In vitro modulation of transcriptional activity of nuclear receptors in blue whale (*Balaenoptera musculus*) and fin whale (*Balaenoptera physalus*) by environmental contaminants

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I Abbreviation list

2,3,7,8-TCDD	2,3,7,8-tetraklorodibenzo-p-dioksin
A230/260/280	Absorbance with 230/260/280 nm
AA	Amino acid
ABC	ATP-binding cassette
AGE	Agarose gel-electrophorese
AhR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor translocator
ATP	Adenosine triphosphate
BaP	Benzo(a)pyrene
bHLH	Basic helix loop helix
bp	Base pairs
BFR	Brominated flame retardants
bw	Blue whale
CALUX	Chemical activated luciferase gene expression
cDNA	Complementary DNA
CFDA-AM	5-carboxyfluorescein diacetate acetoxymethyl ester
CYP	Cytokrome P450
DBD	DNA binding domain
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di(2-ethylhexyl) phthalate
Dexa	Dexamethasone
DiDP	Diisodecyl phthalate
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotides
DRE	Dioxin response element
EC50	Effective concentration 50
ED	Endocrine disruptors

EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
FICZ	6-formylindolo[3,2-b]carbazole
Fwd	Forward
GST	Glutathione-S-transferase
GR	Glucocorticoid receptor
HAH	Halogenated aromatic substances
L	DNA-Ladder
LB	Lysogeny broth
LBD	Ligand binding domain
LRA	Luciferase reporter gene assay
MSA	Multiple sequence alignment
NAD(H)P	Quinone oxidoreductase 1
NRs	Nuclear receptors
ONPG	Ortho nitrophenyl-β-galactoside
OPFR	Organophosphorus flame retardants
PAS	Per-ARNT-Sim
PBS	Phosphate-buffered saline
PCB 153	2,2',4,4',5,5'-Hexachlorobiphenyl
PCBs	Polychlorinated biphenyls
PCF	Polychlorinated varieties of dibenzofurans
PCDD	Dibenzo-p-dioxins
PCR	Polymerase chain reactions
pDNA	Plasmid DNA
PFAS	Per fluorinated substances
PMSF	Phenylmethylsulfonyl fluoride
POPs	Persistent organic pollutants
PPAR	Peroxisome proliferated activated receptor
PXR	Promiscuous xenobiotic receptor (aka pregnane X receptor)
rDNA	Recombinant DNA
Rev	Reverse
RNA	Ribonucleic acid
Rosi	Rosiglitazone

RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RTMIX	Reverse Transcriptase reaction solution
RXR	Retinoid X receptor
S	Strand
SOC	Super optimal broth with catabolite repression
T3	3,5,3´-triiodothyronine
TBE	Tris-borate-EDTA
TCP	2,4,5-trichlorophenol
THR	Thyroid hormone receptor
totRNA	Total Ribonucleic Acid
UAS	Upstream activation sequence
UGT	UDT-glucuronosyltransferase
XRE	Xenobiotic response element
β-Gal	β-Galactosidase

II Acknowledgements

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Writing a master thesis is truly an extraordinary experience, with your plans almost never working out, and you have no idea of what you are doing half of the time, it is an adventure I'll remember forever.

"If we knew what it was we were doing, it would not be called research, would it?"
— Albert Einstein

Karoline A. Viberg
Bergen, August 2019

III Abstract

Baleen whales, such as blue whales (*Balaenoptera musculus)* and fin whales (*B. physalus*), two giants of the ocean are potentially being impacted by multiple stressors, including exposure to pollutants. They are exposed to a large variety of contaminants such as persistent organic pollutants (POPs). Both of these species are listed in threatened categories in the IUCN Red List of Threatened Species. Despite their conservation status, there is little information available regarding concentrations and potential adverse effects of persistent organic pollutant (POP). One-way POPs can affect these animals is through their endocrine system and their transcription factors. The health of marine mammals such as whales is generally dependent on a normal functioning immune system, endocrine system and energy metabolism. These processes are regulated by transcription factors such as nuclear receptors: e.g. glucocorticoid receptor (GR), thyroid hormone receptor beta (THRB) and the peroxisome proliferator-activated receptor gamma (PPARG). Another transcriptional mediator of xenobiotic effects is the Aryl hydrocarbon receptor (AhR). To study the transcriptional activity of blue and fin whale, GR, THRB, PPARG, and AhR when exposed to legacy POPs, *in vitro* luciferase reporter gene assays were performed. AhR was cloned and sequenced, and a luciferase gene reporter assay was attemptably established without success so far. However, a multiple sequence alignment of blue whale AhR with several other marine mammals, including human and mice showed a high level of identity between the species, indicating that the receptor would respond in a similar way across species. The three nuclear receptors previously cloned were successfully studied in luciferase gene reporter assays, where the results indicated no agonistic effects of many of the tested toxic compounds (pp`DDT, pp`DDE, pp`DDD, DEHP, DINP and POPs mixture) that are abundant in whale blubber. Additionally, multiple sequence alignments showed strong conservation of the ligand binding domain of these receptors between blue and fin whales (identical), killer whales, white whales, polar bears and humans. This Suggests a similar activation of the receptors in the different species.

Additional studies would be advantageous to understand if the POPs detected in blue and fin whale blubber have antagonistic effects, in addition to conduct further agonistic studies of the POPs and contaminants mixtures not yet studied.

1 Introduction

1.1. Our oceans today- affected by environmental contaminants?

We live in a time were living standards and consumption are rapidly increasing. The increased usage of the world´s resources is creating a growing pressure on the planet and its ecosystems. This development is creating several new challenges and environmental issues for our society. One of the fastest growing problems is environmental contaminants. This is a classification of chemical substances that is quickly becoming frequently used in our society, and the awareness around them is increasing. In Europe alone there are over 22500 registered chemicals, and the majority of them can act as environmental contaminants (European Union, 2019; Lampa et al., 2012). Environmental contaminants consist of a varied group of chemicals, where several (e.g. mercury, lead and asbestos) can be toxic even at low concentrations (Lanphear, 2017; Zahir et al., 2005). These contaminants often originate from various human activities, such as incorrect handling of human waste (garbage, fishing equipment etc.) effluent discharges, agriculture, in addition to marine and land industries (Bakke et al., 2013; Völkel, Mosch et al., 2009). These chemical substances may not only affect the area to which they are released, but also on a much wider scale. Ever since Rachel Carson's first book "the sea around us" came out in 1951, and the acid rain in 1970 there has been a growing consciousness on long-range transportation of contaminants (Singh et al., 2008; Carson, 1951). Ocean and air circulation in addition to migrating organisms, rivers, and transpolar ice drift are the major transport routes than make it possible for environmental contaminants originally released by land-based industries, to end up in remote areas such as the Arctic and polar areas (Julshamn et al., 2013; Macdonald et al., 2000; Rigèt et al., 2010). Since the Earth's surface consists of more than 70% water (Gleick, 1993) and the majority of the water masses are marine environments, most of the world's contaminants end up in the ocean. The growing amount of different varieties of environmental contaminant in marine environments, results in increasing negative effects on marine organisms (Cole et al., 2011; Moore, 2008). Contaminants of high concern for marine ecosystems are persistent organic pollutants, oil pollution, emerging contaminants and plastic associated contaminants. Many of these compounds have been shown to biomagnify in the food web and thus end up at high concentrations in top predators. For instance, research conducted on killer whales (*Orcinus orca*) have shown alarmingly high concentrations of the environmental contaminants, persistent organic pollutants (POPs) in particular, making it one of the most contaminated species on earth potentially threatening some populations with extinction due to reproductive failure (Buckman et al., 2011; Desforges et al., 2018; Muñoz-Arnanz et al., 2019).

Marine litter is also of high concern to marine mammals such as whales. There have been several incidents of marine mammals with plastics in this digestive system, or wrapped around them e.g. “The plastic whale” (Cole et al., 2011; Derraik, 2002; Fossi et al., 2017, 2016; Lislevland, 2017; Moore, 2008).

Among cetaceans (odontocetes=toothed whales, and mysticetes=baleen whales) particularly within mysticetes there is little knowledge concerning POPs contamination. Two baleen whales that are possibly affected by environmental contaminants are blue whales (*Balaenoptera musculus*) and fin whales (*Balaenoptera physalus*). Both species are widely distributed throughout the world’s oceans, and primarily due to the massive hunt during the whaling era they are both listed under the International Union for Conservation of Natures (IUCN) Red List of Threatened Species as vulnerable and endangered, respectively (Branch et al., 2007; Carwardine, 2002; Cooke, 2018; Hoyt, 2017; Hsu et al., 2013). As opposed to killer whales, which are toothed whales, blue and fin whales have baleen plates instead of teeth. This allows them to filter huge mouthfuls of water, making them filter feeders. One of the differences between the filter feeding blue and fin whales is their diet. Blue whales feed only on krill and other small crustaceans, while fin whales eat mostly fish (Haug, n.d.-a, n.d.-b; Sars, 1875). Because fin whales feed on a higher trophic level than blue whales, fin whales are expected to be exposed to higher concentrations of biomagnifying pollutants throughout the food chain in similarity with humpback whales (Metcalfe et al., 2004; Pinzone et al., 2015). Several studies have shown that baleen whales contain high levels of POPs, in their blubber, compared to e.g. humpback whales (*Meganoptera novaeangliae*) and southern right whales (*Eubalaena australis*) (Fossi et al., 2014a; Gauthier et al., 1997; Metcalfe et al., 2004; Muñoz-Arnanz et al., 2019; Torres et al., 2015).

1.2 Persistent organic pollutants (POPs)

POPs are a group of chemical substances that are resistant to biochemical and physical degradation. Many POPs are nonpolar molecules and highly lipophilic, which enable them to accumulate in the fatty tissues of organisms (Shen et al., 2005; Macdonald et al., 2000; Rigét et al., 2010). Due to these physio-chemical properties, POPs remain in the environment for a long period of time and therefore have a high probability of bioaccumulating in organisms (Giesy & Kannan, 1998; Rigét et al., 2010; Sonne et al., 2014; Wilson et al., 2016). Bioaccumulation occurs if the organism’s detoxification mechanisms are unable to metabolize

and excrete the compound faster than it is absorbed from the environment, as illustrated in Figure 1 (Heindel et al., 2017). The concentration of POPs increases with higher trophic levels, which leads to a higher concentration in top predators (high trophic levels) than in prey (lower trophic levels), a process known as biomagnification (Martineau et al., 1987; Muir et al., 1992 & 1996; Parkinson & Ogilvie, 2008).

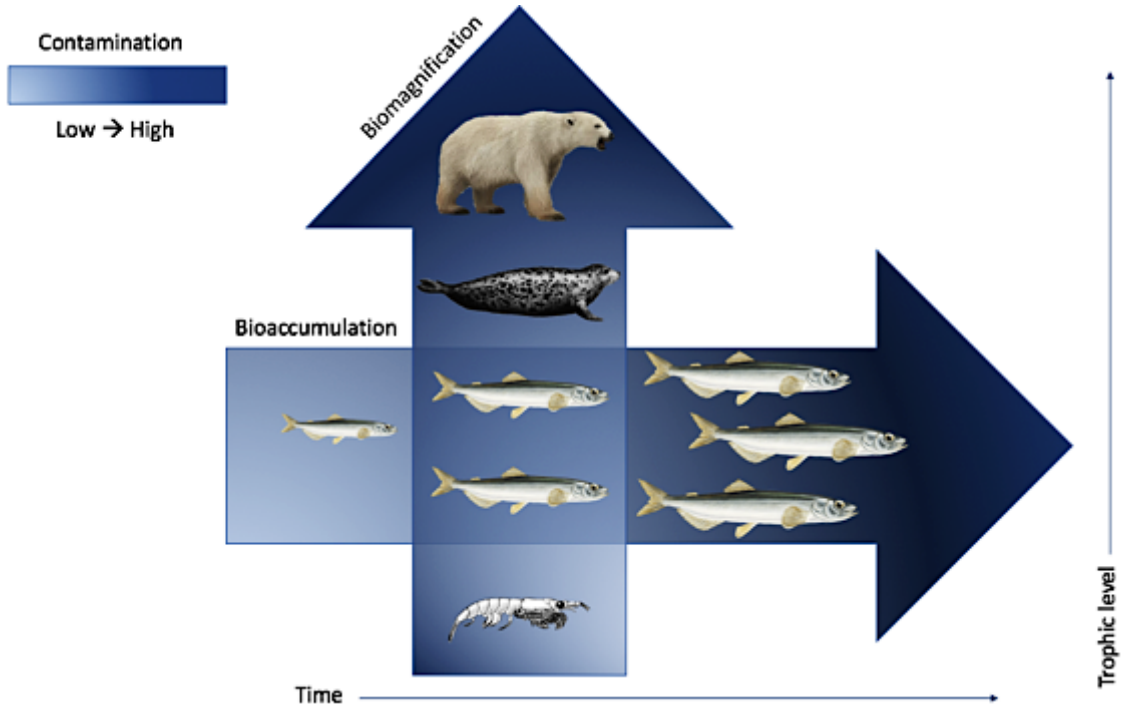


Figure 1. Illustration of biomagnification and bioaccumulation of lipophilic environmental contaminants in marine food webs. The vertical arrow illustrates the increase of lipophilic environmental contaminants in higher trophic levels, this is called biomagnification. The horizontal arrow shows the increase of lipophilic environmental contaminants within an organism over time, a phenomenon termed_bioaccumulation. This leads to animals on top of the food chain and older individuals have higher concentrations of environmental contaminants than young individuals and organisms low in the food chain. Illustration source: modified from Alexander Klevedal Madsen 2016 (Madsen, 2016).

Due to their numerous negative effects on organisms, an increased awareness has led to several management actions to prevent further release of these contaminates into the environment. A result of these actions is the Stockholm Convention, which is a global treaty of strict regulation or elimination of POPs that entered into force in 2004 (Lallas, 2001; Stockholm Convention Secretariat United Nations Environment, 2017). This treaty contains the first twelve regulated POPs so called "the dirty dozen" (heptachlor, chlordane, chlordecone, dieldrin, hexachlorobenzene, toxaphene, Polychlorinated biphenyls (PCB), dichlorodiphenyltrichloroethane (DDT), endrin, mirex, aldrin, and polychlorinated dibenzodioxins and furans) in addition to new POPs which have been added later (Lallas, 2001; Stockholm Convention Secretariat United Nations Environment, 2017).

Many of the toxic chemicals present in biota today have previously been produced in very large quantities, for instance as pesticides and industrial chemicals. PCBs were used in many different industries due to their high chemical stability, low acute toxicity, and their ability to act as electric insulators (Borja et al., 2005). Another group, dioxins, were emitted to the environment through burning of waste produced by humans, and burning of fossil fuels (exhaust) (White & Birnbaum, 2009). In a factory in Seveso, Milan, Italy, the production of 2,4,5-trichlorophenol (TCP) in the 1970s made large amounts of the unfortunate by-product 2,3,7,8-tetrachlorodibenzodioxin (TCDD) (Cattabeni et al., 1986). Within a few days many animals in the area died, and several hundred humans where affected with nausea, chloral acne etc. (Cattabeni et al., 1986). The Seveso accident is one of several incidents causing the usage of POPs to decrease (Andersson et al., 2004; Borja et al., 2005). Despite the decline in use, legacy POPs (POPs that have been and remain in the environment for a long period of time (Cabrerizo et al., 2018) in particular are still found in the environment today (Cabrerizo et al., 2018; karl et al., 2009; Mrema et al., 2013).

1.2.1 Emerging contaminants

An unfortunate consequence to the banning of several POPs, is the creation of new, and similar chemicals. The toxicological data on many of these new compounds is not yet complete, and several of them have a similar structure and properties to the already banned chemicals. Chemicals that are known to have negative effects on organisms (Flint et al., 2012; Kidd et al., 2007), such as organophosphorus flame retardants (OPFR), brominated flame retardants (BFR), in addition to per fluorinated substances (PFAS), and phthalates (Barroso et al., 2019). Phthalates are widely used in several industries and are known to have adverse health effects on reproduction and respiration. They have also been associated with carcinogenic processes in humans (Ventrice et al., 2013) and will be studies further in this thesis in addition to three POPs.

1.2.2 Endocrine disruptors

All POPs are classified as toxic, where almost all, such as organochlorine insecticide DDT or PCBs used in electrical equipment etc. are also defined as endocrine disruptors (ED) (Bhandari et al., 2015; Godfray et al., 2019; Kabir et al., 2015; Nilsson, 2000). Endocrine disruption indicates that the chemical or chemical mixtures can interfere with normal endocrine function of an organism (Diamanti-Kandarakis et al., 2009; Kabir et al., 2015).

Originally, EDs were thought to exert their actions primarily through specific nuclear receptors (NR) such as oestrogen receptor or androgen receptor, but previous studies have shown that EDs are also able to act through several other NRs (e. g. glucocorticoid receptor (GR), peroxisome proliferated activates receptor (PPAR), thyroid hormone receptor (THR)) and the aryl hydrocarbon Receptor (AhR) in addition to other mechanisms (Diamanti-Kandarakis et al., 2009; Goksøyr, 2006; Kabir et al., 2015). There has previously been shown a significant association between hormone levels and tissue concentrations of contaminants in marine mammals. For instance in polar bears (*Ursus maritimus*) and white whales (*Delphinapterus leucas*) there has been found an association between altered plasma thyroid hormone concentrations and pollutant exposure (Kabir et al., 2015; Kidd et al., 2012; Villanger et al., 2011). However, there is limited knowledge about the effect endocrine disruptors have on whales, and especially in large baleen whales such as the blue and fin whale.

Several POPs that have been proven to have endocrine disrupting effects also act as carcinogens, and can cause damage to the immune system, such as 2,3,7,8-TCDD, PCBs, DDT, dichlorodiphenyldichloroethylene (DDE) and dichlorodiphenyldichloroethane (DDD) (Figure 2)(Bertazzi et al., 1998; Cedervall et al., 2012; Mostafalou et al., 2013; Mrema et al., 2013).

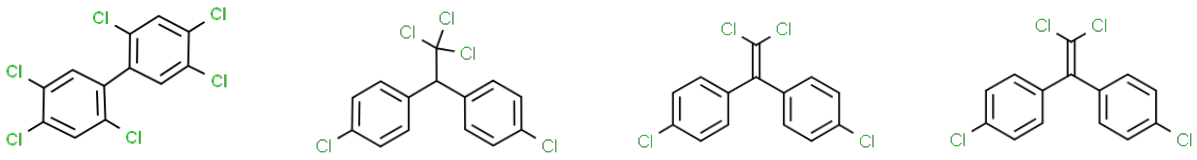


Figure 2. Chemical structure of 2,3,7,8-tetrachlorodibenzodioxin (TCDD), polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (pp`DDT), dichlorodiphenyldichloroethylene (pp`DDE) and dichlorodiphenyldichloroethane (pp`DDD).
Four POPs known to cause damage on organisms, chemical structures from chemspider.com

As previously mentioned, a large amount of anthropogenic chemicals end up in aquatic environments for example through waste water, air currents, and general dumping of human waste (Gallo et al., 2018; Rhind, 2009). Organisms associated to aquatic environments, such as whales, are therefore susceptible to exposure of harmful chemicals (Houtman, 2010; Rasheed et al., 2019). Previous research suggest that baleen whales (e.g. blue and fin whales), along with

other cetaceans (e.g. killer whales), are highly exposed to a large variety of POPs such as PCBs, chlorinated pesticides (e.g. pp`DDT), but also emerging environmental contaminants causing adverse health effects (Fossi et al., 2014a; Fossi et al., 2010a; Muñoz-Arnanz et al., 2019). These POPS may particularly influence the endocrine system, immune system, and reproduction (Bossart, 2011; J.-P. W. Desforges et al., 2016; Yordy et al., 2010).

1.2.3 POPs in whales

As previously mentioned, a large amount of anthropogenic chemicals end up in aquatic environments (Gallo et al., 2018; Rhind, 2009), organisms associated to aquatic environments, such as whales, are therefore susceptible to exposure of harmful chemicals (Houtman, 2010; Rasheed et al., 2019). Blue and fin whales are of high interest in toxicological studies because they feed on different trophic levels, travel very large distances, their magnitude of bioconcentration processes as a result of the massive amount of prey that they consume and have long life spans (up to 90 years) (Aguilar et al., 2018; Muñoz-Arnanz et al., 2019; Sears et al., 2009). So far there are only a few studies that have reported POPs concentrations in blue and fin whales where DDT and PCBs dominate (Table 1) (Fossi et al., 2014a; Gauthier et al., 1997; Metcalfe et al., 2004; Trumble et al., 2013).

Table 1. overview of some POPs found in blue whale and fin whales outside of Svalbard between 2014-2018.

Compound	Concentration (nM)	
	Blue whale	Fin whale
DDD	75	92
DDE	206	286
PCB 138	33	57
PCB153	51	80

Only a few studies have been conducted on fin whale (to my knowledge non on blue whales) to investigate biomarkers (western blot of CYP1A1, CYP2B) of toxicological effects in relation to pollutant concentrations (Fossi et al., 2010a, 2014; Das et al., 2017; Muñoz-Arnanz et al., 2019). Due to logistical challenges the collection of samples from a large number of individuals for correlative studies is difficult, therefore an alternative approach is to study how contaminants modulate the function of transcription factors involved in biotransformation, endocrine disruption, lipid metabolism- and hormone-receptors. Where one approach to better

understand how POPs influence e.g. marine mammals such as whales is through studying their biotransformation.

1.3 Biotransformation

Mammals have complex systems that are responsible for metabolizing and excreting endogenous and exogenous compounds, such as POPs. These systems consist of several enzymatic reactions, creating the "chemical defence mechanism" of the organism (Parkinson & Ogilvie., 2008). In biotransformation, a series of reactions modify and convert substances to excrete them more easily from the organism. Normally this results in more water-soluble and more polar compounds, but biotransformation may also produce more reactive by-products, which could be harmful to the organism. The processes of biotransformation are divided into three phases, each phase involves a series of different reactions, enzymes and transport proteins (Figure 3) (Dekant, 2009; Houtman, 2010; Sousa et al., 2018).The reactions in phase I includes: Oxidation, reduction and hydrolysis, where the three main catalysing enzymes are dehydrogenases, epoxide hydrolases, and cytochrome P450 monooxygenases (CYP). The combination of these reactions converts fat-soluble and non-polar substances into less fat-soluble and polar substances. The following step is phase II, which is performed by conjugating enzymes such as quinone oxidoreductase 1 (NAD(H)P), UDP-glucuronosyltransferases (UGT), and glutathione S-transferases (GST), which further increases the substances polarity, water-solubility and size by conjugating the phase I metabolites to endogenous compounds, or facilitated excretion. The last phase of biotransformation (phase III), is responsible for the transport of the water-soluble substances out of the cell. This often happens through ATP-binding cassette (ABC) proteins that actively transport the metabolites out of the cell (Houtman, 2010). The expression of all the proteins involved in biotransformation can be regulated through specific transcription factors.

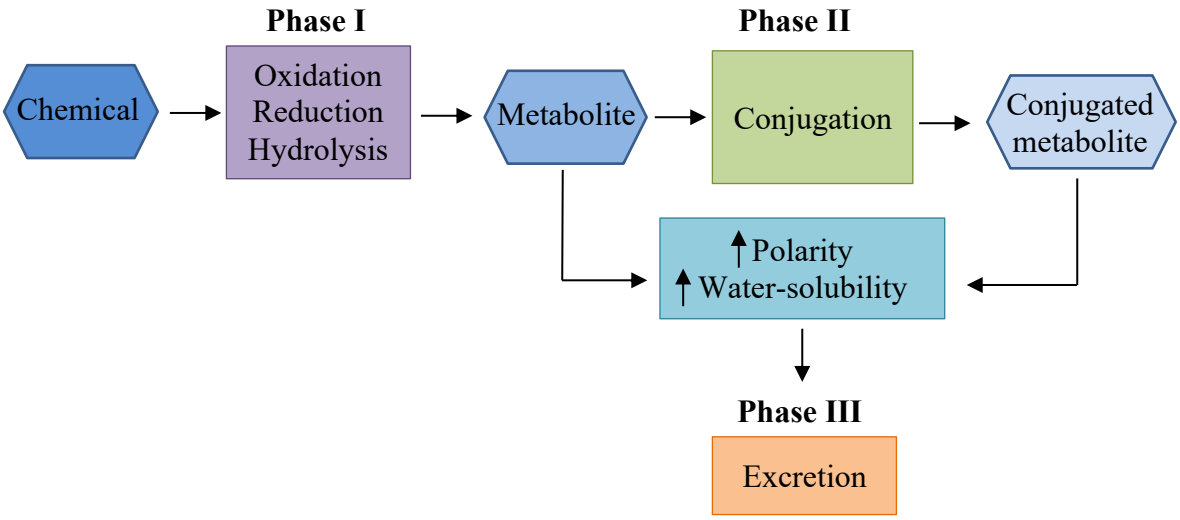


Figure 3 The different phases of biotransformation. The three phases in biotransformation increase a chemicals polarity and water solubility through several reactions. This results on the chemical being transformed and excreted from the organism.

1.4. Transcription factors

Transcription factors are proteins that bind to DNA and facilitate the transcription of specific genes. In order to understand how organisms, respond to POPs it is important to study the receptors that are activated by xenobiotic compounds. Two important groups of transcription factors in regulating the chemical defence and endocrine system are: Nuclear receptors (NRs) and bHLH-PAS receptors (Aryl hydrocarbon receptor (AhR)) (Ma, 2008; Xu et al., 2010). The basic helix loop helix (bHLH) and a Per-ARNT-Sim (PAS) domain-receptors normally function as dimeric DNA-binding protein complexes, forming homo- or heterodimers (Crews, 1998). Common for all the transcription factors mentioned above is that they recognize and bind to specific DNA-sequences, called the response elements. When the majority of the receptors bind to a ligand, they interact with the response element, and in many cases create a dimer that is important in the control of the expression of a gene e.g. nuclear receptors.

1.5 Nuclear receptors

Nuclear receptors are one of the largest receptor-super-families in vertebrates consisting of ligand-activated transcription factors. They have important roles in natural development, homeostasis, reproduction and metabolism in organisms (Ma, 2008; Xu et al., 2010; Sala et al., 2018). In addition, they are often linked to pathologies such as cancer, metabolic diseases and inflammation (Sala et al., 2018). Ligand-activated NRs regulate transcription by binding of

small lipophilic endogenous compounds, which are further divided into seven subfamilies, NR0-NR6 (Zhao et al., 2015). Nearly all NRs (except two in NR0B) contain six functional domains (Figure 4) (Eide et al., 2018; Germain et al., 2006; Zhao et al., 2015).

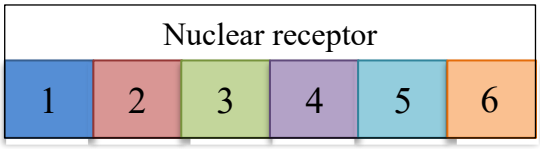


Figure 4 The six functional domains of NRs. 1 + 2 = Variable N-terminal regulatory domain, 3 = Conserved DNA-binding domain (DBD), 4 = Variable hinge regions,5 = Conserved ligand binding domain (LBD) and 6 = Variable C-terminal domains (Eide et al., 2018; Germain et al., 2006; Zhao et al., 2015).

Previous studies have shown that diverse chemicals found in the environment such as pharmaceuticals, pesticides and other synthetic molecules can mimic the endogenous compounds that bind to LBD in NRs and mediate signals leading to toxic responses (Grün et al., 2006; Janošek et al., 2006). There are several examples where interactions with e.g. pesticides have led to birth defects, cancer and developmental neurotoxicity through NRs (oestrogen and androgen receptor)(Huang et al., 2011; World Health Organization, 2002). GR, THRB and PPARG are central in mediating endocrine responses, but little is known regarding how they are affected for POPs, as well as their importance for whales. I will in this thesis focus on the three important NRs: glucocorticoid receptor (GR), thyroid hormone receptor (THRB), and peroxisome proliferated-activated receptor gamma (PPARG). The three nuclear receptors are through heat-shock proteins tethered in the cytoplasm of the cell, and when a ligand binds to the receptors it leads to recruitment of coregulators before releasing the receptor from heat-shock proteins. This enables them to translocate and dimerize to the nucleus where they can positively or negatively regulate gene expression (Sever, 2013; Surma et al., 2015).

1.5.1 Glucocorticoid receptor (GR)

The GR is a nuclear receptor that is expressed in every cell type (in humans) and may affect responses in different tissues such as adipose, skeleton muscle and dermal tissues (Akner et al., 1994; Bellingham et al., 1992; Carson-Jurica et al., 1990; Seckl et al., 2004). In the nucleus GR homodimerizes, where the actions of glucocorticoids upon a target gene is determined by a macromolecular complex with specific coactivator and corepressor proteins. GR is important in numerous physiological possesses such as adaptation to stress, behaviour, immune function, energy metabolism and reproduction (Jenssen, 2006; Wingfield et al., 2003). The release of

glucocorticoids alters an organisms physiological state in response to environmental conditions (Ricklefs et al., 2002; Wingfield et al., 2003). The receptor is known to be activated by several steroids (such as fluorotrisol, dexamethasone, and cortisol), in addition to POPs such as the methyl sulfonyl metabolites of PCBs (Akner et al., 1994; Johansson et al., 1998)

1.5.2 Thyroid hormone receptor (THRB)

The THRB is also an important nuclear receptor that is bound to DNA resided inside the nucleus, and highly expressed in adipose and skeleton muscle tissue (Ribeiro et al., 2010). Generally, it is heterodimeric with the retinoid X receptor (RXR). THRB is central in normal brain development in addition to being involved in maintenance and development of the endocrine system (e.g. the thyroid gland) etc. (Sever et al., 2013). Interestingly, THRB has previously been shown to be an unintended target for several contaminants that humans and animals are continuously exposed to such as phthalates, PCB, BFR and benzo(a)pyrene (BaP) (Zhao et al., 2015; Zoeller, 2005).

1.5.3 Peroxisome proliferated-activated receptor gamma (PPARG)

Similar to THRB, PPARG is bound to DNA resided inside the nucleus, heterodimers, and is bound to ligands in the same way (Sever, 2013; Surma , Zielinski, 2015). In previous studies PPARG has been found to be most abundantly expressed in adipose tissue, but also in skeletal muscle (Vidal-Puig et al., 1997). The receptor is essential in pathophysiological and physiological events such as cell differentiation, in addition to having an important role in adipocyte differentiation, and lipid homeostasis (Morais et al., 2006), which previously have been shown to be activated by environmental contaminants such as PCBs and organochlorine pesticides (Routti et al., 2016) and bis(2-ethylhexyl) phthalate (DEHP) (Ernst et al., 2014).

1.6 Aryl hydrocarbon receptor (AhR)

AhR on the other hand differs from the NRs because it is a cytoplasmatic ligand-activated transcription factor and belong to a separate gene family. It regulates the expression of a diverse set of genes, e.g. CYP1A through binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Beischlag et al., 2008; Hahn, 1998). The receptor is a member of a family of transcription factors who have a bHLH and a PAS domain. When AHR is activated by a ligand, it translocates into the cell nucleus from the cytosol, at the same time as the chaperones p23, HSP90 and XAP2 are released (Tsuji et al., 2014). Inside the nucleus AhR

couples up with its heterodimeric partner aryl hydrocarbon receptor nuclear translocator (ARNT), often referred to as the aryl hydrocarbon receptor complex (Abnet et al., 1999; Andreasen et al., 2002). ARNT is also a bHLH-PAS protein that is necessary for the gene-regulating activity of AhR (Brunnberg et al., 2003). The formed AhR-complex recognizes and binds to a xenobiotic response element (XRE) on DNA, which then activates gene transcription (Tsuji et al., 2014) (Figure 5). The activation of the receptor complex by a ligand regulates the expression of many enzymes involved in both phase I (CYP superfamily CYP1A in particular), phase II (GSTA2, UGTA1 and UGT1A6) and phase III of biotransformation (Ma, 2008; S. Xu et al., 2010).

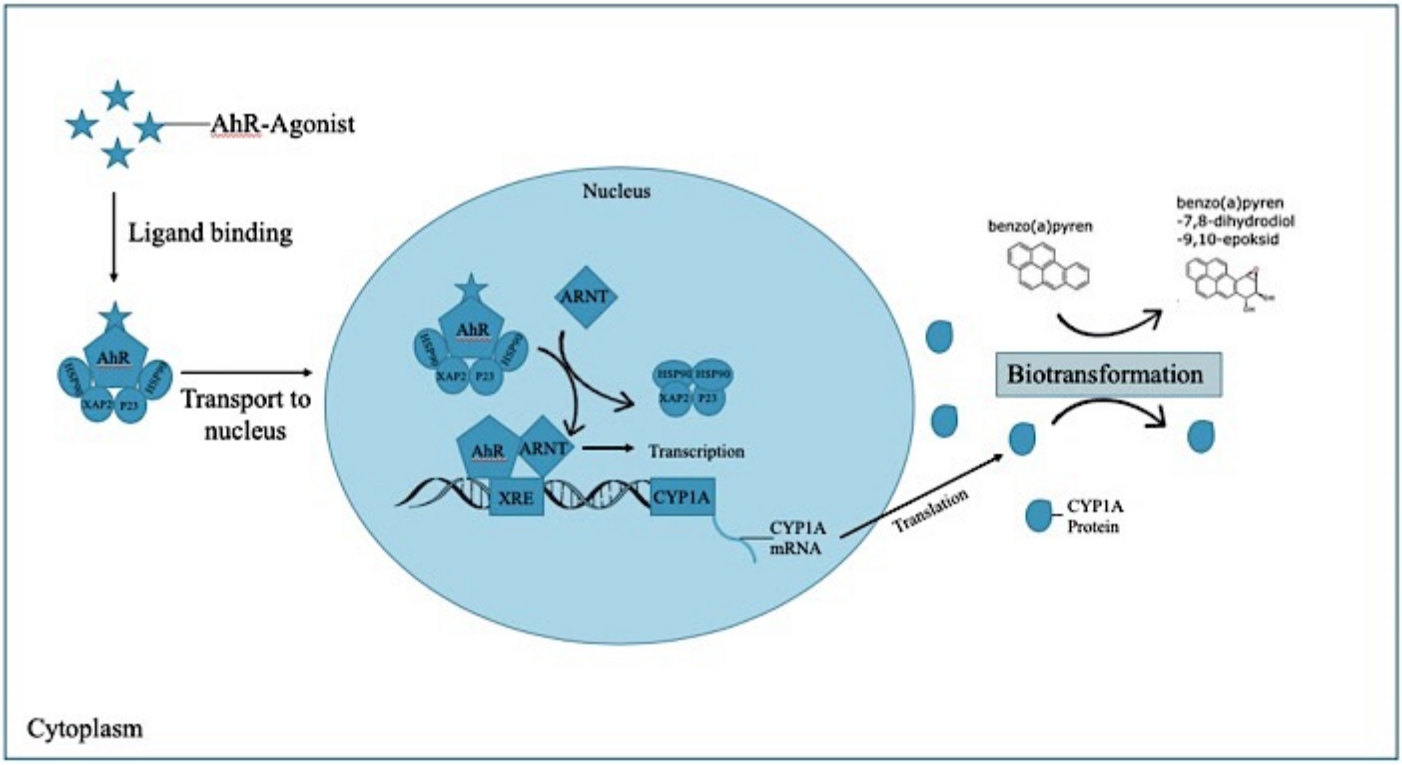
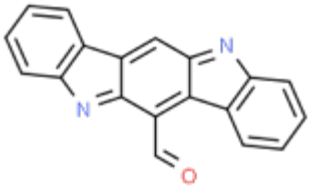


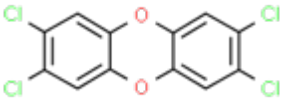
Figure 5 Schematic overview of ligand induced AhR-activation. AhR is located in the cytoplasm in a complex with HSP90, XAP2 and p23. When a ligand binds to the receptor it is translocated into the nucleus, where the cofactors are released and AhR dimerizes with ARNT. AhR-ARNT binds to XRE located upstream from the target gene e.g. CYP1A and initiates the transcription of the gene. CYP1A is for instance very important in the bioactivation of benzo(a)pyrene to benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide. Based on figure from Alexander Klevedal Madsen (2016)(Madsen, 2016).

The AhR signalling pathway is known to be activated by many different exogenous and endogenous substances, where the most common ligands for AhR are dioxins and dioxin-like substances (Denison & Heath-Pagliuso, 1998). These are mainly a group of polychlorinated varieties of dibenzofurans (PCF), biphenyls (PCB), and dibenzo-p-dioxins (PCDD), which are all halogenated aromatic substances (HAH) with anthropogenic origin and have planar configurations (Figure 6). Two of the most potent ligands are 2,3,7,8-TCDD and 6-

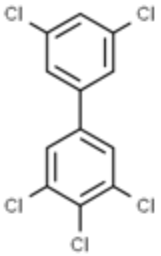
formylindolo(3,2b)carbazole (FICZ), which have often been used as control agonists in experiments with AhR (Beischlag et al., 2008; Ehrlich et al., 2018). The AhR is often associated with an organism's response to environmental contaminants e.g. POPs. This makes the research of AhR thus more important, in a world with increasing amounts of POPs.



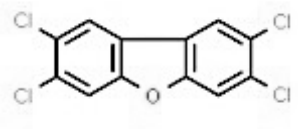
6-Formylindolo[3,2-b]carbazole



2,3,7,8-Tetrachlorodibenzo-p-dioxin



3,3',4,5,5'-Pentachlorobiphenyl



2,3,7,8-Tetrachlorodibenzofuran

Figure 6 Compounds known to activate the AHR- signalling pathway. AHR is known to be activated by numerous ligands such as 6-formylindolo[3,2-b] carbazole (on top) and dioxin/dioxin-like substances (at the bottom). Chemical structures from chemspider.com

1.7 Studies of ligand activation of nuclear receptors and AhR

The three nuclear receptors studied in this thesis have previously been analyzed in e.g. rodents, polar bears and Baikal seals (*Pusa sibirica*) (Crofton, 2004; Johansson et al., 1998; Kim et al., 2002; Routti et al., 2016; Xu et al., 1999). In similarity with the NRs there are also several previous studies conducted on AhR and ARNT e.g. in fish, seals and some toothed whales (Kim et al., 2005; Wilson et al., 2005; Zhou et al., 2010), but to my knowledge this is the first study that investigate the effects POPs have on blue and fin whales NRs (GR, THRB and PPARG) and AhR (Lühmann, 2018).

To study the ligand activation of transcription factors, an *in vitro* reporter gene assay is often used (Hansson et al., 2008; Karchner et al., 2005). In this study I used a luciferase reporter

assay, and for the NRs, a Gal4/UAS-based system was employed. In this system a fusion protein of the DNA binding-domain (DBD) from the yeast protein Gal4, together with the functional ligand binding domain of the desired transcription factor is used. Plasmids encoding species-specific nuclear receptor, together with a reporter gene (luciferase) and a control plasmid encoding β-galactosidase are transfected into an eukaryote cell line with COS-7 cells. The expression of reporter gene is controlled by one or several upstream activation sequences for Gal4 (UAS). When a nuclear receptor binds to a ligand it causes the Gal4-DBD- receptor-LBD fusion protein to bind to UAS of the reporter gene plasmid. This induces an expression of the reporter gene and gives a dose dependent response (Figure 7). One of the advantages with this reporter gene assay is that it is independent from the receptor's natural partner protein and response element. This luciferase gene reporter assay has previously also been used to characterize the ligand binding and activation of nuclear receptors in for example polar bears (Lille-Langøy et al., 2015; Routti et al., 2016, 2019).

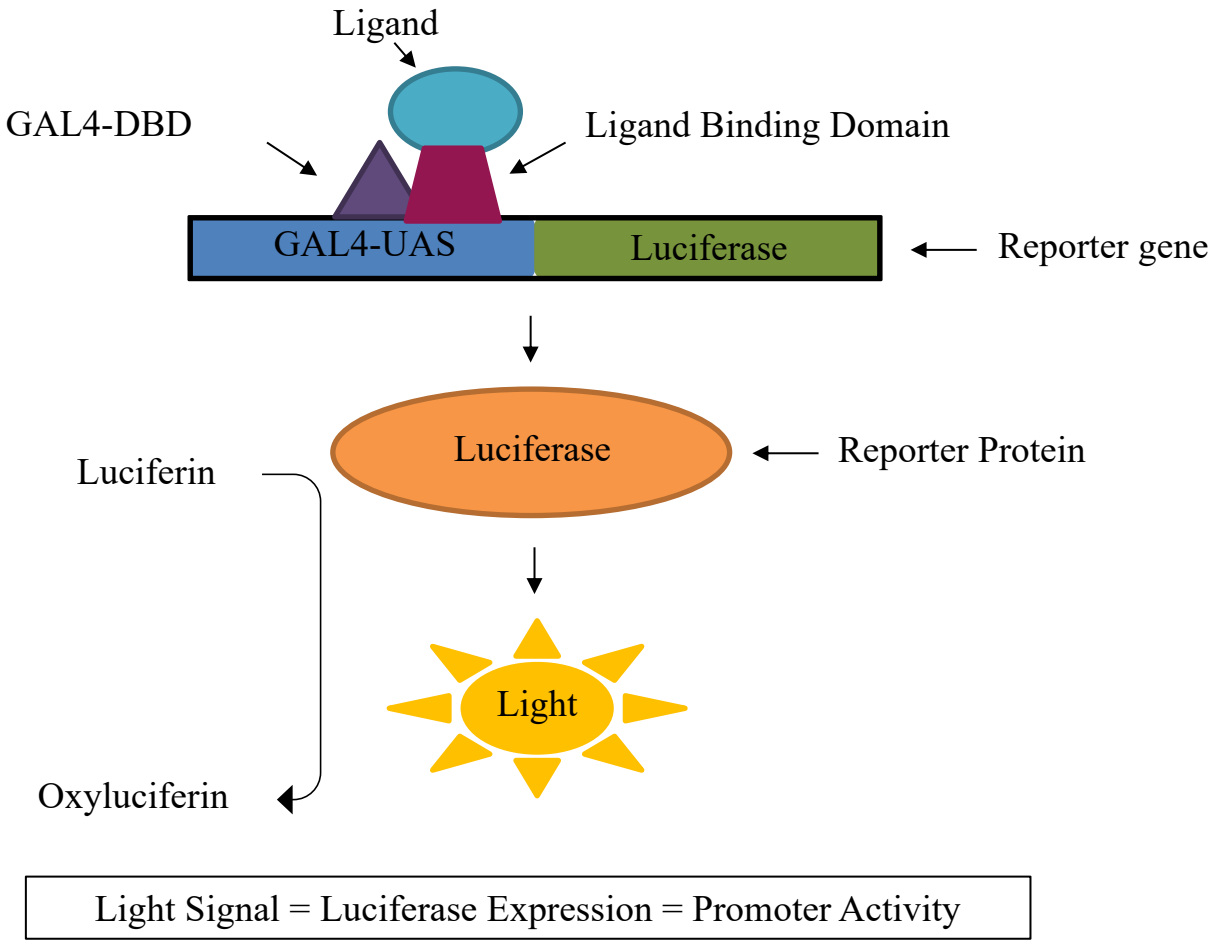


Figure 7. ***In vitro*** **luciferase reporter gene assay.** When a ligand binds to a nuclear receptor it causes the GAL4-DBD-NR-LBD binder UAS to bind to UAS. This induces an expression of the Luciferase and gives a dose dependent transcription factor activity.

The first time *AhR* was discovered as a transcription factor and receptor protein was in the 1970s, where 2,3,7,8-TCDD was shown to induce cytochrome P450 (later identified as CYP1A1) in several different organisms (Poland et al., 1973). In a study conducted by Poland and co-workers binding of radioactive 2,3,7,8 TCDD in liver cytosol was detected (Poland et al., 1976). The DNA- binding and ligand-binding mechanisms, translocating of the receptor, and transcription of target genes of AhR was later discovered by Denison et al. (1984)(Denison et al., 1984). After the receptor had been isolated (making it possible to determine parts of the amino acid sequence), the cDNA-sequence was cloned for the first time (in mice)(Bradfield et al., 1991; Burbach et al., 1992). To enable ligand binding and activation studies different reporter systems were developed. Where different cell lines containing parts of the promoter-region of human CYP1A and a stabile transfected luciferase gene, often referred to as a CALUX-system (Garrison et al., 1996; Murk et al., 1996; Postlind et al. , 1993). This was a revolutionary method enabling quantification and detection of dioxin like and other chemicals. The system has been improved and is now able to detect very low concentrations (picograms) of e.g. 2,3,7,8,-TCDD (Brennan et al., 2015; He et al., 2011; Zhao et al., 2010).

To study the activation of AhR a similar *in vitro* gene reporter assay was used. Also, in this assay a cell line of COS-7 cells were transfected with plasmids containing the luciferase reporter gene, together with species specific AhR and ARNT, and the reporter gene (pGudLuc6.1 with 4x DRE) is under control of one or more response-elements for AhR e.g. CYP1A.

1.8 Study species

The studied species in this thesis are blue whales and fin whales, the two largest animals in the world. One of the first scientists to describe blue whales was Georg Ossian Sars (Sars, 1875). The blue whale was originally believed to be between 25-33 meters long and have later been proven to become more than 27 meters long and up to 120 tons, while fin whales grow up to 22 meters and 80 tons (Sars, 1875). These two species are truly giants of the ocean, both species are widely distributed throughout the world's oceans. As mentioned earlier blue and fin whales are baleen whales (*Mysticeti*) and part of the family *Balaenopteridae.* They are distinguished from right whales (*Eubalanea glacialis*) by their slender body, the dorsal fin being placed at the far back, in addition to their long narrow flippers. Blue whale`s reach reproductive maturity at age 5-15, while fin whale around 6-8 years of age. Little is known about fin whale's reproduction biology and season, but blue whales are suspected to mate late fall through winter

(October-March in the northern hemisphere, May-August southern hemisphere). Blue and fin whales are rarely seen in groups but communicate with one another through loud vocalizations that can be heard over large distances. The blue whale has one of the world's largest "calls" which can be heard up to 1600 km away and is believed to help them navigate in the deep ocean (Hsu et al., 2013; Sears & Perrin, 2009; Whale, 2006). Despite blue and fin whale's global distribution, there is little toxicological data on these species, including POPs.

1.9 Aim

As mentioned there is still a lot of gaps in our knowledge concerning the effect environmental contaminants have on important mechanisms within organisms (e.g. biotransformation (AhR), immune system (GR), brain development (THRB), lipid metabolism (PPARG) etc.), and these gaps continue to grow as long as new chemicals are being developed. This knowledge is required to be able to guide regulatory authorities to where strict chemical regulation is required, in addition to indicate what action is needed to best protect marine organisms and wildlife.

In this thesis the effect of POPs on nuclear receptors and AHR activation in blue whale (*Balaenoptera musculus*) and fin whale *(Balaenoptera physalus*) were further investigated. In addition to this, phylogenetic analyses were conducted to understand the level of identity/similarity of the different nuclear receptor between species. The overall aim was to better understand the impact environmental contaminants such as POPs may have on transcription factors in these marine mammals.

This was done with the following sub-goals:

- Cloning of the coding DNA- sequence of AhR in blue whale
- Analyse and compare the blue whale AhR sequence to other species
- Verify an *in vitro* luciferase gene reporter assay with blue whale GR, PPARG, THRB, and AhR-ARNT
- Study the agonistic effect of 5 POPs abundant in blue and fin whale blubber, in addition to a synthetic POP mixture on the three NRs and AhR.

2 Materials

2.1 List of chemicals

Table 2. Chemical list

Name	Producer
10X Loading buffer	*TaKaRa*
2-log DNA Ladder	*New England Biolabs*
5-CFDA-AM	*Thermofisher Scientific*
Agarose	*Sigma-Aldrich*
Ampicillin-sodium salt	*Sigma-Aldrich*
ATP (Adenosine 5'-trifosfat)	*Sigma-Aldrich*
Boric acid	*Sigma-Aldrich*
Chloroform	*Sigma-Aldrich*
Coenzyme A	*Fisher Scientific*
Dexamethasone	*Sigma-Aldrich*
DDT, DDE, DDD	*Sigma-Aldrich*
DINP	*Sigma-Aldrich*
DEHP	*Sigma-Aldrich*
D-Luciferine firefly	*Biosynth*
DMSO	*Sigma-Aldrich*
Dulbecco's Modified Eagle's Medium (high glucose, with phenol red)	*Sigma-Aldrich*
Dulbecco's Modified Eagle's Medium (high glucose, without phenol red)	*Sigma-Aldrich*

EDTA (Ethylenediaminetetraacetic acid disodium salt dehydrate)	*Sigma-Aldrich*
EGTA (Ethylene glycol-bis(2-aminoethylether)- N, N, N', N'-tetra acetic acid	*Sigma-Aldrich*
Ethanol	*Sigma-Aldrich*
Fetal Bovine Serum (FBS)	*Sigma-Aldrich*
6-formylindolo[3,2-b]carbazole (FICZ)	*Enzo*
Formamide	*Sigma-Aldrich*
GelRed	*Botium*
Isopropanol	*Sigma-Aldrich*
ONPG (2-Nitrophenyl β-D-galactopyranoside)	*Sigma-Aldrich*
Opti-MEM. I Reduced Serum Medium	*GibcoTM*
PCB 101	*Sigma-Aldrich*
PCB 118	*Sigma-Aldrich*
PCB 153	*Sigma-Aldrich*
Penicillin-Streptomycin	*Sigma-Aldrich*
Phosphate-buffered saline (PBS) 10X	*Sigma-Aldrich*
PMSF (Phenylmethanesulfonyl fluoride)	*Sigma-Aldrich*
Rosiglitazone	*Sigma-Aldrich*
SOC Outgrowth media	*New England Biolabs*
Tris Borate EDTA (TBE)	*Sigma-Aldrich*
3,5,3´-triiodothyronine (T3)	*Sigma-Aldrich*
TransIT®-LT1	*Mirus Bio LLC*
Tri reagent	*Sigma-Aldrich*
Trypan Blue solution 0.4%	*Sigma-Aldrich*
Trypsin-EDTA Solution 1X	*Sigma-Aldrich*
Trypton	*Merck*

Yeast extract	*Fluka*
β-Mercaptoethanol	*Sigma-Aldrich*

2.2 Solutions

Table 3. Lysogeny broth – LB medium/LB agar

Component	*Concentration (g/L)*
Sodium chloride (NaCl)	10
Trypton	10
Yeast extract	5
Deionized H2O	-
(Agar)	(15)

Components were dissolved in deionized water and autoclaved for 20 min at 121℃, and to prevent microbial contamination 100U/mL of ampicillin was added to the growth media before use. Agar was only used for the agar-plate, and not for the media.

Table 4. Tris borate EDTA (TBE) buffer 5X

Component	*Concentration (g/L)*
Trizma base	0.45 M
Boric acid	0.45 M
EDTA	0.01 M
H_2O	-

Table 5. TBE Agarose gel

Component	*Concentration (g/L)*
TBE buffer (5X)	0.5 X
Agarose	0.4-1%

2.3 Ligand activation assays

Table 6. Lysis buffer (1X)

Component	Concentration
Tris-PO4, pH 7,8	25 mM
Glycerol	15%
CHAPS	2%
L-α-Phosphatidylcholine	1%
Bovine serum albumin	1%

Table 7. Reaction solution lysis

Component	Concentration
Lysis buffer	1X
EGTA	4mM
$MgCl_2$	8mM
PMSF	0.4mM
DTT	1mM

Table 8. β-galactosidase base buffer (10X)

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

Table 9. Reaction solution β-galactosidase

Component	Concentration
β-galactosidase base buffer (10X)	1X
β-Mercaptoethanol	52.9 mM
ONPG	8.6 mM

Table 10. Luciferase base buffer (4X, pH 7.8)

Component	Concentration
Tricine	80 mM
$(MgCO_3)4 \bullet Mg(OH)2 \bullet 5H_2O$	4.28 mM
EDTA	0.4 mM
$MgSO_4$	10.68 mM

Table 11. Reaction solution luciferase activity

Component	Concentration
Luciferase base buffer (4X, pH7.8)	1X
ATP	0.5 mM
DDT	5
Coenzyme A	0.15 mM
Sodium luciferin	0.5 mM
Deionised H_2O	-

2.4 List of kits and cell-lines used

Table 12. List of kits used

Kit	Supplier
NucleoBond® Xtra Mini & Midi plasmid purification kit	Macherey-Nagel
NucleoSpin® Gel and PCR Clean-up kit	Macherey-Nagel
StrataClone Blunt PCR Cloning Kit	Angilent

Table 13. List of cell lines used

Cell line	Supplier
COS-7 cells	Eukaryote expression (African green monkey)
StrataClone Solo Pack competent cells	Prokaryote cloning (E. coli)

2.5 Primers and Primers

Table 12. Overview of the primers used in the PCR amplifications. Primers were designed based on the minke whale *AhR* sequence

Gene	Use	Sequence (5'-3')	ID
AhR	Amplification of 5′-segment (AhR_{start})	F: GGAAAGCTTATGAACAGCAGCAG	MT1738
		R: CTTGTTGCATCATGGCATTC	MT1781
		R: GGCCAATCTGCTCATGTTTC	MT1782
	Amplification of 3`- segment (AhR_{end})	F: ATCCCAGTTCCCTCCTGAAT	MT1783
		F: ATGTTGCACCAATGGGAAGT	MT1784
		F: AGGATTCCCTCAATCCCAGT	MT1785
		R: CCTCTCGAGTTACAGGAATCCAC	MT1737

Table 13. Overview of the plasmids used in the luciferase gene reporter assays.

	Concentration (ng/µL)	$A_{260/280}$
pCMX_whale THRB	1027	1.86
pCMX_whaleGR	1013	1.89
pCMX_whalePPARG	1659	1.88
pCMX_whaleAhR	765	1.84
Human-Arnt	3388	1.93
(MH100)x4 tk luc	2322	1.92
pCMV_BGAL	2365/1497(AhR)	1.90
pGudLuc6.1	1216	1.87

Table 14. overview of the plasmid mixes created for the transfections in the luciferase gene reporter assays.

						Reactions	
Whale GR					**1**	**1000**	
	ca 1:20	**Plasmid**	**C_{pDNA} (ng/µL)**	**m_{pDNA} (ng)**	**V_{pDNA} (µL)**	**V_{pDNA} (µL)**	
	(MH100)x4 tk luc		2322	48.75	0.021	**20.99**	
	pCMV-BGAL		2365	48.75	0.021	**20.61**	
	pCMX_whaleGR		1013	2.50	0.002	**2.47**	
				100.00	0.044	44.08	
					Add MQ-H2O	**55.92**	µL
					Total volume	100.00	µL
					Concentration	1000.0	ng/µL

Whale THR					**1**	**1000**	
	ca 1:20	**Plasmid**	**C_{pDNA} (ng/µL)**	**m_{pDNA} (ng)**	**V_{pDNA} (µL)**	**V_{pDNA} (µL)**	
	(MH100)x4 tk luc		2322	48.75	0.021	**20.99**	
	pCMV-BGAL		2365	48.75	0.021	**20.61**	
	pCMX_whaleTHR		1027	2.50	0.002	**2.43**	
				100.00	0.044	44.04	
					Add MQ-H2O	**55.96**	µL
					Total volume	100.00	µL
					Concentration	1000.0	ng/µL

Whale PPARG				1	1000	
	ca 1:20	Plasmid	C_{pDNA} (ng/µL)	m_{pDNA} (ng)	V_{pDNA} (µL)	V_{pDNA} (µL)	
		(MH100)x4 tk luc	2322	48.75	0.021	**20.99**	
		pCMV-BGAL	2365	48.75	0.021	**20.61**	
		pCMX_whalePPAR	1659	2.50	0.002	**1.51**	
				100.00	0.043	43.11	
					Add MQ-H2O	**56.89**	µL
					Total volume	100.00	µL
					Concentration	1000.0	ng/µL

					Reactions		
Whale AhR				1	300		300
1:10	Plasmid	C_{pDNA} (ng/µL)	m_{pDNA} (ng)	V_{pDNA} (µL)	V_{pDNA} (µL)		
	GudLuc 1B	1216	30,00	0,025	**7,40**		**7,40**
	pCMV-BGAL	1497	20,00	0,013	**4,01**		**4,01**
	Human Arnt	3388	6,00	0,002	**0,53**		**0,53**
	Whale AhR	765	3,00	0,004	**1,18**		**1,18**
	pcDNA 2	1213	41,00	0,034	**10,14**		**10,14**
			100,00	0,078	23,26		**23,26**
			100,00	Add MQ-H2O	**6,74**	µL	**6,74**
				Total volume	30,00	µL	30,00
				Concentration	1000,0	ng/µL	1000,0

2.6 Software and online tools

Table 15. List of software and online tools

Software	Application	Provider
Clustal Omega	Sequence alignments	EMBL-EBI
Ensembl	Genome database	(Cunningham et al., 2014)
Excel version 16.28	Data treatment and statistics	Microsoft
Jalview	Visualization of alignments	(Waterhouse et al., 2009)
Mega 7	Phylogenetic analyzes	(Tamura et al., 2013)
PowerPoint version 16.28	Figures	Microsoft
Prism 8	Figures	GraphPad
UniProt	Protein database	(Consortium, 2014)
ExPASy Translate tool	Sequence translation	SIB Bioinformatics

2.7 List of equipment

Table 16. List of equipment

Equipment	Application	Provider
Bürker haemocytometer	Cell counting	Marienfeld

ChemiDoc^{TM} XRS+ System	Gel scan	Bio-Rad
DOPPIO Thermal Cycler	PCR Thermo Cycler	VWR
EnSpire 2300 Multilabel Reader	Plate reader	PerkinElmer
G:BOX	Gel doc imaging system	Syngene
HS 501 Digital	Platform shaker	IKA-Werke
NanoDrop 1000	Spectrophotometer	Thermo Scientific
PowerPacTM HC	High-current power supply	Bio-Rad
Thermomixer compact	Heatblock	Eppendorf
Z 216 MK microliter centrifuge	Centrifuge	Hermle
Chromato-vue TM-20 transilluminator	Agerose gel visualization	UVP, San Gabriel

2.8 Chemicals

In this study nine different chemicals were used as ligands. PCB 153-101-118, 3,5,3´-triiodothyronine (T3), dexamethasone, and rosiglitazone were purchased from Sigma Aldrich (St. Louis, Missouri), while pp´-DDT, pp´-DDE, pp´-DDD from Chem Service (West Chester, Pennsylvania). The test compounds were dissolved in dimethyl sulfoxide (DMSO), except dexamethasone (water). The final concentration of solvent (DMSO) in exposure solutions was 0.5 % (v/v). The two phthalates DINP and DEHP were also purchased from Sigma-Aldrich.

2.9 Synthetic mixture

To be able to simulate the actual exposure situation in whales in the best way possible, a synthetic mixture was prepared. The mixture consisted of the 4 most abundant POPs (table17) (hexachlorobenzene, trans-nonachlor, PCB 153 and PCB 158) measured in blue and fin whale blubber. The POPs concentrations in the synthetic mixture were based on measured blubber concentrations from 28 blue and fin whales collected outside of Svalbard between 2014-2017 (Tartu et al., in preparation 2019).

Table 17. Chemical composition of synthetic POPs mixture(200X).

Chemical	Concentration (uM)
Hexachlorobenzene	3840
Trans-nonachlor	2760
PCB-153	2280

3 Methods

To enable ligand activation studies of blue whale and fin whale GR, THRB, PPARG and AhR, several preparatory steps were conducted (Figure 8). The result from each step was quality controlled, before continuing to the next step. In brief, RNA was extracted from blubber samples taken outside of Svalbard (2014-2018), concentrations of different contaminants were analysed in the blubber samples. Further, the AhR receptor was cloned from RNA and later used in a luciferase gene reporter assay and in sequence analysis. In addition, three previously cloned NRs were studied with a luciferase gene reporter assay, in order to investigate their transcriptional activity triggered by different ligands.

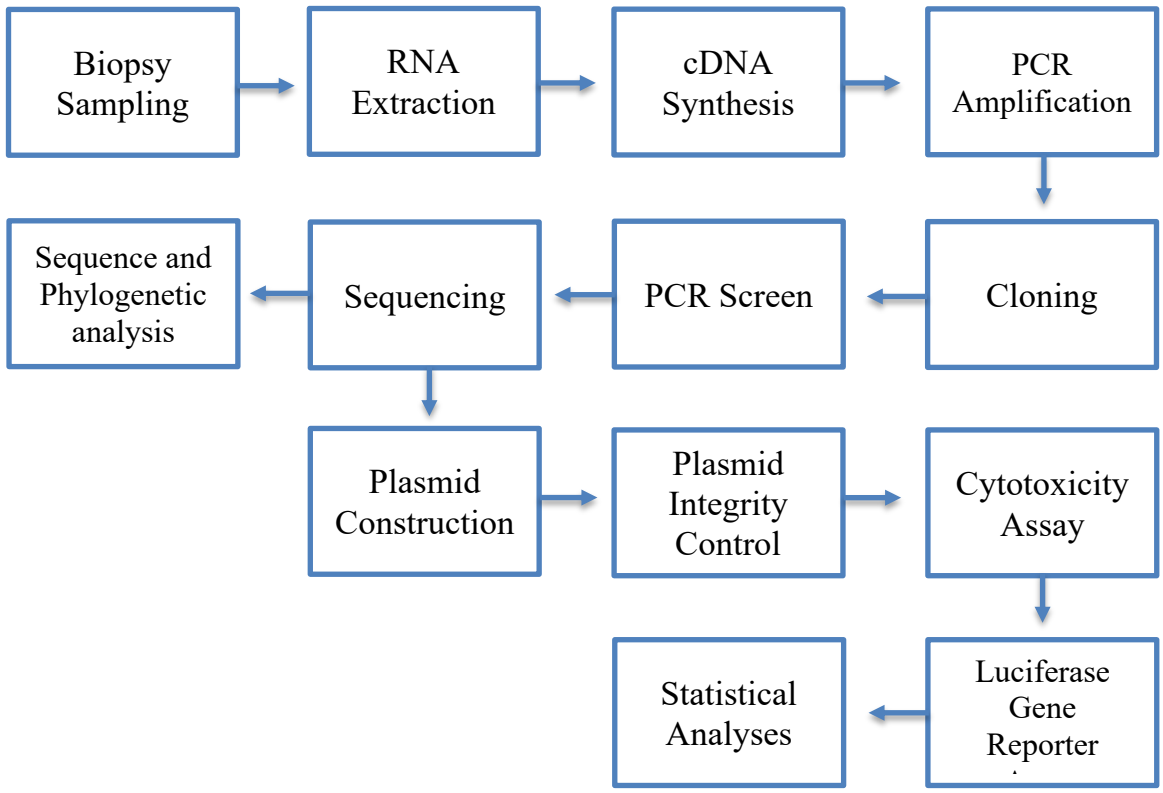


Figure 8. Workflow overview. Blue whale tissues were collected and homogenized. RNA was extracted and transcribed into cDNA by reverse transcriptase. The AhR encoding genes were amplified in two fragments by PCR, using the cDNA as template. Amplified genes fragments were then cloned by transforming *E.coli* cells. To confirm that the right genes fragments had been cloned a PCR screen and sequencing was conducted. The fragments were then ligated together. Successfully amplified plasmids containing the desired receptors, were then used in a luciferase-gene-reporter assay, to conduct activation analysis of different environmental contaminants *in vitro*. A cell viability assay was used to investigate if any of the tested chemicals had cytotoxic effects, at the concentrations used in the gene-reporter assays. Statistical analysis of the result was the finishing step of this study.

3.1 Biopsy sampling of blue whales

Blue whale biopsies where collected in Isfjorden at Svalbard between August-October 2014-2018. The samples were taken with a crossbow from approximately 20 meters or less away. The arrow had a hollow tip (biopsy needle) and a floating element attached to the end, with a rope connecting the top of the arrow to the crossbow, to prevent biopsy losses (Figure 9). The samples were removed from the tip of the arrow and divided into several pieces. These were then placed in individual tubes containing RNAlater. The tubes were kept at 4 ℃ for approximately 24 hours, before being stored at -80℃ until RNA extraction.

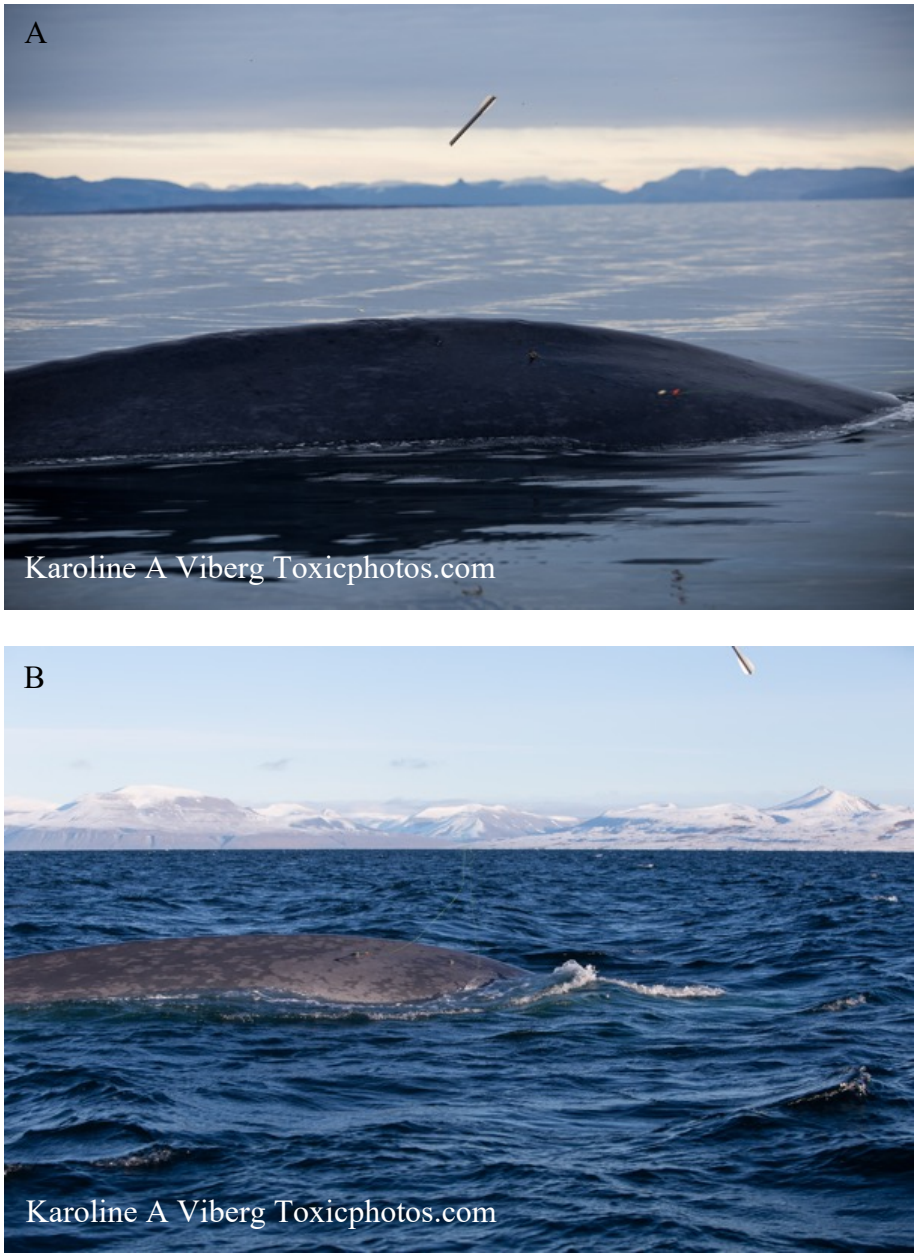


Figure 9 Blubber sampling of blue whale. 18 blue whale and 12 fin whale samples were collected around Svalbard from August- September 2014-2018. The samples were taken with a biopsy arrow, with a rope attached to it. Photo: Karoline A. Viberg, Toxicphotos.com

3.2 RNA isolation

In this study, we only used samples of blue whale blubber collected in 2018 for RNA extraction. RNA was extracted from blue whale biopsies (blubber and skin) using TRI-Reagent® (Sigma-Aldrich) according to the protocol recommended by the producer. This method is an improved version of the original RNA extraction method developed by Chomczynski in 1987 (Chomzynski & Sacchi, 1987). TRI-Reagent® consists of isothiocyanate, phenol, and guanidinium, which denatures protein and dissolves biological material, while the RNA remains intact by inhibiting RNAse activity. The RNA used in this thesis was extracted from two homogenized blue whale blubber samples (Method 3.1). Sample #1 was mainly adipose tissue, sample#2 was a mix of adipose tissue and skin. In each sample 50µg blue whale blubber was homogenized with TRI-Reagent® and chloroform was added to phase-separate the samples, before being centrifuged at 12000g for 15 min at 4℃. The centrifugation creates three different layers containing RNA in the aqueous phase, DNA in the interphase, and proteins in the lower organic phase (Figure 10).

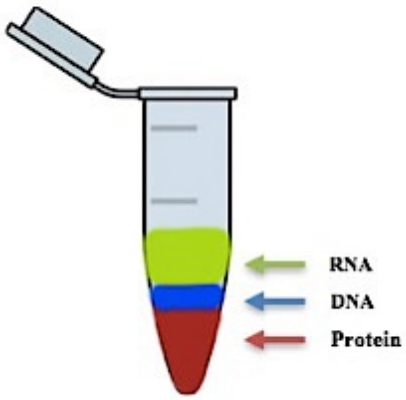


Figure 10. Schematic overview of a blue whale blubber sample post homogenization, centrifugation, and addition of Tri-Reagent®. The extraction process results in blue whale tissue being separated into three different phases. The first phase contains RNA, the interphase contains DNA, and the lowest phase contains proteins.

To extract the RNA the supernatant was carefully removed into a new microcentrifuge tube, and added 0,5 mL isopropanol (100%), before subsequent centrifugation. The pellet created by the centrifugation was washed with ethanol (75%), placed sideways to air dry, before being resuspended in deionized water. The purity and concentration of the RNA was measured with a spectrophotometer (Method 3.3.1), and an agarose-gel-electrophoresis (AGE) (Method 3.3.2) was used to assess the integrity of the RNA. The RNA was then stored at -80℃ untill further use.

3.3 Quality control of RNA

After the RNA had been extracted from the blue whale blubber samples, its quality was ensured through several steps.

3.3.1 NanoDrop – Spectrophotometric Measurements

The purity and concentration of DNA and RNA used in this study was measured with a Nanodrop1000 spectrophotometer ($A_{260nm}/A_{260/280}$-ratio, Thermo Scientific). DNA and RNA concentrations are found by measuring the ultraviolet absorbance at 260 nm. The purity of DNA and RNA may be evaluated by the ratio between the absorbance at 260/280 nm, and 230/260 nm (Okamoto & Okabe, 2000). An $A_{260/280}$ ratio between 1.8-2.0 indicates a pure DNA or RNA, however the $A_{260/280}$ ratio may also be influenced by the ionic strength and pH of the spectrophotometric solution, a low $A_{260/230}$ value indicates a low contamination of phenol and proteins (Wilfinger, Mackey, & Chomczynski, 1997).

3.3.2 Agarose gel electrophoresis

After the concentration was measured and the purity established an agarose gel electrophoresis (AGE) was used to control the integrity of the RNA, this method was also used after several other steps later in this study. Depending on the number of nucleotides in the molecules, the agarose concentration in the gel in this study varied from 0,5% - 0,8% (2000-3000 nucleotides). To visualize the samples a colour substance (GelRed) was added to the gel, before being poured into the electrophorese chamber, and covered with Tris-borate-EDTA 0,5 buffer (TBE). Before the samples were loaded a 10x loading buffer was added. The loading buffer increases the density of the samples and makes them sink to the bottom of the well (not used for AGE of dreamTaq PCR, because the loading buffer is already in the PCR buffer). To know the size of the nucleotides a 2LOG DNA-Ladder (NEB) with a known size was used as a size reference. The gel was run for 35-45 minutes (depending on the size of the sample) at 110 volts. A gel Doc™ EZ imager (Bio-Rad) was used to photograph the gel, to visualize the different bands on the gel.

3.3.3 RNA electrophoresis

To measure the quality of the total RNA the AGE had to be specialized for RNA. The highest percentage of the RNA is ribosomal RNA (rRNA) (>80%), consisting of mainly 28S and 18S subunits, which are mainly what are visible on an agarose gel. The RNA quality was assessed by separating 200 ng of each sample (sample#1 & sample#2) totalRNA in a 0.75% agarose gel.

Next, deionized H_2O, 10x loading buffer and formamide (50% formamide) were added to the samples. Formamide is added to disrupt secondary structures, and to denature the total-RNA, the samples were then heated in a water bath with 70℃ for 10 minutes, before being loaded on to the gel and run for 45 minutes with 110 volts.

3.4 cDNA synthesis

From the quality-controlled RNA blue whale-cDNA was synthesized by using the SuperScript® IV Reverse Transcriptase (Invitrogen) method. Blue whale-cDNA was later used as template in the polymerase chain reactions (PCR) (RNA- complimentary DNA) (Ochman et al., 1988). In the SuperScript® IV Reverse Transcriptase (Invitrogen) kit, there was used a reaction mix of dNTP, Oligo $d(T)_{20}$ primer, mRNA (500ng) and nuclease free water (table18). Oligo $d(T)_{20}$ primer are oligonucleotides made from thymine bases, who can hybridize to the poly-A tail of mRNA. The reverse transcriptase enzyme uses mRNA transcripts as template, and together with oligo $d(T)_{20}$ primers the cDNA strands can be synthesized. The cDNA reaction solution was prepared according to table 13. Prior to addition of the enzyme, RNA was denatured by being heated for 5 minutes at 65°C using a Thermo Cycler (DOPPIO Thermal Cycler with dual 48 well blocks, VWR), then placed on ice for 1 minute.

Table 18 Reaction solution for reverse transcription

Component	**Concentration/Amount**
Total RNA	500 ng
Oligo $d(T)_{20}$ primer	500ng
Deoxynucleoside triphosphate (dNTP) mix	0.5mM
Nuclease-free water	→13 µL

The Reverse Transcriptase reaction solution (RTMix) was made accordingly to table 19 and, combined with the RNA-mix, the mix was then incubated for 30-60 minutes at 50°C, and 10 minutes at 80°C in the Thermal Cycler. The cDNA was used immediately in a PCR reaction or stored at –20°C until further use.

Table 19 cDNA synthesis mix for reverse transcription

Component	Concentration/Amount
5X SuperScript® IV Buffer	4 µL
100 mM DTT	1 µL
SuperScript® IV Reverse Transcriptase	1X (1 µL)

3.5 Amplification of blue whale AhR by polymerase chain reaction (PCR)

When the cDNA-template was successfully synthesized PCRs could be performed. In this thesis, PCR was used to amplify and isolate the genes encoding for blue whale AhR and ARNT. Every cycle included three steps:

1. Denaturation; at 98°C to break any hydrogen bonds between the base pairs.
2. Annealing; to allow the primers to anneal the template strands by forming hydrogen bonds the temperature was lowered.
3. Extension; the temperature was then raised to the optimum temperature of the DNA-polymerase, to allow the primer sequences to facilitate the starting point of where DNA-polymerase should assemble the dNTP

The first fragment of AhR (AhR_{start}=nucleotide 1-1551) had already been amplified by PCR using blue whale-cDNA as template. To complete the full blue whale AhR-sequence (AhR_{Total}=nucleotide 1-2563) an overlapping fragment AhR_{end} (AhR_{end}nucleotide 1542- 2563) consisting of an overlapping fragment of AhR_{start} & AhR_{end} in addition to the remaining nucleotides. AhR_{end} was amplified in the same manner AhR_{start}.

The PCR reactions were set up and run in thermal cycles according to the producer's instructions, using a Thermal Cycler (DOPPIO Thermal Cycler with dual 48 well blocks, VWR)(Table 20).

Table 20 PCR thermal cycle program.

Cycles		Temperature	Duration
	Initial denaturation	95°C	2 minutes
35	Denaturation	95°C	30 seconds
	Annealing	55°C	30 seconds
	Extension	72°C	1 minute/kb
	Final extension	72°C	5 seconds

3.6 Purification of PCR products by gel extraction

To make sure that the PCR resulted in the desired product, the PCR products were separated and analysed by AGE (3.3.2). The DNA fragments where made visible by using UV-light from a chromate-vue TM-20 transilluminator (UVP, San Gabriel). The bands of the expected size for the desired product was cut out of the gel and purified using NucleoSpin® PCR and Gel clean-up kit (Macherey-Nagel) according to the producer's instructions.

3.7 Molecular cloning

To replicate large amounts of recombined DNA (rDNA) *in vivo,* a plasmids ability to replicate separately from bacterial chromosomal DNA was used to transform rDNA into a prokaryotic host cell. The rDNA was constructed *in vitro* by combining the PCR products with a cloning vector using StrataClone Blunt PCR cloning kit (Agilent), based on the producer's instructions.

3.7.1 PCR cloning

The first step in the cloning process was to ligate the AhR fragments into the StrataClone Blunt PCR Cloning Vector with a Topoisomerase I ligase, forming phosphodiester bonds creating a linear vector^{ori}AhR vector$^{amp/kan}$(Fig 7)(Table 21).

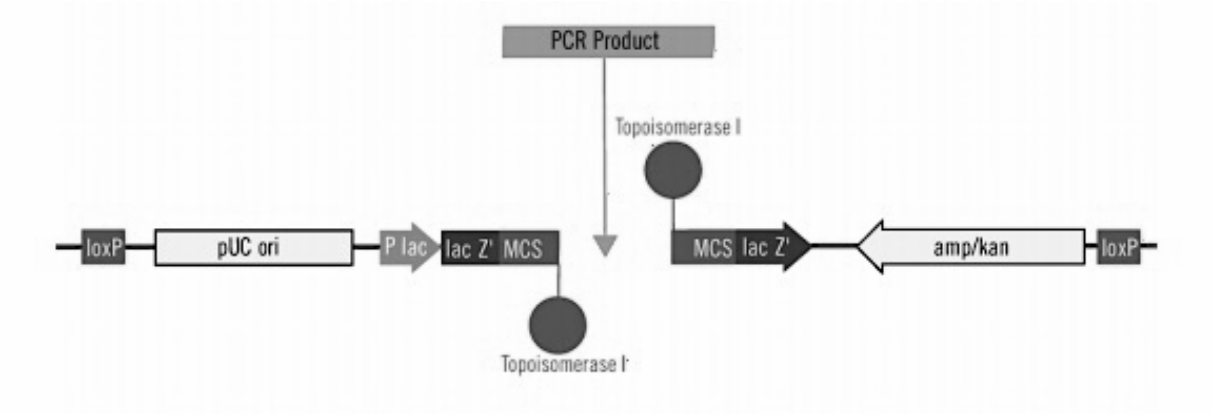


Figure 11 Linear Strata Clone Blunt PCR Cloning Vector (pSC-Bamp/kan). The AhR PCR product is ligated into the cloning vector between two multiple cloning sites. Source: StrataClone Blunt PCR cloning kit (Agilent) Manual.

Table 21 StrataClone Blunt PCR cloning kit (Agilent) – Ligation mix

Component	Volume
StrataClone Blunt Cloning Buffer	3.0 µL
Purified PCR product	0.5 µL
StrataClone Blunt Vector mix	1.0 µL

The buffer and the vector mix were added together in a master mix, before the PCR template. This was then incubated at room temperature for 5 minutes and subsequently put on ice.

3.7.2 Transformation of E. coli

To transform the StrataClone solo pack competent Strataclone-*E.coli*, with the PCR products from the ligation mixture a heat-shock procedure was conducted. In this method, transformation reactions were heat-shocked 42°C for 45 seconds and then transferred directly on ice, causing changes in the fluidity of the cell membrane enabling bound DNA to be taken up in the cells. Prior to being used the StrataClone-*E.coli* cells had been treated with a salt solution to make them susceptible for extracellular DNA, they are also transiently expressing Cre recombinase, which mediates the recombination of the linear StrataClone Blunt PCR Cloning Vector (pSC-Bamp/kan) into a circular plasmid (Figure 12).

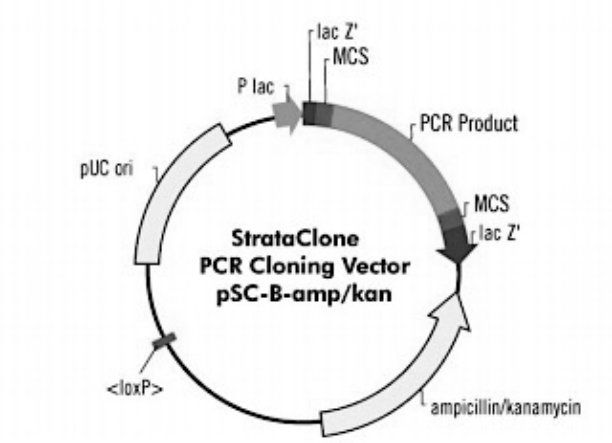


Figure 12 Circular StrataClone Blunt PCR cloning vector (pSC-B-amp/kan). Source: StrataClone Blunt PCR cloning kit (Agilent) Manual.

The heat-shocked bacteria were added 150 µL pre-heated (42°C) SOC-media and incubated for 1 hour at 37°C at 300 rpm, before being plated onto selective lysogeny-broth-agar (LB-Agar) containing ampicillin (0.1 mg/mL) in petri dishes. The plates were then incubated at 37°C for

24 hours.

3.7.3 Identification of positive transformants by PCR screening

After the plating and incubation of the transformed cells, single colonies were grown on LB-media containing ampicillin to ensure that only the bacteria that had acquired recombined plasmid DNA from step 3.7.2. (and thus, containing the ampicillin-gene making the antibiotic-resistant) survived. Even though the cells contained the plasmid, it is not certain that they contained the insert of interest. It is for instance possible that the vector re-ligated without the DNA. To control for this, a PCR screening of single colonies with primers that bind to the cloning vector of each side of the insert was conducted with DreamTaq DNA polymerase (Thermos Scientific) according to table 22. The colony PCR-products where evaluated with AGE (Method 3.3.2), and the colonies containing the cells with the desired DNA-fragment where selected and inoculated in liquid LB-media (with 0.1 mg/ml ampicillin) at 37°C for 24 hours at 250 rpm, before the plasmids where purified (Method 3.10) and verified by sequencing (Method 3.11).

Table 22 Reaction solution for PCR screening of colonies using DreamTaq DNA polymerase

Component	**Concentration/Amount**
Template	Appr. 1-2 colony
dNTP	200 µM
Forward and reverse primer	0.5 µM
DreamTaq buffer	1X
DreamTaq DNA polymerase	5 U/µL
Deionized water	→20 µL total volume

The PCR screen was run in thermal cycles (table 23) using a Thermal Cycler (DOPPIO Thermal Cycler with dual 48 well blocks, VWR).

Table 23 Colony PCR thermal cycler program

Cycles		**Temperature**	**Duration**
	Initial denaturation	95°C	2 minutes
35	Denaturation	95°C	30 seconds
	Annealing	55°C	30 seconds
	Extension	72°C	1 minute/kb

3.8 Small-scale plasmid purification

After the PCR cloning and before sequencing and construction of plasmids, a purification of plasmid DNA was performed according to the producer's protocol. In this thesis, small-scale plasmid preparation (mini prep) was used (NucleoSpin® Plasmid easy pure kit). The cells where then lysed to destroy the cells and desaturate DNA. Further a neutralizing buffer was added to the lysate, to separate cell wall debris, and desaturated chromosomal DNA from the bacteria, and proteins from the plasmid-DNA (that has regained a double-twisted configuration making it possible to separate it from other cell components). To ensure the binding of plasmid-DNA to the silica membrane, the membrane was washed with a buffer supplemented with ethanol (EtOH). This also removes leftover lysate and other impurities. The plasmidDNA (pDNA) was then released from the membrane and eluted by adding an elution-buffer. The concentration of the pDNA was then measured spectrophotometrically with Nanodrop1000 ($A_{260nm}/A_{260/280}$-ratio, Thermo Scientific).

3.9 Sequencing

The cloned and purified plasmid DNA was then sequenced by the sequencing facility at the department of biological sciences, University of Bergen. This laboratory uses an automated Sanger DNA- sequencing with a 3730XL analyzer (Applied Biosystems^{TM}). The sequencing is based on amplifying the DNA of interest by using a mix of deoxynucleotides and fluorescence labelled dideoxynucleosides (dNTPs). The dNTPs lack a 3`-hydroxyl group required for elongation of DNA-molecule and insertion of dNTPs causes the polymerization to terminate. A random adding of dideoxynucleosides creates fragments of different sizes that are separated by electrophoresis and detected by using fluorescence. The dNTP finalizes every fragment, and the nucleotides position can be decided based on the size of these fragments. It is possible to sequence approximately 900 base pairs in each reaction, if the fragment is longer several primers had to be used. Before being delivered to the sequencing laboratory for further analysis, the purified plasmids from 3.8 where amplified according to the Big-Dye terminator v 3.1 protocol described in table 24 and run in thermal cycles (DOPPIO Thermal Cycler with dual 48 well blocks, VWR) (table 24 & 25).

Table 24 BigDye terminator v 3.1 protocol for DNA-sequencing.

Component	Concentration/Amount
DNA template	200 ng
Big-Dye sequencing buffer	1X
Forward and reverse plasmid/fragment specific primers	3.2 pmol
Big-Dye 3.1	1 µL
Deionized water	→10 µL total volume

After the reaction 10 µL of deionized water was added to the reactions, before sequencing was performed.

Table 20 Thermal cycle program for DNA-sequencing

Cycle		Temperature	Duration
	Initial denaturation	96°C	5 minutes
35	Denaturation	96°C	10 seconds
	Annealing	50°C	5 seconds
	Extension	60°C	4 minutes

3.10 Measuring activation of TFs by luciferase assay

When the desired receptors had been successfully cloned and sequenced, I continued with measuring the transcriptional activation of AhR, GR, THRB and PPARG. A luciferase gene reporter assay was used to study all the different transcription factors, but there are a few differences between the assays used with AhR compared to the assays used with the NRs.

3.10.1 Measuring activation of NRs by GAL4-UAS based luciferase assays

To study blue whale GR, THRB, and PPARG ability to be activated by different environmental contaminants was tested *in vitro* in a COS-7 simian kidney cell line, with a UAS/GAL4-DBD based luciferase reporter gene assay. The COS-7 cells are co-transfected together with a luciferase reporter plasmid, that is further regulated by a thymidine kinase promoter together with a Gal4-Upstream activation sequence, in addition to the desired receptor (GR, THRB, and PPARG) (Paguio, Stecha, Wood, , Fan, 2010) (Figure 13). Luciferase activity was measured as luminescence and reflect the level of transcription activity induced by the test compounds

via the different nuclear receptors in the transfected cells. The amount of luciferase is thereby dependent by the degree of TF activation (Brasier et al., 1988).

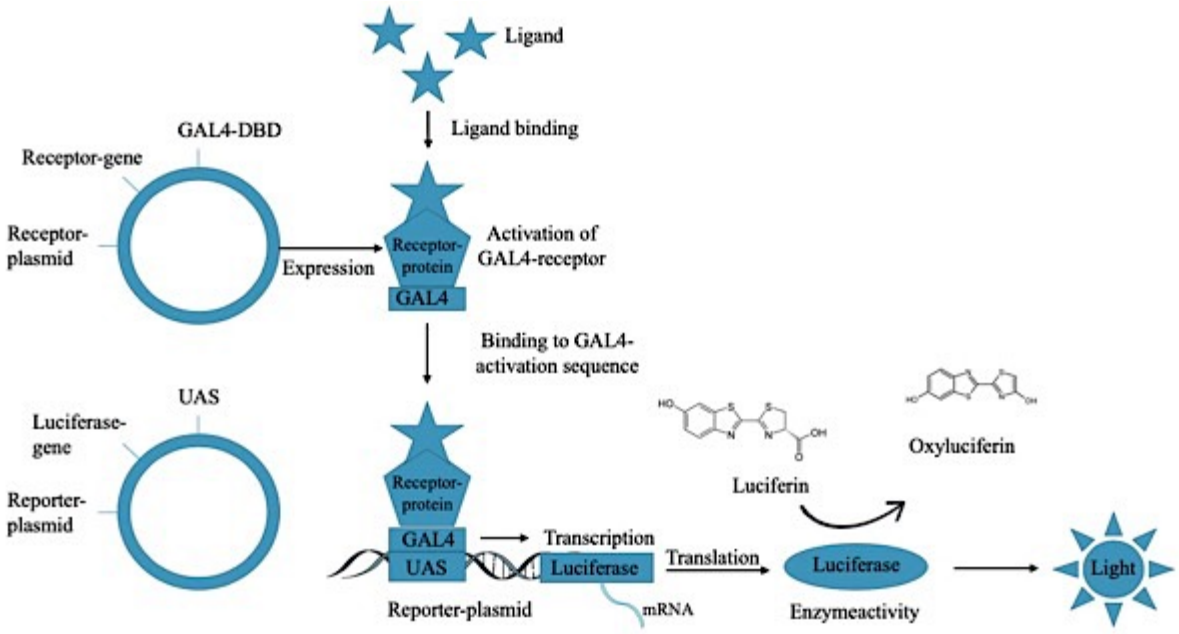


Figure 13 Schematic overview of the principle behind ligand activation experiments using the Gal4-DBD/UAS-system. Plasmids with reporter-gene (luciferase) and receptor-gene (GR, THRB & PPARG) are transferred to COS-7 cells by transfection. The resulting fusion protein of GAL4-DBD and NR-LBD can be activated by ligand binding. Gal4-DBD binds to UAS located upstream from the luciferase-gene, which induces the expression of luciferase. Luciferase catalyses the transformation of luciferin to oxyluciferin, producing light that can be quantified.

3.10.2 Measuring activation of AhRs by luciferase assays

Unlike the Gal4 system where only the hinge and LBD of the desired NR is used, the AhR system uses the entire receptor. The reporter plasmid is also a bit different, instead of having a response element with an UAS (upstream activation sequence) that is recognized and binds to Gal4-DBD (DNA binding domain) the AhR- system contains a promotor with DRE-response elements, in addition to ARNT. Even though the Gal4-system is able to demonstrate activation of NRs, the AhR- system is closer to the *in vivo* situation due to its utilization of the entire receptor and not just the hinge-LBD.

3.10.3 Cultivation of COS-7 cells

The first step to establishing the luciferase gene reporter assays is to cultivate COS-7 cells. The cells used in this study had been stored in a freezing media in liquid nitrogen until used. After the cells were thawed, and 10mL growth media was added (DMEM-10% FBS, table 3) before they were centrifuged at 250xg for 5 minutes to remove dimethyl sulphoxide from the freezing

medium. Next, the excess media was removed, and the pellet resuspended in 10 mL growth media, and finally seeded out on to cultivation plates before incubation 37 °C in 5 % CO_2.
When the cells had a confluency between 70-80% they were split. The sub-culturing of the cells was done by removing the growth media, washing with 1X PBS (pH 7.4) twice, and treating the cells with trypsin-EDTA (0,05 % trypsin, 0,02 % EDTA) for 1 minute at RT. The trypsin was then removed, and the cells incubated at 37 °C in 5 % CO_2 for 5 minutes. The trypsination dissociates the cells from the bottom of the petri dish enzymatically, this allowed us to resuspend the cells in fresh growth media and split them into new petri-dishes with the desired dilution, which in this study was 1:20 for GR, THRB and PPARG, and 1:20 for AhR. All handling of the COS-7 cell cultures were done implementing sterile techniques.

3.10.4 Cytotoxicity assay

To make sure that the different compounds tested did not have cytotoxic effects on COS7 membrane integrity and metabolic activity, cytotoxicity assays were preformed according to the method developed by Schreer et al. (Schreer et al., 2005). The membrane integrity was measured using fluorogenic dye 5-carboxyfluorescein diacetate (CFDA-AM, Sigma Aldrich), and metabolic activity was measured by the fluorescence indicator dye resazurin. As a positive control in the cell viability assay Triton X-100 a non-ionic detergent that solubilizes membrane proteins was used, a chemical known to be cytotoxic for mammalian cells. Three independent experiments with three replicates per chemical was performed.

3.10.5 Luciferase reporter gene assay - seeding of COS-7 cells

The COS-7 cells cultivated in 3.10.3 where harvested at 70-80% confluency through trypsinization as described above (3.10.3), before being resuspended in 10 mL fresh growth media. The cell density was determent by counting the cells in a hemocytometer (Marienfield) under a microscope (Leica DM IL inverted microscope). A mix of 100 µL trypan blue (an azo dye to colour the cells) and 100 µL cell suspension was loaded onto the hemocytometer to determine the cell density and the further dilution to obtain the desired number of cells (10000 cells/well total). 100 µL of growth media with a known (0.1 cells/mL) cell density was added to each well in 96 well plates, and subsequently incubated at 37°C in 5 % CO_2 for 24 hours.

3.10.6 Luciferase reporter gene assay – transient transfection of COS-7 cells

After the cells 24h incubation the desired plasmids were transfected into the cells. During a transfection exogenous DNA is introduced into a eukaryotic cell, but it is not incorporated into

the cell's genomic DNA and will therefore only remain there for a couple of days. Due to this the luciferase reporter gene assay was terminated the second day after transfection. The transfections were performed essentially as described by the producer with the following adaptations:

After the 24 hour incubation, the growth media was removed, and the cells where added a mix of reduced media (Opti-MEM I) and DMEM-10%FBS, in addition to plasmid (receptor-plasmids used in this study were constructed by Lene Øygarden and Roger Lille-Langøy in our lab (Table 7)) and transfection reagent (TransIT-LT1 (Mirus Bio))(Table 21). The transfection reagent is made from a mix of proteins, lipids and polyamines that facilitate transport of plasmid-DNA over the cell membrane. The relationship between the receptor plasmid and reporter plasmid 1:20 (Table 7&8 overview of the plasmids used in the luciferase assays). The plasmid-DNA was added TransIT-LT1 reagent and diluted with Opti- MEM I, before being incubated for 30 minutes at room temperature, then added DMEM-10% FBS. After adding the transfection mix and the growth media the cells where incubated at 37°C in 5 % CO_2 for 24 hours.

Table 21 Transfection mixture protocol using TransIT-LT1

Component	Amount per well (96 well plate)
Opti-MEM I	9.0 µL
Plasmid mix [1000ng/ µL]	0.1 µL
TransIT-LT 1	0.2 µL
Cell growth media (table 2)	92 µL

The plasmid mix was created based on the relationships described above and the plasmids concentration (Materials Table 8).

3.10.6.1 Reporter and control plasmids

In this study the reporter plasmids mh(100)x4 tk luc (GR, THRB, PPARG) and PGudLuc6.1 (AhR) was used (Azam et al., 1995) (table 7), in addition to the control plasmids (pCMV-β-Gal). the reporter and control plasmids were prepared from glycerol stock solutions (kept at -80 °C) of previously transformed *E. coli* cells. Overnight cultures were made, and plasmids purified the next day through midi-prep (3.10).

3.10.7 Luciferase reporter gene assay – Exposure to test compounds

The transfection was terminated (after 24 hours) by the removal of the transfection media and addition of exposure medium. All the different exposure medias consisted of test compounds

(Table 22 & 23) dissolved in DMSO and diluted by desired concentration in DMEM-10% csFBS (Charcoal stripped fetal bovine serum (has had non-polar materials removed Thermofisher, without phenol red). The concentrations of the control agonists for GR, THRB and PPARG are shown in table 22.

Table 22 An overview of the different concentrations of the control agonists used in the exposure studies of blue whale GR, THRB, and PPARG. The table represents a 96 well plate, three replicates of each known agonist were used.

	GR			**THRB**			**PPARG**		
	Dexamethasone (nM)			T3 (nM)			ROSI (nM)		
Row→	1	2	3	4	5	6	7	8	9
A	200			120			50000		
B	100			60			25000		
C	20.0			12			5000		
D	4.0			2.4			1000.0		
E	0.8			0.5			200.0		
F	0.16			0.1			40.0		
G	0.032			0.02			8.0		
H	DMSO			DMSO			DMSO		

To dilute the ligands a dilution line A-G was made in a 96 well plate with five times dilution between each row. The last row (H) was used as a non-exposed control and contained only DMSO. Row A-H was made with a 2x concentration of the final concentration of ligand and DMSO. The 2X ligand solutions were diluted 1:1 in the wells of the culturing plates. The final concentration was made by adding 100 µL from row the dilution in row A to100 µL DMEM-10% csFBS in every well on the cell plate. The cells where then exposed at 37°C in 5 % CO_2 for 24 hours.

The concentrations of the different compounds and synthetic POP mixtures used in this study is listed in Table 23 and were based on chemical analysis of blue and fin whale blubber conducted by Tartu et al. (Tartu et al. in preparation, 2019).

Table 23 An overview of the different compound-concentrations used in the exposure studies of blue whale GR, THRB, and PPARG. Three replicates of each compound were used.

PP`DDT (nM)	PP`DDE (nM)	PP`DDD (nM)	POPs-mix (nM)	DEHP (nM)	DINP (nM)
50000	50000	50000	200	50000	50000
25000	25000	25000	100	25000	25000
5000	5000	5000	10	5000	5000
1000	1000	1000	2	1000	1000
200	200	200	1	200	200
40	40	40	0.5	40	40
8	8	8	0.1	8	8
DMSO	**DMSO**	**DMSO**	**DMSO**	**DMSO**	**DMSO**

DMSO = 0mM of the test compound

3.10.8 Luciferase reporter gene assay – Luciferase and β-galactosidase measurements

After 24h the exposure media was removed, and 125 µL non-denaturation lysis reagent was added to the wells (table 7). The lysis solution inhibits protease activity, dissolves cell membranes, and stabilizes proteins. The well-plate was then incubated for 30 minutes at room temperature on a "shaker", before 50 µL lysate was transferred onto two different 96 well plates, one transparent (Nunc^{TM}) and one white (Nunc^{TM}). The transparent plate was used to measure absorbance and the white plate for luminescence. The luminescence plates where added 100 µL luciferase-reaction solution (table 11) to each well, and luminescence measurements were carried out immediately using EnSpire 2300 Multilabel Rader (PerkinElmer). The plates used for absorbance were added 100 µL β-Gal-reaction solution (table 9) to each well and incubated at room temperature for approximately 20 minutes until the solution turned yellow, the absorbance in the wells were then measured at 420 nm were carried out immediately using EnSpire 2300 Multilabel Rader (PerkinElmer).

3.10.8.1 Quantification of luciferase activity

In this study the enzyme luciferase is utilized to help measuring the activity of the desired receptors. Luciferase is an enzyme that can be found in several places in nature, from fireflies to jellyfish (Jones, 1999; Oba et al., 2012). It is often used in *in vitro* cell experiments as a reporter gene. The luciferase enzyme used in this study catalyses the transformation of luciferin to oxyluciferin by using ATP and O_2. The light (560nm) created by this reaction was measured luminometrically, by using an EnSpire 2300 Multilabel Reader (Perkin Elmer). In this study

the plasmids mh(100)x4 tk luc (GR, THRB, PPARG) and PGudLuc6.1 (AhR) was used (Azam et al., 1995) (Table 13).

3.10.9 Quantification of β-galactosidase

To correct for differences in transfection efficiency between wells in the luciferase assays, the mh(100)x4tk luc and pCMX-GAL4-DBD-GR/THRB/ PPARG plasmids where co-transfected with pCMV-β-GAL- plasmids, which contains the gene encoding for β-galactosidase (β-Gal). The reaction catalysed by β-gal results in the cleavage of ortho-nitrophenyle-β-galactosidase (ONPG) into ortho-nitrophenol from and galactose, the latter of which has a yellow colour that can be quantified spectrophotometrically by measuring the absorbance at 420 nm. The measured values from the luciferase activity was normalized by dividing it on the absorbance from β-gal activity.

3.11 Data analysis and statistics

The values from the luciferase assays were normalized for each well by dividing the luciferase activities by the corresponding β-Galactosidase activities, this was done to account for differences in transfection efficiency. Furthermore, the fold activation value was calculated by dividing the normalized luciferase signal for each well, with the average of the control samples (containing only vehicle solution). By doing this the resulting value could be denoted as fold induction in ligand induced luciferase activity compared to the solvent control. GraphPad Prism8 was then used to visualize graphs displaying the difference in fold change in luciferase activity, caused by each tested ligand at different concentrations, also including the standard error of the mean. To calculate the significant fold induction in the means of the different test concentrations over the control means a paired T-test was used.

3.12 Sequence analysis

The coding nucleotide sequence for blue whale AhR was translated to the protein coding sequence by using the online translator ExPASy (“ExPASy - Translate tool,” n.d.) (Swiss Institute of Bioinformatics, Resource Portal). The protein sequence was then compared with other available species from Enseml and Uniprot (“Ensembl” n.d.; “UniProt,” n.d.), and to compare the different sequences Clustal Omega (“Clustal Omega -EBI,” n.d.; Thompson et al, 1997 was used with standard settings. This website is an online internet-based program used for multiple sequence alignment and can be used to compare large dataset. To visualize the alignments and create phylogenetic trees JalView (3.12.1) together with MEGA 7 was used.

3.12.1 Construction of phylogenetic tree

MEGA 7 was used to construct the phylogenetic tree in this study. Sequences were obtained and compared as described above (Method 3.12), before a neighbour-joined tree was constructed in MEGA 7.

3.13 Student contribution

I participated in the blue whale blubber sample field work at Svalbard in October 2018. I fixed the samples with RNA later before I performed the RNA extraction. I conducted all the steps described in the methods, (unless otherwise specified) except for the chemical analysis (conducted by Tartu et al.2019 in preparation, NILU) and the sequencing chromatography (conducted by the sequencing facilities at UiB). I conducted the statistical analyses and the multiple sequence alignments. Additionally, I made a poster of the key findings in this thesis and the project it is a part of at two conferences: NSFT (Norsk Selskap for Farmakologi og Toksikologi) Winter Meeting 2019, Beitostølen, January 2019 and PRIMO20 (20th Pollutant Responses in Marine Organisms Symposium), Charleston, SC, USA, May 2019. At PRIMO20 I also gave a podium presentation of our work (See Appendix IV). At PRIMO2019 I also gave a podium presentation of our work.

4 Results

In this chapter the results obtained in this thesis will be presented. The results are based on the methods described in chapter 3.

4.1 Molecular cloning of blue whale AhR

The first step to enable ligand activation studies of AhR is molecular cloning as described in the Methods 3.2-3.9.

4.1.1 RNA isolation from blue whale blubber

The quality and integrity of the RNA extracted from the blue whale sample appeared to be adequate, as both 28S and 18S ribosomal were present (Figure 14). The concentration of the total RNA was measured with Nanodrop1000 (A_{260nm}, $A_{260/280}$-ratio, Thermo Scientific) and listed/reported in Table 24.

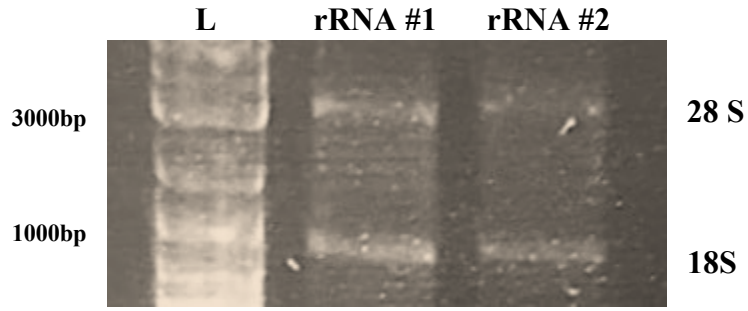


Figure 14 Assessing the integrity of blue whale RNA using agarose gel electrophoresis. Total RNA extracted from blue whale blubber tissue was separated on a 0.5X TBE 0.7% agarose gel stained with GelRed. The subunits for the different sedimentations are indicated at 28S and 18S, the length on the DNA ladder **L** (standard 2log DNA ladder (50ng/µL)) is indicated at 3000 and 1000 base pairs, and the different blubber tissues are labelled.

Table 24. Spectrophotometric measurements of extracted total RNA from blue whale adipose tissue.

Sample	Concentration (ng/ µL)	A260/280	A260/230
Blubber 1	377.6	1.94	1.61
Blubber/skin 2 (with skin)	714.3	1.98	1.60

An A260/280 relationship of 1.94 and 1.98 indicates that the RNA-samples are free of contamination of proteins. An A260/230 relationship of 1.61 and 1.60 illustrates some contamination of phenol and chaotropic salts. Despite of this the RNA- samples were of sufficient quality so a downstream cDNA synthesis was conducted.

4.1.2 cDNA synthesis and PCR amplification

Parts of the AhR nucleotide sequence had previously been cloned and validated by Lene Øygarden in our lab. The remaining fragment of blue whale-AhR sequence (1870 to 2629 bp) was still unknown. The complete AhR sequence could not be amplified in one piece and was therefore amplified in two fragments (Methods 3.7). The two different fragments of blue whale AhR were amplified by PCR from the cDNA template and subcloned in *E.coli* (methods 3.9). An overview of the different fragments amplified is shown in Figure 15.

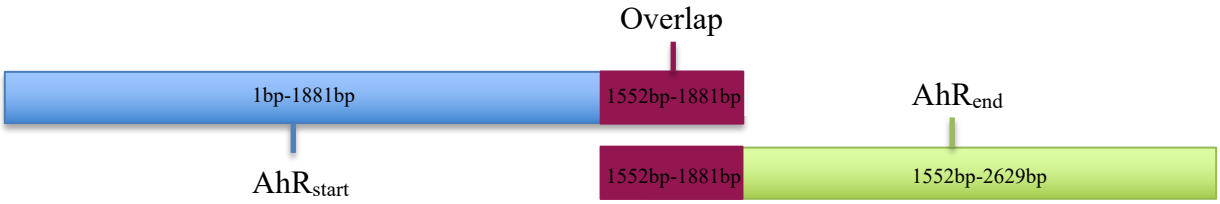


Figure 15 The full sequence of blue whale AhR. Two fragments (blue and green) of blue whale-AhR were amplified separately, but with an overlapping part (pink) in the end of the blue fragment and in the beginning of the green fragment. The blue and pink part is further referred to as AhR_{start}, while the pink and green part is referred to as AhR_{end}.

Blue whale AhR was amplified by PCR (Methods 3.5) from cDNA (Methods 3.4) where the resulting PCR products were evaluated by AGE. As seen in Figure 16, the migration of the amplicons corresponded well to the expected size of the fragments (AhR_{start} 1881 bp, AhR_{end} 1012 bp) based on known *AhR* sequences from minke whale (NCBI accession number for minke whale *AhR:* XP_007164937, 2571bp (857kDa)).

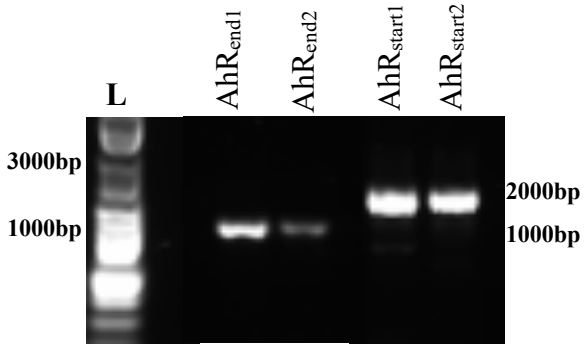


Figure 16 Agarose gel electrophoresis of AhR fragments amplified from blue whale blubber cDNA. To visualize the DNA fragments an 0.5 TBE, 0.7% agarose gel, stained with GelRed was used. The expected lengths of the different fragments were AhR_{start} 1869bp, and AhR_{end}= 1087. The ladder used was a standard 2log DNA ladder= L. On this gel 3 µL ladder (50ng/µL), and 2 µL PCR product was used.

4.1.3 Cloning of blue whale AhR and colony screening

The gel extracted PCR products were ligated into a pSC-A cloning vector and transformed into competent *E.coli* cells. The single colonies that contained plasmid with products of the expected length (positive transformants) could be separated from those not containing the plasmid through two steps:

1. Seeding the cells on an agar plate containing ampicillin, then incubating at 37°C overnight ensuring that presence of ampicillin.
2. Positive transformant were identified by PCR-screen using vector specific primers (see Methods 3.7.3). The screening products were then separated by AGE, where some colonies appeared to hold pSC-A plasmids with *AhR* fragments (AhR_{start}>2000bp, AhR_{start}>1000bp) (figure 17).

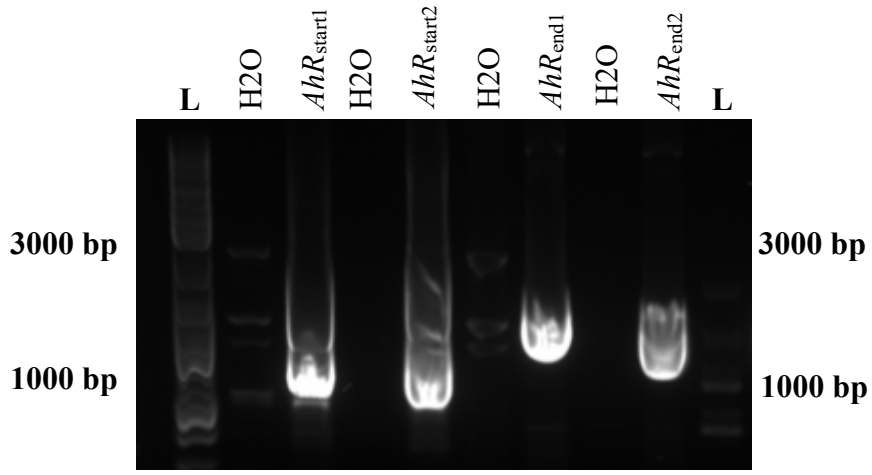


Figure 17 PCR screening of positive transformants containing *AhR* fragment 1 and 2. To visualize the colony PCR amplifications a 0.5 TBE, 0.7% agarose gel, stained with GelRed was used. The ladder in this AGE was a 2log DNA ladder = L, 150 ng ladder, and 2 µL PCR product or water was used.

The colonies $AhR_{star1\&2}$ and $AhR_{end1\&2}$ (Figure 17) were then extracted from the positive transformed bacteria by mini-prep (Methods 3.8), and further sequenced to verify insertion of the AhR fragments (Methods 3.9). The products from the sequence reactions were analysed by the sequencing facility at UIB, and the sequences showed that the PCR products contained AhR sequences, according to the minke whale (NCBI accession number Minke-*AhR*= XM_007164875, 5878 bp) found in NCBI database.

4.1.4 Sequencing and analysis

The two blue whale-*AhR*-fragments were compared to minke whale *AhR* by multiple sequence alignment (MSA). The cloned blue whale AHR nucleotide sequences were translated into protein sequences *in silico* using ExPASy translate tool and were then aligned in Clustal Omega (EMBL-EBI). The sequences were then visualized in Jalview (Method 3.12. & 3.12.1) (Figure 18). Minke whale sequence appear to be an incomplete sequence with two "X", when compare to other species the "X" seems to be replaced by proline (Appendix III). All of the important amino acids for DNA binding and ligand binding of *AhR* in minke whale is conserved in blue whale. In addition to the identical AA the basic helix loop helix, PAS and ligand binding domain is conserved between the two species. There are also some differences in the sequence, six AA present in minke whale sequence, are not present in the blue whale sequence. These AA are outside the important domains, but their function is not yet known. Due to the high level of identity between minke and blue whale, it is assumed that the cloning of blue whale-*AhR* was successful.

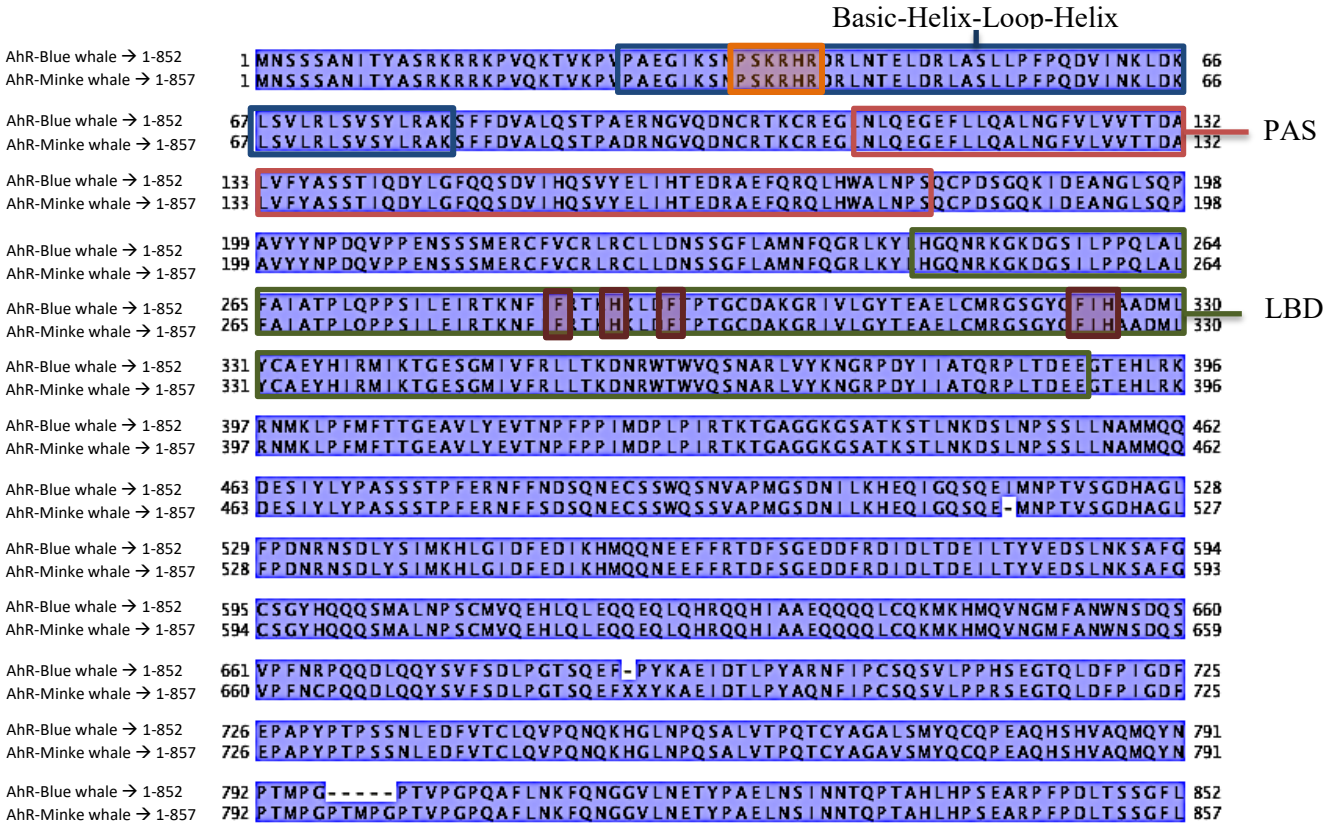


Figure 18 Sequence alignment of blue whale *AhR* and minke whale *AhR*. The encoding genes for minke whale *AhR* was retrieved from NCBI genome database (NCBI accession number minke-*AhR*= XM_007164875, 5878 bp). The sequences were visualized with Jalview (2.10.5), where blue whale and minke whale had a 99,2% identity. The conserved amino acids are marked in blue, and the division of the ligand binding domain (LBD), the PAS-domain and the Basic-helix-loop-helix domain was done

after (Andreasen et al., 2002). Amino acids that are important for DNA-binding are marked in orange, and amino acids important for ligand-binding of TCDD are marked in purple (Bacsi & Hankinson, 1996; Swanson & Yang, 1996).

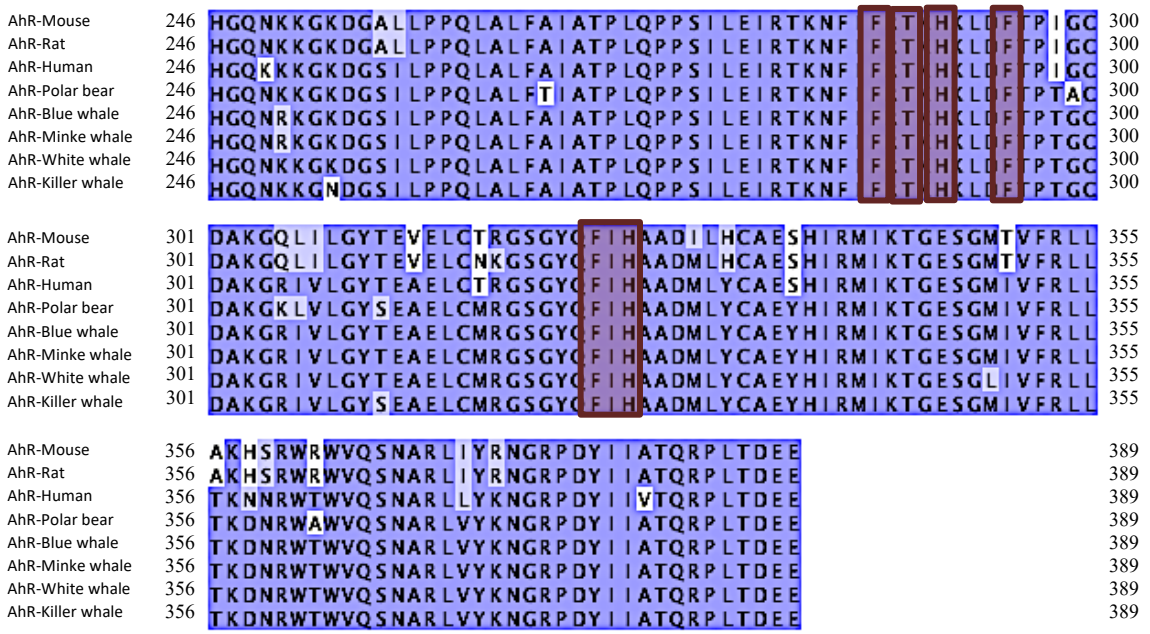


Figure 19 Multiple sequence alignment of *AhR*-LBDs. The encoding genes for the various*AhR*-LBDs were retrieved from NCBI genome database (NCBI accession number minke-*AhR*= XM_007164875, Human-*AhR*= NM_001621.5, Mouse-*AhR*=NM_013464.4, Rat-*AhR*= NM_013149.3, Polarbear-*AhR*= XM_008686532.1, White whale-*AhR*= XM_022587570.1, Killer whale-*AhR*= XM_004263467.2), or cloned from blue whale blubber. The conserved amino acids are marked in blue, and the amino acids important for ligand binding to TCDD are marked in red (Bacsi et al., 1996; Pandini et al., 2009; Swanson et al., 1996).

In mammalian AhR the amino acids P35, S36, K37, R38, H39, R40 have been shown to be essential for AhR to bind to the response element (Bacsi & Hankinson, 1996; Swanson & Yang, 1996). These amino acids were conserved in all the different species that were compared (Figure 19). In the ligand binding domain, there are several specific amino acids that in previous studies have been shown to be important for ligand binding of TCDD. In mammals these are F40, H44, F48, F79, I80, and H81. In all the AhR-sequences that were analysed in this MSA the important amino acids for ligand binding of TCDD were identical (Bacsi & Hankinson, 1996; Swanson & Yang, 1996). Because the important AA for ligand binding in AhR were identical between all the compares species it is expected that they would be activated in the same way. There are however also some differences between the ligand binding domain sequences. Most of the differences are between the marine mammals and mouse, rat and human.

The marine mammals have less differences in AA than e.g. human compared to the marine mammals.

The sequences of GR, THRB and PPARG blue and fin whale LBD, had previously been determined by Lene Øygarden in our lab. The cloned sequences were in this study compared with other species in an MSA. GR was compared with minke whale (*Balaenoptera acutorostrata scammoni*), white whale (*Delphinapterus leucas*), human (*Homo sapiens*), mouse (*Mus musculus*), killer whale (*Orcinus orca*), rat (*Rattus norvegicus*), and polar bear (*Ursus maritimus*) (Figure 20). The alignment showed that the LBDs of blue and fin whale GR were identical, in addition to a high degree of evolutionary conservation when compared to other species. Blue whale and fin whale-GR was identical to minke whale, white whale and Killer whale, as well as showing a high similarity to white whale, human, polar bear, mouse and rat (99.6%, 96.0%, 95.6%, 94.0%, 95.2% identity, respectively)

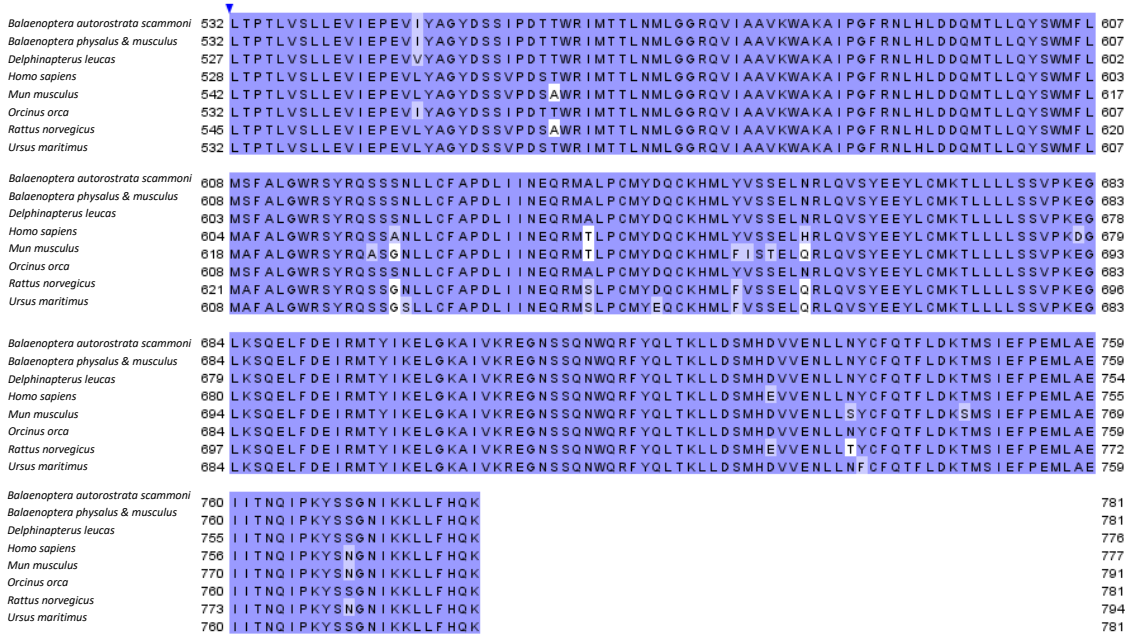


Figure 20 Multiple sequence alignments of GR LBD. The encoding genes for the various GR-LBDs were retrieved from NCBI genome database (NCBI accession numbers: minke whale (*Balaenoptera acutorostrata scammoni*)= XP_007194225.1, white whale (*Delphinapterus leucas*)= XP_022445700.1, human (*Homo sapiens*)= BAH02307.1, mouse (*Mus musculus*)= ABF57998.1, Killer whale (*Orcinus orca*)= XP_004280264.1, rat (*Rattus norvegicus*)= AAL66772.2, and polar bear (*Ursus maritimus*)= XP_008689674.1) or previously cloned by Lene Øygarden. The sequences were visualized with Jalview (2.10.5), where the LBD of fin whaleGR was identical minke whale, white whale and Killer whale.

In similarity with blue and fin whale-GR, blue whale and fin whale-THRB LBD was also compared with minke whale (*Balaenoptera acutorostrata scammoni*), white whale

(*Delphinapterus leucas*), human (*Homo sapiens*), mouse (*Mus musculus*), killer whale (*Orcinus orca*) and rat (*Rattus norvegicus*) (Figure 21). Blue and fin whale-THRB were identical and had a 99.83% identity with minke whale, as well as killer whale, white whale and human.

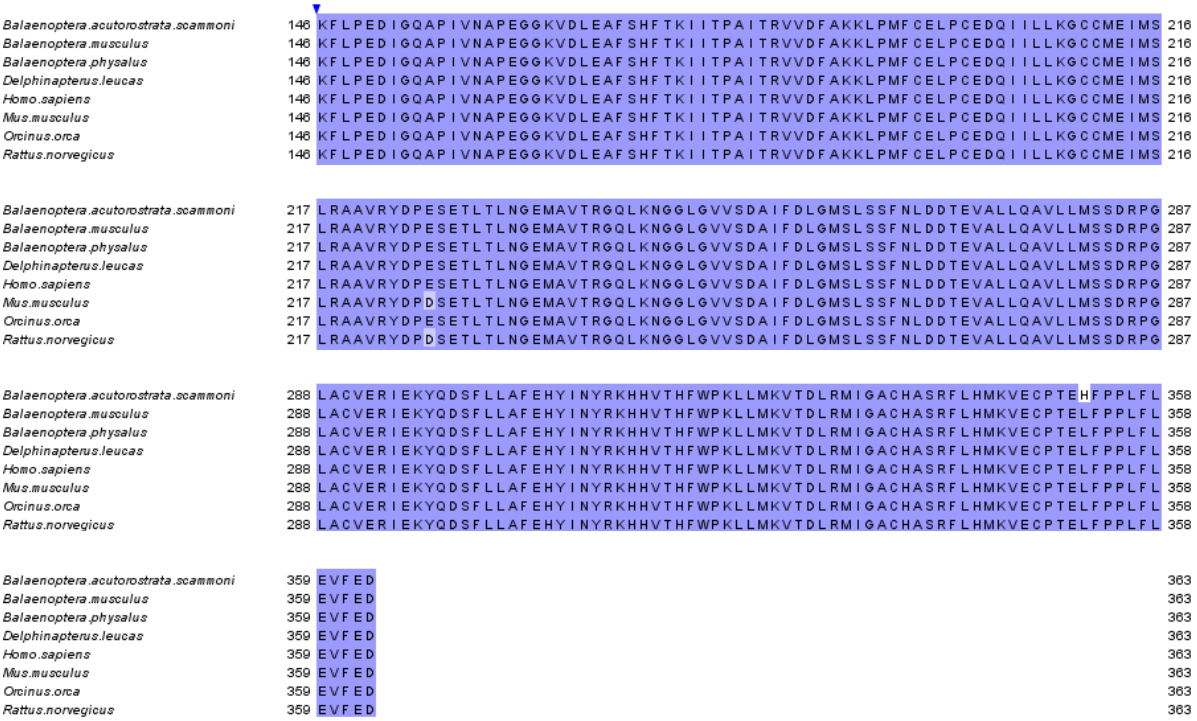


Figure 21 Multiple sequence alignments of THRB LBD. The encoding genes for the various THRβ-LBDs were retrieved from NCBI genome database (NCBI accession numbers: minke whale= XP_007173928.1, white whale=XP_022410384.1, human=NP_001341644.1, mouse=XP_011243047.1, Killer whale= XP_012389258.1 and rat NP_001257783.1. The sequences were visualized with Jalview (2.10.5), where and blue whale (*Balaenoptera musculus*), fin whale (*Balaenoptera physalus*) had a 100% identity with Killer whale, white whale and human.

Unlike blue and fin whale-GR and blue and fin whale-THRB, blue and fin whale-PPARG LBD was identical to all the compared species, except for mouse and rat (Figure 22).

Figure 22 Multiple sequence alignments of PPARG LBD. The encoding genes for PPARG LBD from Fin whale (*Balaenoptera physalus*) and blue whale (*Balaenoptera musculus*) were aligned with white whale (*Delphinapterus leucas*), human (*Homo sapiens*), mouse (*Mus musculus*), Killer whale (*Orcinus orca*), rat (*Rattus norvegicus*) and polar bear (*Ursus maritimus*). The various PPARG -LBDs were retrieved from NCBI genome database (NCBI accession numbers: white whale XP_022418477.1, human NP_005028.4, mouse XP_006505800.1, Killer whale XP_004284335.1, rat NP_037256.1 and polar bear XP_008696091.1). The sequences were visualized with Jalview (2.10.5), where the LBD of fin and blue whale- PPARG was identical to all the compared species except for mouse and rat.

4.1.5 Phylogenetic analysis

After conducting the MSA of the ligand binding domain of AhR from different mammalian species, a phylogenetic tree was constructed by the neighbour-joining tree method (Figure 23). As illustrated in figure 16 the identity between blue whale, minke whale and white whale AhR-LBD are the ones that have from an evolutionary perspective changed the least compare to the remaining specie.

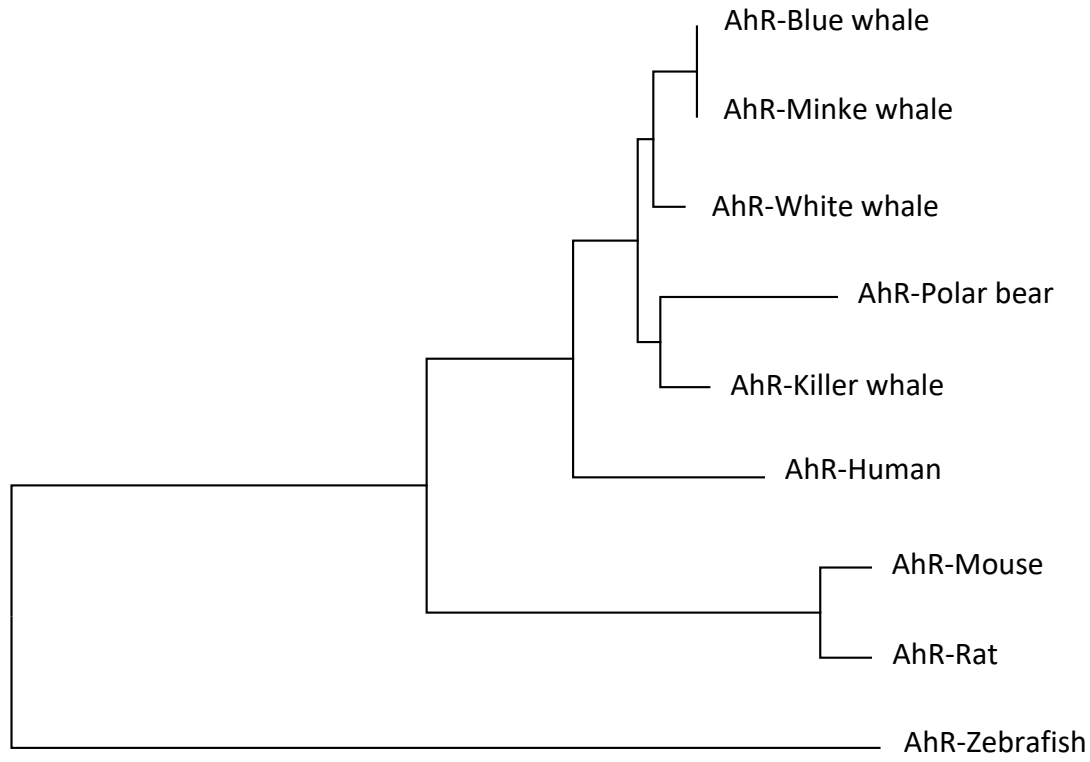


Figure 23 Neighbour-joining tree of AhR encoding sequence. The analysis involved nine LBD-amino acid sequences and illustrates the evolutionary relationship between different mammalian AhR-LBD with zebrafish as an outlier. Blue whale and minke whale were identical, in addition to a high identity with white whale, polar bear and killer whale.

Having conducted the phylogenetic analysis, I continued to study the transcription-factors through measuring their ligand activation trough luciferase gene reporter assays.

4.2 Measuring ligand activation of transcription factors using luciferase reporter assays

In this thesis the ligand activation of the different transcription-factors was quantified by measuring the luciferase expression in the gene reporter assay described in Method 3.12. Because blue and fin whales' sequences proved to be identical in the three NRs, and are

therefore expected to be activated in the same way I only continued with luciferase assays for blue whale.

4.2.1 Cytotoxicity

To ensure that the different compounds tested in the luciferase assays did not affect the metabolic activity or the membrane integrity of the cells, a cytotoxicity assay was performed. In the assays 0.1% Triton X-100 was used as a positive control. Exposure to triton X-100 decreased membrane integrity with 98%, as well as reduced metabolic activity by 95%. All the tested POPs were compared to the control with only DMSO (point 0 in Figure 22, and neither of the test compounds and control agonists used in the luciferase assays showed any significant effect on membrane integrity or metabolic activity of the COS-7 cells (example of no cytotoxic effect of pp`DDT (Figure 24).

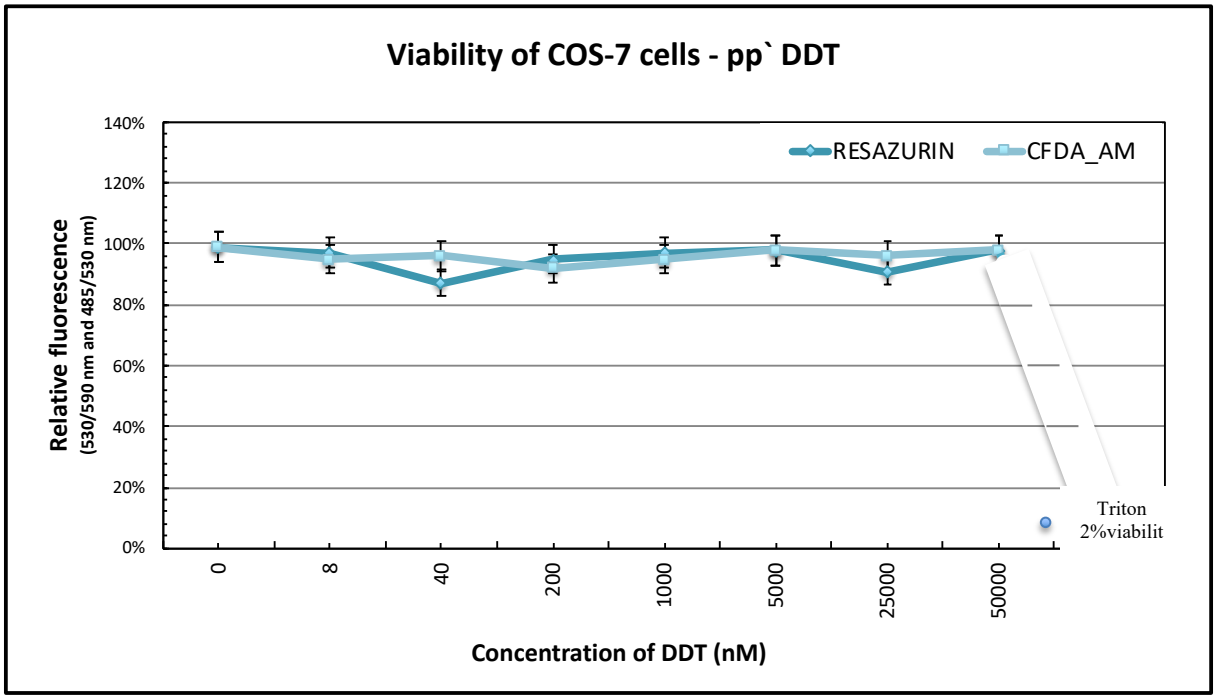


Figure 24. Cytotoxic effect of one of the test-components (pp`DDT) used in the luciferase assays. 0.1% Triton X-100 was used as a positive control in 3 independent cytotoxicity assays. It decreased membrane integrity with 98% and reduced metabolic activity by 95%. The different test compounds used in the luciferase assays did not show a cytotoxic effect.

4.2.2 Measuring ligand activation of blue whale AhR, GR, THRB and PPARG

Having established that none of the tested POPs had a cytotoxic effect on the COS-7 cells, I continued with the luciferase assays. Initially I performed ligand activation assays on the AhR-ARNT but did not observe any increase in luciferase activity in cells exposed to the control

compound TCDD (Figure 25). Because of blue and fin whale sequences identity, I will further present the data as blue whale-NR (bw). Due to limited time for problem solving, the transcriptional activity of blue whale (bw) glucocorticoid (bwGR), thyroid hormone (bwTHRB), and peroxisome proliferated receptor (bwPPARG)

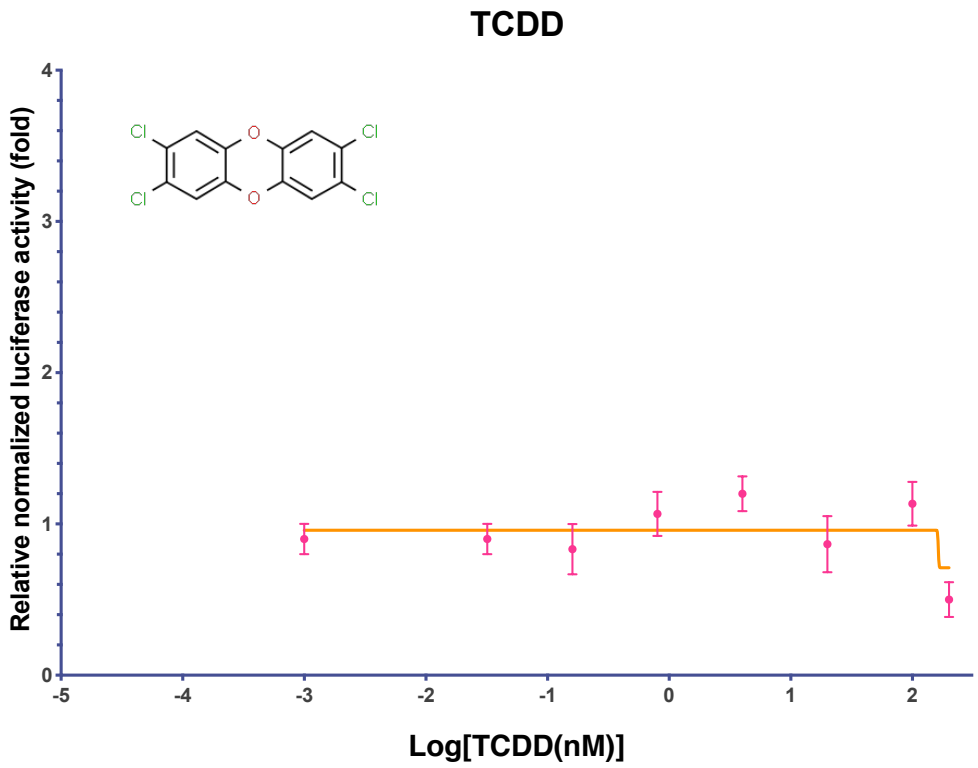


Figure 25 Attempt to study *in vitro* activation of blue whale AhR by the known agonists TCDD. The effect of the known agonists on blue whale AhR was not measurable in a luciferase assay with COS-7 cells in this study. The graphs were made in Prism 8 (version 8.1.2) with the differences illustrated with standard deviation (SD), where the downfall is not statistically significant The dose response is relative to cells only exposed to the control DMSO.

The ligand activation of Gal4-DBD-GR/THRB/PPARG was in this study quantified my measuring the expression of luciferase in the reporter gene assay described in method 2.7.

4.2.3 Evaluation of plasmids for AhR, GR, THRB and PPARG LRA assays

Before initialising the luciferase assays all the different plasmids used in the reporter gene assays (reporter plasmid ((mh100) x4 tk luc), reference plasmid (pCMV-β-Gal), Gudluc, AhR, GR, THRB, and PPARG) were quality controlled with an AGE. The plasmids were mainly in a supercoiled conformation and was therefore suitable for further use (Figure 26). The supercoiled conformation is needed for efficient transfection into the COS-7 cells.

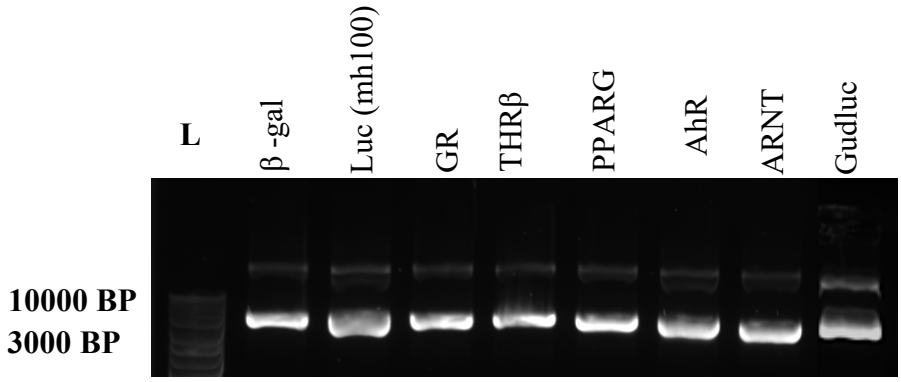


Figure 26 Quality control of plasmids used in the luciferase reporter assays with NRs and AhR. The reporter plasmid ((mh100) x4 tk luc), reference plasmid (pCMV-β-Gal), Gudluc, AhR, GR, THRβ, and PPARG plasmid were separated on an AGE to ensure that the majority of the plasmids were double-twisted and could be used further in the luciferase gene reporter assays. 200 ng of each plasmid were loaded together with a loading buffer, and a 2-log DNA ladder.

4.2.4 Establishing positive controls for ligand activation of bwGR, bwTHRB, and bwPPARG

To test the sensitivity of the luciferase activation assays, with bwGR, bwTHRB and bwPPARG, positive controls were established (Method 3.10.1). The measured luciferase activity was normalized against β-Gal- activity (Method 3.10.9), and ligand activation of Gal4-DBD-GR/ THRB /PPARɣ is described as a fold change in the expression of the receptor gene between the test compound exposed cells and those only exposed to solvent/DMSO. For this initial test, compound previously known to activate mammalian GR, THRB and PPARG were used. Dexamethasone (DEXA) is a known agonist for the human glucocorticoid receptor and as expected also activated blue whale GR strongly to a maximum activation at 26-fold at 20 nM and (Figure 27a). For thyroid hormone receptor β triiodothyronine (T3) was used as control agonist. T3 produced maximum activation was of 24.4-fold change at 120 nM. For PPARG rosiglitazone (ROSI) was used as a known agonist, here the maximum activation was at 17 at 25 µM.

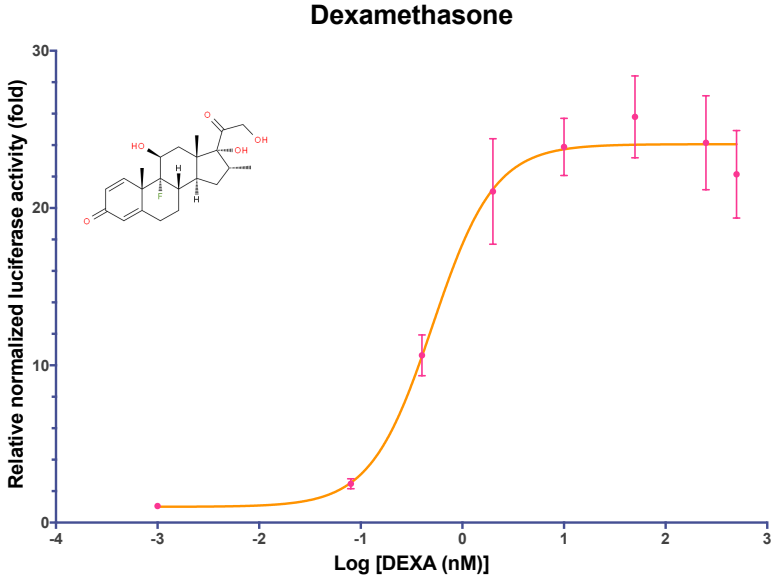


Figure 27 a

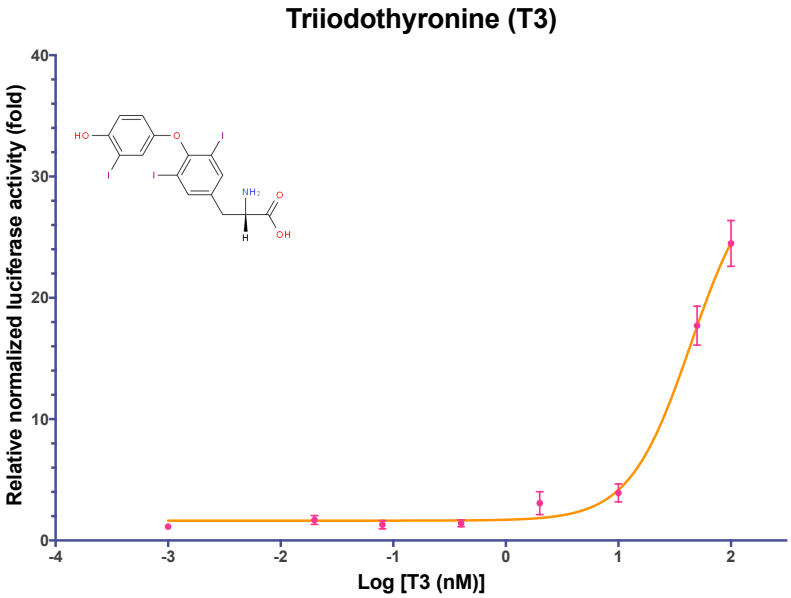


Figure 27 b

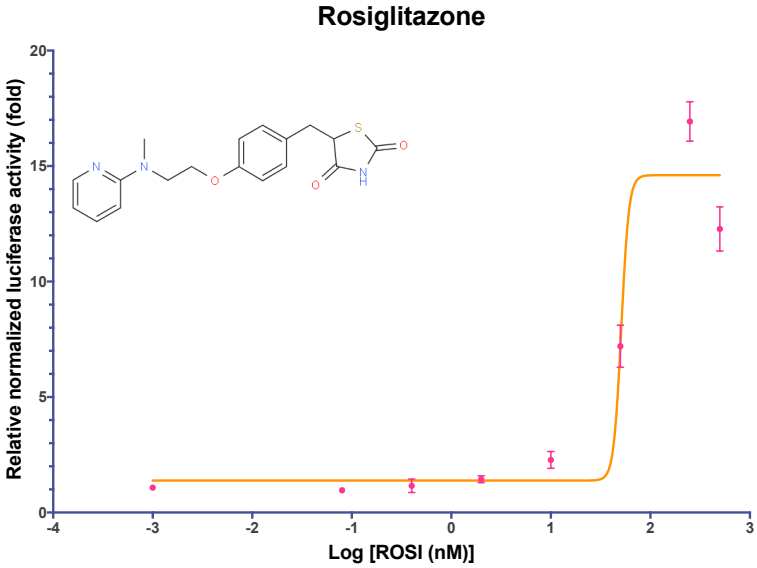


Figure 27c

Figure 27 *In vitro* activation of blue whale GR, THRB and PPARG by known agonists. The effect of a known agonists on blue whale GR (a),THRB (b) and PPARG (c) measured in a luciferase assay with COS-7 cells. The graphs were made in Prism 8 (version 8.1.2) with the means are shown with standard deviation (SD). The dose response is relative to cells only exposed to the control DMSO.

The EC50 values and the maximum fold change activation of blue whale GR, THRB and PPARG are summarized in table 25. This demonstrated that the luciferase gene reporter assay

worked, and could be used further for testing the agonistic abilities of the desired POPs (DDT, DDE, DDD, DEHP, DINP, POPs mix)

Table 25 an overview over the maximum activation (in fold) and EC50 of GR, THRB, and PPARG in a luciferase assay by known agonists. The three known agonists Dexa (GR), T3 (THRB) and ROSI (PPARG) triggered transcriptional activity in all of the tested receptors, and the EC50 value was obtained from THRB and PPARG. THRB had not yet reached its top at the highest concentration, and the EC50 is therefore not conclusive.

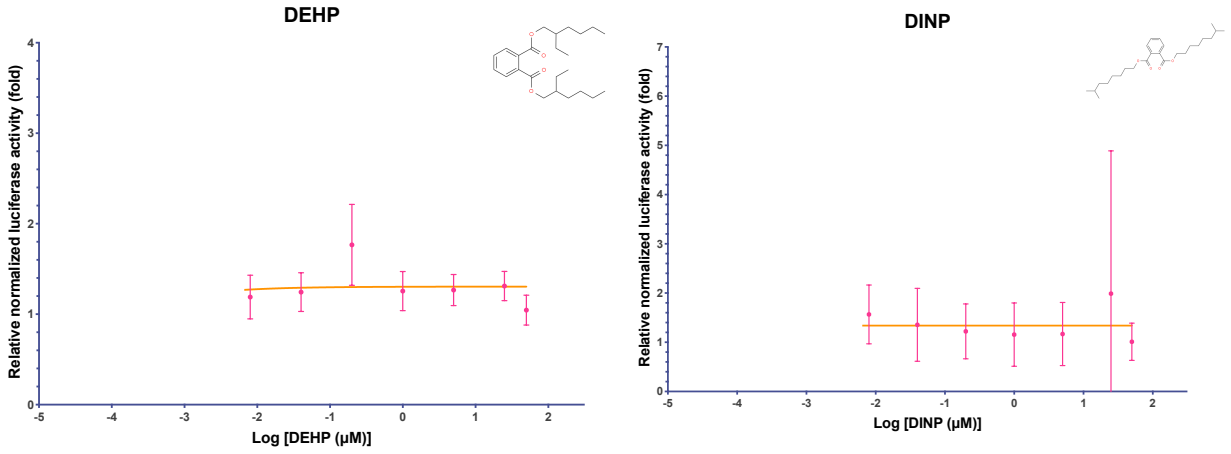
	GR	SD	**THRB**	SD	**PPARG**	SD
EC50	**12.90**		**12.24**		**8.48**	
MAX Activation	**25.80**	6.89	**24.48**	5,66	**16.93**	5.62

4.2.7 Transcriptional activation of whale GR, THRB and PPARG by POPs

Having verified that the luciferase assay worked, I continued with test the activation of bwGR, bwTHRB and bwPPARG by selected test compounds and a synthetic mixture that mimics the POP content in whale blubber.

4.2.7.1 Transcriptional activation of whale GR by POPs

The five tested pops and the synthetic mix did not trigger transcription activity in bwGR. However, DDT, DDE, DDD, and the POPs mixture showed a decrease in luciferase activity, this may indicate that the three pops and the mix act as antagonists in bwGR (Figure 28). To verify this further analysis is necessary.



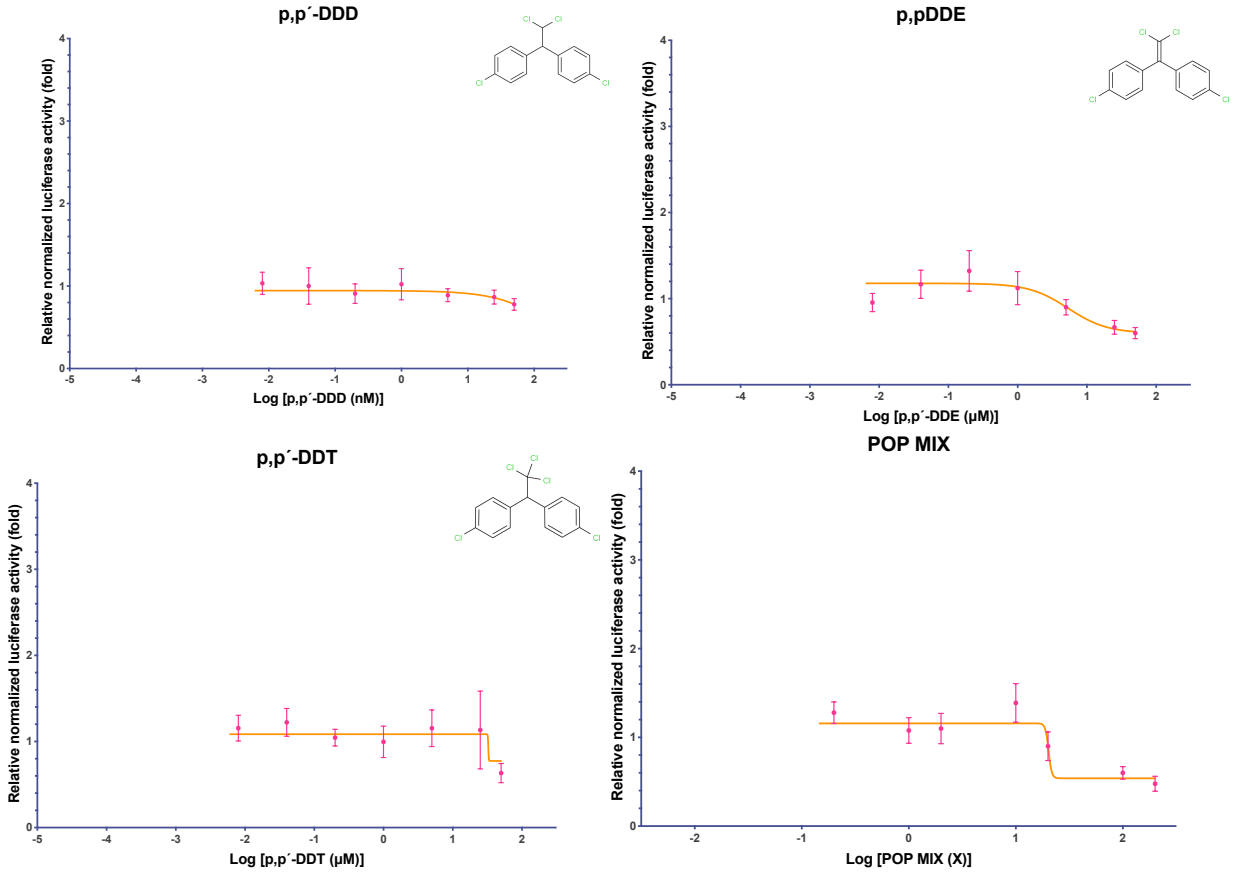


Figure 28 Transcriptional activation of bwGR by different components and a mixture. COS-7 cells were transfected with pCMX-Gal4-bwGR and exposed to five selected compounds and a synthetic mixture at different concentrations for 24 hours. Each datapoint represents the average of three independent experiments (three replicates per experiment), with standard error for each point. The activation of Gal4-bwGR is shown as a fold change in relative normalized luciferase units, of cells exposed to the different test compounds, in comparison to cells exposed only to DMSO.

4.2.7.2 Transcriptional activation of whale THRB by POPs

In similarity with bwGR the thyroid receptor was also not activated by the selected POPs and POPs mixture (Figure 29). However, the phthalate DINP caused a decrease in luciferase activity at the two highest concentrations, which may indicate it acting as an antagonist instead of an agonist. This cannot be said for certain without further investigation.

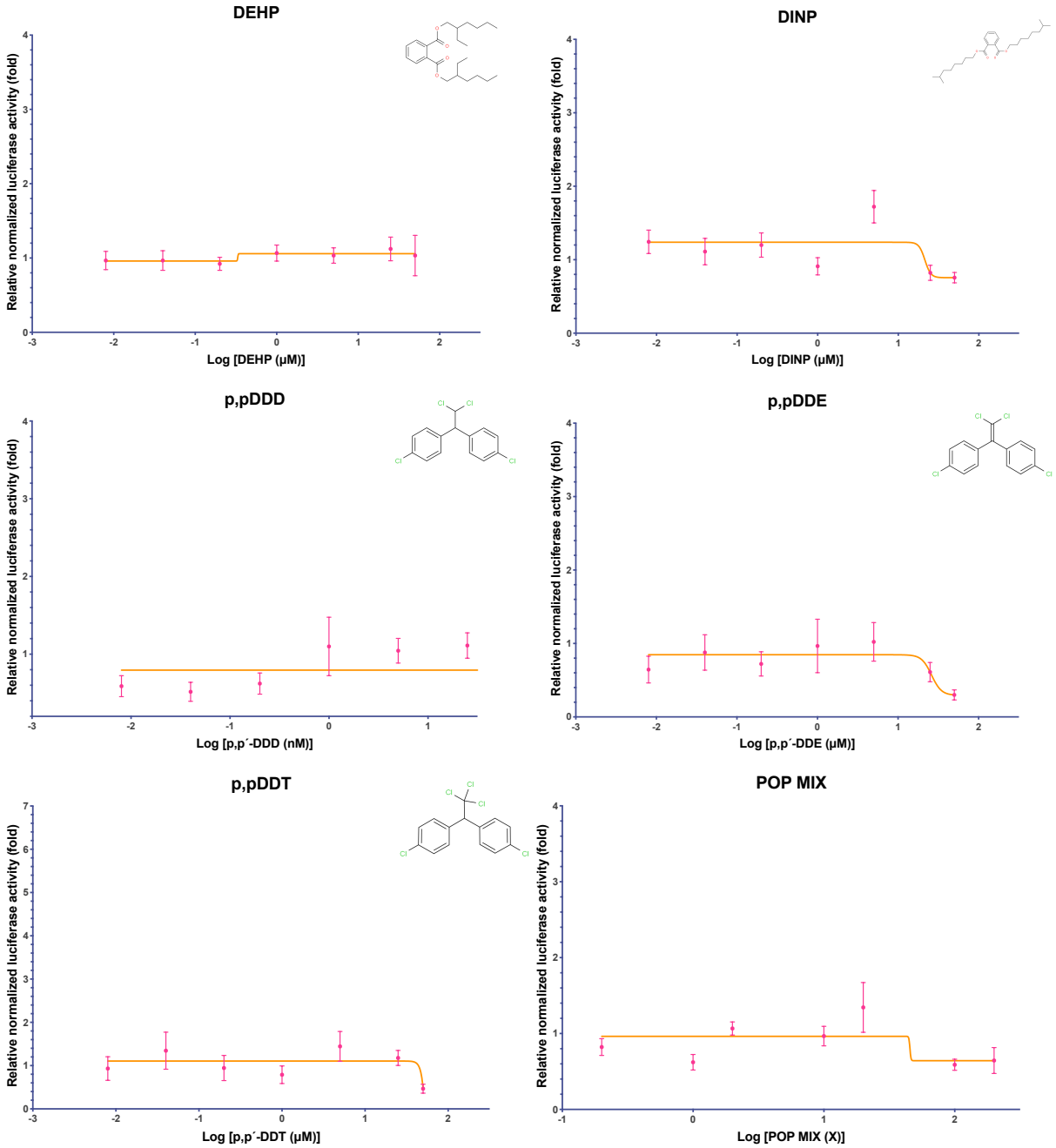


Figure 29 Transcriptional activation of bwTHRB by different components. COS-7 cells were transfected with -Gal4-bwTHRB and exposed to seven different chemicals at different concentrations for 24 hours. Each datapoint shows the average of three independent experiments, with standard error for each point. The activation of Gal4-bwTHRβ is shown as a fold change in relative normalized luciferase units, of cells exposed to the different test compounds, in comparison to cells exposed only to DMSO.

4.2.7.3 Transcriptional activation of whale PPARG by POPs

In addition to bwGR and bwTHRB bwPPARG did also not show an induced transcriptional activation by the tested POPs (Figure 30). The POPs mixture however caused a slight decrease in luciferase activity, which may indicate that the synthetic mix may act as an antagonist instead of an agonist.

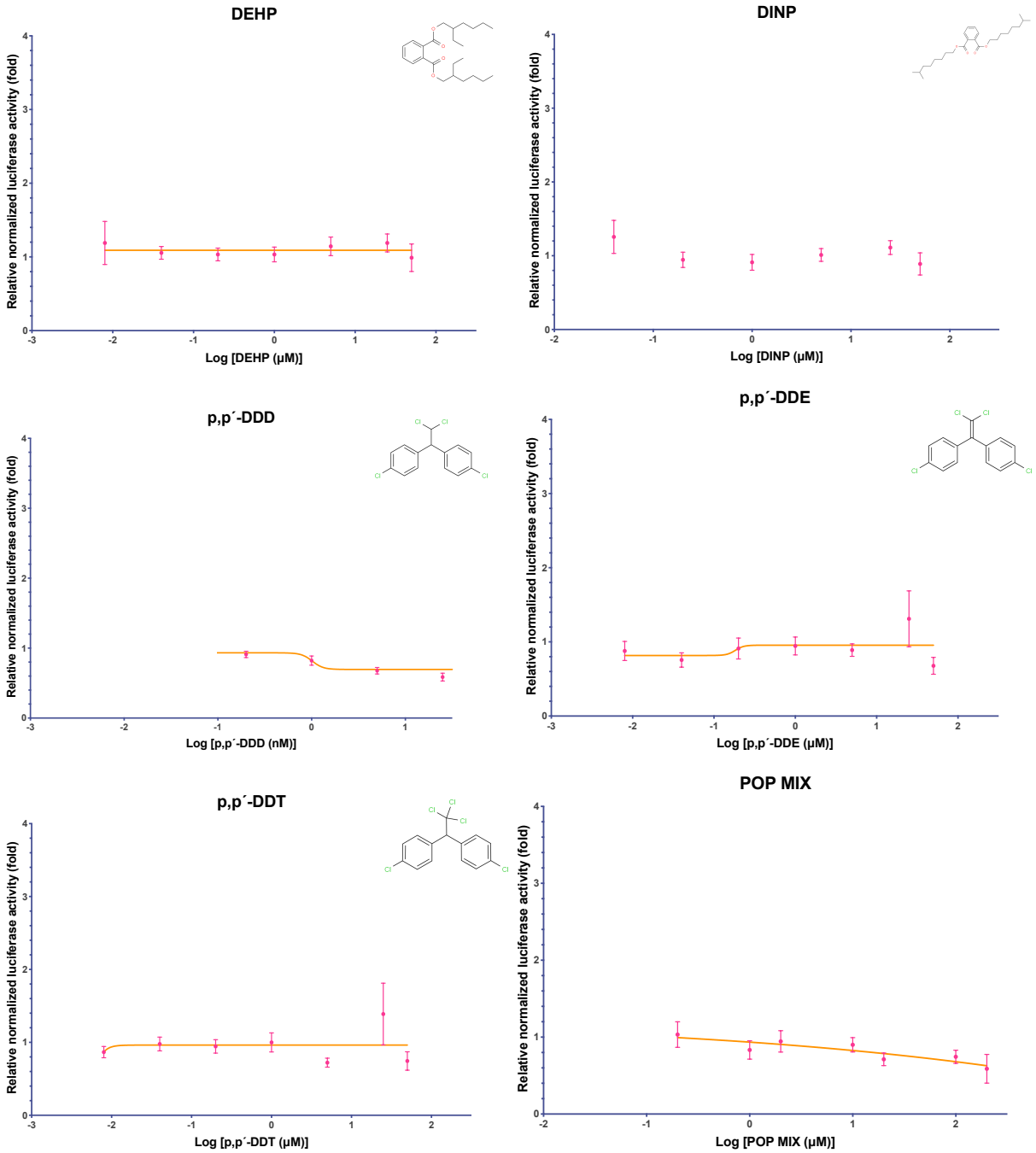


Figure 30 transcriptional activation of bwPPARG by different components. COS-7 cells were transfected with pCMX-Gal4-bwPPARG, and exposed to seven different chemicals at different concentrations for 24 hour. Each datapoint shows the average of three independent experiments, with standard error for each point. The activation of Gal4-bwPPARɣ is shown as a fold change in relative

normalized luciferase units, of cells exposed to the different test compounds, in comparison to cells exposed only to DMSO.

4.2.8 Summary

As seen above, none of the tested components triggered transcriptional activation of the tested nuclear receptors. Several gave a small decrease in luciferase activity (DDT, DDE, DDD, DEHP and DINP) (Figures 28-30 and table 26), but with a weak statistical significance.

Table 26 *In vitro* activation of GR, PPARG, THRB and AhR by control agonists or environmental pollutants in a COS7-based luciferase reporter gene assay. Effects are presented as estimates of fold change at max exposure concentration.

Max response in luciferase activity related to solvent treated cells														
	Control agonist	SD	**DDT**	SD	**DDE**	SD	**DDD**	SD	**DEHP**	SD	**DINP**	SD	**POPs MIX**	SD
Receptor														
Concentration→	**[20μM]**		**[50μM]**		**[50μM]**		**[50μM]**		**[50μM]**		**[50μM]**		**[50μM]**	
GR	**22.5**	6.81	**0.6**	0.33	**0.6**	0.14	**0.7**	0.10	**1.0**	0.50	**1.0**	0.24	**0.4**	0.10
Concentration→	**[100μM]**		**[50μM]**		**[50μM]**		**[50μM]**		**[50μM]**		**[50μM]**		**[50μM]**	
THRB	**24.5**	5.66	**0.4**	0.31	**0.3**	0.21	**1.3**	0.38	**1.0**	0.30	**0.7**	0.21	**0.6**	0.51
Concentration→	**[25μM]**		**[50μM]**		**[50μM]**		**[50μM]**		**[50μM]**		**[50μM]**		**[50μM]**	
PPARG	**12.3**	5.62	**0.7**	0.13	**0.6**	0.10	**0.6**	0.09	**0.9**	0.14	**0.8**	0.21	**0.5**	0.12

5. Discussion

In this thesis the main focus was to study the transcriptional activity of GR, THRB, PPARG and AhR in blue and fin whale when exposed to POPs. Due to identical NR sequences in blue anf fin whale, I only continued with blue whale after the MSA. The three nuclear receptors and AhR are known to be activated by numerous endogenous and exogenous compounds e. g. known environmental contaminants such as POPs, and thereby regulate the expression of important genes involved in biotransformation, adipogenesis, reproduction and brain development (Heitzer et al., 2007; Li et al., 2012; Oakley et al., 2013; Strömqvist et al., 2012; Tsuji et al., 2014; Zhang et al., 2000). Blue whale AhR has to my knowledge never been cloned from cDNA, nor have *in vitro* activation studies on these receptors in baleen whales been conducted before. Cloning and sequencing of AhR from animals (e.g. white whale and Baikal seals (*Phoca sibirica*)) and humans have increased the knowledge and understanding of how AhR works (Burbach et al., 1992; Emas et al., 1994; Kim et al., 2002). However, the functional and structural characteristics of AhR in marine mammals are still poorly understood. To validate the cloned gene sequence of blue whale AhR and to study the identity of GR, THRB and PPARG between different mammalian species phylogenetic analysis were carried out. A pairwise sequence alignment confirmed the identity between the cloned blue whale AhR and minke whale, and a multiple sequence alignment, together with a neighbour-joining tree confirmed the level of identity between blue whale and fin whale GR, THRB and PPARG, and other mammals. Further luciferase gene reporter assays were established and the transcriptional activation of the four receptors studies. The results obtained in this thesis will be further discussed here.

5.1 PCR amplification of blue whale AhR

In this study amplifying a full-length blue whale AhR was not possible. One of the explanations to why the amplification of AhR didn't work could be suboptimal PCR conditions. This depends on a number of different factors such as a good template, the temperature in the annealing-step of the reaction, and primer design. The template used in the PCRs was cDNA synthesised from extracted RNA from Blue whale blubber samples. A challenge with cloning long transcripts is to synthesise a complete cDNA (Hawkins et al., 2003). However, an AGE later confirmed the integrity of the full length AhR this indicates that a complete cDNA had successfully been constructed and used in the PCRs. Another important factor to obtain a successful PCR is primer design. When designing a primer there are many factors to take into

consideration: when used in a PCR the primers should be between 18-25 nucleotides long, they need to be specified from 5´to 3´end, the 3´and need to end with cytosine or guanine etc. Quite a few primers (12 pair) were used in this study in order to find the pairs that gave the desired results. Several primer pairs were able to amplify two smaller fragments of the AhR, demonstrating that the primers used recognize and binds whale AhR and suggests that, when combined correctly, they should be able to amplify the full length AhR. Despite of this it was in this study not possible to amply the complete AhR reading frame. In addition to the primers being an important factor to successfully amplify the full length AhR, the annealing temperature is also essential to obtain a specific primer-binding. Several different temperatures around the primers «theoretically optimal temperature» were tested, with successful results for the two smaller fragments of AhR, but not with the full length AhR. This may imply that there are other reasons in addition to temperature, template and primers to why the amplification of the full-length blue whale-AhR was unsuccessful so far. The length may be one of the issues why the full length AhR proved difficult to amplify, where the polymerase used might not be optimized for the size of the full length AhR. In this study both Dreamtaq (Thermo Scientific™) and Phiusion polumerases (Thermo Scientific™) were used. In addition the AhR sequence has a relatively high GC level, which may lead to a higher melting point that can create stable secondary structures, that terminate the PCR (Mamedov et al., 2008). However, the complete blue whale AhR was in the end successfully amplified by Roger Lille-Langøy in our lab, and could be used further in luciferase assays, and phylogenetic analysis.

5.2 Sequence analysis and phylogeny

AhR is from an evolutionary perspective a very interesting transcription factor, because it is well represented in all vertebrate groups (Hahn, 2002), and AhR homologues have also been characterized in several invertebrates e. g. sea anemone *N. vectensis* (Reitzel et al., 2014). however, some AhR orthologs seem to be functionally different. The invertebrate homologues are unlike vertebrates AhRs not able to bind beta naphthoflavone or 2,3,7,8-TCDD (Butler et al., 2001; Reitzel et al., 2014). This might be because the important amino acids in several mammals found in the multiple sequence alignments (Figure 19) are only partly conserved in invertebrates. The main function of AhR in invertebrates is in the development of the organism. In similarity with invertebrates, the AhR in mammals and other chordates also has an important physiological (both in development and other processes) role, in addition to being a xenosensor (Nebert, 2017). This may indicate that the receptor started as a physiological regulation protein,

and then evolved as a receptor and gene regulator for several exogenous compounds (Pohjanvirta, 2011).

AhR may vary in length between different species, bwAhR had a coding sequence of 2241bp which encoded a protein of 747 AA. Compared to minke whale-AhR with 2571bp (857 AA) the bwAhR is only a few amino acids smaller. In the multiple sequence alignment of the functional regions of bwAhR and minke whale-AhR, both the PAS and bHLH- domain showed a high degree of identity between the two species. This was expected seeing that these parts of AhR are important for dimerizing with ARNT, XAP2, p23, and HSP90, DNA-binding, and ligand binding. The MSAs also showed a high level of conservation of amino acids known to be of importance when the receptor binds to a ligand or to DNA (in mice). The deletions in the blue whale sequence (Figure 18), were not located in areas important for ligand or DNA-binding and are thus not likely to affect the protein function. The high conservation and level of identity between the different species may indicate that the receptors have similar functions and respond to ligands in a similar way. From a toxicological perspective, this indicates that both humans and blue whales' response to POPs through AhR would be similar, and the research conducted on one the two species is applicable to the other as well.

The MSA of the ligand binding domain of GR, THRB and PPARG conducted in this study have shown a 100% identity in each receptor between blue whales and fin whales (blue whale-GR=fin whale-GR etc.) (Figure 20-22 in the Results), and a very strong conservation between the compared species including white whale, human, mouse, killer whale, rat and polar bear. This finding is in accordance with the notion the LBD of nuclear receptors are generally well evolutionary conserved between species (except for e.g. PXR) (Gronemeyer et al., 2004). When compared to other species the LBD of blue and fin whale-GR was identical to two toothed whales, killer whale and white whale, and another baleen whale, minke whale. It also showed a 94-96% identity to human, polar bear, mouse and rat. The LBD of blue and fin whale-THRB in similarity to blue and fin whale-GR alignment showed a high identity to several of the compared species, and was identical to killer whale, polar bear, human and white whale. Only one amino acid in THRB was substituted in minke whale, rat and mouse. The function of this amino acid is to my knowledge not known. Blue and fin whale-PPARG had fewer differences of AAs in the LBD sequence (than GR and THRB) and was identical with all the compared species except for mouse and rat. Because of the high level of identity between the compared

species, it is likely that the receptors are not very receptable for evolutionary change in addition to responding to ligands in similar ways, like previously also assumed for AhR.

To study ligand activation of transcription factors in blue whales a modified version of CALUX luciferase gene reporter assay was the best option due to its ability to illustrate transcription activation through luciferase activity.

5.3 Functionality of the luciferase gene reporter assay

The reporter-system used in this study is a well-established system for measuring ligand activation of nuclear receptors, due to its reduction of cross talk of nuclear receptors and low risk of cross- reactivity caused by other cellar pathways (Routti et al., 2016; Lümann, 2018), but has to my knowledge not been frequently used with AhR. When conducting studies using the Gal/UAS-system to detect possible agonist of NRs is crucial that the sensitivity is high, to detect weak agonists at low concentrations. A limitation with the luciferase gene reporter assay in this study is that that only the LBD and the hinge was used from the different NRs, not the whole sequence. Minor conformational changes in the LBD can therefore not be excluded (Raucy et al., 2013). Another factor that may affect this type of experiment, such as exposure time. In this study an exposure time of 24 hours was used, but in other studies a shorter exposure time have shown higher activation (GAL4-DBD-AhR in rats (Backlund et al., 2004)). 12 hours exposure of 2,3,7,8-TCDD in a CALUX system adapted to dioxins, gave a higher activation than a 24-hour exposure in rats. Another study have also shown a higher activation with a shorter exposure, here with B(a)P, where 6 hours gave a higher activation than 24hours of exposure (Pieterse et al., 2013). Seeing that uptake etc. may vary depending on each chemical, it can be advantageous to optimize the exposure-time for different chemical groups.

Another factor that may affect the results is the choice of cell line. This may be important to obtain maximal ligand activation and sensitivity. The COS-7 cells used in this study have previously been used for receptor-characterization of PXR, PPARA and PPARG from different species using the Gal4/UAS-system (Bainy et al., 2013; Chamorro-García et al., 2012; Lille-Langøy et al., 2015). Despite the fact that the COS-7 cell line is known as an suitable method when studying nuclear receptor activation (Bainy et al., 2013), it was in 2004 demonstrated by Backlund et al that the activation of Gal4-DBD-rat-AhR by 2,3,7,8- TCDD varied between H4IIE and HEA1-C12 cell lines (Backlund et al., 2004). Using H4IIE from rats they observed

a 22-fold activation, while in HEPA1-C12 from mice only a 3-fold activation was observed (both hepatoma cells). Seeing that ligand binding and activation of AhR is dependent on several co-factors (e.g. p23, HSP90 and XAP), it is possible that inefficient binding of these co-factors present in the COS-7 cells could affect the result. “Wrong” choice of cell line may also be the case in this study, where the construction of a blue whale-AhR luciferase assay with FICZ and TCDD as known agonists, has not yet been successful. However, there are a few differences between these two studies. Backlund et al. (2004) used a different assay where AhR in fusion with Gal4 and ARNT-plasmids were not used, making the assay even more dependent on the cell line compared to the luciferase assay used in this study (Backlund et al., 2004). Unlike with AhR a successful luciferase assay was established for GR, THRB and PPARG but no activation of the studied NRs by the tested environmental contaminants was observed. One of the explanations may in similarity with AhR be the choice of cells used. However, previous studies conducted on chicken, ring necked pheasant and Japanese quail AHR1 with COS-7 cells have proven successful (Farmahin et al., 2012). Therefore, it seems unlikely that the choice of cell line would affect the results of now significant agonism on the three NRs by the tested POPs.

The last factor that may have an impact on the ligand-binding is incubation-temperature. In previous studies it has been shown a higher activation at temperatures lower than what was used in this thesis. Zhao et al. (2010) observed a higher activation of AhR where the COS7-cells were exposed at 33ºC to 2,3,7,8-TCDD compared to the cells exposed at 37 ºC (245 +/-24 at 33 ºC, 17 8+/-1 at 37 ºC) (Zhao et al., 2010). The difference may be due an increase in AhR activity at lower temperatures compared to at high temperatures (37 ºC), which can be explained by the reporter gene (luciferase) having a higher activation at lower temperatures (Zhao et al., 2010). This implies that the temperature used in this study may also be a contributing factor to why no activation was seen in the AhR-LRA. However, the luciferase assays used to study GR, THRB and PPARG appear to be functioning at an incubation-temperature of 37 ºC, questioning the importance of a lower temperature, to obtain a successful AhR assay. In addition to the temperature question, ARNT may be a contributing factor, in this thesis a human ARNT was utilized. Because of the high level of identity between human ARNT and blue whale ARNT this shouldn’t be a problem (appendix V) but may in total act as a contributing factor. When combining the factors mentioned above (exposure time, cell-line, incubation-temperature and species specific ARNT), it is apparent how many parameters that may affect how well different gene reporter systems work, and what need to be taken into consideration when conducting studies utilizing such systems.

5.3.1 Ligand activation assays of blue whale GR, THRB and PPARG.

The ligand activation studies of blue whale GR, THRB and PPARG constructs *in vitro* allowed the assessment of both single POPs and a synthetic mixture of POPs of their abilities to agonistically activate the three studied nuclear receptors.

In a luciferase gene reporter assay COS-7 cells were used together with the desired receptor (GR, THRB, PPARG, or AhR) and a reporter gene. In this study such an assay was not successfully established for AhR, but was successfully validated and utilized for bwGR, bwTHRB and bwPPARG.

However, no agonistic effect was observed in the three NRs by the five test components (pp´DDT, pp´DDE, pp´DDD, DINP and DEHP) and the POPs mix tested. Even though the concentration magnitudes of the tested POPs were higher than the levels previously measured in blue and fin whale blubber from different locations (Fossi et al., 2014b; Metcalfe et al., 2004; Muñoz-Arnanz et al., 2019; Tartu et al. 2019). The three blue whale NRs have also previously been studied in our laboratory by Lühmann et al. (2019)(Lühmann et al., 2019). Here the luciferase gene reporter assay was established, and agonistic and antagonistic effects of several legacy POPs were tested. Lühmann et al. observed low agonistic and antagonistic effects on blue whale and fin whale GR, THRB, and PPAR, but the effect was only observed on level higher than those measured in the blue and fin whale blubber samples (Lühmann et al., 2019).

Previous studies conducted on a mammalian (using U2OS cells from human) reporter gene assay with human-GR also showed no activation by different POPs (including pp`DDE (see Wilson et al., 2016)), however pp`DDE was found to decrease GR activity by 72% (Wilson et al., 2016). A study conducted by Li et al. (2012) also indicates that the studied NRs can act as sensor molecules for exogenous compounds (Li et al., 2012). An explanation to why increased transcriptional activity was not detected may be that the ligands tested act as antagonists instead of agonists. As seen in figure 28 in the Results pp´DDE, pp´DDT, pp´DDD and the POPs mix caused a small decrease of luciferase activity at the highest concentrations, indicating that they may function as antagonists in blue whale-GR. The same was found for THRB (Figure 29) with DINP, and with PPARG (Figure 30) and the POPs mix.

In a previous study conducted by Routti et al. (2016) pp´-DDE was shown to act as an antagonist to PPARG, in addition to this Lühmann et al. (2019) demonstrated a low antagonistic effect on

THRB of few POPs (PCB 101&138, op`DDE and oxychlordane)(Lühmann et al., 2019; Routti et al., 2016). Moriyama et al. (2002) also studied antagonistic behaviour of human-THRB in a luciferase gene reporter assay, where T3 was used as an agonist to test the antagonistic abilities of Bisphenol A (Moriyama et al., 2002). To understand if the three POPs and two phthalates tested in this study act as antagonists to bwGR, bwTHRB and bwPPARG such an experimental set up could be a promising approach (Moriyama et al., 2002). Similar non activated agonistic PPAR results were also found by Söderstrøm (2017), also here none of the tested POPs triggered activation of cod PPARs (Söderström, 2017). These findings are in line with the results found in this thesis, although further investigating would be advantageous.

No activation of the three NRs by the tested POPs might also be the case for other marine mammals such as killer whales or polar bears. Because the ligand binding domain of the three tested nuclear receptors were identical to e.g. killer whales, it is expected that the nuclear receptors will have similar activation patterns. The concentrations used in the *in vitro* studies were therefore compared to concentrations previous studies have measured in another marine mammal: the polar bear. Fat tissue samples taken from polar bears have shown PCB concentrations on up to 10.3 µM. This exceeds the highest concentration used in this thesis, in addition to previous studies conducted by Lühmann et al. (2019) and McKinney et al. (2011) where week agonistic effects were observed at concentrations lover than 10 µM (Lühmann et al., 2019; McKinney et al., 2011). Previous studies from the pacific ocean have also shown a positive correlation of PCB concentrations and the expression of THR in killer whales, implying that POPs may impact thyroid hormone homeostasis (Buckman et al., 2011). Which indicates that the negative effects of POPs on marine organisms not only affects the exposed individual, but also possible offspring through maternal exposure (Buckman et al., 2011).

As mentioned earlier there are large knowledge gaps in how environmental contaminants affect marine mammals. There is little knowledge on *in vivo* feedback mechanisms in marine mammals that are triggered by the change in transcription activity. This restricts the *in vitro* data from this study to be extrapolated into *in vivo* effects. *In vitro* data can be used to study specific steps e.g. transcriptional activation, but it is challenging to use *in vitro* data to simulate a living organism, since several processes are intertwined with one another.

Another issue that appears when studying free-ranging organisms, is that they are not only exposed to a single chemical at a time, but a mixture of many chemicals. This problem was

addressed in previous studies (Desforges et al., 2017; Routti et al., 2016) where Desforges et al. (2017) extracted contaminants from blubber were utilized in *in vitro* experiments using immune cells, and Routti et al (2016) extracted contaminants from polar bear tissue also in *in vitro* experiments (Routti et al., 2016). This type of experiment provides more realistic exposure scenarios but is difficult to conduct on baleen whales due to the amount of blubber needed to prepare a sufficient amount of extract (Desforges et al., 2017 used 30g blubber, while Tartu et al 2019 used 0.1-0.5g). Other studies have also shown sex-related, and physiological state related differences in contamination levels, indicating that the levels on which the synthetic mix was made is not always accurate depending on the sex and physiological state the animal is in (Muñoz-Arnanz et al., 2019; Pinzone et al., 2015). Synthetic mixtures are often applied at different concentrations, which may minimise the differences in contaminant levels between animals. The synthetic mixture used in this study was created based on the measured levels of contaminants, found in blue and fin whale blubber. Despite the levels of contaminants varying between individuals, the composition often remains the same, thus making the synthetic mixture more accurate.

One of the challenges with drawing solid conclusions on whether the tested contaminants trigger agonistic or antagonistic effects in free ranging animals, is that the physiological state of the animal is not known, the level of contaminants could vary depending on age and sex etc. where sex can be particularly important because female marine mammals transfer pollutants from heir blubber to their offspring during lactation (Butterworth, 2017), in addition to the challenges with deciding what contaminants to put in the mixture. Because of these limitations the effects that the tested compounds may have on transcriptional activation of NRs remain incompletely understood, especially considering emerging contaminates and mixture effects. However, in Desforges et al. (2017) study the complex mixture showed a lower effect level compared to the single compounds. This may also be the case in our study, since the single compounds had no agonistic effect it might be expected that the mixture wouldn't ether. On the other hand there are several studies proving that a mix of compounds have a greater effect than the compounds alone known as the cocktail effect (Cedergreen, 2014; Celander, 2011; Shaw, 2014).

Disruptive effects by POPs on bwGR, bwTHRB and bwPPARG could negatively affect the adaptation to stress, maintenance and development of the endocrine system, lipid homeostasis and metabolism, thermoregulation as well as reproduction. In this thesis it has been shown that

blue whale bwGR, bwTHRB and bwPPARG can be activated by known agonists, demonstrating that blue and fin whale bwGR, bwTHRB and bwPPARG, which have identical LBD sequences, are ligand-activated. This may indicate that the studied NRs can act as sensor molecules for exogenous compounds, in similarity to what has been shown in previous studies. However, the results from this study indicates that agonistic effects on the transcriptional activity of blue and fin whale-GR, blue and fin whale-THRB, blue and fin whale-PPARG by the tested contaminants in free ranging blue and fin whale is unlikely.

6 Conclusion

In this thesis AhR from blue whale was cloned and sequenced, the coding sequence was made up of 2241bp which encoded a protein of 747 AA. The *AHR* proved to be difficult to amplify in full length, due to structural, PCR, primer, temperature difficulties etc. The translated coding sequence of bwAhR proved to be well evolutionary conserved when the LBD was compared to other species, where important amino acids involved in ligand and DNA binding were identical between blue, fin, and minke whale, as well as rat, mouse, human, polar bear and white whale. Based on the cloned sequence a gene reporter assay was developed, but this has so far not been responsive to typical AhR agonists such as TCDD or FICZ.

The nuclear receptors GR, THRB and PPARG had previously been cloned and sequenced in the lab. Here, a multiple sequence alignment was conducted to compare the blue and fin whale sequences of GR, THRB and PPARG to other species. The MSA showed that blue and fin whales' sequence are identical, in addition to high level of identity between the other compared species (blue, fin, and minke whale, as well as rat, mouse, human, polar bear and white whale). Because of the high conservation of the LBD of GR, THRB and PPARG. The results obtained in this study, may also be relevant for other species, thus the receptors are expected to act in the same way.

A gene reporter assay was then verified for each of the different NRs. The assay demonstrated that blue whale and fin whale- GR, THRB and PPARG are functional proteins that have the ability to bind and be activated by a ligand *in vitro*. In the gene reporter assays the receptors were activated by a known agonist for each receptor (dexamethasone, T3, and rosiglitazone, respectively). The tested POPs detected in blue and fin whale blubber did not trigger a significant activation of the three NR in this study, and so far, antagonistic effect have not yet been investigated. But because of GR, THRB and PPARGs ability to be activated by known agonist and upregulate transcription of several genes that are important in many physiological processes (e.g. metabolism, development and the immune responses), further analysis would be advantageous, to understand to what extent the NRs have a role as xenosensors.

7 Future work

It was in this thesis shown that blue whale GR, THRB and PPARG can be ligand-activated and studied *in vitro* with a gene reporter assay. A gene reporter assay for bwAhR was on the other hand not successfully established. To be able to study bwAhR further work needs to be conducted, and then investigate bwAhRs ability to bind and be activated by different ligands.

To better understand the NRs role in addition to what amino acids and structures that are responsible for ligand activation in the three NRs modulation may be a good approach. It has in previous studies on AhR been shown that modulation of the ligand binding domain can be used to better understand the ligand binding abilities of a receptor (Bisson et al., 2009). This together with mutation-studies may prove useful, when attempting to understand what amino acids and structures that decide the receptors ligand binding abilities. It may also be interesting to develop a ligand binding/structure-model as a useful tool to understand what factors that are central for activation and binding in NRs.

In this thesis it was proven that the Gal4/UAS- system can be used to study ligand-activation of bwGR, THRB and PPARG. Due to the variables discussed earlier there are three main factors that may improve the Gal4/UAS- system: Choice of cell line, temperature, and exposure time. It may be of interest to optimize these parameters to obtain a Gal4/UAS- system with higher activation and sensitivity, in addition to detection of weak agonists. Antagonism would also be very interesting to investigate, seeing that none of the tested environmental contaminants gave any significant agonistic effect.

Further studies on agonistic and antagonistic abilities of the POPs detected in blue whale and fin whale blubber (both single compounds and mixtures), in addition to cell-studies could also help build a better understanding of what mechanisms that are involved in regulation of biotransformation and other processes. In a study conducted by Fossi et al (200) fibroblast cell lines were used in an alternative *in vitro* method to study contaminants effect on several cetaceans (e.g. fin whale)(Fossi et al., 2000). Such a cell line is currently under development in our lab and could serve as a natural progression to this study.

These future studies can increase our knowledge of how defence and sensory systems in different organisms work and have evolved over time, and help us understand how blue and fin whales respond to environmental contaminants within the animal and in their habitat, which can prove to be vital knowledge to best conserve and manage these giants.

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Appendix I

Table 37 overview of environmental contaminants found in blue and fin whales by Tartu et al. in preparation 2019

Compound	Blue Median	nM Min	Max	Fin Median	nM Min	Max
HCB	96	31	142	116	44	146
a-HCH	4	2	6	6	3	7
b-HCH	17	5	37	19	5	28
g-HCH	2	1	3	2	1	3
oxy-chlordane	16	5	40	23	6	40
t-chlordane	0	0	0	0	0	0
c-chlordane	9	2	15	8	5	15
t-Nonachlor	60	20	154	85	27	143
c-Nonachlor	23	8	61	32	10	56
Mirex	2	1	7	3	2	4
o,p'-DDT	38	10	154	51	17	128
p,p'-DDE	34	8	121	23	11	30
o,p-DDT	28	11	79	36	18	63
o,p-DDE	75	23	258	92	32	162
p,p'-DDD	11	8	19	11	5	15
o,p-DDD	206	66	657	286	92	581
PCB 28	0	0	0	4	4	4
PCB 52	24	6	63	43	14	81
PCB 99	16	5	45	27	9	47
PCB 101	21	5	45	36	10	65
PCB 105	4	3	7	7	3	8
PCB 118	24	7	66	40	14	71
PCB 138	33	12	102	57	22	113
PCB 153	51	20	156	80	30	162
PCB 180	14	6	53	21	10	57
PCB 183	3	2	10	4	2	12
PCB 187	14	5	49	19	9	48
PCB 194	4	4	4	6	6	6
Toxaphenes:						
#26	28	7	73	45	17	82
#32						
#38						
#40	19	5	45	32	17	55
#42	61	12	83	58	32	110
#50	62	13	202	114	41	232

Appendix II

Table 31 & 32 An overview of the different concentrations used in the exposure studies of blue whale GR. Each table represents a 96 well plate, there were used two 96 plates for each receptor in this study, three replicates of each ligand were used.

	1	2	3	4	5	6	7	8	9	10	11	12
A	200			50000			50000			50000		
B	100			25000			25000			25000		
C	20.0			5000			5000			5000		
D	4.0			1000.0			1000.0			1000.0		
E	0.8			200.0			200.0			200.0		
F	0.16			40.0			40.0			40.0		
G	0.032			8.0			8.0			8.0		
H	DMSO			DMSO			DMSO			DMSO		
	DEXA (nM)			DDT (nM)			DDE (nM)			DDD (nM)		

Table 32 second 96 well plate for GR

	1	2	3	4	5	6	7	8	9	10	11	12
A	200			50000			50000					
B	100			25000			25000					
C	10			5000			5000					
D	2			1000.0			1000.0					
E	1*			200.0			200.0					
F	0.5			40.0			40.0					
G	0.1			8.0			8.0					
H	DMSO			DMSO			DMSO					
	POPs mix			DEHP (nM)			DINP(nM)					

Table 33 &34 An overview of the different concentrations used in the exposure studies of blue whale THRβ Each table represents a 96 well plate, there were used two 96 plates for each receptor in this study, three replicates of each ligand were used.

	1	2	3	4	5	6	7	8	9	10	11	12
A	120			50000			50000			50000		
B	60			25000			25000			25000		
C	12			5000			5000			5000		
D	2.4			1000.0			1000.0			1000.0		
E	0.5			200.0			200.0			200.0		
F	0.1			40.0			40.0			40.0		
G	0.02			8.0			8.0			8.0		
H	DMSO			DMSO			DMSO			DMSO		
	T3 (nM)			DDT (nM)			DDE (nM)			DDD (nM)		

Table 34 second 96 well plate for THRB

	1	2	3	4	5	6	7	8	9	10	11	12
A	200			50000			50000					
B	100			25000			25000					
C	10			5000			5000					
D	2			1000.0			1000.0					
E	1*			200.0			200.0					
F	0.5			40.0			40.0					
G	0.1			8.0			8.0					
H	DMSO			DMSO			DMSO					
	POPs mix			DEHP (nM)			DINP(nM)					

Table 35 & 36 An overview of the different concentrations used in the exposure studies of blue whale PPARγ. Each table represents a 96 well plate, there were used two 96 plates for each receptor in this study, three replicates of each ligand were used.

	1	2	3	4	5	6	7	8	9	10	11	12
A	50000			50000			50000			50000		
B	25000			25000			25000			25000		
C	5000			5000			5000			5000		
D	1000.0			1000.0			1000.0			1000.0		

E	200.0	200.0	200.0	200.0
F	40.0	40.0	40.0	40.0
G	8.0	8.0	8.0	8.0
H	DMSO	DMSO	DMSO	DMSO
	ROSI (nM)	DDT (nM)	DDE (nM)	DDD (nM)

Table 36 second 96 well plate for PPARγ

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	200			50000			50000					
B	100			25000			25000					
C	10			5000			5000					
D	2			1000.0			1000.0					
E	1*			200.0			200.0					
F	0.5			40.0			40.0					
G	0.1			8.0			8.0					
H	DMSO			DMSO			DMSO					
	POPs mix			DEHP (nM)			DINP(nM)					

Appendix III

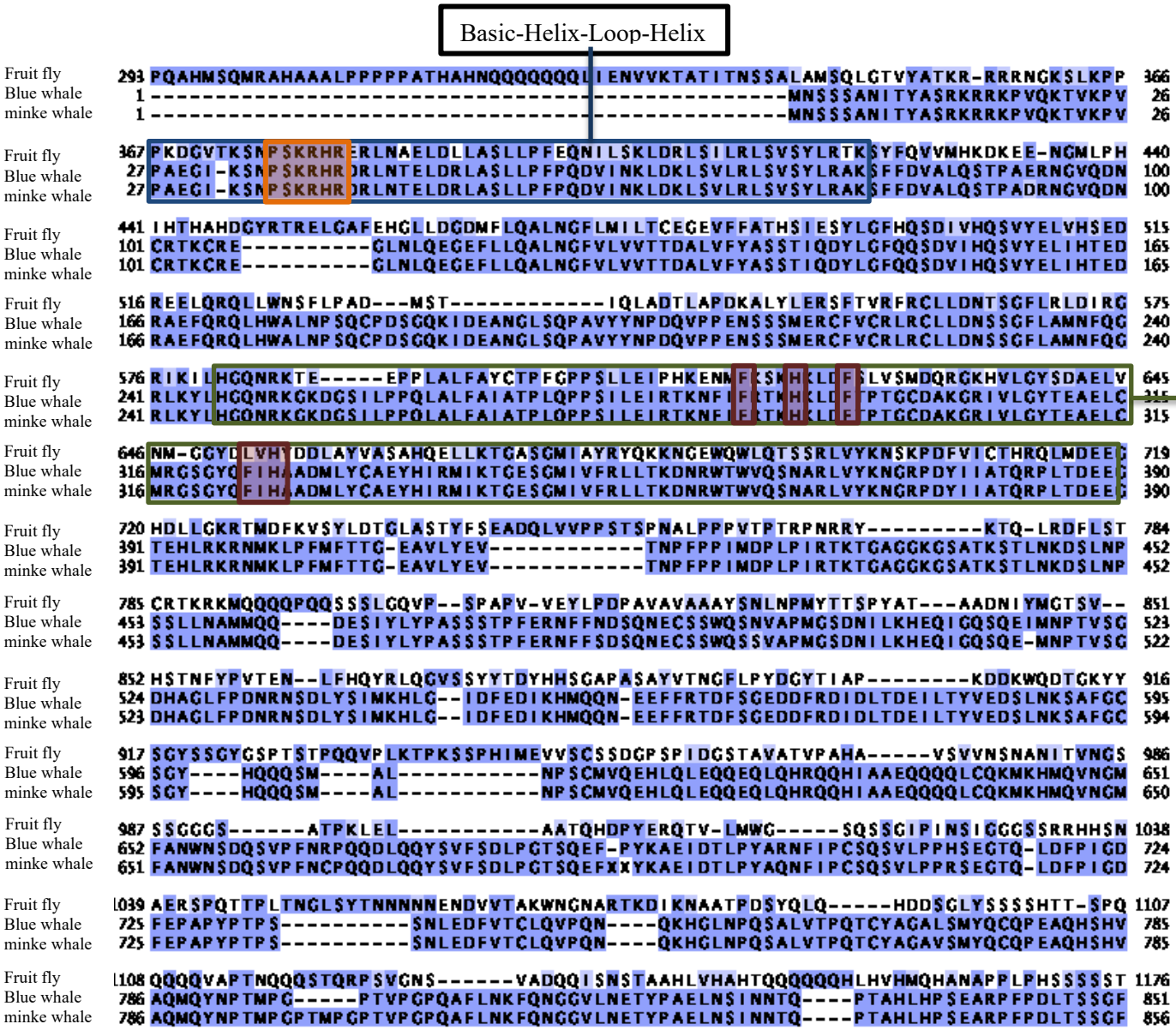

Figure 37 MSA of invertebrate (fruit fly) and blue &minke whale. A comparison of the amino complete sequences of AhR was conducted to investigate if the important amino acids marked in the results are also conserved in invertebrates as well as mammals. Amino acids that are important for DNA-binding are marked in orange, and amino acids important for ligandbinding are marked in purple (Bacsi & Hankinson, 1996; Swanson & Yang, 1996).

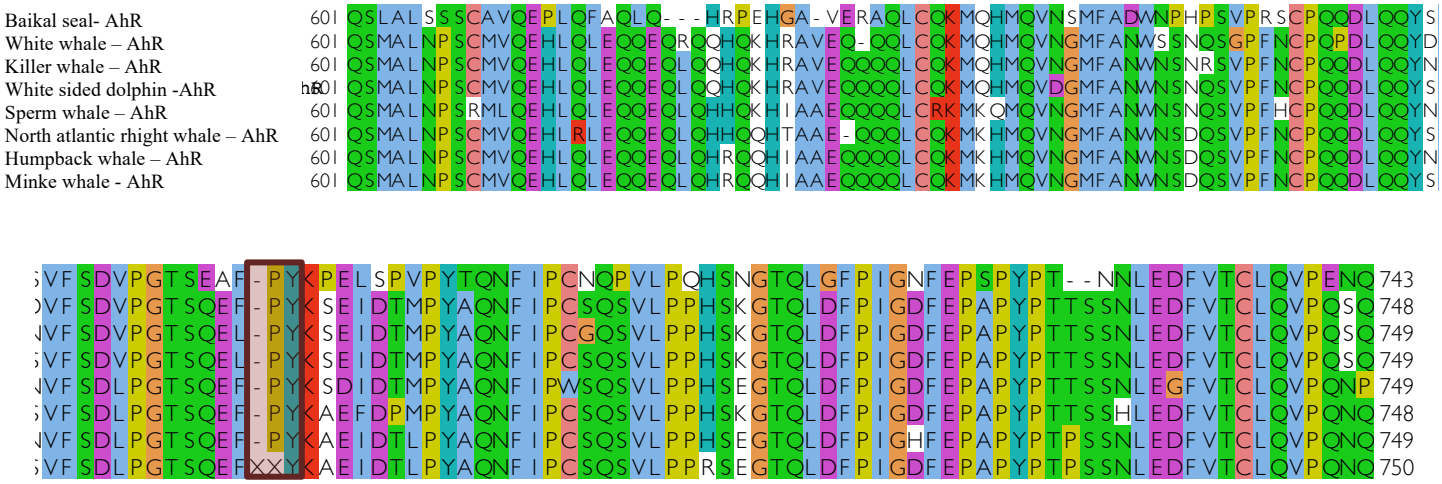

Figure 38 MSA of AhR in marine mammals. A comparison of the amino acid sequences of AhR was conducted to investigate if the "Lost" amino acids in minke whale marked in purple are identical in the other compared species. The lost amino acids in the incompleate Minke whale sequese appear to be a P.

Appendix IV

In vitro modulation of transcriptional activity in nuclear receptors of fin and blue whales by environmental pollutants

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Background & objectives

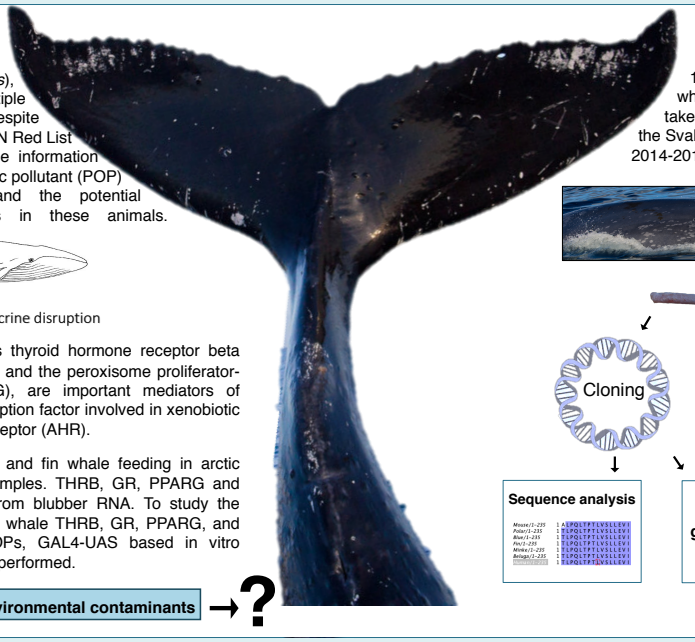
Why? Blue whales (*Balaenoptera musculus)* and fin whales (*B. physalus*), are potentially being impacted by multiple stressors and pollutant mixtures. Despite being listed as threatened on the IUCN Red List of Threatened Species, there is little information available regarding persistent organic pollutant (POP) concentrations in their blubber, and the potential toxicological effect s of pollutants in these animals.

POPs → Endocrine disruption

What? Nuclear receptors, such as thyroid hormone receptor beta (THRβ), glucocorticoid receptor (GR) and the peroxisome proliferator-activated receptor gamma (PPARG), are important mediators of endocrine disruption. Another transcription factor involved in xenobiotic responses is the Aryl hydrocarbon receptor (AHR).

How? Levels of pollutants in blue and fin whale feeding in arctic waters were analyzed in blubber samples. THRB, GR, PPARG and AhR were cloned and sequenced from blubber RNA. To study the transcriptional activity of blue and fin whale THRB, GR, PPARG, and AHR when exposed to legacy POPs, GAL4-UAS based in vitro luciferase reporter gene assays were performed.

Nuclear receptors response to environmental contaminants → ?



Material & methods

18 blue whale and 12 fin whale blubber-samples were taken with a cross bow around the Svalbard archipelago between 2014-2018.

Cloning

Chemical analysis to obtain contaminant concentrations levels in blue and fin whale blubber.

Sequence analysis

Luciferase-gene reporter assay

No or weak agonistic effect of environmental contaminants found in blue whale blubber on transcriptional activity of PPARɣ and THRβ were observed. No detected effect on GR.

Known agonists for GR, PPARɣ and THRβ

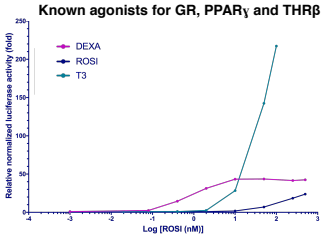


Figure 1 ***In vitro*** **activation of blue whale GR, PPARɣ, THRβ and AhR by known agonists.** The effect of two known agonists on blue whale GR and THRB, measured with a luciferase assay on COS-7 cells.

Fold increase in luciferase activity related to solvent treated cells [max]

Receptor	Known agonist	DDT [50μM]	DDE [50μM]	DDD [50μM]	DEHP [50μM]	DINP [50μM]	POPs MIX [200x]
GR	43,1	1,0	1,0	1,0	0,9	1,0	0,9
THRβ	288,1	0,9	1,1	1,2	1,7	1,2	1,3
PPARɣ	31,5	1,1	1,1	0,9	1.1	1.1	0,9

Table 1 ***In vitro*** **activation of GR, PPARɣ, THRβ and AhR by known agonists or environmental pollutants in a COS7-based luciferase reporter gene assay.** Effects are presented as estimates of fold change at max consentration exposhure

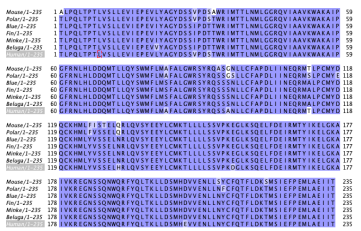


Figure 2 multiple sequence alignment of the ligand binding domain (LBD) in different mammals. Only minor differences were found in the LBD between blue and fin whale, human, mouse, minke and beluga whale.

Key findings

- ❖ Blue and fin whale GR, PPARɣ, THRβ and AhR sequences were obtained and GAL4-based luciferase assays were established
- ❖ Sequence alignments indicated only minor differences between the species
- ❖ No or weak agonistic effects of contaminants found in blubber of blue and fin whale were observed in the reporter assays with GR, PPARɣ and THRβ
- ❖ Further studies are being performed with blue and fin whale AhR

Future research

Comparison of blue and fin whale to other species, in relation to their habitat

Acknowledgements

The Fram Centre Hazardous Substances Program, the Norwegian Research Council, the Norwegian Polar Institute and the University of Bergen

Figure 39. Poster from PRIMO 20 Charleston 2019

Appendix V

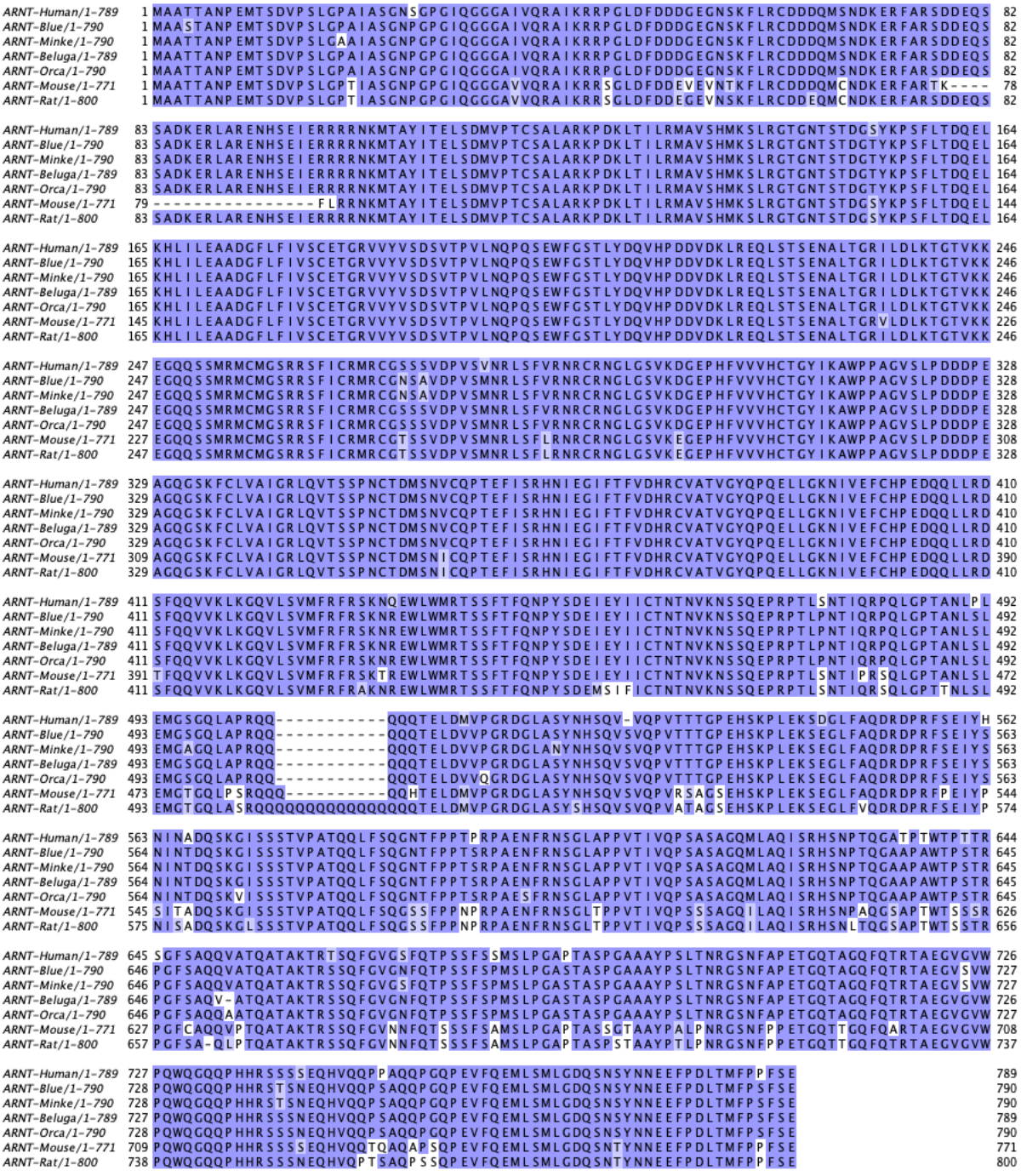


Figure 40 MSA of Blue whale ARNT with other species. A comparison of the amino complete sequences of ARNT was conducted to investigate if the identity of amino acids from different mammal species.