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Ligand activation of aryl hydrocarbon receptor 2 (AhR2) from Atlantic cod (*Gadus morhua*) by polycyclic aromatic hydrocarbons (PAHs)

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Preface

This master thesis is the result of cooperation between the University of Stavanger (UiS) and the University of Bergen (UiB), made possible by my professional supervisor, Daniela M. Pampanin from the International Research Institute of Stavanger (IRIS). It is part of the research project 'iCod 2.0: Integrative environmental genomics of Atlantic cod (*Gadus morhua*)', financed by The Research Council of Norway and UiB, with the project number 244564/E40.

I would like to thank my internal supervisor from UiS, Daniela M. Pampanin and my external supervisor from UiB, Odd André Karlsen for making this thesis work possible. Thank you for all the time you spent on my work, and for all the time and patience given me when I had all kinds of questions. Daniela, you made it possible for me to do this work in Bergen, all though I belonged to UiS, thereby relieving me of much stress from living separately from my partner in Bergen. Odd André, you seemed always available for helping me, even though your schedule was tight, and you always gave me positive, uplifting feedbacks to work with.

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Moreover I would like to thank my partner, Magnus In't Veld Bendiksen, and my family and friends for great support and understanding during these months, and thank you, Karianne Skogland Enerstvedt for your personal uplifting spirit and literature guidance. Your efforts have all made a huge difference to me.

Bergen, June 2017

Unni Liknes

Abstract

Petroleum products are the major and most widespread class of contaminants in the marine environment. Anthropogenic sources are heavy contributors, like discharges of industrial and urban effluents, shipping, offshore oil drilling, oil refineries and accidental oil spills. Polycyclic aromatic hydrocarbons (PAHs) and their metabolites are among the most toxic components of petroleum products, and are a cause of great concern in the marine environment, due to their toxicity and persistence in sediments. PAH compounds represents a high risk to aquatic organisms, and ultimately to humans through fish and shellfish consumption and have been regarded as high priority for environmental pollution monitoring.

Cytochrome P_{450} (CYP1A) is an important enzyme in the biotransformation of PAHs, and is highly induced by the activation of the aryl hydrocarbon receptor (AhR). The metabolism of PAHs by CYP1A often causes an increase in toxicity, as reactive metabolites with the ability to cause crucial cellular damage are produced. Information about bioavailability of PAHs and biological responses is essential in order to assess the risk these contaminants pose to the environment and to advice adequate strategies for protection of biological resources, including those for human consumption.

The Atlantic cod (*Gadus morhua*) is an economically and ecologically important teleost species. Its genome was recently sequenced and annotated, making it an attractive model for analyzing the effects of environmental contaminants in the marine environment. The widespread distribution of Atlantic cod in the North Atlantic Ocean makes this species vulnerable to effluents from human activities.

Luciferase reporter gene assays, like the UAS/GAL4-based system used in this thesis, is a common *in vitro* method used to study ligand activation of transcription factors, such as AhR. Seven PAHs were selected to see if they could bind to and activate the cod AhR2. These PAHs were unsubstituted chrysene, along with its alkylated compounds 1-, 2-, 3- and 6-methylchrysene and (1R,2R)-1,2-dihydrophenatrene-1,2-diol and (1R,2R)-1,2-dihydronaphtalene-1,2-diol, which are the *trans*-dihydrodiols of phenanthrene and naphthalene respectively. Alkylated PAH derivatives exist in various forms, and these alkylated forms have been reported to be more toxic than their unsubstituted congeners. *Trans*-dihydrodiols are the major PAH oxidation products formed and excreted to bile in fish.

All ligands chosen proved to be able to activate cod AhR2 *in vitro*, especially the alkylated and oxidized PAHs, making them plausible to cause adverse effects in the marine environment.

Abbreviations

Table 1: Overview of abbreviations

Abbreviation	Full name
(ss)FBS	(Super stripped) fetal bovine serum
1-Met	1-Methylchrysene
2-Met	2-Methylchrysene
3-Met	3-Methylchrysene
6-Met	6-Methylchrysene
AGE	Agarose gel electrophoresis
AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
BaP	Benzo(a)pyrene
bHLH	Basic helix-loop-helix
BNF	Beta-naphtoflavone
Chr	Chrysene
COX	Cyclooxygenase
CYP	Cytochrome P_{450}
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRE	Dioxin response element
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'tetraacetic acid
EPA	Environmental Protection Agency
ER	Estrogen receptor
FAO	Food and agriculture organization
FICZ	6-formylindolo(3,2-b)carbazol
GAL4	Fungal transcription factor
HAH	Halogenated aromatic hydrocarbons
HSP90	Heat shock protein 90
IRIS	International Research Institute of Stavanger
LB	Lysogeny broth
MDR-1	Multi drug resistant gene
MQ-H_2O	Deionized water
NaCl	Sodium chloride
Naph-diol	(1R,2R)-1,2-Dihydronaphtalene-1,2-diol
NOK	Norwegian Krone
PAH	Polycyclic aromatic hydrocarbon
PAS	PER-ARNT-SIM
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzo-p-dioxins

PCDF	Polychlorinated dibenzofurans
Phen-diol	(1R,2R)-1,2-Dihydrophenatrene-1,2-diol
RNA	Ribonucleic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TEF	Toxic equivalent factor
UAS	Upstream activating sequence
UiB	University of Bergen
UiS	University of Stavanger
XAP2	X-associated protein 2
XRE	Xenobiotic response element

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1 Introduction

In the marine environment, petroleum products are the major and most widespread class of contaminants. Heavy contributors are often man-made sources, like discharges of industrial and urban effluents, shipping, offshore oil drilling, oil refineries and accidental oil spills (Medeiros et al. 2005). Development of industrial and urban centers has increased levels of petro-chemical products in the environment in the recent decades, particularly in estuaries and marine coastal areas (Lima et al. 2007). Among the most toxic components of petroleum products are the polycyclic aromatic hydrocarbons (PAHs) and their metabolites. They are a cause of great concern in the marine environment due to their toxicity and persistence in sediments (Trisciani et al., 2011). Rather than dissolving in water, their hydrophobic character makes them rapidly associate with particles, thus ending up in the sediments (Antizar-Ladislao, B. 2009; Baumard et al. 1999; David et al. 2009).

1.1 Polycyclic aromatic hydrocarbons

PAHs are aromatic hydrocarbons with the presence of benzene rings in their structure. As well as containing from two to eight conjugated ring systems, they can have a range of substituents such as alkyl, nitro and amino groups in their formation. Nitrogen, sulfur and oxygen atoms can also be present in their ring system. Many PAHs are planar molecules consisting of two or more benzene rings directly linked together (Figure 1). Although crude oils are dominated by non-aromatic hydrocarbons, it also contains significant amounts of PAHs. Incomplete combustion of organic materials, such as coal, oil, gasoline, trees and cigarettes, also forms PAHs (Walker, C.H. 2012). Natural products, such as steroids, which have been chemically converted over time, are the precursors for PAHs found in crude oil (Pampanin & Sydnes, 2013).

PAHs present in marine environment are divided into two groups, the pyrogenic and petrogenic, based on their origin. The pyrogenic are formed by incomplete combustion of organic material and are usually composed of larger ring systems (Feng et al. 2009; Lang et al. 1962; Lang et al. 1964). The sources of pyrogenic PAHs are e.g. forest fires, incomplete combustion of fossil fuels, and tobacco smoke (Lang et al. 1962; Lang et al. 1964; Wakeham et al. 1980). Petrogenic PAHs are naturally present in crude oil, coal, and some oil products (Feng et al. 2009; Lang et al. 1962; Lang et al. 1964; Laughlin et al. 1979; Harvey, R. G. 1996; Achten et al. 2010). PAHs near shores origin from sewage, road runoff, the smelter industry and oil spills, while PAHs offshore enter the water through oil seeps, oil spills, and discharge of produced water from offshore oil installations (Durand et al. 2004; Beyer et al. 1998; Næs & Oug, 1998; Smith & Levy, 1990; Mascarelli, 2010; Redondo & Platonov, 2009; Utvik, 1999) (Pampanin & Sydnes, 2013).

Due to their carcinogenic, mutagenic, and ubiquitous properties, PAH compounds represent a high risk to aquatic organisms, and ultimately to humans through fish and shellfish

consumption. For these reasons, they have been regarded as high priority for environmental pollution monitoring (Pampanin et al. 2016, Antizar-ladislao, 2008, Hausken et al. 2014, Byrne et al., 2015, Di Guilio et al., 2015). For this purpose, the US Environmental Protection Agency (EPA) has made a list of 16 unsubstituted PAHs that are on a priority pollutant list (Keith & Telliard, 1979), chosen as the most important PAHs to analyze for. Among these are chrysene, naphthalene, phenanthrene and benzo(a)pyrene, where naphthalene is present in the highest concentration in crude oil. The contents of total PAHs in crude oil differ from different sites from 0.83% in North Sea crude oil, to 1.47% in Exxon Valdez crude oil (Aas et al. 2000; Deepthike et al. 2009). In addition, alkylated derivatives of mother compounds exist in various forms, and these alkylated forms have been reported to be more toxic than their unsubstituted congeners (Rhodes, 2005; Carls et al. 2008; Billiard et al. 1999) (Pampanin and Sydnes, 2013). 1-, 2-,3- and 6-methylchrysene are all examples of methylated forms of chrysene (Figure 1).

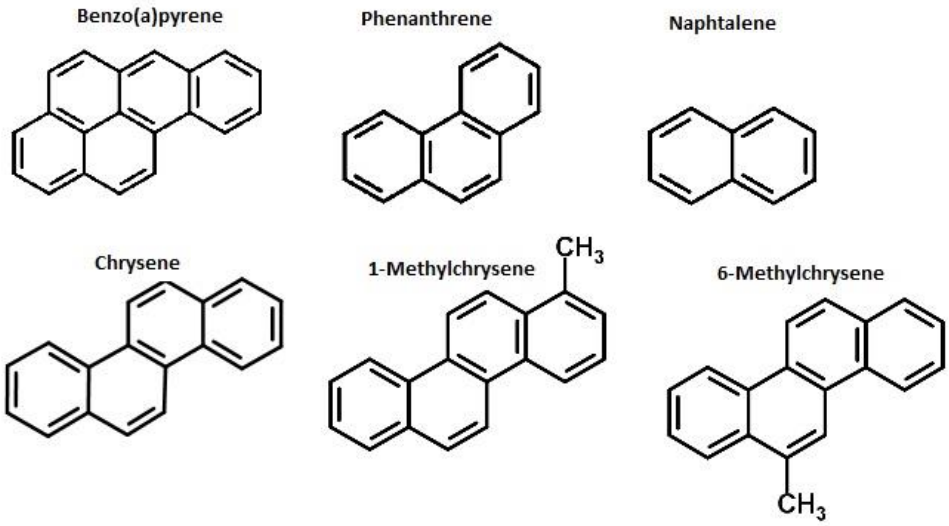


Figure 1: The structures of some unsubstituted PAHs, along with two alkylated forms of chrysene (1-methylchrysene and 6-methylchrysene). Structures modified from ChemSpider.

PAHs are found all over the world, in sediments of many marine and freshwater harbors, wherever there is oil pollution and combustion wastes. Even remote ocean locations are contaminated with PAHs (Elovaara et al. 2007). These lipophilic pollutants have relative short half-lives, and therefore do not have the tendency to pass along food chains or be biomagnified. Fish, birds and mammals metabolize them rapidly by monooxygenase enzyme systems. However, some invertebrates present in the lower trophic levels (e.g., *Mytilus edilus*, mussels) have poor ability to metabolize PAHs, and therefore bioconcentrate and/or bioaccumulate such compounds. Even though PAHs do not biomagnify to a large extent, some PAHs are subject to metabolic activation (Walker, 2012).

PAHs have low water solubility (Walker, 2012), and it is assumed that most require metabolic activation to become harmful (Conney, 1982). They are stored and metabolized in fatty tissue, like in cod-liver (Boström et al., 2002), and are also oxidized there by enzymes that exhibit aryl hydrocarbon hydroxylase activity forming more water soluble epoxides and diols (Boyd et al. 1987). (1R,2R)-1,2-dihydrophenatrene-1,2-diol and (1R,2R)-1,2-dihydronaphtalene-1,2-diol are the *trans*-dihydrodiols of phenanthrene and naphtalene respectively. *Trans*-dihydrodiols are the major PAH oxidation products formed and excreted to bile in fish (Figure 2) (Pampanin et al. 2016).

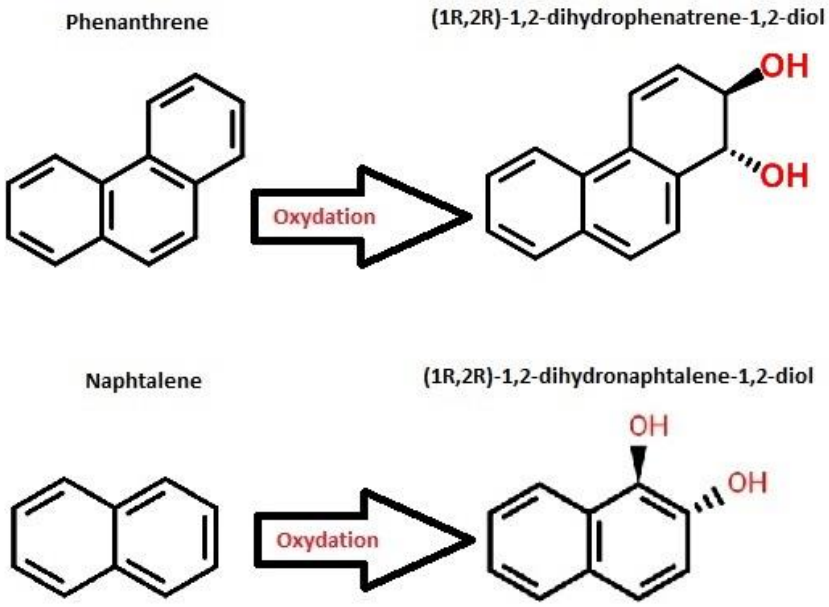


Figure 2: Two PAHs (phenanthrene and naphthalene) and their corresponding, more reactive *trans*-dihydrodiols ((1R,2R)-1,2-dihydrophenatrene-1,2-diol and (1R,2R)-1,2-dihydronaphtalene-1,2-diol respectively) formed by oxidation. Structures modified from ChemSpider.

Hydrophilic metabolites are predominately excreted via bile (Varanasi et al., 1989, Aas et al., 2000). During cellular detoxification *in vivo*, the intermediates formed are often far more toxic than the mother compounds (Conney, 1982). Formation of adducts can occur as a result of PAH metabolites having a high affinity towards DNA, RNA and protein molecules. Since repair mechanisms in fish is not highly developed, this may lead to permanent lesions such as DNA adducts and carcinogenesis (Figure 3) (Pangrekar et al, 2003).

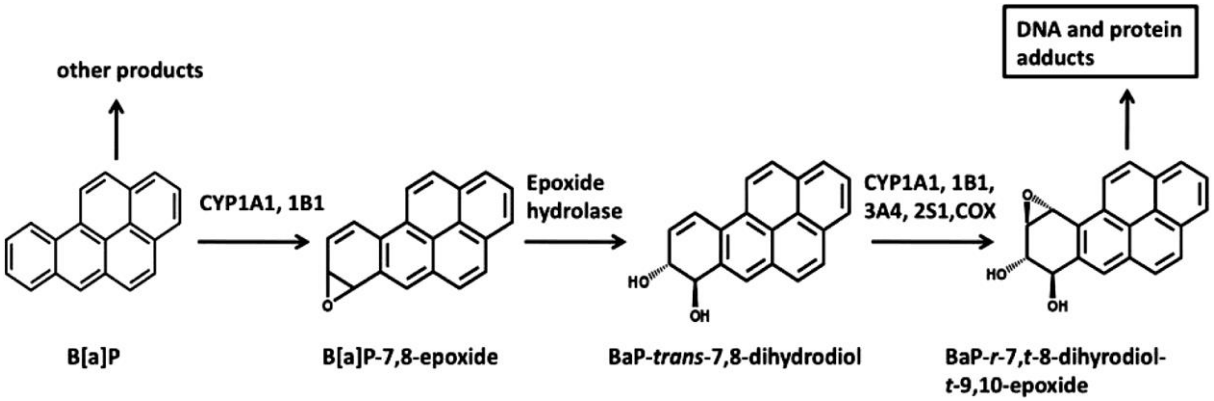


Figure 3: Benzo(a)pyrene activation pathway. In the first step, catalyzed by CYP1A or CYP1B, BaP-*trans*-7,8-epoxide is formed, followed by hydrolysis to the BaP-*trans*-7,8-dihydrodiol (BaP-7,8-diol) catalyzed by epoxide hydrolase. The latter metabolite is further epoxidized to the mutagenic BaP-*r*-7,*t*-8-dihydrodiol-*t*-9,10-epoxide (BaP-diol-*t*-epoxide), which is extremely reactive towards DNA and proteins. This reaction is catalyzed by various CYP enzymes as well as cyclooxygenase (COX). BaP-diol-*t*-epoxide is very unstable, because it rapidly undergoes hydrolysis to BaP-*r*-7,*t*-8,*t*-9,*t*-10-tetrahydrotetrol (r7,t8,t9,t10-tetrol) and BaP-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrotetrol (r7,t8,t9,c10-tetrol), whose detection is indicative of BaP-diol-*t*-epoxide formation. The process of BaP bioactivation usually involves cytochromes P_{450}, particularly members of the CYP1 family. Illustration obtained from HighWire Open Platform.

1.2 Biotransformation

A xenobiotic is a compound that is foreign to an organism, and does not play a role in the organism's normal biochemistry. When the concentration of the xenobiotic exceeds a certain level in cells, it may trigger responses designed to protect the organism against potential toxic effects. Lipophilic xenobiotics must be transformed into more water-soluble molecules in order to be excreted from the organism. A number of enzymes are induced to increase the rate of this biotransformation. Prominent among these enzymes are the monooxygenases denoted cytochrome P_{450} (CYP). CYPs are present in the livers of vertebrate animals and exist in a number of inducible forms. A group of enzymes, designated cytochrome P_{450} family 1 (CYP1A), interact particularly with PAHs. However this metabolism by a CYP can cause increased activation of carcinogens, despite its' purpose is to detoxify the xenobiotics (Walker, 2012).

Biotransformation is divided into 3 phases, phase I, II and III reactions. Phase I reactions includes hydrolysis, reduction and oxidation, which unveils or add a functional group enabling phase II reactions (Figure 4). The CYP-system is especially important phase I enzymes. They are a group of membrane-bound heme-proteins which catalytically detoxify or activate a wide specter of chemicals by monooxygenase reactions. CYP1A, CYP3A and CYP2E are central CYP-families in the biotransformation. Due to their inducibility, CYP-enzymes are often used as biomarkers, where CYP1A is one of the most frequently used biomarker for exposure to environmental toxicants (Hestermann et al. 2000, Goksøyr, 1995).

Phase II reactions, enhance different substances water solubility through enzymatic conjugation of endogenous molecules, and includes glucoronidation, sulphonation,

metylation, acetylation and conjugation with glutathione or amino acids (Figure 4). Phase III consists of transporting the metabolites out of the cells using specific transport proteins, such as MDR-1 (multi drug resistant gene), for further elimination from the organism.

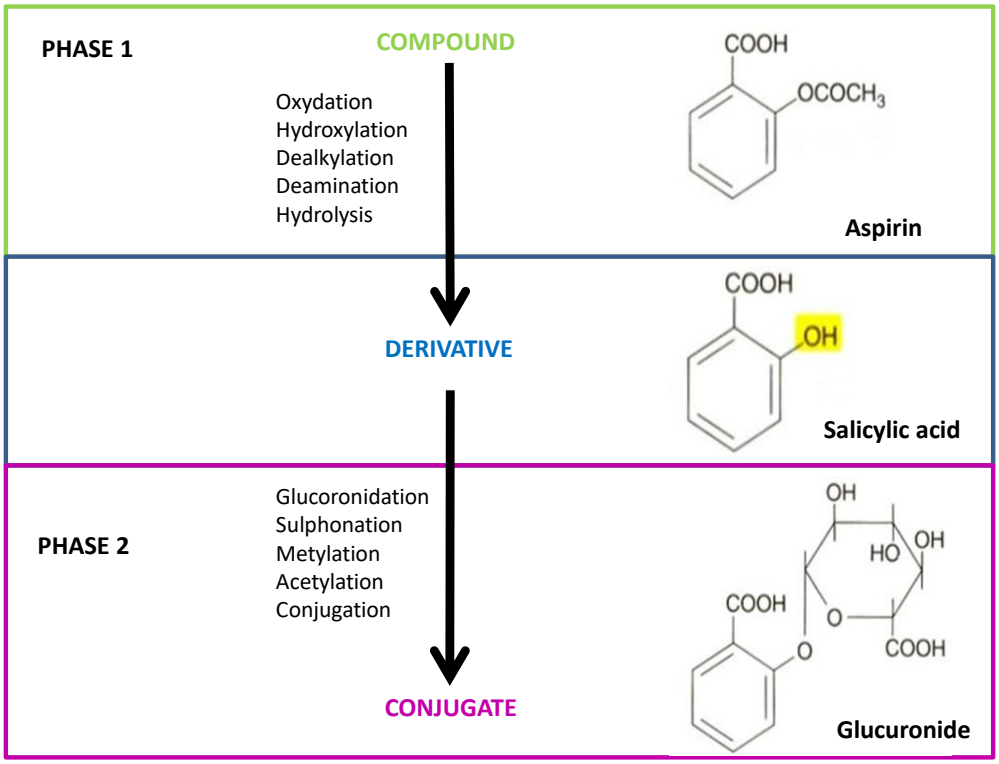


Figure 4: Phase I and II of the biotransformation of a compound. Phase I consists of altering the compound into a derivative by oxidation, hydroxylation, dealkylation, deamination or hydrolysis, making conjugation possible in phase II, and further excretion in phase III.

Biotransformation of toxic compounds in most cases leads to loss of toxicity (detoxification) and is protective to the organism. In many cases though, this metabolism causes an increase in toxicity (activation). Particularly oxidation of carcinogens can lead to the production of highly reactive metabolites that bind to cellular macromolecules, like DNA. In this way biotransformation can convert relatively inert molecules that alone cause no toxic effects into reactive metabolites with very short biological half-lives, but with the ability to cause crucial cellular damage (Figure 3) (Walker, 2012).

In fish up to 99% of PAHs is converted into metabolites within 24 hours after uptake, and therefore only a small amount of PAHs accumulate in their tissue (Vuontisjärvi et al. 2004). The hydrophobic PAH compounds increase expression of phase I and II enzymes by binding to cellular receptors like the aryl hydrocarbon receptor (AhR) (Poland et al., 1976; Okey, 1990). In order to assess the risk these contaminants pose to the environment and advice adequate strategies for protection of biological resources, including those for human consumption, information about bioavailability of PAHs and biological responses is essential (Trisciani et al., 2011).

1.3 Aryl hydrocarbon receptor (AhR)

The AhR is a ligand activated transcription factor that regulates a series of enzymes involved in the biotransformation of xenobiotics. It is one of several chemical/ligand-dependent intracellular receptors that can stimulate gene transcription in response to xenobiotics in vertebrates (Denison et al. 2003). Since its discovery (Poland et al., 1976) it has been given a great deal of attention due to its role linked to toxicity caused by dioxins, especially the highly toxic substance 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Today we know that AhR binds a broad specter of ligands, and plays a central role as a xenobiotic sensor in a wide range of different organisms (Moyer et al. 2016; Zhu et al. 2014; Hao & Whitelaw, 2013; Barouki et al. 2007).

The AhR is a member of a superfamily of transcription factors, belonging to the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) subgroup (Iain et al. 2014). The bHLH domain is important for binding to the DNA and dimerization with its partner protein, the PAS/bHLH-protein aryl hydrocarbon receptor nuclear translocator (ARNT) (Figure 5) (Fukunaga et al. 1995; Gu et al., 2000). The PAS domain acts like a binding site for other PAS proteins, including ARNT. It consists of two subdomains, PAS A and PAS B, each made up by about 50 amino acids each. What separates PAS B apart from other bHLH/PAS proteins, is that it also contains a ligand binding domain (Fukunaga et al. 1995; Reisz-Porszasz et al. 1994; Wang et al. 1995) (Endresen, 2016).

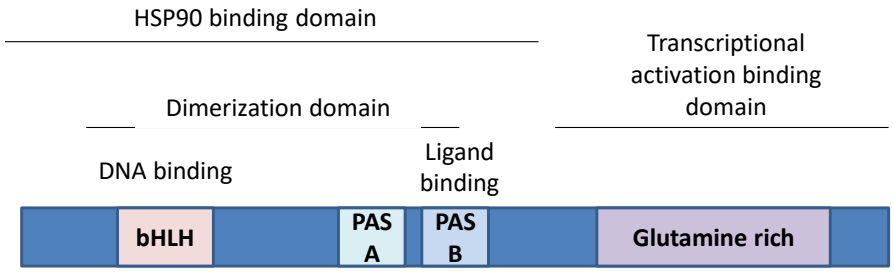


Figure 5: The structure of AhR and its functional domains for binding to cofactors and other PAS-proteins.

AhR exists in cell cytoplasma in a complex with a heat shock protein 90 (HSP90) dimer and a chaperone denoted X-associated protein 2(XAP2). When binding to an agonist (such as PAHs), the AhR complex enters the cell nucleus where ARNT mediates HSP90 displacement, and formation of the AhR-ARNT heterodimer, which can bind to dioxin-responsive elements (DRE) upstream for AhR target-genes. Co-activators can be recruited by both AhR and ARNT, leading to transcription of a wide variety of genes. CYP1A is a primary target gene for AhR, and is almost totally dependent on AhR activity for expression. As shown in Figure 6, this can result in formation of DNA adducts, and subsequent mutagenesis as CYP1A metabolizes various pro-carcinogens, like BaP (Iain et al., 2014).

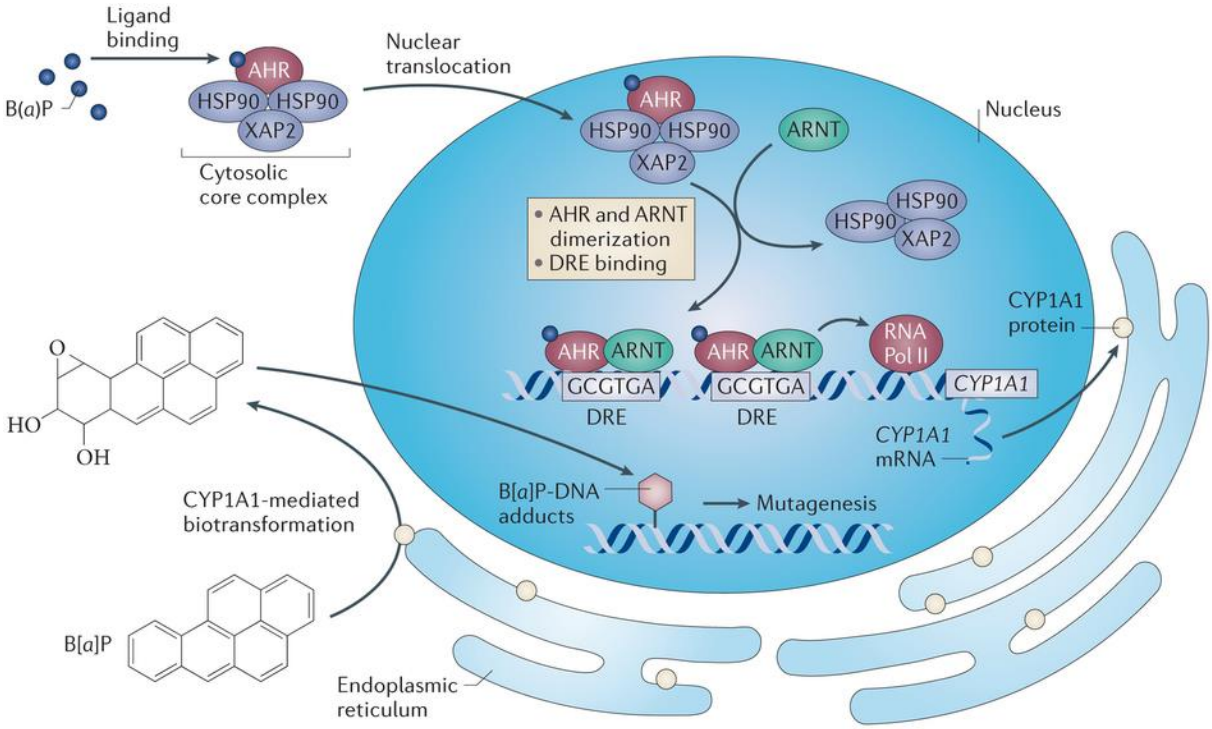


Figure 6: Activation of AhR by benzo(a)pyrene (BaP). The cytosolic core complex AhR-HSP90-XAP2 enters the cell nucleus after binding to an agonist. ARNT mediates HSP90 displacement and subsequent AhR-ARNT dimerization and binding to DRE. CYP1A is a primary target gene for AhR, leading to formation of DNA and mutagenesis.

The classical mechanism for AhR activation (as illustrated in Figure 6), results in the transcription of a number of target genes, including CYP1A, CYP1B, CYP2A, UDP-glucoronosyltransferases and glutathione S-transferases (Whitlock, 1999; Barouki et al. 2012). By inducing the transcription of different biotransformation enzymes, AhR is a very important xenobiotic sensor. In addition, ligand activated AhR also has the ability to interact and affect the functioning of other transcription factors and nuclear receptors (such as preventing estrogen receptor (ER) expression, and stimulation of ER decomposition), intracellular signaling pathways, cell proliferation, and the cell cycles (Denison et al., 2011; Othake et al. 2007; Safe et al. 1998).

Through the genome sequencing of numerous different species, it has become evident that AhR possesses an important physiological role beyond being a xenobiotic sensor. It turns out the AhR is strictly conserved from vertebrates to invertebrates (Hahn, 2002). This extended role can be linked to the immune system and differentiation of T-cells (Abel & Haarmann-Stemman, 2010), normal growth and development of the liver, immune system functioning, and development of the heart (Schmidt et al. 1996; Fernandez-Salguero et al. 1995; Thackaberry et al. 2002). In invertebrates, an AhR isoform not linked to detoxification of foreign substances exist. It has been shown that this AhR is essential for the development of eyes and wings (Céspedes et al. 2010) (Endresen, 2016).

1.3.1 Aryl hydrocarbon receptor in fish

Where mammals only have one AhR-encoding gene, several genes are found in fish, separated into the AhR1 and AhR2 subfamilies. AhR1 is orthologous with the AhR found in mammals (Hahn, 2002). It is presumed that the two different genes arose as a duplication of AhR in early vertebrate development, where mammals later on lost the AhR2 variant. Ahr2 in fish has proven to be the most dominant variant that are expressed in many different tissues (Abneet et al. 1999; Karchener et al. 1999; Tanguay et al. 1999; Hansson & Hahn, 2008). It has also in some teleost species been shown that the affinity for binding ligands and become activated by exogenic substances is higher for AhR2 than AhR1. In zebrafish (*Danio rerio*), AhR2 binds to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) with a higher affinity than the AhR1b (Karchner et al. 2005). Several different AhR-genes have been detected in different species of fish, including 6 (2 AhR1 and 4 Ahr2) in Salmon (*Salmo salar*) (Hansson and Hahn, 2008), 2 in Rainbow-trout (*Oncorhynchus mykiss*) (Abnet et al. 1999), 2 in mummichog (*Fundulus heteroclitus*) (Karchner et al. 1999) and 3 in zebrafish. Two genes encoding AhR1b and AhR2 have been found in the Atlantic cod (*Gadus morhua*) genome. Recently the AhR2 was cloned, and characterized as a xenobiotic sensor in cod (Madsen, 2016). Through studies of ligand activation with a reporter gene assay, the cod AhR2 shows an affinity for known AhR-agonists like benzo(a)pyrene (BaP), 6-formylindolo(3,2-b)carbazol (FICZ) and β-naphtoflavone (BNF) (Madsen, 2016).

1.3.2 AhR ligands

The AhR signaling pathway is known to be activated by both endogenous and exogenous compounds. Dioxins and other dioxin-like substances are among the classical ligands. Mainly of anthropogenic origin, this group of classical ligands consists of halogenated aromatic hydrocarbons (HAH), which are made up by polychlorinated variants of dibenzo-p-dioxins (PCDD), dibenzofurans (PCDF) and biphenyls (PCB) (Kulkarni et al., 2008). Of the dioxin like compounds, TCDD is considered as one of the compounds with highest potential for AhR-mediated toxicity, and has been used as a reference to determine the toxic equivalent factor (TEF). TEF is an indicator of toxic potential for the dioxin like compounds compared to TCDD (Van den Berg et al., 1998, Van den Berg et al., 2006).

Another large group of compounds able to bind to the AhR are the PAHs. Especially substances containing 4 or more benzene rings, like BaP and benzo(k)fluoranthene, can induce CYP1A in fish. An array of natural compounds, able to bind to and activate the AhR, has also been found, including different substances via the diet from plants and their derivatives (carotenoids and flavonoids). Tryptophan-derivatives are formed through biological and physiochemical processes and several have proven to be able to bind to AhR, including FICZ, indigo and indirubin. Tetrapyrroles and arachidonic acid metabolites can also bind to the AhR and have been proposed as endogenic ligands (Denison & Nagy, 2003; Nguyen & Bradfield, 2007).

1.3.3 AhR-mediated toxicity

Toxic effects caused by AhR ligand activation is mainly linked to the classical mechanism for AhR (Figure 6) (Denison et al. 2011). Fish are especially sensitive to exposure to HAH- and PAH-compounds in early stages of development, and the sensitivity for toxicants varies between different species (Doering et al. 2013). In vivo exposure to dioxins give toxic endpoints in early developmental stages, like yolk sac edema, craniofacial deformities, hemorrhaging, reduced reproductiveness and death (Henry et al. 1997; Clark et al. 2010; Yamauchi et al. 2006). Exposure to PAHs in early development stages revealed similar toxic endpoints with zebrafish as with exposure to dioxins (Billiard et al. 2006; Incardona et al. 2004). A reduction in growth and craniofacial- and jaw deformities within herring after the Exxon Valdes oil-spill accident has also been observed (Carls et al. 1999).

1.4 Atlantic cod

The Atlantic cod is an economically and ecologically important species in the North Atlantic Ocean, and has emerged as an important model organism for ecotoxicological studies. The Atlantic cod genome was recently sequenced and annotated (Star et al. 2011). A sequenced genome facilitates comprehensive toxicogenomic analyses, making Atlantic cod an attractive model for analyzing the effects of environmental contaminants in the North Atlantic Ocean (Karlsen et al. 2011; Yadetie et al. 2013). The Atlantic cod is widespread, and this makes it vulnerable to effluents from human activities such as coastal industries and offshore petroleum exploration. It is also a benthopelagic species, meaning it inhabits the water just above the seafloor, making it more exposed to chemicals accumulated in sediments, like the PAHs (Eide et al. 2014).

Many different populations of Atlantic cod exist, and they are mainly divided into coastal- and Arctic cod (Skrei). The coastal cod populations have relatively low ranges of movement, and spawn mainly in the fjords and a bit further out at sea. The main part of the coastal cod is found North of Stadt, where both the catch quota and the reproductive population have been reduced from 1997 to 2013. However, the population shows signs of improvement in recent years (Bakketeig et al. 2015). Unlike the coastal cod, the Arctic cod migrates over great distances to and from their spawning grounds. The largest population in Norway is the Northeastern Arctic cod, which lives in the Barents Sea, spawning all along the coast of Norway to Møre, with main spawning ground in Lofoten and Vesterålen. As the dominating predatory fish in the Barents Sea and an important prey for among other the minke whale and Greenland seal, Atlantic cod plays an important role in marine ecosystems (Link et al., 2009). Figure 7 shows the Atlantic cods' occurrence in the world's oceans.

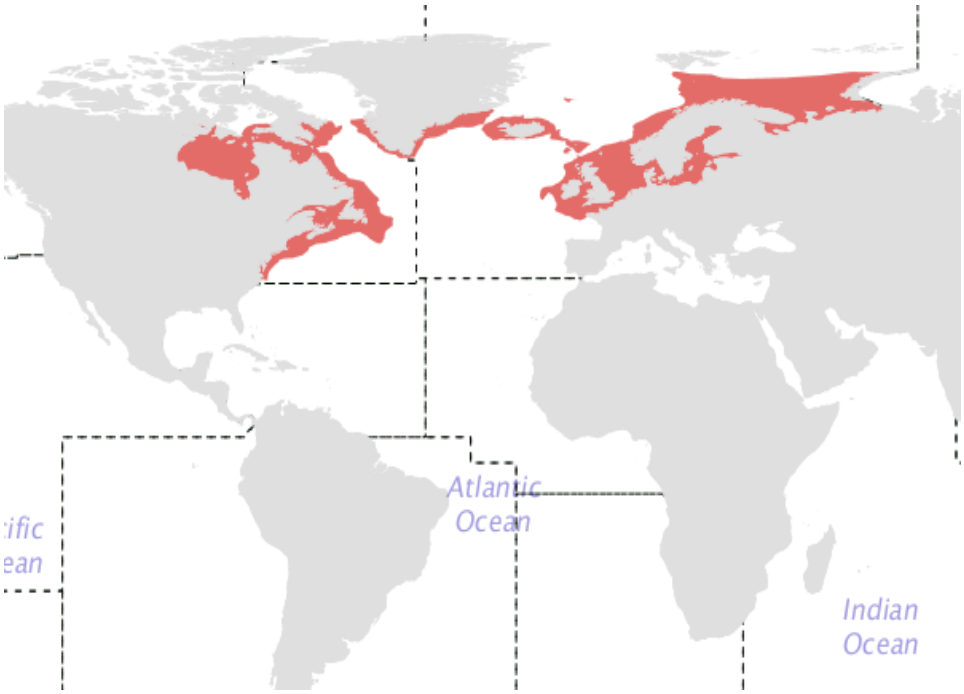


Figure 7: The extent of occurrence of the Atlantic cod (*Gadus morrhua*) in the world's oceans. Illustration obtained from Food and Agriculture Organization of the United Nations (FAO).

The Northeastern Arctic cod population has experienced a dramatic increase in recent years, with an estimated number of over 3 million tons (Bakketeig et al. 2015). The Norwegian cod catch alone was in 2013 more than four hundred thousand tons, with an estimated sales-value of over four billion NOK (Directorate of fisheries, 2015). Although the Northeastern Arctic cod population has increased, other populations of cod have been significantly reduced compared to earlier. Fishery is an important reason for this, but also climate and other factors could have an effect on this decrease (Link et al., 2009). Being an ecological and economical important teleost, increased knowledge about how this species is affected by different factors, including emissions of man-made compounds, is of great interest. The spawning area for the Northeastern Arctic cod itself is located in an area now under interest for future oil exploration, and expected increase in shipping traffic. Emission of oil could negatively affect cod, and importantly, oil has proven to induce negative effects in cod larvae, including upregulation of AhR2- and CYP1A-encoding genes (Olsvik et al., 2011).

1.5 Luciferase reporter gene assays

The luciferase reporter gene assay is a widespread and sensitive *in vitro* method used to study ligand activation of different transcription factors, including AhR (Windal et al. 2005). There are several different types of luciferase gene reporter assays, and two well-known are the Chemical Activated Luciferase Gene Expression (CALUX) and the ARNT dependent reporter gene assay. For the latter method, a eukaryotic cell line, such as COS-7, is transiently

transfected with plasmids containing a reporter gene (luciferase), along with species specific ARNT and AhR. The reporter gene is controlled by one or more response elements recognized by AhR, such as DRE. After ligand binding, AhR dimerizes with ARNT, and the heterodimer induces the expression of the reporter gene (Figur 8, part 1). This method has been used for the characterization of AhR from several different fish species (Abnet et al. 1999b; Andreasen et al. 2002a; Hansson & Hahn 2008; Karcher et al. 2005).

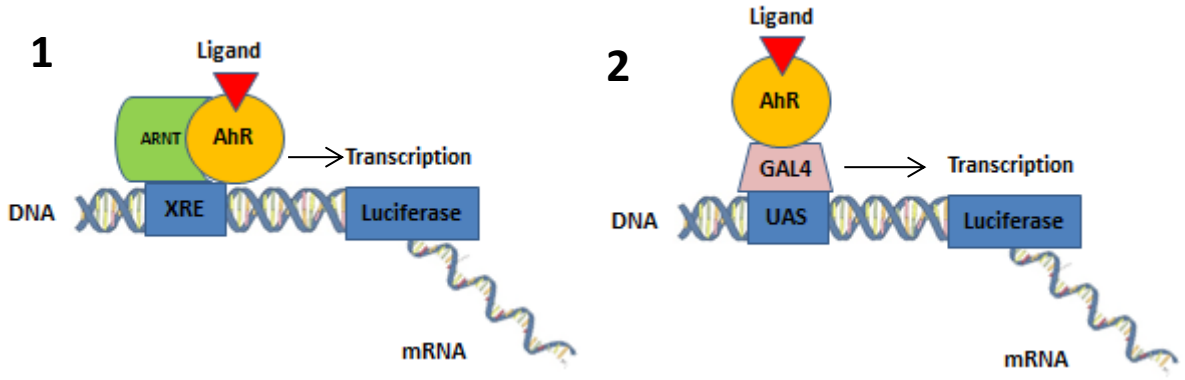


Figure 8: Two different *in vitro* gene reporter systems for studying ligand binding and transactivation of AhR. 1) When binding to a ligand, the AhR (receptor) dimerizes with ARNT, which binds to the response element DRE and thereby inducing the expression of luciferase (reporter gene). 2) When binding to a ligand, the fusion protein GAL4-AhR binds to the response element for GAL4, the UAS, thus inducing the expression of luciferase.

The UAS/GAL4-based assay used in this thesis is not dependent of ARNT and endogenous DRE response elements. In this assay, COS-7 cells are being co-transfected with a reporter- and a receptor plasmid (Figure 8, part 2). The reporter plasmid contains a luciferase-gene controlled by 4 up-stream activating sequences (UAS). The receptor plasmid contains the gene sequence for AhR, fused to the yeast GAL4 DNA-binding domain. This leads to the production of the AhR-GAL4-DBD fusion protein, which when activated by a ligand will bind the UAS in the reporter plasmid and thereby initiating transcription of the luciferase-gene. This system has been used earlier to study ligand activation of AhR from cod and rat (*Rattus norvegicus*), along with a great variety of nuclear receptors from e.g. polar bear (*Ursus maritimus*) (Madsen, 2016; Lille-Langøy et al. 2015; Backlund & Ingelman-Sundberg, 2004).

1.6 Aim of thesis

AhR were recently cloned from Atlantic cod and functionally characterized by establishing an AhR-GAL4-DBD/UAS-based luciferase reporter gene system (Madsen, 2016). The aim of this thesis was to use this reporter gene assay to study whether a selected set of different PAHs are able to bind and activate the AhR2 receptor *in vitro*. The theme of the thesis originated from the International Research Institute of Stavanger (IRIS), in cooperation with

the University of Stavanger, and the University of Bergen. The effect many pollutants have on the environment is poorly understood, and discovering effective ways for early detection of toxicants is important. The results from this thesis is expected to contribute to this understanding, by providing knowledge of the Atlantic cods response to, and potential effects of, toxic chemicals in its environment.

2 Materials

2.1 Chemicals, buffers and reagents

Table 2: List of the chemicals used

Substance	Supplier
10 x Loading buffer	TakaRa
10 x Phosphate buffer solution (PBS), pH 7,4	Sigma-Aldrich
2-Mercaptoethanol	Sigma-Aldrich
2-Nitrofenyl β-D-galactopyranosid (ONGP)	Sigma-Aldrich
5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester (5-CDFA-AM)	Thermo Fisher Scientific
Adenosin 5'-triphosphate disodium salthydrate (ATP)	Sigma-Aldrich
Agar Agar	Merck
Agarose	Sigma Aldrich
Ampicillin sodium salt	Sigma Aldrich
Boric acid	Sigma Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Calciumchloride dihydrate ($CaCl_2 \cdot H_2O$)	Merck
CHAPS	Sigma-Aldrich
Coenzyme A	Fisher Bioreagents
Dimethyl sulfoxide (DMSO)	Sigma Aldrich
Disodium hydrogenphosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$)	Sigma-Aldrich
Dithiothreitol (DTT)	Sigma Aldrich
D-Luciferin Firefly	Biosynth
Dulbecco's Modified Eagle's Medium (high glucose, phenol red free)	Sigma Aldrich
Dulbecco's Modified Eagle's Medium (high glucose, with phenol red) (DMEM)	Sigma Aldrich
Ethanol	Sigma Aldrich
Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'tetraacetic acid (EGTA)	Sigma-Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich
Fenylmetanesulfonyl fluorid (PMSF)	Sigma-Aldrich
Fetale Bovine Serum (FBS)	Sigma Aldrich
Gelred	Botium
Glycerol	Sigma-Aldrich
Isopropanol	Kemetyl
L-α-phosphatidylcholine	Sigma-Aldrich
L-glutamine	Sigma-Aldrich
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)	Sigma-Aldrich
Magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)	Sigma-Aldrich
Magnesium-Carbonathydroxide pentahydrate ($(MgCO_3)_4 \cdot Mg(OH)_2$)	Sigma-Aldrich
Methanol	Sigma-Aldrich
Opti-MEM I Reduced Serume Medium	Gibco
Penicillin-Streptomycin	Sigma-Aldrich
Potassiumchloride (KCl)	Sigma Aldrich
Potassiumphosphate, monobasic (KH_2PO_4	Merck

Resazurin sodium chloride	Sigma-Aldrich
Sodium chloride (NaCl)	Merck
Sodium dihydrogenphosphate monohydrate ($NaH_2PO_4 \cdot 2H_2O$)	Sigma-Aldrich
Sodium pyruvate	Sigma-Aldrich
TransIT-LT1	Mirus
Tricine	Sigma-Aldrich
Tris/HCl-buffer, pH 8,5	Macherey-Nagel
Triton X-100	Sigma-Aldrich
Trizma base	Sigma-Aldrich
Trizma phosphate dibasic (Tris-PO_4)	Sigma-Aldrich
Trypan blue, 0,4% solution	Sigma-Aldrich
Trypsin-EDTA (0,05 % Trypsin, 0,02 % EDTA)	Sigma-Aldrich
Trypton plus	Sigma-Aldrich
Yeast extract	Fluka

2.2 Cell lines

Table 3: Cell line

Name	Descpription	Source
COS-7	Kidney cells from African green monkey (*Cercopitheus aethiops)*	Gluzman (1981)

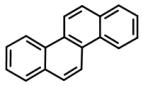
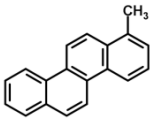
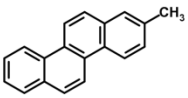
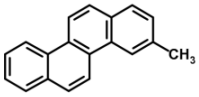
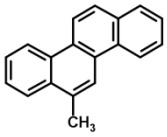
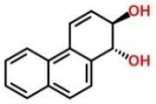
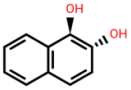
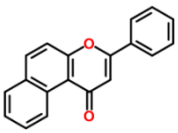
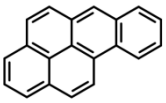
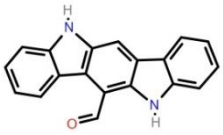
2.3 Plasmids

Table 4: List of plasmids used

Name	Descpription	Source
(mh100)x4tk luciferase	Reporter plasmid	Forman et al., 1995
pCMV β-galaktosidase	Control plasmid	Blumberg et al., 1998
pCMX GAL4-AHR2	Reseptor plasmid Cod Ahr2	Madsen, 2016

2.4 Ligands

Table 5: Overview of the ligands. All ligands used are polycyclic aromatic hydrocarbons, except from FICZ , which is a photoproduct of tryptophan, and BNF, a synthetic flavonoid. Stock solutions were prepared with varying amount of DMSO, depending on the compounds solubility. This resulted in different concentrations for the stock solutions. Structures obtained from ChemSpider.

Name	Structure	Formula	Supplier
Chrysene (Chr)		$C_{18}H_{12}$	UiS
1-Methylchrysene (1-Met)		$C_{19}H_{14}$	UiS
2-Methylchrysene (2-Met)		$C_{19}H_{14}$	UiS
3-Methylchrysene (3-Met)		$C_{19}H_{14}$	UiS
6-Methylchrysene (6-Met)		$C_{19}H_{15}$	UiS
(1R,2R)-1,2-Dihydrophenatrene-1,2-diol (Phen-diol)		$C_{14}H_{12}O_2$	UiS
(1R,2R)-1,2-Dihydronaphtalene-1,2-diol (Naph-diol)		$C_{10}H_{10}O_2$	UiS
Beta-naphtoflavone (BNF)		$C_{19}H_{12}O_2$	Sigma Aldrich
Benzo-a-pyrene (BaP)		$C_{20}H_{12}$	Sigma Aldrich
6-formylindolo(3,2-b)carbazol (FICZ)		$C_{19}H_{12}N_2O$	AH Diagnostics (Enzo Life Science)

2.5 Growth media

2.5.1 Bacterial growth medium

Table 6: Lysogeny Broth cultivation medium for *Escherichia coli* (LB)

Substance	Concentration
NaCl	10 g/L
Trypton	10 g/L
Yeast extract	5 g/L
Ampicillin	100 mg/L

Autoclaved before use in 121°C for 90 minutes

2.5.2 Growth medium for COS-7 cells

Table 7: Medium used for cultivation of COS-7 cells

Substance	Concentration
Dulbecco's Modified Eagle's Medium (DMEM)*	0.87 x
Fetal bovine serum (FBS)*	8.70%
Penicillin-streptomycin	86 U / 0.86 mg/mL
L-Glutamine (200 mM)	3.48 mM
Sodium pyruvate (100 mM)	0.87 mM

*When exposing to ligands, phenol-free DMEM was used along with super stripped FBS

2.6 Solutions

2.6.1 Buffers and solutions for agarose gel electrophoresis

Table 8: 5 x Tris-borate - EDTA (TBE) buffer (pH 8.8)

Substance	Concentration
Trizma base	54 g/L
Boric acid	27.5 g/L
EDTA (0,5 M)	20 mL/L
MQ-H_2O	-

Table 9: 0.7% TBE agarose gel solution

Substance	Concentration
TBE-buffer	1 x
Agarose	0.7 %

2.6.2 Ligand activation solutions

Table 10: Cell lysis buffer (1x)

Substance	Concentration
Tris-PO_4 (pH 7.8)	25 mM
Glycerol	15%
CHAPS	2%
L-α-Phosphatidylcholine	1%
Bovine serum albumin (BSA)	1%

Table 11: Lysis reagent

Substance	Concentration
Cell lysis buffer (1x)	1 x
EGTA	4 mM
DTT	1 mM
$MgCl_2$	8 mM
PMSF	0,4 mM

Table 12: β-galactocidase buffer (10 x)

Substance	Concentration
Na_2HPO_4	60 mM
NaH_2PO_4	40 mM
KCl	10 mM
$MgCl_2$	1 mM

Table 13: β-galactocidase reagent

Substance	Concentration
β-galactocidase base buffer (10 x)	1 x
β-mercaptoethanol	52.9 mM
ONGP	

Table 14: Luciferase buffer (4 x, pH 7,8)

Substance	Concentration
Tricine	80 mM
$(MgCO_3)•Mg(OH)_2•5H_2O$	4.28 mM
EDTA	0.4 mM
$MgSO_4$	10.68 mM

Table 15: Luciferase reagent

Substance	Concentration
Luciferase base buffer (4 x, pH 7.8)	1 x
MQ-H_2O	-
DTT	5 mM
ATP	0.5 mM
Coenzyme A*	0.15 mM
D-luciferin*	0.5 mM

*Added directly before use

2.6.3 Cytotoxicity assay

Deionized H_2O used in all solutions.

Table 16: Solution I

Substance	Concentration
NaCl	1711 mM
KCl	34 mM
$Na_2HPO_4*H_2O$	50 mM
$KH_2PO_4*H_2O$	13 mM
H_2O	-

Adjusted to pH 7.4 with 6 M HCl and autoclaved

Table 17: Solution II

Substance	Concentration
$MgCl_2•6H_2O$	49.2 mM
H_2O	-

Autoclave

Table 18: Solution III

Substance	Concentration
$CaCl_2•2H_2O$	90 mM
H_2O	-

Autoclave

Solution I+II gives 10x DPBS (pH 7.4)

Table 19: 1xDPBS (pH 7.4)

Substance	Concentration
Solution I+III	10%
Solution III	10%
H_2O	80%

Table 20: Resazurin solution

Substance	Concentration
Resazurin (0.15 mg/mL)	20%
CDFA-AM (4 mM in DMSO)	0.1%
DMEM	79.9%

2.7 Kit

Table 21: Kit used for purifying plasmid DNA

Product	Description	Supplier
NucleoBond Xtra Midi/Maxi	Plasmid DNA purification	Macherey-Nagel

2.8 Instruments

Table 22: Instruments used

Product	Description	Supplier
Avanti J-26 XP Centrifuge	Centrifuge (large volume)	Thermo Scientific
Bürker's chamber	Cell counting	Marienfeld
EnSpire 2300 Multilabel Reader	Plate reader	PerkinElmer
G:BOX	Gel reader	Syngene
Heraeus Multifuge X3R centrifuge	Centrifuge	Thermo Scientific
HS 501 Digital	Plate for shaking	IKA-Werke
Leica DM IL inverted microscope	Cell related work	Leica
MilliQ A10 Advantage	Deionized H_2O	Merch Millipore
NanoDrop 1000 Spectrophotometer	Spectrophotometer	Thermo Scientific
New Brunswick Galaxy 170 R	CO_2-incubator	Eppendorf
PowerPac HC	Gel electroforesis	BioRad

2.9 Software

Table 23: Software used

Software	Descrption	Source
EndNote	Reference management system	Thomson
EnSpire Manager	Plate reader	PerkinElmer
Excel 2010	Statistical analyses	Microsoft
GeneSnap Image	Visualization of agarose gel	Syngene
ND-1000	Plasmid concentration	Thermo Scientific
Paint	Illustrations	Microsoft
Prism 7	Statistical analyses and graphs	GraphPad
PowerPoint 2010	Illustrations	Microsoft
Word 2010	Word prossesing software	Microsoft

3 Methods

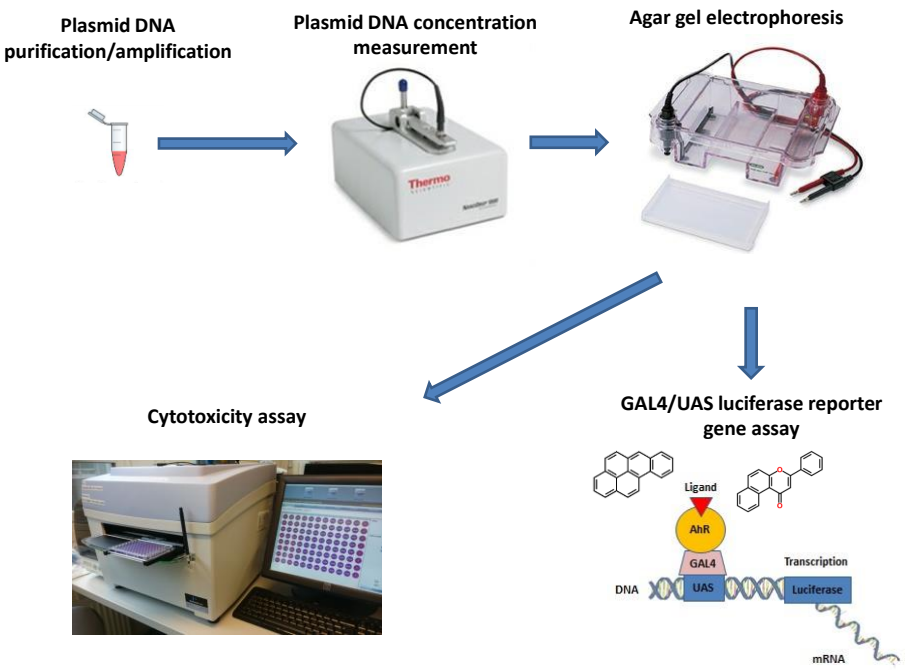


Figure 9: An overview of the methods used in this thesis. Plasmid DNA was purified and concentrations measured using a NanoDrop spectrophotometer. Agarose gel electrophoresis (AGE) was used for assessment of plasmid conformations. The luciferase gene reporter assay and cytotoxicity assays were carried out for studying ligand activation of cod AhR2 and putative toxic effects on cells during exposures, respectively.

3.1 Plasmid DNA purification

Receptor plasmid (pCMX GAL4-AhR2), reporter plasmid ((Mh100)x4tk luciferase) and control plasmid (pCMV β-galaktosediase) were separately produced in *Escherichia coli* and purified using the Nucleobond Maxi/Midi kit. Overnight cultures in LB media were made by adding *E. coli* from a glycerol stock solution (-80°C) previously transfected with the respective plasmids. The cultures were added ampicillin (ampicillin resistance gene in plasmids) and incubated under constant shaking (250 rpm) at 37°C overnight (or 7-8 hours). Cell cultures were subsequently centrifuged at 3500 x *g* for 10 min (4°C). Nucleobond Midi/Maxi kit from Macherey-Nagel was used to purify the DNA plasmids from the overnight culture by following the manufacturer's procedure. In short, after removal of the supernatant, bacterial cells were lysed by adding a NaOH/SDS solution. When neutralized shortly after with neutralization buffer (NEU), cell components, proteins, and chromosomal DNA precipitated and were removed by centrifugation. Plasmid DNA now existed in the supernatant that were further purified and concentrated with columns provided in the kit. Plasmids were eluted from the columns by adding an alkaline buffer and precipitated by adding isopropanol (100%). Plasmid DNA precipitates were collected by centrifugation for 30 min at 15 000 x *g*. The pellet, containing plasmid DNA was washed with ethanol (100%),

centrifuged for 5 min at 15 000 x *g* and left to dry at room temperature. The dried pellet was finally resuspended in the preferred amount of MQ-H_2O (300-400 µL). DNA concentration was measured spectrophotometrically using a NanoDrop-1000 (Thermo Scientific), and plasmid quality was assessed using agarose gel electrophoresis.

3.2 Agarose gel electrophoresis

Plasmid DNA can exist in different conformations. A supercoiled form is preferred, because it is more easily taken up by cells during transfection compared to plasmids that have a relaxed form. Agarose gel electrophoresis (AGE) was used to assess the conformation of the purified DNA plasmids. Supercoiled plasmids move more rapidly through the pores in the agarose gel, and plasmids conformations can therefore be visualized by using AGE. The method separates nucleic acids based on size in an electrical field. Deprotonized nucleic acids are negatively charged, and will migrate towards the positive pole. Size and conformation determine how far they travel in the gel. The gel was made from a 0.7% agarose solution added GelRed (for visualization of plasmids) and set to polymerize for 20 min with a comb imbedded for creating the sample wells. After the gel had polymerized, TBE buffer was gently poured over, completely covering the gel. An amount of 100 ng DNA plasmids was mixed with MQ-H_2O and 1 µL loading buffer (total volume of 10 µL) and applied to the wells. 5 µL of 2-log DNA Ladder was used as a molecular weight standard. The electrophoresis was conducted for 40 minutes at 100 V using a PowerPac HC (Bio-Rad). By using UV-light and G:BOX (Syngene) the plasmids were visualized and photographed.

3.3 Cytotoxocity assay

To assess if any of the ligands used in exposure experiments affected the viability of the COS-7 cells, two endpoints were studied. A combination of resazurin and 5-carboxyflourescein diacetate, acetoxymethyl esther (CFDA-AM) was used as an indication of metabolic activity and cell membrane integrity, respectively (Shreer et al. 2005). Both resazurin and CFDA-AM is transformed into fluorescence compounds by living cells and can therefore be detected fluorometrically. Metabolically active cells have the ability to convert resazurin into a fluorescent and colorimetric indicator. Damaged and non-viable cells have lower metabolic activity, and generate proportionally lower signals. CDFA-AM is an esterase substrate able to permeate cell membranes. Viable cells have enzymatic activity that activates the reagents' fluorescence and retain the fluorescent product. Thus, the CDFA-AM is a measure of both enzymatic activity, and cell membrane integrity (Thermo Fischer Scientific).

The cytotoxicity assay was performed with cells exposed in the exact same manner as for the luciferase reporter gene assay GAL4/UAS, except from the cells were not transfected with DNA plasmids. COS-7 cells were seeded as described in 3.5.2 and incubated in 37°C in 5%

CO_2 for 48 hours. Cells were thereafter exposed to the same ligands at identical concentrations used in the luciferase reporter gene assay (described in 3.5.4) and incubated in 37°C in 5% CO_2 for 24 hours. After removal of medium, 1xDPBS (pH 7,4) was used to wash the cells. 100 µL resazurin/CFDA-AM solution was added to the wells before incubation of one hour in 37°C in 5% CO_2 under protection from light. Controls were included in all plates, including wells with only cells and medium (no exposure) and cells plus DMSO only (solvent control). As a positive control for cytotoxicity, cells were exposed to 0.1% Triton X-100. Fluorescence was measured at 570/585 nm (excitation/emission) for resazurin and 492/517 (excitation/emission) for CFDA-AM. Values are converted into percentage, relative to the DMSO exposed cells (set to 100%). There were 4 replicates from each ligand concentration in 3 experiments (n=12). Cells that were only exposed to medium, DMSO only and Triton X-100 had up to 8 replicates in each experiment (n=24).

3.4 Cultivation and maintaining COS-7 cells cultures

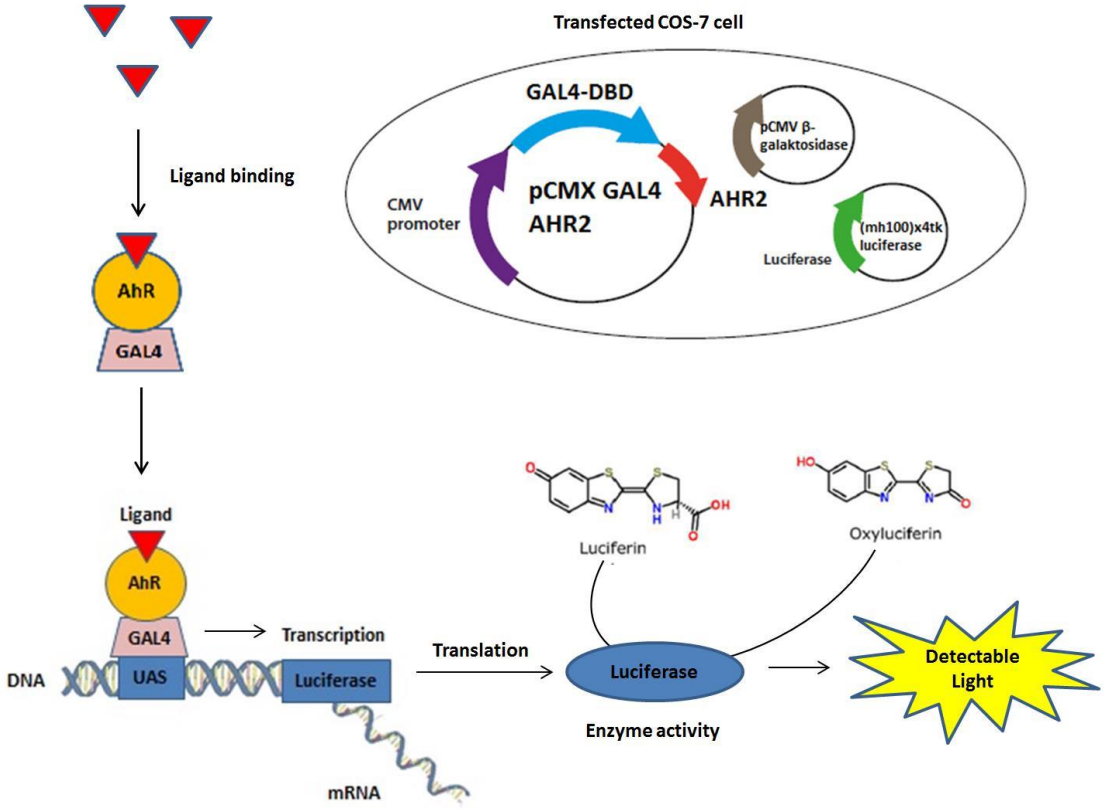
All handling of cells was done with sterile techniques inside a sterile laminar flow (LAF)-bench. COS-7 cells stored in frozen medium (DMEM 10% FBS, 5% DMSO) in liquid nitrogen were thawed and transferred to fresh DMEM-10% FBS growth medium. Centrifugation at 250 x *g* for 5 min removed DMSO, and the pellet was resuspended in DMEM 10% FBS. Cell suspension was transferred into 10 cm Petri dishes and cultivated at 37°C and 5% CO_2. At approximately 80% confluency (estimated by using light microscope) the cells were split in order to not overpopulate the dish and die. Old cultivation medium was removed, and cells washed with 1xPBS (pH 7.4). To break intercellular interactions and attachment to the bottom of the Petri dish, Trypsin-EDTA (0.05% trypsin, 0.02% EDTA) was added to the cells and left for incubation for 60 seconds. After removing the Trypsin-EDTA, cells were incubated for 5 min at 37°C and 5% CO_2. 10 mL growth medium was added, and dilutions ranging from 1:2 – 1:20 (dependent on desired cell density) were transferred into new Petri dishes for further incubation at 37°C and 5 % CO_2.

3.5 GAL4/UAS luciferase reporter gene assay

3.5.1 Principle

To measure ligand-induced activation of cod AhR2, a luciferase reporter gene assay was used. In this procedure, a reporter plasmid, containing the reporter gene luciferase and a receptor plasmid, encoding recombinant GAL4-DBD-AhR2, were transfected into COS-7 cells together with a control plasmid containing a β-galactosidase encoding gene. The control plasmid, and hence measured β-galactosidase-activity, is subsequently used for normalization of plasmid transfection efficiency. The luciferase gene in the reporter plasmid is regulated by four upstream activation sequences (UAS) specific for the GAL4-DBD. After transfection of

COS-7 cells, the fusion protein GAL4-DBD-AhR2 is produced. When a ligand binds to the AhR2 fused to the GAL4-DBD, a conformational change is induced and GAL4-DBD-AhR2 binds to the UAS in the reporter plasmid. This induces the expression of the reporter gene luciferase, which when translated into a protein can catalyze the conversion of D-luciferin into oxiluciferin, which is a light producing reaction (luminescence). This light is detectable in a luminometer, enabling the quantification of the activation of the receptor protein (Figure 10).



Figur 10: A schematic overview of the principle behind the GAL4-DBS/UAS-assay. The reporter-, receptor- and control plasmids are transfected into COS-7 cells. When binding to a ligand, the receptor protein (AhR2) fused to the GAL4-DBD is activated. GAL4-DBD binds to UAS upstream the luciferase gene in the reporter plasmid, inducing the expression of luciferase. Luciferase catalyzes the reaction in which luciferin is converted to oxyluciferin, emitting a quantifiable light signal detactable in a luminometer.

3.5.2 Seeding of COS-7 cells

COS-7 cells with an approximate density of 80% confluency were treated with trypsin-EDTA before being resuspended in 5 mL DMEM-10% FBS. A small volume of this cell solution was mixed 1:1 with trypan blue, and cell number determined by using a heamocytometer (Bürker) and a light microscope. Based on the cell counting, 5000 cells were applied into each well of a 96-well plate and incubated at 37°C, 5% CO_2 for 24 hours.

3.5.3 Transfection of COS-7 cells

Old cell medium was removed before the cells were transfected with a plasmid mix containing pCMV β-galaktosidase control plasmid, (Mh100)x4tk luciferase reporterplasmid, and the receptor plasmid pCMX GAL4-AhR2. This was performed by using the transfection reagents TransIT-LT1. This reagent contains lipids, proteins and polyamines, which facilitates the transport of the plasmid-DNA over the cell membrane. DNA plasmid in a concentration of 100 ng/well was diluted in Opti-MEM I and TransIT-LT1 reagent was added. The mixture was incubated in roomtemperature for 30 minutes, before being added DMEM-10% FBS. Cells were added this transfection mix and growth medium, and incubated at 37°C, 5% CO_2 for 24 hours.

Table 24: Contents of the mixture used for transfection of the COS-7 cells.

Reagent	Amount per well
Opti-MEM I	9 µL
Plasmid-mix (pCMV β-galaktosediase + (Mh100)x4tk luciferase + pCMX GAL4-AHR2)	0.1 µL
TransIT-LT1	0.3 µL
DMEM-10% FBS	92 µL
Total	101.4 µL

3.5.4 Ligand treatment of COS-7 cells

After 24 hours, old medium was removed, and cells were exposed to ten different ligands in different concentrations. All ligands were dissolved in DMSO. Dilutions of the ligands were made in phenol-free DMEM 10% ssFBS and DMSO, with final concentration of DMSO no higher than 0.5%. This maximum concentration of DMSO limited the maximum concentrations that could be used for some of the ligands. This was due to different solubility of PAHs, resulting in different content of DMSO in the stock solutions. Growth medium with DMSO was used as a negative control, and the ligands BNF, BaP and FICZ, which are known to activate cod AhR2 (Madsen, 2016), were used as positive controls for the reporter gene assay. Every exposure concentration had three replicates from three individual experiments (n=9). Plates were incubated at 37°C, 5% CO_2 for 24 hours.

Table 25: An overview of the different ligands used, together with the highest and the lowest concentration for each compound. All ligands were diluted with a factor of 5.

Ligand	Highest conc. (µM)	Lowest conc. (µM)
Chrysene	50,0	$3{,}2 \cdot 10^{-3}$
1-Methylchrysene	50,0	$3{,}2 \cdot 10^{-3}$
2-Methylchrysene	200,0	$1{,}3 \cdot 10^{-2}$
3-Methylchrysene	200,0	$1{,}3 \cdot 10^{-2}$
6-Methylchrysene	100,0	$6{,}4 \cdot 10^{-3}$
(1R,2R)-1,2-Dihydrophenatrene-1,2-diol	50,0	$3{,}2 \cdot 10^{-3}$
(1R,2R)-1,2-Dihydronaphtalene-1,2-diol	200,0	$1{,}3 \cdot 10^{-2}$
Beta-naphtoflavone	4,0	$2{,}6 \cdot 10^{-4}$
Benzo(a)pyrene	20,0	$1{,}3 \cdot 10^{-3}$
6-formylindolo(3,2-b)carbazol	0,10	$6{,}4 \cdot 10^{-6}$

3.5.5 Measurement of luciferase- and β-galactocidase activity

Old medium was removed after 24 hours of incubation, and cells were added a lysis reagent to destroy cell membranes, stabilize proteins and inhibit protease activity. Plates were thereafter gently shaken in room temperature for 30 minutes. 50 µL of the lysate was transferred to a 96 well white luminescence plate, and another 50 µL of the cell lysate to a 96 well clear absorbance plate. The clear plate was added 100 µL β-galactosidase reagent, and incubated in room temperature until a yellow color appeared after about 20 minutes. Absorbance was measured at a wavelength of 405 nm in a PerkinElmer Enspire plate reader. The white luminescence plate was added 100 µL of D-luciferin per well and emitted light was measured immediately using a PerkinElmer Enspire plate reader. To consider possible differences in transfected amount of plasmids between different wells, the luciferase activity was normalized against measured β-galactosidase activity in its corresponding well.

4 Results

4.1 Agarose gel electrophoresis of plasmids

DNA-plasmids were purified as described in 3.1 and assessed with agarose gel electrophoresis to determine their conformation before use in the luciferase reporter gene assay. Receptor plasmid (pCMX GAL4-AhrR), reporter plasmid ((mhx100)4xtk luciferase) and control plasmid (pCMV β-galactosidase) were applied to an agarose gel and the electrophoresis was carried out as described in 3.2. The majority of each of the plasmids appeared to be in a supercoiled conformation and therefore suited for further use in the luciferase gene reporter assay (Figure 11).

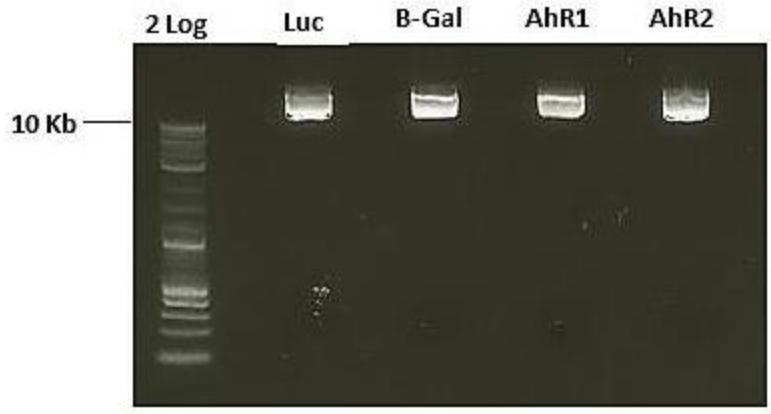
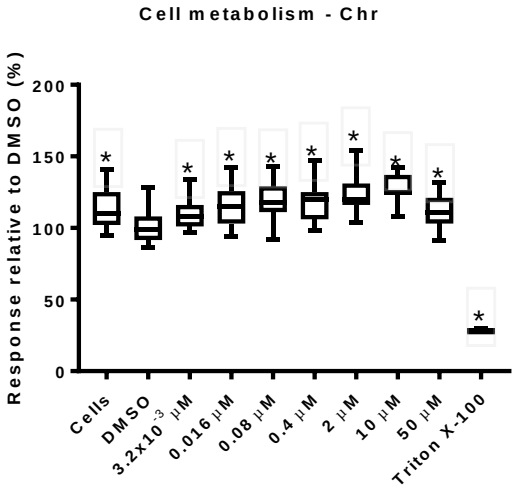


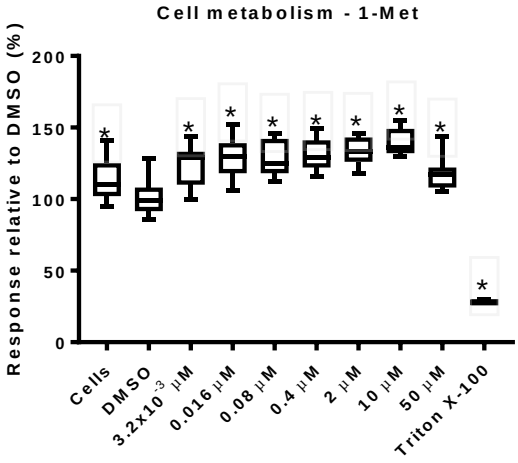
Figure 11: Assessment of plasmid conformation by AGE. As indicated over the gel, the reporter plasmid, (mh100)x4tk luciferase (Luc), control plasmid, pCMV β-galaktosidase (B-Gal), and the receptor plasmid, pCMX GAL4-AhR2 (AhR2) were separated by AGE. 2 Log is the DNA Ladder (0.1-10.0 kB). The receptor plasmid, pCMX GAL4-AhR1 (AhR1) was excluded from this study.

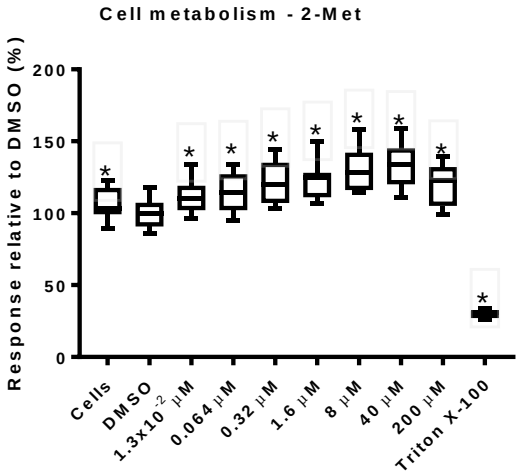
4.2 Cytotoxicity assay

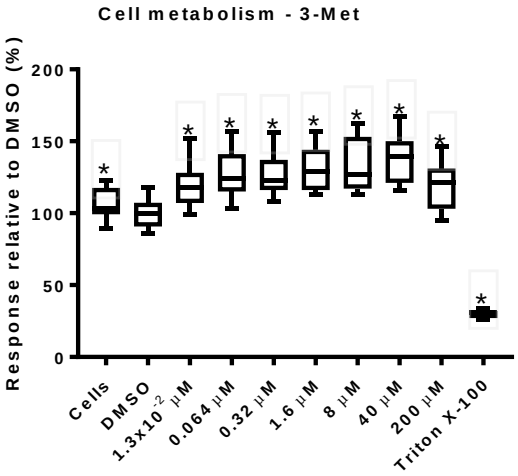
4.2.1 Resazurin assay for assessing metabolic activity

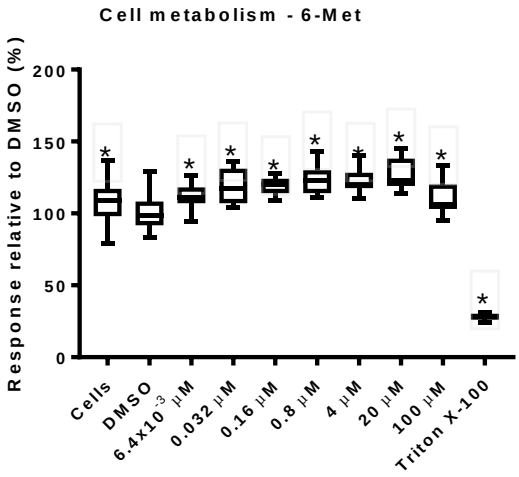
Cytotoxicity was measured after exposure of COS-7 cells to the different PAHs and AhR2 model-agonists. Similar responses were seen for all of the compounds, and none of them seemed to have a negative impact on the cell metabolism (Figure 12). Triton X-100 was the only compound that significantly lowered metabolic activity (less than 100%), which also was in line with Triton X-100 being the positive control for cytotoxicity. All compounds at the concentrations tested seemed to have a stimulating impact on COS-7 cell metabolism. From these results it appeared that none of the PAHs were toxic for COS-7 cell metabolism for any of the concentrations used.

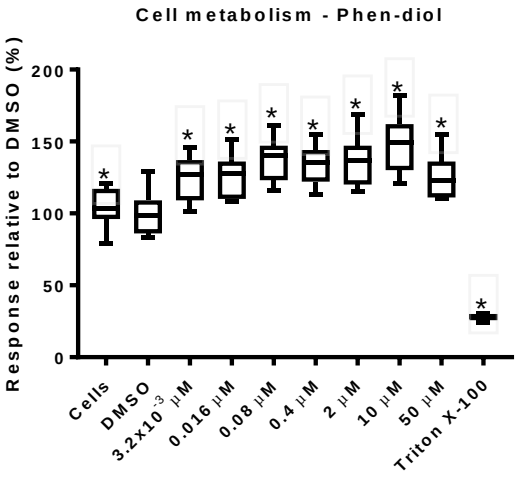

Cell metabolism - Chr
Response relative to DMSO (%)
0
50
100
150
200
Cells
DMSO
3.2x10 -3 µM
0.016 µM
0.08 µM
0.4 µM
2 µM
10 µM
50 µM
Triton X-100

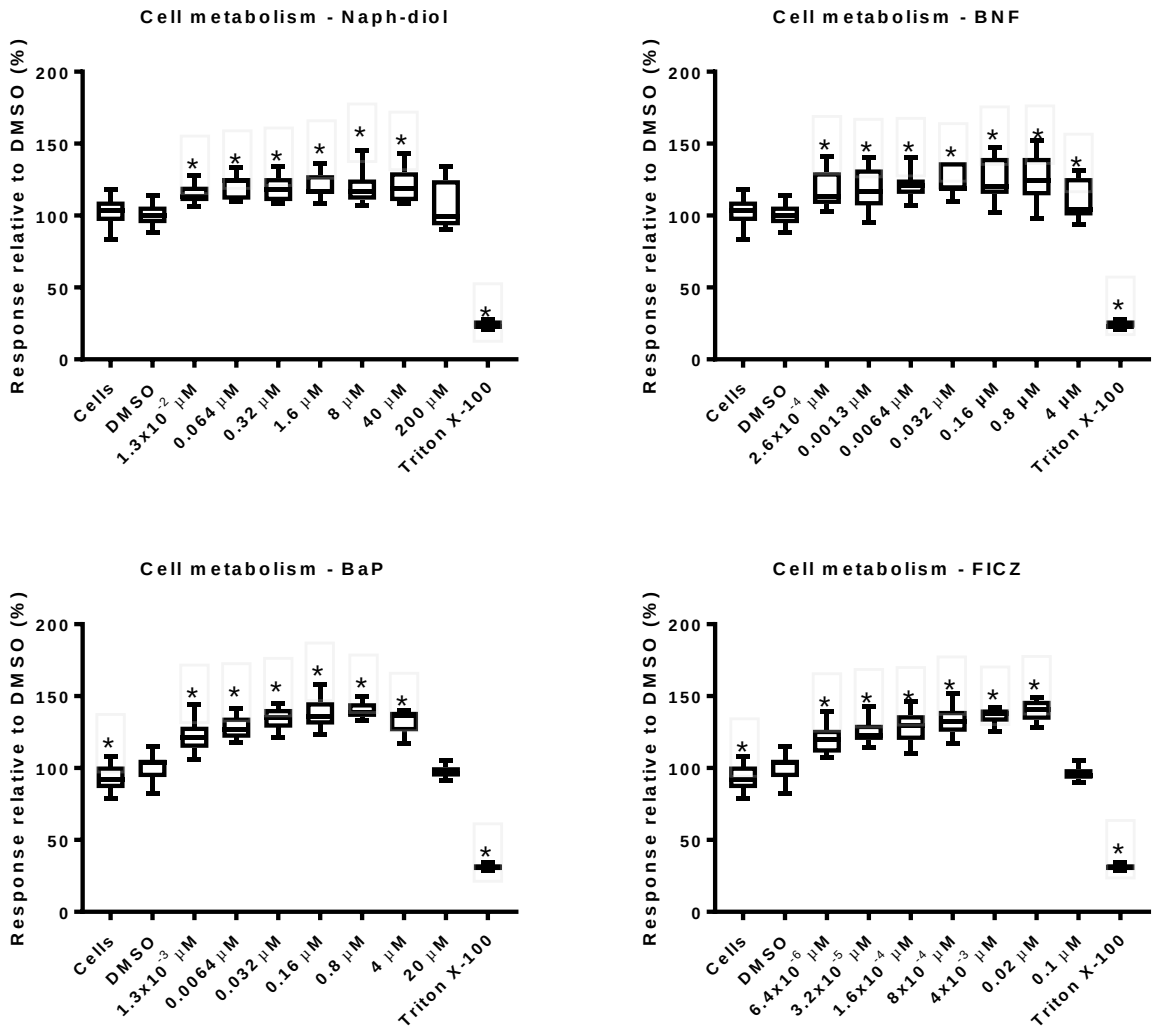

Cell metabolism - 1-Met
Response relative to DMSO (%)
0
50
100
150
200
Cells
DMSO
3.2x10 -3 µM
0.016 µM
0.08 µM
0.4 µM
2 µM
10 µM
50 µM
Triton X-100


Cell metabolism - 2-Met
Response relative to DMSO (%)
0
50
100
150
200
Cells
DMSO
1.3x10 -2 µM
0.064 µM
0.32 µM
1.6 µM
8 µM
40 µM
200 µM
Triton X-100


Cell metabolism - 3-Met
Response relative to DMSO (%)
0
50
100
150
200
Cells
DMSO
1.3x10 -2 µM
0.064 µM
0.32 µM
1.6 µM
8 µM
40 µM
200 µM
Triton X-100


Cell metabolism - 6-Met
Response relative to DMSO (%)
0
50
100
150
200
Cells
DMSO
6.4x10 -3 µM
0.032 µM
0.16 µM
0.8 µM
4 µM
20 µM
100 µM
Triton X-100

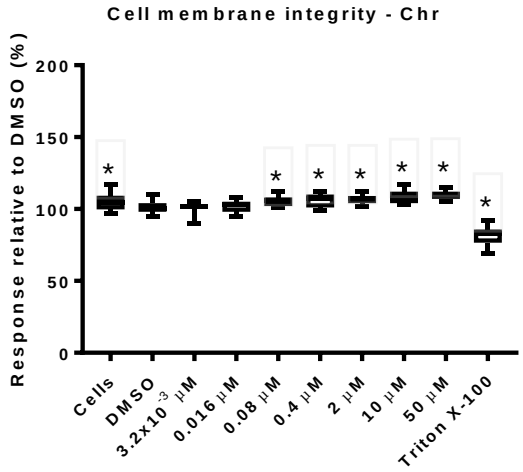

Cell metabolism - Phen-diol
Response relative to DMSO (%)
0
50
100
150
200
Cells
DMSO
3.2x10 -3 µM
0.016 µM
0.08 µM
0.4 µM
2 µM
10 µM
50 µM
Triton X-100

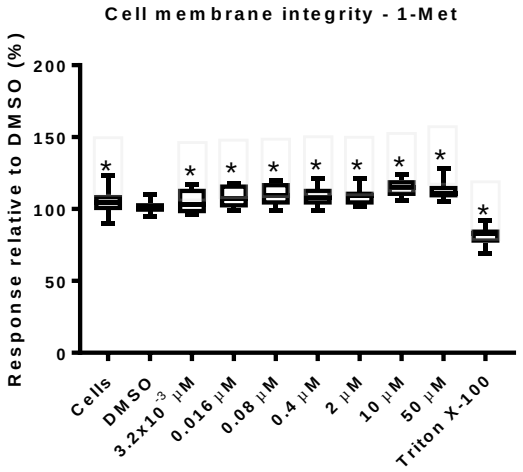


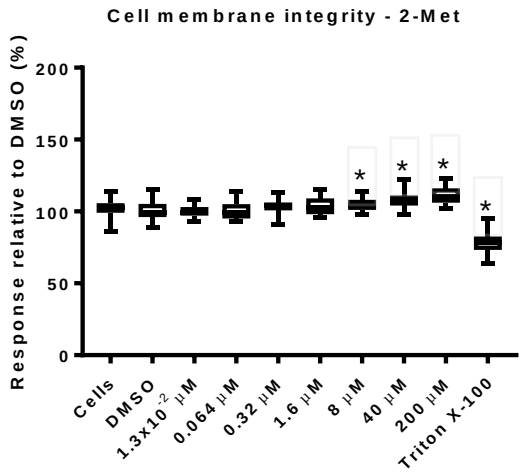
Figur 12: Cytotoxicity measurement of metabolic activity (resazurin) for COS-7 cells exposed for 24 hours to different PAHs and AhR model-agonists. The same concentrations as tested in the ligand activation assay were used and are indicated in the figures. Each box shows the average of at least 4 replicates from 3 different experiments (n=12-24) with standard deviations and mean. The responses are shown (in %) as relative to the response for cells exposed to DMSO (solvent control). "Cells" (x-axis) represents cells that were only grown in medium (no exposure). Triton X-100 (0.1%) was used as a positive control for cytotoxicity. *indicates statistical significance ($p<0.05$, ttest) when compared to the DMSO solvent control.

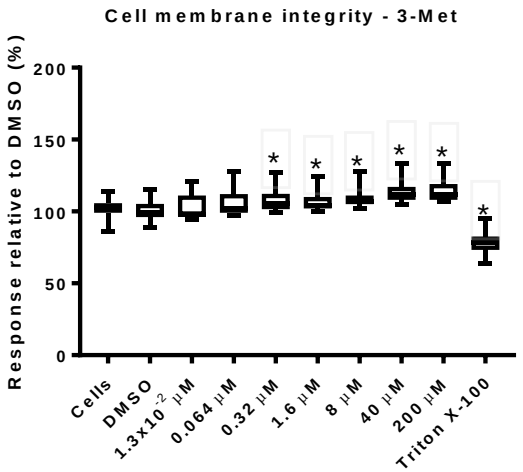
4.2.2 CDFA-AM assay for assessing cell membrane integrity

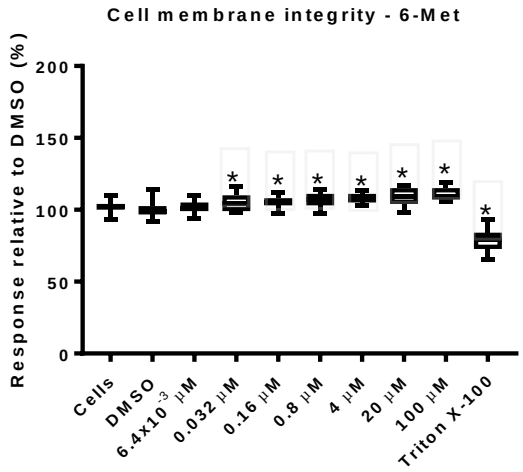
Similar results as for the resazurin metabolic activity assay were observed also for the CDFA-AM cell membrane integrity assay (Figure 13). All compounds gave similar responses, and none seemed to cause a significant negative effect on the COS-7 cell membranes. Triton X-100 exposure resulted in significant decrease (below 100%) in COS-7 cell integrity and enzymatic activity, compared to the cells only exposed to DMSO. Most PAHs in the concentrations tested gave results slightly higher than 100%, and from this it appeared none of the compounds in any concentrations used in the assay had a toxic effect on the COS-7 cell membranes.

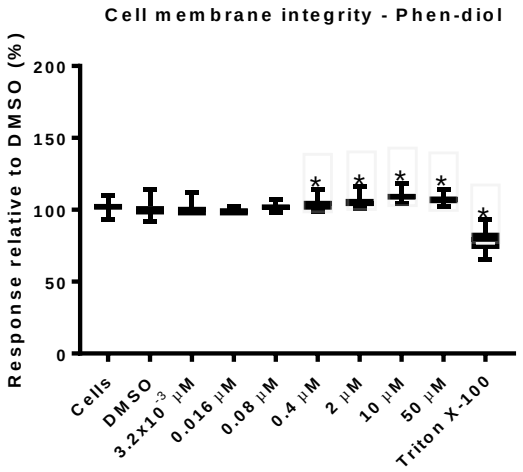

Cell membrane integrity - Chr
Response relative to DMSO (%)
200
150
100
50
0
Cells
DMSO
3.2x10 -3 µM
0.016 µM
0.08 µM
0.4 µM
2 µM
10 µM
50 µM
Triton X-100

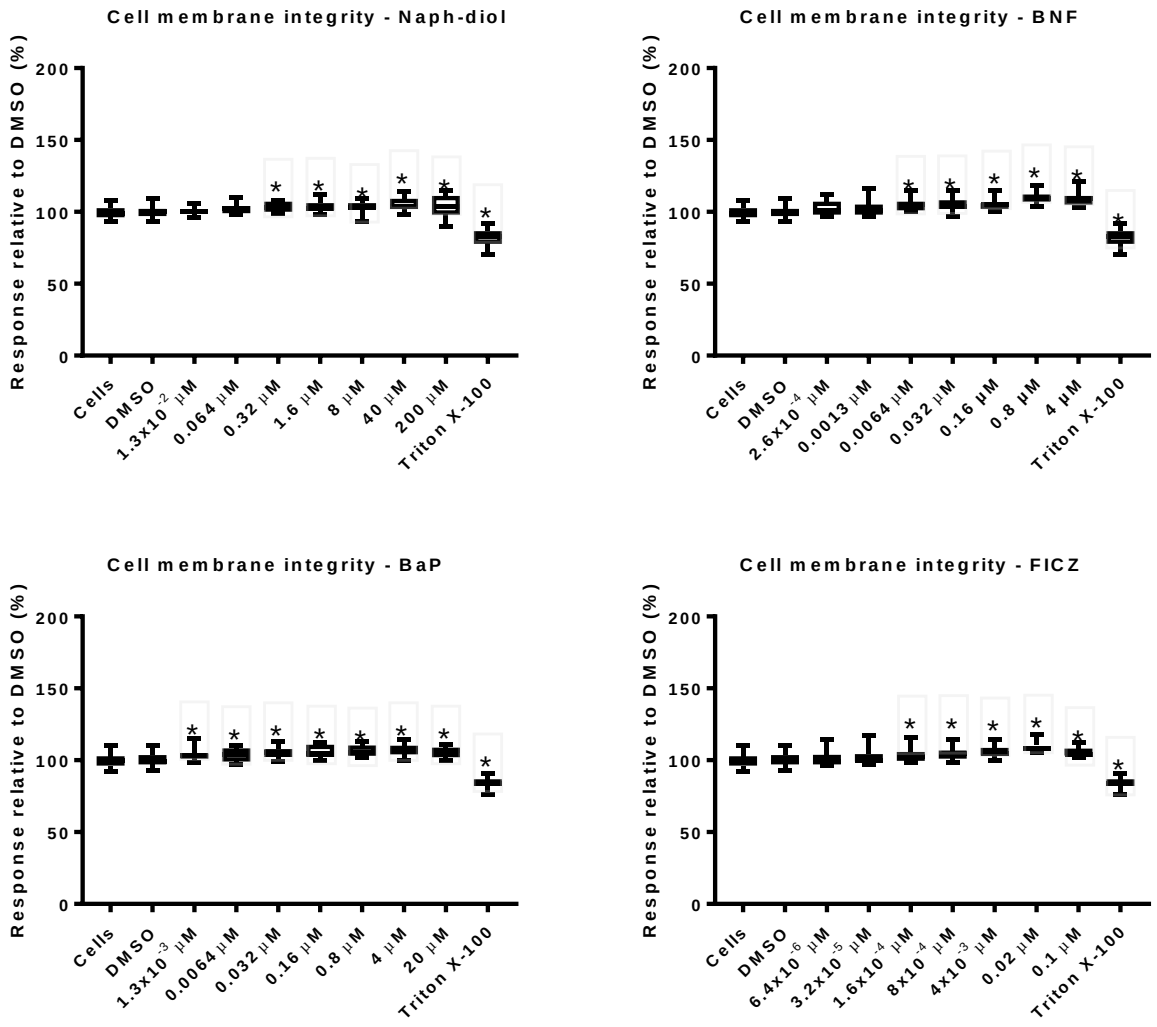

Cell membrane integrity - 1-Met
Response relative to DMSO (%)
200
150
100
50
0
Cells
DMSO
3.2x10 -3 µM
0.016 µM
0.08 µM
0.4 µM
2 µM
10 µM
50 µM
Triton X-100


Cell membrane integrity - 2-Met
Response relative to DMSO (%)
200
150
100
50
0
Cells
DMSO
1.3x10 -2 µM
0.064 µM
0.32 µM
1.6 µM
8 µM
40 µM
200 µM
Triton X-100


Cell membrane integrity - 3-Met
Response relative to DMSO (%)
200
150
100
50
0
Cells
DMSO
1.3x10 -2 µM
0.064 µM
0.32 µM
1.6 µM
8 µM
40 µM
200 µM
Triton X-100


Cell membrane integrity - 6-Met
Response relative to DMSO (%)
200
150
100
50
0
Cells
DMSO
6.4x10 -3 µM
0.032 µM
0.16 µM
0.8 µM
4 µM
20 µM
100 µM
Triton X-100


Cell membrane integrity - Phen-diol
Response relative to DMSO (%)
200
150
100
50
0
Cells
DMSO
3.2x10 -3 µM
0.016 µM
0.08 µM
0.4 µM
2 µM
10 µM
50 µM
Triton X-100



Figur 13: Cell membrane integrity cytotoxicity test (CDFA-AM) for COS-7 cells exposed for 24 hours to different PAHs and AhR model-agonists. The same concentrations as tested in the luciferase ligand activation assay were used and are indicated in the figures. Each box shows the average of at least 4 replicates from 3 different experiments (n=12-24) with standard deviations and mean. The responses are shown (in %) as relative to the response for cells exposed to DMSO (solvent control). "Cells" (x-axis) represents cells that were only grown in medium (no exposure). Triton X-100 (0.1%) was used as a positive control for cytotoxicity. *indicates statistical significant difference ($p<0.05$, ttest) when compared to the solvent control (DMSO).

4.3 Ligand activation of Atlantic cod AhR2

4.3.1 Ratio between receptor and reporter plasmid

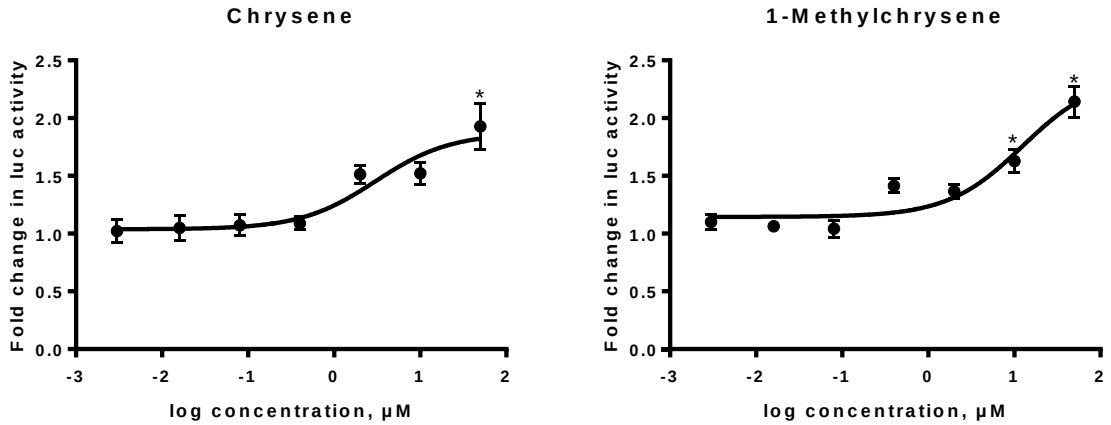
In order to examine activation of cod AhR2 by the selected PAHs, COS-7 cells were transfected as described in 3.5.3 and exposed to increasing concentrations of the different compounds. In this experiment, seven different PAHs were examined (Chr, 1-Met, 2-Met, 3-Met, 6-Met, Phen-diol and Naph-diol). The PAH compound, BaP and two other well-known

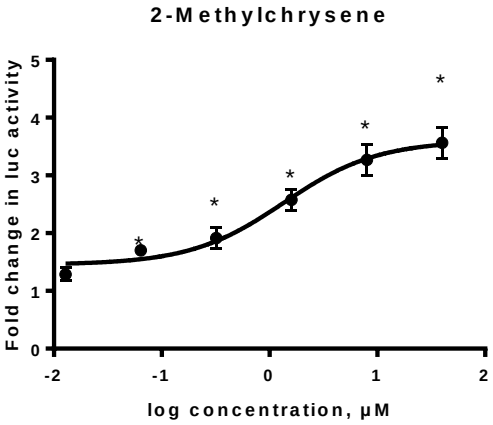
cod AhR2 agonists (BNF and FICZ) were used as positive controls for the assay. The optimal ratio between receptor and reporter plasmid was chosen based on previous work in the Environmental Toxicology laboratory (Madsen, 2016), and from this decided to be 1:20 (receptor:reporter).

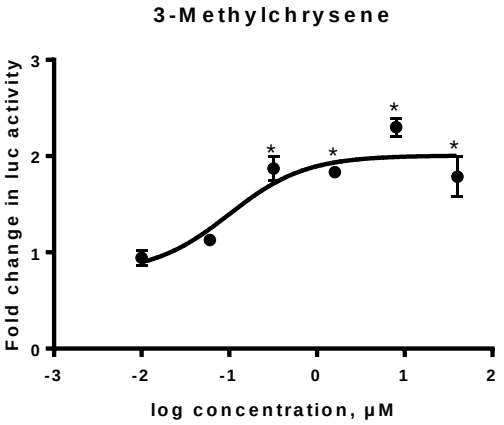
4.3.2 Ligand activation results

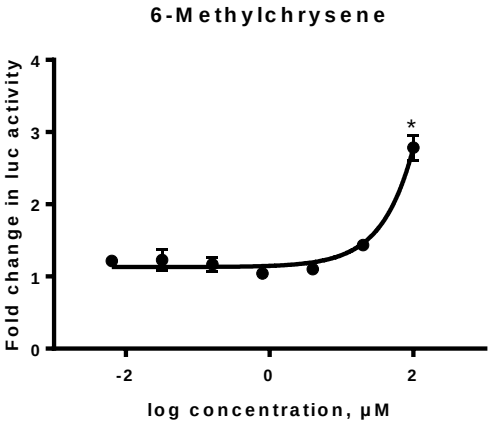
COS-7 cells transfected with pCMX-GAL4-cod AhR2 were exposed to 8 different PAHs and two AhR model compounds (BNF and FICZ) in increasing concentrations for 24 hours. All of the test compounds showed the ability to activate the cod AhR2 (Figure 14). Exposure to 1-Met, 6-Met, Phen-diol, Naph-diol and BaP, showed an increase in fold change luciferase activity with increasing concentrations compared to the DMSO control, but for these compounds a plateau of activation was not reached. When exposed to Chr, 2-Met, 3-Met, BNF and FICZ, a similar gradual increase in fold change luciferase activity was observed with increasing concentrations, but a certain plateau of maximal fold change activation was achieved.

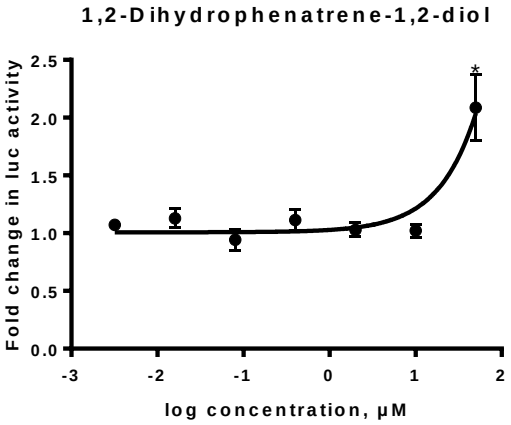
Maximum fold activation, highest minimum significant activation at the lowest concentrations, as well as the half maximum effective concentration (EC50) for each compound is summarized in Table 26. Activations with a fold change equal to, or higher than 1.5 fold compared to DMSO control and with a $p<0.01$ (ttest) are considered statistically significant.

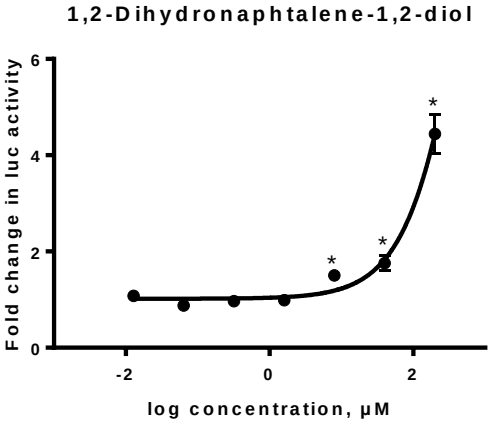


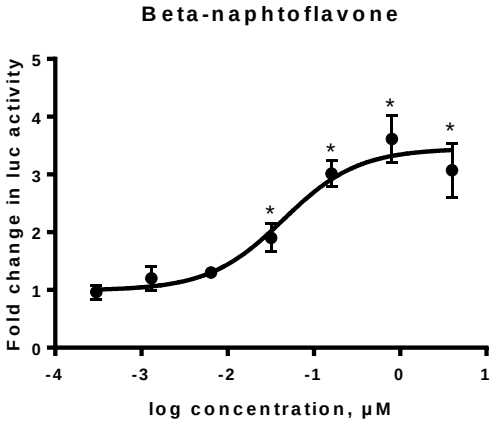

2-Methylchrysene
Fold change in luc activity
log concentration, µM


3-Methylchrysene
Fold change in luc activity
log concentration, µM


6-Methylchrysene
Fold change in luc activity
log concentration, µM


1,2-Dihydrophenatrene-1,2-diol
Fold change in luc activity
log concentration, µM


1,2-Dihydronaphtalene-1,2-diol
Fold change in luc activity
log concentration, µM


Beta-naphtoflavone
Fold change in luc activity
log concentration, µM

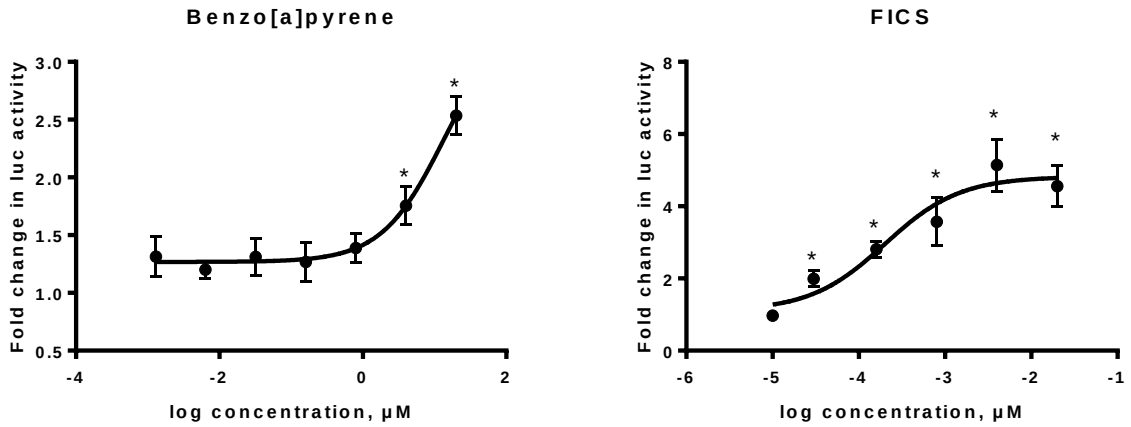


Figure 14: Luciferase reporter gene assay with cod Ahr2. The figure shows COS-7 cells transfected with pCMX-GAL4-cod Ahr2, and exposed to 8 different PAHs and two AhR model compounds (FICZ and BNF) in increasing concentrations for 24 hours. A five-fold dilution series of each ligand was used. Each point (concentration) shows the average of three replicates from three individual experiments (n=9). Activation of cod Ahr2 is shown as fold change in luciferase activity between cells exposed to the PAH, and cells exposed only to DMSO (solvent control). A dose-response curve is drawn based on a log(agonist) vs. response (three parameters) regression model in PRISM (GraphPad). The bars over/under points indicate the standard error of mean (SEM), and * indicates statistical significance ($p<0.01$, ttest) for points ≥1.5 fold activation compared to the DMSO solvent control.

Table 26: Results of maximum fold change in luciferase activity, and minimum significant activation (P<0.01, ttest) of fold changes ≥1.5. The well-known AhR model agonists BNF, BaP and FICZ are highlighted in yellow. Maximum fold activations and minimum significant activation at the lowest concentration are shown. EC50 is stated for the compounds reaching a plateau in activation of AhR2.

Substance	Activation (fold)	Concentration (µM)	EC50 (µM)	T-test (P-value)
Maximum activation:				
Chrysene (Chr)	1.9	50.0	25.0	<0.01
1-Methylchrysene (1-Met)	2.1	50.0	-	<0.01
2-Methylchrysene (2-Met)	3.6	40.0	20.0	<0.01
3-Methylchrysene (3-Met)	2.3	8.0	4.0	<0.01
6-Methylchrysene (6-Met)	2.8	100.0	-	<0.01
(1R,2R)-1,2-Dihydrophenatrene-1,2-diol (Phen-diol)	2.1	50.0	-	<0.01
(1R,2R)-1,2-Dihydronaphtalene-1,2-diol (Naph-diol)	4.4	200.0	-	<0.01
Beta-naphtoflavone (BNF)	3.6	0.8	0.4	<0.01
Benzo-a-pyrene (BaP)	2.5	20.0	-	<0.01
6-formylindolo(3,2-b)carbazol (FICZ)	5.1	0.004	0.002	<0.01
Minimum significant activation (p<0.01) **(≥1.5)**				
Chrysene (Chr)	1.9	50.0		<0.01
1-Methylchrysene (1-Met)	1.6	10.0		<0.01
2-Methylchrysene (2-Met)	1.7	0.064		<0.01
3-Methylchrysene (3-Met)	1.9	0.32		<0.01
6-Methylchrysene (6-Met)	2.8	100.0		<0.01
(1R,2R)-1,2-Dihydrophenatrene-1,2-diol (Phen-diol)	2.1	50.0		<0.01
(1R,2R)-1,2-Dihydronaphtalene-1,2-diol (Naph-diol)	1.5	8.0		<0.01
Beta-naphtoflavone (BNF)	1.9	0.03		<0.01
Benzo-a-pyrene (BaP)	1.8	4.0		<0.01
6-formylindolo(3,2-b)carbazol (FICZ)	2.0	$3.2 \cdot 10^{-5}$		<0.01

5 Discussion

In this thesis a selection of PAHs were used to study whether or not they have the ability to activate cod AhR2 *in vitro*, using a luciferase GAL4/UAS-based reporter gene system. The compounds that were tested were a selection of known agonists, including FICZ, BaP and BNF, and two-to-four-ring PAHs that not previously have been used with this ligand activation assay. These include chrysene and alkylated versions of chrysene, as well as two hydroxylated versions of naphthalene and phenanthrene. All of the substances were able to activate cod AhR.

FICZ gave the highest fold change (5.1) in luciferase activity relative to DMSO at 0.004 µM, and also had the minimum significant activation at the lowest concentration of the test compounds (32 pM) (Table 26). Out of the 7 PAH compounds tested (Chr, 1-Met, 2-Met, 3-Met, 6-Met, Phen-diol and Naph-diol), Naph-diol gave the highest fold change of 4.4 at a concentration of 200 µM. However, 2-Met gave a fold change of 3.6 at a much lower concentration (40 µM), thus appearing to be a more potent agonist. 2-Met also gave a minimum significant activation of 1.7 at the lowest concentration (64 nM) of all the PAH compounds that were tested (Table 26). 6-Met had the highest minimum significant activation of all the test compounds at 2.8 µM, however, this was also the concentration for its' maximal activation. Due to DMSO toxicity during exposure of COS-7 cells, this was the highest concentration possible for 6-Met exposures in this thesis. The same pattern was also observed for Phen-diol. EC50 is stated for the compounds reaching a plateau in the dose-response curve, indicating half of the maximal effective concentration for the compound. Since all of the compounds did not reach a plateau in fold activation, it is not possible to compare the EC50 values for the test compounds (Table 26).

5.1 Known agonists to AhR2

BNF, BaP and FICZ have earlier shown ability to activate cod AhR2 (Madsen, 2016, Endresen 2016), and were therefore included in this thesis as positive controls for the ligand activation. Results of these compounds will be compared mainly to these two master theses, as the same reporter gene assay was used. These compounds are known to activate AhR 2 in other teleosts like Killifish (BNF) and zebrafish (BaP and FICZ) (Eide et al. 2014; Karchner et al. 2002; Incardona et al. 2011; Jönsson et al. 2009).

The dose-response curves for these known agonists, showed similar responses as presented in these two earlier master theses, where BaP's dose response curve showed no tendency of reaching a plateau, as observed for the BNF and FICZ curves. The tryptophan-derivative FICZ activates at very low concentrations (32 pM), the lowest of all the ligands used in this assay, and it has previously been proposed to be a possible endogenous ligand of AhR2 (Denison and Nagy, 2003). FICZ also activates AhR from zebrafish (zfAhR1b and zfAhR2) and salmon at very low concentrations (Jönsson et al. 2009; Madsen, 2016; Bemanian, 2003). Compared to earlier experiments with the cod AhR2, The maximum fold activation of FICZ

was reached at a lower concentration (0.004 µM) compared to the two other master theses (0.02 µM). However, the relative standard deviation (RSD) for this value is relatively high, so it is likely that this observation is due to variations in the luciferase assay.

BaP results showed slightly lower fold activations than the two other master theses, and also did not give the highest fold activation in this thesis. Maximum fold activation (2.5) was much lower than observed in the other two theses (4.9 and 18.2). Since the curve showed no sign of leveling out however, it is possible a higher level would induce higher fold activation, but the toxic DMSO levels at higher concentration would be an issue.

BNF results in this thesis also showed lower maximum fold activations at higher concentrations than for the other two master theses. All in all, the results of these AhR model-agonists were similar to earlier results, perhaps with a slightly lower sensitivity for the luciferase reporter gene assay in this thesis.

5.2 PAHs as potential agonists to AhR2

5.2.1 Chrysene and its alkylated forms

PAHs and their alkylated forms are frequently detected in environment contaminated with spilled oil, due to being common constituents of crude oil (Pampanin and Sydnes, 2013). When released into the environment, the crude oil undergoes a series of weathering processes, encompassing a variety of physical and biochemical alterations, such as evaporation, photo-oxidation, solubilization, alkylation and microbial degradation (Neff et al., 2009). In addition to be potent mutagens, carcinogens or tetragens (Collins et al. 1998; Hong et al. 2012; Machata et al. 2001a), numerous of studies have also shown PAHs could induce dioxin-like responses via activation of the AhR (Eichbaum et al. 2014; Horii et al. 2009; Villeneuve et al. 2002). Several PAHs, such as benzo(a)pyrene and chrysene, have been reported as strong AhR agonists. In sediments contaminated with crude oil, it has been reported that the composition of alkylated PAHs is altered during weathering, and at the same time, influencing the potency of AhR-mediated effects (Hong et al., 2012). Relatively little is known about the toxicities of alkylated PAHs, compared to PCDDs, PCDFs, PCBs and other PAHs and therefore more studies on toxicity characteristics of PAHs and their alkylated forms are important for better understanding the impacts of oil contamination in the environment.

The dioxin-like compounds' (including PAHs) interaction with the AhR can influence expression of many genes, thereby inducing diverse biological or toxic effects in a wide range of species and tissues (Ma, 2001; Motto et al. 2011). These inter-species differences in sensitivities can be explained and predicted from the binding affinity of dioxin-like ligands to the AhR (Doering et al. 2014; Herv et al. 2010; Prokipcak et al. 1990). Ligands relative potencies to activate the AhR are directly proportional to their binding affinity to the AhR (Kramer & Giesy, 1999), so the greater occupancy proportion on the receptor, the greater

probability of the receptor-ligand complex interacting with the dioxin response element (DRE) on DNA (Farmahin et al. 2013; Lee et al. 2013; Larsson et al. 2014).

Earlier studies showed that binding potencies of a given PAH were significantly altered by alkylation, but that the difference in binding affinity could not be simply explained by number of alkylations. Greater relative potency for AhR activation among PAHs has been observed with chrysenes, especially 1-methylchrysene. (Lee et al. 2015). This seems to correspond to the results shown in this thesis, where all of the alkylated chrysenes (1-, 2-, 3-, and 6-methylchrysene) resulted in higher maximum fold activation (2.1, 3.6, 2.3 and 2.8, respectively) than chrysene itself (1.9). Of these, 2-methylchrysene had the highest maximum fold activation (3.6). The minimum significant fold activation results were more similar to each other. However, the concentrations of the PAHs needed for significant minimum activation varied greatly, and 2-Met came out the most potent here too at the lowest concentration. It seems the position of the methyl group may have an impact for the compounds potency and maximal activation of the AhR. Differences in relative potency for AhR activation among chrysene and its alkylated forms could be explained by differences among binding distances in the ligand binding domain of the AhR caused by alkylation (Lee et al. 2015).

5.2.2 *Trans*-dihydrodiols

In fish, PAHs accumulate in fatty tissue, like the liver, and are metabolized there. Metabolism occurs within hepatocytes where PAHs are oxidized, making them more water-soluble and potentially more reactive (Varanasi et al. 1989, Aas et al. 2000). The major PAH oxidation products formed and excreted to bile in fish are the *trans*-dihydrodiols (Pangrekar et al. 2003). For the Atlantic cod the chrysene diol (1R,2R)-1,2-dihydrochrysene-1,2-diol accounts for up to 88% of chrysene metabolites found in bile. The naphthalene-diol (1R,2R)-1,2-dihydronaphthalene-1,2-diol makes up the majority of the naphtalene metabolites present in bile (Pampanin et al. 2016). Naph-diol resulted in the highest fold activation (4.4) of all the test compounds, but at 200 µM it also had the highest concentrations used in this test and appear not to be a very potent AhR agonist.

Phenanthrene has earlier proven to induce CYP1A in marine medaka- (*O. melastigma*) and zebrafish embryo, but not in cod liver (Goksøyr et al. 1986; Incardona et al. 2005; Mu et al. 2012). Even though inducing CYP1A mainly happens via AhR, toxic effects related to phenanthrene are AhR independent (Incardona et al. 2005). Phenanthrene induces CYP1A in many fish species, but no activation was observed in either cod AhR2 or salmon AhR2β (Madsen, 2016). The phenanthrene-diol, (1R,2R)-1,2-dihydrophenatrene-1,2-diol (Phen-diol), proved to be more potent than its parent compound in this thesis. However, Phen-diol only gave one significant fold activation value at the highest concentration used (50 µM). With the curve showing no signs of leveling out, higher concentrations could possibly result in higher fold activations for this compound. Toxic DMSO levels restricted any higher concentrations in this thesis.

5.3 PAHs in fish from the North Sea

The Norwegian Water Column Monitoring programme (WCM) performs investigations into the potential biological effects of offshore oil and gas activity on organisms living within the water column of the Norwegian continental shelf. Caged mussels are monitored on a yearly basis at specific locations, and fish every three year. The methods used for monitoring the effects are still under development (Miljødirektoratet). All of the oil companies in the Norwegian sector with produced water discharges are obliged to perform biological effects monitoring offshore and this work has been performed for the last 20 years.

Since fish are able to metabolize PAHs from their tissue, the metabolized product is excreted from the cell and ends up in the bile of fish. Bile metabolites are therefore preferred matrix for measuring PAH exposure. This measurement however, only represents exposure over the last few days (2-8) and 2 weeks at the most, making it hard to establish a dose-response relationship of PAH in field monitoring studies of the marine environment. (Brooks et al. 2015). Mean levels of summarized PAH metabolites found in cod liver from the Norwegian Sea were generally low at Tampen (37±20 ng/g bile) and at the Egersund Bank (81±75 ng/g bile) in 2011. Summarized PAH metabolites found in haddock bile from the Norwegian Sea and Barents Sea in 2014 were also low (85-184 ng/g bile and 96-113 ng/g, respectively) (Grøsvik et al. Havforskningsinstituttet). Although the total concentrations of PAHs found in fish bile in the North Sea probably are lower than the minimal concentrations needed for significant activation for any PAH in the *in vitro* method in this thesis, the fish bile concentrations are fleeting, and may have been higher at a recent time, before sampling. Also it is possible that *in vivo* activation of the AhR may be induced by lower concentrations than used in this thesis.

5.4 The luciferase gene reporter assay used

Several different types of gene reporter assays are used to study the ligand activation of AhR2, where the CALUX method is widespread (Murk et al. 1996). The UAS/GAL4 luciferase gene reporter assay used in this thesis is another approach. These types of reporter gene assays have proven to be efficient and sensitive methods for mapping possible AhR ligands (Takeuchi et al. 2008; Nagy et al. 2002).

The sensitivity for the UAS/GAL4 luciferase gene reporter assay appeared to be lower in this study, compared to the two earlier theses by Madsen and Endresen. Most compounds responded with lower fold activations at similar concentrations and some at higher concentrations even. Also maximum fold activations were slightly lower.

Standard deviations were relatively high for some of the results. Measured values varied greatly between replicates in the same experiment, as well as between different experiments. This might be explained by the fact that each experiment lasted for 4 days, and the time from

the first results, until the last results therefore took weeks to produce. This might make the system vulnerable for methodological differences.

Since this is an *in vitro* method, the ligand activation of cod AhR2 is not conducted in its physiological surroundings, and choosing cell lines with different species- and tissue differences can affect optimal function and activation response. Ligand activation of cod AhR may be affected by the incubation temperature at 37°C, and may have an optimal temperature lower than this. The GAL4-DBD domain, fused to the codAhR2 may itself affect the conformation of AhR and its ligand binding capacity. Since the UAS/GAL-system is independent of the ARNT, a lack of interaction between the two PAS-domains, may also affect the results (Backlund & Ingelman-Sundberg, 2004).

6 Conclusion

In this thesis, ligand activation of cod AhR2 has been studied by exposure to both well-known AhR ligands, and selected PAHs from EPAs priority pollutants list. Known AhR ligands resulted in a dose-response relationship as seen in earlier studies, functioning as positive controls for this UAS/GAL4 luciferase reporter gene assay. Importantly, all of the PAHs included in this study were able to activate AhR. For some of the compounds, a plateau of fold activation was reached, whereas others did not reach maximum fold activation. The binding potencies of the PAHs were higher in alkylated or oxidized PAH compounds, compared to their unsubstituted variant, resulting in greater relative potency for AhR activation. Given all the PAHs ability to activate cod AhR2 in this study, these compounds may have the potential to cause toxic effects on cod in its natural environment, especially the alkylated and oxidized PAHs.

6.1 The way from here

There is always room for improvement and optimization of the UAS/GAL4 system when it comes to increased sensitivity and activation response, intentionally leading to improvement in studies of weak agonists and very low exposure concentrations (He et al. 2011). Also, increased knowledge about the differences in AhR-activation profiles between species can be achieved by reporter gene systems with AhR orthologs from different species.

Fish liver is one of the main targets of pollutants due to its high metabolic activity related to the transformation and detoxification of contaminants (Lang et al. 2006). It is the main site of detoxification in cod and expresses many components of the biotransformation system, such as nuclear receptors (xenobiotic sensors) and cytochrome P_{450} (CYP) enzymes. Many environmental compounds tend to accumulate in the liver of vertebrates. Precision-cut liver slices have largely been used in pharmacological and toxicological studies. This method constitutes a valuable tool for overcoming the limitations of isolated cell systems, such as accelerated dedifferentiation (Groneberg, 2002; Vermeir 2005). For several days they maintain tissue integrity (cell-cell and cell-matrix interactions) and viable cell populations in culture, thus allowing evaluation of a global tissue response (Van de Bovenkamp et al. 2007). Several studies using microarrays confirmed that PCLS more closely predicted *in vivo* toxicity than isolated hepatocytes or established cell lines (Boess et al. 2003; Elferink et al. 2008). PCLS therefore provides a good alternative to an *in vitro* system for toxicological studies of the Atlantic cod liver.

Different *in vitro* methods provide mechanism-based analysis of a biological process at molecular levels. Often they can be performed relatively quickly and in a large scale, allowing rapid analysis of a large number of chemicals. However, *in vivo* studies are important to study intact animals' response to chemicals. These are often time-consuming, expensive, limited in the number of chemicals to be analyzed, and difficult in detailed mechanistic analysis. The metabolism by CYP1A is often dependent upon organ, tissue, route and time of administration, age, gender, coadministered drugs or agents, and certain pathophysiological

conditions (Ma & Lu, 2007). *In vitro* studies of cod AhR is likely influenced by temperature, the type of cells used for transfection, and a range of other physiological factors in the intricate biological system of an intact animal. Extrapolation of results from animal studies of CYP1A inductions to humans is a complex process that requires, ultimately, the direct proof from human studies (Ma & Lu, 2007).

7 References

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