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Azoles and Contaminants in Treated Effluents Interact with CYP1 and CYP19 in Fish

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Abstract
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Numerous contaminants are present in mixtures in the aquatic environment. Among these are the azoles, a group of chemicals that includes both pharmaceuticals and pesticides. Azole fungicides are designed to inhibit lanosterol 14-demethylase (cytochrome P450 (CYP) 51), while other azoles are intended to inhibit aromatase (CYP19), i.e. the enzyme catalyzing biosynthesis of estrogens. In fish, a variety of CYP enzymes are involved in biotransformation of waterborne contaminants, and in metabolism of endogenous compounds including steroidal hormones. The induction of CYP1A protein and 7-ethoxyresorufin *O*-deethylase (EROD) activity are common biomarkers for exposure to aryl hydrocarbon receptor (AhR) agonists in fish. We developed an assay to measure inhibition of CYP1A activity (EROD) in three-spined stickleback and rainbow trout gill tissue *ex vivo*. Several azole fungicides were found to be potent inhibitors of CYP1A activity. A wastewater effluent containing high concentrations of pharmaceuticals was also shown to inhibit CYP1A activity. Further, several azoles inhibited CYP19 activity in rainbow trout brain microsomes *in vitro*. Azole mixtures reduced both CYP1A and CYP19 activity monotonically and in an additive way. Given the additive action of the azoles, studies to determine adverse effects of azole mixtures on CYP-regulated physiological functions in fish are needed. Induction of EROD and of gene expression of *CYP1* in several organs was observed in an *in vivo* exposure with the same effluent shown to inhibit EROD. This finding could imply that there was a mixture of AhR agonists and CYP1A inhibitors in the effluent. Finally, wastewater treatment technologies were evaluated using biomarker responses in rainbow trout exposed to effluents of different treatments. The results from chemical analysis together with the biomarker results show that ozone and granulated active carbon treatment removed most pharmaceuticals, as well as AhR agonists and other chemicals present in the regular effluent. This part of the thesis demonstrates that biomarkers in fish such as induction of CYP1 gene expression are applicable to evaluate the efficiency of different treatment technologies for wastewater.

Keywords: Azole, fungicide, chemical, CYP1A, CYP19, EROD, aromatase, effluent, STP, wastewater, fish, stickleback, rainbow trout

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Beijer, K**., Abrahamson, A., Brunström, B., Brandt, I., CYP1A inhibition in fish gill filaments: A novel assay applied on pharmaceuticals and other chemicals, *Aquatic Toxicology*, 96 (2010) 145-150.

II **Beijer, K**., Jönsson, M., Shaik, S., Behrens, D., Brunström, B., Brandt, I., Azoles inhibit cytochrome P450 enzymes in rainbow trout involved in biotransformation and steroid hormone synthesis additively. Manuscript.

III **Beijer, K**., Gao, K., Jönsson, M.E., Larsson, D.G., Brunström, B., Brandt, I., 2013. Effluent from drug manufacturing affects cytochrome P450 1 regulation and function in fish. *Chemosphere* 90, 1149-1157.

IV **Beijer, K**., Shaik, S., Björlenius, B., Lindberg R., Brunström, B., Brandt, I., Reduction of pharmaceuticals and other contaminants in sewage treatment effluents by active carbon filtration and ozonation- *Evaluation using biomarker responses in fish and chemical analysis*. Manuscript.

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Abbreviations

αNF	α- naphthoflavone
B[a]P	Benzo[a]pyrene
βNF	β-naphthoflavone
CYP	Cytochrome P450
DMSO	Dimethyl sulfoxide
FICZ	6-formylindolo[3,2-b]carbazole
PETL	Patancheru Enviro Tech Ltd (treatment plant)
PCB126	3,3',4,4',5-pentachlorobiphenyl
STP	Sewage treatment plant
TCDD	2,3,7,8-tetracholorodibenzo-p-dioxin

Introduction

Environmental contaminants

Pharmaceuticals

Pharmaceuticals are increasingly detected in sewage effluents and receiving waters and have thus lately become a focus of attention as environmental pollutants (Daughton and Ternes, 1999; Fent et al., 2006; Heberer, 2002; Nikolaou et al., 2007). Pharmaceuticals can be released to the environment by consumers' use, disposal, and use in hospitals. Several pharmaceuticals have been found in Swedish sewage treatment plants (STP) effluents and surface waters (Fick et al., 2011). In rainbow trout exposed to effluents from three Swedish STPs, 14 of 25 pharmaceuticals analyzed were detected in plasma of fish (Fick et al., 2010).

Pharmaceuticals are produced in large quantities, and it is only recently shown that substantial amounts may be discharged from the production plants. In the US, discharges from pharmaceutical manufacturing facilities led to concentrations of pharmaceuticals 10-1000-fold higher than normally found in wastewater treatment plant effluents (Phillips et al., 2010). A study in Patancheru, India, showed extremely high pharmaceutical concentrations in the effluent from a wastewater treatment plant receiving wastewater from about 90 pharmaceutical manufacturers (Larsson et al., 2007). This huge release has resulted in the contamination of surface, ground, and drinking water in the Patancheru area (Fick et al., 2009). Furthermore, veterinary pharmaceuticals may enter the environment directly; after having passed the animal body they reach the soil and subsequently ground or surface water. In fish farming, antibiotics are distributed directly to the water.

Pharmaceuticals have a high biological activity and selectivity, and are designed to target specific metabolic and molecular pathways in humans and animals. Many STPs cannot purify the water from all pharmaceutical substances, and consequently the substances are released into the aquatic environment (Heberer, 2002). These substances can affect biological processes at low concentrations and can - as a matter of fact - become a problem when they reach the aquatic environment.

Azole fungicides

Azole fungicides have been found in effluents from STPs, rivers, lakes and in muscle tissue of fish caught close to STP outlets (Belenguer et al., 2014; Fick et al., 2011; Kahle et al., 2008; Lindberg et al., 2010; Peng et al., 2012; Stamatis et al., 2010). Azoles are five-membered heterocyclic compounds containing one or more nitrogen atoms in the ring. Azole fungicides are designed to block the synthesis of ergosterol by inhibiting lanosterol 14-demethylase (cytochrome (CYP) 51) which is essential for cell membrane integrity in fungi. Azole compounds including imidazoles, triazoles, benzimidazoles and benzotriazoles (example of azole structures are given in *Figure 1*) have broad applications and are used in medicine, agriculture, materials' protection and more. They are used in agriculture for control of fungi in vegetable and fruit production and as biocidal products for wood preservation, in paints, concrete, and roofs. In medicine they are applied in the treatment of systemic or dermal fungal infections. In addition, certain azoles have been developed as aromatase inhibitors for treatment of estrogen-responsive breast tumors in pre- and postmenopausal women.

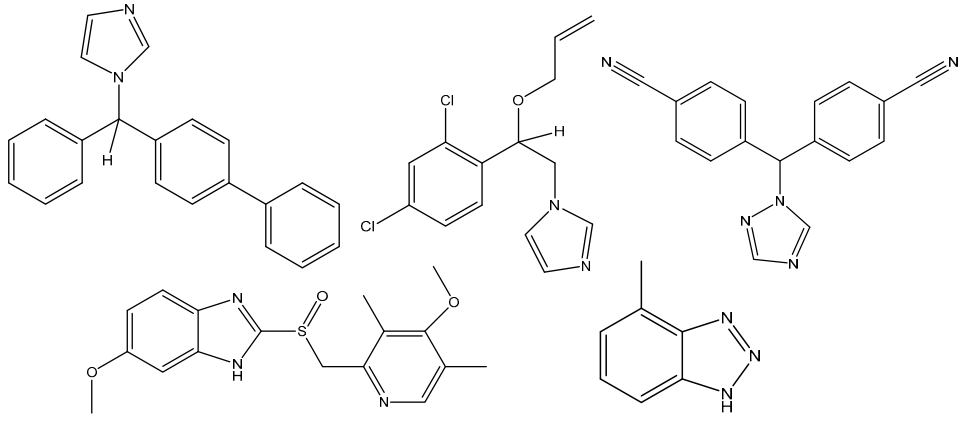

Figure 1. From left to right structural formulas of the imidazoles bifonazole, imazalil, the triazole letrozole (first row), the benzimidazole omeprazole, and the benzotriazole 4-methyl-1H-benzotriazole (second row).

The cytochrome P450 superfamily

The CYP monooxygenases are a superfamily of heme-containing enzymes involved in metabolism of xenobiotic compounds such as chemical pollutants, pharmaceuticals, and endogenous compounds such as steroids, fatty acids, and prostaglandins. In fish, there are 18 families of CYP genes (CYP1, CYP2,

CYP3, CYP4, CYP5, CYP7, CYP8, CYP11, CYP17, CYP19, CYP20, CYP21, CYP24, CYP26, CYP27, CYP39, CYP46 and CYP51) and numerous subfamilies, reviewed by Uno et al. (2012). CYPs catalyze phase I reactions, and convert lipophilic substances to more water-soluble substances mainly by oxidation.

CYP1(A) and induction

The role of CYP1A is in most cases detoxification and a first step towards excretion. Expression of CYP1A enzyme can be induced by xenobiotics. The classical mechanism is reviewed by Beischlag et al. (2008) and Denison et al. (2011), and starts when a compound enters the cell and binds to the cytosolic aryl hydrocarbon receptor (AhR). Following binding of a ligand to the AhR, the AhR-ligand complex translocates to the nucleus, and dimerizes with the AhR nuclear transporter (ARNT). The ARNT-AhR-ligand complex induces the transcription of genes for instance coding for biotransformation enzymes such as CYP1A. Recently, several nonclassical mechanisms of AhR action have also been proposed, involving interaction and cross talks of the AhR with other nuclear proteins and signaling factors, reviewed by Denison et al. (2011).

To assess exposure to AhR ligands in fish, the aryl hydrocarbon hydroxylase (AHH) assay (Payne and Penrose, 1975) and the 7-ethoxyresorufin-O-deethylase (EROD) assay have been used, reviewed by Whyte et al. (2000). EROD is commonly measured in liver microsomes and was first determined in rat liver microsomes (Burke and Mayer, 1974). Expression of CYP1A and EROD activity are highly inducible in liver, gills and some other tissues in fish (Gao et al., 2011; Jönsson et al., 2006; Ortiz-Delgado et al., 2005; Sarasquete and Segner, 2000; Smolowitz et al., 1991; Smolowitz et al., 1992). A protocol for determining EROD activity in gill filament tips of rainbow trout was first described by Jönsson and co-workers (2002).

Induction of *CYP1*-transcripts used as a biomarker

The mRNA expression of *CYP1A* is known to be highly inducible by a number of AhR agonists in various fish species. Apart from *CYP1A*, members of subfamilies *CYP1B*, *CYP1C* and *CYP1D* have been identified in fish. In three-spined stickleback, mRNA of *CYP1A*, *CYP1B1* and two forms of *CYP1C* (*CYP1C1* and *CYP1C2*) have been identified (Gao et al., 2011), and in rainbow trout (*Oncorrhynchus mykiss*), mRNA of two forms of *CYP1A* (*CYP1A1* and *CYP1A3*) (Berndtson and Chen, 1994; Råbergh et al., 2000), *CYP1B1*, and three forms of *CYP1C* (*CYP1C1*, *CYP1C2* and *CYP1C3*) have been found (Jönsson et al., 2010).

Recently, the transcription of these new CYP1 enzymes has been studied, and it has been demonstrated that *CYP1B* is inducible in fish by AhR agonists

(Jönsson et al., 2010; Jönsson et al., 2007; Willett et al., 2006; Yin et al., 2008; Zanette et al., 2009). In addition, *CYP1C1* can be strongly induced in killifish, zebrafish and three-spined stickleback after exposure to PCB126; however, *CYP1C2* seems to be much less responsive to AhR agonists (Gao et al., 2011; Jönsson et al., 2007; Zanette et al., 2009). In rainbow trout, the three *CYP1C* forms seem to be equally inducible by PCB126 (Jönsson et al., 2010). *CYP1D1* is not inducible by PCB126 or TCDD in fish (Goldstone et al., 2009; Zanette et al., 2009). Although *CYP1B* and the *CYP1C* forms are inducible by AhR agonists, their role in biotransformation still needs to be clarified in fish.

AhR agonists and CYP1 inducers

"Classical" AhR ligands are hydrophobic and planar substances, and include halogenated aromatic hydrocarbons (HAHs) such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and polycyclic aromatic hydrocarbons (PAHs). There are other compounds such as YH439, thiobendazole, omeprazole, 1,5-diaminonaphthalene and guanabenz that induce AhR-dependent gene express ion. These compounds are "nonclassical" AhR ligands because they can contain only a single unsaturated ring and must not have hydrophobic and planar characteristics; in addition there are conflicting reports stating that they do or do not bind to the AhR, reviewed by Denison and Nagy (2003). Furthermore, naturally occurring dietary chemicals can also induce CYP1A, e.g., curcumin (Ciolino et al., 1998a), some flavonoids (Gradelet et al., 1996a; Gradelet et al., 1996b) and carotenoids (Canivenc-Lavier et al., 1996; Ciolino et al., 1998b). A variety of endogenous compounds have been reported to be AhR agonists including tetrapyrroles, arachidonic acid metabolites and indole-containing compounds such as FICZ (6-formylindolo[3,2-b]carbazole) formed from tryptophan (Rannug et al., 1995), indigo and indirubin, reviewed by Denison et al. (2003). Hu and co-workers (2007) found that 81 of 137 compounds including pharmaceuticals, toxicants, industrial chemicals or biochemical standards induced CYP1A1 in rat liver, kidney and heart *in vivo.* However, only a few of the substances were confirmed to bind or activate the AhR *in vitro,* and therefore the unspecificity of CYP1A as a biomarker of AhR activation has been discussed.

Aromatase (CYP19)

There are two major types of enzymes in the pathways of steroid hormone synthesis (steroidogenesis): CYP enzymes and hydroxysteroid dehydrogenases (HSDs), reviewed for human and mouse by Payne and Hales (2004). Steroid biosynthesis pathways have been outlined for zebrafish (*Figure 2*) but only some enzymes have been proven to catalyze postulated reactions (Tokarz et

al., 2013). In these pathways, steroids are generated from cholesterol. The biosynthesis of estrogens is catalyzed by aromatase (CYP19). This enzyme converts C19 androgens into C18 estrogens, reviewed by Akhtar et al. (2011). In fish, aromatase is expressed primarily in ovary and brain. Many teleost fish express two *CYP19* genes (*CYP19A* and *CYP19B*) coding for the proteins CYP19A and CYP19B. In rainbow trout, *CYP19B* is mainly expressed in brain, whereas *CYP19A* is mainly expressed in ovary (Tanaka et al., 1992; Valle et al., 2002). Many fish species have very high brain aromatase activity compared to other vertebrates (Borg et al., 1987; Diotel et al., 2010; Goncalves et al., 2008; Timmers et al., 1987). Due to this high aromatase activity in brain fish is a suitable model for inhibition studies of aromatase *in vitro* and *in vivo*.

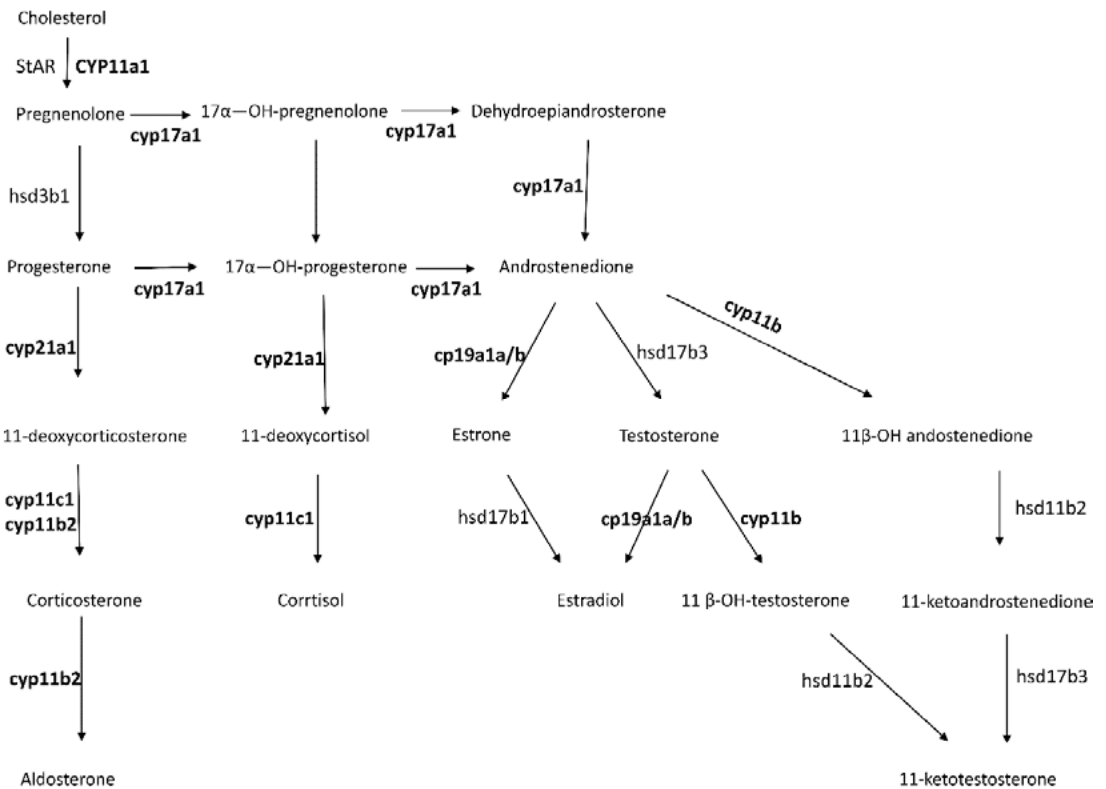

Figure 2. Proposed steroid biosynthesis pathway in zebra fish (*Danio rerio*) (Tokarz et al., 2013)

Aims

This thesis is part of the MistraPharma research program that among other things intends to identify human pharmaceuticals that could be of concern to aquatic ecosystems and to propose improved wastewater treatment technologies. The overall objective of my thesis was to identify human pharmaceuticals and other chemicals that might be a concern to aquatic ecosystems in terms of interference with levels and activities of CYP enzymes, in particular CYP1A and CYP19. Furthermore, wastewater treatment technologies developed in the MistraPharma program were evaluated using biomarker responses and chemical analysis in fish. The specific aims were to:

- Develop a gill filament EROD assay to study inhibition of EROD activity in three-spined stickleback and rainbow trout.
- Identify potent inhibitors of gill CYP1A in fish among pharmaceuticals and other compounds
- Determine the inhibitory effect on gill CYP1A of an effluent containing high levels of pharmaceuticals and to characterize the biological activity of this effluent using biomarker responses in stickleback
- Define potencies of azole fungicides to inhibit activity of CYP19 in brain of rainbow trout
- Evaluate additional STP technologies using biomarkers in rainbow trout

Methods

Test systems

Three-spined stickleback

Stickleback is a small teleost fish distributed over the northern hemisphere, the spread of which includes almost all European countries in limnic, brackish and marine environments. Besides, the genome of stickleback is fully sequenced, which is an advantage when studying gene expression. Stickleback have a male-specific reproductive protein, spiggin, which is a glue protein that males use to build nest. Therefore, stickleback is a good model for studying reproductive disturbance due to its character of detecting both androgenic (induction of spiggin) and estrogenic (induction of vitellogenin) action (Katsiadaki et al., 2002). In this thesis I used these two biomarkers to test if an effluent had androgenic or estrogenic activity (paper III). Stickleback is an indigenous species to European countries and can therefore be used in laboratory and field studies. It adapts readily to laboratory conditions and can also spawn in the laboratory.

In the experiments, adult stickleback were caught in Öresund at the Swedish south-west coast, brought to the Evolutionary Biology Centre's fish facility at Uppsala University, and held in flow through tanks supplied with copper-free tap water. In order to keep the fish in a non-reproductive state, they were held at a short day length (8 h light/16 h dark) and at a low temperature 8-13˚C.

Rainbow trout

Rainbow trout is one of the most commonly used fish in aquatic toxicology. This wild cold-water species has a native habitat in the Pacific Ocean from northwest Mexico to Alaska. They are also cultivated throughout the world and have been introduced in many countries for food or sport. Rainbow trout are easily obtained from local hatcheries and can be maintained in big tanks and clean flow-through water. The hatchery-reared form is a freshwater species. *In vivo* exposures of rainbow trout, primary cultures of trout liver cells, cell lines such as rainbow trout liver cell line (RTL-W1) (Lee et al., 1993), and rainbow trout gonad cell line (RTG-2) (Wolf and Quimby, 1962) are often used to study e.g. EROD activity, gene expression of *CYP1A* or estrogenic activity of chemicals, reviewed by Fent (2001). We used rainbow trout gill

filaments *ex vivo* and brain microsomes *in vitro* to study inhibition of enzymes involved in biotransformation and estrogen synthesis. In addition, we used *in vivo* exposure and examined gene expression in liver of estrogen sensitive markers (zona pellucida mRNA levels) to determine if STP effluents had estrogenic activity.

In this thesis, juvenile rainbow trout were purchased from local hatcheries. They were fed daily with pellets and held at 8-13˚C in flow through tanks supplied with copper-free tap water.

Chemicals

Not only several azoles applied as pharmaceutical fungicides (bifonazole, clotrimazole, fluconazole, letrozole, ketoconazole, miconazole, omeprazole and sulfamethoxazole), as agricultural fungicides (bitertanol, flusilazole, imazalil, prochloraz, propiconazole and tebuconazole), or as fungicides used in the protection of wood (propiconazole and tebuconazole) were examined. But the pharmaceuticals, carbamazepine, diclofenac, disulfiram and ellipticine were also tested. Moreover, other chemicals such as the flavonoid acacetin, benzo[a]pyrene (B[a]P), β-naphthoflavone (βNF), caffeine, and indigo were included.

The effluent water from the treatment plant in Patancheru (PETL), studied in paper III, contained high concentrations of pharmaceuticals; a complete list of concentrations is published elsewhere (Carlsson et al., 2009; Larsson et al., 2007).

The STP effluents in Käppala and Uppsala were subjected to chemical analysis of pharmaceuticals (IV).

Experiments

Experiments were performed to determine *ex vivo* inhibition of CYP1A activity in gills of stickleback and rainbow trout by several azoles and other compounds (I and II), as well as an effluent containing high levels of pharmaceuticals (III). Inhibition of CYP19 activity by azoles was studied *in vitro* using rainbow trout brain microsomes (II). Moreover, mixtures of azoles were studied using the *ex vivo* CYP1A and *in vitro* CYP19 inhibition assays (II).

Biological activity of effluents was investigated by exposing three-spined stickleback or rainbow trout to different effluents. Endpoints such as CYP1A activity (EROD), mRNA expression of *CYP1* genes and mRNA expression of other marker genes were analyzed.

EROD (CYP1A)

Induction assay

In paper III, we used the gill EROD assay, adapted to gill arches of three-spined stickleback. Briefly, excised gill filament arches were placed in 12-well tissue culture plates containing reaction buffer (HC buffer supplemented with 7-ethoxyresorufin and dicumarol). At two time points, aliquots of buffer from each well was taken and fluorescence was determined using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. EROD activity was calculated as the mean of duplicate measurements for each fish and expressed as pmole resorufin formed per filament and minute.

In paper IV, we used the EROD assay developed for rainbow trout gill filament tips (Jönsson et al., 2002). For each fish, ten gill filament tips were prepared for each of two wells.

Inhibition assay

The inhibition model of gill EROD activity in stickleback is described in paper I. To induce CYP1A activity, fish were exposed to waterborne βNF for 24 h. Then gill filament arches were exposed for 2 h to the inhibitors or the PETL effluent in the buffer. EROD activity was then measured following the protocol for induction of CYP1A as described above. The test substances or the PETL effluent were present in the buffers throughout the assay.

The inhibition model used in rainbow trout is described in paper II. A similar assay as that described above in stickleback (I) was used, except that induction of CYP1A activity was achieved by exposing excised gill filament tips *ex vivo* to the AhR-ligand FICZ for 6 h. Subsequently, the CYP1A-induced gill filaments were exposed to azoles for 2 h and thereafter the protocol was identical to the protocol described in paper I.

Aromatase (CYP19)

Inhibition assay

Preparation of brain microsomes was done according to Hinfray et al. (2006) by pooling female and male rainbow trout brains. The total amount of microsomal protein was determined using BSA as a standard with a BCA protein assay reagent kit.

Following microsome preparation, CYP19 activity was analyzed using the tritiated water assay, mainly according to Hinfray et al. (2006). In short, the release of tritiated water during conversion of [1β-^{3}H (N)] androst-4-ene-3,17-dione to estrone was measured. DMSO (used for the control CYP19 activity) or azoles dissolved in DMSO were added to a buffer containing a NADPH-

generating system. Brain microsomal protein was added to the reaction mixture and incubated for 1 h at room temperature. The reaction was then started by addition of substrate ([1β-^{3}H (N)] androst-4-ene-3,17-dione). After 45 min the reaction was stopped by adding chloroform. The aqueous layer was removed and mixed with charcoal. The supernatant was removed and mixed with a scintillation cocktail and radioactivity was measured for 10 min. CYP19 (aromatase) activity was expressed as fmole estrone formed per min and mg protein.

Quantitative (real-time) PCR

The procedure for qPCR analysis is described in detail in paper III and IV. Total RNA was prepared and DNase-treated. The quantity and purity of RNA was determined, and the 260/280 and 260/230 nm ratios were generally 2 or above. RNA integrity was checked via gel electrophoresis and confirmed to be good. Subsequently, cDNA was synthesized. Gene-specific quantitative PCR primers for the genes of interest were synthesized for three-spined stickleback (III) and rainbow trout (IV). Quantitative PCR was conducted using cDNA samples in duplicate with the following protocol: 95°C for 10 min, followed by 35 cycles of 95°C for 15 sec and 62°C for 45 sec. At the end of each PCR run a melt curve analysis was performed in the range from 55°C to 95°C. Mean values of PCR efficiency (E) were determined and relative mRNA expression of target genes was calculated using the $E^{-\Delta\Delta CT}$ method (using a reference gene).

Results and discussion

Inhibition of Cytochrome P450 enzymes

CYP1A

Several substances including pharmaceuticals, azole fungicides, other chemicals and finally an effluent containing high levels of pharmaceuticals were demonstrated to inhibit the catalytic activity of CYP1A (summarized in *Figure 3*).

The most potent inhibitor of CYP1A activity appeared to be ellipticine which eliminated activity to a non-detectable level. Ellipticine is an antitumor drug and a highly efficient inhibitor, in particular of CYP1A (Aimova and Stiborova, 2005; Lesca et al., 1978). The azoles bifonazole, bitertanol, clotrimazole, imazalil, ketoconazole, miconazole and prochloraz were shown to be potent inhibitors. Concentration-response relationships were established for all of them (paper I and II); IC_{50} values were determined for bifonazole, clotrimazole, imazalil, miconazole, and prochloraz (paper II, Table 1). The benzimidazole omeprazole was a weak inhibitor compared to the other azoles. Another CYP1A inhibitor with similar potency as the azoles was the flavonoid acacetin found in plants and used in oriental medicine. Flusilazole, tebuconazole and 4- and 5-methyl-1H-benzotriazole significantly inhibited CYP1A activity but seemed to be weaker inhibitors at the concentrations tested. Compounds that did not significantly inhibit CYP1A activity at the concentrations studied were B[a]P, βNF, caffeine, carbamazepine, diclofenac, disulfiram, indigo, the triazole letrozole and sulfamethoxazole.

The method was applied to test an effluent from a treatment plant in Patancheru, India, containing extremely high concentrations of pharmaceuticals (paper III). The effluent inhibited the βNF-induced CYP1A activity monotonically; decreased activities were obtained for dilutions to 2.5, 5, 10, 20 and 40 % (*Figure 4*). Of the compounds analyzed in the PETL effluent, the fluoroquinolone ciprofloxacin showed the highest concentration (31 mg/L; 94 μM) (Larsson et al., 2007). We suspected that ciprofloxacin and the other fluoroquinolones detected in the effluent could be a contributing factor to the inhibitory effects. Results from our experiments showed that 10 μM (3.3 mg/L) ciprofloxacin had significantly lower activity than the βNF-induced CYP1A activity (*Figure 5*). This concentration (10 μM, 3.3 mg/L) can be compared with the concentration of 10 % PETL effluent in paper III (about 3.1 mg/L

ciprofloxacin). However, 10 % PETL effluent (3.1 mg/L ciprofloxacin) appeared to inhibit CYP1A stronger than 3.3 mg/L ciprofloxacin, suggesting that the effluent contained also other contaminants that inhibited CYP1A activity. Fluoroquinolones are known to inhibit CYP1A activity *in vitro* in liver microsomes of rainbow trout, zebrafish, dog, human, and rat (McLellan et al., 1996; Regmi et al., 2005; Smith et al., 2012). Enrofloxacin has been shown to strongly decrease hepatic CYP-dependent monooxygenase activities including EROD in sea bass (*Dicentrarchus labrax*), when administered as an acute intraperitoneal dose of 3 mg/kg (Vaccaro et al., 2003).

We showed that inhibition of CYP1A can be studied in gill tissue of two fish species, indicating that the methods developed could be a general tool in various fish species to detect inhibition of CYP1A activity by waterborne xenobiotics. Moreover, several compounds such as acacetin, bifonazole, bitertanol, imazalil, flusilazole, tebuconazole, 4- and 5-methyl-1H-benzotriazole, previously not known to interfere with the fish CYP1A enzyme activity, were identified as inhibitors.

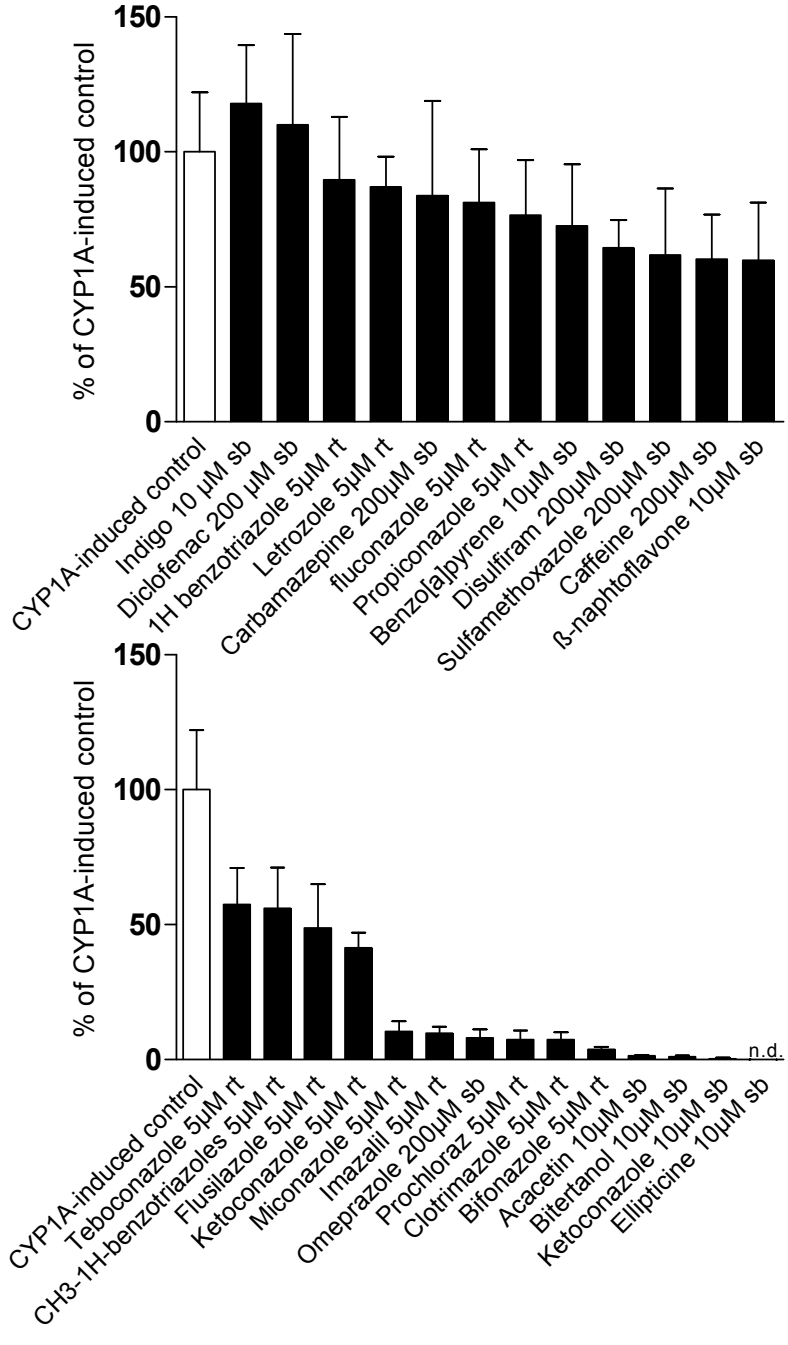

Figure 3. Summary of all chemicals tested for inhibition of CYP1A activity. CYP1A activity for each chemical is expressed as % of the induced control. Inhibition assays with stickleback gills or rainbow trout gills were used and denoted sb or rt in the figure. Concentrations between 5 and 200 µM were used; n.d. indicates activity not detected.

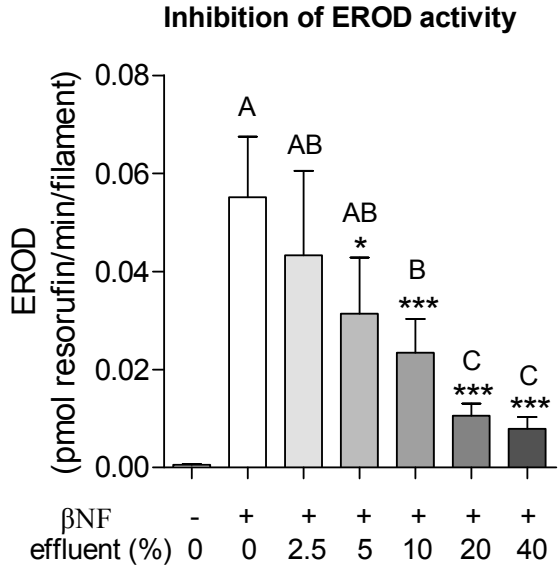

Figure 4. Inhibition of gill CYP1A activity (EROD) by PETL effluent containing high levels of pharmaceuticals, paper III (Beijer et al., 2013).

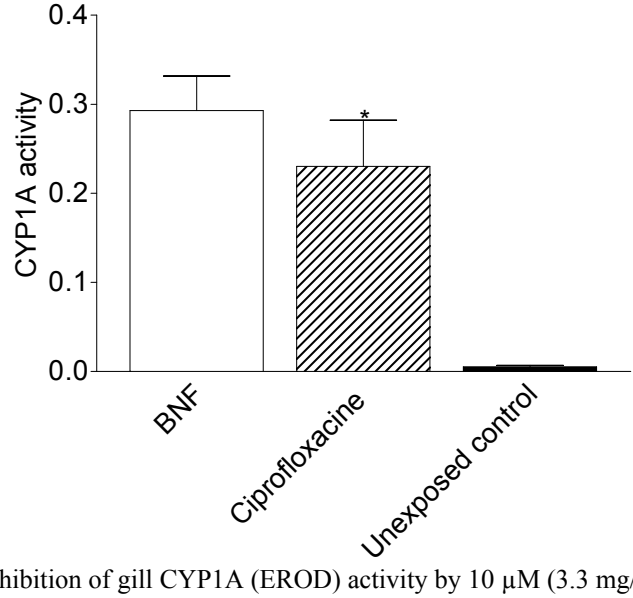

Figure 5. Inhibition of gill CYP1A (EROD) activity by 10 µM (3.3 mg/L) ciprofloxacin; activity was inhibited by 21 %. Rainbow trout were exposed for 24 h *in vivo* to 1 µM βNF or carrier (unexposed control); gill filaments were dissected and exposed *ex vivo* for 2 h to ciprofloxacin or carrier only (βNF, unexposed control) in the buffer. Then the EROD assay was performed with ciprofloxacin present in the buffer. Statistical difference between the βNF-induced control and ciprofloxacin was analyzed using unpaired t-test; the asterisk indicates significant difference ($p < 0.05$). CYP1A activity was calculated as pmole resorufin/filament and minute.

CYP19

The azoles found to be the most potent inhibitors of CYP1A activity in paper II also decreased brain CYP19 activity. These azoles, bifonazole, clotrimazole, imazalil, miconazole and prochloraz, and the well-known aromatase (CYP19) inhibitor letrozole were confirmed to inhibit rainbow trout brain CYP19 activity with IC_{50} values ranging between 0.02 and 3.3 μM (Table 1).

Azoles inhibit CYPs additively and have different selectivity and potency towards specific CYPs

Two azole mixtures containing the five most potent inhibitors were tested for inhibition of CYP1A or CYP19 activity. Both mixtures inhibited CYP activities in a concentration-dependent fashion; the experimentally determined IC_{50} values of these mixtures were similar to the estimated IC_{50} values, assuming additivity. The conclusion of these results is that the azoles inhibited the CYP-enzymes in an additive fashion.

Another study in line with our results suggests that a mixture of three azoles acts in an additive way when inhibiting cortisol secretion, and the observed effect was suggested to be mediated via inhibition of steroidogenic CYP enzymes (Ohlsson et al., 2010). The same mixture displayed a biphasic dose-response curve when inhibiting aldosterone secretion. When the chemicals in the mixture were examined as single compounds, prochloraz and ketoconazole displayed biphasic patterns while imazalil displayed a monotonic dose-response pattern. This finding could indicate that azole fungicides have different selectivity and potency towards specific CYP enzymes. Interestingly, our results show that the tested azoles did not have the same potency to inhibit activities of CYP1A and CYP19, instead varying potencies were obtained with clotrimazole being the strongest inhibitor of CYP19 followed by letrozole, imazalil, miconazole and bifonazole (Table 1). The most potent inhibitor of CYP1A was bifonazole followed by imazalil, prochloraz, clotrimazole and miconazole. To determine whether a chemical interferes with CYP enzymes it is however not enough to study one CYP enzyme. The same chemical can have high potency to inhibit the activity of one CYP enzyme and at the same time lower potency to inhibit the activity of another CYP enzyme. This is shown by our experiments where e.g. letrozole inhibited CYP19 activity with an IC_{50} of 0.06 μM but did not inhibit CYP1A activity at all, and clotrimazole had 60 times higher potency to inhibit CYP19 than CYP1A activity. On the contrary, bifonazole had 10 times higher potency to inhibit CYP1A than CYP19 activity. In my opinion, it would be interesting to study inhibition of a few selected azoles on all CYP enzymes (where assays are available in fish) and to determine potencies of the azoles towards different CYP enzymes. Admittedly, this would imply a lot of labor but, as a reward, sensitive target CYPs could be identified.

Table 1. *Apparent IC_{50} values for inhibition of CYP1A or CYP19 by different azoles.*

Endpoint	IC_{50} (μM)					
	bifonazole	imazalil	prochloraz	clotrimazole	miconazole	letrozole
CYP1A	0.1	0.9	0.9	1.2	1.5	x
CYP19	1.1	0.3	3.3	0.02	0.6	0.06

What consequences could inhibition of CYP enzymes have?

CYP1A is involved in phase I biotransformation. Thus, inhibitors of CYP1A activity can inhibit biotransformation resulting in bioaccumulation of other compounds. This was demonstrated when fish were co-treated with clotrimazole and B[a]P and showed higher concentration of B[a]P compared to fish exposed to B[a]P alone (Levine et al., 1997). If the compound that is not properly metabolized is toxic, this could directly lead to an increase of adverse effects. Such a scenario was observed when killifish (*Fundulus heteroclitus*) were co-exposed to βNF or B[a]P and CYP1A inhibitors (αNF, fluoranthene or piperonly butoxide), resulting in elevated embryo toxicity such as cardiac deformities, tail shortening, and hemorrhaging (Wassenberg and Di Giulio, 2004; Wills et al., 2009). When juvenile rainbow trout were co-treated with ethinylestradiol and ketoconazole, an elevated estrogenic response measured as an elevated plasma vitellogenin level compared to treatment with ethinylestradiol alone was observed (Hasselberg et al., 2008). This increased sensitivity to ethinylestradiol treatment was probably due to inhibition of xenobiotic metabolizing enzymes (CYP1A and CYP3A). A biomarker such as EROD could underestimate exposure, when fish are exposed to mixtures of inhibitors and inducers of CYP1A activity. This was found in fish when co-treatment with the CYP inhibitor fluoranthene reduced B[a]P-induced EROD activity to 50% (Willett et al., 2001). Inhibition of CYP1A activity can also lead to decreased toxicity if bioactivation is required to make a compound toxic. This is illustrated in a study where βNF-preexposed rainbow trout were exposed to H^3-B[a]P and the formation of adducts in the gill filaments were localized by autoradiography (Jönsson et al., 2004). Adduct formation was completely blocked in fish exposed to ellipticine for 30 min before the H^3-B[a]P exposure. This shows that inhibition of CYP1A activity prevents adduct formation.

Further, I believe that shunting of precursors to other pathways could be one of the biggest concerns with CYP inhibiting chemicals. A noteworthy example of interference with metabolic pathways is that exposure to azole fungicides can result in masculinization of fish (Kinnberg et al., 2007; Singh and Singh, 2013; Sun et al., 2007; Uchida et al., 2004). The proposed mechanism behind this masculinization is that inhibition of CYP19 activity suppresses the formation of estrogen resulting in increased androgen levels potentially leading to development of more males (Uchida et al., 2004). This example directly

shows that interference with metabolic systems (e.g. steroidogenesis) can have detrimental consequences for a fish population such as a skewed sex ratio towards males. What the consequences would be if other pathways are changed remains unknown but further studies on azole mixtures are needed to investigate long term effects of e.g. developmental exposure on metabolic enzymes, reproductive system or on other physiological functions in fish or other aquatic organisms.

Induction of CYP1 enzymes

PETL effluent induces expression and activity of CYP1 enzymes

Sticklebacks were exposed to 0.8, 1.6 and 3.2% dilutions of PETL effluent for 24 h, and all concentrations significantly induced EROD in the gills (paper III). In addition, mRNA expression of *CYP1A* was up-regulated by almost all concentrations in all organs. *CYP1B1, CYP1C1* and *CYP1C2* (only at 0.8%) were only induced in gills. These findings on CYP1A induction are in agreement with earlier observations that CYP1A enzyme activity and mRNA expression were induced in livers of rainbow trout (*Oncorhynchus mykiss*) exposed to 0.2% of PETL effluent in a flow-through system for 5 days (Gunnarsson et al., 2009b). These results indicate that the effluent contained potent AhR agonists and indeed PAHs were detected in the effluent at low levels (Carlsson et al., 2009). However, the high CYP1A induction can only partly be explained by these AhR agonists.

Furthermore, our data show that *CYP1A* mRNA is highly inducible in stickleback brain. This finding is consistent with other studies showing high *CYP1A* inducibility in brain of different fish species (Chung-Davidson et al., 2004; Gao et al., 2011; Jönsson et al., 2007). Induced expression of *CYP1B1* in gills following exposure to AhR agonists has been observed earlier (Gao et al., 2011; Jönsson et al., 2007).

A time course study was performed transferring the exposed fish to clean water in order to investigate if the *CYP1A* induction was caused by persistent or transient inducers. These results demonstrate that expression of all *CYP1* transcripts studied (*CYP1A*, *CYP1B1*, *CYP1C1* and *CYP1C2*) were highest in gills shortly after exposure had started (6 h) and returned to control levels after nine days in clean water. In liver, *CYP1A* induction was weak but more persistent compared to gill; induction decreased until the end of the exposure but did not reach control level. Induction of *CYP1B1*, *CYP1C1* and *CYP1C2* in liver was low or absent. Since induction of *CYP1* transcripts was lower in liver than in gills these findings suggest that a first pass metabolism took place in the gills leading to lower concentrations of inducers in the liver. Experience

in our group tells that *CYP1A* induction caused by readily metabolized compounds such as indigo rapidly decreases after transfer to clean water (Gao et al., 2011). Therefore, the compounds responsible for the observed *CYP1* induction by PETL effluent are probably readily metabolized since the *CYP1* induction was quickly eliminated after transfer to clean water.

CYP1 induction and other biomarkers used to evaluate STP technologies

In the final study of this thesis, a mobile pilot plant (*Figure 6*) equipped with additional technology such as granular active carbon (GAC) filtration and ozonation was evaluated regarding its ability to reduce various contaminants from effluent water released from the Käppala (Lindingö, Stockholm) and Uppsala municipal STPs. The mobile plant was developed and operated by our collaborator in the MistraPharma program, Berndt Björlenius and his co-workers, at the Royal Institute of Technology, Stockholm. The reduction of contaminants was recorded by measuring specific pharmaceuticals in the water and by applying a set of biomarkers measuring integrated responses of unknown chemical mixtures in the water. The selected biomarkers should detect if the effluents caused e.g. induction of various CYP enzymes (e.g. by AhR agonists such as PAHs), oxidative stress, and estrogenic responses in exposed fish.

Increased CYP1A catalytic (EROD) activity and *CYP1A1, CYP1A3* and *CYP1C3* mRNA expressions were observed in gills of fish exposed to Käppala effluent, compared to fish maintained in tap water. Increased EROD activity was also recorded in gills of fish exposed to regular Uppsala effluent (no qPCR analysis was performed in Uppsala). Expression of *CYP1A1* and *CYP1A3* mRNA was induced also in the liver, although at a lower magnitude than in the gills. We assume that these effects were predominantly caused by other chemicals than pharmaceuticals, presumably by polycyclic aromatic hydrocarbons (PAHs). Induction of CYP1A protein and mRNA in fish is a commonly used biomarker for waterborne AhR agonists. Urban runoff from e.g. roads entering the STP as municipal wastewater may contain a variety of PAHs (Blanchard et al., 2004; Vogelsang et al., 2006). In addition, natural occurring AhR-active compounds such as humic acids (Andersson et al., 2010), flavonoids or carotenoids, reviewed by Denison and Nagy (2003), could contribute to the CYP1 inductions observed.

We observed no significant increases in mRNA expression for biomarkers responding to oxidative stress (*HSP 70*, *SOD*) and metal exposure (*MT*) in regular Käppala effluent. Since also the estrogen responsive biomarkers *ZP1, ZP2* and *ZP3* mRNA remained unaffected, Käppala effluent most likely contained very low concentrations of estrogenic compounds. Alternatively, it is possible that estrogenic responses were blocked by other compounds in the water. This result is notable because estrogenic activity, measured as an up-

regulation of *ZP*s or *VTG* gene expression, is frequently observed in fish exposed to STP effluents (Cuklev et al., 2012; Gunnarsson et al., 2009a).

GAC filtration was efficient in removing pharmaceuticals from the water and to decrease biomarker responses caused by unknown chemicals in the water. In fish exposed to GAC-filtered effluents, induction of *CYP1* genes and EROD activity was absent and their respective levels were equal or lower than levels recorded in Käppala tap water. Therefore, it seems as GAC efficiently removed AhR agonists in the regular effluent.

The results of chemical analysis and the biomarker responses confirm that also ozonation is effective in reducing a variety of pharmaceuticals and other contaminants in effluent water. In Käppala, levles of gill EROD activity and *CYP1* mRNA expression in gill and liver of fish, exposed to ozonated effluent, were very low and equal to or lower than those in fish held in Käppala tap water. Induction of EROD activity in gills of fish exposed to ozone-treated effluent in Uppsala was also lower compared to regular effluent. Therefore, it appears that ozone treatment caused a substantial reduction of AhR agonists in the effluent.

In conclusion, this study has demonstrated that both GAC filtration and ozonation significantly decreased both numbers and concentrations of APIs present in regular effluent, as well as biomarker responses in fish exposed to the effluent. Eventually, such treatments could therefore present a path forward to further increase removal of biologically active contaminants in Swedish STPs.

Figure 6. The pilot plant with technology for additional treatment of STP effluent developed by Berndt Björlenius at the Royal Institute of Technology, Stockholm. (Photo by Berndt Björlenius).

Concluding remarks

The gill is the first organ of the fish to be in contact with, absorb and metabolize waterborne xenobiotics. We therefore established a new method to study inhibition of the catalytic activity of CYP1A in gill tissue of two fish species, the three-spined stickleback and the rainbow trout. Our results demonstrate that gills are sensitive to CYP1A inhibitors. Therefore, I conclude that our method is a useful and sensitive technique to identify compounds that inhibit CYP1A activity and to show the presence of CYP1A inhibitors in water samples.

We applied the new model to an environmentally relevant exposure scenario, and found that CYP1A activity was inhibited by the PETL effluent. The CYP1A inhibitor ciprofloxacin was detected in the PETL effluent at concentrations even higher than the therapeutic plasma level in humans (Larsson et al., 2007). It is possible that the observed inhibition of CYP1A activity by the PETL effluent increases bioavailability and toxicity of other compounds present in the effluent such as PAHs or other toxic substances metabolized by CYP1A. Therefore, elevated bioavailability and toxicity of certain compounds in the effluent because of inhibited CYP activity may have contributed to previously reported adverse effects of the PETL effluent, such as mortality of zebrafish larvae, and growth reduction in tadpoles (Carlsson et al., 2009). CYP1A measured as EROD activity is present in very early life stages in zebrafish and in tadpoles (Jönsson et al., 2011; Jönsson et al., 2007; Otte et al., 2010).

Activity of CYP19 was found to be inhibited by the same azoles as tested in the CYP1A assay. These results confirm that azole fungicides inhibit other CYP enzymes than the original target enzyme (CYP51). Today, there is evidence that azole fungicides interfere with numerous CYP enzymes involved in biotransformation and steroid biosynthesis e.g. 1A, 3A4, 11B1, 17, 19, and 21. In humans, interactions of various pharmaceuticals with antifungal azoles are well known and are believed to be common since azole fungicides are potent inhibitors of CYP isoenzymes and P glycoprotein (Groll and Walsh, 2007; Wang et al., 2002). Besides, it has also been reported that some azoles can disrupt the rate-limiting step in steroidogenesis. Econazole and miconazole have been demonstrated to block the steroidogenic acute protein (StAR) post-transcriptionally (Walsh et al., 2000). StAR is a transport protein regulating cholesterol transfer to the inner membrane of mitochondria which is rate limiting in the production of steroid hormones.

The results on azole mixtures showed that CYP enzymes are inhibited in an additive way which is also supported by earlier studies on other steroidogenic functions. This is an important finding because azoles are widely used as human and veterinary pharmaceuticals, pesticides and biocides; they are released into the aquatic environment and may act in concert to interfere with CYP-regulated physiological functions in fish.

The PETL effluent inhibited catalytic CYP1A activity *in vitro* and induced CYP1A activity *in vivo*; the induction was also verified on the transcript level. This implies that there was a mixture of AhR agonists and CYP1A inhibitors in the PETL effluent. Possibly, inhibition and induction could be caused by the same chemical. Some azoles are known to both decrease catalytic CYP1A activity and induce the expression of CYP1A measured as EROD activity, CYP1A protein or *CYP1A* mRNA (Navas et al., 2004; Sturm et al., 2001). There are authors stating that CYP1 induction could even be caused by inhibition of CYP1A activity leading to decreased metabolism of endogenous AhR agonists. Such an example is presented by a mechanism via the endogenous compound FICZ. This compound is both a potent AhR ligand and a substrate for CYP1A1, CYP1A2 and CYP1B1, meaning that FICZ can induce expression of CYP1 proteins and on the same time be metabolized by these proteins. It has been shown that if metabolism of FICZ is disrupted by inhibitors of CYP1A, low levels of FICZ are sufficient to induce CYP1A1 mRNA and enzyme activity (Wincent et al., 2012). When studying effects of complex mixtures of chemicals such as the PETL effluent, it is important to keep in mind that these mixtures can interfere with CYP1 both as inhibitors and inducers. Inhibition and induction of CYPs might alter absorption, distribution, biotransformation and excretion of exogenic and endogenic compounds and can consequently change the properties of these compounds to become e.g. more or less toxic.

A complex mixture of natural and synthetic chemicals was also studied in the final study. Biomarker responses were studied in fish exposed to regular STP effluent, GAC-filtered or ozone-treated effluent in order to evaluate improved wastewater treatment technologies. The improved technologies removed several contaminants detected in regular effluent and decreased induction of EROD activity and *CYP1* gene expression. In conclusion, this study shows that chemical analysis together with biomarkers in fish are applicable to evaluate treatment technologies in wastewater management and can be used to improve Swedish STPs.

Svensk sammanfattning

Denna avhandling är en del i forskningsprogrammet MistraPharma som syftar till att bland annat identifiera humanläkemedel som kan utgöra en risk för det akvatiska ekosystemet och att föreslå förbättrade reningstekniker av avloppsvatten. Syftet med min avhandling är att identifiera läkemedel och andra kemikalier som stör den katalytiska aktiviteten av cytokrom P450 (CYP)-enzymer, mer specifikt CYP1A och CYP19, hos fisk. Dessutom utvärderades effektiviteten av de förbättrade reningsteknikerna med hjälp av fiskar som exponerats för avloppsvatten från olika vattenreningstekniker. Biomarkörer, såsom CYP1A-aktivitet och mRNA nivåer av gener som uppregleras av läkemedel, Ah-receptor agonister, oxidativ stress, östrogena ämnen och metaller, mättes i lever och gälfilament hos exponerade fiskar.

Läkemedel, bekämpningsmedel och andra kemikalier är föroreningar som återfinns i vattendrag, sjöar och hav, ofta tillsammans i en blandning. Azolfungicider är svampbekämpningsmedel som används i läkemedel och träskyddsmedel, samt i frukt- och grönsaksodlingar för att kontrollera svampangrepp. De flesta azoler verkar genom att hämma enzymaktiviteten av CYP51, som svamparna behöver för att bygga upp cellmembranet. Ett fåtal azoler används också för att behandla östrogenberoende cancerformer genom att hämma aktiviteten av aromatas (CYP19), ett enzym som katalyserar bildandet av östrogen. Det är tidigare känt att azoler hämmar den katalytiska aktiviteten av andra CYP-enzym förutom de tilltänkta målenzymen CYP51 eller CYP19. Exempel på CYP-enzymer som kan hämmas av azoler är CYP1, CYP3, CYP11, CYP17, CYP19 och CYP21. CYP-enzymer metaboliserar kroppsfrämmande (exempelvis miljögifter) och kroppsegna ämnen (exempelvis hormoner, fettsyror, och prostaglandiner). Induktion av CYP1As proteinnivå har länge använts som en biomarkör för miljögifter som binder till Ah-receptorn, exempelvis polyklorerade dioxiner, polycykliska aromatiska kolväten (PA-Her) och polyklorerade bifenyler (PCBer). Induktion av CYP1As proteinnivå kan mätas som en ökning av 7-etoxyresorufin *O*-deetylas (EROD) aktivitet och bestäms oftast i lever. Inhibering av CYP1As aktivitet kan därför mätas som en minskning i EROD-aktivitet.

I denna avhandling utvecklades en EROD-metod för att mäta kemikaliers CYP1A inhibering *ex vivo* i gälfilament på storspigg och regnbågslax. Ett flertal azolfungicider, cancermedicinen ellipticin och flavonoiden bitertanol var potenta hämmare av CYP1A-aktiviteten i storspigg eller regnbågslax. I regnbågslax utfördes koncentrations-respons försök för fem azoler med avsikt att

bestämma IC_{50} (50 % inhibitory concentration) värden. Bifonazol var den mest potenta, därefter imazalil, prokloraz, klotrimazol och mikonazol.

Den nya gälfilamentsmetoden användes för att studera CYP1A-aktiviteten i renat avloppsvatten från ett reningsverk i Patancheru som ligger utanför Hyderabad i Indien. Genom reningsverket passerar processvatten från 90 läkemedelstillverkare och tidigare studier har uppmätt extremt höga nivåer av läkemedel i det renade avloppsvattnet. Flera utspädningar av avloppsvatten testades (2,5; 5; 10; 20 och 40 %) och alla minskade CYP1A-aktiviteten i gälfilament på storspigg. Samma avloppsvatten, från reningsverket i Patancheru, som hämmade CYP1A-aktiviteten *ex vivo*, inducerade CYP1A-aktiviteten (EROD) och genuttrycket av *CYP1* i flera organ *in vivo*. Fiskar exponerades i 24 timmar för 0.8, 1.6 och 3.2 % avloppsvatten utblandat i kranvatten, varefter CYP1A-aktivitet i gälfilament och mRNA nivåer av *CYP1*-gener bestämdes i gälar, lever och hjärna. De båda resultaten (d.v.s. hämning av CYP1A-aktiviteten *ex vivo*, induktion av den samma *in vivo*) verkar motsägelsefulla men har tolkats som att det fanns en blandning av Ah-receptor-agonister och CYP1A-inhibitorer i det renade avloppsvattnet.

Azoler hämmade inte bara CYP1A utan också CYP19-aktiviteten i hjärnmikrosomer i regnbågslax. För sex azoler bestämdes IC_{50}-värden. Klotrimazol var den kraftigaste CYP19-hämmaren, följd av letrozol, imazalil, mikonazol, bifonazol och prokloraz. I ett försök att studera hur azoler samverkar, bestämdes IC_{50} för blandningar av azoler. Två blandningar gjordes, en blandning med en koncentration av azoler (C_{50}), som skulle hämma 50 % av CYP1A-aktiviteten, och en som skulle hämma 50 % av CYP19-aktiviteten, under förutsättning att de verkade additivt. Dessa blandningar testades sedan tillsammans med högre och lägre koncentrationer ($1/9*C_{50}$, $1/3*C_{50}$, C_{50}, $3* C_{50}$, och $9* C_{50}$). På så sätt erhölls koncentrations-responskurvor och experimentella IC_{50}-värden kunde bestämmas. De erhållna IC_{50}-värdena stämde väl överens med den predikterade koncentrationen (C_{50}) för både CYP1A och CYP19-aktivitet. Slutsatsen av försöken är att de undersökta azolerna i blandningarna hämmar CYP-aktiviteter på ett additivt sätt. Den additiva hämningen av azoler är oroväckande, eftersom azoler finns i en cocktail i miljön. Studier har även visat att azoler kan mätas i muskelvävnad av fisk vilket tyder på att de bioackumuleras i fisk. Därför finns det behov av studier som undersöker negativa effekter av azolblandingar på CYP-reglerade funktioner hos fisk.

En ökning av CYP1-protein och mRNA nivåer noterades i gälar och lever på regnbågar som exponerades för renat avloppsvatten även i det sista delarbetet av denna avhandling. I detta arbete använde vi oss av biomarkörer som skulle fånga upp om avloppsvattnet innehöll läkemedel, Ah-receptor-agonister, östrogena ämnen, metaller eller ämnen som orsakar oxidativ stress. Biomarkörerna var EROD-aktivitet i gälfilament och mRNA-uttryck av ett flertal gener, däribland *CYP1* och *CYP3*, som mättes i lever och gälfilament hos exponerade regnbågslaxar. Syftet med arbetet var att utvärdera ifall de förbättrade reningsteknikerna; granulerat aktivt kol (GAC) och ozonering, kunde ta

bort fler aktiva läkemedelsubstanser än konventionella reningsmetoder. De förbättrade reningsteknikerna utvecklades av vår samarbetspartner Berndt Björlenius (Kungliga Tekniska Högskolan) inom MistraPharma-programmet och installerades i en mobil container som placerades både i Käppala (Lidingö, Stockholm) och Uppsala reningsverk. I containern fanns tankar med ett genomflödessystem för att kunna exponera fiskar för vatten från de olika reningsteknikerna. Läkemedelsanalyser på avloppsvattnet visade att ett flertal läkemedel som uppmättes i renat avloppsvatten avlägsnades med de förbättrade reningsmetoderna. Inga tecken på CYP1-induktion i varken GAC- eller ozonbehandlat avloppsvatten kunde påvisas med hjälp av biomarkörerna. Detta innebär att behandlingar med GAC eller ozonering troligtvis eliminerade Ah-receptor-agonister. Avslutningsvis pekar denna studie på, att biomarkörer i fisk kan utnyttjas för att utvärdera reningsmetoder som är tänkta att förbättra dagens reningsverk.

Sammantaget har resultaten i min avhandling visat att en del azoler, som främst är utvecklade för att hämma enzymaktiviteten av CYP51, även är potenta hämmare av CYP1A eller CYP19-aktiviteten i fisk. Eftersom azoler, i likhet med andra kemikalier, förekommer i blandningar i miljön är ett av de viktigaste fynden att azoler hämmar CYP1A- och CYP19-aktiviteter på ett additivt sätt. Betydelsen av detta är att även om enskilda azoler detekteras i låga koncentrationer i vattnet och därför troligtvis inte påverkar aktiviteten av CYP-enzymer hos fiskar, skulle summan av alla azoler som finns i vattenmiljön faktiskt kunna påverka CYP-enzymers aktiviteter. För att kartlägga om detta utgör ett hot för miljön måste fler studier göras på vattenlevande djur, exempelvis genom exponering i tidiga eller känsliga utvecklingsstadier. Avslutningsvis, verkar induktionen av CYP1A-protein och *CYP1*-genuttryck i gälar på fiskar vara en användbar biomarkör för att mäta förekomsten av Ah-receptor agonister i avloppsvatten.

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