Anne Skjetne Mortensen

Toxicogenomics of Aryl Hydrocarbon- and Estrogen Receptor Interactions in Fish:

Mechanisms and Profiling of Gene Expression Patterns in Chemical Mixture Exposure Scenarios

Thesis for the degree philosophiae doctor

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Norwegian University of Science and Technology
Faculty of Natural Sciences and Technology
Department of Biology

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Summary

Almost without exception, biological processes such as overt morphological changes, development (both reproductive and growth), toxicological responses and clinical manifestation to disease, have molecular basis. From our perspective (i.e. toxicological perspective), the evidence of receptor-mediated mechanisms of xenobiotic-induced effects is provided if the effect is tissue specific, predictable, if increases in the transactivation of specific genes can be demonstrated, transcriptional responses occur rapidly, compounds bind reversibly to intracellular macromolecules or compounds are stereo-specific. Thus, the primary objective of toxicological *in vitro* studies on cells and tissues is to characterize cellular and molecular substrates and pathways that contribute to adverse effects in an organism after toxicant exposure. The estrogenic and xenobiotic biotransformation gene expressions are receptor-mediated processes that are ligand structure-dependent interactions with estrogen-receptor (ER) and aryl hydrocarbon receptor (AhR). The anti-estrogenic activities of AhR agonists have been reported *in vitro* and *in vivo* studies. In teleost species, exposure to AhR agonists has been associated with reduced vitellogenin (Vtg) synthesis or impaired gonadal development. Recently, several studies have shown that AhR-agonists directly activate ERs and induce estrogenic responses in mammalian *in vitro* systems. The overall objective of this thesis was to develop diagnostic gene and protein response tools in the study of the molecular mechanisms of gene expression patterns of xenoestrogens and xenobiotic interactions in wildlife species. Contaminants known to be estrogenic (ethynylestradiol; EE2 and nonylphenol; NP) and/or anti-estrogenic (PCBs), either by direct ER or indirect AhR mechanistic pathways, were used as model xenobiotics and evaluated either singly or in combination using *in vitro* and *in vivo* test systems.

Suppressive subtractive hybridization (SSH) was used to create a cDNA library of clones containing differentially expressed genes from Atlantic salmon (*Salmo salar*) separately exposed to ER and AhR agonists. Based on differentially expressed genes from the library, a targeted cDNA array (SalArray) was developed. Cellular *in vitro* systems, like cell and tissue models, facilitate the investigation of the direct molecular mechanisms accounting for predictable adverse effects of xenobiotic compounds on wildlife and humans. Consequently, in the studies presented primary hepatocyte cultures were isolated from the liver of trout and salmon by the collagenase perfusion method. The targeted SalArray and quantitative real-time PCR (q-PCR) were used to demonstrate that exposure of salmon hepatocytes to the ER-agonist NP singly or in combination with the AhR-agonist PCB77

produced differential gene expression patterns in salmon liver. Exposure of hepatocytes to NP mainly altered genes involved in the estrogenic pathway, including genes involved in steroid hormone synthesis and metabolism. The anti-estrogenic properties of PCB77 were demonstrated in the array analysis as NP induced gene expressions decreased by exposure of hepatocytes to PCB77. Our data showed a reciprocal inhibitory interaction between ER- and AhR-agonists. PCB77 produced anti-estrogenic effects by decreasing the mRNA expression of ER-responsive genes, and NP produced anti-AhR mediated effects as inhibitor of AhRR, Arnt, CYP1A1 and UGT expression. *In vivo* exposure of salmon to EE2 produced a significant decrease of CYP1A1 expression and these effects paralleled EROD activity and AhRR mRNA, suggesting a direct role of EE2 in controlling the cellular detoxification machinery.

While a clear pattern of negative effects on ER-mediated gene expression was found in hepatocytes exposed to PCB77, exposure of cells to the more potent AhR-agonist and dioxin-like PCB126 induced transcriptional activation of ER signalling demonstrated by increased Vtg and ERα mRNA and ERα protein levels. The decreased levels of ERα and Vtg expression in cells treated with PCB126 in the presence of ICI is novel, indicating a possible, but not conclusive "ER-hijacking" not previously reported in any fish species or lower vertebrate. Different gene expression patterns were obtained at similar time-interval with fish from different seasons, demonstrating the complexity of AhR-ER interactions. Thus, the direct estrogenic actions of PCB126 observed contribute new insight on the complexity of the mechanisms involved in ER-AhR crosstalk, prompting a new wave of discussion on whether AhR-mediated anti-estrogenicity is an exception, rather than a rule of action. This thesis demonstrates a complex mode of interactions between two different classes of ligand-activated receptors and provides novel mechanistic insights on signalling pathways. Therefore, the degree of simultaneous interactions between the ER and AhR gene transcripts demonstrated support the concept of cross-talk between these signalling pathways, in addition to generating new hypotheses that need to be evaluated empirically.

Papers included in the thesis

The thesis is based on the following papers that will be referred to in the text by their Roman numerials:

I. Mortensen AS and Arukwe A (2007). Effects of 17α-Ethynylestradiol on Hormonal Responses and Xenobiotic Biotransformation System of Atlantic Salmon (*Salmo salar*). *Aquatic toxicology* 85: 111-123.

II. Mortensen AS, Tolfsen CC and Arukwe A (2006). Gene expression patterns in estrogen (nonylphenol) and aryl hydrocarbon receptor agonists (PCB-77) interaction using rainbow trout (*Oncorhynchus Mykiss*) primary hepatocyte culture. *J Toxicol Environ Health A* 69(1-2):1-19.

III. Mortensen AS and Arukwe A (2007). Targeted salmon gene array (SalArray): a toxicogenomic tool for gene expression profiling of interactions between estrogen and aryl hydrocarbon receptor signalling pathways. *Chem Res Toxicol* 20(3):474-488.

IV. Mortensen AS and Arukwe A (2007). Interactions between estrogen- and Ah-receptor signalling pathways in primary culture of salmon hepatocytes exposed to nonylphenol and 3,3',4,4'-tetrachlorobiphenyl (congener 77). *Comp Hepatol* 6:2.

V. Mortensen AS and Arukwe A (2007). Activation of estrogen receptor signalling by the dioxin-like Aryl hydrocarbon Receptor agonist, 3,3',4,4',5-Pentachlorobiphenyl (PCB126) in salmon in vitro system. *Toxicol Appl Pharmacol.* **In press**.

Abbreviations

3β-hsd	3β-hydroxysteroiddehydrogenase
3-MC	3-methylcholanthrene
3'-UTR	3'-untranslated region
AF	Activation function
AhR	Aryl hydrocarbon receptor
ANF	α-naphthoflavone
Arnt	Aryl hydrocarbon receptor nuclear translocator
AhRR	Aryl hydrocarbon receptor repressor
bHLH-PAS	Basic-helix-loop-helix, Period-ARNT-Single-minded
BNF	β- naphthoflavone
CYP1A1	Cytochrome P450 1A1
DBD	DNA binding domain (DBD)
DMSO	Dimethyl sulfoxide
E2	17β-estradiol
EDCs	Endocrine-disrupting chemicals
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	Estrogen receptor
ERE	Estrogen responsive element
GSH	Glutathione (γ-Glu-Cys-Gly)
GST	Gluthatione-S-transferase
ICI	ICI182,780 (Faslodex or Fulvestrant)
LBD	Ligand binding domain (LBD)
NADPH	Nicotinamide adenine dinucleotide phosphate
NP	Nonylphenol
PAHs	Polycyclic aromatic hydrocarbons
PCB	Polychlorinated biphenyls
PCB77	3,3',4,4'-tetrachlorobiphenyl
PCB126	3,3',4,4',5-pentachlorobiphenyl
PCDDs	Polychlorinated dibenzo-*p*-dioxins
PCDFs	Polychlorinated dibenzofurans

POPs	Persistent organic pollutants
PVDF	Polyvinylidene fluoride
PXR	Pregnane X receptor
q-PCR	Quantitaitive real-time polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSH	Suppression subtractive hybridization
StAR	Steroid acute regulatory protein
Tam	Tamoxifen
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
UGT	Uridine-diphosphate glucuronosyltransferase
Vtg	Vitellogenin
XRE	Xenobiotic response element
Zr-protein	Zrp; *zona radiata* protein

Introduction

The ER signalling pathway

Communications between cells are required to maintain homeostasis and a normal progression of an organism's development. Substances used in cell signalling include endogenous steroid hormones and other endocrine and paracrine compounds (Colborn et al., 1993). Signalling compounds produced by one cell can direct the course of development and thereby determine the immediate and future functioning of another cell or group of cells. For instance, brain, ovaries, adrenal glands and the placenta in mammals produce steroid hormones that play major roles in the development of sexual characteristics of the offspring (Colborn et al., 1993).

Synthetic chemicals, natural plant and animal compounds with the ability to modulate an organism's endocrine system are generally referred to as endocrine-disrupting chemicals (EDCs) (Bogi *et al.*, 2003; Ankley *et al.*, 2005). EDCs are widely distributed in the environment and have been associated with developmental, general health and reproductive problems in wildlife and laboratory animals (Colborn *et al.*, 1993; Guillette and Gunderson, 2001; Fossi *et al.*, 2004). There are also speculations that these chemicals may be affecting in similar ways (Golden et al., 1998). The outcome of exposure to EDCs is dependent on the specific developmental stage of an organism. For example, exposure of progeny (eggs, embryo or fetus) to EDCs could change the course of development (through an organizational process) and fitness, while effects of exposure in adulthood could be displayed in progeny (Guillette and Gunderson, 2001; Lintelmann et al., 2003). Critical exposures during early life stages could result in delayed effects that are not fully expressed until maturity (Colborn et al., 1993). It is generally assumed that exposure to EDCs after maturity will not permanently disrupt functioning of hormone responsive (Colborn et al., 1993).

The endogenous estrogens 17β-estradiol (E2), estriol and estrone (Figure 1) are predominantly produced in ovarian cells and transported to their target organs through the bloodstream. Estrogens control gene expression through interactions with estrogen receptors (ERs) that regulate several endogenous pathways (Smith, 1998; Wierman, 2007). In addition, estrogens are involved in growth regulation either directly or indirectly by interactions with growth promoting factors (Trudeau *et al.*, 2005; Elango *et al.*, 2006). EDCs could either activate or inhibit the estrogenic cell signalling pathways, by mimicking natural hormones and binding directly to intracellular hormone receptor proteins (Janosek et al., 2006). Anti-

hormonal effects are observed when a compound prevents or alter hormonal binding to intracellular hormone receptors and thereby inhibiting trans-activation of cellular processes (Jansen *et al.*, 1993; Denslow *et al.*, 2004). Additionally, some EDCs are capable of altering the metabolism of natural hormones or modifying the synthesis and function of the intracellular hormone receptor proteins (Janosek et al., 2006). The estrogenic and anti-estrogenic activities of several chemicals have been reported in several studies (Legler *et al.*, 1999; Smeets *et al.*, 1999; Navas and Segner, 2000a; Gutendorf and Westendorf, 2001; Abdelrahim *et al.*, 2003; Rankouhi *et al.*, 2004).

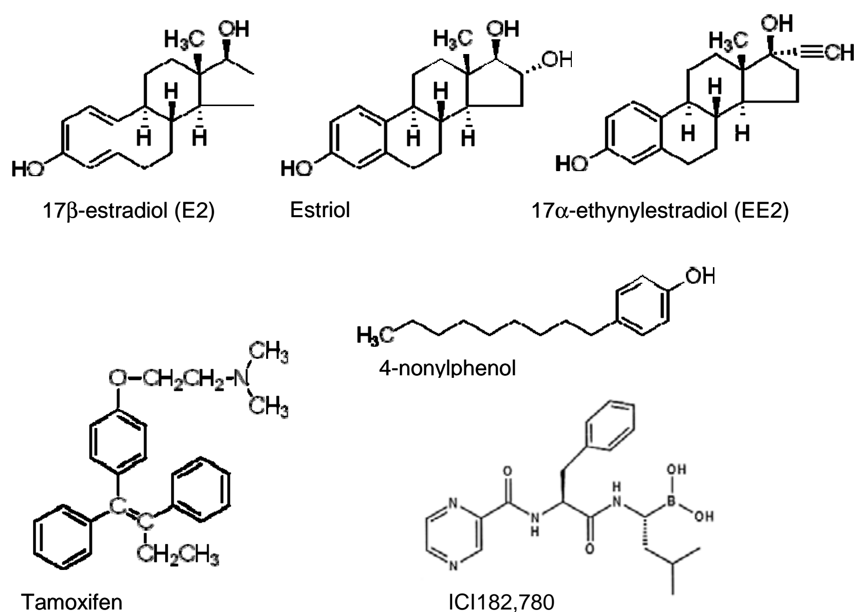

Figure 1. Chemical compounds known to bind to the estrogen receptor. 17β-estradiol (E2), estriol, 17α-ethynylestradiol (EE2), 4-nonylphenol (NP), Tamoxifen and ICI182,780 (Fulvestrant).

Industrial chemicals, pharmaceuticals and personal care products, pesticides and surfactants are common and ubiquitous contaminants in aquatic environments. Therefore, chemical interactions after exposure to complex mixtures of environmental pollutants may have profound consequences on aquatic organisms (Mumtaz *et al.*, 2002; Brian *et al.*, 2005). The relative importance of the influence of complex chemical mixtures on biological systems is not well understood or quantified mechanistically. In the aquatic environment, the effects of EDCs on reproduction have been reported in several invertebrate and vertebrate species. For example, tributyltin (TBT) (an organotin compound used primarily as a biocide in antifouling paints for ships, boats and fishing nets) exert masculinizing effects in zebrafish (McAllister

and Kime, 2003). Altered steroid hormone levels and abnormal male and female gonads were observed in juvenile alligators exposed to organochlorine contaminants in Lake Apopka (Florida) (Guillette and Gunderson, 2001). DDT and its metabolites were shown to alter population structure by causing eggshell thinning as well as endocrine and reproductive toxicity in wild birds (Forsyth et al., 1994) and variable effects on steroid hormone receptors (Kelce et al., 1995).

Several exogenous compounds are found to have estrogenic effects in fish, these include phytoestrogens, synthetic estrogens like the 17α-ethynylestradiol (EE2) used in birth control pills and several other synthetic substances (xenoestrogens) such as alkylphenols, insecticides, phthalates and hydroxylated (OH)-metabolites of polychlorinated biphenyls (PCBs) (Petit *et al.*, 1997; Matthews *et al.*, 2000). The causative agent of estrogenic effects on fish in rivers and estuaries appears to be the natural steroids 17β-estradiol (E2) and estriol, EE2 as well as alkyl phenols derived from poly-ethoxylated phenols (Segner *et al.*, 2003; Rankouhi *et al.*, 2004; Jobling *et al.*, 2006). Examinations of fish from heavily contaminated rivers in the United Kingdom have uncovered synthesis of Vtg, inhibition of testicular growth in male fish and combined male and female gonadal characteristics (ovotestis), abnormal glands and duct and morphological changes of secondary sexual structures that may lead to impaired reproductive effects (Kirby *et al.*, 2003; Kavanagh *et al.*, 2004; Elango *et al.*, 2006; Jobling *et al.*, 2006).

EDCs mediate their effects on target cells through ER-dependent or independent processes. The ERs are members of the nuclear steroid/thyroid/retinoic acid super-family of ligand-activated transcription factors (Hewitt and Korach, 2002; Wierman, 2007). These receptors are involved in regulation of different aspects of development, differentiation, growth, homeostasis and metabolism in the cells by activation or repression of gene expression (Zilliacus *et al.*, 1995; Nilsson *et al.*, 2001). The ER possess five domains with specific functions, namely: 1) The trans-activation domain, named the activation function 1 (AF1), 2) the DNA binding domain (DBD), 3) a variable hinge region (H), 4) the ligand binding domain (LBD) that includes activation function 2 (AF2) and 5) a variable C-terminal region (C) (See figure 2). The DBD and LBD domains are highly conserved, while the AF1, the hinge region and the C-terminal are less conserved (Leaver et al., 2007).

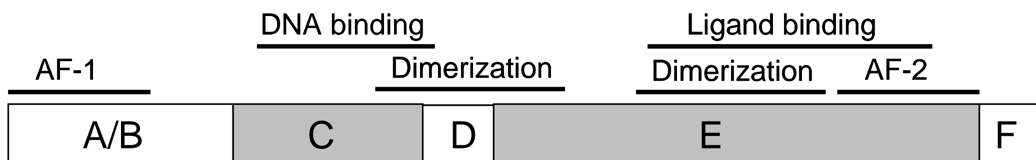

Figure 2. Schematic representation of estrogen receptor domains. The ER possess five domains encoding specific functions. Modified from Matthews and Gustafsson (2003).

In the unbound state, the ERs are located in both the cytoplasm and nucleus associated with chaperone complexes that include heat shock protein 90 (hsp90) and other co-factors (Aranda and Pascual, 2001). The chaperone complex recognizes unliganded ERs and stabilizes the receptors to a conformation that favours ligand binding (Buchner, 1999). Upon ligand binding, the receptor dissociates from the associated chaperone-complex, dimerizes with another ER and subsequently bind to estrogen responsive elements (ERE) in the regulatory upstream region of the initiation site of ER controlled genes (Figure 3) (Aranda and Pascual, 2001; Sabo-Attwood et al., 2004). As the ER-complex binds to ERE, transcriptional co-factors that alter the chromatin structure of DNA are recruited and RNA polymerase II initiates mRNA transcription (Matthews and Gustafsson, 2003). Both co-activators and co-repressors are recognised in regulating ER controlled gene transcription (Klinge, 2000). The resulting mRNA transcripts are translated into polypeptides and modified into active proteins (Figure 3). In fish, ERs mediates the transcription of the expression of egg yolk precursor protein (vitellogenin; Vtg) and eggshell proteins (*zona radiata* proteins; *Zr*-proteins) (Hyllner *et al.*, 1991; Oppen-Berntsen *et al.*, 1992; Arukwe *et al.*, 2000). These proteins are later transported to ovaries and incorporated in the eggs (Arukwe and Goksoyr, 2003).

The DNA-binding sites of ERs (i.e. the EREs) consist of specific palindromic DNA sequences of the same hexameric DNA core motif (5'-AGGTCAnnnTGACCT) (Klinge, 2000). The ERs bind as dimers to the response element and each monomer interacts with a half site sequence within the ERE (Zilliacus et al., 1995). The dimerization of nuclear receptors is a common mechanism to increase binding site affinity, specificity and diversity. The possibility for this is achieved as a result of a) the cooperative binding of receptor dimers, b) the lower frequency of two hexamer binding motifs separated by a spacer compared to that of single hexamers and c) because heterodimers might have distinct recognition sites compared to homodimers (Klinge, 2000).

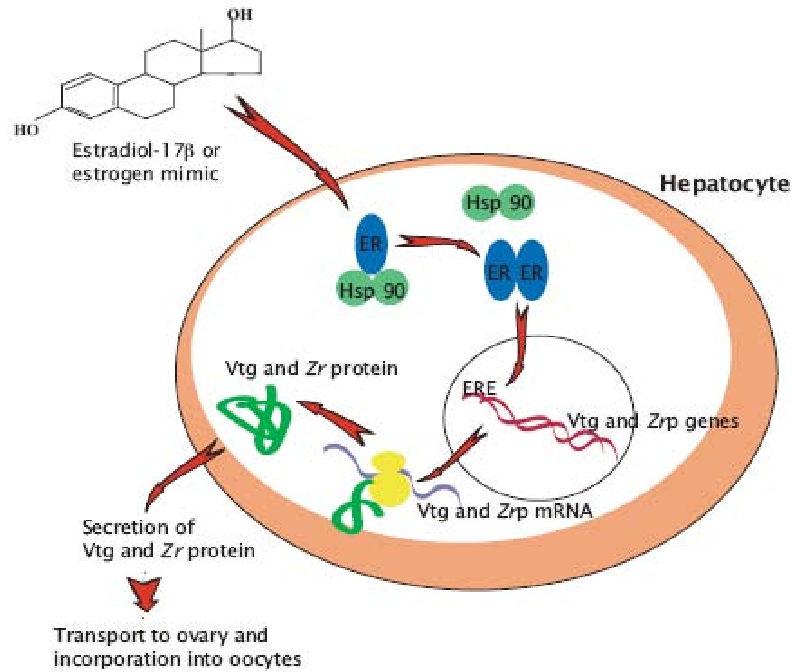

Figure 3. Estrogen stimulated oogenic protein synthesis. E2 or an estrogen mimic bind to ER, this results in dissociation of ER from hsp-90 chaperones, receptor dimerization and activation of gene expression after binding to the ERE. Figure from Arukwe and Goksøyr (2003).

In fish, three ER-isotypes have been characterized, namely the ERα, ERβ and ERγ (i.e. ESR2b in zebrafish) (Menuet *et al.*, 2002; Hawkins and Thomas, 2004; Sabo-Attwood *et al.*, 2004; Greytak and Callard, 2007). The specific functions of the individual receptors are still not clarified. However, the receptor expression patterns varies between different tissues, life stages and hormonal or toxicological exposures (Jobling et al., 1996) and the ER isotypes have different ligand binding affinities and trans-activation properties (Menuet et al., 2004).

The piscine ERs are known to control the mRNA transcription of several genes involved in growth, development and reproduction. Observed increases in ERE regulated gene expression in fish liver is tightly coupled to estrogen-dependent up-regulation of ERα expression both at the mRNA and protein level (Menuet et al., 2004; Meucci and Arukwe, 2006). Increased endogenous E2 levels generally parallel hepatic production of Vtg and *Zr*-protein that are transported in the bloodstream to the ovaries and incorporated in the eggs (Arukwe, 2001). The extracellular oocyte envelope of vertebrates contains three protein homologs referred to as zona radiata proteins (*Zr*-proteins) that are conserved from fish to mammals (i.e. zona pellucida). Zr-proteins respond to estrogen and xenoestrogen exposure with increased expression levels (Arukwe et al., 1997b). Xenoestrogen exposure could

interfere with the timing of oogenesis in immature and adult females and in the worst case scenario induce oogenesis in immature and/or male fish (Arukwe, 2001). Another protein produced in response to increased E2-levels is vigilin or high-density lipoprotein-binding protein (Goolsby and Shapiro, 2003). Vigilin is a ubiquitous protein that is highly conserved in eukaryotes and has been assigned a diversity of biological roles, including chromosome partitioning at mitosis, protein translation and control of mRNA metabolism (Goolsby and Shapiro, 2003). In african clawed frog (*Xenopus laevis*) the stability of Vtg mRNA transcripts is enhanced once E2-induced vigilin binds specifically to a 3'-untranslated region (3´-UTR) segment of the Vtg mRNA, thereby protecting the mRNA from degradation (Dodson and Shapiro, 2002).

Aryl Hydrocarbon Receptor (AhR) signalling pathway

Xenobiotic compounds that alter normal energy-yielding metabolism could represent a hazard to the organism if not eliminated or inactivated. The chemical properties of the compounds affect their biological and/or toxicological fate (Xu et al., 2005). For example, highly polar or volatile compounds may be excreted unchanged (Van der Oost et al., 2003). Non-polar lipophilic compounds could either be retained (stored in lipophilic compartments) or converted to more hydrophilic species spontaneously or enzymatically, before excretion (biotransformation) (Van der Oost *et al.*, 2003; Leaver *et al.*, 2007). The enzymatic metabolism of xenobiotics has been characterized as a biphasic process in which the compound first undergoes functionalisation (oxidation, reduction or hydrolysis) also called phase I metabolism (Mansuy, 1998; Guengerich, 2001). The metabolite could then undergo subsequent conjugation with highly water-soluble, endogenous substrates catalysed by transferases in a phase II metabolic process (Xu et al., 2005). While some xenobiotics undergo only phase I metabolism, others already hold functional groups appropriate for conjugation. However, metabolism is not always a detoxification or inactivation process, as several xenobiotics are metabolically converted to a potentially more reactive and harmful substances (Stearns et al., 1995; Yun et al., 1995).

The majority of phase I biotransformation in eukaryotes are oxidation reactions catalyzed by membrane bound monooxygenases located in the smooth endoplasmatic reticulum of the cells (Ma and Baldwin, 2000). Among these, the cytochrome P450 (CYP)-dependent monooxygenases are dominant (Guengerich, 2001). The CYP-enzymes play important roles in the biotransformation of both endogenous (steroids, fatty acids, bile acids

and prostaglandins) and exogenous (natural plant products, drugs and pollutants) substances (Bard, 2000). Expression and activity of CYPs are tightly regulated and can be induced by their respective substrates involving transcription and translation activation (Arukwe, 2002). The CYP-enzyme inserts one oxygen atom from molecular oxygen (O_2) to a substrate via the enzyme's heme group, and the reaction requires two electrons supplied by NADPH (Guengerich, 2001). The classification of CYP-enzymes is based on proposed evolutionary relationships between genes determined from the degree of amino acid sequence identity between pairs of enzymes in mammalian species (Nebert et al., 1987). The classification includes the designation of "CYP" followed by gene family (Arabic number), subfamily (capital letter) and specific gene (Arabic number) (Nebert et al., 1987). Transcriptional activation of many CYPs is regulated by distinct mechanisms. For example, CYP1A1, CYP1A2 and CYP1B1 are regulated via the AhR (Rowlands and Gustafsson, 1997; Ma, 2001). The pregnane X receptor (PXR) induces expression of enzymes of the CYP3A family and the constitutively androstane receptor (CAR) is involved in CYP2B regulation (Waxman, 1999; Wei et al., 2000; Xu et al., 2005). In fish, CYP1A1-mediated processes are regarded as the most important reaction of xenobiotic transformation to reactive intermediates. Important substrates or inducers are dioxin-like compounds such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), non-*ortho* polychlorinated biphenyls (PCBs) and poly aromatic hydrocarbons (Safe et al., 1985).

In phase II metabolism, a water soluble group is added directly to an endogenous or xenobiotic compound or to the phase I metabolite by covalent binding. Conjugation reactions involving uridine-diphosphate glucuronosyltransferase (UGT) and gluthatione-S-transferase (GST) are extremely important mechanisms in handling xenobiotics (Clarke et al., 1992; Henderson et al., 1998). The UGTs are a multigene family of endoplasmatic reticulum-bound enzymes that conjugate UDP-glucuronic acid with lipophilic acceptors (Findlay et al., 2000). Members of the UGT1 and UGT2 subfamilies are important in xenobiotic metabolism (Leaver et al., 2007). Different UGTs have both dissimilar and overlapping substrate specificities. For example, UGT1 has a broad spectrum of substrates which include bilirubins, carboxylic acids, amines and phenols and UGT2 is involved in steroid and bile acid conjugation (Tukey and Strassburg, 2000; George and Taylor, 2002). Since conjugation is an energy dependent reaction, the enzymes are associated with high-energy compounds (Tukey and Strassburg, 2000). Chemical compounds that induce CYP1A biotransformation enzymes simultaneously regulate UGT-forms that conjugate planar substrates (Leaver et al., 2007). In

fish, the mRNA transcription of both CYP1A1 and certain isotypes of UGT is mediated through the AhR (Gu et al., 2000).

GSTs belong to a superfamily of multifunctional proteins with fundamental roles in oxidative stress responses and in cellular detoxification of a wide range of endogenous and exogenous compounds (Frova, 2006). Several GSTs (but not all) are enzymes that catalyze the transfer of the tripeptide glutathione (GSH: γ-Glu-Cys-Gly) to a broad variety of substrates including xenobiotics (Frova, 2006). GSH is the co-substrate of GST reactions as well as an important antioxidant, reductant and radical scavenger (Jensson *et al.*, 1986; Bartling *et al.*, 1993). The conjugation reactions allow for subsequent renal excretion and removal of potentially harmful metabolites from the organism. GSTs are divided into three main subfamilies; the soluble or cytosolic (soluble) GSTs, the microsomal GSTs and plasmid-encoded bacterial phosphomycin-resistance GSTs (Frova, 2006). The cytosolic GSTs are found in all aerobic organisms (Frova, 2006). The active enzymes consist of either homo- or hetero-dimers and the GST monomers are divided into seven classes in vertebrate species (alpha, mu, pi, zeta, theta, omega and sigma) based on the primary structure, physiological structure (intron number and position) and immuno-reactivity (Henderson et al., 1998). Studies on pi-GST double knockout mice indicate that the pi-class GSTs have no critical physiological function but have major roles in the xenobiotic defence involving detoxification of carcinogenic compounds such as PAH metabolites (Hu *et al.*, 1997; Henderson *et al.*, 1998).

Persistent organic pollutants (POPs) that include polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and organochlorine pesticides (OCPs) are common and persistent environmental contaminants (Weber and Goerke, 2003). These compounds are characterized by low water and high lipid solubility that lead to bioaccumulation in adipose tissues (Pierce et al., 2007). Some POPs are also considered to be endocrine disrupters, which by altering the hormonal system can damage the reproductive and immune systems of exposed individuals as well as their offspring (Reijnders, 1986; Fossi *et al.*, 2002; Lintelmann *et al.*, 2003).

TCDD and structurally related compounds have been shown to have strong AhR binding affinity (Hahn, 1998). AhR is a member of the basic-helix-loop-helix Per, ARNT, Sim (bHLH-PAS) family of ligand activated transcription factors that also include factors involved in the hypoxia response, development of the central nervous system and day-night adaptations (Gu et al., 2000). AhRs are ubiquitously expressed in most organs and found in

the cytoplasm in complex with hsp90 chaperones and immunophilin-like proteins that participate in masking the nuclear localization signal present at the N-terminus of the AhR protein (Pollenz, 2002). Following ligand-binding, the AhR dissociates from the hsp90-complex and translocates to the nucleus where it forms a heterodimer with another bHLH-PAS protein - the AhR nuclear translocator (Arnt) (Gonzalez and Fernandez-Salguero, 1998). The AhR-Arnt heterodimer binds to xenobiotic response elements (XREs) which is regulatory DNA sequences found upstream of AhR regulated genes (Okey, 2007). Transcription is initiated when co-activators bridges between the AhR-Arnt complex and the TATA box-associated factors (TAFs), thereby activating transcription by recruiting RNA polymerase II (Figure 4) (Kobayashi et al., 1997). The XREs consists of two half sites 5'-TNG-3' and 5'-GTG-3' (N designate any nucleotide), which bind to amino acids within the AhR and Arnt, respectively (Kobayashi et al., 1997). The DNA-binding amino acids of bHLH-PAS proteins have been identified by domain mapping and amino acid substitution experiments (Karchner et al., 2006). Several genes involved in metabolism and degradation of lipophilic and persistent compounds contain XREs in their promoter regions among them CYP1A1, CYP1A2, UGT and AhR-repressor (AhRR) (Whitlock, 1999; Ma, 2001). The fact that AhR-null mice is resistant to TCDD toxicity confirms the direct involvement of AhR in TCDD biotransformation (Gonzalez and Fernandez-Salguero, 1998).

As previously stated, the metabolism of xenobiotic compounds could lead to formation of more detrimental substances. The AhR signal transduction pathway is regulated in order to determine the duration and magnitude of the regulatory response firstly by competition with AhRR, and secondly by depletion of AhR protein through 26S proteasome activity. Structurally, AhRR belongs to the bHLH-PAS transcription factor family and suppresses AhR mediated transactivation by competing with AhR for heterodimer formation with Arnt (Gu et al., 2000). Thus, the expression of AhRR is induced by the AhR, since transcription of AhRR is mediated through binding of the AhR-Arnt complex to XRE in the 5'- regulatory sequence of the AhRR gene (Mimura *et al.*, 1999; Karchner *et al.*, 2002). The regulation of AhR protein levels involves proteosomal degradation, since after exposure to TCDD a rapid down-regulation of AhR proteins in rodent liver was observed (Pollenz, 1996; Franc *et al.*, 2001). The AhR-Arnt complex induces AhR degradation through the ubiquitin-proteasome pathway. Ubiquitin is covalently attached to the ligand activated AhR and the ubiquinated receptor is subsequently degraded by the 26S proteasome complex (Ma and Baldwin, 2000). Proteasomal degradation is ligand dependent with stronger degradation for compounds possessing higher AhR affinity (Ma and Baldwin, 2000). The entire process is selective and does not affect the

stability of Arnt (Pollenz, 1996). Proteasomal degradation together with increased AhRR levels represents cellular control mechanisms necessary to maintain cellular homeostasis. The role of proteosomal degradation in receptor protein levels is also described for other nuclear receptors such as the ERs (Alarid, 2006).

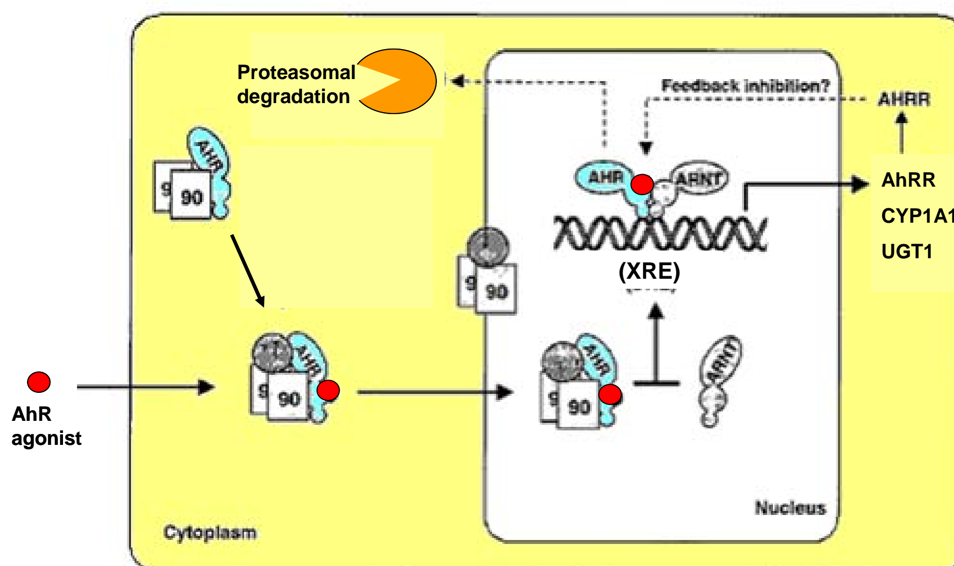

Figure 4. Model of AhR mediated gene expression. The AhR resides in the cytoplasm bound to hsp90 dimers that mediates its structure in a ligand binding form. After activation by its ligand, AhR translocates from cytoplasm into the nucleus and exchanges its chaperones for Arnt. The AhR-Arnt heterodimer binds to the xenobiotic response element (XRE) with the base sequence TNGCGTG and activates transcription of down-stream target genes. Among the activated target genes, the CYP-enzyme and UGT1 are involved in the adaptive response, and the AhR repressor (AhRR) is able to form a feedback inhibition loop by competing with AhR for the binding of Arnt. Ligand activation of AhR induces the proteasome degradation pathway. Modified from Gu (2000).

The physiological role of AhR is yet to be fully characterized. The high degree of AhR conservation suggests an important fundamental role in cellular physiology (Hahn, 1998; Gu *et al.*, 2000; Hahn, 2002). The involvement of AhR in cell cycle regulation provided evience on the influence of AhR ligands on cell proliferation, differentiation, and apoptosis, but the molecular mechanism by which the AhR affects the cell cycle is not fully understood (Chang et al., 2007). A number of endogenous ligands have been reported to activate AhR which suggests that the AhR contains a rather promiscuous ligand binding site (Denison and Nagy, 2003). In addition, the activation of un-liganded AhR by phosphorylation has been suggested (Ikuta et al., 2004). The conversion of l-tryptophan to indole-3 pyruvate in mouse tissue

extracts followed by the spontaneous reaction of indole-3-pyruvate with water produces a large number of compounds acting as AhR agonists and indicated a physiological role of AhR in the metabolism of endogenously generated compounds (Bittinger et al., 2003). AhR double knockout (AhR null (-/-)) mice have been used to determine the physiological role of AhR, and studies showed that the AhR is required for vascular development regulation in liver and other organs (Gonzalez and Fernandez-Salguero, 1998) establishing a role of AhR in development and physiological homeostasis. Recently in our laboratory, we showed that the expression of retinoid acid receptor α-isoform (RARα) apparently required the activation of AhRα by PCB-77 in salmon liver (Nordbø and Arukwe, 2007).

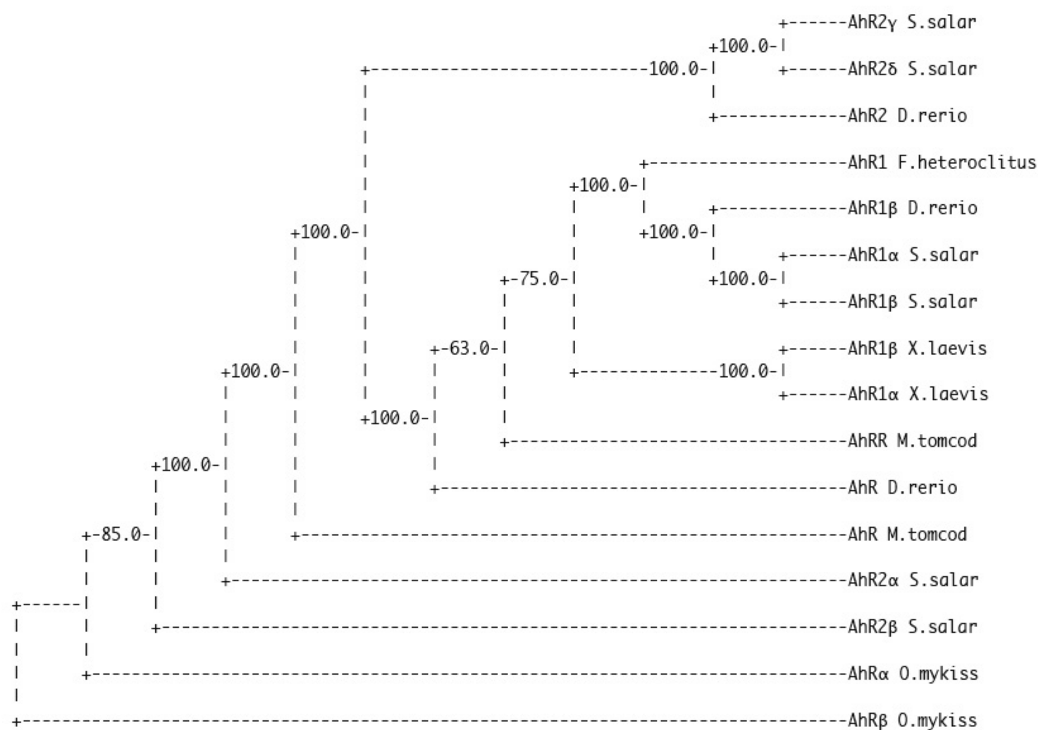

Figure 5. Phylogenetic analysis of the amino acid sequences of AhR of five teleost and one frog species. The amino acid sequences of teleost AhRs and one AhRR were aligned using ClustalW analysis, and Bootstrap values based on 100 samplings were shown above each branch. Positions with gaps were excluded and corrections were made for multiple substitutions. Protein accession numbers used; *O.mykiss* AhRα (AAC95335.2), AhRβ (AAC95336.4), *S.salar* AhR1α (AAS00539.1), AhR1β (AAS00540.1), AhR2α (AAP46168.1), AhR2β (AAP46169.1), AhR2δ (AAO18424.1), AhR2γ (AAL12247.1), *Danio rerio* AhR (NP_571103.1), AhR1β (NP_001019987.1), AhR2 (NP_571339.1) *Fundulus heteroclitus* AhR1 (AAR19366.1), *Microgadus tomcod* AhR (AAC05210.1), *Xenopus laevis* AhR1α (AAV49747.1), AhR1β (AAV49748.1) and the *M.tomcod* AhRR (AAV87644.1).

To our knowledge, only one AhR isotype has been characterized in mammals (Hahn, 2002). In contrast, two distinct evolutionary AhR gene lineages assigned AhR1 and AhR2 with several gene products (α, β, δ and γ in salmon) have been described in teleost species (Yamauchi *et al.*, 2005; Hahn *et al.*, 2006). The explicit role of each AhR isotype is not fully understood. However, the differential AhR expression patterns in different tissues of teleost species indicate explicit roles for each AhR subtype (Yamauchi et al., 2005). Phylogenetic analysis of the AhR amino acid sequences from different fish species (Zebrafish, Killifish, Atlantic salmon, Red seabream and Medaka) (Figure 5) indicates both isotype and species specific functions of piscine AhR (Yamauchi et al., 2005). The differences in AhR isotype function and activity further contribute to the complex regulation of AhR mediated gene transcription (Hahn et al., 2006).

ER-AhR interactions

The endocrine disruptive effects, including anti-estrogenic activities of AhR agonists are well documented (Safe et al., 1991). Inhibitory AhR and ER cross-talk have been thoroughly described in human breast cancer cells, rodent uterus and mammary tumour cells (Safe *et al.*, 1991; Kharat and Saatcioglu, 1996; Wormke *et al.*, 2000b; Abdelrahim *et al.*, 2003). In fish hepatocytes, negative AhR effects on estrogenic responses are reported from both *in vivo* and *in vitro* studies (Smeets *et al.*, 1999; Navas and Segner, 2000a; Arukwe *et al.*, 2001b; Rankouhi *et al.*, 2004; Vaccaro *et al.*, 2005). Several hypothesis have been proposed for the observed anti-estrogenicity of AhR agonists (see Figure 6): 1) reduction or complete inhibition of ER mediated transcription after interactions between the nuclear AhR-complex with target sequences in the regulatory promoter area of ERE regulated genes, 2) induced transcription of modifying enzymes (e.g. CYP1A1) through ligand activated AhR that can directly inhibit E2 induced gene expression or indirectly inhibit the ER-mediated transcription through other factors, 3) induction of modifying enzymes that could inhibit the effects of E2 induced proteins, growth factors or oncogenes on cellular growth and differentiation, 4) activated AhR may induce proteasomes that degrade both ER and AhR or 5) the modifying enzymes could affect cellular growth and differentiation directly (Safe et al., 1991). Other suggested mechanisms of AhR induced anti-estrogenicity are AhR-mediated induction of proteosomal degradation of the ERs (Wormke et al., 2003) and competition between AhR and ER for common transcriptional cofactors such as the Arnt (Brunnberg et al., 2003). Recently,

Matthews et al (2007) showed that TCDD and PCB126 produced inhibition of ERα-dependent expression of pS2 in human breast cancer cells and these response paralleled ERα recruitment to the *CYP1A1* or *CYP1B1* genes followed by reduced recruitment of ERα to the pS2 promoter.

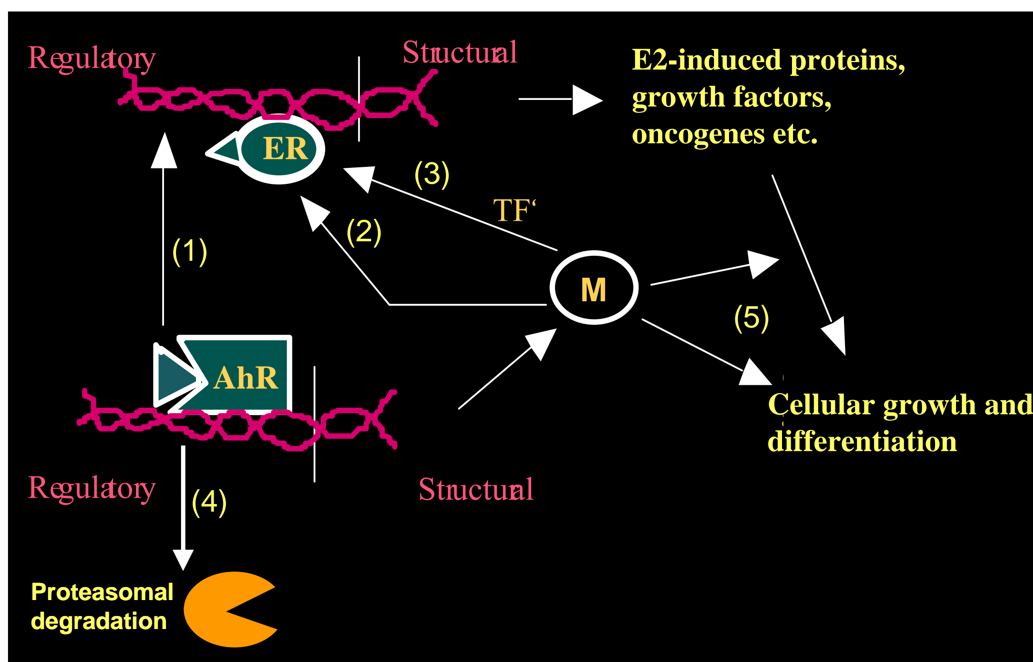

Figure 6. Proposed mechanisms of action of observed anti-estrogenic effects mediated by activated AhR. Activated AhR could inhibit ER mediated transcription directly by interfering with ER-ERE binding (1), activated AhR may induce modulating enzymes (M) that directly interfere with ER activity (2), or indirectly inhibit the ER-mediated transcription through other factors (TF) (3), activated AhR may induce proteasomes that degrade both ER and AhR (4) or M could affect cellular growth and differentiation directly (5). Modified from Safe et al. (1991).

Current research on ER-AhR interactions indicate that AhR-agonist mediated anti-estrogenic activities documented in mammalian and fish cell-based systems could represent an exception rather a rule for these chemicals. Previously, Arukwe et al. (2001b) reported *in vivo* that fish exposure to combined AhR-agonist (3,3',4,4'-tetrachlorobiphenyl, PCB77) and ER-agonist (nonylphenol, NP) resulted in both increase and decrease of NP-induced responses by PCB-77. These responses depended on the dose ratio of both chemicals, sequential order of exposure and influenced by seasonal changes (Arukwe *et al.*, 2001b). Several other studies have used multiple approaches to study estrogenic action in cell systems (i.e. ligand competitive binding assay, receptor induced transcription, quantification of mRNA expression, estrogen dependent

cell growth, pull down assays and yeast two hybrid (Y2H) systems). It has been demonstrated that one substance can possess both estrogenic and anti-estrogenic qualities depending on the cell system and the dose-ratio of exogenous and endogenous compounds (Ohtake *et al.*, 2003; Abdelrahim *et al.*, 2006; Elango *et al.*, 2006; Liu *et al.*, 2006).

One potential molecular mechanism behind AhR mediated estrogenicity that was recently proposed is the "hijacking" of ER by activated AhR-Arnt complex, followed by mRNA transcription of ERE-regulated genes (Figure 7) (Ohtake et al., 2003). The mechanisms of ER-hijacking are confirmed by other studies showing direct interactions between ER and the activated AhR-Arnt complex (Pearce *et al.*, 2004; Abdelrahim *et al.*, 2006; Liu *et al.*, 2006). In mice, stimulated estrogen production through AhR activation of Cyp19 gene expression was observed as well as initiation of ER mediated gene expression by AhR co-activation (Baba et al., 2005). While some studies suggest that AhR mediated estrogenicity requires un-liganded ERs (Ohtake *et al.*, 2003; Boverhof *et al.*, 2006), other reports imply that the presence of ER ligands is indifferent to the AhR mediated activation of ERE transcription of E2 regulated genes by AhR (Nesaretnam et al., 1996). Therefore, the use of *in vitro* versus *in vivo* studies must be evaluated when investigating mechanisms behind ER-AhR crosstalk.

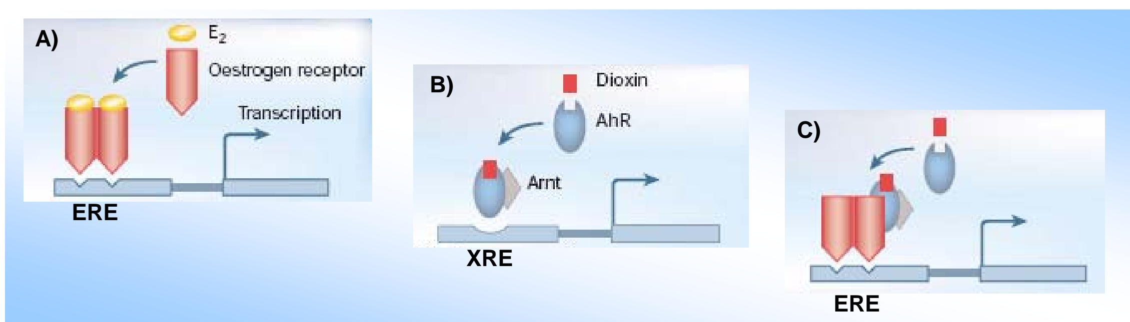

Figure 7. The ligand activated AhR-Arnt complex binds to and activates (hijack) ER. A) Eestrogen (E2) regulates cellular processes by binding to estrogen receptors in the nucleus. The activated receptors dimerize and bind to specific estrogen response elements (ERE) of the target genes and thereby induce gene transcription. B) The ligand activated AhR forms a complex with Arnt and mediates mRNA expression via the XRE. C) AhR ligands can mimic the effects of estrogens through a mechanism that involves the activation of ER by a transcriptionally active AhR–Arnt complex. The mechanism is called "ER-hijacking". Modified from Brosens and Parker (2003).

Toxicogenomics and application of microarrays technology

Previously, mechanistic toxicological questions have been approached mainly by studying the function(s) and expressions of individual genes or gene products (hypothesis-driven approach). This reductional approach has been rewarding, leading to the discovery of an impressive number of biological principles for the effects of environmental contaminants (Arukwe, 2007). However, many fundamental questions still remain concerning the mechanisms behind observed effects, mainly because gene products function singly or in combination. Hence, the biological processes leading to effects should be considered as complex networks of interconnected components (Volz et al., 2006). The combination of ***toxicology***; the study of the nature and effects of poisons, with ***genomics***; the investigation of the way organismal genetic make-up, the genome, translates into biological functions (*toxicogenomics*; most often associated with mammalian toxicology) has become a scientific discipline. Molecular and cellular events are causal and occur in a defined temporal sequence, and these molecular events branch out into different directions, up- and down-regulation of genes and proteins (i.e. molecular profiles of gene and protein expression patterns). Hence, molecular approaches have the potential to serve as predictors of toxicological (i.e endocrine) effects and to provide crucial and reliable information for specific mechanisms of hormonal action, including xenoestrogens on organismal health and reproduction (Arukwe, 2007). This implies that environmental contaminants may induce genomic responses in an organism. Depending upon the severity and duration of the toxicant exposure, genomic measures may be short-term toxicological responses leading to impacts on survival and reproduction (i.e. parental and offspring fitness). Thus, toxicogenomic approaches have emerged as a key and mainstream technique in screening new and emerging endocrine disruptors sigly or in combination, because it may reveal genetic signatures in organisms that can be used to predict their effects on the endocrine systems (Iguchi *et al.*, 2006; Arukwe, 2007; Denslow *et al.*, 2007). Therefore, the development of powerful molecular tools represents unprecedented opportunities to elucidate biological responses to environmental toxicants and stressors at the genomic level.

In the environment, chemical interactions may have profound consequences since organisms are exposed to complex mixtures of environmental pollutants. However, these complex interactions have only recently become the focus of systematic investigations (Arukwe *et al.*, 2001b). There is no doubt that "omics-based" (i.e. genomics, proteomics and metabolomics) biomarkers (of exposure to environmental hazards, of effects to environmentally-induced cellular and molecular changes and of genetic susceptibility) are

revolutionizing the science of risk assessment. As a paradigm shift in investigative toxicology, toxicogenomic approaches are replacing the current ***hypothesis-driven*** toxicology research based on "is my gene or protein affected by a given exposure?" with a ***discovery-driven*** research based on "what genes or proteins are affected by a given exposure?". Microarrays technologies are effective tools for understanding the mechanisms leading to toxicological effects. Data derived from microarrays are used to full potential when applied in the investigation of behaviour and/or functions of biological network as well as some of its particular components individually (Ge *et al.*, 2003; Arukwe, 2007).

Quantitative (real-time) polymerase chain reaction (Q-PCR) and northern blotting are methods frequently applied for analysing alteration of gene expression on the single gene level, while microarray technologies provide high throughput assays for analysis of the transcriptome (mRNA transcribed in the cells of the system). Transcriptome analyses by microarrays have facilitated significant advances in the understanding of the molecular responses in cell and tissues after perturbations and damage caused by xenobiotics (Aardema and MacGregor, 2002). Unquestionably, the gene-array technology is a powerful tool to be used in toxicology with potential to reveal genetic signatures in organisms that can be used to predict toxicity and effects of xenobiotic compounds (Larkin *et al.*, 2003b; Mazurais *et al.*, 2005; Iguchi *et al.*, 2006).

Study outline and objectives

In organisms, biological processes such as response to chemical exposure, cellular growth and organogenesis are mediated by processes of differential gene expression. An understanding of the molecular regulation of these processes requires the identification of relevant subsets of differentially expressed genes of interest to be cloned, and studied in details. Thus, the first interactions between contaminants and organisms occur at the molecular and cellular levels and these happen prior to clinical manifestation. Therefore, changes in gene expression as a result of environmental stressors and the subsequent molecular processes that lead to adverse health effects may be used as quantitative markers ("biomarkers") for cellular, organismal and population effects.

The overall objective of this thesis is to develop diagnostic gene and protein response tools in the study of the molecular mechanisms of gene expression pattern of xenoestrogen and xenobiotic interactions in wildlife species using differential gene-expression profiling in *in vitro* and *in vivo* systems. Contaminants known to be estrogenic (EE2 and NP) and/or anti-estrogenic (PCBs), either by direct ER or indirect AhR mechanistic pathways, were used as model xenobiotics and evaluated either singly or in combination using *in vitro* and *in vivo* test systems. The research addressed the following specific sub-objectives:

- *In vivo* studies, using juvenile salmon as model fish species of the xenoestrogen-induced modulation of xenobiotic systems (**Paper I**).
- Development of a targeted custom gene array for gene expression profiling of xenoestrogen and xenobiotic interaction (**Paper III**).
- *In vitro* studies, using hepatocyte cultures from model fish species (salmon and trout), of the molecular mechanisms and gene expression patterns for xenoestrogen and xenobiotic interactions (**Paper II-V**).

There are many potential stressors regulating the composition and relative abundance of fish communities in a given system. Endocrine modulators have the potential to affect reproduction, growth and development in fish. The long-term benefit of this thesis is to quantify the relative importance of endocrine modulators by mechanism(s) and their interaction in a mixture with environmental chemicals with affinity to the AhR. In addition, this thesis was designed to develop new hypotheses for empirical evaluations on ER-AhR mechanisms of interactions through the production of the targeted gene array. These specific objectives applied chemicals and species models in assessing exposure to and prediction of

effects of contaminants at the individual and the population levels in aquatic systems. The specific objectives are explained in the following hypothesis:

Hypothesis #1: The comparative responses of gene and protein expression biomarkers indicative of adverse health and reproductive effects in fish species demonstrate differential and parallel hepatic-specific patterns in control and exposed individuals.

Hypothesis #2: Hepatic gene expression profile will show chemical exposure specific (i.e. ER and AhR agonists) patterns that will be dependent on concentration (or dose) and time of exposure.

Hypothesis #3: Differences in the profile of gene and protein biomarker responses, including overexpression of certain biomarker genes in exposed individual tissue, will be indicative, prognostic and predictive of chemical susceptibility and adverse health and reproductive effects.

Material and Methods

Atlantic salmon as a model system

Fish, especially Atlantic salmon (*Salmo salar*) form the basis for economically important fisheries and aquaculture for Norway and several other countries. Salmon is an anadromous species that utilizes the best of two worlds (freshwater and seawater). Given the occurrence and distribution of pharmaceuticals and surfactants that affect the fish endocrine systems in the environment, this species provides an excellent and interesting model for the present study. By focusing on salmon and the integral aspects of the estrogenic and xenobiotic biotransformation pathways, this thesis has increaseed our understanding of the mechanisms and interactions of pollutants that affect the general fitness and disrupt organismal endocrine systems. It will also enhance our understanding of potential consequences of human consumption of contaminated fish resources. Salmon enjoys a number of natural advantages as an extremely important cultivated species: a well known reproduction biology, short egg and larval stages, rapid growth, good feed utilisation, suitable behaviour ("tame"), and, as far as we know, good health. Salmon also appears to adapt well to traditional sea-cages for aquaculture technology.

In this thesis, we have mainly used Atlantic salmon as a model organism to increase the understanding of the mechanisms and interactions of pollutants with different mode-of-action that affect the general fitness and disrupt the endocrine systems. Fish are known to contain more copies of genes than mammals and a ratio of 2:1 has been proposed (Jaillon et al., 2004). Atlantic salmon is a tetraploid organism, and evolution has provided this species with duplications of several genes that has single copy in other species (Allendorf and Thorgaard, 1984). The tetraploidity of salmon demand careful evaluation of gene expression studies since there is an increased number of pseudogenes (both functional and non-functional) in these organisms (Allendorf and Thorgaard, 1984).

In vitro techniques as a tool for mechanistic studies

The use of *in vivo* studies in toxicological studies focuses on absolute endpoints which do not allow discrimination of the underlying modes of action (Tiffany-Castiglioni et al., 1999). The direct effects of compounds *in vivo* are masked by pharmacokinetic distribution as well as secondary effects attributed to other unidentified factors (Soars et al., 2007b). The application

of *in vivo* studies are further complicated by the variation in genetic background between individuals and ethical questions concerning animal research since a large number of individuals are needed to perform statistically valuable experiments (Ge et al., 2003). *In vitro* systems, like cell and tissue models, facilitate and hence are widely used for investigating the direct molecular mechanisms accounting for adverse effects of xenobiotic compounds on wildlife and humans (Soars et al., 2007b). The primary objective of toxicological studies on cells and tissues *in vitro* is to characterize cellular and molecular substrates and pathways that contribute to adverse effects on the organism after toxicant exposure. Exposures to various compounds form a broad spectrum of responses that reflects the hierarchy of biological organisation (Tiffany-Castiglioni et al., 1999). At one end of the spectrum is the interaction between molecules leading to molecular responses and on the other, the whole organism that may or may not be affected by the exposure (Ge et al., 2003; Jansen, 2003). In the middle, there may be a formidable gap in knowledge (Ge et al., 2003). Since the liver is the most important organ in xenobiotic biotransformation, an ideal model system for xenobiotic biotransformation should accurately resemble biotransformation *in vivo* in the liver (Brandon et al., 2003; Navas and Segner, 2006). Both continuous growing cell cultures (cell lines) and primary cultures (cells taken directly from animals) have been used in mechanistic toxicological studies (Navas and Segner, 2006).

The principal approach when selecting an *in vitro* system for mechanistic studies is to limit the scope of questions asked so that the appropriate system or model can be chosen. The cell phenotype should be normal or relevant to the proposed mechanism of action (Soars et al., 2007a). While many continuous cell lines do not undergo the stages of development, maturation and aging and therefore stay young forever, others are reliably getting older (Tiffany-Castiglioni et al., 1999). The stage of differentiation of cell lines can be manipulated by growth factors that influence maturity or phenotype. Established hepatic cell lines have a relatively stable phenotype and are easier to culture and maintain compared to primary hepatocytes. One drawback of using cell lines in biotransformation studies is that, albeit having relatively stable enzyme concentrations, the expression levels of most phase I and phase II enzymes are low in these cells (Brandon et al., 2003). Primary cell cultures are obtained from tissues of individual animals and have a limited lifespan. As primary hepatocytes in culture provide the closest *in vitro* model to animal liver, they are well adapted for investigating xenobiotic transformation, enzyme induction and inhibition and biotransformation-mediated toxicity (Soars et al., 2007b). However, a number of liver-specific functions, like cytochrome-P450 activity, are reduced during isolation and with time

(Segner, 1998; Brandon *et al.*, 2003). The use of primary hepatocytes encounters the problem of considerable inter-individual variations, but the problem could be overcome by using mixtures of hepatocytes from multiple donors (Brandon et al., 2003; Soars et al., 2007b).

To find the parallel between whole animal (*in vivo*) models and those not involving the use of whole animals such as *ex vivo* models (tissues from exposed animals) or *in vitro* models (cells and tissues that do not involve exposed animals), one has to include the interpretation of various *in vitro/ex vivo* experimental systems (Brandon et al., 2003). Particularly, an investigation of endpoints indicative of toxicities that occur under specific concentration regiments, with comparison to *in vivo* models (Tiffany-Castiglioni et al., 1999) is of primary significance. Knowledge of the system used, including the factors that may contribute to variability and phenotypic limitations are of vital importance, especially when attempts are made to extrapolate to events that occur in whole animals. In circumstances where the effects of exposure appear to be similar in an *in vivo* and *in vitro* experiment, the magnitude of the effects could differ, making extrapolation to *in vivo* events difficult (Tiffany-Castiglioni et al., 1999). There are times when the *in vivo/in vitro* difference is not resolvable. Ideally, *in vivo* studies should follow *in vitro* results whether the effects of the cell treatments are identified or not.

The correlation between the concentrations needed to produce effects *in vitro* and those relevant to *in vivo* toxicities are not always comparable. There are a number of reasons why differences in concentrations occur and to disregard data solely because they were obtained at higher concentrations than those expected in animals is unwise due to the difference in uptake and metabolic mechanisms in cells and whole animals (Tiffany-Castiglioni et al., 1999). The *in vitro* system chosen is of most importance. For example, continuous cell lines are extremely robust and not susceptible to lethal effects of many toxicants except at high concentrations (Brandon et al., 2003). In tissue cultures, the ability to penetrate the tissue is dependent on the method used and the concentrations of the compounds in question. In primary cell and tissue cultures, other factors like the exposure regimen, animal age and sex, the capacity for compensatory mechanisms and the tissue preparation method involving inclusion or elimination of certain cell types contribute to the effects observed after treatment (Shankland et al., 2007). Time response observations are valuable and should be incorporated as one of the essential controls when performing *in vitro* experiments (Navas and Segner, 2006). The actual age (in hours), of the culture and the developmental stage of the system may cause experimental variability (McGinnity et al., 2006; Navas and Segner, 2006). Although *in vitro* methods are not able to provide information

about behaviour of compounds in real organisms (e.g. pharmacokinetics), they are a strong tool for assessment of specific toxicity mechanisms and/or for screening a large number of chemicals (such as agrochemicals, pharmaceuticals or environmental contaminants) (Janosek et al., 2006).

Liver perfusion technique

Primary hepatocytes are popular *in vitro* systems for drug biotransformation research due to their strong resemblance of *in vivo* liver (Brandon et al., 2003). In the present studies (**Paper II-V**) hepatocytes were isolated from the liver of trout and salmon by the collagenase perfusion method described by Howard and coworkers (1967) and Berry and Friend (1969) and later modified for fish by Andersson et al. (1983). In the initial perfusion step, the liver is cleared of blood using a buffered salt solution containing a chelating agent such as EGTA to remove Ca^{2+} (Calsium is involved in blood coagulation and cell-cell connections). In the second step, Collagenase, which dissolves intercellular collagen, is added to the solution. In salmonids the perfusion is attained through the well developed portal vein. The enzymatic digestion is followed by mechanical disruption when the cells are gently massaged through filter mesh of 150 µm. The cells are collected in medium and washed by several low-speed centrifugations (50 x g for 2 minutes). The cell suspension obtained by perfusion contain mainly hepatocytes which occupy 80 % of the original liver volume (Segner, 1998).

Suppressive subtractive hybridization (SSH) and SalArray analysis

We generated a targeted cDNA library by performing suppressive subtractive hybridization (SSH) with liver samples from juvenile salmon exposed separately to ER-agonists (NP and E2) and AhR-agonists (TCB and BNF). The SSH experiment was performed in the forward and reverse directions to obtain up-regulated and down-regulated genes, respectively. Recombinant colonies were then picked and their cDNA insertions were sequenced before submitted to GenBank database under the title "SalArray SSH cDNA library". The 300-clone salmon membrane cDNA array (SalArray) was constructed of clones with unique expression patterns either as up- or down-regulated in the SSH. All clones were PCR amplified and verified by agarose gel electrophoresis. Clones were spiked with *Arabidopsis thaliana* mRNA as a control for loading differences and array was printed by EcoArray Incorporated (Alachua Fl, USA) under contract. Hybridization of the arrays was performed using P^{33}-labeled cDNA

probes. Radioactivity was scanned using a Phosphoimager FLA-2000 (Fuji), and the spot-density data for each membrane were individually quantified by Array Gauge v2.1 (Fuji Film). The general background of each membrane was subtracted from the average spot-intensity values for the duplicate spots on the membrane. Thereafter, the background-normalized spot-intensity values were further normalized with the *A. thaliana* spikes on the same membrane.

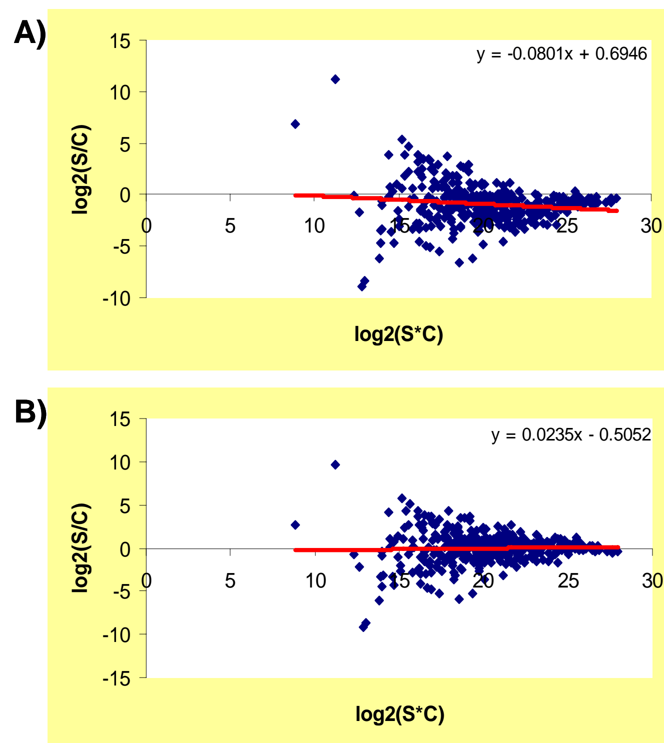

Figure 8. Ratio intensity plots (RI-plots) showing loess normalization. Normalization was performed to minimize the effects of systematic errors between arrays. First, the product of spot signal intensity between exposed samples (S) and the respective control sample (C) were log2 transformed, and then, the ratio between S and C was found and log2 transformed. Presentation of data prior to loess normalization (A) and after loess normalization (B) showing the trend-line function in the top right corner of each figure. Note that in panel B, the distribution of data is centred about y = 0.

Expression levels of genes were expressed as fold change relative to control by dividing the signal intensity of exposed samples (S_i) by the signal intensity in the respective control sample (C_i). The ratio was log2 transformed, and the measured $\log_2(S_i/C_i)$ ratio was visualized as a function of the $\log_2(S_i{*}C_i)$ product intensities in a ratio-intensity plot (RI plot) (Figure 8A). Finally, loess normalization was performed to minimize systematic deviations (RNA quality, probe labeling, hybridization, and development of image) in the $\log_2(S_i/C_i)$ ratio values of spot intensity levels between exposed samples and controls (Figure 8B). The

loess and spike normalizations were the only data normalization performed on the array data, since other options such as normalization with a suite of reference genes did not give useful results. The normalized ratio $\log_2(r^*)$ was evaluated, and stringent criteria were used to filter for genes that were regulated at least 0.3-fold as compared to their respective controls.

Quantitative real-time PCR

Quantitaitive real-time polymerase chain reaction (qPCR) is an established technique for determining the number of specific mRNA transcripts in biological samples. The method has high sensitivity, large dynamic range, the potential for high troughput as well as accurate quantification (Bustin, 2004). Total cDNA for the qPCR reactions were generated from DNase-treated total RNA using a combination of random hexamer and poly-T_{18} primers. The expression of individual gene targets was analyzed and every DNA amplification reaction contained controls lacking cDNA template to determine the specificity of target cDNA amplification. Briefly, cycle threshold (*Ct*) values obtained were converted into mRNA copy number using standard plots of *Ct* versus log copy number. The criterion for using the standard curve is based on equal amplification efficiency with unknown samples and this is checked prior to extrapolating unknown samples to the standard curve. The standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest. Data obtained from triplicate runs for target cDNA amplification were averaged and expressed as % of the solvent control sample quantity. Standard errors were calculated, statistical differences among treatment groups were tested using analysis of variance (ANOVA) and comparison of different exposure treated and control groups were performed using Tukey's multiple comparison test.

To obtain reliable results with biological significance, it is important that qPCR data are normalized with a proper internal control. In this regard, prudent reference gene selection is important in evaluating relative gene expression in samples (Czechowski et al., 2005). Several laboratories including ours have observed that the use of reference genes (so called house-keeping genes) in toxicology vary considerably between different experimental conditions and exposures (Brown et al., 2004; Bustin and Nolan, 2004). The effect of xenobiotic exposure on altered gene expression is dependent on the severity and duration of the toxicant exposures; therefore the assumption that certain genes have a constant level of expression regardless of experimental conditions has become a misconception of reasonable

concern. The considerable variability of so-called housekeeping genes across different experimental conditions could lead to misinterpretation of the expression profile of a target gene (Arukwe, 2006). One example is the widely use β-Actin gene for normalization, we have found that exposure of salmon to DDE or NP decreases and increases (respectively) β-Actin mRNA liver (Arukwe, 2006) and similar effect have been observed in several experiments and other so-called housekeeping genes in our laboratory. We are yet to find a house-keeping gene that does not vary according to experimental conditions.

Immunochemical techniques

Immunochemical detection methods represent sensitive laboratory techniques widely used to detect and quantitate antigens (proteins) present in samples. It can be quantitative (with a standard curve) or semi-quantitative (without a standard curve). The competitive Enzyme-Linked Immunosorbent Assay (ELISA) used for plasma Vtg quantitation (**Paper I**) is a robust and reproducible method for quantifying Vtg in plasma. The method is based on competition between known concentrations of purified Vtg and unknown plasma samples for a polyclonal Arctic char Vtg antibody as previously described by Meucci and Arukwe (2005). Immunochemical analyses of ERα protein levels were performed using Western blotting (**Paper V**). Cellular homogenates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemli (1970). The protein was then transferred to a polyvinylidene fluoride (PVDF) membrane and salmon ERα proteins were detected using primary polyclonal antibodies against human ERα amino acids 154-174 (Accn. No. NM_000125) and visualized after incubation with peroxidise conjugated secondary antibody and substrate for chemiluminescent detection.

General discussion

Targeted SalArray gene chip

As described in **paper III**, we constructed a custom cDNA library containing 300 clones of transcripts from Atlantic salmon exposed to combined ER- and AhR-agonists. The cDNA library was used for developing a toxicological targeted membrane cDNA array (SalArray). When genomic data for an organism is not available, the SSH method is a helpful tool for generating cDNA libraries containing DNA sequences of interest (Brown et al., 2006). The SalArray cDNA chip contained transcripts from genes that were either up- or down-regulated in salmon liver exposed to ER- and AhR-agonist.

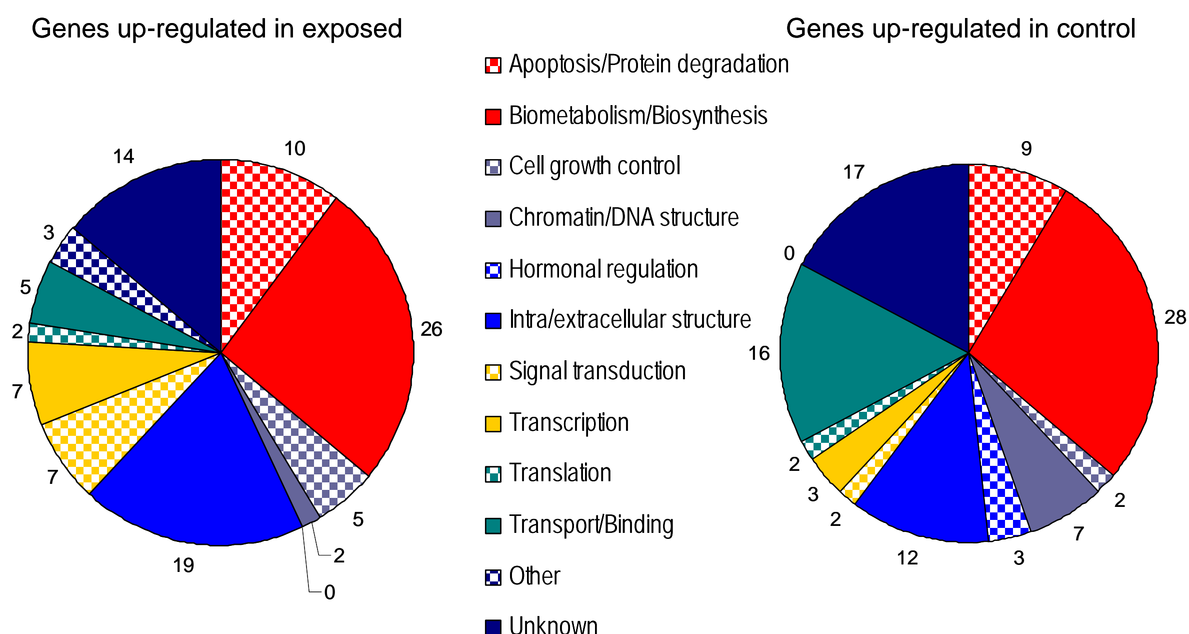

Figure 9. Distribution and functional category assignment of genes identified as either up- or down-regulated in response to ER- or AhR-agonist exposure.

When assigning the transcripts, we observed that a wide range of biological pathways were represented in the library confirming that in toxicology, almost without exception, gene expression in most pathways is altered as either a direct or indirect result of toxicant exposure (Volz et al., 2006). The distribution and functional category assignment of genes identified as either up- or down-regulated in response to combined ER- and AhR-agonist exposure is shown in Figure 9. However, several genes known to be involved in the transcriptional regulation of xenobiotic biotransformation and steroid metabolism (e.g. AhRs, ERs,

aromatase isotypes and the steroidogenic acute regulatory (StAR) protein) were not represented due to low expression levels. Hence, transcripts from these genes were amplified by PCR using specific primers from conserved regions of the respective genes based on sequence informations in the GenBank. The PCR-products were cloned into *Escherichia coli* plasmids and added to the array. All sequences were annotated and thereafter submitted to GenBank.

Gene arrays are commercially available for several mammalian species as well as zebrafish and Rainbow trout (Larkin *et al.*, 2003b; Finne *et al.*, 2007; Pomati *et al.*, 2007). Our SalArray contained transcripts from 300 clones which is a small number compared to commercially available arrays containing 40 000 genes. Comparatively, the targeted array approach contained a unique selection of genes generated by the SSH method. Thus, the array provided the required information for screening of toxicologically interesting candidate genes after exposure to environmental chemicals. Microarray analyses generate enormous amounts of data. Like most new technologies and approaches, interdisciplinary collaborations accross scientific disciplines are required for handling informations of this magnitude (Ge et al., 2003). Sophisticated methods for data storage and analyses are therefore the biggest obstacle in array analyses (Ge et al., 2003). Although several software packages are available for array analysis, these approaches and algorithms are yet to be standardized and their full potential can only be realized by trained staffs (Aardema and MacGregor, 2002). Therefore, the targeted arrays approach (with few, but toxicologically relevant genes) was a reasonable approach for our toxicological questions of interest. Commercial microarrays are usually used in a systems biology approach where the main goal is to investigate the effects of perturbations on network interactions (holistic approach). Given that targeted array was a reductional approach, the 300 clone SalArrays provided adequate information to uncover novel genes to answer the toxicological questions asked and for the generation of new hypotheses.

Thus, the combination of the analytical power of SSH, qPCR and hepatocytes culture was used to evaluate the interactions between the ER- and AhR-signalling pathways (**Paper III**). Primary cultures of salmon hepatocytes were exposed to NP and PCB77, given singly or in combination. Previously, we found that the concentrations used were optimal for *in vitro* studies for ER-AhR interactions in salmonids (**Paper II**). Altered gene and protein levels as a result of toxicological insult are expected to occur at concentrations below those that produce pathological alterations (Aardema and MacGregor, 2002). However, in order to reveal molecular mechanisms involved in ER-AhR interactions, the compound concentrations in this study needed to exceed the capacity of the cellular defence mechanisms. The SSH technique

favours the enrichment of high abundance transcripts and therefore is susceptible to a high false positive rate (Larkin et al., 2003a). Figure 10 presents a Venn diagram of the global expression patterns of transcripts of solvent control and cells exposed to NP and PCB77.

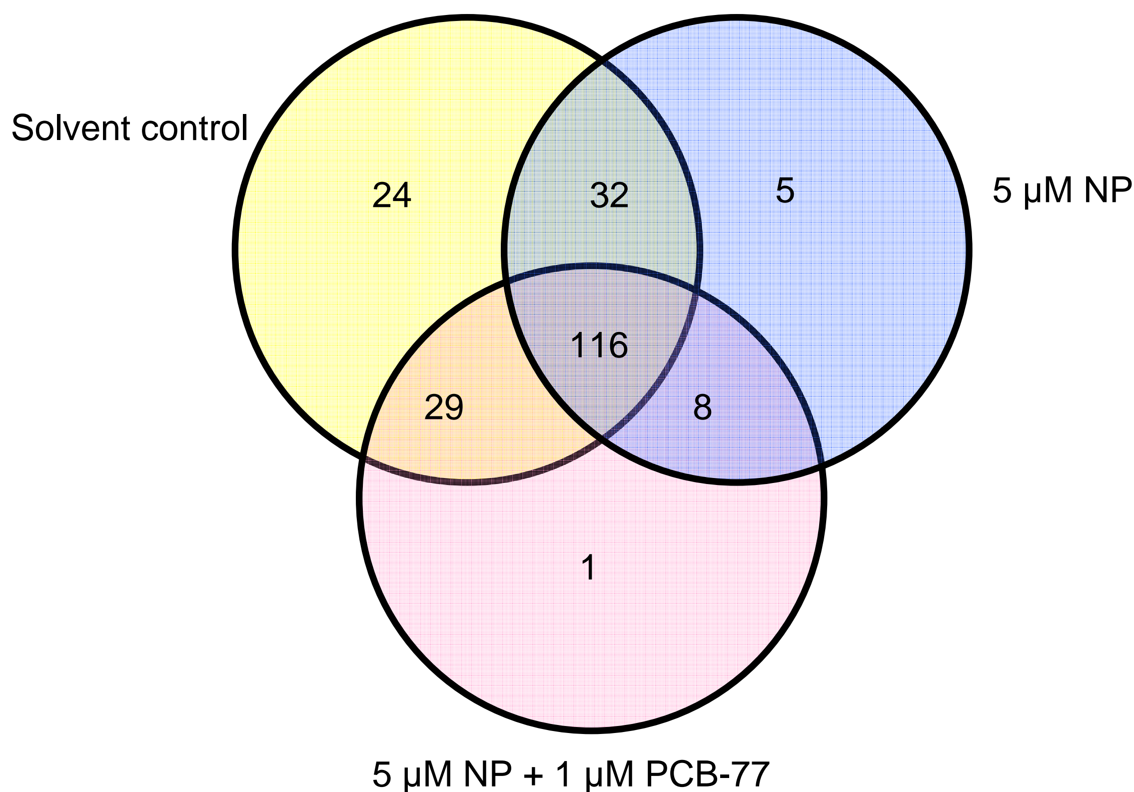

Figure 10. Venn diagram of the global expression patterns of transcripts of solvent control and cells exposed to NP and PCB77.

The array results showed that several genes involved in the steroid hormone synthesis and metabolism were increased in hepatocytes exposed to PCB77 singly or in combination with NP. Specifically; 3β-hydroxysteroid-dehydrogenase (3β-HSD), androgen receptor, CYP11α, CYP11β, CYP19α, CYP19β, in addition to the StAR protein were modulated. Among these, only CYP19α and β (aromatase) increased in response to NP (**Paper III**). PCB77 is an AhR agonist, previously it was shown that AhR deficient mice had insufficient synthesis of E2 and AhR was found to be a regulator of aromatase in female mice (Baba et al., 2005). The effects of NP and PCB77 on hepatic steroidogenic pathways represent the first report on the parallel expression of aromatase gene isoforms, StAR, CYP11β and 3β-HSD in fish liver and the subsequent modulation by an estrogen mimic. The teleost liver is not a typical steroid

producing organ, but rather a steroid metabolizing organ. Therefore, the physiological role or consequences of NP-mediated expression of aromatase-isoforms, StAR and 3β-HSD in salmon liver is yet to be established. Nevertheless, the induction of steroidogenic enzymes and proteins are highly tissue- and cell-type specific and is controlled by different promoters and second messenger pathways. These pathways may provide various targets for interaction with xenobiotics and studies. Even though the expression of steroidogenic enzymes and proteins in fish hepatic tissues has been established the functional role of steroidogenesis yet to be recognized (Wang and Ge, 2004; Zhou et al., 2005).

In general, the targeted SalArray gene chip demonstrated that exposure of salmon to NP singly or in combination with PCB77 produced differential gene expression pattern in salmon liver. Array analysis showed that exposure of hepatocytes to NP mainly altered genes involved in the estrogenic pathway, including genes involved in steroid hormone synthesis and metabolism. The anti-estrogenic properties of PCB77 were demonstrated in the array analysis as genes induced by NP were decreased by PCB77. To study the effects of PCB77 on ER-mediated transcription, hepatocytes were treated for 48 h with tamoxifen (Tam; 1 μM) and ICI182, 780 (ICI; 1 μM). The effect of AhR on ER-mediated transcription was investigated by blocking AhR activity with α-naphthoflavone (ANF; 0.1 and 1 μM). Quantitative real-time PCR confirmed the changes in expression of ERα, ERβ, Vtg, *Zr*-protein and vigilin for the ER-pathway and AhR2α, AhR2β, AhRR, Arnt, CYP1A1, UGT1 as well as a 20S proteasome β-subunit for the AhR-pathway. We found that exposure to NP and PCB77 both singly and in combination produced gene expression patterns that were negatively influenced by individual receptor antagonist. PCB77 caused decreased ER-mediated gene expression and NP caused decreased AhR-mediated responses. Inhibition of AhR with ANF did not reverse the effect of PCB77 on ER-mediated transcription suggesting that AhRs do not have a direct role on PCB77-mediated decreases of ER-mediated responses. In contrast, the inhibition of ER with Tam and ICI reversed the transcription of AhR-mediated responses (except AhRR). Taken together, the findings in the present study demonstrate a complex mode of ER-AhR interaction, possibly involving competition for common co-factors. This complex mode of interaction is further supported by the observation that the presence of ER-antagonists potentiated the transcription of AhR isoforms.

Modulation of Estrogen Receptor signalling pathway

The xenobiotic effects on the expression patterns of ERα, ERβ, Vtg, *Zr*-protein and vigilin were investigated with qPCR, while Vtg and ERα were analysed using immunochemical detection techniques (ELISA and Western immunoblots, **Paper I and V,** respectively). In classical endocrinology, it is assumed that estrogens and their mimics mediate their effects in liver through binding to the ERs. In teleost species ER mediates the expression of several genes involved in growth and development, including Vtg and *Zr*-protein. We investigated the effects of exposure to EE2 in salmon and to NP on primary hepatocytes of trout and salmon (**Paper I-V**).

Three ER-isotypes (ERα, ERβ and ERγ) have been described (Matthews and Gustafsson, 2003; Sabo-Attwood et al., 2004). The specific functions of each receptor isotypes are still not clarified in teleost species. However, the receptor expression patterns varies between different tissues, life stages and hormonal or toxicological exposures (Sabo-Attwood et al., 2004). ERα and ERβ exhibit differences in their transcriptional responses to EE2 and NP (more so for salmon ERs; **Paper III-V**), indicating isotype-specific differences in the ER LBD (Barkhem et al., 1998). The ERs have two activation domains, namely a constitutive activation function-1 (AF-1) and a hormone dependent (AF-2) domain. These two domains function in synergy but may also function independently in certain cell and promoter context (Nilsson et al., 2001). The ERβ mRNA expression pattern in salmon (**Paper III- V**) did not parallel that of ERα, Vtg or vigilin. Furthermore, the intrinsic transcriptional activity of ERβ in liver was minimal compared to ERα (**Paper IV and V**). For example, a human variant of ERα(-) Ishikawa endometrial cell line were unresponsive to E2, despite their expression of ERβ, reflecting the low transcriptional activity of ERβ, compared to ERα (Shipley and Waxman, 2006).

In fish, the responses of target genes are directly dependent on the cellular ER concentration (Specker and Sullivan, 1994; Navas and Segner, 2006). The ERs are the rate limiting transcription factor for mediating estrogenic responses (Arukwe et al., 2001a). While minor alterations in ERα and ERβ expression levels were observed in liver of salmon exposed to EE2 for 3 and 7 days (**Paper I**), the expression levels of ERα increased in response to NP in trout and salmon primary hepatocyte cultures and of ERβ in trout hepatocytes (**paper II-V**). As described in **paper I,** a possible EE2 masking of effects on ER expression levels was suspected.

Exposures of salmon hepatocytes to NP produced a substantial ER expression after 12 h, and thereafter, slight increases were observed at 24, 48 and 72 h post-exposure (**Paper IV and V**). The genes encoding the ER are constitutively expressed to produce basal levels of the receptors sufficient for maintaining its own expression (Flouriot et al., 1996). The ER expression levels are therefore auto-regulated. An increase in E2 levels requires additional receptors for activation of target genes. Thus, a rapid expression of ERs is brought about by binding of ERs to EREs upstream the ER gene (Pakdel et al., 1997; Yadetie et al., 1999),resulting in the availability of sufficient ER levels for initiating oogenic protein gene regulations. However, complete regulation of oogenesis includes a complex biochemical network involving the HPGL-axis feedback process (Arukwe and Goksøyr, 1998).

Analysis of cellular ERα protein levels was performed by western blotting using antiserum against human ERα (hERα). Our data showed a protein band of approximately 68 kiloDalton (kDa) in control and exposed samples (**Paper V**). The rabbit-anti-hERα antiserum was generated against amino acids 154 -171 that correspond to the DNA binding C-domain of human ER1 (Accn no. NM000125) (Sabo-Attwood et al., 2004). The use of hERα in detecting salmon ERα by immunoblotting was evaluated by aligning human and salmon ERα amino acid sequences (Figure 12). There is a high degree of conservation between the amino acid sequences of Atlantic salmon and human in the DB-domain. Given the high degree of conservation in thatn domain, antisera generated for hERα is suitable for detecting ERα from several vertebrate species, including Atlantic salmon, Rainbow trout, zebrafish (*D.rerio*), African clawed frog (*X. laevis*) and Chicken (*Gallus gallus*). Furthermore, the hERα antibody will probably detect the salmon ERβ due to their amino acid sequence similarities (see Figure 11).

Our data showed increased levels of ER mediated gene expression (Vtg, *Zr*-protein, ERα, ERβ and vigilin) as well as increased plasma Vtg and cellular ERα protein levels after treatment with EE2 and NP (**Paper I-V**). Vtg and Zr-protein are biomarkers of exposure to estrogenic chemicals and the responses observed in this study are in accordance with previous reports (Arukwe and Goksøyr, 1998; Celius et al., 2000; Fossi et al., 2002; Meucci and Arukwe, 2005). The induction of vigilin by NP in salmon hepatocytes (**Paper III-V**) supports its role of maintaining cellular Vtg mRNA levels before translation. The stability of liver Vtg mRNA is regulated by binding of vigilin to the 3'-untranslated region (3´-UTR) segment of Vtg mRNA, thereby obstruct mRNA degradation (Dodson and Shapiro, 2002). The regulation of the ERs and their downstream products share important common features, but they exhibit

striking differences in their transcriptional kinetics in response to estrogens reflecting different sensitivities to the liganded ER (Flouriot et al., 1996). For example in **Paper III**, a 3-fold increase in ERα expression and a 35-fold Vtg expression were observed after exposure of hepatocytes to NP. A slight increase in cellular ER protein levels will promptly increase the expression of numerous gene products (including Vtg).

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                                      DBD
NM_000125     RRASTNDKGSMAMSAKT----RYCAVCNDYASGYHYGVWSCGCKAKRSGH     140
S.salar       SKCTSNRSYAAAGSGVRVMANTRYCAVCSDASGYHYGVWSCGCKAKRSGH     149
O.mykiss      SKCASDRSYSAAGSGVRVMANTRYCAVCSDASGYHYGVWSCGCKAKRSGH     150
D.rerio       sgk------------------TRCAVCSDYASGYHYGVWSCGCKAKRSGH     132
X.laevis      GRRMSSANDKGSMSTKT----RYCAVCSDYASGYHYGVWSCGCKAKRSGH     141
G.gallus      RRMSSTNTKGSSMSTKT----RYCAVCNDYASGYHYGVWSCGCKAKRSGH     146
S.Salar ERb   RVSWAHASSSKRKCSSTVSGKAHCAVCHDYASGYHYGVWSCGCKAKRSGH     125

NM_000125     NDYMCATNCTDKNRR-KSCACRRKCYVGMMKGGRKDRRGGRMKHKRRDDG     189
S.salar       NDYMCATNCTMDRNRRKSCACRRKCYVGMVKGGRKDRGGRVRKDKRYCGA     199
O.mykiss      NDYMCATNCTMDRNRRKSCACRRKCYVGMVKGGRKDRGGRVRKDKRYCGA     200
D.rerio       NDYVCATNCTDRNRR-KSCACRRKCYVGMMKGGRKDRGGRSVRRRRRSSN     181
X.laevis      NDYMCATNCTDKNRR-KSCACRRKCYVGMMKGGRKDRRGGRMKHKRKKND     190
G.gallus      NDYMCATNCTTDKNRRKSCACRRKCYVGMMKGGRKDRRGGMMKKRRDSRN     196
S.Salar ERb   NDYCatnc--TDKNRRKSCACRRKCYVGMTKCGMRRDRSSYRGHRRVGRT     173
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Figure 11. Protein sequences representing amino acids of the DNA binding domain (DBD) for the estrogen receptor alpha (ERα) for *S.salar*, *O.mykiss*, *D.rerio*, *X.laevis* and *G.gallus* aligned with the human ERα (Accession no. NM_000125). The DBD is indicated with black lines and the sequence used for antibody synthesis (hER aa 154-171) is boxed in red.

For mechanistic evaluations, ER antagonists (Tam and ICI) were used for investigating the involvement of ER in ER-AhR interactions (**Paper III and V**). A unique aspect of both ER isoforms is that the partial and absolute ER antagonists (Tam and ICI, respectively) produced significant decreases of the expression pattern in presence of NP (**Paper III**). In teleost species, the expression profile of ERα and ERβ shows that both isoforms are expressed in fish liver (Sabo-Attwood et al., 2004) with different binding capacity and ability to induce transcription of E2-mediated genes (Menuet et al., 2004). The results in **paper III** confirmed the different potency of the ER antagonists. For example, Tam partially and ICI completely inhibited ERα, ERβ, Vtg and *Zr*-protein mRNA expression in hepatocytes. While Tam inactivates the ligand dependent AF2-domain and leaves AF-1 active, ICI completely inactivates both the AF1- and AF2-domains and mediates degradation of the receptor protein (Wormke et al., 2003). Thus, the result indicates that the ER mediated expressions are not simply controlled on the transcriptional level. For example, while the

absolute ER antagonist (ICI) reduced the ERα mRNA and ERα protein levels at 12 h postexposure, the ERα mRNA and protein levels were different at 24 and 48 h post-exposure (**Paper V**).

Modulations of AhR and downstream responses

The evaluation of AhRs and their donstream regulated responses was focused at the transcriptional level and activity levels (**Paper I**). Primary hepatocytes retain inducible expression of genes in xenobiotic transformation pathways (Segner, 1998). The interpretation of data concerning the AhR and its role in toxicology is complex. The complexity of the AhR regulation include 1) expression of several AhR genes in salmon, 2) the rapid degradation of AhR proteins in response to ligand activation and 3) the involvement of AhR in endogenous pathways. Comparative genomic analyses of sequenced genomes have revealed that AhR genes are more diverse in non-mammalian vertebrates compared to mammalian species (Hahn et al., 2006). In several fish species, both AhR1 and AhR2 genes have been characterized. For example, AhR2α, AhR2β, AhR2γ and AhR2δ are the four distinct AhR2 genes in salmonids species (salmon and rainbow trout). In addition, salmon genome contains two genes of AhR1, which are presumably non-functional (Hansson et al., 2003; Hansson et al., 2004). It has been suggested that rainbow trout AhR2α and AhR2β differ in their promoter preference and may regulate distinct sets of genes (Abnet et al., 1999). In salmon hepatocytes exposed to PCB126, apparently similar expression patterns were observed for the phylogenetically related AhR2γ and AhR2δ isotypes (Figure 12), in contrast to the different expression patterns observed for AhR2α and AhR2β (**Paper V** and Figure 12). Specifically, AhR2α mRNA expression did not show PCB126 related expression, AhR2β showed a pattern that was concentration-specific (increasing above control at 1 and 50 pM PCB126, and decreasing below control at 10 pM PCB126). In contrast, while 1 pM PCB126 decreased both AhR2δ and AhR2γ (total inhibition for AhR2γ), 10 and 50 pM PCB-126 had indifferent effect on these AhR variants (Fig. 12). Interestingly, while AhR2β mRNA expression maintained the effect from PCB126 exposure alone, in the presence of NP; the AhR2α, AhR2δ and AhR2γ showed a reducing trend in the presence of NP. Thus, a hypothetical evaluation of the salmon AhR genes suggests that different isoforms may have different ligand-dependent and -independent functions in responses to environmental stresses/stressors and during development.

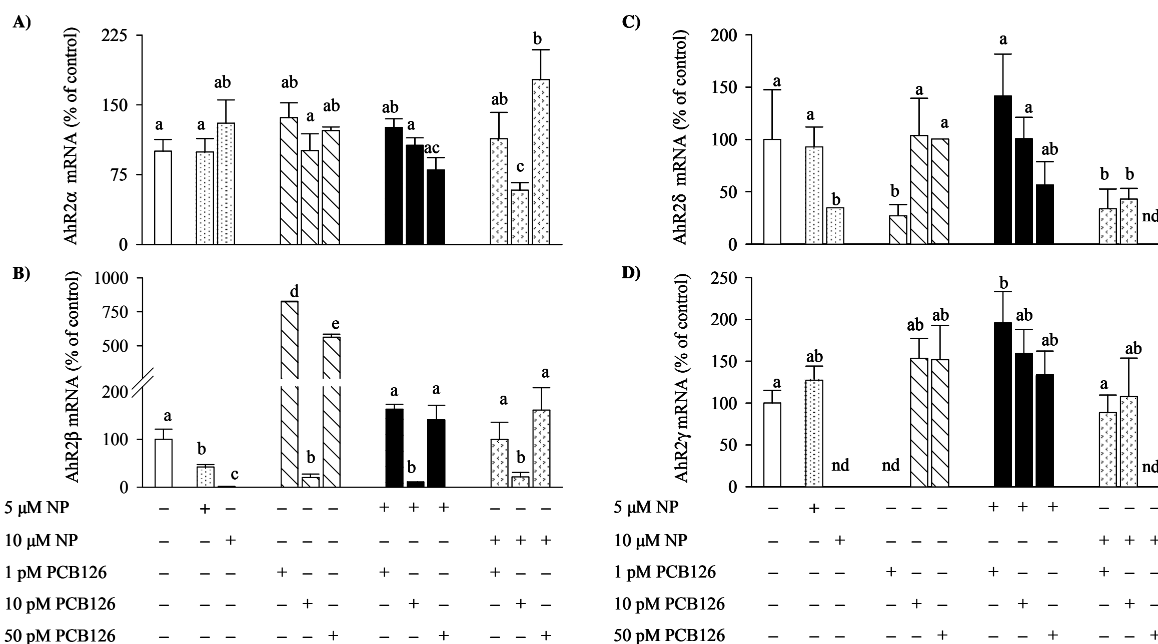

Figure 12. Expression of AhRα (A), AhRβ (B), AhRδ (C) and AhRγ (D) mRNA in salmon hepatocytes exposed to NP (5 and 10 μM) and PCB126 (1, 10 and 50 pM) singly or in combination. Cells were harvested 48 h post-exposure and mRNA levels were quantified using real-time PCR with gene specific primer pairs. The data are given as percentage (%) of solvent control ± standard error of the mean (SEM: n=3). Different letters denote exposure group means that are significantly different for the respective mRNA expression using ANOVA followed by Tukey's multiple comparison test ($p<0.05$). No mRNA transcripts were detected in samples labelled "nd".

Nevertheless, it should be noted that in **paper II,** we investigated the mRNA expression of AhR2α and AhR2β mRNA in rainbow trout hepatocytes where the receptors showed a complex expression pattern in response to PCB77 treatment. The primer pairs used in **Paper III** were designed based on sequences published on Rainbow trout (AhRα; AF065137 and AhRβ; AF065138) in a study by Abnet and colleagues (1999) where AhR2α and AhR2β were able to activate XRE driven reporter genes after exposure to TCDD. Unfortunately, the names published in GenBank were AhRα and AhRβ and not AhR2α and AhR2β. Therefore, erroneous designations for these receptors were used in paper **I, II, III and IV**. Due to the conserved nucleotide sequences between Rainbow trout and Atlantic salmon, the same analytical setups were used to quantify the AhR2α and AhR2β mRNA in samples from Atlantic salmon (**Paper I, III, IV and V**). Quantification of AhR1 expression was not conducted since the AhR1 clade is not functional in zebrafish and is primarily

expressed in brain, gonad and heart in *F.heteroclitus* (Andreasen et al., 2002; Hahn et al., 2006).

The role of the AhR as a ligand-dependent transcription factor for genes in the biotransformation pathways is well known (Hahn, 1998; Gu *et al.*, 2000). The activation of the receptor, dimerization and binding to the XRE has been studied in AhR deficient mice, yeast-two-hybrid-systems (Y2H) and binding affinity studies after TCDD exposures (Klinge *et al.*, 1999; Beischlag *et al.*, 2002; Suzuki and Nohara, 2007). The transcriptional expression analysis presented in this thesis does not indicate direct regulation of the AhRs in response to the AhR-agonists used in the different experiments (**Paper I-V**). For example, minor changes in AhR2α mRNA expression were observed in hepatocytes exposed to PCB77 or PCB126 alone (apparently concentration dependent for PCB77; **Paper IV**). On the other hand, the AhR2β transcription was altered in a random manner that reflects exposure condiotion and time (**Paper II, IV and V**). Transcriptional increases and decreases at certain AhR ligand concentrations were observed, exemplified by the decrease in cells exposed to 0.01 μM PCB77 (**Paper IV**) and 10 and 50 pM PCB126 (**Paper V**). Our findings are in accordance with previous studies in fish showing conflicting results in ligand-dependent AhR expression patterns. For example, in zebrafish embryo and liver cell line, TCDD induced a dose-dependent increase of AhR2 mRNA expression (Tanguay et al., 1999). Similar effects were also observed in rainbow trout where the AhR2 and AhR2β were elevated in gonadal cell line and kidney tissue (Hahn, 1998). In addition, these authors did not observe increases in mRNA expression of either AhR2 or AhR2β mRNA after TCDD exposure in rainbow trout liver or spleen (Abnet et al., 1999). Elsewhere, TCDD or PCB77 doses did not affect transcriptional changes of AhR2 mRNA expression in Atlantic tomcod liver (Roy and Wirgin, 1997).

We speculate that the concentration-specific up- and down-regulation of AhR mRNA levels observed in **paper I-V** could reflect a possible mechanism of receptor regulation that is influenced by chemical concentration and/or individual receptor function. In rodent liver exposed to TCDD, a rapid down-regulation of AhR mRNA was observed and thereafter followed by a gradual up-regulation that paralleled AhR protein. This effect was suggested to be due to proteasome mediated degradation through the ubiquitin-proteasomal pathway (Pollenz and Buggy, 2006). Degradation of AhR by 26S proteasome was shown be AhR-ligand dependent and this was stronger for compounds with higher affinity to the AhR (Wormke *et al.*, 2003; Alarid, 2006; Pollenz and Buggy, 2006). In **paper III and IV** we quantified the 20S proteasome subunit mRNA showing that exposure to AhR ligand alone did

not alter the mRNA expression. The choice of proteasome was based on its differential expression pattern on our subtractive cDNA library after exposure to ER- and AhR-agonists (**Paper III**). In the absence of proteasome activity measurement, the proteasome degradation pathway cannot be ruled out at this time.

Exposure of hepatocytes with the synthetic flavonoid 7,8-benzoflavone (α-naphthoflavone; ANF) significantly decreased the AhR mRNA expression, more so for the higher ANF concentration (**Paper III**). In mammalian cell lines, ANF is found to compete with TCDD for cytoplasmic AhR binding, leading to reduced AhR availability for TCDD followed by decreased CYP1A mRNA levels and enzyme activity (Merchant et al., 1990). The results from **paper III** indicate that the ANF not only competes with TCDD for cytoplasmic AhR binding, but also down-regulates AhR transcription.

The AhR dimerization partner Arnt is a multifunctional protein; In addition to its function as a dimerization/activation partner for several bHLH-PAS proteins, it is found to be a co-factor for other nuclear transcription factors including the ERs (Gu *et al.*, 2000; Wormke *et al.*, 2000a; Brunnberg *et al.*, 2003). Due to the multiple roles of Arnt, only minor alterations in Arnt mRNA expression are expected in response to xenobiotic exposures (Gu *et al.*, 2000; Brunnberg *et al.*, 2003). The Arnt expression patterns observed in **paper III and IV** showed that PCB77 exposure first induced Arnt at low concentrations and thereafter a concentration-specific decrease was observed. Unlike the AhR, Arnt protein levels is not degraded in response to TCDD exposure (Pollenz, 1996) and in cells exposed to the more potent AhR agonist, PCB126, a concentration dependent increase of Arnt mRNA levels is observed (**Paper V**). However, on the basis of sequence homology with one of the ER co-factors (p160), it was shown that Arnt functions as a co-activator of ER and this effect was due to the C-terminal domain and not the conserved bHLH or PAS domains (Brunnberg et al., 2003). In addition, although the Arnt contains a less complex activation domain compared to AhR, the activation domains of AhR and Arnt are located in the carboxy-terminal of both genes (Sogawa et al., 1995). During CYP1A1 (and other genes) activation, the Arnt activation domain does not contribute to the activation of AhR complex (Ko et al., 1996). The transcription of several genes are activated through the AhRs and these genes contain one or several XREs in their upstream promoter region (Zeruth and Pollenz, 2007). We investigated the expression patterns of AhRR, CYP1A1, UGT1 and GSTpi type, all of which are controlled by AhRs (**Paper I-V**). The consistency between AhRR, CYP1A1 and UGT1 (UDPGT) expression patterns suggests that this repressor singly may have caused the decrease in CYP1A1 and UGT1 levels (**Paper IV**). The AhRR-Arnt heterodimerization may

negatively regulate AhR driven gene expression through transcriptional repression (Karchner et al., 2002). In accordance with our data, the modulation of CYP1A1 by NP, E2, and BNF was recently shown to parallel the AhRR gene expression (Maradonna et al., 2004). This is supported by the fact that the bHLH-PAS proteins usually associate with each other to form heterodimers, AhR/Arnt or AhRR/Arnt, and bind the XRE sequences in the promoter regions of the target genes to regulate their expression. CYP1A1 is a well known biomarker for xenobiotic exposure and its induction is measured on the transcriptional level, the protein level and the enzyme activity is measured by the EROD activity (Mortensen and Arukwe, 2007). Our data show that AhR ligands (PCB77 and PCB126) induced CYP1A1 gene expression in an apparent time-specific (**Paper IV**) and concentration-dependent (**Paper II, III and V**) manner. In **paper II**, the expression of a single CYP1A1 transcript was verified using northern blotting. The presence of mRNA transcripts is not synonymous with increased biotransformation capacity which could be measured with the EROD assays (Arukwe *et al.*, 2001b). However, the quantity of CYP1A1 mRNA transcripts is an excellent indicator of activated AhR after xenobiotic exposures. Quantification of UGT1 and GST is frequently used for monitoring the activity of the xenobiotic metabolism after xenobiotic exposures (Xu et al., 2005). The expression of UGT1 generally paralelled CYP1A1 in PCB77 treated cells, but with a lower induction rate of UGT (**Paper III and IV**). However, in treatment scenarios involving complex mixtures (PCB77 and NP in combination) the expression pattern of UGT1 diverged from that of CYP1A1.

Bi-directional ER-AhR Interactions

AhR-mediated anti-estrogenicity

Anti-estrogenic effects of AhR agonists have been described in breast cancer cells, rodent uterus and mammary tumours (Safe et al., 1991). In fish, exposure to AhR agonists has been associated with reduced Vtg synthesis or impaired gonad development in both *in vivo*- and *in vitro* studies (Anderson *et al.*, 1996; Arukwe *et al.*, 2001b; Navas and Segner, 2006). Results obtained in the thesis show that exposure to AhR agonists produced anti-estrogenic effects by decreasing the mRNA expression of ER-responsive genes (**Paper II, III and IV**). In **paper IV,** we showed that PCB77 decreased the expression of NP-induced transcription of ERα, Vtg and *Zr*-protein in a concentration- and time-specific manner. Additionally, we found that the anti-estrogenic effects of the AhR agonist PCB77 were evident at several concentrations

from 0.001 μM to 10 μM (**Paper II and IV**). Results from **paper III** showed that while the partial inhibition of AhR with α-ANF did not reverse the effect of PCB77 on ER-mediated transcription, treatment of cells with ER antagonists (Tam and ICI) reversed the transcription of AhR-mediated responses. These data indicate that the anti-estrogenicity observed after PCB77 treatment is not caused by the AhRs alone but includes other mechanisms.

The anti-estrogenic effects of AhR agonist have proven to be tissue specific. Results obtained using one specific cell type (e.g. breast cancer cell line) may not be achieved when another cell line is used (Franc et al., 2001). Mechanistic studies on hepatocytes are valuable in toxicology since hepatocytes are central in detoxification as well as vitellogenesis in oviparous organisms (Smeets *et al.*, 1999; Navas *et al.*, 2004). The suppression of Vtg-protein by AhR-agonist is comparable to their order of potency to induce CYP1A1 (Smeets et al., 1999). In **paper II,** we observed a direct negative relationship between CYP1A1 and ERα (r^2 =-0.55), ERβ (r^2 =-0.27), Vtg (r^2 =-0.33) and Zr-protein (r^2 =-0.60). Since increased CYP1A1 expression is mediated through the ligand activated AhR, PCB77 mediated anti-estrogenicity is probably dependent on binding of ligand to AhR. Increased CYP1A1 mRNA expression is synonymous with increased CYP1A1 protein levels but not necessarily enzyme activity (Mortensen and Arukwe, 2007). Therefore, it is not likely that PCB-mediated anti-estrogenicity involves the CYP1A1 protein directly, but the effects are mediated through activated AhR-Arnt complexes. This statement is supported by data from Ohtake et al. (2003) that demonstrated the interactions between activated AhR-Arnt complex with unliganded ER in 3-MC treated cells and data from Klinge and co-workers (2000) showing that AhR interacted directly with ERα in a ligand specific manner.

The presence of ERs is required in AhR mediated transcription via XREs (Matthews et al., 2007). A shortage of available ERs would result in a negative relationship between AhR-mediated (CYP1A1 and UDG1) and ER-mediated (Vtg and *Zr*-protein) gene products. Hence, we suggest that AhR does not have a direct role in PCB mediated anti-estrogenicity, but involves competition between the AhR- and estrogenic pathways for ER proteins and/or other cofactors. This is supported by our findings that the ER agonists Tam and ICI further potentated PCB77 mediated anti-estrogenic effects (P**aper III**). Contrasting results were presented by Nodland and co-workers (1997) where cells constitutively expressing ERs showed TCDD inhibited Vtg expression. We suggested that the observed anti-estrogenic of PCB77 exposure could be caused by mechanisms that interfere with the mRNA stability of E2 responsive gene products (**Paper II**). AhR agonists could also be involved in regulating post

transcriptional events such as degradation and stabilisation of mRNA species (Flouriot et al., 1996). Possible roles and mechanisms of AhR induced mRNA destabilisation is reviewed by (Dodson and Shapiro, 2002). However, the expression of vigilin mRNA follows a parallel pattern of Vtg expression (**Paper III-IV**). Since PCB77 alone does not decrease vigilin mRNA (**Paper IV**), we assume that the role of Vtg mRNA stability is not a major contributor in observed AhR-mediated anti-estrogenicity.

ER-hijacking by activated AhR-Arnt complex

Generally, the molecular mechanisms behind estrogen-related effects of typical AhR agonists appear to involve ER-AhR crosstalk. However, there is no universal mechanism of ER-AhR interaction that is generally accepted as the mode-of-action for these chemicals. The fact that some AhR agonists induce endometriosis and estrogen-dependent tumors indicates that they may possess estrogenic activities (Ohtake et al., 2003). In **paper V**, we show that PCB126 activated mRNA transcription for ERα and its controlled genes (Vtg and vigilin) and proteins (ERα) in the absence of ER agonist. These findings are comparable with previous data from our laboratory demonstrating a positive effect of AhR agonists on estrogenic responses in salmon liver (Arukwe *et al.*, 2001b). In addition, the findings of PCB126 mediated activation ERα response is also in accordance with recent reports demonstrating similar effects in mammalian *in vitro* systems (Pearce *et al.*, 2004; Abdelrahim *et al.*, 2006; Liu *et al.*, 2006). We therefore concluded that the AhR agonist mediated anti-estrogenic activities that have been well documented in mammalian cell systems and some fish studies might be an exception rather than the rule for these chemicals.

The first experiment was conducted to investigate the AhR-ER interactions using a potent and dioxin-like AhR agonist (PCB126) and a xenoestrogen (NP) showing that PCB126 induced estrogenic responses above NP and control levels. To examine the involvement of ERs on these interactions, we conducted another experiment using ICI as antagonist for ERs. The results of experiment 2 showed elevated ERα and Vtg mRNA and protein levels (ERα) after exposure to PCB126 alone (**Paper V**). These responses were decreased in the presence of ICI at 12 and 24 h, indicating the involvement of ERα. However, the findings in experiments 1 (performed for only 48 h) and 2 (at 48 h) showed discrepant data for the investigated variables). There are conflicting reports on the direct activation of ER by AhR agonists. For example, while TCDD and PCB77 were shown to elicit estrogenic responses via direct ER binding (Nesaretnam et al., 1996), other studies showed that PCB77 did not produce

estrogenic responses (Ramamoorthy et al., 1999), and TCDD does not bind the ER (Klinge et al., 1999). Therefore, the "ER-hijacking" mechanism involving the activation of unliganded ER by ligand-activated AhR or a coactivator relationship between these signalling pathways was proposed (Ohtake et al., 2003).

Experiment 1 was performed using saltwater adapted fish (in the autumn) under a 48 h fixed time interval that was chosen based on previous experiments that showed a stable culture condition and optimal response time in our laboratory (**Paper IV**). The stronger stimulation of Vtg and ERα expression in experiment 1 probable reflects different composition of endogenous and exogenous substances and physiology of the experimental fish since experiment 2, utilized hepatocytes isolated from freshwater adapted salmon (in the winter). The variation of timing and degree of ER stimulation observed in the experiments in the present study are in agreement with previous results showing that Atlantic salmon produced seasonal pattern of xenoestrogen response that are influenced by dose and sequential order of exposure to PCB77 and NP, singly or in combination (Arukwe *et al.*, 2001b). In addition, the estrogenicity of AhR agonists is shown to be both cell and tissue specific in mammalian systems (Nesaretnam *et al.*, 1996; Ohtake *et al.*, 2003; Boverhof *et al.*, 2006).

Modulation xenobiotic biotransformation pathway

The xenobiotic metabolizing system involves important biological processes that may be subject to chemical disruption. With basis in the previously discussed ER-AhR interactions, we have to consider whether the interactions between the AhR and ER involve bi-directional crosstalk. In **paper I**, we investigated the *in vivo* effects of EE2 on hepatic phase I and II biotransformation and estrogenic pathways. In fish the estrogenic potency of EE2 is 10-50-fold higher than that of 17β-estradiol and estrone most likely due to its longer half-life and tendency to bioconcentrate (650- and 10,000-fold in whole-body tissues and bile, respectively) (Larsson et al., 1999; Segner et al., 2003). Previously the effects of EE2 on fish have been investigated in liver, brain and gonad (Brown et al., 2004; Katsu et al., 2007; Larkin et al., 2007). The expression of Vtg and Zr-protein mRNA showed concentration dependent increases at day 3 post exposure. The effects on the ER mediated gene expression patterns in liver were minor, reflecting the organism's ability to biochemically buffer xenobiotic exposures (Brandon et al., 2003). The expression patterns of genes involved in AhR mediated transcription showed concentration specific responses to EE2 exposures (**Paper I**).

The expression of CYP1A1 is dependent on gender and season (Forlin and Haux, 1990) and EROD activity has been shown to decrease with increasing cellular E2 levels during sexual maturation in fish (Navas and Segner, 2000b). Additionally, environmental pollutants known to mimic the actions of estrogen (like NP) modify the responses of several CYP-isozymes in both hepatic and extra-hepatic organs of fish (Arukwe et al., 1997a; Navas and Segner, 2000b). In **paper II**, we observed that NP decreased PCB77 induced CYP1A1 transcription in Rainbow trout hepatocytes. While exposure of salmon hepatocytes to NP alone did not alter the transcription of genes in the AhR pathways compared to solvent control, NP had a negative effect on PCB77 and PCB126 induced transcription of genes involved in phase I and II reactions (**Paper III-V**). Previously Arukwe and co-workers (Arukwe et al., 1997a) reported negative effects on phase I and phase II enzyme activities (EROD and UGT-assays) in microsomal fractions of salmon treated with NP The anti-AhR signalling effects of NP could be due to direct binding of NP to the CYP1A1 protein leading to inhibition of EROD activity. In Atlantic cod, NP was a potent inhibitor of EROD activity and CYP1A1 protein levels (Hasselberg et al., 2004). However, NP binding to the CYP1A1 enzyme would only explain decreases in EROD activity and not the decreased CYP1A1 mRNA concentrations. Our results indicate that the ER-NP complex interferes with the AhR transcriptional machinery either directly by binding to EREs in proximity of XREs or indirectly by protein-protein interactions. Several XRE controlled genes have complete or partial EREs within their 5'promoter regions (Matthews et al., 2007). The requirement for ER in AhR mediated transcription is supported by the results presented in **paper IV**, as treatment of cells with Tam or ICI reversed the negative NP effects on AhR mediated transcription. Additionally both Tam and ICI alone significantly reduce the basal CYP1A1 mRNA levels in salmon hepatocytes (unpublished results). These results together with recent reports on ER-requirement in AhR-mediated transcription (Matthews et al., 2007), indicate bi-directional AhR-ER crosstalk. However, the complete mechanism by which estrogenic compounds regulate the CYP system is still not fully elucidated.

Concluding remarks

The anti-estrogenic effects of xenobiotic compounds, which induce synthesis of CYP1A, have been demonstrated in both *in vivo* and *in vitro* studies. The potent dioxin-like AhR agonists PCB77 and PCB126 were used in combination with the ER-agonist NP to investigate the AhR-ER interactions. Pharmaceuticals are ubiquitous pollutants in the aquatic environment where their potential effects on non-target species like fish have only recently become subject of systematic investigations. In **paper I**, we investigated the effects of a synthetic pharmaceutical and endocrine disruptor EE2 on phase I and II biotransformation system and hormonal responses in the liver of juvenile salmon. The effect of EE2 on the CYP1A1 gene expressions paralleled EROD activity and AhRR mRNA, suggesting a direct role of EE2 in controlling the cellular detoxification machinery. The findings showed that EE2 also induced variations in hepatic biotransformation and hormonal response pathways in fish.

Generation of a toxicological cDNA library of clones containing differentially expressed genes from Atlantic salmon separately exposed to ER and AhR agonists was accomplished using suppressive subtractive hybridization (SSH). A targeted gene array (SalArray) was developed based on the clones in the cDNA library (**Paper III**). Using the targeted SalArray, we demonstrated that exposure of salmon to the ER agonist NP singly or in combination with the AhR agonist PCB77 produced differential gene expression patterns in salmon liver. Array analysis showed that exposure of hepatocytes to NP mainly altered genes involved in the estrogenic pathway, including genes for steroid hormone synthesis and metabolism. The anti-estrogenic properties of PCB77 were demonstrated in the array analysis as genes induced by NP were decreased by PCB77. Overall, our targeted array was a valuable tool for diagnostic screening and generation of new hypothesis that is currently under empirical evaluation in our laboratory.

Most mechanistic events in toxicology are remote from direct observation in vivo. Hence, examinations of molecular mechanisms involved in ER-AhR interactions were conducted on primary hepatocyte cultures of trout and salmon. While a clear pattern of negative effects on ER-mediated gene expression were found in hepatocytes exposed to the AhR agonist PCB77 (**Paper II-IV**), exposure of cells to the more potent dioxin-like AhR-agonist PCB126 induced transcriptional activation of ERα and estrogenic responses in the absence of ER-agonists (**Paper V**). Thus, the direct estrogenic actions of PCB126 observed contribute with new insight of the mechanisms involved in ER-AhR crosstalk, prompting a

new wave of discussion on whether AhR-mediated anti-estrogenicity is an exception, rather than a rule of action.

Exposure of hepatocytes to NP and PCB77 both singly and in combination produced gene expression patterns that were negatively influenced by individual receptor antagonists. PCB77 caused decreased ER-mediated gene expression, and NP caused decreased AhR-mediated responses. In **paper III**, inhibition of AhR with ANF did not reverse the effect of PCB77 on ER-mediated transcription suggesting that AhRs do not have a direct role on PCB77-mediated decreases of ER-mediated responses. In contrast, the inhibition of ER with Tam and ICI reversed the transcription of AhR-mediated responses (except AhRR). Taken together, the findings in **paper III** demonstrate a complex mode of ER-AhR interaction, possibly involving competition for common cofactors. This complex mode of interaction is further supported by the observation that the presence of ER antagonists potentiated the transcription of AhR isoforms and their mediated responses when PCB77 was given alone.

The results indicated that the anti-estrogenicity observed after PCB77 treatment is not caused by the AhRs alone, but probably includes other mechanisms. Expression analysis of AhR isotypes show that the cellular AhR levels are not regulated as a direct response to agonist concentrations (**Paper II, III and V**). A hypothetical evaluation of the salmon AhR genes suggests that different isoforms may have different ligand-dependent and -independent functions in responses to environmental stress and during development. This hypothesis was supported by the observation that in salmon hepatocytes exposed to PCB126 (singly and in combination with NP), apparently similar expression patterns were observed for the closely related AhR2γ and AhR2δ isotypes, in contrast to the different expression patterns observed for AhR2α and AhR2β.

The ability of ER and AhR to influence each others transcriptional activity appears to involve multiple factors. Supported by previously published results, our results suggest that ER concentrations play a role in mediating this cross-talk and they verify a bi-directional crosstalk between the ER and AhR receptors in Atlantic salmon hepatocytes. The findings of this thesis demonstrate a complex mode of ER-AhR interactions that is dependent on time, endogenous substances and the individual chemical. We have not uncovered a universal mechanism that can explain the mode of action, but the results presented in this thesis contribute with novel knowledge on ER-AhR interactions in teleost fish. We propose that the total outcome of chemical mixture exposure scenarios is dependent on toxicological factors (e.g. relative concentration relationship between the ER and AhR agonists), endogenous factors (e.g. composition of cell metabolites and proteins) and physiological factors (e.g.

tissue type, developmental stage) and seasonal changes (autumn vs. winter). Hence, the complex nature of chemical interactions in biological systems may have more adverse effects on the health, fitness and reproduction than previously anticipated.

Future perspectives

The data from this thesis has contributed with novel understanding on ER-AhR interactions in teleost fish and there is general appreciation showing no universal mechanism(s) to explain the mode of action, most likely because there is no such universal mechanism. It should also e appreciated that the total outcome of chemical mixture exposure scenarios is dependent on toxicological, physiological and environmental factors. Given that an integral aspect of the data presented here was centred on transcript expression, the role of protein modification still remains uncertain. Therefore, an immediate future studies on AhR-ER interactions in Atlantic salmon are being pursued in our laboratory. The focus will be on the interactions between AhR and ER proteins and their ability to mediate gene expressions or transcript stability. We are investigating nuclear receptor degradation in response to AhR-ligand treatments of hepatocytes. Exposure to AhR ligand induces proteosomal degradation of nuclear receptors via the ubiquitin-proteasomal pathways. To test the mechanistic role of AhR on the PCB126-mediated estrogenicity, we are using chemicals that inhibit protein synthesis (cycloheximide), proteasome degradation (MG132) and inhibit AhR (3',4'-dimethoxyflavone: 3',4'-DMF) in an in vitro system. This study will uncover the involvement of AhR-induced proteasomes in controlling the ER and AhR protein levels in salmon hepatocytes.

One important question concerning the mechanisms behind AhR-ER crosstalk in fish is how the receptors interact with each other or cofactors, particularly at the promoter region. Future studies will evaluate the direct interaction between ER and AhR in *in vitro* using cells constitutively expressing labelled recombinant salmon ERs and/or AhRs. Then, immunological methods will be used to evaluate the direct interactions between the receptor proteins. Additionally, evaluation of mRNA species stabilisation and degradation via RNA inhibition could provide novel explanations for transcriptional changes observed in response to xenobiotic exposures. MicroRNAs (miRNAs) are generated by endonucleolytic cleavage of hairpin precursor transcripts, miRNAs can inhibit translation of target mRNAs with complementory sequences (Baulcombe, 2005). Dissection of the complex relationships between miRNAs on one hand and potential mRNA targets on the other should be powerful tools for dissecting the potential involvement of miRNA regulation in AhR-ER crosstalk.

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Paper I

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Effects of 17α-ethynylestradiol on hormonal responses and xenobiotic biotransformation system of Atlantic salmon (*Salmo salar*)

Anne S. Mortensen, Augustine Arukwe *

Department of Biology, Norwegian University of Science and Technology (NTNU), Høgskoleringen 5, 7491 Trondheim, Norway

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Abstract

Pharmaceuticals are ubiquitous pollutants in the aquatic environment where their potential effects on non-target species like fish has only recently become subject of systematic investigations. In the present study, experiments were undertaken to examine the effects of a synthetic pharmaceutical endocrine disruptor, ethynylestradiol (EE2), given in water at 5 or 50 ng/L and sampled at days 0 (control), 3 and 7 after exposure, on hepatic phase I and II biotransformation and hormonal pathways of juvenile salmon using quantitative (real-time) polymerase chain reaction (qPCR), Vtg ELISA and 7-ethoxyresorufin *O*-deethylase (EROD) catalytic activity. Our data show that EE2 produced time- and concentration-specific modulation of estrogen receptor isoforms (ERα, ERβ) and androgen receptor-β (ARβ). EE2 produced a concentration-specific induction of vitellogenin (Vtg) and *zona radiata* protein (*Zr*-protein) at day 3 after exposure. At day 7, Vtg and *Zr*-protein mRNA (and plasma Vtg protein) expression were significantly decreased in the group given 5 ng EE2/L, compared to dimethyl sulfoxide (DMSO) control group. In the xenobiotic biotransformation pathway, EE2 produced a significant increase of aryl hydrocarbon receptor-α (AhRα) at day 3 in the group given 5 ng EE2/L and AhRβ was decreased at the same concentration at day 7. While CYP3A was not significantly affected by EE2 exposure, the CYP1A1, AhR nuclear translocator (Arnt) and AhR repressor (AhRR) mRNA showed an apparent EE2 concentration and time-dependent decrease. The expression of uridine diphosphoglucuronosyl transferase (UGT) and glutathione *S*-transferase class pi-like (GST*pi-like*) mRNA were decreased after exposure to 50 ng EE2/L at both day 3 and 7 after exposure. The effect of EE2 on the CYP1A1 gene expressions paralleled effect on EROD and AhRR mRNA, suggesting a direct role of EE2 in controlling cellular detoxification machinery. Interestingly, the carrier vehicle, DMSO produced significant time-dependent induction of estrogenic (ERα, Vtg and *Zr*-protein) responses, compared with blank (i.e. without DMSO) controls at day 7 post-exposure. The effect of DMSO totally underscored the observed EE2 effect at day 7 after exposure. In general, these findings support previous reports on the endocrine effects of EE2, in addition to effects on hepatic biotransformation system. In view of the data presented here and our recent studies, the use of DMSO as carrier vehicle in endocrine toxicological experimental studies should be re-evaluated.
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Keywords: Pharmaceuticals; Ethynylestradiol; Cytochrome P450; Endocrine pathways

1. Introduction

Ethynylestradiol (EE2) is a pharmaceutical and potent endocrine modulator known to be present in the aquatic environment at biologically active concentrations (Rotchell and Ostrander, 2003; Nash et al., 2004). In sewage treatment work (STW) effluents, steroidal estrogens are believed to, at least in part, be responsible for the feminized responses in some wild fish species in reports from the United Kingdom (Jobling et al., 2002). The concentration of EE2 reported in effluents and surface waters from Europe range between 0.5 and 7 ng/L (Larsson et al., 1999) and concentrations of up to 50 ng/L have been reported (Aherne and Briggs, 1989). In the United States, a survey of 139 streams showed that several rivers had concentrations >5 ng/L with an extreme EE2 concentration up to 273 ng/L reported at some riverine sites (Kolpin et al., 2002). Despite the lower EE2 concentrations in surface waters compared to natural steroidal estrogens, its estrogenic potency in fish *in vivo* studies is 10–50-fold higher than that of estradiol-17β (E2) and estrone (E1) (Segner et al., 2003) most likely due to its longer half-life and tendency to bioconcentrate (650- and 10,000-fold in whole-body tissues and bile, respectively) (Larsson et al., 1999). Although these authors did not observe any indication of EE2 de-ethynylation, it is possible that bilary

* Corresponding author. Tel.: +47 73 596265; fax: +47 73 591309.
E-mail address: arukwe@bio.ntnu.no (A. Arukwe).

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EE2 may produce repeated exposure through enterohepatic circulation and/or enzymatic deconjugation.

The cytochrome P450 (CYP) enzymes play a central role in the oxidative metabolism or biotransformation of a wide range of exogenous and endogenous compounds (Nelson et al., 1996). Specifically, the CYP1, CYP2 and CYP3 enzyme superfamilies metabolize a wide variety of compounds. Glucuronidation and conjugation by hepatic uridine diphosphate glucuronosyltransferase (UGT) and glutathione *S*-transferase (GST) are major pathways for the inactivation and elimination of endogenous compounds but also significant for other lipophilic compounds such as certain xenobiotics (Leaver et al., 1992). The expression of CYP1A1, UGT and GST are regulated by the ligand-dependent basic helix–loop–helix–Per–Arnt–Sim (bHLH–PAS) transcription factor, aryl hydrocarbon receptor (AhR) through which agonists cause altered gene expression and toxicity (Bradshaw et al., 2002; Nelson et al., 1996). Because of their roles in the detoxification and activation of foreign compounds, alteration of the expression of hepatic CYPs and phase II enzymes markedly affects the potential risks and benefits of xenobiotics and is important from a toxicological point of view (Williams et al., 1998). The interaction (crosstalk) between estrogen receptors (ERs) and AhRs has been reported in several teleost and mammalian studies, suggesting a possible agonistic and/or antagonistic expression of the respective receptor regulated genes may occur. For example, in fish, there are reports showing a reduction in Vtg expression after exposure to AhR agonists (Anderson et al., 1996; Mortensen et al., 2006). The exact mechanism(s) of anti-estrogenicity is not well known. Although induced metabolism of E2 may explain some of the anti-estrogenic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds at relatively high concentrations or doses, there are several observations that do not support the hypothesis and these has been summarized by Safe et al. (1991). Recently, a new mechanism involving the direct binding of AhR agonists activated AhR–Arnt complex to the ER followed by activation of the ERE that results to estrogenic responses was demonstrated by Ohtake et al. (2003), who showed that 3-methylchloranthrene (3MC) activated transcription of ER-signalling through ERE in a luciferase reporter plasmid assay with MCF-7 cells.

Studies on endocrine disruptors, including synthetic pharmaceutical estrogens, such as EE2, have mainly focused on reproductive steroids and other receptor-mediated effects (such synthesis of oogenic proteins), but little is known about the effects and mechanisms of endocrine modulators on steroid and xenobiotic metabolizing systems. Research on endocrine toxicology has mainly focused on estrogenicity that involves direct estrogen receptor mediated effects. In addition to direct receptor-mediated estrogenicity, modulation of steroid and xenobiotic biotransformation system is an important biological process in organisms that may be subject to chemical disruption with equally or more severe consequences for organismal health. Therefore, the present study was undertaken to investigate the effect of the pharmaceutical endocrine disruptor, EE2 on hepatic phase I and II biotransformation and estrogenic pathways of salmon using quantitative PCR and CYP1A1 mediated catalytic activity. Our hypothesis is that exposure of salmon to EE2 may induce differential time- and concentration-dependent transcriptional and activity changes in the AhR and ER-signalling gene expression patterns.

2. Materials and methods

2.1. Chemicals

17α-Ethynylestradiol and dimethyl sulfoxide (molecular biology grade with 99.9% purity) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Trizol reagent for ribonucleic acid (RNA) purification and TA Cloning kit were purchased from Invitrogen Corporation (Carlsbad, CA, USA). IScript cDNA synthesis kit and iTAQ™SYBR® Green Supermix with ROX were purchased from Bio-Rad Laboratories (Hercules, CA, USA) and GeneRuler™ 100 base pairs (bp) deoxynucleic acid (DNA) ladder and deoxynucleotide triphosphates (dNTPs) were purchased from Fermentas GmbH (St. Leon-Rot, Germany).

2.2. Fish and exposure

Immature Atlantic salmon (mean weight and length 10 ± 2.5 g and 9 ± 2 cm, respectively) were obtained from Lundamo hatcheries (Trondheim, Norway) and kept and exposed in 70 L aquariums at 7 ± 0.5 °C and for a 14-h light:10-h dark photoperiod at the Department of Biology, Norwegian University of Science and Technology (NTNU) animal holding facilities. In order to test the hypothesis that EE2 will modulate the expression of hepatic phase I and II biotransformation system in parallel with hormonal responses in a concentration- and time-dependent manner, four groups of (18 individuals per group) fish were exposed to waterborne EE2 at concentrations of 5 or 50 ng/L and one group serving as solvent control was exposed to the carrier vehicle dimethyl sulfoxide (DMSO: 7.5 ppb). The last group represented the blank without the solvent (DMSO). The final concentration of DMSO was the same in all exposure groups. Fish were exposed once under static (without water replacement) and aerated environmental condition. When toxicity factors such as bioaccumulation, bioconcentration, biotransformation and rapid adhesion to solid materials are considered, the EE2 concentrations used in the present study represent environmentally relevant concentrations (see Section 1). Six individuals from each exposure group (from two different tanks) and group without the solvent (i.e. blank group) were sacrificed at sampling day 0 (experimental control with solvent), day 3 and day 7 after exposure. A separate 7 days exposure experiment was performed using waterborne nonylphenol (NP: documented ER-agonist) at 5 μg/L dissolved in ethanol (EtOH: 0.5%) and the same fish group as in the first experiment, under identical environmental conditions. After sacrifice, the liver was excised and weighed and snap frozen in liquid nitrogen until processed. During the experimental period, fish were starved and six fish per exposure group (from two different tanks) were sacrificed for gene expression and enzyme activity determinations, respectively. No fish mortalities or other EE2 related toxicological effects were

observed. For sampling, the fish were anaesthetized with benzocaine (5 mg/L), and blood was collected before sacrifice, After sacrifice, the liver was excised and weighed, then processed as explained below.

2.3. Determinations of enzyme activity and protein analysis

Enzyme activity of EROD was measured from microsomal fraction. Microsomal fraction (10 μL) was added to the wells of a black fluorometric plate containing 140 μL of NAPDH (0.25 mg/mL) and incubated at 37 °C for 10 min. The reaction was then started by adding 1 μL of ethoxyresorufin (180 μM in DMSO) and the fluorescence was measured in a microplate reader for 20 min (extinction: 535 nm; emission: 590 nm) according to Burke and Mayer (1974). Enzyme activities were expressed as pmol resorufin/min/mg protein. As a quality control, two known samples were assayed in parallel with all assay series to assure the consistency of the results obtained with unknown samples. All enzyme activities were analyzed at room temperature. Total amount of microsomal protein was determined with the method of Bradford (1976), using bovine serum albumin (BSA) as standard. All enzymes and protein measurements were simplified using a Synergy HT microplate reader from Bio-Tek Instruments Inc. (Winnoski, Vermont, USA) for absorbance and fluorescence readings.

2.4. Enzyme-linked immunosorbent assay (ELISA) analysis of vitellogenin

A quantitative vitellogenin (Vtg) ELISA was performed using a polyclonal Arctic charr Vtg antibody as previously described by Meucci and Arukwe (2005). Purified Vtg protein was used to coat the plates and for preparation of the standard curve. Briefly, purified salmon Vtg was serially diluted to obtain standard concentrations between 3 and 1000 ng/mL. Standards and diluted plasma samples were incubated for 1 h at 37 °C with an equal volume of the primary antibody (diluted 1:5000). Triplicate aliquots of standards and samples (200 μL) were added to 96-well microtiter plates previously coated with Vtg (100 ng/mL overnight at 4 °C) and incubated for 1 h at 37 °C. The plates were washed with Tween–phosphate-buffered saline (TPBS) and a 1:2000 dilution of goat anti-rabbit peroxidase-conjugated secondary antibody (Bio-Rad) was added and incubated for 1 h at 37 °C. Levels of Vtg in samples were measured colorimetrically at 492 nm using *o*-phenylenediamine dihydrochloride (OPD) as substrate with a Synergy HT microplate reader from Bio-Tek Instruments Inc. Vtg ELISA Absorbance values (expressed as optical density, OD) were converted to the proportion of antibody bound (B) expressed as a percentage in the zero standard by the following equation: $B\ (\%) = ((\mathrm{OD} - \mathrm{NSB})/(\mathrm{OD}_0 - \mathrm{NSB})) \times 100$ (where OD is the absorbance of a given sample or standard, OD_0 the absorbance of the zero standard and NSB is the non-specific binding absorbance value). Binding percentage values were logit transformed [$\mathrm{logit}\, B = \log_{10}(B/(1 - B))$] and plotted against log dose to achieve a linear transformation of standard and plasma dilution curves. In evaluating the detection limit of the ELISA assay, the minimum amount of Vtg that produced a response significantly different from OD_0 was 2 ng/mL with 90% binding. The range of the standard curve was between 2 and 500 ng/mL, with 50% of binding around 35 ng/mL. ELISA values for Vtg obtained from control and exposed fish are expressed as mean ± standard error of the mean (S.E.M.).

2.4.1. RNA purification and cDNA synthesis

Total RNA was purified from liver tissues homogenized in Trizol reagent according to manufacturer's protocol. Total cDNA for the real-time PCR reactions were generated from 1 μg DNase-treated total RNA from all samples using poly-T primers from iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad).

2.4.2. Primer optimization, cloning and sequencing

The PCR primers for amplification of 96–391 base pairs (bp) gene-specific PCR-products were designed from conserved regions of the studied genes. The primer sequences, their amplicon size and the optimal annealing temperatures are shown in Table 1. Prior to PCR reactions, all primer pairs were used in titration reactions in order to determine optimal primer pair concentrations and their optimal annealing temperatures. All chosen primer pair concentrations used at the selected annealing temperatures gave a single band pattern for the expected amplicon size in all reactions. PCR products from the genes to be investigated were cloned into pCR2.1 vector in INVαF′ *E. coli* (Invitrogen). Each plasmid was sequenced using ABI-prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Department of Biology, NTNU Norway. Sequences were confirmed using NCBI nucleotide BLAST software (http://www.ncbi.nlm.nih.gov/BLAST).

2.5. Quantitative (real-time) polymerase chain reaction (PCR)

Quantitative (real-time) PCR with gene-sequence primer pairs (Table 1) was used for evaluating gene expression profiles. For each treatment, the expression of individual gene targets was analyzed as described previously (Arukwe, 2005), using the Mx3000P Real-time PCR System (Stratagene, La Jolla, CA, USA). Each 25 μL DNA amplification reaction contained 12.5 μL of iTAQ™SYBR® Green Supermix with ROX (Bio-Rad), 1 μL of cDNA and 200 nM of each forward and reverse primers. The three-step real-time PCR program included an enzyme activation step at 95 °C (5 min) and 40 cycles of 95 °C (30 s), 55–60 °C depending on target gene, see Table 1 (30 s) and 72 °C (30 s), followed by a melting analysis at 95 °C for 1 min, 55 °C for 30 s and thereafter decreasing fluorescence detection with increasing temperature between 55 and 95 °C. Controls lacking cDNA template (minus RT sample) were included to determine the specificity of target cDNA amplification as described previously (Arukwe, 2005; Mortensen et al., 2006). Cycle threshold (C_t) values obtained were converted into mRNA copy number using standard plots of C_t versus log copy number. In our laboratory, we do not use the so-called housekeeping genes as their expressions have been shown in several stud-

Table 1
Primer pair sequences, accession numbers, amplicon size and annealing temperature conditions for genes of interest used for real-time PCR

Target gene	Primer sequence[a]		Amplicon size (nucleotides)	Annealing temperature (°C)	GenBank accession number
	Forward	Reverse			
ERα	TCCAGGAGCTGTCTCTCCAT	GATCTCAGCCATACCCTCCA	173	55	DQ009007
ERβ	GAGCATCCAAGGTCACAATG	CACTTTGTCATGCCCACTTC	126	59	AY508959
ARβ	ATGCTAGGGAGGATGCCC	CCATGGGGAACATGTGGT	121	60	DQ367886
Vtg	AAGCCACCTCCAATGTCATC	GGGAGTCTGTCCCAAGACAA	391	57	DY802177
Zr-protein	TGACGAAGGTCCTCAGGG	AGGGTTTGGGGTTGTGGT	113	55	AF407574
AhRα	AGGGGCGTCTGAAGTTCC	GTGAACAGGCCCAACCTG	82	60	AY219864
AhRβ	GACCCCCAGGACCAGAGT	GTTGTCCTGGATGACGGC	96	65	AY219865
AhRR	TTCCTCCAGGGACAGAAGAA	ATGGAGGGCAGCAGAAGAG	98	60	DQ372978
ARNT	AGAGCAATCCCAGGGTCC	TGGGAGGGTGATTGAGGA	107	60	DQ367887
CYP1A1	GAGTTTGGGCAGGTGGTG	TGGTGCGGTTTGGTAGGT	76	60	AF364076
UGT	ATAAGGACCGTCCCATCGAG	ATCCAGTTGAGGTCGTGAGC	113	55	DY802180
GST*pi*	CGCATTGACATGATGTGTGA	TGTCGAGGTGGTTAGGAAGG	121	57	DQ367889
CYP3A	ACTAGAGAGGGTCGCCAAGA	TACTGAACCGCTCTGGTTTG	146	55	DQ361036

[a] Sequences are given in the 5′–3′ order.

ies to vary with exposure condition and time (Arukwe, 2006). In the absence of the so-called housekeeping genes (Arukwe, 2006), the criterion for using the standard curve is based on equal amplification efficiency with unknown samples and this is usually checked prior to extrapolating unknown samples to the standard curve. The standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest. Data obtained from triplicate runs for target cDNA amplification were averaged and expressed as ng/μg of initial total RNA used for reverse transcriptase (cDNA) reaction and finally expressed as percentage of control in the graphs.

2.5.1. Statistical analysis

Comparison of different concentrations of EE2-treated and control groups were performed using Dunnett's method. Statistical differences among treatment groups were tested using multiparametric analysis of variance (ANOVA) after testing homogeneity of variance and normal distribution. For all the tests the level of significance was set at $p < 0.05$, unless otherwise stated.

3. Results

3.1. Modulation of hormone receptors and signalling pathway

Please note that the blank group (i.e. without DMSO) were analyzed but not included in the figures as the DMSO group represents the true experimental control. The expression of basal hepatic estrogen receptor isoforms (ERα and ERβ), androgen receptor (ARβ), Vtg and *Zr*-protein genes were evaluated in juvenile salmon after exposure to waterborne EE2 concentrations and at different time intervals (Fig. 1). It should be noted that several AR isoforms has been characterized in fish, the primer pair sequences used in the present study, was designed to amplify a gene fragment for the ARβ gene isoform. The ERα expression was significantly decreased after exposure to 50 and 5 ng EE2/L at day 3 and 7, respectively (Fig. 1A). For ERβ mRNA, EE2 exposure produced a 50% reduction at day 3 in the group exposed to 50 ng/L (Fig. 1B). An interesting and parallel pattern of oogenic protein gene expression that was dependent on time and EE2 concentration was observed. Salmon exposure to EE2 produced an apparent concentration-dependent increase of Vtg and *Zr*-protein mRNA expression at day 3 (Fig. 1C and 1D, respectively). At day 7, a significant decrease of Vtg and *Zr*-protein mRNA expression was observed when fish were exposed to 5 ng EE2/L, compared to solvent control (Fig. 1C and 1D, respectively) and thereafter increased with 50 ng EE2. Compared to ER-isoforms, ARβ mRNA expression showed significant transcript reduction after exposure to 50 ng EE2 (Fig. 1E) at day 3 and 7.

Fish exposure of to EE2 for 3 days produced the increase of plasma Vtg levels in a concentration-dependent manner with a 2.2-fold significant increase at 50 ng EE2/L, compared to the control (Table 2). After 7 days, exposure to 50 ng EE2/L resulted in a significant decrease of Vtg, compared to the control (Table 2). Overall, it should be noted that the carrier vehicle (DMSO) used in the present study caused significant time-dependent reduction of ERα mRNA and elevations of Vtg and Zr-protein mRNA (Fig. 2A) and plasma Vtg protein (Table 2) levels after 7 days of exposure compared to 3 days of exposure. To compare the effect of DMSO on estrogenic responses, we performed a separate experiment using juvenile salmon exposed for

Table 2
Quantitative enzyme immunosorbent assay (ELISA) analysis of plasma Vtg levels (μg/mL) in juvenile Atlantic salmon exposed to waterborne dimethyl sulfoxide (DMSO: carrier vehicle control) and ethynylestradiol (EE2) at 5 and 50 ng/L and sampled at day 3 and 7 post-exposure

Exposure group	Day 3	Day 7
Control	6.1 ± 1.3[a]	18.6 ± 0.89[a]
5 ng EE2/L	9.8 ± 2.2[b]	20.5 ± 1.3[a]
50 ng EE2/L	13.6 ± 0.15[c]	14.4 ± 1.3[b]

Data are given as mean ± standard error of the mean (S.E.M.: $n = 5$). Different superscript letters (a–c) indicate means that are significantly different at the respective time intervals (ANOVA, $p < 0.01$). Blank control samples had ELISA value of 1.6 ± 1.4 μg/mL.

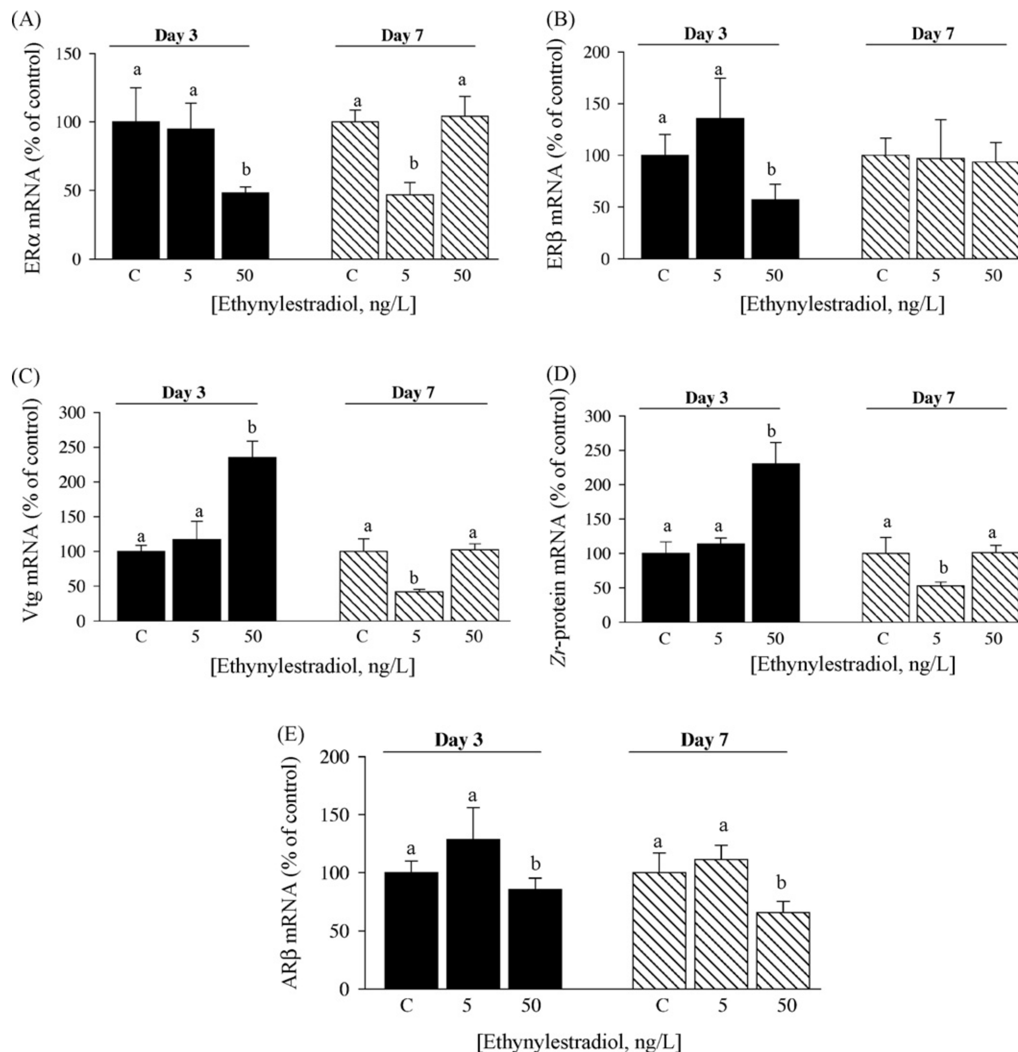

Fig. 1. Effects of 17α-ethynylestradiol (EE2) on hepatic estrogen receptors (ERα, A and ERβ, B), vitellogenin (Vtg, C), eggshell *zona radiata* protein (*Zr*-protein, D) and androgen receptor-β (ARβ, E) mRNA levels in juvenile Atlantic salmon. Real-time PCR of mRNA expression levels with gene-sequence primer pairs of control (DMSO), 5 and 50 ng EE2/L after 3 and 7 days exposure. All values represent the mean ($n = 6$) ± standard error of the mean (S.E.M.). Different letters denote exposure groups that are significantly different ($p < 0.05$), analyzed using multiparametric analysis of variance (ANOVA). C, Experimental control with DMSO.

7 days to NP dissolved in ethanol and Vtg mRNA analysis from this study is shown in Fig. 2B.

3.2. Modulation of Ah-receptors and signalling pathway

The expression of basal hepatic Ah-receptor isoforms (AhRα and AhRβ), AhR nuclear translocator (Arnt), AhR repressor (AhRR), CYP1A1, CYP3A, GST and UGT genes were evaluated in juvenile salmon after exposure to waterborne EE2 concentrations. Exposure of salmon to EE2 produced 3.5-fold significant increase of AhRα mRNA expression at day 3 in the group exposed to 5 ng/L, compared to solvent control (Fig. 3A) and 50 ng EE2 did not affect AhRα at the same time interval. At day 7, a significant decrease of AhRα was observed in the group exposed to 50 ng EE2/L, compared to solvent control (Fig. 3A). At day 3, the group exposed to 50 ng EE2 showed a non-significant reduction of AhRβ mRNA, compared to solvent control and 5 ng (Fig. 3B). A different, but similar to ERα, Vtg and *Zr*-protein expression pattern of EE2 effect was observed for AhRβ mRNA expression at day 7, where a significant decrease was observed in the group given 5 ng EE2 (Fig. 3B). In contrast, fish exposure to EE2 did not produce effect on the Arnt mRNA expression (Fig. 3C). On the contrary, the expression of AhRR showed apparent EE2 concentration and time-specific decrease, albeit not significant (Fig. 3D). The expression of CYP1A1 mRNA was significantly decreased in a time- and concentration-dependent manner after exposure to EE2 (Fig. 4A). The CYP3A expression showed a slight but non-significant increase at day 3 post-exposure, also at day 7 after exposure to 5 ng EE2 (Fig. 4B). For the two phase II enzyme genes (GST and UGT) studied, EE2 produced a concentration-specific mRNA decrease at days 3 and 7 after exposure to 50 ng EE2/L, compared to solvent control (Fig. 4B and C, respectively).

Enzyme activity levels for EROD activity are shown in Fig. 4E. A significant decrease in EROD activity was observed after 5 ng EE2/L exposure at day 3 post-exposure (Fig. 4E). At day 7, an apparent concentration-specific decrease, albeit non-significant, was observed (Fig. 4E).

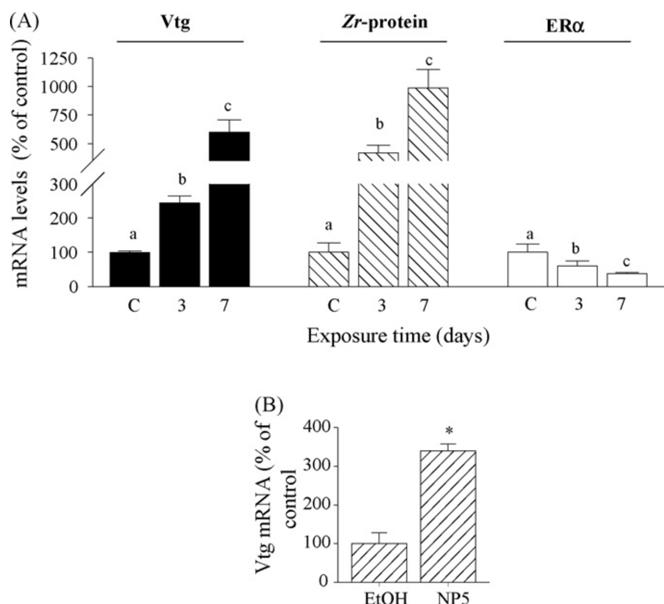

Fig. 2. Effects of carrier solvents on estrogenic responses. (A) Time-dependent transcriptional changes of hepatic vitellogenin (Vtg: left panel), eggshell *zona radiata* protein (*Zr*-protein: middle panel) and estrogen receptors (ERα: right panel) in juvenile Atlantic salmon exposed to 7.5 parts per billion (ppb) of the carrier vehicle (DMSO). Real-time PCR of mRNA expression levels with gene-sequence primer pairs. All values represent the mean ($n = 6$) ± standard error of the mean (S.E.M.). Different letters denote exposure groups that are significantly different ($p < 0.05$), analyzed using multiparametric analysis of variance (ANOVA). "C" in this figure represents experimental control without DMSO or blank control. (B) Vtg mRNA analysis of a separate experiment using juvenile salmon exposed for 7 days to 5 μg/L nonylphenol (NP5) dissolved in ethanol (EtOH).

4. Discussion

The present study investigated the effects of EE2 on phase I and II biotransformation system and hormonal responses in the liver of juvenile salmon. Our data based on nominal exposure concentrations show that EE2 produced a time- and concentration-specific transcriptional decrease of ER isoforms (ERβ and ERα) and ARβ, and increased Vtg and *Zr*-protein gene expressions (also Vtg protein) in salmon liver at day 3 post-exposure. At the same time interval (day 3), EE2 produced concentration-specific effect on AhR isoform (AhRα and AhRβ), Arnt and AhRR, CYP1A1, CYP3A, UGT and GST mRNA expressions. At day 7 post-exposure, EE2 produced increases in the studied hormonal parameters (including Vtg protein), but these effects were totally underscored by the unexpected increase of these responses by the carrier solvent (DMSO). The increase in Vtg and *Zr*-protein mRNA expressions (also Vtg protein) paralleled a decrease in CYP1A1 (mRNA and activity) and apparent increase in CYP3A at day 3 post-exposure. These findings show that the synthetic pharmaceutical endocrine disruptor and ubiquitous environmental pollutant also induces variations in hepatic biotransformation and hormonal response pathways in fish.

4.1. Effect on hormonal responses

The present results demonstrated that EE2 decreased ER-isoform gene expressions and increased plasma Vtg level and Vtg/*Zr*-protein mRNA levels. The molecular basis for Vtg gene and protein expression shows that the Vtg gene (and

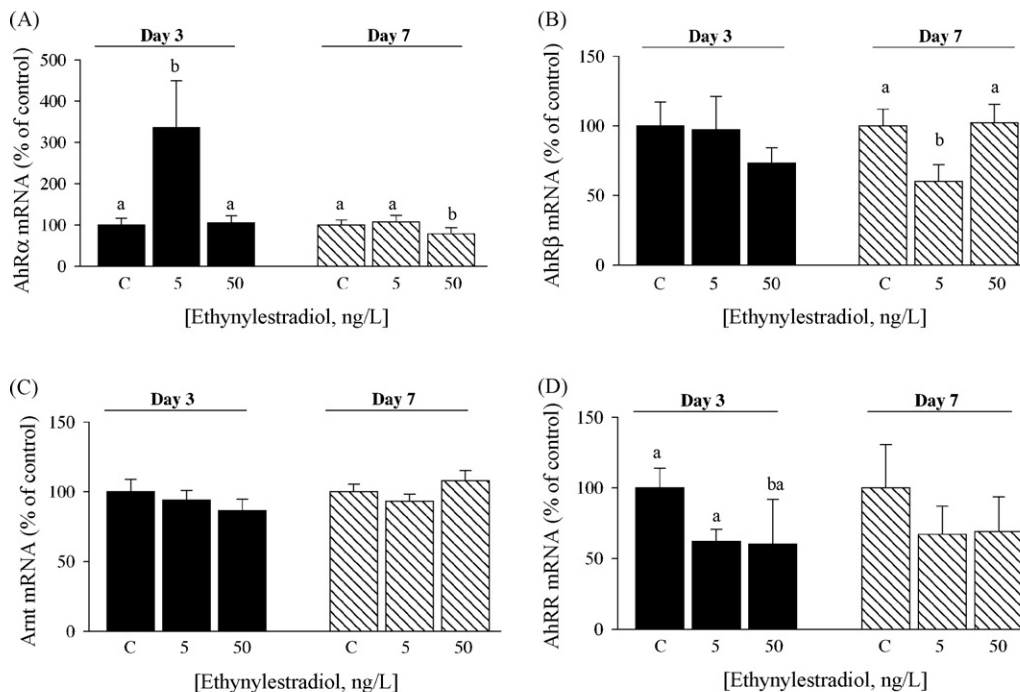

Fig. 3. Changes in hepatic aryl hydrocarbon receptors (AhRα, A and AhRβ, B), AhR nuclear translocator (Arnt, C) and AhR repressor (AhRR, D) mRNA levels of juvenile Atlantic salmon after exposure to 17α-ethynylestradiol (EE2) concentrations. Real-time PCR of mRNA expression levels with gene-sequence primer pairs of control (DMSO), 5 and 50 ng EE2/L after 3 and 7 days exposure. All values represent the mean ($n = 6$) ± standard error of the mean (S.E.M.). Different letters denote exposure groups that are significantly different ($p < 0.05$), analyzed using multiparametric analysis of variance (ANOVA). C, Experimental control with DMSO.

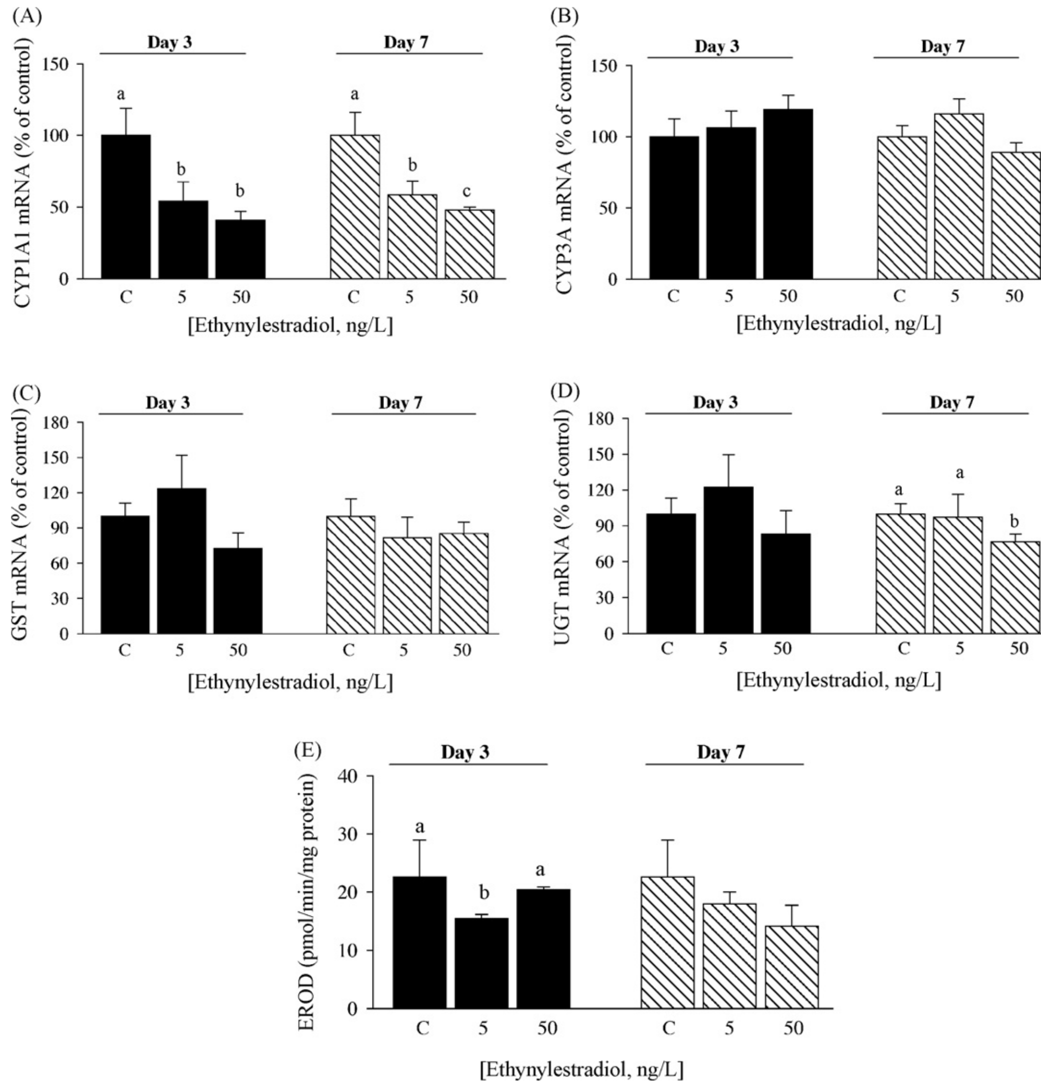

Fig. 4. Hepatic CYP1A1 (A), CYP3A (B), glutathione *S*-transferase (GST, C), uridine diphosphoglucuronosyl transferase (UGT, D) mRNA and (E) EROD activity levels of juvenile Atlantic salmon after exposure to 17α-ethynylestradiol (EE2) concentrations. Real-time PCR of mRNA expression levels with gene-sequence primer pairs and enzyme activity of control (DMSO), 5 and 50 ng EE2/L after 3 and 7 days exposure. All values represent the mean ($n = 6$) ± standard error of the mean (S.E.M.). Different letters denote exposure groups that are significantly different ($p < 0.05$), analyzed using multiparametric analysis of variance (ANOVA). C, Experimental control with DMSO.

Zr-protein) activations are receptor-mediated responses that are ligand structure-dependent interactions with ER, probably involving all isoforms, in addition to other co-activators. The maintenance of Vtg synthesis and concentration in oviparous species, including fish, is achieved through the activation of estrogen receptors by E2 or E2 mimics (Specker and Sullivan, 1994). Therefore, the induction of Vtg and Zr-protein synthesis in response to estrogens and their mimics has been described in several fish species (Arukwe et al., 2001; Flouriot et al., 1997; Yadetie et al., 1999). In the present study, EE2 produced an opposing effect on ER-isoforms and ARβ (decreased) and Vtg and *Zr*-protein mRNA levels (increased) in salmon liver. It is well known that Vtg induction in the liver is mediated through binding of ligand–ERα and ligand–ERβ complex to consensus ERE promoter region in DNA, resulting in increased mRNA transcription and subsequent translation followed by post-translational modification to yield a mature protein that is detectable in plasma samples (Pakdel et al., 1991; Ryffel, 1978). It is not known whether both ER isotypes contribute equally to the regulation of Vtg gene. Despite the negative correlation between ERs and oogenic protein and mRNA levels, the data do not show a direct mechanistic contribution of ER isotypes to protein regulation. These observations indicate that the ER isotypes may not be contributing equally to estrogen-dependent gene regulation. It also shows that the transcriptional increase of the ERs may not have a major role in the transcriptional regulation of Vtg and *Zr*-protein genes in EE2 exposed fish, suggesting that basal ER levels might be enough to initiate oogenic protein gene regulations. However, when basal ER levels are depleted the transcriptional machinery is turned on for refuelling (auto-regulation) and continued oogenic protein gene synthesis (Bowman et al., 2002; Yadetie et al., 1999; Pakdel et al., 1997). Furthermore, all the suggested mechanisms may be connected to an indirect pathway involving the

hypothalamus–pituitary–gonadal–liver axis through a feedback process that regulates receptor levels.

4.2. Effect on the biotransformation system

A number of hepatic CYPs in fish, mice and rats are expressed in a sex-specific manner (Arukwe and Goksøyr, 1997; Larsen et al., 1992; Waxman et al., 1985), suggesting a possible regulatory role by sex steroids in their expression. It is known that the post-pubertal expression of several sex-specific CYP forms is regulated via neonatal programming by gonadal steroids (Waxman et al., 1985). In this study, 5 ng EE2 produced a significant induction of AhRα at day 3 post-exposure (50 ng EE2 caused a reduction at day 7). Otherwise, the AhRβ, Arnt and AhRR showed apparent EE2 concentration-specific decrease at day 3 post-exposure (also AhRR at day 7). The effect of EE2 on AhRR directly paralleled CYP1A1 (partially with GST and UGT) mRNA and EROD activity levels at day 3 and 7 after exposure. The temporal down-regulation of EROD activity, CYP1A1, GST and UGT mRNA levels by EE2 suggests an adaptive response to high cellular levels of estrogen-like compound. This speculation is supported by the fact that CYP1A1 catalytic activities have been shown to decrease with increasing cellular estrogen levels during sexual maturation in several fish species (Andersson and Förlin, 1992; Arukwe and Goksøyr, 1997; Stegeman and Hahn, 1994). The parallel effect on AhRR suggests a concomitant effect of EE2 in controlling cellular detoxification process. Furthermore, it is also possible that there may be compensatory changes or auto-regulation through feedback mechanism in gene expression, particularly for AhRα that was induced at day 3 with 5 ng EE2. Generally, our data are in accordance with previous studies showing that estrogens (such as E2) and estrogenic compounds significantly suppressed hepatic CYP1A1 mRNA levels, EROD activity and CYP1A1 protein in *in vivo* and *in vitro* experiments using Atlantic salmon (Arukwe et al., 2000; Navas and Segner, 2000). There are several hypotheses explaining the CYP1A1 down-regulation by E2 and their mimics. For example, steroid hormones can bind to the CYP1A1 protein (Chan and Hollebone, 1995), and through this binding, E2 or the metabolites generated from E2 may inhibit the catalytic activity of P4501A1 protein (Arukwe and Goksøyr, 1997). Navas and Segner (2000) hypothesized that the inhibitory action of E2 could be mediated, at least in part, through the hepatic estrogen receptor (ER) where the ER-E2 complex can interfere with the CYP1A1 gene directly or alternatively may interact with the AhR, and indirectly regulate CYP1A1 gene expression through binding the XRE. Estrogens and their mimics may control the recruitment of ER and possibly other co-activators, besides activating the detoxification pathway. In a recent study it was shown that E2 exert its effects by activating the AhR–Arnt heterodimer, which is able to interact with the unliganded ER, leading to the induction of estrogenic pathway (Ohtake et al., 2003). In accordance with a recent study by Maradonna et al. (2004), our study showed that the decrease of CYP1A1 system by EE2 paralleled the decrease in AhRR gene expression. Liganded AhR complex has been found to activate gene expression of AhRR, which inhibits AhR function by competing with AhR for its nuclear dimerization partner, the Arnt and subsequent binding to the XRE sequence. Thus, the AhR function is regulated by the feedback inhibition of AhRR. Although the basic–helix–loop–helix–PAS (Per–AhR/Arnt–Sim homology sequence) of transcription factor usually associate with each other to form heterodimers, AhR/Arnt or AhRR/Arnt and bind the XRE sequences in the promoter regions of the target genes to regulate their expression, the complete mechanism by which estrogenic compounds regulate the CYP system is still not fully elucidated.

Steroid metabolism in lower vertebrates is catalyzed by specific CYP3A enzymes (Stegeman, 1993; Zimniak and Waxman, 1993). The ability of CYP3A to hydroxylate steroids is often related to sex (Zimniak and Waxman, 1993). It has been suggested that CYP3A proteins are constitutively expressed in fish (Celander et al., 1989), regulated during sexual maturation (with males showing higher protein levels than females) and metabolize endogenous substrates like testosterone and progesterone at 6β-position (Klotz et al., 1986; Miranda et al., 1991; Stegeman, 1993). In the present study, EE2 did not produce a significant effect on CYP3A mRNA expression, although an apparent concentration decrease of ARβ was observed. Recent findings have demonstrated that steroid hormone mimics modulate CYP3A-mediated catalytic activities and mRNA levels in a similar manner as natural steroid hormones (Arukwe et al., 1997; Hasselberg et al., 2005). Therefore, the effect of EE2 on CYP3A and ARβ observed in the present study is proposed to represent an androgenic effect of this chemical.

In the absence of catalytic data for UGT and GST due to the small sample size, the mRNA expression data from the present study (showing apparent concentration-specific decrease) are in accordance with a previous report demonstrating the effects of EE2 on phase II enzymes (Sole et al., 2000). The effect of EE2 on UGT and GST mRNA observed in the present study may have several physiological explanations due to the integral roles of phase II biotransformation enzymes in regulating steroid hormone homeostasis in organisms. The UGTs (and GSTs) are a multigene enzyme family that plays significant roles in the excretion of both endogenous and xenobiotic compounds (Clarke et al., 1992). In fish, several UGT gene isoforms have been described, with prototypical substrates such as bilirubin, testosterone, and phenolic xenobiotic (Clarke et al., 1992). Nucleotide sequence data has shown that as many as 10 different UGTs are present in zebrafish, with nucleotide similarities to some mammalian UGT gene families (George and Taylor, 2002). The primer pair sequences used in our real-time PCR assay was designed based on up-regulated UGT and GST sequences in subtracted salmon cDNA library in our laboratory (Mortensen and Arukwe, 2007) and the primers spanned the conserved regions of fish UGT1 and GST*pi*. For example, it was reported previously using fish hepatic microsomes that an organotin compound (tributyltin, TBT) at concentrations as low as 5 μM, selectively inhibited UDP-glucuronidation of testosterone (but not 17β-estradiol) (Morcillo et al., 2004). The formation of glucuronides in fish may provide a physiological means for controlling hormone action through their excretion and removal (George and Taylor, 2002) or cellular transport in a

receptor-inactive form. These data show that EE2 can modulate both direct ER-mediated processes and drug/xenobiotic metabolizing systems and thereby expanded the previous study by Arukwe and Goksøyr (1997). It is still subject to speculation whether a given XRE sequence will function as a transcriptional activator enhancer or silencer and this may depend on the role as a specific promoter. This hypothesis needs to be tested experimentally with regard to pharmaceutical endocrine disrupting chemical such as EE2. The differential regulation of CYP1A1 and AhR-isoforms demonstrated in the present study may have some deleterious health consequences. Although xenobiotic-metabolizing enzymes, such as CYP1A1, protect the body against adverse effects, there may be other consequences associated with activating these receptors. For example, significant induction of CYP enzymes by environmental chemicals may lead to activation of protoxicants and alterations of the metabolism of drugs and endogenous substances (Guengerich, 1992, 1999; Nelson et al., 1996; Stegeman and Hahn, 1994).

It should be noted that the present study was performed during a 7-day exposure period under static and nominal EE2 concentration conditions. Without changing the fact these systems were modulated by EE2, the relevance of these findings in terms of ecotoxicological and pharmacological consequences will depend on the environmental concentration of EE2, bioconcentration/bioaccumulation and synergistic interactions with other pollutants (note the differences in most gene expression patterns at days 3 and 7 after exposure). The possible change in EE2 concentration between day 3 and 7 coupled with the effect of the carrier vehicle (DMSO) would be expected to be more severe for the lower nominal concentration than for the higher concentration and this may explain some of the differences in gene expression observed for the concentrations at the different time intervals.

4.3. *Effects of dimethyl sulfoxide (DMSO)*

In recent studies, it was reported that estrogenic responses in juvenile salmonids were modulated in both *in vivo* (Lyssimachou and Arukwe, 2007; Lyssimachou et al., 2006) and *in vitro* (Mortensen and Arukwe, 2006; Osborne et al., 2007) studies by DMSO, a commonly used carrier vehicle in toxicological studies. In accordance with these studies, a critical observation in the present study is the fact the carrier vehicle (DMSO) modulated the hormonal gene responses at day 7 post-exposure. The 3-day exposures typically showed concentration-related effects in hepatic hormonal responses, but generally no significant effect after 7 days exposure, compared with control. However, when the effect of ethanol was evaluated after 7 days exposure of juvenile salmon to NP, no negative effect of ethanol on NP-induced hepatic Vtg mRNA expression was observed (see Fig. 2B). Despite the fact the EE2 and NP study utilized two different chemicals, it should be noted that both chemical are strong agonists to the ER. In the NP experiment, separately designed to study the effects of NP on neurosteroidogenic pathways, we showed that ethanol modulated brain steroidogenic enzyme genes in salmon *in vivo* experiment (Arukwe, 2005), suggesting that the effect of ethanol or other carrier solvents may be tissue and parameter dependent responses. It does seem that EE2 produced a concentration-dependent effect on hormonal responses that was totally underscored by DMSO at day 7. It is unlikely that the environmental stress in the exposure tanks might have contributed to the effect of DMSO observed in the present study since the experimental animals were closely monitored during the study period and showed apparently good condition. Furthermore, the use of flow-through exposure system should have been a preferable condition for such a lipophilic compound and definitely could not have uncovered the effect of DMSO in this study and other studies referred above. Despite the effect of DMSO, the time-dependent differences in the hormonal and biotransformation responses observed in the present study might be explained by the physiological role and susceptibility of the investigated variables on the reproductive and xenobiotic metabolizing systems.

We chose to DMSO as carrier solvent as opposed to ethanol in this study because of evidence that alcohol may activate steroidogenic enzymes (Arukwe, 2005) and promote the conversion of testosterone to estradiol in mature female tilapia (*Oreochromis niloticus*) and their ovaries (Kim et al., 2003). Kazeto et al. (2003) exposed juvenile zebrafish to EE2 and nonylphenol dissolved in DMSO (0.1%, v/v) for 3 days and found that EE2 and nonylphenol induced P450aromB gene expression in a concentration-dependent manner. Elsewhere, Alberti et al. (2005) exposed adult zebrafish to 17β-estradiol and nonylphenol dissolved in DMSO (0.02%, v/v) for 11 days and found a strong Vtg gene expression in the liver of male fish at high E2 (500 ng/L) and nonylphenol (250 μg/L) concentrations. In view of the above studies and the present study, it is clear that DMSO has the potential of modulating the endocrine system, in addition to other effects. Recently, Hutchinson et al. (2006) critically reviewed the acute and chronic effects of carrier solvents in aquatic organisms. It could be argued that DMSO modulation of endocrine responses may be due to its absorption-enhancing properties, as it is widely used as a carrier for drugs across cell membranes (Hui et al., 2001). Thus, DMSO could produce a time-dependent movement of estrogens or their mimics into the hepatocytes, and subsequently produce time-dependent increases in ER activation with enhanced vitellogenesis and zonagenesis. Therefore, the use of DMSO as carrier vehicle in both *in vitro* and *in vivo* fish endocrine disruption studies should be re-evaluated, particularly in the brain.

5. Conclusions

The combined effect of pharmaceutical endocrine disruptors on biotransformation and hormonal pathways might have a more serious consequence for the organism than endpoints like egg yolk and eggshell protein inductions (Arukwe and Goksoyr, 2003). In general, an integration of the findings in the present study with (a) the concentration of EE2 reported in effluents and surface waters from Europe (range between 0.5 and 7 ng/L; Larsson et al., 1999); (b) concentrations of up to 50 ng/L that was reported by Aherne and Briggs (1989) and (c) the United States survey of 139 streams showing that several rivers had concentration range of 5–273 ng/L (Kolpin et al., 2002) pharmaceuticals

in the environment represent a more serious health concern both to humans and wildlife.

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Paper II

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Interactions Between Estrogen- and Ah-Receptor Signalling Pathways in Primary Culture of Salmon Hepatocytes Exposed to Nonylphenol and 3,3',4,4'-Tetrachlorobiphenyl (Congener 77)

Anne S Mortensen and Augustine Arukwe*

Address: Department of Biology, Norwegian University of science and Technology (NTNU), Høgskoleringen 5, 7491 Trondheim, Norway

Email: Anne S Mortensen - anne.mortensen@bio.ntnu.no; Augustine Arukwe* - arukwe@bio.ntnu.no

* Corresponding author

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Abstract

Background: The estrogenic and xenobiotic biotransformation gene expressions are receptor-mediated processes that are ligand structure-dependent interactions with estrogen-receptor (ER) and aryl hydrocarbon receptor (AhR), probably involving all subtypes and other co-factors. The anti-estrogenic activities of AhR agonists have been reported. In teleost fish, exposure to AhR agonists has been associated with reduced Vtg synthesis or impaired gonadal development in both *in vivo*- and *in vitro* studies. Inhibitory AhR and ER cross-talk have also been demonstrated in breast cancer cells, rodent uterus and mammary tumors. Previous studies have shown that AhR-agonists potentiate xenoestrogen-induced responses in fish *in vivo* system. Recently, several studies have shown that AhR-agonists directly activate ERα and induce estrogenic responses in mammalian *in vitro* systems. In this study, two separate experiments were performed to study the molecular interactions between ER and AhR signalling pathways using different concentration of PCB-77 (an AhR-agonist) and time factor, respectively. Firstly, primary Atlantic salmon hepatocytes were exposed to nonylphenol (NP: 5 μM – an ER agonist) singly or in combination with 0.001, 0.01 and 1 μM PCB-77 and sampled at 48 h post-exposure. Secondly, hepatocytes were exposed to NP (5 μM) or PCB-77 (1 μM) singly or in combination for 12, 24, 48 and 72 h. Samples were analyzed using a validated real-time PCR for genes in the ER pathway or known to be NP-responsive and AhR pathway or known to be PCB-77 responsive.

Results: Our data showed a reciprocal inhibitory interaction between NP and PCB-77. PCB-77 produced anti-NP-mediated effect by decreasing the mRNA expression of ER-responsive genes. NP produced anti-AhR mediated effect or as inhibitor of AhRα, AhRR, ARNT, CYP1A1 and UDPGT expression. A novel aspect of the present study is that low (0.001 μM) and medium (0.01 μM) PCB-77 concentrations increased ERα mRNA expression above control and NP exposed levels, and at 12 h post-exposure, PCB-77 exposure alone produced significant elevation of ERα, ERβ and *Zr*-protein expressions above control levels.

Conclusion: The findings in the present study demonstrate a complex mode of ER-AhR interactions that were dependent on time of exposure and concentration of individual chemicals (NP and PCB-77). This complex mode of interaction is further supported by the effect of PCB-77 on ERα and ERβ (shown as increase in transcription) with no concurrent activation of Vtg (but *Zr*-protein) response. These complex interactions between two different classes of ligand-activated receptors provide novel mechanistic insights on signalling pathways. Therefore, the degree of simultaneous interactions between the ER and AhR gene transcripts demonstrated in this study supports the concept of cross-talk between these signalling pathways.

Background

Halogenated organic contaminants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) are notorious environmental pollutants that cause acute and chronic toxicity [1]. Several of these compounds including planar PCBs, exert their biological effects through the aryl hydrocarbon receptor (AhR or Ah-receptor). The AhR is a ligand activated transcription factor that regulates the activation of several genes encoding phase I and II biotransformation enzymes [2]. The AhR belongs to the family of basic helix-loop-helix (BHLH)/Per-ARNT-Sim (PAS) proteins that are characterized by two conserved domains, the N-terminal bHLH and the PAS domain [2,3]. Cytochrome (CYP) P450 enzymes (CYP1A1, 1A2, 1B1) are involved in the metabolism of a wide variety of structurally different chemicals that include many drugs and xenobiotics, through the AhR [2,3]. For example, the molecular mechanism of CYP1A activation has been extensively studied. Prior to ligand binding, the cytosolic form of the AhR is associated with a chaperone complex consisting of heat shock protein 90 (hsp90) and several other co-chaperones [2,3]. Upon ligand binding, the AhR is released from the hsp90 complex and translocated into the nucleus where it dimerizes with a structurally related protein, the AhR nuclear translocator (ARNT). The AHR/ARNT complex binds with high affinity to specific DNA sequences known as dioxin or xenobiotic response elements (DREs or XREs) located in the regulatory regions of target genes leading to their activation and expression. In addition to CYP enzymes, phase-II enzymes such as uridine-diphosphate glucuronosyltransferase (UDPGT) are now known to be inducible through the AhR [2,3] and these responses are putatively controlled through the AhR repressor (AhRR: [2]). Thus, AhR controls a battery of genes involved in the biotransformation of xenobiotics [2,3].

In oviparous animals, accumulation of yolk materials into oocytes during oogenesis and their mobilization during embryogenesis are key processes for successful reproduction [4,5]. Similarly, the envelope (*zona radiata* or *Zr*) surrounding the animal egg plays significant roles in the reproductive and developmental processes; firstly as an interface between the egg and sperm, and secondly as an interface between the embryo and its environment [4,5]. Vitellogenesis and zonagenesis are estrogen receptor (ER)-mediated estradiol-17β (E2)-induced hepatic synthesis of egg yolk protein (Vtg) and eggshell protein (*Zr*-protein) precursor, respectively, their secretion and transport in blood to the ovary and their uptake into maturing oocytes [4,5]. The ERs (ERα and ERβ) are members of the nuclear receptor (NR) gene superfamily. The ERs bind to estrogen response elements (EREs) and activate transcription in an estrogen concentration-dependent manner [6]. This transcriptional activation requires the recruitment of co-activator complexes [6]. Xenoestrogens, such as nonylphenol (NP) were shown to induce hepatic expression of Vtg and *Zr*-protein genes in immature and male fish [7]. NP predominantly occurs as a degradation product of nonylphenol ethoxylate (NPE), found in many types of products, including detergents, plastics, emulsifiers, pesticides, and industrial and domestic cleaning products.

There are many potential xenobiotics and xenoestrogens in aquatic systems (*e.g.*, pharmaceuticals, pesticides, surfactants and personal care products). Thus, in the environment, chemical interactions may have profound consequences since organisms, including fish, are exposed to complex mixtures of environmental pollutants [8]. These complex interactions have only recently become the focus of systematic investigations both in laboratory and elsewhere [8,9]. The anti-estrogenic activities of AhR agonists have been reported [10]. In fish, exposure to AhR agonists has been associated with reduced Vtg synthesis or impaired gonad development in both *in vivo*- and *in vitro* studies [11,9,12]. Inhibitory AhR-ER cross-talk has been demonstrated in breast cancer cells, rodent uterus and mammary tumors [13].

The relative importance of the influence of contaminants on biological systems is not well-understood or quantified mechanistically in complex chemical mixtures. PCB-77 is a documented AhR agonist with anti-estrogenic activity and was previously shown to increase and decrease (depending on dose ratios, season and sequential order of administration) NP-induced responses in Atlantic salmon (*Salmo salar*) *in vivo* system [11]. In toxicological sciences, almost without exception, gene expression is altered as either a direct or indirect result of toxicant exposure. Depending upon the severity and duration of the toxicant exposure, genomic analysis may be short-term toxicological responses leading to impacts on survival and reproduction (parental and offspring fitness). Therefore, gene expression profiling has become a powerful tool in molecular biology with potential to reveal genetic signatures in organisms that can be used to predict toxicity of these compounds [14]. Therefore, the present study was designed with the objective of investigating the concentration- and time-dependency of interactions (cross-talk) between the ER and AhR signalling pathways using molecular approaches. In addition, we wanted to establish in parallel, the time-dependency of the potential bi-directional cross-talk between these two signalling pathways.

Results

Based on previous studies in our laboratory, we selected 5 genes (ERα, ERβ, Vtg, *Zr*-proteins and vigilin) belonging to the ER-pathway or known to be ER-responsive and 7

genes (AhRα, AhRβ, AhRR, ARNT, CYP1A1, UDPGT and a proteasome subunit) in the AhR-pathway or known to be AhR-responsive for quantitative analysis using real-time PCR with gene specific primers. Several subtypes of ARNT and UDPGT have been characterized in fish and the primer sequences used in the real-time PCR assays were designed based on conserved regions of these genes.

Concentration-dependent expression of ER-responsive genes

Exposure to NP alone significantly elevated ERα expression (Fig. 1A). The low PCB-77 concentration (0.001 μM) produced a significant 2-fold decrease of ERα, compared to control and thereafter a concentration-specific increase of ERα mRNA expression was observed (Fig. 1A). When 1 μM PCB-77 was given in combination with NP, an elevated ERα expression above NP level was observed (Fig. 1A). In contrast, exposure to 0.01 μM PCB-77 in combination with NP produced decreased ERα mRNA below NP level (Fig. 1A). For ERβ, exposure to NP alone produced a significant increase of transcript level (Fig. 1B). When hepatocytes were exposed to 1 μM PCB-77 alone or in combination with NP, ERβ mRNA was not altered (Fig. 1B). In contrast, exposure to 0.001 and 0.01 μM PCB-77 alone produced significant increase of ERβ, and when these PCB-77 concentrations were given in combination with NP, ERβ mRNA was significantly decreased only in the 0.01 μM PCB-77 group (Fig. 1B).

The expression pattern of Vtg was induced 19-fold after exposure to NP alone (Fig. 1C). While PCB-77 alone did not alter the expression levels of Vtg mRNA, the combined exposure with NP produced a PCB-77 concentration-specific decrease of NP induced Vtg expression (Fig. 1C). Particularly, exposure of hepatocytes to NP in combination with medium PCB-77 concentration (0.01 μM) produced a total inhibition of Vtg mRNA expression (Fig. 1C). The expression *Zr*-protein showed a similar pattern with Vtg (Fig. 1D). While exposure to NP alone produced a 3.7-fold increase of *Zr*-protein mRNA, the combined exposure with PCB-77 exposure produced significant PCB-77 concentration-specific decrease of *Zr*-protein, compared with NP exposure alone (Fig. 1D). PCB-77 exposure alone produced significant decrease of *Zr*-protein mRNA expression, compared with solvent control (Fig. 1D). Exposure to PCB-77 concentrations singly or in combination with NP produced minor changes, albeit not significant in vigilin mRNA expression (Fig. 1E). Exposure to PCB-77 concentrations singly or in combination with NP produced non-significant changes in proteasome mRNA expression (Fig. 1F).

Concentration-dependent expression of AhR-responsive genes

Exposure of hepatocytes to PCB-77 alone produced a significant concentration-dependent increase of AhRα mRNA. While NP alone did not alter AhRα expression, combined NP and PCB-77 at 0.01 and 1 μM caused decreases of AhRα mRNA, compared with PCB-77 exposure alone (Fig. 2A). The expression of AhRβ was significantly decreased after exposure to PCB-77 alone, compared with control (Fig. 2B). Exposure to combined NP and all PCB-77 concentrations showed decreased expression of AhRβ mRNA, significant in 0.001 and 0.01 μM PCB-77 concentrations, compared to PCB-77 exposure alone (Fig. 2B). For AhRR, exposure to PCB-77 alone produced a concentration-dependent increase of AhRR mRNA expression and the presence of NP caused only slight decreases of PCB-77 mediated effects on AhRR expression (Fig. 2C). NP exposure alone did not significantly alter the expression of AhRR mRNA (Fig. 2C). A different expression pattern was observed for ARNT (Fig. 2D). Exposure to the low PCB-77 concentration (0.001 μM) produced a 4.2-fold increase of ARNT mRNA expression and thereafter a PCB-77 concentration-dependent decrease was observed (Fig. 2D). While NP exposure alone produced a slight, albeit not significant, elevation of ARNT mRNA, combined exposure with 0.001 and 0.01 μM PCB-77 produced respective significant decrease and increase of ARNT mRNA expression, compared with the respective PCB-77 concentration alone (Fig. 2D).

The expression pattern of CYP1A1 showed significant PCB-77 concentration-dependent induction and combined exposure with NP produced significant reduction of CYP1A1 mRNA expression, compared with PCB-77 exposure alone (except with 0.001 μM PCB-77; Fig. 2E). NP exposure alone did not alter CYP1A1 mRNA expression (Fig. 2E). Exposure to PCB-77 produced a concentration-specific increase and combined exposure with NP produced significant reduction of UDPGT mRNA expression, compared with PCB-77 exposure alone (except with 0.001 μM PCB-77; Fig. 2F). NP exposure alone did not significantly alter UDPGT mRNA expression (Fig. 2F).

Time-dependent expression of ER-responsive genes

Exposure of hepatocytes to NP alone or in combination with PCB-77 caused an apparent time-dependent increase of ERα mRNA expression (Fig. 3A). At 12 h post-exposure, NP exposure singly produced a significant (11-fold) increase of ERα, while combined exposure with PCB-77 slightly reduced (albeit not significant) the NP effect on ERα at the same time interval (Fig. 3A). Although the expression ERα was reduced at 72 h, compared to 12 h, in the NP exposure group alone, the combined exposure with PCB-77 produced significant 2-fold reduction of ERα, compared with NP exposure alone at the same time

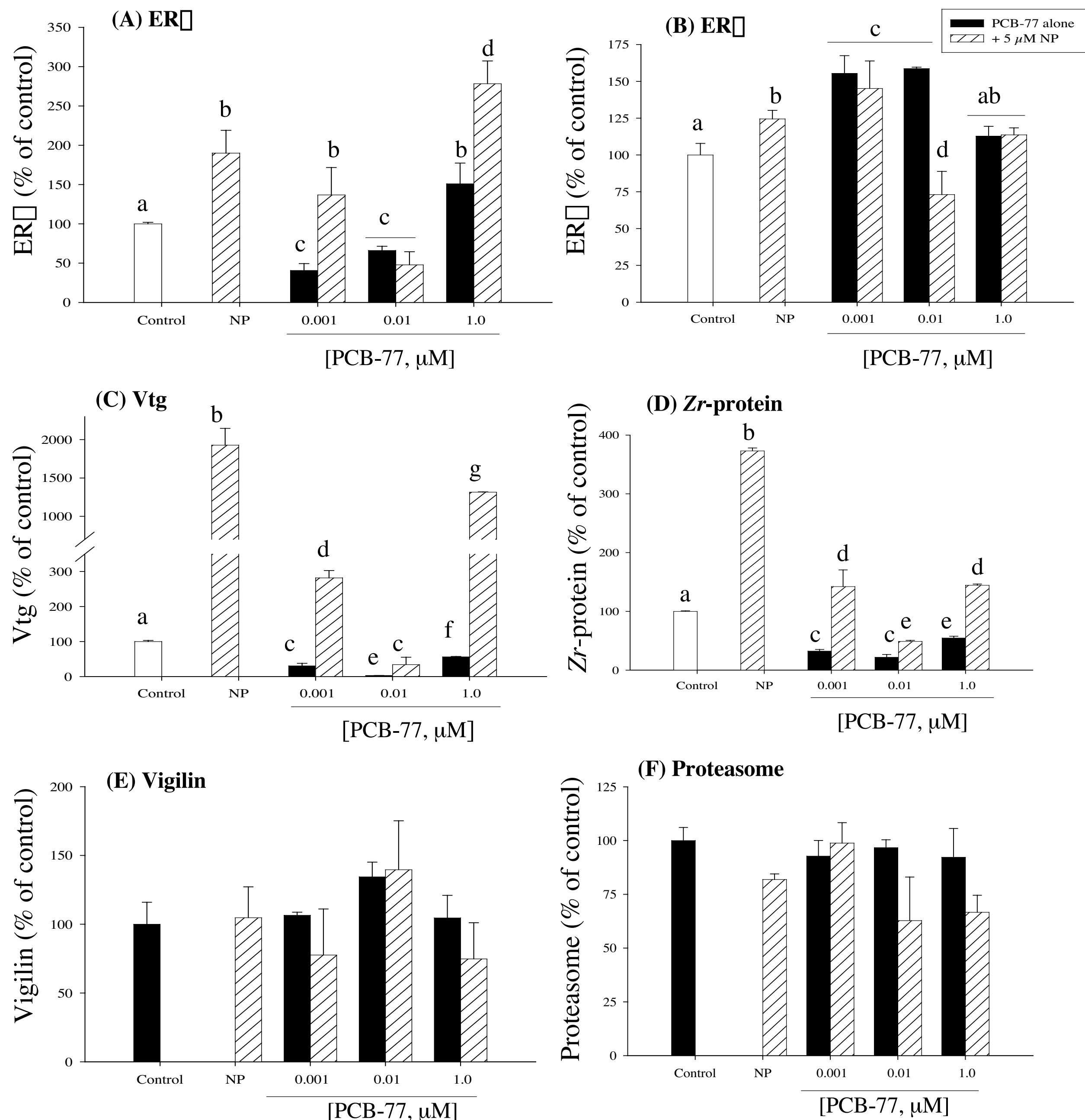

Figure 1

Expression of ERα (A), ERβ (B), Vtg (C), *Zr*-protein (D), vigilin (E) and 20S proteasome subunit (F) mRNA in primary culture of salmon hepatocytes exposed for 48 h to 5 μM NP and PCB-77 at 0.001, 0.01 and 1 μM, singly and in combination. Messenger ribonucleic acid (mRNA) levels were quantified using quantitative (real-time) PCR with gene specific primer pairs. The data are given as % of the solvent control ± standard error of the mean (n = 3). Different letters denote exposure group means that are significantly different for the respective mRNA expression using ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

interval (Fig. 3A). When hepatocytes were exposed to PCB-77 alone, a 3.5-fold increase of ERα mRNA expression was observed at 12 h, and thereafter the expression was reduced below control levels at 24, 48 and 72 post-exposure (Fig. 3A). The expression of ERβ mRNA followed a similar pattern with ERα, but with higher PCB-77 effect

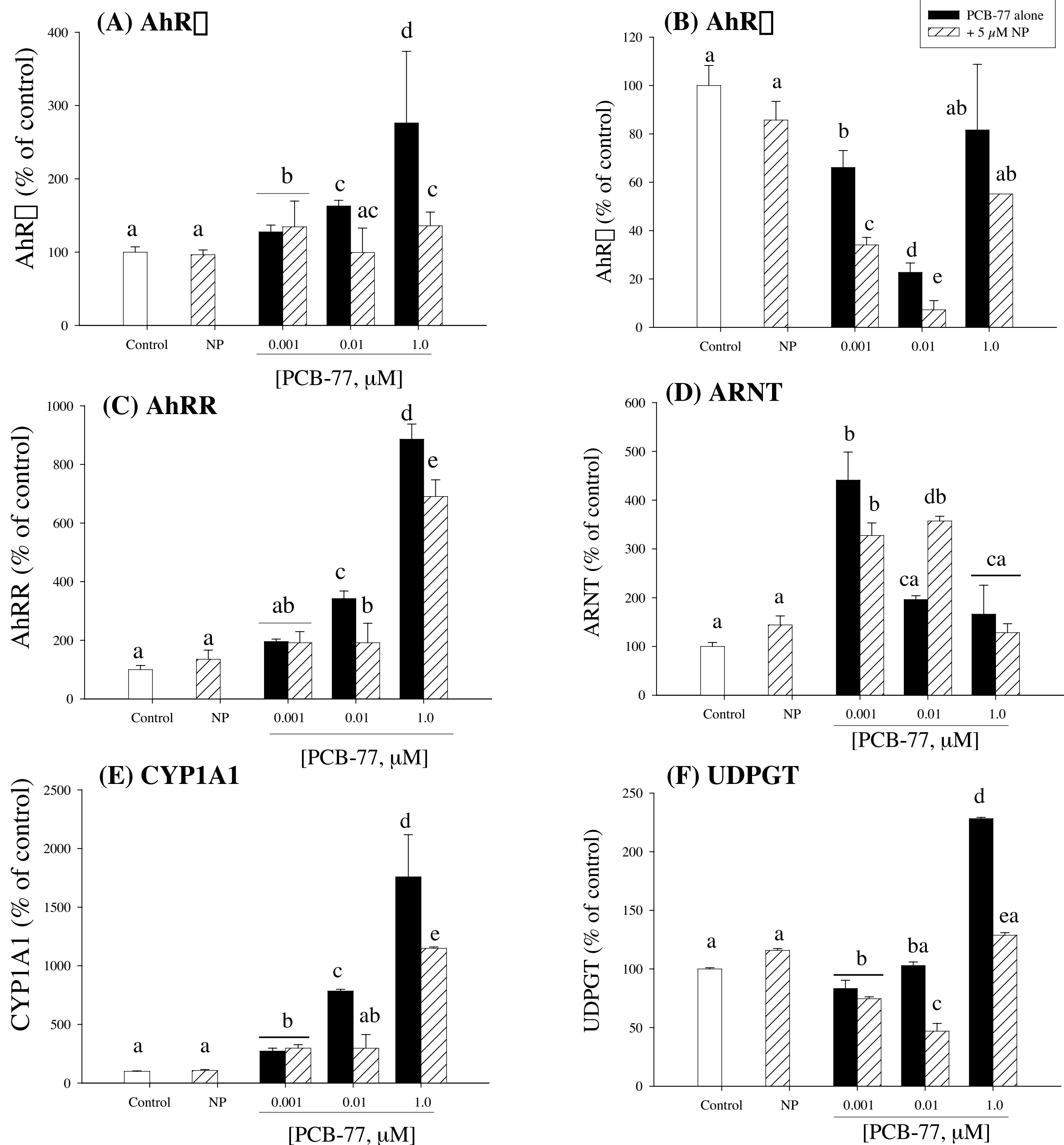

Figure 2

Expression of AhRα (A), AhRβ (B), AhRR (C), ARNT (D), CYP1A1 (E) and UDPGT (F) mRNA in primary culture of salmon hepatocytes exposed for 48 h to 5 μM NP and PCB-77 at 0.001, 0.01 and 1 μM, singly and in combination. Messenger ribonucleic acid (mRNA) levels were quantified using quantitative (real-time) PCR with gene specific primer pairs. The data are given as % of the solvent control ± standard error of the mean (n = 3). Different letters denote exposure group means that are significantly different for the respective mRNA expression using ANOVA followed by Tukey's multiple comparison test (p < 0.05).

(Fig. 3B). Exposure to NP alone produced a significant 11-fold increase of ERβ at 12 h post-exposure and combined NP and PCB-77 exposure resulted to 6-fold reduction compared with NP exposure alone at the same time interval (Fig. 3B). When PCB-77 was given alone, a 4.5-fold increase of ERβ mRNA expression was observed at 12 h after exposure (Fig. 3A). Otherwise, exposure to NP and PCB-77 singly or combined caused minor but variable effects on ERβ mRNA levels at 24, 48 and 72 h after exposure (Fig. 3B).

The expression of Vtg was massively induced (20-fold) after exposure to NP alone at 12 h post-exposure, compared with solvent control (Fig. 3C). Thereafter, Vtg expression in NP-exposed cells showed a time-dependent decreasing trend, albeit massively induced compared to control, at 24, 48 and 72 h after exposure (Fig. 3C). PCB-77 alone produced significant increase of Vtg expression at 24 h post-exposure, compared to control (Fig. 3C). When hepatocytes were exposed to NP and PCB-77 in combination, the NP-induced Vtg expression was reduced at all exposure time points (Fig. 3C). The mRNA expression of *Zr*-proteins increased 3-fold in NP exposed hepatocytes at 12 h post-exposure and decreased back to control level at 24 h (Fig. 3D). Thereafter, a time-dependent increase of *Zr*-protein mRNA, peaking at 72 h, was observed in the NP treated group alone (Fig. 3D). PCB-77 caused significant decreases of *Zr*-protein mRNA expression at 12 and 72 h after exposure, compared to NP treated groups alone (Fig. 3D). When PCB-77 was given alone, a 2-fold increase of *Zr*-protein mRNA was observed at 12 h post-exposure, and thereafter a time-specific decrease was observed (Fig. 3D).

Time-dependent expression of AhR-responsive genes

Compared to solvent control, NP caused variable effect on AhRα, producing a 2-fold significant reduction at 72 h post-exposure (Fig. 4A). The AhRα expression increased 2-fold at 12 and 48 h after exposure with PCB-77 alone and combined NP exposure did not produce significant differences, except at 72 h when NP caused 2-fold decrease of PCB-77 induced AhRα expression (Fig. 4A). In contrast, the expression levels of AhRβ mRNA were not significantly affected over time with NP (Fig. 4B). When PCB-77 was given alone, a 2- and 8-fold increase of AhRβ mRNA expression was observed at 24 and 72 h after exposure, respectively (Fig. 4B), while the combined exposure with NP significantly decreased these effects at the corresponding time intervals (Fig. 4B). For AhRR, NP exposure slightly increased the mRNA level at 24 h, but this effect decreased thereafter with time (Fig. 4C). Exposure of hepatocytes to PCB-77 produced a time-specific significant increase of AhRR mRNA expression and these effects were not significantly affected when PCB-77 was given in combination with NP (Fig. 4C). For ARNT, a different pattern of NP-PCB-77 effect was observed (Fig. 4D). NP induced a 2.5-fold significant increase of ARNT at 12 h, and thereafter a 2-fold decrease at 24 h post-exposure was observed, compared to control (Fig. 4D). The ARNT expression in NP exposed group alone returned to control levels at 48 and 72 h post-exposure (Fig. 4D). Exposure to PCB-77 alone produced a 2-fold significant decrease and increase of ARNT mRNA expression at 48 and 72 h, respectively, compared to control (Fig. 4D). When PCB-77 was given in combination with NP, PCB-77 caused respective significant decrease (at 12 and 48 h) and increase (at 24 and 72 h) of NP-mediated ARNT mRNA expression (Fig. 4D). Exposure to PCB-77 singly produced a time-dependent induction of CYP1A1 mRNA reaching 45-fold at 72 h after exposure (Fig. 4E). When hepatocytes were exposed to combined PCB-77 and NP, the PCB-77-induced CYP1A1 mRNA expressions were significantly reduced reaching 15-fold at 72 h post-exposure (Fig. 4E). The UDPGT mRNA expression levels followed a different pattern compared with CYP1A1. NP exposure alone produced a 3.8-fold increase and 1.5-fold decrease of UDPGT expression at 12 and 24 h after exposure, respectively (Fig. 4F). The expression pattern of UDPGT in PCB-77 exposed group alone was generally similar to NP exposure alone, but with non-parallel abundance at 12 and 72 h after exposure. Combined PCB-77 and NP exposure produced decreased UDPGT mRNA expression level at 12 h compared with NP exposure alone. At 72 h, the UDPGT expression was significantly increased in the combined PCB-77 and NP exposure group, compared with NP exposure alone (Fig. 4F).

Discussion

In the present study, we investigated the ER-AhR interactions and their mediated signalling pathways using agonists for these receptors, genomic methods and *in vitro* system. In our laboratory, we have previously reported that PCB-77, an AhR agonist with known anti-estrogenic activity, caused increases and decreases of *in vivo* ER-mediated NP-induced Vtg and *Zr*-protein gene and protein expression patterns in Atlantic salmon [11]. We found that the *in vivo* responses were dependent on PCB-77 and NP dose ratios and sequential order of exposure and interestingly influenced by seasonal changes [11]. In a recent study, we showed that the partial inhibition of AhR with α-naphthoflavone (ANF) did not reverse the effect of PCB-77 on ER-mediated transcription suggesting that AhRs does not have a direct role on PCB-77 mediated decreases of ER-mediated responses; and the inhibition of ER with tamoxifen (Tam – partial ER antagonist) and ICI 182,780 (ICI – absolute ER antagonist) reversed the transcription of AhR-mediated responses, except AhR repressor (AHRR) [15]. Taken together, these findings demonstrate a complex mode of ER-AhR interaction that is dependent on time- and the individual chemical (NP

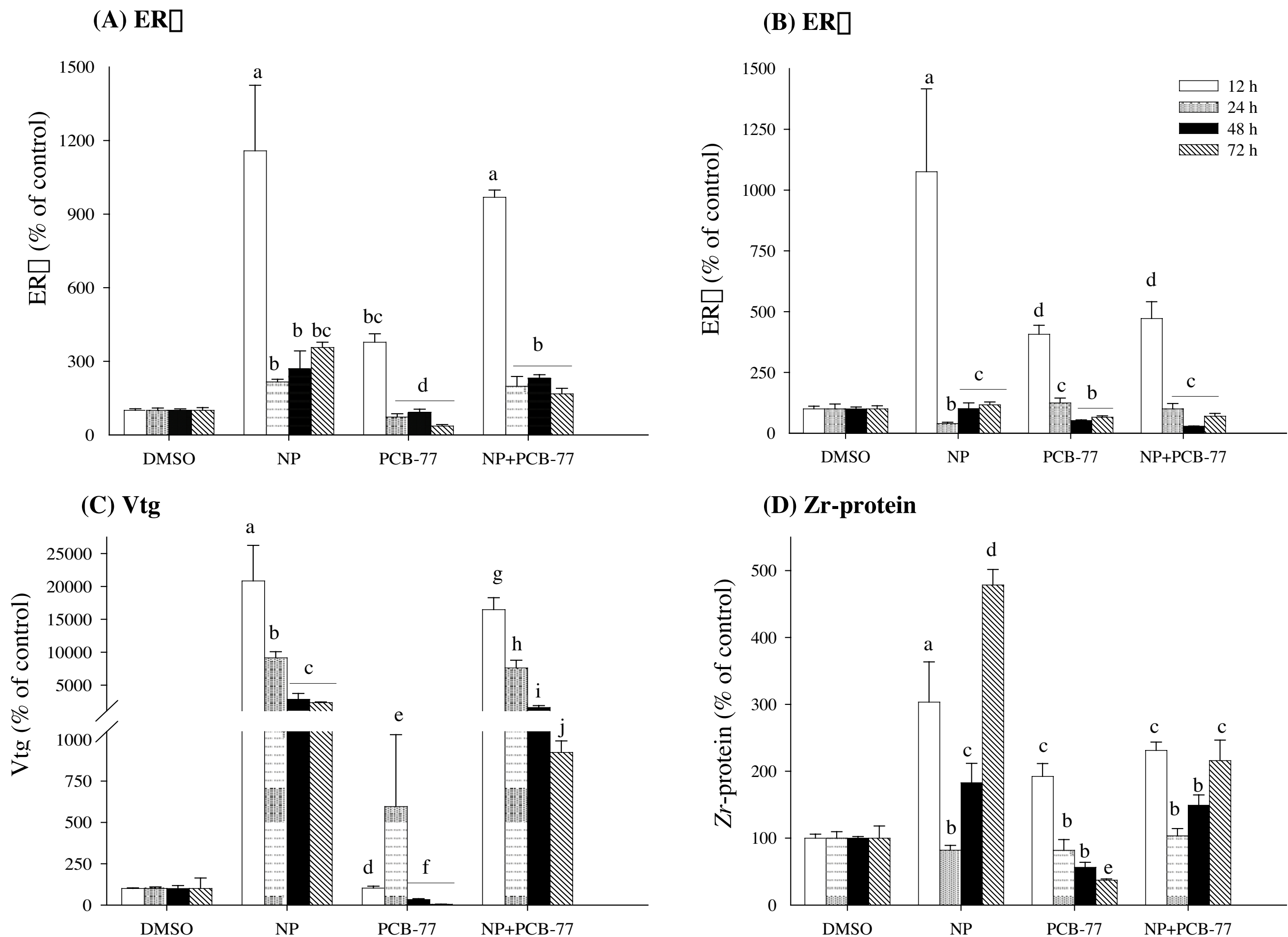

Figure 3

Time-dependent expression patterns of ERα (A), ERβ (B), Vtg (C) and *Zr*-protein (D) mRNA in primary culture of salmon hepatocytes exposed to 5 μM NP and 1 μM PCB-77, both singly and in combination. Hepatocytes were sampled at 12, 24, 48 and 72 hours post-aexposure. Expression of mRNA levels was quantified using quantitative (real-time) PCR with gene specific primer pairs. The data are given as % of the solvent control ± standard error of the mean (n = 3). Different letters denote exposure group means that are significantly different for the respective mRNA expression using ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

and PCB-77) concentrations. In order to further characterize the molecular mechanism(s) behind these responses, the analytical power of quantitative (real-time) PCR and salmon primary hepatocyte culture was used with one concentration of NP (5 μM) and different concentrations of PCB-77 (0.001, 0.01 and 1 μM) to study the time-dependent expression patterns of relevant genes in the ER and AhR signalling pathways. Our data show a bi-directional ER-AhR interaction that is dependent on time and PCB-77 concentration.

Modulation of ER responsive genes

The biological effects of estrogens and their mimics, such as NP are mediated through the ERs. At present, three ER subtypes have been isolated in teleosts. The mRNA transcription of ERα and ERβ, and three estrogen responsive genes (Vtg, *Zr*-protein and vigilin) were studied using real-time PCR. We found that exposure of hepatocytes to NP and PCB-77 singly or in combination produced distinct expression patterns of each ER subtypes, albeit less than NP induced levels. Both ER subtypes (α and β) were significantly altered by NP exposure singly. In mammals, the tissue and cell specific roles of ER isotypes have been described [16]. Tight relationship between the ERα gene isoform expression and Vtg synthesis in a number of teleost species have been reported and strongly suggest that this particular ER plays the dominant role in regulating vitellogenesis [17-19]. In this study, PCB-77 was anti-

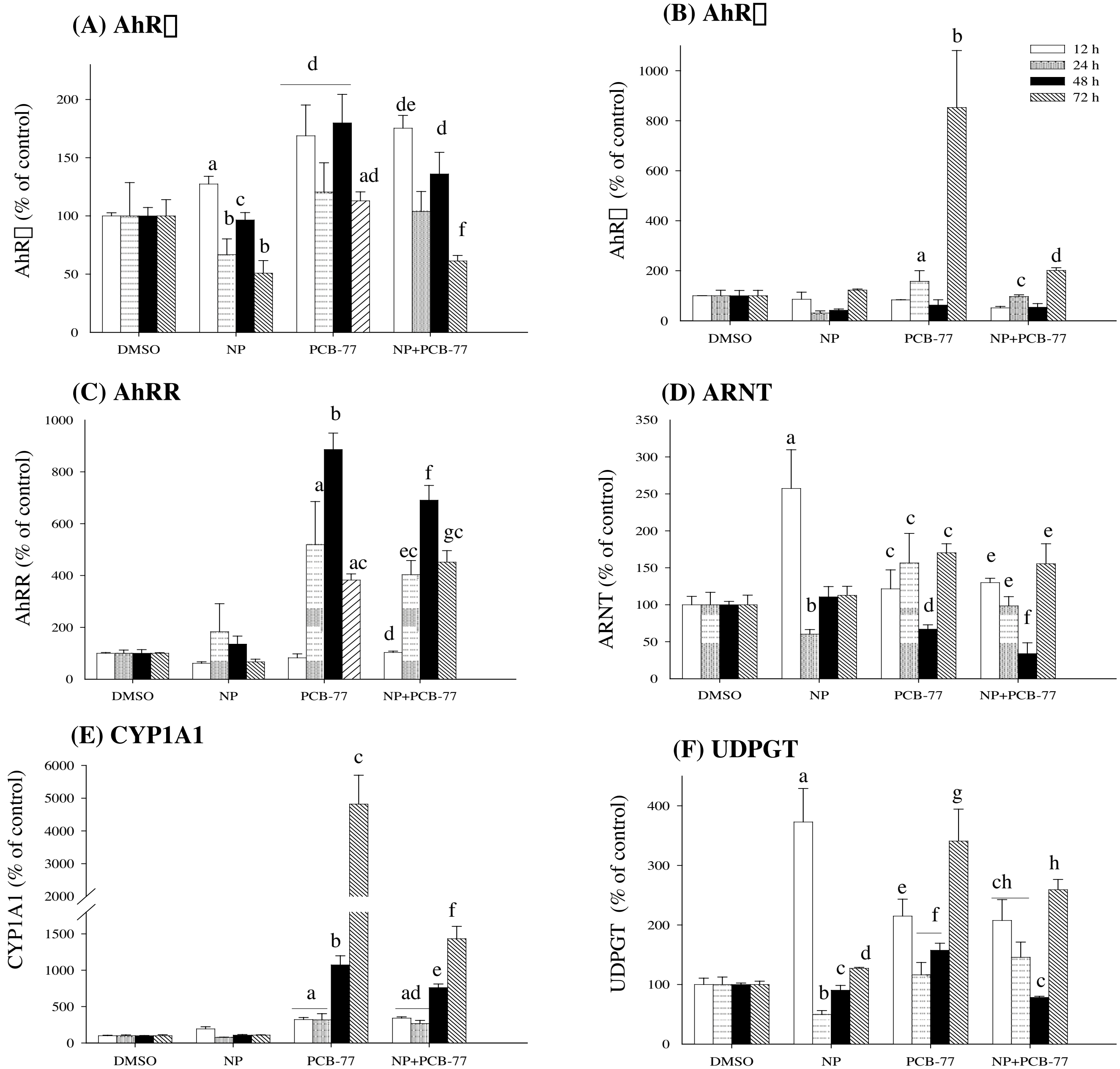

Figure 4

Time-dependent expression patterns of AhRα (A), AhRβ (B), AhRR (C), ARNT (D), CYP1A1 (E) and UDPGT (F) mRNA in primary culture of salmon hepatocytes exposed to 5 μM NP and 1 μM PCB-77, both singly and in combination. Hepatocytes were sampled at 12, 24, 48 and 72 hours post-exposure. Expression of mRNA levels was quantified using quantitative (real-time) PCR with gene specific primer pairs. The data are given as % of the solvent control ± standard error of the mean (n = 3). Different letters denote exposure group means that are significantly different, for the respective mRNA expression using ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

estrogenic on NP induced Vtg and *Zr*-protein expression in a time-specific manner and these effect showed a parallel pattern of expression with ERα gene expression [20].

Modulation of AhR responsive genes

We investigated the effects of NP on PCB-77-induced AhR signalling. It should be noted that in this study AhRα and

AhRβ are used synonymously with AhR1 and AhR2, respectively. We observed that PCB-77 produced effects on AhR signalling by transcriptional changes of AhR-subtypes (AhRα and AhRβ), ARNT, AhRR, CYP1A1, UDPGT and 20S proteasome subunit. The effects on AhR signalling pathway were dependent on time of exposure and PCB-77 concentration, and were negatively affected by NP. In accordance with the present study, the induced transcription of phase I and II biotransformation enzymes by PCB-77 has previously been reported [9]. The expression of AhRα and AhRR followed a parallel pattern with CYP1A1 and UDPGT after exposure to PCB-77 concentrations. On the contrary, AhRβ and the AhR nuclear dimerization partner, ARNT were differentially affected. For ARNT expression, we observed that a decreased expression pattern with increasing PCB-77 concentration. The overall function of ARNT is not fully understood in teleost, while in mammalian cells, this protein appears to be constitutively active [2]. Although the biochemical and molecular properties of AhR has been characterized in mammalian cells, there are still uncertainties concerning the regulation, interactions with other proteins and transcriptional properties of AhRs [21]. In zebrafish (*Danio rerio*) embryo and liver cell line, TCDD induced a dose-dependent increase of AhR2 mRNA expression [22]. Similar effect was also observed in rainbow trout where the AhR2 and AhR2β were elevated in gonadal cell line and kidney tissue [21]. In addition, these authors did not observe increases in mRNA expression of either AhR2 or AhR2β mRNA after TCDD exposure in rainbow trout liver or spleen [23]. Elsewhere, TCDD or PCB-77 doses did not affect transcriptional changes of AhR2 mRNA expression in Atlantic tomcod (*Microgadus tomcod*) liver [24].

As a transcription factor, the normal physiological and toxicological significance of the multiple AhRs and their associated proteins in many fish species is yet to be fully characterized. In view of the present study and others [25], a comparison of the *in vivo* endogenous response with *in vitro* reporter assays that have utilized different AhR subtypes from rainbow trout suggests that AhRα may account for the CYP1A1 induction by PCB-77 in our system [21]. It has been shown that the amino acid sequence of AhR1 is most closely related to mammalian AhRs which mediate the molecular response after exposure to halogenated aromatic hydrocarbons [26]. The AhR1 (or AhRα) mRNA is nearly undetectable in many tissues that exhibit TCDD (and related compounds)-inducible CYP1A1 expression, implying that AhR2 (or AhRβ) is capable of mediating this response [25]. The transcriptional capability of bHLH-PAS family of transcription factors is yet to be fully understood and their individual *in vivo* functions are still subject of current discussions.

ER-AhR interactions

Several reports have shown that AhR ligands possess anti-estrogenic properties [11,27,28]. A direct *in vitro* ligand specific interaction between AhR and ERα has been reported by Klinge and co-workers [29]. In our laboratory, a bi-directional ER-AhR interaction has been reported in rainbow trout *in vitro* system [9]. Herein, we show that PCB-77 decreased the expression of NP-induced transcription of ERα, Vtg and *Zr*-protein in a concentration- and time-specific manner. Interestingly, PCB-77 alone significantly increased ERβ expression. Studies of TCDD ability to bind to ER demonstrated that this strong AhR agonist did not compete with E2 for binding to the ER [30]. Four possible mechanisms have been suggested for the anti-estrogenic actions of AhR agonists: 1) increased rate of E2 metabolism; 2) decreased cellular ER isoform levels; 3) suppression of E2 induced transcription; and 4) ER-AhR competition for transcriptional co-factors [31]. Recently, a new mechanism of action termed "ER-hijacking" that defies the above named mechanisms has been postulated [32]. ER-hijacking describes the ability of AhR ligands to activate ER-regulated transcription independent of ER-ligands and has raised the possibility that several xenoestrogens may indeed have estrogenic properties through activation of AhR-ER complex [33]. In fish, we first reported this alternative mode of action for AhR agonists, using PCB-77 and salmon *in vivo* system in 2001 [11]. In that report, we proposed that although the mechanisms by which AhR-agonists induce CYP1A and mediate their antiestrogenic effects seem to be well understood, it could be argued that these mechanisms may be the exception (with regard to estrogen mimics) rather than the rule for the actions of TCDD and related compounds there seem to be ER isoform preferences that favour the α-isoform. Today, several reports have demonstrated that AhR agonists directly induce estrogenic activity through AhR-ERα interactions [[33-35]; Mortensen and Arukwe, in prep]. However, there seem to be ER isoform preferences that favour the α-isoform. For example, a human variant of ERα(-) Ishikawa endometrial cell line were unresponsive to E2, despite their expression of ERβ, reflecting the low transcriptional activity of ERβ compared to ERα [32,33]. Herein, high PCB-77 concentration produced an increase of ERα (also at 12 h post-exposure), above control and statistically equal to NP levels, and in combination with NP produced elevated ERα above NP and control levels. PCB-77 produced an increase of ERβ that was concentration specific, it is possible that AhR agonists, such as PCB-77 may "hijack" both ER subtypes that does not result in the activation of Vtg (but *Zr*-protein at 12 h) response.

When these potential mechanisms are put into context of the present study, degradation of endogenous E2 (or NP) by metabolizing enzymes induced by AhR may lead to decreased ER-mediated transcription. The involvement of

CYP1A1 in E2 metabolism was previously investigated in female carp by Smeets and co-workers [36] and reported that the anti-estrogenicity of different AhR ligands in female carp was found to be mediated through the AhR, not involving the CYP1A1. This is in accordance with the present study, showing no clear pattern of decreased ER, Vtg or *Zr*-protein gene expression in response to increased CYP1A1 gene or enzyme activity (measured as 7-ethoxyresorifin O-deethylase, EROD- data not shown) after treatment with PCB-77.

The ER degradation by proteasomes induced by AhR has been explained as another possible anti-estrogenic mechanism [37,38]. In addition to activating AhR, TCDD is found to rapidly reduce the level of AhR protein in cells and mechanistic studies have established that the turnover is mediated through the 26S proteasome, involving ubiquitination of AhR and requires the transcription activation domain of AhR [39,40]. Our data does not support these speculations since despite being expressed there is no direct relationship between a 20S proteasome β-subunit quantified in this study with ERα expression levels. On the contrary, a partial relationship was observed between the proteasome subunit and AhR subtypes, AhRR, CYP1A1 and UDPGT in the combined NP and PCB-77 at 0.01 and 1 μM concentrations. This discrepancy might be caused by the possibility that we may have quantified the wrong proteasome subunit. The choice of proteasome in the present study was based on its differential expression pattern on our subtractive cDNA library after exposure to ER- and AhR-agonists [15]. Furthermore, while the proteasome hypothesis provided us with a rationale for measuring the proteasome gene expression, it should be noted that changes in gene expression are generally not a surrogate for changes in protein degradation due to proteasome degradation. Thus, the proteasome hypothesis should be studied at the protein level.

Previous report have shown that mouse hepatic cell line lacking functional AhR due to mutations in the ARNT, lost ER trans-activation potential in the presence of TCDD due to a sharp decrease in its ability to bind to an ERE [41]. Elsewhere, TCDD prevented reporter gene expression in Xenopus Vtg A2 regulatory sequences even when cells were transiently over-expressing ER, suggesting that the mechanism does not involve ER down-regulation by TCDD [42]. While treatment with E2 increased ER-ERE complex formation, TCDD alone did not have an effect and the binding of ER to ERE was completely lost in cells simultaneously treated with both E2 and TCDD. These observations led the authors to conclude that TCDD was no longer anti-estrogenic in the mutated cell line since AhR was required for the ability of ER to trans-activate from the ERE [41]. When these finding are compared to the data in the present study where PCB-77 produced an apparent concentration-specific increased and decrease of ERα and ARNT, respectively, it is plausible to suggest that PCB-77 mediated anti-NP effect does not involve the down-regulation of ERα expression.

Another possible target for AhR-mediated anti-estrogenicity is the mRNA stability of ER and its transcriptional downstream products (Vtg and *Zr*-proteins). RNA gel mobility shift assays has shown that an estrogen-inducible mRNA stabilizing protein that bound specifically to Vtg mRNA in an area previously implicated in estrogen-mediated stabilization of Vtg mRNA [43]. The stability of mRNA is determined by site-specific mRNA endonuclease activities [44]. The endonuclease catalyzed mRNA decay is regulated through the binding of RNA-binding proteins to target mRNAs that prevent their cleavage by endonucleases [45]. Vigilin, or high density lipoprotein-binding protein, is an ubiquitous protein in vertebrate cells [43]. For example, the stability of liver Vtg mRNA *in Xenopus laevis* is regulated by an E2-induced vigilin that binds specifically to a 3'-untranslated region (3'-UTR) segment of the Vtg mRNA and protects it from degradation [43]. In the present study, the expression of vigilin mRNA in NP exposure singly or in combination with PCB-77 concentration did not produce parallel expression pattern with Vtg or *Zr*-protein. Interestingly, the low PCB-77 exposure alone or in combination with NP that produced an almost total inhibition of Vtg and *Zr*-protein levels showed the highest vigilin expression. We are performing further studies to explain this discrepancy. However, it should be noted that 0.01 μM PCB-77 produced a consistent, but complicated pattern of effect in both ER and some AhR mediated responses (see Figs. 1, 2).

On the AhR signalling pathway, we observed that the NP decreased the transcription of AhRα, AhRβ, AhRR, ARNT, CYP1A1 and UDPGT to below PCB-77 exposed levels in a PCB-77 concentration- and time-specific manner, indicating that NP has anti-AhR signalling effects. Interestingly, the expression of AhRβ and ARNT showed a different pattern of effect in PCB-77 exposure alone and in combination with NP. We observed PCB-77 exposure first induced ARNT at low concentration and thereafter a concentration-specific decrease was observed. ARNT functions as a dimerization partner for several proteins in the bHLH-PAS protein superfamily [2,28], therefore, only minor alterations in ARNT gene expression could be expected in response to xenobiotic exposures. However, on the basis of sequence homology with an ER transcription factors p160, it was shown that ARNT functions as a co-activator of ER and this effect was due to the C-terminal domain and not the conserved bHLH or PAS domains [28]. In addition, although the ARNT contains a less complex activation domain compared to AhR; the activation domains of AhR and ARNT are located in the carboxy-terminal of

both genes [46]. During CYP1A1 (and other genes) activation, the ARNT activation domain does not contribute to the activation of AhR complex [47].

In general, the present data are consistent with previous studies showing that NP (*i.e.*, estrogen mimic) and E2 significantly suppressed hepatic CYP1A1 mRNA levels, EROD activity and CYP1A1 protein in *in vivo* and *in vitro* experiments using several teleost species [48,49]. Based on the possible mechanisms explained above, we hypothesize that NP can bind the CYP1A1 protein [50], and through this binding, NP or its metabolites may inhibit the CYP1A1 expression [51]. Alternatively, the effect of NP could partially be mediated by the liver ERs through a process that may involve the ER-NP complex interfering with the AhR transcription machinery either directly or with the CYP1A1, or indirectly through bind to the XRE and regulating AhR-induced gene expression. In addition, NP may control the recruitment of ER and possibly other co-activators, besides activating the detoxification pathway.

The consistency between AhRR, CYP1A1 and UDPGT expression pattern suggests that this repressor singly may have caused the decrease in CYP1A1 and UDPGT levels. The AhRR-ARNT heterodimerization may negatively regulate AhR driven gene expression through transcriptional repression [52]. In accordance with our data, the modulation of CYP1A1 by NP, E2, and BNF was recently shown to parallel the AhRR gene expression [53]. Any of the above mentioned mechanisms might have caused the NP effect on AhR signalling. This is supported by the fact that the BHLH-PAS (Per-AhR/ARNT-Sim homology sequence) of transcription factor usually associate with each other to form heterodimers, AhR/ARNT or AhRR/ARNT, and bind the XRE sequences in the promoter regions of the target genes to regulate their expression.

Conclusion

The findings in the present study demonstrate the interactions between NP and PCB-77 in primary culture of salmon hepatocytes. The AhR-agonist (PCB-77) functioned as anti-NP-mediated effect, and NP functioned as anti-AhR-mediated effect or as inhibitor of AhRα, AhRR, ARNT, CYP1A1 and UDPGT expression. Overall, the findings demonstrate a complex mode of ER-AhR interactions that were dependent on the time of exposure and individual chemical (NP and PCB-77) concentrations. A novel aspect of the present study is that low (0.001 μM) and medium (0.01 μM) PCB-77 concentrations increased ERβ mRNA expression above control and NP levels, and at 12 h post-exposure, PCB-77 exposure alone produced significant elevation of ERα, ERβ and *Zr*-protein expressions above control levels. Nevertheless, a retrospective evaluation of the data presented here showed that 12 h could have been a better exposure time for the concentration study since it was at this time point most unique responses were observed. However, the choice of our exposure time was based on previous studies in our laboratory (and elsewhere) that have produced significant interactions between NP and PCB-77 is fish primary hepatocyte culture. In our laboratory, we are still performing studies on cross-talk between the ER-AhR signal transduction systems and underlying mechanism(s) by which xenobiotics and xenoestrogens interact with each other. This complex interaction between two different classes of ligand-activated receptors provides novel mechanistic insights on signalling pathways.

Methods

Chemicals and reagents

4-nonylphenol (NP; 85% of p-isomers) was purchased from Fluka Chemika-Biochemika (Buchs, Switzerland). The impurities in 4-nonylphenol consist mainly of phenol (8–13%), tripropylene (~1%) and 2,4-dinonylphenol (~1%). 3,3',4,4'-Tetrachlorobiphenyl (PCB-77; 99.7% pure) was purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Dulbecco minimum essential medium (DMEM) with non-essential amino acid and without phenol red, fetal bovine serum (FBS), L-glutamine and TA cloning kit were purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), 100× penicillin-streptomycin-neomycin solution, collagenase, bovine serum albumin (BSA), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), ethyleneglycol-bis-(β-aminoethylether) N, N'tetraacetic acid, (EGTA), 0.4% trypan blue were purchased from Sigma Chemical (St. Louis, MO, USA). E.Z.N.A. total RNA kit for ribonucleic acid (RNA) purification was from Omega Bio-Tek (Doraville, GA, USA). IScript cDNA synthesis kit and iTAQ™ SYBR® green supermix with ROX were purchased from Bio-rad Laboratories (Hercules, CA, USA). GeneRuler™ 100 base pairs (bp) DNA ladder and deoxynucleotide triphosphates (dNTPs) were purchased from Fermentas GmbH (St. Leon-Rot, Germany).

Collagenase perfusion, isolation and culture of hepatocytes

Juvenile Atlantic salmon (*Salmo salar*) of approximately 400–500 g were supplied by Marine Harvest AS, Dyrvik, Norway and kept at the animal holding facilities at the Biology Department, NTNU. Fish were supplied with continuously running saltwater at a constant temperature of 10°C. Prior to liver perfusion all glassware and instruments were autoclaved before use. Solutions were filtration sterilized by using 0.22 μm Millipore filter (Millipore AS, Oslo, Norway). Hepatocytes were isolated from 3 individuals (triplicate exposures) by a two-step perfusion technique with modifications as described by Andersson

and co-workers [54]. The cell suspension was filtered through a 150 μm nylon monofilament filter and centrifuged at 50 × g for 5 min. Cells were washed three times with serum-free medium and finally resuspended in complete medium. Following collagenase perfusion and isolation of hepatocytes, viability of cells was determined by the trypan blue exclusion method. A cell viability value of > 90% was a criterion for further use of the cells. Cells were plated on a 35 mm Primaria culture plates (Becton Dickinson Labware, USA) at the recommended density for monolayer cells of 5 × 10^6 cells in 3 ml DMEM medium (without phenol red) containing 2.5% (v/v) FBS, 0.3 g/L glutamine, and 1% (v/v) penicillin-streptomycin-neomycin solution. The cells were cultured at 10°C in a sterile incubator without additional O_2/CO_2 for 48 hr prior to chemical exposure.

Exposure of hepatocytes

After 48 h pre-culture, two separate experiments were performed. Firstly, we evaluated the effects of different PCB-77 concentrations on NP mediated effects. Secondly, we investigated the time-response pattern of these effects. Both NP and PCB-77 concentrations were chosen based on previous experiments. These studies showed that these concentrations are optimal *in vitro* concentrations for ER-AhR interactions in salmonids [9]; Mortensen and Arukwe, submitted). In the first experiment, hepatocytes were exposed (triplicate plates for each exposure group) for 48 h to 0.01% DMSO (control), 5 μM NP and 0.001, 0.01 and 1 μM PCB-77 singly and also in combination. In the second experiment, hepatocytes were exposed (triplicate plates for each exposure group) for 12, 24, 48 and 72 h to 0.01% DMSO (control), 5 μM NP and 1 μM PCB-77 singly and also in combination. In both experiments, media were replaced with fresh media containing the respective test chemical and concentrations every 24 h. Media and cells were harvested after exposure and lysed in E.Z.N.A lysis buffer for total RNA isolation according manufacturers protocol (Omega Bio-Tek).

Quantitative (real-time) PCR

Total cDNA for the real-time PCR reactions were generated from 1 μg total DNase-treated RNA from all samples using poly-T primers from iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad). Quantitative (real-time) PCR was used for evaluating gene expression profiles. For each treatment, the expression of individual gene targets was analyzed using the Mx3000P REAL-TIME PCR SYSTEM (Stratagene, La Jolla, CA, USA). Each 25-μL DNA amplification reaction contained 12.5-μL of iTAQ™ SYBR® Green Supermix with ROX (Bio-Rad), 1 μL of cDNA and 200 nM of each forward and reverse primers. The 3 step real-time PCR program included an enzyme activation step at 95°C (5 min) and 40 cycles of 95°C (30 sec), 55–65°C for 30 sec, depending on the primers used (see Table 1), and 72°C (30 sec). Controls lacking cDNA template (minus reverse transcriptase sample) were included to determine the specificity of target cDNA amplification as described previously [9,55]. Briefly, cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct versus log copy number. The criterion for using the standard curve is based on equal amplification efficiency with unknown samples and this is usually checked prior to extrapolating unknown samples to the standard curve. The standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest. Data obtained from triplicate runs for target cDNA amplification were averaged and expressed as ng/μg of initial total RNA used for reverse transcriptase (cDNA) reaction. Standard errors were calculated using S-plus statistic software 6.2 (Insightful Corp, USA). Statistical differences among treatment groups were tested using analysis of variance (ANOVA) and comparison of different exposure treated and control groups were performed using

Table 1: Primer pair sequences, accession numbers, amplicon size and annealing temperature conditions for genes of interest used for real-time PCR.

Target Gene	Primer sequence*		Amplicon size (nucleotides)	Annealing temperature (°C)	GenBank accession number
	Forward	Reverse			
ERα	TCCAGGAGCTGTCTCTCCAT	GATCTCAGCCATACCCTCCA	173	55	DQ009007
ERβ	GAGCATCCAAGGTCACAATG	CACTTTGTCATGCCCACTTC	126	59	AY508959
Vtg	AAGCCACCTCCAATGTCATC	GGGAGTCTGTCCCAAGACAA	391	57	DY802177
Zr-protein	TGACGAAGGTCCTCAGGG	AGGGTTTGGGGTTGTGGT	113	55	AF407574
Vigilin	GGGATACGCACAGACACCTT	CCCAGATTCCACAGACACCT	86	60	DY802195
AhRα	AGGGGCGTCTGAAGTTCC	GTGAACAGGCCCAACCTG	82	60	AY219864
AhRβ	GACCCCCAGGACCAGAGT	GTTGTCCTGGATGACGGC	96	65	AY219865
AhRR	TTCCTCCAGGGACAGAAGAA	ATGGAGGGCAGCAGAAGAG	98	60	DQ372978
Arnt	AGAGCAATCCCAGGGTCC	TGGGAGGGTGATTGAGGA	107	60	DQ367887
CYP1A1	GAGTTTGGGCAGGTGGTG	TGGTGCGGTTTGGTAGGT	76	60	AF364076
UDPGT	ATAAGGACCGTCCCATCGAG	ATCCAGTTGAGGTCGTGAGC	113	55	DY802180
Proteasome	TCTTTGACCAGGTTGCACAG	CATACAAAGCTGGTGGCTCA	134	60	DY802110

Tukey's multiple comparison test. The multiparametric ANOVA test was performed after testing for normality and also variance homogeneity, using the Levene's test. For all the tests the level of significance was set at $p < 0.05$, unless otherwise stated.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

ASM carried out the experiments, processed the data and participated in writing the manuscript. AA initiated the study, designed and supervised the study. All authors read and approved the final manuscript

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Activation of estrogen receptor signaling by the dioxin-like aryl hydrocarbon receptor agonist, 3,3′,4,4′,5-Pentachlorobiphenyl (PCB126) in salmon *in vitro* system

Anne Skjetne Mortensen, Augustine Arukwe *

Department of Biology, Norwegian University of Science and Technology (NTNU), Høgskoleringen 5, 7491 Trondheim, Norway

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Abstract

Available toxicological evidence indicates that environmental contaminants with strong affinity to the aryl hydrocarbon receptor (AhR) have anti-estrogenic properties in both mammalian and non-mammalian *in vivo* and *in vitro* studies. The primary objective of the present study was to investigate the interactions between the AhR and estrogen receptor (ER) in salmon *in vitro* system. Two separate experiments were performed and gene expression patterns were analyzed using real-time PCR, while protein analysis was done by immunoblotting. Firstly, salmon primary hepatocytes were exposed to the dioxin-like PCB126 at 1, 10 and 50 pM and ER agonist nonylphenol (NP) at 5 and 10 μM, singly or in combination. Our data showed increased levels of ER-mediated gene expression (vitellogenin: Vtg, *zona radiata* protein: *Zr*-protein, ERα, ERβ and vigilin) as well as increased cellular ERα protein levels after treatment with NP and PCB126, singly or in combination. PCB126 treatment alone produced, as expected, increased transcription of AhR nuclear translocator (Arnt), CYP1A1 and AhR repressor (AhRR) mRNA, and these responses were reduced in the presence of NP concentrations. PCB126 exposure alone did not produce significant effect on AhR2α mRNA but increased (at 1 and 50 pM) and decreased (at 10 pM) AhR2β mRNA below control level. For AhR2δ and AhR2γ isotypes, PCB126 (at 1 pM) produced significant decreases (total inhibition for AhR2γ) of mRNA levels but was indifferent at 10 and 50 pM, compared to control. NP exposure alone produced concentration-dependent significant decrease of AhR2β mRNA. In contrast, while 5 μM NP produced an indifferent effect on AhR2δ and AhR2γ, 10 μM NP produced significant decrease (total inhibition for AhR2γ) and the presence of NP produced apparent PCB126 concentration-specific modulation of all AhR isotypes. A second experiment was performed to evaluate the involvement of ER isoforms in PCB126 mediated estrogenicity. Here, cells were treated with the different concentrations of PCB126, alone or in combination with ICI182,780 (ICI) and sampled at 12, 24 and 48 h post-exposure. Our data showed that PCB126 produced a time- and concentration-specific increase of ERα and Vtg expressions and these responses were decreased in the presence of ICI. In general, these responses show a direct PCB126 induced transcriptional activation of ERα and estrogenic responses in the absence of ER agonists. Although not conclusive, our findings represent the first study showing the activation of estrogenic responses by a dioxin-like PCB in fish *in vitro* system and resemble the "ER-hijacking" hypothesis that was recently proposed. Thus, the direct estrogenic actions of PCB126 observed in the present study add new insight on the mechanisms of ER–AhR cross-talk, prompting a new wave of discussion on whether AhR-mediated anti-estrogenicity is an exception rather than rule of action.

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Keywords: Dioxin-like PCB126; AhR; Estrogen receptor activation; Nonylphenol; Interactions; Fish; *In vitro* system

Introduction

The anti-estrogenic activities of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and related compounds which induce synthesis of cytochrome P4501A (CYP1A) have been demonstrated in both *in vivo* and *in vitro* studies (Anderson et al., 1996a,b; Astroff et al., 1990; Safe et al., 1991). According to Safe et al. (1991) and Spink et al. (1990), the anti-estrogenicity of CYP1A-inducing compounds parallels their AhR binding ability and subsequent

* Corresponding author. Fax: +47 73 596100.
E-mail address: arukwe@bio.ntnu.no (A. Arukwe).

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activation of CYP1A gene, among other transcription products. The estrogen receptors (ERα and ERβ) belong to the nuclear receptor superfamily of transcription factors (Nilsson et al., 2001), while the AhR is a member of the helix–loop–helix–PAS (bHLH–PER–ARNT–SIM) family of gene regulatory proteins (Gu et al., 2000). Upon binding to a ligand the AhR dimerizes with AhR nuclear translocator (Arnt) and the complex translocates to the nucleus where it transactivates mRNA transcription of genes containing XRE (xenobiotic responsive elements) in their upstream regions. Several genes involved in metabolism and degradation of lipophilic and persistent compounds contain XREs in their promoter, including CYP enzymes, uridine-diphosphate glucuronosyltransferase (UGT) and AhR repressor (AhRR) (Gu et al., 2000). Similarly, binding of estrogens or their mimic to the ERs results in dimerization and subsequent binding of cofactors that produce the transactivation of genes (such as vitellogenin; Vtg) with estrogen responsive elements (EREs) upstream of the initiation site (Nilsson et al., 2001). In addition, the endonuclease catalyzed mRNA decay is regulated through the binding of RNA-binding proteins to target mRNAs that prevent their cleavage by endonucleases (Dodson and Shapiro, 2002). Vigilin or high density lipoprotein-binding protein is a ubiquitous protein in vertebrate cells. For example, the stability of liver Vtg mRNA is regulated by an E2-induced vigilin that binds specifically to a 3′-untranslated region (3′-UTR) segment of the Vtg mRNA and protects it from degradation (Dodson and Shapiro, 2002). Thus, ER and AhR are critical for their respective gene expressions. The molecular basis for Vtg and CYP1A1 gene expression shows that these gene activations are receptor-mediated responses that are ligand structure-dependent interactions with respective ER and AhR, probably involving several receptor isoforms. Inhibitory AhR and ER cross-talk have been demonstrated in breast cancer cells, rodent uterus and mammary tumor cells (Safe et al., 1991).

Previously, we reported that *in vivo* exposure of fish to combined AhR agonist (3,3′,4,4′-tetrachlorobiphenyl, PCB77) and ER agonist (nonylphenol, NP) resulted in the potentiation and inhibition (depending on dose ratio, sequential order of exposure and seasonal changes) of NP-induced responses by PCB77 (Arukwe et al., 2001). Based on this study, we suggested that AhR agonist mediated anti-estrogenic activities that have been well documented in mammalian cell-based systems and relatively few studies in fish might be an exception rather than the rule for the mode of action for these chemicals. In the aquatic systems, industrial chemicals, pharmaceuticals and personal care products, pesticides and surfactants are common and ubiquitous contaminants. Therefore, chemical interactions may have profound consequences since organisms, including fish, are exposed to complex mixtures of environmental pollutants (Brian et al., 2005; Mumtaz et al., 2002). These complex interactions have only recently become the focus of systematic investigations both in laboratory and elsewhere using genomic approaches (Arukwe et al., 2001; Brian et al., 2005; Mortensen et al., 2006; Mumtaz et al., 2002). The relative importance of the influence of contaminants on biological systems is not well understood or quantified mechanistically in complex chemical mixtures. Therefore, the present study was designed with the primary objective of investigating the AhR–ER interactions using a potent and dioxin-like AhR agonist (PCB126) and a xenoestrogen (NP). To examine the involvement of ERs on these interactions, we deployed an absolute ER antagonist (ICI182,780). It should be noted that our primary study designed was to test the hypothesis that the dioxin-like PCB126 will produce concentration-dependent anti-NP responses that will parallel AhR activation and subsequent induction P450 responses in primary salmon hepatocyte culture.

Materials and methods

Chemicals and reagents. 4-Nonylphenol (NP; 85% of *p*-isomers) was purchased from Fluka Chemika-Biochemika (Buchs, Switzerland), 3,3′,4,4′,5-Pentachlorobiphenyl (PCB126) from Cambridge Isotope Laboratories (Andover, MA, USA) and ICI182,780 (ICI) from Tocris BioScience (Bristol, England). Dulbecco minimum essential medium (DMEM) with non-essential amino acid and without phenol red, fetal bovine serum (FBS), L-glutamine and TA cloning kit were purchased from Gibco-Invitrogen Life Technologies (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), 100× penicillin–streptomycin–neomycin solution, collagenase, BSA, *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), ethyleneglycol-bis-(β-aminoethylether) *N*,*N*′tetraacetic acid, (EGTA), 0.4% trypan blue and rabbit-anti-hERα antiserum were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany). EZNA total RNA kit for ribonucleic acid (RNA) purification was from Omega Bio-Tek (Doraville, GA, USA). IScript cDNA synthesis kit and iTAQ™SYBR® green supermix with ROX were purchased from Bio-Rad Laboratories (Hercules, CA, USA).

Collagenase perfusion, isolation and culture of hepatocytes. Juvenile Atlantic salmon (*Salmo salar*; body weight of 300–350 g) were supplied by Marine Harvest AS, Norway. In experiment 1 (performed in November 2005) seawater adapted salmon were used and in experiment 2 (performed in February 2007) freshwater adapted salmon were used. The fish were kept at the animal holding facilities at the Biology Department, NTNU. Prior to liver perfusion all glassware and instruments were autoclaved and solutions were filtration sterilized by using 0.22 μm Millipore filter (Millipore AS, Oslo, Norway). Hepatocytes were isolated by a two-step perfusion technique with modifications as previously described (Mortensen and Arukwe, 2007a). The cell suspension was filtered through a 150 μM nylon monofilament and centrifuged at 50×*g* for 5 min. Cells were washed three times with serum-free medium and finally resuspended in complete medium. Following collagenase perfusion and isolation of hepatocytes, viability of cells was determined by the trypan blue exclusion method. A cell viability value of >90% was a criterion for further use of the cells. Cells were plated on a 35 mm TPP Tissue Culture Plates (Techno Plastic Products AG, Switzerland) at the recommended density for monolayer cells of 5×10^6 cells in 3 ml DMEM medium (without phenol red) containing 2.5% (v/v) FBS, 0.3 g/l glutamine, and 1% (v/v) penicillin–streptomycin–neomycin solution. The cells were cultured at 10 °C in a sterile incubator without additional O_2/CO_2 for 48 h prior to chemical exposure.

Exposure of hepatocytes. Two separate exposure experiments were performed. In experiment 1, we evaluated the interaction between the ER and AhR using strong agonists for both receptors. In experiment 2, we wanted to determine whether PCB126 activation of ER and its signaling were dependent on direct activation of ER isoforms. The time–response pattern of PCB126 effects in cells exposed to PCB126 singly or in combination was evaluated with the absolute ER antagonist ICI. Both NP and ICI concentrations were chosen based on previous experiments conducted in our laboratory showing that these concentrations are optimal *in vitro* concentrations for investigating ER–AhR interactions in salmonids (Mortensen and Arukwe, 2007a,b). In the experiment 1, hepatocytes were exposed in triplicate for each exposure group for 48 h to 0.1% DMSO (control), 5 and 10 μM NP or 1, 10 and 50 pM PCB126 singly and also in combination. The exposure time of 48 h was chosen based on previous experiments showing that a 48 h time interval gives a stable culture condition and optimal response time. In the experiment 2, hepatocytes were exposed in triplicate for each exposure group for 12, 24 and 48 h to 0.1% DMSO (control), 1, 10 and

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Table 1
Primer pair sequences, accession numbers, amplicon size and annealing temperature conditions for genes of interest used for real-time PCR

Target gene	Primer sequence[a]		Amplicon size (nucleotides)	Annealing temperature (°C)	GenBank accession number
	Forward	Reverse			
ERα	TCCAGGAGCTGTCTCTCCAT	GATCTCAGCCATACCCTCCA	173	55	DQ009007
ERβ	GAGCATCCAAGGTCACAATG	CACTTTGTCATGCCCACTTC	126	59	AY508959
Vtg	AAGCCACCTCCAATGTCATC	GGGAGTCTGTCCCAAGACAA	391	57	DY802177
Zr-protein	TGACGAAGGTCCTCAGGG	AGGGTTTGGGGTTGTGGT	113	55	AF407574
Vigilin	GGGATACGCACAGACACCTT	CCCAGATTCCACAGACACCT	86	60	DY802195
AhR2α	AGGGGCGTCTGAAGTTCC	GTGAACAGGCCCAACCTG	82	60	AY219864
AhR2β	GACCCCCAGGACCAGAGT	GTTGTCCTGGATGACGGC	96	65	AY219865
AhR2δ	AGGGGCGTCTGAAGTTCC	GTGAACAGGCCCAACCTG	139	60	AF495590
AhR2γ	GACCCCCAGGACCAGAGT	GTTGTCCTGGATGACGGC	139	60	AY052499
AhRR	TTCCTCCAGGGACAGAAGAA	ATGGAGGGCAGCAGAAGAG	98	60	DQ372978
Arnt	AGAGCAATCCCAGGGTCC	TGGGAGGGTGATTGAGGA	107	60	DQ367887
CYP1A1	GAGTTTGGGCAGGTGGTG	TGGTGCGGTTTGGTAGGT	76	60	AF364076
20S Proteasome	TCTTTGACCAGGTTGCACAG	CATACAAAGCTGGTGGCTCA	134	60	DY802110

[a] Sequences are given in the 5′–3′ order.

50 pM PCB126 singly or in combination with 1 μM ICI182,780. In both experiments, media were replaced with fresh media containing the respective test chemical and concentrations every 24 h.

Quantitative (real-time) PCR. Cells for total RNA isolation were harvested in E.Z.N.A lysis buffer according to manufacturer's protocol (Omega Bio-Tek). The integrity of the RNA samples was verified by spectrophotometric analysis and formaldehyde agarose gel electrophoresis. Total cDNA for the quantitative real-time PCR (Q-PCR) reactions was generated from 1 μg total DNase-treated RNA from all samples using a combination of random hexamer and poly-T primers from iScript cDNA Synthesis Kit as described by the manufacturer (Bio-Rad). The expression of individual gene targets was analyzed using the Mx3000P REAL-TIME PCR SYSTEM (Stratagene, La Jolla, CA, USA). Every 25-μl DNA amplification reaction contained 12.5 μl of iTAQ™YBR® Green Supermix with ROX (Bio-Rad), 5 μl of diluted cDNA and 200 nM of each forward and reverse primers. The 3-step real-time PCR program included an enzyme activation step at 95 °C (5 min) and 40 cycles of 95 °C (30 s), 55–65 °C for 30 s, depending on the primers used (see Table 1), and 72 °C (30 s). Controls lacking cDNA template were included to determine the specificity of target cDNA amplification as described previously (Mortensen and Arukwe, 2007b). Briefly, cycle threshold (Ct) values obtained were converted into mRNA copy number using standard plots of Ct versus log copy number. The criterion for using the standard curve is based on equal amplification efficiency with unknown samples and this is usually checked prior to extrapolating unknown samples to the standard curve. The standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest. Data obtained from triplicate runs for target cDNA amplification were averaged and expressed as ng/μl of initial total RNA used for reverse transcriptase (cDNA) reaction. This absolute quantification method is a well-validated procedure in our laboratory as we do not use the so-called housekeeping genes because of their parallel modulation pattern with experimental samples both in our laboratory (Arukwe, 2006) and elsewhere (Steele et al., 2002).

Immunoblotting analysis of protein levels. Cells for immunochemical studies were washed in 1 ml of 0.1 M sodium phosphate buffer (containing 0.15 M KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT) and 10% glycerol at pH 7.4) then homogenized in 150 μl of the same buffer with 4–6 up and down strokes using a potter-elvehjem type teflon glass homogenizer. The homogenate was centrifuged for 30 s at 12,000×*g* at 4 °C, and the pellets were discarded. Total protein concentrations in samples were determined with the Bradford method using bovine serum albumin (BSA) as standard and the protein measurements were simplified using a Synergy HT microplate reader from Bio-Tek Instruments Inc. (Winnoski, Vermont, USA) for absorbance reading. Immunochemical analyses of ERα protein levels were performed using Western blotting. Proteins (10 μg) were separated by 7.5% separating sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The gel was then transferred to PVDF membranes and incubated with the primary polyclonal antibodies against human ERα amino acids 154–174 (IgG fraction of antiserum, Sigma-Aldrich) diluted 1:2000. The rabbit-anti-hERα antiserum was generated against amino acids that are included in the conserved DNA binding domain of human ER1 (Accn no. NM-000125) (Sabo-Attwood et al., 2004). After washing, membranes were incubated with peroxidase conjugated goat anti-rabbit antibodies (GAR-HRP; Bio-Rad) diluted 1:3000 using SuperSignal West Pico Chemiluminescent kit (Pierce Biotechnology, IL, USA) and visualized with Eastman KODAK Company's Molecular Imaging Systems (Rochester, NY, USA).

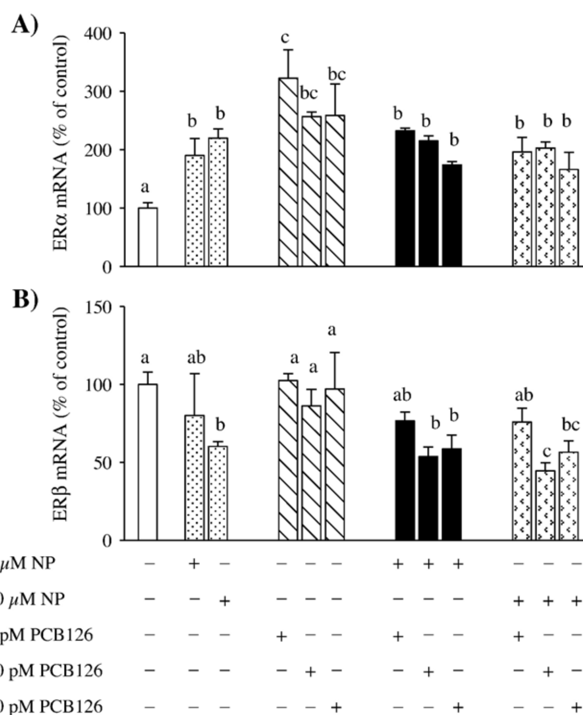

Fig. 1. Transcriptional changes of ERα (A) and ERβ (B) mRNA in salmon hepatocytes exposed to NP (5 and 10 μM) and PCB126 (1, 10 and 50 pM) singly and also in combination. Cells were harvested 48 h post-exposure and mRNA levels were quantified using real-time PCR with gene specific primer pairs. The data are given as percentage (%) of solvent control ± standard error of the mean (SEM: $n = 3$). Different letters denote exposure group means that are significantly different for the respective mRNA expression using ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

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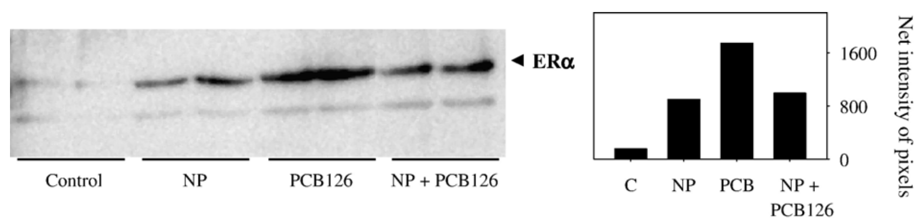

Fig. 2. Representative immunoblot analysis of ERα protein using anti-hERα antiserum in cells exposed for 48 h to carrier solvent control (DMSO), PCB126 (1 pM), NP (10 μM) and combined PCB126-NP at the respective concentrations. Ten micrograms of total cellular protein was loaded per well.

Statistical analysis. Standard errors were calculated using the S-plus statistic software 6.2 (Insightful Corp, USA). Statistical differences among treatment groups were tested using analysis of variance (ANOVA) and comparison of different exposure treated and control groups was performed using Tukey's multiple comparison test. The multiparametric ANOVA test was performed after testing for normality and variance homogeneity, using the Levene's test. For all the tests the level of significance was set at $p<0.05$, unless otherwise stated.

Results

Modulation of ERs and their signaling by PCB126

Analysis of ERα mRNA levels in cells exposed to NP at 5 and 10 μM showed a respective significant 2- and 2.4-fold increase in ERα mRNA levels compared to control (Fig. 1A). Interestingly, exposure of cells to PCB126 at 1, 10 and 50 pM produced significant increases of ERα mRNA levels showing a respective 3-, 2.7- and 2.7-fold increase, compared to the controls (Fig. 1A). A slight decrease of PCB126 induced ERα expression was observed when cells were exposed in combination with NP (Fig. 1A) and this decrease was apparently PCB126 concentration-dependent. For ERβ, the expression patterns were different from that of ERα showing that 5 μM NP did not alter ERβ mRNA levels and a significant 2-fold decrease was observed in cells exposed to 10 μM NP, compared to control (Fig. 1B). While exposure to PCB126 concentrations alone did not alter ERβ expression patterns compared to the control, combined exposure with NP reduced the ERβ transcription in all PCB126 exposure groups (Fig. 1B). A representative immunoblotted ERα protein levels showing the effect of cell exposure to NP (10 μM) and PCB126 (1 pM) singly or in combination is shown in Fig. 2. An ERα protein of approximately 68 kDa, representing Atlantic salmon ERα, was detectable in control and exposed samples. A semiquantitative evaluation of protein band intensity showed that cells exposed to 10 μM NP produced more ERα protein compared to control, less protein compared to 1 pM PCB126 alone or in combination with 10 μM NP (Fig. 2, right panel).

The expression of Vtg mRNA significantly increased in a concentration-dependent manner when salmon hepatocytes were exposed to 5 and 10 μM NP, showing a 14- and 100-fold induction, respectively (Fig. 3A). Treatment of cells with 1, 10 and 50 pM PCB126 produced Vtg mRNA induction above NP levels showing respective 130-, 530- and 160-fold increase (Fig. 3A). Generally, treatment of cells to combined NP and PCB126 concentrations did not alter the effects of PCB126 on Vtg expression, except for 10 pM PCB126 exposure group in combination with 10 μM NP, where a significant decrease of Vtg mRNA was observed (Fig. 3A). *Zr*-protein transcript expression increased in response to NP treatment alone (significantly at 5 μM NP; Fig. 3B). Exposure of hepatocytes to 1, 10 and 50 pM PCB126 alone produced a respective significant 3.3-, 1.5- and 2-fold increase in *Zr*-protein mRNA levels (Fig. 3B) and the presence of 5 μM NP

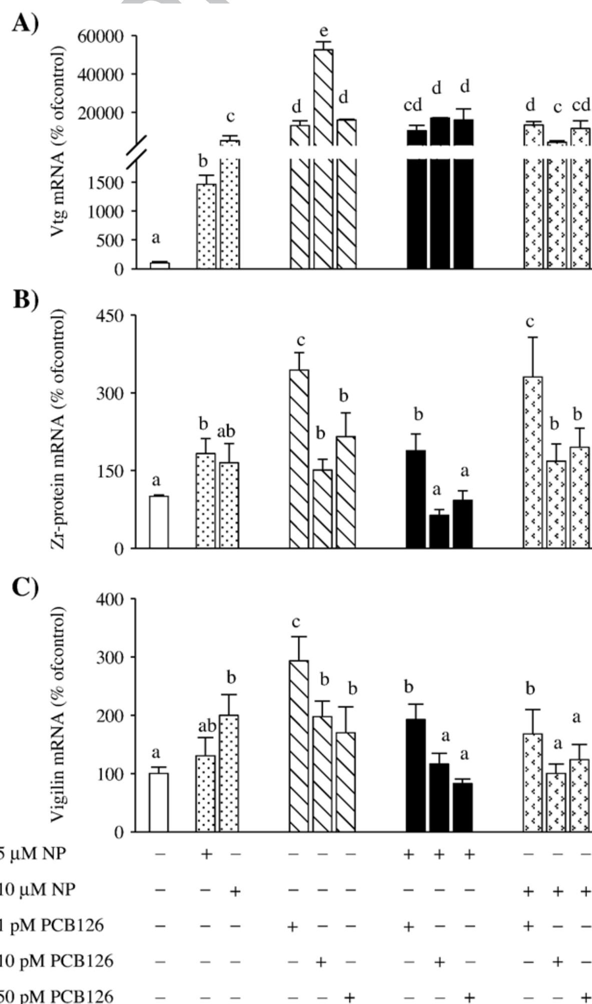

Fig. 3. Transcriptional changes of vitellogenin (Vtg: A), *Zr*-protein (B) and vigilin (C) mRNA in salmon hepatocytes exposed to NP (5 and 10 μM) and PCB126 (1, 10 and 50 pM) singly and also in combination. Cells were harvested 48 h post-exposure and mRNA levels were quantified using real-time PCR with gene specific primer pairs. The data are given as percentage (%) of solvent control ± standard error of the mean (SEM: $n=3$). Different letters denote exposure group means that are significantly different for the respective mRNA expression using ANOVA followed by Tukey's multiple comparison test ($p<0.05$).

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decreased the effects below PCB126 levels. In contrast, combined PCB126 concentrations and 10 μM NP did not produce any *Zr*-protein mRNA transcriptional changes, compared with PCB126 exposure alone (Fig. 3B). The vigilin mRNA expression showed an expression pattern that is apparently similar to ERα and Vtg mRNA expression (Fig. 3C). Specifically, analysis of vigilin mRNA levels in cells exposed to 10 μM NP showed a significant 2-fold increase compared to control (Fig. 3C). Exposure to PCB126 (1, 10 and 50 pM) produced significant increases of vigilin expression showing a respective 3-, 2- and 1.7-fold increase, compared to the control (Fig. 3C). Within the PCB126 concentration exposure groups, a concentration-dependent decrease of vigilin mRNA expression was observed. When PCB126 and NP concentrations were given in combination, a PCB126 concentration-dependent decrease of vigilin mRNA expression was also observed (Fig. 3C).

Effect of ICI on PCB126-mediated estrogenicity

To determine the contribution of ERα on PCB126 mediated estrogenicity, we exposed hepatocytes for 12, 24 and 48 h with PCB126 (1, 10 and 50 pM) singly or in combination with ICI182,780. It should be noted that fish used in the second experiment were freshwater adapted and from a different season, compared to the first experiment. The ERα mRNA expression patterns showed that PCB126 produced a concentration-specific increase of ERα mRNA expression at 12 h exposure, and co-treatment with ICI resulted in a slight (but not significant) decrease of PCB126 induced ERα mRNA levels, except at 50 pM (Fig. 4A). After 24 h of PCB126 treatment, ERα mRNA levels were not significantly altered compared to control, and co-treatment with ICI actually increased ERα expression at 1 pM (Fig. 4A). A significant decrease of ERα mRNA was observed in cells treated with PCB126 concentration for 48 h and combined exposure with ICI brought mRNA expression back to control levels (Fig. 4A). For Vtg mRNA expression, a significant 4.5-fold increase was observed only in cells treated with 10 pM PCB126 for 12 h and combined exposure with ICI reduced Vtg mRNA expression back to control levels (Fig. 4B). A similar effect was observed at 24 h of exposure showing a 7-fold induction of Vtg mRNA expression after exposure to 10 pM PCB126 that was restored to control levels by ICI (Fig. 4B). Exposure to 1 and 50 pM PCB126 for 12 and 24 h produced minor increases of Vtg mRNA expression, compared to control and these increases were reduced in the presence of ICI (Fig. 4B). At 48 h, exposure of cells to PCB126 concentrations alone did not significantly alter Vtg mRNA expression, but combined exposure with ICI reduced Vtg levels (Fig. 4B). Analysis of cellular ERα protein levels showed that cells collected after 12 h

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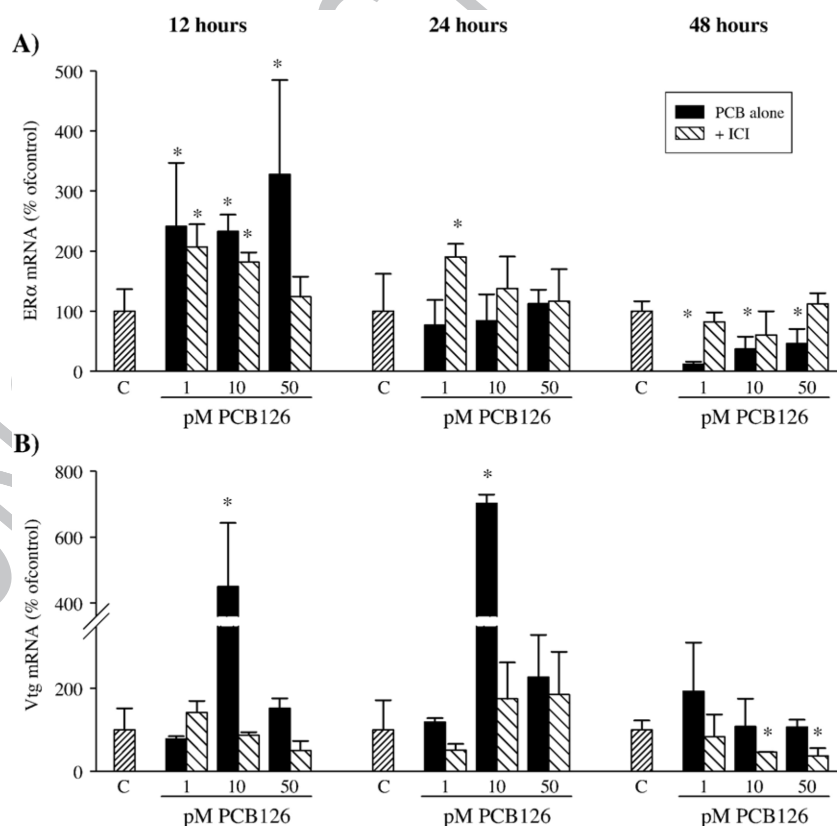

Fig. 4. Changes in ERα (A) and vitellogenin (Vtg: B) mRNA in salmon hepatocytes exposed to PCB126 (1, 10 and 50 pM) singly or in combination with ICI182,780 (ICI: 1μM) at 12, 24 and 48 h after exposure. Cells were harvested 48 h post-exposure and mRNA levels were quantified using real-time PCR with gene specific primer pairs. The data are given as percentage (%) of solvent control ± standard error of the mean (SEM: $n=3$). Asterisks denote exposure group means that are significantly different compared with control using ANOVA followed by Tukey's multiple comparison test ($p<0.05$).

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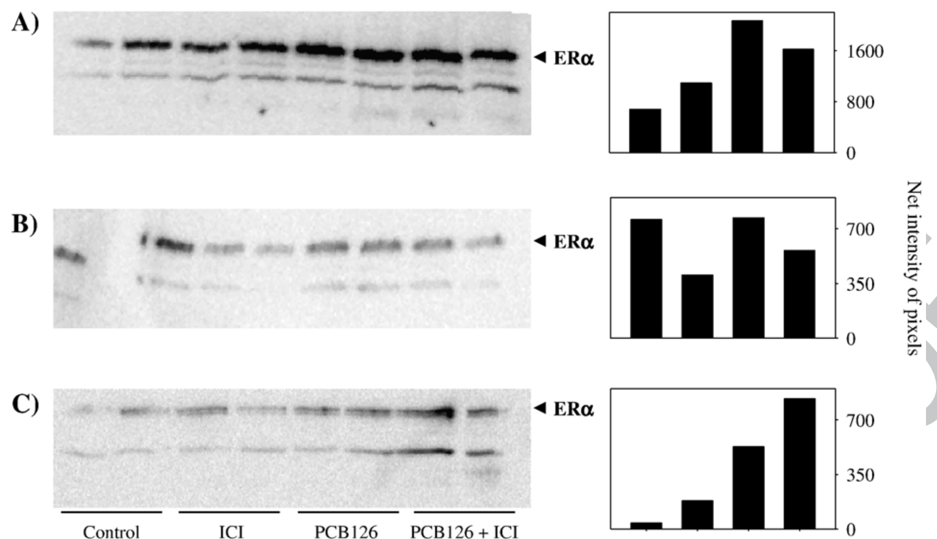

Fig. 5. Representative immunoblot analysis of ERα protein using anti-hERα antiserum in cells exposed for 12 h (A), 24 h (B) and 48 h (C) to carrier solvent control (DMSO), ICI (1 μM), PCB126 (10 pM) and combined PCB126-ICI at the respective concentrations. Ten micrograms of total cellular protein was loaded per well.

exposure either to PCB126 alone or in the presence of ICI (Fig. 5A) showed the highest cellular ERα protein levels, compared to 24 and 48 h (Fig. 5B and C, respectively). Within the different exposure time intervals, cellular ERα protein levels increased in response to treatment with 1 pM PCB126, while the presence of ICI reduced PCB126 mediated increase of cellular ERα protein levels (Fig. 5A, B and C, respectively). Right panels of Fig. 5A, B and C showed a semiquantitative analysis of relative ERα protein intensity.

Modulation of the AhR pathways by NP and PCB126

Exposure of salmon hepatocytes to PCB126 and NP produced AhR isotype-specific mRNA expression patterns. AhR2α

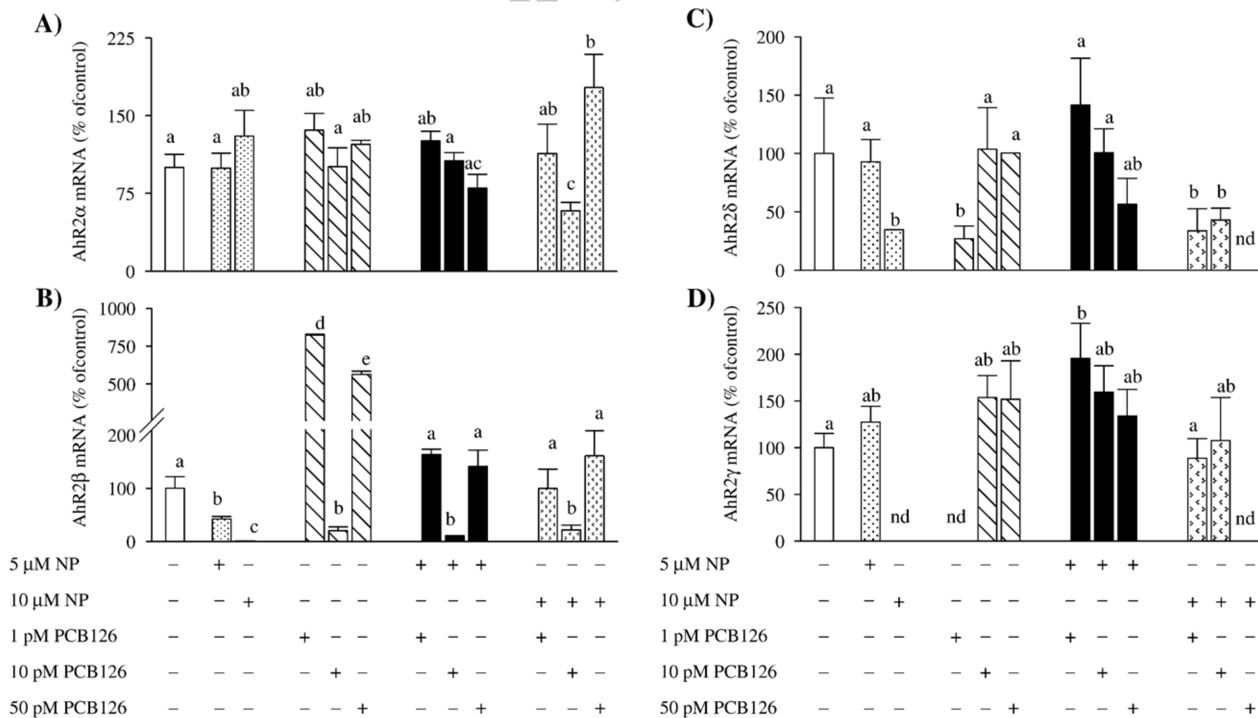

Fig. 6. Expression of AhRα (A), AhR2β (B), AhR2δ (C) and AhR2γ (D) mRNA in salmon hepatocytes exposed to NP (5 and 10 μM) and PCB126 (1, 10 and 50 pM) singly or in combination. Cells were harvested 48 h post-exposure and mRNA levels were quantified using real-time PCR with gene specific primer pairs. The data are given as percentage (%) of solvent control ± standard error of the mean (SEM: $n=3$). Different letters denote exposure group means that are significantly different for the respective mRNA expression using ANOVA followed by Tukey's multiple comparison test ($p<0.05$). "nd" indicates no detected mRNA transcripts.

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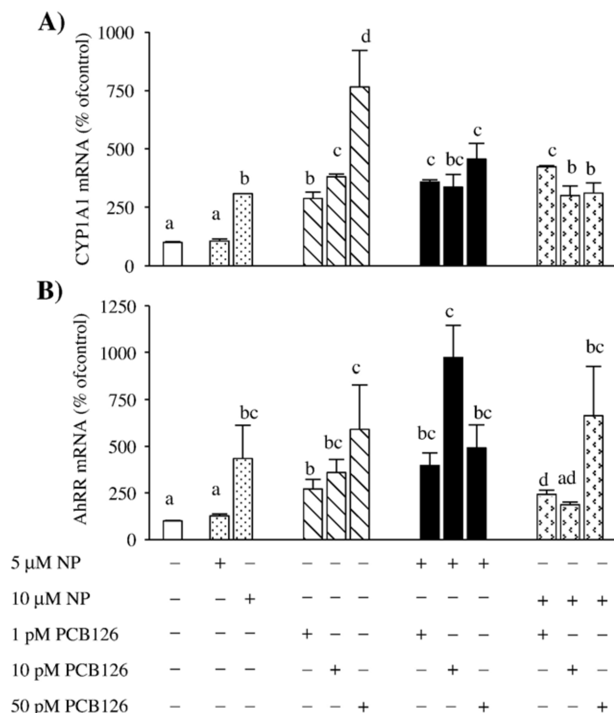

Fig. 7. Transcriptional changes of CYP1A1 (A) and AhRR (B) in salmon hepatocytes exposed to NP (5 and 10 µM) and PCB126 (1, 10 and 50 pM) singly or in combination. Cells were harvested 48 h post-exposure and mRNA levels were quantified using real-time PCR with gene specific primer pairs. The data are given as percentage (%) of solvent control ± standard error of the mean (SEM: $n=3$). Different letters denote exposure group means that are significantly different for the respective mRNA expression using ANOVA followed by Tukey's multiple comparison test ($p<0.05$).

mRNA expression analysis shows that neither NP nor PCB126 significantly altered AhR2α mRNA expression in cells when administered alone, compared to control (Fig. 6A). Combined exposure of 5 µM NP with all PCB126 concentrations produced apparent decreases of AhR2α expression levels with increasing PCB126 concentration (Fig. 6A). While a significant decrease of AhR2α mRNA was observed in cells exposed to 10 µM NP in combination with 10 pM PCB126, increased mRNA level was 2observed when the same NP concentration was given in combination with 50 pM PCB126 (Fig. 6A). On the contrary, AhR2β mRNA showed a different pattern compared to AhR2α after exposure to NP and PCB126 either singly or in combination (Fig. 6B). The transcript pattern of AhR2β mRNA showed a 2-fold reduction and a complete inhibition after treatment with 5 and 10 µM NP, respectively (Fig. 6B). When PCB126 was administered alone, an 8- and 6.5-fold increase of AhR2β mRNA was observed for 1 and 50 pM, respectively (Fig. 6B). On the other hand, cells treated with 10 pM PCB126 alone or in combination with NP showed a significantly repressed AhR2β expression, reaching an almost total inhibition. A decrease of PCB126-induced AhR2β mRNA expression was observed in cells co-treated with 1 and 50 pM PCB126 with NP concentrations, compared with the respective PCB126 concentrations alone (Fig. 6B). Apparently, more similar expression patterns were observed for the phylogenetically related AhR2δ and AhR2γ (Fig. 6C and D, respectively). While exposure to 5 µM NP singly or in combination with PCB126 did not significantly alter AhR2δ mRNA expression, a 2-fold decrease was observed after exposure to 10 µM NP alone (Fig. 6C). Decreased AhR2δ levels were observed after treatment of cells with 1 pM PCB126, but 10 and 50 pM PCB126 produced indifferent effect, compared with control (Fig. 6C). Combined exposure of cells with PCB126 and 10 µM µM NP resulted in decreased AhR2δ expression (total inhibition at 10 µM NP in combination with 50 pM PCB126: Fig 6C). A total inhibition of AhR2γ mRNA expression was observed when cells were exposed to either 10 µM NP or 1 pM PCB126 alone (Fig. 6D). Exposure to 10 and 50 pM PCB126 produced non-significant slight increase of AhR2γ mRNA expression (Fig. 6D). Increased AhR2γ levels, compared to control, were detected after combined 1 pM PCB126 and 5 µM NP treatment (Fig. 6D). In hepatocytes treated with 10 µM NP alone, or in combination to 50 pM PCB126, a total inhibition of AhR2γ transcript was observed (Fig. 6D).

Exposure of hepatocytes to 10 µM NP and PCB126 concentrations alone produced significant concentration-dependent increase of CYP1A1 mRNA showing respective 3-, 3-, 4- and 8-fold induction levels for PCB126 concentrations (Fig. 7A). When NP concentrations and 50 pM PCB126 concentration were given in combination, NP significantly decreased PCB126-induced CYP1A1 mRNA expression, compared 50 pM PCB126 alone (Fig. 7A). An increase in AhRR mRNA was observed in

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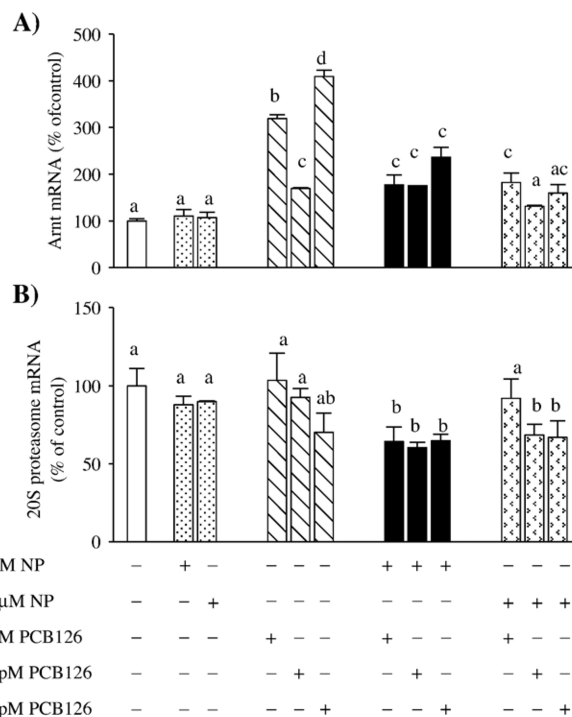

Fig. 8. Expression of Arnt (A) and 20S proteasome subunit (B) mRNA in salmon hepatocytes exposed to NP (5 and 10 µM µM) and PCB126 (1, 10 and 50 pM) singly or in combination. Cells were harvested 48 h post-exposure and mRNA levels were quantified using real-time PCR with gene specific primer pairs. The data are given as percentage (%) of solvent control ± standard error of the mean (SEM: $n=3$). Different letters denote exposure group means that are significantly different for the respective mRNA expression using ANOVA followed by Tukey's multiple comparison test ($p<0.05$).

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cells treated with 10 μM NP or PCB126 concentrations alone, showing a respective 4-, 3-, 3.5- and 6-fold increase (Fig. 7A). Exposure of cells to 5 μM NP in combination with 10 pM PCB126 produced a significant increase of AhRR mRNA levels, compared to 10 pM PCB126 alone (Fig. 7B). On the contrary, exposure of cells to 10 μM NP in combination with 1 and 10 pM PCB126 produced a significant decrease of PCB126 induced AhRR mRNA expression and combined exposure with 50 pM PCB126 did not produce significant effect (Fig. 7B).

For Arnt, the expression pattern increased in a concentration-specific manner (showing significant increase in all concentrations when compared with control) in cells exposed to PCB126 alone (Fig. 8A). Specifically, cells exposed 10 pM PCB126 produced a decrease of Arnt mRNA levels compared to 1 and 50 pM (Fig. 8A). When cells were exposed to PCB126 at 1 and 50 pM in combination with NP concentrations, apparent decrease of Arnt mRNA expression was observed, compared with the PCB126 exposure concentration alone (Fig. 8A). Exposure of cells to NP concentrations alone did not alter Arnt mRNA levels. Analysis of a 20S proteasome mRNA levels showed that exposure of hepatocytes to NP alone did not produce significant alteration of the expression levels (Fig. 8B). When cells were exposed to PCB126, an apparent concentration-dependent decrease in the 20S proteasomal mRNA expression was observed. Decreased levels were also observed in cells exposed to a combined NP and PCB126 concentration, compared to control (Fig. 8B).

Discussion

In teleost, exposure to AhR agonists has been associated with reduced Vtg synthesis or impaired gonadal development in both *in vivo* and *in vitro* studies (Arukwe et al., 2000; Navas et al., 2004; Ramamoorthy et al., 1999). Cross-talk between the AhR and ER that inhibited breast cancer cells, rodent uterus and mammary tumors has also been demonstrated in mammals (Safe et al., 1991; Safe and Wormke, 2003). Previously, we showed that PCB77 increased and decreased NP-induced responses in fish *in vivo* system and these effects were dependent on PCB77 and NP dose ratios and sequential order of exposure and interestingly influenced by seasonal changes (Arukwe et al., 2001). Recently, several studies have shown that AhR agonists directly activate ERα and induce estrogenic responses in mammalian *in vitro* systems (Abdelrahim et al., 2006; Liu et al., 2006; Ohtake et al., 2003; Pearce et al., 2004). Furthermore, we showed in a very recent study that PCB77 at 1 μM concentration alone produced a time-dependent increase of ERα, above control and statistically equal to NP levels, and in combination with NP produced elevated ERα, above NP and control levels (Mortensen and Arukwe, 2007a). However, PCB77 produced an increase of ERβ mRNA that was concentration specific (Mortensen and Arukwe, 2007a), suggesting that AhR agonists, such as PCB77, may induce transcription of both ER subtypes with or without a concomitant or partial activation of estrogenic response. In another separate study, we showed that the partial inhibition of AhR with α-naphthoflavone (ANF; 0.1 μM) reversed the effect of PCB77 on ERβ, Vtg and *Zr*-protein (but not ERα and vigilin) transcription, suggesting that AhRs have a direct role on PCB77 mediated decrease of estrogenic responses; and the inhibition of ER with tamoxifen (Tam; partial ER antagonist) and ICI reversed the transcription of AhR-mediated responses, except for the AhR repressor (AhRR) (Mortensen and Arukwe, 2007b). Taken together, these findings demonstrate a complex mode of ER–AhR interaction that is dependent on time and the individual chemical (NP and PCB77) concentrations. The present study was designed to investigate molecular AhR–ER interactions using the dioxin-like PCB126 in salmon *in vitro* system. We show that exposure of salmon primary hepatocytes to NP and PCB126 singly or in combination produced increased levels of genes controlled by the ER as well as cellular ERα protein levels. Specifically, PCB126 produced increased transcription levels of ERα, Vtg and *Zr*-protein expression, and as expected, together with increased levels of the AhR2β, CYP1A1, AhRR and Arnt mRNA expressions. Thus, the PCB126 mediated activation of ERα and its downstream regulated responses coupled with the negative effect of ICI treatment suggest that this dioxin-like AhR agonist may be hijacking the ER in the absence of ER agonists, with subsequent induction of downstream responses.

Activation of ER signaling by PCB126

PCB126 is one of the most potent dioxin-like AhR agonists (Smeets et al., 1999) and the anti-estrogenic actions of AhR agonists have been described in numerous *in vivo* and *in vitro* studies using mammalian (Harris et al., 1990; Safe et al., 1991; Safe and Krishnan, 1995) and teleost systems (Mortensen and Arukwe, 2007b; Mortensen et al., 2006; Smeets et al., 1999; Vaccaro et al., 2005). Generally, the molecular mechanisms behind estrogen-related effects of typical AhR agonists appear to involve ER–AhR cross-talk. However, there is no universal mechanism of ER–AhR interaction that is generally accepted as the mode of action for these chemicals. The fact that some AhR agonists induce endometriosis and estrogen-dependent tumors indicates that they may possess estrogenic activities (Ohtake et al., 2003). In the present study, we show that PCB126 activated mRNA transcription for ERα and the ERα controlled genes (Vtg, *Zr*-protein and vigilin) and proteins (ERα) in the absence of ER agonist. These findings are in accordance with past and very recent data from our laboratory demonstrating the positive effect of AhR agonists on estrogenic responses in both *in vivo* and *in vitro* (Arukwe et al., 2001; Mortensen and Arukwe, 2007a). In addition, the present findings showing PCB126 mediated activation ERα responses are also in accordance with recent reports demonstrating similar effects in mammalian *in vitro* systems (Abdelrahim et al., 2006; Liu et al. 2006; Pearce et al., 2004). The ER can initiate cellular responses through ligand-dependent, ligand-independent, DNA binding-independent and cell surface (non-genomic) signaling mechanisms (Boverhof et al., 2006). Recently, it was shown that PCB126 does not induce ERE controlled mRNA transcription by direct binding to ERα (Matthews et al., 2007). AhR-mediated estrogenicity that involves direct binding of activated AhR–Arnt complex to the ER followed by activation of the ERE was

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recently demonstrated by Ohtake and co-workers (2003), showing that the AhR ligand 3-methylchloranthrene (3MC) activated transcription of ER signaling through ERE in a luciferase reporter plasmid assay of MCF-7 cells. However, the presence of 17β-estradiol (E2) was shown to reduce 3MC activation of ER responsive genes. Elsewhere, it was shown that TCDD increased, independent of estrogen, the DNA-binding activity of the ER in rats (Chaffin et al., 1996), and treatment of MCF-7 cells with TCDD produced a cell cycle transition from G0/G1 to S-phase and other estrogen-like mitogenic responses (Abdelrahim et al., 2003). In a recent study, it was shown that the AhR–Arnt complex is involved in AhR agonists mediated estrogenicity since ANF abolished TCDD induced growth hormone and prolactin mRNA expression in Rainbow trout pituitary gland culture (Elango et al., 2006). Compared with the study of Ohtake and co-workers (2003), our study showed that combined NP and PCB126 exposure slightly reduced PCB126-induced estrogenicity.

In order to investigate the involvement of ERα in PCB126-induced estrogenicity, hepatocytes were exposed to PCB126 singly or in combination with ICI, showing that ERα and Vtg mRNA and protein levels (ERα) were elevated after exposure to PCB126. These responses were decreased in the presence of ICI at 12 and 24 h, indicating the involvement of ERα. The decision to co-administer ICI with PCB126 was made based on previous studies in our laboratory (Mortensen and Arukwe, 2007b). Given that ICI treatment alone did not inhibit ERα after 48 h, compared to control, it is therefore less likely that pretreatment of cells with ICI may possibly have increased the effect and specificity of the ER antagonist or change the total outcome of our findings. However, the Vtg and ERα mRNA responses were decreased in the presence of ICI at 12 and 24 h, indicating the involvement of ERα. It should be noted that the rabbit-anti-hERα antiserum used in the present study was generated against amino acids 154–171 that correspond to the DNA-binding C-domain of human ER1 (Accn no. NM-000125) (Sabo-Attwood et al., 2004). The use of hERα in detecting salmon ERα by immunoblotting was evaluated by aligning human and salmon ERα amino acid sequences. There is a high degree of conservation between the amino acid sequences of Atlantic salmon and human in the DB domain. Given the high degree of conservation in that domain, antisera generated for hERα are suitable for detecting ERα from several vertebrate species, including Atlantic salmon, Rainbow trout, zebrafish (*D. rerio*), African clawed frog (*X. laevis*) and Chicken (*Gallus gallus*). Furthermore, the hERα antibody detected a consistent 2-band protein pattern (see Figs. 2 and 5), indicating the immunoreactivity of both salmon ERα and ERβ due to their amino acid sequence similarities shown in our alignment evaluation. However, the findings in experiments 1 (48 h) and 2 (48 h) showed discrepant data for the investigated variables. Experiment 1 was performed using saltwater adapted fish (in the autumn) under a 48 h fixed time interval that was chosen based on previous experiments that showed a stable culture condition and optimal response time in our laboratory (Mortensen and Arukwe, 2007a). The stronger stimulation of Vtg and ERα expression in experiment 1 probable reflects different composition of endogenous and exogenous substances and physiology of the experimental fish since experiment 2 utilized hepatocytes isolated from freshwater adapted salmon (in the winter).

The variation of timing and degree of ER stimulation observed in the experiments in the present study are in agreement with previous results showing that Atlantic salmon produced seasonal pattern of xenoestrogen response that is influenced by dose and sequential order of exposure to PCB77 and NP, singly or in combination (2001). In addition, the estrogenicity of AhR agonists is shown to be both cell and tissue specific in mammalian systems (Boverhof et al., 2006; Nesaretnam et al., 1996; Ohtake et al., 2003). Overall, the ICI decrease of PCB126-mediated ERα and Vtg responses suggests that these responses are ER dependent. There are conflicting reports on the direct activation of ER by AhR agonists. For example, while TCDD and PCB77 were shown to elicit estrogenic responses via direct ER binding (Nesaretnam et al., 1996), other studies showed that PCB77 did not produce estrogenic responses (Ramamoorthy et al., 1999), and TCDD does not bind the ER (Klinge et al., 1999). Therefore, the “ER-hijacking” mechanism involving the activation of unliganded ER by ligand-activated AhR or a coactivator relationship between these signaling pathways was proposed (Ohtake et al., 2003).

Despite the fact that the ER–AhR interactions have been extensively studied (Beischlag and Perdew, 2005; Brunnberg et al., 2003), there is still a reasonable level of discrepancy as to the ER coactivator or corepressor function on AhR–Arnt mediated induction of CYP1A1 (Beischlag and Perdew, 2005; Matthews et al., 2005). Contrary to the anti-estrogenicity of TCDD and related compounds, recent studies have demonstrated that TCDD induces the AhR to interact directly with ERα in the absence of estrogen or estrogen-like compounds (Beischlag and Perdew, 2005; Matthews et al., 2007, 2005). In the 1980s and 1990s, there were considerable reports with evidence that TCDD and related compounds have anti-estrogenic properties. This was first reported using inhibited development of spontaneous tumors in female Sprague–Dawley rats by Kociba et al. (1978). For different PCDDs, there was an excellent correlation between the binding affinity of these congeners to the AhR and their ability to down-regulate uterine and hepatic ERs (Astroff et al., 1990). In fish, there were reports showing similar responses using Vtg as a marker for estrogen response (Anderson et al., 1996b). The exact mechanism(s) of anti-estrogenicity is not known. However, it has generally been attributed to increased E2 metabolism by CYP isozymes (Spink et al., 1990). Although induced metabolism of E2 may explain some of the anti-estrogenic effects of TCDD and related compounds at relatively high concentrations or doses, there are several observations that do not support the hypothesis. These data were summarized by Safe et al. (1991) and include the following: (a) anti-estrogenic effects in rodents are not accompanied by decreases in circulating E2 levels; (b) down-regulation of nuclear ER levels occurs at concentrations (1 pM) that do not induce the induction of P450 activities; (c) down-regulation of nuclear ER levels occurs within 2 h after treatment and only minimum induction of CYP was observed within this short time frame; (d) 6-methyl-1,3,8-trichlorodibenzofuran (MCDF), which exhibits minimal CYP1A-

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inducing activity, also produced anti-estrogenic activity. Since TCDD and related compounds do not competitively bind to the steroid ER nor do steroid hormones bind to the AhR (Safe et al., 1991), a different scheme of approach as a working model for the possible mechanisms of TCDD- and related compound-induced anti-estrogenicity was proposed by Safe and co-workers (1991) — (a) the AhR complex itself may directly inhibit estrogen-induced genes, (b) TCDD or related compound may induce modulatory protein(s) which degrade the nuclear ER directly inhibits estrogen-induced gene transcription, (c) inhibits the action of estrogen-induced growth factors or (d) exhibits other anti-mitogenic activities. Therefore, when all the proposed mechanisms and working models are put into context present model of "ER-hijacking" by activated AhR–Arnt complex and the findings from present study, it is still possible that the suggested mechanisms of AhR–ER interactions are both biological and toxicological/pharmacological exception rather than rule of action (Arukwe et al., 2001) with strong implications for cancer therapy, reproductive physiology, molecular toxicology and pharmacological concepts.

Another interesting aspect of the present study is that the expression patterns of ER isotypes showed that ERα and ERβ respond differently to NP and PCB126 treatments. Since ERα is highly inducible by both estrogenic and xenoestrogenic compounds in fish hepatocytes, it is believed to play the dominant role in regulating vitellogenesis (Sabo-Attwood et al., 2004). In previous salmon *in vitro* studies (2007a), while ERα was induced, ERβ mRNA expression was not altered by exposure to NP indicating possible isotype-specific differences in the ER ligand binding domain. The ERs have two activation domains, namely a constitutive activation function-1 (AF-1) and a hormone-dependent (AF-2) domain. These two domains function in synergy but may also function independently in certain cell and promoter context (Nilsson et al., 2001). Despite that the ERβ mRNA expression pattern did not parallel that of ERα, Vtg, *Zr*-protein or vigilin, it is possible that the AhR–Arnt complex could "hijack" both ER isoforms, but ERβ-hijacking does not result in the activation of estrogenic responses (Mortensen and Arukwe, 2007a) due to low intrinsic transcriptional activity of hepatic ERβ, compared to ERα. However, there seem to be ER isoform preferences that favor the α-isoform in regulating vitellogenesis. For example, a human variant of ERα(−) Ishikawa endometrial cell line was unresponsive to E2, despite its expression of ERβ, reflecting the low transcriptional activity of ERβ, compared to ERα (Shipley and Waxman, 2006).

AhR–ER interaction

Our results provide novel aspects of ER–AhR interactions distinct from those proposed earlier in fish. The recruitment of ERα to AhR target genes in the absence on E2 has been described, and this is most likely related to the direct interactions between ER and activated AhR–Arnt (Beischlag and Perdew, 2005; Matthews et al., 2007, 2005). In the present study, exposure to NP alone did not alter AhR2α, Arnt and 20S proteasome subunit mRNA levels but reduced AhR2δ and totally inhibited AhR2γ (at 10 μM) and reduced AhR2β (at 5 and 10 μM) expression. Particularly, we observed a concentration-specific increased expression of Arnt mRNA after PCB126 treatment and the presence of NP decreased this effect. The overall function of Arnt is not fully understood in teleost, while in mammalian cells, this protein appears to be constitutively active (Gu et al., 2000). The AhR and Arnt belong to the bHLH class of transcription factors that usually associate with each other to form heterodimers (Gu et al., 2000). However, on the basis of sequence homology with an ER transcription factors p160, it was shown that Arnt also functions as a co-activator of ER, indicating multiple roles of Arnt in AhR–ER interactions (Brunnberg et al., 2003). The AhR genes are more diverse in non-mammalian vertebrates compared to mammalian species as was revealed through comparative genomic analyses (Hahn et al., 2006). In several fish species, both AhR1 and AhR2 genes have been characterized. For example, AhR2α, AhR2β, AhR2γ and AhR2δ are the four distinct AhR2 genes in salmonid species (salmon and rainbow trout). In addition, salmon genome contains two genes of the AhR1 variant, which are presumably non-functional (Hansson et al., 2003, 2004). It has been suggested that rainbow trout AhR2α and AhR2β differ in their promoter preference and may regulate distinct sets of genes (Abnet et al., 1999). In the present study, exposure to PCB126 apparently produced similar expression patterns for the phylogenetically related AhR2γ and AhR2δ isotypes, in contrast to the different expression patterns observed for AhR2α and AhR2β. Specifically, AhR2α mRNA expression did not show PCB126 related expression, AhR2β showed a pattern that was concentration-specific (increasing above control at 1 and 50 pM PCB126, and decreasing below control at 10 pM PCB126). In contrast, while 1 pM PCB126 decreased both AhR2δ and AhR2γ (total inhibition for AhR2γ), 10 and 50 pM PCB126 had indifferent effect on these AhR variants. Interestingly, while AhR2β mRNA expression maintained the effect from PCB126 exposure alone, in the presence of NP; the AhR2α, AhR2δ and AhR2γ showed a reducing trend in the presence of NP. Thus, a hypothetical evaluation of the salmon AhR genes suggests that different isoforms may have different ligand-dependent and -independent functions in responses to environmental stresses/stressors and during development. Fish are known to contain more copies of genes than mammals and a ratio of 2:1 has been proposed (Jaillon et al., 2004). Atlantic salmon is a tetraploid organism, and evolution has provided this species with duplications of several genes that has single copy in other species (Allendorf and Thorgaard, 1984). Therefore, the tetraploidity of salmon demands careful evaluation of gene expression studies since there is an increased number of pseudogenes (both functional and non-functional) in this organism (Allendorf and Thorgaard, 1984). Indeed, the multiplicity of salmon genome may have direct or indirect influence on biological responses and whether this is true for the observed responses is subject for future toxicological studies.

The AhR expression patterns in different tissues of teleost indicate distinct roles for each AhR subtype (Yamauchi et al., 2005). The concentration-specific up- and down-regulation of AhR2 mRNA levels observed in the present study is suggested to reflect a possible different mechanism of receptor regulation that is influenced by chemical concentration. In rodent liver exposed to TCDD, a rapid down-regulation of AhR mRNA was

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observed and thereafter followed by a gradual up-regulation that paralleled AhR protein. This effect was suggested to be due to proteasome mediated degradation through the ubiquitin–proteasomal pathway (Pollenz and Buggy, 2006). Degradation of AhR by 26S proteasome was shown be AhR ligand dependent and this was stronger for compounds with higher affinity to the AhR (Pollenz and Buggy, 2006). In this study we quantified the 20S proteasome subunit mRNA showing that PCB126 exposure alone did not alter its mRNA expression. However, in cells treated with combined PCB126 and NP concentrations, slight decreases were observed. It is known that receptor functions are cross-regulation through modulation of proteolysis that occurs among nuclear receptors (Wormke et al., 2003). But, PCB126-induced proteosomal ERα protein degradation was not observed in the present study. Despite this observation, the involvement of proteasome degradation of ERα cannot be ruled out since the magnitude and time course of proteasome mediated degradation have been shown to vary between cell types (Pollenz and Buggy, 2006).

Conclusions

Data from the primary study objective to investigate the ER–AhR cross-talk in salmon primary hepatocytes cultures changed the focus of our study to estrogenic effects of dioxin-like PCB congener. We found that exposure of cells to the AhR agonist PCB126 stimulated ER signaling shown by increased Vtg, *Zr*-protein and ERα mRNA and ERα protein levels. The decreased levels of ERα and Vtg expression in cells treated with PCB126 in the presence of ICI indicate an effect resembling the recently proposed "ER-hijacking" in mammalian systems not previously reported in fish species or lower vertebrate. The differences obtained at the similar time interval with fish from different seasons demonstrate the complexity of AhR–ER interaction and emphasized the significance of confounding factors. Since the total outcome of exposure is dependent on toxicological factors (e.g. relative concentration relationship between the ER and AhR agonists), endogenous factors (e.g. composition of cell metabolites and proteins) and physiological factors (e.g. tissue type, developmental stage and seasonal changes, namely autumn vs. winter), the complex nature of chemical interactions in biological systems is of added health consequences than previously anticipated.

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Doctoral theses in Biology
Norwegian University of Science and Technology
Department of Biology

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1974	Tor-Henning Iversen	Dr. philos Botany	The roles of statholiths, auxin transport, and auxin metabolism in root gravitropism
1978	Tore Slagsvold	Dr. philos. Zoology	Breeding events of birds in relation to spring temperature and environmental phenology.
1978	Egil Sakshaug	Dr.philos Botany	"The influence of environmental factors on the chemical composition of cultivated and natural populations of marine phytoplankton"
1980	Arnfinn Langeland	Dr. philos. Zoology	Interaction between fish and zooplankton populations and their effects on the material utilization in a freshwater lake.
1980	Helge Reinertsen	Dr. philos Botany	The effect of lake fertilization on the dynamics and stability of a limnetic ecosystem with special reference to the phytoplankton
1982	Gunn Mari Olsen	Dr. scient Botany	Gravitropism in roots of *Pisum sativum* and *Arabidopsis thaliana*
1982	Dag Dolmen	Dr. philos. Zoology	Life aspects of two sympartic species of newts (*Triturus, Amphibia*) in Norway, with special emphasis on their ecological niche segregation.
1984	Eivin Røskaft	Dr. philos. Zoology	Sociobiological studies of the rook *Corvus frugilegus*.
1984	Anne Margrethe Cameron	Dr. scient Botany	Effects of alcohol inhalation on levels of circulating testosterone, follicle stimulating hormone and luteinzing hormone in male mature rats
1984	Asbjørn Magne Nilsen	Dr. scient Botany	Alveolar macrophages from expectorates – Biological monitoring of workers exosed to occupational air pollution. An evaluation of the AM-test
1985	Jarle Mork	Dr. philos. Zoology	Biochemical genetic studies in fish.
1985	John Solem	Dr. philos. Zoology	Taxonomy, distribution and ecology of caddisflies (*Trichoptera*) in the Dovrefjell mountains.
1985	Randi E. Reinertsen	Dr. philos. Zoology	Energy strategies in the cold: Metabolic and thermoregulatory adaptations in small northern birds.
1986	Bernt-Erik Sæther	Dr. philos. Zoology	Ecological and evolutionary basis for variation in reproductive traits of some vertebrates: A comparative approach.
1986	Torleif Holthe	Dr. philos. Zoology	Evolution, systematics, nomenclature, and zoogeography in the polychaete orders *Oweniimorpha* and *Terebellomorpha*, with special reference to the Arctic and Scandinavian fauna.
1987	Helene Lampe	Dr. scient. Zoology	The function of bird song in mate attraction and territorial defence, and the importance of song repertoires.
1987	Olav Hogstad	Dr. philos. Zoology	Winter survival strategies of the Willow tit *Parus montanus*.
1987	Jarle Inge Holten	Dr. philos Botany	Autecological investigations along a coust-inland transect at Nord-Møre, Central Norway

1987	Rita Kumar	Dr. scient Botany	Somaclonal variation in plants regenerated from cell cultures of *Nicotiana sanderae* and *Chrysanthemum morifolium*
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1988	Hans Christian Pedersen	Dr. philos. Zoology	Reproductive behaviour in willow ptarmigan with special emphasis on territoriality and parental care.
1988	Tor G. Heggberget	Dr. philos. Zoology	Reproduction in Atlantic Salmon (*Salmo salar*): Aspects of spawning, incubation, early life history and population structure.
1988	Marianne V. Nielsen	Dr. scient. Zoology	The effects of selected environmental factors on carbon allocation/growth of larval and juvenile mussels (*Mytilus edulis*).
1988	Ole Kristian Berg	Dr. scient. Zoology	The formation of landlocked Atlantic salmon (*Salmo salar* L.).
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1991	Jan Henning L'Abêe Lund	Dr. philos. Zoology	Reproductive biology in freshwater fish, brown trout *Salmo trutta* and roach *Rutilus rutilus* in particular.
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1994	Kjell Inge Reitan	Dr. scient Botany	Nutritional effects of algae in first-feeding of marine fish larvae
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1999	Torbjørn Forseth	Dr. scient. Zoology	Bioenergetics in ecological and life history studies of fishes.
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1999	Hans Martin Hanslin	Dr. scient Botany	The impact of environmental conditions of density dependent performance in the boreal forest bryophytes *Dicranum majus*, *Hylocomium splendens*, *Plagiochila asplenigides*, *Ptilium crista-castrensis* and *Rhytidiadelphus lokeus*.
1999	Ingrid Bysveen Mjølnerød	Dr. scient. Zoology	Aspects of population genetics, behaviour and performance of wild and farmed Atlantic salmon (*Salmo salar*) revealed by molecular genetic techniques
1999	Else Berit Skagen	Dr. scient Botany	The early regeneration process in protoplasts from *Brassica napus* hypocotyls cultivated under various g-forces
1999	Stein-Are Sæther	Dr. philos. Zoology	Mate choice, competition for mates, and conflicts of interest in the Lekking Great Snipe
1999	Katrine Wangen Rustad	Dr. scient. Zoology	Modulation of glutamatergic neurotransmission related to cognitive dysfunctions and Alzheimer's disease
1999	Per Terje Smiseth	Dr. scient. Zoology	Social evolution in monogamous families: mate choice and conflicts over parental care in the Bluethroat (*Luscinia s. svecica*)
1999	Gunnbjørn Bremset	Dr. scient. Zoology	Young Atlantic salmon (*Salmo salar* L.) and Brown trout (*Salmo trutta* L.) inhabiting the deep pool habitat, with special reference to their habitat use, habitat preferences and competitive interactions
1999	Frode Ødegaard	Dr. scient. Zoology	Host spesificity as parameter in estimates of arhrophod species richness
1999	Sonja Andersen	Dr. scient Bothany	Expressional and functional analyses of human, secretory phospholipase A2
2000	Ingrid Salvesen, I	Dr. scient Botany	Microbial ecology in early stages of marine fish: Development and evaluation of methods for microbial management in intensive larviculture
2000	Ingar Jostein Øien	Dr. scient. Zoology	The Cuckoo (*Cuculus canorus*) and its host: adaptions and counteradaptions in a coevolutionary arms race
2000	Pavlos Makridis	Dr. scient Botany	Methods for the microbial econtrol of live food used for the rearing of marine fish larvae
2000	Sigbjørn Stokke	Dr. scient. Zoology	Sexual segregation in the African elephant (*Loxodonta africana*)
2000	Odd A. Gulseth	Dr. philos. Zoology	Seawater tolerance, migratory behaviour and growth of Charr, (*Salvelinus alpinus*), with emphasis on the high Arctic Dieset charr on Spitsbergen, Svalbard
2000	Pål A. Olsvik	Dr. scient. Zoology	Biochemical impacts of Cd, Cu and Zn on brown trout (*Salmo trutta*) in two mining-contaminated rivers in Central Norway
2000	Sigurd Einum	Dr. scient. Zoology	Maternal effects in fish: Implications for the evolution of breeding time and egg size

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2001	Olga Hilmo	Dr. scient Botany	Lichen response to environmental changes in the managed boreal forset systems
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2002	Mariann Sandsund	Dr. scient. Zoology	Exercise- and cold-induced asthma. Respiratory and thermoregulatory responses
2002	Dag-Inge Øien	Dr. scient Botany	Dynamics of plant communities and populations in boreal vegetation influenced by scything at Sølendet, Central Norway
2002	Frank Rosell	Dr. scient. Zoology	The function of scent marking in beaver (*Castor fiber*)
2002	Janne Østvang	Dr. scient Botany	The Role and Regulation of Phospholipase A_2 in Monocytes During Atherosclerosis Development
2002	Terje Thun	Dr.philos Biology	Dendrochronological constructions of Norwegian conifer chronologies providing dating of historical material
2002	Birgit Hafjeld Borgen	Dr. scient Biology	Functional analysis of plant idioblasts (Myrosin cells) and their role in defense, development and growth
2002	Bård Øyvind Solberg	Dr. scient Biology	Effects of climatic change on the growth of dominating tree species along major environmental gradients
2002	Per Winge	Dr. scient Biology	The evolution of small GTP binding proteins in cellular organisms. Studies of RAC GTPases in *Arabidopsis thaliana* and
2002	Henrik Jensen	Dr. scient Biology	Causes and consequenses of individual variation in fitness-related traits in house sparrows
2003	Jens Rohloff	Dr. philos Biology	Cultivation of herbs and medicinal plants in Norway – Essential oil production and quality control
2003	Åsa Maria O. Espmark Wibe	Dr. scient Biology	Behavioural effects of environmental pollution in threespine stickleback *Gasterosteus aculeatur* L.
2003	Dagmar Hagen	Dr. scient Biology	Assisted recovery of disturbed arctic and alpine vegetation – an integrated approach
2003	Bjørn Dahle	Dr. scient Biology	Reproductive strategies in Scandinavian brown bears
2003	Cyril Lebogang Taolo	Dr. scient Biology	Population ecology, seasonal movement and habitat use of the African buffalo (*Syncerus caffer*) in Chobe National Park, Botswana
2003	Marit Stranden	Dr.scient Biology	Olfactory receptor neurones specified for the same odorants in three related Heliothine species (*Helicoverpa armigera, Helicoverpa assulta* and *Heliothis virescens*)
2003	Kristian Hassel	Dr.scient Biology	Life history characteristics and genetic variation in an expanding species, *Pogonatum dentatum*
2003	David Alexander Rae	Dr.scient Biology	Plant- and invertebrate-community responses to species interaction and microclimatic gradients in alpine and Artic environments
2003	Åsa A Borg	Dr.scient Biology	Sex roles and reproductive behaviour in gobies and guppies: a female perspective
2003	Eldar Åsgard Bendiksen	Dr.scient Biology	Environmental effects on lipid nutrition of farmed Atlantic salmon (*Salmo Salar* L.) parr and smolt

2004	Torkild Bakken	Dr.scient Biology	A revision of Nereidinae (Polychaeta, Nereididae)
2004	Ingar Pareliussen	Dr.scient Biology	Natural and Experimental Tree Establishment in a Fragmented Forest, Ambohitantely Forest Reserve, Madagascar
2004	Tore Brembu	Dr.scient Biology	Genetic, molecular and functional studies of RAC GTPases and the WAVE-like regulatory protein complex in *Arabidopsis thaliana*
2004	Liv S. Nilsen	Dr.scient Biology	Coastal heath vegetation on central Norway; recent past, present state and future possibilities
2004	Hanne T. Skiri	Dr.scient Biology	Olfactory coding and olfactory learning of plant odours in heliothine moths. An anatomical, physiological and behavioural study of three related species (*Heliothis virescens, Helicoverpa armigera* and *Helicoverpa assulta*).
2004	Lene Østby	Dr.scient Biology	Cytochrome P4501A (CYP1A) induction and DNA adducts as biomarkers for organic pollution in the natural environment
2004	Emmanuel J. Gerreta	Dr. philos Biology	The Importance of Water Quality and Quantity in the Tropical Ecosystems, Tanzania
2004	Linda Dalen	Dr.scient Biology	Dynamics of Mountain Birch Treelines in the Scandes Mountain Chain, and Effects of Climate Warming
2004	Lisbeth Mehli	Dr.scient Biology	Polygalacturonase-inhibiting protein (PGIP) in cultivated strawberry (*Fragaria* x *ananassa*): characterisation and induction of the gene following fruit infection by *Botrytis cinerea*
2004	Børge Moe	Dr.scient Biology	Energy-Allocation in Avian Nestlings Facing Short-Term Food Shortage
2005	Matilde Skogen Chauton	Dr.scient Biology	Metabolic profiling and species discrimination from High-Resolution Magic Angle Spinning NMR analysis of whole-cell samples
2005	Sten Karlsson	Dr.scient Biology	Dynamics of Genetic Polymorphisms
2005	Terje Bongard	Dr.scient Biology	Life History strategies, mate choice, and parental investment among Norwegians over a 300-year period
2005	Tonette Røstelien	PhD Biology	Functional characterisation of olfactory receptor neurone types in heliothine moths
2005	Erlend Kristiansen	Dr.scient Biology	Studies on antifreeze proteins
2005	Eugen G. Sørmo	Dr.scient Biology	Organochlorine pollutants in grey seal (*Halichoerus grypus*) pups and their impact on plasma thyrid hormone and vitamin A concentrations.
2005	Christian Westad	Dr.scient Biology	Motor control of the upper trapezius
2005	Lasse Mork Olsen	PhD Biology	Interactions between marine osmo- and phagotrophs in different physicochemical environments
2005	Åslaug Viken	PhD Biology	Implications of mate choice for the management of small populations
2005	Ariaya Hymete Sahle Dingle	PhD Biology	Investigation of the biological activities and chemical constituents of selected *Echinops* spp. growing in Ethiopia
2005	Ander Gravbrøt Finstad	PhD Biology	Salmonid fishes in a changing climate: The winter challenge

2005	Shimane Washington Makabu	PhD Biology	Interactions between woody plants, elephants and other browsers in the Chobe Riverfront, Botswana
2005	Kjartan Østbye	Dr.scient Biology	The European whitefish *Coregonus lavaretus* (L.) species complex: historical contingency and adaptive radiation
2006	Kari Mette Murvoll	PhD Biology	Levels and effects of persistent organic pollutans (POPs) in seabirds Retinoids and α-tocopherol – potential biomakers of POPs in birds?
2006	Ivar Herfindal	Dr.scient Biology	Life history consequences of environmental variation along ecological gradients in northern ungulates
2006	Nils Egil Tokle	Phd Biology	Are the ubiquitous marine copepods limited by food or predation? Experimental and field-based studies with main focus on *Calanus finmarchicus*
2006	Jan Ove Gjershaug	Dr.philos Biology	Taxonomy and conservation status of some booted eagles in south-east Asia
2006	Jon Kristian Skei	Dr.scient Biology	Conservation biology and acidification problems in the breeding habitat of amphibians in Norway
2006	Johanna Järnegren	PhD Biology	Acesta Oophaga and Acesta Excavata – a study of hidden biodiversity
2006	Bjørn Henrik Hansen	PhD Biology	Metal-mediated oxidative stress responses in brown trout (*Salmo trutta*) from mining contaminated rivers in Central Norway
2006	Vidar Grøtan	PhD Biology	Temporal and spatial effects of climate fluctuations on population dynamics of vertebrates
2006	Jafari R Kideghesho	phD Biology	Wildlife conservation and local land use conflicts in western Serengeti, Corridor Tanzania
2006	Anna Maria Billing	PhD Biology	Reproductive decisions in the sex role reversed pipefish *Syngnathus typhle*: when and how to invest in reproduction
2006	Henrik Pärn	PhD Biology	Female ornaments and reproductive biology in the bluethroat
2006	Anders J. Fjellheim	PhD Biology	Selection and administration of probiotic bacteria to marine fish larvae
2006	P. Andreas Svensson	phD Biology	Female coloration, egg carotenoids and reproductive success: gobies as a model system
2007	Sindre A. Pedersen	PhD Biology	Metal binding proteins and antifreeze proteins in the beetle *Tenebrio molitor* - a study on possible competition for the semi-essential amino acid cysteine
2007	Kasper Hancke	PhD Biology	Photosynthetic responses as a function of light and temperature: Field and laboratory studies on marine microalgae
2007	Tomas Holmern	PhD Biology	Bushmeat hunting in the western Serengeti: Implications for community-based conservation
2007	Kari Jørgensen	PhD Biology	Functional tracing of gustatory receptor neurons in the CNS and chemosensory learning in the moth *Heliothis virescens*
2007	Stig Ulland	PhD Biology	Functional Characterisation of Olfactory Receptor Neurons in the Cabbage Moth, */Mamestra Brassicae/* L. (Lepidoptera, Noctuidae). Gas Chromatography Linked to Single Cell Recordings and Mass Spectrometry
2007	Snorre Henriksen	PhD Biology	Spatial and temporal variation in herbivore resources at northern latitudes

2007	Roelof Frans May	PhD Biology	Spatial Ecology of Wolverines in Scandinavia
2007	Vedasto Gabriel Ndibalema	PhD Biology	Demographic variation, distribution and habitat use between wildebeest sub-populations in the Serengeti National Park, Tanzania
2007	Julius William Nyahongo	PhD Biology	Depredation of Livestock by wild Carnivores and Illegal Utilization of Natural Resources by Humans in the Western Serengeti, Tanzania
2007	Shombe Ntaraluka Hassan	PhD Biology	Effects of fire on large herbivores and their forage resources in Serengeti, Tanzania
2007	Per-Arvid Wold	PhD Biology	Functional development and response to dietary treatment in larval Atlantic cod *(Gadus morhua* L.) Focus on formulated diets and early weaning