# Evaluation of primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes suitability as a screening assay for estrogen receptor agonists

En vurdering av primære leverceller fra regnbueørret (*Oncorhynchus mykiss*) sin egnethet som analyseverktøy for østrogenreseptor-agonister

Philosophiae Doctor (PhD) Thesis

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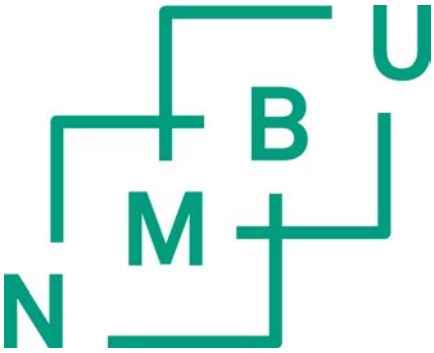


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Maria Hultman

Oslo, April 2016

Sola dosis facit venenum

- *Paracelsus*

# Table of contents

# Summary

Regulatory legislations on chemicals have in recent years become more stringent, requiring more toxicological evaluation. The increased toxicological testing have resulted in a stronger effort to implement the 3Rs (Refinement, Reduction and Replacement) through identifying suitable alternatives (non-animal) to animal testing. Evaluation of alternative methods such as cell-based *in vitro* methods (e.g. continuous cell lines, tissues slices and primary cultures) have shown to be promising, but there are currently few validated alternative bioassays for fish. The present work aimed to evaluate the primary rainbow trout *(Oncorhynchus mykiss*) hepatocyte model's suitability as an ecotoxicological tool in screening of single and mixtures of chemicals with modulatory properties on the estrogen receptor (ER)-activity and auxiliary endpoints such as cytotoxicity and biotransformation (e.g. aryl hydrocarbon receptor (AhR)-activity). The primary hepatocytes sensitivity, reproducibility and responsiveness were assessed for seasonal, donor and assay-related variability to address potential factors affecting the bioassay reproducibility. The hepatocyte model's suitability to characterise environmentally relevant concentrations of estrogens, anti-estrogens and organic compounds was assessed using both single endpoint and broad content approaches (global transcriptomics) after exposure to single compounds and mixtures of these with similar or dissimilar mode of action (MoA).

Compounds estrogenicity and anti-estrogenicity were assessed in the fish hepatocytes using classical estrogen sensitive biomarkers (e.g. ERα and ER-mediated egg-yolk precursor vitellogenin (Vtg) and egg shell zona radiata (*zrp*)) complemented by determination of cytotoxicity (cell membrane stability and metabolic activity), AhR-mediated responses (*ahr*, cytochrome P450 1a (*cyp1a*), enzymatic activity of ethoxyresorufin-O-deethylase (EROD)). Characterisation of chemicals additional MoA in the cells were performed using novel analytical tools such as high-density oligonucleotide salmonid microarray in combination with quantitative real-time polymerase chain reaction (qPCR).

Results from the studies demonstrated primary hepatocytes ability to facilitate detection of ER-mediated responses, biotransformation and cell growth-related gene expression by ER-agonists and acute toxic chemicals during short-term exposures (<96h). The bioassays ability to remain unaffected by variable parameters (robustness) and sensitivity was not affected by seasonal variations in ER sensitivity (Vtg gene and protein expression), but was dependent on individual donor-physiology variability in exposure studies with the model ER-agonist 17α-ethinylestradiol (EE2). The cell-batch variability was however reduced when optimal

exposure time and data normalization was applied, yielding a concentration-dependent Vtg gene and protein expression. In addition to individual donor-physiology, bioassay related factors such as hepatocyte culturing conditions and normalization procedures were identified as possible cause to the observed variability. The variability within the bioassay may be reduced through protocol harmonization (e.g. same-species culturing conditions, cell density, media supplements), resulting in a more robust and reproducible assay for assessing ER active compounds. The primary hepatocytes suitability as a screening tool for ER-agonists were further displayed in measured classical biomarker genes, identifying ER signalling and its associated pathways as the main target of the ER-agonist in the cells. Exposure to the ER-agonist caused similar transcriptional responses *in vitro* as previously reported *in vivo*, suggesting the hepatocytes to facilitate relevant biomarker responses in screening of ER-active compounds.

The suitability of the *in vitro* model in transcriptional and sub-cellular characterization of anti-estrogenic binary and ternary mixtures (AhR-agonist (β-naphtoflavone (BNF), ER-agonist (17 β-estradiol (E2)), partial ER-antagonist (hydroxytamoxifen (OHT))) were performed to better understand how compounds with dissimilar mode of action (MoA) contributed to combined anti-estrogenic effects. The findings displayed significantly increased anti-estrogenic effect in the combined mixture of BNF, OHT and E2, indicative of the individual compounds MoA to contribute to the total anti-estrogenic effect in the primary hepatocytes. The results suggested that the compounds in the mixture induce nuclear receptor-mediated cross-talk involving AhR-mediated transcription of increased *cyp1a* metabolism of the ER-agonist and binding and inhibition of further ER activity by the ER-antagonist. The combined mixture's anti-estrogenic effect is therefore suggested due to the compounds differently acting MoA as they have the same effect (e.g. reduction of Vtg protein) but through differently acting pathways.

To further assess the primary hepatocyte bioassay ability to identify complex and previous un-described ER-agonists, a broad range of uncharacterised mixtures (UCM)-related compounds of naphthenic acids and hydrocarbons were screened for potential estrogenicity. Few compounds induced weak estrogenic activity in the primary hepatocytes as the majority of the tested compounds had a narcotic MoA and reached their water solubility before eliciting any ER-activity. Auxiliary endpoints such as EROD activity could not explain the compounds weak estrogenicity and further supported their narcotic MoA. The potential estrogenicity might however be masked by the compounds highly variable physico-chemical properties that may have affected their bioavailability in the *in vitro* system.

The present work has demonstrated that the primary rainbow trout hepatocyte model is a versatile, multi-endpoint tool for screening ER-agonists using both single biomarker and global gene expression approach. The bioassay provided reproducible results that demonstrate its sensitivity, robustness and responsiveness in ecotoxicological screening of chemicals that modulate the activity of the ER and downstream cellular events. These findings may contribute to a better mechanistic understanding of well-characterised and novel MOA related to single and combined chemical exposures in the *in vitro* fish model. The global gene expression was a good unbiased tool when characterizing the MoA of ER-active chemicals in the primary hepatocytes as it unravelled relevant *in vivo* ER-mediated responses, hence displaying the model's potential to become a (eco)toxicological tool in ER-agonist screening.

# Sammendrag

Regulatorisk kjemikalielovgivning har i de senere årene blitt strengere, noe som krever mer toksikologisk vurdering. Den økte toksikologisk testingen har ført til en sterkere innsats for å implementere de 3Rer (forbedring, reduksjon og erstatning) gjennom å identifisere egnede alternativer til dyreforsøk. Vurdering av alternative metoder som cellebaserte *in vitro* metoder (f.eks kontinuerlige cellelinjer, vev-skiver og primære kulturer) har vist seg å være lovende, men for tiden er det få validerte alternative metoder for fisk. Dette arbeidet evaluerer primære regnbueørret (*Oncorhynchus mykiss*) hepatocytters egnethet som et økotoksikologisk verktøy i screening av enkeltstoffer og blandinger av kjemikalier med modulerende egenskaper på østrogenreseptor (ER)-aktivitet med hjelp av endepunkter som cytotoksisitet, østrogenrespons og biotransformasjon (f.eks aryl hydrokarbon reseptor (AhR)-aktivitet).

Sensitivitet, reproduserbarhet og reaksjonsevne for metoden ble vurdert ved å se på responsvariasjon knyttet til sesong, donorfisk og analysemessige variasjoner, og potensielle faktorer som påvirker bioassayets reproduserbarhet ble identifisert. Hepatocytt-modellens egnethet for å karakterisere miljørelevante konsentrasjoner av østrogener, anti-østrogener og organiske forbindelser ble vurdert ved bruk av både enkelt endepunkter og analyse genuttrykk etter eksponering for enkeltstoffer og blandinger av disse med tilsvarende eller ulik virkningsmekanisme (MoA).

Østrogenisitet og anti-østrogenisitet av kjemikalieblandinger ble vurdert i fiskehepatocytter ved hjelp av klassiske østrogensensitive biomarkører (f.eks ERα og ER-mediert eggeplomme forløper vitellogenin (Vtg) og eggeskallkomponenten zona radiata (ZRP)) supplert med bestemmelse av cytotoksisitet (cellemembranstabilitet og metabolsk aktivitet), arylhydrocarbon reseptor (AhR)-medierte responser (Ahr, cytokrom P450 1a (CYP1A), og enzymatiske aktivitet til etoksyresorufin-O-deetylase (EROD)). Karakterisering av kjemikalier for ytterligere MoA i cellene ble utført ved bruk av nye analyseverktøy som oligonukleotid mikromatrise for laksefisk i kombinasjon med kvantitativ real-tid polymerase kjedereaksjon (qPCR).

Resultater fra studien viste primære hepatocytters evne til å å påvise ER-medierte reaksjoner, biotransformasjon og cellevekst knyttet til genekspresjon etter korttidseksponering (<96h) for ER-agonister og akutt giftige kjemikalier. Bioassayets evne til å forbli upåvirket av variable parametere (robusthet) samt assayets sensitivitet for å detektere østrogene stoffer ble ikke

påvirket av sesongvariasjoner i ER respons (Vtg gen og protein ekspression), men var avhengig av variasjoner i individuell donor-fysiologi i eksponeringsstudier med ER-agonist 17α etinyløstradiol (EE2). Celle-batch variabilitet ble imidlertid redusert når optimal eksponeringstid og data-normalisering ble anvendt, hvilket ga en konsentrasjonsavhengig ekspresjon av Vtg gen og protein.. I tillegg til individuell donor-fysiologi, ble bioassay-relaterte faktorer som hepatocytters dyrkingsforhold og normaliserings-prosedyrer identifisert som mulig årsak til den observerte variasjonen. Variabiliteten i den biologiske metoden kan reduseres ved protokoll-harmonisering (for eksempel av samme arts dyrkningsbetingelser, celletetthet, mediets kosttilskudd), som resulterer i et mer robust og reproduserbart assay for vurdering av ER-aktive forbindelser. Primære hepatocytters egnethet som et screeningverktøy for ER-agonister ble videre vist i målte klassiske biomarkør-gener, som identifiserer ER signalisering og tilhørende signalveier som det viktigste målet for ER-agonist i cellene. Eksponering for ER-agonister forårsaket lignende transkripsjons-responser *in vitro* som tidligere rapportert *in vivo*, noe som bekrefter metodens og biomarkørresponsenes egnethet i screening av ER-aktive forbindelser.

*In vitro*-modellen ble videre brukt i transkripsjon og sub-cellulær karakterisering av anti-østrogeners responser. Både enkeltstoffer og binære blandinger (AhR-agonist (β-naphtoflavone (BNF), ER-agonist (17 β-østradiol (E2)), og delvis ER-antagonist (hydroxytamoxifen (OHT))) ble testet for å bedre forstå hvordan forbindelser med ulik virkning (MoA) bidro til kombinert anti-østrogen effekt. Resultatene viser betydelig økt anti-østrogen effekt i den kombinerte blandingen av BNF, OHT og E2, en indikasjon at de enkelte forbindelsers MoA bidrar til den anti-østrogene effekten av blandingen i de primære hepatocyttene. Videre antydet resultatene at forbindelsene i blandingen induserte nukleær reseptor-formidlet krysstale (cross-talk) mellom AhR og ER, som for eksempel AhR-mediert transkripsjon av økt CYP1A og videre metabolisme av ER-agonist og inhibering av ytterligere ER aktivitet av ER-antagonist. Den anti-østrogene effekten av kjemikalieblandingen er derfor foreslått å komme av at forbindelsene som har ulike MoA påvirker samme endepunkt (f.eks reduksjon av Vtg protein), men gjennom forskjellige signalveier.

For å vurdere primære hepatocytter bioassays evne til å identifisere kompliserte og tidligere ikke beskrevne ER-agonister, ble et bredt spekter av stoffer relatert til ukarakteriserte blandinger (UCM) av naftensyrer og hydrokarboner screenet for potensiell østrogenitet. Få

forbindelser viste svak østrogenaktivitet i de primære hepatocytter da de fleste av disse hadde en narkotisk MoA og nådde grensen for vannløselighet før de utløste noen ER-aktivitet. Understøttende endepunkter som EROD aktivitet kunne ikke forklare forbindelsenes svake østrogenitet og ga støtte til en narkotisk MoA. En østrogen effekt kan heller ikke totalt utelukkes siden de vurderte forbindelsene hadde svært varierende fysikalsk-kjemiske egenskaper som kan ha påvirket den biologiske tilgjengeligheten av forbindelsene i *in vitro*-systemet.

Arbeidet har vist at modellen av primære regnbueørret hepatocytter er et allsidig, multi-endepunkt verktøy for screening av ER-agonister ved hjelp av både enkle biomarkører og global genekspresjon.. Bioanalysen ga reproduserbare resultater som demonstrerer dens følsomhet, robusthet og reaksjonsevne i økotoksikologisk screening av stoffer som modulerer aktivitet av ER og nedstrøms cellulære hendelser. Disse funnene kan bidra til en bedre mekanistisk forståelse av godt karakteriserte og nye MoAs knyttet til enkeltstoffer og kombinerte kjemiske eksponeringer i *in vitro* fisk-modeller. Den globale genekspresjonen var et godt objektivt verktøy til å karakterisere MoA av ER-aktive kjemikalier i primære hepatocytter og identifiserte relevante *in vivo* ER-medierte reaksjoner. Resultatene i dette arbeidet viser modellens potensial til å bli et viktig (øko)toksikologisk verktøy i screening av potensielle ER-modulerende stoffer.

# List of papers

This thesis is based on the following papers, and will be denoted throughout the thesis by their roman numerals (Paper I-IV):

**Paper I**

Evaluation of the sensitivity, responsiveness and reproducibility of rainbow trout (*Oncorhynchus mykiss*) *in vitro* vitellogenin production as a screening assay for estrogen mimics.

**Paper II**

17α-Ethinylestradiol (EE2) effect on global gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes.

**Paper III**

Deciphering combined effects of anti-estrogenic chemicals on vitellogenin production in rainbow trout (*Oncorhynchus mykiss*) hepatocytes.

**Paper IV**

Toxicity of organic compounds associated with unresolved complex mixtures (UCMs) in primary fish hepatocytes.

# Abbreviations

AB – Alamar blue

ABC - ATP-binding cassette

AF - activation function

AhR – Aryl hydrocarbon receptor protein

ANOVA – Analysis of variance

ARNT - Aryl hydrocarbon receptor nuclear translocator

BNF – β-naphthoflavone

CF – Conceptual frame work

CFDA-AM - Carboxyfluorescein diacetate acetoxymethyl ester

CMR - Carcinogenic, Mutagenic and Reproduction toxic

CRC – Concentration response curve

CYP1A - Cytochrome P450 1A protein

DBD – DNA binding domain

DDT – Dichlorodiphenyltrichloroethane

DEG - Differently expressed gene

DES - Diethylstilbestrol

DNA - Deoxyribonucleic acid

EAT – Estrogen, androgen, thyroid

$EC_{10}$ – 10% Effect concentration

$EC_{50}$ - 50% Effect concentration

ECOSAR - Ecological Structure Activity Relationships

EDC – Endocrine disrupting compounds

E2 - 17β-Estradiol

EE2 - 17α-Ethinylestradiol

ELISA – Enzyme linked immunosorbent assay

ERα – Estrogen receptor alpha protein

ERE – Estrogen response element

EROD - Ethoxyresorufin–O-deethylase

EU – European Union

FBS/FCS – Fetal bovine/calf serum

GNRH - Gonadotropin hormone

GO – Gene ontology

GST - Glutathione S-transferase

HPG – Hypothalamus – pituitary – gonad

IC50 – 50% Inhibition concentration

LBD – Ligand binding domain

LC50 – 50% Lethal concentration

LOEC – Lowest observed effect concentration

Log $K_{ow}$ - Octanol-water partition coefficient

MOA – Mechanism of action

MoA – Mode of action

MRP – Multi drug resistance transporter protein

NCoA - Nuclear Receptor Coactivator

NOEC – No observed effect concentration

NR – Nuclear receptor

NRT – None reverse transcriptase control

NTC – No template control

OECD - Organization for eEonomic Cooperation and Development

OHT – 4- Hydroxytamoxifen

PAH – Polycyclic aromatic hydrocarbon

PCA – Principal component analysis

PBT – Persistent, bioaccumulative, toxic

PCB – Polychlorinated biphenyl

PCP – Pentachlorophenol

qPCR – Quantitative real-time polymerase chain reaction

QSAR – Quantitative structure activity relationship

RNA - Ribonucleic acid

3R – Refinement, reduction and replacement

SLIRP – SRA Stem-Loop Interacting RNA Binding Protein

TCDD – 2,3,7,8-Tetrachlorodibenzodioxin

UCM - unresolved complex mixture

UGT - UDP-glucoronosyltransferase

UPLC-MS - Ultra-performance liquid-chromatography tandem mass spectrometer

US-EPA – The United States environmental protecting agency

Vtg – Vitellogenin protein

XRE – Xenobiotic response element

Zrp – egg shell protein zona radiata

**Genes**

*abc* - ATP-binding cassette transporter

*acoxl* - acyl-CoA oxidase-like

*ahrα* - Aryl hydrocarbon receptor

*apo* - apolipoprotein

*arnt* - Aryl hydrocarbon receptor nuclear translocator

*cebpb* - ccaat enhancer-binding protein beta

*cpt1a* - carnitine palmitoyltransferase 1a

*cyp1a* - Cytochrome P450 1A

*dhcr* - lipin-1, 7-dehydrocholesterol reductase

*erα* – Estrogen receptor alpha

*fab* - fatty acid binding protein

*fst* - follistatin

*ghr* - growth hormone receptor

*igfbp* - insulin growth factor binding protein

*nf1b* - squelching nuclear factor 1

*pparγ* - peroxisome proliferator activating receptor γ

*slirp* - SRA Stem-Loop Interacting RNA Binding Protein

*sult* - sulfotransferase

*ugt* - UDP-glucuronosyltransferase precursor

*vtg* – Vitellogenin

*zrp* - egg shell protein zona radiata

# 1 Background

Organic contaminants enter the environment through various anthropogenic activities such as industrial emissions, air deposition, effluents of sewage treatment plants and agricultural run-off (Sumpter, 2005; The Climate and Pollution Agency, 2010). The pollutants either bind to soil and sediment or directly expose organism through air, water or food, during a temporary (acute) or long-term (chronic) duration of time. Acute toxicity is normally caused by exposure to high concentrations of organic chemicals for a short period of time and is most commonly occurring in laboratory studies, but may also occur in the environment after accidental spills or industrial emissions (Safe, 1990). Organic chemicals of environmental concern are often those being identified to be persistent, bioaccumulative, toxic (PBT), carcinogenic, mutagenic and reproduction toxic (CMR) or having endocrine disruptive properties (EDCs) at environmentally relevant exposure concentrations (Diamanti-Kandarakis et al., 2009). Compounds with ED properties are defined as ” … an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations.” (Damstra et al., 2002).

Environmentally relevant EDCs consists of highly heterogeneous chemicals such as industrial solvents and their by-products (polychlorinated biphenyls (PCBs)), plastic softeners (Bisphenol A (BPA)), pesticides (e.g. dichlorodiphenyltrichloroethane (DDT)) and pharmaceuticals (e.g 17α-ethinylestradiol (EE2), diethylstilbestrol (DES), tamoxifen) (Diamanti-Kandarakis et al., 2009). The proposed common traits for EDCs are their small molecular mass (<1000 Daltons), phenolic moiety that mimics endogenous hormones and the occurrence of halogen groups containing bromine and chlorine molecules (Diamanti-Kandarakis et al., 2009).

Each year numerous EDCs are subjected to toxicological testing to evaluate their hazard to humans and wildlife. International organizations such as the Organization for Economic Cooperation and Development (OECD), European Union (EU) and US-Environmental protection agency (US-EPA) organize screening programs and guidelines to assess and evaluate compounds with ED properties (Hecker and Hollert, 2011). In recent years, legislations on chemicals have become more stringent, requiring more extensive toxicological evaluation. To meet these requirements various analytical tools have been implemented in the OECD's Conceptual frame work (CF) directive at various levels of complexity such as computational modelling (*in silico*) (CF1-2), non-animal- (*in vitro*) (CF2) and various conventional *in vivo* screening assays (CF3-5). *In silico* modelling such as quantitative

structure-activity relationship (QSAR) has been applied to prioritize and flag potentially harmful substances based on their physico-chemical properties, reactivity, structural and metabolic resemblance to chemicals with well-described toxic effects (Raunio, 2011). The predicted *in silico* toxicity may be confirmed by specific mechanistic data obtained from *in vitro* assays (e.g. specific nuclear receptor binding and affinity, transcriptional activation). These are then followed by more demanding *in vivo* tests to assess single biomarker endpoints (CF3), which may cause multiple mechanistic responses (CF4) giving rise to effects in the *in vivo* life-cycle or trans-generation (CF5). The increased need for toxicological testing has raised both ethical and economical concerns as conventional screening methods using animals (rabbits, mice, rats and fish etc.) may be unethical, time-consuming and require millions of animals (Rovida and Hartung, 2009). In an effort to limit animal testing, regulatory legislations and scientific research have endorsed implementation of William M. S. Russell and Rex L. Burch principle of the 3R`s: Refine, Reduce and Replace, to fulfil the animal welfare legislations (Directive 2010/63/EU, 2010). There are however challenges associated with using alternatives to animals as the methods are often not validated nor commonly accepted as alternatives to whole animal (*in vivo*) testing. Assessment of alternative testing for EDCs is therefore highly warranted as it has the potential to provide high throughput, time and cost-efficient screening of environmentally relevant single organic compounds and their mixtures.

## 1.1 Endocrine disruption in fish

Characterising and understanding EDCs potential effects have been performed through studying their ability to induce endocrine responses by initiate specific molecular and biochemical events termed as the compounds mechanism of action (MOA). The induction of a sequence of key events involving molecular and cellular processes that contributes to potentially adverse functional, anatomical and behavioural changes in the organism may be described as the compounds mode of action (MoA) (Rand et al., 1995) (Fig. 1). Deciphering EDCs specific MOA and the more general MoA is therefore essential as it will not only contribute to the understanding of their adverse toxicity in the individual organism but also potentially predict population level effects (Fig. 1). These adverse effects are dependent on the EDCs uptake, transport, metabolism, sequestration and excretion (toxicokinetics), as well

as their capacity to bind and modulate molecular and cellular targets and organs, causing toxic effects (toxicodynamics) in organisms.

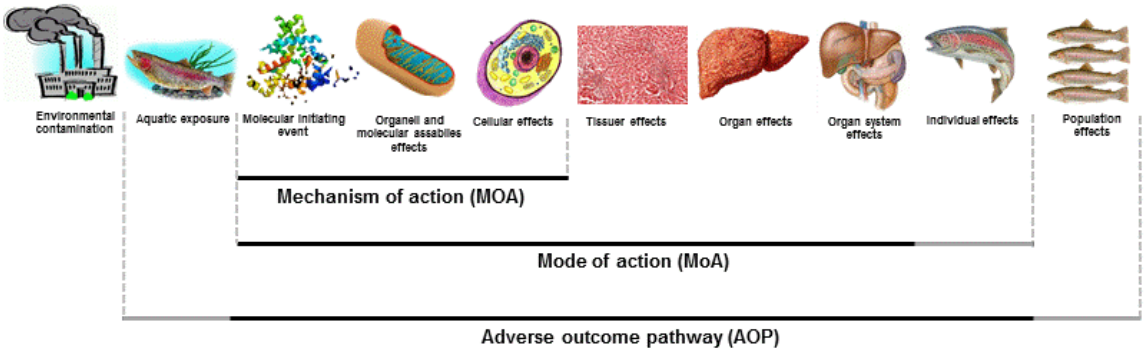


Fig. 1. Representation of relationships between mechanism of action (MOA), mode of action (MoA) and adverse outcome pathway (AOP). The black lines represent the common research concept, while the filled gray line represents the theoretical extent of the concepts. Modified from figure by OECD, 2012.

Since 1980, endocrine disruption in fish has been recognized as an environmental issue in association with e.g. effluents from upstream situated pulp mill industries that cause masculinization in female fish (Howell et al., 1980). EDCs have since then been given frequent attention in association with sewage treatment plants (STP) (Purdom et al., 1994), agricultural run-off and industrial effluents (full review see Mills and Chichester, 2005). Exposure to STP-water and the compounds therein have been associated with adverse estrogenic effects such as reduced growth, fecundity and increased gonad feminization causing skewed sex-ratio which may threaten the survival of the population (Jobling et al., 2005; Lange et al., 2008). These adverse effects may arise due to the disruption of various endocrine-mediated mechanisms associated e.g. with the estrogen-, androgen- and thyroid (EAT) pathway and/or endogenous steroid metabolism in addition to other non-endocrine regulated mechanisms (Arcand-Hoy and Benson, 1998).

Understanding EDCs MOA in fish has been facilitated by studying specific transcriptional regulation of genes and translation of proteins, which reflects the chemicals specific molecular and/or biochemical interaction or target in the cell (biomarker). EDCs such as estrogen mimicking compounds (xenoestrogens) have ER-agonistic (inducing) effects and are the most frequently studied compounds in aquatic toxicology, having multiple well-established estrogen sensitive biomarkers. Biomarkers are frequently used in *in vitro* and *in*

*vivo* toxicology as they may reflect the chemicals ability to induce a molecular event or sub-cellular effect in a concentration dependent manner.

## 1.2 EDCs mode and mechanism of action (MoA/MOA)

Compounds ED effects are concentration dependent and EDCs may therefore cause both general acute toxicity at high concentrations and alter specific endocrine regulation at sub-lethal concentrations. High exposure concentrations of compounds such as ER-agonists may cause adverse toxicity, lethality or cell death through the chemicals non-specific disturbance of biological cell membrane integrity and function (Escher et al., 2002; Krasowski and Harrison, 1999). At lower (sub-lethal) concentrations, chemicals may interfere with the endocrine system either through modulating the classical estrogen-, androgen and thyroid receptor (EAT) pathway or alternative non-steroid receptors (e.g. neurotransmitter receptors), cell-membrane bound receptors (e.g. ER), orphan nuclear receptors (e.g. aryl hydrocarbon receptor (AhR)) and other enzymatic pathways (e.g. steroid biosynthesis and metabolism) (Arcand‐Hoy and Benson, 1998; Damstra et al., 2002). Disruption which may cause adverse effects on the individual organism's normal sexual maturation, growth, stress response, hormone regulation and reproduction (Lange et al., 2008; Sumpter, 2005), ultimately affecting the population dynamics (Scholz et al., 2013).

The toxic MoA of EDCs is often characterised by the tissue-specific adversity and compound selectivity. The compound toxicity may in many cases be explained by the tissue-specific expression of intracellular nuclear receptors (NRs), which mediates the toxicity through various signalling pathways or through genomic activation of target genes and proteins. There are various types of NRs and some of them are main regulators of important signalling pathways such the EAT by e.g. activation of ER and synthesis of estrogen sensitive proteins (Bainy et al., 2013). The transcriptional activity of NRs such as ER belongs to the super family of NRs that are either activated (receptor agonists) or inhibited (receptor antagonists) when binding lipophilic molecules such as EDCs into their ligand-binding pockets. Increased ER activity has previously been associated with adverse health effects in fish, suggesting the importance of better understanding the NRs functionality (Bainy et al., 2013).

### 1.2.1 Estrogen receptor (ER) signalling

ERs are promiscuous nuclear receptors that function as ligand-activated transcription factors in reproductive (e.g. gonads) and non-reproductive tissues (e.g. brain, cardiovascular system, liver, heart and intestine) (Filby and Tyler, 2005; Shanle and Xu, 2011). Depending on the cellular type, ER signalling is involved in numerous pathways related to growth (e.g. insulin growth factor 1, growth hormone), tissue differentiation (e.g. gonad), steroid- and lipid homeostasis (e.g. cholesterol metabolism, lipid synthesis) and reproduction (Bainy et al., 2013; Colborn et al., 1993; Heldring et al., 2007). Disruption of ER signalling and associated pathways has been described after exposure to various EDCs, which bind and modulate the activity of the ER. EDCs with modulatory properties on ER act through either activating (agonists) or inhibiting (antagonists) the receptor and further transcription and translation of downstream target genes and proteins. The disruption of ER activity may give rise to hormonal imbalance in the hypothalamic–pituitary–gonadal (HPG)-axis that contributes to impaired growth and reproduction failure in fish (Arcand-Hoy and Benson, 1998; Colborn et al., 1993; Shved et al., 2008).

### 1.2.2 The estrogen receptor

The ER is composed of 6 distinct functional domains in the DNA (A-F): The trans-activation domain with a ligand independent transcription activation function 1 (AF1) (domain A/B), a DNA binding domain (DBD) (domain C) and a hinge region (domain D), followed by the ligand dependent binding domain (LBD) known as the transcriptional AF2 (domain E) (Matthews and Gustafsson, 2003) and the F domain, which function in vertebrate ERs is poorly understood (Nelson and Habibi, 2013; Olefsky, 2001) (Fig. 2).

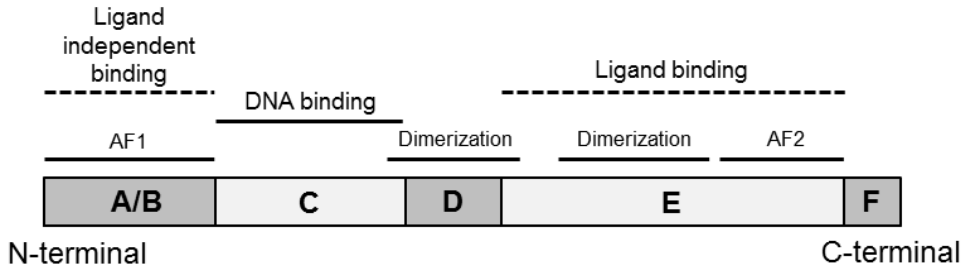


Figure 2. Schematic representation of the estrogen receptor (ER) domains in DNA. The ER consists of totally 6 distinct domains associated either with N- or C-terminal. Abbreviation: AF1/2: Activation function 1/2. Modified from Matthews and Gustafsson (2003).

There are currently three well-described isotypes of ER (ERα1, ERα2, ERβ1(γ)) in teleost fish which have ligand-dependent affinity and tissue distribution. The ERα isotype which have retained its genomic structure and function across vertebrate species, is best characterised and has been proposed as the dominating estrogen responsive receptor (for full review see Nelson and Habibi, 2013). A fourth ER isotype (ERβ2) has been proposed, but has only been identified in rainbow trout (Nagler et al., 2007).

### 1.2.3 Estrogen receptor agonism

Endogenous estrogens estriol, estrone and 17β-estradiol (E2) are ER-agonists produced in ovaries of sexually mature female fish and transported through the bloodstream to estrogen responsive tissue (e.g. liver, pituitary gland, gonad). Once in the estrogen responsive tissue, the ER-agonist binds to ER by displacing the ER-heat shocking protein 90 (Hsp90) through a conformational change of the receptor ligand binding pocket in the AF2 domain. The ER-ligand complex is translocated into the nucleus, where it binds to genomic promotor regions containing an estrogen response element (ERE) (Boelsterli, 2007; O'Malley and Tsai, 1992) (Fig. 3). Upon binding to the ERE, the ER-ligand complex recruits various transcriptional co-factors and RNA polymerase II which alters the chromatin structure of DNA and enables mRNA transcription of ER and ER-mediated down-stream genes (Matthews and Gustafsson, 2003). The transcribed mRNA is transported out of the nucleus into the cytosol, where it is translated into proteins by ribosomes and transported to their target site e.g. ovaries (Arukwe et al., 2000; Mommsen and Walsh, 1988).

Chemicals that modulate the activity of the ER are dependent on their ability to bind to the ER ligand-binding pockets (AF2 region in the LBD) (Fig. 2) and change the conformational position of the ligand-dependent short helical region (helix 12). The ligands ability to stabilize the receptor through the conformational position of the helix 12 is crucial for the specific recruitment and binding efficiency of co-regulators to ER-ligand complex (for full review see Heldring et al., 2007 and Shanle and Xu, 2011). The recruitment and binding efficiency of co-regulators has been proposed to be compound specific (McDonnell and Wardell, 2010), involving more than 300 potential co-regulator proteins in mammalian species (Lonard and O'Malley, 2012), although still poorly described in fish.

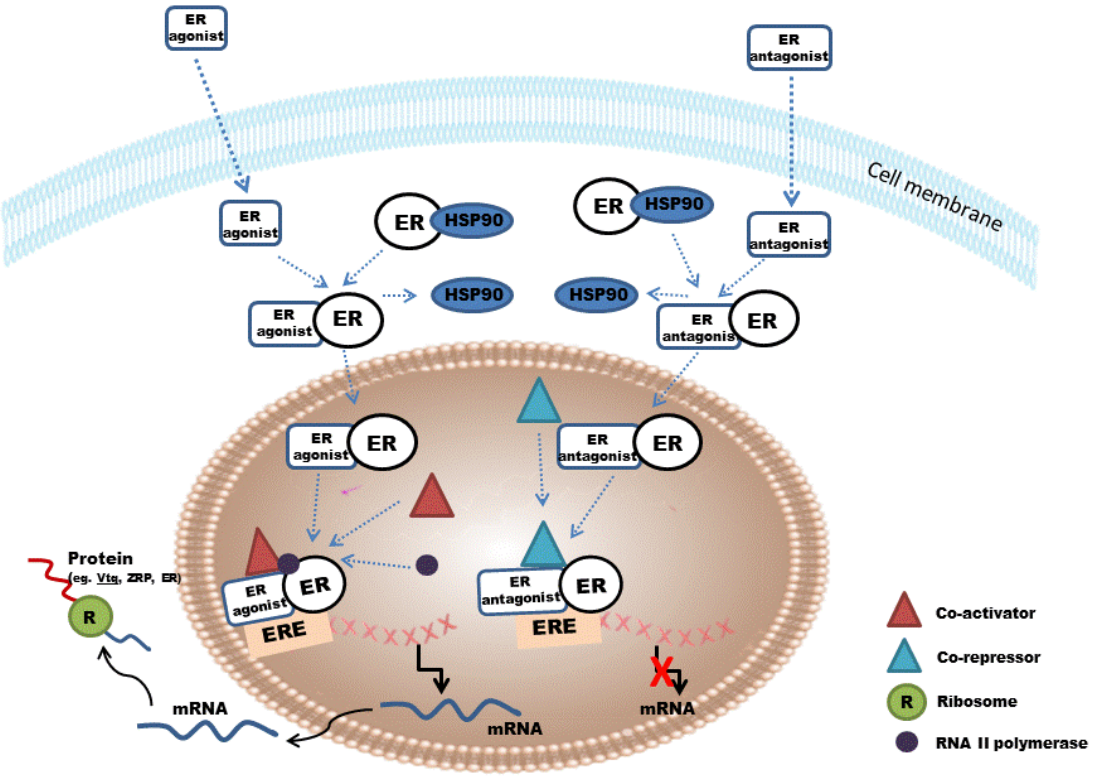


Figure 3. Simplified overview of genomic estrogen receptor (ER) mechanism when exposed to ER-agonist or ER-antagonist. Modified from Boelsterli (2007).

The genomic activation of ER in fish has been proposed to be primarily responsible for phenotypic effects such as increased plasma estrogen levels (Jobling et al., 2005; MacLatchy et al., 2003), decreased testis size and ovotestis in males and reduced ovarian growth (Jobling et al., 2005; Pawlowski et al., 2004). Adverse effects have been associated with reduced growth and fecundity, disrupted lipid metabolism and feminization in fish (Ibabe et al., 2005; Jobling et al., 2005; Pawlowski et al., 2004; Shved et al., 2008). These adverse effects have been associated with up-regulation of ERα during enhanced levels of estrogens or exposure to xenoestrogens. The ER-agonists affect ER and its down-stream target genes such as the egg-yolk precursor protein vitellogenin (*vtg*), Vtg stability RNA-binding protein vigilin and egg shell protein zona radiata (*zrp*), by transcriptionally activate and initiate translation causing a potential disruption of vitellogenesis (e.g. oogenesis) in fish (Arcand-Hoy and Benson, 1998; Arukwe et al., 1997; Nagler et al., 2010).

The Vtg and Zrp are estrogen sensitive proteins synthesized in the liver of fish and other oviparous vertebrates and are expressed in sexually mature females during oogenesis. The *vtg* and *zrp* gene transcripts are present but not naturally expressed proteins in juveniles or male

fish, but may be induced upon exposure to endogenous E2 or other ER-agonists (Arukwe et al., 1997; Hyllner et al., 1991; Purdom et al., 1994). Vitellogenin (gene and protein) is the most commonly measured biomarker in juvenile and male fish as an indicator of xenoestrogens in the aquatic environment (Heppell et al., 1995; Mommsen and Walsh, 1988; Purdom et al., 1994).

### 1.2.4 Estrogen receptor antagonism

In contrast to ER-agonists, suppression of vitellogenesis has been associated with exposure to ER-antagonists, causing reduced ER-mediated Vtg production in females which results in adverse effects such as reduced fecundity and consequently reduced reproductive success (Ankley et al., 2002; King Heiden et al., 2006). Compounds with anti-estrogenic effects may act through dissimilar MoA as either directly or indirectly modulate the activity of the ER and downstream cellular events.

Opposed to ER-agonists, ER-antagonists (e.g. 4-hydroxytamoxifen (OHT)) have variable sized bulky side chains which upon binding to the ER are not fully contained within the ligand-binding pocket. The side chains sterically hinders the helix12 from aligning into an agonist conformational position (Heldring et al., 2007; McDonnell and Wardell, 2010) (Fig. 3), consequently resulting in less or no recruitment of co-factors to the ER-ligand complex and no transcriptional activation of ER at the ERE (Heldring et al., 2007). There are various types of ER-antagonists which partially (Type I: binds to AF2) or fully (Type II: binds AF1 and AF2) binds and saturate the ER. Partial ER-antagonists such as OHT modulate AF2 in its LBD through a conformational position change of the ligand-dependent short helical region, causing instability in the ligand binding pocket of the receptor. Such instability will partly compromise recruitment of ER co-activators and may instead result in recruitment of ERα co-repressors such as the small SRA binding protein (SLIRP) (Hatchell et al., 2006), interfering with the ERE transcriptional activation of ER and target genes. *In vitro* exposure to OHT co-exposed with an ER-agonist may therefore result in partly suppressed transcriptional activation of ER-mediated genes (for full review see Macgregor and Jordan, 1998) and proteins (Petersen and Tollefsen, 2012).

### 1.2.5 Biotransformation

Endogenous estrogens, EDCs and other organic xenobiotics initiate various defence mechanisms in organisms through receptor recognition by the nuclear aryl hydrocarbon (also called “dioxin”) receptor (AhR). The AhR is abundantly distributed and expressed in the kidneys, heart, spleen and liver (Boelsterli, 2007) and has been proposed involved in normal physiological and developmental processes (McMillan and Bradfield, 2007) and metabolism of steroids (e.g. estrogen) (Scornaienchi et al., 2010). Several of these compounds interfere indirectly with the ER, resulting in reduced Vtg synthesis and impaired gonad development (Wannemacher et al., 1992) by transcriptional initiation and translation of AhR mediated proteins (e.g. cytochrome P450 1A) and enzymes (e.g. Ethoxyresorufin–O-deethylase (EROD)). Endogenous estrogens (e.g. E2) and xenobiotic compounds such as model AhR-agonists 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) and β-naphthoflavone (BNF) are metabolized mainly in the liver by CYP1A and transformed into less harmful and more water-soluble metabolites to efficiently be excreted from the body. The detoxification mechanism consists of phase I, II and III biotransformation enzymes, where phase I enzymes are mainly represented by the CYP-family. Phase II biotransformation consists of conjugation reactions which involves transforming endogenous steroids and xenobiotics to more water-soluble compounds through methylation (e.g. methyltransferase), glucuronidation (e.g. UDP-glucuronosyltransferases), and sulphation (e.g. sulfotransferases) before being excreted from the cells through Phase III membrane transporters of the multidrug resistance protein (MRP) family.

Initially, the AhR is activated upon presence of AhR-agonists such as planar PAHs (e.g. BNF) and dioxins, which displaces the AhR-heat shock protein 90 (Hsp90)-chaperone complex and forms a receptor-ligand complex (Fig. 4). The AhR-ligand complex is translocated into the nucleus, where it forms a heterodimer complex with the AhR nuclear translocator (ARNT), binds and initiates transcription upon recruitment of co-activators and RNA polymerase II in the dioxin/xenobiotic responsive element (DRE/XRE) in the DNA promotor region. Genes containing the XRE domain are CYP1A (phase I), glutathione S-transferase (GST), UDP-glucoronosyltransferase (UGT) (Phase II) and excretion proteins such as ATP-binding cassette (ABC) (Phase III) amongst others, that are transcribed and transported out of the nucleus to be translated into functional proteins (Fig. 4).

Biotransformation may however in some cases bioactivate compounds (e.g. brominated biphenyls, tamoxifen) into more reactive-metabolites (e.g. dihydroxylated bromobiphenyl,

hydroxytamoxifen) creating an ER-active hydroxyl group (-OH) which may modulate the activity of the ER (Mürdter et al., 2011; van Lipzig et al., 2005).

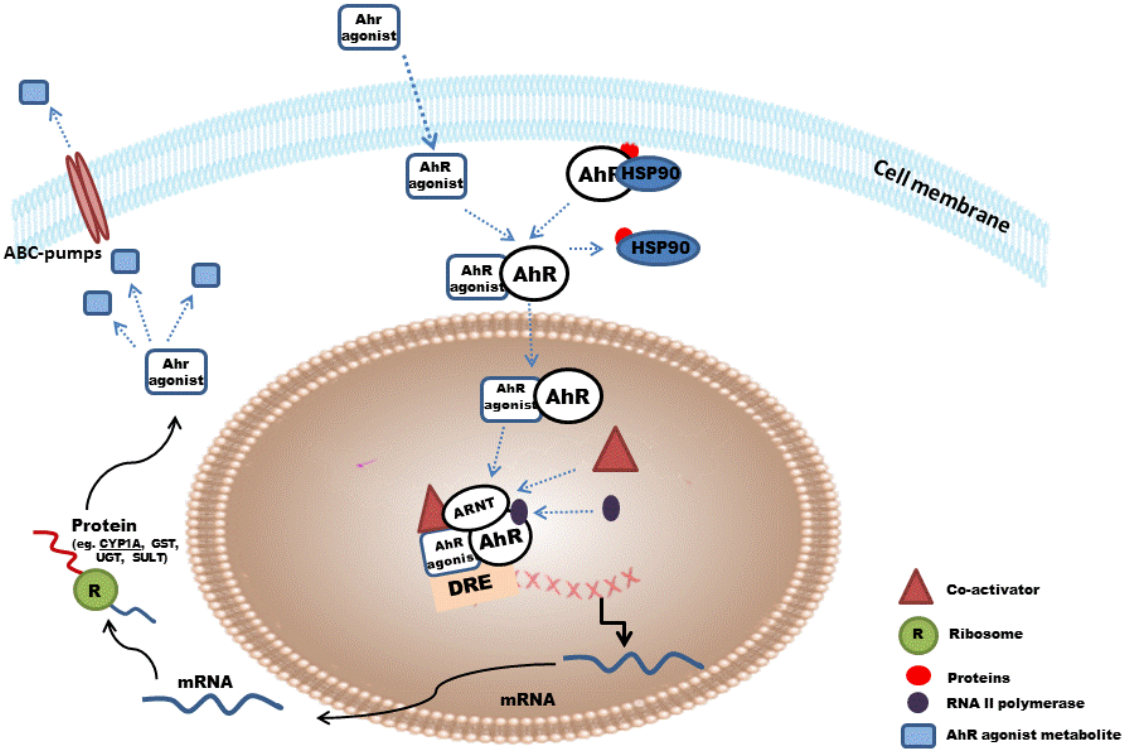


Figure 4. Simplified overview of genomic AhR mechanism when exposed to AhR-agonist. The AhR-agonistic MOA is based on Denison and Nagy, 2003.

### 1.2.6 AhR and ER cross-talk

Cross-talk between the ER and AhR in fish has been proposed in several studies (Anderson et al., 1996; Gräns et al., 2010) as an AhR-mediated inhibition of ER-mediated gene transcripts, resulting in anti-estrogenic effects (for full review see Safe and Wormke, 2003). The interaction between AhR and ER is believed to occur as both uni- and bidirectional receptor cross-talk (Bemanian et al., 2004; Matthews and Gustafsson, 2006; Mortensen and Arukwe, 2007), however the underlying MOA is still not fully understood. The cross-talk has also been proposed to be dependent on the exposure chemical, concentration and in which sequence the compound was added in a mixture (Mortensen and Arukwe, 2007).

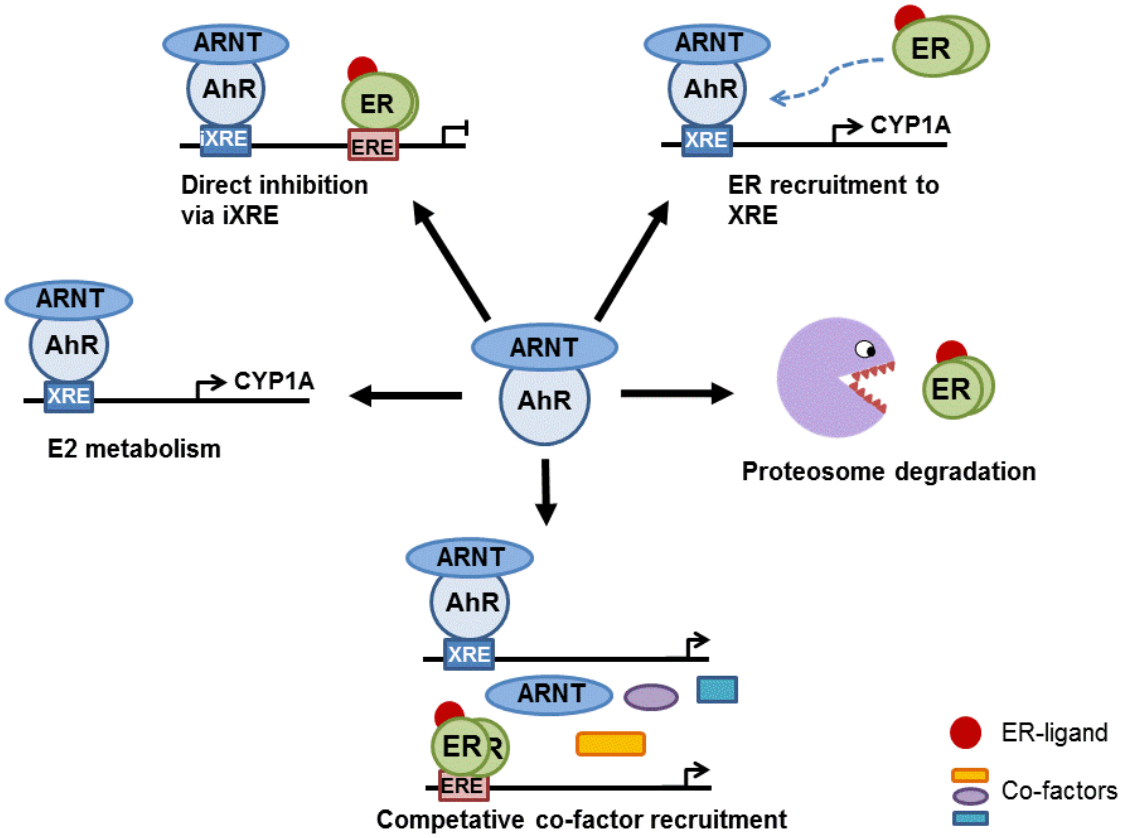


Figure 5. Proposed cross-talk mechanisms between the ER and AhR signalling pathways. The AhR has been reported to interfere with ER activation through several mechanisms: AhR-ARTN complex binding to the iXRE present in ER target genes, recruitment of unbound ER to the active AhR/ARNT complex in the XRE, AhR mediated proteasome degradation of ER, competitive recruitment of the same co-regulators and AhR/ARNT activated E2 metabolism. Abbreviations: AhR – Aryl hydrocarbon receptor; ARNT – Aryl hydrocarbon nuclear translocator; ER – Estrogen receptor; XRE – Xenobiotic response element; iXRE – inhibitory xenobiotic response element; ERE – Estrogen response element; CYP1A – Cytochrome P450 1A. Modified from Safe and Wormke, 2003.

Several different AhR-ARNT mediated activities have been proposed to contribute to the anti-estrogenic effects by interacting directly or indirectly with ER through a NR cross-talk. Mechanisms proposed for this AhR-mediated cross-talk are induction of estrogen metabolism through CYP1A (Safe and Wormke, 2003), proteasome degradation of the ER (Ohtake et al., 2003; Safe and Wormke, 2003), competition of common transcription co-factors such as ARNT (Brunnberg et al., 2003; Rüegg et al., 2008), direct suppression of ER transcription (Bemanian et al., 2004) or by upstream situated XRE which inhibits ER activation (Matthews and Gustafsson, 2006) (Fig. 5).

In addition to these characterised cross-talk mechanisms, nucleus based AhR-ARNT have been proposed to recruit and use unbound ERα as a transcriptional co-regulator, resulting in less transcriptionally active estrogen responsive genes (Matthews et al., 2005).

## 1.3 Alternative (*in vitro*) approaches to assessing ER-agonists and antagonists in fish

Alternative testing using non-animal test systems are defined by absolute to partial replacement of live animals in research (Goldberg and Frazier, 1989) and may consist of embryo-, or *in vitro* methods such as cell-based and cell-free techniques (Bols et al., 2005; Scholz et al., 2013; Tollefsen et al., 2003). Approaches such as cell-based techniques (e.g. immortalized and freshly isolated cells), tissues and fish embryos have been proposed as suitable assays for screening EDCs (Bols et al., 2005; Eide et al., n.d.; Finne et al., 2007; Scholz et al., 2013). The advantage of using *in vitro* bioassays is their ability to facilitate chemicals organ-specific MOA and screen a large number of chemicals simultaneously by using few or no animals at all (Bols et al., 2005). More specifically, cell lines and primary cell cultures have facilitated a better understanding of chemicals e.g. ER-agonists MOA, improving the understanding of apical toxicological effects occurring *in vivo* (Bols et al., 2005). Furthermore, *in vitro* facilitate cost-efficient concentration-dependent responses, which provides a basis for estimating the compound toxicity at different measurements of effect (e.g. the chemical concentration when 10% or 50% effect is observed (EC50)), results obtained from depicted concentrations-response curve (CRC) (Fig. 6) (Walker et al., 2012). The use of concentration response relationships may also provide a more precise description of the low dose effects in addition to the classical no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) which is derived from statistical hypothesis testing of the tested concentrations.

Primary tissues, monolayers and 3D-spheroid cell cultures derived from various organs (e.g. gills, liver, kidney) have demonstrated to retain biotransformation, detoxification and partial endocrine responsiveness (Avella et al., 1999; Baron et al., 2012; Cravedi et al., 1998; Eide et al., n.d.; Pelissero et al., 1993; Segner and Cravedi, 2000). Despite the potential of being good screening tools for EDCs (e.g. ER-active compounds), challenges such as lack of whole organism toxicokinetic and toxicodynamic regulation have limited the *in vitro* model implementation as an alternative to animal testing. Some of the *in vitro* models limitations

originate from their restricted number of target sites that may facilitate toxicological responses, not fully representing the diversity and/or complexity of the target sites *in vivo* (Schirmer, 2006). In addition, criticism towards the *in vitro* assays robustness has raised uncertainties to whether this alternative approach is suitable as a partial or full replacement for *in vivo* fish testing. Assessment of *in vitro* methods sensitivity, reproducibility and robustness in screenings of ER-active compounds is therefore warranted to better understand their limitations and future potential as an (eco)toxicological screening tool.

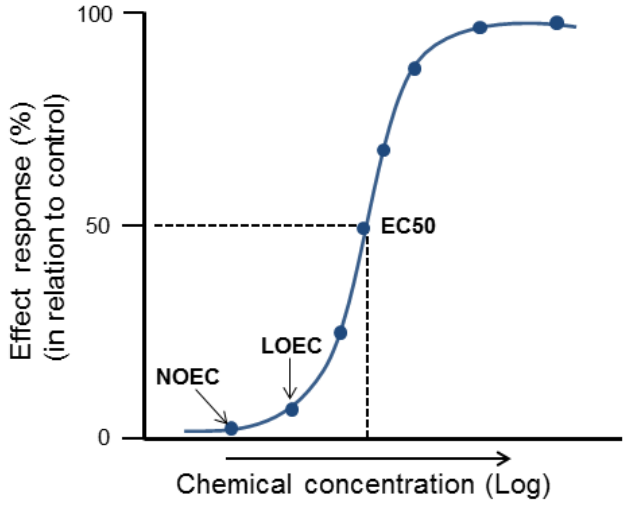


Figure 6. Organism effect response plotted against the chemical exposure concentration. Determination of no effect concentration (NOEC) may only be obtained when the lowest observed effect concentration (LOEC) is known as there would otherwise not be any indication of a toxic concentration. The NOEC and LOEC are determined using statistical hypothesis testing. Modified from Walker et al., 2012.

### 1.3.1 Primary hepatocytes

Many *in vitro* assays are of hepatic origin as the liver is a key organ for many processes such as maintaining the internal homeostasis through regulating metabolic (e.g. metabolism of sex steroids, biotransformation of xenobiotics) and physiological (e.g. reproduction) processes. The liver is often targeted by xenobiotics (*i.e* foreign substances) and has therefore been extensively studied in environmental toxicology (Bickley et al., 2009). Cells or tissues derived from the liver retain many of its native properties (e.g. biotransformation, metabolism, lipid metabolism) and well-established *in vitro* models such as primary hepatocyte cultures have successfully been used when screening chemicals with ER-activity and acute toxic properties in fish (Navas and Segner, 2006, 2000; Pelissero et al., 1993; Tollefsen et al., 2008a, 2008b).

The primary hepatocyte bioassay is a well-characterised method for screening of ER-agonists and antagonists. The liver cells retain organelle function, membrane stability, metabolic activity, detoxification response, partial endocrine response and lipogenesis for approximately <5-8 days of culturing (Braunbeck and Storch, 1992; Navas and Segner, 2006; Segner, 1998; Segner et al., 1994; Segner and Cravedi, 2000; Tollefsen et al., 2003). The hepatocytes have retained their native function of ER ligand binding regions and receptor activity of the liver, and share many functional similarities with the liver *in vivo* (Mortensen and Arukwe, 2006; Pelissero et al., 1993; Tollefsen et al., 2003). The hepatic monolayers have, in similarity to the *in vivo* liver, the ability to initiate and produce estrogen sensitive ER-mediated biomarkers such as Vtg, ZRP and Vigilin, hence proposed as suitable screening tools for screening environmentally relevant ER-agonists (Mortensen and Arukwe, 2007). In addition, detoxification responses such AhR-mediated CYP1A and EROD activity have successfully been performed in primary hepatocytes, demonstrating the assay versatility and multi-endpoint applicability when screening for AhR-agonists with anti-estrogenic effects (Navas and Segner, 2000; Pesonen and Andersson, 1997; Smeets et al., 1999). Furthermore, primary hepatocytes may account for compound cytotoxicity which is supportive of compounds potentially narcotic MoA when lack or decrease of other sub-lethal endpoint responses are observed (Schreer et al., 2005; Tollefsen et al., 2012, 2008a, 2008b).

The hepatocytes offer a high-capacity, small-scale bioassay, which is easily maintained under semi-sterile conditions and applicable to various endpoint analysis formats (e.g. molecular, sub-cellular) when screening ER-active compounds and environmental extracts (Farmen et al., 2010; Petersen and Tollefsen, 2012, 2011; Takemura, 2002; Tollefsen et al., 2008b). However, low reproducibility and variable sensitivity of the primary hepatocytes ER-response has been addressed as a concern, and the bioassay's predictability and suitability as a screening tool for EDCs has been questioned (Bols et al., 2005; Scholz et al., 2013). These concerns originate from primary hepatocytes low reproducibility within assays (intra-assay) and between assays (inter-assay) when compared towards more homogenous continuous cell lines (Bols et al., 2005). The primary cultures are more differentiated than cell lines and will better reflect the *in vivo* scenario as multiple cell-donors are used. There is therefore a need to assess and potentially standardize the primary hepatocyte method for testing of single chemical effects, synthetic mixtures and complex environmental samples.

# 2 Aim and objectives

The aim of this work was to investigate primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes suitability as a model for screening of single compounds and mixtures of ER-agonists and anti-estrogenic chemicals. The work was performed by pursuing the following objectives:

- Evaluating the sensitivity, responsiveness and reproducibility of Vtg gene and protein expression in primary rainbow trout hepatocytes as a biomarker for estrogenicity after exposure to the model ER-agonist 17α-ethinylestradiol (EE2).

- Characterising additional ER-relevant MoA of EE2 in rainbow trout hepatocytes by global transcriptional analysis.

- Characterising AhR-agonist (BNF) and ER-antagonist (OHT) single and combined anti-estrogenic effect on E2-induced Vtg protein expression by analysing AhR and ER-mediated transcriptional responses in primary rainbow trout hepatocytes during a short term exposure.

- Assessing primary rainbow trout hepatocytes ability to characterise ER-agonists in a diverse group of unresolved complex mixtures (UCMs) of hydrocarbons, measuring Vtg protein expression, EROD activity and cytotoxicity.

# 3 Methods

In the following chapter, all experimental studies, biological-, chemical analyses, statistics and bioinformatics used in the present PhD thesis will be presented. In order to evaluate a feasible alternative to fish *in vivo* experiments, an *in vitro* bioassay derived from rainbow trout was consistently used through-out the thesis as the experimental model. The bioassay was evaluated in terms of its sensitivity, reproducibility and responsiveness towards model ER-agonists, and used when characterising non-classical anti-estrogenic effects in single and simple mixtures of chemicals. Characterisation of a number of highly versatile and potentially ER-active compounds was performed using a combination of molecular and functional classical biomarkers with supportive endpoints measuring AhR-agonism and general narcosis.

## 2.1 Experimental studies

### 2.1.1 Choice of *in vitro* model

Rainbow trout has frequently been used as an ecotoxicological model both *in vivo* and *in vitro* as it has well-described toxicological and endocrine responses for EDCs, and acute toxic compounds, both singly and in mixtures (Benninghoff and Williams, 2008; Hook et al., 2010, 2008; Petersen and Tollefsen, 2012, 2011). The present work primarily evaluated the performance of the primary hepatocyte model when exposed to compounds interacting with the activity of the ER. Additional supportive endpoints such as cytotoxicity and AhR-mediated responses were measured as they are known to affect ER-mediated responses.

### 2.1.2 Experimental setup

The primary rainbow trout hepatocytes estrogen response was evaluated in three separate transcriptomic studies (Paper I, II & III), followed by a fourth study applying the assay in screening of compounds with potential ER activity (Paper IV) (Fig. 7). In the first study, primary rainbow trout hepatocytes ER sensitivity, responsiveness and reproducibility were assessed by measuring estrogen sensitive vitellogenin (Vtg) after exposure to the ER-agonist 17α-ethinylestradiol (EE2). To exclude compound cytotoxicity, cell viability (membrane integrity and metabolic activity) was assessed by measuring the emitted fluorescence of hydrolysed carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) in intact cell membranes and the reduction of resazurin to resorufin of Alamar Blue (AB) by the metabolically active cell mitochondria. The induction of Vtg gene and protein expression was determined by quantitative real-time polymerase chain reaction (qPCR) in addition to indirect

enzyme-linked immunoabsorbent assay (ELISA), respectively. Chemical analysis was performed to verify the exposure concentrations of EE2 in media and cells after 0-48h of exposure (Paper I). The material from the 48 hour EE2 exposure study was further used to characterise additional ER-relevant MoA, using a combination of a global oligonucleotide microarray and qPCR (Paper II). In the third study, the anti-estrogenic action of a set of single compounds and a simple synthetic mixture of ER and AhR-agonists (17β-estradiol and β-naphthoflavone) and type I ER-antagonist (4-hydroxytamoxifen (OHT)) were assessed after 48 and 96 hours. The protein response was determined by Vtg ELISA in the sampled cell media (96h), whereas the cells were subjected to qPCR analysis (48h) (Paper III).

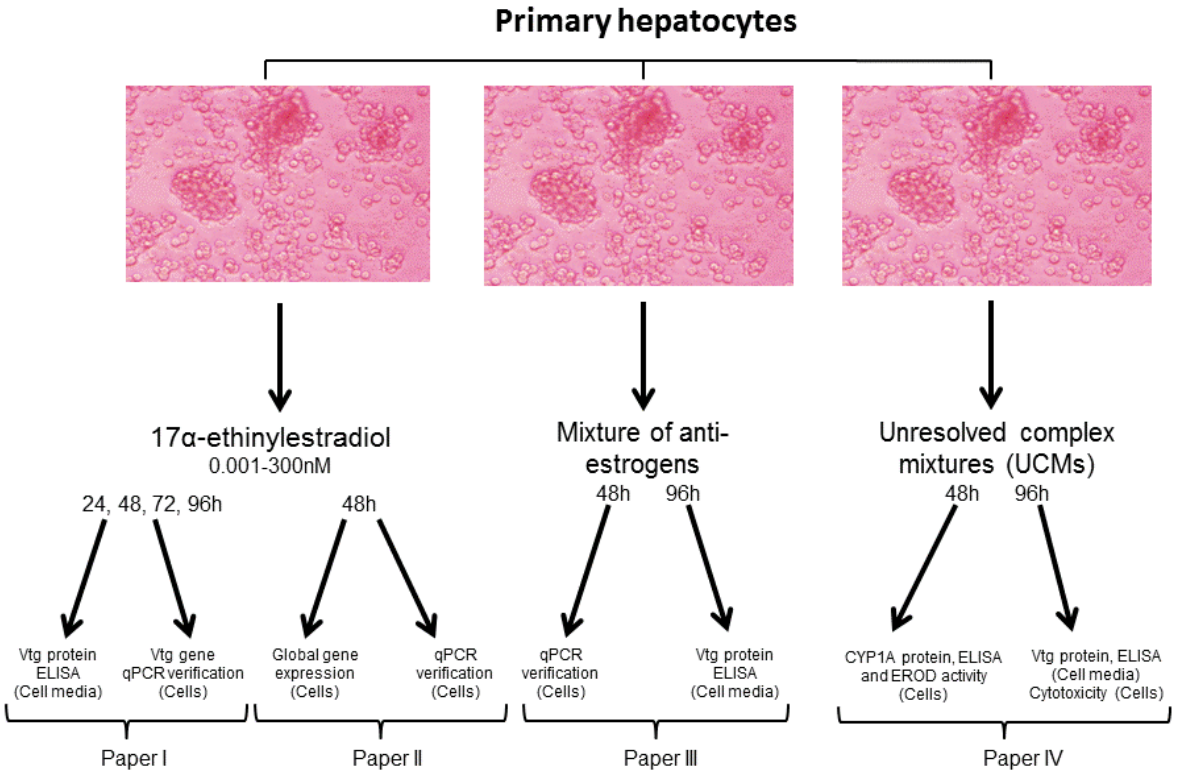


Figure 7. The experimental setup for the papers (I-IV) included in the present work. Two exposure studies were independently conducted to characterise single compounds and mixtures with estrogenic and anti-estrogenic mode of action (MoA) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes (Paper I-III). The third study characterised the potential estrogenic MoA of compounds associated with unresolved complex mixtures of hydrocarbons (UCMs) (Paper IV). Abbreviations: Vitellogenin (Vtg), Cytochrome P450 1A (CYP1A), ethoxyresorufin-*O*-deethylase (EROD) and quantitative real-time polymerase chain reaction (qPCR).

In the last study (Paper IV), primary rainbow trout hepatocytes were exposed to a number of single chemicals associated with the unresolved complex mixtures (UCMs) of hydrocarbons to characterise their potential toxic MoA and in particular the potency to modulate the activity

of ER. The compounds modulatory properties on ER were assessed by measuring Vtg protein expression and several other supportive endpoints such as cell membrane stability, metabolic- and ethoxyresorufin-O-deethylase (EROD) activity after 48 and 96h of exposure. To support the *in vitro* results, the compounds MoA and baseline toxicity for *in vivo* fish were classified and predicted using the Russom model (Russom et al., 1997) and Ecological Structure Activity Relationships, ECOSAR in the Chemprop ver. 2 software (http://www.ufz.de/index.php?en=6738) respectively.

### 2.1.3 Choice of chemicals

The choice of chemicals for paper I and II were based on their well-described properties to modulate the ER. The chemical EE2 was chosen as being an environmentally-relevant ER-agonist in fish (Purdom et al., 1994), which have demonstrated to be highly potent. The selection of an AhR-agonist (BNF) and ER-antagonist (4-OHT) in paper III were based on their differently acting anti-estrogenic MoA on the ER, using non-toxic concentrations based on previously published $EC_{50}$ and 50% inhibition concentration ($IC_{50}$) values for interfering with Vtg production in rainbow trout hepatocytes (Petersen and Tollefsen, 2012). The chemicals associated with the unresolved complex mixtures (UCMs) of hydrocarbons were selected to test the *in vitro* systems applicability on a diverse group of compounds being suspected of endocrine activity (Melbye et al., 2009; Scarlett et al., 2012; Thomas et al., 2009). The selected chemicals displayed a wide range of different physico-chemical properties (hydrophobicity (Log $K_{ow}$), molecular size and structure, volatility and toxic MOA). All tested chemicals are described in the Supplementary Table 1.

## 2.2 Analytical tools

### 2.2.1 Chemical analysis

An ultra-performance liquid-chromatography tandem mass spectrometer (UPLC-MS) was used in the chemical analysis of EE2 (Paper I), due to its accurate, sensitive and reproducible high-performance measurement of low chemical concentrations. Described in brief, all EE2 exposed cells and media were sampled and derivatised to quantify the low EE2 concentration in cell media and the cells themselves. Following derivatization, both media and cell samples

were injected into a UPLC-MS and analysed for EE2 using the method described more in detail in Fox et al. (2011).

### 2.2.2 Transcriptomics

Gene expression is considered as a highly sensitive, multi toxicity endpoint to study, reporting the chemicals early modulatory effects on a molecular level. During the last decade rapid development and improvement of toxicogenomic (OMICS) tools using e.g. transcriptomics have facilitated a better understanding of chemicals MOA and MoA in mammalian (mice, rat, humans) and other vertebrate and invertebrate (teleost fish, crustaceans, nematodes) organisms. The use of transcriptomics have depicted some of the complexities encountered in chemical mixtures (Altenburger et al., 2012), which further have contributed to the understanding of previously, not fully understood molecular mechanisms and adverse effects.

#### *2.2.2.1 RNA isolation and quality control*

Primary hepatocytes were sampled and subjected to RNA isolation and stored at -80$^{o}$C immediately upon isolation to avoid degradation of RNA by RNAses (for further details see Paper I, II and III). RNA was isolated using the RNeasy mini Plus kit (Qiagen GmbH, Hilden, Germany) with on-column DNAse treatment according to a slightly modified version of the manufacturer's protocol (details provided in Paper II). The RNA was quality assessed spectrophotometrically to avoid any interference with the incorporation of fluorescence dye in the microarray and qPCR. The 280/260 (≥2.0) and 260/230 (≥1.8) ratios were measured to verify that the RNA did not contain contamination of phenol and guanine salt, respectively. RNA integrity was assessed with the Agilent BioAnalyzer 2200 (Agilent Technologies) by measuring the 28S/18S peak ratio, which calculates the RNA integrity index (RIN). The acceptable cut-off criteria of RIN was ≥8.0 (Fleige and Pfaffl, 2006), which were applied to all RNA samples used for upstream transcriptomic analysis. Once isolated and quality assured, the RNA was subjected to microarray and/or qPCR analysis.

#### *2.2.2.2 Microarray design*

A custom 60k oligonucleotide microarray (Agilent Technologies, Santa Clara, USA), based on 40,000 unique probes, were previously designed based on the assembly of cGRASP

(web.uvic.ca/grasp/microarray) contingous (contigs) mRNA in combination with Unigene (www.ncbi.nlm.nih.gov/unigene) sequences from two salmonids species (*Salmo salar*: build 31; *O. mykiss*: build 27). The Unigene and mRNA sequences were annotated by blasting against the non-redundant (nr) protein database (NCBI) using blastX, followed by Interpro protein sequence analysis and classification (Hunter et al., 2011), Gene Ontology functional assignment (Ashburner et al., 2000) and mapping the sequences to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa and Goto, 2000) using the application Blast2Go (Conesa et al., 2005) as previously described by Song (2014c). The performance of the oligonucleotide salmonid array has been thoroughly evaluated for different salmonid species and pollutants (Norwegian institute for water research (NIVA), unpublished results; Song et al., 2014) and used in a number of studies (Paper II; Song et al., 2014a, 2014b).

#### *2.2.2.3 One-color microarray sample labelling and hybridization*

Global transcriptional changes were determined by one-color microarray analysis, where fluorescent-tagged copies of RNA are hybridized to complementary oligonucleotide probes fixed on a glass array. The fluorescence intensity of the oligonucleotide probes are determined by the number of RNA copies present in the sample, eliciting higher fluorescence intensity upon increased gene expression. Briefly described, RNA was used to synthesize first strand cDNA which later was used as a template to synthesize fluorescence labelled cRNA, in accordance to the manufacturer's protocol. The labelled cRNA samples were purified using a Qiagen`s RNeasy mini kit and measured spectrophotometrically (Spectrophotometer ND 1000, Nanodrop technologies Inc., Wilmington, USA) applying manufacturers quality cut-off criteria's (cRNA yield: 0.825µg; Cy3 activity/µg cRNA: 6 pmol). Before hybridization onto the microarray, the cRNA was fragmented to a calculated size of 50–200 nt. The fragmented hybridization mix was carefully pipetted onto the gasket slides, the two parts were then closed and fixed with the active array side towards the sample using a hybridization chamber. Each sample was individually hybridized for 17 hours, followed by several washing and drying steps, strictly following the manufacturer protocol. This was immediately followed by scanning in an Agilent high resolution microarray scanner at 3 µm resolution and scanning area of 61×21.6mm. The scanned microarray images were quality assessed for colour saturation and scanning results extracted using Agilent Feature Extraction software v10.7. All microarray raw data was subjected to correction for background signal, flagged for missing

and low quality features, Log2-transformed and expressed as normalized quantiles using GeneSpring software (GX v12.6, Agilent Technologies).

#### *2.2.2.4 Quantitative real-time polymerase chain reaction (qPCR)*

Quantitative PCR was used as an amplification method that monitors the gene amplification in real time using the fluorescent probe SYBRGreen™. In the present work, qPCR was used to quantify gene expression and verify microarray performance. To briefly summarize, isolated RNA from the exposed primary hepatocytes was reverse transcribed to cDNA in order to measure differently expressed gene transcripts (DEGs) from single endpoint (e.g. *erα, vtg, ahrα, cyp1a*) (Paper I and III) and microarray analysis (Paper II). All primers used were optimized to achieve best amplification performance in the qPCR analysis. A total of 3-4 reference (housekeeping) genes were evaluated for each study and one was used to normalize the gene expression. The housekeeping genes used were ubiquitin (Paper I & III) and elongation factor 1α (Paper II). Data normalization was performed using the ΔCq($2^{-\Delta\Delta Cq}$) method (real-time PCR application guide, Bio-Rad) (Paper I) and the Pfaffl method (Pfaffl, 2001) (Paper II and III). All pipetting onto the 96/384-well plate format was performed by automated procedure using a pipetting robot (Biomek® 3000, Beckman Coulter) to avoid errors by human interference. The qPCR analysis was performed on a CFX-96 or CFX-384 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) following the quantitative Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009).

### 2.2.3 Enzyme-linked absorbent assay (ELISA)

Characterisation of the estrogenic or anti-estrogenic effects of the different compounds were performed by determination of Vtg protein expression directly in the primary hepatocyte culture media by enzyme-linked absorbent assay (ELISA). In the present work, an indirect semi-quantitative (capture) ELISA was applied, sampling cell media of exposed primary rainbow trout hepatocytes after 24-96h (Paper I-IV) into a 96-well immunosorbent plate. In brief, the Vtg protein in the cell media adsorb to the plastic walls of a specially designed ELISA microtiter plate where upon a primary monoclonal mouse anti-salmon Vtg antibody are allowed to bind to the Vtg proteins. This was followed by addition of a secondary goat anti-mouse IgG antibody conjugated to the enzyme horseradish peroxidase (HRP) having high affinity for the primary antibody and allowed to bind to the immobilized antigen-antibody

complex. After incubation for 2h, the enzymatic activity of the HRP was measured using the substrate 3,3`,5,5`-tetramethylbenzidine (TMB). The enzymatic reaction was stopped by addition of acid ($H_2SO_4$) and the fluorescence read in a microplate reader at 450 nm to quantify the relative amounts of Vtg.

## 2.3 Data processing

### 2.3.1 Sample quality assurance/assessment

All samples used in the nucleic acid-based methods (qPCR and microarray) in the current thesis have been subjected to a thorough quality control (for details see Paper I, II and III) to meet the quality cut-off criteria of the transcriptional analysis previously described (section 3.2.2.1).

Final quality verification of the microarray data was performed by qPCR verification of relevant DEGs to identify false positives due to cross-hybridizations and/or non-specific saturation of target sequences onto the probes (Morey et al., 2006). To ensure that no primer-dimer formation, water- or genomic DNA contamination were present in the qPCR analysis, non-template (water) controls (NTC), no-reverse transcriptase (no-genomic DNA) control (NRT) and melting curve analysis were performed routinely during analysis.

### 2.3.2 Statistics

The microarray raw data was subjected to a one-way analysis of variance (one-way ANOVA) followed by Tukey's post hoc test using GeneSpring software (GX v12.6, Agilent Technologies). Statistical analysis of data for single endpoint responses was performed in order to determine significant ($p \leq 0.05$) differences between the control and chemical treatments of the hepatocytes. Graphs and basic statistical analysis were conducted with Graphpad Prism (v5.00, GraphPad Software, San Diego California USA). In paper I, III and IV, the Vtg gene (qPCR) and Vtg protein (ELISA) expression and EROD activity was log(10)-transformed and subjected to a one-way ANOVA, followed by a Dunnett`s Post Hoc test. Data presented in Paper III was in addition subjected to a two-way non-parametric ANOVA followed by Tukey's post hoc test to describe the potential mixture interactions occurring among the different treatments.

### 2.3.3 Bioinformatics

The Gene Ontology (GO) functional enrichment analysis was performed with the GeneSpring software (Agilent Technologies, Santa Clara, CA, USA). All network and pathway analysis was performed by mapping the salmonid RNA sequences to their mammalian orthologues and performing analysis using IPA (Ingenuity®Systems, http://www.ingenuity.com). The microarray data (Paper II) was not subjected to false discovery rate (FDR) correction to avoid removing biologically relevant genes (Villeneuve et al., 2011). To avoid generating false positives in the microarray data, qPCR was performed to verify the gene expression analysis for a selection of genes from the microarray.

#### *2.3.3.1 Functional enrichment analysis*

The Functional enrichment analysis was performed to characterise the DEGs involvement in different biological processes, molecular functions and cellular compartments in the organism. The Gene Ontology (GO) based functional analysis included all significantly regulated gene transcripts in the data and assigned them to their major ontologies to get an overview of the gene products involvement in the various physiological processes.

#### *2.3.3.2 Orthologue mapping*

In order to further characterise the global changes in the gene expression of paper II, orthologue-based function analysis was performed with Ingenuity pathway analysis (IPA), mapping the salmonid sequences to their mammalian orthologues using the eukaryotic orthologue database InParanoid (Ostlund et al., 2010). Significant DEGs were successfully mapped 57.7± 3.15% (mean ±SD) towards putative mammalian orthologues (Paper II).

#### *2.3.3.3 Pathway analysis*

Pathway analysis was performed to obtain an overview of the casual connections between putative protein-protein interactions involved in regulatory networks, toxicological and canonical pathways relevant for specific toxicological and biological processes. The information of the pathway analysis contributed to a better understanding of chemicals MoA in exposed primary hepatocytes (Paper II). The ingenuity pathway analysis (IPA), which integrate and interpret the microarray data based on a NCBI expert-curated mammalian-based databases (Ensembl, Entrez Gene, RefSeq, GenBank, UniProt/Swiss-Prot Accession,

GenPrept and UniGene), were used to decipher biological relationships in the data on basis of identification of mammalian orthologues. The pathways analysis only used DEGs that were successfully mapped to their mammalian orthologues. Successfully mapped DEGs were included in the gene (protein-protein interaction) network analysis, toxicity- and canonical pathway analysis.

# 3 Summary of main findings

## 3.1 Paper I: Evaluation of the sensitivity, responsiveness and reproducibility of rainbow trout (*Oncorhynchus mykiss*) *in vitro* vitellogenin production as a screening assay for estrogen mimics.

The primary rainbow trout hepatocyte bioassay suitability as a screening assay for ER-agonists was determined after exposure to the ER-agonist 17α-ethinylestradiol (EE2). In brief, primary hepatocytes of juvenile fish were exposed to 0.001-300nM EE2 to assess the role of exposure duration (24-96h), seasonal variability (January-July), microplate format (6, 24 and 96 well plates) and data normalization (relative potency of positive control) to identify factors affecting the robustness (sensitivity, responsiveness and reproducibility) of the bioassay. Vitellogenin gene and protein expression was measured by qPCR and ELISA, respectively. The cellular biotransformation of EE2 was determined by measuring EE2 in both cells and media by UPLC-MS at defined exposure times.

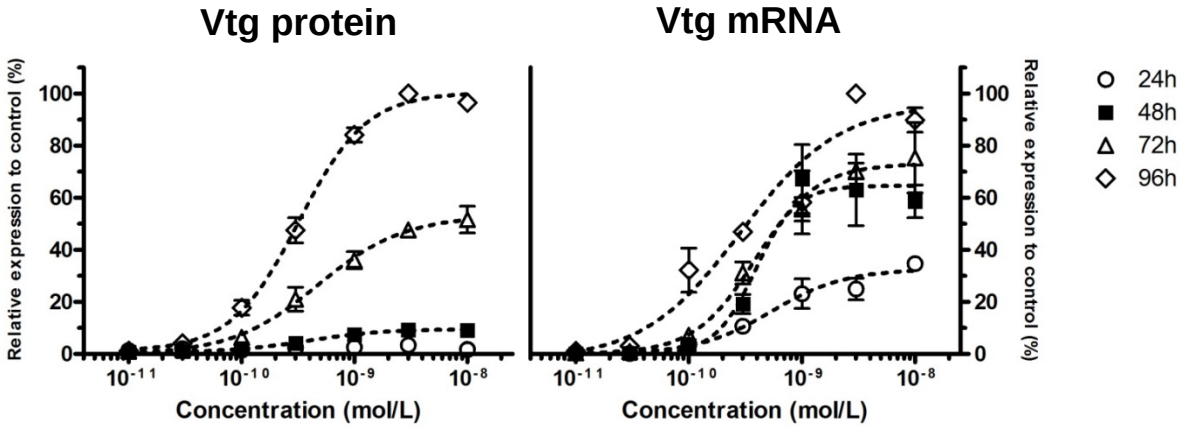


Figure 8. Vitellogenin (Vtg) protein and mRNA induction in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed for 24-96 hours to 17α-ethinylestradiol (EE2). Data (Mean ± SEM) were normalized against solvent control (DMSO) and expressed as percentage of mRNA and protein Vtg maximum (3nM EE2 at 96h), the lines represent non-linear regression curve fit to experimental data from minimum 3 independent cell isolations performed between January-July.

The results suggest primary rainbow hepatocytes to be sensitive, responsive and reproducible when normalized to the maximum Vtg expression by EE2 or E2 and minimum vtg induction

of the solvent control (DMSO). EE2-induced Vtg gene and protein expression was observed in a concentration dependent manner in hepatocytes exposed for 24- and 48 hours respectively. The exposure duration of 48h (gene expression) and 96h (protein expression) were identified to optimal as yielding the lowest inter-replicate variability and highest quality ($r^2 \geq 0.8$) concentration response curves (CRCs) (Fig. 8). In conclusion, the bioassay response was not affected by well plate format or seasonal changes in measured Vtg sensitivity or responsiveness when applying suitable data normalization. Furthermore, the cells high biotransformation capacity for EE2 demonstrated the importance of performing re-exposure of cells exposed for more than 48h.

The current study reported that use of optimal exposure duration and normalization procedure yielded consistent and reproducible data.The study demonstrated that primary hepatocytes are a robust assay when measuring Vtg mRNA and protein expression as it was highly estrogen sensitive, responsive and reproducible when exposed to EE2.

## 3.2 Paper II: 17α-Ethinylestradiol (EE2) effect on global gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes

To further address primary hepatocytes ability to characterise the MoA of EE2, cells exposed for 48 hours (paper I) was used in paper II to perform a global gene expression analysis using a one-color microarray and qPCR. In brief, data of the global gene expression analysis was subjected to functional enrichment analysis and orthologue mapping to better understand gene transcripts involvement in different molecular and cellular processes associated with exposure to EE2.

The results from the study showed that the 60k cDNA microarray identified a total of 1098 differently expressed genes (DEGs) in the primary hepatocytes across all exposure concentrations. A concentration dependent increase of DEGs was observed, identifying 66 (0.03nM), 114 (0.3nM), 468 (3nM) and 992 (30nM) DEGs as significantly regulated.

Classical estrogen biomarker genes such *Vtg*, egg shell protein zona radiata (*zrp3 & 4*), follistatin (*fst1*) and estrogen receptor α (*erα*) were all up-regulated in a concentration dependent manner on the microarray and in the qPCR analysis, indicative of ER-responsive

genes being the main target of EE2. The ER signalling pathway was manually assembled based on well-known ER-mediated responses from the microarray analysis (Fig. 9). Several of these DEGs were enriched and mapped to ED-relevant Gene Ontology (GO) functions such as reproductive process in a multicellular organism, hormone receptor binding and lipid and fatty acid metabolism (e.g. lipid localization) (Fig.10).

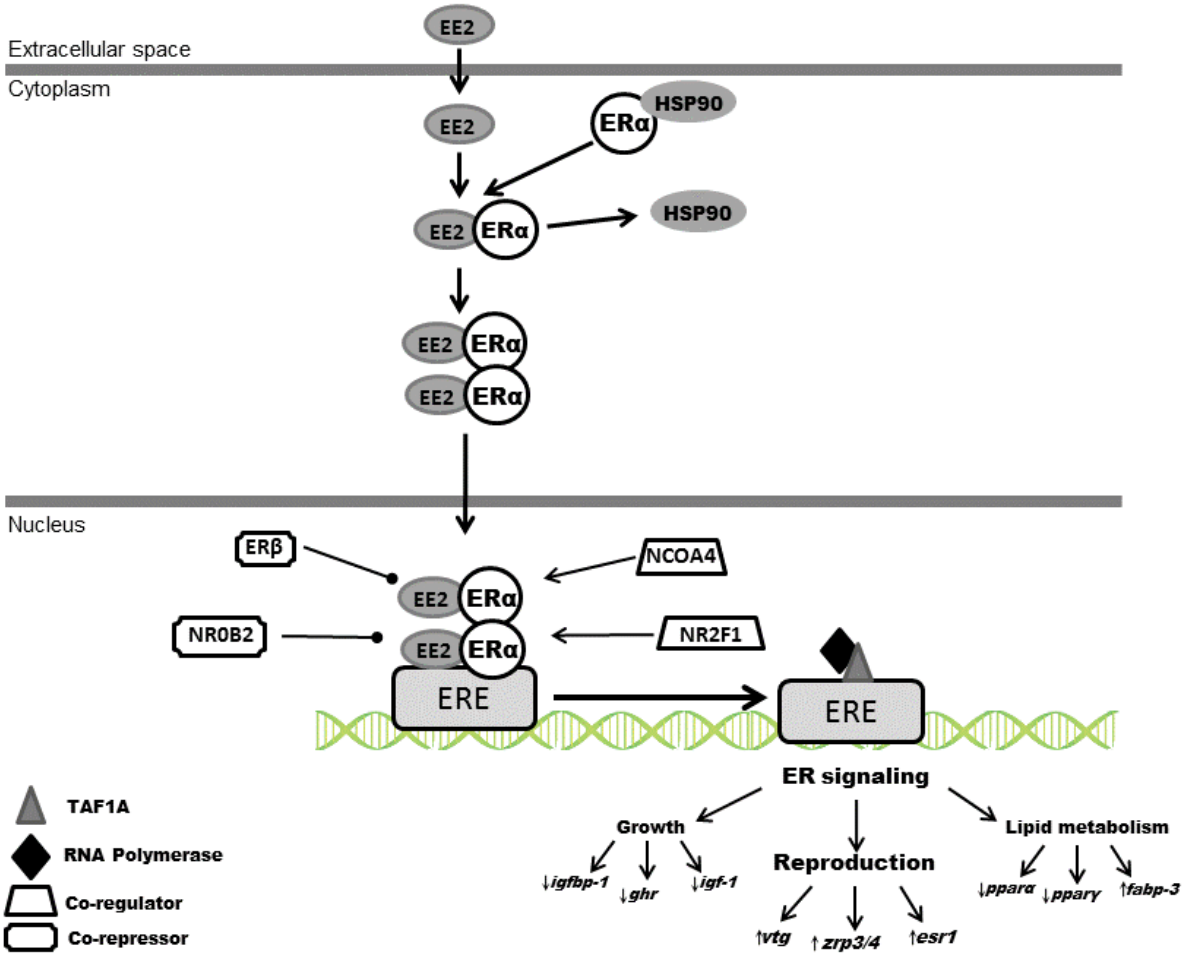


Figure 9. Estrogen receptor (ER) signalling pathway in fish based on differential gene expression in primary rainbow trout hepatocytes exposed to 17α-ethinylestradiol (EE2) for 48h. Abbreviations: HSP90 - heat shocking protein 90; ERE – Estrogen responsive element; NCOA4 - nuclear receptor coactivator 4, NR2F1- nuclear receptor subfamily group member 1; NR0B2 - nuclear receptor subfamily group member 2 ; IGFBP-1 – insulin growth factor binding protein 1; IGF-1 – insulin growth factor 1; GHR – growth hormone receptor; VTG – vitellogenin; ZRP3/4 – zona radiata protein 3/4; FST1 – follistatin 1, *PPAR*α/γ – peroxisome proliferator-activated receptor α/γ, FABP-3 – fatty acid binding protein 3.

Pathway analysis based on mammalian orthologues identified a total of 22, 14, 71 and 77 pathways significantly enriched in cells exposed to 0.03, 0.3, 3 and 30nM EE2, respectively. The identified DEGs were associated with down-regulation of well-known genes involved in xenobiotic biotransformation phase I and II (cytochrome P450 1A (*cyp1a*), sulfotransferase 6b (*sult6b*)) in intermediate (3nM) and high (30nM) concentrations of EE2. Although simultaneously, up-regulation of phase II and III (UDP-glucuronosyltransferase 2A1 precursor (*ugt2a1*) ATP-binding cassette transporter 11b (*abc11b*)) transcripts in the same range of EE2 concentrations indicated induced compound metabolism. Furthermore, DEGs associated with lipid homeostasis (peroxisome proliferator activating receptor γ (*pparγ*), fatty acid binding protein 3 (*fab3*), apolipoprotein F (*apof*), prostanglandins) and growth (insulin growth factor binding protein 1 (*igfbp1*), growth hormone receptor (*ghr*),) suggested that the overall transcriptional regulation of cell growth, lipid metabolism and synthesis were also affected by EE2. This was further supported by concentration-dependent enrichment of toxicologically relevant pathways such as insulin receptor signalling, calcium signalling, glucocorticoid signalling, gonadotropin hormone (GNRH) signalling, and growth hormone signalling.

In conclusion, the global gene expression analysis characterised the MoA of EE2 using primary hepatocytes. The present study demonstrated that EE2 modulate gene transcripts associated with ER-signalling, ER-mediated cellular responses, lipid metabolism and cellular growth in the hepatocytes. The results indicate that the primary hepatocytes are suitable for characterising the MoA of EE2 using global gene expression analysis.

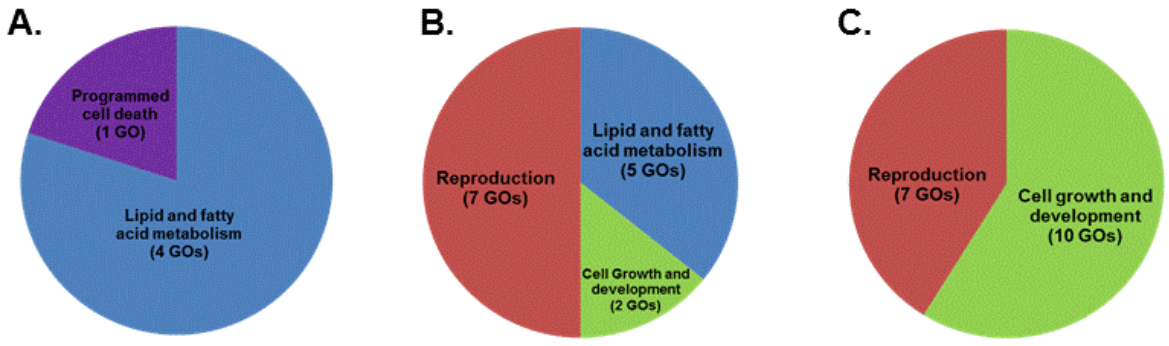


Figure 10. Major functional categories of over represented Gene Ontology (GO) biological processes regulated in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after 48 hours of exposure to 17α-ethinylestradiol (EE2). Figures A, B and C represent EE2 concentrations 0.3, 3 and 30nM respectively.

## 3.3 Paper III: Deciphering combined effects of anti-estrogenic chemicals on vitellogenin production in rainbow trout (*Oncorhynchus mykiss*) hepatocytes

Paper III focused on characterising how single and mixtures of compounds with differently acting MoA reduced E2-induced vitellogenin protein expression in primary hepatocytes as a measure of anti-estrogenicity. Investigation of how the compounds alone or in mixture elicit their anti-estrogenic effects was conducted by analysing AhR- and ER-mediated transcriptional responses with supportive genes involved in the AhR-ER nuclear receptor cross-talk. The primary hepatocytes were also assessed for their suitability as a screening tool for anti-estrogenic compounds with differently acting MoA. The primary hepatocytes were exposed for 48 hours to single and mixtures of the AhR-agonist β-naphtoflavone (BNF) and ER-antagonist 4-hydroxytamoxifen (OHT)) in absence and presence of 17β-estradiol (E2). The cells were then subjected to qPCR and Vtg ELISA analysis (Fig. 7). Analysis of AhR- (*ahrα, cyp1a*) and ER responsive genes (*erα, vtg-1, zrp*) were performed to describe the compounds anti-estrogenic MoA. In addition, the putative genes *arnt* and *cullin 4b* were analysed as previously proposed involved in AhR-mediated AhR-ER cross-talk.

### 3.3.1 ER-signalling

#### *3.3.1.1 Vitellogenin protein expression*

The compounds BNF, OHT and BNF+OHT caused a reduction of 40%, 25% and 60% respectively in E2-induced Vtg protein expression, when compared to the positive control E2 (Fig.11, Table 1). The significant anti-estrogenic effect of the BNF+OHT mixture indicated that the compounds caused a combined effect on ER-mediated Vtg protein expression in presence of E2. No clear evidence of a significant interaction occurring amongst the treatments were identified by a two-way ANOVA statistical test (Fig. 11), possibly due to the variable Vtg protein expression amongst the cell-batches used. The Vtg protein accommodates multiple MoA's and to decipher the single and combined compounds anti-estrogenic MoA an analysis of ER- and AhR-mediated responses was conducted.

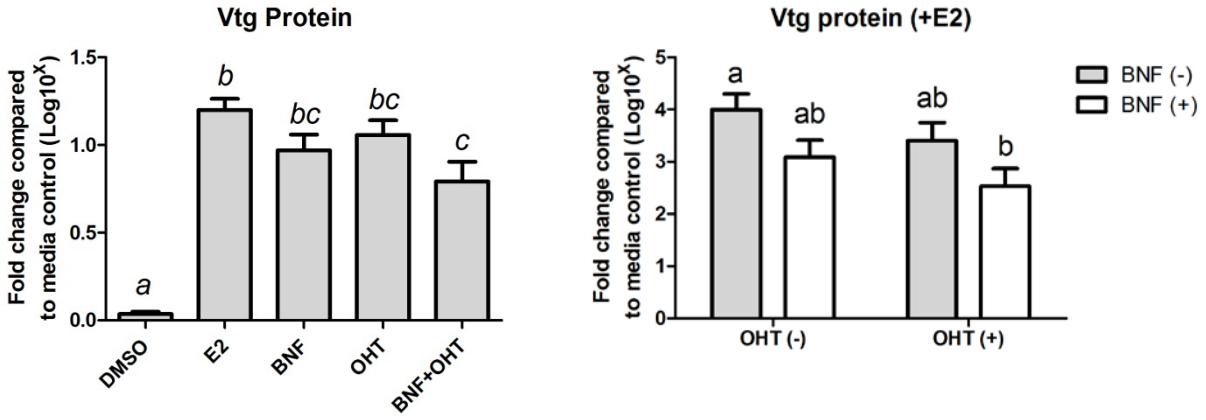


Figure 11. Vitellogenin protein expression in primary hepatocytes exposed to solvent control dimethyl sulfoxide (DMSO), 17β-estradiol (E2: $6.3E^{-10}$ mol/L), β-naphtoflavone (BNF: $1.10E^{-7}$ M), hydroxytamoxifen (OHT: $4.71E^{-9}$M) and a mixture of BNF and OHT in presence of E2 for 96h. Data is presented as fold change of media control and represent the mean of 3 individual experiments ± standard deviation. The statistical analysis for the left graph was performed using a one way-ANOVA with a Tukey's post hoc test while the right graph presents a two-way ANOVA using Bonferroni post hoc test. The different letters denote treatments which are significantly ($p > 0.05$) different from each other in both the one-way and two-way ANOVA.

#### *3.3.1.2 ER-mediated transcription*

The compounds BNF, OHT and a mixture of these caused an apparent reduction of E2-induced *erα* and its target genes *vtg-1 and zrp* in cells exposed for 48h (Table 1). The compounds reduced all the ER-mediated responses in a similar manner as that reported for Vtg protein expression at 96h, indicative of the close coherence between the molecular and sub-cellular regulation of ER activity in the cells. The compounds differently acting MoA both modulated the activity of the E2 induced ER. None of the anti-estrogens activated the ER in absence of E2, indicative that neither OHT nor BNF have the capacity to alone modulate the ER activity in the primary hepatocytes.

#### *3.3.1.3 AhR-mediated transcription*

To characterise how the compounds interfere with ER activity by interfering with biotransformation, the expression of the genes *ahrα* and *cyp1a* were assessed. The *ahrα* was identified to not be differently expressed by any of the compounds or solvent control. However, AhR-mediated *cyp1a* gene expression was significantly up-regulated in all treatments containing BNF both in presence and absence of E2 (Table 1). These results suggested *cyp1a*-mediated metabolism to contribute to the more than anticipated reduction of E2-induced Vtg protein expression in the mixture of BNF+OHT.

Table 1 - Fold change of transformed gene data compared towards the solvent control (DMSO). The data was subjected to a one-way ANOVA with a Tukey post hoc test. The different letters (a,b) denote treatments which are significantly ($p > 0.05$) different from each other.

| | | **ER mediated** | | | | **AhR mediated** | |
|---|---|---|---|---|---|---|---|
| | | **Vtg protein** | ***erα*** | ***vtg-1*** | ***zrp*** | ***ahrα*** | ***cyp1a*** |
| ***E2*** | | 10.85[a] | 16.42 [a(a)] | 4341 [a(a)] | 14.58 [a(a)] | 1.4 [a(a)] | 0.6[a(a)] |
| ***BNF*** | ***(+)E2*** | 6.62 [a] | 16.28 [a] | 3865 [a] | 11.18 [a] | 3.49 [a] | 19.53[b] |
| | ***(-)E2*** | - | 1.36 [(b)] | 3.01 [(b)] | 0.54 [(b)] | 1.7 [(a)] | 12.9 [(b)] |
| ***OHT*** | ***(+)E2*** | 8.06 [a] | 14.52 [a] | 4140 [a] | 12.98 [a] | 1.41 [a] | 1.80 [a] |
| | ***(-)E2*** | - | 1.27 [(b)] | 2.0 [(b)] | 1.29 [(b)] | 0.50 [(a)] | 1.62 [(a)] |
| ***BNF+OHT*** | ***(+)E2*** | 4.34[b] | 11.86 [a] | 3406 [a] | 8.05 [a] | 3.02 [a] | 20.01[b] |
| | ***(-)E2*** | - | 1.75 [(b)] | 0.9 [(b)] | 1.75 [(b)] | 2.44 [(a)] | 17.03 [(b)] |

To describe the mixture BNF+OHT potential interaction and thus its combined anti-estrogenic effect, a two-way ANOVA was performed. The were no evidence of an significant interaction occurring amongst the treatments in neither ER- (*erα, vtg-1* and *zrp*) or AhR-mediated (*ahrα* and *cyp1a*) genes (data not shown) that could explain the greatly reduced Vtg protein expression. The genes *arnt* and *cullin 4b* were found to not be differently expressed in any of the treatments (data not shown), indicative of not having a crucial function in the present study.

In summary, OHT likely caused direct interaction with the binding and activation of the ER, whereas BNF activated AhR-mediated increase in biotransformation activity (*ahrα* and *cyp1a*) that indirectly may have reduced E2 in the bioassay. The combination of the two caused an apparent increase in anti-estrogenicity that could indicate that both MoA were affecting the suppression of Vtg production. The primary hepatocytes were considered to be a suitable screening tool for anti-estrogenic compounds with direct and indirectly acting MoA. However, due to large inter-cell batch variations few endpoints and treatments were determined as significantly different from each other.

## 3.4 Paper IV: Toxicity of organic compounds associated with unresolved complex mixtures (UCMs) in primary fish hepatocytes

The toxicity of organic compounds associated with UCMs of hydrocarbons is poorly understood and the MOA of most of these compounds are mainly unknown. Despite this, presence of these compounds in petroleum industry such as sand-oil processes and produced water have been identified and proposed as a potential environmental threat in and around areas of emissions. The more polar compounds associated with UCM are components often found in crude oil and degraded petroleum such as aliphatic naphthalenes, aliphatic monocyclic-, aliphatic tricyclic-, monocyclic di-, monocyclic tri-, monoaromatic-, polycyclic monoaromatic-, monocyclic thiophenic carboxylic-, monoaromatic thiophenic alkanoic-, monoaromatic thiophenic carboxylic- and diaromatic thiophenic alkanoic acids, chemicals groups which were assessed in paper IV. The tested UCM compounds were screened for their potential to induce ER-activity, but also assessed for their applicability in the *in vitro* format as they consist of a highly diverse group of chemicals with variable physico-chemical properties (e.g. Log $K_{ow}$ 0.13 - 9.08). Assessment of the hepatocyte suitability as an ER-screening tool for highly complex chemicals was performed by characterising the compounds estrogenic (Vtg protein) potency and their cytotoxicity after 96 hours. The compounds AhR potency was assessed as EROD activity after 48 hours of exposure. To support the *in vitro* results, compounds specific MoA and baseline toxicity was predicted for *in vivo* fish by the Russom classification and ECOSAR respectively.

The main MOA of the tested UCM compounds were cytotoxicity, however some caused AhR mediated toxicity and only a few were estrogenic. The few estrogenic chemicals were naphthalenes and polycyclic monoaromatic acids, and they were active at high concentrations (Table 2). The estrogenic compounds had low ER potency, suggestive of few compounds being ER active in combination with low water solubility as effects generally occurred at concentrations above their predicted solubility limit. The low water solubility of the ER active compounds (Log $K_{ow}$: 4.26-6.77) may potentially have underestimated the toxicity due to limited bioavailability in the bioassay exposure solutions.

Table 2. Cytotoxic (metabolic activity), aryl hydrocarbon receptor (EROD activity) and estrogenic (vitellogenin induction) potency of compounds associated with the Unresolved Complex Mixture (UCM) hydrocarbons. The data displays the compounds predicted water solubility, predicted baseline lethal toxicity in fish (LC50), and experimental effect concentrations (EC10, EC50 and 20% efficiency) for cytotoxicity (96h), EROD activity (48h) and estrogenic (96h) in the rainbow trout (*Oncorhynchus mykiss*) hepatocytes. The data was derived from minimum 3 independent studies.

| **Compound** | **Predicted values** | | **Experimental values** | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | **Cytotoxicity (96h)** | | | **EROD activity (48h)** | | | **Vtg (96h)** | |
| | **Water sol. (mM)** | **Fish toxicity (96h)** $LC_{50}$, mM [a] | **$EC_{50}$ (95% CI), mM** [b] | **$EC_{10}$, mM** | **>20% effect** [c] | **$EC_{50}$ (95% CI), mM** | **$EC_{10}$, mM** | **≥ 20% Effect** | **$EC_{50}$ (95% CI), mM** | **≥ 20% Effect** |
| **Aliphatic naphthalenes** | | | | | | | | | | |
| 2,6-dimethylnaphthalene | 0.030[g] | 0.021 | n.a. | n.a. | YES | n.a. | n.a. | NO | 63 | YES |
| 2-ethyl-6-methylnaphthalene | 0.0063[g] | 0.010 | 0.17 (0.14 - 0.21) [d] | 0.12 [d] | YES | n.a. | n.a. | NO | na. | NO |
| **Aliphatic monocyclic acids** | | | | | | | | | | |
| 3-cyclohexylpentanoic acid | 0.23[h] | 0.095 | n.a. | n.a. | YES | n.a. | n.a. | NO | n.a. | NO |
| 4-(4'-n-butylcyclohexyl)butanoic acid | 0.0046 | 0.0085 | 0.077 (0.063 – 0.096) [d] | 0.027 | YES | n.a. | n.a. | NO | n.a. | NO |
| 4-(4'-i-butylcyclohexyl)butanoic acid | 0.0053 | 0.010 | 0.13 (0.098-0.18) [d] | 0.026[d] | YES | n.a. | n.a. | NO | n.a. | NO |
| 4-(4'-s-butylcyclohexyl)butanoic acid | 0.0053 | 0.011 | 0.097 (0.082 – 0.12) [d] | 0.030 [d] | YES | n.a. | n.a. | NO | n.a. | NO |
| **Aliphatic tricyclic acid** | | | | | | | | | | |
| 1-adamantanecarboxylic acid | 3.3[h] | 0.83 | 9.4 (6.7-13)[d,j] | 0.29 | YES | n.a. | n.a. | NO | n.a. | NO |
| **Monocyclic di-acid** | | | | | | | | | | |
| (1R, 3S)-(+)-Camphoric acid | 16[g] | 4.9 | n.[d]. | n.[d]. | NO | n.a. | n.a. | NO | n.a. | NO |
| **Monocyclic tri-acid** | | | | | | | | | | |
| 1,3,5-trimethyl-cyclohexane-1,3,5-tricarboxylic acid | 67[h] | 12 | 24 (14-40)[d,j] | 1.6[d] | YES | n.a. | n.a. | NO | n.a. | NO |
| **Monoaromatic acids** | | | | | | | | | | |
| 4-(4'-n-butylphenyl)butanoic acid | 0.031 | 0.023 | 0.35 (0.29 – 0.43) [d] | 0.14 [d] | YES | 0.55 | n.a. | YES | n.a. | NO |
| 4-(4'-i-butylphenyl)butanoic acid | 0.036 | 0.028 | 0.40 (0.31 – 0.52) [d] | 0.079 [d] | YES | n.a. | n.a. | NO | n.a. | NO |
| 4-(4'-s-butylphenyl butanoic acid) | 0.036 | 0.025 | 0.17 (0.13 – 0.22) [d] | 0.040 [d] | YES | n.a. | n.a. | NO | n.a. | NO |
| 4-(4'-t-butylphenyl)butanoic acid | 0.039 | 0.025 | 0.27 (0.23 – 0.31) [d] | 0.11 [d] | YES | n.a. | n.a. | YES | n.a. | NO |
| (iso-butylphenyl)pentanoic acid | 0.011 | 0.012 | 0.29 (0.26 – 0.32) [d] | 0.12 [d] | YES | n.a. | n.a. | NO | n.a. | NO |
| **Polycyclic monoaromatic acid** | | | | | | | | | | |
| 6-cyclohexyltetralin | $5.1E^{-4}$ [i] | 0.0020 | 0.17 (0.12-0.24) [d] | 0.095 [d] | YES | n.a. | n.a. | NO | 0.16 | YES |
| 7-cyclohexyl-1-isoamyltetralin | $9.3E^{-7}$ | $3.7E^{-5}$ | 0.12 (0.091 – 0.16) [d] | 0.036 [d] | YES | 0.26 | n.a. | YES | n.a. | NO |
| **Monocyclic thiophenic carboxylic acid** | | | | | | | | | | |
| 4,5,6,7-Tetrahydro-1-benzothiophene-2-carboxylic acid | 0.22[h] | 0.20 | n.a. | n.a. | YES | n.a. | n.a. | NO | n.a. | NO |
| 4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid | 0.22[h] | 0.27 | n.a. | n.a. | YES | n.a. | n.a. | YES | n.a. | NO |
| **Monoaromatic thiophenic alkanoic acid** | | | | | | | | | | |
| 3-methylbenzo[b]thiophene-2-acetic acid | 1.4 [h] | 0.13 | 0.61 (0.48 - 0.77) | 0.25 | YES | 0.50 (0.39 - 0.64) [e,f] | 1.3 | YES | n.a. | NO |
| Benzo[b]thiophene-3-acetic acid | 5.3 [h] | 0.41 | 1.9 (1.1 - 3.1) [e] | 0.10 | YES | 1.6 (0.7-3.5) | 0.095 | YES | n.a. | NO |
| **Monoaromatic thiophenic carboxylic acid** | | | | | | | | | | |
| Benzothiophene-2-carboxylic acid | 1.1[h] | 0.35 | 2.0 (1.1-3.6)[d,j] | 0.15 | YES | n.a.[f] | n.a. | NO | n.a. | NO |
| **Diaromatic thiophenic alkanoic acid** | | | | | | | | | | |
| 4-dibenzothiophen-2'yl-4-hydroxybutanoic acid | 0.016[g] | 0.057 | n.a. | n.a. | NO | n.a. | n.a. | NO | n.a. | NO |

**Abbreviations:** a – 50% lethal concentration. b – 10 or 50% effect concentration. c – 20% or higher effect is observed in measured endpoint. d- above predicted water solubility. e – predicted, effect concentration predicted by the concentration-response curve at higher than tested concentrations. f – potential masking by cytotoxicity. g- Predicted by ECOSAR. h- Predicted with ALOGPS based on LogKow. i- (Smith et al., 2001). j - outside the tested concentration range, estimated value. n.a. not applicable. Not possible to model a concentration-response curve with $R^2 \geq 0.7$.

EROD activity were induced by some monoaromatic thiophenic alkanoic and carboxylic acids, monoaromatic acids and a few polycylic monoaromatic acids being identified to be weakly estrogenic, indicating a potential masking of ER-activity by these AhR-agonists. The water solubility was however reached for several of the tested compounds before becoming cytotoxic, thus potentially limiting the ability to identify the compounds true MOA. The compounds MOA and baseline toxicity was therefore predicted *in vivo* which further verified the *in vitro* correctly predicted narcotic MoA (data not shown).

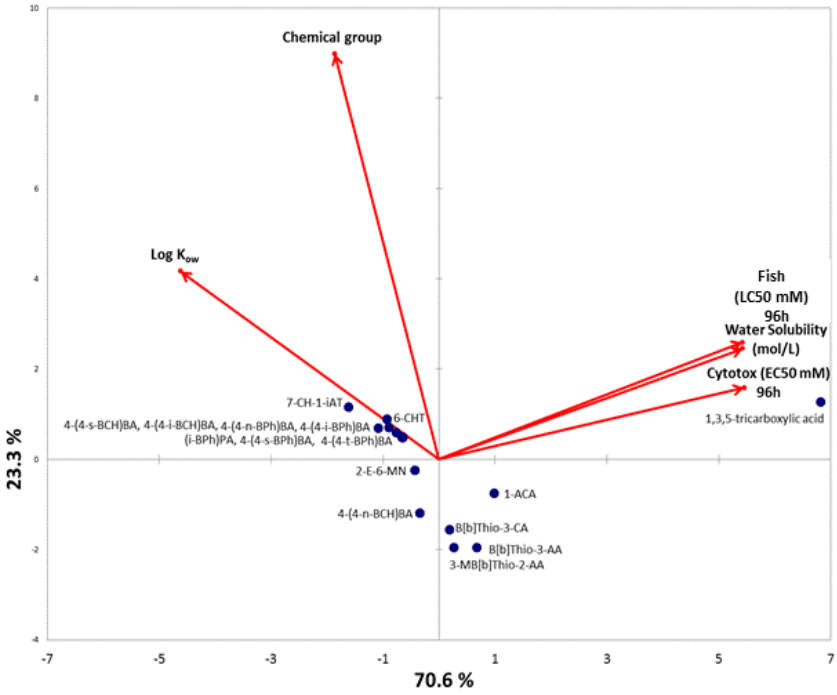


Figure 14. Principal component analysis (PCA) of physico-chemical properties, predicted acute toxicity at 50% effect concentration ($EC_{50}$) in fish and measured cytotoxicity $EC_{50}$ in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes when exposed to compounds associated with UCM hydrocarbons.

The relationship between compounds physico-chemical properties and their experimental (cytotoxicity) and predicted baseline toxicity, was depicted in a principal component analysis (PCA) (Fig. 14). The PCA further displayed the compounds variable hydrophobicity which potentially may introduce challenges for the *in vitro* system as difference between the experimental and predicted baseline toxicity were observed.

In conclusion, few compounds associated with UCM hydrocarbons elicited estrogenic effects or other specific MoA effects at tested concentrations herein. The lack of compound ER

activity may originate in inactive compounds or limited solubility, the later challenging the *in vitro* assay applicability in screening of hydrophobic compounds. However, the predicted narcotic MoA was consistent with the experimental findings in present study suggesting this as the primarily MoA in the hepatocytes.

# 4 General Discussion

Implementation of alternative approaches in the chemical regulatory framework of OECD, EU and US-EPA is highly warranted due to ethical and economic reasons, but few validated ecotoxicological bioassays exist for aquatic organisms such as fish. Evaluation of cell-based *in vitro* methods for fish such as primary cultures (e.g. hepatocytes) have shown promising results, demonstrating to retain many of the native properties of their derived organs (Braunbeck and Storch, 1992; Pedersen and Hill, 2000; Segner and Cravedi, 2000). However, the use of primary hepatocytes has proved challenging due to various assay-related factors (e.g. media supplements, incubation temperature) (Navas and Segner, 2006; Scholz et al., 2013) and chemical factors (e.g. solubility, partitioning behaviour). The present work therefore assessed the primary hepatocytes robustness by studying their sensitivity, reproducibility and performance in characterising ER-agonists and differently acting anti-estrogens through deciphering their MoA and assessing the interactions between different chemicals.

## 4.1 Hepatocytes as a multi-endpoint screening assay

Use of cell-based *in vitro* methods in toxicological testing has contributed to a better understanding of chemicals MOA whilst implementing the 3Rs (National research council (NRC), 2007). The primary rainbow trout hepatocyte model has previously demonstrated to be versatile when assessing various endpoints including screening for (anti)estrogenic and/or acute toxic properties during short-term (<96h) exposures (Gräns et al., 2010; Navas and Segner, 2006; Petersen and Tollefsen., 2012; Tollefsen et al., 2008a, 2008b). Monolayer cultures of primary hepatocytes is a well-characterised bioassay which facilitates ER and AhR-agonistic and antagonistic compounds modulatory properties on the nuclear receptors ER and AhR along with acute toxicity (e.g. cytotoxicity) and various sub-lethal (biomarker) endpoints (Bickley et al., 2009; Gräns et al., 2010; Mortensen and Arukwe, 2008; Petersen and Tollefsen, 2012; Tollefsen et al., 2008a, 2008b). In addition to single endpoint assessment, application of global gene expression analysis and expert-curated bioinformatics tools have greatly facilitated the assessment of chemicals MOA *in vitro* and potential MoA *in vivo*. The transcriptional regulation and its down-stream effects have contributed to a higher level of understanding of cellular regulation and associated biological functions (e.g. GO, gene network and pathway signalling) in the hepatocytes (Finne et al., 2007). The bioassays

offer a versatile multi-endpoint approach of screening a large numbers of chemicals, however limitations such as low reproducibility and variable sensitivity for compounds such as ER-agonists have restricted the bioassays potential as a reliable screening tool for ER-agonists and antagonists. The robustness of the bioassay to accurately predict ER-agonistic and antagonistic effects has been questioned, and thus giving rise to uncertainties of its reliability when assessing single and estrogen sensitive biomarkers (Scholz et al., 2013). The present work has therefore evaluated the primary rainbow trout hepatocytes suitability to assess and characterise the MoA of chemicals that affect the activity of the ER.

### 4.1.1 Reproducibility and sensitivity of the primary hepatocytes bioassay

Vitellogenin is a well-established protein biomarker for identifying ER-active substances *in vivo* and has been successfully measured in primary fish hepatocytes due to the cells metabolic capacity and estrogen sensitivity (Navas and Segner, 2006; Pelissero et al., 1993). Variable estrogen sensitivity and responsiveness of Vtg gene and protein expression (LOEC: $10^{-12}$–$10^{-7}$ mol/L) have contributed to the primary hepatocytes low bioassay reproducibility (Navas and Segner, 2006). This low reproducibility has mainly been attributed to the donor fish physiological condition (e.g. variable hormone production and metabolism) as it has been suggested dependent on seasonal rearing conditions and maturation status (Bon et al., 1997; MacKay et al., 1996; Mackay and Lazier, 1993; Rankouhi et al., 2004). In addition to seasonal differences, interspecies differences in metabolism (Lindholst et al., 2003) and differences in cell culturing conditions (e.g. media supplements, incubation temperature, cell density, volume-surface ratio), exposure conditions (well-plate size, dosing procedure), analytical endpoint (semi-quantitative and quantitative ELISA) and normalization procedures have been reported to affect expression of Vtg (Kim and Takemura, 2003; Navas and Segner, 2006; Pawlowski et al., 2000; Tollefsen et al., 2003).

Seasonally dependent hormone regulation and species-specific metabolic rate are factors that are representing a factor of natural variation that cannot be influenced, but somewhat reduced through use of juvenile or male donor fish as improving the inter-laboratory assay performance (Bickley et al., 2009; Navas and Segner, 2006; Tollefsen et al., 2003). Furthermore, factors related to *in vitro* cell culturing conditions, dosing and normalization procedures have been suggested being key to the assays performance.

#### *4.1.1.1 Donor related factors*

The present work assessed seasonal variability as the potential source of variation in Vtg sensitivity and responsiveness of primary rainbow trout hepatocytes exposed to estrogen and estrogen-mimicking (EE2) compounds (Paper I). Variable levels of Vtg protein has previously been associated with seasonal and temperature changes in sexually mature fish (Bon et al., 1997; MacKay et al., 1996; Mackay and Lazier, 1993). The present work therefore investigated if ER sensitivity and responsiveness of Vtg were seasonally dependent in hepatocytes isolated from juvenile rainbow trout in the period of January to July. The results indicated no seasonal differences in the NOEC of Vtg gene or protein expression when exposed to either estrogen or EE2 (Paper I). Similarly, Bickley et al., (2009) could neither establish any seasonal dependent regulation of Vtg in E2 exposed male primary hepatocytes, suggesting the variable Vtg to instead be due to the physiological history of individual donor-fish. For this reason, the present work addressed the impact of the donor fish physiological condition and how this may be reduced mainly using appropriate data normalization but also more homogenous (donor-independent) bioassays. Application of appropriate data normalization such as the relative potency (Vtg levels as a percentage of relative maximum induction of EE2 or positive estrogen (E2) control) demonstrated to reduce the donor-related variability in the 95% confidence interval of the $EC_{50}$ (non-normalized: 50%, normalized: 31%) when normalized towards 96h exposure, making the data comparable to published Vtg responses (Paper I). Harmonization of normalization procedures, such as that demonstrated herein, is anticipated to reduce the large uncertainty and improve comparability across laboratories and species (Navas and Segner, 2006). However further improvement of donor-related variability may be performed by use of the same uniform strain of fish (Rankouhi et al., 2004) or more homogenous cell bioassays such as cryopreserved primary hepatocytes. The cryopreserved cells have in certain cases demonstrated to be more advantageous than freshly isolated hepatocytes as frozen cells may be isolated in large cell batches, endure extended storage and may be distributed to various labs, potentially increasing the homogeneity of measured endpoints (Markell et al., 2014; Mingoia et al., 2010). Standardization of methods used would if implemented, improve the primary hepatocytes reproducibility and hence credibility as a tool in screening of compounds with modulatory properties on the ER (Navas and Segner, 2006; Scholz et al., 2013).

#### *4.1.1.2 Exposure duration*

To assess the performance of the Vtg assay, the optimal exposure time for both gene and protein expression were assessed when exposed to the model ER-agonist EE2 (Paper I). The results showed that Vtg gene and protein expression were equally sensitive endpoints, both yielding reproducible, high quality CRCs already after 48h of exposure to EE2 and E2 (Paper I and II). However, less variable protein expression and improved magnitude of induction was observed after prolonged exposure (72-96h) (Paper I), thus suggesting 96h as the optimal exposure duration when measuring Vtg protein production in primary hepatocyte cultures. The transcriptional regulation of Vtg was however optimal at shorter exposure durations (48-72h), yielding high-quality CRCs already after 24h (Paper I). After 96h of exposure to both EE2 and E2, Vtg sensitivity (LOEC) and responsiveness ($EC_{50}$) of both gene and protein expression was highly comparable with other studies with estrogens using primary rainbow hepatocytes (Paper I, Supplementary table A). The comparability of Vtg expression to data from other teleost species did however prove more challenging as the donor physiology (e.g. biotransformation rate of compound, Vtg sensitivity) are different amongst species (Lindholst et al., 2003). Indeed, the primary rainbow trout hepatocytes demonstrated to be highly metabolically active, depleting 96% of 3nM EE2 in the media within 48h of exposure (Paper I) and thus verified that biotransformation is likely a potential confounding factor when comparing data from different fish species, fish strains, life stages, and even different labs. Unpublished results from our lab also show that exposure to E2 for 72 and 96h without chemical replenishment yielded relatively low Vtg protein expression in the rainbow trout hepatocytes compared to those being chemically re-exposed after 48h (Petersen et al., unpublished), and agree with reports of rapid metabolism (half-life of <2h) of E2 in primary rainbow trout hepatocytes (Miller et al., 1999). The present studies demonstrate that chemical replenishment in exposures >48h may also be of critical importance in securing high quality CRCs for EE2, and potentially also other chemicals being susceptible to biotransformation in fish. It has been amply demonstrated that accounting for chemical loss and/or changes in chemical bioavailability due to compound metabolism is required to reduce bioassay artefacts in *in vitro* toxicity testing (Groothuis et al., 2015; Kwon et al., 2009). The results therefore are suggestive of the species-specific metabolism and culturing conditions to contribute to the variable Vtg expression across laboratories and species.

#### *4.1.1.3 Bioassay related factors*

The primary hepatocyte bioassay is normally maintained under conditions that mimic the cellular environment by offering attachment factors to allow cells to anchor to the plastic. In addition, media additives provide factor pertinent to normal growth and differentiation, and testing (plate) formats that accommodate the research objectives to be pursued are often different from study to study. Based on literature, culturing conditions such as cell density, incubation temperature and growth media supplements have an effect on the sensitivity and responsiveness in various assay endpoints with primary hepatocyte cultures (Petersen and Tollefsen, 2015 unpublished data; Kim and Takemura, 2003; Pawlowski et al., 2000; Tollefsen et al., 2003). Cell cultures containing growth media supplements (e.g. fetal bovine/calf serum (FBS/FCS), Ultroser SF) have generally displayed a lower sensitivity to chemicals and larger inter-assay variance than serum-free assays (Kramer et al., 2012). The reduced bioavailability has been proposed due to the serum interference with the bioavailability of the test compounds through lipophilic binding and/or partition (Kramer et al., 2012; Tollefsen et al., 2003). Such interference may influence the overall *in vitro* bioassay sensitivity (Kramer et al., 2012), and result in underestimation of chemicals biological activity and toxicity. Reporting measured instead of nominal chemical concentrations *in vitro* is therefore recommended (Groothuis et al., 2015; Kramer et al., 2012) as large molecular size, high hydrophobicity and high volatility may limit the freely dissolved chemical (e.g. adsorption to plastic or media supplements) in the exposure media (Brown et al., 2001; Schreiber et al., 2008). Furthermore, interference of media supplements may not only influence the chemical sensitivity of the bioassays (Kramer et al., 2012), but also interfere with the sensitivity of the analysis of e.g. Vtg and CYP1A in certain ELISA formats due to protein/serum-antibody competition (Goksøyr, 1991). Although this may reduce the assay`s sensitivity with several order of magnitude (Tollefsen, 2003), the use of more advanced ELISA formats such as sandwich ELISAs may alleviate the effects of such artefacts (Gan and Patel, 2013). The use of serum-free cell and exposure media should therefore be encouraged, and may possibly reduce artefacts associated with inter-assay variability. Other cell media supplements such as the pH indicator phenol red has been proposed to introduce artefacts in estrogenic bioassays as suggested being a weak ER-agonist and has therefore been suggested avoided in all screenings of EDCs (Berthois et al., 1986; Navas and Segner, 2006; Welshons et al., 1988). Subsequent studies have demonstrated that phenol red's estrogenicity was not due to the dye itself, but to lipophilic impurities in the dye preparation (Bindal et al., 1988). No differences in Vtg baseline or maximum response were observed in primary rainbow trout

hepatocytes exposed to estrogen when comparing phenol and phenol-free media (unpublished results), and is in agreement with results from previous studies on primary hepatocytes (Moreno-Cuevas and Sirbasku, 2000; Oğuz and Ünal, 2011).

## 4.2 Use of primary hepatocytes in characterizing MoA of ER-agonists

Characterisation of chemicals MoA has demonstrated to be challenging in aquatic toxicology due to the limited knowledge obtained by the directed single endpoint approach. Nevertheless, assemblies of single-endpoint bioassays such as those implemented in the US Toxcast screening program (Dix et al., 2007) generate highly valuable information that further facilitates the characterisation of chemicals MoA (US-EPA, 2011). Moreover, the ultimate aim for a number of *in vitro* bioassays is to offer a high-throughput testing format for screening of EDCs that provide information relevant for characterising the MoA or performing extrapolations to *in vivo* bioactivity. The hepatocytes suitability as a screening tool for characterising MoA of ER-active compounds was assessed in present work (Paper I-IV) on both a molecular and sub-cellular level by linking the activity of molecular targets (e.g. transcriptional nuclear receptor activation) to their potential MoA.

### 4.2.1 ER-agonists

Primary hepatocytes have in numerous studies, including the present work (Paper I-IV), demonstrated to be sensitive to both single compound and simple mixtures of estrogen and ER-agonists when applying single (e.g. ERα/β, Vtg, zrp and vigilin) and multiple (e.g. global gene expression) endpoint approaches (Finne et al., 2007; Gräns et al., 2010; Mortensen and Arukwe, 2008; Petersen and Tollefsen, 2012; Rankouhi et al., 2004; Tollefsen et al., 2008b). The advantage of using primary hepatocytes compared to other *in vitro* methods (e.g. cell lines) is their partially retained *in vivo* liver functions such as native metabolic capacity (Pedersen and Hill, 2000; Pesonen and Andersson, 1997; Segner, 1998) and ER-response (Pelissero et al., 1993). Previous hepatocyte studies have reported relevant *in vivo* concentrations of xenoestrogens (e.g. EE2) to bind and activate ER, giving rise to transcriptional activation of target genes and protein translation of e.g. Vtg (Finne et al., 2007; Petersen and Tollefsen, 2011; Rankouhi et al., 2004). The present work therefore assessed the

primary rainbow trout hepatocytes potential to describe ER-mediated and other affected processes during exposure to the ER-agonist EE2.

#### *4.2.1.1 Estrogen receptor signalling*

In the present work, global gene expression analysis identified ER signalling as the main MOA of EE2. The ER effects on down-stream molecular targets and cellular processes were characterised in the hepatocytes (Paper II) in a similar way as reported elsewhere for estrogen exposed fish (Colli-Dula et al., 2014; Doyle et al., 2013; Harding et al., 2013; Hook et al., 2007; Levi et al., 2009). However, the mammalian ER signalling pathway was not significantly enriched at any EE2 concentration despite regulation of well-characterised ER-responsive genes in fish (e.g. *erα, vtg, zrp3, fst1, igfbp-1*). The ER function in fish may however be different from mammals as fish gene and gene functions may not be conserved over large evolutionary spans, therefore introducing uncertainties regarding the cross-species orthologue mapping performed in the gene enrichment analysis. The ER signalling pathways was therefore manually assembled for fish based the microarray data and available literature (Paper II). The global transcription analysis revealed comparative expression of estrogen sensitive genes to previous studies with E2 and EE2 exposed fish (Doyle et al., 2013; Levi et al., 2009), indicative of the primary hepatocyte model's suitability for characterisation of ER-agonists MoA using classical estrogen biomarker genes.

Deviations from the ideal monotonic CRC of Vtg protein expression was observed in the present study at high exposure concentrations of EE2, findings similar to previous xenoestrogen studies in primary fish hepatocytes (Petersen and Tollefsen, 2011; Rankouhi et al., 2004). Similar to that reported by Petersen and Tollefsen (2011), the present study demonstrated that high concentrations of EE2 (10-30nM) caused an apparent reduction (approximately <23 %) in both Vtg gene expression and protein production. This reduction has been proposed as a result of the ERα gene being suppressed, which subsequently reduces down-stream transcription of *vtg* (Paper I and II). The transcriptional regulator small heterodimer partner (*shp*), an ERα repressor in mammals (Ehrlund and Treuter, 2012), was significantly up-regulated at the highest EE2 concentration (Paper II), indicative of a conserved role in ER repression between mammals and fish. The SHPs function in fish is at present still controversial (Park et al., 2007), and further studies to elucidate the role of SHP and other repressors on Vtg gene and protein expression is clearly warranted.

#### *4.2.1.2 Biotransformation*

Besides classical estrogen biomarkers, the ER-agonist affected Phase I, II and III biotransformation genes in the primary hepatocytes. Inducing Phase II transcripts such as *ugt2a*, which is involved in conjugation of hydroxylated substrates from the Phase I metabolism of xenogenous compounds (Gao et al., 2014). Induction of Phase III transcripts such as cellular xenobiotic efflux transporters (*abc* and *abc11*) at the highest (30nM) EE2 concentration suggested that primary hepatocytes actively excrete EE2 and its metabolites from the cells. Interestingly, the phase I detoxification gene *cyp1a* was down-regulated in a concentration-dependent manner by EE2 at an intermediate concentration (3nM), similar to *in vivo* zebrafish after waterborne exposure to this potent xenoestrogen (Hoffmann et al., 2006). Previous studies investigating E2 and EE2 exposed *in vitro* fish hepatocytes have proposed that down-regulation of *cyp1a* gene were associated with an up-stream uni- or bi-directional negative nuclear receptor cross-talk between the AhR and ERα (Bemanian et al., 2004; Gräns et al., 2010; Mortensen and Arukwe, 2007, 2006). However, the *ahr* transcript was not significantly expressed on the microarray analysis, thus suggesting alternative up-stream AhR regulators such as the ERα-recruited squelching nuclear factor 1 (*nf1b*) to be a potential suppressors of the *cyp1a* (Ricci et al., 1999). Although the biological function of *nf1b* is still not fully characterised in fish, deciphering its modulatory role on AhR may be warranted to better understand if there is a uni- or bi-directional negative nuclear receptor cross-talk between the AhR and ERα (see section “6.2.4 AhR-ER cross-talk” for details).

#### *4.2.1.3 Other signalling pathways*

Additional estrogen sensitive mechanisms such as lipid and cholesterol regulation were altered in the EE2 exposed primary hepatocytes, and specifically observed as increased lipid and cholesterol biosynthesis (e.g. lipin-1, 7-dehydrocholesterol reductase (*dhcr*)) and reduced metabolism (e.g. peroxisome proliferator-activaror receptor α/γ (pparα/γ), ccaat enhancer-binding protein beta (*cebpb*), carnitine palmitoyltransferase 1a (*cpt1a*), acyl-CoA oxidase-like (*acoxl*)). The present work has illustrated primary hepatocytes to facilitate a unbiased assessment of the MoA of EE2, identifying putative DEG involved in various processes (e.g. lipid-cholesterol- biosynthesis, transportation, metabolism and vitellogenesis), previously described in estrogen studies on fish and mammals (Doyle et al., 2013; Hoffmann et al., 2006; Kersten, 2005; Levi et al., 2009; Wang et al., 2010).

In addition to classical ER-mediated pathways, EE2 suppressed genes associated with cellular growth (e.g. IGF-1 and GH pathways). Suppression of the IGF-1 and GH pathways was observed at intermediate and high concentrations (3-30nM), enriched by putative down-regulated genes such as *ghr*, *igfbp-1* and follistatin (*fst*). Several of these transcripts are associated with energy constraints due to the induced vitellogenesis (Davis et al., 2008), resulting in reduced growth and development in estrogen exposed fish (Shved et al., 2008). These findings illustrate the primary hepatocytes versatility and potential for studying more complex and less characterised endocrine disrupting MOA in fish.

### 4.2.2 Anti-estrogens effect on ER

Exposure to ER-antagonists and AhR-agonists has in both *in vivo*- and *in vitro* fish studies been associated with anti-estrogenic effects such as reduced Vtg protein synthesis or impaired gonad development and decreased egg production (Anderson et al., 1996; King Heiden et al., 2006; Navas and Segner, 2000). The anti-estrogenic effect of ER-antagonists (e.g. OHT, ICI) and AhR-agonists (e.g. BNF, PCB126 and TCDD) has successfully been described in primary fish hepatocytes using well-characterised ER-mediated gene and protein biomarkers (e.g. *erα*, *vtg* and *vigilin*) (Gräns et al., 2010; Mortensen and Arukwe, 2007; Petersen and Tollefsen, 2012; Zhao et al., 2006).

In agreement with previous *in vitro* studies, BNF and OHT caused both when single and combined an anti-estrogenic effect in primary hepatocytes co-exposed with E2 (Paper III). The anti-estrogenic effect has previously been suggested as compound derived cytotoxicity (Navas and Segner, 2000), however the present study used non-cytotoxic exposure concentrations (Petersen and Tollefsen, 2012), suggesting the anti-estrogenic effect to be due to the compounds modulatory activity on the ER. The BNF and OHT anti-estrogenic effect has been described as AhR- and ER-mediated respectively (Navas and Segner, 2000; Smeets et al., 1999), a statement supported by the present study as both mRNA expression of *erα* and *vtg-1* and subcellular Vtg protein were reduced in presence of E2.

The BNF+OHT mixture caused a stronger apparent reduction of E2-induced Vtg protein expression when compared to the single exposure of BNF and OHT in presence of E2 (Paper III), showing that both compounds contribute to the anti-estrogenic effect of the mixture. In a recent study these two differently acting anti-estrogens were suggested to contribute to a more than additive anti-estrogenic effect (Petersen and Tollefsen, 2012), a statement that could not

be supported in the present study. However, the study design of the current study limited the possibility to assess the mixture effects in terms of additivity and deviations from additivity, as only interactions at one combination of concentrations and compounds could be assessed. No significant interaction between the compounds could be identified when performing a two-way ANOVA. The lack of interaction between the compounds may possibly be explained by the cell batches variable Vtg expression as normalization was performed towards media control (Paper III) instead of the recommended use of relative potency of a positive control (Paper I). These findings display the importance of performing appropriate data normalization using relative potency of a positive control (e.g. 10nM E2) as it considerably improved data reproducibility of both Vtg mRNA and protein in Paper I.

Overall, the two differently acting anti-estrogens caused a reduction of Vtg protein expression, indicating the assay suitability for screenings of anti-estrogens, independent of MoA. Although, the analysis of Vtg protein could not decipher how the compounds elicited their anti-estrogenic MoA, several AhR- and ER-mediated mechanisms have been suggested involved in a proposed NR- mediated cross-talk (Gräns et al., 2010; Navas and Segner, 2000). The present study therefore assessed BNF, OHT and a mixture of these anti-estrogens on the activity of the ER, identifying E2-induced *erα*, *vtg-1* and *zrp* mRNA expression as parallel expressed with the Vtg protein expression. OHT belongs to a group of selective estrogen receptor modulators (SERMs) which function as a partial ER-antagonist by acting both as an ER-agonist or antagonist depending on tissue and environmental context (Wu et al., 2005). However the present work confirmed OHT to have anti-estrogenic effects related to being a full ER-antagonist in hepatocytes as it suppressed ER-mediated mRNA expression in presence of E2 and caused no estrogenic effect when exposed alone (Paper III).

### 4.2.3 AhR-ER cross-talk

Anti-estrogenic chemicals may have similar target effects through several different MoA, and when combined into a mixture the different MoA might cause more than additive anti-estrogenic effect. Mixtures of differently acting anti-estrogens may cause a potential AhR-ER cross-talk (Matthews and Gustafsson, 2006), involving specific repression on ER through co-repressors, enhance *cyp1a*-mediated E2 metabolism, interaction with estrogen-mediated DNA response elements and/or having a cross-talk with other transcriptional factors (Matthews et al., 2005; Swedenborg and Pongratz, 2010). The present study therefore assessed the involvement of AhR-mediated responses in the anti-estrogenicity of BNF and OHT.

The present work deciphered BNF's AhR-mediated anti-estrogenic MoA by assessing the compound's potential to enhance E2 metabolism through activation of AhR-mediated transcription (*ahrα, cyp1a*), a mechanism involved in the NR-mediated cross-talk (Paper III). The typical AhR-agonist BNF caused an increased transcriptional activity of AhR-mediated genes *ahrα*, *cyp1a* both in presence and absence of E2. The anti-estrogenic effect of BNF has previously been proposed to be AhR-mediated (Navas and Segner, 2000), and potentially involving the transcriptional activation of the ER through AhR-ER cross-talk (Swedenborg and Pongratz, 2010). The present findings indicated that BNF induced modulation of AhR-mediated responses in cells co-exposed with estrogens, thus having the main MoA as the *cyp1a* mRNA was significantly induced compared to the solvent control. The findings further implied that BNF initiated *cyp1a*-mediated metabolism of E2 in an AhR-ER cross-talk (Safe and Wormke, 2003), potentially leaving less bioavailable E2 to activate the ER (Anderson et al., 1996). The present study did not experimentally verify the CYP1A protein expression or the metabolized concentration of E2, however a recent study proposed CYP1A to be primarily responsible for E2 metabolism in *in vivo* fish (Scornaienchi et al., 2010). However contradicting, exposure to E2 with and without BNF was not associated with enhanced E2 metabolism despite increased CYP1A mediated EROD-activity in primary rainbow trout hepatocytes (Navas and Segner, 2000). Although noteworthy, the study did not measure the initial E2 concentration in the media, potentially reporting inaccurate concentrations of metabolized E2 between the treatments with and without BNF. In addition, the study used 159 times higher (1μM) E2 concentration than that used in present study (Paper III), potentially introducing high exposure concentration effects that could directly affect the metabolic rate of E2.

Interestingly, despite OHT's ER-dependent MOA it has been suggested to induce AhR-mediated responses (e.g. *ahr*, *cyp1a1*) in human MCF7 breast cancer cells when exposed to 1.0 $E^{-7}$ M OHT (DuSell et al., 2010). No evidence of such AhR-mediated response was identified in present study, as *ahrα* and *cyp1a* remained unaffected by OHT both in presence and absence of E2. The AhR-agonistic response of OHT may therefore be both concentration- and tissue dependent as the present study used 42 times lower ($2.36E^{-9}$M) concentration of OHT and used a different *in vitro* model than that reported by DuSell et al. (2010) (Paper III).

In similarity to BNF, the mixture of BNF+OHT caused an induction of AhR-mediated *cyp1a* transcription in both presence and absence of E2, indicative of BNF being the main contributor to the AhR-mediated MoA in the mixture. It is therefore suggested that

BNF+OHT more than anticipated anti-estrogenic effect (Petersen and Tollefsen, 2012) was caused by BNF's *cyp1a*-mediated E2 metabolism and OHT's antagonistic binding to ER. However other cross-talk mechanisms such as increased AhR-ligand binding of XRE upstream of the ERE resulting in less transcription of ER (Matthews and Gustafsson, 2006) or co-activator competition (e.g. Nuclear Receptor Coactivator (NCoA)) in the hepatocytes are also provide possible explanations to the anti-estrogenic effects of the mixture. The AhR-ER cross-talk in present study was suggested uni-directional, as E2 had no effect on the AhR-mediated responses in any of the treatments (Gräns et al., 2010). Due to the variable ER- and AhR-responsiveness amongst the cell batches no final conclusion could be made on which cross-talk mechanism that caused the anti-estrogenic effect of the mixture, thus encouraging additional studies to be performed.

### 4.2.4 Screening of uncharacterised compounds

The present work has illustrated the importance of characterisation the MoA of environmentally relevant AhR and ER-agonists and antagonists chemicals using *in vitro* methods. However the acute toxicity of compounds may sometimes mask their potential to modulate ER-activity in primary fish hepatocytes (Grung et al., 2007) or by AhR (Sherry et al., 2005), resulting in false-negative results *in vitro* which underestimate the compounds MoA *in vivo*. To avoid such false-negative results it is essential to apply a multi-endpoint approach on both molecular and sub-cellular level of response as compensatory mechanisms may partly or fully mask specific gene expression or enzymatic activity in complex samples (Celander, 2011). The present work therefore applied a multitude of endpoints using primary rainbow trout hepatocytes to screen the potential estrogenic potency of hydrocarbons associated with UCMs (Paper IV). The present work determined, as previous study by Headley and McMartin (2004), that compounds associated with UCMs cause their overall toxicity through a non-specific membrane interaction (narcotic) MoA (Paper IV). The results were confirmed as few of the tested compounds exerted sub-lethal effects on ER-mediated Vtg production in the primary hepatocytes, which generally occurred above the compound's water solubility. Compounds with AhR-agonistic effects may as well have masked the ER-mediated responses through the proposed AhR-ER cross-talk, thus underestimating the compounds potentially estrogenic effects (Sherry et al., 2005). Characterising compounds MOA has however demonstrated challenging for complex compounds in UCMs of aryl

hydrocarbons as the predicted hormonal activity was highly dependent on the compound structure (Scarlett et al., 2012). Similar compounds and mixtures of these have previously demonstrated difficult to characterise using *in vitro* bioassays as the individual components have several different MoA and highly variable physico-chemicals properties (e.g. high log $K_{ow}$) (Jones et al., 2011; Lacaze et al., 2014; Tollefsen et al., 2012).

The results of present work are however, indicative of the compounds having general narcotic MoA, which also was indicated by the other supportive endpoints measured (e.g EROD activity) (Table 2) and Russom's classification model. However, masking of the UCMs potential ER-agonistic MoA through cytotoxicity was not excluded as the present work (Paper IV) identified several of the compounds to be weak estrogens and simultaneously have cytotoxic properties. The increased cytotoxicity may therefore potentially explain the concentration-dependent decrease in Vtg and EROD-activity in several chemical groups (Table 2), as observed elsewhere (Petersen and Tollefsen, 2011; Rankouhi et al., 2004; Tollefsen et al., 2008b). The majority of UCMs reached their solubility limit before becoming cytotoxic, challenging the reliability testing of compounds with challenging physicochemical properties (e.g. high hydrophobicity, high volatility and the compounds molecule size and polarity) by potentially underestimating their toxicity (Riedl and Altenburger, 2007).

The present work (Papers III and IV) illustrated the challenges of applying the appropriate endpoints that would correctly reflect the compound's MoA *in vitro* (e.g. primary hepatocytes) in addition to determining adverse effects in an organism, especially for complex environmental samples and mixtures of chemicals. To improve *in vitro* testing and overcome these challenges, additional *in vitro* methods such as specific ER/AR (e.g. yeast with human recombinant ER/AR) and AhR screening (e.g. continuous cell line Calux-DR) and receptor binding (e.g. ER, AR, thyroid) assays should be considered as complementing assays to unravel individual MoA. Several of these less complex assays would probably provide a clearer bioanalytical signal of the compounds true MoA as they would not encounter the mechanistic difficulties of the nuclear-receptor cross-talk occurring in the primary hepatocytes.

## 4.3 *In vivo* and *in vitro* extrapolations

The primary hepatocyte bioassay have shown promise as a suitable alternative for screening of single and mixtures of acute toxic and ER/AhR-agonist and antagonist chemicals as demonstrated in Paper I – IV. The hepatocytes have in present work and previous studies demonstrated to retain high metabolic activity (Pedersen and Hill, 2000; Segner and Cravedi, 2000), detoxification response, partial endocrine response and lipogenesis, thus retaining many native functions of the liver (Navas and Segner, 2006; Pesonen and Andersson, 1997; Segner, 1998; Segner and Cravedi, 2000; Tollefsen et al., 2003). However, *in vitro* bioassays (e.g. primary hepatocytes) have only a limited amount of available target sites in the cells when compared to *in vivo,* and will therefore not represent the same diversity of response as the whole organism (Schirmer, 2006). This is demonstrated as *in vitro* models lack the complexity of organ-organ feed-back mechanisms of neurotransmitting signals, toxicodynamic interactions of the various metabolic processes and the full response of the endocrine system of organs *in vivo*.

To assess the differences between *in vitro* and *in vivo* estrogen response, identification was performed of potentially confounding factors (e.g. bioassay-related). The present work reported that the primary rainbow trout hepatocytes yielded Vtg LOEC and $EC_{50}$ values comparable to previous estrogen *in vivo* studies in rainbow trout (Thomas-Jones et al., 2003; Verslycke et al., 2002), deviating no more than a factor of 2 fold from *in vivo* (Paper I). Further comparability between primary hepatocytes and *in vivo* fish suggested that ER-agonists interfere with various molecular and subcellular processes in a similar manner as previously described for exposed fish (Paper I and II). Although comparable on a molecular and subcellular level of estrogen response in the liver, *in vitro* predictions of adverse biological effect are difficult as many estrogenic effects *in vivo* are not directly mediated through the liver ER but instead via the HPG-axis (Schlenk, 2008).

Besides the physiological limitations of hepatocytes, other assay-related factors such as use of nominal exposure concentrations has limited the ability to assess the robustness of the *in vitro* system as proposed in paper I and elsewhere (Navas and Segner, 2006; Tollefsen et al., 2008a). The *in vitro* bioassays robustness is still challenged by the exposure format (e.g. low exposure concentrations, rapid compound biotransformation and plastic-adhesive chemicals) and may sometimes contribute to the large differences observed when compared to *in vivo* ER responses.

The hepatocyte model may instead of strictly mimicking *in vivo* fish be an advantage proxy when predicting acute responses occurring specifically in the liver, such as cytotoxicity and sub-lethal biomarkers (e.g. AhR, CYP1A, EROD, ER, Vtg, Zrp, etc.). However predicting effects of complex chemical mixtures and environmental samples have proven challenging for primary hepatocytes as the compounds true toxic potency may be underestimated due to cells nuclear receptor interactions (e.g. nuclear cross-talk) (Paper III) and bioassay-specific artefacts (e.g. media supplements, cell density and incubation temperature) associated with challenging physico-chemical properties (e.g. hydrophobicity, adsorption to plastic well, precipitation, change of pH) (Paper I and IV). All these limitations combined might be a result of the *in vitro* bioassay reduced sensitivity towards individual and mixtures of chemicals in comparison to *in vivo*, indicative of the cells incapability to facilitate the chemicals modulatory effects on the target sites or other assay related limitations. To improve *in vitro* testing and overcome these challenges, the assessment of a diverse selection of endpoints, understanding of individual compounds MoA and the establishment of exposure concentration of chemicals should be applied (Schirmer, 2006), as performed by the present work. The primary hepatocyte model has in the papers included herein displayed its multi-versatile applicability as an alternative tool to *in vivo* when screening compounds with potential ER-activity. However, the extrapolation between *in vitro* and *in vivo* should still be used with caution until all potential interfering factors are identified and compensated for either experimentally or by computational modelling.

# 5 Future prospects

The ultimate aim for a number of *in vitro* bioassays is to offer a high-throughput testing format for screening of chemicals MoA and perform extrapolations to their *in vivo* bioactivity. The present work assessed the primary hepatocytes reproducibility, sensitivity and applicability to characterise ER-active substances MoA using a multi-endpoint approach. The variability of estrogen sensitivity, responsiveness and reproducibility across donor fish, laboratories and species was described in present work and addressed as due to lack of harmonized protocols (e.g. incubation temperature, cell density, media supplements, data normalization), which if implemented would improve the bioassay reproducibility.

Further reduction of inter-assay variability may be performed through chemical verification of internal cell or media concentrations as the present work illustrated large deviations between nominal and measured concentrations already after a short period of time (Paper I). Difficulties with maintaining exposure concentration under static *in vitro* conditions have given rise to alternative chemical administration techniques. Future work on primary hepatocytes should therefore be performed using partition-driven administration, which maintains the free chemical fraction throughout the exposure by the passive dosing of chemically spiked inert polymer (e.g. silicone) (Brown et al., 2001; Kwon et al., 2009; Mayer et al., 1999). The technique is highly appropriate for hydrophobic chemicals ($\log K_{ow}$3-6) (Kwon et al., 2009) and has successfully been applied in various *in vitro* models and compounds (Booij et al., 2011; Kwon et al., 2009; Smith et al., 2009), potentially solving some of the challenges with chemical bioavailability and solubility in *in vitro* bioassays observed in the present work.

Finally, the transcriptional and subcellular studies of present work identified classical biomarkers to not fully accommodate and characterise the different chemicals MoA. However, future implementation of unbiased approaches such as global transcriptional analysis may provide MOA insight, which presents many avenues for additional research both on mechanisms and on novel biomarkers *in vitro*.

# 6 Conclusion

The present thesis demonstrated that cultures of primary rainbow trout hepatocytes are suitable for screening of chemicals that modulate the activity of ER. However, protocol harmonization, chemical exposure and normalization procedures are fundamental issues that need to be addressed before it may be considered a reliable (eco)toxicological screening tool. From the present work it can be concluded that implementing standardization of intra-species culturing conditions and exposure procedures would reduce inter-laboratory variability, increasing the performance of primary hepatocytes reported sensitivity and responsiveness towards chemicals.

The application of transcriptomics revealed the bioassays true potential and limitations to accommodate ER – mediated responses when exposed to ER-agonists and anti-estrogens with differently acting MoA. The primary hepatocytes also displayed versatility for characterising the MOA of ER-agonists, showing the potential of its multi-endpoint applicability as well as development for novel biomarkers that might better explain the *in vivo* data.

The present work showed that the primary hepatocyte model is suitable for screening single compounds and simple mixtures of these, independent of their MoA. The multi-endpoint approach facilitated the present work to characterise differently acting anti-estrogens MoA by analysing well-characterised ER- and AhR transcriptional responses in addition Vtg protein. The characterisation of the anti-estrogens MoA and mixtures combined toxicity further contributed to the understanding on how differently acting compounds may cause a stronger anti-estrogenic effect when in mixture.

Characterisation of potential ER-agonistic compounds with highly diverse activity, solubility and volatility challenged the exposure format of the *in vitro* system, as many compounds reached their water solubility before eliciting any toxic effect. By using the multi-endpoint approach several compounds were characterised as ER-agonists, however the general MoA was determined as narcotic, supporting previous QSAR predictions and classification models.

In conclusion, this PhD thesis has contributed to the understanding of primary hepatocytes potential applicability in acute toxicity, characterisation and prediction of estrogen and anti-estrogenic chemicals MoA through successfully implementing a combination between cellular and molecular approaches in testing of a selection of ER-agonists and antagonists.

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# Supplementary information

Supplementary table 1. Compiled list of chemicals for Paper I-IV

| Compound | CAS # | Abbreviation | Log $K_{ow}$ | Anticipated MoA/MOA | Paper |
|---|---|---|---|---|---|
| 17α-Ethinylestradiol | 57-63-6 | EE2 | 3.67-4.12 | ED | I & II |
| 17β-Estradiol | 50-28-2 | E2 | 3.94-4.01 | ED | III |
| β-naphtoflavone | 6051-87-2 | BNF | 4.68 | AhR | |
| 4-hydroxytamoxifen | 68047-06-3 | OHT | 5.82 | ED | |
| 2,6-dimethylnaphthalene | 581-42-0 | 2,6-DMN | 4,26 [b] | NR | IV |
| 2-ethyl-6-methylnaphthalene | 7372-86-3 | 2-E-6-MN | 4,26 [b] | NR | |
| 3-cyclohexylpentanoic acid | 13126-82-4, 5456-30-4, 5962-88-9 | 3-CHPA | 4.32 | NR | |
| 4-(4'-n-butylcyclohexyl)butanoic acid | | 4-(4-n-BCH)BA | 4.81 | NR | |
| 4-(4'-i-butylcyclohexyl)butanoic acid | | 4-(4-i-BCH)BA | 5.65 | NR | |
| 4-(4'-s-butylcyclohexyl)butanoic acid | | 4-(4-s-BCH)BA | 5.65 | NR | |
| 1-Adamantanecarboxylic acid | 828-51-3 | 1-ACA | 3.15 | NR | |
| (1R,3S)-(+)-Camphoric acid | 124-83-4 | Camphoric acid | 1.78 | NR | |
| 1,3,5-Trimethyl-cyclohexane-1,3,5-tricarboxylic acid | 118514-35-5 | 1,3,5-tricarboxylic acid | 0.13 | NR | |
| 4-(4'n-butylphenyl)butanoic acid | | 4-(4-n-BPh)BA | 5.8 | NR | |
| 4-(4'-i-butylphenyl)butanoic acid | | 4-(4-i-BPh)BA | 4.72 | NR | |
| 4-(4'-s-butylphenyl)butanoic acid | | 4-(4-s-BPh)BA | 4.72 | NR | |
| 4-(4'-t-butylphenyl)butanoic acid | 24475-36-3 | 4-(4-t-BPh)BA | 4.69 | NR | |
| (iso-butylphenyl)pentanoic acid | | (i-BPh)PA | 5.22 | NR | |
| 6-cyclohexyltetralin | | 6-CHT | 6.77[b] | NR | |
| 7-cyclohexyl-1-isoamyltetralin | | 7-CH-1-IAT | 9.08[b] | NR | |
| 4,5,6,7-Tetrahydro-1-benzothiophene-2-carboxylic acid | 40133-07-1 | 1-Bthio-2-CA | 3.66 | NR | |
| 4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid | 19156-54-8 | B[b]Thio-3-CA | 3.66 | NR | |
| 3-methylbenzo[b]thiophene-2-acetic acid | 1505-52-8 | 3-MB[b]Thio-2-AA | 2.97 | NR | |
| Benzo[b]thiophene-3-acetic acid | 1131-09-5 | B[b]Thio-3-AA | 2.42 | NR | |
| Benzothiophene-2-carboxylic acid | 6314-28-9, 527-72-0, 900791-89-1 | BThio-2-CA | 2.87 | NR | |
| 4-dibenzothiophen-2'yl-4-hydroxybutanoic acid | | 4-DBThio-BA | 3.29[b] | NR | |

**Abbreviation**
ED – Endocrine disruptive; AhR – Aryl hydrocarbon agonist; NR – Not reported

[a]based on concentration-response curves obtained in Petersen and Tollefsen, (2012)

# Paper I

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# Evaluation of the sensitivity, responsiveness and reproducibility of primary rainbow trout hepatocyte vitellogenin expression as a screening assay for estrogen mimics

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ABSTRACT

Vitellogenin (Vtg) induction in primary fish hepatocytes has been proposed as an *in vitro* screening assay for ER agonists and antagonists, but has not yet been used extensively as a high-throughput screening tool due to poor reproducibility, sensitivity and overall feasibility. The present work has evaluated the role of seasonal variation, normalization, optimal culture and assay conditions on the sensitivity, responsiveness and reproducibility of *in vitro* vtg gene mRNA and protein expression in rainbow trout (*Oncorhynchus mykiss*) primary hepatocytes using the xenoestrogen 17α-ethynylestradiol (EE2) as a test chemical. The results show that primary hepatocytes display a relatively high individual and seasonal variation in both *Vtg* mRNA and protein induction potential, although less variance was observed in assay sensitivity. Data normalization of assay response to maximum (3 nM EE2) and minimum (DMSO) Vtg production dramatically reduced this variance and led to improved assay reproducibility. A time-dependent response was observed both for mRNA and protein expression, reaching maximum Vtg induction after 96 h of exposure, although reproducible concentration response curves for both Vtg mRNA and protein could be obtained already after 48 h. A need for chemical re-exposure of the hepatocytes was identified to be important for sustaining exposure concentrations in extended studies (>48 h), whereas different plate formats (96, 24 or 6 wells) did not affect the bioassay performance. In conclusion, standardization of hepatocyte bioassay and test conditions as well as data-normalization procedures are proposed to be instrumental for more consistent and comparable results in future use of this *in vitro* assay.



## 1. Introduction

The impact of endocrine disrupting chemicals (EDCs) on aquatic wildlife has been an issue of environmental concern for many years (Purdom et al., 1994). Endocrine disruptors, such as estrogen mimics, are known to interfere with the endocrine system through activation of the estrogen receptor (ER) and cellular responses associated with normal sexual maturation and differentiation in fish (Hook et al., 2007). In recent years the potential challenge of environmental EDCs has been acknowledged and various international organizations (*e.g.* OECD, US-EPA, Japanese Environmental Agency) have proposed regulatory frameworks and test approaches to assess the potential hazard and risk of EDCs. The recent proposal of an OECD conceptual framework (CF) for testing and assessing EDCs has proposed to categorize different assays into 5 levels ranging from computational efforts (CF1) and *in vitro* screening (CF2) to *in vivo* testing (CF 3–5) based on their ability to address both mode of action (MoA) and adverse endpoints of regulatory concern (OECD, 2010). The OECD conceptual framework for ED testing and assessment proposes *in vitro* assays as a tool to prioritizing and characterize EDCs MoA, but suggest performing *in vivo* (CF level 4–5) studies for assessment of adversity and in-depth characterization of the MoA (OECD, 2010). However, it is expected that the international effort to address EDCs will also increase the demand for toxicological testing (Hecker and Hollert, 2011). Implementation of the European Union chemical legislation REACH alone is estimated to generate a need for hazard assessment of over 30,000 single chemicals for various toxic properties, potentially requiring the use of millions of test animals (ECHA newsletter; Rovida and Hartung, 2009). Although the true number of chemicals that may require *in vivo* testing in REACH may still be unknown, the potential

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demand for an increase in regulatory testing clearly calls for larger implementation of the 3R's (Refinement, Reduction and Replacement) in animal testing and further development and evaluation of alternative (non-animal) methods for EDC testing.

*In vitro* methods such as mammalian continuous cell lines and transgenic organisms (yeast and cells) have been proposed as suitable high-throughput screening (HTS) assays for EDCs at the OECD CF2 level (OECD, 2010). Development of *in vitro* methods for non-mammalian species such as fish has also been proposed, but only the fish embryo toxicity test has been validated for regulatory purposes (OECD, 2012b). Nevertheless, induction of the estrogenic biomarker vitellogenin (Vtg) in fish hepatocytes is along with estrogen receptor/androgen receptor (ER/AR) binding affinity-, aromatase and steroidogenesis assays identified as potential candidates for inclusion as OECD CF level 2 assays (OECD, 2010). Primary cultures of fish hepatocytes retain many of the innate properties of the liver such as biotransformation activity, detoxification and ER-mediated responses (Pedersen and Hill, 2000; Segner and Cravedi, 2000). Thus, vitellogenin induction in primary cultures of fish hepatocytes have successfully been implemented in screening of the ER agonistic and antagonistic properties of single chemicals, complex synthetic mixtures and environmental extracts (Kim and Takemura, 2003; Petersen and Tollefsen, 2011; Tollefsen et al., 2003, 2006). The success of these *in vitro* bioassays has been ascribed to a well-defined endocrine MoA in oviparous fish, involving xenoestrogen binding to and activation of the ER (Mortensen and Arukwe, 2006; Petersen and Tollefsen, 2012; Tollefsen et al., 2002), transcription of *Vtg* (Mortensen and Arukwe, 2006; Scholz et al., 2004; Smeets et al., 1999) and subsequent translation into the functional protein (Tollefsen et al., 2003), which can be monitored and quantified by a variety of different methods. Although clearly showing a screening potential, the feasibility of using these assays in EDC screening has been questioned due to low reproducibility, variable sensitivity and overall feasibility as high-throughput screening tools (Navas and Segner, 2006; Scholz et al., 2013). This applies in particular to the inter-species variability in *Vtg* mRNA and protein response often observed when exposed to the same xenoestrogen (Rankouhi et al., 2004). The physiology of the donor fish has been proposed to be a major contributor to differences in estrogen sensitivity, where mature individuals induce Vtg at a larger magnitude than juveniles (Navas and Segner, 2006). The differences in reproducibility and sensitivity among cell batches and species may also be caused by differences in fish strains and cell isolation procedures within and among laboratories (Navas and Segner, 2006; Rankouhi et al., 2004). Further, cell culture and bioassay conditions have been shown to also affect the assay performance and analytical determination of Vtg (Kim and Takemura, 2003; Pawlowski et al., 2000; Tollefsen et al., 2003), thus illustrating the importance of assay protocol optimization and harmonization. As primary hepatocytes have demonstrated to be a highly versatile multi-endpoint experimental model (Farmen et al., 2010; Finne et al., 2007; Petersen and Tollefsen, 2011; Tollefsen et al., 2006, 2008a), a thorough evaluation of assay performance and suggestions for improvements to facilitate larger implementation in EDC screening is highly warranted.

The aim of the present study was to evaluate the feasibility of primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes as a potential screening assay for ER agonists. This was achieved by evaluating the role of seasonal variation in the *Vtg* (mRNA and protein) sensitivity and responsiveness (*e.g.* induction potential), optimal culture and assay conditions (exposure time and well format), and the role of data normalization procedures using the model xenoestrogen 17α-ethynylestradiol (EE2). Chemical analysis of internal cell concentrations and media was additionally determined to assess the role of chemical depletion on bioassay performance. In addition, species-species and inter-laboratory differences in estrogen sensitivity were compared to identify key factors affecting the bioassay performance.

## 2. Materials and methods

### 2.1. Chemicals

All solvents and materials used in this study were obtained from the following sources: 17α-ethynylestradiol (EE2, ≥98%, CAS 57-63-6), d2 labeled estradiol (d2-E2, CAS 53866-33-4) (>99%), sodium bicarbonate (CAS 144-55-8), sodium carbonate (CAS 497-19-8), ammonium acetate (CAS 631-61-8), dansyl chloride (CAS 605-65-2) and copper sulfate ($CuSO_4{\cdot}5H_2O$, CAS 7758-99-8) were all purchased from Sigma–Aldrich (St. Louis, MI, US). Methanol (MeOH), acetone and toluene were of HPLC grade or analytical-reagent grade and obtained from Merck KGaA (Darmstadt, Germany). Dansylation buffer (pH 9.0) was prepared by dissolving 0.5 g of sodium carbonate and 4.2 g sodium bicarbonate in 200 ml of HPLC grade water. All test chemicals (with the exception of copper sulfate which was prepared freshly in cell media) were dissolved in dimethylsulfoxide (DMSO) and stored at −20 °C in the dark until use.

### 2.2. Rainbow trout

Juvenile rainbow trout (200–500 g) from the same fish stock were obtained from the Valdres rakfisk BA hatchery (Valdres, Norway) and kept at the Department of Biosciences, University of Oslo for a minimum of 4 weeks prior to the start of the studies. The fish were kept at 6 ± 2 °C, pH 6.6, 100% oxygen saturation under a 12 h light/12 h dark photoperiod. The fish were fed daily with commercial pellets (Skretting, Stavanger, Norway) that corresponded to approximately 0.5% of total body weight.

### 2.3. Hepatocyte isolation

Fish were collected from their rearing tanks in the period January–July (1–3 fish/sampling), terminated by a blow to the head and the abdominal cavity exposed by dissection. Only juvenile fish (with no visual gonads) were used in the 2-step hepatic cell isolation procedure described by Tollefsen et al. (2003). The *in situ* perfusion of the liver (5 ml/min, 10–15 min, 4 °C) was performed using a calcium-free perfusion buffer (4.8 mM KCl, 1.2 mM $MgSO_4$, 122 mM NaCl, 11 mM $Na_2HPO_4$, 3.3 mM $NaH_2HPO_4$, 3.7 mM $NaHCO_3$, 4 °C) with 26 μM EGTA to remove the blood from the liver and to disrupt cell–cell interactions. Digestion of connective tissues and dislodgement of cells were performed by perfusion (5 ml/min, 10–15 min, 35–40 °C) with a calcium-containing (1.5 mM $CaCl_2$) and EGTA-free buffer containing collagenase (0.3 mg/ml). The liver cells were thereafter transferred to a sterile glass beaker containing ice-cold calcium-free perfusion buffer with 0.1% BSA, then homogenized by successive filtering through a 250 μm and 100 μm nylon mesh and centrifuged (500 rpm for 4, 3 and 3 min, 4 °C) before being resuspended in a serum-free L-15 medium containing amphotericin (0.25 μg/ml), streptomycin (100 μg/l), penicillin (100 Units/ml), L-glutamine (0.29 mg/ml) and $NaHCO_3$ (4.5 mM). A final filtration through a 100 μm nylon mesh was performed prior to assessing the cell viability (>80%) using a Bürker counting chamber and trypan blue:cell suspension (2:1). The cell suspension was thereafter diluted to 500 000 cells/ml, seeded in 6- (6 ml), 24- (1.25 ml) or 96-well (200 μl) Primaria™ microtiter plates (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) and incubated in ambient atmosphere at 15 °C. This temperature has previously been observed to yield the most sensitive concentration–response

relationships for *Vtg* in rainbow trout hepatocytes (Tollefsen et al., 2008b).

#### 2.3.1. In vitro exposure

After 24 h of acclimation of the cells in the wells, 50% of the media was removed and replaced with cell media spiked with the test chemical EE2 (0.001–300 nM), positive control for cytotoxicity (0.078–10 mM $CuSO_4$) and solvent control (0.1% DMSO) in triplicate. The cells were subsequently re-exposed after 48 h of exposure to compensate for any depletion of the chemical from the medium. At the end of exposure, 100 μl of cell media was sampled from each well and transferred to individual Maxisorp Nunc-immunoplates (Nunc, Roskilde, Denmark), sealed (Nunc, Roskilde, Denmark) and frozen for subsequent Vtg protein analysis by enzyme-linked immunosorbent assay (ELISA). The cells were analyzed for cytotoxicity (96 well format) and total RNA was isolated (24 well plate format) for subsequent gene expression analysis. Sampling for Vtg protein and gene expression was performed after 24, 48, 72 and 96 h exposure and all samples were frozen at −80 °C prior to analysis.

### 2.4. Cytotoxicity

Cytotoxicity was determined by measuring metabolic activity and cell membrane integrity, using Alamar Blue (AB) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) according to the method described by Tollefsen et al. (2008a). The cell media was removed from each well and replaced with 100 μl of Tris buffer (50 mM, pH 7.5) containing 5% AB and 4 μM CFDA-AM, followed by incubation in the dark on an orbital shaker set to 100 rpm for 30 min (room temperature). Fluorescence was measured at excitation–emission pairs of 530–590 nm (AB) and 485–530 nm (CFDA-AM) by a Victor $V^3$ multilabel counter (Perkin Elmer, Waltham, MA, USA). The viability data was expressed as relative cytotoxicity, where the data was normalized between solvent control (100% viability) and 0.1 M $CuSO_4$ (0% viability).

### 2.5. Capture enzyme-linked immunosorbent assay (capture ELISA)

Vitellogenin was measured by a capture ELISA previously described by Tollefsen et al. (2003). In brief, frozen microtiter plates with samples were thawed at 4 °C, 100 μl of positive control (plasma samples with 3–3000 ng/ml Vtg) was applied to the control wells and the plates were incubated in the dark overnight (16 h). The capture ELISA was carried out using a mono-clonal mouse anti-salmon Vtg antibody (BN-5, 1:6000× diluted in PBS with 1% BSA, Bioscense Laboratories, Bergen, Norway) and a second antibody goat anti-mouse IgG (1:6000× diluted in PBS with 1% BSA, Bio-Rad, Herculeas, CA, USA) followed by addition of HRP enzyme substrate (TMB plus, KEMENTEC diagnostics, Taastrup, Denmark) to start the color development. The plates were thereafter incubated in the dark at room temperature. The color development was stopped after 15–20 min with 50 μl 1 M $H_2SO_4$ and the plates were measured at 450 nm using a Thermomax microplate reader (Molecular Devices, USA) within 20 min after $H_2SO_4$ addition. The relative Vtg expression was calculated as percentage of maximum vtg induction (3 nM EE2) at 96 h of exposure or at individual exposure durations of 24, 48, 72 h (3–30 nM EE2) by normalizing against the solvent control, plotted and fitted to a sigmoidal concentration–response curve with a variable slope (Eq. (1)).

$$\log(X) = \frac{\log(Top - Bottom/Y - Bottom) - 1}{Hill\ Slope} + \log EC50 \quad (1)$$

In Eq. (1) the bottom value represents the minimum Vtg induction (solvent control) and the top value represents the maximum Vtg induction, fixed at 0 and 100 respectively. The hill slope represents the steepness of the curve, *X* value represents the fitted relative Vtg response and *Y* is the experimental Vtg response obtained from the Vtg ELISA analysis.

### 2.6. RNA isolation and quality assessment

Total RNA was isolated using Qiagen RNeasy Plus mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer instructions. Further concentration and purification of resulting RNA were performed with the ZYMO ZR-96 RNA Clean & Concentrator™ (Zymo Research, The Epigenetic company, USA). RNA concentration was measured spectrophotometrically (Spectrophotometer ND 1000, Nanodrop technologies Inc., Wilmington, USA) using the following quality cut-off criteria: 280/260 ratios of >2.0 and 260/230 ratios of >1.6. The RNA integrity of the samples was checked using Agilent BioAnalyzer RNA 6000 nano series kit (Agilent Technologies, USA). All samples had RIN values >8, indicative of high RNA integrity (Fleige and Pfaffl, 2006).

#### 2.6.1. Quantitative real time PCR (qPCR)

Quantitative polymerase chain reaction (qPCR) was performed for the target gene Vtg and the reference gene ubiquitin (ubiq). Total RNA (0.5–1 μg) was reverse transcribed to cDNA using Quanta qScript™ cDNA Synthesis Kit (Quanta Biosences Inc., Gaithersburg, USA) according to the manufacturer's instructions. Primer pair optimization was performed using a 5-step dilutions series (5–100 ng) in a 96 well plate format on a CFX-96 thermal cycler (Bio-Rad laboratories Inc., USA). The fluorescent dye SYBR®Green Supermix (Quanta Biosences Inc., Gaithersburg, USA) was used in the amplification reaction, where duplicates of 10 ng sample cDNA/well was pipetted in a final reaction volume of 20 μl/well. The vitellogenin primer pair was obtained from Celius et al. (2000) and the ubiquitin primer sequences were designed with Beacon designer™ and both were produced by Eurofins MWG synthesis GmbH (Ebersberg, Germany). The genes accession numbers and optimized primer assays are presented in Table 1. All corresponding primers had a non-template control (NTC) to exclude any contamination of primer in the amplification reaction. A melting curve was determined for each primer to confirm specific amplification of each sample. Accepted threshold cycle (Cq) value of NTC was set to be either non-detectable (N/A) or Cq value >30, ensuring a non-significant amount of quantified primer product. Ubiquitin was identified as a stable gene over all the treatments and used as a reference gene in normalization of gene signals by the $\Delta C_q$ ($2^{-\Delta\Delta Cq}$) method (real-time PCR application guide, Bio-Rad) (Table 1).

### 2.7. EE2 chemical analysis

Two milliliter of cell medium from a 24-well cell culture plate was transferred to a 10 ml glass reaction vial and 10 ng of internal standard (d2-E2) was added before the solution was extracted with 1.5 ml toluene by shaking for 5 min. The phases were allowed to separate and a 1 ml aliquot of the toluene layer was transferred to a 1.5 ml HPLC vial. Similarly, 10 ng of internal standard (d2-E2) was added to a 450 μl cell suspension which was extracted with 0.8 ml of toluene and a 0.5 ml aliquot of the toluene layer was transferred to a 1.5 ml HPLC vial. The toluene was evaporated to dryness under nitrogen. Derivatization of EE2 and d2-E2 was performed according to a previously published method by Fox et al. (2011). The dried sample extract was resuspended in 200 μl of 250 mM sodium carbonate/sodium bicarbonate buffer and 200 μl of 1 mg/ml dansyl chloride in acetone. After mixing, the solution was incubated for 30 min at 65 °C. Thereafter 50 μl of MeOH was added and the sample analyzed directly on UPLC–MS using a Waters BEH C8 (1.7 μm, 100 mm × 2.1 mm) with a Waters Acquity UPLC module (Waters Micromass, Manchester, UK). Analyte separation was achieved

**Table 1**
Genes, primer sequences, accession numbers and analysis protocol used for the qPCR analysis.

| Target gene | Primer sequence | Amplification protocol | Primer conc. (nM) | Acc. no |
|---|---|---|---|---|
| *Ubiquitin* | | | | |
| *-forward* | 5′-ACAACATCCAGAAAGAGTCCAC-3′ | Cycle 1: 95 °C 3 min, cycle 2: 95 °C 20 s, 56,0 °C 20 s, 72 °C 20 s | 400 | AB036060 |
| *-reverse* | 5′-AGGCGAGCGTAGCACTTG-3′ | | 400 | |
| *Vitellogenin* | | | | |
| *-forward* | 5′-GAGCTAAGGTCCGCACAATTG-3′ | Cycle 1: 95 °C 3 min, cycle 2: 95 °C 20 s, 61.4 °C 20 s, 72 °C 30 s | 700 | X92804 |
| *-reverse* | 5′-GGGAAACAGGGAAAGCTTCAA-3′ | | 700 | |

by linear gradient elution, starting from MeOH–water containing 2.6 mM ammonium acetate 20:80 v/v, rising to 98% MeOH over 9 min, held for 3 min, then switched back to the start-eluent. The UPLC system was coupled to a Waters Premier XE triple quadrupole mass spectrometer operating with an electrospray ionization (ESI) interface. Typical ESI parameters were a spray voltage of 2.5 kV, desolvation temperature at 400 °C, source temperature at 100 °C and cone gas and desolvation gas at 50 and 800 L $N_2$/h, respectively. The mass spectrometer was operated in MS/MS mode with argon as collision cell gas. Ionization and MS/MS collision energy settings were optimized while continuously infusing (syringe pump) 100 ng/ml of the derivatized standards at 5 μl/min. Detection of the dansyl derivatized analytes was performed by multiple reaction monitoring (MRM) in positive ionization mode; EE2 dansyl 530.2 > 171.1 and d2E2 dansyl 508.2 > 171.1.

### 2.8. Data analysis and statistics

Graphic design and statistical analysis were performed with Graphpad Prism Version 5.04 software (GraphPad Software Inc., San Diego, CA, USA). The mean Vtg protein and mRNA expression with standard error of the mean (SEM) were fitted in a concentration–response curve by non-linear regression. All data were log-transformed to fulfill criteria of normality and equal variances among groups. All data were tested with Bartlett's test for equal variances followed by statistical analysis ($p < 0.05$) using the *t*-test for pairwise comparison or analysis of variance (ANOVA) with Dunnett's *post hoc* test for concentration–response curves.

## 3. Results

The reproducibility, sensitivity and responsiveness of *Vtg* mRNA and protein expression in the rainbow trout primary hepatocytes were assessed by evaluating the effect of seasonal variation, assay conditions (exposure time, and well format) and data normalization procedures. Verification of EE2 exposure concentration was performed to determine the role of cellular biotransformation and chemical loss of EE2 at different exposure concentrations.

### 3.1. Cytotoxicity

No cytotoxicity was observed in cells exposed to EE2 at any concentration or duration of exposure (24–96 h) when compared to the solvent control (0.1% DMSO) and the cell media alone (data not shown).

### 3.2. Vitellogenin mRNA and protein expression

The solvent (DMSO) was found not to produce a significant increase in *Vtg* mRNA or protein response at any exposure time when compared to the response of the cell media alone (data not shown).

#### 3.2.1. Seasonal variation

An inter-individual and seasonal (January–July) variation was observed in both *Vtg* protein and hepatic mRNA expression (Fig. 1). The inter-assay variability in *Vtg* mRNA and protein expression had no apparent coherence with season. The Vtg protein expression was described by concentration–response curves (CRCs) with $r^2 > 0.7$ at 96 h of exposure (Fig. 1). When compared (Fig. 2), the sensitivity of the protein response measured as the No Observed Effect Concentration (NOEC) varied 3-fold (0.03–0.1 nM) and apparent maximum Vtg expression varied 2.4-fold (8.4–19.9-fold change from control). A larger inter-assay variation was observed in *Vtg* mRNA induction, where the NOEC varied 33-fold (0.003–0.1 nM) while the responsiveness was highly variable (74–10 000-fold). A 6–23% reduction in *Vtg* mRNA and protein expression from the maximum levels was observed at concentrations above 30 nM EE2 (data not shown), but were considered being outside of the applicability range of the assay and therefore omitted from further analysis.

#### 3.2.2. Effect of exposure duration

Evaluation of exposure duration was performed to assess whether it had an effect on *Vtg* mRNA and protein expression by exposing the cells for 24, 48, 72 and 96 h to EE2 with re-exposure after 48 h. A clear increase in Vtg protein expression was observed after 24 h of exposure, although the data did not yield a high quality CRC due to low level of induction and high variation in the Vtg response (Fig. 3). Production of the Vtg protein increased with the duration of exposure, illustrated by a high quality CRC in the range 0.03–10 nM from 48 h exposure. Maximum Vtg protein expression was obtained after 96 h of exposure to EE2 and an apparent increase in assay responsiveness was observed with prolonged exposure time (Fig. 3 and Table 2). A significant induction of the hepatic *Vtg* mRNA expression was observed already after 24 h exposure to 1 nM EE2 (Fig. 3), yielding a high quality CRC in the concentration range 0.03–10 nM EE2. The data show an apparent increase in assay sensitivity (24 h EC10: 0.0743 nM, EC50: 0.923 nM; 96 h EC10: 0.0285 nM, EC50: 0.381 nM) with prolonged duration of exposure (Table 2), although these differences were not identified as being significantly different. Determination of the coefficient of variation (CV, %) for Vtg mRNA at 1 nM EE2 (Fig. 3), showed an irregular decrease that was not associated with the exposure duration (*i.e.* 48% at 24 h, 39% at 48 h, 14% at 72 h and 27% at 96 h), which in protein expression was less variable with prolonged exposure (*i.e.* 77% at 24 h, 41% at 48 h, 17% at 72 h and 8% at 96 h). A deviation from ideal concentration-dependent response was observed as a reduction in Vtg expression for both protein and mRNA expression at concentrations in the range from 10 to 30 nM EE2 (results not shown).

When normalized against the maximum Vtg response at 24, 48, 72 and 96 h exposure to EE2, the data yielded high quality CRCs in both protein and mRNA expression in the concentration range 0.01–10 nM (Fig. 4 and Table 2). Vitellogenin protein and mRNA was expressed in a consistent manner between 48–96 h (protein) and 24–96 h (mRNA) (Fig. 4) with similar EC-values at 24–72 h, but displaying an apparent increase in Vtg responsiveness over time (Table 2). An apparent temporal increase in sensitivity,

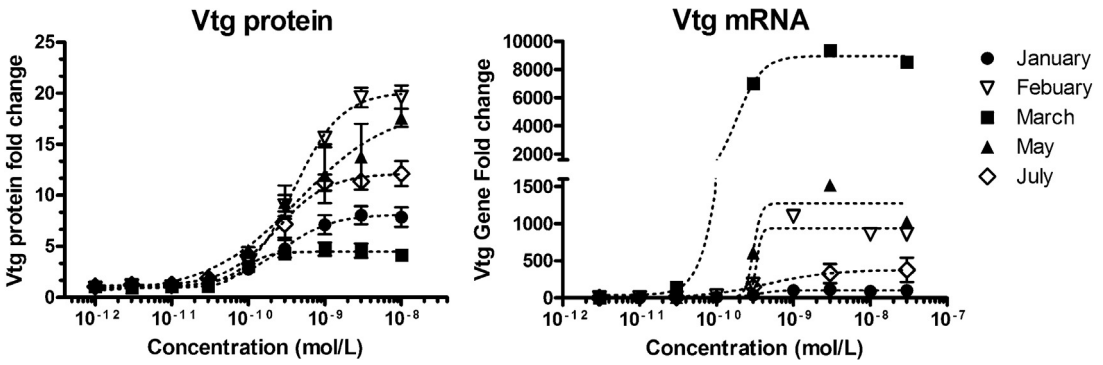


**Fig. 1.** Seasonal variation in the induction of vitellogenin (Vtg) protein and mRNA production in rainbow trout (*Oncorhynchus mykiss*) hepatocytes after exposure to 17α-ethynylestradiol for 48 h (mRNA) and 96 h (protein). Data were normalized against solvent control (DMSO) and fitted to sigmoidal concentration–response curves using non-linear regression.

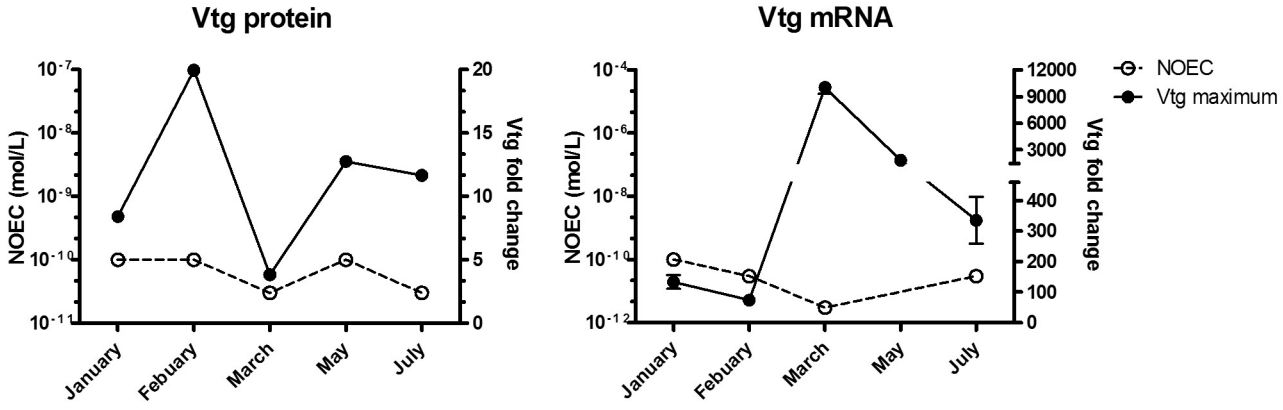


**Fig. 2.** Seasonal variation in the sensitivity (No Observed Effect Concentration, NOEC) and responsiveness (maximum induction) of vitellogenin (Vtg) protein and mRNA production in rainbow trout (*Oncorhynchus mykiss*) hepatocytes after exposure to 17α-ethynylestradiol for 48 h (mRNA) and 96 h (protein). The data is presented as the mean of NOEC and Vtg fold change was normalized against the control (L-15 medium) and represent data from minimum 1–3 independent cell isolations per month.

measured as a reduction in the $EC_{10}$, was observed for both protein and mRNA expression (Table 2). The cells exposed to EE2 for 24 h did not produce a consistent Vtg protein expression and was thus omitted from the analysis. Vitellogenin protein expression varied considerably after 24 h of exposure despite normalization, but was greatly improved and comparable from 48 to 96 h of exposure. A large degree of consistency was found between the shape of the CRCs (Fig. 4) and ECs for protein and mNA expression after 96 h exposure (Table 2).

### 3.2.3. *Effect of plate format*

The effect of well size exhibiting different volume/total surface ratios on induction of Vtg protein production was assessed by exposing the cells to EE2 for 48 and 96 h in 96, 24, and 6 well plates. No significant differences in sensitivity or responsiveness were observed in the Vtg protein induction (non-normalized data, results not shown) or the Vtg CRCs (normalized data, Fig. 5) for the different plate formats after 48- and 96 h exposure.

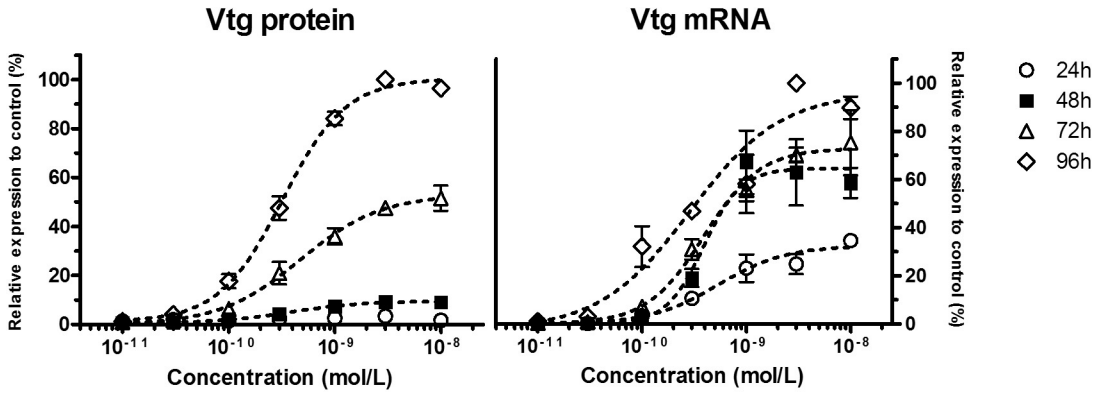


**Fig. 3.** Vitellogenin (Vtg) protein and mRNA induction in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed for 24–96 h to 17α-ethynylestradiol (EE2). Data (mean ± SEM) were normalized against solvent control (DMSO) and expressed as percentage of mRNA and protein Vtg maximum (3 nM EE2 at 96 h). The lines represent non-linear regression curve fit to experimental data from minimum 3 independent cell isolations performed between January and July.

**Table 2**
Calculated effect concentrations (EC) and maximum fold induction for vitellogenin (Vtg) protein and mRNA in rainbow trout (*Oncorhynchus mykiss*) heptatocytes exposed to 17α-ethynylestradiol. The EC-values were determined from data normalized against solvent control (0%) and the maximum Vtg induction at 10–30 nM EE2 (100%) for the indicated exposure time.

| Endpoint | Time (h) | EC10 (mol/L) | EC50 (mol/L) | EC90 (mol/L) | Fold induction[a] | Hill slope[b] |
|---|---|---|---|---|---|---|
| Protein | 24 | – | – | – | – | – |
| | 48 | 8.97E$^{-11}$ | 3.83E$^{-10}$ | 1.63E$^{-09}$ | 1.98 | 1.51 |
| | 72 | 9.52E$^{-11}$ | 3.76E$^{-10}$ | 1.49E$^{-09}$ | 6.41 | 1.60 |
| | 96 | 5.37E$^{-11}$ | 2.42E$^{-10}$ | 1.09E$^{-09}$ | 10.2 | 1.46 |
| mRNA | 24 | 7.43E$^{-11}$ | 9.23E$^{-10}$ | 1.14E$^{-08}$ | 61.87 | 0.87 |
| | 48 | 7.33E$^{-11}$ | 6.59E$^{-10}$ | 5.93E$^{-09}$ | 101.83 | 1 |
| | 72 | 7.56E$^{-11}$ | 6.11E$^{-10}$ | 4.94E$^{-09}$ | 267.90 | 1.05 |
| | 96 | 2.85E$^{-11}$ | 3.81E$^{-10}$ | 5.09E$^{-09}$ | 247.28 | 0.85 |

[a] Maximum vtg fold induction compared to solvent control.
[b] Hill slope is obtained from the sigmoidal dose response curve fit.

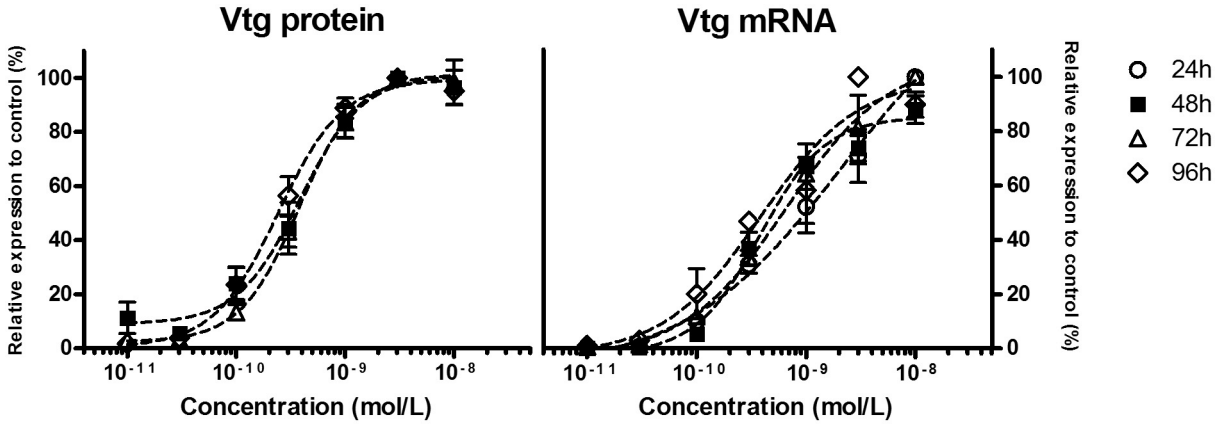


**Fig. 4.** Vitellogenin (Vtg) protein and mRNA expression in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 17α-ethynylestradiol for 24–96 h. Data (mean ± SEM) were normalized against solvent control (DMSO) and the EE2 concentrations (3–10 nM) yielding maximum Vtg response at each exposure time. The lines represent non-linear regression curve fit to experimental data from minimum 3 independent cell isolations performed between January and July.

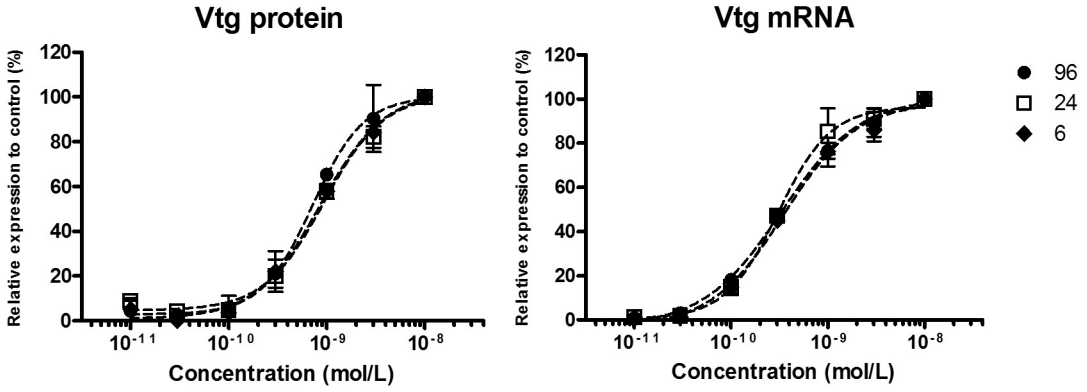


**Fig. 5.** Relative vitellogenin protein expression in rainbow trout (*Oncorhynchus mykiss*) hepatocytes in different (6, 24, 96) well formats when exposed to 17α-ethynylestradiol (EE2) for 48 and 96 h. Data (mean ± SEM) were normalized against solvent control (DMSO) and EE2 (10 nM) and presented as percentage of individual Vtg maximum at respective well size format after 48 and 96 h. The lines represent non-linear regression curve fit to experimental data from minimum 3 independent cell isolations performed between January and July.

### 3.3. Chemical depletion of EE2 in the bioassay

Verification of EE2 exposure concentration was performed in the 24-well format. When exposed to 3 nM EE2 in a time-dependent (0–48 h) manner, cells were found to contain 70.7 times higher concentration of EE2 than in the media within an hour of exposure (Fig. 6A). The EE2 concentration in the cells was fairly stable for 8 h, before decreasing over time. Concentrations of EE2 in the media decreased already from the start of the exposures and throughout the 48 h exposure period. When primary hepatocytes were exposed to 3 and 30 nM EE2 for 0 and 48 h (Fig. 6B), measured EE2 concentrations in media were identified at 0 h to be 74 and 93% of nominal EE2 concentration, albeit as little as 4 and 9% of nominal EE2 concentrations could be measured in media after 48 h exposure (Fig. 6B).

## 4. Discussion

Knowledge about the potential impact of endocrine disrupting chemicals (EDCs) on humans and the environment has increased substantially the last 20 years with the establishment of international EDC screening programs and regulatory guidelines (Hecker

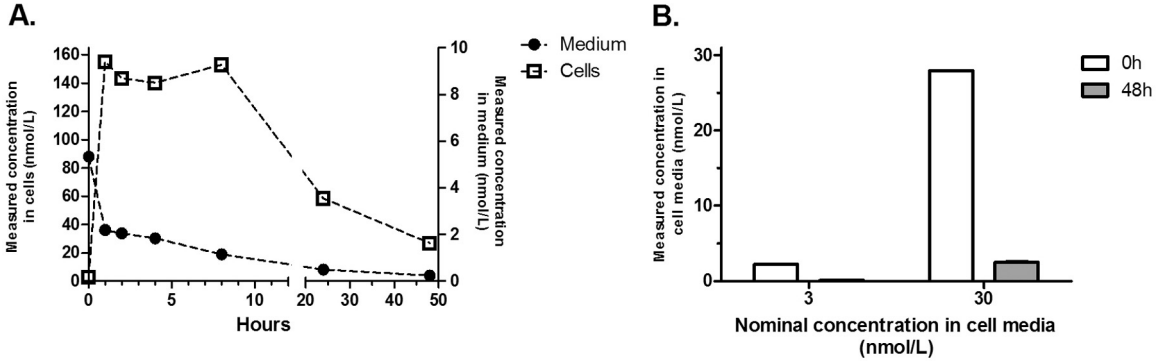


**Fig. 6.** Reduction of 17α-ethynylestradiol (EE2) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes (cells) and L-15 medium (medium) exposed to 3 nM EE2 for 48 h (A) and comparison between nominal and measured concentrations of 3 and 30 nM EE2 between start (0 h) and the end (48 h) of the experiment (B).

and Hollert, 2011). As the regulations for EDCs develop, a higher number of chemicals will require regulatory testing and tiered approaches using alternative (*in silico* and *in vitro*) methods to characterize EDCs are likely to surface as alternatives to resource-demanding and ethically challenging *in vivo* tests. Various international organizations are working toward a larger implementation of the 3R's into integrated Approaches for Testing and Assessments (IATAs) and these initiatives often involve using *in silico* methods such as (quantitative) structure activity relationship, structural alerts and category formation, and *in vitro* assays such as estrogen receptor (ER)- and androgen receptor (AR)-binding assay (OECD, 2012a; US-EPA, 2009). Well-characterized *in vitro* methods such as the primary fish hepatocyte assay has shown to be a promising tool in the screening of estrogenic and anti-estrogenic chemicals, complex mixtures and environmental monitoring as they retain many of their native metabolic and biochemical properties (Petersen and Tollefsen, 2012, 2011; Segner and Cravedi, 2000; Tollefsen et al., 2006; Tollefsen and Nilsen, 2008). However, large variations in sensitivity, responsiveness and reproducibility have been reported among laboratories and species (Navas and Segner, 2006) and thus proposed to limit the applicability of this assay within regulatory settings (Combes et al., 2006). Use of laboratory-specific strains of fish and lack of standardized guidelines, have made direct comparison between data and assays challenging. The present paper evaluated key properties of the performance of the primary hepatocyte assay, such as quantification of the Vtg response at the protein and mRNA level, role of seasonal variation, assay conditions (*e.g.* well plate format) and data normalization procedures to identify the assay's applicability as a potential screening assay for ER-agonists.

### 4.1. Seasonal variation

It is well documented that *in vivo* Vtg production varies throughout the season and maturation status of the fish (Bon et al., 1997; Larsson et al., 2002). This has led to the use of either male or juvenile fish in *in vivo* and *in vitro* studies for EDCs (Bickley et al., 2009; Tollefsen et al., 2003). Use of juvenile or male fish as performed in the present and a number of studies elsewhere is thus expected to reduce a large source of variability, increase sensitivity toward estrogens, improve assay performance and allow inter-assay comparability (Bickley et al., 2009; Navas and Segner, 2006). Despite such improvements, considerable variability in the maximal Vtg response (*i.e.* the fold induction) at the protein and mRNA level was observed after exposing primary hepatocytes from rainbow trout to EE2 in the present study. This level of variance in Vtg response has previously been reported in hepatocytes from bream (*Abramis brama*) and common carp (*Cyprinus carpio*) (Bickley et al., 2009; Rankouhi et al., 2004) and proposed being associated with a number of factors such as seasonal differences in the host fish physiology, rearing temperature and photoperiod, and *in vitro* culture conditions (Clark et al., 2005; Pawlowski et al., 2000). Changes in the basal activity of Vtg have previously been associated both with the seasonal changes in rearing temperatures by affecting the estrogen sensitivity of the liver and capacity to regulate *ER* and *Vtg* transcription, translation and post translation events downstream binding and activation of estrogen responsive elements (EREs) (MacKay et al., 1996; Mackay and Lazier, 1993). Elevated temperatures have also been demonstrated to affect the general cellular metabolism and specifically increase the expression of *Vtg* and *ER* mRNA both *in vitro* and *in vivo* (MacKay et al., 1996; Mackay and Lazier, 1993; Pawlowski et al., 2000). Other cellular processes such as biotransformation involving the aryl hydrocarbon receptor (AhR) and Cytochrome P450 1A (CYP1A) may directly or indirectly be affected by changes in ER activity (Gräns et al., 2010; Mortensen and Arukwe, 2007) as well affect the transcriptional activation of the *ER* (Klinge et al., 1999) by potential unidirectional cross-talk (Bemanian et al., 2004). Besides temperature, maturation status of the donor fish has been proposed to affect the estrogen response in fish hepatocytes (Bickley et al., 2009; Smeets et al., 1999). Changes in *Vtg* expression and concentrations of nuclear and cytosolic ER with a factor up to 4 have been reported, although no correlation between the amount of natural estrogen E2 and induced nuclear and cytosolic ER could be determined (Smith and Thomas, 1991). Furthermore, the activity of the CYP-family enzymes is also reported to be closely associated with the reproductive cycle and seasonal maturation of fish (Koivusaari et al., 1984; Larsen et al., 1992), potentially affecting the biotransformation of the test compound. Despite suggestions of a number of factors that may affect regulation of circulating steroids and their biological activity (Clark et al., 2005; Pawlowski et al., 2000), performance of the current study under a static photoperiod (*in vivo* rearing: 12 h light/12 h dark, *in vitro* culturing: in the dark), minor variations in temperature (*in vivo* rearing: $6 \pm 2\,^{\circ}C$, *in vitro* culturing: $15 \pm 0.5\,^{\circ}C$) and use of juvenile fish for all cell isolations have likely minimized any differences in responsiveness between different cell batches. It is therefore suggested that individual differences between cell batches are difficult to minimize further and any improvements in the assay performance will have to be performed through data normalization procedures (Bickley et al., 2009; Smeets et al., 1999).

### 4.2. Data normalization

Variation in production of Vtg has been proposed to be reduced by normalizing either to basal Vtg production to obtain relative fold induction or to fit the responses within a minimum (basal) to maximal response relations to derive relative potencies (Bickley et al., 2009; Navas and Segner, 2006; Rankouhi et al., 2004). The

Vtg production may also be reported as total quantity of protein or amount of RNA (ng)/well (Navas and Segner, 2006), but such normalization procedures will not minimize any data variations arising from different studies, although clearly improve the ability to perform absolute quantification. The strategy of the current exposure and analysis approach were developed to enhance sample throughput capacity (24 or 96 well format) and rapid detection by capture ELISA (protein detection) and/or qPCR (mRNA detection), and data normalization was best served by the relative potency approach. Implementation of this normalization procedure clearly improved the quality of the CRCs, minimized the inter-assay (*e.g.* cell batch) variation, and increased the reproducibility of both mRNA and protein data considerably. Furthermore, the data normalization greatly improved the previously reported inter-cell batch variation, yielding high-quality CRCs for both Vtg mRNA and protein for different exposure durations. The variability in the 95% confidence interval of $EC_{50}$ was greatly improved at 96 h when data was normalized (non-normalized: 50%, normalized: 31%), further increased the reproducibility and reliability of the primary hepatocyte assay. The variation in Vtg protein expression between replicates ranged from 25% to 6% when data were normalized at 96 h, which was consistent with previous studies with primary salmon (*Salmo salar*) hepatocytes (Tollefsen et al., 2003). Although the current study used between four and seven donor fish, a minimum of four assays were normally found to be sufficient to obtain high quality CRCs for both *Vtg* mRNA and protein expression.

### 4.3. Bioassay-specific factors

#### 4.3.1. Effect of exposure duration

Regulation of *Vtg* mRNA and protein production is a sensitive and time-dependent translation process from the molecular (gene) to the subcellular (protein) response. Hepatocytes exposed to EE2 for 24–96 h expressed high-quality CRCs for Vtg mRNA already after 24 h exposure when normalized using relative expression as performed for fish hepatocytes elsewhere (Bickley et al., 2009; Finne et al., 2007). In contrast, clear induction and high-quality CRCs for Vtg protein expression was first observed after 48 h exposure. This is in compliance with exposure studies with Atlantic salmon hepatocytes exposed to E2 (Tollefsen et al., 2003), and likely reflect the time delay between transcription, translation and subsequent protein synthesis (Scholz et al., 2004) and transport of the protein to the exterior of the hepatocyte. Although gene expression was initiated earlier than 24 h, consistent induction of both *Vtg* mRNA and protein production may require as much as 48 h of exposure to produce high-quality CRCs (Bickley et al., 2009; Gagné and Blaise, 1998; Scholz et al., 2004; Tollefsen et al., 2003). Prolonging the exposure duration to 72 and 96 h improved the magnitude of both the mRNA and protein *Vtg* response (Table 2). Optimal exposure time was identified as 96 h whereupon both the *Vtg* protein and mRNA expression showed an apparent increase in the assay sensitivity, responsiveness and reduced CV, consistent with previous studies performed on primary salmon hepatocytes exposed to E2 (Tollefsen et al., 2003). However, extending the duration from 48 to 96 h of exposure, required chemical re-exposure as the concentration of EE2 in the wells was reduced to less than 50% after 24 h of exposure and almost depleted within 48 h (Fig. 6A). Such rapid depletion has previously been observed for E2 and ascribed to high biotransformation activity in rainbow trout hepatocytes and liver preparations (Miller et al., 1999; Schmieder et al., 2004). These observations are in agreement with observations that E2 requires re-exposure after 48 h to maintain reproducible CRCs (results not shown). The observed relationship between nominal and measured concentration of EE2 obtained herein suggests a concentration-independent depletion of EE2 (Fig. 6B), thus confirming that the hepatocytes are highly metabolically capable (Pedersen and Hill, 2000; Segner and Cravedi, 2000). Despite the clear advantages of having metabolically active cells to mimic the natural biotransformation and thus potentially detect both estrogenic mother compounds and their metabolites (Nillos et al., 2010; Pedersen and Hill, 2000; Segner and Cravedi, 2000), the rapid depletion occurring may challenge accurate calculation of effective concentration or potencies for metabolically susceptible EDCs (Lindholst et al., 2003). Although routine measurement of exposure concentrations in *in vitro* bioassays are clearly a complicating factor that will limit sample throughput, lack of consistency between *in vitro* potency and *in vivo* toxicity (Tollefsen et al., 2008a) may require analytical or computational corrections to adjust for chemical depletion in bioassays such as primary hepatocytes.

#### 4.3.2. Plate format

The different plate formats used in chemical exposures often reflects the samples need for the down-stream endpoint analysis. Determination of viability and high-throughput use of various biochemical probes are often conducted in the 96 well plate formats (Farmen et al., 2010; Tollefsen et al., 2008b), whereas sublethal effects such as gene expression may require either 24 or 6 well formats to ensure sufficient biological material for analysis using most cDNA construction protocols (Finne et al., 2007). Use of different plate formats with different volume to surface ratios may thus introduce confounding bioassay factors such as differences in sorption to and possible interaction with the plastic surface (Schreiber et al., 2008). Such confounding factors may potentially affect the freely dissolved concentration of hydrophobic chemicals (log Kow >3) in the bioassay and result in overestimation of the chemicals actual concentration in the assay (Brown et al., 2001; Mayer et al., 1999; Riedl and Altenburger, 2007). Despite potential for such confounding factors, no significant difference in Vtg protein production was observed in assays run in 6, 24 or 96 well formats with different total surface-volume ratio after 48 and 96 h of exposure. Although this holds true for EE2, larger discrepancies may be expected for chemicals that are more hydrophobic and volatile than EE2.

### 4.4. Bioassay performance

Exposure to EE2 for 24–96 h caused a sensitive, responsive and reproducible induction of *Vtg* mRNA and protein expression similar to that observed for primary salmonid hepatocytes (Olsen et al., 2005; Tollefsen et al., 2003). However, deviation from ideal CRCs for both *Vtg* protein and mRNA expression at higher concentrations of EE2 (10–30 nM) in the primary rainbow trout hepatocytes suggest that EE2 causes complex cellular responses that are not accounted for by pure receptor-mediated responses alone. This has been reported in rainbow trout, bream and carp hepatocytes previously, although the reason for such discrepancy is currently not understood (Petersen and Tollefsen, 2011; Rankouhi et al., 2004). Unpublished global transcriptional analysis using materials generated in the same bioassay as used herein show a 14% lower expression of *ERα* at 30 nM compared to the second highest concentration (3 nM), thus suggesting that part of the reduction in Vtg observed is mediated through regulation of the activity of the ER (results not shown). Phase II biotransformation enzymes, involved in the steroid homeostasis (UDP-glucuronosyl transferase) and phase III biotransformation (ABC-family transporters), involved in steroid transport were also induced transcriptionally at this concentration, thus may potentially lead to a reduction in the cellular level of EE2 and consequently also *ER* and *Vtg* expression. Other factors such as concentration-dependent ER desensitization, receptor competition, receptor down-regulation and/or negative endocrine feedback loops, receptor cross-talk and induction of sex steroid-binding proteins (SBPs) that limit cellular access of EE2 (Foucher et al., 1991; Gräns et al., 2010; Nagel et al., 1997, 1998; Vandenberg

**Table 3**
Bioassay conditions, the No Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and 50% Effect Concentration ($EC_{50}$) for vitellogenin protein induction in different primary hepatocyte cultures exposed to 17β-estradiol (E2) and 17α-ethynylestradiol (EE2). The relative binding affinity (ER-RBA) for EE2 is reported for comparative purposes.

| | Chemical | Exposure (h) | Temperature | Cell density (cells/cm$^2$) | Media supplements | NOEC (mol/l)[e] | LOEC (mol/l) | EC50 (mol/l) | ER RBA (%) | References |
|---|---|---|---|---|---|---|---|---|---|---|
| Cold water species (*Salmo salar, Oncorhynchus mykiss*) | E2 | 48 | 14–18 °C | 1.0E$^6$ | No | 1.0E$^{-10}$ | 1.0E$^{-9}$ | NR | – | Pawlowski et al. (2000) |
| | | 96 | 12–18 °C | 2.5E$^5$–1.0E$^6$ | No | 1.0E$^{-13}$–1.0E$^{-10}$[b] | 1.0E$^{-12}$–1.0E$^{-9}$ | 2.6E$^{-11}$–6.29E$^{-10}$[b] | – | Tollefsen et al. (2003), Olsen et al. (2005) Pawlowski et al. (2000) and Petersen and Tollefsen (2011) |
| | EE2 | 96 | 12 °C | 2.5E$^5$ | No | NR | NR | 6.2E$^{-11}$ | – | Tollefsen et al. (2003) |
| | | | | – | – | – | – | – | 55–88.9 | Tollefsen et al. (2002) and Denny et al. (2005) |
| Warm water species (*Abramis brama , Carassius auratus, Cyprinus carpio, Oryzias latipes, Oreochromis mossambicus, Oreochromis niloticus, Ictalurus punctatus, Pimephales promelas*) | E2 | 48 | 25–28 °C | 2.08E$^5$–3.75E$^5$ | No | 3.7E$^{-8}$–1.0E$^{-5}$ | 1.8E$^{-7}$–1.0E$^{-4}$ | 4.7E$^{-7}$[b] | – | Riley et al. (2004), Kim and Takemura (2003) and Liu et al. (2007) |
| | | 96 | 20–24 °C | 3.0E$^5$–3.125E$^6$ | 1–2% Ultroser[c,d], 5% FBS[a] | 1.0E$^{-8}$–1.0E$^{-7}$ | 2.0E$^{-9}$–1.0E$^{-6}$ | 5.0E$^{-8}$–2.12E$^{-6}$[b] | – | Bickley et al. (2009), Rankouhi et al. (2004), Smeets et al. (1999) and Zhao et al. (2006) |
| | EE2 | 96 | 24 °C | 7.03E$^5$–3.125E$^6$ | 1% Ultroser | 1.0E$^{-8}$–1.0E$^{-7}$ | 1.0E$^{-7}$–1.0E$^{-6}$ | 3.0E$^{-8}$–2.0E$^{-7}$ | – | Rankouhi et al. (2004) |
| | | | | – | – | – | – | – | 166.4–515.7 | Nimrod and Benson (1997) and Denny et al. (2005) |

NR - not reported; FBS - fetal bovine serum

[a] Bickley et al. (2009)

[b] All included studies do not report this value.

[c] Smeets et al. (1999).

[d] Rankouhi et al. (2004).

[e] Highest reported value that is not significantly different from the solvent control.

**Table 4**
Lowest Observed Effect Concentration (LOEC) for vitellogenin induction in cold and warm water species exposed to 17β-estradiol (E2) and 17α-ethynylestradiol (EE2).

| | | Exposure time | LOEC – E2 (mol/l) | LOEC – EE2 (mol/l) | References |
|---|---|---|---|---|---|
| Cold water species (*Oncorhynchus mykiss*) | *in vivo* | <14 days | $5.14E^{-11}$ (14 ng/L) | $3.37E^{-12}$–$3.37E^{-10}$ (1–100 ng/L)[a] | Verslycke et al. (2002) and Thomas-Jones et al. (2003) |
| Warm water species (*Cyprinodon variegatus*, *Cyprinus carpio*,*Danio rerio*, *Oryzias latipes*, *Pimephales promelas*) | *in vivo* | <110 days | $3.67E^{-10}$–$7.34E^{-10}$ (100–200 ng/L) | $3.37E^{-12}$– $3.37E^{-10}$ (1–100 ng/L) | Folmar et al. (2000), Gimeno et al. (1998), Huang et al. (2010), Jobling et al. (2003), Pawlowski et al. (2004) and Scholz et al. (2004) |

[a] EE2-water exposure studies.

et al., 2012) are additional explanations to the relative reduction in the Vtg response at high EE2 concentrations.

### 4.5. Inter-laboratory and inter-species differences

Large variances in estrogen sensitivity (LOEC: $10^{-12}$–$10^{-7}$ M) and Vtg responsiveness have previously been reported in primary hepatocytes among different laboratories and donor species (Supplementary Table A) (Navas and Segner, 2006). In an attempt to address this issue, a comparison of relevant *in vitro* bioassays (primary hepatocytes, liver slices, liver homogenate) was performed to identify potentially contributing factors to the observed intra- and inter-species estrogen sensitivity of the bioassays (Suppl. Table A). The ER-sensitivity has previously been suggested as one of the contributing factors to the inter-species discrepancies of *in vitro* Vtg responses (Rankouhi et al., 2004). However when comparing ER relative binding affinity (ER-RBA) for EE2 in four different cold- and warmwater species it differed no more than 10-fold (Table 3) as previously stated by Denny et al. (2005). Somewhat higher differences (<200-fold) were observed when comparing the Lowest Observed Effect Concentration (LOEC) for *in vivo* Vtg-induction in the same species (Table 4, Suppl. Table B). Interestingly, the LOECs for rainbow trout *in vitro* and *in vivo* Vtg-induction differed with no more than 0-19.5-fold for E2, which was within the concentration range of the LOEC for *in vivo* Vtg-induction in coldwater species alone (Tables 3 and 4). However, orders of magnitude differences (2-1 000 000-fold) were observed when comparing the *in vitro* Vtg-sensitivity in primary hepatocytes from cold- and warmwater species (Table 3), thus suggesting that factors related to the *in vitro* bioassays were the main reason for the large discrepancy. Bioassay factors such as cell incubation temperature, cell density, culture plate formats and especially media supplements (*e.g.* fetal bovine serum, Ultroser SF) varied greatly within and among the bioassays done on warm-water species. Such *in vitro* culturing factors have previously been demonstrated to significantly affect Vtg induction, as well as metabolism and bioavailability of the exposure chemicals (Pawlowski et al., 2000; Tollefsen et al., 2003). Additionally, the selection of *Vtg* transcripts (*e.g.* those of *Vtg 1*, *Vtg 3*) used in the gene expression analysis may contribute to the observed discrepancies as they may have different estrogen responsiveness (Martyniuk et al., 2007). The present comparison suggests that inter-laboratory factors and bioassay conditions (*e.g.* incubation temperature, media supplements, cell density *etc.*) are the largest contributors to the observed variability of Vtg sensitivity and responsiveness among and within species. In addition to these confounding bioassay factors, different analytical approaches for determining Vtg and normalization procedures are applied across laboratories, making the results difficult to compare (Navas and Segner, 2006). It is evident that there is still a need for intra-species protocol standardization of rearing temperatures, cell isolation procedures, bioassay conditions, analytical approaches and data normalization procedures to reduce inter-assay and inter-laboratory variability.

### 4.6. Future directions for use of fish hepatocytes

The present work has demonstrated that assays with rainbow trout primary hepatocytes are sensitive, reproducible and responsive when the cells are exposed to the estrogen receptor agonist EE2. The variable capacity of primary hepatocytes to induce Vtg gene either at mRNA or protein level could not fully be explained by either seasonal or variable inter-cell batch endocrine regulation. Although differences in incubation temperature and inter-cell batch variation might have affected the Vtg response, this variation was dramatically reduced by data normalization. The present paper also briefly addressed the inter-lab and inter-species variability in Vtg sensitivity and concluded that there is a need for larger standardization of the primary hepatocyte culture and exposure protocols. Future assay standardization should consider use of serum-free exposure media, fixed intra-species incubation temperatures, standardized bioassay cell density and verification of chemical exposure concentrations to account for depletion of chemicals in the bioassays. Chemical bioavailability of hydrophobic chemicals has proven particularly challenging due to various bioassay factors (*e.g.* chemical depletion, well format, wall sorption) in which partition-driven administration dosing may alleviate some of the problems (Booij et al., 2011; Kwon et al., 2009; Riedl and Altenburger, 2007; Schreiber et al., 2008; Smith et al., 2009). Development of continuous liver cell lines or use of cryopreserved primary hepatocytes, which retain properties of freshly isolated hepatocytes (Markell et al., 2014; Mingoia et al., 2010), may additionally reduce inter-lab and inter-assay variance to a level that accommodates suitable high-throughput screening formats (Bols et al., 2005; Petersen and Tollefsen, 2012, 2011; Tollefsen et al., 2003, 2008b).

## 5. Conclusion

The present study has shown primary rainbow trout hepatocytes to be a sensitive, reproducible and responsive *in vitro* model. The confounding effect of inter-individual-, inter-assay- and seasonal variations in *Vtg* protein expression and mRNA induction were reduced when data normalization procedures were implemented. Standardization of protocols for cell culture conditions, exposure procedures, chemical verification and data normalization has the potential to reduce inter-assay and inter-laboratory variance in use of primary hepatocytes for screening of ER-agonists. Implementation of more harmonized efforts in bioassay testing with primary hepatocytes should thus be expected to increase the assay's potential as an experimental model. The present proposal for optimization and standardization of protocols will hopefully facilitate improvement of the assay's robustness, reproducibility, sensitivity and render this assay more applicable to lower tier regulatory testing strategies (*e.g.* an OECD CF2 assay).

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2014.12.013.

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# Paper II

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# 17α-Ethinylestradiol *(EE2)* effect on global gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes

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ABSTRACT

The potential impact of endocrine disrupting chemicals (EDCs) in the aquatic environment has driven the development of screening assays to evaluate the estrogenic properties of chemicals and their effects on aquatic organisms such as fish. However, obtaining full concentration–response relationships in animal (*in vivo*) exposure studies are laborious, costly and unethical, hence a need for developing feasible alternative (non-animal) methods. Use of *in vitro* bioassays such as primary fish hepatocytes, which retain many of the native properties of the liver, has been proposed for *in vitro* screening of estrogen receptor (ER) agonists and antagonists. The aim of present study was to characterize the molecular mode of action (MoA) of the ER agonist 17α-ethinylestradiol (EE2) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes. A custom designed salmonid 60,000-feature (60k) oligonucleotide microarray was used to characterize the potential MoAs after 48 h exposure to EE2. The microarray analysis revealed several concentration-dependent gene expression alterations including classical estrogen sensitive biomarker gene expression (*e.g. estrogen receptor α, vitellogenin, zona radiata*). Gene Ontology (GO) analysis displayed transcriptional changes suggesting interference of cellular growth, fatty acid and lipid metabolism potentially mediated through the estrogen receptor (ER), which were proposed to be associated with modulation of genes involved in endocrine function and reproduction. Pathway analysis supported the identified GOs and revealed modulation of additional genes associated with apoptosis and cholesterol biosynthesis. Differentially expressed genes (DEGs) related to impaired lipid metabolism (e.g. *peroxisome proliferator-activated receptor α and γ*), growth (*e.g. insulin growth factor protein 1*), phase I and II biotransformation (*e.g. cytochrome P450 1A, sulfotransferase, UDP-glucuronosyltransferase and glutathione S-transferase*) provided additional insight into the MoA of EE2 in primary fish hepatocytes. Results from the present study suggest that biotransformation, estrogen receptor-mediated responses, lipid homeostasis, growth and cancer/apoptosis in primary fish hepatocytes may be altered after short-term exposure to ER-agonists such as EE2. In many cases the observed changes were similar to those reported for estrogen-exposed fish *in vivo*. In conclusion, global transcriptional analysis demonstrated that EE2 affected a number of toxicologically relevant pathways associated with an estrogenic MoA in the rainbow trout hepatocytes.


## 1. Background

Compounds that modulate the endocrine system and cause adverse effects causally related to these changes are known as endocrine disrupting chemicals (EDCs). These chemicals may enter the environment through anthropogenic activities such as effluents of sewage treatment plants, industrial processes and agricultural run-off (Sumpter, 2005). During the past decade, increasing awareness of the adverse effects of EDCs in wildlife and human has given rise to the implementation of stricter legislations in international regulatory organizations worldwide (Hecker and Hollert, 2011). Adverse effects such as impaired reproduction in fish, reproductive disorders and various cancer types (*e.g.* breast and ovary cancer) in human and other mammals have been associated with EDCs such as 17α-ethinylestradiol (EE2), dichlorodiphenyltrichloroethane (DDT) and bisphenol A (BPA) (Benninghoff and Williams, 2008; Purdom et al., 1994; Soto and Sonnenschein, 2010; Vom Saal et al., 2007). Characterization of a chemical's mode of action (MoA) involving interference with specific molecular, cellular and biochemical changes, behavioral alterations and adverse effects are often laborious and expensive due to extensive use of

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animals (Aardema and MacGregor, 2002). Although the use of single biomarker screening approaches has facilitated the understanding of MoAs of EDCs, the knowledge obtained from such biomarker studies is still limited, as the response of a single endpoint may not always represent complex biological responses at higher levels of organization.

In recent years, the development of broad-content screening approaches such as transcriptomics has made it possible to characterize the global gene expression changes after exposure to single EDCs and mixture of these in different *in vitro* and *in vivo* experimental models (Finne et al., 2007; McHale et al., 2010; Wang et al., 2010; Yang et al., 2007). Deciphering complex molecular interactions of chemicals using transcriptomic studies has enabled detailed studies on the *in vivo* responses of (xeno) estrogens in common laboratory species such as zebrafish (*Danio rerio*), fatheaded minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), but also non-model species such as coho salmon (*Oncorhynchus kisutch*) (Harding et al., 2013; Hook et al., 2008; Levi et al., 2009; Villeneuve et al., 2011; Wang et al., 2010). Many of these studies involve the analysis of estrogenic responses, which typically involve the binding and activation of the estrogen receptor (ER) and of genes containing estrogen response elements (ERE) to initiate multi-organ endocrine responses in fish. The ER signaling pathway may further regulate the expression of several classical estrogenic biomarker genes such as the egg-yolk precursor protein *vitellogenin* (*vtg*), egg envelope proteins *zona pellucida* and *zona radiata protein* (*zrp*) (Arukwe et al., 1997; Sumpter and Jobling, 1995). The endocrine modulatory effects of EDCs have been extensively studied *in vivo,* including the characterization of MoA associated with hormone binding, lipid and cholesterol metabolism and steroidogenesis, immune function and ion homeostasis in fish (Colli-Dula et al., 2014; Flores-Valverde et al., 2010; Hook et al., 2007; Kausch et al., 2008; Levi et al., 2009; Wit et al., 2010). Various estrogen-mediated responses have also been observed in biomarker studies in various piscine *in vitro* (non-animal) bioassays, and consequently led to the proposal of using these experimental models as screening assays for environmental estrogens and antiestrogens (Björkblom et al., 2008; Hultman et al., 2015; Kordes et al., 2002; Navas and Segner, 2006; Rankouhi et al., 2004; Tollefsen et al., 2003).

Use of alternative approaches provided by *in vitro* methods have offered rapid screening methods and facilitated better understanding of the MoA of chemicals whilst implementing the 3R's (refinement, reduction and replacement) into toxicological testing (National research council (NRC), 2007). *In vitro* hepatic models such as primary hepatocytes have demonstrated to be advantageous proxies for the assessment of *in vivo* bioactivity as the cells retain many of the native hepatic functions including biotransformation, detoxification and ER-mediated responses (Flouriot et al., 1993; Pedersen and Hill, 2000; Pesonen and Andersson, 1997; Segner and Cravedi, 2000). The hepatocytes have previously demonstrated their potential for toxicological screening of cellular toxicity, endocrine disruption (ED) and bioaccumulation in various assay formats including suspension, monolayer and 3-dimensional spheroid cultures (Baron et al., 2012; Hultman et al., 2015; Mingoia et al., 2010; Smeets et al., 1999; Tollefsen et al., 2008a). Despite the broad applicability of such assays, thorough characterization of the MoAs and concentration-dependent global changes of gene and protein expression are generally lacking, and effort to provide this for EDCs is highly warranted.

The objectives of this study were (1) to characterize the molecular MoAs of the ER-agonist 17α-Ethinylestradiol (EE2) in primary rainbow trout (*O. mykiss*) hepatocytes after a short-term (48 h) *in vitro* exposure; (2) to determine the concentration-dependent transcriptional changes occurring, and (3) to evaluate the potential of primary hepatocytes to predict *in vivo* hepatic responses in fish.

## 2. Material and methods

### 2.1. Chemicals

17α-Ethinylestradiol (EE2, ≥98%, CAS 57-63-6) and sodium bicarbonate (CAS 144-55-8) were purchased from Sigma–Aldrich (St. Louis, MI, US). The test chemical was dissolved in dimethylsulfoxide (DMSO) and stored in the dark at −20 °C until use.

### 2.2. Fish

Sexually immature rainbow trout (200–500 g) from the same fish stock were obtained from the Valdres rakfisk AB hatchery (Valdres, Norway) and reared at the Department of Biosciences, University of Oslo (Norway) for a minimum of 4 weeks prior to the start of the studies. The fish were maintained in tap water at 6 ± 2 °C, pH 6.6, 100% air saturation and light regime of 12 h light/12 h dark. Rainbow trout were fed daily with commercial pellets (Skretting, Stavanger, Norway) corresponding to approximately 0.5% of total body weight.

### 2.3. Cell culture and exposure

A total of four donor fish were collected (January–July, 2012) and terminated by cephalic concussion, followed by immediate dissection to expose the abdominal cavity. Only juvenile fish (with no visual gonads) were used in a 2-step hepatic cell isolation procedure as described by Tollefsen et al. (2003) and modified for studies on gene expression by Hultman et al. (2015). The viability of primary hepatocytes (>80%) was assessed using a Bürker counting chamber and trypan blue:cell suspension (2:1). The cell suspension was diluted to 500,000 cells/ml in serum-free L-15 medium with phenol-red containing amphotericin (0.25 g/ml), L-glutamine (0.29 mg/ml), streptomycin (100 g/l), penicillin (100 Units/ml) and $NaHCO_3$ (4.5 mM), seeded in 24-well Primaria™ microtiter plates (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) with a density of 625,000 cells/well and incubated in ambient atmosphere at 15 °C. After 24 h of acclimation to the test wells, 50% of the medium was removed from the cells and replaced with media spiked with EE2 (0.03, 0.3, 3 and 30 nmol/l (nM)) or solvent control (0.1% DMSO) in triplicate. The chemical exposure concentration in the medium at the start of the experiment was verified by ultra-performance liquid-chromatography tandem mass spectrometer (UPLC-MS) analysis on derivatized EE2 and d3 labeled estradiol (d2-E2) and described in detail by Hultman et al. (2015). The measured concentrations ranged between 75% and 93% of the nominal concentrations (Hultman et al., 2015). After 48 h exposure, the test medium was removed and the cells were sampled and lysed with RNeasy lysis buffer (Qiagen GmbH, Hilden, Germany) and stored at −80 °C for later RNA isolation and gene expression analysis.

### 2.4. Gene expression analysis

#### 2.4.1. Microarray analysis

A high-density (60,000-feature) custom salmonid oligonucleotide microarray (Agilent Technologies, City, Country; GEO accession number: GPL18864) was used to study the global transcriptional changes in the rainbow trout hepatocytes. The performance of the array was thoroughly evaluated for different salmonid species and pollutants (Song et al., 2012). Prior to microarray hybridization, the frozen primary hepatocytes were subjected to RNA extraction using Qiagen RNeasy Plus mini kit with on-column DNAs treatment (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions with minor modifications to accommodate high RNA purity and yield. The modifications included extended incubation time of RNA membrane-bound

**Table 1**
Genes, primer sequences, accession numbers and analysis protocol used for the qPCR analysis.

| Target gene | | Primer sequence | Amplicon size | Annealing temperature | Primer conc. (nmol/l) | Efficiency (%) | Acc. no | Reference |
|---|---|---|---|---|---|---|---|---|
| *ef1α* | -forward | 5′-AGCCCAATCAGCCTGAGAGCTA-3′ | NR | 62 °C | 600 | 107.5 | NM.001141909.1[a] | Gabillard et al. (2006) |
| | -reverse | 5′-GCTGGACAAGCTGAAGGCTGAG-3′ | | | 600 | | | |
| *pparα* | -forward | 5′-CTGCGAGCTGGATGACAGTGA-3′ | 195 | 55 °C | 500 | 95.0 | AY494835 | Cruz-Garcia et al. (2002) |
| | -reverse | 5′-GGCAAGTTTTTGCAGCAGAT-3′ | | | 500 | | | |
| *pparγ* | -forward | 5′-GACGCGCGGTCAGTACTTTA-3′ | 171 | 60 °C | 700 | 94.2 | AY356399.1 | Cruz-Garcia et al. (2002) |
| | -reverse | 5′-ATGCTCTTGGCCAACTCTGT-3′ | | | 700 | | | |
| *esr1* | -forward | 5′-CCCTGCTGGTGACAGAGAGAA-3′ | NR | 61 °C | 270 | 99.9 | AJ242741 | Nagler et al. (2007) |
| | -reverse | 5′-ATCCTCCACCACCATTGAGACT-3′ | | | 620 | | | |
| *vtg* | -forward | 5′-GAGCTAAGGTCCGCACAATTG-3′ | NR | 61.4 °C | 700 | 110 | X92804 | Celius et al. (2000) |
| | -reverse | 5′-GGGAAACAGGGAAAGCTTCAA-3′ | | | 700 | | | |
| *ghr-1* | -forward | 5′-CGTCCTCATCCTTCCAGTTTTA-3′ | NR | 62 °C | 500 | 97.0 | NM.001124535.1[a] | Gabillard et. al (2006) |
| | -reverse | 5′-GTTCTGTGAGGTTCTGGAAAAC-3′ | | | 500 | | | |
| *igfbp-1* | -forward | 5′-AGTTCACCAACTTCTACCTACC-3′ | NR | 62 °C | 700 | 107.2 | NM.001124561.1[a] | Gabillard et. al (2006) |
| | -reverse | 5′-GACCACTCACACTGCTTGGC-3′ | | | 700 | | | |
| *cyp1a* | -forward | 5′-TCCTGCCGTTCACCATCCCACACTGCAC-3′ | NR | 57 °C | 700 | 90.5 | U62797.1 | Gräns et al. (2010) |
| | -reverse | 5′-AGGATGGCCAAGAAGAGGTAGACCTC-3′ | NR | | 700 | | | |

Abbreviations: *ef1α—elongation factor 1 α*; *pparα—peroxisome proliferator-activated receptor α*;*pparγ—peroxisome proliferator-activated receptor γ*; *esr1—estrogen receptor 1/α*; *vtg—vitellogenin*; *ghr-1—growth hormone 1*; *igfbp-1—insulin growth factor protein 1*; *cyp1a—cytochrome P450 1A*.
[a] Acc. No. was not provided by reference.

washing buffer (2–3 min) and increased centrifugation time and speed (35 s, 9700 g), with a final removal of excess fluid from the O-ring prior to RNA elution. Protocol modifications were performed in order to remove excess guanine salts and avoid the need for further clean-up/purification of the samples. The RNA concentration was measured spectrophotometrically (Spectrophotometer ND 1000, Nanodrop technologies Inc., Wilmington, USA) with quality cut-off criteria as follows: 280/260 ratios of >2.0 and 260/230 ratios of >1.8. The RNA integrity was assessed using Agilent Bioanalyzer RNA 6000 nano series kit (Agilent technologies, Santa Clara, CA, USA), with samples obtaining RIN value >8.0 passing the quality cut-off criteria (Fleige and Pfaffl, 2006). Technical replicates of RNA were pooled and the four biological replicates (individual batches of cells from different fish) were used in the subsequent analysis. The biological replicates were subsequently subjected to cDNA synthesis (input: 50 ng total RNA), linear amplification, cRNA synthesis and Cyanine-3 (Cy-3) labelling strictly performed according to Agilent's "One-color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling) protocol, Version 6.5 May 2010". The Cy-3 labelled samples were hybridized on the microarray, followed by washing and scanning in a high resolution microarray scanner (Agilent technologies, USA) at 3 μm resolution and scanning area of 61 × 21.6 mm.

### 2.4.2. Quantitative real time PCR (qPCR)

Verification of differentially expressed genes (Table 1) was performed by quantitative real-time polymerase chain reaction (qPCR) essentially as described by Hultman et al. (2015). Synthesis of cDNA was performed by reverse transcription of total RNA (0.5–1 μg) using Quanta qScript™ cDNA Synthesis Kit (Quanta Biosences Inc., Gaithersburg, USA) according to the manufacturer's instructions. The primer optimization was performed with a CFX-384 thermal cycler (Bio-Rad laboratories Inc., USA) using a 5-step dilution series (2.5–50 ng/reaction) with pooled template cDNA. SYBR® Green Supermix (Quanta Biosences Inc., Gaithersburg, USA) was used in the qPCR amplification reaction, where technical triplicates of 10 ng template/reaction were used in the final mastermix reaction (20 μl/reaction). The primers were obtained from previously published papers (Table 1) and were purchased from Eurofins MWG synthesis GmbH (Ebersberg, Germany). Primers were optimized for concentrations and annealing temperature in order to yield an amplification efficiency of 90–110%. The qPCR protocol was performed as following, Cycle 1: 95 °C for 3 min, Cycle 2–40: 95 °C for 20 s, followed by the specific primer annealing temperature for 20 s and 72 °C for 20 s. All primers run had a none-template control (NTC) and a no-reverse transcriptase control (NRT) to exclude any contamination of primer or presence of genomic DNA in the mastermix or the RNA sample that may influence the qPCR analysis. The qPCR analysis reported no amplification of either NRT or NTC for any of the primers used. In the end of the qPCR protocol a melt curve analysis was performed, verifying that no primer-dimer formation or unspecifically amplified products were formed in the samples. Accepted threshold cycle ($C_q$)-value of NTC was set to be either non-detectable (N/A) or $C_q$ value >30 with minimum 7 cycles in between template replicate and NTC. The expression of elongation factor 1α (EF1α) was relatively stable in all treatments and was therefore used as a reference gene (Table 1). Data normalization was performed using the Pfaffl method (Pfaffl, 2001).

## 2.5. Statistics and bioinformatics

### 2.5.1. Microarray

Scanned microarray images were quality assessed and extracted using Agilent Feature Extraction software v10.7 (Agilent Technologies). The raw data were subjected to normalization, including correction for background signal, flagged for missing and low

quality features, followed by statistical analysis using GeneSpring GX v12.6 (Agilent Technologies). Determination of differentially expressed genes (DEGs) was performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test ($p < 0.05$). Statistics were not corrected for Benjamin and Hochberg (BH) false discovery rate (FDR) correction to avoid potential loss of biologically relevant data (Villeneuve et al., 2011). The Venn diagrams were generated using Venny (Oliveros, 2007) and the Gene Ontology (GO) enrichment analysis was performed in Cytoscape v2.8 (Smoot et al., 2011) using the application Bingo v.2.4 (Maere et al., 2005). Pathway and protein–protein interaction network analyses were performed using Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity) on basis of identification of mammalian orthologs by the standalone Inparanoid 4.1 algorithm (Ostlund et al., 2010) implementing BLAST 2.2.27+ binaries from the NCBI expert-curated mammalian-based databases Ensembl, Entrez Gene, RefSeq, GenBank, UniProt/Swiss-Prot Accession, GenPrept and UniGene.

#### 2.5.2. qrtPCR analysis

Prior to statistical analyses, the qPCR data was normalized against a reference gene followed by the Grubb's outlier test (Burns et al., 2005) to exclude clearly erroneous values (10 out of 140 values removed). The statistical analyses were performed in Graphpad Prism v5.04 (Graphpad Software, Inc., San Diego, CA, USA), applying a one-way ANOVA test, followed by Tukey's post hoc test. The significant level was set to $p < 0.05$ for all statistical tests.

## 3. Results

### 3.1. Global transcriptional changes

A total of 1098 differentially expressed gene (DEG) transcripts (707 up-regulated and 391 down-regulated) were identified to be regulated in the EE2 exposed cells when compared to the control. Concentration-dependent transcriptional changes were further determined using Tukey HSD post hoc test. A total of 66 (up-regulated: 27, down-regulated: 39), 114 (up-regulated: 84, down-regulated: 30), 468 (up-regulated: 301, down-regulated: 167) and 992 (up-regulated: 695, down-regulated: 297) genes were identified to be differentially expressed after exposure to 0.03, 0.3, 3 and 30 nM EE2, respectively (Fig. 1). The Venn diagram analysis identified 3 up-regulated DEGs being commonly regulated across all treatments (*i.e. transposable element Tc1 transposase* (*tca1*), *diaphanous homolog 2* (*diap2*) and *uncharacterized protein*) and 2 down-regulated (*i.e. dedicator of cytokinesis protein 9* (*dok9*) and *protein naked cuticle homolog 2-B* (*nkd2b*)). The cells exposed to 3 and 30 nM EE2 had a high number of common DEGs being regulated (246 DEGs, 179 up- and 67 down-regulated). Both total number (Fig. 1) and the expression of the DEGs were found to be concentration-dependent and the complete list of DEGs can be found in Supplementary Table A.

### 3.2. Gene Ontology-based functional enrichment analysis

The biological roles of the DEGs were first characterized by functional Gene Ontology (GO) analysis and grouped into molecular functions, biological processes and cellular components (Table 2). Assigning the biological roles for the gene products naturally supposes that they are the same in rainbow trout hepatocytes as in mammals, which the GO is based on. The total numbers of overrepresented GO biological processes and molecular functions were found to be affected in a concentration-dependent manner between 0.3 and 30 nM EE2. Increased enrichment of DEGs related to functional categories such as lipid and fatty acid metabolism was observed between 0.3 and 3 nM EE2, whereas biological processes related to reproduction and the regulation of the endocrine system were mainly observed between 3 and 30 nM EE2. A complete list of GO terms associated with the DEGs can be found in Supplementary Table B.

### 3.3. Ortholog-based functional enrichment analysis

The DEGs were further mapped to their mammalian orthologs to better understand their biological roles using well-curated mammalian databases, assuming that the functions of the gene products are conserved between mammals and fish. A total of 54.3% (0.03 nM), 52% (0.3 nM), 57.4% (3 nM) and 58.5% (30 nM) of the rainbow trout DEGs were successfully mapped to mammalian orthologs (see Supplementary Table B for a complete list of mapped orthologs). The gene network, toxicity and canonical pathway analysis revealed a concentration-dependent increase of enriched gene/protein networks and pathways.

#### 3.3.1. Gene networks

The list of successfully mapped orthologs was subjected to protein–protein interaction (PPI)-based gene network analysis to obtain a better overview of the functional processes associated with the DEGs identified (Table 3). The gene networks of 0.03 nM EE2 were mainly related to cell signaling, molecular transport and nucleic acid metabolism, whereas 0.3 nM EE2 regulated DEGs associated with cellular function and maintenance, small molecule biochemistry and cellular development. The 3 nM and 30 nM EE2 exposure predominantly resulted in transcriptional changes associated with hematological disease, hereditary disorder, carbohydrate metabolism and post-translational modification, lipid metabolism and alterations of small molecule biochemistry in the cells, respectively.

#### 3.3.2. Toxicity pathways

The toxicity pathway analysis was performed to better understand the potential MoAs of EE2 based on the mapping of DEGs to well-characterized mammalian toxicity pathways. No toxicity pathways were identified to be commonly affected across all EE2 concentrations (Fig. 2). Exposure to 0.03 nM EE2 predominantly altered the expression of DEGs associated with toxicity pathways related to organismal injury and abnormalities. One toxicity pathway (negative acute phase response proteins) was found to be affected by 0.3 nM EE2, whereas 3 nM EE2 led to alterations of a number of DEGs associated with lipid metabolism, endocrine system development and function, as well as cellular growth and proliferation. A majority of the identified DEGs in the 30 nM EE2-exposed hepatocytes were related to processes such as cell death and survival, cardiovascular disease, cellular growth and proliferation. The complete list of toxicity pathways is provided in Supplementary Table C.

#### 3.3.3. Canonical pathways

Canonical pathway analysis was performed to obtain more detailed knowledge on the potential MoAs of EE2. The number of toxicologically relevant canonical pathways increased in an apparent concentration-dependent manner (Table 4). A total of 10, 5, 28 and 36 pathways were uniquely affected by 0.03, 0.3, 3 and 30 nM EE2, respectively. Two canonical pathways (CCR3 Signaling in Eosinophils and Protein Kinase A Signaling) were commonly affected at all EE2 concentrations (Supplementary Table D). The cells exposed to 3 nM and 30 nM EE2 responded by modulating DEGs involved in cell growth and development, immune response, intracellular and second messenger signaling, apoptosis and metabolic pathways. Exposure to 0.03 nM EE2 led to the enrichment of DEGs in pathways linked to intracellular and second messenger signaling (e.g. glucocorticoid receptor- and

**Table 2**
Common Gene Ontology (GO) processes over-represented in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after 48 h exposure to 17α-ethinylestradiol (EE2). The exposure to 0.03 nmol/l did not yield any significant enrichment of GOs.

| nmol/l | GO-ID | GO namespace | *P*-value (<0.05) | No. of DEGs | Total features on array | GO term | Functional category |
|---|---|---|---|---|---|---|---|
| 0.3 | GO:0019216 | P | 2.69E$^{-02}$ | 5 | 270 | Regulation of lipid metabolic process | Lipid and fatty acid metabolism |
| | GO:0019395 | P | 2.69E$^{-02}$ | 4 | 154 | Fatty acid oxidation | |
| | GO:0034440 | P | 2.69E$^{-02}$ | 4 | 154 | Lipid oxidation | |
| | GO:0046320 | P | 3.65E$^{-02}$ | 3 | 74 | Regulation of fatty acid oxidation | |
| | GO:0043154 | P | 4.15E$^{-03}$ | 4 | 46 | negative regulation of caspase activity | Programmed cell death |
| | GO:0043028 | F | 3.65E$^{-02}$ | 3 | 74 | Caspase regulator activity:regulation of caspase activity | |
| | GO:0043027 | F | 1.28E$^{-02}$ | 3 | 35 | Caspase inhibitor activity: regulation of caspase activity | |
| 3 | GO:0060397 | P | 2.57E$^{-02}$ | 3 | 13 | JAK-STAT cascade involved in growth hormone signaling pathway | Cell Growth and development |
| | GO:0046543 | P | 4.19E$^{-03}$ | 3 | 5 | Development of secondary female sexual characteristics | Reproduction |
| | GO:0032504 | P | 1.72E$^{-02}$ | 21 | 838 | Multicellular organism reproduction | |
| | GO:0048609 | P | 1.72E$^{-02}$ | 21 | 838 | Reproductive process in a multicellular organism | |
| | GO:0000003 | P | 2.24E$^{-02}$ | 28 | 1344 | Reproduction | |
| | GO:0022414 | P | 2.76E$^{-02}$ | 27 | 1330 | Reproductive process | |
| | GO:0045136 | P | 3.22E$^{-02}$ | 3 | 15 | Development of secondary sexual characteristics | |
| | GO:0046544 | P | 2.57E$^{-02}$ | 2 | 3 | Development of secondary male sexual characteristics | |
| | GO:0019395 | P | 2.18E$^{-02}$ | 8 | 154 | Fatty acid oxidation | Lipid and fatty acid metabolism |
| | GO:0034440 | P | 2.18E$^{-02}$ | 8 | 154 | Lipid oxidation | |
| | GO:0010876 | P | 2.18E$^{-02}$ | 14 | 446 | Lipid localization | |
| | GO:0006635 | P | 2.57E$^{-02}$ | 6 | 89 | Fatty acid beta-oxidation | |
| | GO:0006631 | P | 2.76E$^{-02}$ | 15 | 539 | Fatty acid metabolic process | |
| | GO:0015645 | F | 2.57E$^{-02}$ | 5 | 55 | Fatty acid ligase activity | |
| | GO:0035257 | F | 3.67E$^{-02}$ | 8 | 182 | Nuclear hormone receptor binding: hormon receptor binding | Regulation of endocrine system |
| | GO:0005102 | F | 3.94E$^{-02}$ | 31 | 1661 | Receptor binding: Hormon receptor binding | |
| | GO:0035258 | F | 6.38E$^{-03}$ | 7 | 83 | Steroid hormone receptor binding: hormon receptor binding | |
| | GO:0050681 | F | 2.57E$^{-02}$ | 5 | 59 | Androgen receptor binding: hormon receptor binding | |
| 30 | GO:0040008 | P | 3.97E$^{-02}$ | 18 | 753 | Regulation of growth | Cell Growth and development |
| | GO:0040008 | P | 3.79E$^{-05}$ | 40 | 753 | Regulation of growth | |
| | GO:0040007 | P | 6.67E$^{-05}$ | 56 | 1294 | Growth | |
| | GO:0016049 | P | 2.25E$^{-03}$ | 29 | 576 | Cell growth | |
| | GO:0001558 | P | 2.37E$^{-03}$ | 24 | 435 | Regulation of cell growth | |
| | GO:0035264 | P | 7.27E$^{-03}$ | 13 | 171 | Multicellular organism growth | |
| | GO:0045454 | P | 1.51E$^{-02}$ | 12 | 164 | Cell redox homeostasis | |
| | GO:0030308 | P | 2.79E$^{-02}$ | 13 | 208 | Negative regulation of cell growth | |
| | GO:0045926 | P | 3.11E$^{-02}$ | 14 | 239 | Negative regulation of growth | |
| | GO:0007275 | P | 4.21E$^{-02}$ | 191 | 7536 | Multicellular organismal development | |
| | GO:0060397 | P | 1.16E$^{-02}$ | 4 | 13 | JAK-STAT cascade involved in growth hormone signaling pathway | |
| | GO:0000003 | P | 2.25E$^{-03}$ | 52 | 1344 | Reproduction | Reproduction |
| | GO:0032504 | P | 2.37E$^{-03}$ | 37 | 838 | Multicellular organism reproduction | |
| | GO:0048609 | P | 2.37E$^{-03}$ | 37 | 838 | Reproductive process in a multicellular organism | |
| | GO:0022414 | P | 4.91E$^{-03}$ | 50 | 1330 | Reproductive process | |
| | GO:0046543 | P | 9.60E$^{-03}$ | 3 | 5 | Development of secondary female sexual characteristics | |
| | GO:0019953 | P | 2.53E$^{-02}$ | 30 | 737 | Sexual reproduction | |
| | GO:0032355 | P | 3.63E$^{-02}$ | 8 | 91 | Response to estradiol stimulus | Regulation of endocrine system |
| | GO:0042562 | F | 4.19E$^{-02}$ | 7 | 73 | Hormone binding | |

Abbrevations: P—biological process; F—molecular function; DEGs —differently expressed genes.

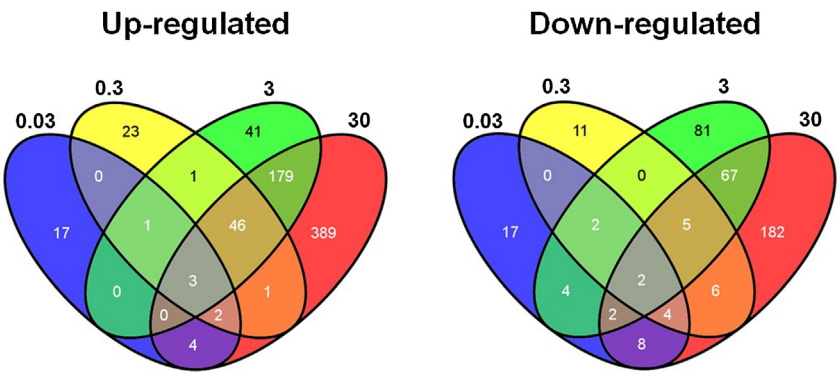


**Fig. 1.** Differentially (up- and down-) expressed genes (DEGs) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 0.03, 0.3, 3 and 30 nmol/l 17α-ethinylestradiol (EE2) for 48 h.

**Table 3**
Top gene networks (score>10, supporting genes >10) and supporting differently expressed genes (DEGs) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after 48h exposure to 17α-ethinylestradiol (EE2).

| nmol/l | Top network function | Score | DEGs |
|---|---|---|---|
| 0.03 | Cell signaling, molecular transport, nucleic acid metabolism | 80 | 34 |
| 0.3 | Cellular function and maintenance, small molecule biochemistry, cellular development | 109 | 46 |
| 3 | Hematological disease, hereditary disorder, carbohydrate metabolism | 172 | 98 |
| | Cellular assembly and organization, cellular function and maintenance, lipid metabolism | 100 | 65 |
| | Cellular development, cellular growth and proliferation, cell cycle | 91 | 63 |
| | Cell death and survival, post-translational modification, cardiac necrosis/cell death | 11 | 14 |
| 30 | Post-translational modification, lipid metabolism, small molecule biochemistry | 121 | 89 |
| | Embryonic development, organ development, organismal development | 104 | 81 |
| | Cellular assembly and organization, cellular function and maintenance, cellular movement | 99 | 80 |
| | Post-translational modification, cell signaling, hereditary disorder | 85 | 70 |
| | Amino acid metabolism, small molecule biochemistry, connective tissue disorders | 82 | 69 |
| | Cell cycle, tissue morphology, cancer | 63 | 58 |
| | Cell morphology, cellular function and maintenance, cell cycle | 63 | 59 |

Score: The score takes into account the number of Network Eligible molecules in the network, their relevance and its size, as well as the total number of Network Eligible molecules analyzed and the total number of molecules in the Ingenuity Knowledge Base that could potentially be included in networks (definition by IPA).
DEGs: differently expressed genes.

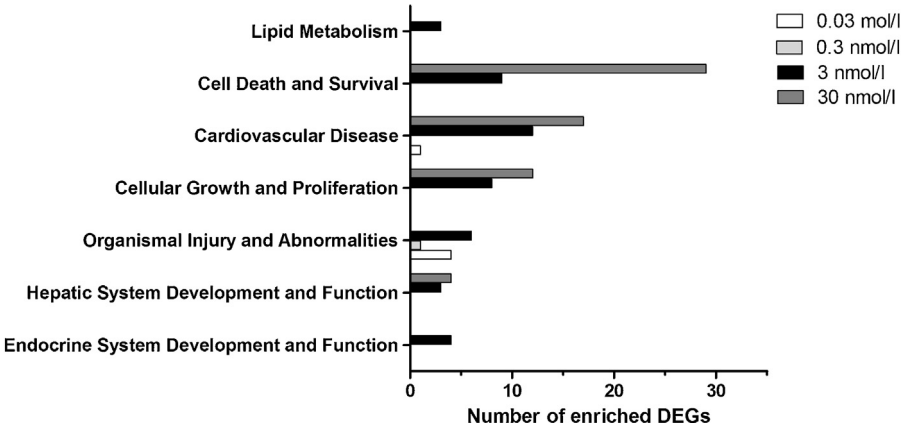


**Fig. 2.** Toxicity pathways associated with the modulation of differently expressed genes (DEGs) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 17α-ethinylestradiol (EE2) for 48 h. All chemical concentrations having significant enrichment of relevant toxicity pathways are in the figure represented by individual bars.

insulin receptor signaling), whereas DEGs regulated by 0.3 nM EE2 were enriched in the apoptotic signaling pathway. In the cells exposed to 3 nM EE2, gene transcripts related to cell growth and development (*e.g.* Growth hormone (GH) and Insulin growth factor-1 (IGF-1) signaling), apoptosis (*e.g.* death receptor signaling), intracellular and second messenger signaling (*e.g.* Glucocorticoid receptor and Calcium signaling) and nuclear receptor signaling (*e.g.* Pregnane X receptor/Retinoic X receptor (PXR/RXR) activation) were mainly affected. Primary hepatocytes exposed to the highest (30 nM) concentration of EE2 exhibited enrichment of DEGs associated with regulation of cell growth and development (*e.g.* Corticotropin-Releasing Hormone Signaling), neurotransmitters and other signaling pathways (*e.g.* Gonadotropin-releasing hormone (GNRH) signaling), nuclear receptor signaling (*e.g.* Peroxisome proliferator-activated receptor (PPAR) signaling) and disease-specific pathways (e.g. molecular mechanism of cancer). A detailed list of the canonical pathways can be found in Supplementary Table D.

**Table 4**
A selection of relevant canonical pathways and supporting differentially expressed genes (DEGs) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes after 48 h exposure to 17α-ethinylestradiol (EE2). Arrows indicates ↑ up- and ↓ down-regulation of DEGs. Full description of gene symbols is found in Supplementary Table A.

| nmol/l | Apical toxicological category | Ingenuity canonical pathway | *p*-value[a] | Ratio[b] | Supporting DEGs |
|---|---|---|---|---|---|
| 0.03 | Intracellular and second messenger signaling | Glucocorticoid receptor signaling | 1.35E$^{-2}$ | 1.00E$^{-2}$ | *jak1*↓,*pou2f1*↓,*nppa*↓ |
| | | Insulin receptor signaling | 2.69E$^{-2}$ | 1.34E$^{-2}$ | *jak1*↓, *ppp1cb*↑ |
| 0.3 | Apoptosis | Apoptosis signaling | 2.09E$^{-2}$ | 2.00E$^{-2}$ | *rock1*↑, *xiap*↑ |
| 3 | Apoptosis | Death receptor signaling | 3.16E$^{-2}$ | 4041E$^{-02}$ | *diablo*↓, *xiap*↑, *faslg*↓ |
| | | Apoptosis signaling | 2.04E$^{-2}$ | 4.00E$^{-02}$ | *rock1*↑, *diablo*↓, *xiap*↑, *faslg*↓ |
| | Disease-specific pathways | Estrogen-dependent breast cancer signaling | 3.89E$^{-2}$ | 4.11E$^{-2}$ | *stat5a*↑, *stat5b*↑, *creb5*↑ |
| | Immune response | IL-6 signaling | 4.90E$^{-2}$ | 3.23E$^{-2}$ | *grb2*↑, *crp*↑, *stat3*↓, *cebpb*↓ |
| | Intracellular and second messenger signaling | Calcium signaling | 2.04E$^{-2}$ | 2.76E$^{-2}$ | *myl2*↑, *ryr3*↑,*hdac10*↓, *asph*↑, *cabin1*↑,*creb5*↑ |
| | | Glucocorticoid receptor signaling | 1.26E$^{-2}$ | 2.68E$^{-2}$ | *stat5a*↑, *pou2f1*↓, *grb2*↑, *stat3*↓, *cebpb*↓, *nppa*↓, *stat5b*↑, *polr2i*↓ |
| | | Phospholipase C signaling | 6.76E$^{-3}$ | 3.02E$^{-2}$ | *myl2*↑, *grb2*↑, *gnb2l1*↑, *hdac10*↓, *gng3*↓, *pla2g12b*↑, *rhof*↓, *creb5*↑ |
| | Nuclear receptor signaling | PXR/RXR activation | 4.47E$^{-2}$ | 3.02E$^{-2}$ | C*cpt1a*↑, *abcb11*↑, *igfbp1*↓ |
| | | TR/RXR activation | 1.82E$^{-2}$ | 3.67E$^{-2}$ | *tshb*↑, *ncoa4*↑, *pfkp*↑, *tbl1xr1*↓ |
| | | Androgen signaling | 4.37E$^{-2}$ | 2.76E$^{-2}$ | *gnb2l1*↑, *ncoa4*↑, *gng3*↓, *polr2i*↓ |
| | | LPS/IL-1 mediated inhibition of RXR function | 4.79E$^{-2}$ | 2.45E$^{-2}$ | *cpt1a*↑, *acsl5*↑, a*bcb11*↑, *slc35a2*↑, *fabp3*↑, *acox3*↑ |
| | Metabolic pathways | Thyroid hormone biosynthesis | 3.47E$^{-2}$ | 1.43E$^{-2}$ | *iyd*↓ |
| 30 | Disease-specific pathways | Molecular mechanisms of cancer | 2.75E$^{-2}$ | 3.87E$^{-2}$ | *map2k4*↓, *rapgef1*↑,*jak1*↓,*grb2*↑,*xiap*↑, *cdc25b*↑, *ptk2*↓, *gnai2*↑, *amtor3*↓, *prkcd*↓, *arhgef11*↑, *bmp6*↑, *rhof*↓, *diablo*↓, *ptch2*↑ |
| | Cell growth and development | Corticotropin releasing hormone signaling | 2.40E$^{-2}$ | 4.83E$^{-2}$ | *gnai2*↑, *prkcd*↓, *npr1*↓, *jund*↑, *opn1sw*↑, *ptch2*↑, *creb5*↑ |
| | Neurotransmitters and other nervous system signaling | GNRH signaling | 5.62E$^{-3}$ | 5.88E$^{-2}$ | *map2k4*↓, *ptk2*↓, *gnai2*↑, *grb2*↑, *prkcd*↓, *map3k8*↑, *opn1sw*↑, *map3k2*↑, *creb5*↑ |
| | Nuclear receptor signaling | PPAR signaling | 3.16E$^{-2}$ | 5.61E$^{-2}$ | *il33*↑, *nr2f1*↑, *stat5a*↑, *nr0b2*↑, *grb2*↑, *stat5b*↑ |
| | Intracellular and second messenger signaling | Insulin receptor signaling | | | *eif2b1*↑, *grb2*↑*jak1*↓, *ppp1cb*↑, *ppp1r7*↓, *ppp1r3c*↑, *rapgef1*↑ |
| | Metabolic pathway | CDP-diacylglycerol biosynthesis I | 5.75E$^{-3}$ | 1.11E$^{-1}$ | *gpam*↑, *cds1*↓, *cds2*↓ |
| Commonly regulated between 3 and 30 nmol/l[c]l | | | | | |
| | Immune response | CXCR4 signaling | 5.50E$^{-3}$ | 5.75E$^{-2}$ | *map2k4*↓, *ptk2*↓, *rock1*↑, *gnai2*↑, *myl2*↑, *prkcd*↓, *arhgef11*↑, *gng3*↓, *rhof*↓, *opn1sw*↑ |
| | | CCR3 signaling in eosinophils | 2.63E$^{-2}$ | 5.22E$^{-2}$ | *rock1*↑, *gnai2*↑, *prkcd*↓, *ppp1cb*↑, *gng3*↓, *pla2g12b*↑, *opn1sw*↑ |
| | Intracellular and messenger signaling | Tec kinase signaling | 6.03E$^{-4}$ | 6.52E$^{-2}$ | *map2k4*↓, *ptk2*↓, *gnai2*↑, *stat5a*↑, *jak1*↓, *prkcd*↓, *stat3*↓, *gng3*↓, *stat1*↑, *stat5b*↑, *rhof*↓, *itk*↓ |
| | | Protein kinase A signaling | 1.74E$^{-2}$ | 4.16E$^{-2}$ | *ptptg*↓, *myl2*↑, *ppp1r3c*↑, *ppp1cb*↑, *gng3*↓, *ptpn5*↑, *creb5*↑, *cdc25b*↑, *prk2*↓, *rock1*↑, *gnai2*↑, *add3*↑, *flnc*↑, *ppp1r7*↓, *prkcd*↓, *prch2*↑, *opn1sw*↑ |
| | Neurotransmitters and other nervous system signaling | Neurotrophin/TRK signaling | 2.82E$^{-2}$ | 6.58E$^{-2}$ | *map2k4*↓, *grb2*↑, *ntrk1*↑, *map2k5*↑, *creb5*↑ |
| | Cell growth and development | Oncostatin M signaling | 1.82E$^{-4}$ | 1.71E$^{-1}$ | *stat5a*↑, *jak1*↓, *grb2*↑, *stat3*↓, *stat1*↑, *stat5b*↑ |
| | | Growth hormone signaling | 7.76E$^{-3}$ | 7.69E$^{-2}$ | *stat5a*↑,*ghr*↓, *prkcd*↓, *stat3*↓, *stat1*↑, *stat5b*↑ |
| | | IGF-1 signaling | 3.63E$^{-2}$ | 5.61E$^{-2}$ | *ptk2*↓, *jak1*↓, *grb2*↑, *stat3*↓,*igfbp1*↓, *cyr61*↑ |
| | Disease-specific pathways | Breast cancer regulation by stathmin1 | 3.98E$^{-4}$ | 6.54E$^{-2}$ | *grb2*↑, *ppp1r3c*↑,*tubb2a*↑, *ppp1cb*↑, *gng3*↓, *rock1*↑, *gnai2*↑, *ppp2cb*↑, *ppp1r7*↓, *prkcd*↓, *ppm1l*↑, *arhgef11*↑, *tuba3e*↑, *opn1sw*↑ |

[a] *p*-value is defined to $p < 0.05$
[b] Ratio is calculated as the number of regulated molecules in a given pathway that meet cutoff criteria, divided by total number of molecules that make up that pathway (definition by IPA).
[c] Represents 30 nmol/l EE2.

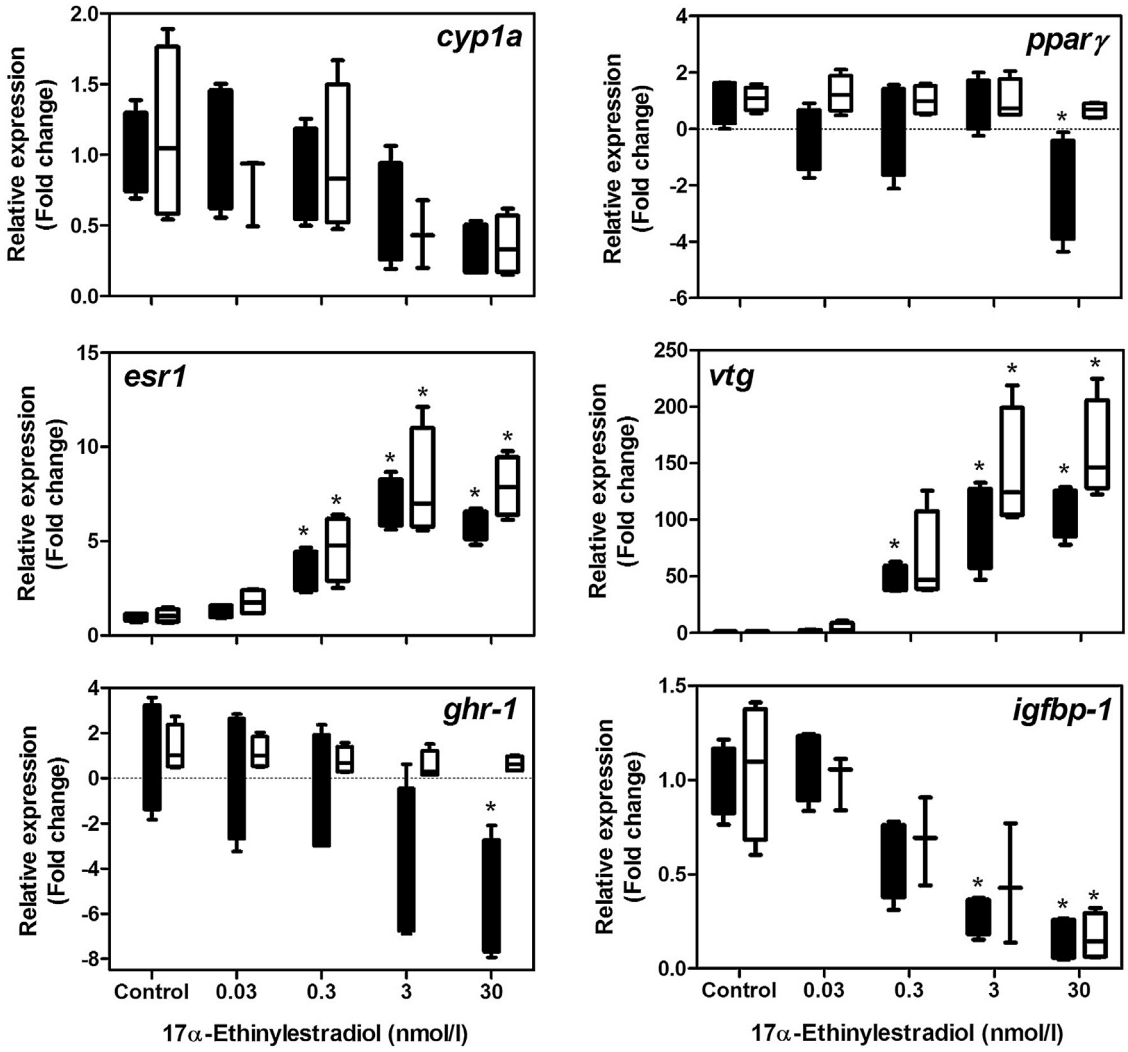


**Fig. 3.** A comparison of gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 17α-ethinylestradiol (EE2) for 48 h. Data (Mean ± SEM) depicts quantitative real time polymerase chain reaction, qPCR (open boxplots, $n = 3–4$) and microarray (filled boxplots, $n = 4$). *Denotes genes being significantly different ($p < 0.05$) from the solvent control. Abbrevations: *cyp1a1*—cytochrome P450 1a1; *esr1*—estrogen receptor 1; *ghr-1*—growth hormone receptor 1; *igfbp-1*—insulin growth factor binding protein 1; *pparγ*—peroxisome proliferator-activated receptor γ; *vtg*—vitellogenin.

### 3.4. Quantitative real-time polymerase chain reaction verification

Verification of the microarray results was performed on a selection of DEGs involved in key biological processes such as ER regulation (*esr1, vtg1*), biotransformation (*cytochrome P450 1A* (*cyp1a*)), lipid metabolism/homeostasis (*pparγ*) and cellular growth (*growth hormone receptor 1* (*ghr-1*), *IGF binding factor 1* (*igfbp-1*)). The selected genes were consistently expressed in a concentration-dependent manner in coherence with the microarray data, although some differences in the magnitude of expression were observed. In addition, *pparα* was verified as regulated using qPCR, despite not being identified as significantly altered on the microarray. Among the genes analyzed, four (*esr1, vtg1*, *igfbp-1* and *pparα*) were verified to be significantly regulated when compared to the solvent control (Figs. 3 and 4). An apparent concentration-dependent regulation was also observed in the remaining genes, and found to be in general agreement with the microarray data.

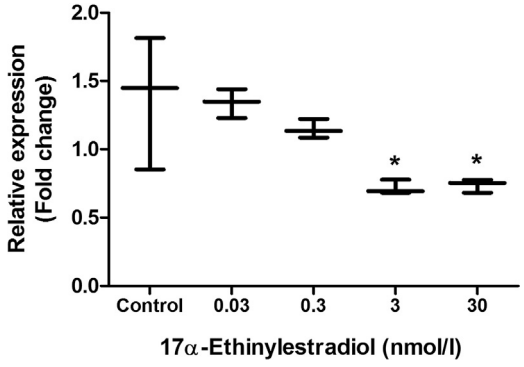


**Fig. 4.** Gene expression of *peroxisome proliferator-activated receptor α* (*pparα*) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 17α-ethinylestradiol (EE2) for 48 h. Data (Mean ± SEM) depicts quantitative real time polymerase chain reaction, qPCR (open boxplots, $n = 3$). *Denotes the gene being significantly different ($p < 0.05$) from the solvent control.

## 4. Discussion

Exposure to ER-agonists such as EE2 has been reported to affect a number of estrogen sensitive genes and their proteins product in both *in vivo* and *in vitro* fish models (Arukwe et al., 1997; Finne et al., 2007; Hultman et al., 2015; Sumpter and Jobling, 1995). The majority of available studies with EDCs have been focused

on a few responses associated with the endocrine functions in fish, thus contributing to the development and evaluation of a number of estrogen sensitive biomarkers suitable for laboratory and field-based studies (Arukwe et al., 1997; Folmar et al., 2000; Harries et al., 1997; Purdom et al., 1994; Sumpter and Jobling, 1995). Although this approach has been highly successful, most of these studies have limited their approaches to the MoA characterization using single or small suites of biomarkers, which only provide a snapshot of the biological responses to EDCs. The present study has implemented a broad-content transcriptomic (microarray) analysis to provide an unbiased characterization of the complex and concentration-dependent cellular transcriptional responses in rainbow trout hepatocytes after exposure to the ER-agonist EE2. The exposure duration (48 h), and bioassay protocol have previously been identified as optimal for determination of Vtg gene and protein expression in rainbow trout hepatocytes exposed to non-cytotoxic concentrations of EE2 (Hultman et al., 2015). Dimethylsulfoxide (0.1%) was used as the solvent control in these studies as it has been shown to not affect Vtg gene and protein expression (Hultman et al., 2015).

The present study identified clear concentration-dependent responses in DEGs (Figs. 1, 3 and 4), GOs (Table 2) and pathways (Fig. 2; Table 3) relevant for a number of potential toxic MoAs (Figs. 5A–D and 6 ). The lowest concentration of EE2 (0.03 nM) affected a limited number of DEGs (66) mainly associated with cellular signaling, cellular transport, and biotransformation, whereas 0.3 nM EE2 modulated about twice as many DEGs (114) that were related to cellular metabolism of fatty acids and lipids, cellular development, and apoptosis. Ten times higher concentrations of EE2 (3 nM) led to regulation of 466 DEGs predominantly associated with cellular processes such as growth, development, metabolism, apoptosis, but also to nuclear receptor signaling, endocrine regulation, reproductive functions, immune functions and cancer development. The highest concentration of EE2 (30 nM) affected a high number of DEGs (992), whereof many were associated with similar functions as those observed for 3 nM, but modulation of DEGs associated with cell cycle regulation neuro-signaling were also observed. Overall, the lowest concentration of EE2 (0.03 nM) was considered to only marginally affect toxicologically-relevant processes, whereas a number of processes relevant for endocrine disruption in fish was identified at higher EE2 concentrations (Fig. 2) and discussed in detail below.

### 4.1. Estrogen receptor signaling

Estrogens bind to and activate the ER and ER-mediated signaling pathways, which may regulate sexual development and reproduction in fish. The genomic ER signaling pathway is well characterized and includes homodimerization of the ligand and ER heat shock protein complex, translocation of the dimer into the nucleus, and recruitment of co-regulators to the ERE-promoter region leading to induced or suppressed transcription of downstream genes (Figs. 3 and 6). Exposure of the primary rainbow trout hepatocytes to EE2 in the present study resulted in a concentration-dependent up-regulation of genes encoding ERα (*esr1*), transcriptional regulators (e.g. *nuclear receptor coactivator 4* (*ncoa4*), *nuclear receptor subfamily group member 1* (*nr2f1*), *nuclear receptor subfamily group member 2* (*nr0b2*)), and genes encoding downstream reproductive-relevant hepatic proteins such a vitellogenin (*vtg1)* and zona radiata proteins (*zrp3 and zrp4*) (Figs. 3 and 5B–D) whereof several have previously been reported to respond to estrogens (Colli-Dula et al., 2014; Doyle et al., 2013; Hoffmann et al., 2006; Mortensen and Arukwe, 2007). Many of these genes (i.e. *esr1, vtg and zrp*) have been proposed as biomarkers for estrogenicity in fish (Arukwe et al., 1997; Heppell et al., 1995; MacKay et al., 1996; Sumpter and Jobling, 1995), and the present microarray and qPCR analysis (Supplementary Table A, Fig. 3) confirmed that they were highly responsive to estrogens at low concentrations *in vitro* (NOEC of 0.03–0.3 nM EE2). Other estrogen responsive genes such as *follistatin* (*fst*), *fatty acid binding protein 3* (*fabp3*) and *nitric oxide synthase interacting protein* (*nosip*) were also regulated in the primary hepatocytes. Although the roles of these genes were not studied in detail herein, they have previously been reported to be regulated in the liver of fish after exposure to E2 and EE2 (Gunnarsson et al., 2007; Levi et al., 2009).

Interestingly, the microarray analysis revealed that *nr0b2* (also known as *the small heterodimer partner, SHP*), the product of which is a potential transcriptional repressor of *esr1* and downstream genes (Ehrlund and Treuter, 2012), was significantly up-regulated after exposure to 30 nM EE2. Although controversy still exists with regard to the actual role of *nr0b2* in fish (Park et al., 2007), up-regulation of *nr0b2* may provide a potential explanation for the slight reduction in transcriptional activation observed for *esr1* and *vtg1* after exposure to the highest EE2 concentration (Fig. 3). Although the repression of the vtg gene and protein expression at high estrogen concentrations have been amply demonstrated elsewhere (Hultman et al., 2015; Petersen and Tollefsen, 2011; Rankouhi et al., 2004), further investigations will be required to properly elucidate the role of *nr0b2* in piscine ER signaling.

Enrichment of hepatic DEGs in additional estrogen and ER-mediated pathways were identified to be associated with several reproductive processes involving calcium (*e.g.* oocyte formation), GNRH (*e.g.* hormone regulation) and androgen signaling (Table 4) that is relevant also for other organs. These observations agree well with responses to estrogens in fish tissues such as the pituary (Harding et al., 2013). The GO-based functional analysis further identified calcium signaling being affected which is involved in various key regulatory processes in the cell such as cell death (Pretorius and Bornman, 2005), molecular transport, reproduction, GNRH signaling and hormone signaling (Harding et al., 2013; Jobin and Chang, 1992).

### 4.2. Biotransformation

Biotransformation is a key step in the detoxification in organisms and important for the reduction of intracellular concentrations of both xenobiotics and compounds of endogenous origin (Newman, 2009). Several genes involved in the biotransformation of (xeno) estrogens were affected in the hepatocytes at low (0.03 nM), intermediate (0.3 and 3 nM) and high (30 nM) concentrations of EE2. The present study identified differential modulation of genes involved in phase I (*cyp1a* and *catechol-O-methyl transferase (comt))*, phase II biotransformation (*glutathione S-transferase (gst), sulfotransferase (sult6b1), UDP-glucuronosyltransferase (ugt1b5, ugt2a1*)) and phase III-multidrug transport (resistance) (*atp-binding cassette (abc*, *abcb11)).* The induction of *ugt2a1* (Supplementary Table A) was consistent with increased hepatic glucurononyl conjugation of hydroxylated substrates from phase I biotransformation as previously described for endogenous steroids and xenobiotic compounds in fish (Gao et al., 2014). Subsequent induction of the bile salt pump *abc* and *abcb11,* which are involved in hepatic cellular efflux transport of xenobiotics (Luckenbach et al., 2014) and reported to be induced by EE2 (Finne et al., 2007), confirm that EE2 was also actively excreted from the hepatocytes. Down-regulation of other phase II biotransformation genes such as *gst, sult6b1,* and *ugt1b5*, suggest that these pathways play less important roles in the biotransformation of estrogens in fish (Kurogi et al., 2013; Mortensen and Arukwe, 2007; Skillman et al., 2006; Solé et al., 2000; Sovadinová et al., 2014; Wang et al., 2014).

Interestingly, the *cyp1a* gene, which is normally induced by exposure to xenobiotics in fish (Schlenk et al., 2008), was suppressed in a concentration-dependent manner in both the

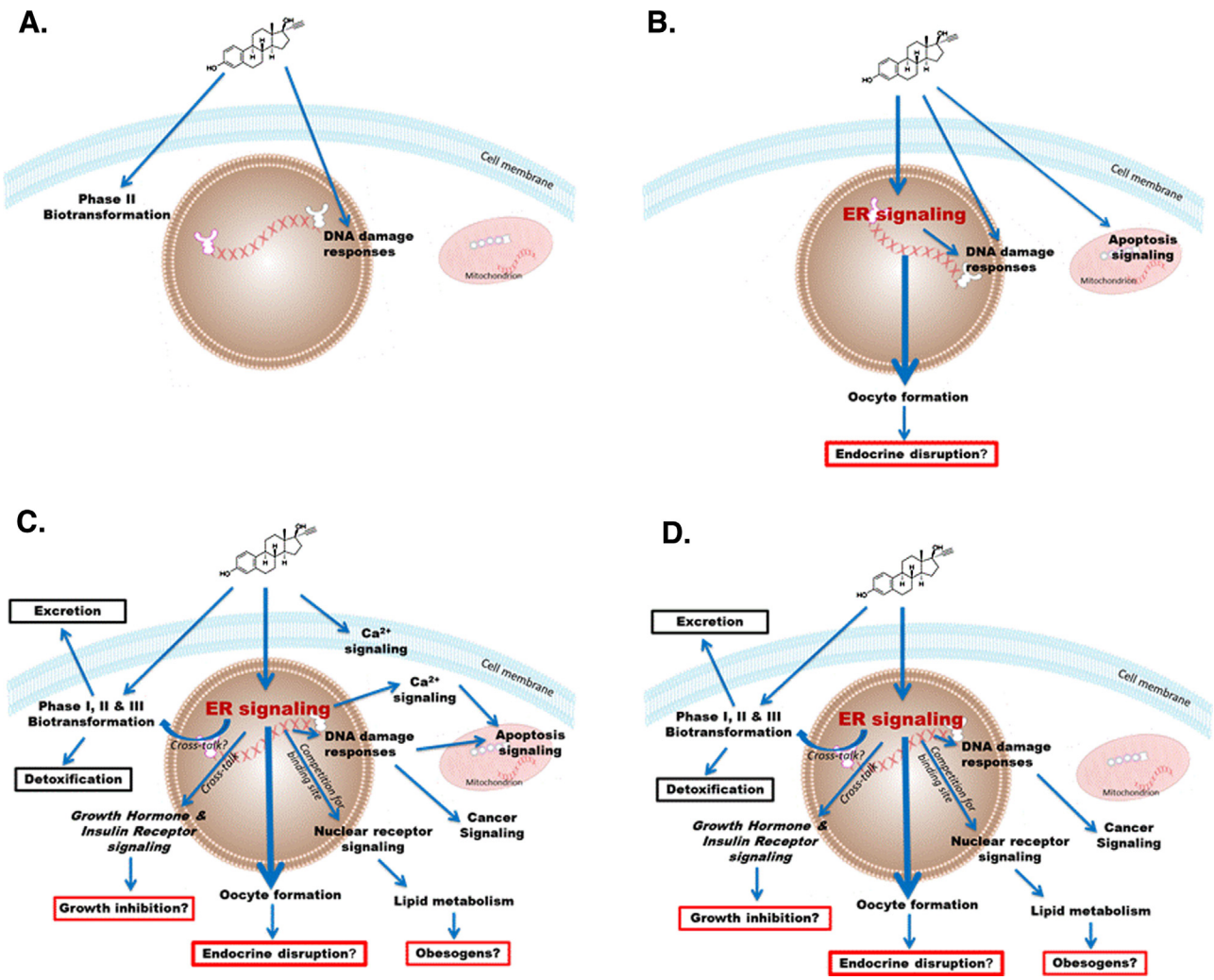


**Fig. 5.** Concentration-dependent modulation of toxicity (canonical) pathways and supporting differently expressed genes (DEGs) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed to 0.03 (A), 0.3 (B), 3 (C) and 30 (D) nmol/l 17α-ethinylestradiol (EE2) for 48 h.

microarray and qPCR analysis (Fig. 3). Down-regulation of *cyp1a* has previously been associated with estrogen exposure and proposed to be the result of a uni- or bi-directional negative crosstalk between the upstream nuclear receptors ER and aryl hydrocarbon receptor (AhR) (Bemanian et al., 2004; Gräns et al., 2010; Skjetne Mortensen et al., 2006). The present microarray study shows indication of such cross-talk as up-regulation of the ERα-recruited squelching *nuclear factor 1* (*nf1b*) may reduce AhR mediated *cyp1a* expression (Ricci et al., 1999). However the *ahr* gene transcript, assumed to be central in the AhR-ER cross-talk (Bemanian et al., 2004; Matthews et al., 2005; Ohtake et al., 2003; Safe and Wormke, 2003) was not significantly expressed. Although this lack of positive verification may have been due to high inter-replicate variation, additional studies to decipher the role of AhR in modulating the ER activity in fish may be warranted. This seems also to be the case for the CYP P450 isoform 2M1 (CYP2M1), a major regulator of lauric acid hydroxylation, which has been reported to be suppressed by estrogenic compounds (Sovadinová et al., 2014). However, this gene was not identified to be differentially regulated in the present study. Nevertheless, the present results clearly demonstrated that primary hepatocytes retain many of their native detoxification properties and agree with previous suggestions elsewhere (Finne et al., 2007; Hultman et al., 2015; Segner, 1998; Segner and Cravedi, 2000).

### 4.3. Lipid and cholesterol homeostasis

Disruption of steroidogenesis and lipid metabolism in fish exposed to estrogens may lead to accumulation of lipids, impairment of hormone, glucose and cholesterol homeostasis in various organs (Koren et al., 1982; Tocher, 2003), affecting vitellogenesis and normal oocyte formation and development (Doyle et al., 2013; Levi et al., 2009; Luckenbach et al., 2008). Steroid, lipid (*e.g.* apolipoprotein and high density lipoprotein (HDL) cholesterol) and fatty acid homeostasis (metabolism, biosynthesis, uptake, and transport) is mainly regulated in the liver by *pparα* (Lee et al., 2003; Tocher, 2003), which in the present study was transcriptionally suppressed by EE2 in a concentration-dependent manner (Fig. 4). Estrogens such as E2 and EE2 are not PPAR-ligands themselves, but may indirectly interact with PPARs through crosstalk with the ER (Keller et al., 1995). This receptor crosstalk has been proposed to be involved in competitive binding of shared transcriptional co-regulators upon estrogen exposure (Wang and Kilgore, 2002) in addition to incomplete PPAR binding to the ERE resulting in reduced transcriptional activation of PPAR (Keller et al., 1995), as indicated in the present work. Another abundantly expressed PPAR in the fish liver is the *pparγ*, the product of which is responsible for peroxisomal β-oxidation of fatty acids in fish (Ruyter et al., 1997), and may therefore aid the uptake and to some extent also metabolism of lipids in the liver. The present study identified that *pparγ* was sig-

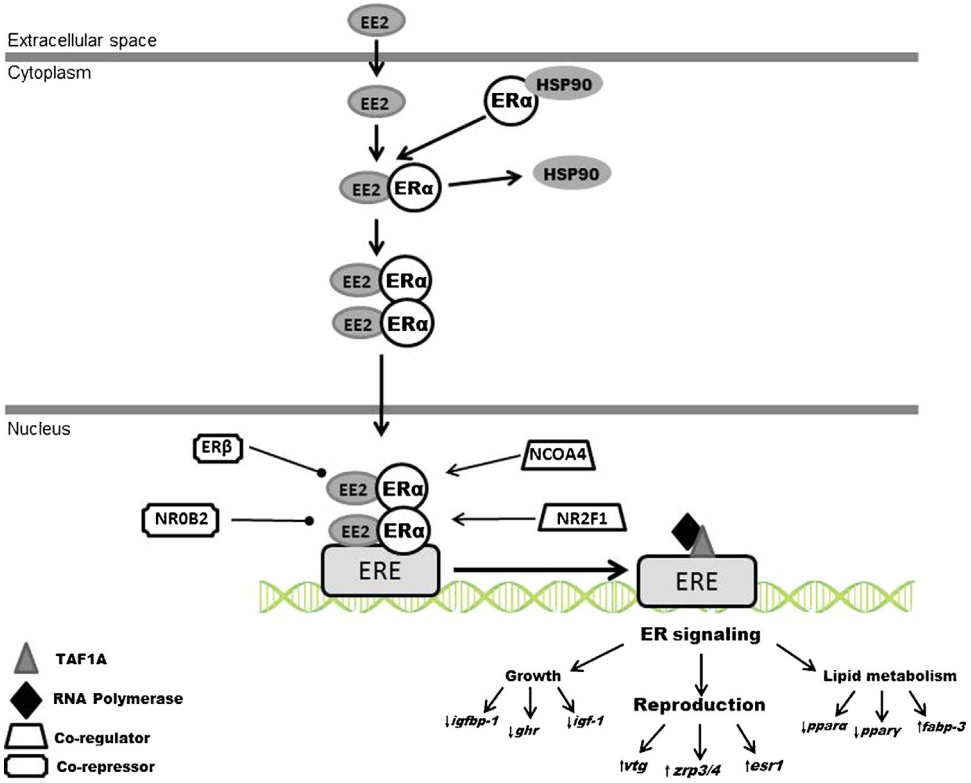


**Fig. 6.** Estrogen receptor (ER) signaling pathway in fish based on differential gene expression in primary rainbow trout hepatocytes exposed to 17α-ethinylestradiol (EE2) for 48 h. Abbrevations: HSP90—heat shocking protein 90; ERE—Estrogen responsive element; NCOA4—nuclear receptor coactivator 4, NR2F1—nuclear receptor subfamily group member 1; NR0B2—nuclear receptor subfamily group member 2; TAF1A—TATA box-binding protein-associated factor RNA polymerase I subunit A; *igfbp-1—insulin growth factor binding protein 1*; *igf-1—insulin growth factor 1*; *ghr—growth hormone receptor*; *vtg—vitellogenin*;*zrp3/4—zona radiata protein 3/4*; *esr1 - estrogen receptor 1*; *pparα/γ—peroxisome proliferator-activated receptor α/γ*, *fabp-3 – fatty acid binding protein 3*. The pathway is modified from Lanzino et al. (2005), Petit et al. (1999) and Sanyal et al. (2002).

nificantly suppressed only at the highest EE2 concentration (30 nM) (Fig. 3), hence suggesting that *pparγ* is less susceptible to EE2-induced modulation than *pparα* in the hepatocytes. Nevertheless, suppression of *pparα* and *pparγ* in primary hepatocytes exposed to estrogens is suggestive of chemical interference with key regulators of lipid metabolism in fish hepatocytes.

Impaired *pparα* expression may also affect transcriptional activation of *apolipoprotein A-I* (*apoa1*) and *A-II* (*apoa2*) in fish (Tocher, 2003), transcripts which the present study did not identify as significantly altered. These apolipoproteins have a major role in the uptake and transport of lipids across the cellular membrane and into the blood, where they function as co-regulators in transport, lipoprotein uptake, lipid metabolism or act as inhibitors of catabolic pathways (Erkelens, 1989; Kingsbury and Bondy, 2003). In fish, apolipoproteins consist of various classes including *apolipoprotein a* (*apoa*) and *apolipoprotein e* (*apoe*) which regulates the incorporation of lipids and lipoproteins in the oocyte (Luckenbach et al., 2008). Suppression of *apo1a* and *apoe* in liver tissue has previously been associated with exposure to E2 and EE2 (Hoffmann et al., 2006; Martyniuk et al., 2007; Wit et al., 2010), which ultimately may modify uptake and transport of cholesterol and lipids during vitellogenesis and oocyte formation (Doyle et al., 2013; Hoffmann et al., 2006). None of these genes were identified as significantly regulated in the microarray analysis in the present study, an observation in line with previous transcriptional studies in primary hepatocytes (Finne et al., 2007; Sovadinová et al., 2014) and whole fish (Doyle et al., 2013; Levi et al., 2009) exposed to EE2 and E2. The lack of modulation of *apo1* and *apoe* may suggest that lipid transport and uptake is maintained during vitellogenesis, hence allowing normal oocyte development (Doyle et al., 2013), which has previously been demonstrated to be compromised in fish after EE2 exposure (Schäfers et al., 2007).

Interestingly, genes associated with increasing biosynthesis and cellular uptake of cholesterol such as *apof* (also known as *lipid transfer inhibitor protein (ltip)*), *angiopoietin-related protein 3, 7-dehydrocholesterol reductase* (*dhcr*), *low-density lipoprotein receptor* (*ldlr*) and *neutral cholesterol ester hydrolase 1* (*nceh1)* were all up-regulated in the EE2-exposed hepatocytes. These genes are associated with increased cholesterol biosynthesis and transport during vitellogenesis in fish (Hoffmann et al., 2006; Kersten, 2005; Levi et al., 2009) and may be modified through ERα-mediated interference by negative feedback regulation of cholesterol biosynthesis (Wang et al., 2006).

Overall, the present study found xenoestrogen-mediated molecular changes associated with lipid and cholesterol homeostasis which previously has been proposed to affect fish through disruption of sex steroid biosynthesis (Levi et al., 2009) and potentially cause delayed sexual maturation and subsequent reproductive success in fish (Schäfers et al., 2007).

### 4.4. Cellular growth and development

Impaired cellular growth and development in juvenile and adult fish has previously been associated with exposure to low concentrations of estrogenic compounds (Ashfield et al., 1998; Schäfers et al., 2007; Shved et al., 2008). The present study identified concentration-dependent modulation of genes associated with negative cell growth regulation (e.g. *ghr-1, igfbp-1, myostatin*) (Table 4, Figs. 3 and 5C and D, Supplementary Table B and E), whereof several genes have been reported regulated in EE2

exposed fish elsewhere (Martyniuk et al., 2007; Shved et al., 2008). Impaired cell and organism growth has previously been correlated with disruption of steroid homeostasis and reproduction in fish, potentially due to energetic constraints introduced by simultaneously occurring energy demanding processes such as reproduction, growth (Davis et al., 2008) and detoxification of xenoestrogens (Schäfers et al., 2007). Although not studied in detail, this may also be applicable to the primary hepatocytes studied herein as they likely have limited energy/lipid storage capacity and lack the ability to compensate large energy losses by mobilization of nutrients from other tissues. However, additional explanations such as nuclear receptor cross-talk between the ER and GH/IGF-1 systems leading to suppression of *ghr, igfbp-1* genes has been proposed to explain estrogen-induced impairment of growth during sexual differentiation in fish (Davis et al., 2008; Nelson and Habibi, 2013; Shved et al., 2008). The findings of the present *in vitro* study indicate that primary fish hepatocytes retain the molecular regulatory networks specifically associated with IGF-1 and GH signaling, and may thus provide valuable insight into how these processes are affected after exposure to xenoestrogens in fish.

### 4.5. Cancer and other signaling pathways

Estrogens exert carcinogenic effects in mammals by ER signaling and transduction pathways associated with inhibition of apoptosis and increased cell proliferation (Pearce and Jordan, 2004; Yager and Davidson, 2006). Evidence of estrogen-induced liver carcinogenesis and perturbation of cell cycle regulation has been demonstrated in E2-exposed fish and been proposed to result from interference with putative genes associated with cell cycle regulation such as G1-S and S-G2 phase transition (Lam et al., 2011), both being mechanisms that regulate mitosis and cellular growth. The present study identified that several affected pathways and GOs were associated with suppression of cell death (caspase activity, death receptor and apoptosis signaling) supported by the suppression (*direct inhibitor-of-apoptosis protein-binding protein with low pI* (*Diablo*), *tumor necrosis factor ligand superfamily member 6* (*faslg*)) and induction (*X-linked inhibitor of apoptosis* (*xiap*)) of putative DEGs, which further have been associated with a number of cancer types (Reed, 2003). Interestingly, the present study identified enrichment of several additional pathways associated with cancer (*i.e.* breast cancer regulation by *stathmin 1*, molecular mechanism of cancer and estrogen-dependent breast cancer) and regulation of DEGs (*reticulon 1/3* (*rtn1/3*), *pim-1/3 oncogene* (*pim1/3*) and *breast cancer metastasis-suppressor 1-like* (*brms1*)), whereof several have been reported modulated in fish exposed to estrogens (Colli-Dula et al., 2014; Harding et al., 2013; Levi et al., 2009). However, estrogens have also been proposed to induce oxidative DNA damage (Lam et al., 2011) through production of reactive metabolites that subsequently may cause mutagenic, carcinogenic and genotoxic effects (Roy et al., 2007; Russo et al., 2003). Interestingly, putative DEGs involved in DNA damage such as *breast cancer 1* (*brca1*) were suppressed. This seems to contradict the behavior of *brca1* in E2-exposed fish (Lam et al., 2011). The induction of *brca1* is primarily mediated by estrogens binding to and activating the ER, but recent studies have suggested that recruitment of unliganded AhR to the proximal *brca1* transcriptional binding domain is required to potentiate its expression (Hockings et al., 2006). Lack of AhR regulation (Supplementary Table A and D) after exposure to EE2 in this study suggested that AhR may not respond in a similar manner as that seen *in vivo*. However, the decreased transcription of the genes encoding the death receptor (*faslg*) and proteins involved in apoptosis signaling (*Diablo* and *xiap*) may suggest that EE2 is associated with estrogen-mediated inhibition of apoptosis and induced cell proliferation in primary hepatocytes as observed *in vivo* (Harding et al., 2013; Lam et al., 2011; Levi et al., 2009).

### 4.6. Primary hepatocytes as a screening model for estrogen mimics

Rainbow trout hepatocytes have been suggested to be a rapid and high-throughput assay for the identification of estrogenic and antiestrogenic properties of chemicals (Hultman et al., 2015; Navas and Segner, 2000; Smeets et al., 1999; Tollefsen et al., 2008b), and recently demonstrated to yield highly reproducible responses to estrogenic biomarkers such as Vtg expression both at the gene and protein level (Hultman et al., 2015). The present study demonstrated that the rainbow trout hepatocyte bioassay, in combination with the global transcriptional analysis, may provide better understanding of the MoAs of EE2. The main advantages of applying this approach to rainbow trout hepatocytes are: low amount of biological material required for the analysis, rapid and unbiased MoA characterization and low biological variation. The primary hepatocytes displayed well-characterized biomarker gene responses, which was generally in agreement with that occurring *in vivo* in fish after exposure to E2 and EE2. This applies in particular to the *in vivo* expression of DEGs associated with estrogen receptor signaling (*e.g. esr1, vtg1, zrp*), biotransformation (*e.g. cyp1a, abcb11*), lipid and cholesterol metabolism (*e.g. dhcr, fabp3, ldlr*), growth (*e.g. igfbp-1*, *ghr*), and cancer/apoptosis (*rtn1/3, pim1/3, xiap, diablo*), which were conserved and responsive in the rainbow trout hepatocyte model. The observed *in vitro* transcriptional changes increased in number of DEGs identified and their responses to EE2, a finding also seen when analyzing functional (GO) enrichment and pathway analysis. Interestingly, the sensitivity of the hepatocytes to well-known biomarkers such as *vtg, esr1* and *cyp1a* also seemed to be in accordance with that observed in *in vivo* exposure studies with 0.87–125 ng/l EE2 (Doyle et al., 2013; Gunnarsson et al., 2007; Hoffmann et al., 2006; Martyniuk et al., 2007; Skillman et al., 2006). The *in vitro* to *in vivo* extrapolation performed followed principles demonstrated by Skillman et al. (2006) by using measured liver cell concentrations of EE2 in the hepatocyte assay (see Hultman et al., 2015). Using a fixed water:liver EE2 accumulation ratio derived from a 48 h *in vivo* exposure study to 125 ng/l EE2 (Skillman et al., 2006), the *in vitro* Lowest Observed Effect Concentration (LOEC = 0.3 nM, fig. 3) were estimated to correspond to an external water exposure concentration of 6.4–9.6 ng/l in the present study. These external exposure concentrations has been demonstrated not only to be in the range of sensitivity observed *in vivo* (Gunnarsson et al., 2007; Martyniuk et al., 2007), but also considered to be environmentally relevant (Larsson et al., 1999).

Although molecular methods such as transcriptomics have greatly improved our ability to perform global response assessments, transcriptional regulation will not account for posttranscriptional modifications leading to alterations of downstream molecular events such protein expression, cellular signaling, and metabolic activity (Schirmer et al., 2010). However, close correlation of Vtg gene and protein expression were observed in the same experimental models (Hultman et al., 2015), and suggest that certain transcriptional responses are descriptive for the activity occurring at the functional level. As a thorough evaluation of the predictability of transcriptional changes to other levels of organization was not conducted in the present study, it is recommended that such effort is undertaken in the future characterization of MoAs of EE2 and other xenoestrogens. The use of primary hepatocytes also has some limitations for larger scale implementation in MoA assessment of EDCs as the bioassay is restricted to hepatic responses and does not necessarily reflect MoAs relevant for other organs (*e.g.* oocyte formation) and adverse effects (*e.g.* feminization) at the whole organism level. However, this may also

be advantageous as the primary hepatocytes reflect key molecular events in the liver and can thus be studied without influences from other organs involved in the hypothalamus-pituary-gonad axis. Overall, rainbow trout hepatocytes seem to represent a feasible complement to *in vivo* testing as displaying many central hepatic MoA relevant for endocrine disruption, and in particular the response to ER-agonists.

## 5. Conclusion

The present study showed that the primary rainbow trout hepatocyte bioassay can be a suitable and responsive *in vitro* model when using a broad content transcriptomic analysis to characterize the effects of the ER-agonist 17α-ethinylestradiol (EE2). The potential toxic mechanisms of EE2 in the primary hepatocytes were successfully characterized and found to affect gene expression related to biotransformation, lipid metabolism and ER signaling and ER-mediated cellular responses. The potential of using primary hepatocytes for assessment of estrogen mimics was supported by the clear concentration-dependent enrichment of functional categories (GO), pathways and DEGs associated with the well-known MoAs of EE2 (*e.g.* induction of estrogen signaling, suppression of biotransformation and growth, disruption of lipid homeostasis, and cancer) *in vivo*. Furthermore, well-characterized estrogen-responsive biomarker genes (*e.g. esr1, vtg, zrp*) were identified to be differentially expressed in accordance with several previous *in vitro* and *in vivo* estrogen studies. The primary hepatocyte model is thus proposed to represent a promising complement to *in vivo* testing of EDCs, and in particular for estrogen mimics that affect central liver functions associated with endocrine regulation and reproductive processes in fish.

## Availability of supporting data

The raw data sets supporting the results of this article are available in the Gene Expression Omnibus (GEO) repository, accession number: GSE68335 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE68335 Other data sets supporting the results of this article are included in attached excel sheet.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2015.10.004.

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# Paper III

# Deciphering combined effects of anti-estrogenic chemicals on vitellogenin production in rainbow trout (*Oncorhynchus mykiss*) hepatocytes

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# Abstract

The aquatic environment is constantly exposed to a complex mixture of anthropogenic chemicals which may cause endocrine disruption (ED) in aquatic organisms such as fish. EDCs with anti-estrogenic properties has received less attention than estrogenic compounds, but may cause reduced oocyte formation in female fish by different modes of action (MoA) such as suppressing the estrogen receptor (ER) mediated egg-yolk precursor protein vitellogenin (Vtg). The MoA of many anti-estrogens are still not fully deciphered and use of *in vitro* methods such as primary hepatocyte bioassays have proven suitable for characterising the effect of anti-estrogens individually and in mixtures. The aim of the present study was to decipher the single and combined anti-estrogenic MoA of the aryl hydrocarbon receptor (AhR)-agonist β-naphtoflavone (BNF) and ER-antagonist hydroxytamoxifen (OHT) on Vtg protein in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes using the enzyme-linked absorbent (ELISA) assay. Transcriptional analysis of ER-responsive genes (*estrogen receptor α* (*erα*), *egg shell protein zona radiata* (*zrp*), *vitellogenin -1* (*vtg-1*)) and supportive AhR-mediated genes (*aryl hydrocarbon receptor 2α* (*ahrα*), *cytochrome p450 1a* (*cyp1a*)) using quantitative real-time polymerase chain reaction (qPCR) was performed to further decipher the compounds MoA. All compounds tested caused a reduction of the ER-mediated Vtg protein production, and a mixture of BNF and OHT caused the strongest inhibition of E2-

induced expression of ER-responsive genes (*erα, zrp* and *vtg-1*) and protein (Vtg) production. The present study identified that albeit OHT and BNF suppressed the activity of the ER-responsive genes (*erα, zrp* and *vtg-1*), BNFs MoA were likely associated with AhR-ER cross-talk through *cyp1a*-mediated induction of E2 metabolism. The large anti-estrogenic effect of the binary mixture was proposed caused by a combination of direct inhibitory action on the ER (OHT) and stimulation of E2 metabolism in the hepatocytes. In conclusion, the present study partially deciphered the two differently acting anti-estrogens single and mixture effect using a multi-endpoint approach. The present study has displayed the primary hepatocyte model's suitability for screening of anti-estrogens individually and in simple mixtures, independent of their MoA.

# 1 Background

Aquatic organisms are constantly exposed to mixtures of organic chemicals from various anthropogenic sources of emission (e.g. industry emission, air deposition, sewage treatment plants, land run-off etc.). These compounds affect the aquatic organisms through different mode of action (MoA), whereof some may modulate the endocrine system beyond homeostasis and cause endocrine disruption (Sumpter, 2005). Endocrine disrupting chemicals (EDCs) have the potential to affect reproduction, immune response and development, raising concern for wild life and humans (Casanova-Nakayama et al., 2011; Kavlock and Ankley, 1996; Tyler et al., 1988).

EDCs may modulate molecular targets in the endocrine system by disturbing the homeostasis of hormone-regulating processes such as the hypothalamus-pituitary-gonad (HPG) axis through the estrogen, androgen or thyroid (EAT) signaling pathway (Munn and Goumenou, 2013). The best characterised EDCs are estrogen receptor (ER) agonists, which in many tissues bind and activate the ER to initiate a series of cellular events. Upon ligand binding, the ER-ligand complex is translocated into the nucleus where it initiates transcriptional activation of the ER and modulate downstream target genes containing the estrogen responsive element (ERE) (Filby and Tyler, 2005; Matthews and Gustafsson, 2003; Shanle and Xu, 2011). In female fish, activation of the ER induce vitellogenesis in the liver by increasing the transcriptional activity and synthesis of the egg-yolk precursor protein vitellogenin (Vtg), egg shell protein zona radiata (zrp), vigilin and follistatin (Arukwe et al., 2000; Hyllner et al.,

1991). The synthesized proteins (e.g. Vtg) are then transported via the blood stream to the gonads where they have an essential role in oogenesis (Tyler et al., 1988). However, induction of vitellogenesis (e.g. vtg, zrp) do not occur in juvenile or male fish, consequently these genes and proteins in liver or blood are used as estrogenic biomarkers to detect chemicals with ER modulatory properties (Sumpter and Jobling, 1995; Tollefsen et al., 2003).

Although, the main focus has been on ER-agonistic compounds, several organic pollutants exert anti-estrogenic effects by suppressing the expression of the ERs, Vtg, zrp and vigilin in fish (Gräns et al., 2010; Mortensen and Arukwe, 2008; Petersen and Tollefsen, 2012; Rankouhi et al., 2004), and by doing so act as functional anti-estrogens. Some of these anti-estrogenic compounds cause adverse effects such as masculinization and reduced fecundity in female fish (Andersson et al., 1988; Bugel et al., 2011; King Heiden et al., 2006).

Directly acting antiestrogens such as estrogen receptor antagonists (e.g. 4-hydroxytamoxifen (OHT) and fluvestrant (ICI)) binds to the ER, and disrupts the ER signaling in all ER-containing tissues and may activate recruitment of co-repressors and block the action of co-regulators (Dobrzycka et al., 2003). The ER-antagonists may also bind to the activation function 1 (AF1) and/or 2 (AF2) in the ligand binding domain (LBD) of the ERE, causing full (AF1 and AF2) or partial (AF2) inhibition of ER transcription (for full review see Aranda and Pascual, 2001). Interestingly, all chemicals with anti-estrogenic properties are not necessarily causing their effects through a ligand-binding mechanism, but may indirectly elicit anti-estrogenicity by nuclear receptor (NR) cross-talk (Matthews and Gustafsson, 2006). One type of NR crosstalk has been described in several *in vitro* and *in vivo* models (for full review see Matthews and Gustafsson, 2006; Safe and Wormke, 2003; Swedenborg and Pongratz, 2010) as a uni- or bi-directional crosstalk between the aryl hydrocarbon receptor (AhR) and ER (Gräns et al., 2010; Mortensen and Arukwe, 2007). This AhR-ER crosstalk involve several mechanisms including AhR-mediated metabolism of estrogens (Safe and Wormke, 2003), AhR-mediated competing for common transcriptional co-regulators (Brunnberg et al., 2003; Rüegg et al., 2008), and AhR-mediated proteasome degradation of the ER (Bemanian et al., 2004; Ohtake et al., 2003; Safe and Wormke, 2003). Although the general mechanisms of the AhR-ER cross-talk has been proposed in mammalian systems (Safe and Wormke, 2003), the MoA is still not fully understood in fish and knowledge about how combinations of EDCs in complex mixtures may cause effects are largely unknown. It is becoming increasingly clear that the combined effects of anti-estrogenic compounds is complex (Mortensen and Arukwe, 2007), and demonstration of synergy between classical pollutants and ER-antagonists on the

suppression of ER-mediated processes in fish bioassays (Petersen and Tollefsen, 2012) suggest that effort to decipher the MoA of these anti-estrogens are highly warranted.

The aim of the study was to characterise the antiestrogens β-naphtoflavone (BNF) and hydroxytamoxifen (OHT) direct and indirect modulatory effects on ER-responsive genes (*estrogen receptor α* (*erα*), *egg shell protein zona radiata* (*zrp*),*vitellogenin -1* (*vtg-1*)) and Vitellogenin protein (Vtg), separately and in combination in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes. Genes associated with the AhR-activity (*aryl hydrocarbon receptor 2α* (*ahrα*), *cytochrome p450 1a* (*cyp1a*)) was also monitored to unravel the potential NR cross-talk. Transcriptional and protein analysis, using quantitative real-time polymerase chain reaction (qPCR) and enzyme-linked absorbent (ELISA) assay were used to characterise the mixtures MoA and the potential nuclear receptor cross-talk.

# 2 Material and methods

## 2.1 Chemicals and exposure concentrations

The chemicals, β-naphtoflavone (BNF, CAS: 6051-87-2), 4-hydroxytamoxifen (OHT, CAS: 68047-06-3) and 17 β-estradiol (E2, CAS: 50-28-2) were obtained from sigma Aldrich (St Lois, MI, US). All compounds were dissolved in DMSO and kept at -20°C when not in use.

## 2.2 Fish

Juvenile rainbow trout (200-500g) were obtained from the Valdres rakfisk AB hatchery (Valdres, Norway) and reared at the Department of Biology, University of Oslo (Norway) for a minimum of 4 weeks prior to the study. The donor fish used in the study were from the same fish stock, maintained in tap water at 6±2$^{o}$C, pH 6.6, 100% oxygen saturation and light regime of 12h light/12h dark. Rainbow trout were fed daily with commercial pellets (Skretting, Stavanger, Norway) corresponding to approximately 0.5% of total body weight.

## 2.3 Primary cultures of rainbow trout hepatocytes, exposure and sampling

Totally 6 juvenile rainbow trout size 200-500 grams were terminated by a blow to the head, followed by visual inspection of the maturity status by assessing the gonads. Only juvenile fish with no visual development of the gonads were subjected to liver perfusion. The liver was perfused using a two-step perfusion method as described in Tollefsen et al. (2003). The viability of the cells in the suspension was assessed by the trypan blue exclusion test and only cell isolations with ≥ 90% viability were used for the experiments. Cells were diluted to a final concentration of 500 000 cells/ml and seeded in 24 well plates (1.25 ml/well). After 24h of acclimatisation, cells were exposed to single (E2, BNF, OHT), binary (E2 + OHT, E2 + BNF, BNF + OHT) or ternary (E2 + OHT + BNF) mixtures of the compounds or a solvent control (DMSO 0.01%). The exposure concentrations were chosen on basis on previously observed sub-lethal concentrations for 50% inhibition of E2 induced Vtg protein production (E2: $6.3E^{-10}$ M) by the differently acting anti-estrogens BNF (AhR-agonist) and OHT (partial ER-antagonist) (BNF $IC_{50}$: $1.10E^{-7}$ M ; OHT $IC_{50}$: $4.71E^{-9}$M) in rainbow trout primary hepatocytes (Petersen and Tollefsen, 2012). An overview of the test concentrations are shown in suppl. table 1.

Cells were re-exposed after 48h to maintain exposure concentrations as shown to be crucial to obtain high-quality concentration-response curves (CRC). Cells and cell culture media were sampled at 48h and 96h for gene and protein analysis respectively. At the end of exposure, cell culture media was transferred in triplicates (3 x 100µl) to a Maxisorb nunc-immunoplate (Nunc, Roskilde, Denmark), and frozen at -80°C for subsequent vitellogenin analysis, whereas cells were sampled for subsequent RNA isolation after the supplier instructions from the Qiagen RNeasy Plus mini kit (Qiagen GmbH, Hilden, Germany).

## 2.4 Enzyme linked absorbent assay (ELISA)

Vitellogenin was measured in the primary hepatocyte media by a capture (semi-quantitative) ELISA, previously described by Tollefsen et al. (2003). In brief, the frozen microtiter plates containing media sampled after 96h of exposure, were thawed at 4°C, followed by application of 100µl of positive standard dilution (rainbow trout Vtg) in empty wells and further incubated in the dark over night at 4°C (>16h). The capture ELISA was performed using a monoclonal mouse anti-salmon Vtg (BN-5, Biosense Laboratories, Bergen, Norway)

antibody, followed by the secondary antibody goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA), both applied in a 1:6000 dilution and incubated 2h respectively. A HRP enzyme substrate (TMB plus, KEMENTEC diagnostics, Taastrup, Denmark) was added to the wells following appropriate washing in order to start the color development. After 15 minutes of incubation in the dark at room temperature, the color development was stopped by addition of 50µl of 1M $H_2SO_4$. The absorbance was measured within 20 min at 450nm using a Thermomax microplate reader (Molecular Devices, USA). The relative Vtg expression was calculated as percentage of maximum vtg induction ($6.3E^{-10}$M E2) at 96h of exposure by normalising the individual treatment against the basal expression in the solvent control.

## 2.5 Quantitiative Real time PCR (qPCR)

The gene expression analysis was performed using quantitative polymerase chain reaction (qPCR). Synthesis of cDNA was performed through reverse transcription of total RNA (0.5-1µg) using Quanta qScript™ cDNA Synthesis Kit (Quanta Biosences Inc., Gaithersburg, USA) according to the manufacturer's instructions and outlined for use with primary rainbow trout hepatocytes in Hultman et al. (2015). The primer optimisation was performed using a template pool consisting of 5-step dilution series (5-100 ng/reaction) in a 384 well plate format on a CFX-384 thermal cycler (Bio-Rad laboratories Inc., USA). SYBR®Green Supermix fluorecence dye (Quanta Biosences Inc., Gaithersburg, USA) was used in the qPCR amplification reaction, where triplicates of 10ng template/well was used in the final mastermix reaction (20 µl/reaction). The primers were designed using the NCBI accession number (Table 1) and the primer design software "Primer 3 Input version 0.4.0" and were produced by Eurofins MWG synthesis GmbH (Ebersberg, Germany). All primers run had a non-template control (NTC) and a no-reverse transcriptase control (NRT) as to exclude any contamination of the mastermix and to exclude any presence of genomic DNA in the sample. Primer dimers or unspecific amplified products were also assessed by applying a melting curve in the end of the qPCR analysis. Accepted Cq-value of NTC was set to be either non-detectable (N/A) or Cq value >30 with minimum 7 cycles in between template replicate and NTC. Ubiquitin was stable in all treatments and was therefore appointed as a housekeeping gene (table 2). Data normalisation was performed using the Pfaffl method (Pfaffl, 2001).

Table 1. Species, gene name, primer sequences, product size, accession numbers and analysis protocol used for the qPCR analysis.

| *Species* | Name | Sequence | | Amplicon size (bp) | Acc. No. | Annealing Temperature ($^{o}$C) | Primer conc. (nM) | Efficiency (%) | Reference |
|---|---|---|---|---|---|---|---|---|---|
| *O.mykiss* | *ubiquitin* | *Forward* | 5´-ACAACATCCAGAAAGAGTCCAC-3´ | NR | AB036060 | 55 | 400 | 103 | Hultman et al., 2015b |
| | | *Reverse* | 5´-AGGCGAGCGTAGCACTTG-3´ | | | | 400 | | |
| *O.mykiss* | *erα* | *Forward* | 5´-CCCTGCTGGTGACAGAGAGAA-3´ | NR | NR | 61 | 270 | 109 | - |
| | | *Reverse* | 5´-ATCCTCCACCACCATTGAGACT-3´ | | | | 620 | | |
| *O.mykiss* | *vtg-1* | *Forward* | 5´-GAGCTAAGGTCCGCACAATTG-3´ | NR | X92804 | 61 | 700 | 105 | Celius et al., 2000 |
| | | *Reverse* | 5´-GGGAAACAGGGAAAGCTTCAA-3´ | | | | 700 | | |
| *S.salar* | *zrp* | *Forward* | 5´- TGACGAAGGTCCTCAGGG -3´ | 113 | AF407574 | 59.4 | 500 | 90 | Arukwe et al., 2007 |
| | | *Reverse* | 5´- AGGGTTTGGGGTTGTGGT -3´ | | | | 500 | | |
| *S.salar* | *ahrα* | *Forward* | 5´- AGGGGCGTCTGAAGTTCC -3´ | 96 | AY219864 | 57.6 | 700 | 90 | Mortensen et al., 2006 |
| | | *Reverse* | 5´- GTGAACAGGCCCAACCTG -3´ | | | | 700 | | |
| *S.salar* | *arnt* | *Forward* | 5´-AGAGCAATCCCAGGGTCC-3´ | 107 | DQ367887 | 57.6 | 700 | 93 | Mortensen et al., 2006 |
| | | *Reverse* | 5´-TGGGAGGGTGATTGAGGA-3´ | | | | 700 | | |
| *O.mykiss* | *cyp1a* | *Forward* | 5´-TCCTGCCGTTCACCATCCCACACTGCAC-3´ | NR | U62797.1 | 57 | 700 | 91 | Gräns et al., 2010 |
| | | *Reverse* | 5´-AGGATGGCCAAGAAGAGGTAGACCTC-3´ | | | | 700 | | |
| *S.salar* | *cul4b* | *Forward* | 5´-AAACAGCTACGGGTGGTTTG-3´ | 175 | NM_001139983 | 59.4 | 900 | 95 | - |
| | | *Reverse* | 5´-AAACATAGGTGCGGTCCAAG-3´ | | | | | | |

## 2.6 Statistics

### 2.6.1 ELISA and qPCR analysis

All qPCR data was normalised against the reference gene Ubiquitin and stated as relative expression prior to statistical analysis. All data are normalised against their individual cell media control and presented as fold change. The statistical analysis was performed using Graphpad Prism v5.04 (Graphpad Software, Inc., San Diego, CA, USA) prior to any statistical tests all data was log-transformed to meet the criteria's of normality, and thereafter applying a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test across all tested treatments, with a significant p-value of $<0.05$ for all executed tests. To investigate whether there was an interaction amongst the tested treatments a two-way ANOVA was performed followed by Bonferroni post hoc test, with a significant p-value set at $<0.05$.

# 3 Results

A selection of relevant genes was analysed in order to decipher the anti-estrogenic effects of the compounds individually and in mixtures. All relevant data are presented as figures herein, further details may be found as supplementary information (Supplementary Figure 1, 2 and Table 2).

## 3.1 Effects on ER signaling

### 3.1.1 Vitellogenin protein

17β-estradiol caused a significant induction of Vtg protein expression (10.9 fold change) when compared towards the solvent control after 96h of exposure (Figure 1). The E2-induced Vtg protein expression was apparently reduced with 40% and 25% by BNF and OHT, respectively. The mixture of these two (BNF+OHT) caused a 60% reduction, in E2-induced Vtg production. The reduced Vtg protein indicated all the treatments to modulate the ER-mediated responses on a subcellular level, although not all were identified to be statistically different from the positive control due to variable expression amongst cell batches (Fig.1). The two-way ANOVA (Table 2) identified BNF to cause a significant ($p=0.0254$) reduction of E2-induced Vtg protein (Fig. 1, Table 2), albeit no significant interaction was identified between the compounds BNF and OHT (Table 2). Due to the compounds suppression effect

on vitellogenesis, further investigation of potential anti-estrogenic MoAs and potential interactions was performed on a suite of ER-mediated responses by qPCR.

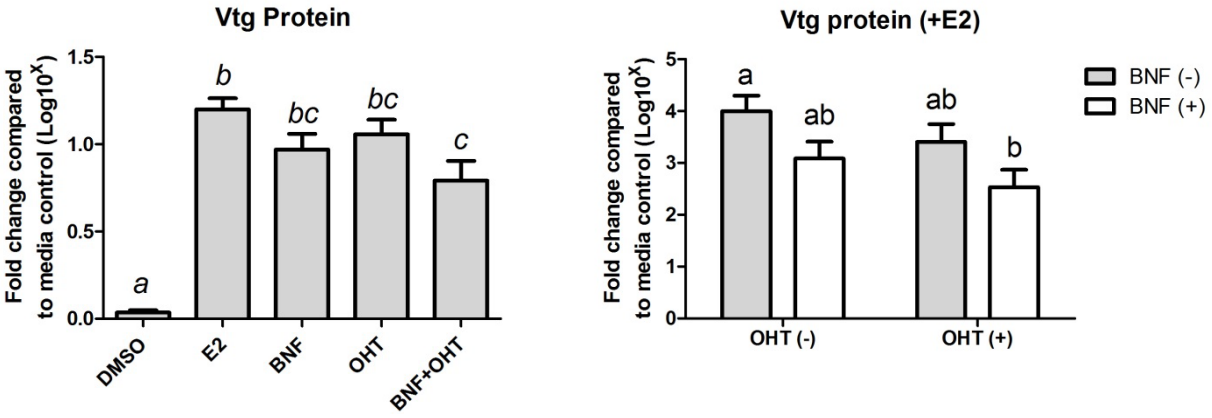


Figure 1. Vitellogenin protein expression in primary hepatocytes exposed to solvent control dimethyl sulfoxide (DMSO) and 17β-estradiol (E2: $6.3E^{-10}$ mol/L) alone and in combination with β-naphtoflavone (BNF: $1.10E^{-7}$ M),hydroxytamoxifen (OHT: $4.71E^{-9}$M), and a mixture of these (BNF and OHT) for 96h. Data is presented as fold change of media control and represent the mean of 3 individual experiments ± standard deviation. The statistical analysis for the left graph was performed using a one way-ANOVA with a Tukey's post hoc test while the right graph presents a two-way ANOVA using Bonferroni post hoc test. The different letters denote treatments which are significantly ($p > 0.05$ ) different from each other in both the one-way and two-way ANOVA.

### 3.1.2 Estrogen receptor α (*erα*)

The positive control E2 caused a significant hepatic induction of *erα* gene expression (16.4 fold change) compared to the solvent control after 48h exposure (Fig. 2). A reduction of 11%, 1% and 26% of the E2-induced *erα* gene expression was observed for BNF, OHT and a mixture of these (BNF+OHT), respectively. In similarity with the Vtg protein, the reduced *erα* expression indicated that the compounds modulate the activity of the ER-mediated responses. The two-way ANOVA did however not identify any significant differences or anti-estrogenic interactions among the treatments in presence of E2 (Table 2). In absence of E2, *erα* gene expression was apparently unaffected by BNF, OHT and the mixture of these (BNF+OHT) (Supplementary fig. 1), indicating that the compounds do not act as classical ER-agonists.

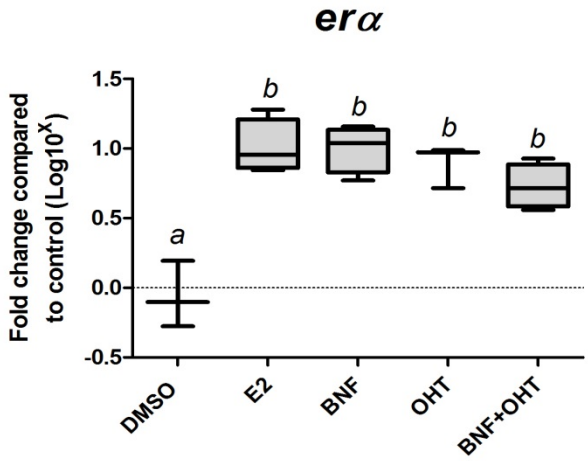


Figure 2. Estrogen receptor α transcription in primary rainbow trout (*Oncorhynchus mykiss*) hepatocyte after exposure to solvent control dimethyl sulfoxide (DMSO) and 6.3E$^{-10}$M 17β-estradiol (E2), 5.5E$^{-8}$M β-naphtoflavone (BNF), 2.36E$^{-9}$M 4-hydroxytamoxifen (OHT) and mixture of these (BNF+OHT) when co-exposed with E2 for 48h. Data represent the mean of 3-4 individual experiments ± standard deviation. The statistical analysis was performed using a one way-ANOVA with a Tukey's post hoc test where the different letters denote treatments which are significantly ($p > 0.05$) different from each other.

### 3.1.3 Vitellogenin-1 (*vtg-1*)

The positive control E2 caused a significant induction of the *vtg-1* gene expression (4341 fold change) when compared towards the solvent control (Fig. 3, Supplementary Table 2). In contrast, the anti-estrogens caused a reduction of the E2-induced transcriptional expression of *vtg-1* in a comparable manner as *erα* (Fig. 3). In similarity to *erα* expression, the E2-induced *vtg-1* gene expression was reduced with 10, 5 and 22% by BNF, OHT and the mixture of these (BNF+OHT) respectively, albeit none of the apparent changes were determined to be statistically different from E2 control.

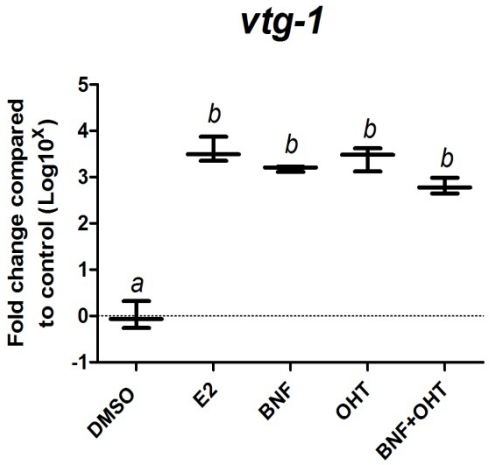


Figure 3. Vitellogenin-1 gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocyte after exposure to solvent control dimethyl sulfoxide (DMSO), 6.3E$^{-10}$M 17β-estradiol (E2), 5.5E$^{-8}$M β-naphtoflavone (BNF), 2.36E$^{-9}$M 4-hydroxytamoxifen (OHT) and mixture of these (BNF+OHT) when co-exposed with E2 for 48h. Data represent the mean of 3 individual experiments ± standard deviation. The statistical analysis was performed using a one way-ANOVA with a Tukey's post hoc test where the different letters denote treatments which are significantly ($p > 0.05$ ) different from each other.

The two-way ANOVA revealed that treatments containing BNF caused a significant (p=0.0032) reduction in E2-induced *vtg-1* expression, findings coherent with the observed suppression of Vtg protein production (Table 2). Furthermore, no interaction (p=0.0658) was identified between the two compounds when assessed as *vtg-1* expression (Table 3). The same treatment groups had no affect the *vtg-1* gene expression in the absence of E2 (Supplementary fig. 1).

### 3.1.4 Zona radiata (*zrp*)

The ER downstream target gene *zrp* was significantly induced (35 fold change) after exposure to the positive control E2 (Fig. 4). The treatments BNF, OHT and the mixture of these (BNF+OHT) apparently reduced the E2-induced *zrp* gene expression with 24%, 11% and 48%, respectively. Interestingly, the two-way ANOVA revealed all treatments containing BNF led to a significant (p=0.015) reduction of E2-induced *zrp* expression. However, no interaction was identified between the two compounds when assessed as *zrp* gene expression (Table 2). The compounds BNF, OHT and a mixture of these (BNF+OHT) did not affect the *zrp* expression in absence of E2, suggestive of the compounds not displaying ER-agonistic properties at the concentrations tested (Supplementary fig. 1).

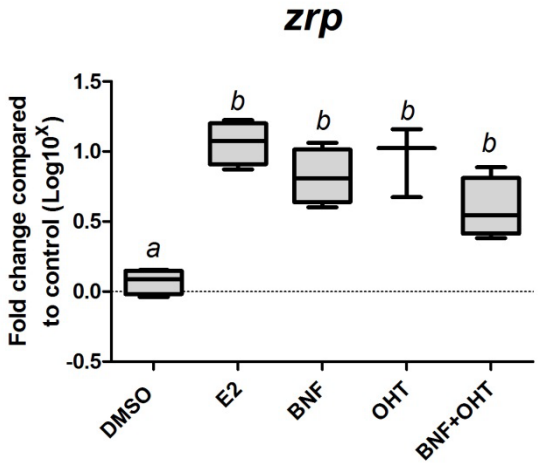


Figure 4. Zona radiata gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocyte after exposure to solvent control dimethyl sulfoxide (DMSO), $6.3E^{-10}$M 17β-estradiol (E2), $5.5E^{-8}$M β-naphtoflavone (BNF), $2.36E^{-9}$M 4-hydroxytamoxifen (OHT) and mixture of these (BNF+OHT) when co-exposed with E2 for 48h. Data represent the mean of 3-4 individual experiments ± standard deviation. The statistical analysis was performed using a one way-ANOVA with a Tukey's post hoc test where the different letters denote treatments which are significantly ($p > 0.05$ ) different from each other.

## 3.2 Aryl hydrocarbon receptor (AhR) signaling

### 3.2.1 Aryl hydrocarbon receptor 1β (*ahrα*)

The typical AhR-agonist BNF caused an apparent induction of *ahrα* gene expression in presence of E2 when compared towards the solvent control (Fig. 5), whereas neither OHT nor E2 caused an effect when exposed alone. The BNF+OHT mixture caused a minor induction of *ahrα*, albeit not statistically different from the solvent control. Similar findings were identified in cells co-exposed with E2, suggestive of E2 not being able to modulate the *ahrα* mRNA expression in presence of an AhR-agonist. The two-way ANOVA revealed that there were no interactions between the compounds on *ahrα* gene expression (Table 2).

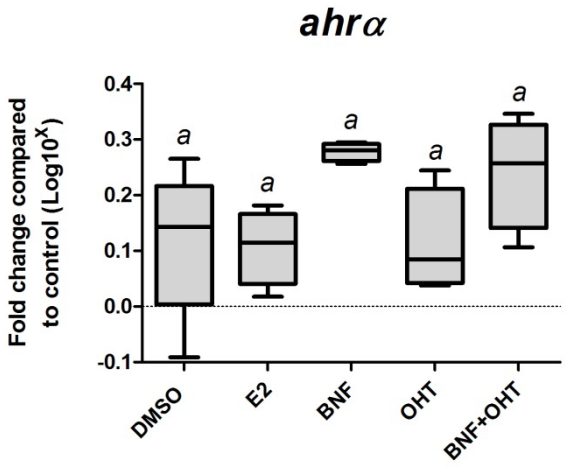


Figure 5. Gene expression of aryl hydrocarbon receptor α (*ahrα*) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocyte after exposure to solvent control dimethyl sulfoxide (DMSO), $6.3E^{-10}$M 17β-estradiol (E2), $5.5E^{-8}$M β-naphtoflavone (BNF), $2.36E^{-9}$M 4-hydroxytamoxifen (OHT) and mixture of these (BNF+OHT) in presence of E2 for 48h. Data represent the mean of 4 individual experiments ± standard deviation. The statistical analysis was performed using a one way-ANOVA with a Tukey's post hoc test where the different letters denote treatments which are significantly ($p > 0.05$ ) different from each other.

### 3.2.2 Cytochrome P450 1a (*cyp1a*)

The treatments BNF and BNF+OHT caused a significant induction of *cyp1a* gene expression when co-exposed with E2 (19.5 and 20.0 fold change, respectively) when compared towards the solvent control (Fig. 6). The compound OHT did not cause any transcriptional changes in *cyp1a*, consistent with the *ahrα* expression for treatments both with and without E2. As seen for cells co-exposed with E2, BNF and BNF+OHT in absence of E2 caused a significant induction of *cyp1a* gene expression (12.9 and 17.0 fold change, respectively) (Supplementary

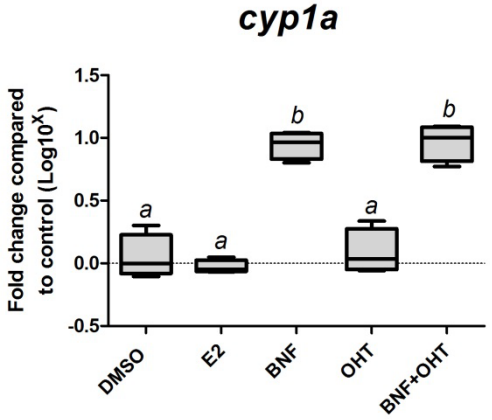


Figure 6. Transcriptional expression of cytochrome P450 1a (*cyp1a*) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocyte after exposure to solvent control dimethyl sulfoxide (DMSO), $6.3E^{-10}$M 17β-estradiol (E2), $5.5E^{-8}$M β-naphtoflavone (BNF), $2.36E^{-9}$M 4-hydroxytamoxifen (OHT) and mixture of these (BNF+OHT) in presence of E2 for 48h. Data represent the mean of 4 individual experiments ± standard deviation. The statistical analysis was performed using a one way-ANOVA with a Tukey's post hoc test where the different letters denote treatments which are significantly ($p > 0.05$ ) different from each other.

Fig. 2, Table 2). Interestingly, the two-way ANOVA revealed that BNF caused a significant induction of *cyp1a* gene expression, both in absence and presence of E2 (Table 2). The two-way ANOVA further identified that there was no interaction between the compounds when analysing *cyp1a* expression, thus suggesting that BNF is the main cause of *cyp1a* induction (Table 2). The *cyp1a* transcription was unaffected by co-exposure of E2, indicative of E2 not contributing to the analysed AhR-mediated responses in the present study.

Table 2. A two-way ANOVA comparing gene expression in factorial treatments exposed to E2, BNF, OHT and BNF+OHT with and without presence of E2, OHT and BNF. The presented F value represents the ratio of the experimental effect compared to the given 'error', while $p<0.05$ was determined as statistically significant.

| | | **F (p)** | | | | | |
|---|---|---|---|---|---|---|---|
| | | **Vtg protein** | ***erα*** | ***vtg-1*** | ***zrp*** | ***ahrα*** | ***cyp1a*** |
| E2+ | BNF | **7.516 (0.0254)*** | 0.5966 (0.4562) | **17.22 (0.0032)*** | **8.27 (0.0151)*** | 0.3513 (0.5644) | **201.7 (< 0.0001)*** |
| | OHT | 3.104 (0.116) | 4.073 (0.0686) | 5.111 (0.0537) | 2.615 (0.1342) | 0.006081 (0.9391) | 1.146 (0.3055) |
| | B+O | 0.003 (0.9586) | 0.256 (0.6229) | 0.7906 (0.3999) | 0.3239 (0.5807) | 1.193 (0.2962) | 0.5032 (0.4917) |
| E2- | BNF | NR | 0.958 (0.3564) | 0.294 (0.6022) | 0.0838 (0.7796) | 1.357 (0.2776) | **243.1 (< 0.0001)*** |
| | OHT | NR | 0.8394 (0.3863) | 0.9596 (0.356) | 0.2945 (0.6022) | 2.244 (0.1725) | **7.356 (0.0301)*** |
| | B+O | NR | 0.4153 (0.5373) | 0.0838 (0.7796) | 0.9596 (0.356) | 0.4547 (0.5191) | 4.016 (0.0851) |
| OHT+ | E2 | NR | **74.31 (< 0.0001)*** | **288.3 (<0.0001)*** | **50.53 (< 0.0001)*** | 0.8253 (0.3902) | 0.1791 (0.6821) |
| | BNF | NR | 1.934 (0.1945) | 3.458 (0.1) | 1.012 (0.3382) | 0.2949 (0.6019) | 4.245 (0.0695) |
| | E2+BNF | NR | 2.807 (0.1248) | 0.1137 (0.7447) | 2.041 (0.1836) | 1.725 (0.2254) | 3 (0.1173) |
| OHT- | E2 | NR | **13.5 (0.0079)*** | **930.1 (< 0.0001)*** | **175 (< 0.0001)*** | 0.3689 (0.5521) | 3.148 (0.0994) |
| | BNF | NR | 2.558 (0.1538) | 0.005006 (0.9449) | 0.4339 (0.5216) | 1.33 (0.2657) | 0.6221 (0.4444) |
| | E2+BNF | NR | **10.94 (0.013)*** | 1.939 (0.1913) | 0.7002 (0.4178) | 0.2774 (0.6057) | 0.0296 (0.866) |
| BNF+ | E2 | NR | 0.7473 (0.4098) | **17.19 (0.0032)*** | 3.077 (0.1133) | 0.2718 (0.6135) | **136.7 (< 0.0001)*** |
| | OHT | NR | **86.44 (< 0.0001)*** | **1014 (< 0.0001)*** | **53.5 (< 0.0001)*** | 0.0006483 (0.9802) | 1.156 (0.3103) |
| | E2+OHT | NR | 1.612 (0.2361) | 4.538 (0.0658) | 3.617 (0.0896) | 0.8818 (0.3698) | 0.6934 (0.4265) |
| BNF- | E2 | NR | 0.5587 (0.4681) | 0.299 (0.5954) | 3.216 (0.0946) | 0.01185 (0.915) | **244.8 (< 0.0001)*** |
| | OHT | | **110.3 (< 0.0001)*** | **421.3 (< 0.0001)*** | **168.3 (< 0.0001)*** | 0.3689 (0.5541) | 0.006437 (0.9372) |
| | E2+OHT | NR | 0.6988 (0.4183) | 3.608 (0.084) | 2.631 (0.1271) | 0.2266 (0.6419) | 2.507 (0.1357) |

**Abbreviations**

E2 - 17β-estradiol; BNF – β-naphtoflavone; OHT – Hydroxytamoxifen; B+O – BNF+OHT; *ahr1β* – aryl hydrocarbon receptor 1β; *cyp1a* – cytochrome P450 1a; *erα* – estrogen receptor α; *zrp* – zona radiata; *vtg-1* – vitellogenin 1; Vtg protein – vitellogenin protein; NR – No data reported.

# 4 Discussion

Understanding of the combined effects of ER agonists and antagonists becomes increasingly important when addressing complex mixtures and environmental samples (Grung et al., 2007; Petersen and Tollefsen, 2011, 2012). Use of *in vitro* bioassays such as primary hepatocytes in combination with transcriptional tools has successfully been used for screening of ER-agonists and antagonists, as the hepatocytes retain native liver functions such as biotransformation activity, AhR and ER mediated responses (Hultman et al., 2015b; Pedersen and Hill, 2000; Petersen and Tollefsen, 2012; Segner and Cravedi, 2000). The bioassay has

also demonstrated to be suitable for screening both single and complex mixtures of ER and AhR agonists and antagonists when using single endpoint approach (Hultman et al., 2015a, 2015b; Navas and Segner, 2000, 2006; Pedersen and Hill, 2000; Petersen and Tollefsen, 2011, 2012). However single endpoint approaches using e.g. Vtg protein have not yet characterised the MoA of typical ER-antagonists and identified why mixtures of typical ER-antagonists and AhR-agonists act synergistic on ER-mediated responses (Petersen and Tollefsen, 2012). The present study therefore assessed mixture effects of the anti-estrogenic AhR-agonist BNF and ER-antagonist OHT by investigating their modulatory properties on AhR and ER responsive genes and protein and their previously proposed cross-talk in primary rainbow trout hepatocytes.

## 4.1 ER signaling

The present study showed that exposure to BNF, OHT and a mixture of these had an apparent reduction on the E2-induced Vtg protein expression (Fig. 1), findings similar to previous primary hepatocyte studies (Gräns et al., 2010; Navas and Segner, 2000; Smeets et al., 1999). The suppression of E2-induced Vtg protein expression has previous been suggested caused by cytotoxicity (Navas and Segner, 2000). However, non-cytotoxic exposure concentrations were used in present study (Petersen and Tollefsen, 2012), thus proposing the compounds anti-estrogenic effect to strictly be dependent on their ability to directly or indirectly modulate the activity of the ER.

The compounds anti-estrogenic effect was the greatest in the mixture of BNF+OHT, where the mixture reduced Vtg protein significantly towards the positive control E2 (Fig. 1). This was expected based on the concept of concentration addition. Mixtures of indirect and direct anti-estrogens (BNF and OHT) have previously demonstrated to cause more than additive effects on inhibition of the E2-induced Vtg protein expression in teleost hepatocytes (Petersen and Tollefsen, 2012), a statement not supported in present study. As the concentrations used in this study were based upon a previous study and a different fish batch, caution should be taken when assessing the combined effects as predictions for additivity is highly dependent on high quality concentration response parameters. Thus, any slight difference in sensitivity between the fish batches or small differences in preparation of exposure solutions will make the combined effect predictions uncertain.

The anti-estrogenicity of simple or complex mixtures of compounds with differently acting MoA are still not fully understood as the effects have been proposed dependent on compound, order of exposure and individual concentrations in a mixture (Mortensen and Arukwe, 2007). Previous hepatocyte studies have in agreement with the present findings demonstrated BNF, OHT and a mixture of these to reduce E2-induced Vtg protein expression (Navas and Segner, 2000; Petersen and Tollefsen, 2012). The Vtg protein may accommodate multiple anti-estrogenic mechanisms in the hepatocytes but without fully deciphering the compounds specific anti-estrogenic MoA. The present study therefore analysed ER-mediated (*erα, vtg-1* and *zrp*) (Fig.2-4) and supportive AhR-mediated (*ahrα* and *cyp1a*) transcriptional responses with the objective to characterise how the compounds anti-estrogenic MoA is mediated.

The E2-induced hepatocytes co-exposed with BNF, OHT and BNF+OHT caused transcriptional expression of *erα* (Fig. 2), *vtg-1* (Fig. 3) and *zrp* (Fig. 4) that was parallel to the Vtg protein, illustrating *erα* to have a direct role in regulating downstream target genes. Several of the treatments reduced both Vtg gene and protein expression in a similar manner, illustrating the close coherence between molecular and subcellular response of the estrogen sensitive biomarker, as previously reported elsewhere (Hultman et al., 2015b). The ER-antagonist OHT is a well-described anti-estrogen which competitively bind and partly inhibit ER transcription (Macgregor and Jordan, 1998). Interestingly, OHT is a selective estrogen receptor modulator (SERMs) which act as ER agonist or antagonist depending on tissue in mammalians (Wu et al., 2005). However, the present study concluded that OHT alone caused no induction of *erα* or ER-mediated down-stream target genes *zrp* and *vtg-1* in the primary hepatocytes, findings consistent with OHT eliciting only ER-antagonistic properties in teleost hepatocytes as reported elsewhere (Petersen and Tollefsen, 2012; Smeets et al., 1999).

In agreement with previous teleost hepatocyte studies, BNF caused a reduction of E2-induced *erα*, *vtg-1*, *zrp* transcription and Vtg protein expression (Supplementary table 2) (Gräns et al., 2010; Navas and Segner, 2000; Petersen and Tollefsen, 2012; Smeets et al., 1999). There is currently limited knowledge on the AhR-agonists anti-estrogenic MoA as compounds such as BNF are not ER-ligands (Arcaro et al., 1999; Ebright et al., 1986). However the anti-estrogenic effect of BNF is suggested to suppress the activity of ER via AhR-mediated mechanisms, referred to as a nuclear receptor cross-talk (Navas and Segner, 2000). This putative nuclear receptor cross-talk involves CYP1A-mediated induction of metabolism of active estrogens to water soluble conjugates with potentially lower estrogenic potential. Although several AhR-agonist metabolites have weak affinity for ER by competitively

binding to and initiating or inhibiting transcription (Ebright et al., 1986; Tran et al., 1996), BNF and/or its potential metabolites did not induce ER-mediated activity of *erα, zrp* and *vtg-1* nor suppressed the basal (constitutive) activity of these genes when exposed alone in the present study. BNF were only capable of causing anti-estrogenic effects in presence of E2, thus suggesting that the MoA involved interference with the ER-mediated activation of down-stream genes and signaling pathways.

To further characterise the two anti-estrogenic compounds MoA, a mixture of BNF and OHT was assessed for its potential to contribute to a combined anti-estrogenic effect. The mixture had as observed for Vtg protein the largest suppression on E2-induced genes (*erα, zrp* and *vtg-1*) in the hepatocytes. Knowledge about the potential MoA of the two compounds suggest that the anti-estrogenic effect of the mixture was likely due to interference both with the binding to the ER (i.e. OHT) and by modulating ER-mediated signaling and/or metabolism of E2.

## 4.2 AhR signaling

To decipher the anti-estrogenic MoA of BNF, OHT and BNF+OHT, the compounds AhR-mediated responses were assessed. The AhR-agonist BNF was the only treatment apparently increasing *ahrα* gene expression both in absence and presence of E2, suggestive of the other treatments causing less effect on the *ahrα* transcription. Another reason for the present study's low transcriptional activity of *ahr* may be the AhR-isotype (*ahrα*) is less responsive than other more specific AhR-isotypes (e.g. *ahr2β)* (Gräns et al., 2010). However the AhR downstream target gene *cyp1a* was more responsive than *ahrα* as BNF and the mixture of BNF+OHT both in absence and presence of E2 caused a significant up-regulation, whereas the ER-agonist OHT had no effect on *cyp1a* (Fig. 6).

In agreement with the present findings, fish hepatocytes co-exposed to BNF and E2 caused a similar increase in the CYP1A-mediated EROD activity (Navas and Segner, 2000), confirming BNF to be the contributing factor to the AhR-mediated *cyp1a* transcription in the present study. AhR-agonists such as BNF bind to and activate the nuclear dioxin/xenobiotic response elements (DRE/XRE), which initiates AhR-mediated transcription of *ahr* and down-stream genes such as *cyp1a* and translation into CYP1A. Several studies have reported PAHs to increase metabolism of E2 (Arcaro et al., 1999), thus potentially reducing the presence of E2 in estrogen responsive tissue (Gierthy et al., 1988). Surprisingly, presence of E2 in cells

exposed to BNF and the BNF+OHT mixture had no significant effect on the E2-induced *cyp1a* transcription when compared to cells not treated with E2, indicative of neither *ahrα* nor *cyp1a* being affected by the presence of E2 (Fig. 5 and 6, table 2). Interestingly, CYP1A is proposed to be primarily responsible for E2 metabolism in fish *in vivo* (Scornaienchi et al., 2010). It is therefore suggested that BNF alone mediates its anti-estrogenic MoA by inducing *cyp1a* expression, causing increased CYP1A-mediated metabolism of E2 in the hepatocytes (Miller et al., 1999). In contradiction to this, a primary hepatocyte study reported BNF-induced EROD activity and *cyp1a* transcription to not cause enhanced metabolism of E2 (Navas and Segner, 2000), introducing uncertainties in the proposed *cyp1a*-mediated metabolism of the present study.

The mixture's large anti-estrogenic effect on the E2 induced genes was further suggested caused by the presence of OHT which in a recent *in vitro* study induced *ahr, arnt, cyp1a1* and *cyp1b1* gene expression in human breast cancer cell line MCF7 when exposed to 0.1 $E^{-6}$ M OHT (DuSell et al., 2010). The present study did not demonstrate any induction of *ahrα* or *cyp1a* by OHT in presence or absence of E2, suggesting OHT's AhR-mediated responses (DuSell et al., 2010) to be concentration or potentially tissue dependent. It is therefore proposed that the OHT concentration used herein only elicited direct modulatory properties on ER by reducing E2 sensitive genes (*erα, vtg-1* and *zrp)* and protein (Vtg) expression.

## 4.3 Nuclear receptor cross-talk

The proposed AhR-mediated cross-talk is constituted by several different mechanisms involving competition for common transcription co-factors (e.g. aryl hydrocarbon receptor translocator protein (ARNT)) (Brunnberg et al., 2003; Rüegg et al., 2008), induction of E2 metabolism by CYP1A (Safe and Wormke, 2003), proteasomal degradation of the ER (e.g. cullin 4b (CUL4b)) (Ohtake et al., 2003; Safe and Wormke, 2003), and direct suppression of ER transcription via AhR-ligand binding XRE upstream of ER (Matthews and Gustafsson, 2006). The present study assessed several of these mechanisms by analysing gene expression of *arnt* and *cul4b*, however none of the genes were apparently affected by the differently acting anti-estrogens (Supplementary table 2). The co-regulator *arnt* is a dimerization partner for several basic helix-loop-helix (bHlH)- Per-AhR/ARNT-Sim homology sequence (PAS) protein super families (e.g. AhR) (Gu et al., 2000) and a suggested co-activator for ERα, based on the sequence homology to other ER transcription factors in mammalians (Brunnberg

et al., 2003). However, no significant correlation between *arnt* and *erα* transcription was identified in any of the treatments in the present study, suggestive of *arnt* not having an essential role as an ER co-activator in the present hepatocyte study. One explanation for the unchanged expression is that *arnt* may be associated with its heterodimer partner AhR, as the transcriptional expression of *ahrα* was unaffected in the majority of treatments. However, gene expression does not always reflect the translated protein and its activity and should therefore be studied at the protein level to assess the role of *arnt* as a nuclear receptor co-activator. Furthermore, the low transcriptional activity of *ahrα* may also be associated with the unaffected transcription of *cul4b*, which was assessed for its essential role in the AhR-promoted ubiquitin ligase complex which supposedly increases the proteolysis of ER*α* in mammals (Ohtake et al., 2009). However, as sequences and gene functions may not be conserved over the larger evolutionary spans, the function and involvement of *cul4b* in the AhR-ER nuclear-crosstalk in fish has still to be characterised. The present study therefore suggests that additional work not covered herein should be performed to determine these genes and proteins function in the AhR-ER cross-talk in fish.

## 4.4 Screening anti-estrogens using primary hepatocytes

The present study demonstrated primary hepatocytes to successsfully accommodate differently acting anti-estrogens by assessing Vtg protein expression. However, the single biomarker approach did not characterise the compounds anti-estrogenic MoA. Use of multiple endpoints, as applied herein, is therefore recommended in order to better characterise compounds anti-estrogenic MoA. This approach will lead to a better understanding of how simple and more complex mixtures affect organisms in the aquatic environment. Use of *in vitro* models (e.g. primary hepatocytes) as a screening tool for mixtures might facilitate a better understanding of their MoA without the compensatory regulation and interference of the *in vivo* hypothalamus-pituary-gonad (HPG)-axis, potentially leading to a better characterisation of combined effect of anti-estrogens in the aquatic environment. However, the present study demonstrated that the individual primary hepatocyte cell-batches may introduce large variations in the gene and protein expression, hampering the interpretation of the anti-estrogens single and mixture MoA. The present study illustrate that it is crucial to perform inter-cell batch normalisation using a positive control such as E2, as to obtain more homogenous and reproducible results as previously described by Hultman et al. (2015b). Future studies should therefore account for this and perform intra-cell batch normalisation.

# 5 Conclusion

The present study showed that the differently acting anti-estrogenic compounds BNF, OHT and a mixture of these all reduced the ER-mediated Vtg protein expression, independent of their specific anti-estrogenic MoA. The compounds anti-estrogenic potency caused a similar reduction of E2 induced genes *erα*, *zrp* and *vtg-1*, illustrating their ability to modulate the activity of both ER-sensitive genes and proteins. The strongest anti-estrogenic effects were, as expected, reported in the mixture of BNF and OHT, albeit due to variable ER-responsiveness amongst the cell batches the difference was not statistically significant. The anti-estrogenicity of BNF were mainly proposed caused by induction of AhR-mediated CYP1A biotransformation of E2, whereas OHT likely interfered directly with ER-binding and activation of downstream ER-dependent molecular events. The combination of the two were most likely caused by a combination of the two fundamentally different MoA, and were demonstrated to apparently cause a larger response than that of the single stressors on Vtg protein expression. The present study also assessed the involvement of specific genes involved in putative AhR-ER crosstalk, however none could be directly associated with the compounds anti-estrogenic MoA and their involvement remains inconclusive. In summary, the present study showed that the primary rainbow trout hepatocyte model is a versatile bioassay for screening anti-estrogens, independent of their MoA.

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# 7 Supplementary information

Supplementary Table 1. Overview of mixture exposure concentrations and anticipated effects

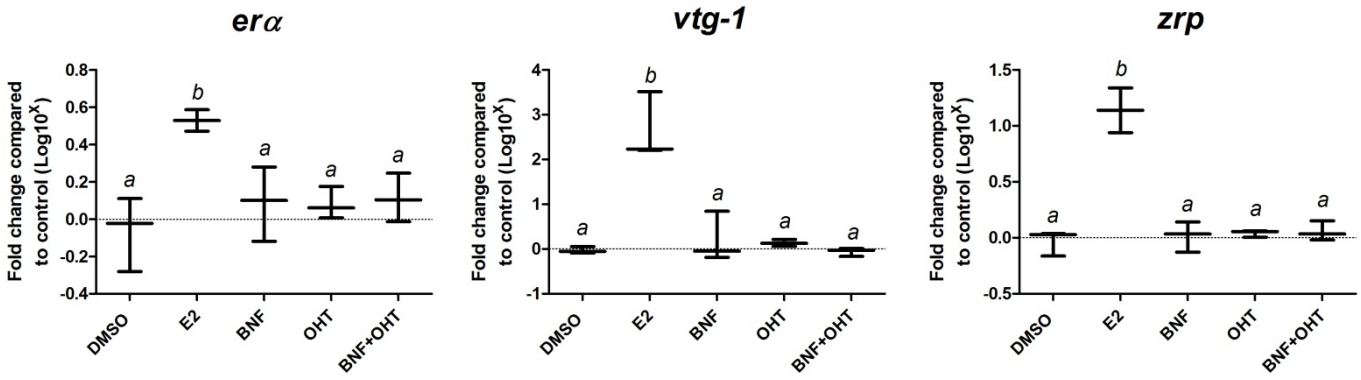
| Mixture | OHT+E2 (mol/L)[a] | BNF+E2 (mol/L) [a] | E2 (mol/L) [a] |
|---|---|---|---|
| OHT+E2 | $2.36E^{-9}$ | | $6.3E^{-10}$ |
| BNF+E2 | | $5.5E^{-8}$ | $6.3E^{-10}$ |
| OHT+BNF+E2 | $2.36E^{-9}$ | $5.5E^{-8}$ | $6.3E^{-10}$ |

a – Concentrations are based on 50% inhibition concentration (IC50) on E2 ($6.3E^{-10}$ M)-induced Vtg protein production (Petersen and Tollefsen, 2012).

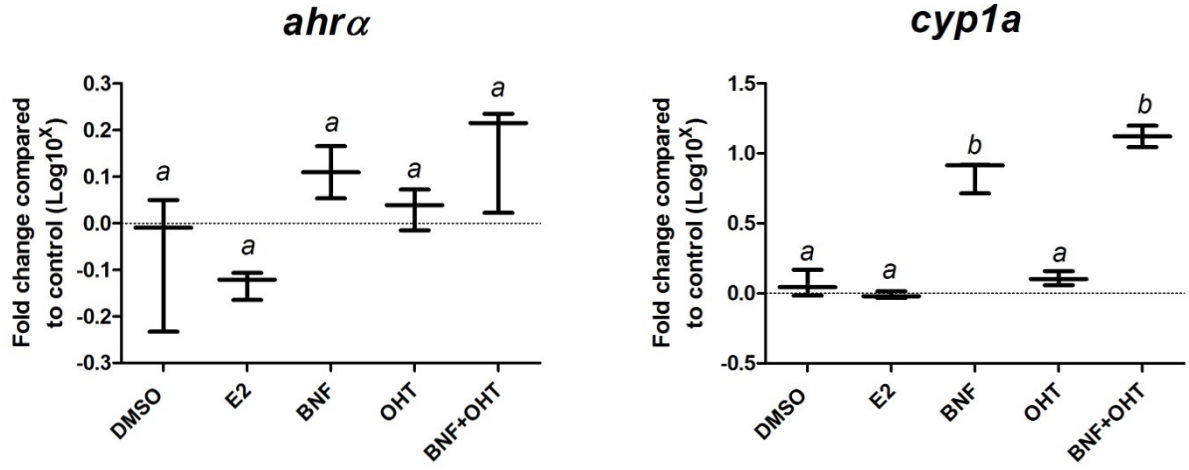
Supplementary Table 2 - Fold change of transformed gene data compared towards the solvent control (DMSO). Data represent the mean of 3-4 individual experiments. The statistical analysis was performed using a one way-ANOVA with a Tukey's post hoc test. Values that are significantly different from each other are denoted with *.

| | | **ER mediated** | | | | **AhR mediated** | | **NR-cross-talk** | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Vtg protein** | ***erα*** | ***zrp*** | ***vtg-1*** | ***ahrα*** | ***cyp1a*** | ***arnt*** | ***cul4b*** |
| ***E2*** | | 10.85* | 16.42 (*) | 14.58(*) | 4341(*) | 1.4 | 0.6* | 0.85 | 1.12 |
| ***BNF*** | ***(+)E2*** | 6.62 | 16.28 | 11.18 | 3865 | 3.49 | 19.53** | 1.029 | 1.22 |
| | ***(-)E2*** | - | 1.36 (**) | 0.54(**) | 3.01(**) | 1.7 | 12.9(*) | -2.29 | -1.72 |
| ***OHT*** | ***(+)E2*** | 8.06 | 14.52 | 12.98 | 4140 | 1.41 | 1.80* | 1.09 | 0.80 |
| | ***(-)E2*** | - | 1.27(**) | 1.29(**) | 2.0(**) | 0.50 | 1.62(**) | -2.21 | -2.22 |
| ***BNF+OHT*** | ***(+)E2*** | 4.34** | 11.86 | 8.05 | 3406 | 3.02 | 20.01** | 0.59 | 0.82 |
| | ***(-)E2*** | - | 1.75(**) | 1.75(**) | 0.9 (**) | 2.44 | 17.03(*) | -3.64 | -3.12 |

**Abbreviations:** erα – estrogen receptor α; zrp – zona radiata; vtg-1 – vitellogenin -1; ahrα – aryl hydrocarbon receptor 1 β; cyp1a – cytochrome p450 1a;arnt; aryl hydrocarbon receptor translocator ; cul4b – cullin 4b.




Supplementary Figure 1. Gene expression of putative transcripts included in the estrogen receptor signaling (ER) pathway in primary rainbow trout (*Oncorhynchus mykiss*) hepatocyte after exposure to $6.3E^{-10}$M 17β-estradiol (E2), $5.5E^{-8}$M β-naphtoflavone (BNF), $2.36E^{-9}$M 4-hyroxytamoxifen (OHT) and mixture of these (BNF+OHT) for 48h. Data represent the mean of 3-4 individual experiments ± standard deviation. The statistical analysis was performed using a one way-ANOVA with a Tukey's post hoc test.




Supplementary Figure 2. Gene expression of aryl hydrocarbon receptor α (*ahrα*) and cytochrome P450 1A (*cyp1a*) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocyte after exposure to $6.3E^{-10}$M 17β-estradiol (E2), $5.5E^{-8}$M β-naphtoflavone (BNF), $2.36E^{-9}$M 4-hyroxytamoxifen (OHT) and mixture of these (BNF+OHT) for 48h. Data represent the mean of 3-4 individual experiments ± standard deviation. The statistical analysis ($p \leq 0.05$) was performed using a one way-ANOVA with a Tukey's post hoc test.

# Paper IV

# Toxicity of organic compounds from unresolved complex mixtures (UCMs) to primary fish hepatocytes

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# Abstract

Many environmental matrices contaminated with organic pollutants derived from crude oil or degraded petroleum, contain mixtures so complex that they are typically unresolved by conventional analytical techniques such as gas chromatography (GC). The resulting chromatographic features have become known as 'humps' or unresolved complex mixtures (UCMs). Over the last 25 years, efforts to resolve and thus identify, or at least to produce average structures, for some UCM components, have proved fruitful. Numerous non-polar UCM hydrocarbons and more polar UCM acids have been synthesised or purchased from commercial suppliers. The prospect of assessment of the ecotoxicological effects and characterisation of the modes of action (MoA) of these environmental pollutants has thus arisen. In the present study, a number of potential UCM chemicals were assessed for cytotoxic effects (membrane disruption and metabolic activity), activation of the aryl hydrocarbon receptor (AhR) and activation of estrogen receptor (ER) in primary rainbow trout hepatocytes (*Oncorhynchus mykiss*), to characterise the toxic mode of action (MoA) of this diverse group of chemicals. The tested chemicals had different hydrophobicity (Log $K_{OW}$), water solubility, and displayed different structural features. The results from the *in vitro* screening indicate that the predominant toxic

MoA was cytotoxicity, and $EC_{50}$ values for cytotoxicity were obtained for 16 compounds and ranged from 77 µM-24 mM, whereof aliphatic monocyclic acids, monoaromatic acids, polycyclic monoaromatic acids and alkylnaphthalenes were the most toxic. The observed cytotoxicity correlated well with the chemicals Log $K_{OW}$ and water solubility, potentially due to most chemicals having a non-specific toxic MoA. Interestingly, a few compounds induced the ER-mediated production of vitellogenin (Vtg) and the AhR-mediated Ethoxyresorufin-O-deethylase (EROD) enzymatic activity, and by doing so suggesting that they may act as estrogen mimics and 'dioxin-like' compounds in fish. The tested UCM compounds appear to be a highly diverse group of chemicals with multiple MoA that may potentially cause adverse effects in fish if exposed to sufficiently high concentrations in the environment. Additional studies to determine if these compounds may cause adverse effects *in vivo* are highly warranted to identify specific compounds of concern.

# 1 Introduction

Many environmental matrices (e.g. water, sediments, biota) contaminated with organic pollutants derived from crude oil or degraded or refined petroleums, such as some lubricating oils, contain mixtures so complex that they are typically unresolved by conventional analytical techniques such as gas chromatography (GC). The resulting gas chromatographic features have become known as 'humps' or unresolved complex mixtures (UCMs), a term first introduced to describe the hydrocarbons found in sewage discharge waters from a large municipal sewage treatment plant, surface sediments and hard shell clams (Farrington and Quinn, 1973). These authors later attributed these UCM features of their chromatograms to contamination from biodegraded oils and/or lubricating oils, following comparison with data for biodegraded reservoir crude oils of the Bell Creek Formation on the border of Montana and Wyoming (reviewed by Farrington and Quinn, 2015).

Over the four decades since 1973, efforts to chromatographically resolve and thus identify individual compounds in UCMs (Frysinger et al., 2003; Gros et al., 2014), or to use degradative methods to produce average structures for some UCM components (Gough and Rowland, 1990; Thomas, 1995; Warton et al., 1999) have proved at least partially fruitful for the hydrocarbons.

The advent of multidimensional GC coupled to modern mass spectrometers (e.g. GCxGC-MS) has also led to partial resolution (usually following derivatisation) even of UCMs of more polar petroleum constituents, such as petroleum acids e.g. naphthenic acids (NAs)(Bowman et al., 2014; Swigert et al., 2015). Such components become more quantitatively important following biodegradation of petroleum e.g. following oil spills (Ruddy et al., 2014), or in oil sands processing; reviewed by Brown and Ulrich (2015).

Numerous examples of non-polar UCM hydrocarbons and more polar UCM acids and hetero compounds, have been synthesised or purchased from commercial suppliers (Rowland et al., 2011b; Smith et al., 2001; West et al., 2014). The prospect of ecotoxicological testing and characterisation of the toxic modes of action (MoA) of these UCM-type components has thus arisen to properly assess their potential for causing adverse effects under ecologically relevant exposure scenarios. One of the methods suitable for screening the toxicity and modes of action (MoA) of UCM compounds is the use of *in vitro* bioassays. These assays offer a high-throughput and multi-endpoint testing capacity. Primary hepatocytes from fish has previously been demonstrated to be a highly versatile multi-endpoint screening assay by assessing a range of endpoints ranging from assessment of cytotoxicity to characterisation of molecular MoA (Finne et al., 2007; Hultman et al., 2015a, 2015b; Petersen and Tollefsen, 2011; Tollefsen et al., 2008a). This analysis include determination of potential ER agonists and antagonist compounds through measuring their potential binding to and activation/inhibition of the estrogen receptor (ER) and downstream processes such as the ER-mediated production of the egg-yolk precursor protein vitellogenin (Vtg) (Petersen and Tollefsen, 2011, 2012; Tollefsen et al., 2008b). Furthermore, analysis of potential aryl hydrocarbon receptor (AhR) agonists of single compounds (e.g. dioxin-like compounds) and complex mixtures of chemicals will be performed through measurement of the AhR-mediated cytochrome P450-dependent monooxygenase modulation of 7-ethoxyresorufin-O-deethylase (EROD) enzyme activity (Melbye et al., 2009; Segner and Cravedi, 2000). In addition to characterising the MoA, primary hepatocytes have been applied in the screening of cytotoxicity of individual synthetic naphthenic acids and mixtures of these (Tollefsen et al., 2012). As the current knowledge of the toxicity of UCMs is limited, conducting multi-endpoint screening of relevant UCM components are highly warranted.

The objective of the present study was to screen a number of synthetic UCM-related chemicals for cytotoxic, 'dioxin-like' and estrogenic properties in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes to characterise the toxic MoAs of this diverse group of chemicals. The chemicals tested were chosen to secure a broad chemical applicability domain by including chemicals with large differences in hydrophobicity (e.g. Log $K_{OW}$), solubility and displaying structural diversity.

# 2 Materials and methods

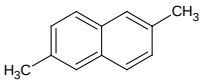
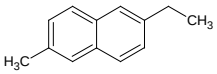
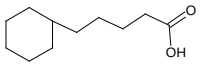
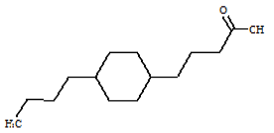
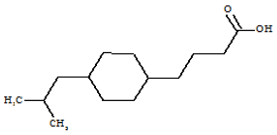
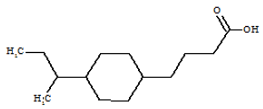
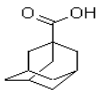
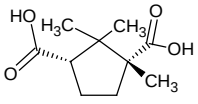
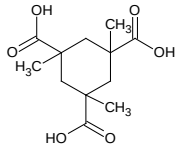
## 2.1 Chemicals

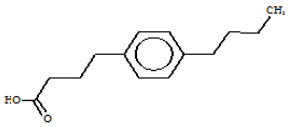
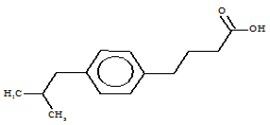
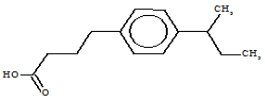
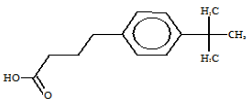
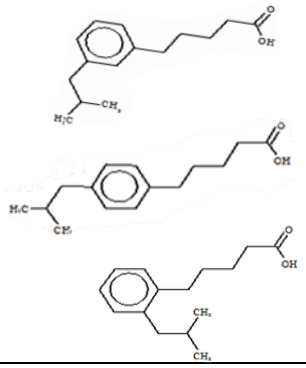
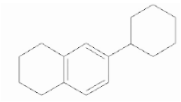
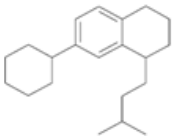
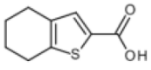
Twenty two substances (Table 1) were chosen for the study. The alkynaphthalenes, 1-Adamantanecarboxylic acid, 3-cyclohexylpentanoic acid, (1R,3S)-(+)-Camphoric acid, 1,3,5-Trimethyl-cyclohexane-1,3,5-tricarboxylic acid, 4,5,6,7-Tetrahydro-1-benzothiophene-2-carboxylic acid, 4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid, 3-methylbenzo[b]thiophene-2-acetic acid and Benzo[b]thiophene-3-acetic acid were obtained from Sigma, whereas 4-(4'-t-butylphenyl)butanoic acid was obtained from Molport (Riga, Latvia). The additional tested compounds were synthesised at Plymouth University, England (Sturt, 2001; Smith, 2002). Chemicals used as positive controls, such as 17β-estradiol (E2, ≥98%, CAS: 50-28-2) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, 10 μg/mL in toluene, CAS: 1746-01-6) were obtained from Sigma–Aldrich (St. Lois, MI, US), while $CuSO_4.5H_2O$ (CAS. 7758-99-8) was purchased from VWR (Merck, city, country). All compounds and standards, with exception of $CuSO_4$ which was spiked into the growth media, were dissolved in dimethylsulfoxide, DMSO (99%, Sigma-Aldrich) and stored in the dark at -20°C until use.

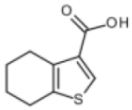
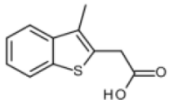
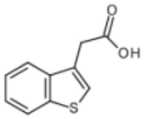
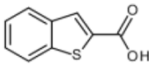
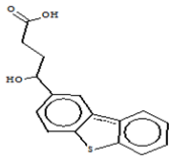
## 2.2 Rainbow trout

Juvenile rainbow trout, *O. mykiss* (size 200-500 g) were purchased from Valdres Ørretoppdrett (Valdres, Norway) and kept at the Institute of Biology at the University of Oslo (Norway) for a minimum of 4 weeks prior to the first experiment. The water parameters were 9 ± 3˚C, 100 % oxygen saturation, pH 6.6 and a 12 h light/12 h dark cycle. The fish were fed daily with pellets (Skretting, Stavanger, Norway) corresponding to approximately 0.5 % of total body mass.

Table 1. Chemicals tested in a multi-endpoint rainbow trout (*Oncorhynchus mykiss*) hepatocyte assay. The name, abbreviation, CAS RN, producer, purity, molecular weight (MW), SMILES and log$K_{OW}$ are shown for all the tested compounds.

| Chemical name | Abbreviation | CAS RN[a] | MW | SMILES | LogKoW | Chemical structure |
|---|---|---|---|---|---|---|
| **Aliphatic naphthalenes** | | | | | | |
| 2,6-dimethylnaphthalene | 2,6-DMN | 581-42-0 | 156.23 | Cc1ccc2cc(C)ccc2c1 | 4.26 [b] |  |
| 2-ethyl-6-methylnaphthalene | 2-E-6-MN | 7372-86-3 | 170.26 | Cc1ccc2cc(ccc2c1)CC | 4.26 [b] |  |
| **Aliphatic monocyclic acids** | | | | | | |
| 3-cyclohexylpentanoic acid | 3-CHPA | 13126-82-4, 5456-30-4, 5962-88-9 | 184.28 | O=C(O)CCCCC1CCCCC1 | 4.32 |  |
| 4-(4'-n-butylcyclohexyl)butanoic acid | 4-(4-n-BCH)BA | NR | 226.36 | C(=O)(O)CCCC1CCC(CCCC)CC1 | 4.81 |  |
| 4-(4'-i-butylcyclohexyl)butanoic acid | 4-(4-i-BCH)BA | NR | 226.36 | C(=O)(O)CCCC1CCC(CC(C)C)CC1 | 5.65 |  |
| 4-(4'-s-butylcyclohexyl)butanoic acid | 4-(4-s-BCH)BA | NR | 226.36 | C(=O)(O)CCCC1CCC(C(C)CC)CC1 | 5.65 |  |
| **Aliphatic tricyclic acid** | | | | | | |
| 1-Adamantanecarboxylic acid | 1-ACA | 828-51-3 | 180.25 | O=C(O)C12CC3CC(C1)CC(C2)C3 | 3.15 |  |
| **Monocyclic di-acid** | | | | | | |
| (1R,3S)-(+)-Camphoric acid | Camphoric acid | 124-83-4 | 200.24 | C[C@]1(CC[C@H](C(=O)O)C1(C)C)C(=O)O | 1.78 |  |
| **Monocyclic tri-acid** | | | | | | |
| 1,3,5-Trimethyl-cyclohexane-1,3,5-tricarboxylic acid | 1,3,5-tricarboxylic acid | 118514-35-5 | 258.27 | OC(=O)C1(C)CC(C)(CC(C)(C1)C(=O)O)C(=O)O | 0.13 |  |

| Chemical name | Abbreviation | CAS RN[a] | MW | SMILES | LogKoW | Chemical structure |
|---|---|---|---|---|---|---|
| **Monoaromatic acids** | | | | | | |
| 4-(4'n-butylphenyl)butanoic acid | 4-(4-n-BPh)BA | NR | 220.31 | c1(CCCC)ccc(CCCC(=O)O)cc1 | 5.8 |  |
| 4-(4'-i-butylphenyl)butanoic acid | 4-(4-i-BPh)BA | NR | 220.31 | c1(CCCC(=O)O)ccc(CC(C)C)cc1 | 4.72 |  |
| 4-(4'-s-butylphenyl)butanoic acid | 4-(4-s-BPh)BA | NR | 220.31 | c1(C(C)CC)ccc(CCCC(=O)O)cc1 | 4.72 |  |
| 4-(4'-t-butylphenyl)butanoic acid | 4-(4-t-BPh)BA | 24475-36-3 | 220.31 | C(C)(C)(C)c1ccc(CCCC(=O)O)cc1 | 4.69 |  |
| (iso-butylphenyl)pentanoic acid | (i-BPh)PA | NR | 234.34 | c1(CCCCC(=O)O)ccc(CC(C)C)cc1<br>c1(CCCCC(=O)O)cc(CC(C)C)ccc1<br>c1(CCCCC(=O)O)c(CC(C)C)cccc1 | 5.22 |  |
| **Polycyclic monoaromatic hydrocarbons** | | | | | | |
| 6-cyclohexyltetralin | 6-CHT | NR | 214.35 | C3CCC(c2ccc1CCCCc1c2)CC3 | 6.77[b] |  |
| 7-cyclohexyl-1-isoamyltetralin | 7-CH-1-IAT | NR | 284.49 | c12c(C(CCC(C)C)CCC1)cc(C1CCCCC1)cc2 | 9.08[b] |  |
| **Monocyclic thiophenic carboxylic acid** | | | | | | |
| 4,5,6,7-Tetrahydro-1-benzothiophene-2-carboxylic acid | 1-Bthio-2-CA | 40133-07-1 | 182.24 | c1c2c(sc1C(=O)O)CCCC2 | 3.66 |  |

| Chemical Name | Abbreviation | CAS RN[a] | MW | SMILES | LogKow | Chemical structure |
|---|---|---|---|---|---|---|
| **Monocyclic thiophenic carboxylic acid** | | | | | | |
| 4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid | B[b]Thio-3-CA | 19156-54-8 | 182.24 | c1c(c2c(s1)CCCC2)C(=O)O | 3.66 |  |
| **Thiophenic alkanoic acids** | | | | | | |
| 3-methylbenzo[b]thiophene-2-acetic acid | 3-MB[b]Thio-2-AA | 1505-52-8 | 206.26 | Cc1c2ccccc2sc1CC(=O)O | 2.97 |  |
| Benzo[b]thiophene-3-acetic acid | B[b]Thio-3-AA | 1131-09-5 | 192.23 | c1ccc2c(c1)c(cs2)CC(=O)O | 2.42 |  |
| **Monoaromatic thiophenic carboxylic acid** | | | | | | |
| Benzothiophene-2-carboxylic acid | BThio-2-CA | 6314-28-9, 527-72-0, 900791-89-1 | 178.21 | c1ccc2c(c1)cc(s2)C(=O)O | 2.87 |  |
| **Diaromatic thiophenic alkanoic acid** | | | | | | |
| 4-dibenzothiophen-2'yl-4-hydroxybutanoic acid | 4-DBThio-BA | NR | 286.35 | C1=CC=C2C(=C1)C3=C(S2)C=CC(=C3)C(CCC(=O)O)O | 3.29[b] |  |

**[a]RN numbers obtained from chemspider (http://www.chemspider.com); [b]Predicted with ECOSAR (http://www.epa.gov/oppt/newchems/tools/21ecosar.htm); NR – Not reported.**

## 2.3 Isolation and exposure of hepatocytes

Primary cultures of rainbow trout hepatocytes were obtained by a 2-step perfusion of livers from juvenile fish as described in Tollefsen et al. (2003). Cell viability was determined with the trypan blue exclusion test and isolations with ≥85 % viability were diluted to 500 000 cells/ml and plated into 96-well Primaria™ plates, 200 µl/well (Falcon, Becton Dickinson Labware, Oxnard, CA, USA). Cells were incubated at 15˚C for 24 h prior to replacement of half the volume of media with media containing the solvent control (DMSO, 0.1-1%, v/v) or increasing concentrations of the test chemicals or standards.

In order to characterise the MoA, the endpoints cytotoxicity (96h), induction of Vtg (96h) and EROD (48h) were determined in the hepatocytes to obtain information about the acute toxic potency, the estrogenic potential and "dioxin" activity, respectively. Positive controls (E2 for Vtg, TCDD for EROD, and $CuSO_4$ for cytotoxicity) were used for all assays to calculate the relative responses of the different chemicals (see below for details).

After 48 h exposure, media was removed from cells targeted for EROD analysis, where upon the cells were frozen and stored at -80$^{o}$C until further analysis. Cells to be targeted for cytotoxicity and Vtg analysis were re-exposed for additional 48 h. At the end of the 96 h exposure period, 100 µL growth media from each well were transferred to Maxisorp Nunc-immunoplates (Nunc, Roskilde, Denmark), sealed with sealing tape (Nunc, Roskilde, Denmark) and frozen at -80˚C for subsequent analysis of Vtg. The cytotoxicity was measured directly in the microplate wells at the end of the exposure period.

## 2.4 Cytotoxicity determination

Cytotoxic effects were measured as decrease in metabolic activity and/or membrane integrity essentially as described by Schreer et al. (2005) using the two probes: Alamar blue (AB) and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM), respectively. After 96 h exposure, the exposure media was replaced with Tris buffer (50 mM, pH 7.5) containing 5% AB and 4 µM CFDA-AM. Plates were incubated in the dark at room temperature on an orbital shaker (100 rpm, 30 min) and fluorometric readings were performed with a Victor $V^3$ multilabel counter (Perkin Elmer, Waltham, MA, USA) using excitation and emission wavelength pairs of 530-590 (AB) and 485-530 (CFDA-AM). The cell viability was expressed relative to the solvent control (100% cell viability) and the positive control exposed to 0.01 M $CuSO_4$ (maximum loss of viability, 0%).

## 2.5 Ethoxyresorufin-O-deethylase (EROD) activity analysis

EROD activity was determined directly in the microplate wells by the cellular conversion of ethoxyresorufin (ER), a substrate for the CYP1A isoenzymes, to resorufin (RR) essentially as described in Tollefsen et al. (2006). In brief, the microplates containing cells were thawed on ice

before being incubated for 15 minutes in 50 mM Tris buffer containing 0.1 M NaCl, 20 µM dicumarol, 2 µM ER, 100 µM beta-NADPH (200 µl pr. well). Fluorescence was measured with excitation and emission wavelength pair of 530 nm and 595 nm, respectively. The protein concentrations were measured with the Bradford method, using bovine gamma-globulin (Bio-Rad, Hercules, CA, USA) as protein standard. The cellular EROD activity was expressed relative to the solvent control (no induction of EROD activity, 0%) and the positive control exposed to 0.3 nM TCDD (maximum EROD induction, 100%) after normalisation to protein content.

## 2.6 Vitellogenin analysis

Vitellogenin was measured directly in the growth media according to the method described by Tollefsen et al. (2003). Plates containing cell culture media collected after 96 h exposure were thawed for a minimum 4 h at 4 ˚C before 100 µl standards (rainbow trout Vtg) was applied to assigned (empty) wells and the plates were further incubated overnight (16 h) in the dark at 4˚C. Vitellogenin capture ELISA was performed with the monoclonal mouse anti-salmon Vtg (BN-5, Biosense Laboratories, Bergen, Norway) and the secondary antibody goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA) both diluted 1:6000 in PBS containing 1 % BSA. After the final washing step, a HRP enzyme substrate (TMB plus, KEMENTEC diagnostics, Taastrup, Denmark) was added to initiate the color development and the reaction was stopped after 15 min. by addition of 50 µl $H_2SO_4$ (1 M). The colour absorbance was measured at 450 nm using a Thermomax microplate reader (Molecular Devices, USA). The cellular Vtg production was expressed relative to the solvent control (no induction of Vtg production, 0%) and the positive control exposed to 30 nM 17-β-estradiol (maximum induction of Vtg production, 100%).

## 2.7 Data analysis

The responses of the tested compounds were analysed with GraphPad Prism v6.01 software (GraphPad Software Inc., San Diego, CA, USA). The measured endpoints are all expressed as percent of induction/reduction towards their solvent control and their individual positive controls. A sigmoidal concentration-response curve (CRC) with variable slope was fitted to experimental data using the equation 1. As the responses were normalised from 0-100, the bottom and top values were constrained to 0 and 100 respectively.

$$Y = Bottom + \frac{(Top-Bottom)}{(1+10\text{^}((Log\ EC50-X)*Slope))} \quad (1)$$

The quality of the fit was determined and $R^2$ values > 0.7 were considered indicative of a good model fit (Lundstedt et al., 1998). The $EC_{50}$ and $EC_{10}$ values derived from the CRCs were used to establish fixed effect concentrations and assess the potency of the different test chemicals. Where the observed response did not reach a 50% reduction or induction, the modeled CRCs were used to estimate the effect concentrations. In cases where the CRC could not be fitted, but exposure resulted in a >20% response compared to control, these data were also reported.

A PCA analysis was applied to identify correlations between different physico-chemical properties ($LogK_{ow}$ and water solubility), chemical grouping (see table 2), predicted baseline toxicity in fish ($EC_{50,}$ see effect and MoA predictions below) and the observed cytotoxicity to primary rainbow trout hepatocytes ($EC_{50}$). Computations were performed using XLSTAT2015® with a p-value <0.05 considered to be statistically significant. Variables identified as relevant for the present study were further analysed in a linear regression and correlation analysis.

A linear regression analysis was performed using GraphPad Prism v6.01 software (GraphPad Software Inc., San Diego, CA, USA), reporting the regression line's Goodness of fit, followed by a Pearson correlation analysis ($R^2$). No statistically verified outliers were identified in the data set when applying GraphPad Prism outlier-test, however data points identified as apparently visually different was marked with a gray ring. The analysed data was log(10)-transformed prior to the regression analysis.

## 2.8 Lethality and MoA predictions

Effect concentrations ($LC_{50}$) for baseline toxicity (lethality) in fish using the Ecological Structure Activity Relationships (ECOSAR) and MoA based on Russom classification (Russom et al., 1997) were predicted on basis of quantitative structure activity relationship (QSAR) models using the Chemprop ver. 2 software (http://www.ufz.de/index.php?en=6738).

# 3 Results

All test chemicals were screened for cytotoxicity and induction of Vtg production and EROD activity. All endpoint analysis yielded high quality concentration response curves ($R^2 > 0.92$) for the positive controls $CuSO_4$ (cytotoxicity), E2 (Vtg induction) and TCDD (EROD activity) (Data not shown).

## 3.1 Cytotoxicity

The acute toxicity (96 h) in fish was predicted using Chemprop. The Russom classification model predicted all 22 compounds to have a narcotic MoA. However, the model demonstrated to only be applicable for the two compounds 2,6-DMN and 2-E-6-MN. The remaining compounds were classified as being on the border but outside the applicability range of the classification. Fish $LC_{50}$-values (base line toxicity) were estimated using the built-in ECOSAR model in Chemprop. The predicted $LC_{50s}$ for baseline toxicity and evaluation of whether being within the applicability domain of the QSAR model in fish are given in Table 2. The chemicals which were predicted as least toxic of the 22 compounds were Camphoric acid and 1,3,5-tricarboxylic acid, also being the most hydrophilic compounds (Table 1 and 2). The $LC_{50}$ for 8 of the 22 compounds were estimated to be above the predicted water solubility limit generally by a factor of 2-27 fold (7-CH-1-IAT by a factor of 97 849 fold), two of which were predicted to be the most toxic (6-CHT with $LC_{50}$ of 2 µM and 7-CH-1-iAT with $LC_{50}$ of 0.037µM). The predicted $LC_{50s}$ for fish varied by more than five orders of magnitude and ranged from 37 nM to 12 mM for the compounds investigated. The compounds with highest predicted toxicity to fish were the polycyclic monoaromatic acids, whereas the monocyclic di-acid and monocyclic tri-acid had the lowest predicted toxicities of those tested.

Concentration-dependent reductions in metabolic activity and membrane integrity in exposed primary hepatocytes occurred at fairly similar concentrations for most chemicals (table 2). The inhibitory effects on metabolic activity seemed to be slightly greater than loss of membrane integrity in most cases (Figure 1), and led to the use of metabolic activity as the most relevant parameter for developing CRCs. Of the 22 compounds assayed, 20 reduced the metabolic activity to less than 80% of the control. High quality CRCs with $R^2 > 0.7$ were obtained for 16 of these compounds. Only three compounds (1,3,5-tricarboxylic acid, 3-MB[b]Thio-2-AA and B[b]Thio-3-AA) displayed $EC_{50}$ values and additional two compounds (1-ACA and BThio-2-CA) yielded

$EC_{10}$ values for cytotoxicity below the predicted limit for water solubility. Based on the CRC-estimated $EC_{50}$ values for cytotoxicity (ranging from 0.077 to 24 mM for metabolic activity), the order of potency was 4-(4-n-BCH)BA > 4-(4-s-BCH)BA > 7-CH-1-iAT > 4-(4-i-BCH)BA > 2-E-6-MN > 4-(4-s-BPh)BA > 6-CHT > 4-(4-t-BPh)BA > (i-BPh)PA > 4-(4-n-BPh)BA > 4-(4-i-BPh)BA > 3-MB[b]Thio-2-AA > B[b]Thio-3-AA > BThio-2-CA > 1-ACA > 1,3,5-tricarboxylic acid (Table 2).

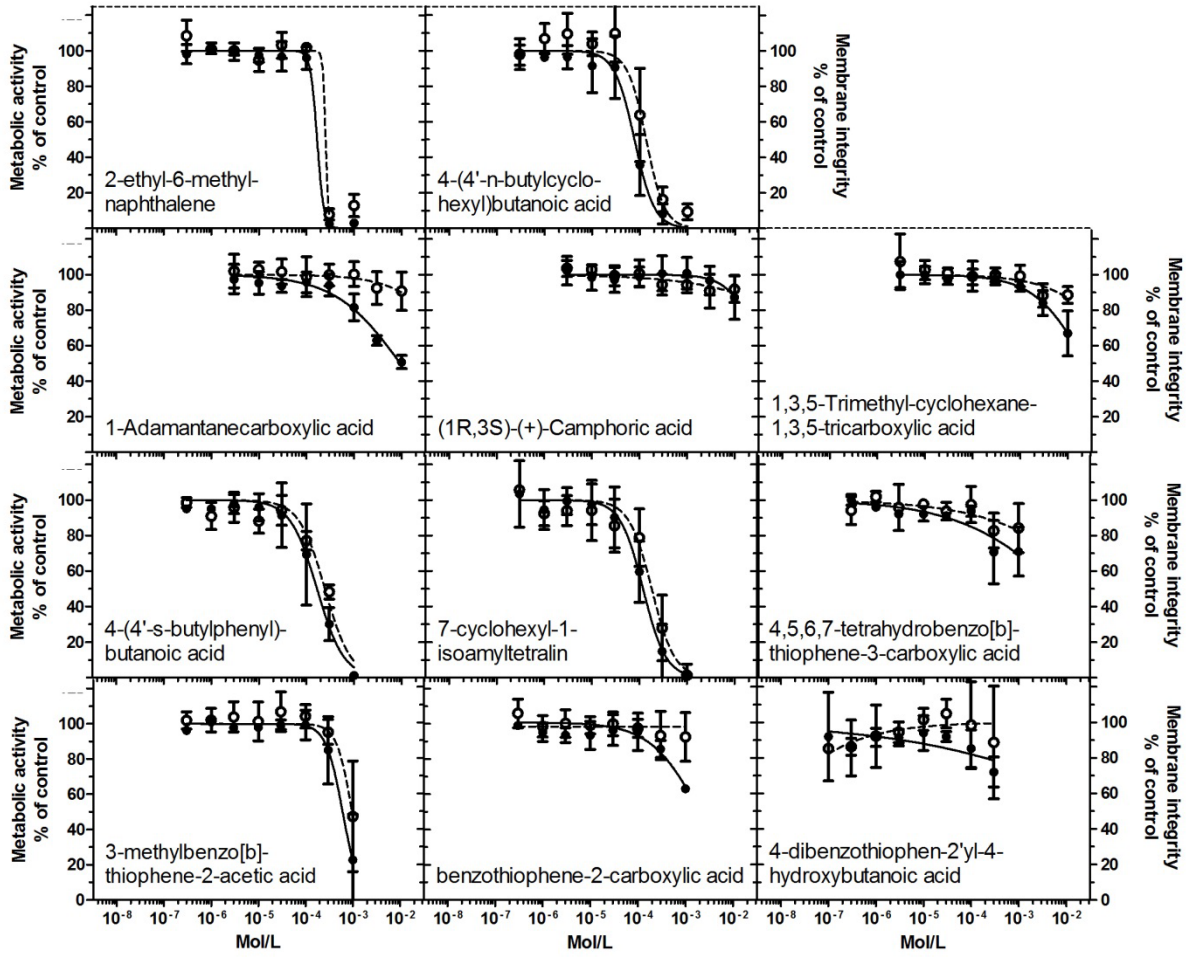



Figure 1. Metabolic activity (●) and membrane integrity (○) as measures for cytotoxicity in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed for 96 h to a selection of compounds associated with the Unresolved Complex Mixture. The data (mean ± standard deviation) represent three independent exposure studies.

The most cytotoxic compounds were aliphatic monocyclic acids, alkylnaphthalenes, polycyclic monoaromatic acids and monoaromatic acids. Compounds eliciting low cytotoxicity, hence no CRCs could be derived, belonged to the chemical groups of alkylnaphthalenes, aliphatic monocyclic di-acids, monocyclic di-acids, and thiophenic acids.

For six of the tested compounds, no $EC_{50}$ could be predicted due to low toxicity at the concentrations tested (up to 1 mM). The ECOSAR predicted toxicity ($LC_{50}$) of these six compounds to fish, ranging from about 20 µM to 5 mM (Table 2). The predicted $LC_{50}$ values for *in vivo* fish toxicity were typically around 1.2 to 20 times lower than the experimental $EC_{50}$ for cytotoxicity in the primary hepatocytes, with some exceptions (e.g. 7-CH-1-IAT: 2560 times difference in toxicity).

## 3.2 EROD activity

The induction of EROD activity, indicative of the activation of the AhR 'dioxin like' effects of chemicals, was analysed after 48 h exposure to the 22 investigated compounds. Of these, 6 compounds (3-MB[b]Thio-2-AA, B[b]Thio-3-AA, B[b]Thio-3-CA, 7-CH-1-iAT, 4-(4-n-BPh)BA, 4-(4-t-BPh)BA) induced the EROD activity to more than 20% of the positive control (Figure 2). However, reduced EROD-activity was associated with induced cytotoxicity (>20%) in the majority of compounds (Figure 1), masking the compounds MoA through onset of cytotoxicity. Of the 6 potential AhR agonists, only three (3-MB[b]Thio-2-AA, B[b]Thio-3-CA and 4-(4-t-BPh)BA) induced EROD activity at concentrations below the predicted water solubility limit. The compounds shown to induce the EROD activity were monoaromatic thiophenic alkanoic and carboxylic acids, polycylic monoaromatic acids and monoaromatic acids (table 2). Worth mentioning is that other compounds belonging to these groups (except monoaromatic thiophenic alkanoic acids) did not induce any EROD activity, indicating substantial differences in potency or even MoA between fairly similar compounds. The $EC_{50}$ values for EROD induction could only be obtained from the concentration response curves for two compounds ($R^2$ >0.7); 3-MB[b]Thio-2-AA ($EC_{50}$ = 0.50 mM) and B[b]Thio-3-AA ($EC_{50}$ = 1.6 mM), both belonging to the group of monoaromatic thiophenic alkanoic acids (Table 2). The compounds $EC_{50}$ values were estimated from the extrapolated CRCs, as 50% EROD induction

was not reached for the concentration range in any compound tested. Full overview of the experimental data for all compounds tested is presented in table 2.

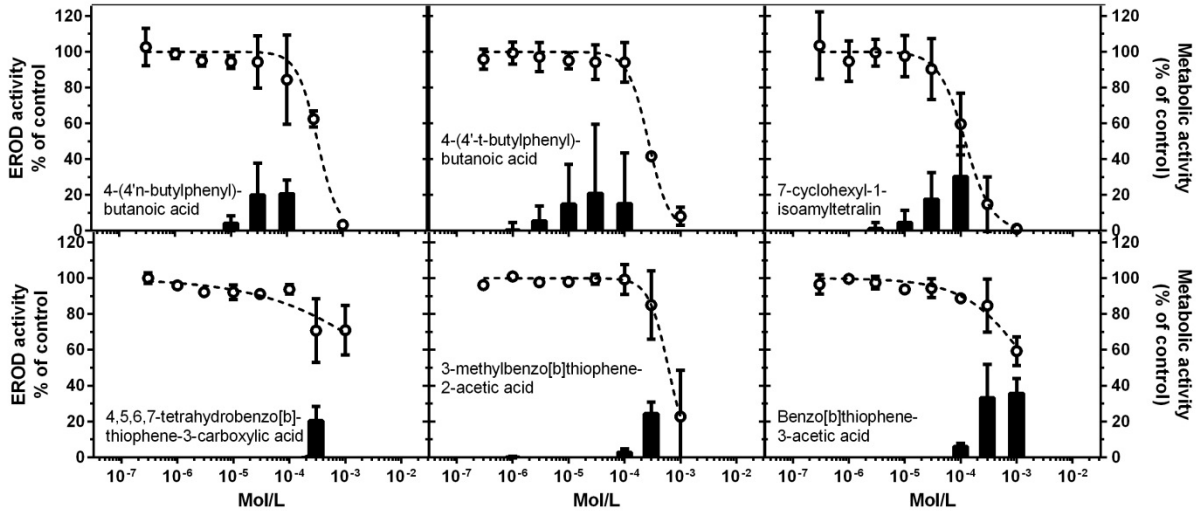



Figure 2. Ethoxyresorufin-O-deethylase (EROD) activity (columns) and metabolic activity (○) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed for 48 and 96 h respectively to Unresolved Complex Mixture (UCM)-related compounds. The data (mean ± standard deviation) consists of a minimum of three individual exposure studies.

## 3.3 Vitellogenin induction

The induction of Vtg, a measure of activation of the ER, was assessed for all 22 investigated compounds. Of the compounds tested, the two compounds 2,6-DMN and 6-CHT induced more than 20% Vtg production compared to the positive control (Figure 3). Only 6-CHT displayed a clear concentration-dependent response up to 0.1 mM, where a sharp reduction in Vtg production due to cytotoxicity occurred. Hepatocytes exposed to 2,6-DMN did not elicit a full Vtg concentration response curve, probably due to solubility issues at the two highest tested concentrations as precipitates were observed in the exposure media. Full overview of the experimental data is presented in table 2.

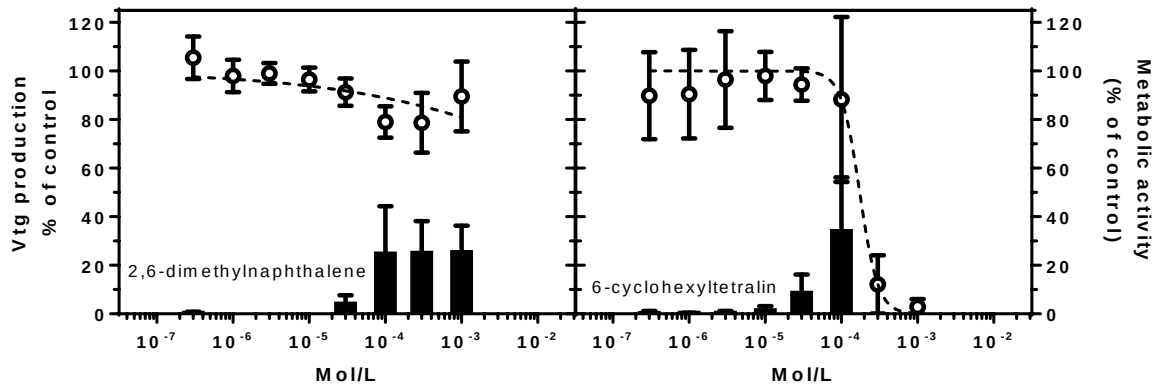



Figure 3. Vitellogenin (Vtg) (columns) and metabolic activity (○) in rainbow trout (*Oncorhynchus mykiss*) hepatocytes exposed for 96 h to the estrogen receptor (ER) agonistic Unresolved Complex Mixture (UCM) compounds 2,6-dimethylnaphthalene and 6-cyclohexyltetralin. The data (mean ± standard deviation) consists of a minimum of three exposure studies.

## 3.4 Principal Component Analysis (PCA)

A PCA was performed to investigate any correlations between the compound related factors (e.g. chemicals physico-chemical properties, chemical grouping) and the predicted lethality in fish and experimental cytotoxicity data (Figure 4). The PCA analysis represented 93% (PCA1:70.6%; PCA2:23.3%) of the total variance, where PCA 2 clearly separated endpoints of observed/predicted toxicity and water solubility, from the hydrophobicity (Log $K_{ow}$) and chemical grouping. The results showed a significant relationship between water solubility of the tested compounds and the predicted and observed toxicity ($EC_{50}$) respectively. Furthermore, factors related to the chemical grouping (structure) demonstrated to be within a moderate but significant proximity of the compounds individual Log $K_{ow}$, predicted ($LC_{50}$) and observed ($EC_{50}$) toxicity.

Table 2. Cytotoxic, estrogenic (vitellogenin induction) and dioxin-like (EROD activity) potency of compounds associated with the Unresolved Complex Mixture (UCM). The data represent the compounds` predicted water solubility, predicted baseline toxicity in fish ($LC_{50}$), and experimental data ($EC_{10}$, $EC_{50}$ and 20% efficiency) for cytotoxicity (96 h), estrogenic (96 h) and dioxin-like (48 h) activity in the rainbow trout (*Oncorhynchus mykiss*) hepatocytes. The data was derived from minimum 3 independent studies.

| Compound | Predicted values | | Experimental values | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Water sol. (mM) | Fish toxicity (96h) | Cytotoxicity (96h) | | | EROD activity (48h) | | | Vtg (96h) | |
| | | $LC_{50}$, mM [a] | $EC_{50}$ (95% CI), mM [b] | $EC_{10}$, mM | >20% effect [c] | $EC_{50}$ (95% CI), mM | $EC_{10}$, mM | ≥ 20% Effect | $EC_{50}$ (95% CI), mM | ≥ 20% Effect |
| **Aliphatic naphthalenes** | | | | | | | | | | |
| 2,6-dimethylnaphthalene | 0.030[g] | 0.021 | n.a. | n.a. | YES | n.a. | n.a. | NO | 63 | YES |
| 2-ethyl-6-methylnaphthalene | 0.0063[g] | 0.010 | 0.17 (0.14 - 0.21) [d] | 0.12 [d] | YES | n.a. | n.a. | NO | na. | NO |
| **Aliphatic monocyclic acids** | | | | | | | | | | |
| 3-cyclohexylpentanoic acid | 0.23[h] | 0.095 | n.a. | n.a. | YES | n.a. | n.a. | NO | n.a. | NO |
| 4-(4'-n-butylcyclohexyl)butanoic acid | 0.0046 | 0.0085 | 0.077 (0.063 – 0.096) [d] | 0.027 | YES | n.a. | n.a. | NO | n.a. | NO |
| 4-(4'-i-butylcyclohexyl)butanoic acid | 0.0053 | 0.010 | 0.13 (0.098-0.18) [d] | 0.026[d] | YES | n.a. | n.a. | NO | n.a. | NO |
| 4-(4'-s-butylcyclohexyl)butanoic acid | 0.0053 | 0.011 | 0.097 (0.082 – 0.12) [d] | 0.030 [d] | YES | n.a. | n.a. | NO | n.a. | NO |
| **Aliphatic tricyclic acid** | | | | | | | | | | |
| 1-adamantanecarboxylic acid | 3.3[h] | 0.83 | 9.4 (6.7-13)[d,j] | 0.29 | YES | n.a. | n.a. | NO | n.a. | NO |
| **Monocyclic di-acid** | | | | | | | | | | |
| (1R, 3S)-(+)-Camphoric acid | 16[g] | 4.9 | n.[d]. | n.[d]. | NO | n.a. | n.a. | NO | n.a. | NO |
| **Monocyclic tri-acid** | | | | | | | | | | |
| 1,3,5-trimethyl-cyclohexane-1,3,5-tricarboxylic acid | 67[h] | 12 | 24 (14-40)[d,j] | 1.6[d] | YES | n.a. | n.a. | NO | n.a. | NO |
| **Monoaromatic acids** | | | | | | | | | | |
| 4-(4'-n-butylphenyl)butanoic acid | 0.031 | 0.023 | 0.35 (0.29 – 0.43) [d] | 0.14 [d] | YES | 0.55 | n.a. | YES | n.a. | NO |
| 4-(4'-i-butylphenyl)butanoic acid | 0.036 | 0.028 | 0.40 (0.31 – 0.52) [d] | 0.079 [d] | YES | n.a. | n.a. | NO | n.a. | NO |
| 4-(4'-s-butylphenyl butanoic acid) | 0.036 | 0.025 | 0.17 (0.13 – 0.22) [d] | 0.040 [d] | YES | n.a. | n.a. | NO | n.a. | NO |
| 4-(4'-t-butylphenyl)butanoic acid | 0.039 | 0.025 | 0.27 (0.23 – 0.31) [d] | 0.11 [d] | YES | n.a. | n.a. | YES | n.a. | NO |
| (iso-butylphenyl)pentanoic acid | 0.011 | 0.012 | 0.29 (0.26 – 0.32) [d] | 0.12 [d] | YES | n.a. | n.a. | NO | n.a. | NO |
| **Polycyclic monoaromatic acid** | | | | | | | | | | |
| 6-cyclohexyltetralin | $5.1E^{-4}$ [i] | 0.0020 | 0.17 (0.12-0.24) [d] | 0.095 [d] | YES | n.a. | n.a. | NO | 0.16 | YES |
| 7-cyclohexyl-1-isoamyltetralin | $9.3E^{-7}$ | $3.7E^{-5}$ | 0.12 (0.091 – 0.16) [d] | 0.036 [d] | YES | 0.26 | n.a. | YES | n.a. | NO |
| **Monocyclic thiophenic carboxylic acid** | | | | | | | | | | |
| 4,5,6,7-Tetrahydro-1-benzothiophene-2-carboxylic acid | 0.22[h] | 0.20 | n.a. | n.a. | YES | n.a. | n.a. | NO | n.a. | NO |
| 4,5,6,7-tetrahydrobenzo[b]thiophene-3-carboxylic acid | 0.22[h] | 0.27 | n.a. | n.a. | YES | n.a. | n.a. | YES | n.a. | NO |
| **Monoaromatic thiophenic alkanoic acid** | | | | | | | | | | |
| 3-methylbenzo[b]thiophene-2-acetic acid | 1.4 [h] | 0.13 | 0.61 (0.48 - 0.77) | 0.25 | YES | 0.50 (0.39 - 0.64) [e,f] | 1.3 | YES | n.a. | NO |
| Benzo[b]thiophene-3-acetic acid | 5.3 [h] | 0.41 | 1.9 (1.1 - 3.1) [e] | 0.10 | YES | 1.6 (0.7-3.5) | 0.095 | YES | n.a. | NO |
| **Monoaromatic thiophenic carboxylic acid** | | | | | | | | | | |
| Benzothiophene-2-carboxylic acid | 1.1[h] | 0.35 | 2.0 (1.1-3.6)[d,j] | 0.15 | YES | n.a.[f] | n.a. | NO | n.a. | NO |
| **Diaromatic thiophenic alkanoic acid** | | | | | | | | | | |
| 4-dibenzothiophen-2'yl-4-hydroxybutanoic acid | 0.016[g] | 0.057 | n.a. | n.a. | NO | n.a. | n.a. | NO | n.a. | NO |

a – 50% lethal concentration. b – 10 or 50% effect concentration. c – 20% or higher effect is observed in measured endpoint. d- above predicted water solubility. e – predicted, effect concentration predicted by the concentration-response curve at higher than tested concentrations. f – potential masking by cytotoxicity. g- Predicted by ECOSAR. h- Predicted with ALOGPS based on LogKow. i- (Smith et al., 2001). j - outside the tested concentration range, estimated value. n.a. not applicable. Not possible to model a concentration-response curve with $R^2 \geq 0.7$.

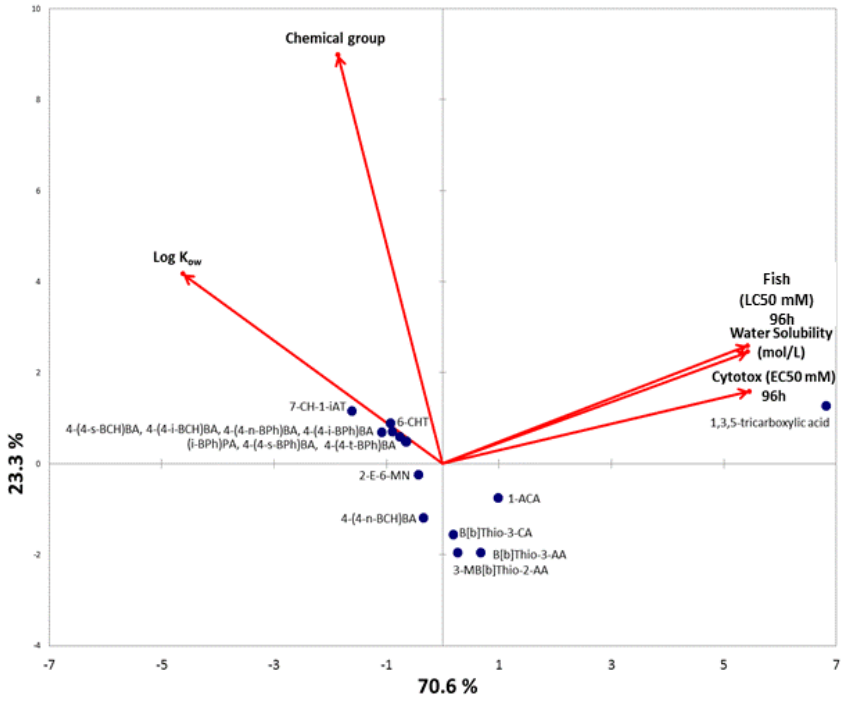



Figure 4. Principal component analysis (PCA) of measured cytotoxicity at 50% effect concentration ($EC_{50}$) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes and predicted acute toxicity at $EC_{50}$ fish when exposed to UCM-related compounds.

The overall PCA reported that there was a close proximity between the compounds physico-chemical properties (e.g. Log $K_{ow}$) and their toxicity (cytotoxicity and predicted baseline toxicity in fish). This was further identified more specifically as a close significant ($p \leq 0.0001$) proximity for the compounds` water solubility and their toxicity to primary hepatocytes. The PCA also reported that there is a close significant ($p \leq 0.0001$) relationship between the observed *in vitro* cytotoxicity and the predicted baseline toxicity to fish (fish $LC_{50}$).

To further investigate the most significant relationships, a linear regression and correlation analysis were conducted between the most relevant data (Figure 5). The correlation analysis reported that the observed cytotoxicity ($EC_{50}$) and the predicted base line toxicity ($LC_{50}$) were significant moderately correlated ($R^2=0.729$, $p \leq 0.0001$). The compound which had the highest deviation between the observed and predicted toxicity was 7-CH-1-IAT (marked with a grey dotted ring in Figure 5). Moreover, the observed $EC_{50}$ was significantly negatively correlated with the predicted log $K_{ow}$ ($R^2=0.626$, $p \leq 0.0003$) of the compounds, whereas a significant positive correlation was reported for the water solubility ($R^2=0.657$, $p \leq 0.0001$).

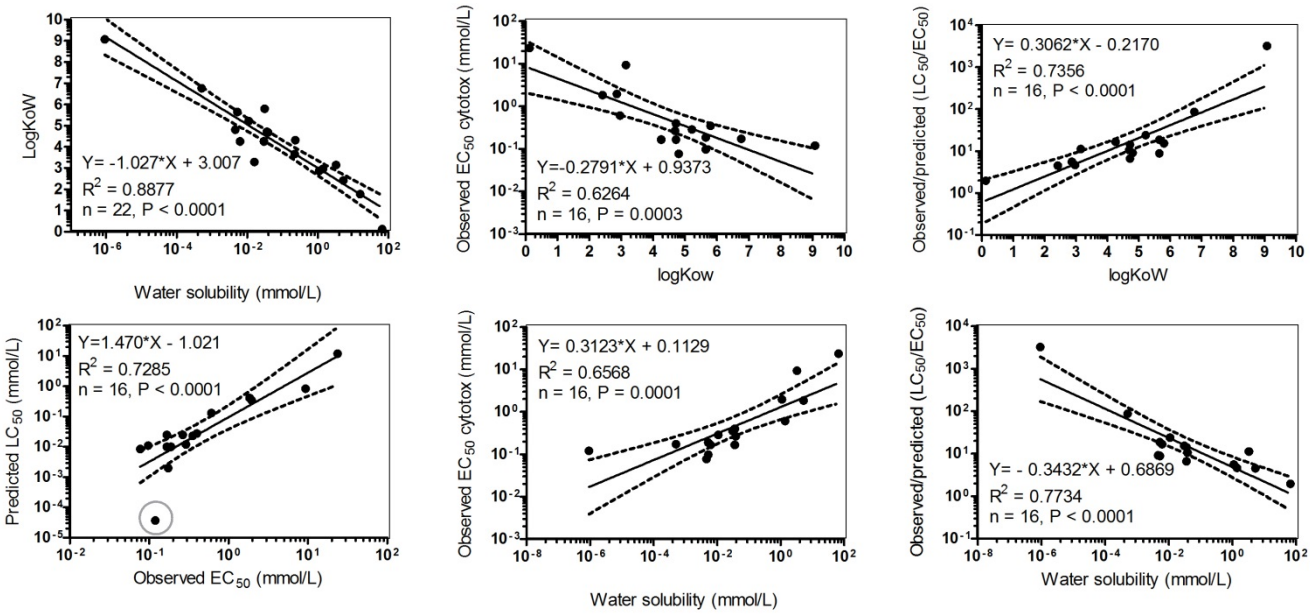



Figure 5. A comparative linear regression and correlation analysis of observed cytotoxicity at 50% effect concentration ($EC_{50}$), predicted lethal concentration ($LC_{50}$) in fish, water solubility and Log $K_{octanol\text{-}water\ (ow)}$ of the unresolved complex mixtures (UCMs). The analysis consist of single factor (Predicted $LC_{50}$ vs. observed $EC_{50}$) more complex composite factors (Ratio between observed and predicted toxicity vs. water solubility and vs. Log $K_{ow}$). The observed cytotoxicity data (mean ± standard deviation) consists of a minimum of three exposure studies measuring the metabolic activity and is expressed as percentage of a positive control (0.01 M $CuSO_4$), towards the solvent control (DMSO). The predicted acute fish toxicity data was obtained from Chemprop. The gray ring depicts an apparent visual outlier.

To further investigate potential deviation between observed and predicted baseline toxicity a ratio was established before plotting it towards the compounds` water solubility and Log$K_{ow}$. The baseline toxicity ratio reported a significant moderate negative correlation between the water solubility ($R^2$=0.773, p≤ 0.0001) and a significant moderate positive correlation with Log $K_{ow}$ ($R^2$=0.736, p≤ 0.0001) of the compounds. These correlations demonstrate that there is a moderate relationship between the compounds toxicity and their water solubility and hydrophobicity (Log $K_{ow}$), and that the difference between the observed and predicted toxicity in this study increases with increasing Log $K_{ow}$.

# 4 Discussion

Accidental oil spills and legal or accidental discharges of processed or refined petroleum fractions may result in exposures of fish to various UCM compounds (Conly et al., 2002; Headley and McMartin, 2004; Swigert et al., 2015). Despite the dominance of UCM pollutants in many petroleum-contaminated samples (Frysinger et al., 2003) and the known toxicity of some UCMs to aquatic organisms such as bivalves (Booth et al., 2007; Donkin et al., 2003; Rowland et al., 2001) and bacteria (Jones et al., 2011), few studies have investigated the toxicity of individual UCM-related chemicals to fish. This is partly due to a historic lack of proper identification of UCM-compounds and limited availability of pure compounds from commercial sources. However, a number of aliphatic, alicyclic and aromatic UCM-related hydrocarbons and several monocyclic, aromatic and alicyclic and hetero-containing aromatic acids have recently been identified in UCMs by GCxGC-MS, and have been successfully synthesised or made available from commercial vendors (Rowland et al., 2011a, 2011c, 2011d; Scarlett et al., 2011; Smith et al., 2001; West et al., 2014; Wraige, 1997).

The compounds are designated as 'UCM-related' herein since the structures of the hydrocarbons have been deduced following degradative studies and are considered 'average' or 'model' structures (Smith et al., 2001; Sturt, 2001; Thomas, 1995) whilst those of the acids have been more firmly identified, usually by comparison of the mass spectra and GCxGC retention times with those of authentic compounds (Rowland et al., 2011d; West et al., 2014). This study is one of the first to characterise the toxicity and MoA of a number of UCM-related compounds to fish cells *in vitro* by assessing their cytotoxic, AhR agonistic and ER agonistic potency in primary rainbow trout hepatocytes to identify compounds of potential environmental concern.

## 4.1 Cytotoxicity and baseline toxicity

Several *in vitro* and *in vivo* studies have reported various toxic effects of NA mixtures (oil sands process water-derived and commercial NA) including embryo deformities, mortality, reduced immune response, oxidative stress, necrosis and cell death (full review see Chao Li, 2014). The present study demonstrated that 20 out of the 22 tested compounds were cytotoxic for the primary rainbow trout hepatocytes, reducing the metabolic ability with more than 20%. Considerable differences in cytotoxicity were however observed for the different groups of compounds tested ($EC_{50}$: 0.077 to 24 mM). Overall, $EC_{50}$ were obtained for a total of 16 of the 22 compound tested, a majority belonging to the monoaromatic acids at concentrations generally around 0.1-0.6 mM,

with some exceptions (Table 2). The reported cytotoxicity concentrations was however only reliable for two compounds (Benzo[b]thiophene-3-acetic acid, 1,3,5-trimethyl-cyclohexane-1,3,5-tricarboxylic acid) as their $EC_{50}$'s were still within their predicted water solubility limits (Table 2). Only 2 of the 22 compounds (Camphoric acid and 4-DBThio-BA) demonstrated to not elicit any cytotoxicity at the tested concentrations, potentially requiring higher exposure concentrations than those used in present study as the predicted baseline toxicity for Camphoric acid was high (Table 2). In an earlier study performed by Scarlett et al. (2012) the predicted fathead minnow (*Pimephales promelas*) base line toxicity of a number of individual NA of different structural groups reported polycyclic acids containing a single aromatic ring to be the most toxic with $LC_{50}$s typically at around 1 µM. These predictions are in agreement with current experimental findings as the polycyclic monoaromatic acids were among the most toxic of the tested compounds ($EC_{50}$= 0.12-0.24 mM), only exceeded by some aliphatic monocyclic acids such as 4-(4'-n-butylcyclohexyl)- and 4-(4'-s-butylcyclohexyl)butanoic acid ($EC_{50}$= 0.077-0.097 mM). Although no $EC_{50}$ was obtained for aliphatic monocyclic acid 3-CHPA in the present cytotoxicity study, it did reduce the cell viability by more than 20% at the highest exposure concentrations, suggestive of toxicity occurring at higher concentrations than those used in this study.

It's well established that different UCMs and UCM components such as NAs may be toxic to fish cells by causing cellular swelling, affecting the cell membranes, metabolic activity (Tollefsen et al., 2012), and mitochondrial and lysosomal integrity (Chao Li, 2014). This narcotic MoA is possibly due to the NAs potential to increase the membrane fluidity in the cell by disrupting the cell membrane lipid bilayer through the insertion of the NAs surfactant-like structures, eventually causing cell death (full review see Chao Li, et al., 2014). The present study measured cytotoxicity as disruption of membrane integrity and metabolic activity, and results were fairly consistent between the two endpoints tested. The metabolic activity of the cells did however report slightly more consistent dose-response curves, probably due to the NAs potential to increase the membrane fluidity, thus interfering with the conversion of non-specific esterase form of CFDA-AM to the fluorescent product, 5-carboxyfluorescein. These results were further supported by the moderate correlation between the physico-chemical parameters affecting partition of chemicals into biological membranes (e.g. $\log K_{OW}$, water solubility and molecular size) and their toxicity in the rainbow trout hepatocytes. These findings have provided additional support for suggesting that baseline toxicity is of high relevance for the tested compounds.

The majority of the tested UCM-related compounds were cytotoxic at relatively high concentrations ($EC_{50}$ ranging from 0.077 to 24 mM) compared to concentrations reported for chemicals with a specific MoA. ).

## 4.2 Induction of EROD activity

Ethoxyresurofin-O-deethylase (EROD) is a well-established biomarker for catalytic measurement of the AhR-mediated cytochrome P450 1A induced detoxification protein by chemicals with dioxin-like properties (Whyte et al., 2000). The EROD activity may act as an early warning signal of AhR agonists that are associated with adverse effects such as immunotoxicity, histopathological lesions and mortality in fish (Whyte et al., 2000). Six of the tested compounds induced EROD activity in primary rainbow trout hepatocytes, whereof all compounds except B[b]Thio-3-CA induced EROD activity in a concentration-dependent manner at non-cytotoxic concentrations. All tested monoaromatic thiophenic alkanoic acids (3-MB[b]Thio-2-AA and B[b]Thio-3-AA) induced EROD activity to > 20% of the positive control. The other 4 compounds inducing the EROD activity were two monoaromatic acids (4-(4-n-BPh)BA and 4-(4-t-BPh)BA), one of the two investigated polycyclic monoaromatic hydrocarbons (7-CH-1-IAT) and one of the two monocyclic thiophenic carboxylic acids (B[b]Thio-3-CA). Interestingly, the $EC_{50}$s for EROD activity were in some cases higher than the $EC_{10}$ for cytotoxicity, thus displaying a potential high-concentration masking of EROD by induced cytotoxicity. Despite this, the present study reports novel findings of compound-specific induction of EROD activity, which are in line with previous *in vitro* studies on unspecified NAs potential to induce CYP1A gene expression (Knag et al., 2013). Few studies have investigated the effect of UCM-related compounds and OSPW extracts containing UCMs such as NAs. However, effects typical of 'dioxin-like' exposure have been observed in early life stages of fathead minnow exposed to OSPW; albeit no significant increase in the AhR-mediated induction of the CYP1A transcript was observed (He et al., 2012). In agreement, no statistical difference in EROD activity was observed in rainbow trout exposed to different pond waters and extracts of naphthenic acids at low milligram per liter (between 1 mg/L and 8 mg/L) concentrations (Leclair et al., 2013). However, increased CYP1A activity was observed in fish exposed in South Bison Pond, Canada, a tailings pond containing aged un-extracted oil sands material, compared to fish exposed in a demonstration pond and reference lake (Arens et al., 2015). Primary hepatocytes exposed to

extracts from oil sand tailing ponds and downstream of oil sand tailing ponds in Athabasca river reported increased EROD activity (Gagné et al., 2011). The extracts used did however contain polycyclic aromatic hydrocarbons (PAHs), which are known potent inducers of AhR-mediated EROD activity in fish (Whyte et al., 2000). The EROD activity in the present study was associated with chemical groups of monoaromatic thiophenic alkanoic acids, polycylic monoaromatic acids and monoaromatic acids, whereof the later have been suggested as substrates for CYP1A2 in humans (Scarlett et al., 2012). The monoaromatic acids role as potential AhR agonists in fish is however still unknown. Besides sharing some structural similarities with AhR agonists, the tested compounds` characteristics are not typical EROD inducers such as 2,3,7,8-Tetrachlorodibenzodioxin (TCDD), polychlorinated biphenyls (PCBs) and benzo[A]pyrene (BaP) (Whyte et al., 2000), suggesting that UCMs might contain non-typical EROD inducing components with the potential to cause AhR-mediated effects in fish.

As only a few of the tested compounds within these chemically diverse groups induced EROD activity, specific physico-chemical (e.g. structure, Log $K_{ow}$ etc.) differences is believed to contribute to the MoA.

## 4.3 Induction of Vtg production

Chemicals with estrogen mimicking properties have the ability to bind and activate the estrogen receptor (ER), initiating down-stream transcriptional activation and translation of estrogen responsive genes and proteins such as the egg-yolk precursor vitellogenin (Vtg). The Vtg protein is a well-established biomarker for (xeno)estrogen pollution primarily measured in juvenile and male organisms as not naturally occurring in these. Induction of Vtg has been associated with adverse effects in fish and mammals such as reduced growth, reproductive success, fecundity and increased ratio of feminisation (Colborn et al., 1993; Janošek et al., 2006; Sumpter and Jobling, 1995). Model ER agonists such as 17β-estradiol, 17α-ethinylestradiol and bisphenol A all have specific structural properties that binds into the hormone binding pocket and induce conformational changes which activates the ER and its downstream targets (O'Malley and Tsai, 1992).

Several (mono)aromatic acids related to UCMs, OSPW fractions and naphthenic acids (NAs) have been suggested to be weak estrogens (He et al., 2012; Scarlett et al., 2012; Wang et al.,

2015). A selection of the UCM-related compounds tested in the present study were therefore anticipated to be at least partial ER agonists, having structural resemblance to NAs predicted to be weak estrogens (Scarlett et al., 2012). The present study could however only report the hydrocarbons 2,6-DMN which is structurally similar to many unresolved branched alkylnaphthalenes present in UCMs (Scarlett et al., 2011) and the 'model' UCM compound 6-CHT (Sturt, 2001) to induce Vtg in the primary hepatocytes by more than 20% from control levels. The majority of the tested compounds did not elicit any ER-mediated effect when measuring Vtg protein. The structural specificity of ER agonists and their affinity for the ER hormone binding cavity is crucial in order to initiate ER-mediated events. Groups of NAs (e.g. (mono)aromatic acids) which are predicted ER-agonists (Scarlett et al., 2012) have structural resemblance to some steroidal chemicals in addition to favorable physico-chemical properties (Reinardy et al., 2013). However, steroidal activity of acids might be variable as estradiol related acids are not necessarily estrogenic (Labaree et al., 2003). The tentative interpretation of the UCMs acids and NAs estrogenicity solemnly based on their structural resemblance to steroidal acids is therefore unreliable and potentially challenging. The lack of estrogenic activity in the majority of the UCM-related compounds tested in this study may therefore be related to their specific structure as the 6-CHT and 2,6-DMN are among the few polycyclic hydrocarbons and naphthalene compounds inducing Vtg in the hepatocytes. Interestingly, several studies have demonstrated OSPW, oil sand (OS), produced water (PW) and commercially available (CA) naphthenic acids to significantly induce transcriptional and translation changes of ERα/β2, Vtg and Cyp19b in modified yeast strains with human ER (YES), primary rainbow trout hepatocytes and zebrafish (*Danio rerio*) embryos (Gagné et al., 2012; Thomas et al., 2004; Wang et al., 2015). However these mixtures of CA-NAs mainly consist of non-complex component structures (8-11 carbons) and have no structural hydrogen deficiency (z=0) and low Log $K_{ow}$, making the NAs highly bioavailable for the cells. The UCMs of present study do on the other hand consist of mainly complex NAs (carbon numbers >11) which have a hydrogen deficiency (z=-2 to -8), suggesting the compound size, structure and polarity to potentially contribute to the lack of estrogenicity in the majority of UCM-related compounds. Nevertheless, there are still uncertainties in predictions of chemicals ED properties (e.g. estrogenicity) based solemnly on their structure and physico-chemical properties, which might only be verified experimentally (Scarlett et al., 2012). This has previously been performed *in vitro* on mixtures and fractions of NAs and PW, demonstrating ER-agonistic, AR-antagonistic (Thomas et al., 2004, 2009) and ER-

antagonistic properties at environmentally relevant concentrations (Leclair et al., 2015). The lack of estrogenicity in the present study might therefore be a result of the UCM-related compounds potential properties to modulate other ED processes, which were not assessed in present study.

The lack of or low ER activity may also be a result of a conflicting MoA as a recent study suggested the water soluble fraction (WSF) of crude oil, containing predominantly naphthalenes and phenols, to inhibit ERα in zebra fish through a suggested nuclear receptor cross-talk with the aryl hydrocarbon receptor (AhR)-CYP1A pathway (Salaberria et al., 2014). Nuclear receptor cross-talks such as the uni-directional AhR-ER (Gräns et al., 2010) have been demonstrated in numerous *in vitro* studies to inhibit ER-activity through various interfering AhR-mediated mechanisms (e.g. ER degradation, competitive co-regulators etc.) (Matthews and Gustafsson, 2006; Safe and Wormke, 2003). The limited Vtg induction might also be explained by the NAs low water solubility hence bioavailability, as effects were generally occurring at concentrations higher than the predicted solubility in water (Table 2). Thus future studies addressing the compounds potential ED modulatory (AR agonistic and antagonistic and ER antagonistic) properties during sub-lethal concentration and within the range of the compounds water solubility limit in order to retrieve reliable and descriptive data that will decipher the UCM NAs potential MoA is warranted.

## 4.4 Chemical properties related to the observed and predicted effects

*In vitro* methods offer rapid screening of chemicals potential toxicity and may contribute to deciphering their MoA. The primary hepatocytes ability to estimate the baseline toxicity was similar to that predicted *in vivo* (Figure 6). Despite being limited by its single-organ response (among other things), only a few discrepancies were observed between the observed and predicted toxicity which complied rather well for highly water soluble compounds (Log $K_{ow}$: 2.42-2.97). More hydrophobic compounds were however more difficult to toxicologically estimate, as their bioavailable concentration in the *in vitro* assay was unknown.

Bioassay relater artefacts such as compounds molecular size, structure, polarity, water solubility, chemical precipitation, and bioavailability challenge the *in vitro* system, which potentially contributes to the deviations observed between the *in vitro* (observed) vs. *in vivo* (predicted)

toxicity for hydrophobic compounds (Figure 6). The experimental cytotoxic effects generally occurred above the predicted water solubility, which in previous *in vitro* studies have been related to reduced chemical bioavailability due to chemical adherence to the plastic well-plate walls (Schreiber et al., 2008), and interaction with proteins and other cell media components (Groothuis et al., 2015; Hestermann et al., 2000; Riedl and Altenburger, 2007). Reporting only nominal concentrations for more hydrophobic compounds (Log $K_{ow}$ >3) might therefore result in underestimation of the compounds toxicity (Riedl and Altenburger, 2007) as suspected for 7-CH-1-iAT (water solubility $9.3E^{-7}$mM, Log $K_{ow}$= 9.08), which experimentally deviated more than 3000 times from its predicted toxicity (Figure 6). In line with the present study, underestimation of highly hydrophobic chemicals` (e.g. TCDD, phenanthrene, pyrene and benzo(a)pyrene, Log Kow: 4.5-6.8) acute toxicity and sub-lethal EC-values has been reported in numerous *in vitro* bioassay's (Groothuis et al., 2015; Hestermann et al., 2000; Riedl and Altenburger, 2007; Schreiber et al., 2008). Studies investigating effects of emerging compounds of concern would therefore greatly benefit from verifying the exposure concentrations both in media and intracellular (*in vitro*) or tissue (*in vivo*), understanding the potentially arising bioassay artefacts for both the *in vitro* and *in vivo* system.

The predictions of acute toxicity (e.g. baseline toxicity) in the present study are based on chemical structures and physico-chemical properties using a quantitative structure-activity relationship (QSAR) model. These predictions have contributed to the understanding of chemicals with unknown MoA when having no or little empirical data (Russom et al., 1997). The acute toxicity predictions performed herein report $LC_{50}$ values for the majority of the UCM-related compounds, although several of which had baseline toxicity above the predicted water solubility (Table 2). The reliability of these chemicals` predicted toxicity should therefore have less relevance when compared towards the experimentally estimated $EC_{50}$ values in the present study. The uncertainty of the predicted baseline toxicity highlights the need for conducting additional experimental studies to improve the QSAR model's predictability of NAs, potentially applying a multi-endpoint approach using relevant *in vitro* bioassays for fish.

## 4.5 Environmental implications

UCMs are known to contain large numbers of chemicals which may vary both in composition and concentrations in the environment dependent on nearby sources and fate. Further investigation of the NAs potential MoA is essential as UCMs containing NAs are more likely to remain in the water phase (e.g. in OSPW) instead of partition to soils or sediments (reviewed by Headley and McMartin , 2004), thus being bioavailable for aquatic organisms. Reports of concentrations as high as 110 mg/L of mixtures of NAs from OSPW have been measured in tailing pond waters (Scott et al., 2008). These concentrations are of environmental concerns as acute (>10 days) $LC_{50}$ -values as low as 25-75 mg/L for a variety of ecologically relevant fish species at different developmental stages (2 months - 2 years) have been reported (Dokholyan and Magomedov, 1984).

The hydrocarbons tested herein are models for those estimated to occur in environmentally relevant UCMs (e.g cyclohexyl tetralin) (Warton et al., 1999) or are analogues of those identified in UCMs by GCxGC-MS (e.g. alkylnaphthalenes and alkyltetralins) (Booth et al., 2008; Scarlett et al., 2011). The compounds investigated also include those belonging to chemical classes known to occur in UCMs found in the derivatised NAs of OSPW (Barrow et al., 2010; Clemente and Fedorak, 2005; Grewer et al., 2010; Headley and McMartin, 2004; Madill et al., 2001; Rogers et al., 2002; Rowland et al., 2011b, 2011c, 2011e; West et al., 2014), and a few of these; 1-ACA (Rowland et al., 2011c), have also been individually identified by GCxGC-MS. The results of the present study clearly show that the compounds` toxicity were in agreement with the predicted acute toxicity for fish with minor exceptions. Results which demonstrate the primary hepatocyte model's applicability as a screening tool of environmentally relevant UCM of NAs, as also reported elsewhere (Tollefsen et al., 2012).

The assessed endpoints were based on present knowledge of the compounds predicted MoA from previous *in vivo* studies (Dokholyan and Magomedov, 1984; Reinardy et al., 2013; Scarlett et al., 2013), and several of the UCM-related compounds elicited more specific sub-lethal effects such as induction of the estrogenic biomarker Vtg and EROD activity. The results are also similar to sub-lethal responses previously observed in *in vitro* and *in vivo* studies on mixtures of NAs (Knag et al., 2013; Wang et al., 2015). In addition to the present findings, assessment of additional endpoints such as genotoxicity (Lacaze et al., 2014), induction of oxidative stress (Farmen et al., 2010) and modulation of steroidal receptors (e.g. androgen- and thyroid receptor) (Leclair et al.,

2015; Thomas et al., 2009) may also have been of relevance to this study in order to fully investigate the potential MoA of UCM related compounds. The genotoxicity of some individual acids and OSPW NAs has previously been observed in primary rainbow trout hepatocytes (Lacaze et al., 2014). The reported sensitivity for DNA damage (significant differences at 5 mg NAs /L, 0.02% v/v OSPW) (Lacaze et al., 2014) occurred below NA concentrations previously found in tailing ponds (Clemente and Fedorak, 2005), raising an environmental concern. DNA damage was observed at concentrations lower than those causing cytotoxicity, demonstrating its suitability as a sub-lethal endpoint in future chemical screenings of NAs potential MoAs.

Even though the environmental concentrations of the individual tested compounds may be lower than the concentrations shown to induce effects in this study, it should be kept in mind that combined effects might occur as additive cytotoxic effects of NAs have previously been observed (Tollefsen et al., 2012).

# 5 Conclusions

The compounds investigated in this study belong to chemical groups known to occur in UCMs. Few studies have been performed that aim to characterise the toxicity and MoA of individual UCM-related compounds. This study indicates that the general MoA of the investigated compounds in primary rainbow trout hepatocytes is narcosis. $EC_{50}$ values for cytotoxicity were obtained for 16 of the 22 tested compounds and ranged from 77µM to 24 mM. A few compounds also exhibited specific MoA such as estrogenicity and ‘dioxin-like’ effects. Even though the concentrations of the individual compounds in UCMs might be below those to induce effects in this study, combined effects might occur as additive effects of mixtures have previously been shown. UCM-related compounds appear to be highly diverse group of chemicals with a potential for a wide range of effects on exposed wild life. Further identification of specific compounds and characterisation of effects and MoA of related compounds are thus important.

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