USING LIGAND-ACTIVATED RECEPTORS FROM ATLANIC COD (*Gadus morhua*) AND ZEBRAFISH (*Danio rerio*) TO ASSESS BIOLOGICAL EFFECTS OF PHARMACEUTICALS IN WASTEWATER IN BERGEN, NORWAY

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Master thesis in environmental toxicology

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August 2021

III. Acknowledgements

This master thesis has been a part of the dCod 1.0 project funded by the Research Council of Norway (project no. 248840) as part of the Digital Life Norway initiative. The master thesis has also been supported by Miljøringens studentstipend 2020.

Samples for this master thesis has been provided by Bergen Vann KF, and I would like to thank the technical staff for sampling and Fereidun Akhoundzadeh for help with organizing and answering all my questions about wastewater and wastewater treatment throughout the past year. Chemical analyses were conducted at NMBU by Ivo Havranek.

I would like to thank my supervisors Ander Goksøyr and Odd André Karlsen. Your enthusiasm, help and support throughout the year, especially in the writing process, has been invaluable. Thank you for the countless interesting papers you have sent me, which have provided me with new insight into several exciting topics.

Next, I would like to thank Rhîan Gaenor Jacobsen for teaching and helping me out in the lab whenever I needed it. A special thanks to Kristianne Hjorth Viken for your support and help with the luciferase and cytotoxicity assays in the lab.

I would also like to thank the rest of the people in the environmental toxicology group, including fellow master student, for interesting lunches, encouragement, helpful input at Monday meetings and helping me out with whatever questions I have had the past year.

Lastly, I would like to give the greatest thank my friends, my parents, my sister Katrine Lovise and Tobias. Thank you for always believing in me and cheering me up whenever I have needed it. I'm forever grateful for your support.

Bergen, August 2021

Ingrid Beate Øpstad Fredriksen

IX. Abbreviations

Abbreviation	Ful name
AF-1	Activator function 1
AOP	Adverse outcome pathway
AR	Androgen receptor
BFR	Brominated flame retardant
CEC	Contaminants of emerging concern
DBD	DNA-binding domain
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC50	Half maximal effect concentration
EDC	Endocrine disrupting compound
EE2	17alpha-ethynylestradiol
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetra acetic acid
ER	Estrogen receptor
FBS	Fetal bovine serum
GR	Glucocorticoid receptor
HR	Hinge region
HRE	Hormone response elements
HSP90	Heat shock protein 90
IC50	Half maximal inhibitory concentration
KE	Key event
LB	Lysogeny broth
LBD	Ligand binding domain
LOEC	Lowest observed effect concentration
LOD	Limit of detection
LOQ	Limit of quantification
LRA	Luciferase reporter gene assay
ME	Matrix effect
MIE	Molecular initiating event
NR	Nuclear receptor
NTD	N-terminal domain
ONP	Ortho-nitrophenol
ONPG	Ortho-nitrophenyl-β-galactosid
PBS	Phosphate buffered saline
POP	Persistent organic pollutants
PPCP	Pharmaceuticals and personal care products
PXR	Pregnane X receptor
RPM	Revolutions per minute
RXR	Retinoid X receptor
SEM	Standard error of mean
TBE	Tris-borate-EDTA buffer
TCPP	Tris (1-chloro-2-propyl) phosphate
UAS	Upstream activation sequence
VTG	Vitellogenin
WW	Wastewater
WWTPs	Wastewater treatment plants
XRE	Xenobiotic response element
β-Gal	β-Galactosidase

X. Abstract

Pharmaceuticals and personal care products are continuously released into the marine environment through wastewater. Understanding the possible biological implications of pharmaceuticals specifically may be particularly important as they are designed to be biologically active, and many of them are known ligands for nuclear receptors. Nuclear receptors are ligand-activated transcription factors and include a multitude of receptors such as the pregnane X receptor (PXR), androgen receptor (AR) and estrogen receptor (ER). PXR has an important role in regulation of enzymes responsible for biotransformation and transport of both xenobiotics and certain endogenous compounds. The AR and ER are vital in development and reproduction as they bind and mediate effects of androgens (e.g., testosterone) and estrogens (e.g., estrone). Common for these nuclear receptors is that they might bind both natural hormones and synthetic compounds such as several pharmaceuticals. The main goal of this thesis was to investigate the ability of mixtures of compounds present in wastewater and sludge to activate or inhibit zebrafish PXR, cod ARα and cod ERα. This was done by utilizing *in vitro* luciferase reporter gene assays where these receptors were exposed to sample extracts from wastewater influents, wastewater effluents, or sludge from five different wastewater treatment plants in Bergen, Norway. Additional targeted chemical analysis was conducted in order to establish what compounds were present in the samples. The reporter gene assays indicated the presence of PXR agonists in multiple wastewater influents and effluents, where several effluents clearly stood out as more potent activators. ARα activation was seen in fewer samples, although for both influents and effluents. No ERα activation was identified for any of the samples. Several wastewater extracts did however induce significant inhibition of cod ERα. None of the sludge samples activated any of the receptors. The targeted chemical analysis detected compounds at variable concentrations. Caffeine was detected at highest concentrations followed by acetaminophen, TCPP, and 5-H diclofenac for both influent and effluent samples. Additionally, metoprolol, carbamazepine, prednisolone, and octocrylene were detected at lower concentrations. Effluent from Knappen wastewater treatment plant (WWTP) was identified as the sample with highest total concentration of compounds. The findings of this thesis suggest that compounds and/or mixtures of compounds present in wastewater influents and effluents from WWTPs in Bergen, Norway can activate and inhibit certain nuclear receptors from zebrafish and Atlantic cod. The methods applied also uncovered elevated cytotoxicity in extracts where chemical analyses were inconclusive, implying that bioassays may work as a useful supplement to chemical analyses by indicating both biological activity and cytotoxicity of wastewater released into the environment. The thesis does however represent a narrow window of information. The targeted chemical analysis, singular sampling day and the three receptors chosen, provides only a compound specific, time specific, and receptor specific image of the possible biological effects of compounds in the wastewater released.

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1. Introduction

1.1 Contaminants of emerging concern

The environment that all living organisms depends upon does not only have the nutrients and compounds needed to sustain life, but it also includes a multitude of chemical pollutants. These chemical pollutants may be categorized in various ways. One way to categorize them is by when they were taken into use and became apparent in the environment. Numerous persistent chemical contaminants that previously were abundantly used and released into the environment are now well-studied and called legacy contaminants (Landis, Sofield & Yu, 2018). The production and use of many of these compounds have been regulated and well managed, although many still persist in the environment (The Stockholm Convention, 2017). On the contrary, emerging contaminants are chemical pollutants that have been released into the environment more recently, which may lack both efficient regulations and thorough toxicity studies. However, there are no clear distinction, and a legacy contaminant may be of emerging concern due to current circumstances that are not yet properly studied. Due to this matter, Sauvé and Desroisers (2014) proposed that *contaminants of emerging concern* (CEC) may be a more appropriate term.

For a compound to be classified as a CEC, the compound (either naturally occurring or manmade) must be suspected to pose a potential risk to organisms, as well as being poorly regulated (Sauvé, S., Desrosiers, 2014). Microplastics, brominated flame retardants (BFRs), per- and polyfluoroalkyl substances (PFAS), and pharmaceuticals and personal care products (PPCPs) can all be considered CECs (Landis, Sofield & Yu, 2018).

1.1.1 Pharmaceuticals

Pharmaceuticals are medicinal drugs used to prevent, treat, or relieve disease or symptoms of disease in humans and animals. They may also be given to sustain or re-establish physiological function. They are commonly taken orally or by inhalation, rectally, as an injection, or added to skin as ointment or a plaster. To understand why, how, and in what form pharmaceuticals are released into the environment, knowledge on the fate of these compounds when they enter our bodies, exerts their purpose, and eventually gets excreted is needed.

The majority of pharmaceuticals influence chemical processes in specific cells, but they may work in different ways (Vennerød & Granås, 2021). In most cases however, to exert the desired effect, the pharmaceutical has to move from where it is administered to a receptor in a target site that stimulates a biological reaction (Eichhorn, 2013). Before the compound reaches the target site, it is influenced by several physical and biological processes within the organism, collectively known as pharmacokinetics. These processes include absorption, distribution, metabolism, and excretion, commonly abbreviated to ADME (Figure 1.1) (Eichhorn, 2013). When excreted, many pharmaceuticals are no longer in their original form. This is due to the metabolization – or biotransformation – that these compounds go through. As drugs are exogenous substances, the body will detect and attempt to excrete them as a defence mechanism. In this process, the liver is vital as it contains a number of enzymes with diverse substrate specificity responsible for catalysing the reactions involved (Landis et al.,2018).

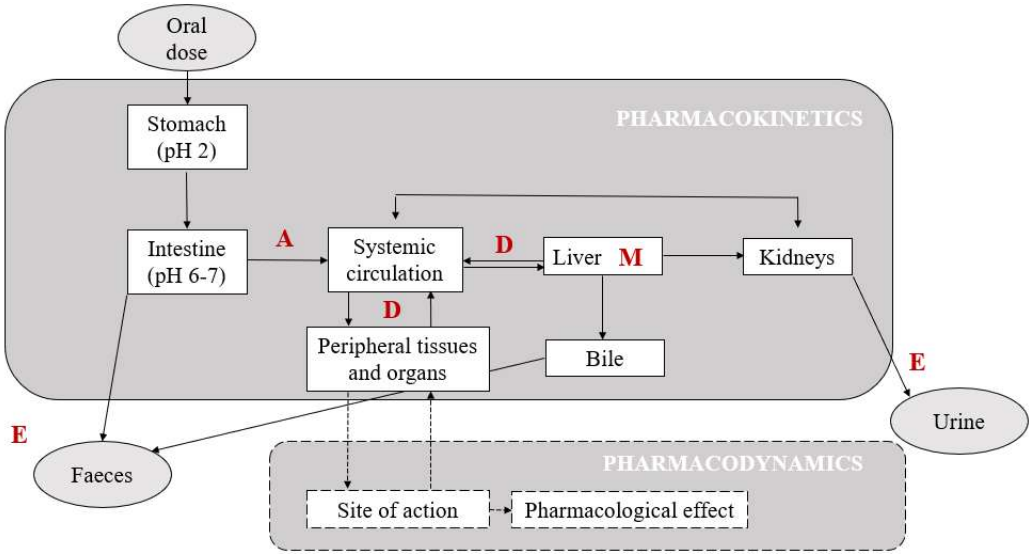


Figure 1.1 Schematic illustration of the pathways of an orally administered pharmaceutical, and the relationship between pharmacokinetics and pharmacodynamics. After administration, the drug is absorbed (A) in the intestine. Distribution (D) to peripheral tissues and organs, as well as the liver, typically occurs via the bloodstream. Metabolism (M) commonly occurs in the liver and kidneys where more water-soluble metabolites are created. Excretion (E) happens through faeces, urine, and in some cases also through the lungs or the skin. Pharmacokinetics describes what the body does to the substance (the ADME-steps), whilst pharmacodynamics describes the biological response to the pharmaceutical. Illustration modified from Peter Eichhorn (2013).

Cytochrome P450 enzymes (CYPs), such as members of the CYP1, CYP2, and CYP3 families, act as phase I oxidising enzymes and introduces polar groups to the xenobiotic compound (Kretschmer & Baldwin, 2005). In phase II, these more polar compounds are conjugated with an endogenous molecule, further promoting hydrophilicity for the metabolite to increase its ability to be excreted. The conjugation reaction usually includes conjugation to glutathione, sulfonation, glucuronidation, amino acid conjugation, or acetylation (Jancova, Anzenbacher, & Anzenbacherova, 2010). Enzymes responsible for phase II reactions are to a large extent transferases such as UDP-glucuronosyltransferases (UGTs/UDPGTs), sulfotransferases (SULTs), N-acetyltransferases (NATs), and glutathione S-transferases (GSTs) (Jancova et al., 2010). Some proteins, such as members of the solute carrier (SLC) and ATP-binding cassette (ABC) families, act as phase III transporters that are responsible for carrying the metabolites across the cell membrane and into the blood stream, urine, and faeces.

Through the biotransformation the xenobiotic is converted into a more water-soluble and excretable form and the metabolite can therefore diffuse/be transported from the liver into the bloodstream. Ultimately, this leads to glomerular filtration at the kidneys and excretion through urine. Another route of excretion is through faecal matter. This occurs either when the pharmaceutical is not completely dissolved and absorbed (and hence in an unchanged form), or due to biliary excretion (Eichhorn, 2013).

As pharmacokinetics describes what the body does to the exogenous compound, pharmacodynamics describes what the compound does to the body. More specifically, it describes the mechanism of action of an exogenous compound. This involves the chemical interaction between the compound and the organism through e.g. receptors and enzymes (Øye & Brørs, 2021). Such an interaction may release a series of responses at multiple levels in the organism, from a cellular level to the whole organism. Further details on biological pathways will be covered in section 1.1.2 Adverse outcome pathways, whereas receptor interactions will be covered in section 1.2 Nuclear receptors.

It is important to note that some pharmaceuticals are only partially or not systemically absorbed at all. A good example of this is clotrimazole which is a broad spectrum antimycotic drug commonly used to

treat dermal infections such as “athlete’s foot” (fungal feet infection) and yeast infections. Together with e.g. compounds used in anti-dandruff shampoos and sunscreens, these chemicals are released unchanged into our sewage water system when washed off our bodies. In areas where wastewater treatment systems are available, sewage containing either the original or metabolised compound ends up in wastewater treatment plants (WWTPs).

WWTPs are not specifically designed to remove pharmaceuticals and a review conducted by Santos et al., 2010, found that efficiencies in removal of such compounds could vary with up to 99 %. In addition to this, the use of pharmaceuticals in Norway has in recent years increased, with a 2.7 % increment in defined daily doses (DDD) in 2020 compared to 2019 (Reseptregisteret, 2021). Assessing the possible environmental side effects of the release of pharmaceuticals through wastewater may therefore be of relevance.

1.1.2 Adverse outcome pathways - AOP

The environment that pharmaceutical-containing wastewater is released into is highly complex and can be studied at multiple biological levels. However, connecting scientific findings on a cellular level to organism, or even population responses, is demanding. Previously, experiments based on mortality, growth and reproduction were used to derive benchmark values when assessing potential adverse outcomes (Russom, Lalone, Villeneuve, & Ankley, 2014). This is highly expensive and ultimately leads to a relative low number of compounds being properly tested.

The Adverse outcome pathway (AOP) is a framework proposed by Ankley et al. 2010, intended to enable mechanistic data to be linked to adverse outcomes at a higher biological level by using existing knowledge. A key application for AOPs is to provide both terminology and a way to structure knowledge from different biological levels that are relevant for risk assessment and management. This could enable a reduced need for expensive and time-consuming *in vivo* testing, and a greater use of new computational, molecular, and *in vitro* methods (Ankley et al., 2010).

The first step in the AOP is the molecular initiating event (MIE), which also is recognized as the first key event (KE1). This step requires, or assumes, that the compound has been absorbed in the organism and distributed to the site of action. KE1 is characterised by e.g. receptor/ligand interaction, DNA binding, or protein oxidation (Ankley et al., 2010) . This marks the beginning of the toxicological pathway (Figure 1.2). KE2 involves the cellular response, and effects may involve gene activation, protein expression, altered signalling or protein depletion. Organ responses are in KE3. Here, altered physiology, disrupted homeostasis, or altered tissue development or function are direct effects. The organ responses may lead to organism responses (KE4), such as lethality, impaired development, or reduced fertility. Progressing from KE4 to KE5 represents the separation between traditional toxicology and environmental toxicology (Landis et al., 2018). Changes in population structure, recruitment, or even extinction of species may be direct outcomes (Ankley et al., 2010). Furthermore, community and ecological structures may be added. These were not included in the original framework, but has been incorporated later (Landis et al., 2018).

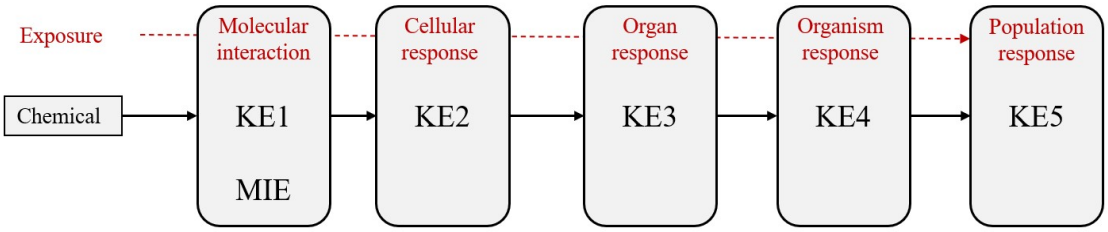


Figure 1.2 Basic principles of an adverse outcome pathway (AOP). The pathway starts with the introduction of a chemical which leads to the molecular initiating event (MIE). This is also denoted as key event one (KE1). The AOP describes a total of five key events connecting molecular interaction to population response. Figure modified from (Landis, Wayne G., Sofield, Ruth M., Yu, 2018)

As previously mentioned, the first step of the toxicological pathway is often the receptor/ligand interaction. In many cases, this interaction involves nuclear receptors.

1.2 Nuclear receptors

Endogenous signal molecules and some exogenous substances, such as pharmaceuticals or endocrine disrupting compounds (EDCs), can bind to receptors in cell membranes or within cells of an exposed organism. Nuclear receptors (NRs) are a family of ligand-activated transcription factors that recognises and binds specific ligands that regulate the expression of various genes involved in numerous physiological processes, such as metabolism and reproduction (Weikum, Liu, & Ortlund, 2018). NRs can be divided into seven nine subfamilies, from NR0 to NR8 (Huang et al., 2015; Wu, Niles, Hirai, & LoVerde, 2007). This thesis will focus on one member of subfamily 1; the pregnane X receptor - PXR, briefly mention one member of subfamily 2; the retinoid X receptor - RXR, and go in detail on three members of subfamily 3; the androgen receptor - AR, the estrogen receptor - ER and the glucocorticoid receptor - GR.

NR1 members are regulated by lipophilic molecules such as thyroid hormones, bile acids and certain types of steroids (Weikum et al., 2018). NR2s are sometimes called orphan receptors where specific ligands important for regulation has not yet been determined, except for 9-cis retinoic acid for RXR (Weikum et al., 2018; Mangelsdorf & Evans, 1995). NR3s are receptors regulated by steroid hormones which derive from cholesterol. Due to the lipophilicity of these hormones they can pass the cell membrane by simple diffusion, and bind to the steroid hormone receptors located in the cytosol (Beato & Klug, 2000). The ligand binding to the receptor induces a conformational change and translocation to the nucleus where regulation of target genes occurs.

Although belonging to the same superfamily, these receptors all work differently and have their own unique role within the cell. However, most NRs have the same general structure (Figure 1.3)(Weikum et al., 2018). At one end, we find the N-terminal domain (NTD). The NTD varies highly, but all contains the activator function-1 region (AF-1) which is responsible for interaction with regulatory proteins (Weikum et al., 2018). The DNA-binding domain (DBD) is located next to the NTD and holds a short motif (P-box) that binds to specific DNA sequences known as response elements (Robinson-Rechavi, Garcia, & Laudet, 2003). The DBD also contains a D-box involved in the heterodimerization and homodimerization of the NRs. (Pawlak, M., Lefebvre, P., Staels, 2012). The DBD in NRs is constructed of two zinc fingers which enable DNA binding (Franco, Li, & Wei, 2003). Between DBD and the ligand binding domain (LBD), there is a hinge region (HR), which is highly flexible and plays an important role in the orientation of the DBD and LBD. Finally, we have the ligand binding domain. The LBD is the site where lipophilic ligands that either activate or repress the transcriptional activity of the receptor may bind. The specific site of binding is called the ligand binding pocket (LBP) and varies between the different receptors (Pawlak, Lefebvre & Staels, 2012). The activator function-2 region (AF-2), which is found in the LBD is, compared to AF-1, highly structured and ligand dependent (Wärnmark, Treuter,

Wright, & Gustafsson, 2003). Coactivators that are ligand dependent have been shown to bind to the AF-2, and most of these have a consensus sequence (LXXLL) providing stability to this interaction (Wärnmark et al., 2003).

Figure 1.3 The functional domains of nuclear receptors. N-terminal domain (NTD) with AF-1, DNA-binding domain (DBD), a hinge region (HR) and the ligand-binding domain (LBD) with AF-2. Illustration based on Weikum, Liu, and Ortlund 2018.

As well as this common general structure, all nuclear receptors, as their name suggests, are in one way or another involved with the cell nucleus. Yet, their localization in the cell and operational mechanism varies. Based on this, they are often divided into four different types: I, II, III and IV (Weikum et al., 2018)(Figure 1.4).

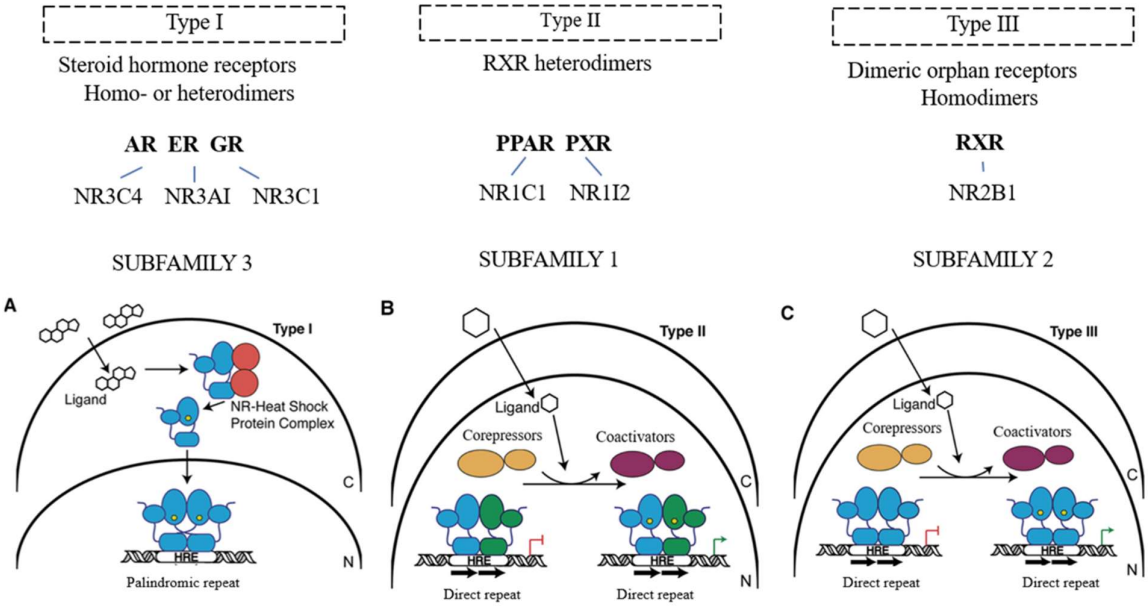


Figure 1.4 Three types of nuclear receptors. Type I nuclear receptors, such as AR, ER and GR, are present in cytosol upon ligand binding. Type II and Type III nuclear receptors, such as PXR and RXR, are present in nucleus. Type IV nuclear receptors are monomeric orphan receptors also present in cell nucleus upon ligand binding (not included in this thesis). Illustration modified from Weikum et al., 2018.

Type I includes steroid receptors (the NR3 subfamily). Receptors of this type are found in the cytoplasm where they are bound to chaperones. When a ligand binds, the receptor-ligand complex forms a homo- or heterodimer (with RXR) and gets translocated to the nucleus where it binds to hormone response elements (HRE) in the DNA. Type II includes members of NR1 subfamily which usually forms heterodimers with RXR. These are found in the nucleus where co-repressors are exchanged with co-activators upon ligand-binding. Type III includes members of NR2 subfamily and have similar mechanisms to Type II receptors. The main difference here is that the members of Type III form homodimers when binding to their HREs.

1.2.1 The androgen receptor (AR)

The androgen receptor (AR, NR3C4) is a steroid hormone receptor and a member of the NR3 subfamily of NRs. It is expressed in a wide range of organs and have been found to have a significant role in reproduction, muscle and bone development and maintenance, and in the immune system (Davey & Grossmann, 2016; Rana, Davey & Zajac, 2014) The AR binds androgens such as testosterone, dihydrotestosterone (DHT), and synthetic compounds with testosterone-like properties, such as the anabolic steroid nandrolone (Figure 1.5) (Neal, 2016). These androgens are key in male development and fertility as well as responsible for some secondary puberty changes in females (Øye & Borén, 2018). Just like the other nuclear receptors, AR contains an N-terminal domain, a DBD, a hinge region and an LBD. Unliganded, the AR is found as a monomer in the cytosol bound to heat shock proteins (HSP90 chaperones). When an androgen binds to the LBD, the AR changes conformation, forms homodimers, and translocates to the nucleus where androgen response elements (AREs) are detected in the genomic DNA (Feng, Qin, He, 2019). Here, coactivators are recruited, and transcription of target genes will follow.

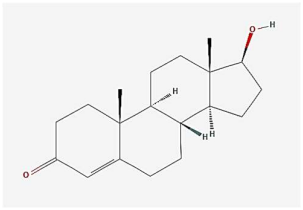
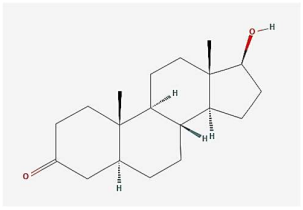
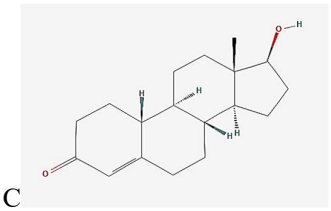
A  B  C 

Figure 1.5 2D structure of some AR ligands. (A) Testosterone (PubChem CID 6013), (B) dihydrotestosterone (PubChem CID 10635), and (C) nandrolone (PubChem CID 9904).

One known target gene of the AR is *probasin*, which is a prostate-specific gene responsible for directing the production of the protein probasin which is found in the epithelium of the prostate (Johnson, Hernandez, Wei, & Greenberg, 2000) ("Probasin," 2008). Studies treating human prostate cancer cell line (LNCaP) with the synthetic androgen R1881, also found that androgen treatment induced several other genes involved in the production of prostatic fluid, as well as prostate-specific antigen (PSA), sorbitol dehydrogenase and vascular endothelial growth factor (VEGF) (DePrimo et al., 2002), further emphasizing its significance in male reproduction. Androgen disrupting chemicals has been found to reduce sperm counts and lead to decreased fertility and testicular and prostate cancer (Luccio-Camelo & Prins, 2011).

Concentration of androgens are significantly lower in females, although they are important in both female development and fertility (Dart, Waxman, Aboagye & Bevan, 2013). A vital role of androgens in females is as a substrate for the aromatization of androgens to estrogens, although the AR is indeed expressed in female tissues such as the mammary gland, uterus and in ovarian follicles as well (Dart et al., 2013; Sen & Hammes, 2010). In female mice, androgens have been found to cause AR induction and expression of miR-125b, a micro-RNA gene which leads to suppression of follicular atresia (the breakdown of ovarian follicles), indicating a regulatory role in female reproduction (Sen et al., 2014). However, elevated levels of testosterone in human females have been linked to polycystic ovarian syndrome (PCOS) causing reduced fertility (Rosenfield & Ehrmann, 2016).

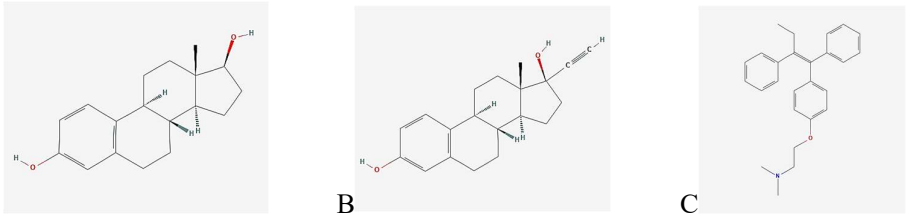
The AR is found across a multitude of invertebrate (Köhler et al., 2007), and vertebrate species, including fish, where two AR subtypes are known (Douard et al., 2008; Ikeuchi, Todo, Kobayashi, & Nagahama, 2001; Takeo & Yamashita, 1999). AR is also found in Atlantic cod (*Gadus morhua*), (LaLone et al., 2013) (Marta Eide et al., 2018). 11-ketotestosterone is an important androgen in fish (Kortner, Rocha, & Arukwe, 2009) which is also found to activate human AR *in vitro* (Imamichi et al., 2016).

In addition to natural ligands, AR in various species can be activated by synthetic compounds (see Appendix, Table 1) such as the flame retardant TBECH (Pradhan, Kharlyngdoh, Asnake, & Olsson,

2013) and the pharmaceutical dexamethasone (Y. Park, Park, & Lee, 2021). Synthetic compounds known to inhibit AR (Appendix, Table 2) includes pharmaceuticals such as flutamide (Martinović-Weigelt et al., 2011) and the plastic additives bisphenol A, S and F (Ekman et al., 2012; C. Park et al., 2020) Although AR is regarded as well conserved across species, differences can be observed in binding affinities for certain compounds (Wilson, Cardon, Gray Jr., & Hartig, 2007; Wu et al., 2010).

1.2.2 The estrogen receptor (ER)

The estrogen receptors (ER, NR3A1, NR3A2) are steroid hormone receptors and members of the NR3 subfamily of nuclear receptors. The ER binds estrogens like 17β-estradiol (E2) and the synthetic estrogen ethynylestradiol (EE2) (Figure 1.6). Many estrogens, such as estradiol, are lipophilic and protein-bound in the blood. This means that only when disassociating from these proteins enables them to diffuse easily through the cell membrane (Mueller & Korach 2005). The unliganded estrogen receptor is located as a monomer in the cytoplasm and bound to heat-shock proteins. Upon binding of a ligand, the heat-shock proteins are displaced, and the ER creates homodimers before binding the estrogen responsive elements on the DNA in the cell nucleus. This results in the transcription of the specific target gene mRNAs, which ultimately leads to translation into the corresponding proteins (Mueller & Korach, 2005).



A B C

Figure 1.6 2D structure of ER ligands. 17β-estradiol/E2 (A) (PubChem CID 5757), Ethynylestradiol/EE2 (B) (PubChem CID5991), tamoxifen (antagonist) (C) (PubChem CID 2733526).

Estrogens and the estrogen receptors are essential in development and fertility. Research done on rats and mice indicates that a disruption in ERα causes infertility in both males and females, whilst disruption in ERβ causes infertility in females only (Rumi et al., 2017). The pS2/TFF1 (trefoil factor 1 protein, present in gastrointestinal mucosa) gene was one of the first genes demonstrated to be controlled by estrogen at a transcriptional level (Brown, Jeltsch, Roberts, & Chambon, 1984). Other well-known target genes of ERα are cathepsin D (CATD), and c-Myc (Shang, Hu, Direnzo, Lazar, & Brown, 2000). C-myc is of particular interest as it is regarded an oncogene (cancer-promoting) which stimulates both cell cycle progression and proliferation (Wang et al., 2011).

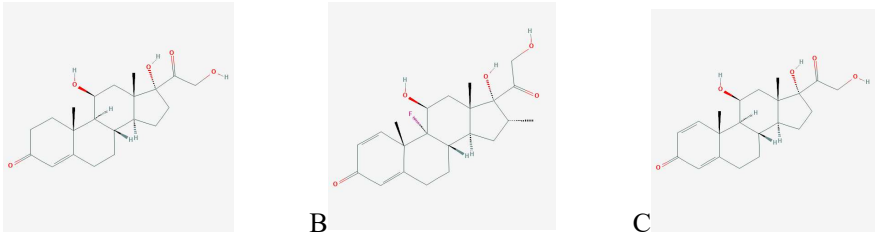
Estrogen receptors have been identified in a multitude of vertebrates such as human (Green et al., 1986), rat (Koike, Sakai, & Muramatsu, 1987), and Atlantic cod (Goksøyr, Sørensen, Grøsvik, Pampaning, Goksøyr & Karlsen, 2021). The receptors have also been found in some invertebrates such as molluscs (Hultin et al., 2014), annelids (Keay & Thornton, 2009), and rotifers (Jones et al., 2017) (Cuvillier-Hot & Lenoir, 2020). In Atlantic cod, three estrogen receptors have been identified, cod ERα (NR3A1), cod Erβ-I (NR3A2), and cod Erβ-II (NR3A3) (Nagasawa, Presslauer, Kirtiklis, Babiak, & Fernandes, 2014).

ER activation by synthetic estrogens in fish is closely linked to the expression of vitellogenin (Vtg), which is the precursor for yolk proteins in oviparous vertebrates (Oppen-Berntsen, Hyllner, Haux, Helvik, & Walther, 1992), making it a suitable indicator of environmental estrogen exposure in male fishes. Synthetic estrogens known to activate ER (in various species) includes the makeup and plastic additive benzophenone (Schreurs, Sonneveld, Jansen, Seinen, & van der Burg, 2005), the UV-filter octocrylene (Kunz & Fent, 2006b) and the synthetic hormone EE2 (Notch & Mayer, 2011). Compounds known to inhibit ER includes the antibacterial and antifungal compound triclosan (Ahn et al., 2008), the

pharmaceutical tamoxifen (Miksicek, 1994), and the polycyclic musk AHTN (Schreurs et al., 2005), among others (Appendix, Table 3 & 4).

1.2.2 The glucocorticoid receptor (GR)

The third, and last, steroid hormone receptor covered by this thesis is the glucocorticoid receptor (GR, NR3C1). GR binds glucocorticoids synthesised by the adrenal cortex which are involved in functions such as metabolism, growth, and development. The receptor is expressed in almost all vertebrate cells (Weikum et al., 2017). Glucocorticoids such as dexamethasone and prednisolone (Figure 1.7) are also used clinically to treat inflammation and autoimmune diseases in humans. Just like the other nuclear receptors, the GR has the same structural domains and is associated with chaperone proteins such as hsp70 and hsp90 (McEwan & Kumar, 2015). Upon ligand binding the GR undergoes a conformational change, separation from its chaperones and translocation to the cell nucleus where it binds the glucocorticoid response elements (GREs). This either leads to an activation or repression of target genes.



A B C

Figure 1.7 2D structure of GR ligands. Cortisol (A) (PubChem CID 5754), Dexamethasone (B) (PubChem CID 5743), Prednisolone (C) (PubChem CID 5755).

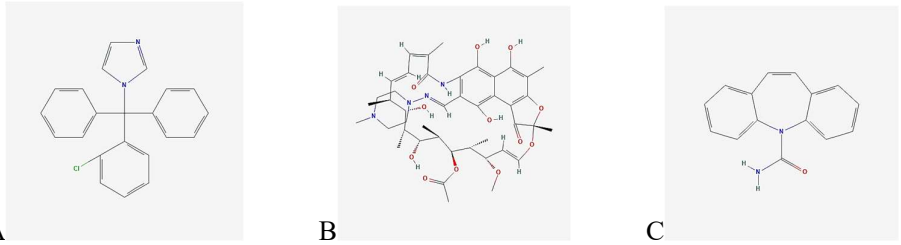
The target genes of the GR includes (among others) genes involved in antiinflammation (Wang et al., 2004), and genes regulating circadian rhythm. This was shown by an increased transcription level of 10 known core clock components following dexamethasone exposure in mice (So, Bernal, Pillsbury, Yamamoto, & Feldman, 2009). The glucocorticoid receptor has been identified in a multitude of vertebrates such as humans (Hollenberg et al., 1985; Rupprecht et al., 1993), rats (Funder, Feldman, & Edelman, 1973), and teleosts, including Atlantic cod (Hori, Rise, Johnson, Afonso, & Gamperl, 2012; Kleppe et al., 2013).

The GR has in recent years gained increased attention as a possible target for EDCs (Zhang, Yang, Liu, Schlenk, & Liu, 2019). The EDCs bisphenol A, dicyclohexyl phthalate, endrin (pesticide) and totylfluanid (fungicide) has been identified as activators of human GR *in vitro* (Sargis, Johnson, Choudhury, & Brady, 2010). Certain members of the flame retardant class polybrominated diphenyl ethers (PBDEs) have also been shown to be antagonists in human GR (Zhang, Wang, Zhu, Liu, & Zhao, 2017) (Appendix, Table 5.).

1.2.4 The pregnane X receptor (PXR)

The pregnane X receptor (PXR, NR1I2) is a nuclear receptor belonging to the type II nuclear receptors. Upon ligand binding it goes through conformational change and forms heterodimers with the retinoid X receptor (RXR). The PXR is found in the nucleus where co-repressors are exchanged with co-activators upon ligand-binding. When bound, the PXR can induce expression of a number of genes responsible for metabolization, oxidation, conjugation and transport of both xenobiotics and endogenous substances (Kliewer, Goodwin, & Willson, 2002). This is important as the receptor may bind both xenobiotics as well as possible harmful endogenous compounds such as bile acids (Kliewer et al., 2002). More specifically, enzymes induced through PXR activation include CYP2B, 2C and 3A (Klaassen & Watkins, 2015). The role of CYPs in metabolization of xenobiotics such as pharmaceuticals is described in more detail in part 1.1.1 Pharmaceuticals.

Due to the structurally wide range of ligands (Appendix, Table 6), the receptor is also commonly referred to as a promiscuous receptor. PXR agonists include compounds such as the plastic additive bisphenol A (Takeshita et al., 2001), the pharmaceuticals clotrimazole, carbamazepine (Lille-Langøy et al., 2015) and dexamethasone (Pacussi, Drocourt, Fabre, Maurel & Vilarem, 2000), and the antibiotic rifampicin (Lille-Langøy et al., 2015) (Figure 1.8), just to mention a few. Although multiple PXR agonists are known, very few antagonists have been reported, with the fungicide ketoconazole being the most researched one (H. Huang et al., 2007; Takeshita, Taguchi, Koibuchi, & Ozawa, 2002).



A B C

Figure 1.8 2D structure of some known PXR ligands. Clotrimazole (A) (PubChem CID 2812), Rifampicin (B) (PubChem CID 135398735), Carbemazepine (C) (PubChem CID 2554)

The PXR has been identified in humans (Kliewer et al., 1998), and numerous vertebrates such as zebrafish (Bainy et al., 2013), pigs (Moore et al., 2002), and polar bears (Lille-Langøy et al., 2015).

However, studies have indicated significant cross-species variations in PXR ligand specificity, especially when comparing mammalian PXRs to avian and zebrafish PXR (Ekins, Reschly, Hagey, & Krasowski, 2008; Krasowski, Ni, Hagey, & Ekins, 2011). Additionally, research on mammalian PXR has found key differences between human and mouse PXR and the agonistic activities of 200 pesticides by using *in vitro* reporter gene assays (Kojima, Sata, Takeuchi, Sueyoshi, & Nagai, 2011). Even within the same species, variations have been found, as those identified in zebrafish by Lille-Langøy et al., 2019. Interestingly, the PXR has been found to be present in e. g. zebrafish, but absent in Atlantic cod, and several other teleosts (such as sticklebacks) belonging to the Gadiformes order (Marta Eide et al., 2018).

1.3 Bioassays

The first manifestations of toxicity are the cellular responses (Fent 2001), and these can be investigated with bioassays. By using bioassays, ligand–receptor interactions can also be studied, and provide key knowledge on the first step in the toxicological pathway, the MIE. Additionally, bioassays can be used to measure the biological activity of complex mixtures of both known and unknown chemicals, even at low concentrations (Leusch et al. 2018). Bioassays may also contribute with knowledge on the potency of a contaminant or mixture of contaminants, which is particularly important as the most abundant contaminants are not necessarily the most biologically active ones (Brack et al. 2005). By increasing the use of *in vitro* (cells or tissue outside the living organism) methods, it could result in a decreased use of bioindicator organisms in environmental monitoring (Pérez-Albaladejo et al. 2016) and thereby contribute positively to the 3R principle of replacement, reduction, and refinement of animal testing. The luciferase reporter gene assay (LRA) is a well-established method used to investigate molecular interactions in a cell.

1.3.1 Luciferase reporter gene assay (LRA)

Luciferase reporter gene assay (LRA) is one commonly applied bioassay-principle in environmental toxicology as it can be utilized to determine if a substance can activate or inhibit a certain receptor. The assay is based on luciferase, which is an enzyme originally used for bioluminescence in fireflies (Brasier,

Tate, & Habener, 1989). Cells are cultivated and transfected with both receptor plasmids and a reporter plasmid containing the luciferase gene. When an agonistic ligand binds to the nuclear receptor investigated, a conformational change will occur, activating the transcription of the luciferase gene, and ultimately translation of the enzyme luciferase. Luciferase oxidates luciferin into oxyluciferin which is a process creating a light signal, which can be quantified (a detailed description can be found in Methods 3.2.3). The assay has been used in research on numerous nuclear receptors from various species, such as human AR (Ermler, Scholze, Kortenkamp, 2011), cod ARα and cod ERα (Goksøyr, Sørensen, Grøsvik, Pampaning, Goksøyr , Karlsen, 2021) and zebrafish PXR (Lille-Langøy et al., 2019). Ultimately, the LRA can give us an indication about whether a particular receptor could be activated or antagonized by a compound or mixture of compounds.

1.3.2 Zebrafish and Atlantic cod as model species

Zebrafish (*Danio rerio*) is a small freshwater fish species belonging to the family Cyprinidae, and is native to waters in India, North Pakistan, Nepal, Bhutan and South Asia (Jagdale, Hude, & Chabukswar, 2020). It is frequently used as an animal model and is popular in both toxicology and biomedical research. Initially, its popularity was due to its small size, fast development, short generation time, their embryonic and larval transparency, and rapid hatching time (Chakraborty, Sharma, Sharma, & Lee, 2016). As more research has been done on this species, its genome has been sequenced, and several important physiological homologies with humans have been identified (Jagdale et al., 2020)(Hsu, Chi-Hsin, Wen, Zhi-Hong, Lin, Cha-Shing, Chakraborty, 2007).

Atlantic cod (*Gadus morhua*) is a marine teleost with a wide distribution in the North Atlantic Ocean and the Barents Sea (Figure 1.9). Due to its presence and position in the coastal marine food chain and ecosystem, it is regarded both as an important species ecologically, but also as an indicator species in pollution monitoring programs and in the Protection of the Marine Environment of the North-East Atlantic (OSPAR) convention (OSPAR Commission, 2009). The genome of the Atlantic cod was first sequenced and annotated in 2011 (Star et al., 2011) and new and updated genome assemblies have been added later. In recent years the species has been used as an indicator species when investigating biological consequences of produced water exposure (Sundt et al., 2012), capped waste disposal sites (Dale et al., 2019) and contaminated sediments (Goksøyr et al. 2021).

Figure 1.9 Distribution of Atlantic cod (red). Illustration from FAO (2021).

1.4 Pharmaceuticals as possible environmental stressors

Norway has the second longest coastline in the world, with a total length of 100,915 km (Regjeringen, 2015). Along this coastline, and by some freshwater lakes and rivers, more than 2500 municipal wastewater treatment plants (WWTP) are located. A majority of these were built between 1970 – 1985, and the treatment goal is removal of organic matter, and not necessarily contaminants. Additionally, 500 planned treatment plants are not yet built (Norwegian Environmental Agency, 2021). This means that

for about 350,000 people in Norway, wastewater is released untreated into the environment. The general rule is also that ships may release untreated sewage 300 m from land (except some stricter rules in certain fjords and highly trafficked areas) (Norwegian Maritime Authority, 2016), further contributing to the release of wastewater into Norwegian waters. As previously mentioned, pharmaceutical usage is on the rise, and an inevitable consequence of this is higher levels of pharmaceuticals being discharged into the environment (Corcoran, Winter, & Tyler, 2010). Even for pharmaceuticals regarded as easily degradable, it can be argued that their continuous input into the environment makes them "pseudo persistent" (Trombini, Hampel, & Blasco, 2019). With this, more knowledge on possible environmental consequences is needed.

The topic has received increased attention the past decades, with research demonstrating outcomes such as collapse in a fish population following synthetic estrogen exposure (Kidd et al., 2007), and androgens in wastewater causing masculinization in female mosquitofish (Huang et al., 2019). Extensive reviews have also reported on an increase in research in the field, but still a general lack of knowledge on pharmaceutical mixtures and ecotoxicological impacts (Mezzelani, Gorbi, & Regoli, 2018).

Zebrafish PXR, cod ARα, and cod ERα have all previously been utilized to assess the presence of contaminants in several places, including in Bergen, Norway. Human and zebrafish nuclear receptors have also been used in *in vitro* bioassays on wastewater and surface water extracts (Neale, Grimaldi, Boulahtouf, Leusch, & Balaguer, 2020) where wastewater-based chemicals such as pharmaceuticals have been found to have a higher affinity to zebrafish PXR compared to human PXR (Neale, Grimaldi, et al., 2020). Research on Norwegian WWTPs using these receptors specifically has not yet been reported.

1.5 Aim of the study

This master thesis aims to investigate the utility of a set of receptor-based *in vitro* bioassays in detecting and monitoring the potency of pharmaceuticals released from WWTPs in Bergen, Norway. Additionally, the study aims at evaluating potential differences in wastewater treatment methods with respect to the efficient removal of pharmaceuticals.

The receptors chosen are from both zebrafish (PXR) and Atlantic cod (ARα, ERα and GR). These receptors are important in a wide array of biological processes, such as the biotransformation of xenobiotics for PXR, development and reproduction for ERα and ARα, and growth and immune responses for GR. This means that any receptor activation or inhibition detected also identifies a molecular initiating event (MIE) with potential adverse biological impacts.

In order to investigate this, the following experiments were planned:

- Extraction and analysis of pharmaceuticals and personal care products in wastewater and sludge samples from five different WWTPs in Bergen (both before and after treatment)
- Investigation of the cytotoxic properties of the extracts using a cell viability assay
- Utilizing luciferase gene reporter assays *in vitro* to investigate whether PPCPs or other compounds present in wastewater or sludge samples may possess agonistic or antagonistic effects on the following receptors:
    - Zebrafish PXR-TL
    - Atlantic cod ARα
    - Atlantic cod ERα
    - Atlantic cod GR

2. Materials

2.1 Chemicals

Table 2.1. Overview of used chemicals with chemical formula and supplier/product number

Name	Chemical formula	Supplier/product number
10x loading buffer		TaKaRa/9157
2-log DNA ladder		New England Biolabs
2-β mercaptoethanol	C_2H_6OS	Sigma-Aldrich/M6250
5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester (5-CFDA, AM)	$C_{25}H_{16}O_9$	Thermo Fischer Scientific/C1354
Adenosine 5′-triphosphate disodium salt trihydrate (ATP)	$C_{10}H_{20}N_5Na_2O_{16}P_3$	Sigma-Aldrich/A2383
Agar-agar		Merch Millipore/101614
Agarose		Sigma-Aldrich/A9539
Ampicillin sodium salt	$C_{16}H_{18}N_3NaO_4S$	Sigma-Aldrich/A9518
Boric acid	H_3BO_3	Merch Millipore/100165
Bovine serum albumin (BSA)		Sigma-Aldrich/A9647
Calcium chloride dihydrate	$CaCl_2 \cdot 2H_2O$	Merch Millipore/102382
CHAPS	$C_{32}H_{58}N_2O_7S$	Thermo Fisher/28299
Coenzyme A trilithium salt	$C_{21}H_{33}Li_3N_7O_{16}P_3S$	Sigma-Aldrich/C3019
Dimethyl sulfoxide (DMSO)	$(CH_3)_2SO$	Sigma-Aldrich/D8418
Disodium hydrogen phosphate dihydrate	$Na_2HPO_4 \cdot 2H_2O$	Sigma-Aldrich/30435-M
DL-Dithiothreitol (DTT)	$HSCH_2CH(OH)CH(OH)CH_2SH$	Sigma-Aldrich/D0632
D-luciferin Firefly	$C_{11}H_8N_2O_3S_2$	Biosynth/L-8200
Dulbecco's Modified Eagle medium (with phenol red)		Sigma-Aldrich/D5671
Dulbecco's Modified Eagle medium (without phenol red)		Sigma-Aldrich/D1145
Erythrosine B	$C_{20}H_6I_4Na_2O_5$	Sigma-Aldrich/200964
Ethanol	CH_3CH_2OH	Sigma-Aldrich/34852
Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA)	$[-CH_2OCH_2CH_2N(CH_2CO_2H)_2]_2$	Sigma-Aldrich/E3889
Ethylenediaminetetraacetic acid disodium salt (EDTA)	$C_{10}H_{14}N_2Na_2O_8$	Merch Millipore/108418
Fetal bovine serum (FBS)		Sigma-Aldrich/F7524
Galactose	$C_6H_{12}O_6$	
GelRed ®	$C_{60}H_{72}I_2N_8O_5$	Biotium/41003
Glycerol	$HOCH_2CH(OH)CH_2OH$	Sigma-Aldrich/G5516
Isopropanol	$(CH_3)_2CHOH$	Kemetyl/603-117-00-0
L-α-phosphatitylcholine		Sigma-Aldrich/P3644
L-glutamine	$H_2NCOCH_2CH_2CH(NH_2)CO_2H$	Sigma-Aldrich/G7513
Magnesium carbonate hydroxide pentahydrate	$(MgCO_3)_4 \cdot Mg(OH)_2 \cdot 5H_2O$	Sigma-Aldrich/G7513
Magnesium chloride	$MgCl_2$	Sigma-Aldrich/M8266
Magnesium chloride hexahydrate	$MgCl_2 \cdot 6H_2O$	Sigma-Aldrich/M9272
Magnesium sulfate	$MgSO_4$	Sigma-Aldrich/M2643
Magnesium sulfate heptahydrate	$MgSO_4 \cdot 7H_2O$	Sigma-Aldrich/63145
Methanol	CH_3OH	Sigma-Aldrich/322415
o-Nitrophenyl β-D-galactopyranoside (ONPG)	$C_{12}H_{15}NO_8$	Sigma-Aldrich/N1127

Opti-MEM ® Reduced Serum Medium		Life Technologies/31985-062
Penicillin-Streptomycin		Sigma-Aldrich/P4458
Phenylmethanesulfonylfluoride (PMSF)	$C_7H_7FO_2S$	Sigma-Aldrich/P7626
Phosphate buffered saline (PBS)		Sigma-Aldrich/P5493
Potassium Chloride	KCl	Sigma-Aldrich/C3019
Potassium Phosphate monobasic	KH_2PO_4	Merch Millipore/529568
Resazurine sodiumsalt	$C_{12}H_6NNaO_4$	Sigma-Aldrich/R7017
Sodium chloride	NaCl	Merch Millipore/106404
Sodium dihydrogen phosphate monohydrate	$NaH_2PO_4 \cdot H_2O$	Merch Millipore/106346
Sodium pyruvate	$C_3H_3NaO_3$	Sigma-Aldrich/S8636
Super Optimal broth with catabolite repression (SOC)		New England Biolabs/B9020S
TransIT®-LT1		Mirus/MIR2300
Tricine	$(HOCH_2)_3CNHCH_2CO_2H$	Sigma-Aldrich/T0377
Triton™ X-100	*t*-Oct-C_6H_4-$(OCH_2CH_2)_xOH$	Sigma-Aldrich/X100
Trizma ® base	$NH_2C(CH_2OH)_3$	Sigma-Aldric/T1503
Trizma® phosphate dibasic	$(C_4H_{11}NO_3)_2 \cdot H_3PO_4$	Sigma-Aldrich/T4258
Trypsin-EDTA solution 1x (0,05% trypsin, 0,02% EDTA)		Sigma-Aldrich/59417c
Trypton		Merch Millipore/111931
Yeast extract		Fluka/92144

2.2 Plasmid preparation

2.2.1 Commercial kits

Table 2.2 Kit used for plasmid preparation

Name	Usage	Supplier
NucleoBond© plasmid purification kit	Plasmid prep (Midi-prep)	Macherey-Nagel

2.2.2 Prepared plasmids

Table 2.3 Overview of plasmids

Plasmid		Reporter gene system
(MH100)x4 tk luc	Reporter plasmid	Gal4/UAS-based
pCMV-β-Gal	Control plasmid	
pCMX-Gal4-ARα (cod)	Receptor plasmid	
pCMX-Gal4-ERα (cod)	Receptor plasmid	
pCMX-Gal4-GR (cod)	Receptor plasmid	
pCMX-Gal4-PXR-TL (zebrafish)	Receptor plasmid	

2.3 Cell lines

Table 2.4 Overview of cell lines

Cell lines	Description
StrataClone SoloPack competent cells	*Escherichia coli* (procaryote)
COS-7-cells	African green monkey kidney cells (eucaryote)

2.4 Cell growth media

Table 2.5 Lysogeny broth (LB) medium

Components	LB medium (concentration)	LB-agar (concentration)
Agar-agar	-	15 g/l
Ampicillin*	-	100 mg/l
MilliQ water	-	-
Sodium chloride	10 g/l	10 g/l
Tryptone	10 g/l	10 g/l
Yeast extract	5 g/l	5 g/l

Solution autoclaved

* Added post autoclavation

Table 2.6 Cell freezing media for COS-7 cells

Component	Concentration
Dulbecco's modified Eagle medium (DMEM) w/phenol red	1X
Fetal bovine serum (FBS)	10%
L-glutamine	4 mM
Sodium pyruvate	1 mM
Penicillin-Streptomycin	100 u/mL
Dimethyl sulfoxide (DMSO)	5%

Table 2.7 Growth media for cultivation of COS-7 cells

Component	Concentration
Dulbecco's modified Eagle medium (DMEM)*	1X
Fetal bovine serum (FBS)**	10%
L-glutamine	4 mM
Sodium pyruvate	1 mM
Penicillin-Streptomycin	100 u/mL

*DMEM w/phenol red used for growth media, DMEM w/o phenol red used during exposure

** Charcoal stripped FBS in exposure media

2.5 Buffers and solutions

2.5.1 Agarose gel electrophoresis

Table 2.8 TBE buffer (tris borate EDTA) 5X

Components	Concentration
Trizma base	0.45 M
Boric acid	0.45 M
EDTA	0.01 M
H_20	-

Table 2.9 TBE agarose gel

Component	Concentration
TBE buffer (Table 2.8)	0.5 X
Agarose	0.70 %
Gel Red ®	0.0002%

2.5.2 Solutions for luciferase reporter gene assay ligand activation

Table 2.10 Cell lysis base buffer (1X)

Component	Concentration
Tris-PO_4 pH 7,8	25 mM
Glycerol	15%
CHAPS	2%
L-a-phosphatidylcholine	1%
BSA	1%

Table 2.11 Cell lysis reagent solution

Component	Concentration
Lysis base buffer (Table 2.10)	1 X
EGTA	4 mM
$MgCl_2$	8 mM
PMSF	0.4 mM
DTT	1 mM

Table 2.12 β-galactosidase base buffer (10X)

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

Table 2.13 β-galactosidase reagent solution

Component	Concentration
β-galactosidase buffer 1X (Table 2.12)	1 X
β-mercaptoethanol	52.9 mM
ONPG	8.6 mM

Table 2.14 Luciferase base buffer (4X, pH 7.8)

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

Table 2.15 Luciferase reagent solution

Component	Concentration
Luciferase base buffer (Table 2.14)	1 X
ATP	0.5 mM
DTT	5 mM
CoA*	0.2 mM
D-luciferin*	0.5 mM

* Added immediately before use

2.5.3 Cell viability

Table 2.16 L-15/ex Solution 1

Component	Concentration
NaCl	133.33 g/l
KCl	6.67 g/l
$MgSO_4 \cdot 7H_2O$	3.33 g/l
$MgCl_6 \cdot 6H_2O$	3.33 g/l
H_2O	-

Table 2.17 L-15/ex Solution 2

Component	Concentration
$CaCl_2$	1.4 g
Deionized-H_2O	100 mL

Table 2.18 L-15/ex Solution 3

Component	Concentration
Na_2HPO_4	1.9 g
KH_2PO_4	0.6 g
Di-H_2O	300 mL

Table 2.19 L-15/ex solution for cell viability measurement

Component	Concentration
Solution 1 (Table 2.16)	34 mL
Solution 2 (Table 2.17)	6 mL
Solution 3 (Table 2.18)	17 mL
Galactose 90	0.8 mL
Pyruvate	0.5 mL
Di-H_20	500 mL

Table 2.20 Resazurin and CFDA-AM viability solution

Component	Concentration
L-15/ex (Table 2.19)	1 X
Resazurin	0.03 mg/mL
CFDA-AM	0.001 mM

2.6 Instruments

Table 2.21 Instruments used

Instrument	Application	Supplier
Bürker haemocytometer	Counting COS-7 cells	Marienfield
ChemiDOC™ XRS+ System	Gel scanning	Bio-Rad
EnSpire 2300 Multimode Reader	Plate reader	PerkinElmer
HS 501 Digital	Platform shaker	IKA-Werke
NanoDrop 1000	Spectrophotometer	Thermo Scientific
PowerPac™ HC	Power supply	Bio_Rad
Aeros multifuse X3R	Centrifuge	Thermo Scientific
Panasonic mco-170aicuv-pe	Incubator	Lab-Tec
ClearAir EuroFlow Class II	Biosafety cabinet	Baker

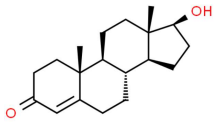
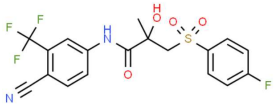
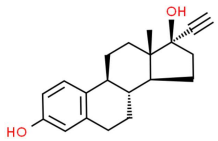
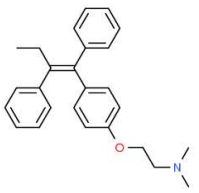
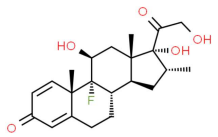
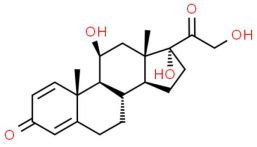
2.7 Software

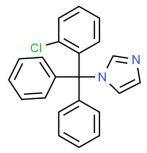
Table 2.22 Software used

Software	Application	Supplier
EnSpire Manager	Plate reader operation	PerkinElmer
Excel 2020	Data analysis and statistics	Microsoft
GraphPad Prism 7	Figures and statistics	GraphPad
Mendeley	Literature citation	Elsevier
PowerPoint 2020	Processing of figures	Microsoft

2.8 Ligands used in luciferase reporter gene assay

Table 2.23 Ligands used in LRA. All structures from chemspider.com

Ligand name	Receptor interaction	Molecular formula	Structure
Testosterone	Cod ARα agonist	$C_{19}H_{28}O_2$	
Bicalutamide	Cod ARα antagonist	$C_{18}H_{14}F_4N_2O_4S$	
EE2	Cod ERα agonist	$C_{20}H_{24}O_2$	
Tamoxifen	Cod ERα antagonist	$C_{26}H_{29}NO$	
Dexamethasone	Cod GR agonist	$C_{22}H_{29}FO_5$	
Prednisolone	Cod GR agonist	$C_{21}H_{28}O_5$	

Clotrimazole	Zebrafish PXR-TL agonist	$C_{22}H_{17}ClN_2$	

3. Methods

3.0 Experimental outline

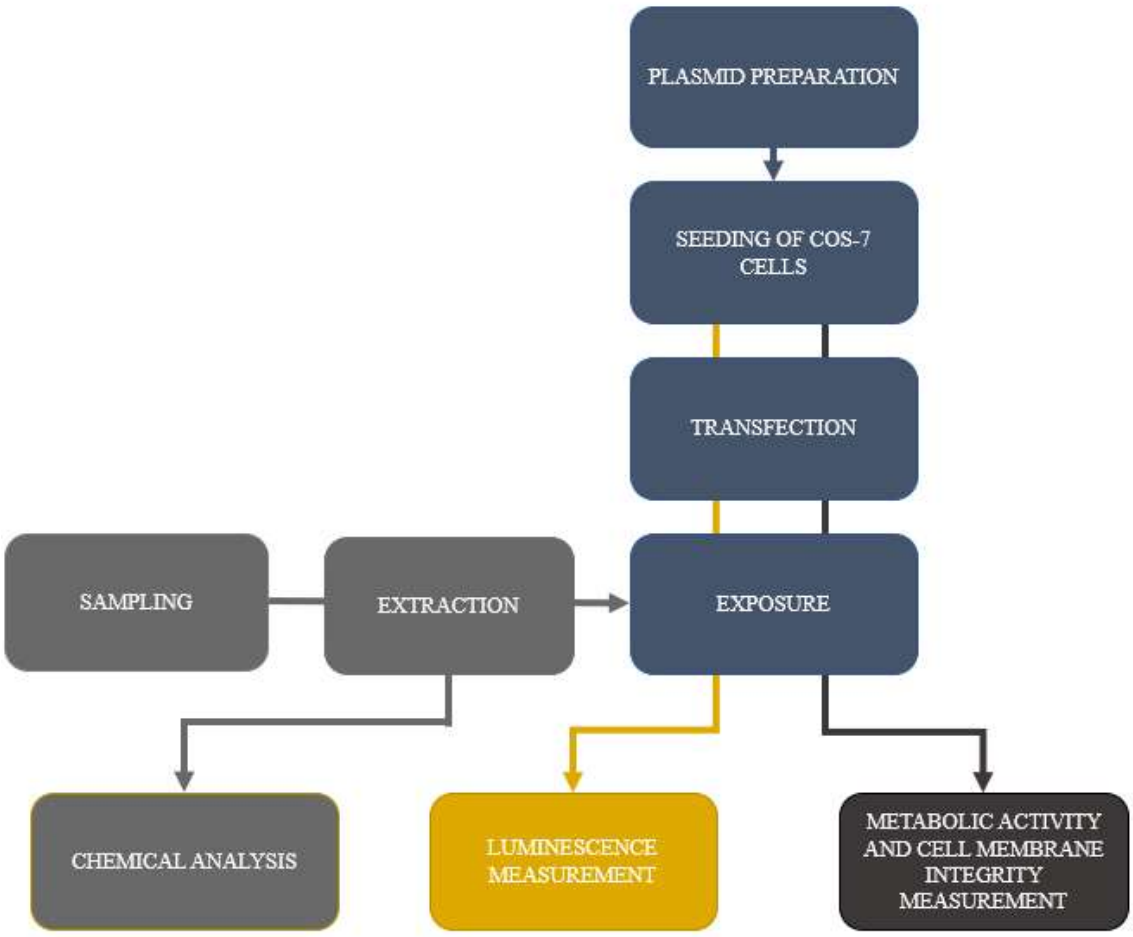


Figure 3.1 Experimental outline. Samples were taken from five different wastewater treatment plants in Bergen, Norway, and extracted by Ivo Havranek, postdoc at NMBU by using Oasis HLB-disks. Plasmids, including AR, ER and PXR, were prepared and transfected into COS-7 cells seeded in 96-well plates. The transfected COS-7 cells were exposed to wastewater or sludge extracts for 24 hours prior to luciferase activity measurement, or cytotoxicity measurement. Metabolic activity and cell membrane integrity were used as indicators of cytotoxicity.

3.1 Collection and treatment of samples

3.1.1 Wastewater samples

Samples (2 litres) were taken before and after treatment for the five treatment plants (Table 3.2). The sampling was based on a 24-hour accredited method conducted by trained and contracted personnel from BergenVann. For 24-hour accredited sampling a multitude of samples are taken from the wastewater stream in a set interval for 24 hours. These samples are continuously added to a pool, which constitutes a representative fraction of the wastewater stream for the past 24 hours. The room in which the pool is stored is kept at < 5°C. All samples are accredited according to this method, except samples taken from Kvernevik due to slightly increased room temperatures (5 – 7 °C) during sampling. This is not expected to influence the results.

All samples before treatment (influents, named station name + in) are completely unprocessed sewage and wastewater (Stage 1, Figure 3.3 - 3.5), whilst samples taken after treatment (effluents, named station name + out) have been treated according to the respective treatments at the given WWTP (Stage 7, Figure 3.3 – 3.5). Kvernevik WWTP, Ytre Sandviken WWTP, Holen WWTP, and Knappen WWTP were all 24-hour samples collected 07.10.2020. Flesland WWTP was 24-hour sample collected 26.10.2020. Samples were kept cold and dark in methanol-washed Nalgene™ Wide-Mouth Lab Quality HDPE Bottles and frozen at -20 °C within 6 hours.

3.1.2 Sludge samples

Sludge samples (2-4 litres) (named station number + sludge) were taken from a pool containing an average of the past month's produced sludge, and the samples are consequently representative of the average for the previous month. All sludge samples represent their stations' sludge average during December 2020. Samples were kept cold and dark in methanol-washed Nalgene™ Wide-Mouth Lab Quality HDPE Bottles and frozen at -20 °C within 6 hours.

3.1.3 Oasis HLB disk extraction and high-performance liquid chromatography (HPLC)

Disk extraction, High Performance Liquid Chromatography (HLPC), and Electrospray ionization Mass spectrometry, was performed by Ivo Havranek, postdoctoral fellow, Faculty of Chemistry, Biotechnology and Food Science at Norwegian University of Life Sciences (NMBU).

Disk extraction. Frozen samples were thawed at room temperature for about 24 hours, shaken thoroughly and a homogenous aliquot of 500 mL for each sample was taken and transferred to a clean 500 mL reagent glass. Oasis HLB columns (6 cc, 500 mg Sorbent per Cartridge, 60 µm) were conditioned with 6 mL MeOH followed by 6 ml Milli-Q H_2O. Internal standards (CBZ d10 – 7.5 ng per 500 mL sample, DEET d10, Metoprolol d7, and Octocrylene d15 on 50 ng per 500 mL sample) and water samples were added. Samples were run through HLB columns at 1-3 droplets/second using a vacuum manifold. Columns were washed with 6 mL 5% MeOH in Milli-Q water. The analytes were eluted with 5 mL 100 % MeOH followed by 5 mL CH_3CN into 12 mL glass tubes. The elutes were evaporated completely using nitrogen and 37 ± 3°C. 1 mL 20% MeOH in Milli-Q water was added to each dried sample and shaken at vortex for 10 seconds. Samples were then added to a microcentrifuge (Costar® Spin-X® Centrifuge Tube Filters, 0.22 µm Pore CA Membrane), and centrifuged at 12 000 rpm for 3 minutes, before being transferred to LC-MS glass with screw caps.

High Performance Liquid chromatography (HPLC). The targeted analysis was performed on an Agilent 1200 series HPLC (Agilent Technologies, Waldbronn, Germany). A Zorbax Eclipse plus C_{18} RRHD (2.1 x 100 mm, 1.8 µm) (Agilent, Palo Alto, USA) with a respective Guard Cartridge (4 µm x 3.0 mm

ID) (Zorbax, Agilent, Palo Alto, USA) was used for chromatographic divisions, whilst the column temperature was kept at 25 ˚C. 10 µL was used as the injection volume. Separations were achieved applying a binary gradient with mobile phase containing of H_2O with 0.1% formic acid (A) and pure CH_3CN (B) with a mobile phase flow rate of 0.35 mL/min (v:v). The initial mobile phase proportion was 100 % (A). B was then linearly increased to 100 % over 8 minutes and kept for 7 minutes. Initial mobile phase conditions were restored over 1.0 minute and the column was equilibrating for 4 minutes resulting in an overall run time of 20 min.

Electrospray ionization Mass spectrometry. An Agilent 6490 (Agilent Technologies, Santa Clara, CA, USA) triple quadrupole mass spectrometer with an Agilent Jet Stream electrospray ion source was used for the detection and quantitative analysis. The ions were monitored in positive and negative dynamic multiple reaction monitoring (dMRM). For instrument control, method validation and quantification, an Agilent MassHunter software (Version B.07.00 /Build 7.0.457.0, 2008) was used.

3.2 The wastewater treatment plants

Samples from WWTPs in this master thesis came from Ytre Sandviken WWTP, Kvernevik WWTP, Holen WWTP, Knappen WWTP and Flesland WWTP, all localized in the Bergen area, Western Norway (Figure 3.2).

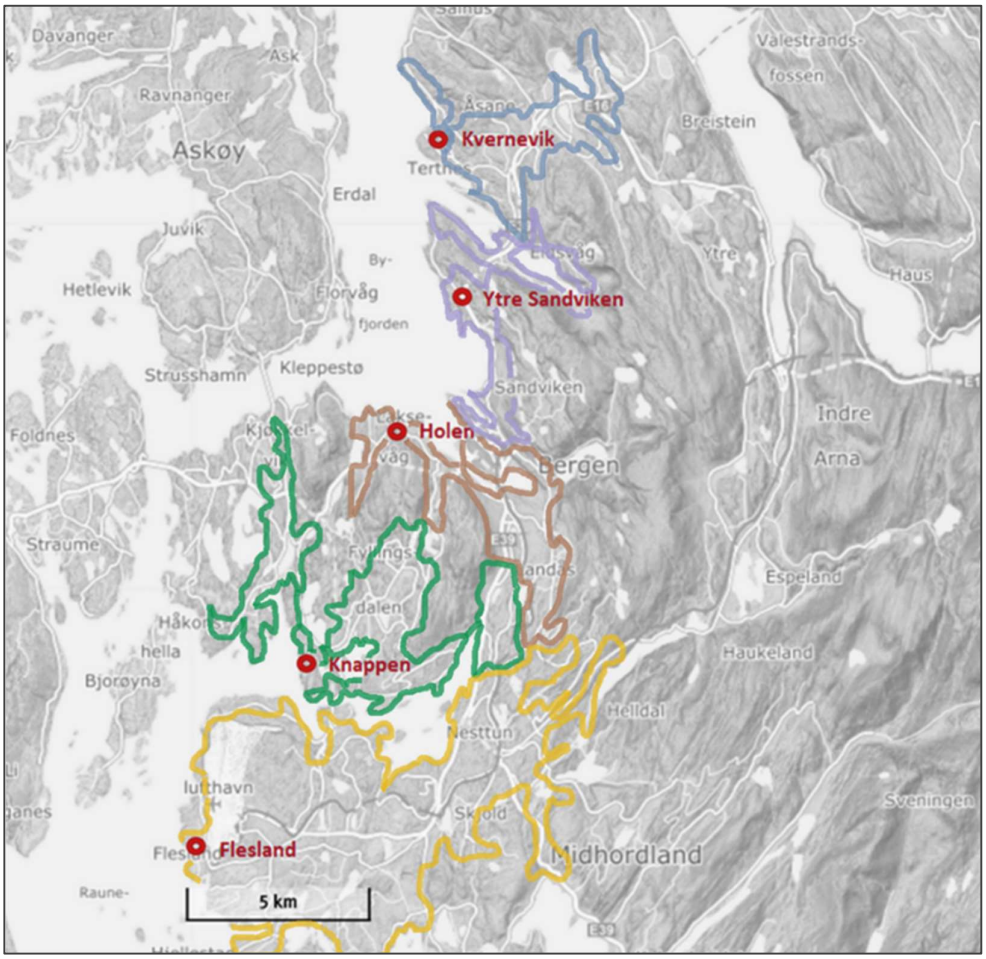


Figure 3.2 Overview of localisation of WWTPs studied. Kvernevik, Ytre Sandviken, Holen, Knappen, Flesland. Blue outlines the approximate collection area for Kvernevik WWTP, purple for Ytre Sandviken, brown for Holen, green for Knappen, and yellow for Flesland. Picture source kartverket.no, with modifications.

3.2.1 Activated sludge-based treatment plants

Kvernevik WWTP is dimensioned for 56,000 population equivalents (pe, defined by the organic biodegradable load having a five-day biochemical oxygen demand (BOD5) of 60 g of oxygen/day (European Commission, n.d.)) and receives wastewater from Haukås, Flaktveit, Tertnes, Ervik and Morvik in Åsane. The treatment process involves a preliminary treatment with a grate, a sand and grease trap and a sedimentation pool based on activated sludge. This is the same system as in Flesland WWTP (Figure 3.3). Flesland WWTP is dimensioned for 152.000 pe and receives wastewater from the Nesttun area to Kokstad, Sandsli, Bergen Airport Flesland, and Blomsterdalen.

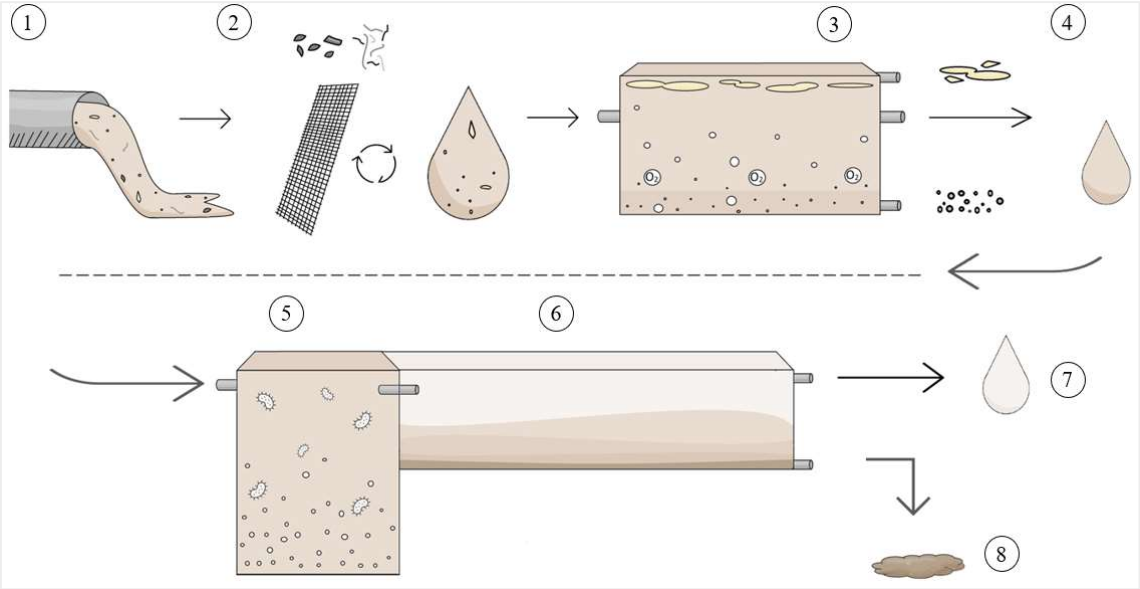


Figure 3.3. Simplified illustration of wastewater treatment in Kvernevik and Flesland WWTPs 1) raw sewage enters the facility, 2) rotating screen (6 mm) traps waste and particles > 6 mm, 3) WW is lead to a sand and grease trap where air is added to facilitate rapid precipitation of sand, while fat floats towards the top and is removed, 4) the WW is directed to the biological treatment unit based on activated sludge, 5) air is injected through the bottom of a deep tank where the WW and activated sludge is present, 6) the mixture ends up in a large sedimentation basin, 7) treated water runs out at the surface of the basin and is released, 8) excess sludge is removed from the bottom of the basin and is added mechanical thickeners and centrifuged before sent to a biogas facility. Illustration based on information from Bergen municipality.

3.2.2 MBBR and Actiflo-based treatment plants

Ytre Sandviken WWTP has a capacity of 44,000 pe, and receives wastewater from the area surrounding Vågen, Skuteviken, Sandviken, Breiviken, Biskopshavn, and parts of Eidsvåg. The treatment process involves a preliminary treatment and a secondary treatment based on both a biological (Moving Bed Biofilm Reactor, MBBR) and a chemical step (Actiflo). This is the same treatment method as in Holen WWTP (Figure 3.4). Holen WWTP is dimensioned for 134,000 pe and receives wastewater from the central parts of Bergen city, Laksevåg, Solheimsviken and Landås area.

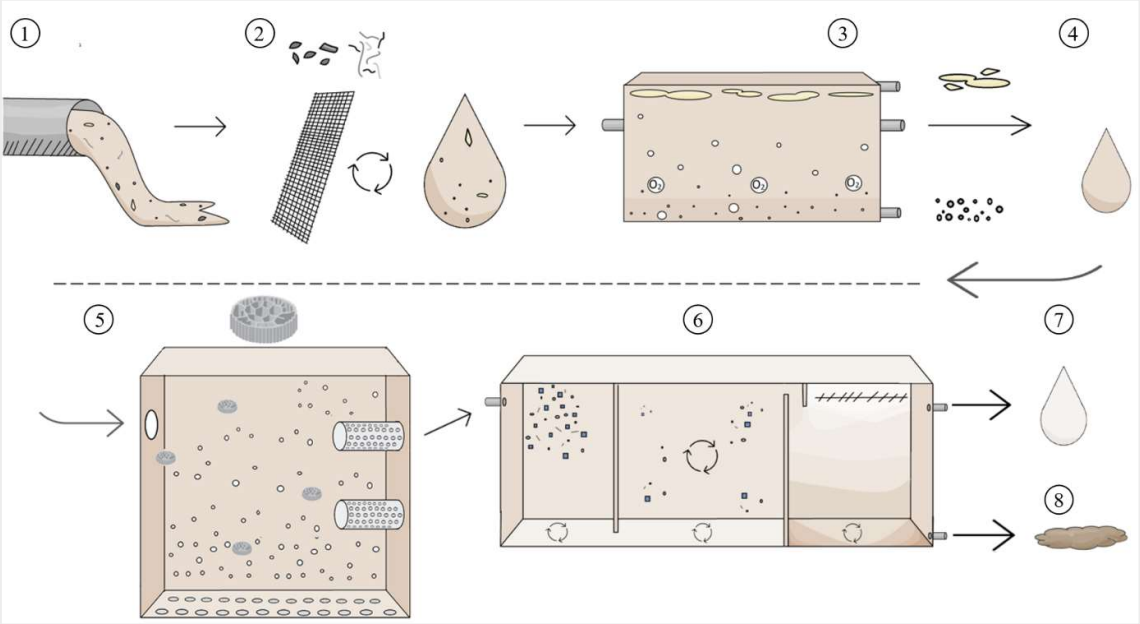


Figure 3.4 Simplified illustration of wastewater treatment in Ytre Sandviken and Holen WWTPs 1) raw sewage enters the facility, 2) rotating screen (6 mm) traps waste and particles > 6 mm, 3) WW is lead to a sand and grease trap where air is added to facilitate rapid precipitation of sand, 4) sand is removed from the bottom of the pool and floating fat is removed at the top, 5) the WW is led to a MBBR (Moving Bed Biofilm Reactor) pool. Air is added and biofilm forms on large surface plastic beads. Due to a short retention time, the biofilm (bacteria) on these beads consumes only the suspended organic matter in the WW and grows till the biofilm drops off the beads and mixes in with the WW before being led to the chemical treatment (Actiflo), 6) Here, ferric chloride, sand and polymer is added. The WW is stirred to further facilitate flocculation of sand and sludge which sediments in the final section of the Actiflo treatment, 7) treated water runs out at the surface of the basin and is released 8) sludge is removed from the bottom of the basin and pumped to Actidyn (sludge thickening). Illustration based on information from Bergen municipality. Drawing of plastic bead from information leaflet about Ytre Sandviken WWTP, provided by Bergen municipality.

3.2.3 Chemical treatment-based treatment plant

Knappen WWTP is dimensioned for 63,000 pe, and receives wastewater from Minde, Fjøsanger, Kråkenes, Bønes, Fyllingsdalen, Hetlevik, Loddefjord and Bjørndalen The treatment process consists of a preliminary treatment and a secondary chemical treatment (Figure 3.5). Knappen WWTP is the only WWTP out of the five plants studied, that does not have a biological treatment step.

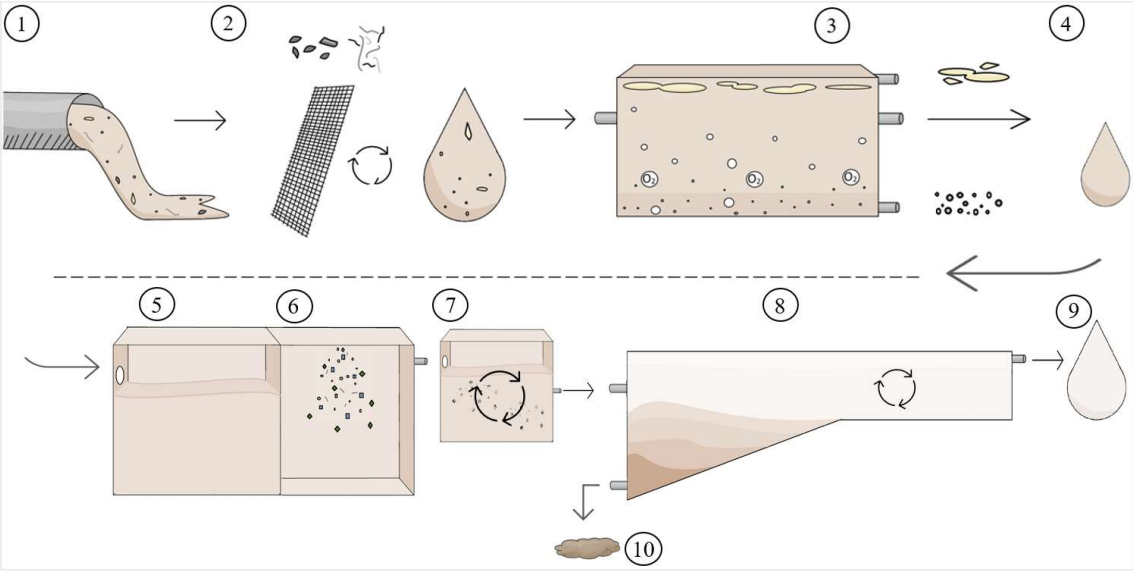


Figure 3.5 Simplified illustration of wastewater treatment in Knappen WWTP 1) raw sewage enters the facility, 2) rotating screen (3 mm) traps waste and particles > 3 mm, 3) WW is lead to a sand and grease trap where air is added to facilitate rapid precipitation of sand, while fat floats towards the top and is removed, 4) the WW is directed to a chemical treatment unit, 5) here, the amount of WW is measured in order to add correct amount of aluminium chloride and ferric chloride in the next step, 6) aluminium chloride and some ferric chloride is added which reacts with phosphate and creates aluminium phosphate particles, 7) the WW is sent through a flocculation chamber, 8) sedimentation pool where sludge and particles of aluminium phosphate sink towards the bottom, 9) treated water runs out of the surface of the basin and is released at 50 m depth in Grimstadfjorden, 9) sludge is removed from the bottom of the basin and is sent to sludge thickening. Illustration based on information from Bergen municipality.

Table 3.2 Summary of treatment methods for the five chosen WWTPs

WWTP	Prelim.	Primary	Secondary		Sludge	Recipient
Flesland	6 mm	Sand and grease collection	Biological treatment. Activated sludge, sedimentation.		Centr. and drainage.	Raunefjorden, 40 m depth.
Holen	6 mm	Sand and grease collection	Biological treatment. MBBR.	Chemical treatment. Actiflo. FeCl, sand and polymer.	Actidyn sludge thickening and centr.	40 m depth, Bergensfjorden
Knappen	3 mm	Sand and grease collection	Chemical treatment. AlCl + FeCl		Sludge thickening and centr.	Grimstadfjorden, 150 m from land, 50 m depth.
Kvernevik	6 mm	Sand and grease collection	Biological treatment. Activated sludge and sedimentation.		Filter belt press, centr and drainage	Bergensfjorden/ Salhusfjorden, 50 m depth.
Ytre Sandviken	6 mm	Sand and grease collection	Biological treatment. MBBR.	Chemical treatment. Actiflo. FeCl, sand. polymer.	Actidyn sludge thickening and centr.	Bergensfjorden

3.3 Plasmid preparation

3.2.1 *Escherichia coli* (*E. coli*) transformation

StrataClone SoloPack competent *E. coli* cells with Mix & Go from Agilent Technologies stored at -80 °C were used. The cells were thawed on ice and added the plasmid pCMX Gal4 zfPXR-TL-LBD (midi 10/5/17) with an original concentration of 2527 ng/L. 25 µL of a 1:100 dilution was then plated on a LB-agar plate containing ampicillin and incubated at 37 °C overnight. The same protocol was used for pCMX Gal4 Cod GR-LBD (mini 09/07/19) with an original concentration of 253.5 ng/uL.

Following the transformation, a colony (positive transformant) was chosen, and transferred to 200 mL LB medium with 200 µL ampicillin in an Erlenmeyer flask. For (MH100)x4tk-luciferase, pCMV-β-galactosidase, and the receptor plasmids for cod ARα-LBD, and cod ERα-LBD, a small amount of previously made glycerol stocks was used. The LB-medium containing ampicillin and positive transformant or glycerol stock was then incubated overnight (19 - 30 hours) in a Multitron standard shaking incubator from Infors HT at 37 °C and 250 rpm.

3.2.2 Plasmid purification

The plasmids must be purified before being used for transfection into COS-7 cells prior to exposure in the luciferase reporter gene assay. Medium-scale plasmid preparation (midi-prep) is a commonly used method to do this.

Following the overnight incubation, the density of the cell culture was evaluated at 600 nm using a cell density meter (Ultraspec 10 Cell Density Meter, Amersham Biosciences). Optical density * cell volume (mL) = 200 ODV was calculated, and the bacteria were harvested by centrifugation (3500 *g* for 5 minutes). The kit used was a NucleoBond® PC100 kit from Macherey-Nagel. The pellet was resuspended in RNase buffer to degrade any RNA present. After RNase treatment, SDS/alkaline lysis liberated the DNA content of the *E. coli* cells, and an acetate containing neutralization buffer was added to facilitate a supercoiled conformation of the plasmid DNA. Chromosomal DNA, proteins, and cell debris were precipitated. The supernatant containing the plasmid was applied to a silica-based column made to bind plasmid DNA. The column was washed afterwards with a buffer containing ethanol in order to remove any possible contamination. To elute the plasmid DNA from the column, an elution buffer was used (provided by the kit). The eluted plasmid DNA was precipitated by adding isopropanol followed by centrifugation and drying of the pellet using ethanol. Lastly, the final pellet was dissolved in 250 µL AE-buffer and plasmid DNA concentration and purity was measured using a Nanodrop instrument.

3.2.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to investigate plasmid conformation. As nucleic acids are negatively charged, agarose gel electrophoresis can separate nucleic acids based on their size and charge. Hence, supercoiled plasmids will effectively be transported through the gel towards the positively charged side. A ladder (standard marker indicating size) was used to confirm correct plasmid conformation. Gels in this master thesis was produced according to Table 2.9 to a final concentration of 1 % agarose.

3.4 Luciferase reporter gene assay (LRA)

A luciferase reporter gene assay (LRA) was used to investigate if compounds in extracts from the wastewater samples could induce agonistic or antagonistic responses in various receptors from cod and zebrafish. The LRA involves three plasmids: a reporter-plasmid, a receptor-plasmid, and a control-plasmid. All of them are transfected into COS-7 cells derived from African green monkey kidney tissue.

The reporter-plasmid (MH(100)x4tk-luciferase) codes for luciferase. Luciferase is an oxidizing enzyme which produces bioluminescence (light) when the substrate luciferin is present (Figure 3.6). For the transcription of luciferase to be controlled, there is a Gal4-activation sequence (UAS) in its promoter-region. The transcription only occurs when a receptor protein containing a Gal-4-DNA binding domain (DBD) goes through a conformational change, is activated, and binds to the UAS in the reporter-plasmid.

The receptor-plasmid contains the receptor of interest. Fused to the ligand binding domain of this receptor is the abovementioned Gal-4-DBD. Upon exposure and activation, the ligand binds to the ligand binding domain (LBD). In turn, this leads to a conformational change in the Gal-4-DBD which is activated. In its activated form, Gal-4-DBD binds the UAS is the reporter-plasmid which leads to the expression and translation of luciferase as described above. Therefore, the binding of a ligand to the receptor works like a light-switch in the reporter plasmid. No (agonistic) binding = no light signal, (agonistic) binding = light signal. This light is measured at 560 nm and quantified after addition of the luciferin substrate.

The third plasmid is the control-plasmid. This plasmid is the pCMV-β-gal and is used to normalize the measured light signal to number of cells as well as transfection efficiency. β-galactosidase hydrolyses ONPG to galactose and ONG which has a bright yellow colour that absorbs light at 420 nm. Due to this, β-gal activity can be measured as absorbance.

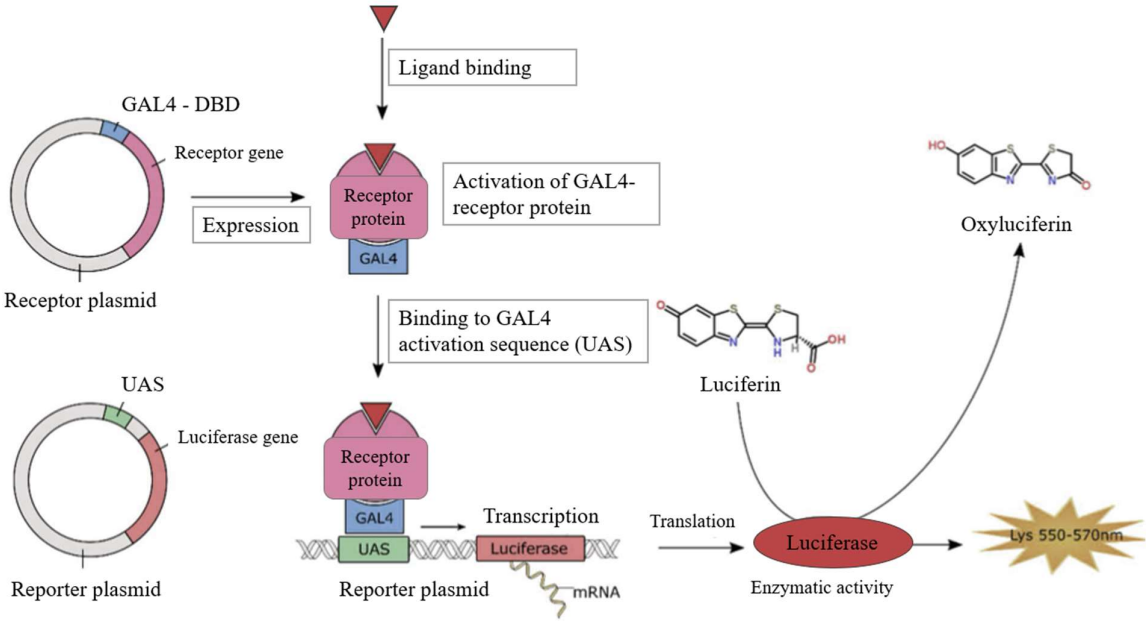


Figure 3.6 Illustration of the principle behind the Gal4-DBD based luciferase reporter gene assay. Receptor plasmid contains the receptor gene and a Gal4-DBD. Upon ligand binding, this plasmid changes conformation, enabling the binding of Gal4 to the activation sequence (UAS) in the reporter plasmid. Downstream from this sequence in the reporter plasmid is the luciferase gene, which gets transcribed and translated. Luciferase is an enzyme which catalyses the reaction where luciferin is transformed to oxyluciferin, a process which results in emitted light at 560 nm. Illustration from Madsen, 2016, (translated).

3.4.1 Cultivation of COS-7 cells

Aliquots of COS-7 cells (Table 2.4) in DMEM-10% FBS and 5% DMSO were stored in liquid nitrogen until thawed and added 10 mL DMEM-10% FBS. Subsequently, the cells were centrifugated for 5 minutes at 250 *g* at rt. Medium was carefully removed and the pellet containing COS-7 cells were resuspended in 10 ml DMEM-10% FBS before plated in petri disks and incubated at 37 °C with 5 % CO_2 until the cells reached a confluency of 60 – 80% and split. Splitting was conducted by removing old medium and washing the cells with 5 ml 1X PBS (pH 7.4) twice before adding 1.5 ml Trypsin-EDTA (0.05% trypsin, 0.02 % EDTA) for the cells to detach from the surface of the plate. Excess trypsin was removed after 45 seconds, and the cells were incubated for approximately 5 minutes at 37 °C with 5 % CO_2. Afterwards, the cells were resuspended in DMEM-10% FBS and added to new plates in a 1:5, 1:10 or 1:20 dilution with new medium.

3.4.2 Seeding

Cells were detached from plates following the same procedure as described under 3.3.1. and counted in a 1:1 solution with Erythrosine B using a Leica DM IL inverted microscope and a haemocytometer. Based on this, 100 uL containing approximately 5000 cells were seeded in each well in 96-well plates and incubated for 18 - 24 hours at 37 °C with 5 % CO_2.

3.4.3 Transfection

Transfection is a method used to introduce foreign DNA into a eukaryotic cell. A transfection mixture (Table 3.4) consisting of Optimem, plasmid mix (Table 3.3), and transIT-LT1 reagent (Mirus Bio) was made and incubated at rt for 30 minutes. After 30 minutes, DMEM-10% FBS was added. The 96 well plates were taken out of the incubator and old medium removed. Each well was then added 101,4 µL of the transfection mixture with fresh DMEM before incubated at 37 °C with 5 % CO_2 for 24 hours.

Table 3.3 Plasmid mixture. Ratios of receptor plasmid 1:10 compared to reporter- and control plasmid.

Plasmid	**Amount (ng)**
(MH100)x4 tk luc	47.62
pCMV-β-Gal	47.62
pCMX-GAL4-receptor plasmid	4.76
Total	100.00

Table 3.4 Transfection mixture. TransIT LT kit (Mirus Bio) based.

Component	**µL per well**
Plasmid mixture (1µg/µL)	0.1
Optimem	9.0
TransIT LT1	0.2
DMEM w/10% FBS	92.1
Total	101.4

3.4.4 Ligand exposure

Old medium was removed from the 96 well plates, and test compounds (Table 3.5 - 3.6) dissolved in DMSO, or WW or sludge extracts (Table 3.7 – 3.9) in water with 20% MeOH, were serially diluted from well A - G in exposure medium. DMSO (for test compounds dissolved in DMSO) was added to the exposure medium (max 0.5 %) to give an equal concentration along the dilution gradient, as well as in the solvent control. Water with a 20% MeOH concentration was added according to the same principle for the extract sample testing.

To assess the chosen receptors' potential for activation by pharmaceuticals, some commonly used control-compounds were selected for testing in the luciferase reporter gene assays (LRAs). Moreover, a few frequently prescribed pharmaceuticals in Norway were tested as well. For testing of agonistic

properties, five compounds were selected (Table 3.5). Three compounds were used for antagonistic testing (Table 3.6).

Table 3.5 Compounds used for *in vitro* agonistic testing on zebrafish PXR-TL, cod ARα, cod ERα and cod GR. All tested at seven concentrations.

Plasmid	Ligand	Highest concentration [µM]	DF
Zebrafish PXR-TL	Clotrimazole	3.0	5
Cod ARα	Testosterone	0.4	5
Cod ERα	EE2	0.4	5
Cod GR	Dexamethasone	100	5
	Prednisolone	100	6

For testing of antagonistic properties, a background activation with either testosterone (ARα) or EE2 (ERα) was used. The concentration of compound used as background activation was maintained constant along the exposure gradient, enabling the solvent control to be a control for background activation. Hence, any reduction in luciferase activity compared to solvent control could imply antagonistic properties of exposure compound or extract.

Table 3.6 Compounds used for *in vitro* antagonistic testing on cod ARα and cod ERα.

Plasmid	Ligand	Highest concentration [µM]	Background activation	DF
Cod ARα	Flutamide	20	0.005 µM testosterone	5
	Bicalutamide	100		5
Cod ERα	Tamoxifen	20	0.003 µM EE2	5

Clotrimazole, Testosterone and EE2 were also analysed in parallel as an assay control.
500 mL sample volumes of WW and sludge were extracted and reconstituted in 1 mL 20% MeOH, thus representing either 500 mL wastewater equivalents/ml (WWEQ/ml) or 500 mL sludge equivalents/ml (SEQ/mL). When exposing the transfected COS-7 cells, a smaller volume of the extracts was diluted in cell medium, with 30 mL WWEQ/mL (Table 3.7) and 30 mL SEQ/mL (Table 3.8) as the highest concentrations used.

Table 3.7 Wastewater extracts used for *in vitro* agonistic testing of zebrafish PXR-TL, cod ARα and cod ERα, as well as antagonistic testing of cod ERα.

Station	Highest conc. mL WWEQ/mL				No. triplicates				DF
	PXR-TL	AR	ER	ER ant**	PXR-TL	AR	ER	ER ant**	
Kvernevik-in	30	-	30	15	1	-	1	1	2
Kvernevik-out	30	15	30*	15	3	3	2	2	2
Ytre Sandviken-in	30	15	30*	15	3	3	2	2	2
Ytre Sandviken-out	30	15	30*	15	3	3	2	2	2
Holen-in	30	15	30*	15	3	3	2	2	2
Holen-out	30	15	30*	15	3	3	2	2	2
Knappen-in	30	15	30*	15	3	3	2	2	2
Knappen-out	30	15	30	15	1	2	1	1	2
Flesland-in	30	15	30*	-	3	3	2	-	2
Flesland-out	30	15	30*	-	3	3	2	-	2

* One triplicate with 30 mL, the other with 15 mL WWEQ/mL

** ER antagonism with 0.003 µM EE2 background activation

Table 3.8 Sludge sample extracts used for *in vitro* agonistic testing. Identical concentrations, dilution and no. of triplicates for zebrafish PXR-TL, cod ARα, and cod ERα.

Station	Highest conc. mL WWEQ/mL				No. triplicates	DF
	PXR-TL	AR	ER	ER ant*		
Kvernevik-sludge	15	15	15	15	2	2
Ytre Sandviken-sludge	15	15	15	15	2	2
Holen-sludge	-	-	-	-	-	-
Knappen-sludge	15	15	15	15	2	2
Flesland-sludge	15	15	15	15	2	2

* 0.003 µM EE2 background activation

3.4.5 Lysis and quantifying luciferase and β-galactosidase activation

3.4.5.1 Lysis

Old medium containing exposure mixture was removed after 24 hours, and each of the wells were added 125 µL lysis buffer with reagent solution (Table 3.11), which was incubated at rt for 30 minutes on a HS501 Digital shaker from IKA-Werke at 95 – 105 shaking frequency. Following this, 50 µL lysate was transferred to each well in a clear 96 well plate and the same volume of the lysate in a white 96 well plate. Clear plates were used for β-gal measurement (absorbance) whilst white plates were used for luciferase measurement (light).

3.4.5.2 β-galactosidase activation

To the clear plate, 100 µL β-galactosidase reagent (Table 3.11) was added to each well, and the plate was incubated for ~ 20 minutes in rt until a bright yellow colour was obtained. The absorbance was measured at 420 nm in an EnSpire 2300 PerkinElmer plate reader.

3.4.5.3 Luciferase activation

To the white plate, 100 µL luciferase reagent (Table 3.11) was added to each well and the luminescence was measured immediately at 560 nm in an EnSpire 2300 PerkinElmer plate reader.

Table 3.11 Lysis, β-galactosidase, and luciferase reagent

Lysis reagent		β-galactosidase reagent		Luciferase reagent	
Components	Conc.	Components	Conc.	Components	Conc.
Lysis base buffer (Table 2.10)	1 X	β-gal. base buffer (Table 2.12)	1 X	Luc. Base buffer (Table 2.14)	1 X
EGTA	4 nM	β-mercaptoethanol	52.9 mM	DTT	5 mM
$MgCl_2$	8 mM	ONPG	8.6 mM	ATP	0.5 mM
DTT	1 mM			CoA	0.5 mM
PMSF	0.4 mM			D-luciferin*	0.5 mM
				MilliQ H_2O	

* Added right before measured.

The data from the EnSpire 2300 plate reader (Perkin Elmer) was firstly treated in Microsoft Excel where the luciferase activity was normalized to the β-gal value of cells deriving from the same well. This was done to adjust for possible differences in transfection efficiency. Furthermore, the normalized luciferase-activity was compared to the solvent control and calculated as fold change in luciferase activity. For further statistical analysis, GraphPad Prism 8 ® was used.

3.5 Cell viability measurement

A cell viability assay was performed to investigate whether any of the compounds or samples were cytotoxic to the exposed COS-7 cells. The cell viability assay follows the same steps as the LRA, except

the cells are not transfected on day 2. Instead, the old medium is removed, and new medium (101.4 µL) added. All cells were exposed to a max concentration of 30 mL WWEQ/mL with a dilution factor (DF) of 2, identical to the procedure used for the LRA. Triton X-100 was used as a positive control for cell death. 24 hours after exposure old medium was removed, the cells washed with 100 µl 1X PBS per well. In order to assess the cytotoxic properties of the extracts, 100 µl/well resazurin and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) solutions was added to the cells. The 96 well plates were placed back into the incubator at 37 °C with 5% CO_2 for 1 hour. Fluorescence was measured with EnSpire plate reader (PerkinElmer) at 530/590 nm for resazurin and 485/530 nm for CFDA-AM. As healthy cells transform resazurin (measuring metabolic activity) and CFDA-AM (measuring membrane integrity) to fluorescence signals, a significant reduction in fluorescence compared to non-exposed cells indicates a possible cytotoxicity.

4 Results

4.1 Assessment of plasmids used in luciferase reporter gene assays.

The integrities of plasmids used in the LRA were initially assessed with spectrophotometry and gel electrophoresis prior to the experiments. For spectrophotometry, absorbance at $A_{260nm/280nm}$ and $A_{260nm/230nm}$ were measured (Table 4.1) in order to indicate the purity of the DNA both in terms of protein- and chemical contamination.

Table 4.1 Plasmid-DNA concentration and purity measured spectrophotometrically

Plasmid	Concentration (ng/µl)	$A_{260nm/280nm}$	$A_{260nm/230nm}$
(MH100)x4tk-luciferase*	1606	1.94	2.38
pCMV-β-galactosidase*	2959	1.90	2.33
zebrafish PXR-TL***	2285	1.91	2.34
cod ARα**	1997	1.93	2.31
cod Erα**	2387	1.93	2.34
cod GR***	2165	1.93	2.33

* Mediprepped multiple times with slight variations in concentration

** Cod ARα prepared by Siri Øfsthus Goksøyr (PhD student, Department of Biological Sciences, UiB). Cod ERα prepared by Rhîan Gaenor Jacobsen (chief engineer, Department of Biological Sciences, UiB) *** Zebrafish PXR-TL and cod GR obtained from Roger Lille-Langøy

Absorbance at A260 nm/280 nm of approximately 1.9 indicates no protein contamination. Values above 2.0 for A260 nm/A230 nm indicates no chemical contamination.

Gel electrophoresis (Method 3.2.3) shows the conformation of the plasmids. Based on the migration of the plasmids in the agarose gel, it appears that the majority of the plasmids have retained a supercoiled conformation (Figure 4.1). A supercoiled conformation is desired as it promotes an efficient transfection into COS-7 cells.

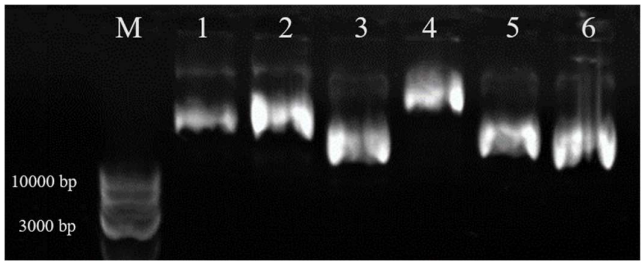


Figure 4.1 Agarose gel electrophoresis of plasmids used in ligand activation experiments. Approximately 200 ng purified plasmids were separated in a 1 % agarose 0.5X TBE gel for 45 minutes. The following plasmids were applied to lane 1 – 6 respectively; (MH100)x4tk-luciferase (1), pCMV-β-galactosidase (2), cod ARα (3), zebrafish PXR-TL (4), cod Erα (5) and cod GR (6). A 2-LOG ladder was applied to lane M as a reference marker.

4.2 Cell viability

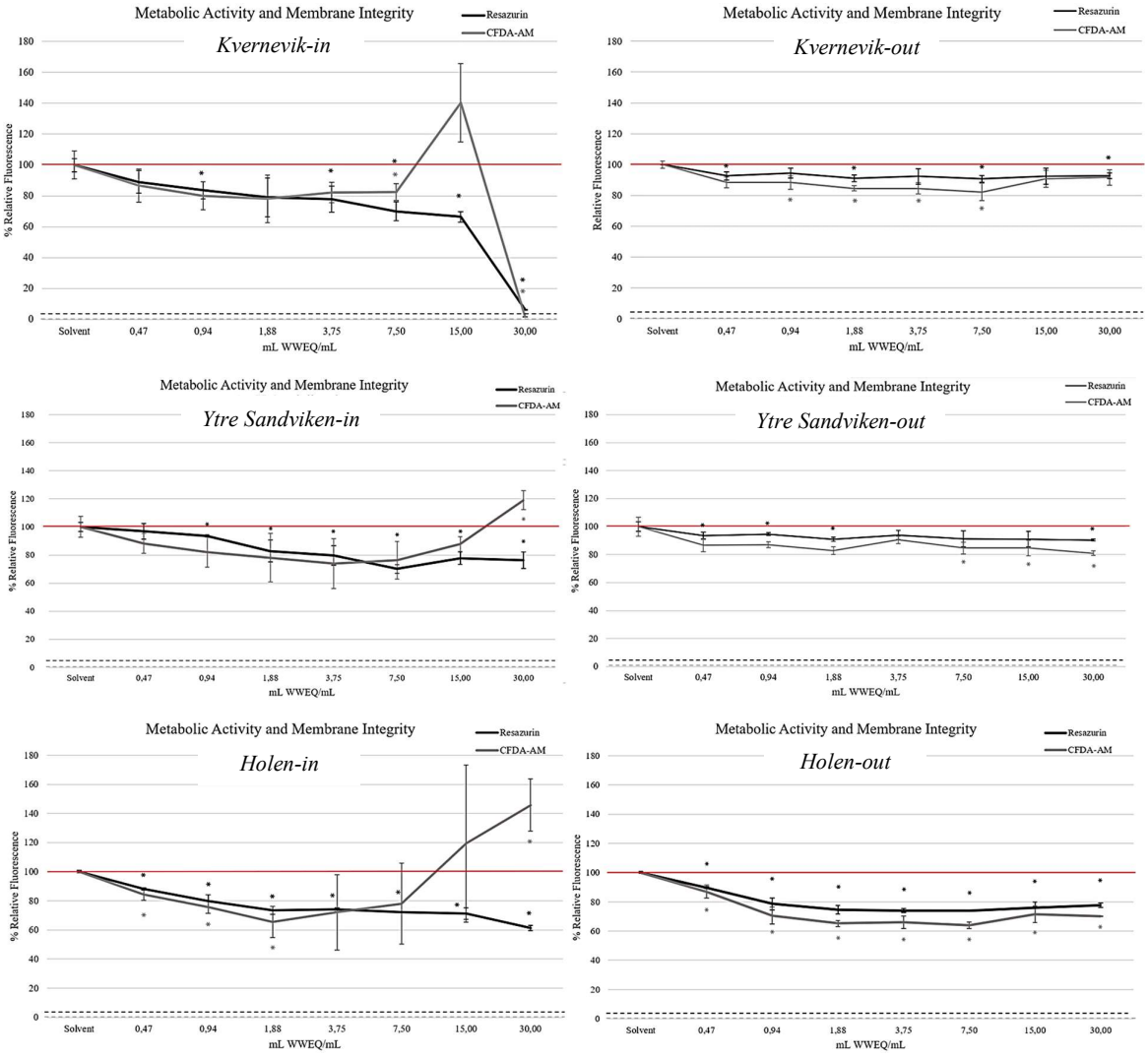
The cytotoxicity of wastewater and sludge sample extracts to COS-7 cells were assessed using a cell viability assay measuring the metabolic activity and cell membrane integrity of the COS-7 cells.

4.2.1 Cytotoxicity of wastewater samples

The extract producing highest measured cytotoxicity was *Kvernevik-in*, where 30 mL wastewater equivalents (WWEQ) /mL resulted in more than a 90 % reduction in fluorescence in both resazurin (metabolic activity) and CFDA-AM (cell membrane integrity) (Figure 4.2). The extract was almost as effective as the positive control (Triton X-100) in producing a cytotoxic response. Generally, the metabolic activity was reduced with between 40 – 20% for the remaining in-samples, except *Flesland-*

in, with a 10 % reduction compared to solvent control. The reduction in metabolic activity of the effluent samples were generally lower with approximately 10 % lower fluorescent signal compared to solvent control. Focusing on the conversion of resazurine, all stations had improved levels of metabolic activity for effluent samples compared to the influent samples.

CFDA-AM, which indicate alterations in cell membrane integrity after exposure gave unresolved results for the influents. Fluorescence levels indicated increased membrane integrity at higher exposure concentrations, especially for *Holen-in*, and *Knappen-in*, where cell membrane integrity was measured to > 140 % compared to solvent control. This trend also appeared in *Kvernevik-in* at 15 mL WWEQ/mL, prior to significantly decreasing at 30 mL WWEQ/mL. The same remarkable results were not seen in the out-samples where all CFDA-AM signals were measured to be below 100 %, but over 75 %.



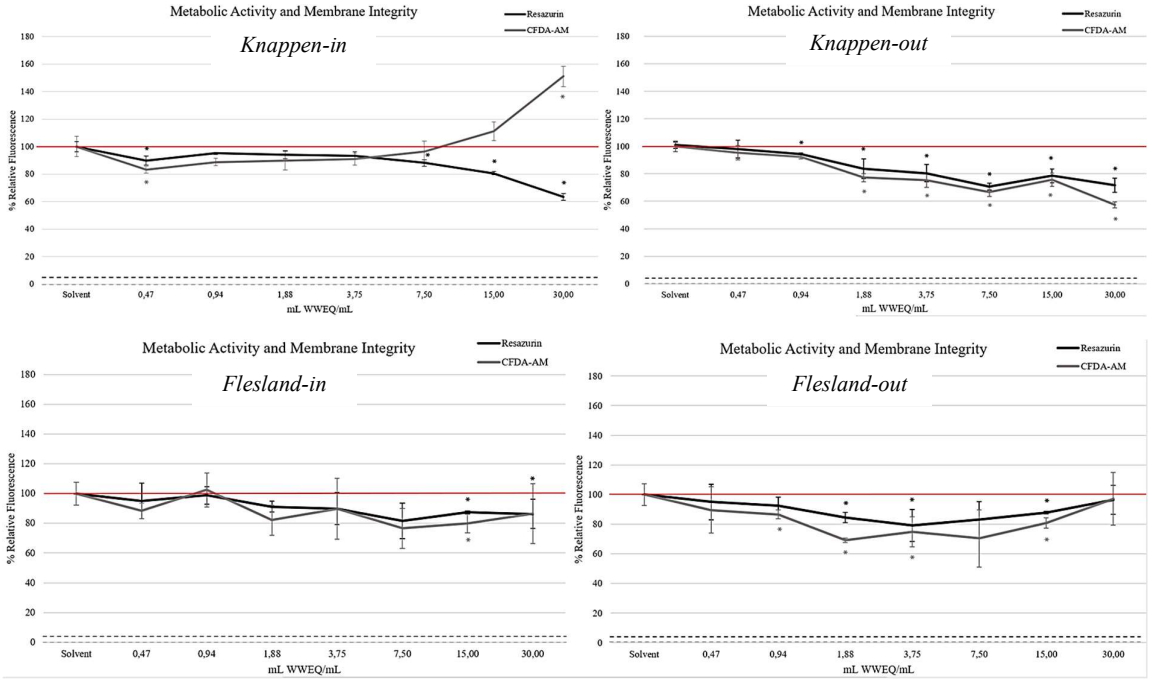


Figure 4.2 Viability of COS-7 cells following 24h exposure to wastewater extracts. COS-7 cells were exposed to wastewater extracts in relevant ranges of concentration used for the LRAs. Solvent (milli-Q H_2O with 20 % MeOH) in DMEM (equivalent to solvent concentration for the samples) was used as a negative control for cytotoxicity. Triton X-100 (1%) was used as a positive control for cytotoxicity, indicated by a black (rezasurin) and grey (CFDA-AM) dotted line in each figure. Cytotoxic responses were defined as change in fluorescent signal compared to solvent control (defined as 100 %, indicated with red line). Black bars indicate metabolic activity measured by resazurin. Grey bars indicate plasma membrane integrity measured by CFDA-AM. Significance shown as * = $p < 0.05$ (TTEST).

4.2.2 Cytotoxicity of sludge samples

The cell viability assay indicated a small reduction in fluorescence signals for resazurin with the highest observed effect in cells exposed to 30 ml SEQ/mL from *Ytre Sandviken* and *Flesland*, both at ~ 80 % relative fluorescence (Figure 4.3). There was also observed increased cell membrane integrity values, but only for *Ytre Sandviken* (115 %), and *Knappen* (155 %). The remaining stations has CFDA-AM results at between 80 – 90 %.

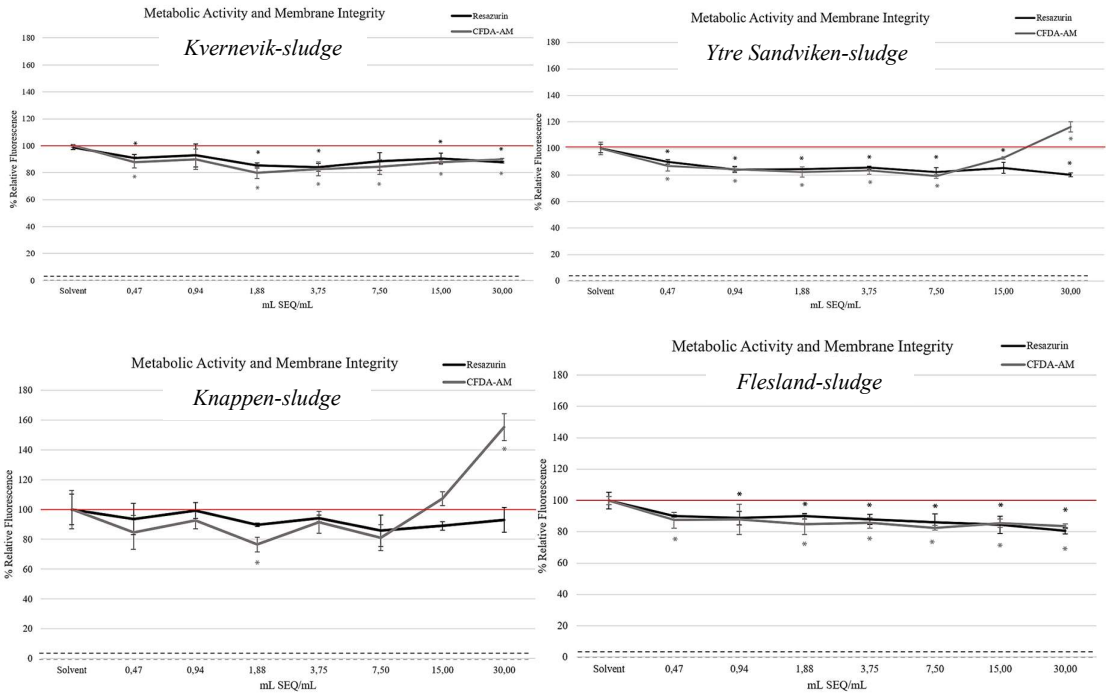


Figure 4.3 Viability of COS-7 cells following 24h exposure to sludge extracts. COS-7 cells were exposed to wastewater extracts in relevant ranges of concentration used for the LRAs. Solvent (milli-Q H_2O with 20 % MeOH) in DMEM (equivalent to solvent concentration for the samples) used as a negative control for cytotoxicity. Triton X-100 (1%) used as a positive control for cytotoxicity, indicated by a black (rezasurine) and grey (CFDA-AM) dotted line in each figure. Cytotoxic responses were defined as change in fluorescent signal compared to solvent control (defined as 100 %, indicated with red line). Black bars indicate metabolic activity measured by resazurin. Grey bars indicate plasma membrane integrity measured by CFDA-AM. Significance shown as * = p < 0,05 (TTEST).

Table 4.2 Heatmap showing relative fluorescence in % compared to control. Resazurin results for two concentrations (15 and 30 mL WWEQ/ml, or mL SEQ/mL) shown for in-samples (a), out-samples (b), and sludge samples (s). Colours ranging from red indicating low metabolic activity, to green indicating metabolic activity at similar levels as solvent control. ± standard deviation for triplicate measurements. No sludge sample was available from Holen.

mL WWEQ/mL mL SEQ/mL		Kvernevik	+/-	Y. Sandv.	+/-	Holen	+/-	Knappen	+/-	Flesland	+/-
-in	15	66,47	3,36	77,76	4,33	71,13	4,53	80,62	1,15	87,43	2,57
	30	6,21	0,26	76,27	5,84	61,38	15,14	63,38	2,67	86,32	1,62
-out	15	92,29	5,18	91,16	5,40	75,82	3,98	78,48	4,92	87,74	0,91
	30	92,79	1,77	90,52	0,66	77,66	1,68	71,63	5,09	96,38	9,72
-sludge	15	90,49	3,92	85,26	4,19	-		89,05	2,79	84,42	5,51
	30	87,87	0,56	80,11	1,42	-		93,02	8,33	80,58	2,02

4.3 Luciferase reporter gene assay (LRA) testing of specific pharmaceuticals

In order to assess the receptors' potential for activation by pharmaceuticals, some commonly used control-compounds were selected for testing in the luciferase reporter gene assays (LRAs). Moreover, a few frequently prescribed pharmaceuticals in Norway were tested, including clotrimazole (for zf PXR-TL), testosterone (cod ARα), EE2 (cod ERα), and dexamethasone and prednisolone (both cod GR). For antagonistic testing, flutamide and bicalutamide were used (cod ARα), as well as tamoxifen (cod ERα). All investigated agonists activated the receptors in a dose-response manner (Figure 4.4 – 4.5). Highest significant activation (E_{max}) and half maximal effective concentration (EC50) for all compounds tested are compiled in Table 4.3.

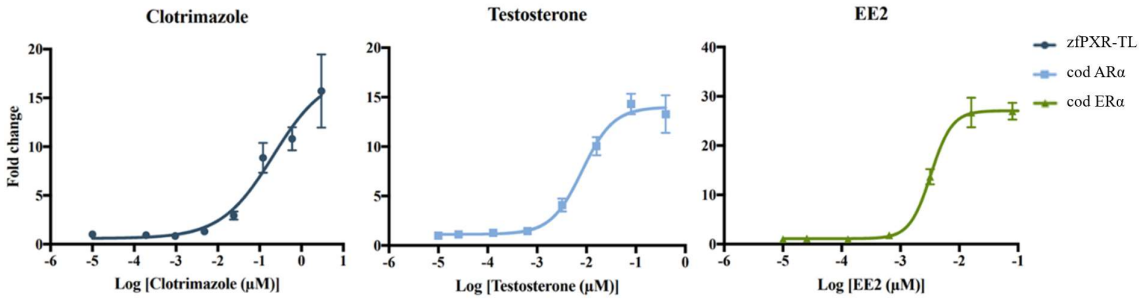


Figure 4.4. Ligand activation of zf PXR-TL, cod ARα, and cod ERα by clotrimazole, testosterone, and EE2, respectively. Receptor plasmid, reporter plasmid ((MH100)x4tk-luciferase) and control plasmid (pCMV-β-galactosidase) were transfected into COS-7 cells. The cells were exposed to seven concentrations of clotrimazole, testosterone, or EE2 for 24 hr. Activation of ligand is shown as relative fold change in luciferase activity in exposed cells compared to DMSO control. Each exposure was done in triplicates and individual experiments were performed three times. Dose-response curves were fitted by non-linear regression, with bars indicating SEM (GraphPad, PRISM v 7.0).

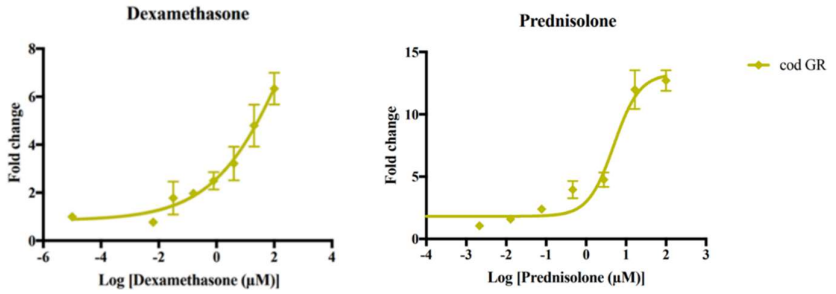


Figure 4.5 Ligand activation of cod GR exposed to dexamethasone and prednisolone. Receptor plasmid, reporter plasmid ((MH100)x4tk-luciferase) and control plasmid (pCMV-β-galactosidase) were transfected into COS-7 cells. The cells were exposed to seven concentrations of dexamethasone (left) and prednisolone (right) for 24 hr. Activation of ligand is shown as relative fold change in luciferase activity in exposed cells compared to DMSO control. Each exposure was done in triplicates and individual experiments were performed three times. Dose-response curve fitted by non-linear regression, with bars indicating SEM (GraphPad, PRISM v 7.0).

As some pharmaceuticals act as antagonists, measurements of antagonistic effects were also performed. Compounds used were flutamide and bicalutamide (cod ARα), and tamoxifen (cod ERα), which have previously been shown to act as antagonists of these receptors (Table 2 and 4, Appendix). Lowest significant inhibition (I_{max}) and half maximal inhibitory concentration (IC50) for all compounds tested stated in Table 4.3. Bicalutamide did not inhibit ARα as expected but appears to have a biphasic response (Figure 4.6).

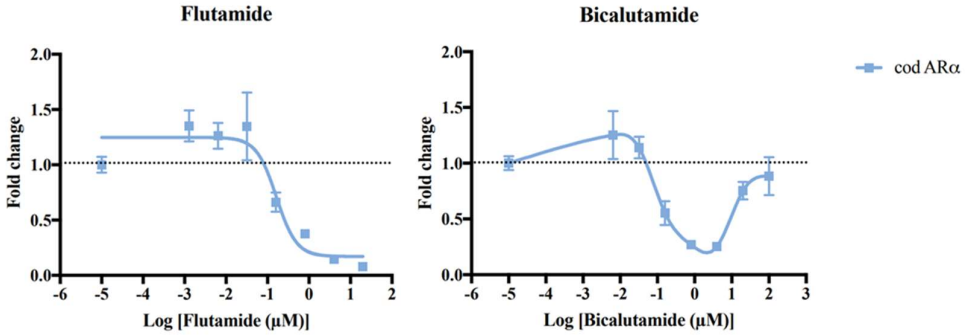


Figure 4.6. Antagonistic effects of flutamide (left) and bicalutamide (right) on cod ARα. Receptor plasmid, reporter plasmid ((MH100)x4tk-luciferase) and control plasmid (pCMV-β-galactosidase) were transfected into COS-7 cells. The cells were exposed to 7 concentrations of flutamide (left) and bicalutamide (right) for 24 hr as well as a fixed concentration of 0.005 μM testosterone (indicated as dotted line). Inhibition of ligand is shown as relative fold change in luciferase activity in exposed cells compared to DMSO control with testosterone. Each exposure was done in triplicates and individual experiments were performed three times. Dose-response curves were fitted by non-linear regression (flutamide) and best fit (bicalutamide), with bars indicating SEM (GraphPad, PRISM v 7.0)

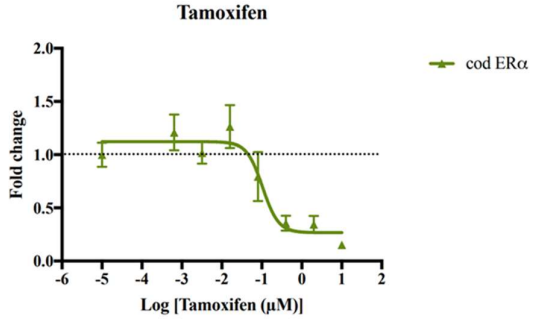


Figure 4.7 Antagonistic effects of tamoxifen on cod ERα. Receptor plasmid, reporter plasmid ((MH100)x4tk-luciferase) and control plasmid (pCMV-β-galactosidase) were transfected into COS-7 cells. The cells were exposed to 7 concentrations of tamoxifen for 24 hr as well as a fixed concentration of 0.003 μM EE2 (indicated as dotted line). Inhibition of ligand is shown as relative fold change in luciferase activity in exposed cells compared to DMSO control with EE2. Each exposure was done in triplicates and individual experiments were performed three times. Dose-response curve was fitted by non-linear regression, with bars indicating SEM (GraphPad, PRISM v 7.0)

Table 4.3 Highest significant activation (E_{max}), inhibition (I_{max}), EC50, and IC50 values for tested compounds. IC50 and EC50 value stated only when max activation/inhibition of receptor has been reached. E_{max} stated for agonists, I_{max} stated for antagonists.

Receptor	Compound		Activation E_{max}	Inhibition I_{min}	p-value	EC50 µM	IC50 µM
PXR-TL	Clotrimazole	Agonist	17.5	-	<0.0001	0.209	-
ARα	Testosterone	Agonist	14.0	-	<0.0001	0.008	-
ERα	EE2	Agonist	27.1	-	<0.0001	0.003	-
GR	Dexamethasone	Agonist	6.3	-	<0.001	-	-
GR	Prednisolone	Agonist	13.3	-	<0.0001	4.965	-
ARα*	Flutamide	Antagonist	-	0.2	<0.0001	-	0.163
ARα*	Bicalutamide	Antagonist/ biphasic	-	0.2	<0.0001	-	-
ERα**	Tamoxifen	Antagonist	-	0.3	0.0017	-	0.104

* Background activation of 0.005 µM testosterone
** Background activation of 0.003 µM EE2

4.3 Receptor interactions by wastewater extracts

To investigate whether compounds in wastewater samples from the five chosen treatment plants could induce agonistic effects on selected receptors, COS-7 cells were transfected and exposed to increasing concentrations of the different extracts (Method 3.2.3). Highest significant activation (E_{max}) and statistical significance for all wastewater extracts tested are summarized in Table 4.5.

4.3.1 Agonistic effects of wastewater extracts

For *Kvernevik-in*, the highest concentration was removed from the graph due to a significantly reduced cell viability (Table 4.2, cytotoxicity). 15 mL WWEQ produced a 1.9-fold activation for zebrafish PXR-TL, but no activation was observed for cod ERα (Figure 4.9, left). Cod ARα was not included in the analyses of *Kvernevik-in* due to insufficient sample volume. For *Kvernevik-out*, 30 mL WWEQ induced 6.3-fold increase in luciferase activity for zf PXR-TL, the highest activation in zf PXR-TL seen in all extracts. No effects were observed with cod ARα and cod ERα (Figure 4.9, right) in *Kvernevik-out*.

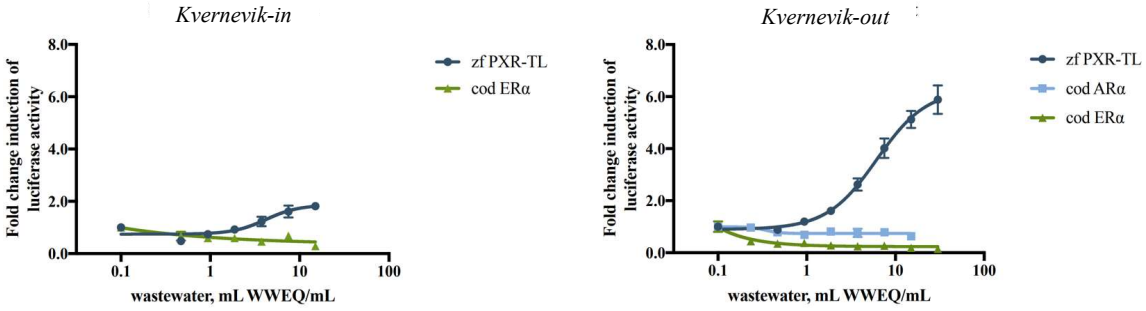


Figure 4.9. Ligand activation of zf PXR-TL, cod ARα, and cod ERα exposed to WW sample from *Kvernevik-in* and *Kvernevik-out*. Receptor plasmid, reporter plasmid ((MH100)x4tk-luciferase) and control plasmid (pCMV-β-galactosidase) were transfected into COS-7 cells. The cells were exposed to 7 concentrations of ww-extracts for 24 hr. Activation of ligand is shown as relative fold change in luciferase activity in exposed cells compared to solvent control. Each exposure was done in triplicates and individual experiments were performed three times for zfPXR-TL and ARα, and twice for cod ERα. Top concentration in *Kvernevik-in* was removed due to cytotoxicity. Cod ARα could not be included in the analyses of *Kvernevik-in* due to insufficient sample volume. Dose-response curves were fitted by non-linear regression, with bars indicating SEM (GraphPad, PRISM v 7.0).

For *Ytre Sandviken-in*, both zf PXR-TL and cod ARα showed an increased activation at 30 ml WWEQ/mL and 15 ml WWEQ/mL exposure, respectively (Figure 4.10, left). No activation cod of ERα was observed. For *Ytre Sandviken-out*, zf PXR-TL and cod ARα showed an increase in luciferase activity of xx.x and xx.x at 30 ml WWEQ/ml and 15 ml WWEQ/ml respectively (Figure 4.10, right). No activation of cod ERα observed.

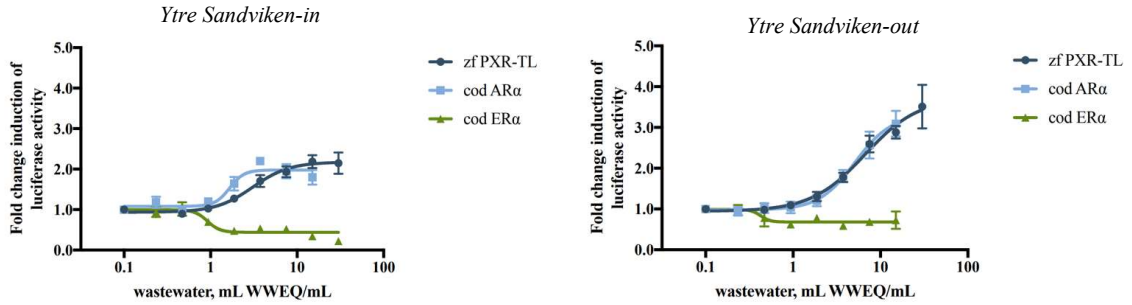


Figure 4.10 Ligand activation of zf PXR-TL, cod ARα and cod ERα exposed to WW sample from *Ytre Sandviken-in* and *Ytre Sandviken-out*. Receptor plasmid, reporter plasmid ((MH100)x4tk-luciferase) and control plasmid (pCMV-β-galactosidase) were transfected into COS-7 cells. The cells were exposed to 7 concentrations of ww-extracts for 24 hr. Activation of ligand is shown as relative fold change in luciferase activity in exposed cells compared to solvent control. Each exposure was done in triplicates and individual experiments were performed three times for zfPXR-TL and ARα, and twice for cod ERα. Top exposure concentration was 30 mL WWEQ/mL for zf PXR-TL and ERα, and 15 mL WWEQ/mL for ARα. Dose-response curves were fitted by non-linear regression, with bars indicating SEM (GraphPad, PRISM v 7.0).

For *Holen-in*, the top concentration of 30 mL WWEQ/mL was removed due to cytotoxicity causing almost 40 % reduction in cell viability. Activation could be observed in PXR-TL at 7.5 and 15 mL WWEQ/mL. For cod ARα, a slight activation could be observed at ~ 2 mL WWEQ/mL, before decreasing at increasing concentrations. *Holen-out* showed a higher activation of PXR-TL compared to the in-sample, although no concentrations here had to be removed due to cytotoxicity. A slight activation of cod ARα was observed. No significant activation of cod ERα was observed.

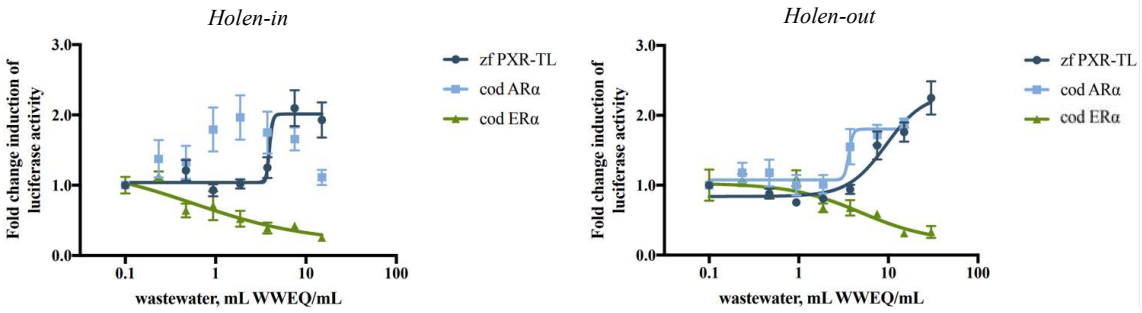


Figure 4.11 Ligand activation of zf PXR-TL, cod ARα and cod ERα exposed to WW sample from *Holen-in* and *Holen-out*. Receptor plasmid, reporter plasmid ((MH100)x4tk-luciferase) and control plasmid (pCMV-β-galactosidase) were transfected into COS-7 cells. The cells were exposed to 7 concentrations of WW-extracts for 24 hr. Activation of ligand is shown as relative fold change in luciferase activity in exposed cells compared to solvent control. Each exposure was done in triplicates and individual experiments were performed three times for zfPXR-TL and ARα, and twice for cod ERα. Top exposure concentration was 30 mL WWEQ/mL for zf PXR-TL and ERα, and 15 mL WWEQ/mL for ARα. Top concentration in *Holen-in* was removed due to cytotoxicity. Dose-response curves were fitted by non-linear regression, with bars indicating SEM (GraphPad, PRISM v 7.0).

Knappen WWTP was the only treatment plant where the highest activation was observed in cod ARα, a trend revealed in both the in- and the out-sample. An activation of zf PXR-TL in the in-sample was observed, although with a larger response in the out sample. No significant activation of cod ERα was observed for neither in- nor the out-sample.

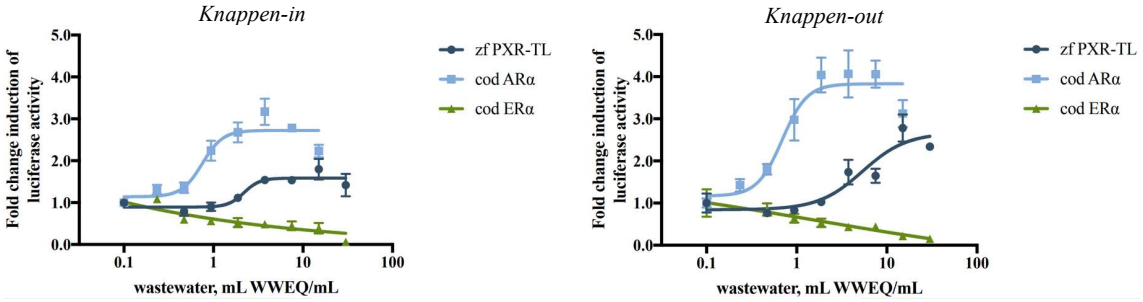


Figure 4.12 Ligand activation of zf PXR-TL, cod ARα, and cod ERα exposed to WW sample from *Knappen-in* and *Knappen-out*. Receptor plasmid, reporter plasmid ((MH100)x4tk-luciferase) and control plasmid (pCMV-β-galactosidase) were transfected into COS-7 cells. The cells were exposed to 7 concentrations of WW-extracts for 24 hr. Activation of ligand is shown as relative fold change in luciferase activity in exposed cells compared to solvent control. Each exposure was done in triplicates and individual experiments were performed three times for zfPXR-TL and ARα, and twice for cod ERα. Top exposure concentration was 30 mL WWEQ/mL for zf PXR-TL and cod ERα, and 15 mL WWEQ/mL for cod ARα. Dose-response curves were fitted by non-linear regression, with bars indicating SEM (GraphPad, PRISM v 7.0)

The final treatment plant that was examined was Flesland. Here, a slight activation in zf PXR-TL could be observed for both the in- and the out-sample. No activation was recorded in cod ARα and cod ERα for neither in- nor out-sample.

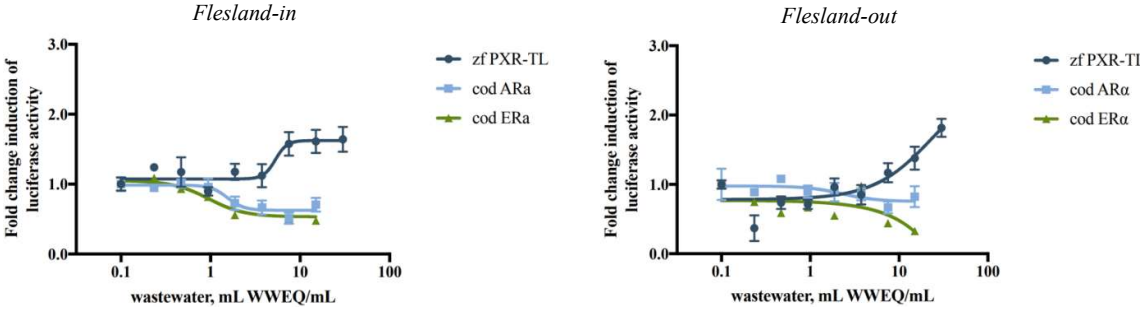


Figure 4.13 Ligand activation of zf PXR-TL, cod ARα, and cod ERα exposed to WW sample from *Flesland-in* and *Flesland*-out. Receptor plasmid, reporter plasmid ((MH100)x4tk-luciferase) and control plasmid (pCMV-β-galactosidase) were transfected into COS-7 cells. The cells were exposed to 7 concentrations of ww-extracts for 24 hr. Activation of ligand is shown as relative fold change in luciferase activity in exposed cells compared to solvent control. Each exposure was done in triplicates and individual experiments were performed three times for zfPXR-TL and ARα, and twice for cod ERα. Top exposure concentration was 30 mL WWEQ/mL for zf PXR-TL, and 15 mL WWEQ/mL for cod ERα and cod ARα. Dose-response curves were fitted by non-linear regression, with bars indicating SEM (GraphPad, PRISM v 7.0)

4.3.2 Antagonistic effects of wastewater extracts

As a multitude of pharmaceuticals act as antagonists on their target receptors, the antagonistic effects of the wastewater samples were also investigated. This was done with a constant background activation with 0.003 µM EE2 for ERα antagonism experiments, including all samples at all concentrations.

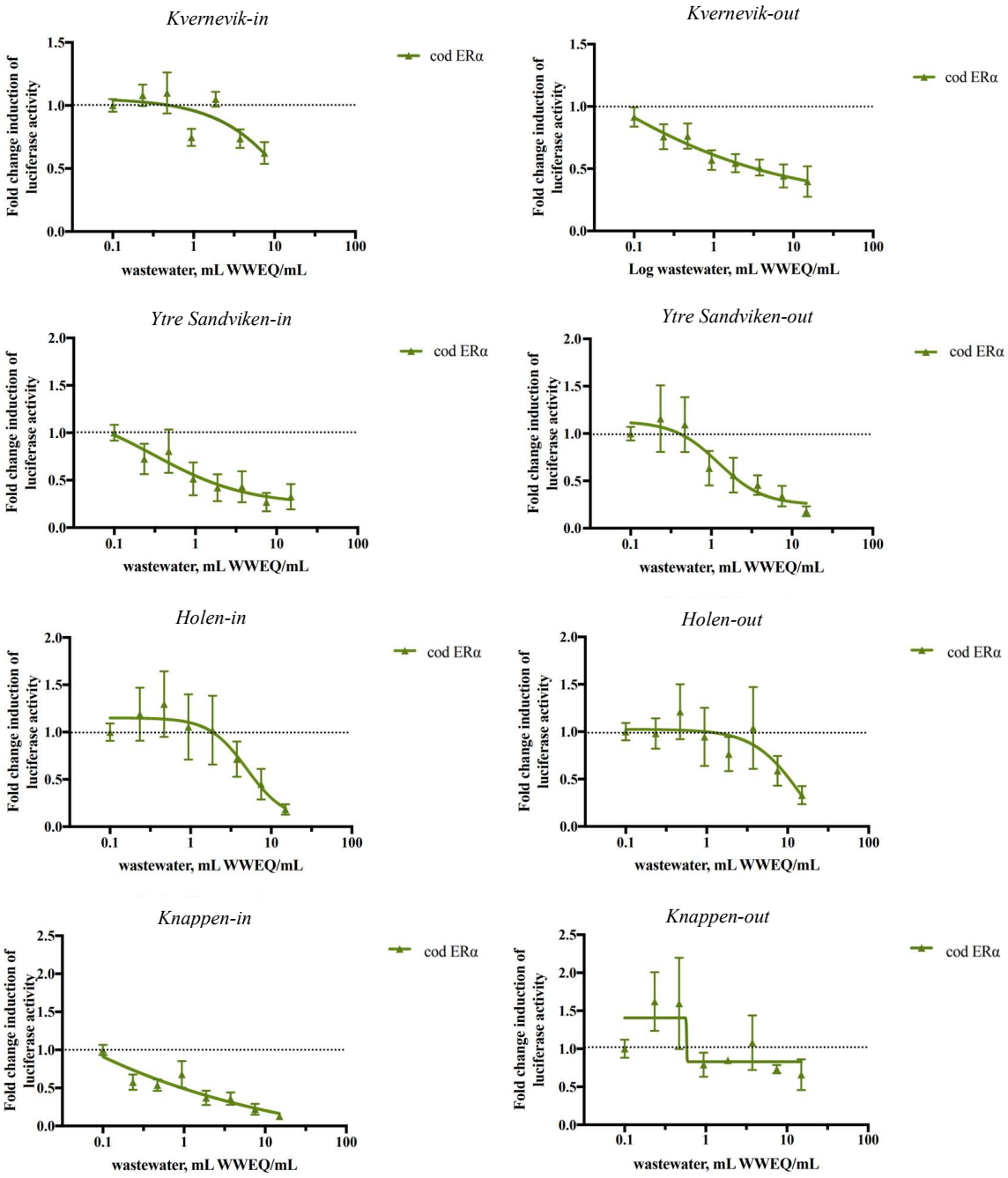


Figure 4.14 Ligand inhibition of cod ERα exposed to wastewater extracts. Receptor plasmid, reporter plasmid ((MH100)x4tk-luciferase) and control plasmid (pCMV-β-galactosidase) were transfected into COS-7 cells. The cells were exposed to a fixed concentration of 0.003 µM EE2 and 7 increasing concentrations of WW-extracts for 24 hr. Inhibition of EE2-mediated activation is shown as relative fold change in luciferase activity in exposed cells compared to solvent control. Each exposure was done in triplicates and individual experiments were performed two times. Top concentration 15 mL WWEQ/mL. Dose-response curves were fitted by non-linear regression, with bars indicating SEM (GraphPad, PRISM v 7.0). Extracts from Flesland were not included due to late arrival of samples.

For all stations, except *Knappen-out*, a clear trend in reduction of luciferase activity was observed at increasing concentrations of wastewater extracts (Figure 4.14). Highest significant inhibition (I_{max}) and for all WW extracts tested are summarized in Table 4.5.

4.3.3 Summary of ligand activation and inhibition of receptors exposed to wastewater extracts

Table 4.5 Maximum response (E_{max}) fold change and half maximal effective concentration (EC50) for receptors exposed to wastewater extracts. E_{max} (best fit value) with concentration (mL WWEQ/mL) and p-value indicating statistical significance.

	Receptor	St.	Maximum response (E_{max})	p-value	EC50
Wastewater samples	PXR-TL	*Kvernevik-in*	1.8	0.5681	4.2
		Kvernevik-out	6.3	<0.0001	6.2
		Ytre Sandviken-in	2.17	0.2033	3.1
		Ytre Sandviken-out	3.73	<0.001	6.6
		Holen-in	2.01	0.0084	~ 4.0
		Holen-out	2.32	0.002	9.4
		Knappen-in	1.58	<0.001	2.2
		Knappen-out	2.67	0.0122	5.4
		Flesland-in	1.62	<0.0001	~ 5.5
		Flesland-out	1.43	0.2002	7.5
	ARα	*Kvernevik-in*	nt*	nt*	nt*
		Kvernevik-out	0.99	0.0137	1.0
		Ytre Sandviken-in	1.98	0.2062	1.7
		Ytre Sandviken-out	3.31	0.1998	5.1
		Holen-in	1.65	0.002	~ 0.5
		Holen-out	1.80	0.0305	~ 3.6
		Knappen-in	2.72	<0.001	0.7
		Knappen-out	3.83	0.1868	0.7
		Flesland-in	0.99	0.2783	1.5
		Flesland-out	0.98	0.2002	1.5
	ERα**				

* nt = not tested

** No activation of ERα in any stations. See table 4.6 for antagonism.

Table 4.6 Maximum response (I_{max}) for cod ERα exposed to fixed concentration of EE2 and wastewater extracts. I_{max} (best fit value) with concentration (mL WWEQ/mL) and p-value indicating statistical significance.

	Receptor	St.	% Reduction compared to solvent control	I_{max}	p-value	IC50
Wastewater samples	ERα-antagonism	*Kvernevik-in*	38 %	0.62	0.4135	-
		Kvernevik-out	77 %	0.23	0.0013	~0.06
		Ytre Sandviken-in	76 %	0.24	0.2046	0.31
		Ytre Sandviken-out	75 %	0.25	0.0331	1.23
		Holen-in	93 %	0.07	0.1075	4.97
		Holen-out	67 %	0.33	0.0001	15.00
		Knappen-in	87 %	0.13	0.0167	0.05
		Knappen-out	17 %	0.83	0.0280	0.58
		Flesland-in	nt*	-	nt*	-
		Flesland-out	nt*	-	nt*	-

*nt = not tested

Radar charts were made to summarize and visualize the WW extract activation and inhibition of the selected receptors (Figure 4.15). *Kvernevik-out* stands out as the WW sample causing the highest activation in PXR, whilst *Knappen-in* and *Knappen-out* stands out as the WW samples with the highest androgenic activity.

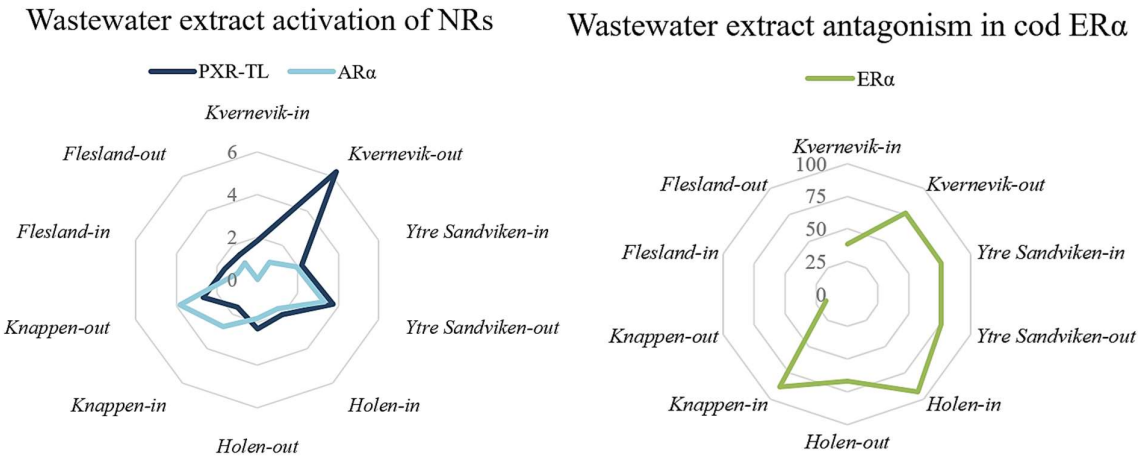


Figure 4.15 Radar chart summarizing receptor activation of PXR and ARα exposed to wastewater extracts (left) and inhibition of ERα (right). Activation: shown as fold change in luciferase activity compared to solvent control (1). Inhibition: shown as percentage reduction compared to solvent control (0%). A 100% reduction indicates high inhibition. Flesland samples not tested for ERα antagonism due to late arrival of extracts.

4.4 Receptor interactions of sludge sample extracts

COS7 cells were transfected with PXR, ARα or ERα to investigate whether compounds in sludge samples from four of the chosen treatment plants could induce agonistic effects. LRA was conducted following the same method as for wastewater samples.

No significant activation was observed for any of the receptors exposed to the sludge sample extracts (Figure 4.16), although a trend towards decreased activation was observed.

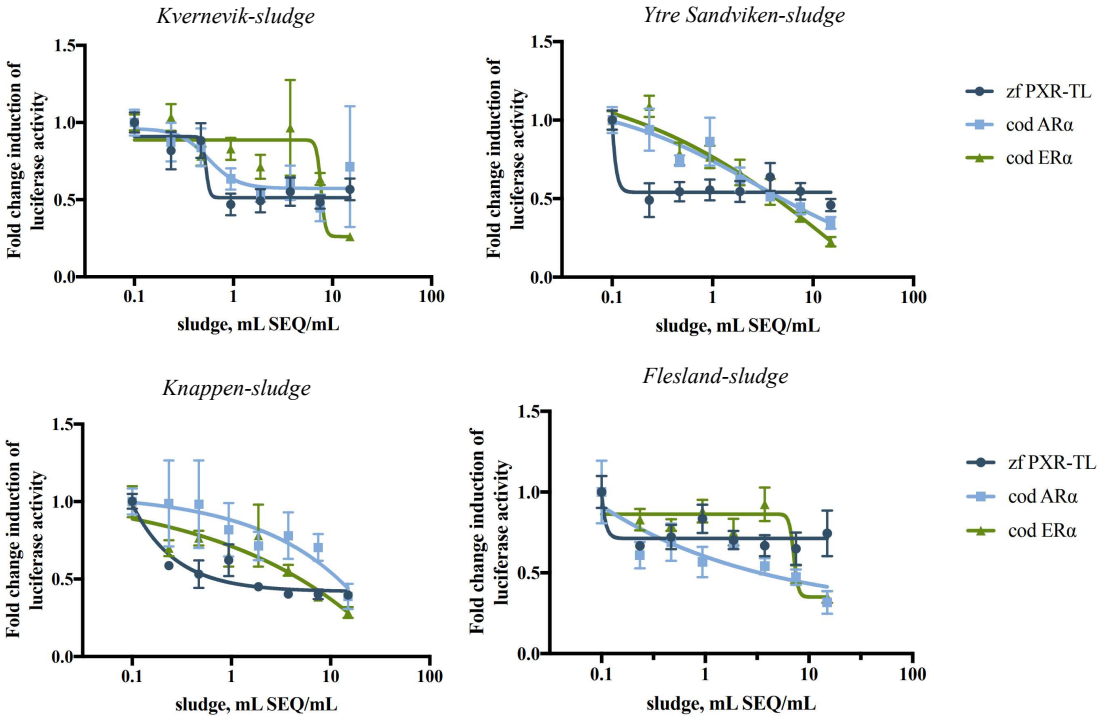


Figure 4.16 Luciferase reporter gene assay exposing zf PXR-TL, cod ARα, and cod ERα to sludge sample extracts from *Kvernevik, Ytre Sandviken, Knappen* and *Flesland* WWTPs. Receptor plasmid, reporter plasmid ((MH100)x4tk-luciferase) and control plasmid (pCMV-β-galactosidase) were transfected into COS-7 cells. The cells were exposed to 7 concentrations of sludge sample-extracts for 24 hr. Activation of ligand is shown as relative fold change in luciferase activity in exposed cells compared to solvent control. Each exposure was done in triplicates and individual experiments were performed two times. Top concentration was 15 mL WWEQ/mL. Dose-response curves were fitted by non-linear regression, with bars indicating SEM (GraphPad, PRISM v 7.0)

Figure 4.17 Summary of receptor activation and inhibition data by WW and sludge extracts. Shades from yellow to darker orange indicating increased E_{max} values, while green indicates no activation. Samples not tested marked as grey.

	PXR			ARα			ERα			ERα-antagonism	
	-in	*-out*	*-sludge*	*-in*	*-out*	*-sludge*	*-in*	*-out*	*-sludge*	*-in*	*-out*
Kvernevik											
Ytre Sandviken											
Holen											
Knappen											
Flesland											

* antagonism, orange indicates significant reduction in receptor activation, green indicates reduction but not statistically significant.

4.5 Chemical analysis of wastewater extracts

The targeted chemical analysis of the wastewater extracts detected acetaminophen, metoprolol, caffeine, prednisolone, carbamazepine, 5-h diclofenac, as well as the flame retardant TCPP and octocrylene (UV-filter) in varying concentrations (Figure 4.18, and Table 4.7). Chemical analysis results for *Kvernevik-in* and *Knappen-in* were inconclusive. The pharmaceutical detected at highest concentrations was acetaminophen, mainly detected at *Ytre Sandviken-in*, *Holen-in* and *Knappen-out*. Caffeine was the non-pharmaceutical detected most frequently with up to 46,779 ng/L in *Knappen -out*. Both caffeine and acetaminophen (Figure 4.19) appear to decrease in concentration in effluents compared to influents. Interestingly, carbamazepine and TCPP appears to have increased concentration in effluents compared to influents.

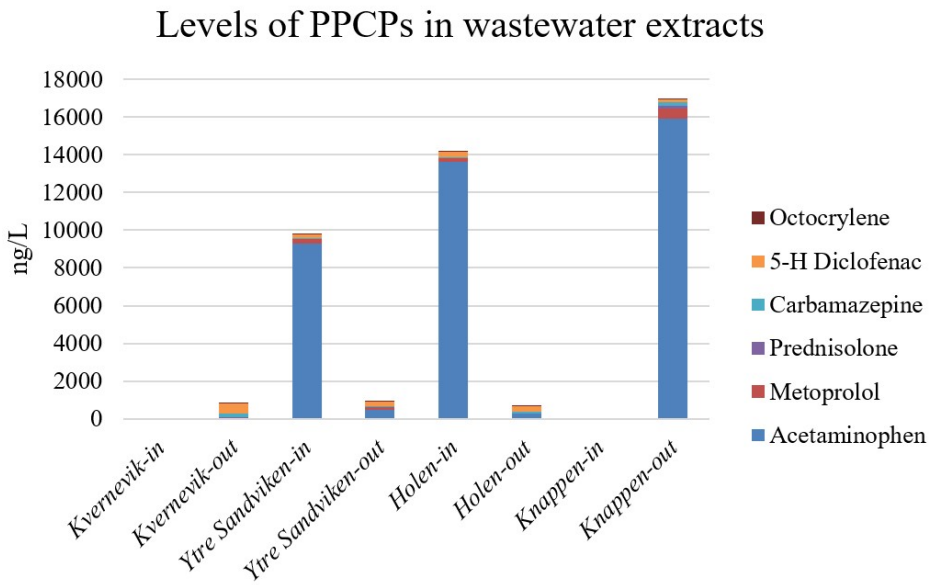


Figure 4.18 Levels of PPCPs in wastewater extracts. Concentrations of six compounds detected in targeted analysis. *Flesland* WWTP was not included due to late arrival of sample. Results for *Kvernevik-in* and *Knappen-in* were inconclusive.

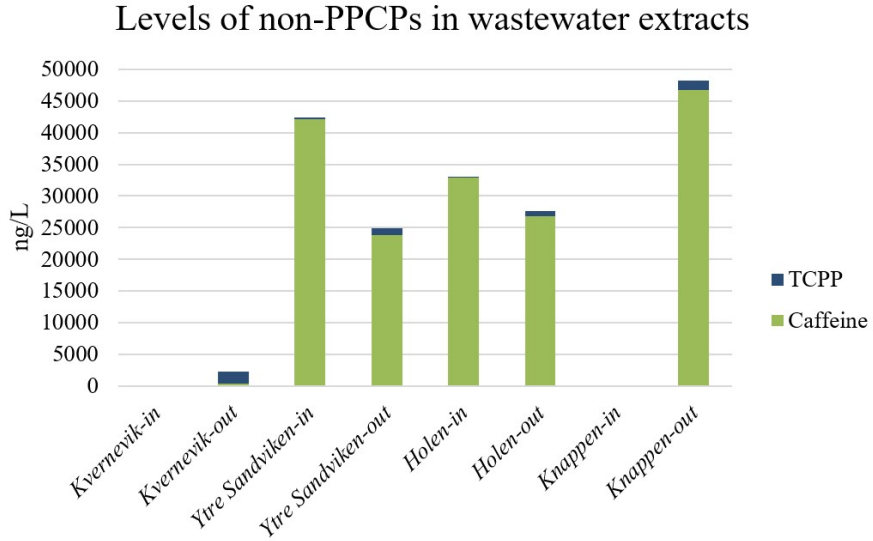


Figure 4.19 Levels of caffeine and TCPP in wastewater extracts from four WWTPs. Flesland WWTP was not included due to late arrival of sample. Results for *Kvernevik-in* and *Knappen-in* were inconclusive.

Table 4.7 Concentrations of compounds detected by targeted analysis in ng/L.

	Acetaminophen	Metoprolol	Caffeine	Prednisolone*	Carbamazepine	5-H Diclofenac	TCPP*	Octocrylene*	Total
*Kvernevik-in***									
Kvernevik-out	55	-	390	18	197	563	1817	45	3086
Ytre Sandviken-in	9311	239	42109	-	43	159	327	17	52206
Ytre Sandviken-out	461	139	23800	-	52	247	1080	20	25798
Holen-in	13604	209	32871	-	38	309	214	17	47261
Holen-out	298	-	26853	-	72	309	815	25	28371
*Knappen-in***									
Knappen-out	15907	519	46779	168	191	169	1465	15	65213
LOD	75	50	28	74	83	68	77	102	
LOQ	227	152	86	227	253	207	231	309	

* Instrumental carry-over for prednisolone, TCPP and octocrylene. See Appendix Table 9 and comment under 5.4 General comments about sampling and chemical analysis.

** Results for these stations were inconclusive

5 Discussion

5.1 Summary of the study

This thesis aimed to investigate how a luciferase reporter gene assay using nuclear receptors from zebrafish and Atlantic cod can be utilized in detecting and monitoring the potency of pharmaceuticals released from WWTPs in Bergen, Norway. COS-7 cells transfected with either zebrafish PXR-TL, cod ARα, or cod ERα were exposed to extracts from wastewater or sludge for 24 hours, followed by measurement and quantification of luciferase activity reflecting receptor activation or inhibition. Cytotoxic properties of the extracts were measured using a cell viability assay. Additionally, targeted chemical analysis of the extracts for a selected set of compounds was conducted at NMBU (by postdoc Ivo Havranek). Differences in cytotoxicity, potency, and receptor interactions between untreated and treated samples obtained from the various WWTPs, have been identified and explored in this thesis.

5.2 Cell toxicity caused by sample extracts

A cell viability assay was used to assess if the extracts were toxic to the cells. Cytotoxicity-assays using various cell lines, have previously been proven useful in detecting extracts inducing cytotoxicity corresponding to a pollution gradient in sediments (Blanco et al., 2018), as well as detecting cytotoxicity in WW samples (Stalter, Magdeburg, Wagner, & Oehlmann, 2011; Xiao, Araujo, Sze, & Stuckey, 2015).

The concentrations used in the cytotoxicity assay in this thesis correspond with concentrations used for the luciferase reporter gene assays (15 mL WWEQ/mL for ARα and ERα antagonism, and 30 mL WWEQ/mL as highest concentrations for PXR and ERα agonism). For the untreated wastewater samples, the average metabolic activity across all stations was reduced to 77 % compared to solvent control at 15 mL WWEQ/mL. At 30 mL WWEQ/mL this was decreased further to an average of 72 % for *Ytre Sandviken-in*, *Holen-in*, *Knappen-in*, and *Flesland-in*. The concentration corresponding to 30 mL WWEQ/mL from *Kvernevik-in* clearly stood out with a dramatically decreased relative metabolic activity to only 6.2 % compared to solvent control. In comparison, the treated wastewater sample extracts had a relative metabolic activity of 85 % and 86 % at 15 mL WWEQ/mL and 30 mL WWEQ/mL, respectively, indicating a clear improvement. For the sludge samples, the average relative metabolic activity at 15 and 30 ml WWEQ/mL was 87 % and 85 %, respectively.

Notably, the cytotoxicity of out-samples was lower than for the in-samples for all stations, indicating a removal of compounds producing cytotoxic responses. This difference in cytotoxicity in in- vs out-samples may also contribute in some extent to the differences seen in activation of the receptors as reduced metabolic activity and/or reduced cell membrane integrity may influence the cells.

The cytotoxicities produced by the influent samples are by themselves also interesting as several inhabitants in Norway are either not connected to wastewater treatment at all, or only to primary treatment plants. This means that there is a chance that cytotoxic effluents are being released into the environment continuously in several locations. An extensive review by Besse, Latour, & Garric (2012) found that a majority of anticancer drugs with cytotoxic properties detected in WW have only been tested *in vitro*, which makes it challenging to evaluate their environmental risk. Elevated levels of disinfectants and antibiotics in WW have also previously been linked to cytotoxicity in human hepatoma HepG2 cells *in vitro* (Giuliani, Koller, Würgler, & Widmer, 1996; Žegura, Heath, Černoša, & Filipič, 2009). However, it appears that linking the cytotoxicity detected in WW samples to environmental impacts requires further research.

In addition to metabolic activity, the cell viability assay also included a measurement of cell membrane integrity. The CFDA-AM is an esterase substrate that can permeate the cell membrane, and once inside the cell the compound is hydrolysed by intracellular esterases, which results in fluorescent metabolites. This process requires a stable cell membrane. Multiple in-samples and a few sludge samples produced

cell membrane integrity values at > 120 % (Figure 4.2). An increased cell membrane integrity appears unlikely. One possible explanation could be that compounds present in the extracts reacts in such a way that it appears to increase the fluorescent signal alone. However, in that case one could expect a steady increase corresponding to increased amount of extract added for the samples. This contradiction is highlighted by the sudden decrease in CFDA-AM at 30 mL WWEQ/mL for *Kvernevik-in*, indicating that the increased values depend on a certain level of metabolic activity. The source of the increase in cell membrane integrity values, whether a result of methodology issues or compounds present in the samples, is yet to be discovered.

5.3 Ligand exposure experiments

To assess if contaminants in wastewater and sludge extracts could induce activation or inhibition of ligand activated receptors from zebrafish and Atlantic cod, *in vitro* reporter gene assays were used. Seven out of ten wastewater extracts activated the zebrafish PXR, although to various extent (Table 4.5). Extracts from both untreated and treated wastewater in Ytre Sandviken WWTP, Holen WWTP, and Knappen WWTP activated cod ARα, while none of the wastewater extracts induced activation of cod ERα. Notably, a general trend of increased activation of receptors by treated samples compared to untreated samples was observed. For cod ERα antagonism studies, most extracts appeared to have antagonistic properties. Sludge sample extracts did not induce activation of any of the receptors tested.

5.3.1 Activation of zebrafish PXR

The control compound clotrimazole activated the zebrafish PXR as expected, with a fold change in luciferase activity of 17.5 (t-test, $p < 0.0001$) compared to solvent control. All samples produced an E_{max} value above 1,4, although *Kvernevik-in*, *Ytre Sandviken-in* and *Flesland-out* were not statistically significant (t-test, $p > 0.05$).

The stations activating zfPXR to the largest extent were *Kvernevik-out* ($E_{max} = 6.3$, $p < 0.0001$), *Ytre Sandviken-out* ($E_{max} = 3.7$, $p < 0.001$), and *Knappen-out* ($E_{max} = 2.6$, $p = 0.0122$). As previously mentioned, it has been shown for several species, including zebrafish, that PXR has multiple agonists (Appendix, Table 6). This complicates the possibility to link receptor activation and chemical analysis, particularly as the chemical analysis in this thesis has been targeted towards relatively few compounds, meaning that several other yet unknown compounds may be present in the extracts. However, the stations with the highest activations of zebrafish PXR correspond to the top three highest concentrations of TCPP with concentrations between 1000 ng/L to 1800 ng/L. TCPP (Figure 5.1) is a flame retardant used in polyurethane foam for isolation (in freezer-rooms, cold-rooms, et cetera), as well as in furniture and various consumer products. The compound has been found to have agonistic properties in human PXR *in vitro* (Kojima et al., 2013), and has by the Danish EPA been added to the List of Undesirable Substances (LOUS) due to its possible mutagenic and reproductive effects (Larsen, Andersen, Lam & Slothuus, 2014). Screening of several Norwegian wastewater plants in 2011 identified TCPP as the dominating organophosphate flame retardant in wastewater effluents with a mean of 600 ng/L, and a maximum concentration detected of 1,160 ng/L (Thomas et al. , 2011). This compound has also been reported in creeks (68.3 – 5102 ng/L), offshore regions (<LOD – 329 ng/L), and in various sediment samples in Korea (Lee, Cho, Choi, & Moon, 2018). A screening program conducted by Akvaplan-niva also detected TCPP in most of the fish species sampled, as well as in 11 out of 14 seabird liver samples from Kongsfjorden and Leifdefjorden, Svalbard, Norway in 2008 (Evenset, Leknes, Christensen, Warner, Ramberger, & Gabrielsen, 2009) indicating a ubiquitous presence of this compound in the environment.

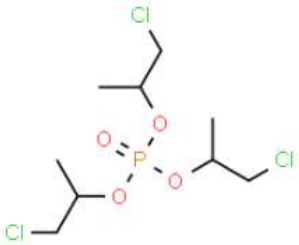


Figure 5.1 Chemical structure of TCPP. Illustration from ChemSpider.

Further research into possible activation of zebrafish PXR could contribute to a more definite answer of what compounds present in the extracts that are responsible for the activation. Nevertheless, the presence of relatively high levels of TCPP and the significant activation of zebrafish PXR indicates that compounds continuously released by WWTPs may pose a risk to marine environments.

5.3.2 Activation of cod ARα

The control compound testosterone activated the cod ARα with an E_{max} = 14.0 (t-test, $p < 0{,}0001$). Due to time constraints and limited sample volumes, cod ARα antagonism was not tested for the extracts, but only investigated for two compounds, i.e. the known antagonist flutamide and the compound bicalutamide. As flutamide, bicalutamide is an antiandrogen used in the treatment of hormone-sensitive prostate cancer (Berg, 2021). It is an androgen receptor antagonist by promoting inefficient recruitment of co-activators and thereby causing the transcription complex to be inactive (Kharlyngdoh, Pradhan, & Olsson, 2018). Flutamide decreased the testosterone-mediated ARα activation to 17 % compared to solvent control (t-test, $p < 0.0001$), demonstrating clear antagonistic properties. Bicalutamide also produced an antagonistic response at concentrations between 0.03 – 0.8 µM, decreasing the testosterone induced activation to 25 % compared to solvent control. At concentrations between 4 - 100 µM the antagonistic effects decreased, reaching 88 % activation at 100 µM. The reason for this biphasic response is unclear, but previous research has identified that the compound can acquire agonistic properties over time (Culig et al., 1999). The possibility of precipitation of bicalutamide at higher concentrations could also be investigated, although no precipitated material was observed with visual inspection during these experiments.

The stations inducing the largest activation of cod ARα were *Knappen-out*, *Ytre Sandviken-out*, and *Knappen-in* (Table 4.5). However, many environmental contaminants known to bind AR are antagonists blocking the binding of natural agonists such as testosterone (Luccio-Camelo & Prins, 2011). One such compound is octocrylene (Figure 5.2). Octocrylene is not a pharmaceutical, but a broad spectrum ultraviolet absorbing compound added to sunscreens and makeup, as well as some plastic packaging. Under *in vitro* conditions, the compound has been associated with some anti-estrogenic, but primarily, anti-androgenic activities in human AR (Kunz & Fent, 2006a). It has previously been detected in WWTPs in Norway with a median concentration in effluents from Tomasjord, Tromsø, of 2 167 ng/L, VEAS, Oslo, with 258 ng/L and HIAS, Hamar, with 158 ng/L, indicating that mechanical treatment alone (as in Tomasjord) is not sufficient for octocrylene removal (Langford, Reid, Fjeld, Øxnevad, & Thomas, 2015). The same study also found that 80% of cod livers sampled from Oslofjord had detectable levels of this compound. Research from 2015 investigated six coastal locations in South Carolina, USA, and found an annual average concentration ranging from 37.6 to 497 ng/L (Bratkovics, Wirth, Sapozhnikova, Pennington, & Sanger, 2015). The levels of octocrylene detected by chemical analysis in this thesis varied between 20 and 45 ng/L (Table 4.7). The highest detected concentration was 45.15 ng/L in *Kvernevik-out*, where a decrease in activation in the cod ARα agonistic assay could be observed (E_{min} = 0.74, $p = 0.0137$). However, antagonistic experiments are needed to confirm antagonism. Concentrations of 25 ng/L and 20 ng/L were detected for *Holen-out* and *Ytre Sandviken-out* respectively, where no significant activation in ARα was observed. *Knappen-in*, the station with the highest

significant activation (E_{max} = 2.7, $p < 0.001$) contained the lowest octocrylene levels measured (except for *Kvernevik-in* and *Knappen-in* where the chemical analysis was inconclusive), indicating a possible correlation between high levels of octocrylene and reduced activation of ARα. Compared to levels detected by Langford et al., 2015, the octocrylene levels in the samples collected in this thesis appear low. It should however be noted that a compound primarily used in sunscreen is likely to have strong seasonal variations. The samples collected and analysed in this thesis were taken in October, implying that the octocrylene usage might not be at its seasonal peak. Additionally, testing for antagonistic activities could contribute with knowledge on possible links between octocrylene and cod ARα activity.

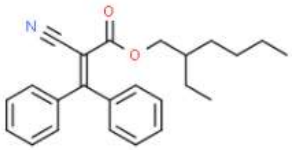


Figure 5.2 Chemical structure of octocrylene. Illustration from ChemSpider.

The activation seen for *Knappen-in* indicates the presence of compounds with agonistic properties in the wastewater, but the targeted chemical analysis conducted here did not identify any compounds described as AR agonists. Previous research has described androgenic activity in environmental samples (Bellet et al., 2012). One of the most important sources of EDCs from WWTPs are natural estrogens and androgens excreted from humans (Liu, Kanjo, & Mizutani, 2009). This correlates with previous research identifying testosterone, dihydrotestosterone and epiandrosterone as partly responsible for androgenic activities detected in WW samples (Bellet et al., 2012). The androgen androstenedione has recently been observed in both wastewater influent and effluent, and has been confirmed as an androgen receptor agonist (Zwart et al., 2020).

As highlighted by Bellet et al, 2012, natural androgens can explain some of the androgenic activity seen in wastewater, but not all. Sampling from three WWTPs in Norway in 2009 detected the flame retardant TBECH at concentrations between 5.4 (±0.5) (Drammen in) and 0,6 (±0.4) (Tromsø out.) ng/L (Nyholm, Grabic, Arp, Moskeland, & Andersson, 2013). Although at low concentrations, this compound has been identified as a potent human AR agonist, as well as inducing androgenic activities and causing negative effects on zebrafish physiology at higher concentrations (Pradhan et al., 2013). TBECH was not one of the compounds included in the targeted chemical analysis in this thesis. However, a possible connection between TBECH in wastewater and androgenic activity of environmental samples has previously been Suggested by Sørensen, 2020, who observed agonistic properties in sediment samples from Fisketorget/Vågen in Bergen in 2019, approximately 3 km from Ytre Sandviken WWTP (Goksøyr et al., 2021).

5.3.3 Activation and inhibition of cod ERα

The synthetic steroid EE2 was used as a positive control agonist, and activated cod ERα as expected, producing an E_{max} of 27.1 (t-test, $p < 0.0001$). Tamoxifen was used as a positive control for antagonism and decreased the EE2-mediated activation by 73% (t-test, $p = 0.0017$).

None of the WW samples were able to activate cod ERα (Figure 4.9 – 4.13, Table 4.5). This corresponds well with the results of the targeted chemical analysis where no known strong estrogenic compounds were found. However, estrogenic compounds such as estrone (E1), 17β-ethynylestradiol (EE2), and estriol (E3) (Liu et al., 2009; Välitalo et al., 2016) are known to be frequently detected in wastewaters worldwide, including in Norway (Thomas, 2007) . Research on eight WWTPs in Finland found that all wastewater effluents had estrogenic effects (Välitalo et al., 2016). However, a number of studies using the *in vitro* estrogen receptor reporter gene assay has detected antiestrogenic activities in environmental samples of wastewater (Conroy, Sáez, Quanrud, Ela, & Arnold, 2007; Ihara et al., 2014). Ihara et al (2014) compared the estrogenic activity to various compounds in wastewater and found that there seems to be a tendency for antiestrogenic compounds that can suppress the activity of natural estrogens,

ultimately indicating that luciferase assays reflect the net estrogenic balance of the samples. Such a possible masking-effect has also been reported in more recent research (Archer, Wolfaardt, van Wyk, & van Blerk, 2020). Altogether, the lack of receptor activation cannot be seen as an indication of no estrogenic compounds in the wastewater alone but should also be seen as an indication that there might be a net balance between estrogenic and antiestrogenic compounds. To assess if there was a net dominance towards antiestrogenic compounds, testing extracts with a background activation of ERα with EE2 was conducted.

Possible antagonism caused by the extracts was measured by using the luciferase reporter gene assay with cod ERα and a fixed background activation with EE2. *Kvernevik-out*, *Ytre Sandviken-out*, *Holen-out* and *Kvernevik-in* were identified as stations causing statistically significant reductions in luciferase activity compared to solvent control. Compounds detected with antiestrogenic properties in the chemical analysis are octocrylene (also an antiandrogen, mentioned in 5.3.2) and diclofenac (Ezechiáš, Janochová, Filipová, Křesinová, & Cajthaml, 2016). Diclofenac (sold under names such as Voltaren and Voltarol in Norway) is an analgetic and anti-inflammatory drug which works by being a non-selective inhibitor of COX1 and COX2-enzymes (Reiter, 2017). It may also inhibit the PPARγ receptor (Gan, 2010). 5-H diclofenac is one of three metabolites of diclofenac in humans and its production is catalysed by CYP2C and CYP3A4 enzymes. Research suggests that diclofenac (and ibuprofen, a widely used non-steroidal anti-inflammatory drug) act as inhibitors of the estrogen receptor pathway *in vitro* (Ezechiáš et al., 2016). More research is needed to identify the definite cause of cod ERα inhibition produced by these extracts, especially in terms of metabolite binding potencies as well as in terms of differences between the treatment methods. The detection of 5-H diclofenac is nevertheless interesting.

The concentrations at which 5-H diclofenac was detected varied between 170 (*Knappen-out*) and 560 ng/L (*Kvernevik-out*), which correspond to levels previously measured in Norway. 5-H diclofenac has formerly been detected at 551 ng/L in Tromsø and 675 ng/L in Oslo (Langford & Thomas, 2011). In seawater specifically, diclofenac concentrations have been reported at 1-4 ng/L (Longyearbyen, Norway) (Kallenborn, Brorström-Lundén, Reiersen, & Wilson, 2018) and up to 241 ng/L on the Northwestern Portuguese coast (Lolić et al., 2015). An extensive review on detected diclofenac concentrations in water, sediments and organisms from 2018 identified a scarcity of research on the occurrence and possible effects of diclofenac in the marine environment (Bonnefille, Gomez, Courant, Escande, & Fenet, 2018). Other commonly detected anti-estrogens in wastewater includes tamoxifen, with levels up to 180 ng/L (Negreira, de Alda, & Barceló, 2014). Research using both *in vitro* and *in silico* modelling identified additionally the anti estrogens raloxifene and bazedoxifene acetate as pharmaceuticals in wastewater with potential to reach fish tissue at relevant concentrations (Pinto, Bloom, & Laurenson, 2019).

5.3.4 Activation of cod GR

Dexamethasone is a known human GR agonist and was used to test if the compound could activate cod GR as well. At 100 µM, an E_{max} of 6.3 was observed, demonstrating the compound's ability to act as a cod GR agonist. Similarly, prednisolone was also tested and produced an E_{max} of 13.3 at 100 µM. Cod GR was not used in testing of wastewater and sludge extracts in this thesis due to time constraints and limited extract volumes. The demonstrated ability of cod GR to bind commonly used pharmaceuticals does nevertheless propose that this receptor might be useful and relevant in future environmental research and monitoring. GR may be particularly interesting in testing of sludge samples, as recent research has detected glucocorticoid activity in all sludge samples from Australian WWTPs (Papa, Dogruer, Bailey, & Leusch, 2022).

5.3.5 Compounds detected by chemical analysis

In addition to the abovementioned compounds, acetaminophen, metoprolol, prednisolone, carbamazepine, and caffeine were detected by the targeted chemical analysis of the WWTP samples.

Acetaminophen (also known as paracetamol) is an analgetic (pain relieving) and antipyretic (fever-reducing) drug. Its mode of action is not completely understood, but it works partly by inhibiting the enzyme COX-2 (cyclooxygenase – 2) and stimulates pain relief by increasing the levels of serotonin in the central nervous system (Spiering, Lisa, Dietrichs, 2020). Although debated, its primary site of action is believed to be the activation of cannabinoid receptors via one of its metabolites (Klinger-Gratz et al., 2018). The compound has been detected in concentrations between 12 – 9,600 ng/L (lagoon, Spain), and between <20 and 930 ng/L in recipient waters in Greenland, Iceland, and the Faroe Islands (Huber et al., 2013; Moreno-González et al., 2014). Influent samples from VEAS WWTP, Oslo, contained paracetamol at the highest maximal concentration of 43,000 ng/L and a median concentration of approx. 3 500 ng/L, with a median in effluent samples of 31 ng/L (Thomas, 2007). The levels detected in samples from the five WWTPs in Bergen ranged from 55 ng/L (*Kvernevik-out*) to 16,000 ng/L (*Knappen-out*). The general trend showed decreasing levels in out-samples compared to in-samples, although in-samples from Kvernevik and Knappen were inconclusive.

Metoprolol (Selo-Zok®) is one of multiple pharmaceuticals that block β-adrenergic receptors, and it works directly on the heart by specifically blocking the β_1-adrenergic receptors. This results in a reduced heartbeat frequency and hence lowers the blood pressure (Alrawaf, 2019). The levels detected in samples from the five WWTPs in Bergen ranged from not detected to 520 ng/L in *Knappen-out*. These levels are comparable to what has previously been detected elsewhere. Metoprolol was identified in all samples, ranging from 51 to 810 ng/L in WW effluents investigated in Greenland Iceland and the Faroe Islands in 2013 (Huber et al., 2013). In Mediterranean sewage water the compound has been detected at about 200 ng/L (Desbiolles, Malleret, Tiliacos, Wong-Wah-Chung, & Laffont-Schwob, 2018).

Prednisolone is a metabolite of the synthetic glucocorticoid prednisone. It is used in treatment of rheumatic diseases, inflammatory bowel disease, severe asthma, and chronic obstructive pulmonary disease (COPD), and as a part of a cancer treatment (Solsvik, 2019). Prednisolone is highly effective at treating inflammation through its binding to the glucocorticoid receptor. The compound has previously been detected in wastewater influents in concentrations between 315 – 1918 ng/L in The Netherlands (Schriks et al., 2010). The levels detected in samples from the five WWTPs in Bergen were for all stations below limit of detection except in *Kvernevik-out* with 18.08 ng/L and *Knappen-out* with 168 ng/L.

Carbamazepine (Tegretol/Trimonil) is a sodium channel blocker used in treatment of epilepsy, specific types of nerve pain, in treatment of bipolar disorders, and alcoholism. It is also a potent inhibitor of the muscarinic and nicotinic acetylcholine receptors, N-methyl-d-aspartate (NMDA) receptors, and adenosine receptors in the central nervous system (Lo, 2014). Carbamazepine was detected in concentrations between 37 ng/L (*Holen-in*) to 200 ng/L (*Kvernevik-out*). Previous research has detected carbamazepine at a mean concentration of 393 ng/L and 294 ng/L in treated wastewater samples from VEAS, Oslo, and Breivika, Tromsø, respectively (Langford & Thomas, 2011). Additionally, the compound was identified in 37 out of 43 samples in coastal and offshore seawater sampled in the Baltic Sea with a median concentration of 2.6 ng/L (Björlenius et al., 2018). The same authors investigated WWTPs in Denmark, Finland, France, Germany, Great Britain, Japan, Poland, Spain, and Sweden, and found that the removal efficiency of carbamazepine varied from 31 - 41 % with more than half of the WWTPs having higher levels of carbamazepine after treatment compared to before treatment. One explanation suggested by the authors of the article is the deconjugation of the compound that might happen through the treatment process (Björlenius et al., 2018). As described in the introduction (1.1.1, Pharmaceuticals), many pharmaceuticals are excreted from our bodies in a conjugated form. Throughout the treatment process, deconjugation may occur due to the presence of bacteria such as *E. coli*, which

exhibits enzymatic activity, and thereby an "increase" the concentrations of the original compound in the effluents can be observed (Gomes, Scrimshaw, & Lester, 2009; Verlicchi, Al Aukidy, & Zambello, 2012). This correlates well with the findings in this master thesis where an increase in carbamazepine was observed in both Ytre Sandviken WWTP (+19.4 %) and Holen WWTP (+90.8%). Carbamazepine is also known for its long half-life in the environment with 82 +/- 11 days (Sanderson et al., 2004).

Caffeine is a compound naturally present in coffee (about 150 mg/cup) and tea (about 50 mg) and is added to various energy drinks and supplements. Caffeine is a stimulant which increases brain activity and decreases drowsiness (Bjørneboe & Johansen, 2021). These effects come as a result of various biochemical processes, but mainly as a result of the caffeine being an antagonist to adenosine receptors (A1, A2A, A2B, and A3), which blocks the binding of adenosine in the nervous system ("Kaffein," 2018). Some coffee and tea enter the wastewater system simply by being discharged. In addition, about 5 % of ingested caffeine is excreted without being metabolized (Magkos & Kavouras, 2005). The levels detected in samples from the five WWTPs in Bergen ranged from 390 ng/L (*Kvernevik-out*) to approx. 47.000 ng/L (*Knappen-out*). In comparison to previously detected levels in wastewater (e.g. 2.000-293.000 ng/L in Tromsø (Weigel et al., 2004)), the concentrations are as expected. Levels of caffeine detected in the environment in Europe has been found in concentrations up to 17.400 ng/L in Romania (Gheorghe, Petre, Lucaciu, Stoica, & Nita-Lazar, 2016), 857 ng/L in Spain (Dafouz et al., 2018), and ranging between 7 – 87 ng/L in seawater in Tromsø, Norway (Weigel et al., 2004).

5.3.6 Sludge samples

None of the sludge samples extracts activated PXR, ARα or ERα. A decrease in luciferase activity was observed for many for the receptors at increased concentrations of the extracts, indicating that assessment of antagonism could provide useful information. This was planned, but not conducted due to late arrival of extracts to our laboratory. Chemical analysis of sludge samples was also not performed due to limited time.

Prediction of distribution of compounds in different environmental compartments is commonly based on Log K_{ow} values, indicating the relationship between the lipophilicity and hydrophilicity of a substance (Hendriks, van der Linde, Cornelissen, & Sijm, 2001). However, some research has found that models based on K_{ow} for environmental fate of pharmaceuticals might not be suitable for prediction of levels in soils and sludge (Lindberg, Fick, & Tysklind, 2010; Tolls, 2001) emphasizing the need for chemical analysis.

Compounds frequently detected in European sludge samples do however include compounds such as diclofenac (1.3 – 429.1 ng/g, detected in 81 % of trials), and ibuprofen (0.2 – 108.2 ng/g, detected in 42.4 % of trials) (Fijalkowski, Rorat, Grobelak, & Kacprzak, 2017). Diclofenac, as previously described, is a known ERα antagonist, and ibuprofen act as an agonist on human PPARγ (Cosgrove et al., 2021). Recent research has also detected glucocorticoid activity in all sludge samples from Australian WWTPs (Papa et al., 2022), emphasizing that the glucocorticoid receptor might be an interesting receptor for investigation of biological activity in sewage sludge samples. Additionally, antimycotics such as the PXR agonist clotrimazole (Svecova et al., 2008) and the PXR antagonist ketoconazole (H. Huang et al., 2007) has been detected in all sludge samples investigated and measured at concentrations between 60 and 1800 µg/kg dry weight in six Swedish WWTPs (Lindberg et al., 2010). The same study concludes that the major route of antimycotics into the environment is through sludge used as fertilizer or landfill material.

The sludge from the investigated WWTPs in Bergen is used in biogas production, and the result is biogas digestates that are commonly used as fertilizers and soil for green areas in connection with infrastructure development (Statens vegvesen, 2019). Research on biogas digestates from several Norwegian production plants has found that the processes within biogas production facilities does not eliminate the

compounds investigated (Ali et al., 2019). Octocrylene and TCPP was even found at levels exceeding the upper limit of quantification threshold (>600 ng/g wet weight, and > 500 ng/g wet weight, respectively)(Ali et al., 2019).

Ultimately, this emphasises that sludge and digestate samples must be included when assessing the possible environmental risk of pharmaceuticals derived from wastewater, since what appears as a removal when comparing in-and out-samples might just be a channelling of compounds from WW to sludge.

5.3.7 Summary of luciferase reporter assay and targeted chemical analysis findings

By summarizing the maximal activations (E_{max}) and inhibitions (I_{max}) observed by the different wastewater extracts, the environmental quality of the various influents and effluents can be assessed. The luciferase assay data partly correspond to the chemical analysis where *Kvernevik-out* showed the highest concentrations of several compounds (Table 4.7, Figure 4.19). Solely evaluating the removal efficiencies between the WWTPs in terms of chemicals detected, Knappen WWTP stands out. Although chemical results for *Knappen-in* were inconclusive, the results from *Knappen-out* identifies it as the only effluent samples where significant amounts of acetaminophen, metoprolol and caffeine are detected. *Knappen-out* also had the highest total concentration of compounds (Table 4.7) Knappen WWTP is the only treatment plant with solely chemical treatment, indicating that biological treatment might be more effective in removal of certain compounds than chemical treatment alone. This is supported by reviews summarizing knowledge on various treatment methods reporting that EDCs and PPCPs to a large extent gets removed through activated sludge and in membrane bioreactors (MBRs)(Ahmed et al., 2017; Hamid & Eskicioglu, 2012).

Initially, multiple samples from the WWTPs were collected, as several samples are needed to distinguish brief and random variations to differences caused by the treatment methods. Although all samples were based on a 24-hour sampling pool, this suggests that differences seen between the wastewater treatment plants could be random and limited for the one sampling day. The in- and out-samples are not taken with the retention time within the treatment plants in mind, although the composition is believed to be relatively representative due to the large masses of water within the plants. Unfortunately, limited time has resulted in only one sampling from each WWTP being analysed in this thesis. For details on what this implies, see 5.4. General comments about sampling and chemical analysis.

5.3.8 Significance of the findings in an Adverse Outcome Pathway perspective

The interaction between a ligand and a receptor is a key event in the adverse outcome pathway (AOP), as described in the introduction. The findings of this thesis illustrate the ability of compounds in both untreated and treated wastewater to bind to nuclear receptors which have important roles in xenobiotic metabolism, reproduction, and development.

PXR is the main transcriptional regulator of CYP3A, which catalyses the first step in the detoxification of xenobiotics (Moore et al., 2002). Induction of CYP3A has also been identified following exposure of clotrimazole to zebrafish PXR, suggesting resemblances in response pathways in zebrafish and mammals (Bresolin, De Freitas Rebelo, & Dias Bainy, 2005). Detoxification of xenobiotics is needed and highly useful for the organism (as described in Introduction 1.1.1) although activation of PXR may cause unwanted side effects as activation of PXR results in upregulation of e.g. transferases, increased biliary excretion of the thyroid hormones T3 and T4, which alters the thyroid hormone system critical in normal development (Brucker-Davis, 1998; D., M., C., & Thomas, 2009; Schuetz, Brimer, & Schuetz, 1998). Teleost thyroid follicles has been found to produce the same thyroid hormones (T3 and T4) as other vertebrate species, including humans (Shkil, Siomava, Voronezhskaya, & Diogo, 2019), indicating an environmental significance. Recent research has also identified activation of PXR as the molecular

initiating event of flame retardant-induced dose-dependent lipid accumulation and mitochondrial dysfunction in human liver cells *in vitro* (Negi, Bajard, Kohoutek, & Blaha, 2021). These factors do altogether suggest that activation of PXR may lead to both cellular and organ responses, although more research is needed to identify if the compounds and activation seen in this thesis are substantial enough to cause organ or organism responses in the environment.

The AR has, as mentioned in the introduction several important roles such as in development and maintenance of the reproductive system (Davey & Grossmann, 2016). Exposed fish to ng/L concentrations of the androgen 17β-trenbolone has been shown to cause changes in endocrine function in both short term and long term exposures, including skewed sex ratios and reduced fertility (Ankley et al., 2018). River waters with very high androgen levels (due to pig farm wastewater input) have been found to cause adverse effects such as masculinization and inhibited vitellogenin levels in female mosquitofish (G.-Y. Huang et al., 2019), and the androgen trenbolone has been identified as a cause of irreversible masculinization of zebrafish at environmentally relevant concentrations (Morthorst, Holbech, & Bjerregaard, 2010). The Atlantic cod androgen receptor activation produced by WW from some of the stations in this thesis suggests that further research is needed to establish if any potential effects on fertility and development could be caused by wastewater on this species specifically.

Vitellogenin has for long been a standard biomarker for estrogenic activity in oviparous vertebrates, as the protein should be detected at negligible levels in males and immature females (Arukwe & Goksøyr, 2003; Oberdörster & Cheek, 2001; Sumpter & Jobling, 1995) and any increased vitellogenin production indicates exposure to estrogenic compounds. No estrogen receptor activation was detected in the samples from the five WWTPs in this thesis, although multiple extracts showed antagonistic properties. One of the compounds with suggested antiestrogenic properties is octocrylene. Exposure of zebrafish to octocrylene revealed accumulation of the compound, and a subsequent microarray analysis identified changes in genes involved in developmental processes, organ development, fat cell differentiation, and metabolism (Blüthgen, Meili, Chew, Odermatt, & Fent, 2014). A search of relevant literature for octocrylene and other potential estrogen receptor inhibitors for Atlantic cod estrogen receptor yielded no related articles. Further research linking estrogen receptor inhibition in Atlantic cod to cellular and organ responses could be highly useful.

Although several of the wastewater effluent samples induced significant changes in some receptors, one should bear in mind that the likelihood of the WW extract compounds reaching its target site within an organism in these concentrations is low. Several abiotic factors influence the effluents when released, such as dilution (Weigel et al., 2004), photodegradation (Andreozzi, Raffaele, & Nicklas, 2003; Buser, Poiger, & Müller, 1998; Fent, Weston, & Caminada, 2006), and temperature (Vieno, Tuhkanen, & Kronberg, 2005). This thesis illustrates how a mixture of compounds may interact with receptors at a molecular level, illustrating the possibility for a molecular initiating event, given that an organism is exposed, the compound is absorbed and distributed to the cite of interaction.

Combined with the elevated cytotoxicity for several of the samples, these findings highlight the importance of authorities evaluating biological potencies, as well chemical composition, when assessing the environmental impact of released WW. The information provided in this thesis could also contribute to knowledge useful when assessing the removal efficiencies of different WWTPs in Bergen.

5.3.9 Comments to the luciferase reporter gene assay

The luciferase reporter gene assay is a commonly used method to investigate ligand activation of nuclear receptors *in vitro*. A Gal4/UAS based system was used in this master thesis, which enables the investigation of interactions between the ligand and nuclear receptor without PXR being dependent on the presence of RXR.

The cell line chosen for this thesis (COS-7), was selected based on its properties as robust and easy to work with. The endogenous expression of the receptors studied are low as COS-7 cells are cells derived from the kidneys, generally regarded as a non-steroidogenic organ. This implies that the receptor activation seen in the experiments would be minimally influenced by the cell line itself. However, different cell lines may have various expression of cofactors available necessary to initiate transcription, and some research has disputed the non-steroidogenicity of the COS-7 cells (Nozaki et al., 2018). This implies that a different response of the zebrafish and Atlantic cod receptors in their natural cells cannot be ruled out.

Comments made about anti-estrogenic compounds masking for estrogenic properties of the wastewater may be relevant for all receptors, implying that the reporter gene assay results should be read as a net receptor activation. What may appear as a "non-significant response" may in fact be a balance between agonist and antagonists in the wastewater sample.

5.4 General comments about sampling and chemical analysis

The samples analysed are from one day only and reflects the biological activity and chemical properties of a limited time period. A significant variation in compounds detected over time has been demonstrated by previous research, exemplified by a transient increase in discovered anabolic steroids and weight loss products in WW following a sports event (Causanilles et al., 2018). Likewise, concentrations of drugs such as cocaine and MDMA has been found to vary significantly between various locations as well as throughout a week (Löve et al., 2018). Compounds used in sunscreens and insect repellents are also more likely to be present in the summer months compared to the autumn (when the samples in this thesis was collected) (Ekpeghere, Kim, Kim & Oh, 2016; Knepper, 2004) These factors as well as differences in retention-time within treatment plants, weather conditions and shifts in demographics, highlights the importance of multiple samples taken throughout a year. Flesland samples can also only to a limited extent be compared to the other samples as this was taken two weeks later and on a different weekday than the others.

The stability of certain analytes in WW has been shown to be influenced by storage temperature, and certain compounds have been found to undergo significant degradation even at low temperatures. At -20°C, the compound clomiphene (antiestrogen) has been observed to have a 25 % decrease after 3 days illustrating the importance of immediate freezing and quick analysis of wastewater samples (Causanilles et al., 2018). For the Flesland samples and sludge samples this may be of particular relevance as they were sampled at a later point, and were stored for approximately 5 months at -20 °C.

Multiple pharmaceuticals are also easily degradable by light. All samples have been treated as light sensitive and have been shielded from light as far as possible, but some light degradation of the compounds cannot be excluded.

The HLB disk method used (at NMBU) in this thesis is frequently used as a method to extract pharmaceuticals from water and wastewater samples (Česen & Heath, 2017; Neale, O'Brien, et al., 2020; Yuan, Jiang, Xia, Zhang, & Zheng, 2013). Variations in recovery between various compounds may however influence the results. Previous research on recovery of various compounds (e.g. UV filters) revealed recoveries varying between 79% and 118 % (Ekpeghere et al., 2016) Recoveries may also influence the LRA and cytotoxicity assays, as several compounds in the wastewater might not be efficiently extracted by the extraction method in the same extent as others. This could influence the final concentrations in the extracts used for exposure experiments.

Shortly before the deadline for handing in the thesis, I was made aware that instrumental carry-over and contamination had occurred for several compounds. Had this been noticed earlier, the average blank sample contamination could have been subtracted from the reported levels in the results section making the reported concentrations more accurate. A table (Table 9) has been added in appendix with the

average contamination in blank samples. The specific compound concentrations reported in this thesis should therefore be interpreted with caution. Instrumental carry-over does however not influence the levels of compounds in the extracts used for LRA and cytotoxicity assays.

5.5 Conclusion

By extracting, analysing, and exposing COS-7 cells transfected with zebrafish PXR-TL, cod ARα and cod ERα to wastewater and sludge sample extracts from five different WWTPs in Bergen, Norway, several environmentally valuable discoveries were made. Multiple WW extracts significantly activated zebrafish PXR, and WWTP effluents did so to a larger extent than influents. Some extracts also activated cod ARα, whilst a majority of WW samples showed antagonistic properties towards cod ERα. No activation was detected in extracts from sludge samples. The use of LRA has uncovered differences in biological potencies in influents and effluents, as well as contributed with knowledge about differences between various WWTPs.

By utilizing nuclear receptors from zebrafish and Atlantic cod, these findings also highlighted the possible adverse consequences that chronic release of wastewater might pose to aquatic wildlife. This is relevant information in an environmental management perspective.

Direct correlation to compounds identified by chemical analysis was not obvious but the different approaches contributed to accentuate separate aspects and properties of wastewater-carried compounds, including detecting significant cytotoxic properties of extracts where the targeted chemical analysis was unable to do so. Furthermore, differences in compound and toxicity removal and activation were found for the individual treatment plants, although more research is needed to establish the significance of treatment method specifically. The singular sampling date, the targeted chemical analysis, and the three receptors chosen, do however only provide a narrow time specific, receptor specific, and compound specific image of the possible biological effects of substances present in the wastewaters released.

5.6 Suggestions for future work

The targeted chemical analysis aimed for specific compounds, meaning that a multitude of unidentified compounds are probably present in the extracts. A non-targeted analysis would provide a more complete picture of the compounds released as a wide variety of unknown compounds could be detected (Sobus et al., 2018). Furthermore, an effect directed analysis (EDA) where biological analysis, fractionation of extracts, and chemical analysis are combined, could be utilized to identify the key compounds responsible for the biological effects (Brack, 2003; Hong, Giesy, Lee, Lee, & Khim, 2016; Samolloff et al., 1983; Schuetzle & Lewtas, 1986).

This thesis focused on a set of three different nuclear receptors from zebrafish and Atlantic cod, although multiple other receptors, such as Ahr (Aranguren-Abadía et al., 2020; Tanguay, Abnet, Heideman, & Peterson, 1999) and PPARs (Marta Eide et al., 2018; Ibabe, Grabenbauer, Baumgart, Fahimi, & Cajaraville, 2002) are present in both species. As a large amount of pharmaceuticals also target G protein-coupled receptors (GPCRs), and mixtures of pharmaceuticals in wastewater have been found to behave additively in GPCR assays (Zhang, Ihara, Nakada, Tanaka, & Ihara, 2020), research on these receptors could also provide useful additional information. Ultimately, including more receptors in such analyses could point to biological responses not seen in this thesis.

The bioassay used is also based on *in vitro* methods. This means that one cannot conclude that the receptors investigated in this thesis would have been activated *in vivo*. Utilizing *ex vivo* methods, e.g. precision cut liver slices, would make it possible to investigate receptor activation in intact Atlantic cod cells (Bizarro, Eide, Hitchcock, Goksøyr, & Ortiz-Zarragoitia, 2016; Eide, Karlsen, Kryvi, Olsvik, & Goksøyr, 2014; Yadetie et al., 2018). Additional *in vivo* methods such as caging of cod in effluent

recipients (Dale et al., 2019) could also contribute in providing valuable links between the molecular initiating events established in this thesis and consequences at higher biological levels.

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Appendix

Table 1. Known AR agonists (non-exhaustive)

Compound name	Species*	Reference
Testosterone	Human, zebrafish	(Øye, Ivar, Borén, 2018)(Jørgensen, Andersen, Bjerregaard, & Rasmussen, 2007)
Dihydrotestosterone (DHT)	Human	(Øye, Ivar, Borén, 2018)(Jørgensen et al., 2007)
Nandrolone	Human	(Legemiddelhåndboken, 2015c)
Chlormadinone acetate	Human	(Siegenthaler, Bain, Riva, & Fent, 2017)
TBECH	Zebrafish	(Pradhan et al., 2013)
Trenbolone	Human	(Y. Park et al., 2021)
Dexamethasone	Human	(Y. Park et al., 2021)
Nandrolone	Human	(Y. Park et al., 2021)
Androstenedione	Zebrafish	(Jørgensen et al., 2007)
11 – Ketotestosterone	Atlantic salmon, rainbow trout, zebrafish	(Imamichi et al., 2016)(de Waal, 2008)(Hossain, Larsson, Scherbak, Olsson, & Orban, 2008)

* this list does not consider the differences in binding affinity to AR between species.

Table 2 Known AR antagonists (non-exhaustive)

Compound name	Species*	Reference
Cyproterone	Human	(Legemiddelhåndboken, 2016)
Bicalutamide	Human	(Legemiddelhåndboken, 2016)
Flutamide	Human, zebrafish	(Legemiddelhåndboken, 2016)(Martinović-Weigelt et al., 2011)
Bisphenol A	Fathead minnows, human	(Ekman et al., 2012)(C. Park et al., 2020)
Bisphenol S	Human	(C. Park et al., 2020)
Bisphenol F	Human	(C. Park et al., 2020)
Benzophenenone-3	Zebrafish	(Schreurs et al., 2005)
3-benzolidene champor	Zebrafish	(Schreurs et al., 2005)
Homosalate	Zebrafish	(Schreurs et al., 2005)
Triclosan	Human	(Ahn et al., 2008)
Octocrylene	Human	(Kunz & Fent, 2006b)
Ketoprofen	Human	(Ezechiáš et al., 2016)
Diclofenac		
ibuprofen		

* this list does not consider the differences in binding affinity to AR between species.

Table 3. Known estrogen receptor agonists (non-exhaustive)

Compound name	Species*	Reference
Benzophenone-3	Zebrafish	(Schreurs et al., 2005)
3-benzolidene champor	Zebrafish	(Schreurs et al., 2005)
Homosalate	Zebrafish	(Schreurs et al., 2005)
E2	Zebrafish	(Menuet et al., 2002)
EE2	Zebrafish, Human	(Notch & Mayer, 2011) (Miksicek, 1994)
Bisphenol A	Human, zebrafish	(C. Park et al., 2020)(Serra et al., 2019)
Bisphenol S	Human	(C. Park et al., 2020)
Bisphenol F	Human	(C. Park et al., 2020)
Et-PABA	Human, Rainbow trout	(Kunz & Fent, 2006a)

* this list does not consider the differences in binding affinity to ER between species.

Table 4. Known ER antagonists (non-exhaustive)

Compound name	Species*	Reference
AHTN	Zebrafish	(Schreurs et al., 2005)
HHCB	Zebrafish	(Schreurs et al., 2005)
Triclosan	Human	(Ahn et al., 2008)
Tamoxifen	Human	(Legemiddelhåndboken, 2015a)
Klomifen	Human	(Legemiddelhåndboken, 2015a)
Fulvestrant	Human	(Legemiddelhåndboken, 2015a)
Tamoxifen	Human	(Miksicek, 1994)
Octocrylene	Human, zebrafish	(Kunz & Fent, 2006a) (Meng, Yeung, & Chan, 2021)
Diclofenac	Human	(Ezechiáš et al., 2016)
Ibuprofen	Human	

* this list does not consider the differences in binding affinity to ER between species.

Table 5. Glucocorticoid receptor ligands (non-exhaustive)

Compound name	Species*	Reference
Cortisone	Human	(Legemiddelhåndboken, 2015b)(Chen, Jia, Snyder, Gong, & Lam, 2016)
Hydrocortisone	Human	(Legemiddelhåndboken, 2015b)
Prednisolone	Human, zebrafish, mice	(Legemiddelhåndboken, 2015b), (Chen et al., 2016) , (So et al., 2009).
Dexamethasone	Human, zebrafish	(Legemiddelhåndboken, 2015b) (Chen et al., 2016)
Triamcinolone	Zebrafish	(Chen et al., 2016)
TMPP**	Human	(Q. Zhang et al., 2017)
TPHP**	Human	
TDBPP**	Human	

* this list does not consider the differences in binding affinity to GR between species.
** antagonist

Table 6. Known ligands for PXR (non-exhaustive)

Compound name	Species*	Reference
4BAB	Human, zebrafish	Lille-Langøy et al. 2019
Bisphenol A	Human	Takeshita et al. 2001
Cafestrol	Mouse	Ricketts et al. 2007
Carbemazepine	Human, polar bear	Lille-Langøy et al. 2015
Clotrimazole	Zebrafish, human, polar bear	Bainy et al. 2013, Lille-Langøy et. al. 2015
DEHP	Human, mouse	Takeshita et al. 2001
Dexamethasone	Human	Pacussi, Drocourt, Maurel,Vilarem, 2000
Diclofenac	*Mugilogobius abei*	Ku et al. 2018
Ketoconazole**	Human	Lille-Langøy et. al. 2015, Takeshita et. al., 2001
Nonylphenol	Mouse	Takeshita et al. 2001
Omeprazole	Human, polar bear	Lille-Langøy et. al. 2015
Pregnenolone 16α-carbonitril	Zebrafish	Bainy et al. 2013
Rifampicin	Human, polar bear	Lille-Langøy et. al. 2015, Takeshita et al. 2001
SR12813	Human, polar bear	Lille-Langøy et. al. 2015
TCPP	Human	Kojima et al., 2013

* this list does not consider the differences in binding affinity to PXR between species.

** antagonism reported by Takeshita, Taguchi, Koibuchi, & Ozawa, 2002 and Huang et al., 2007.

Table 7. Details on concentrations detected by chemical analysis including LOD and LOQ

	Acetaminophen	Metoprolol	Caffeine	Prednisolone	Carbamazepine	5-H Diclofenac	TCPP	Octocrylene
Name	Final Conc.	Final Conc.	Final Conc.	Final Conc.	Final Conc.	Final Conc.	Final Conc.	Final Conc.
	ng/ 500mL	ng/ 500mL	ng/ 500mL	ng/ 500mL	ng/ 500mL	ng/ 500mL	ng/ 500mL	ng/ 500mL
Holen-in	6802.24	104.40	16435.50	0.00	18.78	154.32	106.94	8.42
Holen-out	148.90	0.00	13426.43	0.00	35.84	154.30	407.58	12.48
Kvernevik-in	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Kvernevik-out	27.42	0.00	195.05	9.04	98.67	281.52	908.54	22.57
Knappen-in	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Knappen-out	7953.50	259.44	23389.67	84.02	95.51	84.39	732.30	7.68
Ytre Sandviken-in	4655.54	119.61	21054.41	0.00	21.60	79.58	163.71	8.70
Ytre Sandviken-out	230.42	69.38	11900.04	0.00	25.82	123.42	539.98	10.11
LOD	75.02	50.08	28.31	74.78	83.49	68.23	76.09	101.93
LOQ	227.34	151.77	85.78	226.62	252.99	206.77	230.58	308.88
Blank	5.11	0.00	0.01	0.00	0.00	4.99	249.24	9.47

Table 8. Matrix effect (ME) of the compounds included in the targeted analysis

	slope			
	solvent	**matrix**		**ME**
ACE	0.641	0.526		-17.9
MET	2.347	1.682		-28.3
CAF	9.063	9.054		-0.1
PDS	0.248	0.202		-18.5
CBZ	0.099	0.086		-13.1
5-h DCF	0.360	0.187		-48.1
TCPP	0.358	0.347		-3.1
OCT	0.095	0.066		-30.5

Table 9. Instrumental carry-over. ng/500 mL

	Acetaminophen	Metoprolol	Caffeine	Prednisolone	Carbamazepine	5-h Diclofenac	TCPP	Octocrylene
blank1	0.00	0.00	0.00	0.00	0.00	10.33	806.40	22.52
blank2	0.00	0.00	0.00	1.23	0.00	7.91	280.50	10.80
blank3	0.00	0.00	0.00	0.00	0.00	13.42	2365.81	59.49
Average	**0.00**	**0.00**	**0.00**	**0.41**	**0.00**	**10.55**	**1150.90**	**30.94**
Sd	0.00	0.00	0.00	0.58	0.00	2.25	885.49	20.75
Sd %				141.4		21.4	76.9	67.1