# Interactions between cytochrome P450 and estrogenic compounds in fish

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Department of Zoophysiology

Göteborg University 2004

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# DISSERTATION ABSTRACT

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Contamination by various pollutions is an environmental concern. Many fish populations are continuously exposed to xenobiotics, including endocrine disrupting chemicals. Cytochrome P450 (CYP) enzymes metabolize lipophilic compounds facilitating their excreation, which prevents bioaccumulation. The aim of this thesis was to study effects of estrogenic compounds on CYP, redox status and endocrine responses in fish. Furthermore, to identify possible sites of interaction between two classes of environmental pollutants, 1) estrogenic compounds, *i.e.* alkylphenols, ethynylestradiol ($EE_2$) and 2) antifungal azoles, *i.e.* ketoconazole. We hypothesize that estrogenic compounds and azoles share common routes of excretion in fish through CYP1A and CYP3A.

Atlantic cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*) were exposed orally or by i.p. injections. Effects on hepatic CYP1A and CYP3A protein expression and activities were investigated as well as glutathione, glutathione-related enzymes, vitellogenesis and sex steroid hormone levels.

Alkylphenols induced CYP1A and CYP3A protein expressions in male Atlantic cod, but not in females. Alkylphenols had no effect on CYP1A activities in either males or females. *In vitro* inhibition studies showed that the alkylphenols efficiently inhibited CYP1A activity. In addition, ketoconazole induced CYP1A and CYP3A protein expression, whereas CYP1A and CYP3A activities were inhibited. These results indicate that CYP1A and CYP3A represent sites of interactions between these classes of xenobiotics. Combined exposure of ketoconazole with $EE_2$ increased the responsiveness to $EE_2$ measured as vitellogenesis. Thus, co-exposure to ketoconazole appears to make juvenile rainbow trout more sensitive to $EE_2$ exposure. Combined exposure to ketoconazole and $EE_2$ also decreased circulating androgens. This study shows interactions between ketoconazole and $EE_2$, which affect the endocrine system and that CYP1A and CYP3A may play an important role in this interaction.

Keywords: Cytochrome P450, CYP1A, CYP3A, glutathione, alkylphenols, ethynylestradiol, ketoconazole, vitellogenin, Atlantic cod, rainbow trout, fish
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## LIST OF PAPERS

The thesis is based on the following papers and manuscripts, which are referred to in the text by their Roman numerals:

I Hasselberg, L., Meier, S. and Svardal, A., 2004. Effects of alkylphenols on redox status in first spawning Atlantic cod (*Gadus morhua*). *Aquatic Toxicology* 69:95-105.

II Hasselberg, L., Meier, S., Svardal, A., Hegelund, T. and Celander, M.C., 2004. Effects of alkylphenols on CYP1A and CYP3A expression in first spawning Atlantic cod (*Gadus morhua*). *Aquatic Toxicology* 67:303-313.

III Hasselberg, L., Grøsvik, B.E., Goksøyr, A. and Celander, M.C. Interactions between xenoestrogens and ketoconazole on CYP1A and CYP3A in juvenile Atlantic cod (*Gadus morhua*). *Submitted.*

IV Hasselberg, L., Westerberg, S. and Celander, M.C. Ketoconazole, an antifungal imidazole, makes rainbow trout more sensitive to 17α-ethynylestradiol exposure. *Submitted.*

# ABBREVIATIONS

| | |
|---|---|
| 11-KT | 11-keto-testosterone |
| 2D-GE | 2-dimensional gel electrophoresis |
| AhR | aryl hydrocarbon receptor |
| APE | alkylphenol ethoxylates |
| ARNT | aryl hydrocarbon nuclear translocator |
| BFC | 7-benzyloxy-4-[trifluoromethyl]-coumarin |
| BFCOD | 7-benzyloxy-4-[trifluoromethyl]-coumarin *O*-debenzyloxylase |
| BNF | β-naphthoflavone |
| $C_4$ | 4-*tert*-butylphenol |
| $C_5$ | 4*n*-pentylphenol |
| $C_6$ | 4*n*-hexylphenol |
| $C_7$ | 4*n*-heptylphenol |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| CYP | cytochrome P450 |
| DDT | dichloro diphenyl trichloroethane |
| $E_2$ | 17ß-estradiol |
| $EE_2$ | 17α-ethynylestradiol |
| EDC | endocrine disrupting chemical |
| ELISA | enzyme-linked immunosorbent assay |
| ER | estrogen receptor |
| EROD | 7-ethoxyresorufin *O*-deethylase |
| FMO | flavin-containing monooxygenases |
| G6PDH | glucose-6-phosphate dehydrogenase |
| GnRH | gonadotropin releasing hormone |
| GR | glutathione reductase |
| GSH | reduced glutathione |
| GSSG | oxidized glutathione |
| tGSH | total glutathione (reduced + oxidized) |
| GST | glutathione *S*-transferase |
| GtH | gonadotropin |
| HFC | 7-hydroxy-4-[trifluoromethyl]-coumarin |
| HPLC | high performance liquid chromatography |
| $K_i$ | inhibitor constant |
| $NADP^+$ | oxidized nicotinamide adenine dinucleotide phosphate |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| NP | nonylphenol |
| PAH | polyaromatic hydrocarbon |
| PCB | polychlorinated biphenyl |
| PXR | pregnane X receptor |
| RXR | *9-cis* retinoic acid receptor |
| SDS-PAGE | sodium dodecyl sulphate-polyacrylamide gel electrophoresis |
| T | testosterone |
| UDPGT | uridine diphosphate glucuronyl transferases |
| vtg | vitellogenin |

# INTRODUCTION

## BIOTRANSFORMATION

Lipophilic foreign compounds (xenobiotics) as well as endogenous substances (endobiotics) are metabolized to more water soluble products for facilitated excretion (Nebert, 1994). Biotransformation is usually associated with detoxification and occurs mainly in the liver, but is also prominent in the respiratory and digestive tracts. Biotransformation can be divided into two phases; Phase I and Phase II. Phase I reactions often introduce a reactive group such as hydroxyl, into the molecule (functionalisation). Phase I reactions are typically oxidation, reduction or hydrolysis. The product can be conjugated with an endogenous molecule in phase II reactions (conjugation) (Nebert, et al., 1996). The conjugated product is usually inactive and more water-soluble, thus can be more rapidly excreted. Biotransformation of lipophilic compounds prevents bioaccumulation and the major routes for excreation are via the bile, urine or gill/lung.

Several enzymes are involved in Phase I reactions. For example, Phase I oxidation is primarily catalyzed by cytochrome P450 (CYP) monooxygenases, which belongs to a diverse gene superfamily. Other major Phase I enzymes are the flavin-containing monooxygenases (FMO), epoxide hydrolases, lipoxygenases, cyclooxygenases, peroxidases and reductases (Nebert, 1994). FMOs are important in oxygenation of drugs, pesticides and other environmental chemicals, and they are found in the endoplasmic reticulum membrane of cells. FMOs utilize molecular oxygen and electrons from NADPH to oxidize substrates, such as secondary and tertiary amines, thiocarbamates, thioamides, sulfides, and thiols. Most of the reactions catalyzed by these enzymes produce more polar products that are less toxic than the parent compound. Unlike CYP enzymes, *de novo* synthesis of FMOs cannot be induced, and the activity of FMO enzymes cannot be lowered through the use of mechanism-based inhibitors (Halpert, et al., 1998). FMOs are found in bacteria, invertebrates and vertebrates, including fish, reptiles, amphibians, birds and mammals (Schlenk, 1998).

### *Cytochrome P450 (CYP) monooxygenases*

The CYP gene superfamily are a group of related, heme containing enzymes that are found in nearly all living organisms, animals, plants and microorganisms (Omura, et al., 1993). The catabolic CYP enzymes are predominately located in the liver, in the endoplasmic reticulum of the cells, but CYP also are found in all other tissue studied in mammals. There are over a thousand known CYP genes, although the number in the human genome is

only 57 putatively functional full-length CYP genes, listed on the homepage of P450 nomenclature committee, "Cytochrome P450 homepage" (http://drnelson.utmem.edu/CytochromeP450.html). The heterogeneity of CYP super genefamily is thought to partly reflect the complex interdependence between for example plants and animals which plays an active role in the animal-plant "warfare" (Gonzalez and Nebert, 1990). Thus, plants develop new metabolites that are increasingly toxic to animals, and animals develop new enzymes to metabolize these plant toxins (Nebert, et al., 1989). A number of CYP genes, in particular members in the mammalian CYP2 genefamily, emerged at about the time when terrestrial animals evolved, around 400 million years ago (Gonzalez and Nebert, 1990).

The individual CYP enzymes are grouped together into families and subfamilies based on sequence similarities. A number following the CYP designation identifies the family (genes that have at least a 40% sequence homology), a letter identifies the subfamily (at least a 55% identity), and another number identifies the individual enzyme (Nelson, et al., 1993). Some CYP are constitutively expressed (anabolic CYP), whereas the expression of others (catabolic CYP) can be induced by xenobiotic exposure. CYP that mainly degrade xenobiotics belong to the CYP1, CYP2, CYP3 families and subfamilies.

The CYP reaction is initiated by substrate (RH) binding to the oxidized CYP ($Fe^{3+}$). This binding induces conformational changes around the heme, increasing the heme iron redox potential, by which CYP becomes reduced. Electrons are transferred to the substrate-CYP complex from specific electron carrier proteins by reductase enzymes. The reduced state CYP ($RH\text{-}Fe^{2+}$) has high affinity for oxygen, and one electron at the heme iron is donated to the bound oxygen molecule ($RH\text{-}Fe^{3+}\text{-}O^{2-}$). A second electron stabilizes the ($RH\text{-}Fe^{2+}\text{-}O^{2-}$)-form, one oxygen reacts with hydrogen ions and water is released. The other oxygen is incorporated in the substrate, forming a hydroxyl group (ROH). The substrate is released and CYP is oxidized ($Fe^{3+}$) again (Omura, et al., 1993). The general reaction catalyzed by a CYP enzyme can be expressed as:

$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP^+ + H_2O$$

## CYP1A

Two enzymes comprise the mammalian CYP1A subfamily, CYP1A1 and CYP1A2. The two CYP1A forms found in teleost fish are more closely related to the mammalian CYP1A1 than the CYP1A2 form and have been named

CYP1A1 and CYP1A3 in rainbow trout (*Oncorhynchus mykiss*) (Morrison, et al., 1995; Buhler and Wang-Buhler, 1998; Morrison, et al., 1998). Furthermore, antibodies to fish CYP1A proteins cross-reacted with CYP1A orthologs in liver microsomes from several different fish species as well as rat (Goksøyr, et al., 1991). This shows that CYP1A forms in fish and mammals have a close immunochemical relationship (Stegeman and Hahn, 1994).

Generally, CYP1A protein levels are very low in fish unexposed to chemical inducers (Stegeman and Hahn, 1994). Many compounds can induce CYP1A in fish, for example polyaromatic hydrocarbons (PAHs), and planar halogenated aromatic hydrocarbons including planar polychlorinated biphenyls (PCBs) and dioxins, and in a certain sense they induce their own metabolism (Stegeman and Hahn, 1994). The induction mechanism involves a ligand-activated transcription factor known as the aromatic hydrocarbon receptor (AhR), which is a member of the basic helix-loop-helix family of transcription factors (Hahn, 1998). In mammals, this transcription factor controls the expression of several other genes apart from CYP1A, such as the Phase II enzymes uridine diphosphate glucuronyl transferases (UDPGT) and glutathione *S*-transferase (GST) belonging to the AhR gene battery (Nebert, et al., 1990). The inactive cytosolic AhR exists as a complex that includes two 90-kD heat shock proteins (hsp90) (Hahn, 1998). Upon ligand binding, the cytosolic AhR dissociate from hsp90 and becomes activated. The activated AhR-ligand complex translocates to the nucleus where it associates with the AhR nuclear translocator (ARNT) protein. The ligand-AhR-ARNT complex binds to dioxin or xenobiotic response elements (DRE or XRE) in target gene promoters such as CYP1A genes, resulting in increased transcription of specific DNA sequences (Hahn, 1998).

There are sex differences in hepatic CYP1A expression. Higher activities are generally being present in male fish (Stegeman and Woodin, 1984; Förlin and Haux, 1990). CYP1A activities in microsomes from mature females are reduced during the sexual maturation process and this decrease in females during spawning are believed to be regulated by circulating 17ß-estradiol ($E_2$) (Pajor, et al., 1990; Buhler, et al., 2000). The estradiol derived down-regulation mechanisms in CYP expression are not known, but probably involves the estrogen receptor (ER) (Solé, et al., 2003). A cross-talk between ER and AhR has been described in mammals (Safe, et al., 1991; Klinge, et al., 2000; Safe, 2001) and also in fish (Elskus, 1992; Arukwe, et al., 1997; Solé, et al., 2000b; Navas and Segner, 2001).

### CYP3A

The CYP3A enzymes in fish are orthologs to the mammalian CYP3A forms and are the most abundant of all CYP isoforms (McArthur, et al., 2003). For

example, CYP3A is the dominant hepatic CYP in fish (Celander, et al., 1996) and CYP3A4 represents up to 60% of all hepatic CYP isoforms in humans (Thummel and Wilkinson, 1998). Nearly 50% of all pharmaceuticals are metabolized by CYP3A4 and CYP3A4 is consequently involved in numerous drug-interactions (Thummel and Wilkinson, 1998; Guengerich, 1999). In addition, CYP3A enzymes are responsible for the metabolism of several endogenous sex steroids, *e.g.* testosterone, estradiol, progesterone and androstenedione in both mammals and fish (Klotz, et al., 1986; Snowberger and Stegeman, 1987; Waxman, et al., 1988; Wang and Strobel, 1997; Guengerich, 1999). Another important consideration is the fact that CYP3A enzymes are located in the small intestine and responsible for the majority of first-pass metabolism in mammals (Thummel and Wilkinson, 1998). The CYP3A enzyme is the dominant CYP form expressed in the digestive- and respiratory tracts also in fish (Husøy, et al., 1994; Cok, et al., 1998; Lee, et al., 1998a; Lee, et al., 2001; Hegelund and Celander, 2003).

The CYP3A enzyme expression shows sexual dimorphic differences in fish. Generally higher levels are present in sexually mature males compared to females (Stegeman and Woodin, 1984; Celander, et al., 1989; Hegelund and Celander, 2003). However, sex differences in hepatic CYP3A have been reported where female winter flounder (*Pseudopleuronectes americanus*) showed higher CYP3A levels and activity than males (Stegeman and Woodin, 1984; Gray, et al., 1991). The question whether growth hormone plays a role as a regulator of hepatic CYP in fish is intriguing, as growth hormone is a strong effector of CYP gene expression in mammals (Waxman, et al., 1988; Waxman, et al., 1990; Kawai, et al., 2000).

The CYP3A enzymes have unusually broad substrate specificity and catalytic activity is modulated by a variety of compounds. A unique characteristic of CYP3A is that its catalytic activity for a particular substrate may be stimulated by the addition of another xenobiotic to the *in vitro* incubation mixture (Thummel and Wilkinson, 1998). This is called positive co-operativety or autoactivation (Shou, et al., 1994; Ekins, et al., 1998; Guengerich, 1999).

The most common mechanism for CYP3A induction is transcriptional activation followed by *de novo* synthesis. The orphan nuclear receptor designated the pregnane X receptor (PXR), a relatively new member of the nuclear receptor superfamily, activates CYP3A gene expression in response to diverse chemicals, including drugs, xenobiotics, bile acids, natural and synthetic steroids (Masuyama, et al., 2000; Xie, et al., 2001; Kliewer, et al., 2002; Masuyama, et al., 2002). The ligand-PXR complex forms a heterodimer with *9-cis* retinoic acid receptor (RXR) (Moore, et al., 2002). This complex binds to response elements in CYP3A gene promoter and regulate expression

the CYP3A gene. The existence of a piscine PXR ortholog recently was suggested by cloning of the ligand-binding domain of a zebrafish (*Danio rerio*) PXR gene (Moore, et al., 2002). Thus, PXRs have been found in species ranging from fish to man (Jones, et al., 2000; Moore, et al., 2002).

Certain compounds that are metabolized by CYP3A also act as inhibitors of the CYP3A activity. Binding of an inhibitor, or its metabolite, to CYP3A has been found to result in either competitive or non-competitive inhibition. The most potent reversible CYP3A inhibitors include azole antifungal agents, *e.g.* ketoconazole, propiconazole, clotrimazole and miconazole (Miranda, et al., 1998; Thummel and Wilkinson, 1998; Guengerich, 1999; Hegelund, et al., 2004)

### *Conjugation*

After the Phase I reaction, the molecule is susceptible to conjugation, *i.e.* attachment of a substituent group. Phase II enzymes catalyze conjugation reactions, facilitating the excretion of chemicals by the addition of more polar groups. Phase II enzymes include UDP glucuronyl transferases (UDPGT), glutathione *S*-transferases (GST), sulfotransferases, transaminases, acetyltransferases and methyltransferases (Nebert, 1994). The enzymes UDPGT and GST catalyze conjugation to glucuronic acid and glutathione, respectively. Studies have shown that fish may metabolize xenobiotics by conjugation to both glucuronic acid or glutathione (Ankley and Agosin, 1987).

#### Glucoronic acid

Glucuronide formation, the most common conjugation reaction, involves the formation of a high-energy phosphate compound, uridine diphosphate glucuronic acid (UDPGA). This formation is catalyzed by UDPGT, which has broad substrate specificity, so the reaction occurs with a wide varity of drugs and other xenobiotics (Hanninen, et al., 1984).

#### Glutathione

Detoxification of xenobiotics, as well as endobiotics is an important function of glutathione (GSH), a cysteine containing tripeptide that also serves other several essential functions within the cell, including synthesis of proteins and DNA, transport, enzyme activity, metabolism and protection of cells (Meister and Anderson, 1983). In mammals, GSH is present intracellularly in the millimolar-range and is therefore the main non-protein thiol in most aerobic organisms (Meister and Anderson, 1983). Conjugation of GSH to electrophilic sites on a wide range of substrates is catalyzed by GST (Habig, et al., 1974). The conjugation of GSH to these compounds is the initial step in the

formation of mercapturic acids. GSTs are primarily cytosolic and present at high levels in the liver (Meister and Anderson, 1983).

Glutathione is the predominant cellular defense against oxidative stress (DeLeve and Kaplowitz, 1991). For example, hydrogen peroxide is reduced by GSH in the presence of glutathione peroxidase (GPx) and GSH is oxidized to GSSG. GSSG is then reduced back to GSH by glutathione reductase (GR) at the expense of NADPH (DeLeve and Kaplowitz, 1991). A closed system, a redox cycle is formed (Fig. 1). NADPH is produced in the pentose phosphate pathway, where the key enzyme is the glucose-6-phosphate dehydrogenase (G6PDH) (Eggleston and Krebs, 1974). NADPH is important in providing reducing equivalents in reactions that are critical in protecting against oxidant damage .

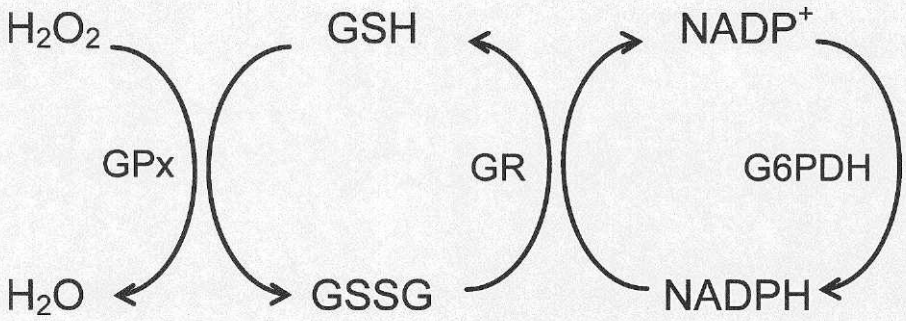



Figure 1. The glutathione redox cycle

The cell can actively transport GSSG out of the cell to protect the cell from a shift in the redox equilibrium (DeLeve and Kaplowitz, 1991). The ratio of reduced to oxidized glutathione (GSH:GSSG) may be decreased as a consequence of direct radical scavenging or increased peroxidase activity (Otto and Moon, 1995). However, normal GSH:GSSG ratios can be maintained by increased GR activity or increased GSH synthesis.

## ENDOCRINE DISRUPTING CHEMICALS

Many anthropogenic compounds present in the environment have been found to affect hormonal functions in various ways (Sumpter, 1998). The endocrine disrupting chemical (EDC) has been described as "An exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones" (Kavlock, et al., 1996). However, another

definition is made by The International Programme on Chemical Safety (IPCS) in 2002, which suggest "An endocrine disruptor is an exogenous substance or mixture that alters the function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations" (Fisher, 2004). In this thesis, the term EDC refers to compounds that fulfil the criterions of both definitions.

### *Environmental estrogenic compounds*

Those EDCs that have estrogenic activities have been denoted as environmental estrogens, ecoestrogens, estrogen mimics, or xenoestrogens and they mimic or interfere with the effects of the female hormone estrogen by binding to the estrogen receptor (ER). Chemicals that can mimic natural estrogens include certain organochlorine industrial compounds such as PCBs and various pesticides including dichloro diphenyl trichloroethane (DDT) and dioxins. In addition to the use in pesticides, these and other estrogenic compounds also are used in plastics, pharmaceuticals, paper and pulp production, textiles, and detergents. Many EDCs are lipophilic and bioaccumulate in living organisms (Ahel, et al., 1993). Environmental estrogenic compounds comprise a structurally diverse group and cannot be identified by structure alone. This makes it hard to predict which natural and synthetic chemicals will act like estrogenic hormones in living organisms.

Over the last few years, a number of studies have been published which support the hypothesis that certain chemicals in the environment constitute a threat to animal reproductive health. In connection with a massive spill of DDT into Lake Apopka, Florida, they found depressed plasma testosterone concentrations and a reduction in penis size in young male alligators (*Alligator mississippiensis*) (Guillette, et al., 1994; Guillette, et al., 1996). In addition, female alligators superovulated, with multiple nuclei in some of the surplus ova with a declining alligator population as a consequence (Guillette, et al., 1994). Furthermore, human epidemiological studies have indicated a decreasing sperm count (Sharpe and Skakkebaek, 1993) and increasing incidence of testicular cancer worldwide in men (Toppari, et al., 1996). There also is an alarming increase in breast cancer incidence (Tyczynski, et al., 2004). Studies have implicated that increased breast cancer incidences may be due to xenoestrogens (Ibarluzea, et al., 2004). It is well known that, under certain conditions, estrogens (natural as well as synthetic) can be tumor promoting, as most breast cancer cell types have ERs (Platet, et al., 2004). However, these evidences for endocrine disruption are being critically questioned. Are the concentrations of environmental estrogenic compounds high enough to cause these problems? Many estrogenic compounds exhibit only weak estrogenic activity and exist at extremely low levels in the environment and thus may not

pose a serious threat to the wildlife. Nevertheless, even though environmental estrogenic compounds have weaker binding affinities to estrogen receptors than the natural estrogen, their concentrations in human blood are generally much higher than the natural estrogen since it is bound up in plasma proteins. The higher concentrations and lower binding to plasma proteins of environmental estrogenic compounds means that they have a greater potency to act as endocrine disrupters.

This study investigates several known environmental estrogenic compounds, including the alkylphenols, (butylphenol ($C_4$), pentylphenol ($C_5$), hexylphenol ($C_6$), heptylphenol ($C_7$), nonylphenol (NP) and the synthetic estrogen 17α-ethynylestradiol ($EE_2$). All of these are referred to as xenoestrogens in this thesis.

## Alkylphenols

Alkylphenols are found in produced water released in the ocean from oil platforms (Brendehaug, et al., 1992; Røe, 1998) (Fig. 2). The estrogenic activity of alkylphenols is well known and has been investigated in several studies (Nimrod and Benson, 1996; Christiansen, et al., 1998; Arukwe, et al., 2000a; Arukwe, et al., 2001). The alkylphenols are shown to interact with ER in an identical way as the natural estrogen 17ß-estradiol, but with a weaker response (White, et al., 1994; Soto, et al., 1995; Yadetie, et al., 1999; Arukwe, et al., 2001). An *in vitro* study showed that the potency of the alkylphenols to activate ER depends on 1) the position and 2) the branching of the alkyl group. The maximum activity (1000 to 6000 fold less potent than estradiol) is found in $C_6$ to $C_8$ para substituted tertiary alkylphenols, but also $C_5$, $C_4$ and $C_3$ phenols are estrogenic (Routledge and Sumpter, 1997).

### *Produced water*

Produced water is continuously released into the ocean as a result of offshore oil production. The amount of produced water increases dramatically with the age of the oil field, and the discharge of produced water from the Norwegian sector reached a volume of more than 120 million $m^3$ in year 2001 (SFT; The Norwegian pollution control authority). Produced water contains both natural compounds and chemicals that have been added through the process or separation line (Brendehaug, et al., 1992). The phenols are one of the larger groups of organic pollutants. The cresol ($C_1$), the most water solvable phenol, stands for around 80%, but phenols with longer alkyl chains, *i.e.* $C_4$ to $C_7$, are also reported in small concentrations of 2 to 237 µg/L in produced water from oil platforms outside the Norwegian coast (Brendehaug, et al., 1992). The acute toxicity towards marine organisms is low for produced water because of the large amounts of sea water in which this discharge is diluted

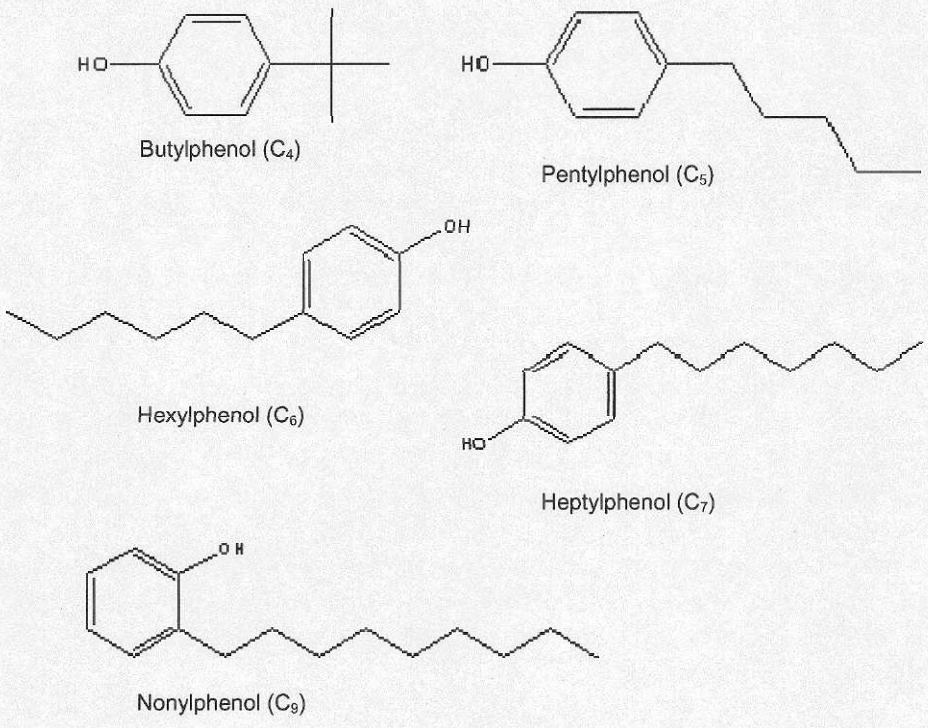



Figure 2. Chemical structures of alkylphenols found in produced water: 4-*tert*-butylphenol ($C_4$), 4*n*-pentylphenol ($C_5$), 4*n*-hexylphenol ($C_6$), 4*n*-heptylphenol ($C_7$) and 2*n*-nonylphenol (NP).

(Røe, 1998). However, alkylphenols can accumulate as they move up the food chain. The bioconcentration factors for alkylphenols $C_4$ to $C_7$ range from 118 to 578 in fish (McLeese, et al., 1981; Freitag, 1985; Tollefsen, et al., 1998). This could lead to unexpected side effects over time and knowledge of the effects from alkylphenols on the environment is still somewhat lacking.

### *Alkylphenol ethoxylates*

Long-chain alkylphenols, *e.g.* octylphenol and nonylphenol, originate from the widespread use of alkylphenol ethoxylates (APE). APEs are used as industrial surfactants, detergents, paints, pesticides, textile and petroleum recovery chemicals, metalworking and personal products. The annual worldwide production of APE is 500,000 tons of which 60% ends up in the aquatic environment (Renner, 1997; Solé, et al., 2000a).

Restriction on the use of APE has arisen since the discovery in 1984 that their metabolites were more toxic to aquatic organisms than the APE themselves. Biodegradation of APE leads to the shortening of the ethoxylate chains to alkylphenol carboxylates leading ultimately to nonylphenol and octylphenol, which both have low water solubility and adsorb to suspended solids and sediments. Nonylphenol in wastewater extracted from digested sewage sludge can pass into rivers (Tyler, et al., 1998).

APEs, nonylphenol and octylphenol has been reported in surface water all around the world (Ying, 2002), as well as in drinking water (up to 40 μg/L) (Berryman, et al., 2004). These alkylphenol doses are not likely to result in a biologically significant body burden in humans. However, the situation may be different in non-mammalian species: alkylphenol precursors can apparently bioaccumulate in certain aquatic organisms, such as mussels (*M. edulis* L.), shrimp (*Crangon crangon*), sticklebacks (*Gasterosteus aculeatus* L.), Atlantic salmon (*Salar salar*) and Atlantic cod (*Gadus morhua*) reviewed by Thiele, et al. (1997), but whether they exert adverse effects due to estrogenicity or other mechanisms is still unclear.

### Ethynylestradiol

Discharges from sewage treatment works contain both natural estrogens such as $E_2$ and estrone as well as the synthetic derivate 17α-ethynylestradiol ($EE_2$), a very potent pharmaceutical estrogen used in oral contraceptive (Fig. 3) (Tyler, et al., 1998). Ethynylestradiol interfere with the effects of estradiol by binding to ER, and it is classified as an EDC. The potency of ethynylestradiol for activation of ER is higher than that for estradiol (Larsson, et al., 1999). For example, ethynylestradiol had one order of magnitude or more higher potency to induce the ER mediated vitellogenesis in adult zebrafish than that for estradiol (Van den Belt, et al., 2004).

Natural estrogens as well as ethynylestradiol are metabolized in the liver by CYP enzymes, CYP1A and CYP3A in particular (Guengerich, 1988; Waxman, et al., 1991; Wang and Strobel, 1997; Thummel and Wilkinson, 1998; Yamazaki, et al., 1998). In humans, estrogens are metabolized at the 2-position to a 2-OH derivate by CYP1A2 (Badawi, et al., 2001). CYP1A2 is more active than CYP3A in catalysing 2-, 4- and 16α-hydroxylation of estradiol (Yamazaki,

et al., 1998). However, since CYP3A levels are approximately 4- to 14-fold higher than CYP1A2 in the liver, the CYP3A4 is the major CYP isoform responsible for hepatic metabolism of estradiol (Badawi, et al., 2001). The 2-hydroxylation of ethynylestradiol in human is primarily catalyzed by CYP3A4 (Guengerich, 1988). This metabolite can undergo further oxidation with subsequent conjugation such as sulphation and glucuronidation prior to excretion (Guengerich, 1988). Enzymes from bacteria such as ß-glucuronidase in sewage treatment works can biotransform ethynylestradiol to its biologically active form again (Tyler, et al., 1998). Ethynylestradiol has been found in sewage effluent water at concentrations of 2 ng/L in Sweden, 7 ng/L in the UK and up to 17 ng/L in Germany (Desbrow, et al., 1998; Belfroid, et al., 1999; Larsson, et al., 1999).

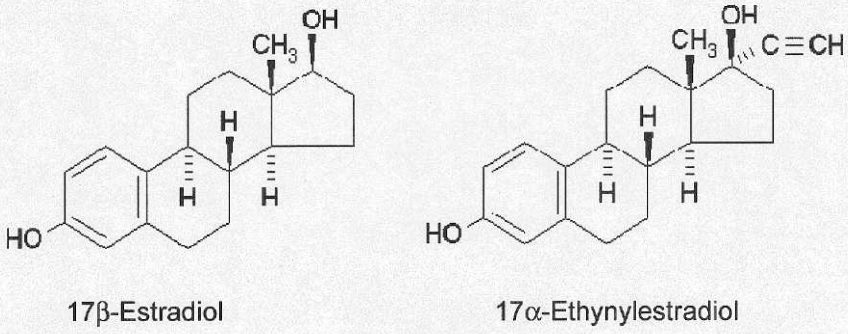



Figure 3. Structure of the natural estrogen 17ß-estradiol ($E_2$) and the synthetic derivative 17α-ethynylestradiol ($EE_2$).

### *Azole fungicides*

Imidazoles and triazoles are used as fungicides, clinically as well as in horticulture and agriculture, and inhibit CYP51 mediated ergosterol biosynthesis in fungus (Vanden Bossche, et al., 1995) (Fig. 4). In addition to disrupting key enzymes in fungus, azoles cause endocrine disruption in mammals and can be classified as EDCs (Kan, et al., 1985; Latrille, et al., 1989). For example, the imidazole ketoconazole reduced the rate of estradiol production in isolated rat ovaries (Latrille, et al., 1989). Vinclozolin and prochloraz are two fungicides with anti-androgenic effects, *i.e.* they interfere with the activity of androgen hormones in male rats (Gray, et al., 1999; Vinggaard, et al., 2002). Fungicides also inhibit drug-metabolizing members of the CYP1, CYP2 and CYP3 gene families in vertebrates (Levine, et al., 1997;

Miranda, et al., 1998; Boxenbaum, 1999; Guengerich, 1999; Venkatakrishnan, et al., 2000; Hegelund, et al., 2004). This may have adverse effects on metabolic clearance of endobiotics and xenobiotics. In humans, prolonged ketoconazole therapy results in decreased clearance of 17ß-estradiol, which may cause gynecomastia in men (Venkatakrishnan, et al., 2000). Thus, it is possible that ketoconazole treatment affect metabolic clearance of steroids also in fish. The triazole propiconazole has been detected in the aquatic environment in drainage canals of a banana plantation in Costa Rica (>20 µg/L) (Castillo, et al., 2000) and in Swedish streams (>1 µg/L) (The Swedish National Chemicals Inspectorate, 1998).

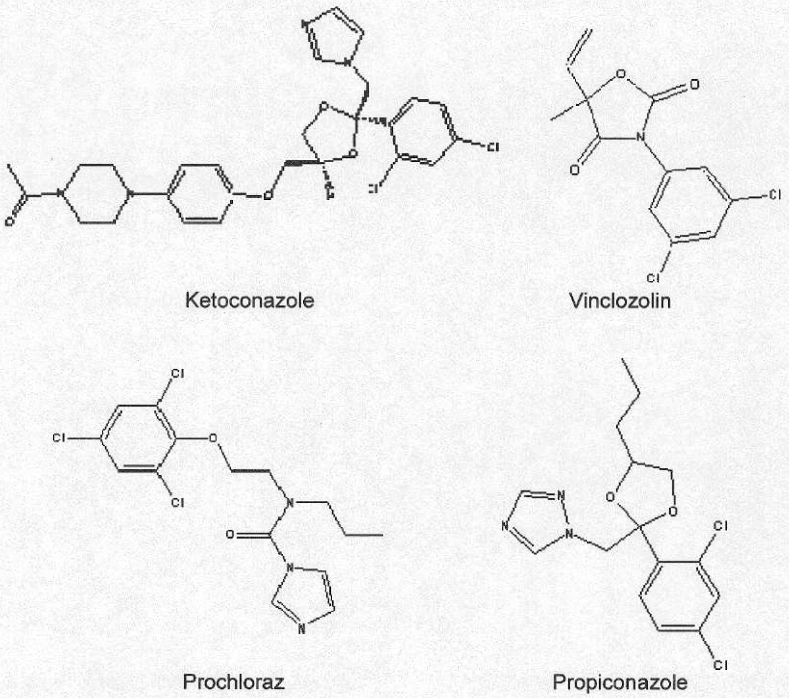



Figure 4. Chemical structures of the antifungal ketoconazole, vinclozolin, prochloraz and propiconazole.

## THE ESTROGEN RECEPTOR

Estrogens are small hydrophobic molecules that can diffuse through cell membranes. Target cells for steroid hormones have intracellular receptors in the nucleus (Brown, 2002). Estrogens act through two known classes of nuclear estrogen receptors (ERs), ERα and ERß. Activated ER binds to estrogen response elements (ERE) in the 5' flanking promoter region on target genes and regulate specific gene expression in the nucleus of the target cell. It is still unknown how these receptors control gene expression in their target cells (Brown, 2002). The ERα and ERß differ in ligand binding affinities to estrogens, tissue distributions, and transactivation properties (Kuiper, et al., 1997; Paech, et al., 1997). For example, *in vitro* studies showed that estradiol-binding to ERα activated transcription, whereas with ERß, estradiol inhibited transcription (Paech, et al., 1997).

A new class of nuclear ER has recently been identified in teleost fish, designated ERγ (Hawkins, et al., 2000). The ERγ have been identified in the Atlantic croaker (*Micropogonias undulates*), and appears to have arisen by gene duplication from ERß early in the teleost linage (Hawkins, et al., 2000). The three ER mRNA transcript subtypes are differently expressed in reproductive organs as well as in other tissues. Thus, ERα transcripts are abundant in the liver and less abundant in testes, ERß transcripts are abundant in liver and testes, less abundant in ovary and barely detectable in muscle. The ERγ transcripts are abundant in the ovary and testes and undetectable in the liver, whereas expression in brain and muscle is low or undetectable (Hawkins, et al., 2000). The differences in tissue distribution between ERα, ß, and γ transcripts suggest that they have different functions. Interestingly, the high levels of ERγ expression in the testes, implies that ERγ mediates effects of environmental estrogens in male fish. However, the possible effects of ER on the observed feminization in male fish exposed to sewage still remains to be elucidated.

## ACTIONS OF ESTROGENIC COMPOUNDS

Estrogenic compounds affect the endocrine system in a number of different ways. Once the compound binds to ER, the mimicker can produce a normal hormone response, cause an abnormal response or block the receptor site, preventing natural hormones from binding. Estrogenic compounds may interact with a plasma membrane ER thought to be responsible for non-genomic actions of estrogens. Activation of plasma membrane ERs have been shown to be associated with regulation of cell membrane ion channels, G-protein-coupled receptors, tyrosine kinases and generation of cyclic AMP and

the activation of mitogen activated protein (MAP) kinase cascades, recently reviewed by Simoncini and Genazzani (2003).

Estrogenic compounds may bind to other receptors and create a novel reaction or interfere directly with normal hormonal action. Other receptors shown to be influenced by estrogenic compounds include AhR, which regulates some ER responsive genes as well as several other genes (Safe, et al., 1998), and PXR, which regulates steroid metabolism (Masuyama, et al., 2000). Furthermore, estrogenic compounds can alter synthesis and metabolism of hormone receptors and natural hormones modifying the endocrine responses. There are reports of endocrine disruptors increasing the expression of ERs in Atlantic salmon (Yadetie, et al., 1999) and in channel catfish (*Ictalurus punctatus*) (Nimrod and Benson, 1997). The mechanism by which the observed increase in ER density occurs is unclear but such a change could mediate estrogenic effects by increasing sensitivity to endogenous estrogens as well as xenoestrogens. Furthermore, steroid hormone biosynthesis involves a number of different steroid hydroxylases and reductases, which also are possible targets for endocrine disrupters.

## REPRODUCTIVE ENDOCRINOLOGY OF FISH

Hypothalamus controls synthesis and release of hormones through the influence of external stimuli, such as photoperiod, temperature, feeding and social factors as well as internal stimuli, such as sex steroid hormones. Secretion of gonadotropin releasing hormone (GnRH) from the hypothalamus stimulates the release of gonadotropins (GtH) from the pituitary. Two forms of GtH, GtH I and GtH II, have been isolated from salmonid pituitaries (Swanson, et al., 1991), which are regarded as homologous to mammalian follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively (Borg, et al., 1998). GtH are responsible for stimulating the synthesis of sex steroids ($E_2$ and testosterone (T) in female fish, T and 11-keto-testosterone (11-KT) in male fish), which in turn act on target tissues to regulate gametogenesis, reproduction, sexual phenotype and behavioral characteristics. Furthermore, GtH also controls oogenesis in fish by binding to receptors on the thecal and granulose cell layer of the follicle (Arukwe, 2001). The thecal cells synthesize T, and the conversion of T to $E_2$ depends on the action of the aromatase enzyme (CYP19) in the granulose layer, referred to as aromatization (Brown, 2002). Plasma $E_2$ binds to ER and triggers the series of steps resulting in the production of vitellogenin (vtg) in the liver (Mommsen and Walsh, 1988). Vtg are released from the liver into the blood and are incorporated into the oocytes, through receptor-mediated endocytosis. The cascade reaction axis that leads to the production of maturation protein is known as hypothalamus-

pituitary-gonadal-liver-axis (HPGL-axis). Hormones provide both positive and negative feedback to the hypothalamus and pituitary (Brown, 2002).

### *Vitellogenesis*

One of the most commonly used biomarker to estimate estrogenic exposure is the induction of vtg synthesis in male or juvenile fish (Folmar, et al., 2002). Although male and juvenile fish do not normally synthesize vtg, the hepatic estrogen receptor and the gene that encodes for vtg is present and expression can be activated by both natural and anthropogenic estrogenic substances (Tyler, et al., 1998).

Vtg proteins are large phospholipoglycoproteins with a molecular weight of 250-600 kD (Arukwe and Goksøyr, 2003), and they are enzymatically cleaved into smaller yolk proteins, which act as nutrient to support the development of the embryo (Tyler, et al., 1998). Vtg levels are usually measured using radio immune assays (RIA) or enzyme-linked immunosorbent assays (ELISA).

## AIMS OF THESIS

Fish are continuously exposed to anthropogenic compounds in their natural environment, including estrogenic compounds. The general aim of this thesis was to study effects of environmental estrogenic compounds on cytochrome P450 (CYP), redox status and endocrine responses in fish. In addition, we identified possible sites of interaction between environmental estrogenic compounds and an antifungal imidazole, *i.e.* ketoconazole.

The specific aims of this thesis were:

- To study effects of estrogenic alkylphenols on the redox status in Atlantic cod, with emphasis on glutathione and glutathione-related enzymes (paper I)

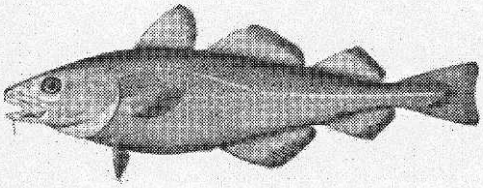
- To study effects of estrogenic alkylphenols and ethynylestradiol on hepatic CYP1A and CYP3A protein expressions and enzyme activities in Atlantic cod (paper II, paper III)

- To study effects of estrogenic alkylphenols and ethynylestradiol, alone and in combination with ketoconazole on hepatic CYP1A and CYP3A enzyme activities in Atlantic cod and rainbow trout *in vitro* and *in vivo* (paper II, paper III, paper IV)

- To study effects of nonylphenol and ethynylestradiol, alone and in combination with ketoconazole on vitellogenesis and circulating sex steroid hormones in Atlantic cod and rainbow trout (paper III, paper IV)

# METHODOLOGICAL CONSIDERATIONS

Different techniques have been used in this thesis to study the effects of environmental estrogenic substances on CYP as well as effects on oxidative stress and steroid hormone homeostasis. These techniques include catalytic assays, using spectrophotometry and fluorometry, Western blot, high performance liquid chromatography (HPLC), 2-dimensional gel electrophoresis (2D-GE) and enzyme-linked immunosorbent assay (ELISA), which are discussed here, although a more detailed description of the methods used are provided in papers I to IV.



## ANIMALS

### *Atlantic cod*

We used hatchery-reared first spawning Atlantic cod (*Gadus morhua*) in our studies with alkylphenols (paper I and II), and hatchery reared juvenile Atlantic cod to study co-exposure to nonylphenol and ketoconazole (paper III). The Atlantic cod exposure studies were conducted in Bergen, Norway.

Atlantic cod is an economically and ecologically important species. It is widely distributed in a variety of habitats, from the shoreline down to the continental shelf (depth range 1 - 600 m). Atlantic cod are omnivorous; they feed on invertebrates and fish, including young cod. Sexual maturity is attained between ages two to four and spawning occurs once a year during winter and early spring. The most important stocks are the Norwegian Arctic stock in the Barents Sea and the Icelandic stock. The populations around Greenland and Newfoundland have declined dramatically. During the past years declining populations along the Swedish coast have generated debates on whether the Atlantic cod are being exterminated. Overfishing seems to be the cause of extermination. However, the environmental contamination may be another factor for diminishing recruitment of Atlantic cod populations.

There are several advantages to using hatchery-reared fish from the same strain such as reduced inter-individual variations due to age, strain, polymorphism, handling, diet, previous environment and exposures. In paper I and II, the fish were exposed by feeding via a sonde inserted directly into the stomach to achieve better control of actual individual exposure. In paper III and IV, the fish were starved and intraperitoneally (i.p.) injected with the test agents.

### *Rainbow trout*

Juvenile hatchery-reared rainbow trout (*Oncorhynchus mykiss*) was used to investigate co-exposure to ethynylestradiol and ketoconazole (paper IV). Rainbow trout is one of the most widely introduced fish species and may be regarded as global in its present distribution. Rainbow trout in the wildlife inhabit fresh water (depth range 1-200 m) with about 12°C in summer and feed primarily on insects, crustaceans, fish eggs and small fish. They are capable of adapting to sea water and can migrate to the ocean where they spend several years of their life. They require moderate to fast flowing, well-oxygenated waters for breeding, but they also live in cold lakes. So far the rainbow trout has been the most widely used species in studies of endocrine disrupting chemicals in aquatic environments (Rose, et al., 2002). It also is commonly used as a model laboratory species to investigate physiological processes or toxicities involving CYP metabolism.

## METHODS

### *Analyzing the glutathione concentration*

The level of glutathione in Atlantic cod liver was measured using HPLC, by a method developed by Svardal, et al. (1990) (paper I). HPLC is used in many laboratories for separation, identification, purification and quantification of various compounds. There are several types of HPLC systems. We used reversed-phase chromatography where the stationary phase is hydrophobic and the mobile phase polar, for example a mixture of water and acetonitrile used in our protocol. We used a fluorescence detector, which is a sensitive HPLC detector. For example, fluorescence sensitivity is 10-1000 times higher than that of the UV detector for strong UV absorbing materials. HPLC is reproducible and a specific method, however, time-consuming.

Using this method, we were able to measure total free glutathione (tGSH) as well as reduced glutathione (GSH) concentration. To reduce oxidized glutathione (GSSG) and for derivatization of free sulfhydryl groups, we used $NaBH_4$ and monobromobimane, respectively.

### *Glutathione-related enzyme assays*

The activities of glutathione reductase (GR), glutathione *S*-transferase (GST) and glucose-6-phosphate dehydrogenase (G6PDH) were measured in Atlantic cod liver S9-fractions (paper I). All three methods allow rapid processing of small samples, which is important when measuring multiple variables. The GR method is based on the cyclic reaction of GSH with 5,5'-dithiobis (2-

nitrobenzoic acid) (DTNB) in the presence of added NADPH (Cribb, et al., 1989). GSH conjugation catalyzed by GST was measured using the substrate 1-chloro-2,4-dinitrobenzene (CDNB). CDNB does not distinguish between the different GST isoforms, due to overlapping substrate specificities of these isoenzymes (Habig, et al., 1974). G6PDH is the rate-limiting enzyme in the pentose phosphate pathway that provides the cell with NADPH. G6PDH was measured using glucose-6-phosphate and $NADP^+$. The increase in absorbance at 340 nm is proportional to G6PDH activity (Deutsch, 1987).

### *7-Ethoxyresorufin-O-deethylase (EROD) activity*

CYP1A activity was measured using a synthetic fluorescent compound, 7-ethoxyresorufin, as a diagnostic substrate (paper II, III and IV). CYP1A enzymes catalyze the formation of the fluorescent product resorufin. The amounts produced resorufin is normally proportional to the CYP1A activity in the sample. We used the protocol for microplates with a high throughput provided in (Hahn, et al., 1993), modified for microsomes by (Yawetz, et al., 1998).

Ethoxyresorufin-*O*-deethylase (EROD) activity has been used for many years in biomonitoring program as an early-warning signal for potentially harmful pollutants. The activity of EROD assay seems to be the most sensitive catalytic probe for determining CYP1A activity in fish (Goksøyr and Förlin, 1992) and this method has a great value as a biomarker in environmental risk assessment in the aquatic environment (van der Oost, et al., 2003).

### *7-Benzyloxy-4-[trifluoromethyl]-coumarin-O-debenzyloxylase (BFCOD) activity*

A specific CYP3A substrate is difficult to find because of the unusually wide substrate specificities of CYP3A enzymes (Guengerich, 1999). However, a protocol was prepared by Miller, et al. (2000) for the mammalian substrate 7-benzyloxy-4-[trifluoromethyl]-coumarin (BFC), which is predominately metabolized by CYP3A4 in humans (Stresser, et al., 2002). This protocol was previously optimized for rainbow trout liver microsomes (Hegelund, Hackzell and Celander, unpublished) and was shown to be a good probe for piscine CYP3A activities (Hegelund, et al., 2004). The CYP3A enzymes catalyze the conversion of BFC to the fluorescent product 7-hydroxy-4-[trifluoromethyl] coumarin (HFC). This 7-benzyloxy-4-[trifluoromethyl]-coumarin *O*-debenzyloxylase (BFCOD) activity was not detected in cod liver S9-fractions (paper II). However, the CYP3A activity could be recorded in cod liver microsomes (paper III) where CYP levels are higher.

### *CYP1A and CYP3A* in vitro *inhibition studies*

Both EROD and BFCOD activities were used to assess the possible inhibition effects of $C_4$ to $C_7$, NP, $E_2$, $EE_2$ and ketoconazole on CYP1A and CYP3A enzymes (paper II and III). We analyzed $IC_{50}$ (median inhibition concentrations) and $K_i$ values on these activities in cod liver microsomes. When measuring CYP1A activity we used liver microsomes from cod exposed to ß-naphthoflavone (BNF), which has high levels of CYP1A enzymes. BNF is an agonist to the AhR, commonly used as a prototypical CYP1A inducer in fish (Celander and Förlin, 1991; Celander, et al., 1993).

### *CYP1A and CYP3A protein blot analyses*

It is important to analyze protein expression levels as a complement to catalytic activity measurements, because of the possible presence of inhibitors that can mask CYP protein levels. The Western blot technique we used is a semi-quantitative method, separating proteins based on size using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (paper II, III and IV). Proteins are then transferred to a nitrocellulose membrane and visualized using specific antibodies and enhanced chemoluminescence (ECL). We used polyclonal antibodies (PAb) against rainbow trout CYP1A and CYP3A raised in rabbits (Celander and Förlin, 1991; Celander, et al., 1996), as well as monoclonal antibody (MAb) 1-12-3 against scup (*Stenotomus chrysops*) CYP1A1 (Park, et al., 1986). The intensities of the protein bands were analyzed using densitometry on scanned fluorograms (Celander, et al., 1996).

### *2-Dimensional gel electrophoresis (2D-GE)*

2D-GE is generally used for the isolation of proteins and can be used for further characterization by mass spectroscopy. By isoelectric focusing in the first dimension proteins are separated by charge (pI) and in the second dimension proteins are separated by size using SDS-PAGE. This allows an efficient separation of proteins, which can be visualized by different staining methods using coomassie blue, silver, zinc, copper or fluorescent stains, such as SyproRuby. In addition, proteins can be transferred to nitrocellulose membrane and detected using specific antibodies (paper III). 2D-gel protein databases are being created and can be used to apply pattern matching techniques. The gel matching is one of the essential and most time-consuming requirements for a quantitative and qualitative data analysis of protein gel images. Nevertheless, the quality of data and the ability to analyze multiple proteins are advantageous.

### *Enzyme-linked immunosorbent assay (ELISA)*

ELISA techniques are commonly used to analyze levels of protein, steroids, drugs etc. in various samples such as plasma, microsomes, urine and saliva by using antibodies coupled to an enzyme that possesses a high turnover number. It is a rapid method, generally highly sensitive and specific and favorably comparable with radio immune assays (RIA). The main advantage is that there is no need for radioactive reagents in ELISA. Recovery rate, dilution linearity, intra-assay and inter-assay variation should be considered when setting up a new ELISA protocol.

One common type of ELISA protocol is based on the sandwich approach. The sandwich ELISA requires two antibodies that bind to two different epitopes on the antigen. In the first step, the primary antibody is coated on the microplate and the standard or samples are added. In the second step, unbound products are washed away and the labeled secondary antibody is allowed to bind to the antigen, completing the sandwich. There is no need to purify antigen in the sample prior to use and there is a high throughput, using for example 96-well microplates. A sandwich ELISA can be non-competitive or competitive. In a competitive sandwich ELISA, an antigen-specific conjugate are added to each well, competing for the limited number of antibody binding sites. After washing, a substrate to the conjugate is added. The product of the enzymatic reaction is colored and can be assessed spectrophotometrically. The amount of color development is inversely proportional to the concentration of sample antigen present in the well.

For measuring Atlantic cod vitellogenin (vtg) we used a non-competitive sandwich ELISA kit employing rabbit PAb against Atlantic cod vtg (paper III). Plasma samples from Atlantic cod were diluted 1:20, 1:5,000 and 1:50,000 in phosphate buffered saline (PBS) with Tween 20 and 1% bovine serum albumin. Samples were compared to purified Atlantic cod vtg protein standards. For analysis of rainbow trout vtg, we used a competitive ELISA using purified rainbow trout vtg as standards and rabbit PAb against Arctic char (*Salvelinus alpinus*) vtg (paper IV) (Larsson, et al., 1999; Parkkonen, et al., 2000). Plasma samples were diluted 1:20, 1:1,000 and 1:5,000 in PBS-Tween containing 1% milk.

The sex steroids, 17ß-estradiol, testosterone and 11-ketotestosterone, were measured using competitive sandwich ELISA kits (paper III and IV). Plasma was first extracted with diethyl ether. Atlantic cod plasma was concentrated (2:1) prior to the measurements, whereas rainbow trout plasma was diluted (1:3) in enzyme immunoassay buffer.

# RESULTS & DISCUSSION

## ALKYLPHENOLS AND THE GLUTATHIONE-DEPENDENT ANTI-OXIDANT SYSTEM

### *Glutathione*

Oxidative stress was in 1991 defined by Sies as "a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage" (Halliwell and Gutteridge, 1999). Oxidative stress could lead to increased formation of reactive oxygen species or decreased capacity of antioxidant defense systems (Halliwell and Gutteridge, 1999). This in turn could result in oxidative damage, alternatively the organism could adapt to oxidative stress by up-regulating the antioxidant defense system. GSH is one of the most important non-enzymatic defenses against oxidative stress (DeLeve and Kaplowitz, 1991).

Atlantic cod of both sexes were force-fed with various doses ranging between 0.02 and 80 ppm of a mixture of alkylphenols ($C_4$:$C_5$:$C_6$:$C_7$ ratio 1:1:1:1) or 5 ppm 17ß-estradiol (paper I). Female Atlantic cod showed increased hepatic total glutathione (tGSH) levels after one week of exposure to alkylphenol mixture (0.02 to 80 ppm), whereas tGSH levels decreased in females after four weeks exposure to 2 ppm alkylphenol mixture (paper I, fig. 1). In males, on the other hand, no effects on tGSH levels were seen after four weeks of exposure.

Increased hepatic GSH concentration in response to exposure to environmental pollutants have been observed in liver, gills and kidney (Gallagher, et al., 1992; Di Giulio, et al., 1993; Hasspieler, et al., 1994). The GSH biosynthesis takes place in the cytosol and is regulated by feedback inhibition. Increased GSH consumption will lead to an increase in synthesis to keep the glutathione homeostasis (Meister and Anderson, 1983). The ability of the cell to reduce GSSG by GR may be overcome under severe oxidative stress. Accumulated GSSG can actively be transported out of the cell to protect itself from a shift in the redox equilibrium (DeLeve and Kaplowitz, 1991).

Normally, almost all (>99%) intracellular glutathione is in its reduced form (Deneke and Fanburg, 1989). Thus, measuring total hepatic glutathione often reflects the reduced levels. The GSH/GSSG ratio can decrease through GSH depletion in connection with phase II conjugation or antioxidant defense. In paper I, the level of tGSH and GSH were measured and the tGSH/GSH ratio was calculated. The tGSH/GSH ratios were similar in controls, 17ß-estradiol and alkylphenol exposed groups. Similar observations have been reported in

rainbow trout where exposure to the PCB tetrachlorobiphenyl increased hepatic GSH concentrations as well as GSSG levels, even though the GSSG/GSH ratio remained unchanged (Otto and Moon, 1995). Atlantic cod had close to 100% of all glutathione in its reduced form after one week in control fish (paper I). The corresponding levels after four weeks were 95% for female and 91% for male Atlantic cod (paper I). Other studies have reported similar results. For example, eels (*Anguilla anguilla*) had 88% of all hepatic glutathione in its reduced form (Pena, et al., 2000), and in rainbow trout (*Oncorhynchus mykiss*) the hepatic glutathione pool consisted of 92% GSH and 4% GSSG (Otto, et al., 1997). The remaining glutathione is present within the cell as mixed disulfides (mainly GSS-protein) and as thioethers (DeLeve and Kaplowitz, 1991).

Male Atlantic cod displayed significantly higher hepatic GSH levels (512 nmol GSH per mg wet weight) compared to females (360 nmol). Sex differences in tGSH concentration also have been reported in a field-collected brown bullhead (*Ameiurus nebulosus*). Other biomarkers including superoxide dismutase and GST activities, also displayed sexual dimorphic expression (McFarland, et al., 1999). These results further illustrate the importance of sex determination in biomonitoring programs.

### *Glutathione reductase*

Glutathione reductase (GR) keeps the GSH-dependent antioxidant system active by reducing GSSG to GSH. The hepatic GR activity in female Atlantic cod exposed to alkylphenol mixture (0.02 to 80 ppm) was affected in a biphasic mode (paper I, Fig. 2). The GR activity had a tendency to increase at lower concentrations of alkylphenols and decreased at higher concentrations. Although the GR activity was reduced in the highest doses of alkylphenol mixture (40 to 80 ppm), GSH was retained in its reduced form (paper I, Fig.1A). Hepatic GR activities were significantly increased (about 80%) in fish of both sexes exposed for four weeks to 0.02 ppm alkylphenol mixture. This observed increase is conceivably involved in GSH homeostasis and increased GR activity indicates exposure to oxidative stress. Higher GR activities were observed in shorthorn sculpin (*Myoxocephalus scorpius*) from a polluted harbour than in fish from a cleaner harbour (Stephensen, et al., 2000). Furthermore, the GR activity was the most responsive enzyme in juvenile rainbow trout injected with the redox cycling compounds paraquat and menadione (Stephensen, et al., 2002). Increased hepatic GR activities were observed in European eel exposed to the herbicide molinate. Induction of GR activity correlated with increased GSH level as well as maintained GSH:GSSG ratio in the liver, resulting in extended survival under oxidative stress (Pena-Llopis, et al., 2001). These results in fish indicate that GR activity as well as tGSH levels

may be used for detecting changes in redox status and could be good biomarkers to assess exposure to oxidative stress.

### *Glucose-6-phosphate dehydrogenase*

NADPH is a reducing agent in the cell, provided by $NADP^+$ specific dehydrogenases such as glucose-6-phosphate dehydrogenase (G6PDH). This enzyme is activated when $NADP^+$ and GSSG levels are increased in relation to NADPH and GSH (Eggleston and Krebs, 1974). Hepatic G6PDH activities were induced in female Atlantic cod after one week exposure to the lowest dose of the alkylphenol mixture, representing higher NADPH-generating capacity. Increasing the dose and exposure times did not affect G6PDH activities, which suggests that the level of NADPH was constant (paper I, Table 1). The G6PDH activity in erythrocytes from Nile tilapia (*Oreochromis niloticus*) from a polluted site in Brazil was increased, although tGSH content decreased (Bainy, et al., 1996). 17ß-Estradiol treatment decreased the G6PDH activity in male Atlantic cod after four weeks of exposure to the same level, as seen in the female control group (paper I, Table 1). Winzer, et al. (2002) have shown that 17ß-estradiol inhibits G6PDH activity in isolated hepatocytes of immature European flounder (*Platichtys flesus*) of both sexes and that male hepatocytes showed higher G6PDH activity compared to females (Winzer, et al., 2002). This could imply that females are more susceptible to oxidative stress and xenobiotic exposure.

### *Glutathione* S*-transferase*

Glutathione *S*-transferases (GST) are abundant in the liver and catalyze conjugation of compounds including Phase I metabolites to endogenous GSH, thereby preventing them from causing oxidative stress (Halliwell and Gutteridge, 1999). The lowest dose of the alkylphenol mixture resulted in a decreased GST activity, using CDNB as substrate in male Atlantic cod, whereas higher doses had no effect (paper I, Table 2). GST activities in female Atlantic cod were not affected. However, using CDNB as substrate for measuring induction of cytosolic GST activity may be less relevant than the induction of certain GST isoenzymes (Sole, et al., 2002).

It has been suggested that glucuronidation (*i.e.* conjugation to glucuronic acid by UDP-glucuronosyltransferase) may be the major pathway in Phase II detoxification in fish (Clarke, et al., 1991; Otto and Moon, 1995). It also is known that glucuronidation is of particular importance in Phase II metabolism of alkylphenols (Lewis and Lech, 1996; Meldahl, et al., 1996; Thibaut, et al., 1998; Arukwe, et al., 2000b; Ferreira-Leach and Hill, 2001).

These results taken together shows that GST and G6PDH activities were not affected to a higher extent in Atlantic cod exposed to alkylphenols.

Nevertheless, tGSH levels and GR activities were affected, suggesting that alkylphenol exposure affects the redox status in Atlantic cod.

## EFFECTS OF ENVIRONMENTAL ESTROGENS ON CYP1A

### *Alkylphenols*

Treatment of male Atlantic cod with the alkylphenol mixture for four weeks resulted in induction of CYP1A protein expression (paper II, Fig. 1). The induction was dose-dependent. No induction of CYP1A protein levels was seen in females. The alkylphenol induction of CYP1A protein levels in male fish was not reflected on CYP1A-mediated EROD activities *in vivo*, possible due to enzyme inhibition. In fact, *in vitro* inhibition studies confirmed that the alkylphenol mixture efficiently inhibited the EROD activity. The individual alkylphenols $C_6$ and $C_7$ seem to be primarily responsible for this inhibitory effect. In female Atlantic cod, treatment with 17ß-estradiol had no effect on either CYP1A protein expression or EROD activity *in vivo*. However, four weeks exposure to 17ß-estradiol resulted in a significant decrease in EROD activity in males, whereas CYP1A protein expression was induced. In cultured rainbow trout hepatocytes, exposure to 4-*tert*-octylphenol and 17ß-estradiol both induced vitellogenesis, but only the 17ß-estradiol treatment resulted in markedly reduced basal EROD activities (Navas and Segner, 2000). Hence, effects on CYP1A catalytic activity vary between different estrogenic compounds and between different studies. Variables such as species, sex, reproductive stage, compound, route of exposure, time of exposure, doses and interactions with other compounds may influence the effects.

### *Xenoestrogens and ketoconazole*

Treatment of juvenile Atlantic cod with ketoconazole (12 mg/kg fish) resulted in a 60% increase in EROD activities (paper III, Fig. 1A). Mixed exposure to ketoconazole (12 mg/kg fish) and nonylphenol (25 mg/kg fish) resulted in a 70% increase in EROD activities as well as an almost 2-fold increase in CYP1A protein levels. Induction of hepatic CYP1A gene expression by exposure to imidazoles and/or triazoles also has been reported in rat, bobwhite quail (*Colinus virginianus*) and trout (Hostetler, et al., 1989; Ronis, et al., 1994; Egaas, et al., 1999; Hegelund, et al., 2004). However, it is possible that induction of EROD activity, partly or completely, is masked by CYP1A inhibition caused by ketoconazole present in the tissue. Chemical data are required before this can be concluded. Possible inhibition of EROD activity is supported in paper III, showing that ketoconazole was a potent non-competitive inhibitor of EROD activity *in vitro*. Ketoconazole and other

imidazoles also have been shown to be potent inhibitors of EROD activities in other vertebrates, *e.g.* in fish (Levine, et al., 1997; Miranda, et al., 1998; Levine and Oris, 1999; Hegelund, et al., 2004), birds and mammals (Ronis, et al., 1994; Ronis, et al., 1998; Guengerich, 2004).

Treatment of Atlantic cod with nonylphenol (25 mg/kg fish) and ethynylestradiol (5 mg/kg fish) resulted in decreased CYP1A activities (40 and 70% respectively) (paper III, Fig. 1A), whereas no effects of these substances were observed on CYP1A protein levels (paper III, Fig. 1B). *In vitro* inhibition studies confirmed that nonylphenol and ethynylestradiol acted as non-competitive inhibitors of the EROD activity. Hence, ketoconazole, nonylphenol, and ethynylestradiol interact with CYP1A enzymes, indicating a possible site for interaction of these different classes of xenobiotics. In addition, ketoconazole treatment induces CYP1A expression, which further may affect this interaction.

Treatment of juvenile rainbow trout with a combination of ketoconazole (100 mg/kg fish) and ethynylestradiol (2.5 µg/kg fish) resulted in more than a 4-fold increase in EROD activities 3, 6 and 12 days post injection (paper IV, Fig. 2). The CYP1A protein levels were elevated 10-fold in rainbow trout exposed to a mixture of ethynylestradiol and ketoconazole 6 days post injection (paper IV, Fig. 1B). This 10-fold increase was not reflected on the CYP1A-activity, suggesting CYP1A inhibition by ketoconazole. Exposure to ketoconazole alone resulted in elevated CYP1A protein expression 3 and 6 days post injection (paper IV, Fig. 1A) and increased EROD activities only 12 days post injection (paper IV, Fig. 2). Ketoconazole treatment has been shown to induce CYP1A gene expression in rainbow trout (Hegelund, et al., 2004). However, imidazoles also inhibits CYP1A mediated EROD activities in fish (Levine et al., 1997; Hegelund et al., 2004). Thus, the elevated CYP1A protein expression appears to be partly masked by CYP1A inhibition caused by ketoconazole presumably present in the tissue. However, it is not clear whether it is the ketoconazole parent compound or a ketoconazole metabolite that may activate the AhR.

There were no effects of the low dose of ethynylestradiol (2.5 µg/kg fish) on the CYP1A activities in juvenile rainbow trout (paper IV, Fig. 2). A significant reduction in CYP1A activity and CYP1A protein has been reported in different fish species after exposure to 17ß-estradiol, or after injection with ethynylestradiol (Arukwe, et al., 1997; Solé, et al., 2000b). In juvenile Atlantic salmon, this reduction was stronger in CYP1A protein levels than in CYP1A activity after administration of either nonylphenol (1-125 mg/kg fish) or 17ß-estradiol (5 mg/kg fish) (Arukwe, et al., 1997). The mechanism responsible for CYP1A inhibition by estrogens is still undefined (Sole, et al., 2002).

## EFFECTS OF ENVIRONMENTAL ESTROGENS ON CYP3A

### *Alkylphenols*

Exposure to the alkylphenol mixture resulted in a dose-dependent elevation of CYP3A protein levels in male Atlantic cod, whereas this treatment had no effect on CYP3A protein levels in females (paper II, Fig. 2). In addition, exposure of male fish to 17ß-estradiol resulted in slightly elevated CYP3A protein levels. Alkylphenols, i.e. nonylphenol and 4-*tert*-octylphenol, have been show to interact with CYP3A enzymes in rat (Lee, et al., 1996; Hanioka, et al., 1999; Hanioka, et al., 2000), implying that these enzymes are involved in alkylphenol metabolic clearance in mammals. *In vitro* inhibition studies revealed that alkylphenols ($C_4$ to $C_7$) are poor inhibitors of CYP3A-mediated BFCOD activity in Atlantic cod (paper II, Table 1). This is in contrast to efficient inhibition by alkylphenols ($C_4$ to $C_7$) on the CYP1A-mediated EROD activity. Expression of CYP3A genes in mammals is mediated by the nuclear PXR, which can be activated by steroids and xenobiotics including nonylphenol (Kliewer, et al., 2002). Nonylphenol was shown to activate the PXR mediated expression of CYP3A1 mRNA in rat and to block the proteasome dependent PXR degradation in mouse mammary cancer cells, resulting in elevated CYP3A expression (Masuyama, et al., 2000; Masuyama, et al., 2002). Thus, it is possible that similar mechanisms are responsible for the observed elevation of CYP3A expression in Atlantic cod.

### *Xenoestrogens and ketoconazole*

Juvenile Atlantic cod exposed to nonylphenol (25 mg/kg fish), ethynylestradiol (5 mg/kg fish) or ketoconazole (12 mg/kg fish) displayed reduced CYP3A activities (paper III, Fig. 2A). Furthermore, mixed exposure to ketoconazole and nonylphenol resulted in a 98% decrease in CYP3A activity, which was greater than the additive effects of these two compounds administrated alone. Thus, nonylphenol and ketoconazole appear to synergistically impair this CYP3A activity *in vivo*. However, mechanism for this possible interaction is not known. Nevertheless, the CYP3A inhibitory effect by ketoconazole is well known and ketoconazole is the most established diagnostic inhibitor, used to assess human CYP3A4 activities *in vitro* (Thummel and Wilkinson, 1998; Stresser, et al., 2002). Studies in fish demonstrate that ketoconazole is a potent inhibitor of hepatic CYP3A activities in killifish (*Fundulus heteroclitus*), rainbow trout and Atlantic cod with $IC_{50}$ values 0.01, 0.1 and 0.3 µM, respectively (Hegelund et al., 2004; paper II). Western blot analyses of CYP3A proteins using PAb against rainbow trout CYP3A revealed the presence of one CYP3A immunoreactive protein band in liver microsomes, with an apparent molecular size around 50 kD in Atlantic

cod (paper III, Fig. 3A). No effect on hepatic CYP3A protein levels was observed in fish treated with ketoconazole and nonylphenol, either alone or in combination. However, by using 2D-GE followed by immunoblotting, two CYP3A immunoreactive protein spots were detected above 50 kD in Atlantic cod microsomes (paper III, Fig. 3B). The more basic isoform appeared to be responsive to ketoconazole treatment, whereas ethynylestradiol and nonylphenol treatment resulted in suppressed expression of the more basic isoform (paper III, Fig. 3B).

The existence of multiple CYP3A genes has been shown in several vertebrate species, including teleosts, summerized in McArthur, et al. (2003). It is conceivable that there are two different CYP3A genes in Atlantic cod and that these genes respond differently to ketoconazole treatment. Further studies are required to elucidate whether these two CYP3A isoforms are different gene products, or due to post-translational modifications such as phosphorylation (Mann and Jensen, 2003). Phosphorylation of several members of the CYP2 gene family, through phosphokinase A, resulted in immediate loss in catalytic activity (Oesch-Bartlomowicz and Oesch, 2003). The shift to a more basic form in this report implies a dephosphorylation of CYP3A upon ketoconazole treatment (paper III). In juvenile Atlantic salmon, multiple CYP3A proteins also were seen, which responded differently to nonylphenol treatment. Nonylphenol (125 mg/kg fish) suppressed the high-molecular CYP3A protein band, whereas lower doses (≤25 mg/kg fish) of nonylphenol appeared to induce this isoform (Arukwe, et al., 1997).

Ethynylestradiol has been shown to act as a mechanism-based inhibitor of CYP3A4 activities (Guengerich, 1988; Lin, et al., 2002). A possible mechanism-based inactivation of CYP3A in Atlantic cod by ethynylestradiol was suggested (paper III). Thus, exposure to ethynylestradiol resulted in significantly reduced (22%) CYP3A levels and ethynylestradiol acted as an uncompetitive inhibitor of microsomal CYP3A activities. However, preincubation of hepatic microsomes with ethynylestradiol for up to 60 min had no significant effect on CYP3A protein content, which implies a different, yet unidentified, mechanism of inhibition (paper III, Fig. 6).

Treatment of juvenile rainbow trout to a low dose of ethynylestradiol (2.5 µg/kg fish) had no effect on CYP3A activities or protein expression in rainbow trout (paper IV). Exposure to ketoconazole (100 mg/kg fish) alone or in combination with ethynylestradiol resulted in 80% decreased CYP3A activities 3 and 6 days post injection (paper IV, Fig. 4). CYP3A protein expression was increased around 1.5-fold by ketoconazole after 6 and 12 days (paper IV, Fig. 3), although this was completely masked by inhibition of CYP3A activities. This temporal effect on CYP3A activity suggest efficient

clearance of ketoconazole, although chemical data are required for confirmation.

## SEXUALLY DIMORPHIC EXPRESSION OF CYP1A AND CYP3A GENES

### *CYP1A*

Sexual dimorphic expression of CYP1A genes in fish have been observed, where male fish usually have higher CYP1A levels than females (Stegeman and Woodin, 1984; Pajor, et al., 1990; Arukwe and Goksøyr, 1997). A cross-talk between ER and AhR has been described in mammals (Safe, et al., 1991; Klinge, et al., 2000; Safe, 2001), where the AhR ligand tetrachlorodibenzo-*p*-dioxin may inhibit ER, and conversely, estradiol affect AhR (Klinge, et al., 2000). In paper II, female Atlantic cod displayed slightly higher CYP1A protein levels than males (paper II, Fig. 1A). Furthermore, treatment of male Atlantic cod with 17ß-estradiol resulted in elevated CYP1A protein levels, whereas this treatment had no effect in females (paper II, Fig. 1B). The gender difference in CYP1A protein expression was not reflected on the EROD activity. Hence, untreated males had 2-fold higher CYP1A activities than females (paper II, Fig. 3 and 4). Furthermore, the sex difference in EROD activity was abolished by treatment with 17ß-estradiol, which resulted in pronounced suppression of CYP1A activity in male Atlantic cod. Treatment with 17ß-estradiol had no effect on EROD activity in females. *In vitro* inhibition studies show that the inhibitory effect of 17ß-estradiol on CYP1A activity was insignificant and thus does not explain the discrepancy between CYP1A protein levels and EROD activities in Atlantic cod. However, possible mechanism-based inhibition have not been investigated in this study. Future studies should investigate the possible AhR-ER cross-talk and other possible effects on post-translatory levels.

### *CYP3A*

Gender differences in CYP3A protein levels have been demonstrated in several investigations in vertebrates, including fish (Stegeman and Woodin, 1984; Celander, et al., 1989; Cok, et al., 1998). There was a pronounced sexually dimorphic expression of CYP3A proteins in adult Atlantic cod liver, with 7-fold higher protein levels in females compared to males. In rainbow trout and winter flounder (*Pseudopleuronectes americanus*), females displayed higher CYP3A expression or CYP3A activities than males (Stegeman and Woodin, 1984; Cok, et al., 1998; Lee, et al., 1998b). However, in rainbow trout during spawning and in adult killifish, males displayed higher CYP3A mRNA and protein expression compared to females (Celander, et al., 1989; Hegelund

and Celander, 2003). Furthermore, no gender differences in CYP3A levels were detected in scup (*Stenotomus chrysops*) (Gray, et al., 1991). The mechanism for the sexual dimorphic CYP3A expression is not fully understood, though it is possible that sex steroids are involved. Treatment with 17ß-estradiol resulted in down-regulation of CYP3A protein expression in immature brook trout (*Salvenius fontinalis*) and testosterone 6ß-hydroxylase activity (presumably CYP3A) in winter flounder (Pajor, et al., 1990; Gray, et al., 1991). In addition, treatment with 17ß-estradiol resulted in induced vitellogenesis and decreased CYP3A mRNA and protein levels in juvenile rainbow trout (Buhler, et al., 2000). An inverse relationship between PXR and ER expression was shown in human breast tumors (Dotzlaw, et al., 1999) and possible cross-regulatory mechanism(s) between PXR and ER cannot be ruled out. However, in the present study in first spawning Atlantic cod, 17ß-estradiol treatment resulted in elevated vitellogenesis and CYP3A protein expression in males (paper II). Thus, this sex steroid appears to be involved in regulation of CYP3A expression in fish, although species differences are apparent. It is not yet clear if 17ß-estradiol mediates a direct effect on CYP3A expression or indirect effect(s) through other hormones, feed-back mechanisms or possible PXR-ER cross-talks in fish.

## *IN VITRO* INHIBITION STUDIES OF CYP1A AND CYP3A ACTIVITIES IN ATLANTIC COD

The possible inhibitory effect of 17ß-estradiol and the alkylphenols ($C_4$ to $C_7$) were determined as the median inhibition concentration ($IC_{50}$) values in liver microsomes (paper II, Table 1). The $IC_{50}$ values for 17ß-estradiol, the alkylphenol mixture and each individual alkylphenol on CYP1A and CYP3A activities were determined in pooled liver microsomes from BNF treated and untreated Atlantic cod, respectively. In addition, the $IC_{50}$ values for these substances were determined in CYP3A4 cDNA expressed baculovirus supersomes, using BFC as substrate (paper II, Table 1).

The inhibitory effect of 17ß-estradiol on CYP1A activity was insignificant ($IC_{50}$=500 µM). However, the alkylphenol mixture ($C_4$ to $C_7$) efficiently inhibited CYP1A activity ($IC_{50}$=10 µM). The inhibitory effects of each individual alkylphenols also were determined. The $IC_{50}$ values for $C_4$, $C_5$, $C_6$ and $C_7$ were 300, 30, 15 and 12 µM, respectively. Thus, the potency for inhibition of CYP1A activity increases with increasing length of the 4-alkylchain. For comparison, the $IC_{50}$ value for ketoconazole on CYP1A activity was 0.6 µM (paper II, Table 1).

Estradiol inhibited CYP3A activity ($IC_{50}$=75 µM). Furthermore, the alkylphenol mixture ($C_4$ to $C_7$) also inhibited CYP3A activity ($IC_{50}$=100 µM). In addition, the inhibitory effects of each individual alkylphenol on CYP3A

activity were weak or insignificant (paper II, Table 1). The $IC_{50}$ values ranged between 60 and 250 μM and the rank order for inhibition were [$C_5 > C_6 > C_7 > C_4$]. For comparison, the $IC_{50}$ value for ketoconazole on CYP3A activity was 0.3 μM. A similar pattern was observed for BFCOD activities in cDNA expressed human CYP3A4 supersomes (paper II, Table 1).

Further *in vitro* inhibition studies using pooled Atlantic cod liver microsomes showed that nonylphenol, ethynylestradiol and a ketoconazole:nonylphenol (1:5) mixture inhibited CYP1A activity, with $IC_{50}$ values ranging from 5 to 20 μM (paper III, Table 1). The CYP3A catalytic activity also was inhibited by ethynylestradiol ($IC_{50}$= 35 μM) and the ketoconazole:nonylphenol (1:5) mixture ($IC_{50}$= 5:25 μM). Exposure to nonylphenol *in vivo* resulted in reduced CYP3A activities in juvenile Atlantic cod (paper III). However, nonylphenol did not inhibit microsomal BFCOD activities *in vitro* in Atlantic cod ($IC_{50}$=180 μM).

For comparison, $IC_{50}$ values for nonylphenol and ethynylestradiol also were determined for BFCOD activities in cDNA expressed human CYP3A4 baculovirus supersomes, compared to the prototypical CYP3A4 inhibitor ketoconazole ($IC_{50}$=0.4 μM). In contrast to Atlantic cod liver microsomes, nonylphenol inhibited the human CYP3A4 BFCOD activity ($IC_{50}$=35 μM) as well as ethynylestradiol ($IC_{50}$=50 μM) (paper III, Table 1).

The inhibitory effects of these compounds were further investigated on hepatic microsomal CYP1A and CYP3A enzyme kinetics (paper III). Thus, $K_i$ values were determined in Dixon plots and ketoconazole was a potent non-competitive inhibitor of both CYP1A and CYP3A activities with $K_i$ values in the sub-micromolar range (paper III, Fig. 4). Ethynylestradiol was a non-competitive inhibitor of CYP1A with $K_i$ from 3.5 to 5.4 μM and an uncompetitive inhibitor of CYP3A with $K_i$ from 54 to 85 μM (paper III, Fig. 5). Nonylphenol was a non-competitive inhibitor of CYP1A activity with $K_i$ around 3.5 μM. There were no effects of preincubation either with ketoconazole or ethynylestradiol (in the presence or absence of NADPH) on hepatic microsomal CYP3A protein levels in this study (paper III, Fig. 6).

Other studies have shown that ethynylestradiol is a mechanism-based inactivator of human liver microsomal CYP with loss of both spectrally detectable CYP and ethynylestradiol 2-hydroxylase activity during incubation in the presence of NADPH (Guengerich, 1988; Lin, et al., 2002). Ketoconazole also is a well known, highly potent inhibitor of CYP3A activity in humans (Venkatakrishnan, et al., 2001). Another antifungal imidazole, clotrimazole, was shown to be a noncompetitive mixed-type inhibitor of EROD activity with an $IC_{50}$ of 190 nM in rainbow trout (Levine and Oris, 1999). Hence, both ethynylestradiol and antifungal imidazoles affect CYP1A and CYP3A enzymes in vertebrates, which implies that these compounds are

involved in metabolic clearance of sex steroid hormones and xenobiotics, including EDCs.

## VITELLOGENESIS

Juvenile Atlantic cod exposed to nonylphenol (25 mg/kg fish), ethynylestradiol (5 mg/kg fish) and the combination of ketoconazole and nonylphenol showed increased plasma vtg levels (paper III, Table 2). However, nonylphenol dependent vitellogenesis was not affected by co-treatment with ketoconazole (paper III, Table 2).

Exposure to ethynylestradiol (2.5 µg/kg fish) had no significant effect 6 days post injection on vitellogenesis in juvenile rainbow trout (paper IV, Fig. 5). However, in combination with ketoconazole (100 mg/kg fish) this threshold-dose of ethynylestradiol resulted in a 4.5-fold increase of plasma vtg concentration (paper IV, Fig. 5). Thus, co-exposure to ketoconazole appears to make rainbow trout more sensitive to low exposure of ethynylestradiol. This effect was time-dependent as it was not observed 3 or 12 days post injection.

Schultz, et al., (2001) observed that a 12 h lag time occurred before increases in plasma vtg concentrations could be detected in male rainbow trout i.v. injected with ethynylestradiol (0.001 to 10.0 mg/kg). They also found maximal plasma levels around 7-9 days post injection. Increasing the ethynylestradiol dose or time of exposure probably resulted in an increase in estrogen receptor recruitment leading to progressively more vtg synthesis (Schultz, et al., 2001). This delay in response is believed to be due to recruitment of ER, which has been suggested by Nimrod and Benson (1997). In another time-course experiment on 17ß-estradiol and ethynylestradiol exposed adult male zebrafish, a major time-dependent increase in the vtg concentration was displayed until day 5 (Rose, et al., 2002). No further increase in vtg levels was not observed until termination of the experiment on day 8. Vitellogenesis is evidently a dynamic process. Temporal aspects as well as situations of mixed exposure should be considered when using this biomarker to assess estrogenic exposure in the laboratory and in the aquatic environment.

## SEX STEROID HORMONES

Treatment with nonylphenol (25 mg/kg fish), ethynylestradiol (5 mg/kg fish) and the combination of ketoconazole (12 mg/kg fish) and nonylphenol (25 mg/kg fish) had no significant effect on 17ß-estradiol, testosterone or 11-keto-testosterone plasma levels compared to control fish (paper III, Table 2). However, co-exposure to ketoconazole and nonylphenol resulted in 51% decrease in testosterone plasma concentration compared to fish treated with

ketoconazole (12 mg/kg fish) alone (paper III, Table 2). Thus, mixed exposure to xenoestrogen and ketoconazole may affect sex steroid homeostasis in Atlantic cod.

Treatments with ethynylestradiol (2.5 µg/kg fish), ketoconazole (100 mg/kg fish) or the ethynylestradiol and ketoconazole mixture had no significant effect on plasma 17ß-estradiol levels in juvenile rainbow trout (paper IV, Table 1-3). Testosterone levels, on the other hand, were reduced 50% after co-exposure to ketoconazole and ethynylestradiol 12 days post injection compared to fish treated with ketoconazole alone (paper IV, Table 3). The combined exposure also decreased plasma 11-keto-testosterone levels compared to fish exposed to ethynylestradiol alone 12 days post injection (paper IV, Table 3). The reason for this anti-androgen effect is not known and analyses of plasma levels of $EE_2$ may provide further insight.

The hypothalamus-pituitary-gonadal axis is a complex system and many mechanisms are involved in the regulation process (Brown, 2002). Response to this feedback may involve ER or brain aromatization of androgens to estrogens (Kime, 1999). Plasma steroid concentration reflects the rate of synthesis as well as the rate of deactivation and excreation by the liver (Kime, 1999). Therefore, low plasma steroid levels could be due to inhibited synthesis or elevated hepatic catabolism. In a previously conducted study, plasma vtg levels increased without a coincident significant increase in plasma estradiol levels in male carp (*Cyprinus carpio*) exposed to municipal sewage effluent. However, circulating testosterone levels were reduced (Folmar, et al., 1996). Phytoestrogens such as ß -sitosterol found in pulp mill effluent are capable of dramatically reducing plasma testosterone levels in goldfish by altering cholesterol availability (MacLatchy and Van Der Kraak, 1995). Testicular pieces from ß -sitosterol treated fish exhibited decreased basal release of both testosterone and pregnenolone, suggesting that depressed plasma steroid levels in males were due to direct effects on testicular steroid production. The decreased levels of pregnenolone produced by the ovary and testis from the ß -sitosterol treated fish further suggest that the sterol may effect cholesterol availability or reduce the activity of the side chain cleavage enzyme cytochrome $P450_{SCC}$ (CYP11), which mediates conversion of cholesterol to pregnenolone (MacLatchy and Van Der Kraak, 1995). It is not impossible that ketoconazole, the very potent CYP1A and CYP3A enzyme activity inhibitor, also is a possible inhibitor of CYP11 in our study. In fact, ketoconazole has been shown to inhibit several steroidogenic CYP enzymes in vertebrates, such as CYP11, CYP17, CYP19 and CYP51 (Weber, et al., 1991; Monod, et al., 1993; Denner, et al., 1995; Kühn-Velten and Löhr, 1996; Lamb, et al., 1999; Monteiro, et al., 2000). Still, CYP1A and CYP3A enzymes are possible candidates involved in metabolic clearance of sex steroids in vertebrates

(Waxman, et al., 1988; Miranda, et al., 1989; Waxman, et al., 1991; Wang and Strobel, 1997; Yamazaki, et al., 1998). Thus, reduced steroid levels reported here 12 days post injection may be due to increased clearance as a result of induced CYP1A and CYP3A protein levels together with declining inhibitory effect of ketoconazole. To conclude, ketoconazole acts as a broad-spectrum CYP inhibitor, which may interfere with metabolic clearance of sex steroid hormones and xenobiotics, including EDCs.

## CONCLUDING REMARKS

The general aim of this thesis was to study effects of environmental estrogenic compounds on cytochrome P450 (CYP), and to identify possible sites of interaction between estrogenic compounds and the model antifungal imidazole, *i.e.* ketoconazole. Produced water from offshore oil production is a source of environmental estrogenic compounds. Produced water contains significant amounts of alkylphenols, which are continuously released into the aquatic environment. Alkylphenols ($C_4$ to $C_7$) induced CYP1A and CYP3A protein expression in male Atlantic cod, but not in females. In contrast, treatment with alkylphenols had no effect on CYP1A activities in males and only slightly induced CYP1A activities in females. Treatment with estradiol resulted in increased CYP1A protein expression but decreased CYP1A activities in males, whereas estradiol had no effect in females. Thus, estradiol and alkylphenols are both estrogenic, but have different effects on CYP1A activities *in vivo*. *In vitro* inhibition studies showed that the alkylphenol mixture (but not estradiol) efficiently inhibited CYP1A activity, suggesting that these two types of estrogenic compounds have diverse effects on CYP1A activities in Atlantic cod. Furthermore, alkylphenols increased oxidative stress responses in Atlantic cod. We found elevated tGSH levels and increased GR activity in alkylphenol treated Atlantic cod. The consequence of the discharges of produced water is still not known, but there is good reason to believe that long-term exposure will have negative effects on marine organisms. It is therefore critical that the discharges of oil and chemicals from produced water are reduced as much as possible.

We identified interactions between ketoconazole and two different types of estrogenic compounds on CYP1A and CYP3A in both juvenile Atlantic cod and rainbow trout *in vivo*. Ketoconazole induced CYP1A and CYP3A protein expression, whereas CYP1A and CYP3A activities were inhibited to various degrees. These studies suggest that induction of CYP1A and CYP3A gene expression can be partly or completely masked by inhibition of catalytic activities *in vivo* in these species. *In vitro* studies using Atlantic cod liver microsomes revealed that ketoconazole acted as a non-competitive inhibitor of both CYP1A and CYP3A activities, ethynylestradiol acted as a non-competitive inhibitor of CYP1A and an uncompetitive inhibitor of CYP3A activities and nonylphenol was a non-competitive inhibitor of CYP1A but did not inhibit CYP3A. Furthermore, interactions between ketoconazole and CYP1A and CYP3A in rainbow trout resulted in increased responsiveness to ethynylestradiol exposure, measured as induction of plasma vtg levels. These results indicate that CYP1A and CYP3A represent sites of interactions between these classes of xenobiotics and that ketoconazole exposure may

increase the sensitivity to exposure of low ethynylestradiol concentrations. Combined exposure to ethynylestradiol and ketoconazole resulted in suppressed levels of circulating androgens. However, the mechanism(s) for this response is still not understood and at this point we can only speculate. Ketoconazole is known to inhibit steroidogenic CYP enzymes, such as CYP11, CYP17, CYP19 and CYP51 in vertebrates. It is possible that ketoconazole affected these steroidogenic CYP enzymes also in this study, resulting in decreased steroid hormone synthesis.

We have studied short-term effect of EDCs in fish. In order to better understand the long-term effects, more research is needed to estimate the possible adverse effect. Furthermore, more research is needed to identify additional EDCs and to develop and optimize new, reliable and sensitive assay systems. For estrogenic compounds, there are features that make risk assessment difficult, such as existence of receptor cross-talk, dependence of effects on sex, age, polymorphism, dietary compounds and complicated toxicokinetics with plasma protein binding. It is therefore important to learn more about interindividual differences, with regards to age, dose, duration of exposure and pharmacogenetics. Another aspect is to learn more about interactions between multiple synthetic chemicals *in vivo*. For example, with azole fungicides, which interact with CYP1A and CYP3A and possible also with steroidogenic CYP enzymes. It is important to move forward with further studies on metabolic clearance of endocrine disrupters and steroid hormones to better understand the molecular and physiological mechanisms of endocrine disruption.

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