetition of normality test was performed on non-normal distributed data. A one-way ANOVA with Dunnett's multiple comparisons test was performed on normal distribution data, and Friedman's test with Dunn's multiple comparisons test was used on non-normal distributed data.

3.2 LUCIFERASE REPORTER GENE ASSAY

3.2.1 EXPERIMENTAL DESIGN

To further investigate the mixtures ability to activate the Ah-receptor, a luciferase reporter gene assay was conducted. Steps were performed every 24 hours, starting with seeding the cells into the wells, transfecting them with the necessary plasmids, ligand treatment and the final luminescence measurements. A cell viability assay was performed to assess the cytotoxicity of the concentrations used, and for this part, the cells were un-transfected, and instead kept in culture for 48 hours between seeding and ligand treatment (Figure 8).

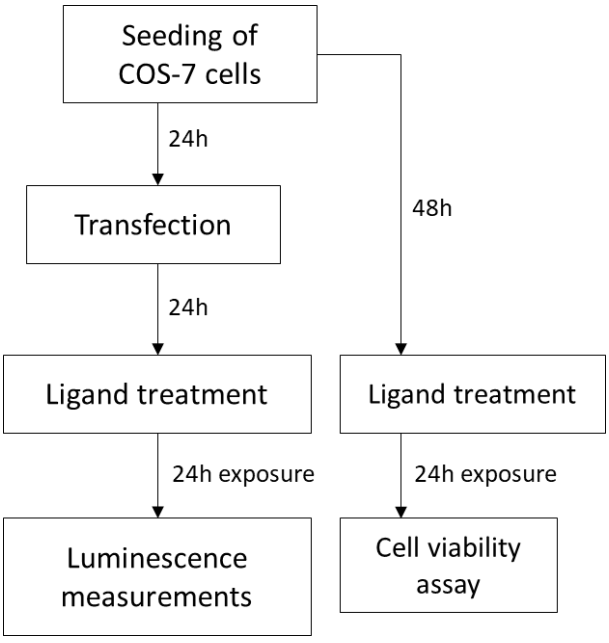


Figure 8: ***Flow chart of the process from seeding out the COS-7 cells to running the luminescence measurements and cell viability assays.***

In the luciferase assay, COS-7 simian cells transiently transfected with Atlantic cod *Ahr2* and Atlantic cod *Arnt* were exposed to mixtures of B(a)P and PFAS. The same compounds were used as in Experiment 2, but with concentrations adapted to a luciferase reporter gene system (Table 33). Cells were also exposed to B(a)P (0.008 - 10 µM) alone as it is a well-known agonist of *gmAhr2a*.

For the co-exposures, [B(a)P] = 0.01 µM (~EC50) was used as the fixed concentration. This concentration was determined based on results from a previous exposure to B(a)P performed by Roger Lille-Langøy (See Appendix A). This concentration is high enough to show activation of the Ah-receptor on its own, but any agonistic or antagonistic effects of the PFAS would still be observable. DMSO (0,55%) was used as solvent control. The concentrations given for the PFAS-mix represent ΣPFAS, which means that the 10 µM exposure consists of 3.33 µM PFOS, 3.33 µM PFOA and 3.33 µM PFNA, and so forth. Each concentration was added in triplicates on each plate, and the experiment was performed three times.

Table 33: Ligand treatments and concentrations

Compound (µM)	*Exposures*					
	Solvent control	B(a)P	B(a)P + PFOS	B(a)P + PFOA	B(a)P + PFNA	B(a)P + PFAS-mix
DMSO (0.55%)	x	x	x	x	x	x
BaP (0.01)		x	x	x	x	x
PFOS (100, 50, 10, 2, 0.4, 0.08)			x			
PFOA (100, 50, 10, 2, 0.4, 0.08)				x		
PFNA (100, 50, 10, 2, 0.4, 0.08)					x	
PFOS (33.3, 16.66, 3.33, 0.666, 0.133, 0.026)						x
PFOA (33.3, 16.66, 3.33, 0.666, 0.133, 0.026)						x
PFNA (33.3, 16.66, 3.33, 0.666, 0.133, 0.026)						x

3.2.2 PLASMID PREPARATION

To prepare the receptor- and control plasmids necessary for the LRA, 250 µl *E. coli* culture previously transformed with the LRA plasmids (and stored in glycerol at -80°C) was added to 250 ml LB medium (w/ 0.1 mg/ml ampicillin) and left to incubate at 250 RPM and 37 °C overnight. The over-night cultures ware collected, and optical density (OD) measured using an Ultrospec 10 cell density meter (Amersham Biosciences) and the plasmids were purified from the overnight culture using the NucleoBond® Xtra Midi plasmid purification kit (Macherey-Nagel) following the producer's protocol. This is a column-based assay, where the supernatant containing the plasmid DNA is applied to a silica column that binds the plasmid but lets the remaining components be washed out with a washing buffer. The plasmids prepared are listed in Table 23. To assess the integrity of the DNA, all five plasmids were controlled using agarose gel electrophoresis. This work was performed by Roger Lille-Langøy. A picture of the gel was obtained using GelDoc™ EX Imager (BioRad) and is attached in Appendix B.

3.2.3 CULTIVATION OF COS-7 CELLS

The COS-7 cells were kept in 37°C, normal atmosphere with 5% CO_2, and split at a confluency of 80-90%. They are adherent and grow as a monolayer in a petri dish. To split the cells, the culture medium was removed, and the cells washed using 1X PBS (pH 7.4). To detach them from the 10 cm cell culture dish, they were treated with 2 ml trypsin-EDTA (0.05% trypsin, 0.02% EDTA) for 1 minute in RT before the excess trypsin was removed and the plate incubated for 5 minutes at 37°C, normal atmosphere with 5% CO_2. To further detach the cells, they were resuspended in culture medium and the medium was pipetted up and down onto the bottom of the dish a few times. The cells were seeded out onto new petri dishes in a 1:10 or 1:20 dilution depending on the confluency and time until seeding.

3.2.4 SEEDING OF COS-7 CELLS

On the first day of the experiment, COS-7 cells were seeded out in a 96-well plate. The culture medium was removed from the cell culture dish, and the cells were washed and treated with trypsin as described in 3.3.3. The cells were then resuspended in culture medium and transferred to a centrifuge tube before the cells were counted. To make sure only living cells were counted, equal amounts of cell suspension and erythrosine B (50µl) was mixed to stain

the dead cells. The cells were then counted using a gridded haemocytometer, and the cell suspension diluted to give a cell density of $10*10^4$ cells/ml. 100 µl of the suspension was transferred to a 96-well plate using an electronic multichannel pipette giving a total number of 10 000 cells per well. They were left to incubate for 24 hours at 5% CO_2 and 37°C before they were transfected with plasmids.

3.2.5 TRANSFECTION

On day two, the cells were transfected with pcDNA3.1_gmAhr2a (receptor plasmid), pcDNA3.1_gmArnt, pGudLuc6.1 (reporter plasmid), pCMV-β-galactosidase (control plasmid), and pcDNA3.1 (expression vector plasmid). A transfection mix was prepared consisting of plasmids, transfection reagent (TransIT®LT-1 transfection kit, Mirus Bio), and serum-free medium (OptiMEM) according to Table 10. It was left to incubate for 30 minutes, before culture medium was added to the transfection mixture. The old culture medium was removed from the 96-well plates, and 100µl of the DMEM transfection medium was added to each well. The plates were then incubated for another 24 hours at 5% CO_2 and 37 °C before ligand treatment.

3.2.6 LIGAND TREATMENT

On day three, 24 hours after the transfection, the cells were treated with the compounds of interest to detect any agonistic or antagonistic effects. To do this, a 2x ligand solution in DMEM w/o phenol red was prepared in a 96-well plate suitable for dilution. The solution was serial diluted once five-fold and then two-fold giving the concentrations shown in Table 33. The old culture medium was then removed from the 96-well plate containing COS-7 cells, and replaced with 100 µl new culture medium (DMEM w/o phenol red and super stripped foetal bovine serum (FBS)) before 100 µl of the 2x dilution series was added giving the desired concentrations in each well. The plate was shaken carefully from side to side a couple of times and again incubated for 24 hours at 5% CO_2 and 37°C before cell lysis and measurement of luciferase- and β-galactosidase activity was performed.

3.2.7 LYSIS AND MEASUREMENTS

On day four, the old medium was removed from the 96-well plate, and the cells were lysed to measure the luciferase- and β-galactosidase-activity. A lysis reagent was prepared as described in Table 14, and 125 µl of this mixture was added to each well before the plates were left to incubate in RT for 30 minutes on gentle shaking. In the meantime, a β-Gal reagent and luciferase reagent was prepared as described in Table 15 and 16 respectively. After 30 minutes of incubation, 50 µl of the lysate from the original 96-well plate was transferred to the wells of one white and one clear luminescence plate. The white plate was used to measure luciferase activity, by adding 100 µl of the luciferase reagent to the 50 µl of lysate. The emitted light was measured immediately using a Perkin Elmer Enspire 2300 Multilabel Plate Reader. To the clear plate, 100 µl of the β-Gal reagent was added to the 50 µl lysate and set to incubate for 20 minutes. The absorbance was measured at a wavelength of 405 nm. This part of the assay was performed to control proper transfection of the plasmids.

3.2.8 CELL VIABILITY ASSAY

To determine if the concentrations used were cytotoxic, a cell viability assay was performed assessing two factors: the metabolic activity, and the cell membrane integrity. The metabolic activity of the cells can be measured using resazurin. This is a low-fluorescent compound that dehydrogenase enzymes in living cells will reduce to form resorufin, which is highly fluorescent. To assess the membrane integrity, 5-carboxyfluorescein diacetate (CFDA-AM) was used. This is a non-fluorescent compound that will cross the cell membrane by passive transport and be converted to the fluorescent dye carboxyfluorescein by non-specific esterases, and the amount of fluorescence will reflect the enzyme activity.

COS-7 cells were seeded out in 96 well-plates as described in 3.3.4 and left to incubate for 48 hours at 5% CO_2 and 37°C. The culture medium was changed after the first 24 hours. After the 48 hours, the cells were treated with the compounds of interest as described in 3.3.6 before additional 24 hours of incubation. Exposure to Triton® X100 (0.5%) was used as a positive control of cytotoxicity. On the last day, the cells were washed in 1X PBS (pH 7.4) before the resazurin-CFDA-AM reaction solution (Table 21) was added. The plates were incubated for 1 hour, before fluorescence (resazurin) was measured at 530/590 nm (ex/em) and at 485/530 nm (ex/em) for CFDA-AM using a Perkin Elmer Enspire 2300 Multilabel Plate Reader.

3.2.9 DATA TREATMENT AND STATISTICS

The LRA readings collected from the EnSpire 2300 Multilabel Reader (PerkinElmer) were initially processed using Microsoft Excel (2007). The ligand induced luciferase activity in each well was normalised by dividing by the corresponding β-galactosidase readings for correction of transfection efficiency. These values were then divided by solvent control (0.5% DMSO) averages to calculate fold induction in ligand induced luciferase activity. In GraphPad Prism V.7.05, normality was tested using D'Agostino & Pearson normality test. A one-way ANOVA with Dunnett's multiple comparisons test was performed on normal distribution data, and Kruskal Wallis test with Dunn's multiple comparisons test on non-normal distribution data.

The cell viability fluorescence reading (both Resazurin- and CFDA-AM-numbers) in each well was divided by the mean of the corresponding DMSO-values to calculate the percentage of viable cells. In GraphPad Prism V.7.05, the same statistical analyses were done as for the LRA readings.

4 RESULTS

4.1 ACTIVATION OF THE AHR AND PPAR SIGNALLING PATHWAYS IN PRECISION CUT LIVER SLICES

To assess if exposure to PFAS and B(a)P could activate the Ahr or PPAR signalling pathways *ex vivo*, precision cut liver slices of Atlantic cod were prepared and exposed to increasing concentrations of PFOS, PFOA, PFNA, and B(a)P. RNA was thereafter isolated from exposed PCLS to assess alterations in expression of Ahr and Pparα target genes with qPCR. The purity and integrity of the RNA-samples were monitored with spectrophotometry and gel electrophoresis, respectively, before cDNA synthesis and qPCR analyses. All the $A_{260/280}$-ratios recorded for the RNA-samples were above 1.8, indicating that the isolated RNA contained low or no contamination of DNA. The $A_{260/230}$-ratios varied more, but most samples were close to 2. The lower $A_{260/230}$-ratios could be due to some contamination of proteins, or a reagent such as phenol. An agarose gel electrophoresis with a 0.75% agarose gel revealed that all samples contained two distinct bands at 1200 and 2300 bp, representing 18S and 28S ribosomal RNA, respectively (Figure 9), and indicating that the RNA has been successfully separated and the integrity of the RNA is intact.

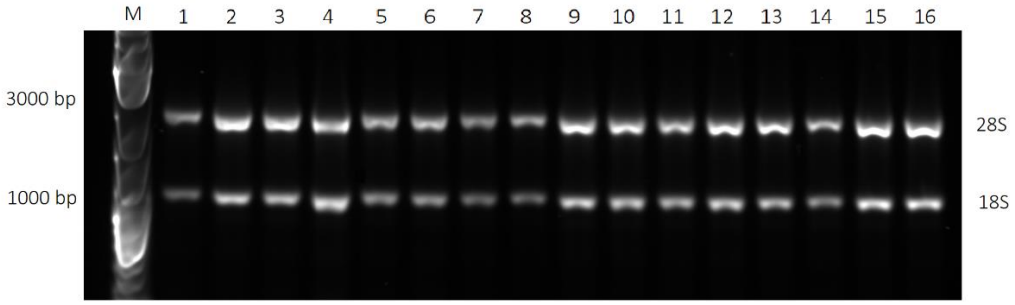


Figure 9: ***Control of RNA integrity.*** *The figure shows a representative selection of samples of RNA isolated from Atlantic cod PCLS analysed with agarose gel electrophoresis. The liver slices were exposed to solvent control, 0.1% DMSO (sample 1-3), 1 µM B(a)P (sample 4-7), 1 µM B(a)P +10 µM PFNA (sample 8, 9), 1µM B(a)P + 100 µM PFNA (sample 10), 1 µM B(a)P + 10 µM PFAS-mix (sample 11, 12), 1 µM B(a)P + 50 µM PFAS-mix (sample 13, 14), and 1 µM B(a)P + 100 µM PFAS-mix (sample 15, 16). 0.5 µl RNA was added to each well of a 0.75% agarose gel. M = 2-log DNA ladder (200 ng) (New England Biolabs).*

Gene expression in the liver slices after exposure was measured using qPCR, assessing the induction of the Ahr target-gene *cyp1a*, and the mammalian Pparα target genes *acox1* and *acly*. B(a)P is known to be a strong activator of the Ahr-pathway and was used as an inducer of *cyp1a* expression. As expected, the qPCR-results demonstrated significantly increased expression of *cyp1a* in comparison to solvent control for all concentrations used (significant for 1 µM and 10 µM) (Figure 10). The LDH assay was used to monitor the viability of the liver slices and showed no apparent cytotoxicity for any of the concentrations (Figure 11). Based on these results, 1 µM B(a)P was chosen as the fixed B(a)P-concentration to be used for the co-exposures with PFAS. This concentration was high enough to induce expression of *cyp1a* and also considered to be low enough for potential mixture effects to be visible and to avoid cytotoxicity in co-exposures.

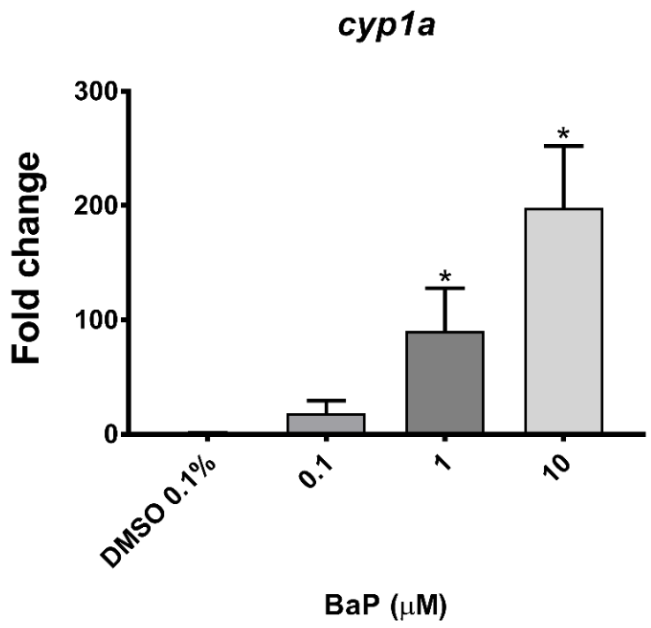


Figure 10: ***Gene expression of cyp1a in Atlantic cod PCLS exposed to B(a)P.*** *PCLS from juvenile cod (n=4) were exposed to B(a)P (0.1, 1 and 10 µM) for 48 hours. The graph shows fold change transcript levels +/- SEM relative to solvent control (0.1% DMSO) in transcription levels of cyp1a normalized to the reference gene arp. Statistical significance is defined as change in gene expression compared to solvent control and is indicated as: * = p ≤ 0.05.*

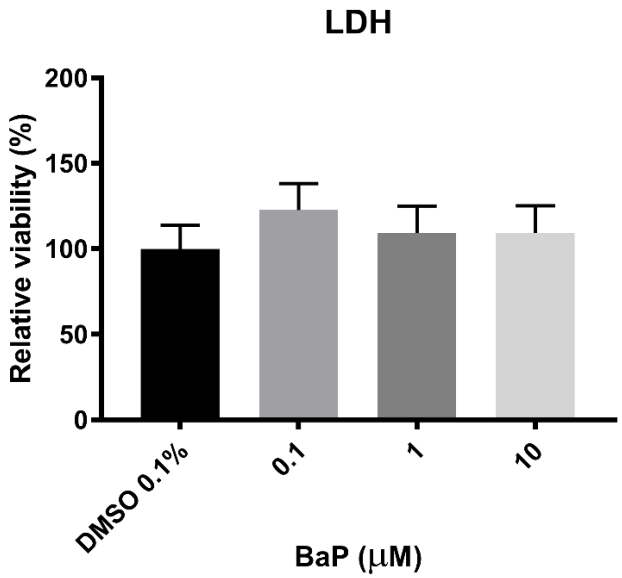


Figure 11: ***Cell viability assay.*** *Culture medium collected from Atlantic cod PCLS-exposures was used to measure LDH activity as an indicator of damaged cell membranes and a proxy of cytotoxicity. The graph shows relative viability (%) +/- SEM in comparison to solvent control (0.1% DMSO). Cytotoxic response was defined as decrease in fluorescence compared to solvent control, which is adjusted to 100%. No significant differences were found using one-way ANOVA.*

When assessing activation of the Ahr signalling pathway, all PFAS exposures of PCLS showed a trend in increase in *cyp1a* expression. The highest concentrations (100 µM) of PFOS and PFOA demonstrated also a statistically significant difference in *cyp1a* expression in comparison to solvent control, with 1.44 and 2.3 in fold change in transcript levels, respectively (Figure 12 A, and B). Furthermore, exposure to PFNA showed significant differences at 10 and 100 µM with a fold change of 1.75 and 2.08 in *cyp1a* expression, respectively. (Figure 12 C). Exposure to a PFAS-mix showed a trend of a dose-dependent increase in *cyp1a* levels, but no statistical significance was found. (Figure 12 D).

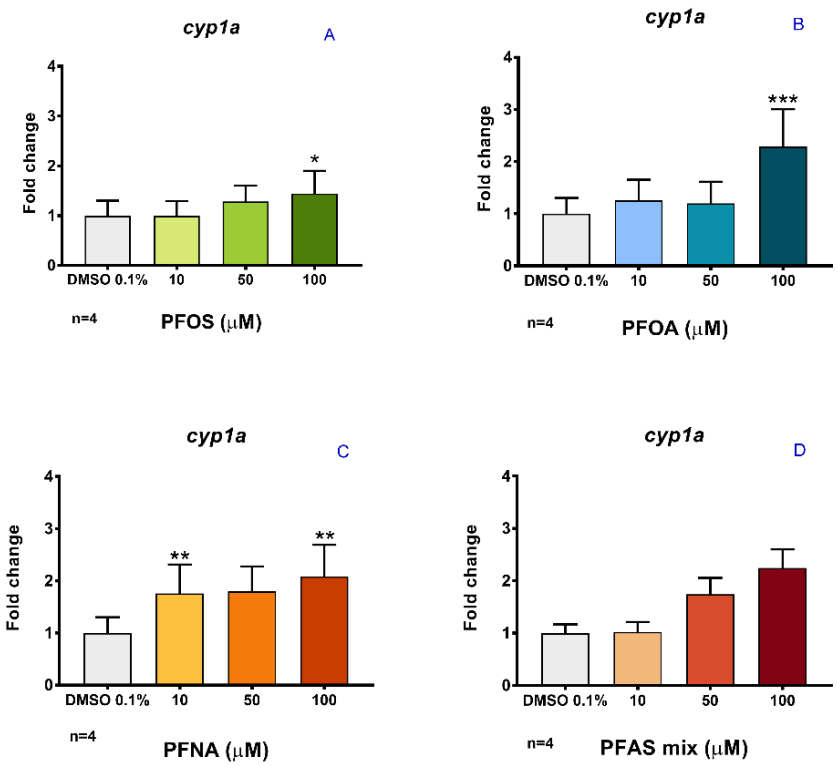


*Figure 12: **Expression of cyp1a in Atlantic cod PCLS exposed to PFOS, PFOA, PFNA and PFAS-mix.** PCLS from juvenile cod (n=4) were exposed to increasing concentrations of PFOS (A), PFOA (B), PFNA (C), and a PFAS-mix (D) (10, 50 and 100 µM) for 48 hours. The graph shows fold change transcript levels +/- SEM relative to solvent control (0.1% DMSO) in transcription levels of cyp1a normalized to the reference gene arp. Statistical significance is defined as change in gene expression compared to solvent control and is indicated as: * = p ≤ .05, **= p ≤ .01, ***= p ≤ .001, ****= p ≤ .0001.*

Activation of the PPARα signalling pathway was also assessed using qPCR for quantifying the expression of *acox1* and *acly,* which are both central genes in lipid metabolism. Besides exposure to PFOS, which showed no increase in the expression of *acox1*, all concentrations of PFNA, in addition to the highest concentration used with PFOA (100 µM), produced significant differences in *acox1* expression in comparison to solvent control (1.77, 1.51 and 1.55 in fold change, Figure 13 C) (1.27 in fold change, Figure 13 B). Neither of the single PFAS exposures, nor exposure to the PFAS-mixture, produced any significant differences compared to solvent control in the expression of *acly* (Figure 14).

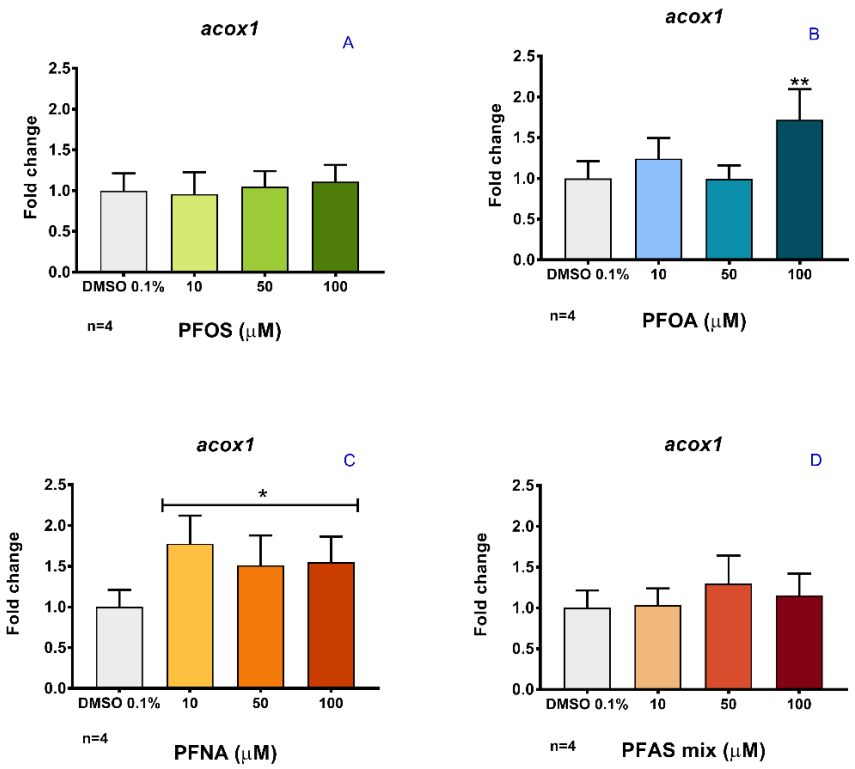


*Figure 13: **Expression of acox1 in Atlantic cod PCLS exposed to PFOS, PFOA, PFNA, and a PFAS-mix.** PCLS from juvenile cod (n=4) were exposed to increasing concentrations of PFOS (A), PFOA (B), PFNA (C), and a PFAS-mix (D) (10, 50 and 100 µM) for 48 hours. The graph shows fold change transcript levels +/- SEM relative to solvent control (0.1% DMSO) in transcription levels of acox1 normalized to the reference gene arp. Statistical significance is defined as change in gene expression compared to solvent control and is indicated as: * = p ≤ .05, **= p ≤ .01, ***= p ≤ .001, ****= p ≤ .0001.*

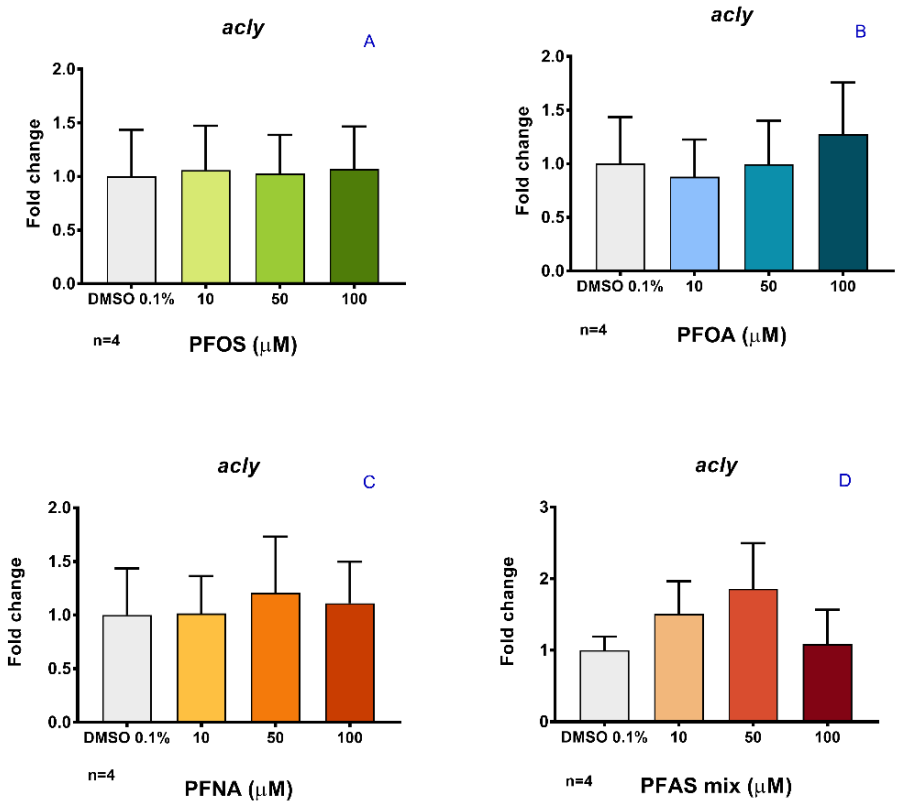


Figure 14: ***Expression of acly in Atlantic cod PCLS exposed to PFOS, PFOA, PFNA and a PFAS-mix.*** *PCLS from juvenile cod (n=4) were exposed to increasing concentrations of PFOS (A), PFOA (B), PFNA (C), and a PFAS-mix (D) (10, 50 and 100 µM) for 48 hours. The graph shows fold change transcript levels +/- SEM relative to solvent control (0.1% DMSO) in transcription levels of acly normalized to the reference gene arp. Statistical significance is defined as change in gene expression compared to solvent control and is indicated as: * = p ≤ .05, **= p ≤ .01, ***= p ≤ .001, ****= p ≤ .0001.*

To detect putative mixture effects, combination exposures of B(a)P and the three PFAS of interest were performed and assessing alterations in the gene expression of the same genes as in the single PFAS exposures. As demonstrated above, B(a)P induced *cyp1a* expression at a concentration of 1 µM. A trend of stronger induction of *cyp1a* compared to B(a)P exposure alone was observed by co-exposure with both single PFAS compounds and the PFAS-mix. For expression of *cyp1a*, all exposure scenarios showed significant difference to solvent control (highest fold change 88, 89, 86, and 123 for B(a)P + PFOS, PFOA, PFNA or PFAS-mix respectively) (Figure 15). However, significant differences between any of the co-exposures and single exposure to B(a)P was only observed for 50 µM PFOA (Figure 15 B).

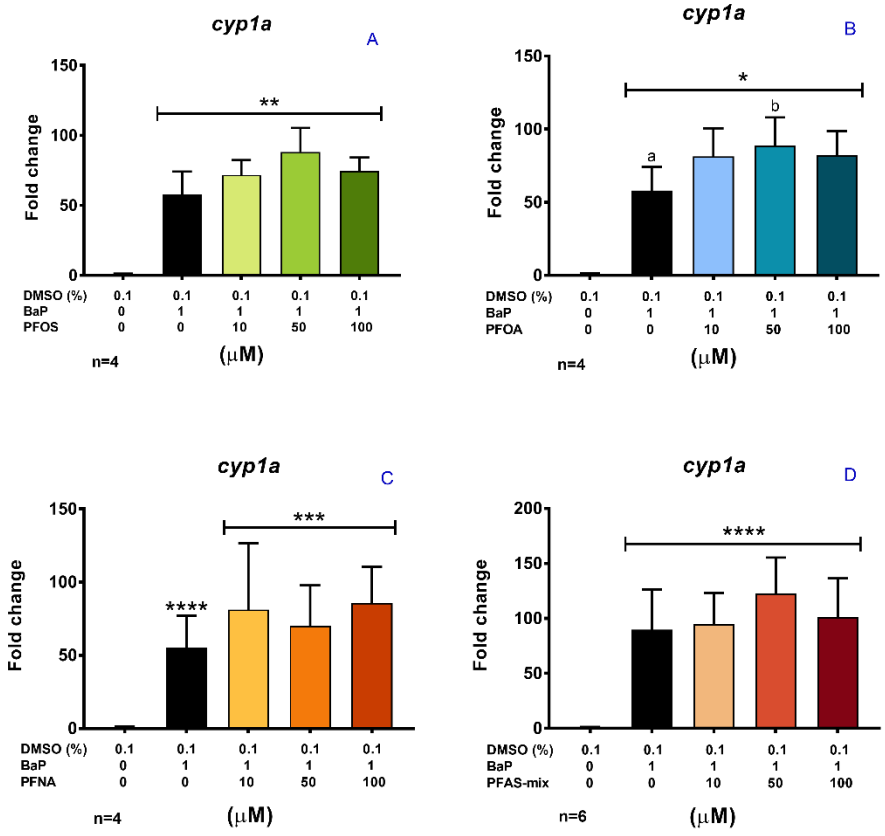


*Figure 15: **Expression of cyp1a in Atlantic cod PCLS co-exposed to B(a)P and PFOS, PFOA, PFNA and a PFAS-mix.** PCLS from juvenile cod (n=4 or n=6) were exposed to a fixed concentration of B(a)P (1 µM) and increasing concentrations of PFOS (A), PFOA (B), PFNA (C), or a PFAS-mix (D) (10, 50 and 100 µM) for 48 hours. The graph shows fold change transcript levels +/- SEM relative to solvent control (0.1% DMSO) in transcription levels of cyp1a normalized to the reference gene arp. Statistical significance is defined as change in gene expression compared to solvent control and is indicated as: * = p ≤ .05, **= p ≤ .01, ***= p ≤ .001, ****= p ≤ .0001. Significant difference to 1 µM B(a)P (p ≤ .05) is indicated as differentiating letters.*

acox1 and *acly* also showed some trends of an increased gene expression with combined PFAS and B(a)P exposures, compared to solvent control and B(a)P exposure alone. However, no statistically significant differences were revealed for expression of *acox1* in exposure to B(a)P + PFOS, PFOA or PFNA. Exposure to B(a)P + 50 and 100 µM PFAS-mix showed significant increase in *acox1* expression compared to single B(a)P-exposure (Figure 16 D). Expression of *acly* did not change significantly in combined exposures to B(a)P and PFOS, PFOA or PFAS-mix in comparison to solvent control or single exposures to B(a)P (Figure 17 A, B, D). The gene expression in 50 µM exposure of PFNA in combination with B(a)P, however, showed to be significantly different to the expression determined in the single exposure of B(a)P (Figure 17 C).

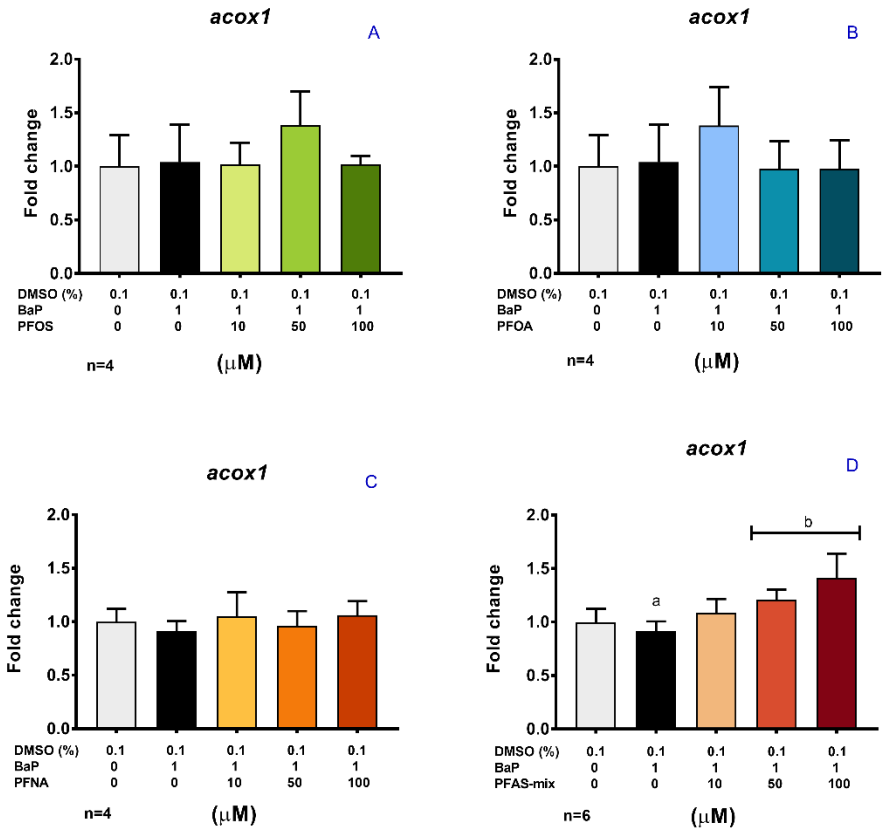


*Figure 16: **Expression of acox1 in Atlantic cod PCLS co-exposed to B(a)P and PFOS, PFOA, PFNA and a PFAS-mix.** PCLS from juvenile cod (n=4 or n=6) were exposed to a fixed concentration of B(a)P (1 µM) and increasing concentrations of PFOS (A), PFOA (B), PFNA (C), or a PFAS-mix (D) (10, 50 and 100 µM) for 48 hours. The graph shows fold change transcript levels +/- SEM relative to solvent control (0.1% DMSO) in transcription levels of acox1 normalized to the reference gene arp. Statistical significance is defined as change in gene expression compared to solvent control and is indicated as: * = p ≤ .05, **= p ≤ .01, ***= p ≤ .001, ****= p ≤ .0001. Significant difference to 1 µM B(a)P (p ≤ .05) is indicated as differentiating letters.*

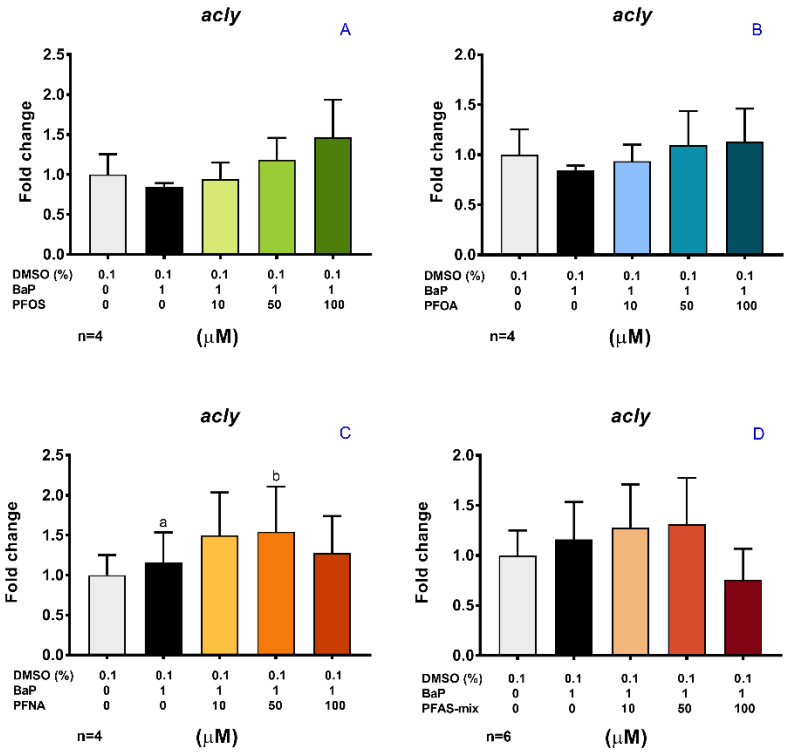


Figure 17: ***Expression of acly in Atlantic cod PCLS co-exposed to B(a)P and PFOS, PFOA, PFNA and a PFAS-mix.*** *PCLS from juvenile cod (n=4 or n=6) were exposed to a fixed concentration of B(a)P (1µM) and increasing concentrations of PFOS (A), PFOA (B), PFNA (C), or a PFAS-mix (D) (10, 50 and 100 µM) for 48 hours. The graph shows fold change transcript levels +/- SEM relative to solvent control (0.1% DMSO) in transcription levels of acly normalized to the reference gene arp. Statistical significance is defined as change in gene expression compared to solvent control and is indicated as: * = p ≤ .05, **= p ≤ .01, ***= p ≤ .001, ****= p ≤ .0001. Significant difference to 1 µM B(a)P (p ≤ .05) is indicated as differentiating letters.*

4.2 CELL VIABILITY ASSAY OF PCLS EXPOSURES

An MTT assay was conducted to assess for putative cytotoxic effects induced by the exposure regimes used with the PCLS. DMSO (0.1%) was used as solvent control and (1%) Triton® X100 was used as positive control for cytotoxicity. Cytotoxicity was defined as the decrease in fluorescence compared to the solvent control (0.1% DMSO). For the MTT assays of B(a)P + PFOS and B(a)P + PFOA exposures, Triton® X100 was not included. The MTT assays showed no cytotoxicity for any of the different exposure regimes (Figure 18 and 19).

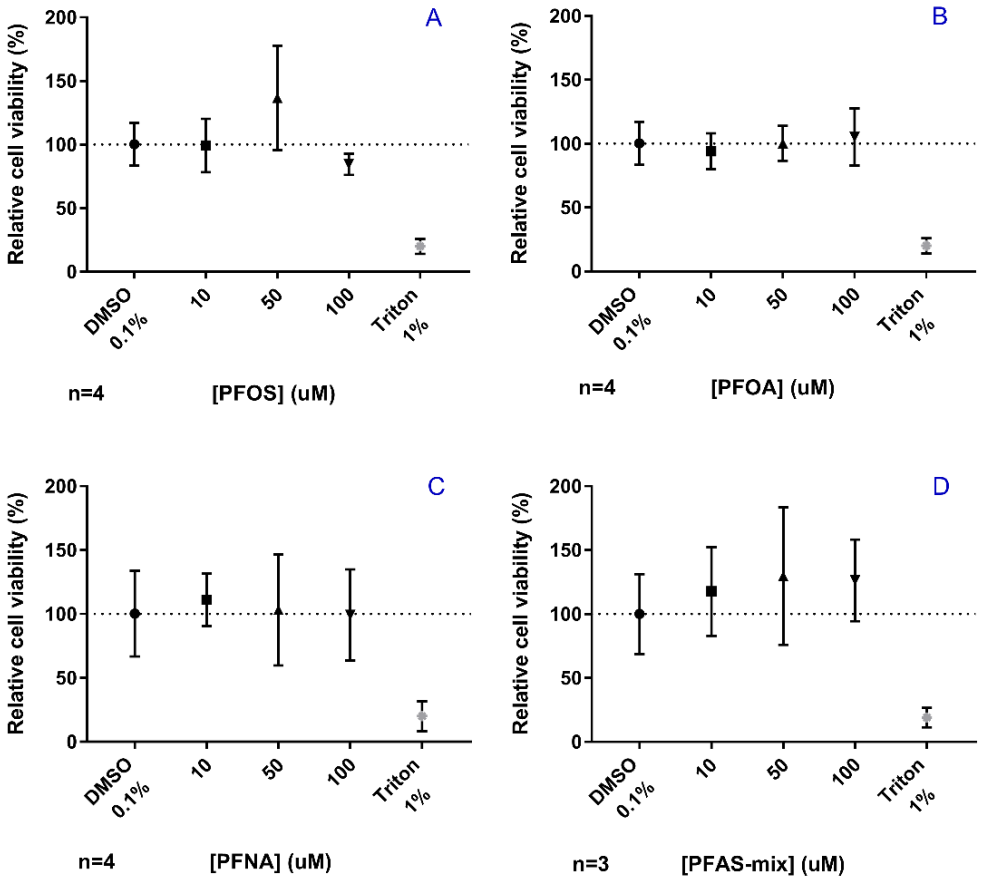


*Figure 18: MTT **cell viability assay.** Atlantic cod PCLS were exposed to the same compounds/mixtures and concentrations used with PCLS in Experiment 1 and indicated in the figure. Culture medium with 0.1% DMSO was used as negative control, and Triton (1%) used as positive control. Cytotoxic response is defined as decrease in fluorescence compared to solvent control (+/- SEM), which is adjusted to 100% and indicated by the dotted line. No significant difference was shown using RM one-way ANOVA or Friedman's test.*

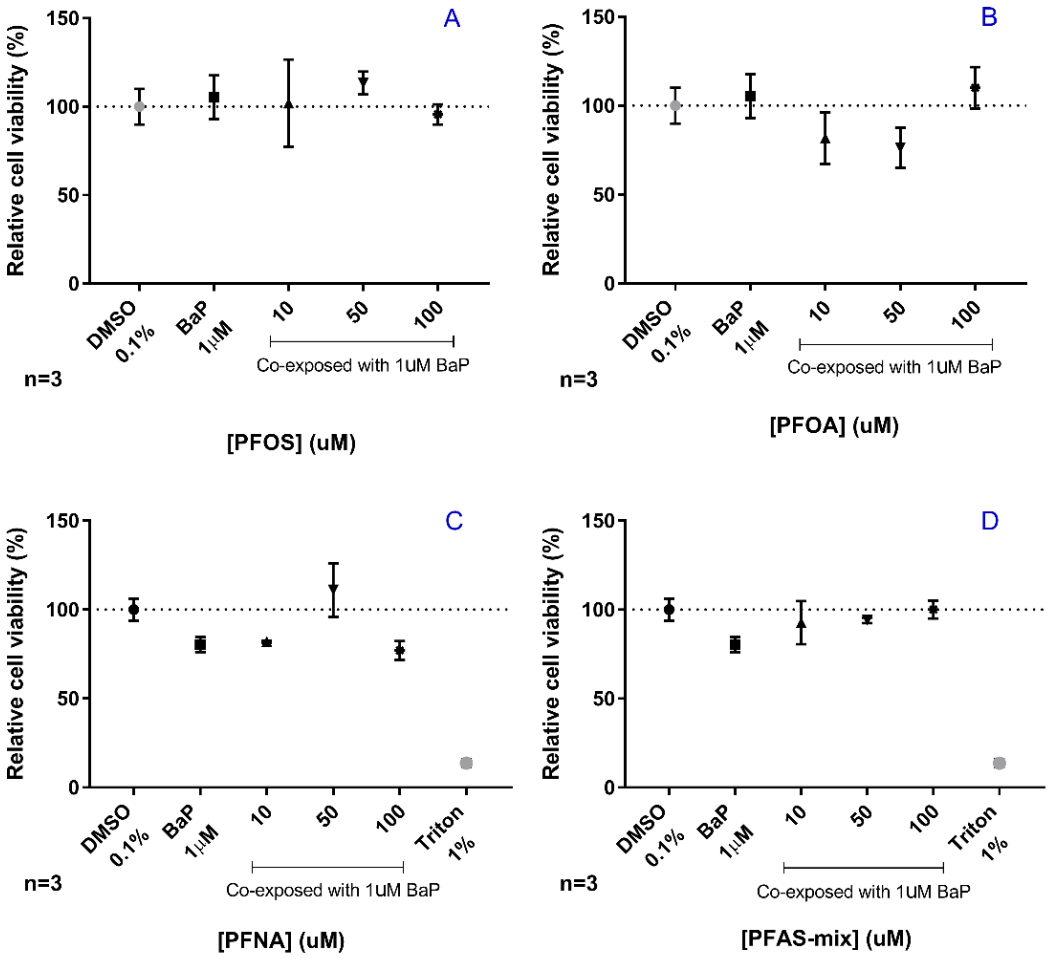


*Figure 19: **MTT cell viability assay.** Precision cut liver slices were exposed to the same compounds/mixtures and concentrations used with PCLS in Experiment 2 and indicated in the figure. Culture medium with 0.1% DMSO was used as negative control, and Triton (1%) used as positive control. Cytotoxic response is defined as decrease in fluorescence compared to solvent control (+/- SEM), which was adjusted to 100% indicated by the dotted line. No significant difference was shown using RM one-way ANOVA or Friedman's test.*

4.3 AHR TRANSCRIPTIONAL ACTIVITY IN COS7-CELLS

The putative effects of B(a)P and PFAS were further investigated using a luciferase reporter gene assay to study modulation of Atlantic cod Ahr2 activation. Transfected COS-7 cells were exposed to the compounds of interest for 24 hours before activation of Ahr2a was measured indirectly using a luciferase reporter gene assay. As expected, the exposure to B(a)P showed a dose-dependent increase in activation of the Ah-receptor (Figure 20 A). All concentrations in each exposure scenario with combinations of B(a)P and PFAS also showed significant difference in activation of Ahr compared to solvent control (0.1% DMSO). However, none of the combined exposures showed significant difference in receptor activation compared to exposure to B(a)P alone (Figure 20 B-E).

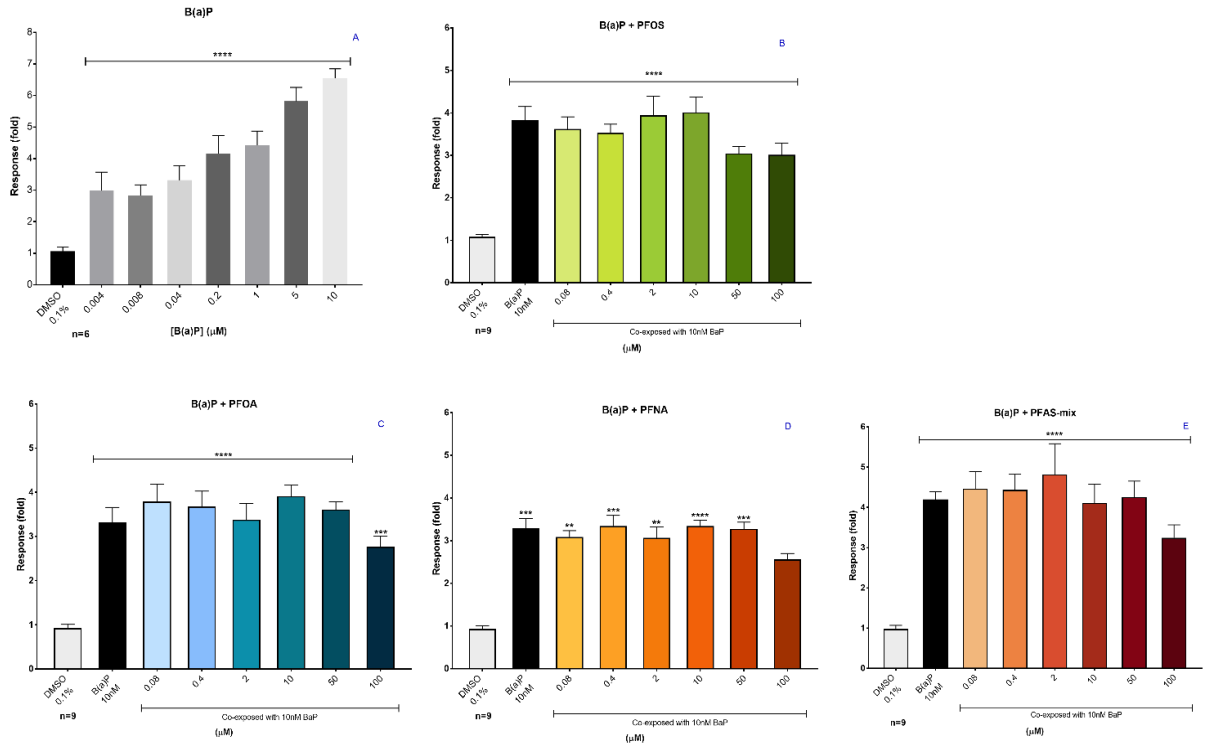


Figure 20: ***Ligand activation in gmAhr2a-transfected COS-7 cells co-exposed to B(a)P in addition to either PFOS, PFOA, PFNA or a PFAS-mix.*** *COS-7 cells (n=9) were transfected with receptor plasmid (pcDNA3.1_gmAhr2a; pcDNA3.1_gmArnt), reporter plasmid (pGudLuc6.1), expression vector (pcDNA3.1) and control plasmid (pCMV-ß-galacosidase). The cells were exposed to a fixed concentration of B(a)P (1 nM) and increasing concentrations of either PFOS, PFOA, PFNA or a PFAS-mix as indicated (0.08, 0.4, 2.0, 10, 50 and 100 µM) for 24 hours (graphs B to E). Exposure to B(a)P (0.004, 0.008, 0.04, 0.2, 1.0, 5.0, and 10 µM, n=6) was done to show activation of Aryl hydrocarbon receptor (graph A). The graphs show fold change +/- SEM relative to solvent control (0.1% DMSO) in activation of Ahr2a. Statistical significance is defined as change in receptor activation compared to solvent control, and is indicated as: * = p ≤ .05, **= p ≤ .01, ***= p ≤ .001, ****= p ≤ .0001.*

4.4 CELL VIABILITY ASSAY OF COS-7 CELL EXPOSURE

To assess if the concentrations used in the luciferase reporter gene assays produced cytotoxic effects in the COS-7 cells, a cell viability assay measuring the metabolic activity and the membrane integrity was conducted. Non-transfected COS-7 cells were exposed to the ligands in the same concentrations as used in the luciferase reporter gene assay. In addition, a positive control for cytotoxicity (Triton 0.1%) was added. Cytotoxicity was defined as significant difference to solvent control (0.5% DMSO). If there was a significant decrease in the viability of the cells, this could affect the receptor activation, and possibly result in a reduced activation profile. The results showed that there was a significant decrease in metabolic activity for most concentrations in all of the four exposure scenarios. All scenarios produced a "dip" in metabolic activity on the medial concentrations, but no cytotoxicity was revealed for the highest concentrations used (100 µM) (Figure 21). The lowest relative fluorescence was 89.7% (B(a)P + PFOS 10µM), 81.4% (B(a)P + PFOA 2µM), 78.2% (B(a)P + PFNA 2µM), and 89.16% (B(a)P + PFAS-mix 0.4µM).

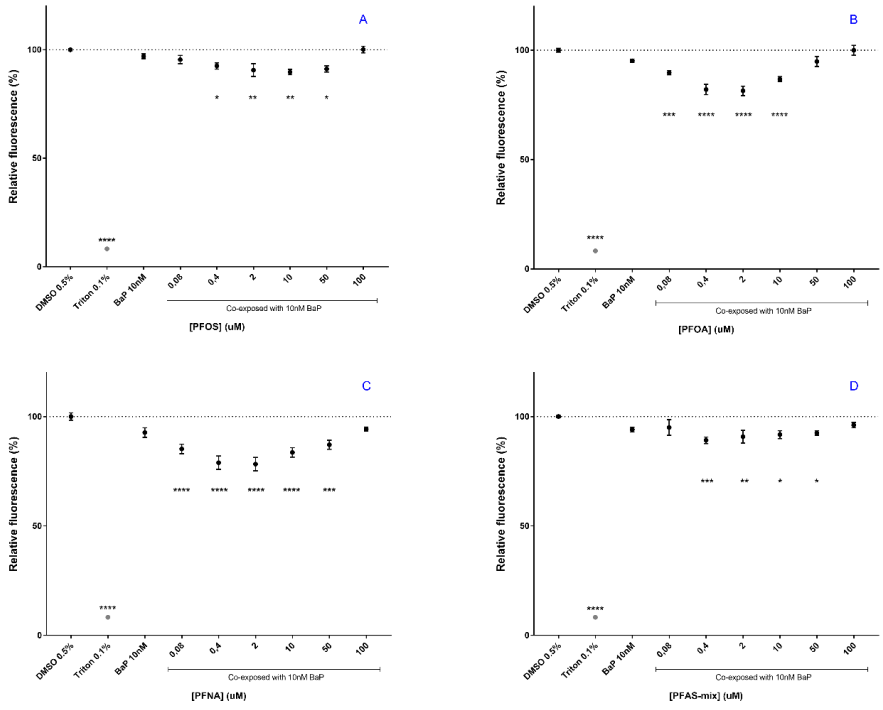


Figure 21: ***Metabolic activity in COS-7 cells after ligand exposure.*** *COS-7 cells were exposed to the same concentrations as used for the luciferase reporter gene assay. DMSO (0.5%) was used as solvent control, and Triton (0.5%) used as positive control. Cytotoxic response is defined as the decrease in fluorescence compared to negative control (0.5% DMSO) (+/- SEM) which is adjusted to 100% and indicated by the dotted line. Significance is indicated as * = p ≤ .05, **= p ≤ .01, ***= p ≤ .001, ****= p ≤ .0001. One-way ANOVA or Kruskal-Wallis test was performed.*

The same “dip” in relative fluorescence was also revealed in the results for the membrane integrity (Figure 22), but for exposure to B(a)P in combination with PFOA, PFNA or PFAS-mix, all concentrations showed to be cytotoxic, including exposure to B(a)P alone. The lowest relative fluorescence was 77.0% (B(a)P + PFOS 10µM), 67.6% (B(a)P + PFOA 10µM), 62.8% (B(a)P + PFNA 2µM), and 77.3% (B(a)P + PFAS-mix 2µM).

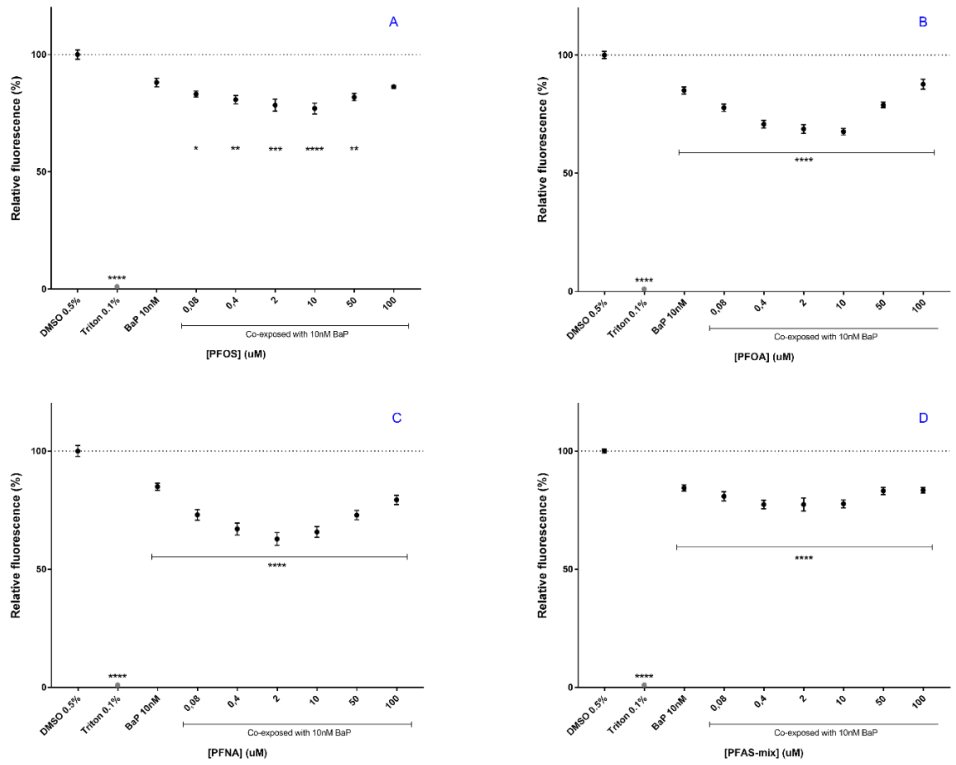


Figure 22: ***Membrane integrity in COS-7 cells after ligand exposure.*** *COS-7 cells were exposed to the same concentrations as used for the luciferase reporter gene assay. DMSO (0.5%) was used as solvent control, and Triton (0.5%) used as positive control of cytotoxicity. Cytotoxic response is defined as the decrease in fluorescence compared to negative control (0.5% DMSO) (+/- SEM) which is adjusted to 100% and indicated by the dotted line. Significance is indicated as * = p ≤ .05, **= p ≤ .01, ***= p ≤ .001, ****= p ≤ .0001. One-way ANOVA or Kruskal-Wallis test was performed.*

5 DISCUSSION

Most contaminants of emerging concern are man-made. When introducing these compounds to consumers and our environment, it comes with a responsibility of obtaining the necessary knowledge of their toxicities and to perform comprehensive risk assessments to limit potential negative impacts of these compounds on nature and wildlife. It is therefore important to have sufficient insights into what the effects of these compounds might be, and if, or how, different combinations of compounds can make an exposure scenario more severe for biota. To do this, extensive testing of toxicity must be performed to set proper safety thresholds and determine Environmental Quality Standards. The recent regulation implemented by EFSA takes these potential effects into account. The 2018-opinion set separate TWI values for PFOA and PFOS, but the new safety threshold of 4.4 ng/kg bodyweight applies to PFAS as a group and thus represent a new framework for evaluating chemical mixtures in food and feed (*Mixtures Methodology Equips EFSA for Multiple Chemicals*, 2019).

Epidemiologic studies of prenatal exposure to PFAS show negative associations between PFAS levels in maternal blood plasma and foetal growth, as well as showing endocrine-disrupting potential through altering foetal gonadotropin levels and thyroid hormones through affecting GH_3 cell growth and proliferation (Long et al., 2013; Nian et al., 2020; Verner et al., n.d.), and altering normal functioning of female gonads (Khan et al., 2020). B(a)P has also been shown to produce developmental toxicity on its own, including in Atlantic cod, where B(a)P can cause premature ovarian failure and tumour genesis (Drwal et al., 2019; Lim et al., 2013). As these chemicals already affect various biological pathways on their own, mixture effects between the two groups could potentially cause adverse outcomes, especially as sensitive periods of an organism such as during the early developmental stages. As limited knowledge exists when it comes to mixture effects, the aim of this study was to characterise toxicological effects in Atlantic cod after co-exposure to three different PFAS molecules and the PAH B(a)P. The focus of this thesis was on potential changes in expression levels of genes relevant for the biotransformation of xenobiotics (*cyp1a*) and the energy metabolism (*acly* and *acox1*), which are two cellular processes that previously have been found to be affected by PAH and PFAS exposure (Krøvel et al., 2008; Long et al., 2013; Takacs & Abbott, 2007; Whitlock, 1999). Receptor activation of Ahr2a was also assessed using a luciferase reporter gene assay.

5.1 ACTIVATION OF THE AHR SIGNALLING PATHWAY

As expected, B(a)P exposure induced *cyp1a* expression in PCLS, which also has been shown previously by (Yadetie et al., 2018). In the same study, B(a)P also demonstrated the ability to interact with other compounds, producing anti-estrogenic effects in PCLS when co-exposed with ethynylestradiol (EE2) (Ibid.). The MTT assay conducted on the PCLS samples in this thesis showed that neither of the concentrations used of B(a)P and PFAS were cytotoxic to the liver slices. Furthermore, the results of the gene expression analyses revealed that there was an increase in the expression of *cyp1a* in PCLS with exposure to increasing concentrations of PFAS, and statistical significance was demonstrated for PFOS, PFOA and PFNA (100 µM). Induction of cytochrome P450 enzymes by PFASs has also been shown in mouse liver through activation of Pparα, and in chicken embryo hepatocyte cultures as a result of various transcriptional responses (Cheng & Klaassen, 2008; Hickey et al., 2009). PFAS-mediated induction of Cyp1a expression has also been demonstrated in several fish species, e.g zebrafish and marine medaka (*Oryzias melastigma*), along with the activation and inhibition of several other genes (Krøvel et al., 2008; Lee et al., 2020). Several studies performed on mammals have shown that PFOS is not able to induce expression of *cyp1a*, while it has been demonstrated to inhabit the ability to induce *cyp1a* in several fish species, suggesting that there are species differences in the transcriptional responses (Ibid.). Krøvel et al. (2008) used Atlantic salmon, and showed that PFOS induced cyp1a in hepatocytes, as was also shown in this thesis on PCLS from Atlantic cod. In this thesis, co-exposure between B(a)P and all four PFAS variations showed trends of an increased *cyp1a* expression that was higher than an additive response, but with statistical significance only for B(a)P + PFOA (50 µM) in comparison to B(a)P alone. This represents an apparent synergistic effect between the three relevant PFAS congeners and B(a)P, indicating that these two groups of compounds potentially could be of a higher risk to wildlife when present in mixtures. Such mixture-specific effects between PFASs and PAHs have also been shown in zebrafish embryos, where mixtures of PFAS and PCB126 induced a downregulation in expression of *cyp1a*, and up-regulation of glutathione peroxidase 1a (*gpx1*) compared to single exposures of PCB126 (Blanc et al., 2017), although the developmental stage and species differences is of relevance when comparing such experiments.

5.2 ACTIVATION OF THE PPARA SIGNALLING PATHWAY

It has previously been shown that the carboxylic PFAS congeners PFOA and PFNA transactivate the Atlantic cod Ppara1 *in vitro*, while the sulfonic PFOS does not (Söderström, 2017). The results from my thesis further support these data, as there was a trend of an increase in expression of the Pparα target genes *acox1* and *acly* after exposure of PCLS to PFOA, PFNA, and the PFAS-mix. For *acox1* significant induction was shown for PFOA and PFNA, while no such trends were observed when exposed to PFOS alone. When it comes to the co-exposures of B(a)P and PFASs, these combinations did not seem to have any further effects on the activation of Pparα, except for the highest concentrations of the PFAS-mix + B(a)P. This exposure regime produced significantly higher expression of *acox1* than B(a)P alone. The same effect was observed for the expression of *acly,* as exposure to 50 µM PFNA was significantly different from the expression of *acly* revealed in B(a)P-exposed PCLS. Although the tendencies that have been shown here are generally weak, these *ex vivo* results are in agreement with the work of (Dale et al., 2020), where activation of the Pparα signalling pathway was revealed by observing upregulation of enzymes in fatty acid degradation pathways after *in vivo* exposure of Atlantic cod to mixtures of PAHs and PFASs.

5.3 AHR TRANSCRIPTIONAL ACTIVITY IN COS7-CELLS

In the luciferase reporter gene assay, B(a)P activated the Atlantic cod Ah receptor as expected and previously demonstrated (Aranguren-Abadía et al., 2020). Based on the PCLS-results, it could also be expected that a similar trend of further receptor activation could be promoted by co-exposures with PFASs, but no further activation of Ahr in combination with the PFAS compounds was revealed. On the contrary, the higher concentrations showed a trend of decrease in receptor activation. These results could be due to cytotoxicity, although the three PFAS-concentrations used in the PCLS-system corresponded to the highest concentrations used in the Luciferase-based system, and the fixed B(a)P concentration used on the COS-7 cells was 100 times lower than the B(a)P-concentration used with the PCLS. However, the cell viability assays performed on the COS-7 cells showed that the concentrations used were in fact cytotoxic. There was a significant reduction in metabolic activity and an even higher reduction in membrane integrity for most concentrations used for all exposure compounds. PFASs are amphiphilic compounds, and have shown to increase the cell membrane permeability in fish

leukocytes after exposure to PFOS, and in microbial membranes of *Aliivibrio fischeri* as they were more permeable to semi-membrane permeable dye after PFAS exposure (Fitzgerald et al., 2017; Hu et al., 2003). Since significant reduction in membrane integrity was observed already at 10 nM B(a)P exposure without the addition of PFAS congeners, the fixed B(a)P concentration was apparently high enough to affect the cell viability. As no cytotoxicity was shown in the PCLS exposures, biotransformation of compounds could be central as this could occur in the liver slices, but not as likely in the COS-7 cell system. The cytotoxicity could explain the reduction in receptor activation measured from the highest concentrations in the luciferase assay, and potentially the lack of any further receptor activation for the lower concentrations.

5.4 COMMENTS ON THE SYSTEMS USED

The two systems used represent two different approaches to answer the questions of interest, yet there are differences in the results, which may be caused by the set-up of the luciferase system. Cytotoxicity was shown in the exposure of COS-7 cells and not in any of the PCLS-exposures despite the fact that the concentrations used on the COS-7 cells were equal to or lower than the concentrations used on the PCLS. The liver slices could simply be more robust than the COS-7 cells as they inhabit most of the complex multi-cellular structure of the liver as found *in vivo*, including erythrocytes, fat-storing cells, and endothelial cells. In addition, the slices have not undergone any form of handling or stressors before ligand exposure such as cell cultures have. One essential difference between the two systems is that biotransformation can occur in the liver slices and most likely to a much lesser extent in the COS-7 cells. This means that some of the compounds used for exposure could be transformed within the cells by enzymes induced through the activation of e.g. the Ahr signalling pathway, such as CYP1A. If e.g. B(a)P is metabolised fast enough, this could result in a difference in the actual concentrations present in the cells of the specific compounds, and the COS-7 cells could in fact have been exposed to higher effective concentrations than the PCLS. Although the luciferase-based reporter gene assay is a well-established system for conducting high-throughput ligand screening it is a limited model as it does not account for metabolic activity, and the concentrations used must be adjusted to avoid cytotoxicity and a loss of cell viability.

5.5 CONCLUSION

In general, the results obtained in this thesis indicate that the PFASs used in the experiments could in fact induce the activation of *cyp1a*, and also enhance the toxicity of B(a)P through stronger induction of *cyp1a*. The PFASs could also possibly interfere with the lipid metabolism through the Pparα signalling pathway. The lack of receptor activation by co-exposure during luciferase reporter gene assay could be explained by high cytotoxicity in most conditions used, which again could be confirmed by adjusting the concentrations used and repeat these experiments. The experiments performed in this master's thesis have accentuated that mixture effects is a field that must be explored further, as there are indications that combinations of B(a)P and the PFASs of interest could indeed give rise to responses beyond additive effects. Regardless of the room for improvement, the results provide additional information that enhances the importance of integrating mixture effects into the toxicity testing and risk assessment of chemicals.

5.6 FUTURE PERSPECTIVES

There are several steps that can be taken to further investigate different combinations of PAHs and PFAS and to see whether mixture effects arise. Even though this thesis was a continuation of an *in vivo* experiment, a natural next step would be to test the findings for these specific combinations *in vivo* to look for systemic responses after sub-chronic or chronic exposures. As there could be differences in effects between organisms, the use of human or other mammalian receptors in similar exposure scenarios could also be interesting for obtaining a clearer picture of the risk these mixtures pose to humans or other organisms. For instance, PFOS has shown to activate human Pparα (Behr et al., 2020) although it does not activate mouse and Atlantic cod Pparα (Söderström, 2017; Takacs & Abbott, 2007). Performing a repeated version of the luciferase reporter gene assay using lower concentrations of B(a)P and/or the various PFAS congeners in order to avoid cytotoxic effects and get more valid results would also be beneficial, especially regarding PFOS-exposure to investigate the mechanisms behind PFOS mediated induction of *cyp1a*. Many of the most common PFASs are also replaced today by short-chained PFASs when new restrictions arise such as the European ban of PFOA

above 0.025 ng/kg product in chemicals and consumer products. Thus, to include shorter-chained PFAS molecules in similar experimental designs, could aid to assess if the replacement of these compounds is an effective approach.

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APPENDIX A

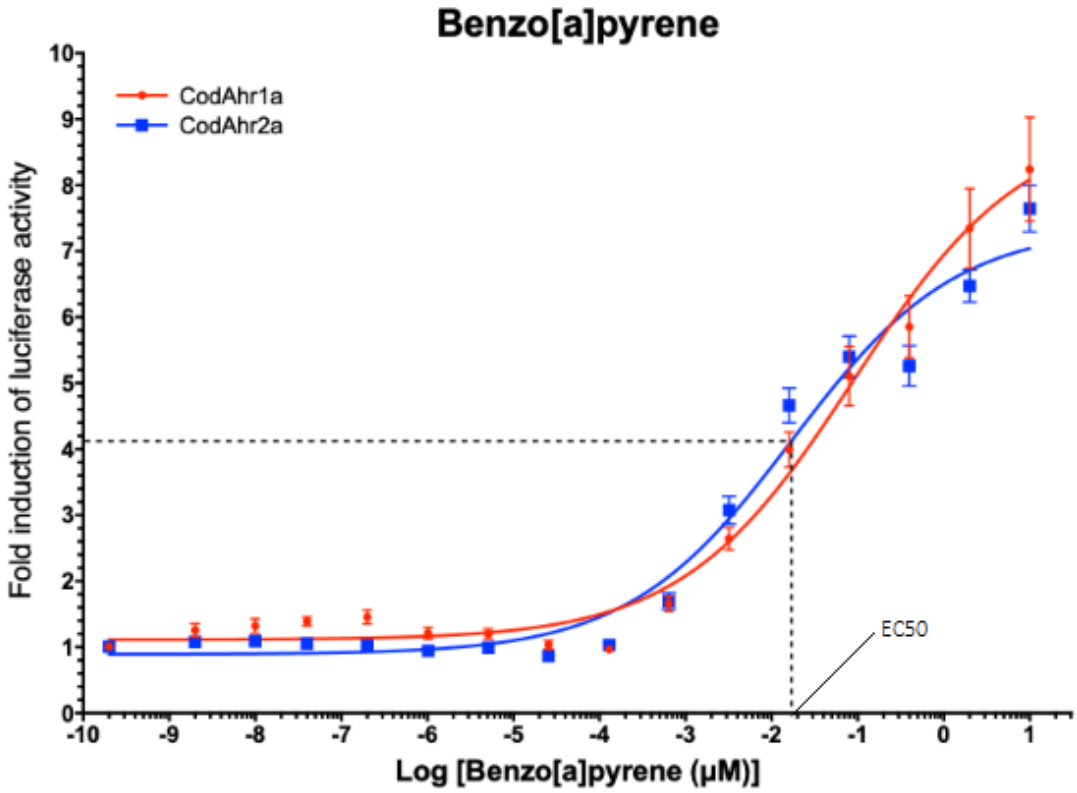

Figure A1: Response in luciferase activity with increasing concentrations of Benzo(a)pyrene.

APPENDIX B

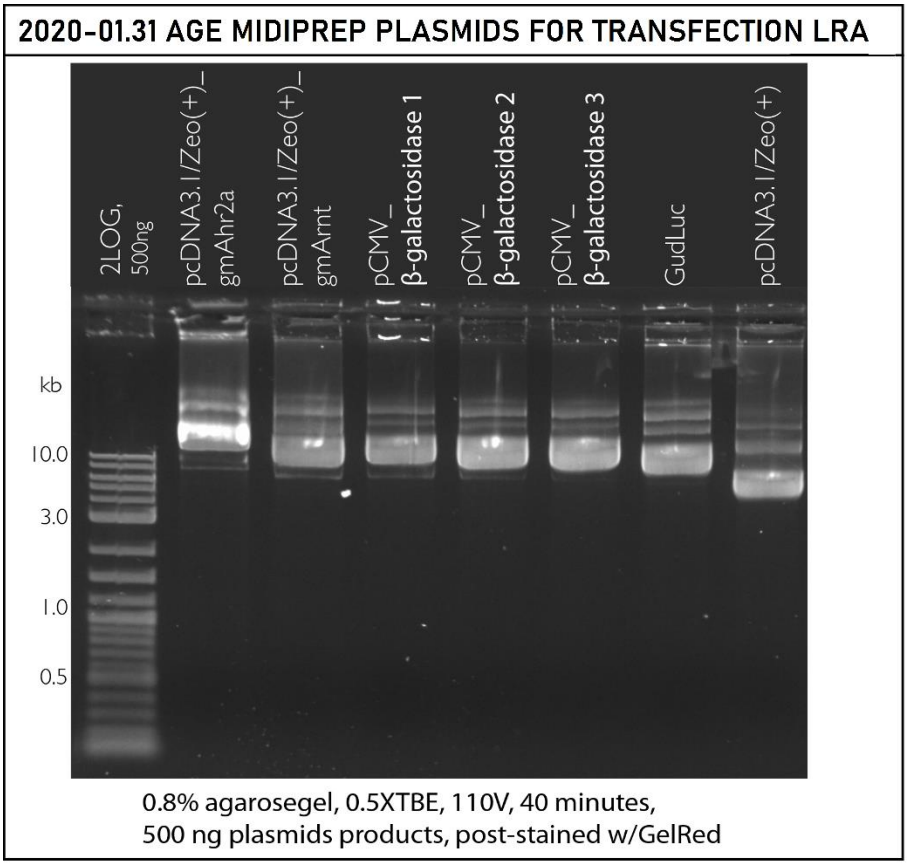

Figure B1: Control of the conformation of midiprep plasmids used for transfection in luciferase reporter gene assay.

M= log 2 referansemarkør (500 ng) (New England Biolabs).

APPENDIX C

Mixture effects of benzo[a]pyrene and perfluoroalkyl substances on the aryl hydrocarbon receptor signalling pathway and energy metabolism of Atlantic cod (*Gadus morhua*)

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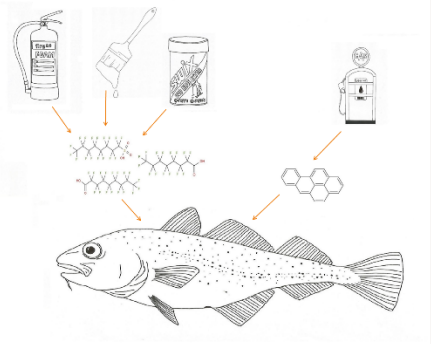
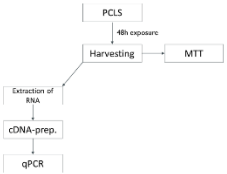
INTRODUCTION

- Benzo[a]pyrene (BaP) is a well-studied polycyclic aromatic hydrocarbon that is known to induce Cyp1a activity through activation of the aryl hydrocarbon receptor (Ahr).
- Perfluoroalkyl substances (PFAS) are synthetic compounds that are persistent in the environment. There are over 5000 PFASs on the market used in both industry and in consumer products such as food packaging, paints, and stain- and water-resistant fabrics.
- Previous research has suggested that PFASs can modulate the uptake and toxicity of other chemicals (Keiter et al., 2016)
- The aim of this project is to study toxicological effects in Atlantic cod (*Gadus morhua*) after co-exposure to BaP and PFASs, focusing on changes in gene expression levels of cytochrome P450 1a (*cyp1a)*, acyl-coA oxidase 1 (*acox1*) and ATP citrate lyase (*acly*). The latter two genes are central in the lipid metabolism of the cod.
- PFOS and PFOA have previously shown to modulate the activity of mouse, human and cod PPARA and PPARB, which are receptors involved in controlling the energy homeostasis (Takacs and Abbott, 2007) (Söderstrøm, 2019).

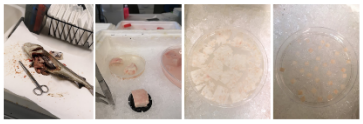


EXPERIMENTAL OUTLINE



METHODS

Precision-cut liver slices (PCLS, Eide et al., 2014) were prepared from the liver of juvenile Atlantic cod. The slices were exposed to BaP in combination with three PFASs, either alone or as a PFAS-mixture. The PFASs tested were PFOS, PFOA and PFNA.

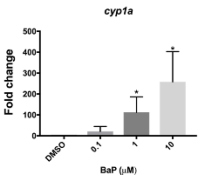


An MTT-assay was conducted to make sure that the concentrations used during exposures were not cytotoxic. Real-time quantitative PCR (qPCR) was carried out to quantify the expression of the genes *cyp1a*, *acox1* and *acly*.

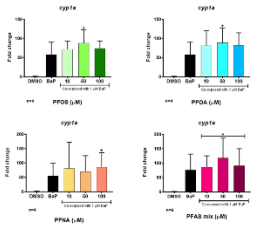
RESULTS

The viability of the liver slices did not change during the different exposure regimes (not shown). BaP induced *cyp1a* expression, and a trend of stronger induction compared to BaP was observed by co-exposure with PFASs, both single compounds and in mixture. Expression of *acox1* and *acly* also showed tendencies of increased expression by both individual- and combined PFAS exposure compared to solvent control and BaP.

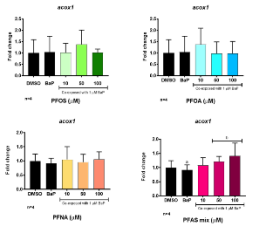
* = expression in exposed cells was significantly different to solvent control. P-value less than 0.05



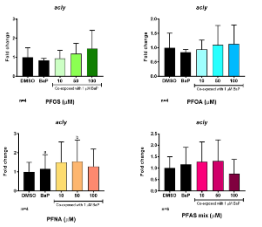
Gene expression of *cyp1a* in PCLS exposed to 0.1, 1 and 100 µM Benzo[a]pyrene. 0.1% DMSO was used as solvent control.



Gene expression of *cyp1a* in PCLS exposed to 1µM Benzo[a]pyrene and 10, 50 and 100 µm of PFOS, PFOA, PFNA or PFAS-mix. 0.1%DMSO was used as solvent control.



Gene expression of *acox1* in PCLS exposed to 1µM Benzo[a]pyrene and 10, 50 and 100 µM of PFOS, PFOA, PFNA or PFAS-mix. 0.1% DMSO was used as solvent control.



Gene expression of *acly* in PCLS exposed to 1µM Benzo[a]pyrene and 10, 50 and 100 µm of PFOS, PFOA, PFNA or PFAS-mix. 0.1%DMSO was used as solvent control.

CONCLUSIONS & FURTHER WORK

- The results indicate that the PFASs used in this experiment could enhance the toxicity of BaP through stronger induction of *cyp1a*.
- PCLS have also been exposed to the PFASs alone and in mixtures without BaP. qPCRs of the same genes will be performed.
- The putative interaction effects of BaP and PFASs will be further investigated using a luciferase reporter gene assay to study modulation of Ahr activation.

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dCod 1.0
decoding the systems toxicology of atlantic cod

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