Detoxification Mechanisms in Fish

-Regulation and Function of Biotransformation and Efflux in Fish Exposed to Pharmaceuticals and Other Pollutants

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Akademisk avhandling för filosofie doktorsexamen i zoofysiologi, som med tillstånd från Naturvetenskapliga fakulteten kommer att offentligt försvaras fredagen den 16 november 2012 kl. 10.00 i föreläsningssalen, Zoologihuset, Institutionen för biologi och miljövetenskap, Medicinaregatan 18 A, Göteborg.

UNIVERSITY OF GOTHENBURG

Department of Biological and Environmental Sciences
Faculty of Science

2012

Cover illustration by Anna Holmberg, Maleviks bildverkstad, Kullavik

Published by the Department of Biological and Environmental Sciences, University of Gothenburg, Sweden

ISBN 978-91-628-8540-3
http://hdl.handle.net/2077/30452

Printed by Kompendiet, Göteborg (http://www.kompendiet.se)

Till Robert och Isak

Dissertation Abstract

It is likely that fish in their natural environment are exposed to mixtures of several pharmaceuticals as well as other pollutants. This may result in adverse effects which are augmented due to the chemical interactions. Such chemical interactions are challenging to predict and increased knowledge on key detoxification mechanisms is needed. In human, adverse drug-interactions can arise by interactions with the pregnane X receptor (PXR) and the target genes cytochrome P450 3A4 (CYP3A4) and P-glycoprotein (Pgp). These genes also exist in fish, but their functions are less understood. The main focus in this thesis was to elucidate whether PXR regulates CYP3A and Pgp in fish, and how pharmaceuticals interact with regulation of these genes and the functions of the proteins. We found weak induction of CYP3A and Pgp genes by two mammalian PXR ligands in rainbow trout hepatocytes. Also, we found weak induction of hepatic PXR, CYP3A and Pgp expressions with PCBs in a killifish population that is non-responsive to CYP1A inducers. To further explore fish PXR activation, rainbow trout PXR was isolated, sequenced and expressed in a reporter assay. The reporter assay resulted in weak or no activation of rainbow trout PXR with a suite of prototypical PXR ligands. A CYP3B gene transcript was sequenced from the *Poeciliopsis lucida* hepatocellular carcinoma (PLHC-1) cell line. Basal expression of CYP3B was low in PLHC-1 cells and it was not responsive to exposure to PXR ligands. We have used both *in vitro* and *in vivo* fish models and we have analyzed gene regulations and protein functions upon pharmaceutical exposures, both as single substance exposures and as a mixture exposure. Several pharmaceuticals were shown to inhibit the CYP1A catalytic functions and to interfere with efflux pumps activities in PLHC-1. Combined exposure of ethinylestradiol with the broad-spectrum CYP inhibitor ketoconazole resulted in increased sensitivity to ethinylestradiol exposure in juvenile rainbow trout. This drug interaction was caused by inhibition of CYP1A and CYP3A enzyme activities in rainbow trout liver. In conclusion, pharmaceuticals affected both functions and regulations of key detoxification proteins in fish. Adverse toxicokinetic interactions via CYP1A and CYP3A inhibitions were demonstrated in rainbow trout.

Keywords:
Fish, PXR, CYP1, CYP3, efflux, pharmaceutical, drug interaction

Populärvetenskaplig sammanfattning

Över 100 olika läkemedelsämnen har påvisats i miljön runt om i världen. Läkemedel utsöndras främst i urinen och dagens reningsverk är inte tillräckligt effektiva på att ta bort dessa ämnen. Således är utloppen från reningsverken en stor källa till förekomsten av läkemedel i miljön. Fiskar riskerar därmed att exponeras för en mängd olika läkemedel och andra miljöföroreningar. Alla läkemedel som introduceras på marknaden måste enligt Europeisk lag riskbedömas med avseende på deras giftighet för alger, kräftdjur och fisk. I denna bedömning undersöks bara varje ämne ett i taget och ingen hänsyn tas till att djur utsätts för komplexa blandningar, som är det troliga scenariot i miljön. Från sjukvården finns många exempel på att olika läkemedel kan interagera med varandra och ge allvarliga läkemedelsinteraktioner när de används tillsammans. Dessa interaktioner beror ofta på att flera läkemedel bryts ner av samma enzymer i levern, och kroppens förmåga att göra sig av med dessa främmande ämnen blir otillräcklig. Leverns avgiftningsfunktion är väl konserverad genom evolutionen och många likheter finns mellan människa och fisk. Enzymfamiljen cytokrom P450 (CYP) har en central roll för att göra både kroppsegna restprodukter och främmande ämnen tillräckligt vattenlösliga för att de ska kunna utsöndras med urin och avföring. En viktig funktion har också de s.k. effluxproteiner som sitter i levercellernas membran och pumpar ut ämnen som är skadliga för cellen.

Denna avhandling handlar om hur läkemedel påverkar CYP-enzymer och effluxproteiner i fisklever. Sådan grundläggande kunskap är viktig för att kunna förutspå vilka blandningseffekter som kan uppstå när fisk utsätts för föroreningar såsom läkemedel i miljön. Avhandlingen består av fyra artiklar som på olika sätt belyser vad som händer i levern hos fiskar som blir utsatta för läkemedel och andra kemikalier, var för sig och i blandning. Fokus har varit på CYP-enzymet CYP3A som är den vanligaste CYP-formen i levern hos fisk och människor. Avhandlingen handlar om hur CYP3A-nivåerna och effluxproteinerna regleras i levern. Det visar sig att fiskarnas CYP3A-nivåer inte regleras lika kraftfullt som de gör hos människor. Det kan bero på den receptor, pregnan-X-receptorn (PXR), som hos människa styr hur mycket CYP3A-enzym det bildas i levern. Vi studerade PXR från regnbågslever och fann att den verkar vara mindre känslig för läkemedel än människans PXR. Detta kan förklara varför fiskarnas CYP3A-nivåer inte påverkas lika mycket när fiskar blir utsatta för läkemedel. Den tydligaste och kanske mest allvarliga effekten vi såg var att flera läkemedel förändrade, och i de flesta fall försämrade, fiskarnas CYP-enzym och effluxfunktioner. Detta är

allvarligt då det kan medföra en försämrad förmåga att göra sig av med kemikalier och därmed ökad känslighet för vissa typer av kemikalier i miljön. Vi såg t.ex. att regnbåge som utsatts för svampmedel hade försämrad CYP3A-enzymkapacitet. Det ledde i sin tur till att dessa fiskar blev sju gånger känsligare för östrogena ämnen som finns i p-piller och som är vanligt förekommande i sjöar och vattendrag. Sammanfattningsvis visar resultaten att fiskars avgiftningsförmåga kan påverkas negativt av läkemedel i miljön. Denna kunskap kan användas för att bättre förstå, och i framtiden förutspå, kombinationseffekter av kemikalier i miljön.

List of publications

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

I. **Wassmur, B, Gräns, J, Norström, E, Wallin, M, Celander, M C (2012)** Interactions of pharmaceuticals and other xenobiotics on key detoxification mechanisms and cytoskeleton in *Poeciliopsis lucida* hepatocellular carcinoma, PLHC-1 cell line.
Toxicology in Vitro http://dx.doi.org/10.1016/j.tiv.2012.10.002

II. **Hasselberg, L, Westerberg, S, Wassmur, B, Celander, M C (2008)** Ketoconazole, an antifungal imidazole, increases the sensitivity of rainbow trout to 17alpha-ethynylestradiol exposure.
Aquatic Toxicology, 86, 256-64

III. **Wassmur, B, Gräns, J, Kling, P, Celander, M C (2010)**
Interactions of pharmaceuticals and other xenobiotics on hepatic pregnane X receptor and cytochrome P450 3A signaling pathway in rainbow trout (*Oncorhynchus mykiss*).
Aquatic Toxicology, 100, 91-100

IV. **Wassmur, B, Gräns, J, Fernandez, M, Zanette J, Woodin, B R, Stegeman, J J, Wilson, J Y, Celander, M C**
Regulation of PXR, CYP3A and Pgp in PCB-resistant killifish (*Fundulus heteroclitus*) in New Bedford Harbor.
Manuscript

Abbreviation list

ABC	ATP-binding cassette
AhR	Aryl hydrocarbon receptor
ANF	α-Naphthoflavone
ARNT	Aryl hydrocarbon receptor nuclear translocator
BFCOD	Benzyloxy-4-[trifluoromethyl]-coumarin-*O*-debenzyloxylase
BLAST	Basic local alignment search tool
BNF	β-Naphthoflavone
CAR	Constitutive androstane receptor
CYP	Cytochrome P450
EROD	Ethoxyresorufin-*O*-deethylase
LBD	Ligand binding domain
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance associated protein
NBH	New Bedford Harbor
NRF2	Nuclear factor-E2-related factor 2
PAH	Polyaromatic hydrocarbons
PCB	Polychlorinated biphenyl
PCB 126	3,3',4,4',5-Pentachlorobiphenyl
PCB 153	2,2',4,4',5,5'-Hexachlorobiphenyl
PCN	Pregnenolone-16α-carbonitrile
PCR	Polymerase chain reaction
Pgp	P-glycoprotein
PLHC	Poeciliopsis lucida hepatocellular carcinoma
PXR	Pregnane X receptor
qPCR	Quantitative polymerase chain reaction
RTH	Rainbow trout hepatoma
RXR	Retinoid X receptor
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
SC	Scorton Creek
STP	Sewage treatment plant
UDP	Uridine diphosphate

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1. Introduction

Fish are exposed to numerous pharmaceuticals present in the aquatic environment. Still, the effects on fish from mixed exposure of several pharmaceuticals are largely unknown. The majority of drug interactions in human are due to shared detoxification pathways and similar drug interactions may occur in fish. This thesis focuses on regulation and function of key proteins in these detoxification pathways in fish exposed to pharmaceuticals.

1.1 Pharmaceuticals in the environment and their effects in fish

Pharmaceuticals are continuously detected in the aquatic environment, most in effluents from sewage treatment plants (STPs), but also in surface and ground water (Heberer 2002, Kümmerer 2009, Verlicchi et al. 2012). Today's STPs are not designed for removal of pharmaceuticals and more than 100 different pharmaceuticals have been found in the environment (Monteiro and Boxall 2010). Consumed pharmaceuticals predominantly leave the body through urine or feces, as the original compound or as metabolites, that enter the environment via STPs. Recently, pharmaceutical industries have been revealed to be responsible for large discharges of drugs to waste water which have been reported to occur in India, Europe and USA (Larsson et al. 2007, Sanchez et al. 2011, Phillips et al. 2010).

1.1.1 Effects of pharmaceuticals in fish

Several studies of toxic effects of pharmaceuticals on aquatic animals have been conducted during the last decades. Environmental occurrence and the studies of adverse effects in fish are summarized in several reviews (Halling-Sørensen et al. 1998, Fent et al. 2006, Corcoran et al. 2010). Environmental concentrations are typically ranging from ng L^{-1} to low µg L^{-1} and are often described to be below the concentrations of obvious acute toxic effects in aquatic animals. However, there are reports of adverse effects in fish for a number of pharmaceuticals. One of the most alarming effects in fish seen so far is from ethinylestradiol, a potent synthetic estrogen used in contraceptive pills and present in STP effluents. An experimental lake in Canada was dosed with environmental concentrations

of ethinylestradiol that resulted in a collapse of the fathead minnow population in the lake (Kidd et al. 2007). Furthermore, feminization of fish downstream of STPs has been reported repeatedly and is likely to be caused by ethinylestradiol present in the effluents (Folmar et al. 1996, Larsson et al. 1999, Jobling et al. 2002). Additionally, the synthetic gestagen levonorgestrel, also used in contraceptive pills, has been shown to impair the reproductive success in fathead minnow (Zeilinger et al. 2009. Levonorgestrel has been detected in STP effluent and lab exposure of rainbow trout to STP effluent resulted in plasma concentrations exceeding human therapeutic concentrations (Fick et al. 2010). In fact, the plasma concentrations in that study exceeded the effluent concentration by approximately four orders of magnitude. This is a clear example of bioaccumulation as fishes breathe large volumes of water and the pharmaceuticals therein, which are often lipophilic, can accumulate in the fish resulting in higher concentration in the fish compared to the surrounding water. Another example of adverse effects by pharmaceuticals is tissue damage in rainbow trout caused by exposure to the non-steroidal anti-inflammatory drug diclofenac, which has been detected in surface water (Schwaiger et al. 2004, Mehinto et al. 2010). As fish share many physiological functions with humans they also have many drug targets in common (Gunnarsson et al. 2008). However, aquatic invertebrates and plants lack many of these drug targets and therefore, the standard risk assessments made on algae and crustaceans are less informative to predict effects in fish. For example, data on effects of estrogenic chemicals from invertebrates, that lack the estrogen receptor, cannot be used to predict estrogenic effects in fish that have estrogen receptors (Gunnarsson et al. 2012). Extrapolation between species within diverse taxonomic groups such as fish with about 32.000 extant species (www.fishbase.org) adapted to different environments should also be based on species-specific molecular knowledge (Celander et al. 2011).

1.1.2 Risk assessments of pharmaceuticals

Since 2006, all new human pharmaceuticals must be tested for chronic toxicity in aquatic animals according to the European medical agency (EMEA) guidelines (www.ema.europa.eu) to be approved for use in the European Union (EU). The toxicity tests are carried out in algae, crustacean

and fish. The recommended fish test is an "early life-stage test", from embryo to free-feeding fish. These chronic toxicity tests are more sensitive compared to the acute toxicity tests previously used. Still, the difference between those tests and a true chronic exposure situation in the environment is large. The standard toxicity tests in fish would need to be accompanied by tests designed to identify more subtle effects, mediated by the respective drug targets (Fent et al. 2006). This thesis focuses on detoxification mechanisms that act as a first line defence against chemicals.

Environmental risk assessments are only made for single chemicals. However in the environment, chemicals end up as mixtures since a large number of pharmaceuticals, as well as other pollutants, are present simultaneously. Accordingly, it has been raised by Boxall et al. (2012) that one of the top 20 questions, in the field of pharmaceuticals in the environment, is how to assess the effects on wild life of exposure to pharmaceuticals in mixtures after long-term exposure to low concentrations. Today, knowledge on effects of pharmaceutical mixtures is based on experience from human drug therapies. These adverse drug interactions are largely due to shared detoxification pathways. Therefore, increased knowledge on detoxification pathways in fish is essential for better predictions of environmental mixture effects (Celander 2011). These types of interactions will be further discussed in section 1.3.

1.2 Detoxification mechanisms

Xenobiotics, *i.e.* foreign substances, including pharmaceuticals can be recognized by receptors that regulate the production of detoxification proteins. The concerted actions of these proteins increase the ability to excrete the xenobiotics and thereby prevent harmful accumulation in the body. The most important organ for detoxification is the liver which metabolizes xenobiotics as well as endogenous compounds, such as steroid hormones (Waxman et al. 1988, Parkinson 1996). The detoxification mechanisms consist of a battery of proteins that can be divided into biotransformation enzymes that are active in phase 1 and 2, and efflux pumps that are active in phase 0 or III (Xu et al. 2005). This thesis focuses on regulation and function of these detoxification proteins in fish exposed to pharmaceuticals and other model substances.

1.2.1 Biotransformation

The transformation of a chemical compound in an organism is defined as biotransformation (Parkinson 1996). It is an essential reaction in the metabolism of both endogenous and xenobiotic compounds in order to convert fat-soluble substances to more water-soluble metabolites that can be excreted from the body. Biotransformation in the metabolism of drugs and other xenobiotics usually proceeds in two phases. In phase 1, cytochrome P450 (CYP) enzymes catalyze for example the hydroxylation of a chemical (Figure 1).

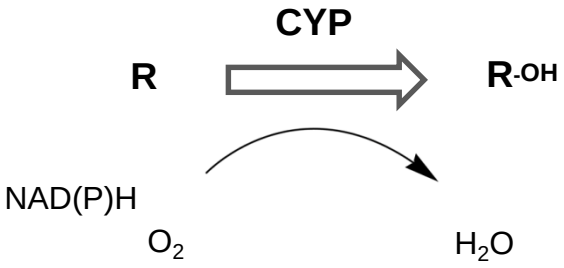


Figure 1. The overall cytochrome P450 (CYP) catalyzed reaction of a chemical R.

The phase 1 reaction thus increases the water solubility and allows the compound to be further processed in phase 2. In the following phase 2 reaction, a polar endogenous group (*e.g.* UDP-glucuronic acid or glutathione) is conjugated to the phase 1 metabolite to further increase water solubility and excreatability (Figure 2). The phase 2 reactions are catalyzed by different transferases *e.g.* UDP-glucuronosyl-transferases, and glutathione-*S*-transferases. The dominant enzymes in phase 1 belong to the CYP superfamily, which is in focus in this thesis.

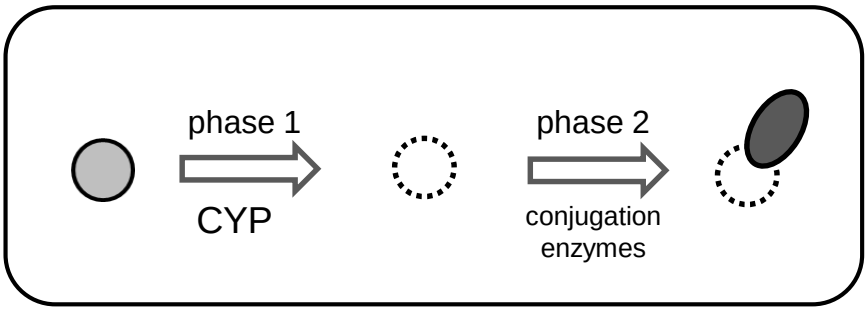


Figure 2. Phase 1 and phase 2 biotransformation of lipophilic organic chemicals.

1.2.2 Phase 1 and the CYP superfamily

The first isoenzyme in the CYP superfamily was described in 1962 as a new cytochrome, *i.e.* a membrane-bound hemoprotein (Omura & Sato 1962). When this protein binds carbon monoxide, it absorbs light at 450 nm, hence the suffix P450. Of the phase 1 enzymes, more than 95% belong to the CYP superfamily (Nebert et al. 1996). A recent analysis of the zebrafish (*Danio rerio*) genome revealed 94 CYP genes which can be divided into the 18 CYP gene families that are also present in human (Goldstone et al. 2010). The gene families are broadly divided into catabolic CYP enzymes, catalyzing the breakdown of both endogenous and foreign substances, and anabolic CYP enzymes that are involved in the biosynthesis of lipophilic compounds like steroids and fatty acids (Guengerich 2005). This thesis focuses on the xenobiotic- and steroid-metabolizing CYP1A and CYP3A subfamilies.

Table 1.
The CYP gene families in zebrafish and humans and their major functions in humans.

	Gene family	Function in humans
Breakdown of xenobiotics and endobiotics "catabolism"	CYP1	xenobiotics and steroid metabolism
	CYP2	xenobiotics and steroid metabolism
	CYP3	xenobiotics and steroid metabolism
	CYP4	xenobiotics and fatty acids metabolism
Biosynthesis "anabolism"	CYP5	thromboxane synthase
	CYP7	bile acid biosynthesis
	CYP8	prostacyclin and bile acid synthesis
	CYP11	steroid biosynthesis
	CYP17	steroid biosynthesis
	CYP19	estrogen biosynthesis - aromatization
	CYP20	unknown function
	CYP21	steroid biosynthesis
	CYP24	vitamin D metabolism
	CYP26	retinoic acid metabolism
	CYP27	bile acid biosynthesis, vitamin D3 activation
	CYP39	cholesterol metabolism
	CYP46	cholesterol metabolism
	CYP51	cholesterol biosynthesis

1.2.3 The major drug and steroid metabolizing CYP3A form

The predominant CYP subfamily in the liver of both humans and fish is the CYP3A subfamily (Thummel and Wilkinson 1998, Celander et al. 1996). Approximately 75% of human drugs are metabolized in humans by CYP enzymes and almost half of these reactions are catalyzed by CYP3A4 (Guengerich 2008). Regulation of CYP3A genes was unknown until a new nuclear receptor was first described in mouse in 1998. It was denoted pregnane X receptor (PXR) since it was first found to be activated by the pregnan steroids (Kliewer et al. 1998). The mammalian PXRs appear to be extraordinary promiscuous as they are activated by a wide range of structurally diverse lipophilic chemicals, including many pharmaceuticals and steroids (Figure 3).

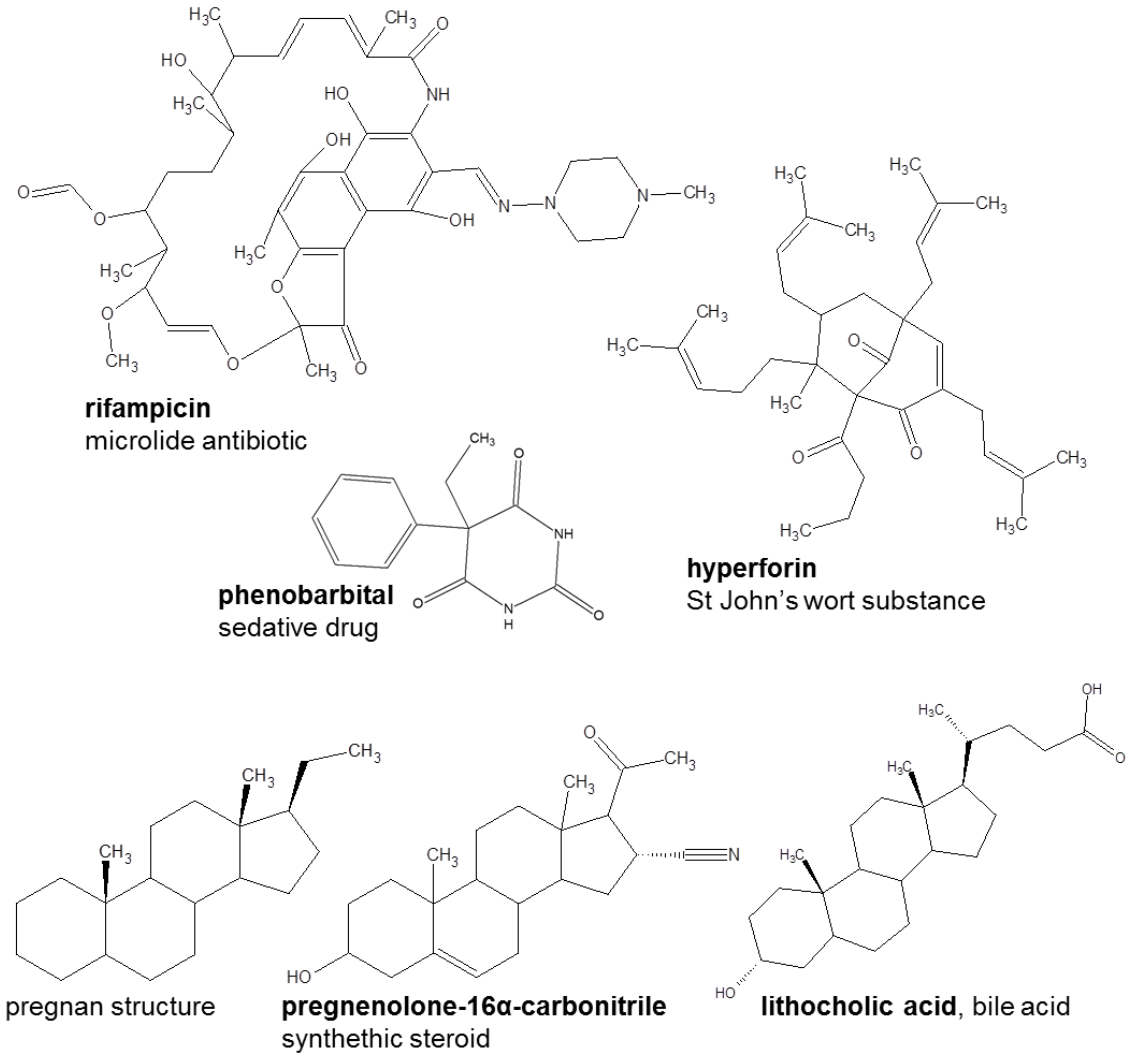


Figure 3. Examples of mammalian PXR ligands.

When human PXR is activated by ligand binding, it dimerizes with the retinoid X receptor (RXR) and the heterodimer functions as a transcription factor to the CYP3A4 gene (Kliewer et al. 1998) (Figure 4). In addition to CYP3A regulation, mammalian PXRs are also involved in regulation of other detoxification genes such as CYP2, phase 2 enzymes and efflux proteins (Maglich et al. 2002). The PXR act as a broad detoxification regulator and is often referred to as a xenosensor.

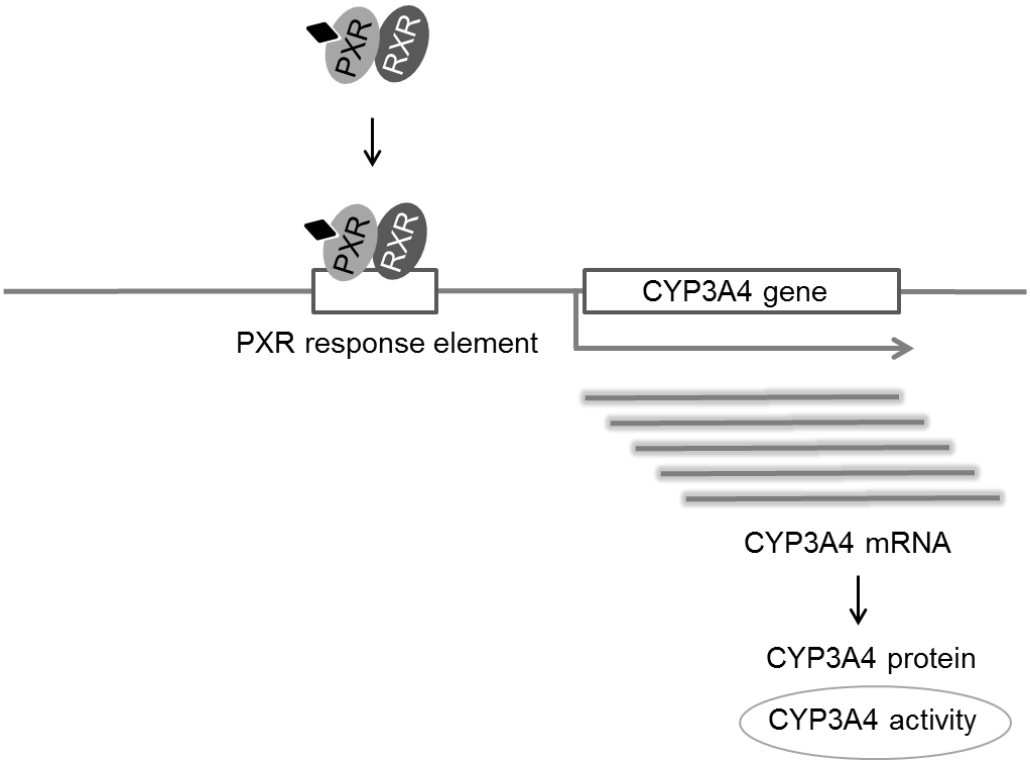


Figure 4. Activation of the human CYP3A4 gene.

1.2.4 Regulation of CYP3A genes in fish

The role of CYP3A in chemical interactions in fish has been less studied compared to mammals. The inducibility of CYP3A expressions in fish is generally lower than that in mammals. For example, about 40% and 2-fold induction of CYP3A expression has been reported in fish liver (Celander et al. 1989, Pathiratne and George 1998, Bresolin et al. 2005), whereas in mouse liver, 2 to 9 fold inductions of CYP3A proteins have been seen upon exposure to PXR ligands (Matheny et al. 2004). At the onset of this thesis work, only two fish PXR sequences were available, from zebrafish (Bainy

and Stegeman 2002, Moore et al. 2002) and from pufferfish (*Fugu rubripes*) (Maglich et al. 2003). A functional study of the ligand binding domain (LBD) of zebrafish PXR was made, using a construct of the zebrafish LBD coupled to the Gal4 DNA binding domain fragment. This reporter construct was activated by certain steroids and a few pharmaceuticals, such as nifedipine, phenobarbital and clotrimazole (Moore et al 2002). However, the classical mammalian PXR ligand pregnenolone-16α-carbonitrile (PCN) did not activate the zebrafish PXR reporter construct. This is in contrast with an *in vivo* study in zebrafish, showing induction of CYP3A expression with PCN, but not with nifedipine or clotrimazole (Bresolin et al. 2005). This illustrates that further studies are needed to determine PXR activation and involvement in CYP3A regulation in fish. This has been addressed in all four papers of this thesis and in particular in **Paper III**.

1.2.5 Functions and regulation of CYP2 and CYP4 gene family members

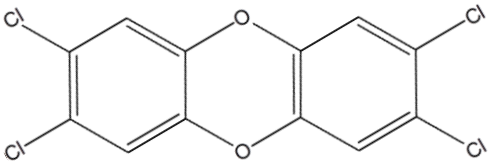
In addition to CYP1A and CYP3A, members of the CYP2 and CYP4 gene families are also involved in xenobiotic metabolism in humans. Vertebrate CYP4 enzymes are mainly involved in metabolism of fatty acids and are regulated by the nuclear peroxisome proliferator activated receptors (Hardwick 2008). Some CYP4 enzymes metabolize xenobiotics and at least CYP4F enzymes metabolize pharmaceuticals (Kalsotra and Strobel 2006). Three subfamilies have been found in fish CYP4F, CYP4T and CYP4V (Kirishian and Wilson 2012). However, information of function of CYP4 enzymes in fish is still lacking. The CYP2 gene family, on the other hand, is the largest and most diverse CYP family in vertebrates and the human CYP2 family includes important drug-metabolizing enzymes. The CYP2 enzymes in fish can metabolize both endogenous and xenobiotic compounds (Schlenk et al. 2008). Of the 12 mammalian CYP2 subfamilies, only two have also been found in fish. Instead, 12 additional CYP2 subfamilies have been reported in fish. Phylogenetic analysis confirmed that the two CYP2 genes in common for mammals and fish are CYP2R and CYP2U. Hence, these subfamilies are likely to be ancestral CYP2 genes (Kirishian et al. 2011). The mammalian drug-metabolizing CYP2B is predominantly regulated by the constitutive androstane receptor (CAR) and CYP2C via multiple nuclear receptors, *i.e.* PXR, CAR, glucocorticoid receptor and vitamin D receptor (Pustylnyak et al. 2007, Chen and Goldstein 2009). Similar to PXR, CAR is

also involved in CYP3A regulation in mammals (Xie et al. 2000). Thus, there are several nuclear receptors that regulate CYP genes involved in xenobiotic metabolism.

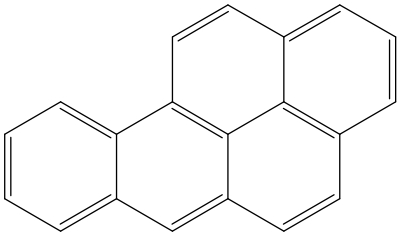
1.2.6 The aromatic hydrocarbon metabolizing CYP1A form

In ecotoxicology, the CYP1A is by far the most studied CYP isoform. Expression of CYP1A is normally low, but is highly induced in fish exposed to polyaromatic hydrocarbons (PAHs), such as petroleum components, and planar halogenated aromatic hydrocarbons such as polychlorinated biphenyls (PCBs) and dioxins (Stegeman and Hahn 1994).

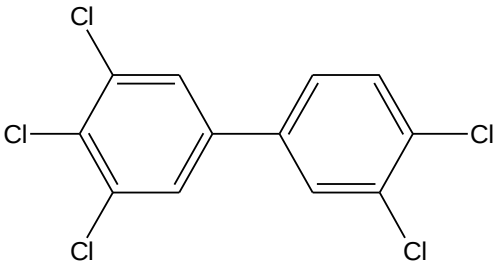
Induction of CYP1A is regulated by the aryl hydrocarbon receptor (AhR). In the absence of a CYP1A substrate, AhR resides in the cytoplasm linked to chaperone proteins. When AhR is activated by a ligand, it is released from the chaperones and translocated to the nucleus, where it dimerizes with aryl hydrocarbon receptor nuclear translocator (ARNT). The AhR-ARNT heterodimer binds to xenobiotic response elements in the promoter of CYP1A gene and induces transcription (Denison and Nagy 2003). Classical AhR ligands are PAHs, like benzo[*a*]pyrene, aromatic hydrocarbons, like the dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), dioxin-like compounds, like 3,3',4,4',5-pentachlorobiphenyl (PCB 126) and the model inducer β-naphthoflavone (BNF) which is a PAH-type chemical (Figure 5). Alternative CYP1A induction pathways have also been suggested in order to explain CYP1A induction by non-classical AhR activators (Delescluse et al. 2000). Induction of CYP1A activity, measured with the ethoxyresorufin-*O*-deethylase (EROD) assay, is one of the most widely used biomarker of exposure in environmental monitoring (Whyte et al. 2000). Pharmaceutical interactions on CYP1A induction and on EROD activities will be discussed in section 4.4 and 4.5, respectively.



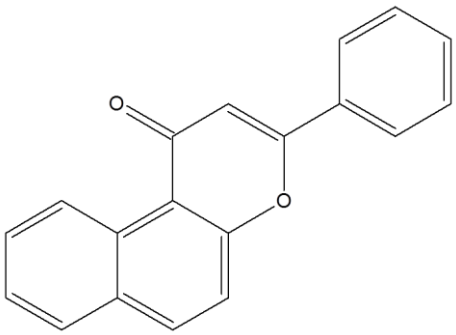
2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)
the ultimate AhR ligand



benzo[*a*]pyrene
polyaromatic hydrocarbon



3,3',4,4',5-pentachlorobiphenyl (PCB 126)
a dioxin-like PCB



β-naphthoflavone (BNF)
PAH-type model substance

Figure 5. Classical AhR ligands.

1.2.7 Cytoskeleton and CYP1A induction

Several nuclear receptors, like AhR and PXR, are translocated upon ligand activation from the cytoplasm to the nucleus. The mechanism for this transport is not fully understood but it has been suggested that AhR translocation is microtubule-dependent as CYP1A induction is limited in cells with depolymerized microtubules (Dvořák et al. 2006). The cell is dependent on the cytoskeleton for proliferation, intracellular transport, adhesion and motility. The cytoskeleton is a collected name for microtubules, intermediate filaments and actin filaments (microfilaments) in the cytoplasm. In **Paper I**, we have investigated whether the integrity of microtubules or actin filaments is affected by pharmaceutical exposures.

1.2.8 The efflux pumps

Efflux pumps are membrane proteins that actively transport a wide range of compounds out of the cells. The first efflux pump that was reported was, the permeability glycoprotein (Pgp) in mutated Chinese hamster ovarian (CHO) cells. These cells are resistant to a wide range of drugs and have been found to have an over-expression of Pgp pumps (Juliano and Ling 1976). High Pgp activities result in decreased accumulation of drugs and multidrug resistance which is a problem in chemotherapy (Ford and Hait 1993).

The Pgp and the related multidrug resistance associated proteins (MRP), are expressed in human tissues (Gillet and Gottesman 2010). They all belong to the large superfamily of ATP-binding cassette (ABC) proteins and they prevent bioaccumulation of a wide range of chemicals (Leslie et al. 2005). The gene coding for Pgp is denoted ABCB1 and MRPs are called ABCC-genes. The Pgp is involved in transportation of un-metabolized xenobiotics (phase 0), whereas MRPs are involved in transportation of conjugated metabolites (phase 3) (Figure 6). However, there are overlap in substrate specificities between Pgp and MRPs (Keppler et al. 1999, Kim 2002). Interactions of pharmaceuticals on efflux pumps were addressed in **Paper I**.

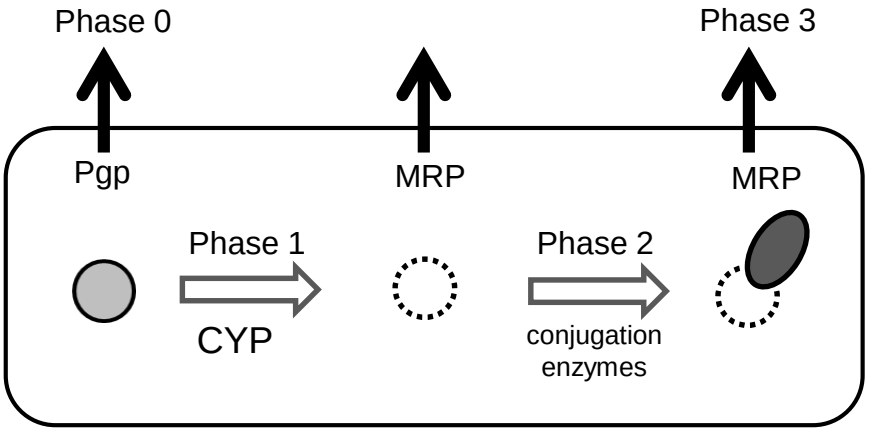


Figure 6. Biotransformation and efflux in a liver cell.

1.2.9 Multixenobiotic resistance and chemosensitizers

Multiresistance characteristics, *i.e.* simultaneous resistance to several xenobiotics due to decreased accumulation within the cells, have been found in organisms living in polluted waters and are described as multixenobiotic resistance (Kurelec 1992). The Pgp has been detected in several aquatic organisms including fish (Bard 2000). Also MRPs have been identified in several fish species (Sauerborn et al. 2004, Zaja et al. 2007, Fischer et al. 2010, Sauerborn Klobucar et al. 2010). Xenobiotic resistance in fish is discussed in section 4.6. Substances that interfere with the efflux pumps are called chemosensitizers and are used in chemotherapy to enhance effects of anti-cancer drugs. In ecotoxicology, chemosensitizers can pose a problem as they can impair the detoxification capacity (Smital and Kurelec 1998).

1.3 Pharmacokinetic interactions

1.3.1 Chemical interactions

It has been highlighted that chemical interactions can result in mixture toxicities that lead to adverse health effects, above those of single substance exposure. Drug interactions due to the same mode of action, *e.g.* a shared drug target, are known as pharmacodynamic interactions whereas drug interactions due to a shared pathway for metabolism and excretion are known as pharmacokinetic interactions (Figure 7). Pharmacokinetic interactions are in focus in this thesis and as other non-pharmaceutical chemicals are also studied, we also use the term toxicokinetic interactions.

1.3.2 Drug interactions caused by CYP3A inhibition

Inhibition of CYP3A activities can increase biological half-lives of chemicals that depend on CYP3A metabolism for their excretions. This can result in increased plasma concentrations and a potential overdose risk (Figure 8). Examples of CYP3A inhibitors are antifungal azoles like ketoconazole. Also, food components can affect CYP3A metabolism and for example bergamottin in grape-fruit juice is known to inhibit intestinal CYP3A

activities, which results in increased plasma concentrations of several drugs in humans (Huang et al. 2004, Paine et al. 2006). For that reason, patients are sometimes recommended to avoid drinking grapefruit juice together with drugs metabolized by CYP3A enzymes. Interestingly, inhibition of CYP3A can be used to enhance certain drug therapies. For example, grapefruit juice consumption efficiently increased the effect of the cancer drug sirolimus as the dose could be decreased to reduce adverse effects of sirolimus with preserved therapeutic effect (Cohen et al. 2012). Drug interaction by CYP3A inhibition was investigated in **Paper II** and **Paper III**.

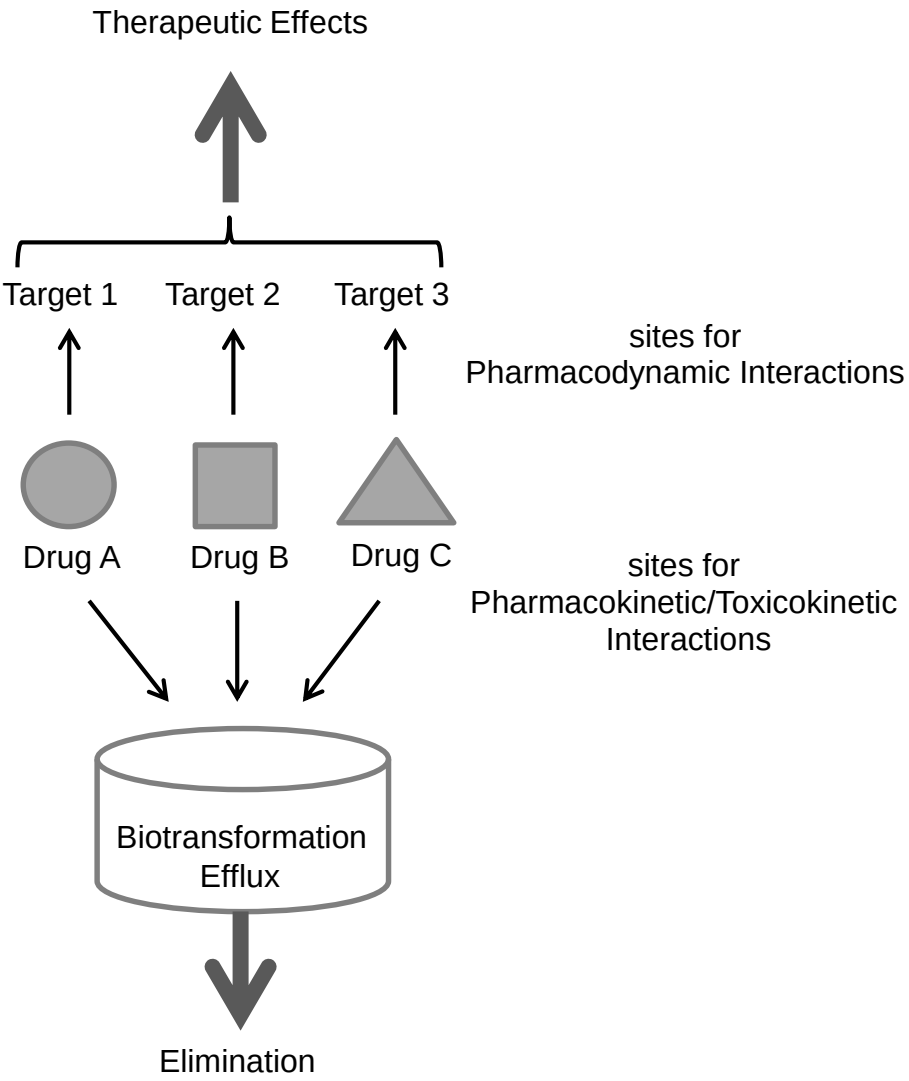


Figure 7. Chemical interactions.
Pharmacodynamic interactions occur when different chemicals have the same or opposite modes of action.
Pharmacokinetic/Toxicokinetic interactions occur when different chemicals share the same elimination pathway.

1.3.3 Drug interactions caused by CYP3A induction

Induction of CYP3A activities can also result in drug interactions (Figure 8). For example, the herbal medicine St John's wort (*Hypericum perforatum*) is used as a mild antidepressant and an alternative to the synthetic drugs with similar effect. Several reports reveal that this herbal medicine reduces the effect of other drugs like oral contraceptives and immunosuppressors (Huang et al. 2004). Thus, a substance in St John's wort, hyperforin, was shown to be an efficient ligand to PXR resulting in induced CYP3A4 gene expression and metabolic elimination in humans (Moore et al. 2000).

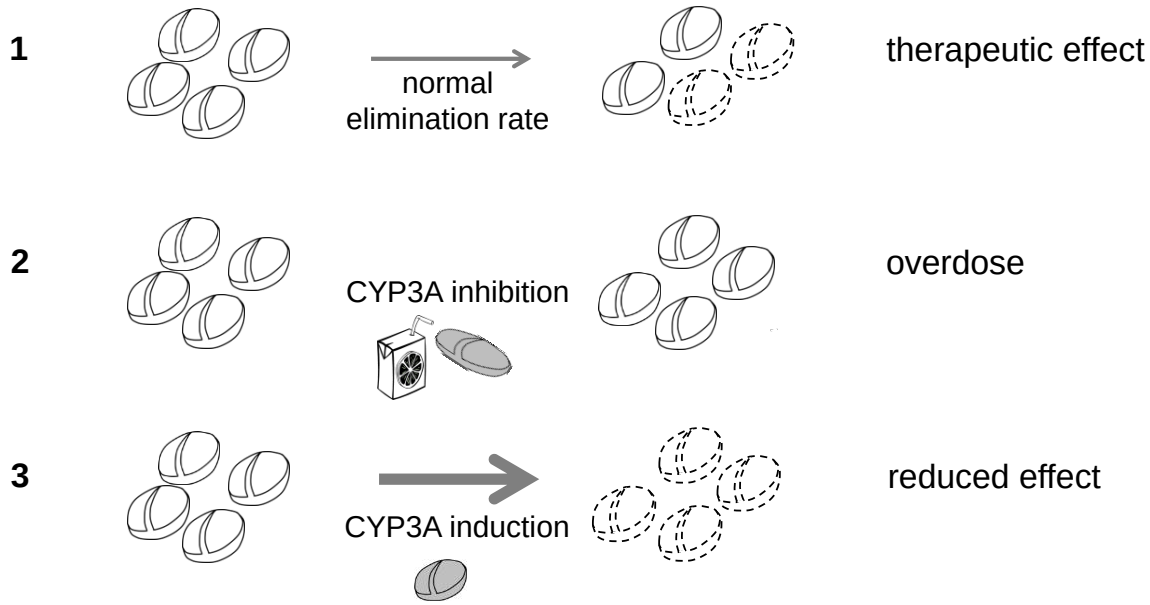
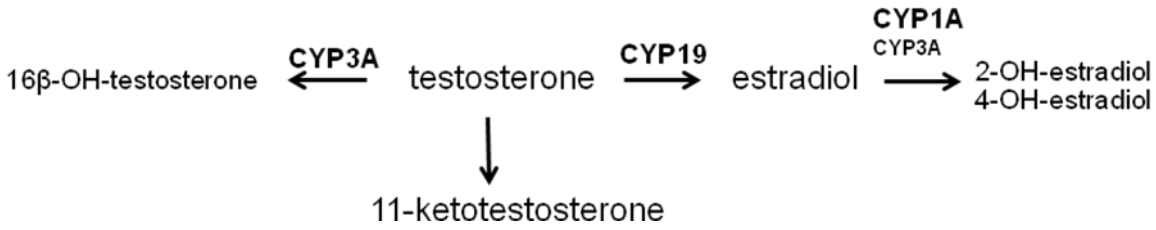


Figure 8. Examples of pharmacokinetic interactions in human by altered CYP3A activity. The tablet with a dashed line illustrates a metabolized pharmaceutical.
1. Normal elimination rate of a pharmaceutical to maintain a therapeutic effect.
2. Decreased elimination rate of a pharmaceutical as a result of inhibition of the metabolic clearance caused by another pharmaceutical or grapefruit juice.
3. Accelerated elimination rate of a pharmaceutical as a result of induction of the metabolic clearance caused by another pharmaceutical.

1.3.4 Drug interactions with sex steroid levels

Sex steroid hormone levels in plasma are chiefly dictated by the rate of hormone biosynthesis, *i.e.* anabolism, and the rate of hormone breakdown, *i.e.* catabolism. Fish steroid catabolism was recently reviewed by James (2011). In fish, as in mammals, CYP3A catalyzes 6β-hydroxylation of testosterone (Lee and Buhler 2002). Estradiol is predominantly metabolized by CYP1 and CYP2 subfamily members. In contrast to mammals, CYP3A is less effective in metabolizing estradiol in fish (Miranda et al. 1989, Scornaienchi et al. 2010). Elevated levels of CYP1A by benso[*a*]pyrene have been shown to increase estradiol hydroxylation (Butala et al. 2004). Accordingly, alteration in the CYP1A or CYP3A activity, such as induction or inhibition due to the presence of drugs or other xenobiotics, may lead to an imbalance in sex steroid levels resulting in endocrine disruption. The major sex steroid hormones in fish are estradiol, progesterone, testosterone and 11-ketotestosterone. Estradiol is formed when testosterone is hydroxylated catalyzed by the enzyme CYP19 aromatase. The following reactions illustrate involvement of CYP enzymes in sex steroid metabolism.



For this reason, inhibition of CYP19 aromatase can result in disrupted sex steroid hormone profiles which may affect the reproductive success (Ankley et al. 2002). In addition, the efflux pumps are involved in the excretion of steroid hormones and their metabolites. Both in mammals and fish *e.g.* channel catfish (*Ictalurus punctatus*), estradiol has been found to be transported by Pgp prior to biliary excretion (Kim and Benet 2004, Kleinow et al. 2004). Accordingly, changes in Pgp activities may also affect the steroid levels. Plasma levels of estradiol, testosterone and 11-keto-testosterone were analyzed after exposure to the wide-spectrum CYP inhibitor ketoconazole in **Paper II**.

1.4 Resistance to environmental pollutants

1.4.1 Chemically resistant fish populations

There are several reports of chemically resistant killifish (*Fundulus heteroclitus*) populations in North America. These populations live and reproduce in areas that are heavily polluted by aromatic hydrocarbons from industrial activities. Examples of such areas, inhabited by killifish, are the New Bedford Harbor, MA and parts of the Elizabeth River, VA (Van Veld and Westbrook 1995, Nacci et al. 1999, Bello et al. 2001).

1.4.2 Mechanisms of chemical resistance

A feature in some of these killifish populations, including that in New Bedford Harbor, is a low CYP1A expression in spite of high PCB exposure. Furthermore, these fish display low or no CYP1A inducibility to CYP1A inducers in the laboratory (Nacci et al. 1999, Bello et al. 2001). The resistance associated with lack of CYP1A inducibility is contradictory as CYP1A is normally induced by aromatic hydrocarbons in order to secure an efficient metabolism that prevents accumulation of these compounds. However, CYP1A metabolism can produce reactive metabolites that are more toxic than the parent compound. In addition, uncoupling of the CYP1A catalytic cycle can result in formation of reactive oxygen species (Schlezinger et al. 1999). Therefore, lack of CYP1A induction can be beneficial in certain situations. The mechanism behind lack of CYP1A induction is hypothesized to involve the AhR as other AhR regulated genes (*e.g.* CYP1B and CYP1C) also have been reported to be non-inducible in these killifish (Oleksiak et al. 2011). Apparently, these resistant fish thus have a compromised CYP1A function, but it is not clear how these fish survive in highly pollutant environments. In addition to planar PCBs that act as AhR ligands, other non-planar PCB congeners are present, some of which act as mammalian PXR ligands. However, it is not known how PXR-CYP3A/Pgp signaling is affected in these fish. This was investigated in **Paper IV** in which expressions of PXR, CYP3A and Pgp were studied in fish, with disrupted AhR signaling, exposed to planar and non-planar PCBs.

2. Scientific Aim

2.1 Overall aim

The overall aim of this thesis was to increase knowledge on key detoxification mechanisms in fish and to identify sites for interactions by pharmaceuticals and other pollutants.

2.2 Specific aims

A key aim was to clarify whether CYP3A expression is regulated by PXR signaling in fish. Further, we wanted to find out whether expression of the PXR, CYP3A and Pgp genes is affected in a killifish population with disrupted AhR-CYP1A signaling, and which is resistant to dioxin-like compounds. Focus was also placed on determining drug interactions on detoxification functions in rainbow trout exposed to CYP1A/CYP3A inhibitors. Finally, we also aimed to find potential markers of adverse effects by pharmaceuticals using other endpoints such as cytoskeleton morphology in fish hepatic cells.

3. Methods

3.1 Animals and cell models

3.1.1 Fish species

 In **Paper I**, we used **guppy** (*Poecilia reticulata*) to isolate a *Poeciliidae* CYP3A sequence that was used in the search for a CYP3A gene in the *Poeciliopsis lucida* hepatocellular carcinoma cell line.

 In **Paper II** and **Paper III**, we used **rainbow trout** (*Oncorhynchus mykiss*), which is a salmonid fish originating from western North America. This species has been farmed since late 19th century and spread worldwide in fish farms. It is a commonly used model for teleosts research including the ecotoxicology field. For **Paper II** and **Paper III**, relatively small rainbow trout (ca 50 and 150 g body weight, respectively) were intraperitoneally injected with test substances. For **Paper III**, livers from larger fish (c:a 500 g body weight) were used to obtain primary cultures of hepatocytes from.

 In **Paper IV**, we used **Atlantic killifish** (*Fundulus heteroclitus*), which inhabits the Atlantic coast-line of North America. This stationary species is extraordinary tolerant to large variations in salinity and temperature, and is able to survive and reproduce in highly polluted areas, sometimes as the only fish species present. Additionally, it has a relatively narrow home range and burrows in the sediment during winters. These features have given the killifish an important role in ecotoxicology research. In the present study, killifish were collected from the highly PCB-polluted area in New Bedford Harbor and from the more pristine area Scorton Creek (SC) on Cape Cod. These fish were further exposed to two PCB congeners in the lab.

3.1.2 Primary cell cultures

Primary cultures of hepatocytes from rainbow trout were used in **Paper III**. Primary cultures were obtained by perfusing the liver with collagenase solution to break up the liver tissue to individual cells, predominantly hepatocytes. The *in vitro* approach of using cell cultures decreases the biological variation of the research model in comparison with using an *in vivo* approach of using whole fish as the cell culture from each donor fish can be used in several exposure experiments. Primary cultures of rainbow trout hepatocytes are typically viable for 5-6 days (Pesonen and Andersson 1991).

3.1.3 Cell lines

In contrast to primary cells, cell lines often originate from a tumor tissue, and are highly proliferative cells that can be maintained in culture for many generations. Accordingly, it should be taken into account that cell lines are not necessarily representatives for normal differentiated cells. Highly proliferating cells may have lost certain functions compared to primary cells as a result of de-differentiation. Despite this, cell lines are useful tools for studying mechanisms of cell functions, including gene regulations.

The *Poeciliopsis lucida* hepatocellular carcinoma (**PLHC-1**) cell line used in **Paper I** is a good *in vitro* model for studies of detoxification mechanisms. This cell line has retained several hepatocyte functions, including CYP1A and efflux activities. The PLHC-1 originates from the desert topminnow, also named clearfin livebearer.

The rainbow trout hepatoma cell line (**RTH-149**) was initially used in **Paper III** for gene expression, but was shown to have an unusual low basal CYP3A27 expression. This cell line was instead used in the PXR reporter assay in **Paper III**.

The human liver hepatocellular carcinoma cell line (**HepG2**) is commonly used for transfection studies with reporter vectors. We used HepG2 and compared it to RTH-149 for the reporter assay in **Paper III**.

3.2 Techniques

3.2.1 Gene analyses

In **Paper I**, we isolated partial transcripts of CYP3A from guppy and CYP1A and CYP3A from PLHC-1 by conventional reverse transcriptase PCR using degenerated primers. These primers were designed against conserved regions found by clustalW sequence alignments. Sequencing services were provided from Eurofins MWG operon, Germany. The obtained sequences were compared to the NCBI database using the basic local alignment search tool (BLAST). In **Paper III**, the complete coding sequence of PXR from rainbow trout was isolated using degenerated primers for a partial sequence followed by the method rapid amplification of cDNA ends (RACE). In RACE one utilizes the known partial sequence for designing gene specific primers directed outwards. Small DNA strands are ligated to the cDNA ends providing a target for a universal primer directed inwards. This enables PCR amplification and following sequencing of the unknown parts upstream and downstream of the partial sequence. The upstream promoter region of the CYP3A27 gene in rainbow trout was isolated using the genome walking method. This technique resembles RACE as small DNA strands are ligated to the genomic DNA providing target for a PCR primer used in combination with a primer targeting the known sequence.

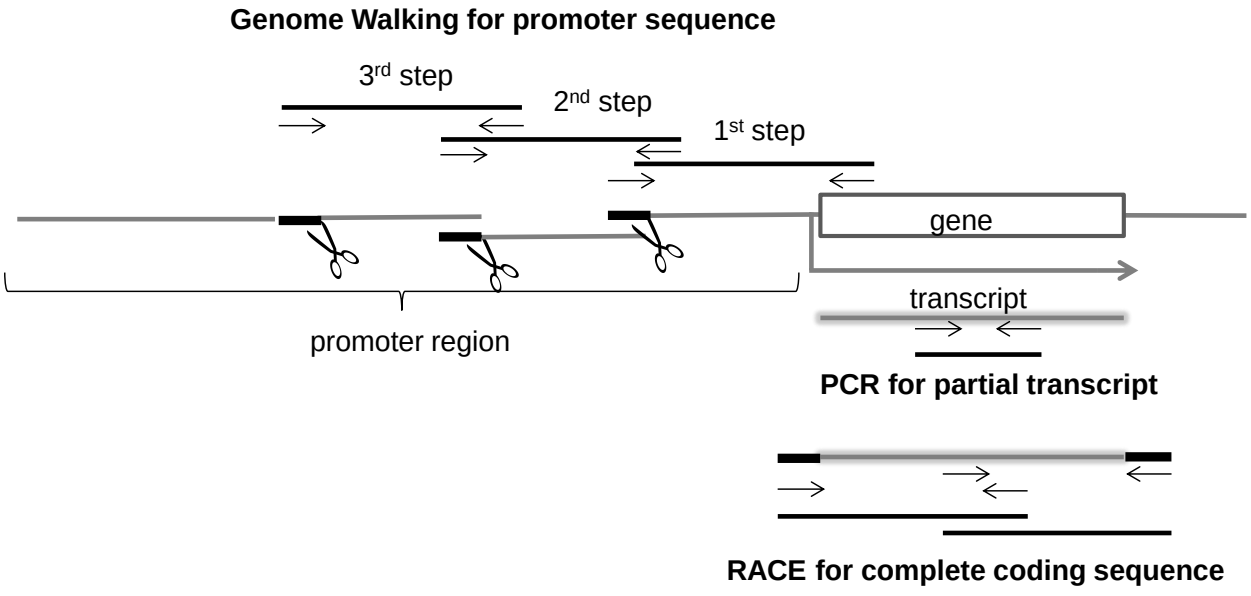


Figure 9. The PCR approaches used for gene identification in this thesis.

3.2.2 Phylogenetic analyses of fish CYP3 gene family and PXR

The phylogenetic analyses were done after acceptance of **Paper I** and **Paper III.** Therefore, the method is described in details here. The analyses were performed by Dr. Joanna Wilson, McMaster University, Hamilton, Ontario.

The deduced amino acid partial CYP3 coding sequences of two guppy species *Poecilia reticulate* and *Poeciliopsis lucida*, zebrafish CYP3A65 and CYP3C1, medaka (*Oryzias latipes*) CYP3B4 and stickleback (*Gasterosteus aculeatus*) CYP3D1 were blasted (blastp) against the NCBI database (non-redundant sequences) with a restriction for taxa Actinopterygii (teleost fish). An additional blastp search (not restricted by taxa) was completed with human CYP3A4 to supply 17 mammalian CYP3 sequences to root the phylogenetic tree; mammalian sequences were no more than 88% dissimilar from CYP3A4. The top 30-50 blast hits from each fish sequence, the mammalian CYP3A sequences, the two guppy CYP3 sequences and sequences annotated from zebrafish (CYP3A65, CYP3C1-4), medaka (CYP3B3-4), and stickleback (CYP3D1) genomes were collated and redundant sequences removed.

The deduced amino acid coding sequence of rainbow trout PXR was blasted (blastp) against the NCBI database (non-redundant sequences). The top 100 hits included sequences from the NR1I2 family; vitamin D receptor, CAR and PXR sequences were from a range of vertebrate species including mammals, fish, and amphibians.

Sequences were aligned with Clustal W using the online server on EMBL-EBI (www.ebl.ac.uk/Tools/msa/clustalw2/) with default settings. The alignment was examined in mesquite 2.75 and regions that were difficult to align were removed from subsequent analyses. ProtTest (2.4 server, darwin.uvigo.es/software/prottest2_server.html) determined the optimal model for phylogenetic analyses using the AIC selection criteria. Maximum likelihood and bootstrap analyses were run on the RaxML black box server (CIPRES science gateway; www.phylo.org) specifying a JTT +I+G+F model. The best tree with bootstraps was rooted with the vitamin D receptor clade (PXR phylogeny) or mammalian CYP3A clade (CYP3 phylogeny) in FigTree.

3.2.3 The PXR reporter assay

Reporter assays enable high-throughput screening of potential gene activators for specific receptors. A reporter vector carries the promoter of the target gene. The promoter is directly connected to the reporter gene, in our case luciferase, which is transcribed when the promoter is activated. The enzyme luciferase oxidizes luciferin resulting in light emission that can easily be detected. The rainbow trout genome is, however, not fully sequenced and a promoter sequence for rainbow trout CYP3A27 is not yet available. Instead, in **Paper III**, we used a human CYP3A4 reporter vector. The reporter vector was transiently transfected to RTH-149 and HepG2 cells. We also overexpressed rainbow trout PXR by co-transfecting the cells with an expression vector carrying the coding sequence of PXR (Figure 10).

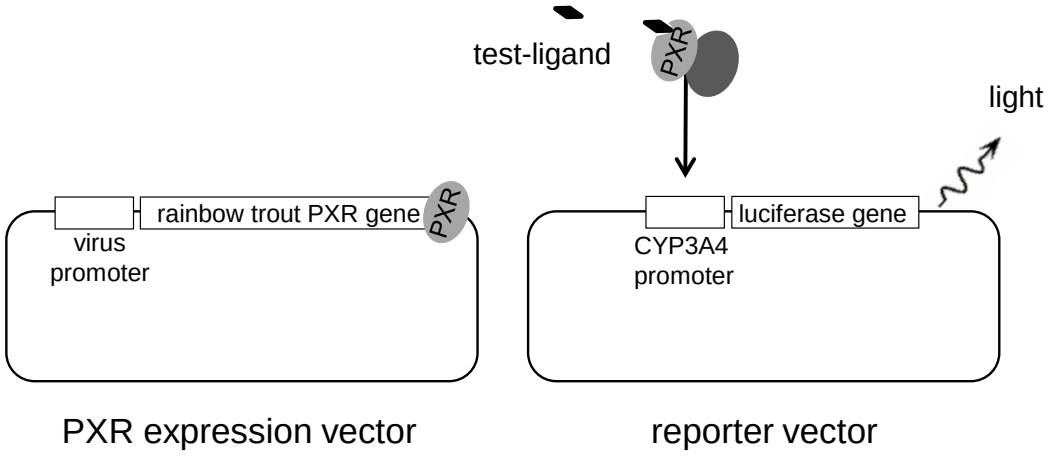


Figure 10. Principals of the PXR reporter assay.

3.2.4 Quantification of mRNA levels

The mRNA levels of detoxification proteins were quantified using quantitative PCR (qPCR) in **Paper I-IV**. In qPCR the amplification is followed in real time, detected using a fluorescent dye. There is a negative correlation between mRNA levels and cycle number that reaches the threshold level (Figure 11). The limitation of all mRNA analysis is that one can only assume that the protein translation will follow and result in a corresponding production of functional proteins. For this reason, the analyses of protein levels and enzyme activities are important complements to address functional effects.

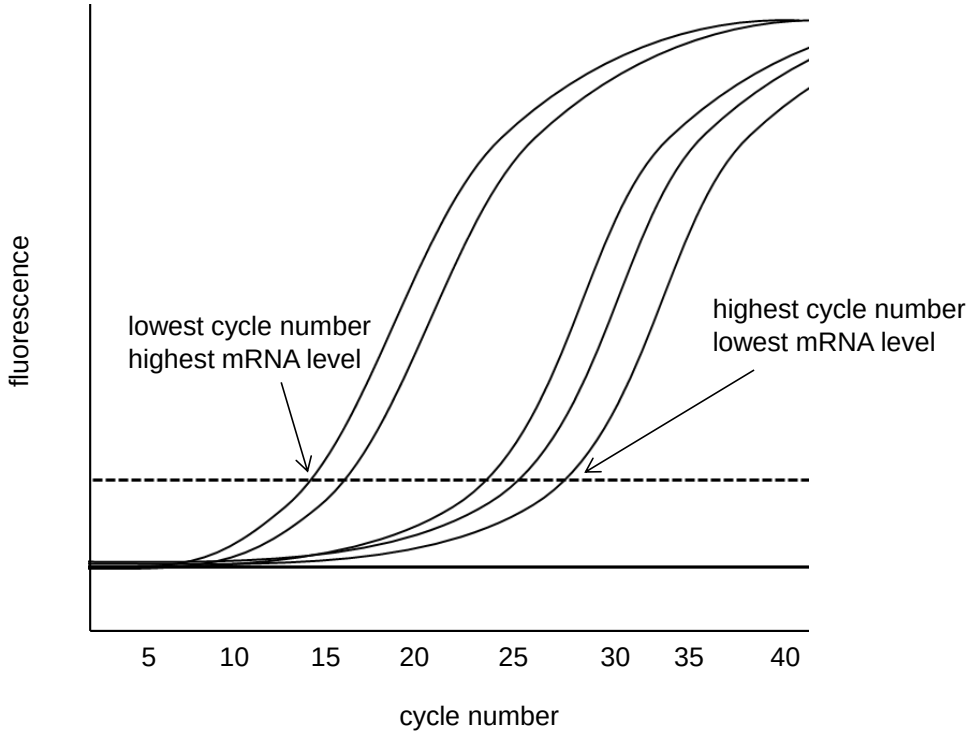


Figure 11. Amplification curves of qPCR.

3.2.5 Quantification of enzyme activities

Enzyme activities were analyzed in paper I, II and III. The CYP1A activities were analyzed using ethoxyresorufin-*O*-deethylase (EROD) activity. The CYP3A activities were analyzed using benzyloxy-4-[trifluoromethyl]-coumarin-*O*-debenzyloxylase (BFCOD) activity.

3.2.6 Quantification of efflux pump activities

In **Paper I**, the efflux pump activities were measured using the fluorescent dye rhodamine123, which is a good substrate for the efflux pumps. This assay does not distinguish between Pgp and MRP1/2 activities. If the test substances interact with efflux activities, the accumulation of rhodamine 123 is altered (Figure 12).

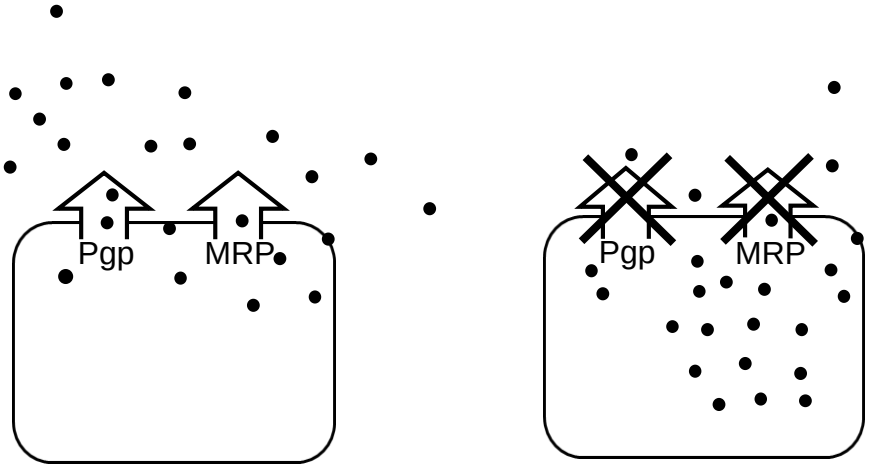


Figure 12. Interactions on efflux activities analyzed by dye accumulation.

3.2.7 Immunochemical analyses

Immunocytochemistry (**Paper I**), Western blot (**Paper II, Paper III**) and enzyme-linked immune sorbent assay (ELISA) (**Paper II**) are analytical methods based on antibody detection. We used immunocytochemistry to stain microtubules (**Paper I**). Microtubules are highly conserved between species and a mouse monoclonal antibody against chicken microtubules was used. To quantify CYP1A and CYP3A protein levels by Western blot, we used polyclonal antibodies raised in rabbits against CYP1A from perch (*Perca fluvatilis*) and CYP3A from rainbow trout (**Paper II, Paper III**). Competitive ELISA protocols were used to analyze plasma levels of vitellogenin, testosterone, 11-ketotestosterone and estradiol (**Paper II**).

Table 2.
Substances used in this thesis and their actions in mammals.

	Substance	Action in mammals
Pharmaceuticals	Clotrimazole	antifungal drug
	Dexamethasone	glucocorticoid receptor agonist
	Diclofenac	non-steroidal anti-inflammatory drug
	Ethinylestradiol	estrogen receptor agonist
	Fulvestrant	estrogen receptor antagonist
	Ibuprofen	non-steroidal anti-inflammatory drug
	Ketoconazole	antifungal drug
	Omeprazole	proton pump inhibitor
	Paracetamol	analgesic drug
	Quinidine	anti-arrhythmic drug
	Rifampicin	macrolide antibiotic drug
	Troleandomycin	macrolide antibiotic drug
Estrogenic pollutant	Bisphenol A	estrogen receptor agonist
Model substances	α-Naphthoflavone (ANF)	aryl hydrocarbon receptor antagonist
	β-Naphthoflavone (BNF)	aryl hydrocarbon receptor agonist
	Lithocholic acid	pregnane X receptor agonist
	Nocodazole	microtubule disruptor
	Pregnenolone-16α-carbonitrile (PCN)	pregnane X receptor agonist

4. Findings and Discussion

4.1 Fish CYP3A genes

4.1.1 Identification of a CYP3 gene in PLHC-1 cells

The PLHC-1 cell line is an established *in vitro* model in ecotoxicology studies (Fent 2001). However, sequence information from the species that it is derived from, the clearfin livebearer, is limited and no PLHC-1 CYP3A gene has been reported. Despite thorough PCR screening using several combinations of degenerated PCR primers, no CYP3A cDNA was amplified in PLHC-1. Therefore, we isolated and sequenced a CYP3A cDNA from liver from the closely related species guppy and by using guppy gene specific primers, a CYP3A-like sequence was finally found in PLHC-1. A protein BLAST search revealed highest sequence identity (69%) with CYP3A40-like protein from Nile tilapia (*Oreochromis niloticus*). However, when compared to zebrafish, it showed 60% sequence identity with CYP3C1 and slightly less *i.e.* 58% with CYP3A65 (**Paper I**). This suggests that the PLHC-1 CYP3 sequence actually may belong to another CYP3 subfamily than CYP3A. In mammals, there is only one CYP3 subfamily, namely CYP3A. In fish in contrast, four CYP3 subfamilies have been discovered and these are CYP3A, CYP3B, CYP3C and CYP3D (Yan and Cai 2010). A phylogenetic analysis of the CYP3 gene family in fish was carried out including the PLHC-1 and the guppy sequences. The guppy sequence clustered within the CYP3A subfamily whereas the PLHC-1 sequence clustered with CYP3B genes (Figure 13). The CYP3A40-like sequence that was most similar in the first BLAST search also clustered with CYP3B genes. Therefore, the PLHC-1 sequence is denoted CYP3B in this thesis.

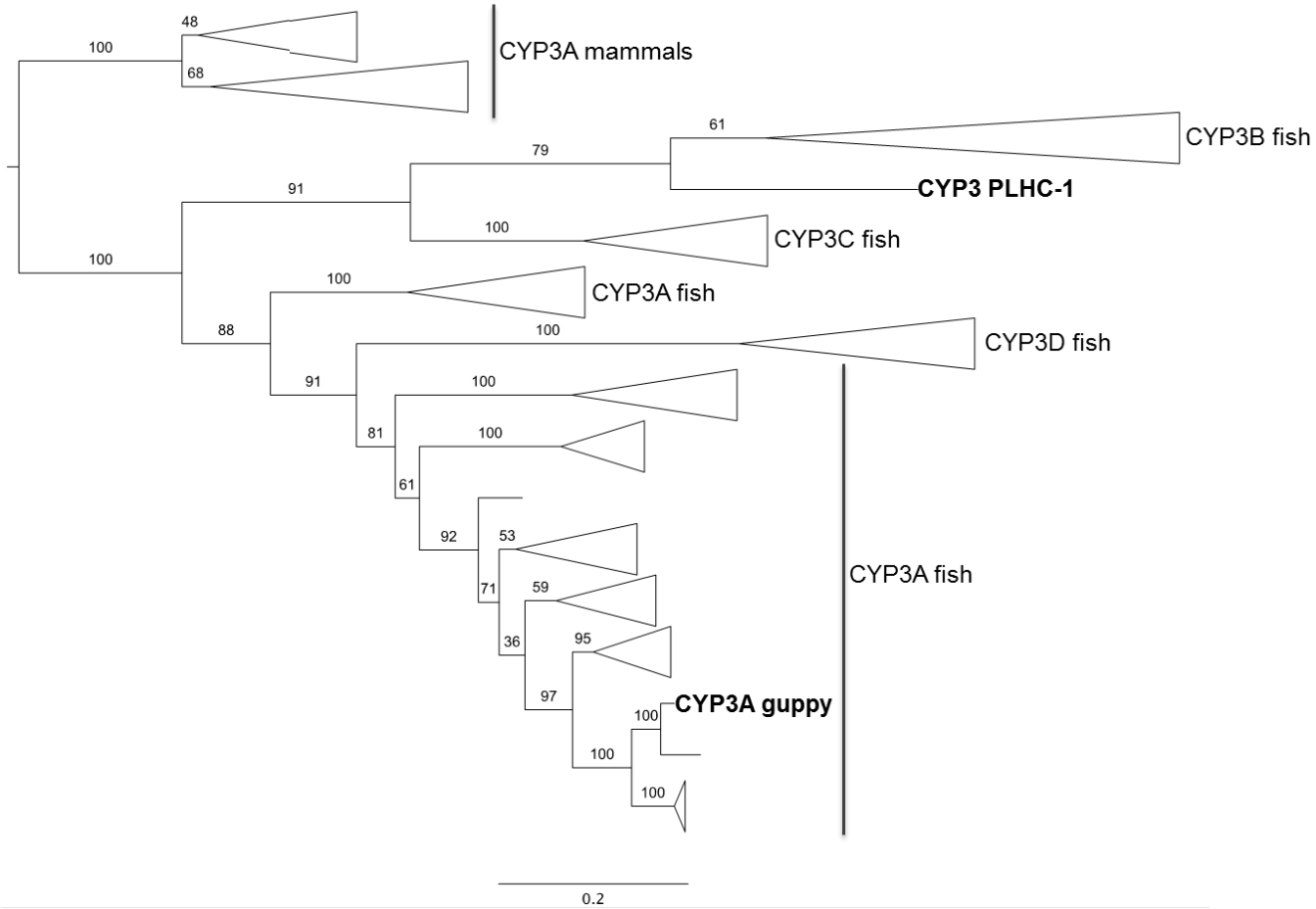


Figure 13. Phylogenetic tree of the fish CYP3 gene family.

4.1.2 Expression of CYP3A and CYP3B in cell lines

Cell lines in general are less differentiated than normal cells (Thibaut et al. 2009). Besides, many cell lines have lower CYP activities (Rodriguez-Antona et al. 2002). The PLHC-1 cell line was shown to have a low basal CYP3B expression (denoted CYP3A in **Paper I**) and it was not affected by any of the pharmaceuticals tested, including the prototypical CYP3A inducers, rifampicin and PCN (**Paper I**). Previous studies showed that exposure to rifampicin, PCN and dexamethasone had no effect on CYP3A-like protein levels in PLHC-1 cells (Celander et al. 1996). Furthermore, CYP3A activities (measured as testosterone 6β-hydroxylations) have been reported to be very low in PLHC-1 (Thibaut et al. 2009). However, increased CYP3A activities (measured as BFCOD activities) were reported in PLHC-1 cells exposed to higher doses of rifampicin (Christen et al. 2009).

Although, gene expressions were not addressed and it cannot yet be ruled out that other CYP isoforms are involved in BFCOD metabolism. In rat hepatocytes, BFCOD activities can be catalyzed by several CYP isoforms, including CYP1A and CYP2B (Price et al. 2000). Furthermore, in rainbow trout cell line (RTH-149), low basal expression of CYP3A and lack of inducibility was evident (**Paper III**). In another rainbow trout cell line 6β-hydroxylation of testosterone was not detected (Thibaut et al. 2009). Low CYP3A gene expressions and poor inducibilities of CYP3A have also been reported in the human HuH7 cell line (Phillips et al. 2005). It was suggested that this was due to a dense chromatin structure in HuH7 cells. This was supported by the CYP3A4 gene expression being relatively unsensitive to DNase treatment. Besides, addition of a histone deacetylation inhibitor, which opens the chromatin structure, increased both basal expression and inducibility of the CYP3A4 gene in HuH7 (Phillips et al. 2005).

Increased CYP3A4 expression and CYP3A activities were seen in HuH7 cells grown in confluence for 5 weeks (Sivertsson et al.). This is likely due to higher differentiation in confluent cells when proliferation is limited. Accordingly, we aimed to differentiate RTH-149 in a similar way. However, no change was seen in basal CYP3A27 expression after 5 weeks compared to proliferating RTH-149 cells (unpublished). We found the PXR expression being lower in RTH-149 cells compared to that in primary hepatocytes from rainbow trout (unpublished), and PXR has so far not been found in PLHC-1 cells. Hence, it is possible, that the low CYP3A expressions in several cell lines are due to poor function of PXR or that the chromatin structure interferes with CYP3A expressions.

4.1.3 Responses to CYP3A inducers in vitro *and* in vivo

As the fish cell lines tested had compromised CYP3 gene expressions, primary hepatocytes from rainbow trout were initially used for testing effects of prototypical CYP3A inducers. Approximately, 2-fold inductions of CYP3A27 mRNA levels were seen in hepatocytes exposed to the mammalian PXR-ligands PCN and lithocholic acid (**Paper III**). Weak inducibility is in accordance with previous studies in fish exposed *in vivo* to PCN (Celander et al. 1989, Pathiratne and George 1998, Bresolin et al. 2005). In addition, cortisol and ketoconazole induce CYP3A expression in rainbow trout

(Celander et al. 1989, Hegelund et al. 2004, **Paper II**). No increased CYP3A activities, assessed with BFCOD activities, were observed in rainbow trout and killifish exposed *in vivo* to dexamethasone, PCN and rifampicin (Smith and Wilson 2010). Higher CYP3A induction has however been observed in hepatocytes from common carp (*Cyprinus carpio*), where a 7-fold induction of CYP3A mRNA level was seen upon exposure to rifampicin (Corcoran et al. 2012). Though, many fish species show weaker CYP3A inducibilities compared to mammals. In human hepatocytes, a 5 to 20 fold induction of CYP3A4 mRNA levels was seen after exposure to rifampicin, phenobarbital, St John's wort extracts and dexamethasone (Komoroski et al. 2004, Phillips et al. 2005). Species differences between humans and rats, in responsiveness to rifampicin and dexamethasone, have been shown to be due to differences in the LBD of the PXR rather than differences in the target CYP3A genes (LeCluyse 2001). It is possible that the differences of CYP3A induction between fish species are a result of differences in PXR. The PXR is further discussed in section 4.2.

4.1.4 Variations in basal CYP3A levels

The low inducibility of CYP3A in fish can be due to differences in regulation and function of the PXR-CYP3A pathway compared to mammals. However, it is also possible that fish already have a sufficiently high basal expression of CYP3A for detoxification and further induction is not needed. We observed that hepatocytes with low basal CYP3A27 gene expression had higher responses to CYP3A inducers compared to cells with higher basal expressions (**Paper III**). Similarly, responsiveness to dexamethasone and rifampicin treatments were higher in zebrafish larvae that had lower basal CYP3A65 expression. Thus, basal expressions of CYP3A65 mRNA increases significantly between 84 and 96 hours post fertilization (hpf) and response to CYP3A inducers was only observed at 84 hpf (Tseng et al. 2005). These two studies both support the hypothesis that lack of CYP3A induction in fish is related to high basal CYP3A gene expressions.

4.1.5 Possible involvement of AhR in CYP3A regulation

In addition to exposure to the PXR ligand rifampicin, exposure the AhR ligand TCDD also induced CYP3A65 in zebrafish larva (Tseng et al. 2005). Blocking the expression of AhR2 with morpholino oligonucleotides, suppressed both basal expression as well as the TCDD-induced expression of CYP3A65. This suggests that AhR2 has a role in CYP3A65 regulation in zebrafish larvae (Tseng et al. 2005). In human cell lines, TCDD also induces CYP3A4, although PXR and not AhR was found to be the active receptor, based on results from reporter assays using silencing RNA for the receptors (Kumagi et al. 2012). In rainbow trout hepatocytes, exposure to the AhR ligand BNF resulted in weak (50%) induction of CYP3A27 mRNA (J Gräns and M Celander unpublished). However, in the rainbow trout PXR reporter assay, a strong down-regulation of the basal reporter activity was seen with BNF, whereas the AhR antagonist α-naphthoflavone (ANF) had no effect on basal reporter activity (J Gräns, B Wassmur and M Celander, unpublished). This illustrates the complexities in AhR and PXR receptor signaling pathways and that they may interfere with each other's actions.

4.1.6 Ketoconazole – a putative PXR agonist in fish

Ketoconazole is referred to as a PXR antagonist in mammals (Huang et al. 2007). However in rainbow trout, a 50% induction of CYP3A protein levels was evident after exposure to ketoconazole (**Paper II**). We found a time difference as this induction was observed 6 days after injection, but not after 3 days. A dose-dependent response to ketoconazole has earlier been shown in rainbow trout, where induction was seen in fish exposed to a low dose, but not in fish exposed to a higher dose (Hegelund et al. 2004). The antagonistic property of ketoconazole has been investigated in fish using primary hepatocytes from carp. Thus, ketoconazole antagonized the rifampicin mediated induction of CYP3A mRNA levels, but had no effect alone on CYP3A expression (Corcoran et al. 2012). It is possible that the observed dose and time differences in CYP3A induction are due to ketoconazole being an antagonist for PXR at high doses and a weak agonist at low doses. Ketoconazole concentrations are likely lower in fish 6 days compared to 3 days post injection, as a result of ketoconazole elimination.

4.2 The PXR – a xenosensor

4.2.1 Isolation of PXR from rainbow trout

In mammals, CYP3A genes are regulated via PXR signaling. The mammalian PXR is unusually promiscuous and responds to a wide range of substances and is referred to as a xenosensor. To characterize PXR in fish, we isolated the complete PXR coding sequence from rainbow trout (**Paper III**). The CYP3A genes have been relatively well conserved during vertebrate evolution (McArthur et al. 2003). However, there is large variation in CYP3A inducibility among mammalian species and it has been proposed that these differences are dependent on PXR species differences (LeCluyse 2001). As discussed in section 4.1.3 above, fish CYP3A genes are less responsive to mammalian PXR ligands. Sequence comparisons of the LBD in PXR from fish and human are provided in Table 3.

Table 3. Sequence comparisons between the LBD in PXR genes.

	Rainbow trout	Fathead minnow	Zebrafish	Killifish	Medaka	Human
Rainbow trout	-	72%	70%	67%	63%	55%
Fathead minnow		-	90%	63%	60%	60%
Zebrafish			-	63%	60%	55%
Killifish				-	75%	53%
Medaka					-	52%
Human						-

4.2.2 Phylogenetic analysis of PXR genes

Thereafter a phylogenetic analysis of the nuclear receptor (NR) 1I family of receptors was performed (Figure 14). This receptor family consists of PXR, CAR and the vitamin D receptors. The tree shows that rainbow trout PXR clusters with other fish PXR, having zebrafish PXR as the closest neighbor.

The relatively low bootstrap value of 66 is likely due to the lack of known PXR genes from other salmonid species. The fish PXR genes form a cluster separate from PXR in amphibians (*i.e.* frogs) and mammals. In contrast to amphibians, birds and mammals, there are no known CAR genes in fish. It has been hypothesized that CAR diverged from PXR in the mammalian lineage from the chicken X receptor (Handschin et al. 2004). A recent report however, suggests that the duplication of an ancestral gene resulting in CAR and PXR took place early in vertebrate evolution and CAR was later lost in the fish lineage (Mathäs et al. 2012).

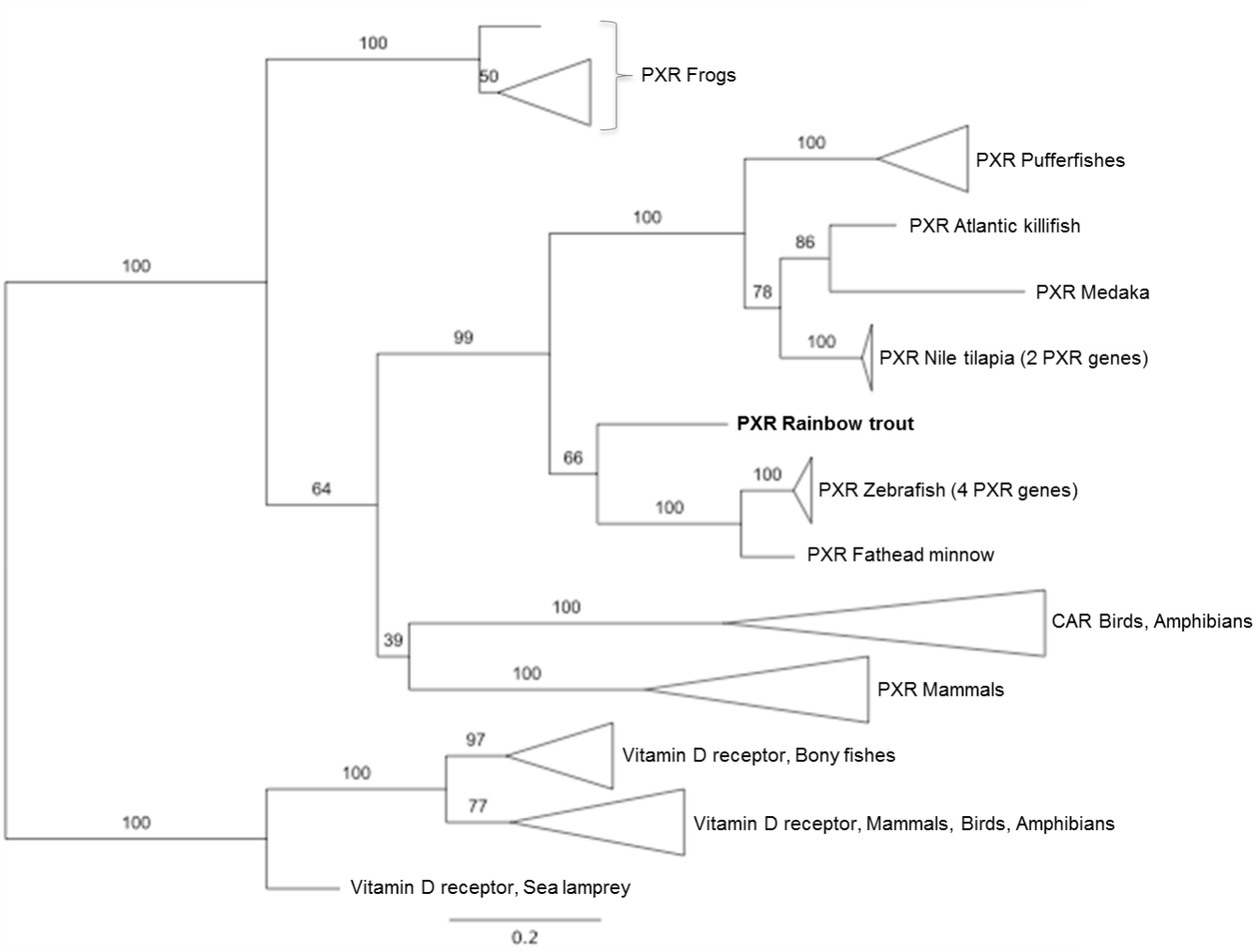


Figure 14. Phylogenetic tree of the nuclear receptor 1I family.

4.2.3 Rainbow trout PXR reporter assay

Reporter assays are useful tools to analyze receptor activation and gene regulation. We developed a reporter assay to screen for ligands for rainbow trout PXR. The rainbow trout CYP3A27 promoter sequence is not known and therefore we used the human CYP3A4 promoter in our reporter assay. The reporter assay showed weak activation with a few ligands (**Paper III**). This result is in line with exposure studies in fish and showing low induction of CYP3A expression (see section 4.1.3). However, we cannot rule out that the rainbow trout PXR may not recognize the human CYP3A4 promoter and that the reporter construct therefore is suboptimal. Furthermore, low activation of the rainbow trout PXR reporter was seen in HepG2 cells, but not in RTH-149 cells (**Paper III**). This might be due to a less efficient reporter assay in the fish cell line that is cultured at a lower temperature, but it is also possible that endogenous human PXR in HepG2 cells interfere with the reporter gene.

In contrast to the weak activation of the rainbow trout PXR reporter assay, zebrafish PXR reporter assays showed 6 to 8-fold activation in response to clotrimazole, used as the positive control (Moore et al. 2002, Milnes et al. 2008). However, clotrimazole did not induce CYP3A mRNA levels in zebrafish *in vivo* (Bresolin et al. 2005). Moreover, in fathead minnow exposed to clotrimazole *in vivo* no induction of CYP3A mRNA was observed (Crago and Klaper 2011). Nevertheless, a fathead minnow PXR reporter assay showed 35-fold activation by clotrimazole (Milnes et al. 2008). The zebrafish and the fathead minnow reporter assays were based on the LBD of fish PXR, and not the complete receptor as in our assay. Thus, the PXR LBDs were fused with the DNA-binding domain of the yeast protein GAL4 and the GAL4 binding element was used instead of a CYP3A promoter. It is likely that the GAL4 reporter assay is more sensitive compared to a full length PXR reporter assay. Higher sensitivities in GAL4 reporter assays for other nuclear receptors compared to assays using complete receptors are reported (Wilkinson et al. 2008). Although, these reporter assays have a potential to be used for screening of PXR ligands, great care should be taken when extrapolating to the *in vivo* situation.

4.2.4 Rainbow trout CYP3A27 promoter sequencing

We sequenced 1000 base pair proximal to the rainbow trout CYP3A27 gene to search for PXR response elements. The search was limited to the mammalian proximal PXR everted repeat 6 response element. It consists of two half-sites, spaced by 6 various nucleotides. The right-hand half-site has the consensus sequence AG(G/T)TCA, whereas the left-hand half-site is less conserved between mammalian CYP3A genes and is reported to have the sequence T(T/G)A(A/G)(C/A)T (Kliewer et al. 2002). We searched for the most conserved half-site in the 1000 base pair upstream sequence of rainbow trout CYP3A27 as well as in zebrafish CYP3A65, medaka CYP3A38, CYP3A40 and fugu CYP3A48. These other upstream regions were collected from the ENSEMBL genome database. An AGGTCA sequence is present in the CYP3A27 and CYP3A48 upstream regions and an AGTTCA sequence is found in medaka CYP3A38. These sequences were not found upstream of zebrafish CYP3A65 or medaka CYP3A40. The less conserved left-hand half-site was not present in the 15 nearest upstream nucleotides. However, there were some similarities in this region between three fish sequences.

CGGCTTCCAAGGTGTAGGTCACCTGTCCAT **CYP3A27**
GAGCTTCAGAGCACAAGTTCAACCATCAGA **CYP3A38**
GTTGCCTTCATTTAGAGGTCAACAAAATTT **CYP3A48**

Whether this is a functional response element for PXR in these fishes must be determined empirically. Since the conserved right-hand half-site is not found in zebrafish CYP3A65 or in medaka CYP3A40, it implies that the putative fish PXR response element is not well conserved.

4.3 Expression of efflux pumps

There is a great substrate overlap between CYP3A4 and Pgp in humans. These proteins are likely to work in concert and constitute a broad defense against accumulation of xenobiotics. The efflux pumps can be up-regulated by chemical exposure to prevent bioaccumulation. We investigated the effects of pharmaceuticals and model substances on regulation of Pgp as

well as MRP1 and MRP2 in fish as these pumps transport pharmaceuticals and their conjugated metabolites in mammals (Borst et al. 2000).

4.3.1 Co-regulation of Pgp with CYP3A

In mammals, PXR regulates expression of Pgp in addition to CYP3A (Harmsen et al. 2009). In PLHC-1 cells, exposure to mammalian PXR ligands rifampicin, troleandomycin and PCN had no effect on Pgp mRNA levels (**Paper I**). In rainbow trout hepatocytes, on the other hand, Pgp mRNA levels were induced by mammalian PXR ligands and displayed a similar pattern as CYP3A27 (**Paper III** and Figure 15). This provides circumstantial evidence for the involvement of PXR in regulation of both Pgp and CYP3A gene in fish, similar to humans.

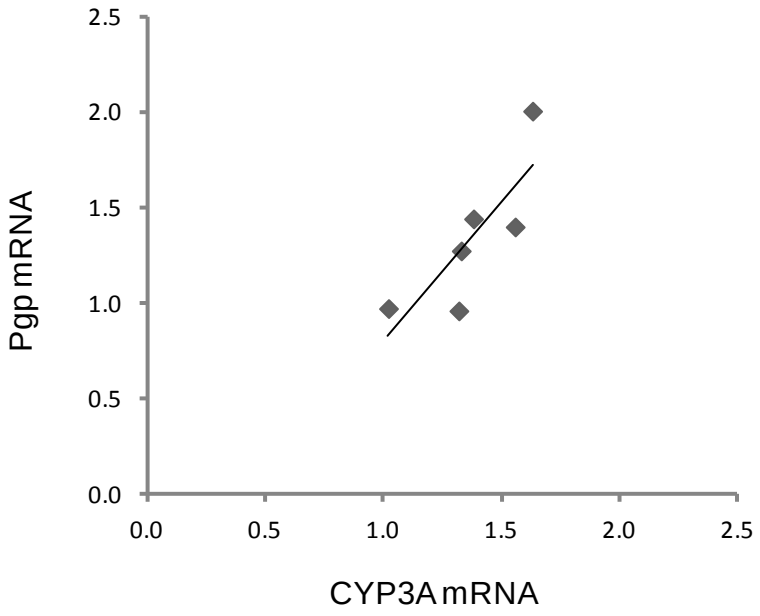


Figure 15. Co-induction of Pgp and CYP3A mRNA in rainbow trout hepatocytes. Based on results from **Paper III**.

4.3.2 Regulation of MRP1/MRP2 expressions

The MRP1 mRNA expression was not affected by chemical exposures, whereas MRP2 was induced by BNF and troleandomycin in PLHC-1 cells (**Paper I** and Figure 16). This is similar to that observed in mammals where MRP1 seems to be less regulated compared to MRP2 (Maher et al. 2005). The observed powerful effect of the PAH-type substance BNF exposure on

MRP2 expression could be either mediated via AhR signaling pathway or via the nuclear factor-E2-related factor 2 (NRF2) and the antioxidant response element. The antioxidant pathway is supported for BNF induction of mouse MRP2 in a reporter assay (Vollrath et al. 2006). In addition, PXR has been shown to be involved in MRP2 regulation in humans. Induced MRP2 expression was reported in human hepatocytes exposed to the rifampicin (Kast et al. 2002). We did not observe any effect on MRP2 expression with rifampicin in PLHC-1 cells, but we did see an induction of MRP2 with troleandomycin (**Paper I**). As rifampicin, troleandomycin is a macrolide antibiotic and a PXR agonist (Yasuda et al. 2008). Interestingly, troleandomycin acted as a rhodamine efflux inhibitor in PLHC-1 cells and it is possible that troleandomycin-PXR-MRP2 signaling was activated in order to prevent bioaccumulation of troleandomycin. In contrast to troleandomycin, BNF that had no effect on rhodamine efflux activities, and therefore is probably not a substrate for efflux pumps, but still BNF induced MRP2 expression. It is possible that BNF and troleandomycin regulates MRP2 expression via different signaling pathways, *e.g.* the antioxidant-NRF2 or via AhR or PXR. This suggests that different efflux pumps are regulated by different mechanisms.

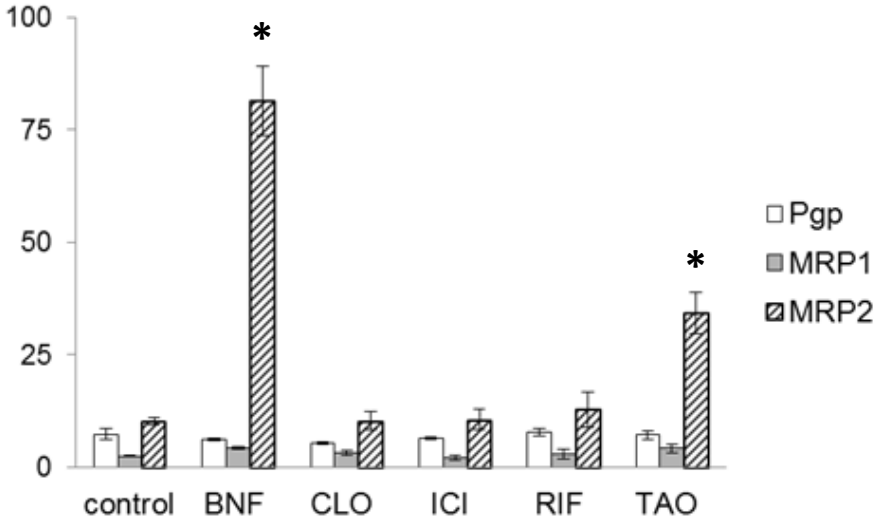


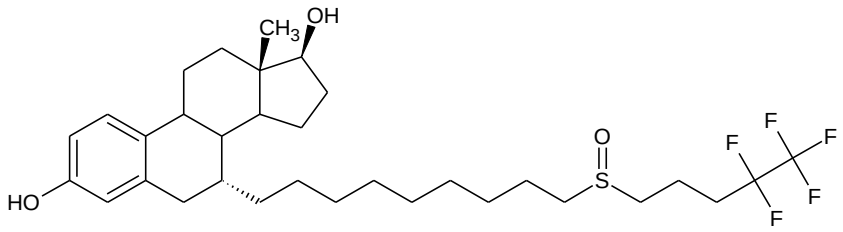
Figure 16. Efflux pumps mRNA levels after 24 h exposure.
Based on results from **Paper I**.

4.4 Regulation of CYP1A genes

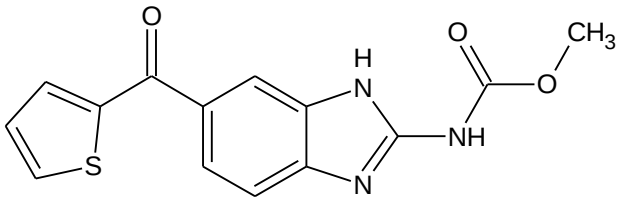
The CYP1A enzyme is important for metabolism of aromatic hydrocarbons and it is typically induced via AhR signaling. The AhR is a promiscuous receptor, although considerably less so than PXR, and the AhR can be activated by structurally diverse compounds (Denison et al. 2011). Induction of CYP1A in fish is a routinely used biomarker to assess exposure of aromatic hydrocarbons in the aquatic environment (Schlenk et al. 2008). This thesis addresses effects of pharmaceuticals on the CYP1A biomarker.

4.4.1 Regulation of CYP1A by non-classical AhR ligands

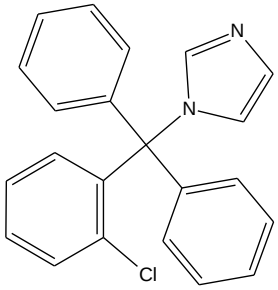
We tested a variety of structurally diverse chemicals and found induction of CYP1A by azoles, *i.e.* clotrimazole, ketoconazole and nocodazole and by the anti-estrogen fulvestrant (**Paper I** and **Paper II**). Induction of CYP1A expression by clotrimazole and ketoconazole has previously been reported in fish (Hegelund et al. 2004, Navas et al. 2004, Hasselberg et al. 2005). None of these substances are typical AhR ligands (Figure 17). Some characterization of clotrimazole as an AhR ligand has been made. It was found that the model AhR antagonist (ANF) is not effective in blocking the CYP1A induction by clotrimazole (Navas et al. 2004). Clotrimazole is not planar in its structure as the classical AhR-ligands that are described to fit the relatively restrictive substrate-binding site of AhR (Denison and Nagy 2003). It was suggested that clotrimazole induced CYP1A by a different mechanism compared to classical AhR signaling (Navas et al. 2004). The mechanism for induction of CYP1A by non-classical AhR ligands such as azoles and fulvestrant is not clear. It cannot be ruled out that AhR activation is mediated by a metabolite, rather than the parent substance. In human hepatocytes, metabolites of the anti-ulcer drug omeprazole have been shown to activate CYP1A induction and involvement of CYP3A metabolism was suggested (Gerbal-Chaloin et al. 2006). Hence, it is possible that catabolic CYP1A/CYP3A enzymes can activate pro-AhR ligands. This illustrates the complexities of the detoxification mechanisms and the possible concerted actions of several proteins involved in detoxification.



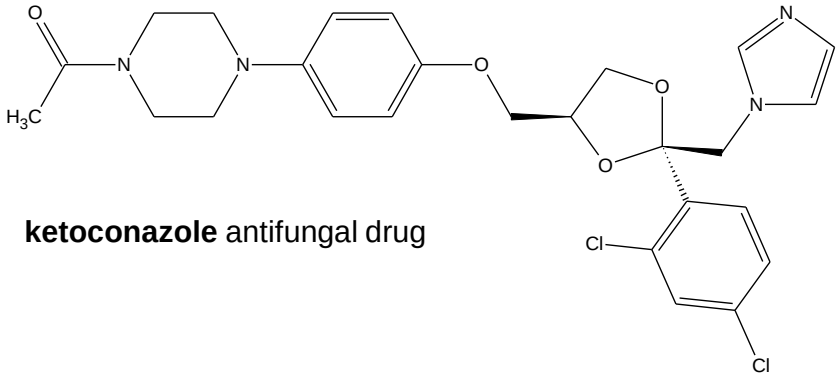
fulvestrant antiestrogenic drug



nocodazole microtubule disruptor



clotrimazole antifungal drug



ketoconazole antifungal drug

Figure 17. Non-classical inducers of CYP1A.

4.4.2 Induction of CYP1A in microtubule disassembled cells

Nocodazole is different from the other investigated azoles as it acts as a microtubule disassembly drug. Previous studies have shown that nocodazole significantly reduces mediated CYP1A induction. It has been suggested that induction of CYP1A is dependent on an intact cytoskeleton for AhR translocation to the nucleus (Dvořák et al. 2006, Vrzal et al. 2008). Hence, the strong CYP1A induction in nocodazole-exposed PLHC-1 cells is surprising as microtubules are disassembled in these cells and the track for translocation thereby removed (**Paper I** and Figure 18). Whether AhR signaling in fish is depending on microtubules or not therefore remains to

be investigated. Our finding that nocodazole induces CYP1A can be explained by one of the following explanations: **i**) AhR translocation is not microtubules dependent. **ii**) AhR translocation is not required for CYP1A induction in this cell line, i.e the AhR is constantly located in nucleus in PLHC-1 cells. **iii**) AhR is successfully translocated before microtubules are disassembled. **iv**) CYP1A induction by nocodazole is independent of AhR. To find out which explanation(s) that may be true, cellular localization and transport mechanisms of AhR needs to be studied.

4.4.3 Effects of pharmaceuticals on cytoskeleton morphology

The cytoskeleton is necessary for basal cellular functions. One of the most important components, the microtubules, enables cell division and intracellular transports. The microtubule disassembling drug nocodazole completely disrupted the microtubule network in PLHC-1 cells. Such a serious consequence was not seen by any of the pharmaceuticals tested. However, the commonly used pharmaceuticals ibuprofen and omeprazole caused microtubule fragmentations (**Paper I** and Figure 18). Similar fragmentations by exposure to antimitotic drugs have previously been reported in mammalian cells (Yang et al. 2010). We observed that common pharmaceuticals affect microtubules morphology and this may have adverse effects on cellular transport mechanisms.

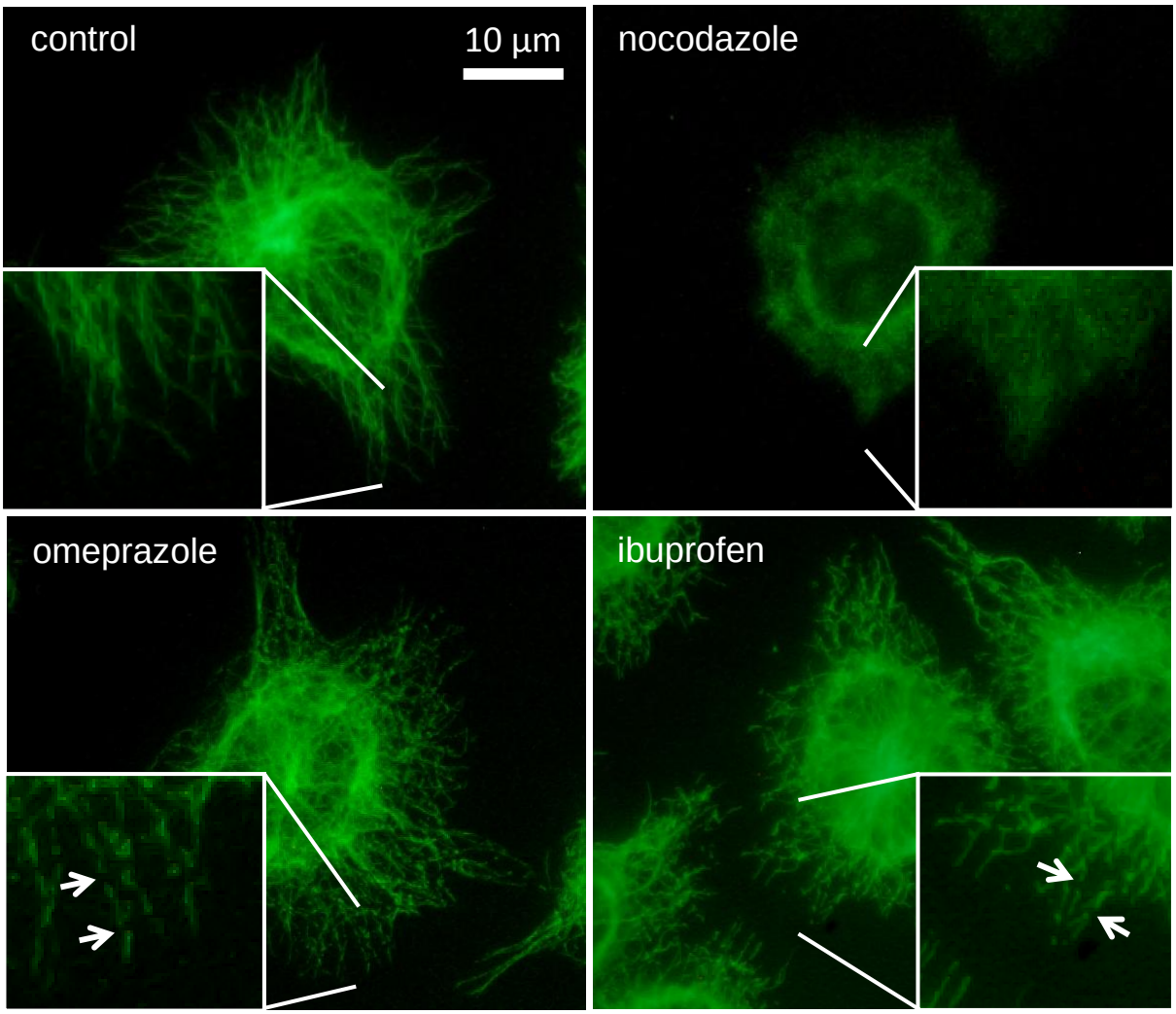


Figure 18. Microtubule morphology in PLHC-1 cells exposed to pharmaceuticals. Arrows indicate fragmentation of microtubules. Photos from **Paper I.**

4.5 Functional studies of CYP enzymes and efflux pumps

4.5.1 Interactions of pharmaceuticals on CYP activities

We investigated the direct effects of pharmaceuticals on CYP1A activities. The imidazoles, clotrimazole, nocodazole, ketoconazole, in a descending order, resulted in strongest inhibition of CYP1A activities. Both ketoconazole and clotrimazole are potent antifungal drugs acting by inhibiting the CYP51-mediated ergosterol synthesis in fungus. The azoles are however not specific to CYP51. Hence, several of the azoles including ketoconazole and clotrimazole, act as broad-spectrum CYP inhibitors. Accordingly, clotrimazole and ketoconazole were shown to be potent inhibitors of CYP1A and CYP3A in fish (Miranda et al. 1998, Hegelund 2003,

Hegelund et al. 2004, Hasselberg et al. 2005, **Paper I**, **Paper II** and **Paper III**). In addition, Fulvestrant that is commonly referred to as an absolute estrogen receptor antagonist in mammals was shown to inhibit CYP1A biotransformation activities (**Paper I**). The CYP1A and CYP3A inhibiting effects are of concern for both impaired detoxification and disturbed steroid metabolism that we addressed in **Paper II**. These results are discussed in section 4.5.3. Omeprazole and rifampicin acted as weak inhibitors on CYP3A activities whereas PCN acted as a CYP3A activator (**Paper III**). The mechanism for activation of CYP3A by PCN is probably by an allosteric cooperativity, similar to that previously described for the AhR antagonist ANF (Harlow and Halpert 1997, Hegelund 2003). In contrast to weak effects on CYP3A induction, we see powerful inhibition of CYP3A by several xenobiotics. Inhibition of key catabolic CYP metabolism results in impaired capacity to metabolize xenobiotics and steroid hormones and can lead to increased sensitivity to chemical exposures and endocrine disruption.

4.5.2 Influence of pharmaceuticals on efflux activities

As mentioned above in section 4.3, efflux pumps transport a wide range of substrates, many of which are also CYP1A/CYP3A substrates. We investigated interactions by pharmaceuticals on rhodamine efflux (**Paper I**). We found that diclofenac and troleandomycin acted as inhibitors of efflux whereas ethinylestradiol increased efflux (Figure 19).

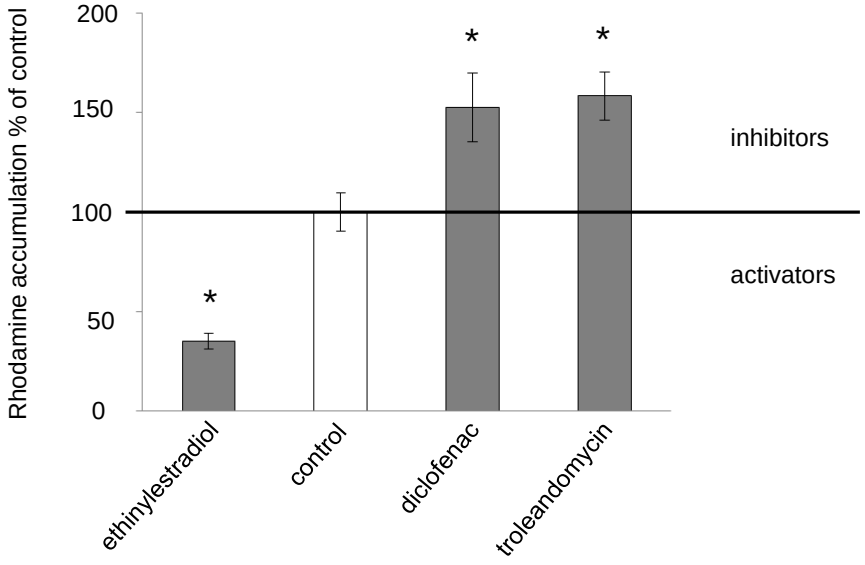


Figure 19. Rhodamine accumulation in PLHC-1 cells. Based on results from **Paper I.**

The inhibition of rhodamine efflux is most likely due to competitive transport of diclofenac and troleandomycin by efflux pumps, competing with rhodamine as substrates. Increased efflux activities in an acute assay like this, *i.e.* without any gene induction being involved, have been reported before (Jin and Audus 2005). The Pgp pump has more than one substrate binding site. Binding of a substrate in one site can allosterically activate transport of another substrate in another site (Shapiro and Ling 1997). The increased rhodamine transport in the presence of ethinylestradiol suggests that ethinylestradiol is a substrate for a different binding site than rhodamine, as exposure to ethinylestradiol facilitates rhodamine efflux. A potential risk with increased efflux activities is depletions of endogenous substances as a result of accelerated efflux. A known risk with efflux inhibitors is that they can act as chemosensitizers and thereby increase cellular levels of xenobiotics in aquatic species (Kurelec 1992). Our results suggest that diclofenac and troleandomycin can inhibit efflux activities and thereby may lead to bioaccumulation of other xenobiotics in situations of exposure to these pharmaceuticals in mixtures.

4.5.3 Mixture effects

Toxicokinetic interactions can result in chemosensitization (Celander 2011). For example, rainbow trout exposed to ketoconazole were seven times more sensitive to ethinylestradiol exposure due to inhibition of CYP1A and CYP3A enzymes by ketoconazole (**Paper II**). In the teleost gizzard shad (*Dorosoma cepedianum*) exposure to clotrimazole resulted in a 10 times higher bioaccumulation of benzo[*a*]pyrene. It was suggested that this was due to inhibition of CYP1A activities (Levine et al. 1997). It is possible that the gizzard shad exposed to clotrimazole also had reduced CYP3A activities, but this was not addressed in that study. The two different studies illustrate the roles of CYP1A and CYP3A enzymes in detoxification of environmental pollutants. Hence, inhibition of catabolic CYP activities can result in endocrine disruption and in increased accumulation of xenobiotics. Though, these different chemicals belong to different classes such as imidazoles, estrogens and PAH and therefore they have different modes of action. Still they all share a common elimination pathway. This can result in adverse toxicokinetic interactions. Therefore, a non-toxic concentration of a single chemical can result in toxic effects when it is present in a mixture, as

a result of toxicokinetic interactions. These types of toxicokinetic interactions are likely to occur in fish, as they are continuously exposed to mixtures in the aquatic environment during their whole lifecycle.

4.6 Resistance to environmental pollutants

Killifish in NBH survive high levels of PCBs by limiting the AhR-CYP1 signaling pathway (Nacci et al. 1999, Bello et al. 2001). We also found lower PXR mRNA levels in fish from NBH compared to fish from SC. Though the difference is small, down-regulation of PXR expression can have an impact on potential PXR target genes that are involved in detoxification mechanisms, since the PXR is a naturally expressed at low levels. It is not clear if reduced PXR expression is beneficial in situations of exposures to high concentrations of PCBs. Nevertheless, hepatic mRNA expressions are inducible for PXR, CYP3A and Pgp in fish from NBH exposed to PCBs in the lab. The PCB congeners used in the lab exposure studies were the planar dioxin-like PCB 126, which is a strong ligand to vertebrate AhR, and the non-planar 2,2',4,4',5,5'-Hexachlorobiphenyl (PCB 153), which is ligand to human PXR (Jacobs et al. 2005, Kopec et al. 2010, Al-Salman and Plant 2012). Despite this structural difference and potential different actions of the two congeners, they equally induced mRNA expressions of PXR, CYP3A and Pgp genes. This implies that PXR activation is involved in regulation of PXR, CYP3A and Pgp genes, but not AhR activation since the NBH fish have a less functional AhR. Our findings suggest that PXR signaling in addition to AhR signaling, is impaired in killifish that reside and reproduce in a heavily PCB polluted area and that these receptors may be involved in chemical resistance.

5. Summary and Conclusion

In this thesis, effects of pharmaceuticals on fish detoxification mechanisms have been addressed. Risk assessments of pharmaceuticals are based on single exposure experiments, but in the environment pharmaceuticals and other pollutants occur in mixtures. It is thus likely that toxicokinetic interactions arise in fish as many of these chemicals occur as mixtures in the aquatic environment and share common elimination pathways. The CYP3A enzyme and the Pgp efflux pumps in mammals are regulated by PXR and are key proteins in detoxification mechanisms. Hence, these are important proteins to study in order to assess risks for toxicokinetic interactions. This thesis focuses on functions and regulations of CYP3A and Pgp in fish, in single and in mixture exposure experiments.

We used the fish cell line PLHC-1 as a model to screen for effects of pharmaceuticals and other chemicals on detoxification enzymes and efflux pumps. The PLHC-1 cell line is an established ecotoxicology *in vitro* model, but no CYP3A gene has so far been found in PLHC-1. We sequenced a CYP3A-like gene and we showed that it belongs to the CYP3B-family. The CYP3B was expressed at low levels and was non-inducible by exposure to the pharmaceuticals tested. No CYP3A gene was found in PLHC-1, most likely because of very low expression or that PLHC-1 cell line lack CYP3A. Therefore, PLHC-1 is not a good fish model for studying effects of pharmaceutical exposure on CYP3A. Nevertheless, PLHC-1 is a good model for studying expression and functions of CYP1A and the efflux pumps. We observed differences between acute and 24h exposure effects for several pharmaceuticals. We found effects on both functions and regulations of CYP1A and efflux pumps by several classes of pharmaceuticals. Our studies reveal possible sites for toxicokinetic interactions and provide new knowledge that is useful for understanding mixture effects in fish.

Drug interactions were demonstrated *in vivo* in rainbow trout exposed to ethinylestradiol and ketoconazole. Co-exposure to ketoconazole increased the effect by ethinylestradiol, compared to exposure to ethinylestradiol alone. This was likely due to an insufficient clearance of ethinylestradiol caused by CYP1A and CYP3A enzyme inhibitions by ketoconazole and it made the fish seven times more sensitive to estrogenic exposure. Our studies illustrate the importance of including studies on inhibition of

detoxification functions in environmental risk assessments of pharmaceuticals and other pollutants that will likely occur as mixtures in the aquatic environment.

It has so far not been established if fish CYP3A genes are also regulated by PXR as they are in mammals. We found that mammalian PXR ligands induced CYP3A in primary hepatocytes from rainbow trout, although weakly compared to that in mammals. To clarify if PXR activation was involved in the weak CYP3A induction we cloned the complete PXR from rainbow trout. Next, we developed a PXR reporter assay to screen for potential fish PXR ligands. The reporter assay confirmed no or weak activation of rainbow trout PXR, by mammalian PXR ligands. The question of whether PXR regulates CYP3A in fish is still open. When analyzing the promoter sequences of rainbow trout and other fish CYP3 genes, no full mammalian PXR response element was found. So far, we can conclude that the inducibility of CYP3A is low in rainbow trout exposed to mammalian PXR ligands. The reason for this may be due to a natural high basal expression of CYP3A genes in fish and as a result of that, a potentially limited induction span. This is supported by our findings in primary cultures, where cells with high basal CYP3A mRNA levels are non-responsive to CYP3A inducers, whereas cells with low basal CYP3A can be induced.

There are examples of fish populations with an extraordinary ability to survive in heavily polluted areas. A classic example is the New Bedford Harbor (NBH) killifish that are tolerant to exposure to high PCB levels. Interestingly, they survive by shutting down parts of their detoxification mechanisms that involves AhR signaling. There are no reports on how PXR and CYP3A gene expressions are affected in these killifish. We show lower expressions of both PXR and Pgp in the fish from NBH, compared to the fish from the reference site. The reason for down-regulation of PXR-CYP3A/Pgp is not understood, but it is possible that these fish are also protected by limiting the PXR signaling pathway, in addition to AhR, upon chronic PCB exposures.

Acknowledgements

Så roligt jag har haft de här åren. Tack alla Ni som hjälpt mig på olika sätt.

Speciellt tack till:

Malin Celander, min handledare, för att jag fick komma till CYP-lab! Tack för all energi och tid du har lagt på att lära mig skriva bättre och för att du alltid verkat övertygad om att avhandlingen blir bra. Tack för stöd, uppmuntran och ALLT du lärt mig på vägen!

Margareta Wallin, min biträdande handledare, för att du alltid tar dig tid att läsa och kommentera när det behövs.

Thrandur Björnsson, min examinator, särskilt tack för kommentarerna på kappan.

Johanna Gräns, min fina pålitliga CYP-vän som alltid hjälper till när det behövs. Extra tack för suveränt resesällskap ute i vida världen.

Linda Hasselberg, som alltid har ett gott råd vad det än gäller, så glad jag är att du kom tillbaka till Zoologen!

Peter Kling, för att du kan svara på alla viktiga frågor om transfektion, DNA-fällning och vilken film man bör se.

Elisabeth Norström, för de fina mikrotubilibilderna, väldigt trevligt sällskap och goda råd på steril-lab.

Kerstin Wiklander, för att du alltid ställer upp och svarar på mina statistikfunderingar.

Lina Gunnarsson Kearney, särskilt tack för fin hjälp under skrivandet av kappan.

Joanna Wilson, thank you for coming here with your lovely family, for the trees, for explaining the basic phylogenetics to me, for comments on the thesis and for answering so many questions.

John Stegeman, thank you for providing the opportunity for me to be involved in the killifish study and for your excellent help with the manuscript.

Tack till ALLA på Zoologen, för att det är så trevligt att arbeta här! För allt härligt sällskap vid fika, lunch och däremellan! Speciellt *Anna Ansebo* för alla viktiga pratstunder och för att du alltid jobbar för att förbättra arbetsmiljön. *Inger Holmqvist* och *Birgitta Wallander* för den fina servicen på kurs-lab, *Jari Parkkonen*, för VICTOR-hjälp när det behövs, *Ann-Sofie Olsson, Lena Sjöblom* och *Jenny Johansson,* för hjälp med det administrativa och *Bernth Carlsson* och *Lilioth Olsson* för att det alltid är lika trevligt att komma in på Zoologen tidiga mornar, och för hjälp med allt möjligt.

Tidigare och nuvarande doktorander, speciellt *Eva Albertsson*, du är så klok och en sådan expert på att prata om allt viktigt i livet, *Anna Holmberg*, som förgyllt så många fikastunder och ritat den fina bilden på framsidan!, *Sara Aspengren* och *Kristin Ödling*, för att ni tog mig med för att fika från allra första dagen, *Henrik Seth*, det är alltid lika trevligt att få en pratstund med dig och *Daniel Hedberg*, för fint sällskap i vårt gamla rum.

Ni studenter som varit på CYP-lab, speciellt *Anna Christoffersson* och *María Fernández* för noggrant, flitigt lab-arbete och trevligt sällskap!

Tina Vallbo och *Jan-Erik Damber*, tack för att jag fick låna qPCR utrustningen på Urologen, Sahlgrenska, innan Zoologen hade sin egen, och för din suveräna tech-support i alla qPCR-frågor Tina!

Dick Delbro, som gav mig mitt första biologjobb, på avd. för kirurgi, Sahlgrenska. Där träffade jag mina fina vänner, *Lena Hultman, Berit Dimming* och *Ann-Sofi Söderling*. Tack för att ni tog så väl hand om mig och lärde mig så mycket!

Mina fina molekylärbiolog-vänner, *Elin Krogh*, *Heléne Gustavsson* och *Åsa Agapiev*, så mycket roligt vi haft under alla år. Jag längtar alltid till våra luncher med era goda råd om allt från kloning till semesterresor.

Mamma! för att du alltid gjort ”allt” för mig, för att du stöttat mig och tragglat läxor med mig under många år. *Pappa*, jag vet att han också hade varit väldigt stolt över den här boken. Min bror *Sven*, tack för att du så pedagogiskt förklarade allt jag behövde veta om fysik, matte och kemi när jag läste naturvetenskapliga basåret.

Robert, min älskade man och bäste vän. Du är fantastisk på så många sätt och du har bidragit så mycket till den här boken. Tack för allt du gör för mig och för din kärlek!

Isak, vår älskade pojke! Vilket tålamod du har haft när jag skrivit Boken. Jag håller med dig, det är ”asa-skönt” nu när den är klar. Tack för att du alltid gör mig så glad! Du är så snäll, rolig och klok. Att få vara din mamma är alldeles fantastiskt!

Financially supported by Faculty of Science University of Gothenburg, FORMAS, Helge Ax:son Johnsons stiftelse, Wilhelm och Martina Lundgrens vetenskapsfond and Adlerbertska forskningsstiftelsen.

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