Effect-directed toxicity assessment of sediments from Bergen harbour (Norway) using luciferase reporter gene and cell-based bioassays

Rune Storli

Master thesis in environmental toxicology

Department of Biological Sciences

University of Bergen

September 2022

ii Acknowledgements

This master thesis has been funded by the Research Council of Norway as a part of the dCod 1.0 project (project no. 248840). It has also been supported by Miljøringens studentstipend 2021.

First, I would like to thank my supervisors Anders Goksøyr and Odd André Karlsen. Your help and guidance have been invaluable, and I truly appreciate the effort that has been put into helping me, especially during the writing process. I would also like to thank Aasim Ali for helping me with the chemistry of this thesis, both teaching me and analysing my samples.

Next, I would like to thank Rhîan Gaenor Jacobsen and Alexander Klevedal Madsen for helping me through the lab work, and always answering my stupid questions that I probably should have known the answers to already.

Thanks to all the people at the Environmental Toxicology group, including fellow master students. Everyone has in some small part or way helped me create this thesis.

Lastly, I would like to thank my friends and family. None of this would have been possible without you helping and motivating me, through the highest highs and the lowest lows. Each word of encouragement and sympathy helped me more than you could ever know.

iii Abstract

Bergen harbour has been a historically polluted area in line with the harbours of many other major coastal cities in Norway and other countries around the world. With pollutants accumulating to toxic levels in organisms inhabiting these areas, the need for targeted and precise ways of remediating polluted sediments is necessary. To achieve this, a better understanding of the composition and bioactivity of the pollutants in the sediments is needed. Effect-based bioassays in combination with fractionation of sediment extracts and targeted and non-targeted chemical analysis can be used to identify the specific compounds responsible of mediating toxicity.

Sediments in the inner part of Bergen harbour (Vågen) has previously been shown to contain discharges from factories and harbour activities. In this study, sediment extracts from this site were chemically fractionated into 10 individual fractions ranging from least polar to most polar by using mixtures of hexane, dichloromethane, and methanol in varying ratios. Luciferase-based reporter gene assays were then used to measure the activation of a selected set of stress-activated receptors from Atlantic cod (*Gadus morhua*) and zebrafish (*Danio rerio*) using the different fractions obtained from the sediment samples.

The receptors assessed were Atlantic cod aryl hydrocarbon receptor 2 (gmAhr2a), androgen receptor alpha (gmAra), estrogen receptor alpha (gmEra), and zebrafish pregnane X receptor (drPxr), which were chosen based on previous work (Goksøyr et al, 2021). The gmAhr2a was activated by all the fractions assessed with the exceptions of the least- and most polar ones, which was in good agreement with the EROD assays showing a similar pattern of Cyp1A activity in PLHC-1 cells exposed to the same fractions. The drPxr receptor was selectively activated by two fractions of intermediate and high polarity, with little or no activation by the other fractions. With the gmAra receptor, most activation was observed by fractions from the higher end of the polarity gradient compared to the gmEra receptor where almost all fractions showed similar, but low activation. The general activation in both the gmAra and gmEra receptors were low compared to the controls. Results from the chemical analysis show that most compounds congregated in fractions 2, 3 and 4. This coincides somewhat with the activation of the Ahr receptors where fraction 4 activated the strongest. For the other receptors this is not the case and further chemical characterization of the fractions is ongoing.

iv Abbreviations

Full name	Abbreviation
Persistent organic pollutant	POP
Organochloride pesticide	OCP
European Union	EU
Contaminants of emerging concern	CEC
Polycyclic aromatic hydrocarbon	PAH
Polychlorinated biphenyl	PCB
Persistent, bioaccumulative and toxic	PBT
Emerging contaminant	EC
Pharmaceuticals and personal care product	PPCP
Endocrine-disrupting compound	EDC
Flame retardant	FR
Artificial sweetener	ASW
Adverse outcome pathway	AOP
Molecular initiating event	MIE
Adverse outcome	AO
Key event relationship	KER
Key event	KE
Nuclear receptors	NR
Thyroid hormone receptor	TR
Retinoic acid receptors	RAR
Peroxisome proliferator activated receptors	PPAR
Reverse-Erb receptors	REV-ERB
Retinoic acid related receptors	ROR
Farnesoid X receptors	FXR
Liver X receptors	LXR
Pregnane X receptor	PXR
Vitamin D receptor	VDR
Steroid hormone receptor	SHR
Androgen receptor	AR
Progesterone receptor	PR
Glucocorticoid receptor	GR
Mineralocorticoid receptor	MR
Estrogen receptors	ER
N-terminal domain	NTD
DNA binding domain	DBD
Hinge region	H
Ligand binding domain	LBD
C-terminal domain	C
Aryl hydrocarbon receptor	AHR
Basic helix-loop-helix, Per-Arnt-Sim	bHLH-PAS
Luciferase reporter gene assay	LRA
Ethoxyresorugin-O-deethylase	EROD
Halogenated organic compound	HOC
Chlorinated aliphatic hydrocarbon	CAH
Chlorophenols	CPhs
Polychlorinated dibenzo-p-dioxin	PCBD
Polychlorinated dibenzofuran	PCDF
Polybrominated diphenyl ether	PBDE
Effect-directed analysis	EDA
Toxicity identification evaluation	TIE

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	TCDD
17α-Ethynylestradiol	EE_2
Adenosine 5'-triphosphate disodium salt trihydrate	ATP
Bovine serum albumin	BSA
Coenzyme A	CoA
Dimethyl sulfoxide	DMSO
DL-Dithiothreitol	DTT
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12	DMEM/F-12
Dulbecco's Modified Eagle medium with phenol red	DMEM w/ phenol red
Dulbecco's Modified Eagle medium without phenol red	DMEM
Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid	EGTA
Ethylenediaminetetraacetic acid disodium salt	EDTA
Fetal bovine serum	FBS
O-nitrophenyl-β-D-galactopyranoside	ONPG
Phenylmethanesulfonylfluoride	PMSF
Phosphate buffered saline	PBS
Accelerated solvent extraction	ASE
Escherichia coli	E. coli
Sediment extract fraction	SEF
Principal component analysis	PCA
Mode of action	MOA

Table of contents

Acknowledgements……ii

Abstract……..iii

Abbreviations……iv

1 Introduction……1

1.1 Sediments as sink and source for pollutants……1

1.1.1 Legacy contaminants……2

1.1.2 Contaminants of emerging concern……3

1.2 Adverse outcome pathway……3

1.3 Nuclear receptors……4

1.3.1 Androgen receptors……5

1.3.2 Estrogen receptors……5

1.3.3 Pregnane X receptor……5

1.4 Aryl hydrocarbon receptor……6

1.5 Bioassays……6

1.6 Halogenated organic compounds……7

1.7 Polybrominated diphenyl ethers……7

1.8 Polycyclic aromatic hydrocarbons……7

1.9 Mixture toxicity……8

1.10 Effect-directed analysis……8

1.11 Aim of thesis……10

2 Materials……11

2.1 Chemicals……11

2.2 Plasmids……12

2.3 Cell lines……13

2.4 Growth medium luciferase assay……13

2.5 Growth medium EROD assay……13

2.6 Buffers and solutions……14

2.6.1 Luciferase……14

2.6.2 EROD assay……15

2.7 Accelerated Solvent Extraction (ASE)……16

2.8 Chromatography……16

2.9 Instruments……17
3 Methods……18
3.1 Sediment extraction and fractionation……18
3.2 Plasmid preparation and purification……19
3.3 Luciferase reporter gene assay……20
3.3.1 COS-7 cell cultivation……21
3.3.2 Seeding……21
3.3.3 Transfection……21
3.3.4 Exposure……22
3.3.5 Lysis……22
3.4 Ethoxyresorufin -O-deethylase (EROD) assays……23
3.4.1 PLHC-1 cell cultivation……23
3.4.2 Seeding……23
3.4.3 EROD activity……23
3.4.4 Protein measurement……23
3.5 Chemical analysis……23
3.5.1 Analysis of Halogenated organic contaminants (HOCs)……24
3.5.2 Analysis of brominated diphenyl ethers (BDEs)……24
3.5.3 Analysis of polycyclic aromatic hydrocarbons (PAHs)……24
4 Results……25
4.1 Luciferase reporter gene assay……25
4.2 Ethoxyresorufin -O-deethylase (EROD) assays……29
4.3 Chemical analysis……30
4.4 Principal component analysis……33
5 Discussion……34
5.1 Summary of the study……34
5.2 general Discussion ……34
5.2 Conclusion……38
5.2 Further studies……39
6 References……39
Appendix……47

1 Introduction

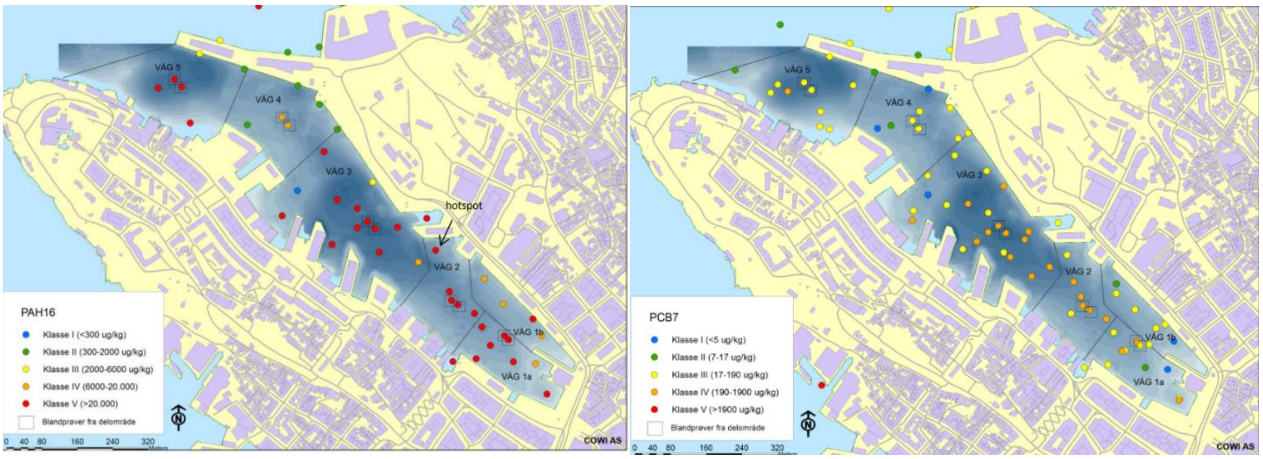
1.1 Sediments as a sink and source for pollutants

Marine sediments represent a diverse habitat for a large and unique range of organisms such as algae, macrophytes, benthic invertebrates, fish, and bacteria. This habitat acts as both a food source and a refuge for these organisms, which in turn are prey for organisms higher up in the food chain (Maher, Batley and Lawrence, 1999). Accumulation of pollutants in the sediments of marine environments can therefore have significant and long reaching effects in form of bioaccumulation and biomagnification. Being on top of the food chain, bioaccumulation and biomagnification can ultimately affect humans. It has been demonstrated that sediments can act as a sink for different compounds like hydrophobic persistent organic pollutants (POPs), including organochloride pesticides (OCPs) and polycyclic aromatic hydrocarbons (PAHs) (Zhang *et al.*, 2009; Tobiszewski and Namieśnik, 2012). Thus, sediments may act both as a source and sink of contaminants in the marine ecosystem

The composition and characteristics of the sediments are crucial for how sedimentation works and what types of compounds that are absorbed into the sediments (Bigus, Tobiszewski and Namieśnik, 2014). Since a lot of the compounds that sediments accumulate are very persistent, many of these can also migrate back in the water after being remobilized. (Kosmehl *et al.*, 2007).

As the dangers of sediment pollution has become evident, the European Union (EU) revised its own directive (EU, 2006) to acknowledge the effect sediment pollution has on water quality and the importance of monitoring and toxicity testing. Even though the EU has designated 33 compounds as priority pollutants with established threshold concentrations, the newfound knowledge of contaminants of emerging concerns (CECs) shows the need for new and effective ways to better understand the composition and potential adverse effects of polluted sediments (EU, 2009).

Many Norwegian fjords have been severely polluted for decades. In 1993, a survey of 120 fjords showed that 90% of them were heavily polluted by one or more pollutants. Industry and harbours have traditionally been placed at the innermost part of the fjords, sheltered from rough seas and weather, and with access to hydroelectric power. With some fjords being very deep, the water exchange in the deepest part of the water column is almost non-existent and released pollutants end up accumulating in the sediments (Jære, 2016; Omsted, 2019). In Bergen, Norway, the waters and sediments in Vågen, which is part of the inner part of Byfjorden, has been a historically polluted area originating from its early origin as a port city. Analyses of the seabed and sediments in this location has revealed high levels of pollutants like PAHs, heavy metals and polychlorinated biphenyls (PCBs) (Figure 1.1). Pollution can be traced to municipal emissions, boat activity, rainwater runoff, and industries. The pollution is complex and the sources are difficult to pinpoint exactly (Miljødirektoratet, 2022).



a) **b)**

Figure 1.1: Levels of PAHs (a) and PCBs (b) in Vågen, Bergen. Colour codes refer to contaminant classification. Figures are from (Bergen Kommune, 2014).

1.1.1 Legacy contaminants

Chemicals can be classified in several different ways depending on their purpose. For example, by their intended allocations like pesticides or pharmaceuticals, by their chemical structure like PCBs or PAHs, their environmental characteristics such as persistent pollutants, or by their mechanism of action, like endocrine disrupting chemicals (Akashe, Pawade and Nikam, 2018). One can also classify pollutants by when they were released into the environment and became a known environmental issue, either as legacy contaminants or emerging contaminants. Legacy contaminants can be defined as contaminants that were previously released into the environment by humans and persist in the environment, are well studied, and have regulations in place for their production and use. Persistent chemicals are chemicals that remain in the environment for long periods of time leading to an extended potential for exposure and subsequent toxicity (Landis, Sofield and Yu, 2018). The category of legacy contaminants encompass among others, persistent, bioaccumulative and toxic (PBT) chemicals like cadmium, lindane, PAHs and PCBs (Hutchinson *et al.*, 2013). These compounds, with the exception of cadmium are covered by the Stockholm Convention on Persistent Organic Pollutants that aims to protect human health and the environment (Porta and Zumeta, 2002). There are also specific directives in the EU to curtail the pollution of water, mainly the Water Framework Directive, which dictates the water quality to protect the use and sustainability of fresh water, groundwater, and coastal waters. These directives and conventions have greatly helped to reduce the introduction of new contaminants in both water and sediments, although they do not have any effect on the pollutants already released into the environment (EU, 2000)

1.1.2 Contaminants of emerging concern

The first reports of emerging contaminants (ECs), also known as contaminants of emerging concern (CECs), date back to 1962. Even though this phenomenon has been known for six decades, it is only in the last two decades that the majority of research on occurrence and detection in the environment has been performed (Rosenfeld and Feng, 2011). CECs encompass compounds such as pharmaceuticals and personal care products (PPCPs), endocrine-disrupting compounds (EDCs), flame retardants (FRs), certain pesticides and artificial sweeteners (ASWs) (Salimi *et al.*, 2017). A notable difference between legacy contaminants and CECs is that while the legacy contaminants are normally regulated globally the CECs are not. While not being regulated, some CECs have the ability to cause toxicological effects like inhibiting growth and mobility in fish, developing antibiotic resistant bacteria, and damaging surrounding ecosystems (Yang *et al.*, 2017; Grenni, Ancona and Barra Caracciolo, 2018).

CECs are in general not defined by the timeframe since they were first discovered but by the amount of research and knowledge about them. Thus, a contaminant may keep its status as a CEC for decades as long as the knowledge about the compound is lacking, even though it is suspected that their toxicity or persistence may affect living organisms (Sauvé and Desrosiers, 2014).

1.2 Adverse outcome pathway

Adverse outcome pathways (AOPs) are a mechanistic representation of the toxicological effects on different levels of biological organization. AOPs consist of a molecular initiating event (MIE), where there is an interaction between a biomolecule and a xenobiotic, a series of intermediate steps and events, and finally an adverse outcome (AO) at a higher biological level (individual, population) (Vinken, 2013). By portraying existing knowledge between the MIE and an AO, the AOP can help determine relevant risk assessment according to biological levels of organizations. Ideally, the aim of an AOP is to link the MIE to the AO by predictive linkages termed as key event relationships (KERs) between measurable and/or observable essential biological changes termed key events (KE) (Villeneuve *et al.*, 2014). The development of the AOP came from the need to describe the pathway of toxicity to a well-quantified endpoint of demographic significance. Compared to this, the toxicity pathway only covers the toxic effects from the cellular responses that is expected to result in adverse health effects (Ankley *et al.*, 2010).

The concept of AOP is based upon the evolution of mechanism and mode of action as there was uncertainties concerning the use of these terms in the field of toxicology (Ankley *et al.*, 2010). Compared to both mode and mechanism of action the scope of an AOP is much broader and can be designed for both different human-relevant toxicological and ecotoxicological endpoints (Vinken, 2013).

1.3 Nuclear receptors

Nuclear receptors (NR) are transcription factors that regulate several important biological processes like cell growth and development, reproduction, and inflammation (Chai and Chen, 2021). Several NRs can act as both metabolic and toxicological sensors. By quickly responding to environmental stimuli, the nuclear receptors are able to induce transcription of metabolic genes and pathways to adapt to new situations (Delistraty, 1997). NRs are DNA-binding proteins that regulate the transcription of genes as a response to cognate ligands. As the nuclear receptors are largely ligand-dependent they represent obvious targets for drug design (Renaud and Moras, 2000).

The NR superfamily can be divided into seven different subfamilies (N0 to N6), which is based on the nomenclature system developed for cytochrome P450s (Auwerx *et al.*, 1999). The NRs in these subfamilies differ by binding ligands with different structures, size, and charge. However, they share a common modular domain structure (Figure 1.3), with the exception of the subfamily N0 (Weikum, Liu and Ortlund, 2018). For this thesis I will focus on subfamilies 1 and 3 which contain the receptors used in the master's project.

Subfamily 1, NR1 is a group containing thyroid hormone receptors (TR), retinoic acid receptors (RAR), peroxisome proliferator activated receptors (PPAR), reverse-Erb receptors (REV-ERB), retinoic acid related receptors (ROR), farnesoid X receptors (FXR), liver X receptors (LXR), pregnane X receptor (PXR) and vitamin D receptors (VDR). The activity of these receptors are mediated by lipophilic signalling molecules, thyroid hormones, fatty acids, bile acids and sterols (Weikum, Liu and Ortlund, 2018).

The subfamily 3, or NR3, consists of steroid hormone receptors (SHRs) like androgen receptors (AR), progesterone receptor (PR), glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and the estrogen receptors (ERs). The steroid receptors are regulators of metabolic, reproductive and developmental processes (Weikum, Liu and Ortlund, 2018)

Figure 1.3: Illustration of the general structure of a nuclear receptor (NR) with the N-terminal domain (NTD), the DNA binding domain (DBD), hinge region (H), ligand binding domain (LBD) and C-terminal domain (C). Figure based on Porter et al., 2019

The general structure of a nuclear receptor is shown in Figure 1.3 with the five regions/domains conserved through the superfamilies. These structures are the N-terminal domain (NTD), the DNA binding domain (DBD), the hinge region, a ligand binding domain (LBD), and lastly a variable C-terminal domain (Porter *et al.*, 2019). In the DBD, two zinc finger motifs act as hooks to allow the binding of DNA within the nucleus. As for the LBD, it differs highly both in specificity and affinity to specific ligands but remains similar in function among the families of NRs. Except for the orphan receptors, the members of the superfamily of NRs are all ligand activated. All the ligands within each subfamily are similar, but it is the classification of these ligands that determines which class the receptors belong to (Porter *et al.*, 2019).

1.3.1 Androgen receptor

The androgen receptor (AR, NR3C4) is part of the NR3 subfamily. It's main responsibility is mediating of the physiological effects of androgens by influencing transcription of androgen responsive genes by binding to specific DNA sequences (Gelmann, 2002). ARs are found in all vertebrates, including fish and human (Ikeuchi *et al.*, 2001). Androgens are important for the male sexual maturation and the maintenance of spermatogenesis alongside gonadotropin regulation. This does not mean that the AR is unimportant for females as it is a requirement for the normal development of fertility, reproductive tract, brain, cardiovascular system and smooth muscle and bone (Heinlein and Chang, 2002; Dart *et al.*, 2013).

1.3.2 Estrogen receptor

The estrogen receptor (ER, NR3A1, NR3A2), part of the subfamily NR3 is a member of the steroid/nuclear receptor superfamily. The mammalian ER is divided into two subtypes that are encoded from two different genes, i.e. alpha or beta (ERα and ERβ). When binding to a ligand, ER goes through conformational changes (activation), to form a ligand-occupied ER dimer (Klinge, 2001). ERs are involved in numerous processes like the menstrual cycle, estrous cycle, tissue growth maintenance and reproduction. The subtypes of ERs have different distribution, both in tissue and cells. ERα is found in the mammary gland, uterus, thecal cells, bone, testes, epididymis, stroma, liver, and adipose tissue. On the other hand, ERβ is found epithelium, bladder, granulosa cells, colon, adipose tissue, and the immune system. While ERα and ERβ are mainly distributed in different areas, but there are overlaps where both are found such as in the cardiovascular and central nervous system (Farzaneh and Zarghi, 2016).

1.3.3 Pregnane X receptor

The pregnane X receptor (PXR/NR1I2) is a ligand activated transcription factor in the nuclear hormone receptor family, specifically in the subfamily NR1. PXR plays an important role in the regulation of cytochrome P450-3A (CYP3A) gene expression in mammalian liver and small intestine, thus facilitating xenobiotic metabolism. PXR has also been shown to play a role in endobiotic metabolism (Watkins *et al.*, 2001; Ihunnah, Jiang and Xie, 2011).

PXR has been shown to be activated by many different compounds encompassing xenobiotics like rifampicin, clotrimazole and hyperforin, both natural and synthetic steroids, and bile acids. Notably, with the exception of the Merlucciidae family, the PXR receptor is not present in Gadiformes, but it has been suggested that the aryl hydrocarbon receptor (Ahr) in the Atlantic cod instead has acquired an extended regulatory role in the expression of some PXR target genes such as *cyp3a* (Eide *et al.*, 2018)**.**

1.4 Aryl hydrocarbon receptor

The aryl hydrocarbon receptor (AHR) is another xenosensing transcription factor belonging to the basic helix-loop-helix, Per-Arnt-Sim (bHLH-PAS) family (Larigot *et al.*, 2018). This receptor has been shown to modulate the responses to environmental stimuli and help to maintain cellular homeostasis (Neavin *et al.*, 2018; Safe *et al.*, 2020). AHR is found in a wide range of cell types in varying concentrations, working as both an activator of metabolism and as a regulator of different cell functions. This includes the immune system, signalling pathways critical to homeostasis, which includes physiological functions such as cell, proliferation and differentiation, gene regulation, cell motility and more (Esser, Rannug and Stockinger, 2009; Feng, Cao and Wang, 2013).

AHR is a ligand-dependent transcription factor that binds to and is activated by dioxins, dioxin like compounds and related chemicals and plays a role in mediating the metabolism of these compounds (Sharma *et al.*, 2021). When binding to a ligand, the cytoplasmic AHR translocate to the nucleus, heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and induces the transcription of numerus AHR-responsive genes including *cyp1a*, which is an important biomarker to mediate biological and toxicological effects (Furue *et al.*, 2014).

1.5 Bioassays

Using bioassays, toxicity can be tested at different levels of organization by employing both *in vivo* (whole organism exposure testing) and *in vitro* (tissue, organs, or cells outside the living organism) methodologies. The advantage of using *in vitro* bioassays is that it is a low cost method that is repeatable for both specific and non-specific toxicological endpoints with almost none of the ethical considerations of *in vivo* testing (van de Merwe *et al.*, 2018; Escher, Peta and Leusch, 2021). *In vitro* bioassays are useful for quantifying the effects of known and unknown chemicals on specific endpoints while also accounting for potential mixture effects and the fact that chemicals that are more potent will have a greater effect, making *in vitro* bioassays risk scaled (Leusch *et al.*, 2018). When applied to a mixture containing different substances with the same mode of action, for example receptor activation, the biological signal is higher than for a single substance making *in vitro* bioassays highly suitable as a screening tool for environmental samples, and also being able to quantify and distinguish between agonistic and antagonistic effects (Wernersson *et al.*, 2011).

Assessing receptor activity using a luciferase reporter gene assay (LRA) and CYP1A activity with ethoxyresorufin-O-deethylase (EROD) assays are one way to construct a bioassay. In the case of this master thesis, several receptor based endpoints and *in vitro* bioassays utilizing cell cultures to detect the existence of chemical compounds and pollutants were used (Belin Tavakoly Sany *et al.*, 2016). LRA utilizes the reporter gene luciferase, an enzyme that catalyses a bioluminescence reaction where the light intensity is measured to determine activation of a receptor, making it a suitable assay for the quantitative measurement of receptor activation (Nakajima and Ohmiya, 2010). The EROD bioassays uses PLHC-1 epithelial cells derived from liver tissue of *Poeciliopsis lucida* (topminnow) to monitor the induction of the enzyme CYP1A which is a xenobiotic metabolizing enzyme and a biomarker for exposure to compounds that bind the AHR (Goksøyr *et al.*, 1992; Petrulis *et al.*, 2000).

1.6 Halogenated organic compounds

Organic molecules that contain fluorine, chlorine, bromine, or iodine atoms are classified as halogenated organic compounds (HOCs). As HOCs have long-term bioaccumulative characteristics and a resistance to both chemical and biological degradation they are considered as POPs. HOCs can be further divided into several other classes of compounds like the organochloride compound family, which is the largest HOC family, consisting of chlorinated aliphatic hydrocarbons (CAHs), chlorophenols (CPhs), PCBs, polychlorinated dibenzo-p-dioxins (PCBDs), and polychlorinated dibenzofurans (PCDFs). HOCs are generally accumulated in fatty tissue where they undergo a biomagnification and bioaccumulation process causing negative impact in organism health and is in humans associated with diabetes, obesity, and endocrine disruption. HOCs also cause alterations in enzyme activity where some show inhibition by HOCs, while others metabolize these compounds. The cytochrome P450 (CYP450) is one family of enzymes affected by HOCs, where CYP1A1, 1A2, 2A1 and 1B1 are induced via AHR by coplanar PCBs and dioxins (Louis, Hallinger and Stoker, 2013; Artabe, Cunha-Silva and Barranco, 2020).

1.7 Polybrominated diphenyl ethers

Polybrominated diphenyl ethers (PBDEs) are used as flame retardants in consumer products like textiles, electronics, and plastics, and are found in both abiotic and biotic environmental samples. Global PBDE productions in 2001 was about 67000 tons and due to their environmental persistence and chemical properties bioaccumulate and biomagnifies in the environment with the highest levels found in aquatic biotopes. PBDEs has been shown to have adverse health effects on humans such as abnormal birth weights, cryptorchidism, and endocrine disruptions. In 2009, PBDEs were designated as a banned chemical in the Stockholm convention on persistent organic pollutants. However, it was not until 2020 that the EU banned the recycling of products containing brominated flame retardants (Darnerud *et al.*, 2001; Hites, 2004; Lorber, 2007; Meeker *et al.*, 2009; Ma *et al.*, 2012). (Darnerud *et al.*, 2001; Hites, 2004; Lorber, 2007; Meeker *et al.*, 2009; Ma *et al.*, 2012; Bich, 2022).

1.8 Polycyclic aromatic hydrocarbons

PAHs consist of aromatic hydrocarbons with two or more fused benzene rings that can be divided into light and heavy PAHs depending on if the compounds contain more or less than four benzene rings. Most PAHs encompass multiple components such as naphthalene, acenaphthylene, acenaphthene, fluorine, phenanthrene, anthracene, fluoranthene, pyrene, benz[α]anthracene, benzo[α]pyrene and indenol(1,2,3-cd) pyrene (Lawal, 2017).

PAH accumulation in the environment is a result of incomplete combustion of organic materials, their low solubility in water, and that they are highly lipophilic. Most PAHs is released as emission from fossil fuel burning such as combustion engines in cars, waste incinerators, oil refining and asphalt production, etc., and has been an established environmental pollutant for decades (Laflamme and Hites, 1978). Studies have shown PAHs to be mutagenic and carcinogenic, cause elevated EROD levels and DNA damage, and cause endocrine disruption in fish. Human exposure of PAHs include uptake from air, dust, food, and dermal contact (Srogi, 2007; Zhang *et al.*, 2016; Lawal, 2017).

1.9 Mixture Toxicity

‘’There is no such thing as a single chemical exposure’’, states Yang et al. (1998). Most environmental contaminants are found as mixtures where both the behaviour and predicted toxicity may not act as it does from how each individual compound behave in the lab. The cocktail effects are where the mixing of different chemicals might alter their effects and cause synergistic interactions, additive effects, antagonistic effects, and potentiation. Some chemicals can enhance the effects of others, producing a joint effect larger than the sum of their individual parts (Yang *et al.*, 1998; Burcham, 2014; Cedergreen, 2014; Sternudd, 2022).

As most ecotoxicological studies focus on exposure and effects of individual compounds, also most regulatory measures are based on single compound toxicity testing. The European Commission has highlighted this lack of knowledge on mixture assessment in 2012 from the lack of exposure information (European Commission - Directorate General Environment, 2012).

1.10 Effect-directed analysis

The complexity of an environmental sample can be staggering, containing thousands of different chemicals and pollutants. Determining which of these compounds that can cause toxicity or be harmful is a daunting task requiring an enormous amount of both time and effort. It is unreasonable and unrealistic to think that analysing each individual compound is plausible. Therefore, a better approach is needed, where the complexity of the sample is reduced while also limiting the possibility of overlooking significant contributors to risk and effect (Brack *et al.*, 2016). This is where effect-directed analysis (EDA) can be employed to meet these challenges. By testing the bioactivity of a mixture using the responses of a cellular system, i.e., biotesting which is defined as ‘’the evaluation of the properties of the subject under investigation in terms of its effect on the biological test system under standard conditions’’ (Goncharuk and Kovalenko, 2012), and combining this with fractionation and chemical analysis it is possible to identify the bioactive chemicals in a complex environmental sample (Brack *et al.*, 2016).

Chromatography is the process of separating different components of a mixture (J. Calvin, 2022). It differs from other separation methods in that a large variety of materials, equipment and techniques can be used. The term chromatography applies to an assortment of different separation techniques of partitioning or distribution of a sample (solute) between a mobile and stationary phase. Chromatography was first applied in the late nineteenth century, but it was not until its importance to the oil industry in the late 1960s that the method evolved into a sophisticated, commercially available technique. The type of chromatography employed in this thesis is liquid column chromatography and will therefore be the focus, but this is just one of the many different types of chromatography techniques one can use to separate the compounds present in a mixture (Ismail and Nielsen, 2010).

In column chromatography the fractionation of the compounds in the mixture occurs because of the differential migration through a closed stationary phase where the analytes can be monitored while the separation is ongoing. The length and diameter of a column determines the resolution and separation of the mixture where a longer and narrower column provides the best result (Ismail and Nielsen, 2010).

Silica as a stationary phase is widely used for chromatography and has been shown to be an absorbent for nonionics and ionics where the typical separation range of silica columns range from nonpolar to medium polarity. Silica also show metal ion retention capabilities by cation exchange (Smith and Pietrzyk, 1984).

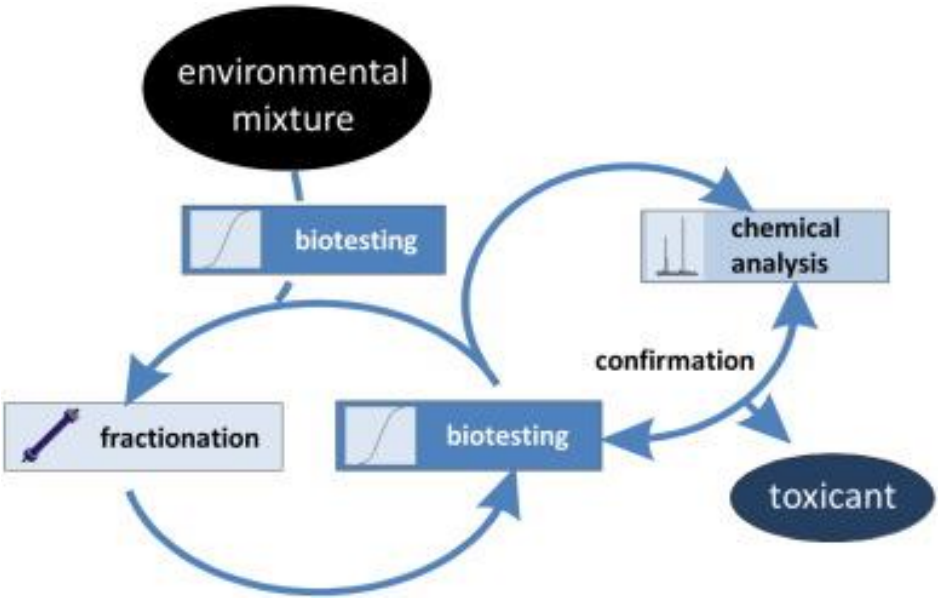


Figure 1.10: Illustration of the general scheme of effect-directed analysis (EDA) where an environmental mixture is biotested, fractionated and biotested again. Chemical analysis is employed to identify toxicants, if the toxicants cannot be identified the sample goes through the cycle again. Figure from Brack *et* al., 2016.

Figure 1.10 outlines the general concept of EDA. This is a process that is repeated several times until the toxicants has been identified. A sample undergoes biotesting before it is further fractionated and biotested again. Lastly, chemical analysis is used to identify the toxicants present. If the toxicants cannot be identified the sample undergoes the whole procedure again, where it is fractionated further followed by more chemical analyses and biotesting to identify the toxicant. This approach has a large area of impact in its usefulness, including drug discovery, toxicology, forensic, and environmental sciences (Brack *et al.*, 2016).

Another approach to identify chemicals and contaminants causing toxicity is the toxicity identification evaluation (TIE). This method and EDA differ significantly in how they approach the assessment of the polluted sample by having different initial assumptions, strategies, methodologies and in most cases endpoints. The most distinct difference in the two approaches are the endpoints. For EDA the typical endpoints are *in vitro* endocrine disruption, mutagenicity, and genotoxicity, while TIE focuses on the organism with survival, growth, and reproduction. While TIE does not target any specific chemicals, EDA usually has an emphasis on organic contaminants. There is also a difference in the degree of specificity of toxicant identification. Whereas EDA has a very high specificity, TIE is more focused around the classes of toxicants while only being moderately specific for individual toxicants. (Burgess *et al.*, 2013).

1.11 Aim of this master thesis

The aim of this master thesis was to study the toxicity of sediments from Vågen, Bergen and examine if effect-directed analysis can be used as an approach to identify the cause of the biological effects observed. This thesis is a continuation of earlier works trying to identify the compounds activating chosen receptors in sediments from several different locations in the Bergen area by S. Goksøyr et al. (2021)(Goksøyr *et al.*, 2021).The sediment from Vågen was chosen as this was the site containing the most chemicals and with the highest receptor activating toxicity. As this sediment had already been tested using bioassays, a correlation between the detected compounds in the chemical analysis with the bioassay data could be envisaged. To achieve the goal, the approach I chose was to:

1. Extract and fractionate the sediments originating from Vågen, Bergen using ASE and gravity column chromatography to obtain ten different effluent mixtures based on polarity.

2. Use bioassays to determine activation of the receptors chosen by each fraction. The bioassays employed were receptor activation measurements using a luciferase reporter gene-based bioassay battery consisting of the stress activated receptors gmAre, gmEra, gm Ahr2a and drPxr from fish expressed in COS-7 cells. Induction of EROD activity was also performed on PLHC-1 cells derived from liver tissue of *Poeciliopsis lucida,* topminnow to measure CYP1A activity.

3. Compare the biological data with the chemical analysis data to try to identify the specific compounds or groups of compounds causing activation of the chosen receptors.

2 Materials

2.1 Chemicals

Table 2.1 Overview of chemicals, their chemical formula and if possible, their supplier

Name	Chemical formula	Supplier
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)	$C_{12}H_4Cl_4O_2$	Sigma-Aldrich
17α-Ethynylestradiol (EE_2)	$C_{20}H_{24}O_2$	Sigma-Aldrich
7-ethoxyresorufin	$C_{14}H_{11}NO_3$	Sigma-Aldrich
B-Naphthoflavone	$C_{19}H_{12}O_2$	
Adenosine 5'-triphosphate disodium salt trihydrate (ATP)	$C_{10}H_{20}N_5Na_2P_3$	Sigma-Aldrich
Agar-agar		Merch Millipore
Ampicillin sodium salt	$C_{16}H_{18}N_3NaO_4S$	Sigma-Aldrich
Androgen		Sigma-Aldrich
Bovine serum albumin (BSA)		Sigma-Aldrich
CHAPS	$C_{32}H_{58}N_2O_7S$	ThermoFischer
Clotrimazole	$C_{22}H_{17}ClN_2$	Sigma-Aldrich
Coenzyme A (CoA)		ThermoFischer
Dichloromethane	CH_2Cl_2	Sigma-Aldrich
Dimethyl sulfoxide (DMSO)	$(CH_3)_2SO$	Sigma-Aldrich
Disodium hydrogen phosphate dihydrate	$Na_2HPO_4•2H_2O$	Sigma-Aldrich
Di-Sodium hydrogen phosphate dihydrate	$Na_2HPO_4*2H_2O$	Sigma-Aldrich
DL-Dithiothreitol (DTT)	$HSCH_2CH(OH)CH(OH)CH_2SH$	Sigma-Aldrich
D-luciferin	$C_{11}H_8N_2O_3S_2$	Biosynth
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12)		ThermoFisher
Dulbecco's Modified Eagle medium with phenol red (DMEM w/ phenol red)		Sigma-Aldrich
Dulbecco's Modified Eagle medium without phenol red (DMEM)		Sigma-Aldrich
Erythrosine B	$C_{20}H_6I_4Na_2O_5$	Sigma-Aldrich
Ethanol	CH_3CH_2OH	Sigma-Aldrich
Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA)	$[CH_2OCH_2CH_2N(CH_2CO_2H)_2]_2$	Sigma-Aldrich
Ethylenediaminetetraacetic acid disodium salt (EDTA)	$C_{10}H_{14}N_2NaO_8$	Merch Millipore
Fetal bovine serum (FBS)		Sigma-Aldrich
Fluorescamine	$C_{17}H_{10}O_4$	Sigma-Aldrich
Glycerol	$HOCH_2CH(OH)CH_2OH$	Sigma-Aldrich
Hexane	$CH_3(CH_2)_4CH_3$	Sigma-Aldrich
Isopropanol	$(CH_3)_2CHOH$	Kemetyl
L-α-phosphatitylcholine		Sigma-Aldrich
L-glutamine	$H_2NCOCH_2CH_2CH(NH_2)CO_2H$	Sigma-Aldrich
Magnesium carbonate hydroxide pentahydrate	$(MgCO_3)_4•Mg(OH)_2•5H_2O$	Sigma-Aldrich

Magnesium chloride	$MgCl_2$	Sigma-Aldrich
Magnesium sulfate	$MgSO_4$	Sigma-Aldrich
Methanol	CH_3OH	Sigma-Aldrich
O-nitrophenyl-β-D-galactopyranoside (ONPG)	$C_{12}H_{15}NO_8$	Sigma-Aldrich
Opti-MEM		ThermoFischer
Penicillin-Streptomycin		Sigma-Aldrich
Phenylmethanesulfonylfluoride (PMSF)	$C_7H_7FO_2S$	Sigma-Aldrich
Phosphate buffered saline (PBS)		Sigma-Aldrich
Potassium chloride	KCl	Sigma-Aldrich
Resorufin sodium salt	$C_{12}H_6NNaO_3$	Sigma-Aldrich
Silica gel, pore size 60		Sigma-Aldrich
Sodium chloride	NaCl	Merch Millipore
Sodium dihydrogen phosphate monohydrate	$NaH_2PO_4*H_2O$	Merch Millipore
Sodium pyruvate	$C_3H_3NaO_3$	Sigma-Aldrich
TransIT-LT1		Mirus
Tricine	$(HOCH_2)_3CNHCH_2CO_2H$	Sigma-Aldrich
Trypsin-EDTA solution 1x (0.05% trypsin, 0.02%EDTA)		Sigma-Aldrich
Tryptone		Merch Millipore
Yeast extract		Fluka

2.2 Plasmids

Table 2.2.1 Plasmid preparation kit

Name	Usage (FB*)	Supplier
NucleoBond© Plasmid purification kit	Plasmid preparation (Midi-prep)	Macherey-Nagel

Table 2.2.2 Plasmid overview

Plasmid	Plasmid type	Reporter gene system
GudLuc C	Reporter	AHR2/ARNT
pCMV-β-Gal	Control	Gal4/UAS
pcDNA3.1 gm ARNT	Dimerization partner	
pcDNA3.1 gm AHR2A	Receptor	
pcDNA3.1/zeo(+)	“Empty DNA”	
(MH100)x4 tk luc	Reporter	
pCMX-Gal4-Arα (cod)	Receptor	
pCMX-Gal4-Erα (cod)	Receptor	
pCMX-Gal4-PXR-TL (zebrafish)	Receptor	

2.3 Cell lines

Table 2.3 Cells employed in this study

Cell lines	Type
PLHC-1	Eukaryote, hepatocyte liver tissue
COS-7	Eukaryote, African green monkey kidney cells
StrataClone Solo Pack Competent Cells	Prokaryote, *Escherichia coli*

2.4 Growth medium luciferase assay

Table 2.4.1 Lysogeny broth (LB) growth medium

Components	LB-agar	LB-medium
Tryptone	10g/L	10g/L
Yeast exctract	5g/L	5g/L
NaCl	10g/L	10g/L
MilliQ water	-	-
Agar-agar	15g/L	X
Ampicillin	100mg/L	100mg/L

Table 2.4.2 Media for COS-7 cell freezing and cultivation

Component	Concentration
DMEM w/ phenol red	1x
FBS	10%
L-glutamine	4mM
Sodium pyruvate	1mM
Penicillin-streptomycin	100u/ml
DMSO*	5%

***DMSO was not used for cultivation

2.5 Growth medium EROD assay

Table 2.5.1 Media for PLHC-1 cell freezing and cultivation

Component	Concentration
DMEM/F-12	1x
FBS	10%
Penicillin-streptomycin	100u/ml
DMSO*	

***DMSO was not used for cultivation

2.6 Buffers and solutions

2.6.1 Luciferase

Table 2.6.1.1 Cell lysis buffer*

Components	Concentration
Tris pH 7.8	25mM
Glycerol	15%
CHAPS	2%
L-α-phosphatidylcholine	1%
BSA	1%

***Components makes a 1X solution**

Table 2.6.1.2 Cell lysis reagent solution

Component	Concentration
Cell lysis buffer	1x
EGTA	4mM
$MgCl_2$	8mM
PMSF	0.4mM
DTT	1mM

Table 2.6.1.3 β-galactosidase base buffer*

Component (FB*)	Concentration
Na_2HPO_4	60mM
NaH_2PO_4	40mM
KCl	10mM
$MgCl_2$	1mM

***Components make a 10X solution**

Table 2.6.1.4 β-galactosidase reagent solution

Component	Concentration
B-galactosidase base buffer	1x
B-mercaptoethanol	52.9mM
ONPG	8.6mM

Table 2.6.1.5 Luciferase base buffer*

Components	Concentration
Tricine	80mM
$(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$	4.28mM
EDTA	0.4mM
$MgSO_4$	10.68mM

***Components make a 4X solution and pH is set to 7.4**

Table 2.6.1.6 Luciferase reagent solution

Components	Concentration
Luciferase base buffer	1x
ATP	0.5mM
DTT	5mM
CoA	0.2mM
D-luciferin	0.5mM

2.6.2 EROD assay

Table 2.6.2.1 Na-phosphate buffer

Components	Concentration
$Na_2HPO_4*2H_2O$	8.8995g/L
$NaH_2PO_4*H_2O$	6.8996g/L

Table 2.6.2.2 7-ER stock solution

Components	Concentration
7-ethoxyresorufin	0.1mg/ml
DMSO	

Table 2.6.2.3 7-ER reagent solution

Components	Concentration
7-ER stock	120µl
Na-phosphate buffer	24.9ml

Table 2.6.2.4 Resorufin stock solution (2mM)

Components
Resorufin sodium salt
Ethanol

Table 2.6.2.5 Fluorescamine stock solution

Components	Concentration
Fluorescamine*	1.5mg/ml

***Fluorescamine stock is diluted to 1:10 in DMSO**

Table 2.6.2.6 Bovine Serum Albumin (BSA)

Components	Concentration
BSA*	1mg/ml
MilliQ water	-

***BSA was prepared by adding 100mg in 100ml of milliQ water**

2.7 Accelerated Solvent extraction (ASE)

Table 2.7: Solvent mixture used in ASE

Solvent	% In mixture
Hexane	50
Dichloromethane	50

2.8 Chromatography

Table 2.8: Chromatography fractionation overview

Fraction	Eluent
1	100% Hexane
2	9:1 H/DCM
3	7:3 H/DCM
4	5:5 H/DCM
5	3:7 H/DCM
6	1:9 H/DCM
7	100% Dichloromethane
8	9:1 DCM/MeOH
9	5:5 DCM/MeOH
10	100% Methanol

*Hexane = H, Dichloromethane = DCM, Methanol = MeOH

2.9 Instruments

Table 2.9: Instrument overview

Name	Use	Manufacturer
Thermo Scientific Dionex ASE 350 Accelerated Solvent Extractor	Sediment extraction	Thermo Scientific
Gravity Column	Chromatography	Sigma-Aldrich
NanoDrop 1000	Spectrophotometer	Thermo Scientific
HS 501 Digital	Platform shaker	IKA-Werke
Panasonic mco-170aicuv-pe	Incubator	Lab-Tec
Multitron Standard shaking incubator	Incubating cells while shaking	Infors HT
Aeros mulitfuse X3R	Centrifuge	Thermo Scientific
EnSpire 2300 Multimode Reader	Plate reader	Perkin Elmer
Hidex Sense plate reader	Plate reader	Hidex
CleanAir EuroFlow Class II	Biosaftey cabinet	Baker
Burker haemocytometer	Cell counting	Marienfield
DM IL inverted microscope	Confluency determination	Leica
Zymark TurboVap II Evaporator	Solvent evaporation	Gemini
Ultraspec 10 cell density meter	Cell density measurement	Amersham Biosciences

3. Methods

The methods used in this thesis is presented in Figure 3.1 as a flowchart where the sediment's path is shown. Firstly, analytes were extracted from the sediment using accelerated solvent extraction (ASE) where it was then further fractionated by gravity column chromatography. Lastly the fractions from the chromatography were used in both EROD and LRA assays and chemically analysed at the Institute of Marine Research using four validated methods.

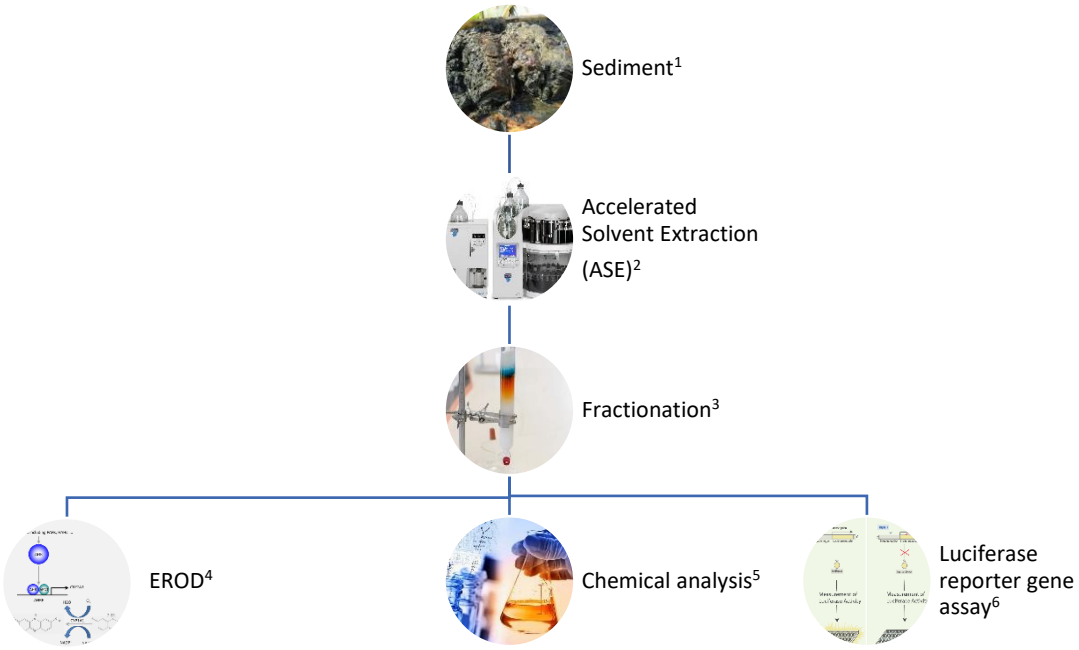


Figure 3.1: Flowchart showing the path of the preparation and analyses of the sediment samples.

Picture sources: (Miljødirektoratet, 2022)[1], *(ThermoFisher, 2022)[2], (Thongdumhyu/Shutterstock, 2022)[3], (Mohammadi-Bardbori and Mohammadi-Bardbori, 2014)[4], (Eurolab, 2022)[5], (Tyasning, 2022)[6]*

3.1 Sediment extraction and fractionation

The sediment sample was collected in Vågen, Bergen, in January of 2018. The sampling collection was done using a van Veen grab. The top one-centimetre layer of sediment was sampled and airdried before 15 grams were extracted by Accelerated Solvent Extraction, ASE (Thermo Scientific Dionex ASE 350 Accelerated Solvent Extractor). ASE cells with a 35 ml volume were filled with a mix of 10% deactivated alumina and silica gel above and below the 15 grams of sediment. Two cycles were performed with the conditions: 100 ºC, 1500 psi, 60% ''flush volume'', and a solvent mix of 1:1 hexane:dichloromethane. Using a TurboVap II (Zymark TurboVap II Evaporator) the sediment extract was reduced to a volume of 0.5 ml.

The sample was then fractionated in a glass chromatography gravity column (33 cm x 24 mm ID; glass-wool plug at the bottom) wet-packed in a mixture containing a slurry of hexane and silica gel. The sediment extract was loaded on top of the column and then eluted with 10 different solvents/solvent-mixtures containing hexane (H), dichloromethane (DCM) and methanol (MeOH) as shown in Table 2.8 and Figure 3.2. All elutions were performed using 50 mL of solvent. All fractions were dried using a TurboVap II and reconstituted in 1 ml of DMSO where the final concentration was equivalent to 15 g of dry weight sediment per ml (eQsed/ml)

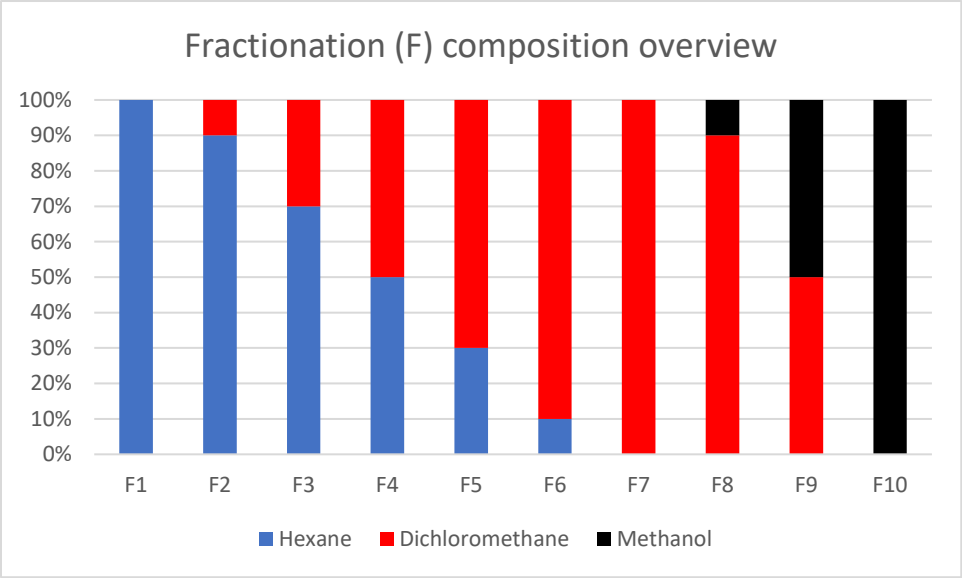


Figure 3.2: Overview of solvent mixtures used in gravity column chromatography to produce ten fractions of the sediment extract. The sediment extract was loaded on top of a gravity column packed with a slurry of hexane and silica gel. Each solvent/solvent-mixture was added at a volume of 50 ml

3.2 Plasmid preparation and purification

The following *Escherichia coli* (*E. coli)* transformation protocol was performed for these plasmids: GudLuc C, pCMV-β-Gal, pcDNA3.1 gmARNT, pcDNA3.1 gmAHR2A, pcDNA3.1/zeo(+), (MH100)x4 tk luc, pCMX-Gal4-Erα and pCMX-Gal4-PXR-TL.

E. coli cells containing the individual plasmids were stored at -80ºC. Cells were thawed and plated on a LB-agar plate with ampicillin (0.1 mg/ml) and incubated on a shaker at 250 rpm for 24-hours at 37ºC. The mix was then transferred to an Erlenmeyer flask and 200 ml of LB medium was added alongside 200 µl of ampicillin. The bacteria were then incubated in a Multitron standard shaking unit for 18-24-hours at 37ºC at 250 rpm.

After incubation, cell density was determined with an Ultraspec 10 cell density spectrophoto meter at 600 nm. This formula was used to calculate 200 ODV:

$$Optical\ density * cell\ volume = 200\ ODV$$

The cells were then harvested by centrifugation at 3500 x g for 5 minutes. Using the NucleoBond® kit the pellet was resuspended in SDS/alkine lysis buffer to free the *E. coli* DNA, then RNase buffer was added to degrade remaining RNA, before a neutralization buffer was mixed in to maintain a supercoiled confirmation of the plasmid DNA. The mix was then loaded onto a silica-based column to bind the plasmid DNA. The plasmid DNA was then eluted using an elution buffer from the NucleoBond® kit before being precipitated using isopropanol and centrifugation. It was then dried using ethanol. The pellet was then reconstituted in 250 µl of AE-buffer before the purity and concentration of the plasmids were determined using a Nanodrop.

3.3 Luciferase reporter gene assay

Luciferase reporter gene assay (LRA) (Figure 3.3) were used to measure responses in receptors from cod and zebrafish. This was done by cotransfecting reporter ((MH100)x4 tk luc/GudLuc C), receptor and control-plasmids (pCMV-β-Gal) into COS-7 simian cells.

The reporter plasmid codes for luciferase which is an oxidizing enzyme producing bioluminescence when in contact with luciferin. The Gal4-activation sequence (UAS) in the promoter region controls the transcription of luciferase. When the receptor protein with the Gal-4-DNA binding domain (Gal-4-DBD) goes through conformational change, activation, and binding to the UAS in the reporter plasmid, transcription occurs.

The receptor plasmid is the plasmid being investigated and is different for all the LRA experiments. The Gal-4-DBD is attached to the ligand binding domain of the receptor plasmid. When activated, the ligand binds to the ligand binding domain (LBD). The Gal-4-DBD is activated by the conformational change. Binding to the UAS in the reporter-plasmid by the Gal-4-DNA leads to expression and translation of luciferase. After the luciferin is added, the emitted light from this reaction can be measured (560nm) to determine activation. For the AHR2/ARNT reporter gene system, full length Ahr2a was used by co-transfecting pcDNA3.1/Zeo(+)-based gmARNT1 plasmids with pcDNA3.1/Zeo(+)-based gmAhr2a.

A control plasmid is used to normalize measured light to cell number and transfection efficiency. ONPG is hydrolysed by β-galactosidase to ONG and galactose. The yellow colour from this reaction absorbs light at 420nm and can therefore be used to measure β-gal activity as absorbance.

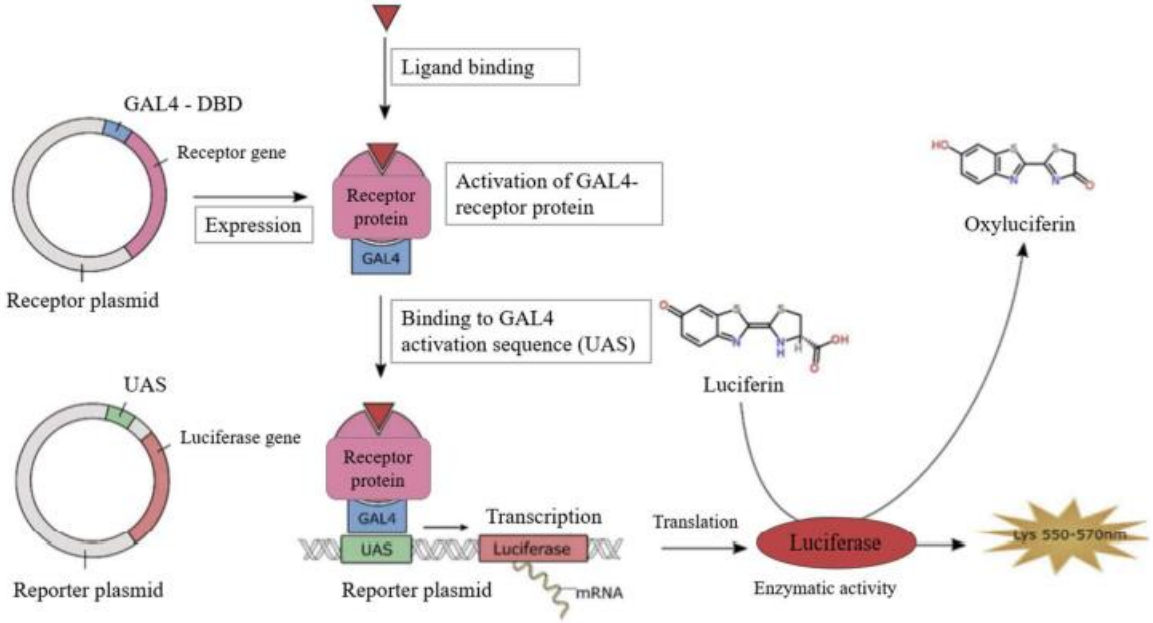


Figure 3.3. Overview of the Gal4-DBD based luciferase reporter gene assay. The receptor plasmid contains the receptor gene and Gal4-DBD. When ligan binding occurs, the plasmid changes confirmation and the Gal4 can bind to the activation sequence (UAS) of the reporter plasmid. Downstream in the reporter plasmid the luciferase gene gets transcribed and translated. When catalysed by the luciferase enzyme, luciferin is transformed to oxyluciferin which results in emitted light at 560 nm. Figure taken from (Fredriksen, 2021).

3.3.1 COS-7 cell cultivation

COS-7 cells are derived from kidney tissue of African green monkeys. Aliquots of COS-7 cells were stored in liquid nitrogen and used as starters to cultivate sufficient cells needed for planned experiments. An aliquot of COS-7 cells was thawed quickly before 10 ml of growth medium (DMEM, 10% FBS) was added and the mixture was centrifuged at 500 x g for 5 minutes. Excess medium was removed, and cells were resuspended in new medium. Cells were then seeded in LB-agar plates and incubated for 18-24 hours at 37 ºC, 5% CO_2 until confluency was determined at 70%. Medium was then removed, and cells were washed in 1X PBS two times before Trypsin-EDTA (0.05% trypsin, 0.02% EDTA) was added before being removed after 45 seconds to detach cells. Cells were then resuspended in growth medium and transferred to new LB-agar plates in desired concentration.

3.3.2 Seeding

Detached cells were mixed with Erythrosine B in a 1:1 ratio and counted using a Leica DM IL inverted microscope and a hemocytometer. 96-well plates were seeded with cells at a density of 500 cells per well with a total volume of 100 µl with growth medium. Cells were incubated for 18-24-hours at 37 ºC, 5% CO_2.

3.3.3 Transfection

The plasmid mix was prepared beforehand (Table 3.3.3.1. For Ahr2a see table 3.3.3.2). Opti-MEM I, TransIT-LT1 and the plasmid mix was used to create a transfection mix (Table 3.3.3.3) that was incubated at RT for 30minutes. Old medium was removed from the 96-well plates and the 101.3 µl of transfection mix was added to each well. Plates where then incubated at 37 ºC, 5% CO_2 for 18-24-hours.

Table 3.1: Plasmid mix overview.

Plasmid	Mass (ng)
(MH100)x4 tk luc	47.62
pCMV-β-Gal	47.62
pCMX-Gal4-Arα*	4.76
pCMX-Gal4-Erα*	4.76
pCMX-Gal4-PXR-TL*	4.76
Total	100.00

***Only one of the receptor-plasmids is added per mix.

Table 3.2: Plasmid mix for Ahr2a overview.

Plasmid	Mass (ng)
GudLuc C	30.00
pCMV-β-Gal	30.00
pcDNA3.1 gm ARNT	3.00
pcDNA3.1 gm AHR2A	3.00
pcDNA3.1/zeo(+)	34.00
Total	100.00

Table 3.3: Transfection mix overview.

Reagents	Volume per well (µl)
Opti-MEM I	9
Plasmid mix	0.3
TransIT-LT1	0.1
DMEM-10%FBS	92

3.3.4 Exposure

Cells were exposed to a 1X ligand-solution dissolved in DMSO at a dilution factor of 1:5 in growth medium without phenol-red. The ligands were made in a 2X concentration and diluted in a deep well plate with one well being used as a control well containing only growth medium and DMSO. Old medium was removed from 96-well plate and 100 µl of 2X dilution mix was added to each well. Lastly, 100 µl of growth medium without phenol-red was added to the wells. The highest concentration of sediment sample used in dilution was 120 mg eQsed/ml. Cells were then incubated for 18-24-hours at 37 ºC, 5% CO_2.

3.3.5 Lysis

Old medium was removed and 125 µl of lysis reagent was added to all wells of the 96-well plate. The plates were then incubated for 30 minutes at room temperature on a shaker. 50 µl of lysate was then added to each well of both a clear 96-well plate and a white luminescence 96-well plate. The clear plate was used for β-galactosidase measurements while the white plate was used for luciferase activity measurements. 100 µl of β-galactosidase 1X substrate was added to each well of the clear plate and incubated at room temperature for a minimum of 20 minutes or until the solution turned bright yellow. Absorbance was measured at 420 nm with a plate reader. In the white plate, 100 µl of luciferase reaction solution was added and the luminescence was measured immediately. β-galactosidase measurements were used to adjust the corresponding luciferase activity for transfection efficiency of the plasmid mix.

3.4 Ethoxyresorufin -O-deethylase (EROD) assays

3.4.1 PLHC-1 cell cultivation

PLHC-1 cells are derived from hepatocyte liver tissue of *Poeciliopsis lucida,* topminnow. Aliquots of cells were stored at -80 ºC. Cells were thawed as quickly as possible, 10 ml of growth medium (DMEM/F-12, 10%FBS and 100 u/ml penicillin-streptomycin) was added and then centrifuged at 500 xg for 5 minutes. Old medium was removed, and pellet was resuspended in 10 ml of medium and transferred to a tissue culture flask and incubated at 30 ºC, 5% CO_2.

Old medium was removed before 0.5 ml PBS was added to wash the cells before being removed. 5 ml of Trypsin-EDTA was then added before letting the cells incubate at room temperature for 5 minutes to detach from the tissue culture flasks. 10 ml of growth medium was added, and cells were diluted in new tissue culture flask in desired concentration.

3.4.2 Seeding

Detached cells were counted using a microscope and cell counting chamber before being seeded at a density of 300000 cells in 500 µl medium per well of a 48-well plate and incubated at 30 ºC, 5%CO_2 for 18-24-hours.

3.4.3 EROD activity

Old medium was removed and 500 µl of new medium containing sediment extract sample was added to each well and incubated at 30 ºC, 5% CO_2 for 24-hours.

The old medium was removed after incubation. The cells were then washed with 250 µl of PBS twice. After removing the PBS, 500 µl of 7-ER reagent solution was added to each well and the plates were immediately wrapped in aluminium foil and incubated at 37 ºC, 5% CO_2 for 15 minutes. The fluorescence was then read at 537/583 nm with a plate reader.

3.4.3 Protein measurement

After the fluorescence was measured the 7-ER reagent solution was removed from the plates and cells were washed with 200 µl of PBS twice. Then, 125 µl of Milli-Q water, 250 µl of Na-phosphate buffer and 125 µl of fluorescamine work solution was added to each well before the plate was incubated on a shaker at room temperature for 5 minutes. The fluorescence was then read at 340/460 nm.

3.5 Chemical analysis

Target, suspect, and non-target analyses of organic contaminants in the sediment extract fractions were performed at the Institute of Marine Research by Dr. Aasim Ali

3.5.1 Analysis of Halogenated organic contaminants (HOCs)

Quantification of the selected halogenated organic contaminants depicted in Figure 4.3.1 was conducted using a gas chromatograph (Agilent 7890) coupled to a triple quadrupole mass spectrometer (Agilent 7010) operated in multiple reaction monitoring (MRM) mode. LOQ for each individual HOC was 0.02 µg kg^{-1} dry weight (dw). The method is validated and quality assurance (QA) and quality control of other accredited methods and IMR were followed.

3.5.2. Analysis of brominated diphenyl ethers (BDEs)

BDEs, shown in Figure 4.3.2, were determined using GC Agilent 6890) coupled to MS (Agilent 7010) operated in selective ion monitoring (SIM) mode. LOQs for PBDEs were 0.03 µg kg^{-1} dw.

3.5.3. Analysis of polycyclic aromatic hydrocarbons (PAHs)

Sediment samples were analysed for the selected polycyclic aromatic hydrocarbons (PAHs) (Figure 4.3.3) using a single quadrupole-mass spectrometry gas chromatography (GC-SQ-MS). The method is accredited by the official Norwegian accreditation body, Norsk Accrediting, according to the European quality assurance (QA) standard NS-EN ISO/IEC-17025. LOQ values for the individual PAHs were 0.5 µg kg^{-1} dw. Details about the method performance can be found elsewhere (Boitsov, Klungsøyr and Jensen, 2020).

For perfluoroalkyl and Polyfluoroalkyl Substances (PFAS), suspect and nontarget screening analyses, a Thermo Scientific Dionex UltiMate 3000 UHPLC coupled to a Orbitrap Exploris 120 (HPLC-q-Orbitrap) equipped with a heated ESI source (HESI, Thermo-Fisher Scientific, CA, USA) was used. Five µL of each sample extract was injected on an C18, 150 x 2.1 mm, 1.8 µm chromatographic column with a pre-filter (2.1 mm ID, 0.2 µm) from Agilent. For the mobile phase, 2mM of ammonium acetate was added to both solvents (water and methanol). The q-Orbitrap was operated in full scan – data dependent MS2 mode for both positive and negative ionizations. MS2 fragmentation was trigged by mass list for PFAS. The details of the HPLC-q-Orbitrap method are shown in the following figure. Targeted analysis of PFAS was conducted using TraceFinder 5.1 (Thermo-Fisher Scientific) software, while for both suspect and nontarget analyses, the Compound Discoverer 3.3 (Thermo-Fisher Scientific) software was utilized.

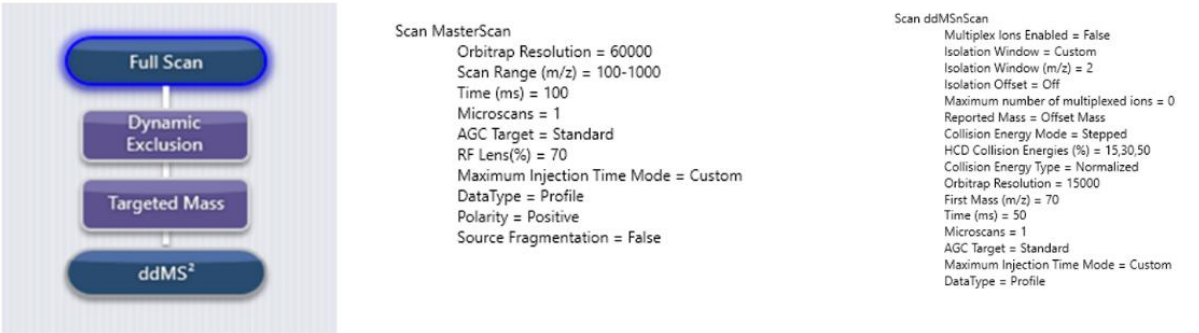
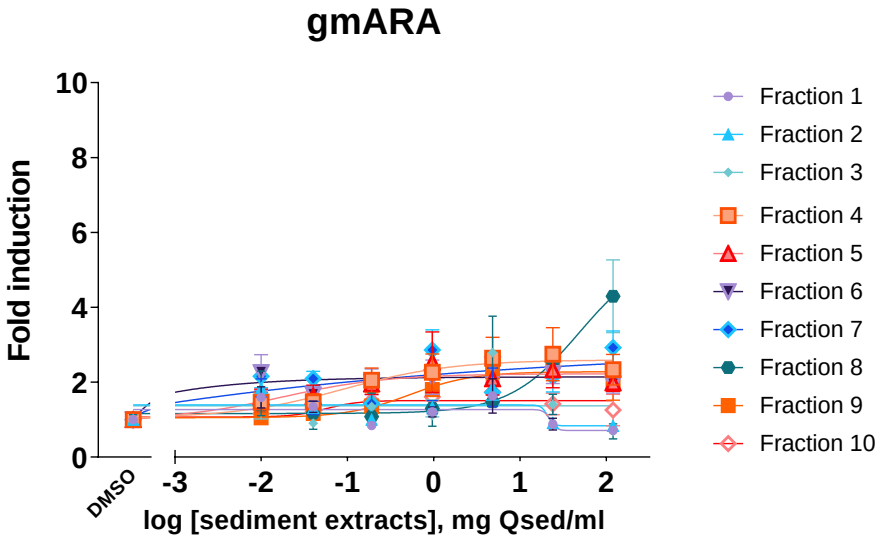


Figure 3.3. HPLC-q-Orbitrap instrumental method parameters

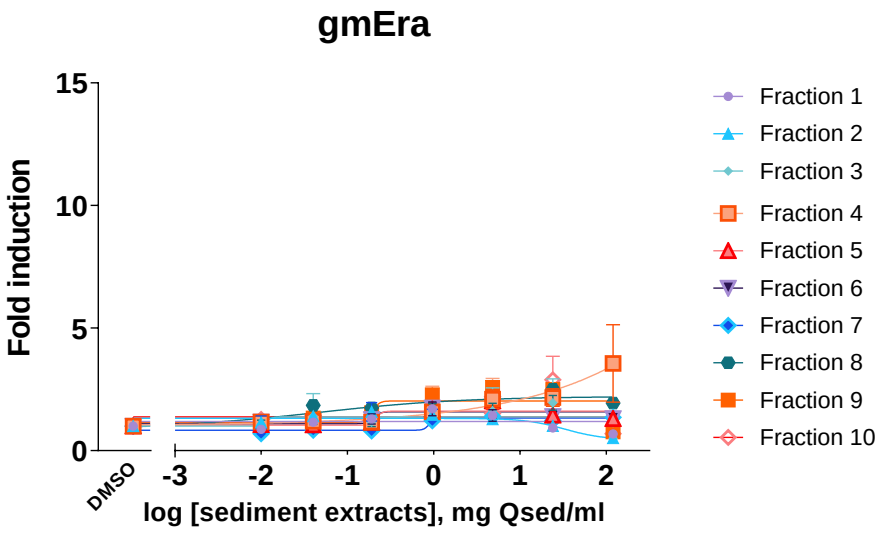
4. Results

4.1 Luciferase reporter gene assay

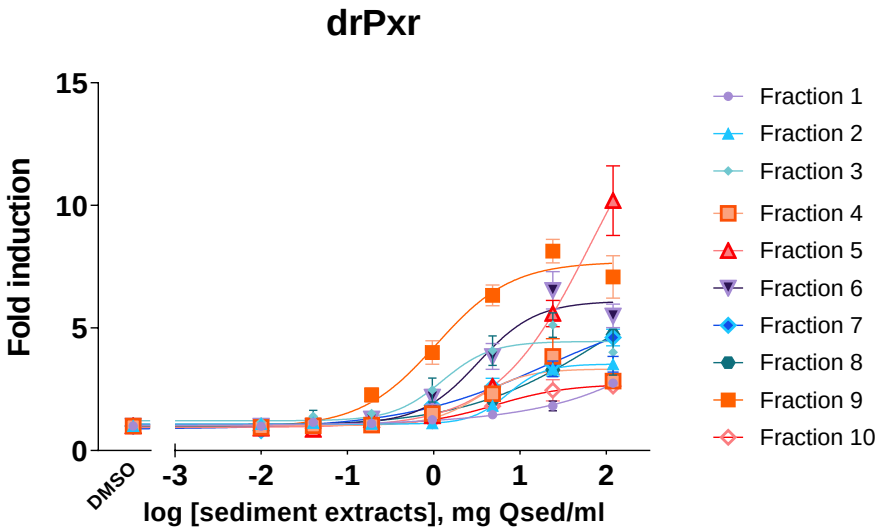
The sediment extract from Vågen was fractionated into ten fractions so that any potential contaminants would be separated based on polarity as described in Methods 3.1. A battery of stress-activated receptors, including gmAra, gmEra, gmAhr2a, and drPxr, were tested for ligand activation with sediment extract fractions (SEFs) using transiently transfected COS-7 cells. Cells expressing the different individual receptors were exposed to serial dilutions (1:5) of SEFs ranging from 0 - 120 mg Qsed/ml. The positive control agonists used for the LRA assays were testosterone (gmAra), clotrimazole (drPxr), EE2 (gmEra) and TCDD (gmAhr2a) (see Appendix A1-A4).



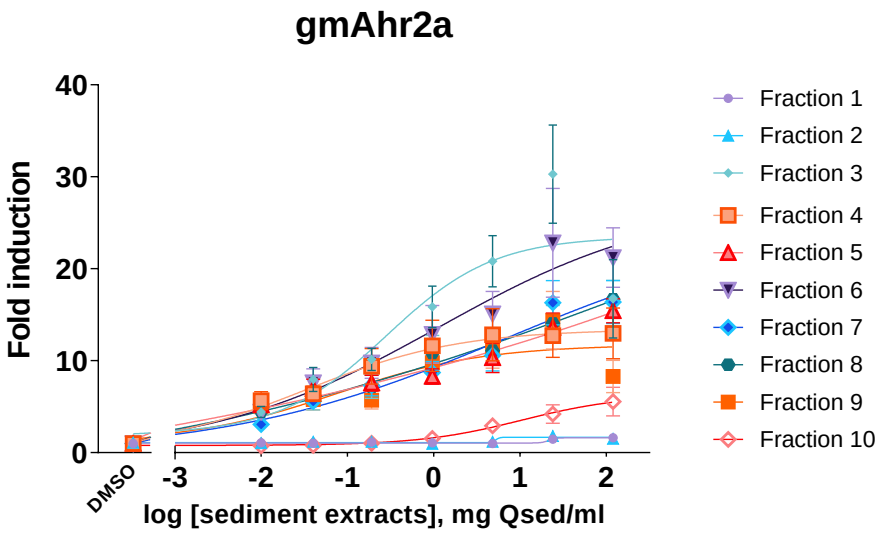
A)


gmEra
Fold induction
15
10
5
0
DMSO
-3
-2
-1
0
1
2
log [sediment extracts], mg Qsed/ml
Fraction 1
Fraction 2
Fraction 3
Fraction 4
Fraction 5
Fraction 6
Fraction 7
Fraction 8
Fraction 9
Fraction 10

B)


drPxr
Fold induction
15
10
5
0
DMSO
-3
-2
-1
0
1
2
log [sediment extracts], mg Qsed/ml
Fraction 1
Fraction 2
Fraction 3
Fraction 4
Fraction 5
Fraction 6
Fraction 7
Fraction 8
Fraction 9
Fraction 10

C)



D)

Figure 4.1.1: Ligand activation of gmAra (A), gmEra(B), drPxr (C) and gmAhr2a (D) by different fractions of a sediment extract from Vågen, Bergen, using luciferase reporter gene assays. COS-7 cells were transfected with either Atlantic cod or zebrafish receptor plasmids and were exposed to ten different fractions prepared from a sediment extract from Vågen. The ligand activation is shown as relative fold change in luciferase activity compared to a DMSO solvent control. Each exposure had three technical replicates and each experiment was repeated three times. The graph was made in PRISM (v 9.3) where a dose-response curve was fitted with non-linear regression. Error bars indicate SEM. Individual graphs for each fraction and positive control can be found in Appendix Figure A.1-A.4.

For the gmAra luciferase assay, the strongest activation was mainly observed in fraction 8 at the highest concentration used (Figure 4.1.1 (A) and Appendix A.1). Furthermore, there is a slight increase in activation in fractions 4, 5 and 9. While fractions 1, 2, 3, 6, 7 and 10 show a twofold activation they also have little to no variation in activation in the different concentrations tested. Notably, fractions 1, 2, and somewhat 3, demonstrated less activation at the highest concentration compared to the lower concentrations used, which may indicate cytotoxic effects.

The luciferase reporter gene assay for gmEra showed little to no activation in fractions 1, 2, 3, 5, 6, 7, and 10 (Figure 4.1.1 (B) and Appendix A.2). Of these fractions, one and two also shows lower activation in the highest concentrations. Fractions 4, 8 and 9 show some activation while fraction 9 also has a lover activation in the highest concentration.

As seen in Figure 4.1.1 (C) and Appendix A.3, the highest fold induction activation in the drPxr receptor is fractions 5 and 9. Of these two, fraction 9 appeared to be the most potent of the two based on the steady increase in activation compared to fraction 5's sharp increase at higher concentrations. There is low activation in fractions 1, 2 and 10 with slightly higher activation for fraction 4, and only slightly higher activation of the fractions 3, 6, 7 and 8.

Figure 4.1.1 (D) and appendix A.4 shows that in the luciferase reporter gene assay for the receptor gmAhr2a there was high activation observed in fractions 3, 4, 5, 6, 7, 8 and 9, where fraction 3 has the highest observed activation of them all. Little to no activation was observed in fractions 1 and 2. In fraction 10 there is activation, especially in the highest concentrations but less compared to the other fractions with activation.

Table 4.1.1 Summary of luciferase reporter gene assay results. Effective concentration (EC50) and maximum response (E_{max}) fold change for receptors used in luciferase reporter gene assay.

Receptor	Fraction	EC50 (best fit value)*	Emax
gmAra	1	-	1.63
	2	-	1.86
	3	-	2.79
	4	0.09	2.74
	5	-	2.53
	6	-	2.47
	7	-	2.92
	8	-	4.29
	9	0.52	2.56
	10	-	1.74
gmEra	1	-	1.70
	2	-	1.58
	3	-	1.94
	4	-	3.55
	5	-	2.02
	6	-	1.90
	7	-	1.40
	8	-	2.50
	9	-	2.58
	10	-	2.88
drPxr	1	-	2.74
	2	-	3.51
	3	1.24	5.12
	4	3.25	3.83
	5	-	10.19
	6	3.25	6.54
	7	-	4.61
	8	-	4.72
	9	1.06	8.13
	10	5.21	2.62
gmAhr2a	1	-	1.61
	2	-	1.82
	3	0.28	30.28
	4	0.03	12.95
	5	-	15.43
	6	-	22.82
	7	6.69	16.30
	8	-	16.68
	9	0.07	14.40
	10	10.44	5.54

***Only predicted for curves reaching a plateau

**All p-values $p<0.05$

4.2 Ethoxyresorufin-O-deethylase (EROD) assay

As previously mentioned, the EROD assay is a sensitive and accurate way to determine AHR activating compounds and to predict the toxicity of chemicals and is why it was used in this master thesis. The EROD assay was performed on PLHC-1 cell derived from liver tissue of the *Poeciliopsis lucida,* topminnow. This assay is used to measure ethoxyresorufin-*O*-deethylase activity in fish and is a well-established biomarker of exposure to planar halogenated and polycyclic aromatic hydrocarbons (PHHs and PAHs). EROD is a sensitive indicator of contaminant uptake in fish and provides evidence of receptor-mediated induction of cytochrome P450-dependant mono-oxygenase by xenobiotic compounds (Whyte and Tillitt, 1995).

From the EROD assay shown in Figure 4.2.1, fraction 1 and 2 produced little to no induction of CYP1a activity, while fraction 10 shows some Cyp1a induction above the levels of fraction 1 and 2. CYP1a induction can be seen in all the other fractions, from 3 - 9. Notably in all of these fractions, the highest concentrations of sediment extract show lower induction than the lower concentrations.

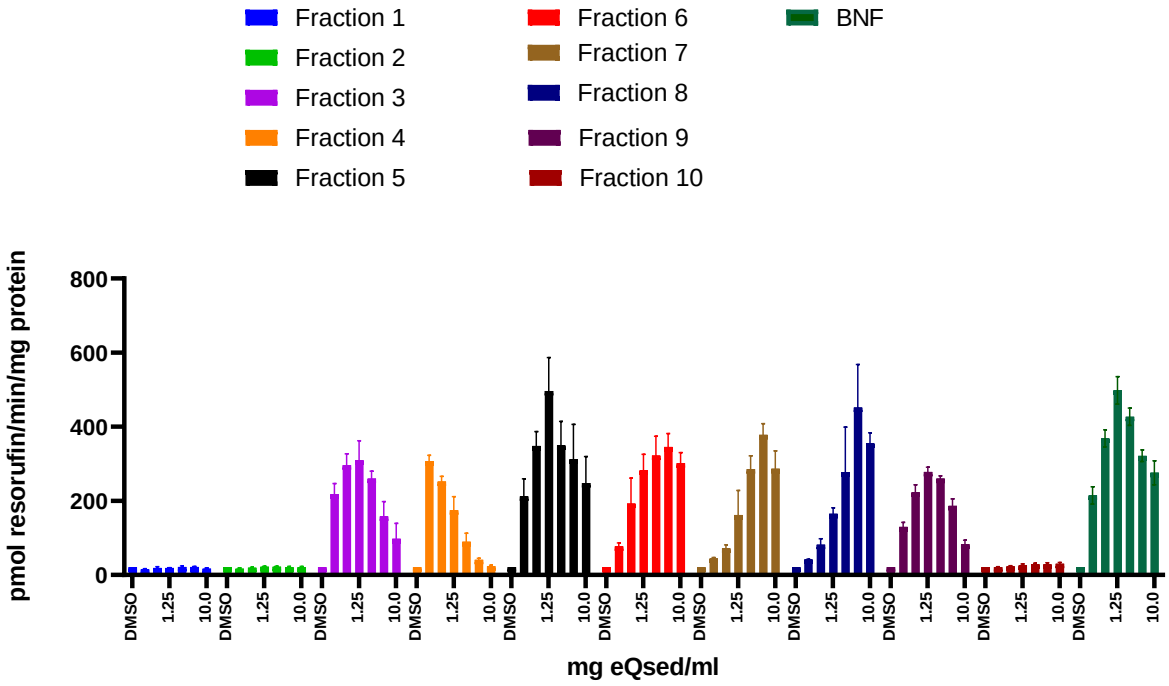


Figure 4.2.1 EROD activity measured by induction of CYP1a receptor measured in PLHC-1 cell after exposure to different fractions of sediment extract originating from Vågen, Bergen. PLHC-1 cells were exposed to ten different fractions of a sediment extract with six different concentrations, 0.3125, 0.625, 1.25, 2.5, 5.0 and 10.0 mg eQsed/ml. The BNF had the same concentration but with µM as unit of measurement. Vågen. The EROD activity is shown as pmol resorufin produced per minute per mg protein. The positive control for EROD activity was BNF, and three duplicates of the experiment was performed. Figure was made in PRISM (v 9.3).

4.3 Chemical analysis

Chemical analysis is an important tool in identifying the compounds activating the receptors in the luciferase reporter gene assays and the EROD assay by comparing data.

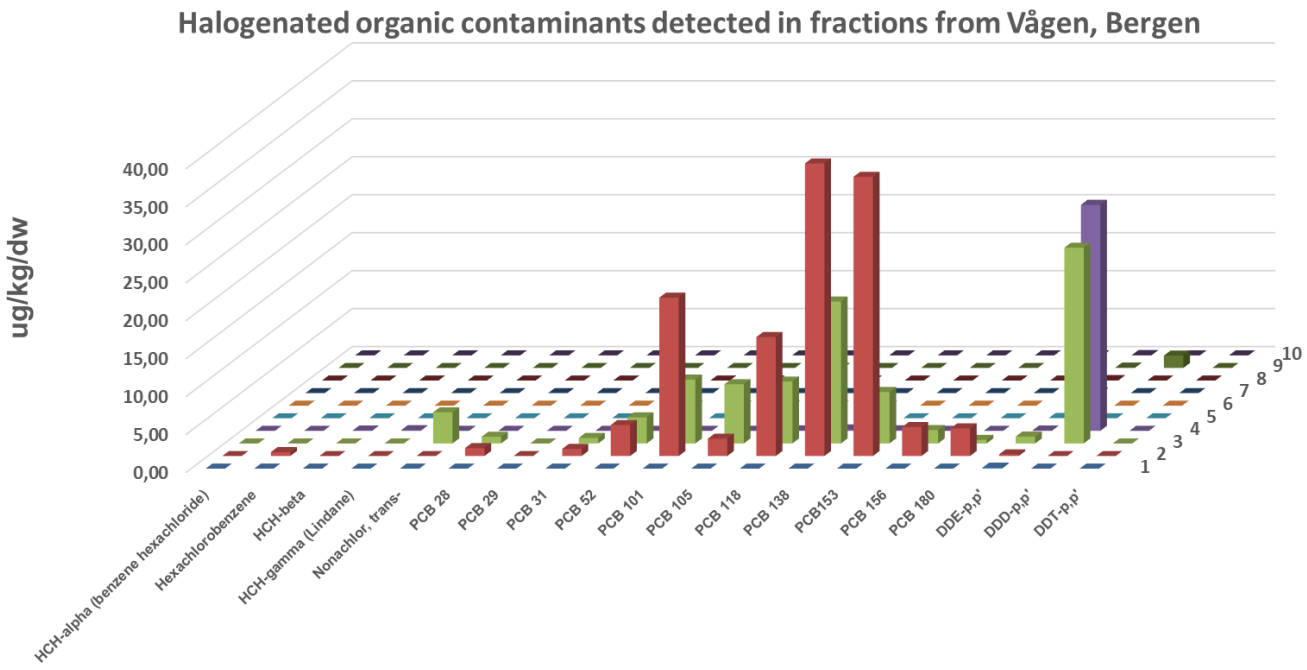


Figure 4.3.1 Chemical analysis of halogenated organic contaminants (HOCs) in fractions obtained from sediment extracts from Vågen, Bergen. Sediment extract from Vågen, Bergen, was fractionated into ten different fractions (z-axis) using gravity chromatography. The results for HOCs were reported in µg per kg per dry weight sediment (dw). The graph was made using Microsoft Excel 2020.

The chemical analysis in figure 4.3.1 shows the presence of PCB 28, 31, 52, 101, 105, 118, 138, 153, 156 and 180 in fractions 2 and 3. Trans-nonachlor and DDE-p,p' is only present in fraction 3 while DDD-p,p' is found in fractions 3, 4 and 9. hexachlorobenzene is only found in fraction 2.

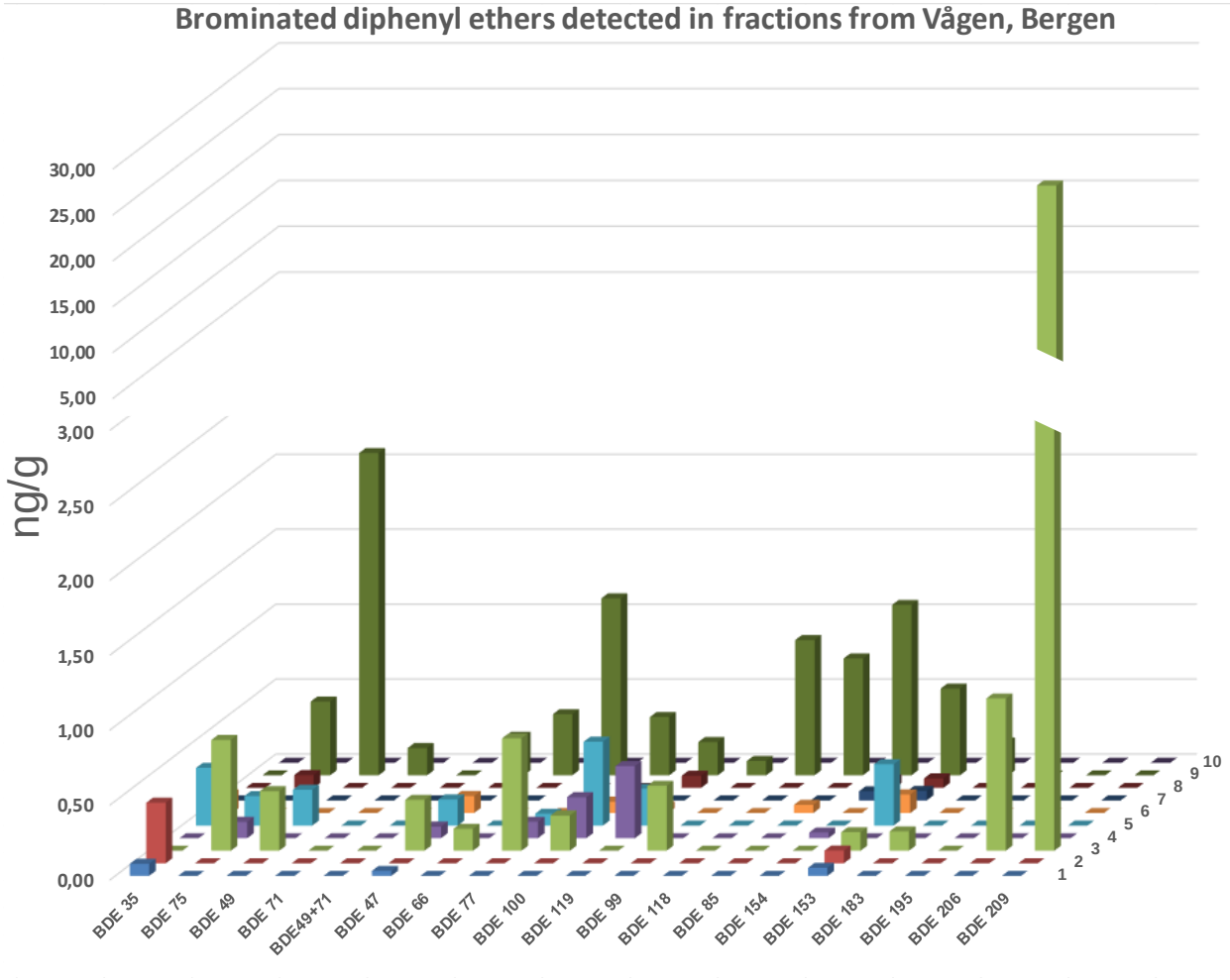


Figure 4.3.2 Chemical analysis of brominated diphenyl ethers (BDEs) in sediment extractions from Vågen, Bergen. Results are presented as nanograms per gram of sample. Graph was made using Microsoft excel 2020.

From figure 4.3.2 we can see a trend of BDEs found mostly in fractions three, four, five and nine with some exceptions, like the presence of BDE 35 and 153 in fraction 1 and 2. There is also a large amount of BDE 75, 49, 77, 100, 119, 118, 85, 154, 153 and 183 found in fraction 9. The most notable find was BDE 209 found in fraction 3 in an amount ten times more than any BDEs found in any of the other fractions.

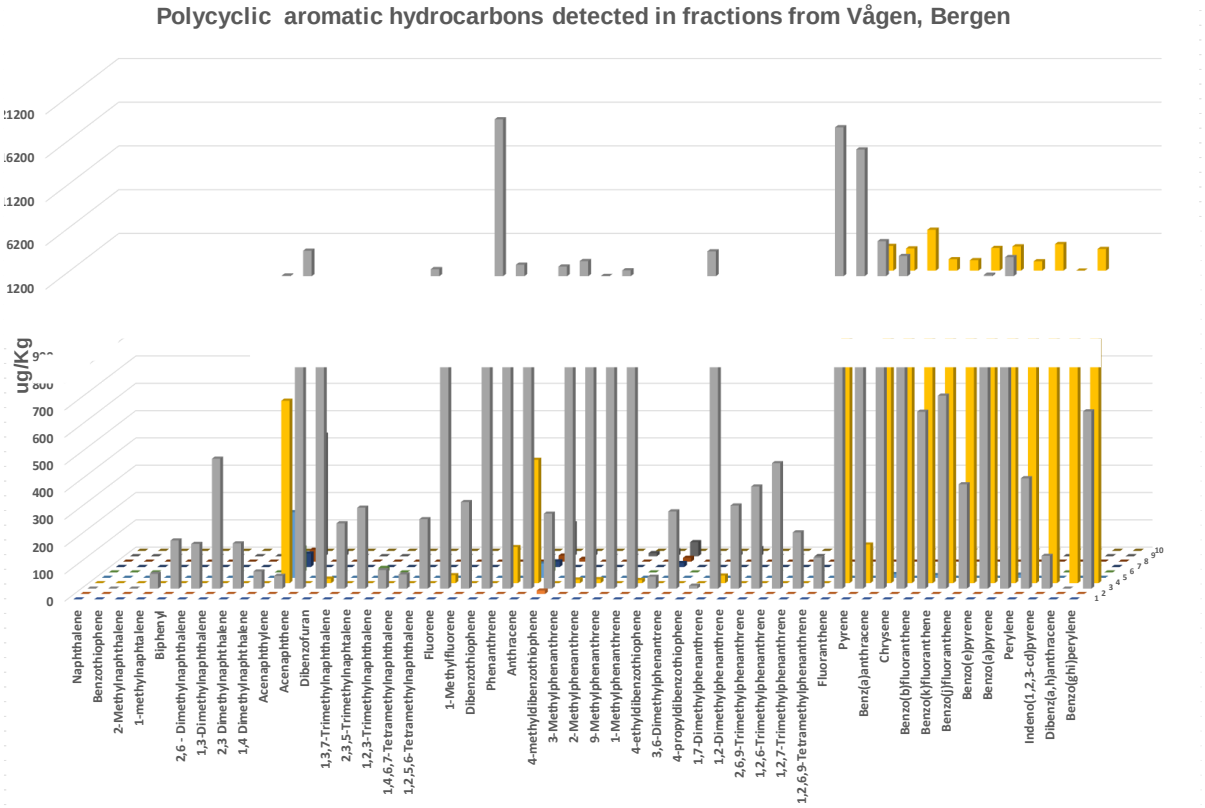


Figure 4.3.3: Chemical analysis of polycyclic aromatic hydrocarbons (PAHs) in fractionated sediment extract from Vågen, Bergen. Results are presented as micrograms per kilo of dry weight sediment. Graph was made using Microsoft excel 2020.

Figure 4.4.3 shows that certain PAHs are very abundant compared to others, this includes especially phenanthrene at 19117 µg/Kg/dw, pyrene at 15677 µg/Kg/dw and fluoranthene at 18206 µg/Kg/dw. There were 27 PAHs found in quantities of above 1200 µg/Kg/dw, where most of these were found in fractions 3 and 4, with trace amounts found scattered in other fractions. Especially acenaphthylene, anthracene and 4-ethyldibenzothiophene were observed in some of the other fractions.

4.4 Principal component analysis

Principal component analysis (PCA) is a way to reduce the dimensionality of data while retaining the variation of the data set (Ringnér, 2008). PCA was chosen as it seemed the best available option to compare the large data sets from the chemical analysis with the biological data from the luciferase and EROD assays into a comprehensive figure.

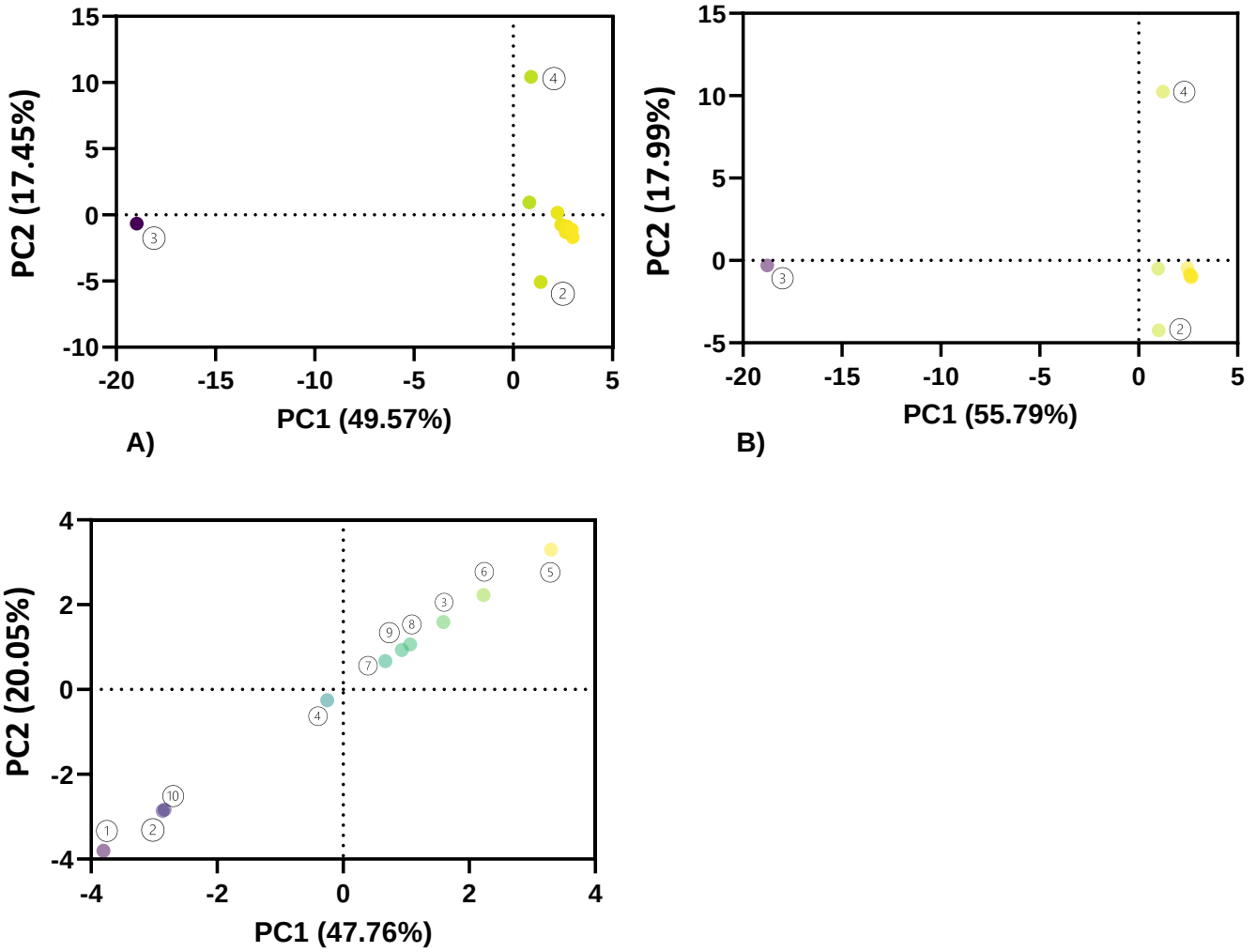


Figure 4.5.1: Multivariate statistical analysis of biological and chemical data, both as separate analyses and combined where the numbers indicate which fraction it is. (A) Principal component analysis (PCA) plot of chemical analysis and biological data (Luciferase reporter gene assays and EROD activity) where PC1 and PC2 explain 67.02% of the data variance. (B) PCA plot of chemical analysis data where PC1 and PC2 explain 73.78% of the data variance. (C) PCA plot of biological data where PC1 and PC2 explain 67.81% of data variance. All PCA plots were created using PRISM (v 9.3).

The principal component analyses (PCA) (Figure 4.5.1) show the biological and chemical data analysed combined and separately (A, B and C) where the combined PC1 and PC2 of each figure explain 67.02%, 73.78% and 67.81% from of data set variance from A, B and C respectively. In plot A the clustering of fractions 1 and 5-10 is more spread out than the same cluster for the chemical data in plot B. Plot C shows the fraction more spread out in an oblique line with fractions 1, 2 and 10 closer together with the other fractions, except fraction 5 at the far, top end.

5. Discussion

5.1 Summary of the study

The goal of this master thesis was to determine whether I could achieve a better understanding of the toxicity and composition of the sediments in Vågen, Bergen. Effect-based bioassays were employed in combination with the chemical fractionation of the sediment extract and chemical analysis to identify the specific compounds or groups of compounds mediating toxicity using stress-activated receptors from Atlantic cod and zebrafish. The strongest activation was found for the gmAhr2a and drPxr receptors, with little to no activation of the gmAra and gmEra receptors. The strongest activating fractions for the drPxr receptor was 5 and 9 while for the gmAhr2a it was 3 and 6 with low activation only seen in fractions 1, 2 and 10.

5.2 General discussion

The extraction conditions of the sediment extract determine what compounds that are present in what fractions. In this thesis, ten different extraction conditions were used, divided into ten fractions based on the polarity of the solvent mixture in the eluent. The separation of different compounds in the same mixture utilizing the different polarities of the various compounds has previously been done by Tang & Row (2020) using deep eutectic solvent-based extraction methods and successful simultaneous separation of high and low polarity compounds (Tang and Row, 2020). The separation of compounds based on polarity is also used in oil reefing techniques as shown by Vargas et al. (2017), where chromatography and varying the polarity of the elution solvent system was used to separate crude oil into six different fractions for further studies (Vargas *et al.*, 2017). However, not all compounds are able to be extracted with the solvents used and would therefor remain in the sediment sample or on the column and not in any of the fractions.

The PXR is a promiscuous receptor activated by a wide variety of compounds (Watkins *et al.*, 2001; Lille-Langøy *et al.*, 2015; Eide *et al.*, 2018). Lille-Langøy et al. (2015) used an *in vitro* luciferase reporter gene assay to show that both human and polar bear PXR were activated by compounds such as pharmaceuticals, non-dioxin like PCBs, and brominated flame retardants (BFRs) (Lille-Langøy *et al.*, 2015). Zebrafish has also been used as a model species for characterizing the PXR receptor as shown by Chen et al. (2016), where the responsiveness of several zebrafish genes, including PXR was tested using dexamethasone, prednisolone and triamcinolone, pharmaceuticals not removed during waste water treatment (Chen *et al.*, 2016). Zebrafish Pxr has also been used to investigate the presence of bioactive pollutants in sediments by Blanco et al. (2018) near industrial zones and cargo ports in Split, Croatia. In a similar manner, Pérez-Albaladejo et al. (2016) assessed the quality of coastal sediment from the black sea using zebrafish-Pxr and PLHC-1 cells where the highest responses were detected in harbour areas (Pérez-Albaladejo *et al.*, 2016).

As earlier studies revealed that PXR is a promiscuous receptor, high activation could be expected in most fractions, but this was not the case. High activation was observed in only three fractions, namely 5, 6 and 9. This does not provide a clear correlation between the chemical analysis and the activation of the receptor. There are however some correlations that can be drawn between the two results. For the BDEs, a large number of these compounds were detected in fraction 9, which contained 14 of the 19 BDEs screened for in the chemical analyses. This could help explain the activation of drPxr by this fraction as BDEs have

previously been shown to activate it. This was shown by Pacyniak et al. (2007), where LRA were used to demonstrate the activation of mice and human PXR by several PBDEs (Pacyniak *et al.*, 2007). Fery et al. (2009) also linked the activation of the PXR of mice and humans with several PBDEs using a similar LRA assay (Fery *et al.*, 2009). The chemical analyses revealed no clear candidate agonist in either fraction 5 or 6, prompting the need for further biotesting to determine the culprit. Compounds such as antibiotics have been proven to activate the human PXR receptor but has not been screened for in this thesis (Wallace *et al.*, 2010). The PCA results show that the most activating fraction is 5 which coincides with that fraction for the Pxr receptor being more potent compared to fraction 9, as seen in the EC50 values in table 4.4.1 and the individual graphs in Appendix A.2. The composition of the eluent used for this fraction (30% hexane and 70% dichloromethane, Figure 3.2) does not give an indication as to why this specific fraction would deviate so much from the rest of the fractions. A study performed by Gao et al. (2008) suggested that introducing a polar chemical group to human PXR agonists could reduce the activation of the receptor via destabilizing in the hydrophobic area interactions in the ligand binding PXR pocket (Gao *et al.*, 2008). If this is also the case with zebrafish Pxr, it could help explain why the more polar compounds did not activate the receptor in this thesis.

The Ahr receptor has been shown to be activated by several types of compounds such as PAHs and flavonoid derivates (Zhu *et al.*, 2019). It has been employed for decades to research toxicity as e.g. shown by Machala et al. (2001), who used luciferase assays to determine aryl hydrocarbon receptor-mediated mutagenic activity (Machala *et al.*, 2001). The Ahr receptor is also widely used in the chemical-activated luciferase expression (CALUX) assay. The CALUX assay is used for the bio-analytical screening of sediments for dioxin-like activity, as shown by Hurst et al. (2004) to determine dioxin-like activity in sediments (Hurst *et al.*, 2004). The Ahr receptor was also used by Stronkhorst et al. (2002). to investigate the presence of dioxin-like compounds in harbour sediment from the Dutch coast, determining the presence of PCBs, PCDDs, PCDFs, PBBs and PBDEs in 20 selected sediment extracts (Stronkhorst, Leonards and Murk, 2002). Screening for Ahr induction and mediated gene expression *in vitro* has also been employed successfully by Yoo et al. (2006), in sediments from inland lakes in Korea, confirming the presence of Ahr activating compounds (Yoo, Khim and Giesy, 2006). The EROD assay is also an established method to determine Cyp1a induction, as shown by Hinger et al. (2011), comparing it with the CALUX method and showing its ability to screen for dioxin-like compounds (Hinger *et al.*, 2011). Lui et al. (2014) used the EROD assay to determine developmental toxicity in zebrafish exposed to PCB126 mediated by the activation of Ahr (Liu *et al.*, 2014).

The gmAhr2a receptor and EROD activity showed clear similarities, with both being activated by the same fractions. There was activation in all fractions except for fractions 1 and 2 with fraction 10 also showing significantly lower activation than the other fractions, but slightly higher than 1 and 2, which were very similar. This contrasts with earlier experiments with the Vågen fraction in S. Goksøyr et al. (2021) where no activation was seen in the EROD assays despite strong activation of Ahr2a indicating the presence of an EROD inhibitor in the sediment extract sample. It was proposed that this could originate from metals found in the sediment samples, as Cd, Hg and some PCBs that has been shown to inhibit EROD activity in some organisms (Beyer *et al.*, 1997; Bozcaarmutlu and Arinç, 2004; Whyte *et al.*, 2008; Goksøyr *et al.*, 2021). Manning et al. (2012), used a luciferase reporter gene assay to demonstrate activation of the aryl hydrocarbon receptor 1 (AHR1) by dioxin-like PCBs such as PCB 126, 77 and 105 (Manning *et al.*, 2012), but this does not seem to translate to this study and Ahr2a with the dioxin-like PCBs mainly contained in fractions 2 and 3, with only fraction 3 showing

activation of the receptor. The observed decreasing Cyp1a induction in the higher concentrations of the SEFs in the EROD assay, most notably in fractions 3, 4, 5 and 9, could be indications of the concentration being toxic to the PLHC-1 cells, but it could also be due to competitive inhibition of the enzymes. Competitive inhibition has been studied by Petrulis & Bunce (1999), where EROD activity declination after reaching a concentration dependent maximum was explained by the competitive inhibition of EROD enzyme-substrate reaction by dioxin-like compounds (Petrulis and Bunce, 1999). With the PAHs found mostly in fractions 3 and 4, it is somewhat surprising that this is not clearly reflected in the LRA of the gmAhr2a receptors, even though not all PAHs are proven to activate the Ahr2a receptor. Lille-Langøy et al. (2021) analysed the activation of Ahr2a by a wide range of PAHs using the same LRA as applied in this thesis, observing that parent compounds such as naphthalene and phenanthrene did not act as agonists while hydroxylated and/or alkylated versions of these PAHs were potent agonists (Lille-Langøy *et al.*, 2021). The correlation between PAHs and biological activity of both EROD assays and LRA has been tested in similar manners earlier by Arrieta et al. (2003), where both assays were used to test dichloromethane-extracted particulate matter and showed similar activation levels (Arrieta *et al.*, 2003). Fraction 3 displayed the highest activation of this receptor with fraction 4 having lower activation than fractions 5, 6, 7, 8 and 9. An explanation for this could be that PAHs, HOCs and BDEs are all found in this fraction and the mixture of these compounds could result in such a high level of activation. Zhou et al. 2019 employed a luciferase reporter assay to detect AHR activity and the potential bio-toxicity towards aquatic animals in pesticides and confirmed a link between AHR activation and Cyp1a induction. This could give a clue to the similar activation pattern of the EROD assay and the LRA with pesticides activating the receptor and promoting the Cyp1a induction (Zhou *et al.*, 2019).

For the gmAR, a clear correlation between the findings in the chemical analysis and the receptor activation was not observed. This is in contrast to studies like Lynch et al. 2017, where a quantitative high-throughput assay was used to detect potential androgen activators, which was then confirmed using a biochemical binding and androgen nuclear translocation assay for identifying multiple androgen agonists (Lynch *et al.*, 2017). The human AR has previously been tested using *in vitro* methods to investigate the luciferase activation of PCBs in mothers milk, discovering that several acted as androgen antagonists (Schrader and Cooke, 2003). For the gmAR, fraction 8 was the most active (E_{max} 4.29), but most compounds were identified in fractions 1 and 2 for the halogenated organic contaminants (HOCs), fractions 3 and 4 for the PAHs, with the brominated diphenyl ethers (BDEs) more spread out, but mostly in fractions 3, 4, 5 and 9. PAHs have been tested by Vinggaard et al. 2000 were it was discovered using a reporter gene assay based on CHO cells that several of these act as antiandrogenic compounds (Vinggaard, Hnida and Larsen, 2000). The lowest activation of the gmAR is in fractions 1, 2 and 10 which also according to the PCA plot C of the biological data represent the three biological outliers. It could be argued as there is little activation in these three fractions across all four receptors and the EROD assay, that the low activation here might stem from other sources. These fractions were eluted using almost entirely hexane or methanol (table 2.8 and Figure 3.2). The polarity of these extremes in this fractionation process can lead to the exclusion of most pharmaceuticals, many of which are EDCs. Many drugs must travel across cell membranes, and to do this they need to be relatively non-polar, while water soluble drugs need to be somewhat polar or else risk binding to tightly to components in food or proteins in blood (Tollefsen *et al.*, 2007). Tollefsen et al. 2007 analysed offshore produced water effluents from solid phase extractions (SPE) where both estrogen and androgen agonists were observed, and with the probability of oil spillage in the Vågen harbour this could be one of the causes of activation in these receptors.

Activation of the ER can be used to detect the potency of EDCs as shown by Gordon et al. (2004) where ER was used in high throughput screening analysis of the estrogenic disrupting potencies of pesticides (Gordon *et al.*, 2004). Furthermore, it has been shown that artificial estrogens such as BPA and selective estrogen receptor modulators induces distinct patterns of gene activation in HepG2 and U2 osteogenic sarcoma cells as shown by Safe et al. (2001) (Safe *et al.*, 2001). Gutendorf & Westendorf (2001) also used several different bioassays to assess the estrogenic and antiestrogenic potential of single and complex compounds (Gutendorf and Westendorf, 2001).

For both the Ar and Er the activation displayed in the fractions are very low. A chemical not screened for could be behind the slight variation in activation of these receptors while there could also be chemicals in the SEFs inhibiting the Ar and Er resulting in a generally low activation across the fractions. There have been studies done that discovered several PCBs acting like both agonists and antagonists for Er activation, like Zhang et al. (2014) where *in vitro* dual- luciferase reporter gene assay was used to determine this (Zhang *et al.*, 2014). There are also some chemicals that could affect both receptors, either similarly or differently, like DDE that works as both an antiandrogenic and estrogenic compound as shown by Hoffmann & Kloas. (2016), where they exposed amphibians to DDE to determine its mode of action (MOA) (Hoffmann and Kloas, 2016). Further testing, fractionating, and chemical screening is needed to determine with more certainty where the activation and or inhibition stems from as there is a number of environmental contaminants that could affect these receptors, like steroids, diethylstilbesterol, flutamide derivates, bisphenol derivates, alkylphenols, parabens DDTs, PCBs, and pesticides among others (Fang *et al.*, 2003; Hong *et al.*, 2007; Luccio-Camelo and Prins, 2011). A source of these EDCs could also potentially be water, either partially cleaned from wastewater treatment plants (WWTP) or runoff water from sewage during heavy rains. Westerhoff et al, 2005 tested WWTP cleaning methods to determine the ability to remove EDCs and PAHs and it was discovered that unless ozone is involved in the cleaning process most of these compounds are not removed (Westerhoff *et al.*, 2005). A high concentration of PAHs in fraction 4 could be the factor affecting the Er activation or it could be a pointer to other pharmaceuticals congregating here which could have some effects.

The PCA results for the biological data (Plot C) show fractions 1, 2 and 10 as negative outliers while fraction 5 is shown as a positive outlier. This indicates that the negative outliers are the fractions activating the receptors the least, while fraction 5 is activating the most. This fits well with fractions 1, 2 and 10 activating low to nothing for all receptors tested. Fraction 5, on the other hand, is not strongly activating any of the receptors, with the exception of drPxr, which could have skewed the results this way. When looking at the PCA plots for both the chemical analysis (Plot B) and the chemical analysis combined with the biological data (Plot A) they are very similar. Here we see that fractions 2, 3 and 4 are the outliers with the rest of the fractions are more clumped together. Looking at the results from the HOC, BDE and PAHs we see that this is where most of the chemicals were observed. However, no direct correlation between these findings and receptor activation could be seen.

5.3 Conclusion

In summary, this study has shown that the sediment samples are complex mixtures of many different chemicals and further fractionation and biotesting is needed to determine the cause of the toxicity more precisely. The results are promising though, showing that by fractionating the sediment extract we see different activations of the receptors tested for and we find that not all chemical compounds screened for are present in all fractions. This can help to further narrow the list of possible contaminants responsible for toxicity.

By employing effect-directed analysis of the sediment extract fractions from Vågen, Bergen, it was discovered that compounds activating the receptors were observed in most of the fractions. A comparison between the chemical analysis and the biological data did not reveal a distinct correlation, indicating that it is not the most common or abundant compound in the environmental mixture that are responsible for activating the receptors.

By using fractionation and EDA the inhibiting effect on EROD previously observed in the Vågen extract has been separated from the compounds activating Ahr, as shown by the parallel activation of gmAhr and induction of EROD activity in PLHC-1 cells.

5.4 Further studies

Future work should entail further fractionation and testing of the fractions proven to affect the receptors, so as to further narrow down the list of potential compounds activating these receptors in this highly polluted sediment. Testing for inhibiting compounds, such as antagonism-testing in a luciferase reporter assay should also be performed. The chemical analysis showed a large quantity of compounds congregating in a few of the fractions which did not correlate with the activity of the bioassays. Determining if this is because the compounds do not influence the receptors or if there are some factors inhibiting the activation of the receptors is necessary.

6 References

Akashe, M. M., Pawade, U. V and Nikam, A. V (2018) 'CLASSIFICATION OF PESTICIDES: A REVIEW', *International Journal of Research in Ayurveda and Pharmacy*, 9(4), pp. 144–150. doi: 10.7897/2277-4343.094131.

Ankley, G. T. *et al.* (2010) 'Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment', *Environmental Toxicology and Chemistry*, 29(3), pp. 730–741. doi: 10.1002/ETC.34.

Arrieta, D. E. *et al.* (2003) 'Aryl hydrocarbon receptor-mediated activity of particulate organic matter from the Paso del Norte airshed along the U.S.-Mexico border', *Environmental Health Perspectives*, 111(10), pp. 1299–1305. doi: 10.1289/EHP.6058.

Artabe, A. E., Cunha-Silva, H. and Barranco, A. (2020) 'Enzymatic assays for the assessment of toxic effects of halogenated organic contaminants in water and food. A review', *Food and Chemical Toxicology*, 145, p. 111677. doi: 10.1016/J.FCT.2020.111677.

Auwerx, J. *et al.* (1999) 'A Unified Nomenclature System for the Nuclear Receptor Superfamily', *Cell*, 97(2), pp. 161–163. doi: 10.1016/S0092-8674(00)80726-6.

Belin Tavakoly Sany, S. *et al.* (2016) 'An overview of detection techniques for monitoring dioxin-like compounds: latest technique trends and their applications'. doi: 10.1039/c6ra11442c.

Bergen Kommune (2014) 'Oppdatert risikovurdering av forurenset sjøbunn i Vågen, Bergen'.

Beyer, J. *et al.* (1997) *Time-and dose-dependent biomarker responses in flounder (Platichthys flesus L.) exposed to benzo[a]pyrene, 2,3,3¢ ,4,4¢ , 5-hexachlorobiphenyl (PCB-156) and cadmium*.

Bich, D. (2022) *The ban on toxic flame retardant stays, chemical industry dismissed by EU court - META*. Available at: https://meta.eeb.org/2022/03/30/the-ban-on-toxic-flame-retardant-stays-chemical-industry-dismissed-by-eu-court/ (Accessed: 14 August 2022).

Bigus, P., Tobiszewski, M. and Namieśnik, J. (2014) 'Historical records of organic pollutants in sediment cores', *Marine Pollution Bulletin*, 78(1–2), pp. 26–42. doi: 10.1016/J.MARPOLBUL.2013.11.008.

Boitsov, S., Klungsøyr, J. and Jensen, H. K. B. (2020) 'Background concentrations of polycyclic aromatic hydrocarbons (PAHs) in deep core sediments from the Norwegian Sea and the Barents Sea: A proposed update of the OSPAR Commission background values for these sea areas', *Chemosphere*, 251, p. 126344. doi: 10.1016/J.CHEMOSPHERE.2020.126344.

Bozcaarmutlu, A. and Arinç, E. (2004) 'Inhibitory effects of divalent metal ions on liver microsomal 7-ethoxyresorufin O-deethylase (EROD) activity of leaping mullet', *Marine environmental research*, 58(2–5), pp. 521–524. doi: 10.1016/J.MARENVRES.2004.03.040.

Brack, W. *et al.* (2016) 'Effect-directed analysis supporting monitoring of aquatic environments — An in-depth overview', *Science of The Total Environment*, 544, pp. 1073–1118. doi: 10.1016/J.SCITOTENV.2015.11.102.

Burcham, P. C. (2014) *An introduction to toxicology*, *An Introduction to Toxicology*. doi: 10.1007/978-1-4471-5553-9.

Burgess, R. M. *et al.* (2013) 'Effects-Directed Analysis (EDA) and Toxicity Identification Evaluation (TIE): Complementary but Different Approaches for Diagnosing Causes of Environmental Toxicity', *Wiley Online Library*. doi: 10.1002/etc.2299.

Cedergreen, N. (2014) 'Quantifying Synergy: A Systematic Review of Mixture Toxicity

Studies within Environmental Toxicology', *PLOS ONE*, 9(5), p. e96580. doi: 10.1371/JOURNAL.PONE.0096580.

Chai, S. C. and Chen, T. (2021) 'Nuclear Receptors', *Reference Module in Biomedical Sciences*. doi: 10.1016/B978-0-12-820472-6.00113-4.

Chen, Q. *et al.* (2016) 'Glucocorticoid activity detected by in vivo zebrafish assay and in vitro glucocorticoid receptor bioassay at environmental relevant concentrations', *Chemosphere*, 144, pp. 1162–1169. doi: 10.1016/J.CHEMOSPHERE.2015.09.089.

Darnerud, P. O. *et al.* (2001) 'Polybrominated diphenyl ethers: Occurence, dietary exposure, and toxicology', *Environmental Health Perspectives*, 109(SUPPL. 1), pp. 49–68. doi: 10.1289/EHP.01109S149.

Dart, D. A. *et al.* (2013) 'Visualising Androgen Receptor Activity in Male and Female Mice', *PLoS ONE*, 8(8). doi: 10.1371/JOURNAL.PONE.0071694.

Delistraty, D. (1997) 'Toxic equivalency factor approach for risk assessment of polycyclic aromatic hydrocarbons', *Toxicological and Environmental Chemistry*, 64(1–4), pp. 81–108. doi: 10.1080/02772249709358542.

Eide, M. *et al.* (2018) 'Independent losses of a xenobiotic receptor across teleost evolution'. doi: 10.1038/s41598-018-28498-4.

Escher, B., Peta, N. and Leusch, F. (2021) *Bioanalytical Tools in Water Quality Assessment: Second Edition - external_content.pdf*. Available at: https://library.oapen.org/viewer/web/viewer.html?file=/bitstream/handle/20.500.12657/50078/external_content.pdf?sequence=1&isAllowed=y (Accessed: 30 May 2022).

Esser, C., Rannug, A. and Stockinger, B. (2009) 'The aryl hydrocarbon receptor in immunity', *Trends in Immunology*, 30(9), pp. 447–454. doi: 10.1016/J.IT.2009.06.005.

EU (2000) 'Official journal of the european communities', *Analytical Proceedings*, 21(6), p. 196. doi: 10.1039/AP9842100196.

EU (2006) *EUR-Lex - 32006L0112 - EN - EUR-Lex*. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=celex%3A32006L0112 (Accessed: 31 May 2022).

EU (2009) *EUR-Lex - 32009L0090 - EN - EUR-Lex*. Available at: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32009L0090 (Accessed: 30 March 2022).

Eurolab (2022) *Special Chemical Analysis*. Available at: https://www.labaratuar.com/en/testler/kimyasal/ozel-kimyasal-analiz/ (Accessed: 24 March 2022).

European Commission - Directorate General Environment (2012) 'Preparatory Action - Development of Prevention Activities to halt desertification in Europe - Service Contract to contribute to the building of Water and Ecosystem accounts at EU level - Final Report 2: Uses & Supply', p. 202. Available at: http://ec.europa.eu/environment/water/blueprint/pdf/WaterEcosystemAccount2.pdf.

Fang, H. *et al.* (2003) 'Study of 202 Natural, Synthetic, and Environmental Chemicals for Binding to the Androgen Receptor', *Chemical Research in Toxicology*, 16(10), pp. 1338–1358. doi: 10.1021/TX030011G/ASSET/IMAGES/LARGE/TX030011GF00019.JPEG.

Farzaneh, S. and Zarghi, A. (2016) 'Scientia Pharmaceutica Estrogen Receptor Ligands: A Review (2013-2015)'. doi: 10.3390/scipharm84030409.

Feng, S., Cao, Z. and Wang, X. (2013) 'Role of aryl hydrocarbon receptor in cancer', *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 1836(2), pp. 197–210. doi: 10.1016/J.BBCAN.2013.05.001.

Fery, Y. *et al.* (2009) ‘Technical pentabromodiphenyl ether and hexabromocyclododecane as activators of the pregnane-X-receptor (PXR)’, *Toxicology*, 264(1–2), pp. 45–51. doi: 10.1016/J.TOX.2009.07.009.

Fredriksen, I. B. Ø. (2021) ‘USING LIGAND-ACTIVATED RECEPTORS FROM ATLANIC COD (Gadus morhua) AND ZEBRAFISH (Danio rerio) TO ASSESS BIOLOGICAL EFFECTS OF PHARMACEUTICALS IN WASTEWATER IN BERGEN, NORWAY’. Available at: https://bora.uib.no/bora-xmlui/handle/11250/2780586 (Accessed: 18 September 2022).

Furue, M. *et al.* (2014) ‘Role of AhR/ARNT system in skin homeostasis’, *Archives of Dermatological Research*, 306(9), pp. 769–779. doi: 10.1007/S00403-014-1481-7/FIGURES/4.

Gao, Y. D. *et al.* (2008) ‘Attenuating pregnane X receptor (PXR) activation: A molecular modelling approach’, *http://dx.doi.org/10.1080/00498250601050412*, 37(2), pp. 124–138. doi: 10.1080/00498250601050412.

Gelmann, E. (2002) ‘Molecular Biology of the Androgen Receptor’, *Article in Journal of Clinical Oncology*. doi: 10.1200/JCO.2002.10.018.

Goksøyr, A. *et al.* (1992) *Detection of cytochrome P450 1A1 in North Sea dab liver and kidney on JSTOR*. Available at: https://www.jstor.org/stable/24832644#metadata_info_tab_contents (Accessed: 14 August 2022).

Goksøyr, S. Ø. *et al.* (2021) ‘Toxicity assessment of urban marine sediments from Western Norway using a battery of stress-activated receptors and cell-based bioassays from fish’, *Environmental Toxicology and Pharmacology*, 87, p. 103704. doi: 10.1016/J.ETAP.2021.103704.

Goncharuk, V. V and Kovalenko, V. F. (2012) ‘Original Russian Text © V’, *Technology*, 34(2), pp. 171–178. doi: 10.3103/S1063455X12020051.

Gordon, J. D. *et al.* (2004) ‘Detection of estrogen receptor endocrine disruptor potency of commonly used organochlorine pesticides using the LUMI-CELL ER bioassay’, *Organohalogen Compounds*, 66, pp. 3068–3075.

Grenni, P., Ancona, V. and Barra Caracciolo, A. (2018) ‘Ecological effects of antibiotics on natural ecosystems: A review’, *Microchemical Journal*, 136, pp. 25–39. doi: 10.1016/J.MICROC.2017.02.006.

Gutendorf, B. and Westendorf, J. (2001) ‘Comparison of an array of in vitro assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens’, *Toxicology*, 166(1–2), pp. 79–89. doi: 10.1016/S0300-483X(01)00437-1.

Heinlein, C. A. and Chang, C. (2002) ‘Androgen Receptor (AR) Coregulators: An Overview’, *Endocrine Reviews*, 23(2), pp. 175–200. doi: 10.1210/EDRV.23.2.0460.

Hinger, G. *et al.* (2011) ‘Some heterocyclic aromatic compounds are Ah receptor agonists in the DR-CALUX assay and the EROD assay with RTL-W1 cells’, *Environmental Science and Pollution Research*, 18(8), pp. 1297–1304. doi: 10.1007/S11356-011-0483-7/TABLES/1.

Hites, R. A. (2004) ‘Polybrominated Diphenyl Ethers in the Environment and in People: A Meta-Analysis of Concentrations’, *Environmental Science and Technology*, 38(4), pp. 945–956. doi: 10.1021/ES035082G/ASSET/IMAGES/LARGE/ES035082GF00008.JPEG.

Hoffmann, F. and Kloas, W. (2016) ‘p,p′-Dichlordiphenyldichloroethylene (p,p′-DDE) can elicit antiandrogenic and estrogenic modes of action in the amphibian Xenopus laevis’, *Physiology & Behavior*, 167, pp. 172–178. doi: 10.1016/J.PHYSBEH.2016.09.012.

Hong, H. *et al.* (2007) 'Comparative molecular field analysis (CoMFA) model using a large diverse set of natural, synthetic and environmental chemicals for binding to the androgen receptor', *https://doi.org/10.1080/10629360310001623962*, 14(5–6), pp. 373–388. doi: 10.1080/10629360310001623962.

Hurst, M. R. *et al.* (2004) 'Determination of dioxin and dioxin-like compounds in sediments from UK estuaries using a bio-analytical approach: chemical-activated luciferase expression (CALUX) assay', *Marine pollution bulletin*, 49(7–8), pp. 648–658. doi: 10.1016/J.MARPOLBUL.2004.04.012.

Hutchinson, T. H. *et al.* (2013) 'Evaluating legacy contaminants and emerging chemicals in marine environments using adverse outcome pathways and biological effects-directed analysis', *Marine Pollution Bulletin*, 74(2), pp. 517–525. doi: 10.1016/J.MARPOLBUL.2013.06.012.

Ihunnah, C. A., Jiang, M. and Xie, W. (2011) 'Nuclear receptor PXR, transcriptional circuits and metabolic relevance', *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, 1812(8), pp. 956–963. doi: 10.1016/J.BBADIS.2011.01.014.

Ikeuchi, T. *et al.* (2001) 'Two subtypes of androgen and progestogen receptors in fish testes', *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 129(2–3), pp. 449–455. doi: 10.1016/S1096-4959(01)00375-X.

Ismail, B. and Nielsen, S. S. (2010) 'Basic Principles of Chromatography', (1), pp. 473–498. doi: 10.1007/978-1-4419-1478-1_27.

J. Calvin, G. (2022) *chromatography | Definition, Types, & Facts | Britannica*. Available at: https://www.britannica.com/science/chromatography (Accessed: 14 August 2022).

Jære, L. (2016) *Miljøgifter i sjøbunn spres til omgivelsene*. Available at: https://www.barentswatch.no/artikler/forurenset-sjobunn/ (Accessed: 14 July 2022).

Klinge, C. M. (2001) 'Estrogen receptor interaction with estrogen response elements', *Nucleic Acids Research*, 29(14), pp. 2905–2919. doi: 10.1093/NAR/29.14.2905.

Kosmehl, T. *et al.* (2007) 'Differentiation between bioavailable and total hazard potential of sediment-induced DNA fragmentation as measured by the comet assay with zebrafish embryos', *Journal of Soils and Sediments*, 7(6), pp. 377–387. doi: 10.1065/JSS2007.11.261.

Laflamme, R. E. and Hites, R. A. (1978) 'The global distribution of polycyclic aromatic hydrocarbons in recent sediments', *Geochimica et Cosmochimica Acta*, 42(3), pp. 289–303. doi: 10.1016/0016-7037(78)90182-5.

Landis, W. G., Sofield, R. M. and Yu, M.-H. (2018) *Introduction to Environmental Toxicology*.

Larigot, L. *et al.* (2018) 'AhR signaling pathways and regulatory functions', *Biochimie Open*, 7, p. 1. doi: 10.1016/J.BIOPEN.2018.05.001.

Lawal, A. T. (2017) 'Polycyclic aromatic hydrocarbons. A review', *http://www.editorialmanager.com/cogentenv*, 3(1). doi: 10.1080/23311843.2017.1339841.

Leusch, F. D. L. *et al.* (2018) 'Analysis of endocrine activity in drinking water, surface water and treated wastewater from six countries', *Water Research*, 139, pp. 10–18. doi: 10.1016/J.WATRES.2018.03.056.

Lille-Langøy, R. *et al.* (2015) 'Environmental contaminants activate human and polar bear (Ursus maritimus) pregnane X receptors (PXR, NR1I2) differently', *Toxicology and Applied Pharmacology*, 284(1), pp. 54–64. doi: 10.1016/j.taap.2015.02.001.

Lille-Langøy, R. *et al.* (2021) 'Substituted Two-to Five-Ring Polycyclic Aromatic Compounds Are Potent Agonists of Atlantic Cod (Gadus morhua) Aryl Hydrocarbon Receptors Ahr1a and

Ahr2a', *Environmental Science and Technology*, 55(22), pp. 15123–15135. doi: 10.1021/ACS.EST.1C02946/ASSET/IMAGES/LARGE/ES1C02946_0004.JPEG.

Liu, H. *et al.* (2014) 'Developmental Toxicity, EROD, and CYP1A mRNA Expression in Zebrafish Embryos Exposed to Dioxin-Like PCB126', *Inc. Environ Toxicol*, 31, pp. 201–210. doi: 10.1002/tox.22035.

Lorber, M. (2007) 'Exposure of Americans to polybrominated diphenyl ethers', *Journal of Exposure Science & Environmental Epidemiology 2008 18:1*, 18(1), pp. 2–19. doi: 10.1038/sj.jes.7500572.

Louis, G. W., Hallinger, D. R. and Stoker, T. E. (2013) 'The effect of triclosan on the uterotrophic response to extended doses of ethinyl estradiol in the weanling rat', *Reproductive Toxicology*, 36, pp. 71–77. doi: 10.1016/J.REPROTOX.2012.12.001.

Luccio-Camelo, D. C. and Prins, G. S. (2011) 'Disruption of androgen receptor signaling in males by environmental chemicals', *The Journal of Steroid Biochemistry and Molecular Biology*, 127(1–2), pp. 74–82. doi: 10.1016/J.JSBMB.2011.04.004.

Lynch, C. *et al.* (2017) 'Identifying environmental chemicals as agonists of the androgen receptor by using a quantitative high-throughput screening platform', *Toxicology*, 385, pp. 48–58. doi: 10.1016/J.TOX.2017.05.001.

Ma, J. *et al.* (2012) 'State of polybrominated diphenyl ethers in China: An overview', *Chemosphere*, 88(7), pp. 769–778. doi: 10.1016/J.CHEMOSPHERE.2012.03.093.

Machala, M. *et al.* (2001) 'Aryl hydrocarbon receptor-mediated activity of mutagenic polycyclic aromatic hydrocarbons determined using in vitro reporter gene assay', *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 497(1–2), pp. 49–62. doi: 10.1016/S1383-5718(01)00240-6.

Maher, W., Batley, G. E. and Lawrence, I. (1999) 'Assessing the health of sediment ecosystems: use of chemical measurements', *Freshwater Biology*, 41(2), pp. 361–372. doi: 10.1046/J.1365-2427.1999.00436.X.

Manning, G. E. *et al.* (2012) 'A luciferase reporter gene assay and aryl hydrocarbon receptor 1 genotype predict the LD50 of polychlorinated biphenyls in avian species', *Toxicology and Applied Pharmacology*, 263(3), pp. 390–401. doi: 10.1016/J.TAAP.2012.07.016.

Meeker, J. D. *et al.* (2009) 'Polybrominated diphenyl ether (PBDE) concentrations in house dust are related to hormone levels in men', *Science of the Total Environment*, 407(10), pp. 3425–3429. doi: 10.1016/J.SCITOTENV.2009.01.030.

van de Merwe, J. P. *et al.* (2018) 'In vitro bioassays reveal that additives are significant contributors to the toxicity of commercial household pesticides', *Aquatic Toxicology*, 199, pp. 263–268. doi: 10.1016/J.AQUATOX.2018.03.033.

Miljødirektoratet (2022) *Bergen havn - sjøbunn - Miljødirektoratet*. Available at: https://www.miljodirektoratet.no/ansvarsomrader/vann-hav-og-kyst/forurenset-sjobunn/bergen-havn/ (Accessed: 31 May 2022).

Mohammadi-Bardbori, A. and Mohammadi-Bardbori, A. (2014) 'Assay for quantitative determination of CYP1A1 enzyme activity using 7-Ethoxyresorufin as standard substrate (EROD assay)', *Protocol Exchange*. doi: 10.1038/PROTEX.2014.043.

Nakajima, Y. and Ohmiya, Y. (2010) 'Bioluminescence assays: multicolor luciferase assay, secreted luciferase assay and imaging luciferase assay', *http://dx.doi.org/10.1517/17460441.2010.506213*, 5(9), pp. 835–849. doi: 10.1517/17460441.2010.506213.

Neavin, D. R. *et al.* (2018) 'The Role of the Aryl Hydrocarbon Receptor (AHR) in Immune and Inflammatory Diseases', *International Journal of Molecular Sciences 2018, Vol. 19, Page 3851*, 19(12), p. 3851. doi: 10.3390/IJMS19123851.

Omsted, G. (2019) *Mye mer miljøgifter i fjordene enn i havet*. Available at: https://forskning.no/forurensning-havet-niva/mye-mer-miljogifter-i-fjordene-enn-i-havet/1331950 (Accessed: 14 July 2022).

Pacyniak, E. K. *et al.* (2007) 'The Flame Retardants, Polybrominated Diphenyl Ethers, Are Pregnane X Receptor Activators', *Toxicological Sciences*, 97(1), pp. 94–102. doi: 10.1093/TOXSCI/KFM025.

Pérez-Albaladejo, E. *et al.* (2016) 'Assessment of the environmental quality of coastal sediments by using a combination of in vitro bioassays', *Marine Pollution Bulletin*, 108(1–2), pp. 53–61. doi: 10.1016/J.MARPOLBUL.2016.04.063.

Petrulis, J. R. *et al.* (2000) *Application of the Ethoxyresorufin-O-Deethylase ( ) EROD Assay to Mixtures of Halogenated Aromatic Compounds*.

Petrulis, J. R. and Bunce, N. J. (1999) 'Competitive inhibition by inducer as a confounding factor in the use of the ethoxyresorufin-O-deethylase (EROD) assay to estimate exposure to dioxin-like compounds', *Toxicology Letters*, 105(3), pp. 251–260. doi: 10.1016/S0378-4274(99)00005-3.

Porta, M. and Zumeta, E. (2002) 'Implementing the Stockholm Treaty on Persistent Organic Pollutants', *Occupational and Environmental Medicine*, 59(10), pp. 651–652. doi: 10.1136/OEM.59.10.651.

Porter, B. A. *et al.* (2019) 'Structure and Function of the Nuclear Receptor Superfamily and Current Targeted Therapies of Prostate Cancer', *Cancers*, 11(12). doi: 10.3390/CANCERS11121852.

Renaud, J. P. and Moras, D. (2000) 'Structural studies on nuclear receptors', *Cellular and Molecular Life Sciences CMLS 2000 57:12*, 57(12), pp. 1748–1769. doi: 10.1007/PL00000656.

Ringnér, M. (2008) 'What is principal component analysis?', *Nature Biotechnology 2008 26:3*, 26(3), pp. 303–304. doi: 10.1038/nbt0308-303.

Rosenfeld, P. E. and Feng, L. G. H. (2011) 'Emerging Contaminants', *Risks of Hazardous Wastes*, pp. 215–222. doi: 10.1016/B978-1-4377-7842-7.00016-7.

Safe, S. *et al.* (2020) 'Aryl Hydrocarbon Receptor (AHR) Ligands as Selective AHR Modulators (SAhRMs)', *International Journal of Molecular Sciences 2020, Vol. 21, Page 6654*, 21(18), p. 6654. doi: 10.3390/IJMS21186654.

Safe, S. H. *et al.* (2001) 'Toxicology of environmental estrogens', *Reproduction, Fertility and Development*, 13(4), pp. 307–315. doi: 10.1071/RD00108.

Salimi, M. *et al.* (2017) 'Contaminants of emerging concern: a review of new approach in AOP technologies'. doi: 10.1007/s10661-017-6097-x.

Sauvé, S. and Desrosiers, M. (2014) 'A review of what is an emerging contaminant', *Chemistry Central Journal*, 8(1), pp. 1–7. doi: 10.1186/1752-153X-8-15/FIGURES/2.

Schrader, T. J. and Cooke, G. M. (2003) 'Effects of Aroclors and individual PCB congeners on activation of the human androgen receptor in vitro', *Reproductive Toxicology*, 17(1), pp. 15–23. doi: 10.1016/S0890-6238(02)00076-X.

Sharma, D. *et al.* (2021) 'Reverse transcription-loop mediated isothermal amplification (RT-LAMP) assay for detection of AhR receptor responsive xenobiotics',

https://doi.org/10.1080/15376516.2021.1884923, 31(5), pp. 359–366. doi: 10.1080/15376516.2021.1884923.

Smith, R. L. and Pietrzyk, D. J. (1984) *Liquid chromatographic separation of metal ions on a silica column*, *Anal. Chem*. UTC. Available at: https://pubs.acs.org/sharingguidelines.

Srogi, K. (2007) 'Monitoring of environmental exposure to polycyclic aromatic hydrocarbons: a review', *Environmental Chemistry Letters 2007 5:4*, 5(4), pp. 169–195. doi: 10.1007/S10311-007-0095-0.

Sternudd, K. (2022) *Cocktail effect makes chemicals more toxic | Karolinska Institutet*. Available at: https://ki.se/en/research/cocktail-effect-makes-chemicals-more-toxic (Accessed: 7 June 2022).

Stronkhorst, J., Leonards, P. and Murk, A. J. (2002) 'Using the dioxin receptor-calux in vitro bioassay to screen marine harbor sediments for compounds with a dioxin-like mode of action', *Environmental Toxicology and Chemistry*, 21(12), pp. 2552–2561. doi: 10.1002/ETC.5620211205.

Tang, W. and Row, K. H. (2020) 'Design and evaluation of polarity controlled and recyclable deep eutectic solvent based biphasic system for the polarity driven extraction and separation of compounds', *Journal of Cleaner Production*, 268, p. 122306. doi: 10.1016/J.JCLEPRO.2020.122306.

ThermoFisher, C. (2022) *Accelerated Solvent Extraction - Dionex Solvent Extractors*. Available at: https://www.chemeurope.com/en/products/127465/accelerated-solvent-extraction-ase-dionex.html (Accessed: 24 March 2022).

Thongdumhyu/Shutterstock, R. (2022) *Chiral Column Chromatography*. Available at: https://www.news-medical.net/life-sciences/Chiral-Column-Chromatography.aspx (Accessed: 24 March 2022).

Tobiszewski, M. and Namieśnik, J. (2012) 'PAH diagnostic ratios for the identification of pollution emission sources', *Environmental Pollution*, 162, pp. 110–119. doi: 10.1016/J.ENVPOL.2011.10.025.

Tollefsen, K. E. *et al.* (2007) 'Estrogen receptor (ER) agonists and androgen receptor (AR) antagonists in effluents from Norwegian North Sea oil production platforms', *Marine Pollution Bulletin*, 54(3), pp. 277–283. doi: 10.1016/J.MARPOLBUL.2006.07.012.

Tyasning, K. (2022) *A Deep Dive Into the Luciferase Assay: What It is, How It Works and More | GoldBio*. Available at: https://www.goldbio.com/articles/article/a-deep-dive-into-the-luciferase-assay-what-it-is-how-it-works-and-more (Accessed: 24 March 2022).

Vargas, V. *et al.* (2017) 'Development of a chromatographic methodology for the separation and quantification of V, Ni and S compounds in petroleum products', *Fuel Processing Technology*, 162, pp. 37–44. doi: 10.1016/J.FUPROC.2017.03.027.

Villeneuve, D. L. *et al.* (2014) 'Adverse Outcome Pathway (AOP) Development I: Strategies and Principles', *Toxicological Sciences*, 142(2), pp. 312–320. doi: 10.1093/TOXSCI/KFU199.

Vinggaard, A. M., Hnida, C. and Larsen, J. C. (2000) 'Environmental polycyclic aromatic hydrocarbons affect androgen receptor activation in vitro', *Toxicology*, 145(2–3), pp. 173–183. doi: 10.1016/S0300-483X(00)00143-8.

Vinken, M. (2013) 'The adverse outcome pathway concept: A pragmatic tool in toxicology', *Toxicology*, 312(1), pp. 158–165. doi: 10.1016/J.TOX.2013.08.011.

Wallace, K. *et al.* (2010) 'The PXR is a drug target for chronic inflammatory liver disease', *The Journal of Steroid Biochemistry and Molecular Biology*, 120(2–3), pp. 137–148. doi:

10.1016/J.JSBMB.2010.04.012.

Watkins, R. E. *et al.* (2001) 'The human nuclear xenobiotic receptor PXR: Structural determinants of directed promiscuity', *Science*, 292(5525), pp. 2329–2333. doi: 10.1126/SCIENCE.1060762/SUPPL_FILE/1060762S2_THUMB.GIF.

Weikum, E. R., Liu, X. and Ortlund, E. A. (2018) 'The nuclear receptor superfamily: A structural perspective'. doi: 10.1002/pro.3496.

Wernersson, A.-S. *et al.* (2011) 'The European technical report on aquatic effect-based monitoring tools under the water framework directive'. doi: 10.1186/s12302-015-0039-4.

Westerhoff, P. *et al.* (2005) 'Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes', *Environmental Science and Technology*, 39(17), pp. 6649–6663. doi: 10.1021/ES0484799/SUPPL_FILE/ES0484799SI20050607_060544.PDF.

Whyte, J. J. *et al.* (2008) 'Ethoxyresorufin-O-deethylase (EROD) Activity in Fish as a Biomarker of Chemical Exposure', *http://dx.doi.org/10.1080/10408440091159239*, 30(4), pp. 347–570. doi: 10.1080/10408440091159239.

Whyte, J. J. and Tillitt, D. E. (1995) 'EROD ACTIVITY Jeff J. Whyte and Donald E. Tillitt', *Signal Transduction*.

Yang, R. S. H. *et al.* (1998) 'Approaches to Developing Alternative and Predictive Toxicology Based on PBPK/PD and OSAR Modeling', *Health Perspectives*, 106, pp. 1385–1393. Available at: https://www.jstor.org/stable/4641206 (Accessed: 7 June 2022).

Yang, Y. *et al.* (2017) 'Occurrences and removal of pharmaceuticals and personal care products (PPCPs) in drinking water and water/sewage treatment plants: A review', *Science of The Total Environment*, 596–597, pp. 303–320. doi: 10.1016/J.SCITOTENV.2017.04.102.

Yoo, H., Khim, J. S. and Giesy, J. P. (2006) 'Receptor-mediated in vitro bioassay for characterization of Ah-R-active compounds and activities in sediment from Korea', *Chemosphere*, 62(8), pp. 1261–1271. doi: 10.1016/J.CHEMOSPHERE.2005.07.007.

Zhang, P. *et al.* (2009) 'One century record of contamination by polycyclic aromatic hydrocarbons and polychlorinated biphenyls in core sediments from the southern Yellow Sea', *Journal of Environmental Sciences*, 21(8), pp. 1080–1088. doi: 10.1016/S1001-0742(08)62385-2.

Zhang, Q. *et al.* (2014) 'Characterization of estrogen receptor α activities in polychlorinated biphenyls by in vitro dual-luciferase reporter gene assay', *Environmental Pollution*, 189, pp. 169–175. doi: 10.1016/J.ENVPOL.2014.03.001.

Zhang, Y. *et al.* (2016) 'Biological impact of environmental polycyclic aromatic hydrocarbons (ePAHs) as endocrine disruptors', *Environmental Pollution*, 213, pp. 809–824. doi: 10.1016/J.ENVPOL.2016.03.050.

Zhou, Y. *et al.* (2019) 'Bioassay system for the detection of aryl hydrocarbon receptor agonists in waterborne pesticides using zebrafish cyp1a1 promoter-luciferase recombinant hepatic cells', *Chemosphere*, 220, pp. 61–68. doi: 10.1016/J.CHEMOSPHERE.2018.12.115.

Zhu, K. *et al.* (2019) 'Aryl hydrocarbon receptor pathway: Role, regulation and intervention in atherosclerosis therapy (Review)', *Molecular Medicine Reports*, 20(6), pp. 4763–4773. doi: 10.3892/MMR.2019.10748/HTML.

7 Appendix

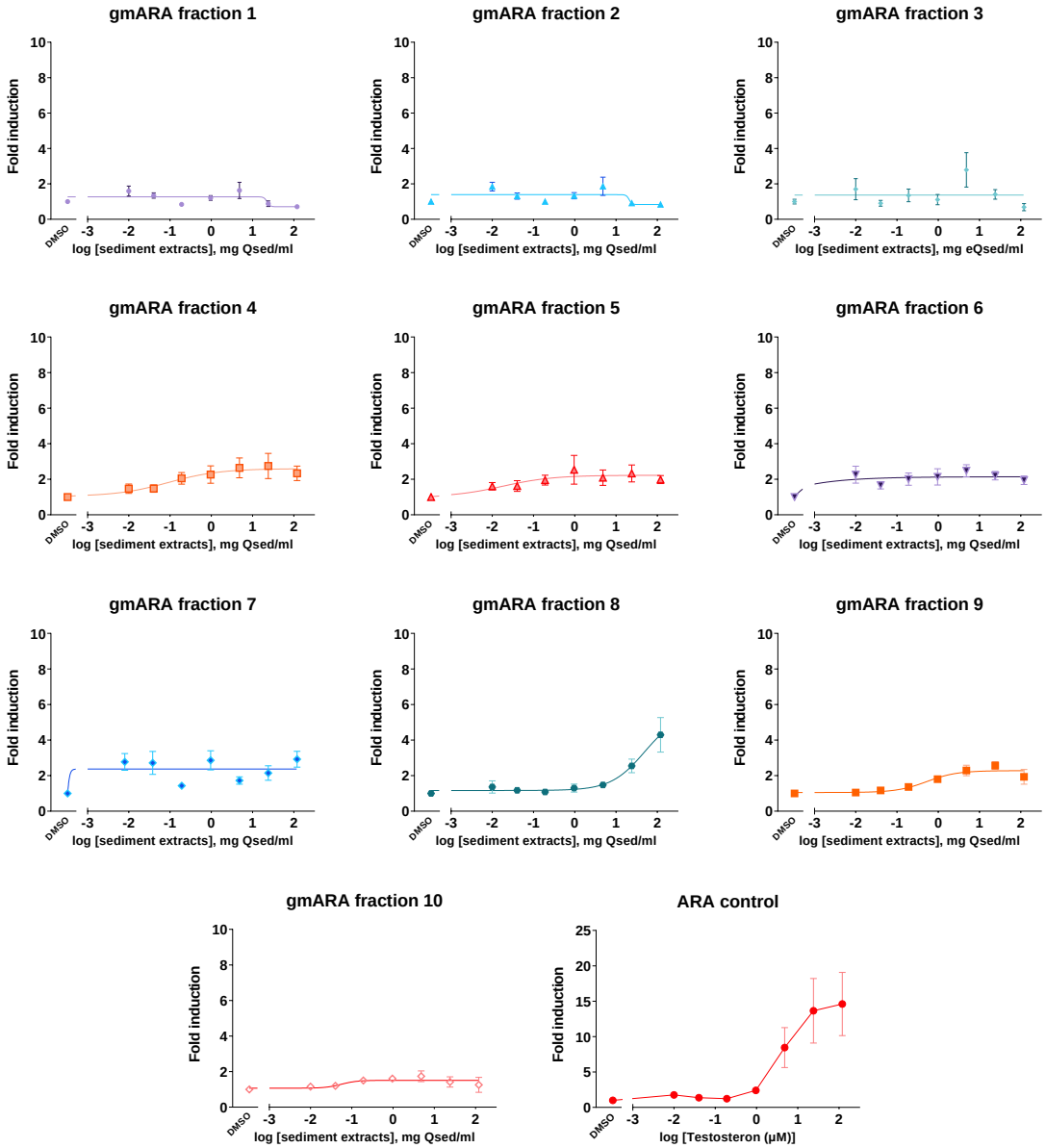


Figure A.1.

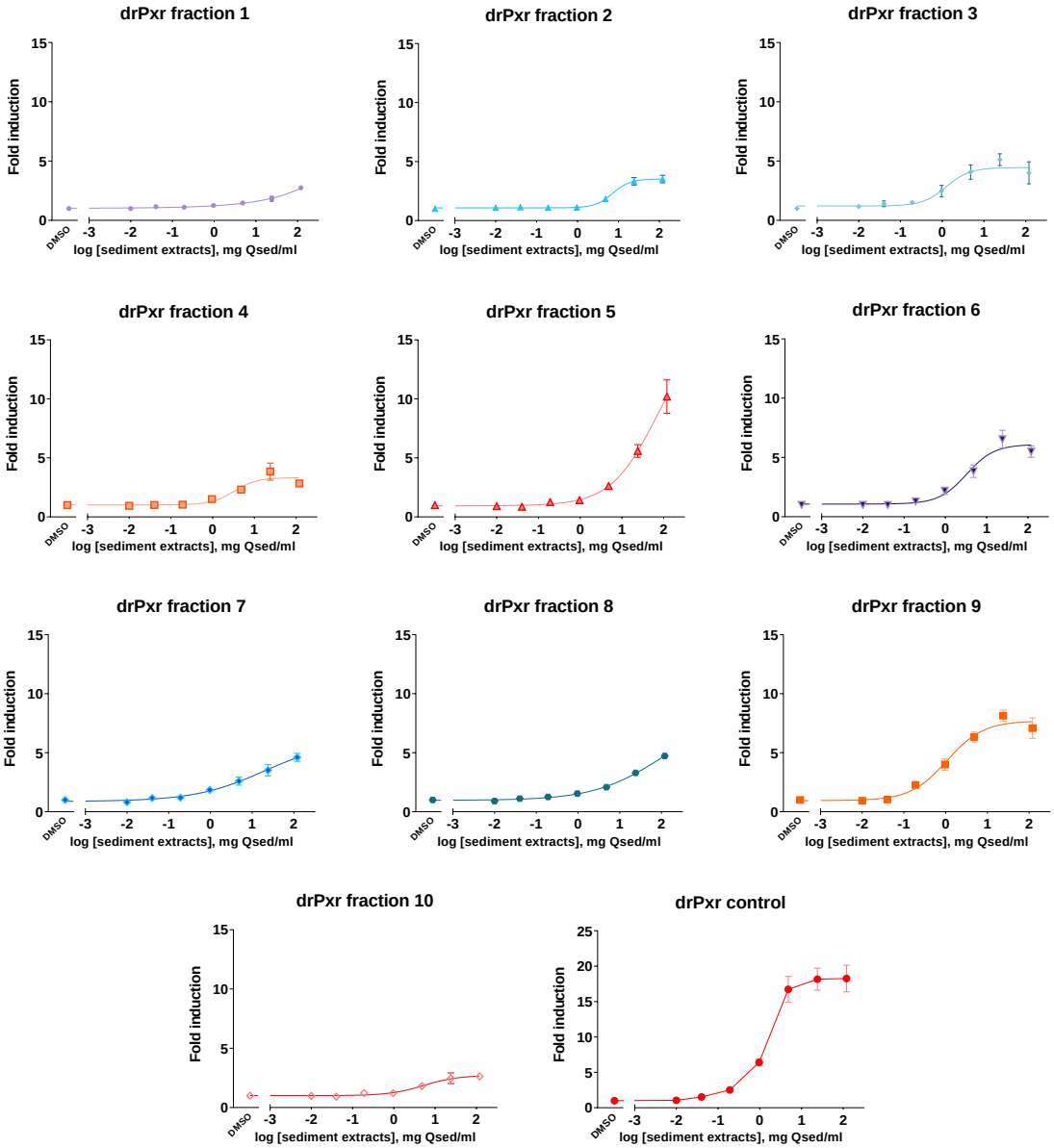

drPxr fraction 1
drPxr fraction 2
drPxr fraction 3
drPxr fraction 4
drPxr fraction 5
drPxr fraction 6
drPxr fraction 7
drPxr fraction 8
drPxr fraction 9
drPxr fraction 10
drPxr control
Fold induction
log [sediment extracts], mg Qsed/ml
DMSO

Figure A.2.

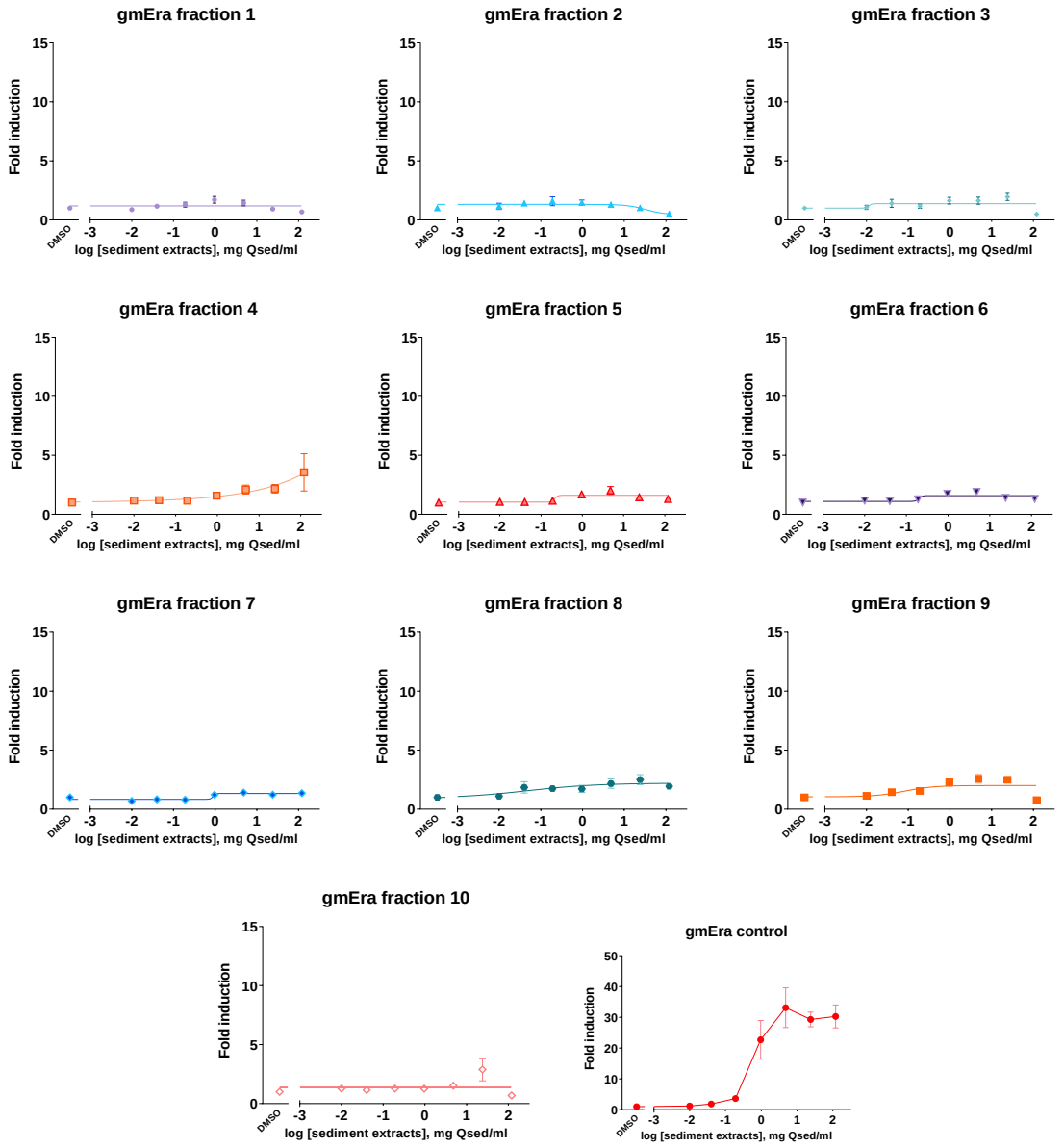

gmEra fraction 1
gmEra fraction 2
gmEra fraction 3
gmEra fraction 4
gmEra fraction 5
gmEra fraction 6
gmEra fraction 7
gmEra fraction 8
gmEra fraction 9
gmEra fraction 10
gmEra control
Fold induction
log [sediment extracts], mg Qsed/ml
DMSO

Figure A.3.

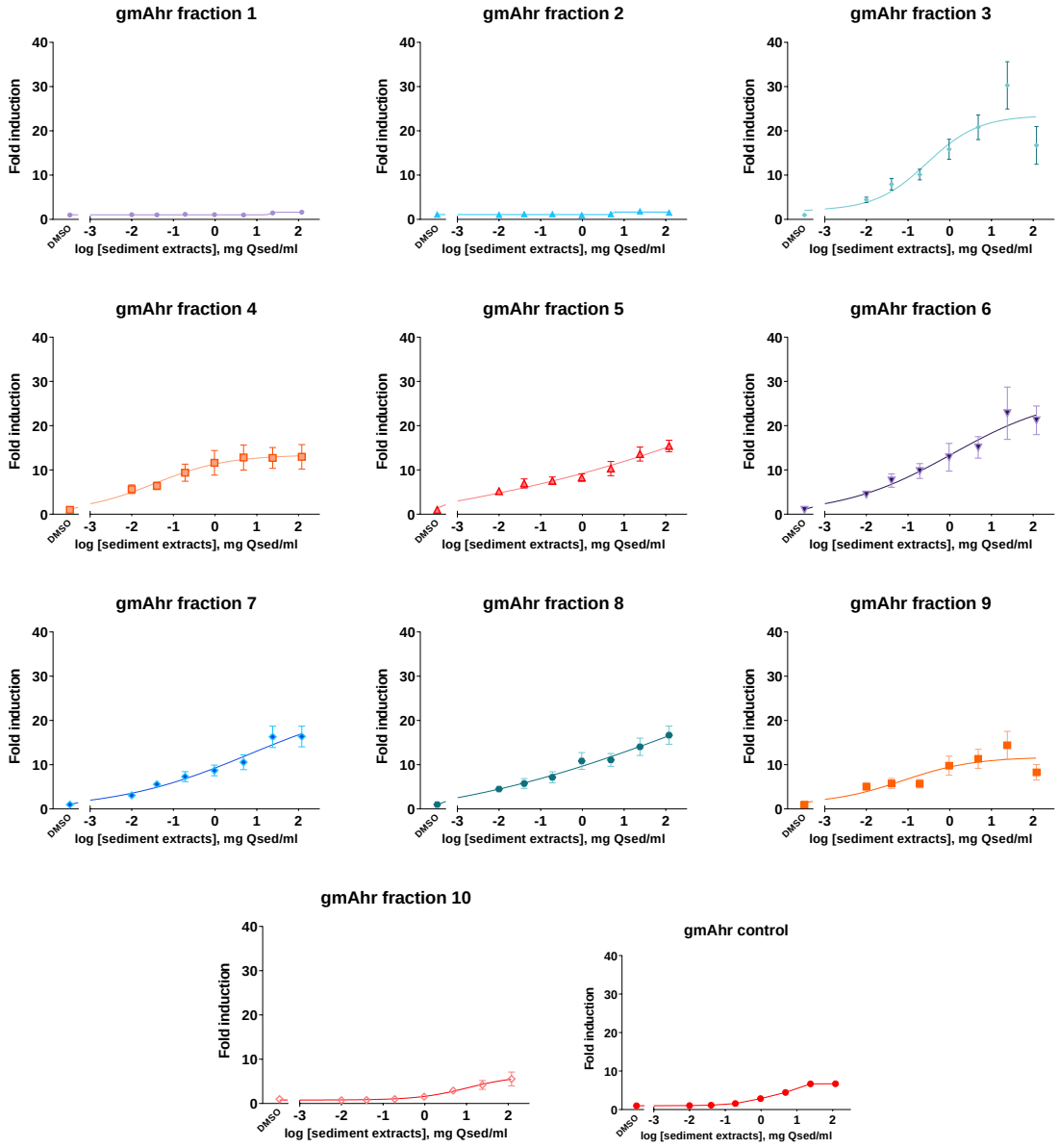

gmAhr fraction 1
gmAhr fraction 2
gmAhr fraction 3
gmAhr fraction 4
gmAhr fraction 5
gmAhr fraction 6
gmAhr fraction 7
gmAhr fraction 8
gmAhr fraction 9
gmAhr fraction 10
gmAhr control
Fold induction
log [sediment extracts], mg Qsed/ml
DMSO

Figure A.4.