ABSTRACT

Title of Dissertation: HEPATIC PHASE I AND II BIOTRANSFORMATION KINETICS IN FISHES: A COMPARATIVE STUDY

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Eight finfish species were selected to test the similarities and dissimilarities on their phase I and II biotransformation capabilities using microsomal and cytosolic fractions of the liver. This research had three main objectives: 1) a comparison among the kinetics of the 8 species using model substrates, 2) farm-raised and laboratory-acclimated specimens of channel catfish, rainbow trout and tilapia were compared to determine similarities and differences in the biotransformation reactions, and 3) the same latter three species were tested to compare *in vitro* hepatic metabolism of albendazole, a drug that undergoes metabolic biotransformation mainly through hepatic phase I reactions.

The comparison among the 8 finfish species showed that some of them had higher biotransformation capabilities than others. For most of the seven phase I- II reactions that were tested; rainbow trout, tilapia, channel catfish and Atlantic salmon had higher enzymatic efficiencies than those showed by of striped bass, hybrid striped bass and bluegill. Largemouth bass shared some enzymatic capabilities with one group or the other. The comparison between lab-acclimated and farm-raised specimens of tilapia,

channel catfish and rainbow trout did not show biologically significant differences among the two groups of fish for the 3 species. All the values found for the kinetics of the reactions were within the ranges of the constitutive expression that has been reported for them and far below those found in other works when inducers (e.g. pollutants) of enzymatic reactions have been used. The phase I-mediated hepatic sulfoxidation of albendazole in tilapia, channel catfish and rainbow trout showed significant differences in $V_{max}$ and $K_m$ values among the three species. However, the catalytic efficacies of the reaction ($V_{max}/K_m$) in the 3 species transforming the parent compound were similar. In addition, albendazole induced EROD activity (2.6 fold) in *in vivo* dosed channel catfish.

The results found in the present study showed that the catalytic efficiency of hepatic phase I-II enzymatic reactions varied among finfish species. Some of them may be categorized as “more efficient metabolizers” than others. This may have important implications in drug metabolism and residue depletion patterns.

# HEPATIC PHASE I AND II BIOTRANSFORMATION KINETICS IN FISHES:

# A COMPARATIVE STUDY

by

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To all the persons in my family who I deeply love:
my wife Martha, my son Andrés Fernando, my mother Josefina,
my sister Victoria Helena and my nephew Daniel Felipe.
To the memory of my father.

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# Chapter I

## Phase I and II biotransformation reactions in fish: a review of the literature

## INTRODUCTION

Piscine species are an increasing focus for manuscripts characterizing the molecular and biochemical aspects of biotransformation with rainbow trout (*Oncorhynchus mykiss*) the most studied. However, biotransformation reactions are considerably less known in fish than in mammals. This chapter presents a review of some of the most relevant aspects of fish biotransformation reactions. Aspects such as gene characterization, tissue and cellular distribution, catalytic activity, and changes in expression due to physiological and environmental factors will be discussed for the phase I–II biotransformation enzymes that are presented. Reports in mammals will be cited in some instances as a way to compare the expression of biotransformation reactions in fish with those of more evolutionarily recent animals.

## PHASE I BIOTRANSFORMATION REACTIONS IN FISH

The best characterized phase I reaction systems in fish consist of two groups of monooxygenases: the heme protein monooxygenases (i.e., cytochrome P450) and the flavoprotein monooxygenases (FMO). These two systems are reviewed in the following sections.

### The Cytocrome P450 System in Fish

The cytochrome P450 system (P450) (EC 1.14.14.1) is a superfamily of heme-containing enzymes that catalyze different types of phase I reactions of both endogenous and exogenous substrates (Ueng and Ueng, 1995; Buhler and Wang-Buhler, 1998). P450 genes are present in virtually all living organisms (e.g., plants,

animals, lower eukaryotes, bacteria) with 3 billion years of evolutionary history (Nelson, 1998). This section describes fundamental aspects of P450 forms in fish.

### *Gene Characterization*

P450 genes in fish species are classified in different families and subfamilies: 1A, 2K, 2M, 3A, 11, 17, and 19. However, a more complete characterization of these genes (sequencing, tissue expression, catalytic activity, etc.) has only been accomplished in very few species. As a result, substrate specificity and functionality of corresponding proteins from the P450 genes have been identified in rainbow trout (*Oncorhynchus mykiss*) (Buhler and Wang-Buhler, 1998) and killifish (*Fundulus heteroclitus*) (Stegeman and Hahn, 1994). The first recognition of the P450 system in fish was attained in the late 1960s (Buhler and Rasmusson, 1968; Dewaide and Henderson, 1968) and by 1998, a total of 40 P450 genes had been cloned from fish (Stegeman and Livingstone, 1998). Table 1 shows some of the P450 genes subfamilies and species that have been studied for gene characterization.

### *Tissue Distribution and Catalytic Activities*

P450 enzymes are localized within the smooth endoplasmic reticulum of cells in different organs including liver, kidney, gills, intestine, brain, heart, gonads, etc. (Stegeman and Livingstone, 1998). However, the liver is the primary site for phase I reactions of most endogenous and exogenous substrates through P450-mediated reactions (Cok et al., 1998).

The P450 system is one of the most versatile biotransformation systems in terms of catalytic capabilities. Common P450-mediated reactions (e.g., oxidation, reduction, hydrolysis, etc.) are accomplished by this heme-containing protein (Stegeman and Livingstone, 1998). The two primary roles of P450 monooxygenases are: a) endogenous molecule (e.g. fatty acids, steroids, prostaglandins, etc.) biosynthesis or degradative oxidation; and b) exogenous compound (e.g. environmental contaminants) oxidative reactions. Other minor P450-mediated reactions include cleavage of C-C and C=N bonds, dehydrations, dehydrogenations and isomerizations (Mansuy, 1998).

The catalytic cycle of P450-mediated reactions involves the transfer of electrons from NADPH via NADPH-cytochrome P450-reductase, a flavoprotein that is present in much lower concentration than P450 (Figure 1). The P450 cycle starts with the binding of the substrate (RH). A second step is accomplished when the heme iron is reduced from the ferric form ($Fe^{3+}$) to the ferrous ($Fe^{2+}$) by the addition of an electron from cytochrome-P450-reductase. Oxygen then binds to the P450 in its ferrous state. The complex is converted to a $Fe^{2+}OOH$ state by the addition of a proton ($H^{+}$) and a second electron derived from the P450 reductase or from CYT b5. A second $H^{+}$ cleaves the $Fe^{2+}OOH$ and produces $H_20$ and the $(FeO)^{3+}$ complex which transfers its oxygen atom to the substrate. When the oxidized substrate is released (ROH), the P450 molecule is ready for a next cycle (Parkinson, 2001). Other side reactions are possible under ceratin conditions but in the interests of simplicity will not be discussed here.

Table 1. Some of the cloned P450 genes in fish species

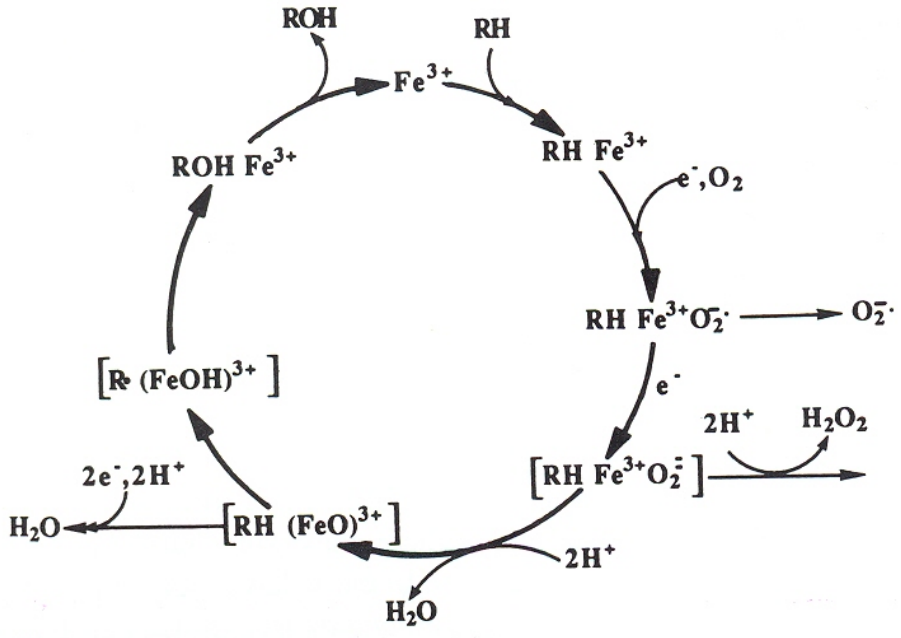
| Species | CYP subfamily | Cloned genes | Reference |
|---|---|---|---|
| Rainbow trout (*Oncorhynchus mykiss*) | CYP1A | ***CYP1A1 - CYP1A3*** | Nelson et al. (1996)<br>Cao et al. (2000) |
| | CYP2K | ***CYP2K1*** | Buhler et al. (1994)<br>Cok et al. (1998) |
| | | ***CYP2K3*** | Miranda et al. (1997) |
| | | ***CYP2K1v2 - CYP2K1v3*** | Buhler & Wang-Buhler (1998) |
| | | ***CYP2K4*** | Yang et al. (1997) |
| | CYP2M | ***CYP2M1*** | Yang et al. (1998a)<br>Cok et al. (1998) |
| | CYP3A | ***CYP3A27 - CYP3A45*** | Buhler & Wang-Buhler (1998)<br>Cok et al. (1998)<br>Lee & Buhler (2003) |
| | CYP4T | ***CYP4T1*** | Falckh et al. (1997) |
| | CYP11A | ***CYP11A1*** | Takahashi et al. (1993) |
| | CYP17 | ***CYP17*** | Sakai et al. (1992) |
| | CYP19 | ***CYP19*** | Tanaka et al. (1992) |
| Channel catfish *(Ictalurus punctatus)* | CYP2X | ***CYP2X1*** | Schlenk et al. (2002) |
| Killifish (*Fundulus heteroclitus*) | CYP3A | ***CYP3A30***<br>***CYP3A56*** | Celander & Stegeman (1997)<br>Hegelund & Celander (2003) |
| | CYP2Ns | ***CYP2N1***<br>***CYP2N2*** | Oleksiak et al. (2000) |

The following sections describe the different P450 subfamilies that have been identified in fish species and the catalytic activities performed by the corresponding products of gene translation.

*CYP1A subfamily*

*CYP1A* gene expression at the translational level is constitutively low in fish that have not been exposed to chemical inducers (Buhler and Wang-Buhler, 1998). The CYP1A subfamily is studied in fish mainly as a biomarker of aquatic pollution due to its inducibility with numerous compounds that are present as water contaminants (Goksøyr, 1995).

Figure 1. The P450 catalytic cycle (Taken from Poulos and Raag, 1992).



*CYP1A* expression is studied through different approaches. All stages from gene transcription to the catalytic activity performed by the corresponding P450 protein can be evaluated (Goksøyr, 1995). Catalytic activity is mainly studied using model reactions such as ethoxyresorufin-*O*-deethylation (EROD) (Whyte et al., 2000) and aryl hydrocarbon hydrolase (AHH) activity (Goksøyr, 1995). A more complete evaluation may include immunoblotting with antibodies raised against purified CYP1A forms and Northern blots with cDNA probes (Buhler and Wang-Buhler, 1998). A recent report on quantitative PCR analysis of CYP1A in wild Atlantic salmon (*Salmo salar*) facilitates the evaluation of induction using minute samples taken from branchial tissue without sacrificing the sampled specimens (Rees et al., 2003). Induction of EROD activity, using gill filaments as the enzyme source, has also been reported in rainbow trout (Jönsson et al., 2006) and the African catfish, *Clarias gariepinus* (Mdegela et al., 2006).

Some of the CYP1A chemical inducers for fish are organic contaminants present in the water column, sediments or in the food. Among these sources are polyaromatic hydrocarbons (PAHs), coplanar polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (TCDDs), dibenzofurans (PCDDs/PCDFs), and other halogenated compounds present in some pesticides and herbicides. CYP1A-mediated biotransformation of many of these substrates yields reactive and genotoxic metabolites (Goksøyr, 1995).

Research on CYP1A induction is mainly accomplished by exposures of sentinel animals in contaminated waters or after application of known inducers under

laboratory-controlled conditions. Some of the studies reporting CYP1A induction are presented below.

An example of CYP1A induction as a biomarker of exposure comes from Haasch et al. (1993). The effects of PCB- and PAH-contaminated rivers on CYP1A expression were studied in caged channel catfish and largemouth bass in the Kinnickinnic River in Milwaukee (WI, USA) as well as wild killifish collected from Newark (NJ, USA). All three species showed CYP1A changes in expression when parameters such as catalytic activity, immunodetection and nucleic acid hybridization were tested. These techniques were useful to detect induction in the expression of CYP1A due to the contaminants present in the polluted waters.

Tilapia has also been used as a bioindicator of CYP1A induction in field studies. PCBs and PAHs that were present as contaminants in sediments from Bahía Chetumal (México) were studied as inducers of CYP1A expression in Nile tilapia (*Oreochromis niloticus*) after intraperitoneal injection of sediment extracts (Zapata-Pérez et al., 2000). Significant increases (186-210 %) in catalytic activity of fish treated with sediments of some of the sampling areas were reported. Other studies using tilapia as a bioindicator of CYP1A inducing effects from contaminants such as bleached kraft mill effluents and PCBs/PAHs were done in Bainy et al. (1999), Leitão et al. (2000), Chen et al. (2001), and Parente et al. (2004).

Although the investigations cited so far indicate the usefulness of CYP1A induction as a biomarker of contaminated waters, other studies indicate that chronic exposure to organic contaminants does not necessarily induce the expression of P450 subfamily

enzymes. One study using oyster toadfish (*Opsanus tau*), a bottom–dwelling fish with a very restricted migratory behavior, was planned to detect CYP1A induction in the Elizabeth River (Virginia, USA). This river has been reported to have a well-defined gradient of PAH contamination. The study indicated no significant differences at the catalytic, immunochemical and immunohistochemical CYP1A expression levels between specimens from a reference-site and chronically-exposed Elizabeth River fish (Collier et al., 1993). Another study by Elskus et al. (1999) using *Fundulus heteroclitus* suggests a reduced expression of CYP1A in fish challenged with PCBs after being chronically exposed to contaminated waters in Newark Bay. In this study, the authors propose that fish that are exposed for prolonged periods of time to organic contaminants have an altered response to CYP1A inducers. This change is considered to be persistent and even heritable. As such, the use of CYP1A expression as a biomarker of contamination could be ambiguous under conditions of chronic or high levels of exposure.

Studies on laboratory-acclimated fish have tested different types of chemical CYP1A inducers through various exposure routes (e.g. waterborne, foodborne, intraperitoneal injection). These studies offer the benefit of a direct association between the inducer and the changes in CYP1A expression. Depending on the species, inducers may not only change CYP1A expression but those of other P450 subfamilies. 3-methylcholanthrene (Ueng and Ueng, 1995), benzo[a]pyrene (Levine and Oris, 1999), PCBs mixtures (Brumley et al., 1995; Gadagbui et al., 1996) and β-naphthoflavone (BNF) (Gadagbui et al., 1996; Rees et al., 2003) are among the most common compounds used to study CYP1A induction in laboratory-maintained fish.

***CYP1C subfamily***

The newest members of CYP1 genes from teleosts were cloned and designated as *CYP1C1* and *CYP1C2* (Godard et al., 2005). However, the physiological significance, inducibility and metabolic functions of *CYP1C* are still unknown (Wang et al., 2006). *CYP1C1* mRNA expression is constitutively higher than *CYP1A* in brain, spleen, eye, gonad. Preliminary results obtained by Wang and collaborators indicate that *CYP1C* is inducible by B[a]P and may be implicated in the bioactivation of carcinogens. Phylogenetic analysis indicates that CYP1Cs and CYP1Bs are a sister clade to the CYP1As (Godard et al., 2005).

***CYP2K and CYP2M subfamilies***

CYP2K was found to be the major constitutive hepatic P450 form in rainbow trout (Williams and Buhler, 1984). Different CYP2K genes have been cloned from various tissues in rainbow trout: *CYP2K1* (liver, brain, blood, intestine) (Buhler et al., 1994; Cok et al., 1998), *CYP2K3* (liver) (Miranda et al., 1997), *CYP2K1v2* - *CYP2K1v3* (liver) (Buhler and Wang-Buhler, 1998), and *CYP2K4* (kidney) (Yang et al., 1997).

CYP2K proteins catalyze the 2-, 6β-, and 16α-hydroxylations of 17β-estradiol, testosterone and progesterone (Miranda et al., 1989). LMC2, one of the proteins obtained from *CYP2K1* translation, catalyzes the (ω-1)- and (ω-2)- hydroxylations of laurate (Buhler et al., 1997) and longer chain fatty acids such as palmitate, myristate and stearate (Buhler and Wang-Buhler, 1998). ω- and ω-1 lauric acid hydroxylations by CYP2K-like proteins have also been reported in tilapia and carp (Ichihara et al.,

1969 and Salhab et al., 1987; cited by Haasch et al., 1998). LMC2 is also highly reactive toward aflatoxin $B_1$ ($AFB_1$) forming $AFB_1$-8, 9-epoxide, a strong hepatic carcinogen. The acute sensitivity of rainbow trout to $AFB_1$ has been attributed to the presence of CYP2K as the major constitutive P450 isoform in this species (Bailey et al., 1984 and Bayley et al., 1996). Rainbow trout is considered the most susceptible animal species to the carcinogenic effects of $AFB_1$. This may have a direct correlation with its high constitutive expression of CYP2K proteins (Hendricks, 1996).

There are sex-, organ- and age-related differences in the CYP2K1 expression in rainbow trout. Mature males expressed LMC2 proteins almost 2-fold higher in liver and 25-fold higher in kidney as compared to mature females. This factor has been important in determining different susceptibilities to $AFB_1$ effects between the two sexes in this species. When immature males and females were compared for the LMC2 protein expression, no differences were found between them (Cok et al., 1998).

The expression of proteins encoded by *CYP2K1* seems to have different patterns in other fish species. *CYP2K1* is also considered the major constitutive P450 form in channel catfish. Using chromatographic methods for the purification of proteins, a single CYP2-related protein designated as CM-HA3 showed 27% identity to both CYP2K and CYP2M forms of rainbow trout. However no significant relationship was found, when using correlational analysis, with all the sexual steroids that were previously mentioned as endogenous substrates of CYP2K forms in rainbow trout (Perkins et al., 2000). The remarkable resistance of catfish to $AFB_1$ carcinogenic effects has also been associated with very low biotransformation capacity of catfish P450 isoforms when $AFB_1$ is biotransformed to the carcinogenic 8,9-epoxide

metabolite (Gallagher and Eaton, 1995). The absence of biotransformation toward this exogenous substrate in catfish confirms that CYP2-like proteins in this species have different substrate affinities as compared to those of rainbow trout. Lower susceptibility to $AFB_1$ effects that are reported in tilapia (Tuan et al., 2002) might have a correlation with lower expression of CYP2-like isoforms. Nevertheless, this has not yet been confirmed in this species.

Induction of lauric acid hydroxylation in response to treatment with peroxisome proliferative agents (PPAs) (e.g. clofibrate, ciprofibrate) was investigated in bluegill and channel catfish (Haasch et al., 1998). Qualitative and quantitative patterns in sub-terminal hydroxylated metabolites after CYP2K-mediated reactions indicated sex- and species-specific differences. Although, immunological similarity has been found between proteins in these two species and those isolated from rainbow trout, chemical identification of hydroxylated products show differences in catalytic reactions among proteins that share CYP2K identity.

The constitutive protein translated from *CYP2M1* in rainbow trout, LCM1, was first identified in livers of specimes that had not been exposed to any kind of known inducers (Miranda et al., 1989). *CYP2M1,* the gene that encodes for LMC1, has been cloned from liver, brain, blood, and intestine in the same species (Yang et al., 1998a; Cok et al., 1998). This protein has cross-reactivity with rat CYP2B1 proteins (Miranda et al., 1990). The corresponding protein from *CYP2M1* was thought at first to be responsible for the same type of ω-hydroxylations made by LMC2 (e.g. CYP2K1 product) but further investigations showed that LMC1 actually catalyzes the ω-6 hydroxylation of laurate (Miranda et al., 1990).

Recent findings in Atlantic salmon (*Salmo salar*) reveal the involvement of CYP2K- and CYP2M-like enzymes in the terminal and sub-terminal hydroxylations of 4n-nonylphenol (4n-NP) (Thibaut et al., 2002). Nonylphenols (NPs) are the predominant components of alkylphenols, a group of compounds used for the manufacturing of surfactants. NPs are part of contaminants known as endocrine disruptors and cause estrogenic effects in fish, amphibians, birds and mammals (White et al., 1994). One of the most interesting aspects about this report is the possible competition for P450 isoforms (e.g. CYP2M-, CYP2K-) between endogenous steroids and exogenous endocrine disruptors such as NPs. This emphasizes the likely implications of pollutants on endogenous biotransformation of steroids and other key substrates for constitutive metabolic reactions.

***CYP2N subfamily***

Oleksiak et al. (2000) reported the cDNA cloning of two novel P450s from *Fundulus heteroclitus* that were designated *CYP2N1* and *CYP2N2*. The former was predominantly found in liver and intestine and the latter in heart and brain. Both protein products play a role in the epoxidation of arachidonic acid yielding epoxyeicosatrienoic acids.

***CYP2P subfamily***

*CYP2P* is a subfamily in which 3 genes have been cloned from killifish (*Fundulus heteroclitus*) (Oleksiak et al., 2003). Structurally, *CYP2Ps* are related to fish *CYP2Ns* and *CYP2Js* in mammals. CYP2Ps transcripts are expressed mainly in liver and

intestine and are responsible for the benzphetamine-N-demethylation and arachidonic acid oxidation. Based on similarities found in gene sequences, catalytic function, tissues distribution and regulation, it has been hypothesized that CYP2J, CYP2N and CYP2P subfamilies are related, vertebrate P450 enzymes involved in arachidonic acid metabolism and probably evolved from a common ancestral gene (Oleksiah et al., 2000, 2003).

***CYP2X subfamily***

Schlenk et al. (2002) cloned a 1470-base pair fragment that encodes a 490-aminoacid protein of approximately 57 kD from liver. This gene was classified as *CYP2X1* by the P450 nomenclature committee. Although this gene has 41% identity with Fundulus *CYP2P2* and 40% identity with *CYP2N2*, no significant correlation was found with catalytic activities that have been recognized for the corresponding proteins of these genes in mammals. At this point, the catalytic function(s) of *CYP2X1* proteins remains unknown.

*CYP3A subfamily*

CYP3A subfamily represents the largest fraction of CYP proteins in liver and intestine of vertebrates (Hegelund and Celander, 2003). *CYP3A* genes that have been cloned in fish species include: rainbow trout *CYP3A27* (liver, brain, blood, intestine) (Buhler and Wang-Buhler, 1998; Cok et al., 1998), rainbow trout *CYP3A45* (liver) (Lee and Buhler, 2003), killilifish *CYP3A30* (liver) (Celander and Stegeman, 1997), and killifish *CYP3A56* (intestine) (Hegelund and Celander, 2003). More recently

Kashiwada and collaborators (2005) reported the functional characterizacion of medaka (*Oryzias latipes*) *CYP3A38* and *CYP3A40,* which are involved in testosterone hydroxylation.

LMC5 is the purified protein obtained from *CYP3A27* in untreated juvenile rainbow trout (Miranda et al., 1989). This protein exhibits a significant 6β-hydroxylation of testosterone and progesterone. This reaction is catalyzed in mammalian systems by the same P450 subfamily (*CYP3A4*). Antibodies raised against CYP3A4 inhibited the 6β-hydroxylation in rainbow trout offering compelling evidence for similarities in catalytic activities between CYP3A4 in mammals and CYP3A27 in trout (Buhler and Wang-Buhler, 1998). CYP3A27 has also been isolated from rainbow trout gastrointestinal tract. Furthermore, levels in the intestinal ceca and proximal descending intestine were found to be from 2- to 6-fold greater than those in the liver. Although the expression of CYP3A27 in other fish species has not been established, various papers suggest that the consequences of a high CYP3A27 expression in the GI tract, reflects a substantial capacity for intestinal metabolism of ingested drugs and other chemicals (Cok et al., 1998; Lee et al., 2001).

*CYP3A45*, a new CYP3A gene, was cloned and compared to previously known CYP3A forms (Lee and Buhler, 2003). *CYP3A45* shows 94% identity with trout *CYP3A27*, 72% with killifish *CYP3A56* and 71% with killifish *CYP3A30*. As it was reported for *CYP3A27*, *CYP3A45* has the major expression site in the intestine of both male and female trout. The encoding protein of this gene has steroid hydroxylase activities toward 6β-, 2β-, and 16β-hydrotestosterone.

*Changes in the expression of P450 due to pathogens and pollutants*

Baseline P450 activity expected in animals under normal, physiological conditions seems to be affected by stressful conditions such as the presence of infectious pathogens and water pollutants.

Recent reports on the effects of pathogens, their immunostimulants (e.g., lipopolysacharides, LPS), and the inflammatory cytokines (IL1, IL6, TNFα) that are involved in disease processes, indicate that they can play some role in regulating cytochrome P450 expression in fish. For example, the activity of cytochrome P450 in 3-MC-induced carp (*Cyprinus carpio*) was inhibited by LPS in liver and head kidney microsomes (Marionnet et al. 2006). This report opens many questions with regard to the way P450 expression can be affected by infectious diseases as compared to non-infected animals.

A new area of much interest in toxicology is involved with the effects of pharmaceuticals as water pollutants and their effects on biotransformation reactions in aquatic organisms. Thibaut and collaborators (2006) demonstrated that anti-depressive drugs such as fluoxetine, fluvoxamine and paroxetine that can be found in sewage effluents can decrease several P450 isoforms (CYP1A, CYP3A-like, and CYP2K-like) in carp (*Cyprinus carpio*) *in vitro*. In addition, anti-inflammatory drugs such as ibuprofen, naproxen and ketoprofen depressed CYP2M-mediated reactions in the same species.

## The Flavin Monooxygenase System in Fish

The flavin monooxygenases (FMOs) (EC 1.14.13.8) are membrane-bound enzymes found in the smooth endoplasmic reticulum of the cell. FMOs catalyze NADPH-dependant metabolism of xenobiotics containing nucleophilic nitrogen-, selenium-, sulfur-, and phosporus-heteroatoms (Hines and McCarver, 2002). FMOs are found in a wide variety of living organisms ranging from bacteria to humans. FMOs are identified as important biotransformation systems for xenobiotics such as thioethers, thiocarbamates, and thiocarbamides. Many of these are chemical components of pesticides that may reach the water column and affect fish populations (Wang et al., 2001; El-Alfy and Schlenk, 2002).

The main physiological role played by FMO seems to be osmoregulation. The oxidation of trimethylamine (TMO) to trimethylamine oxide (TMAO) is the most important reaction catalyzed by FMO in terms of osmoregulation. TMAO is found in large amounts in marine fishes and is thought to play an important role as an intracellular osmolyte and protein stabilizer in the marine or estuarine environment (Groninger, 1964 and Hebard et al., 1982 cited by Schlenk, 1998). The intracellular TMAO : urea ratio goes from 2:1 to 3:1 in elasmobranches (Yancey et al., 1982). An important finding that confirms the interrelation between FMO and osmoregulation is an increase in its expression when fish are acclimated from freshwater to saltwater environments (Schlenk and El-Alfy, 1998).

*Gene Characterization*

Gene characterization of FMOs is better known in mammals than in fish. In the former, 5 different families (FMO 1 through FMO5) encoding 5 unique proteins with different substrate specificities, tissue distribution and regulatory mechanisms have been identified (Lawton et al., 1994). In addition to rat and mouse FMOs, various studies in FMO characterization include pig liver FMOs and both rabbit liver and lung FMOs (Ziegler, 1988).

Attempts on gene characterization of fish FMOs have been made in rainbow trout, tilapia and striped bass species (Schlenk, 1998). A 850-basepair fragment that is 97% and 88% homologous to rabbit liver FMO1 and pig liver FMO1, respectively, is reported in rainbow trout.

Although gene characterization has not been reported in fish, several FMO1- and FMO2-like proteins are expressed in species such as rainbow trout, Atlantic flounder (*P. flesus*) and turbot (*S. maximus*). Unlike mammals, there does not seem to be multiple FMO isoforms in fish (Schlenk, 1998). A reaction of FMO3-like protein that was expressed in Japanese medaka (*Oryzias latipes*), simultaneously exposed to different levels of sexual steroids (e.g. estrogen and testosterone) and the carbamate aldicarb, suggests the presence of this type of FMO isoform. However, full gene characterization has not been accomplished (El-Alfy and Schlenk, 2002).

*Tissue and Cellular Distribution*

FMOs are membrane-bound enzymes found in the smooth endoplasmic reticulum of the cell. In fish, activity is usually greatest in liver but the expression in other organs such as gills and kidney may be more significant when some environmental conditions prevail. In this regard, euryhaline (wide-salinity tolerance) species have higher FMO activity in gills and kidney as compared to stenohaline (narrow salinity tolerance) whose FMO activity is mainly hepatic (Schlenk et al., 1995). Factors affecting FMO expression will be discussed in more detail in further sections.

*Catalytic Activity*

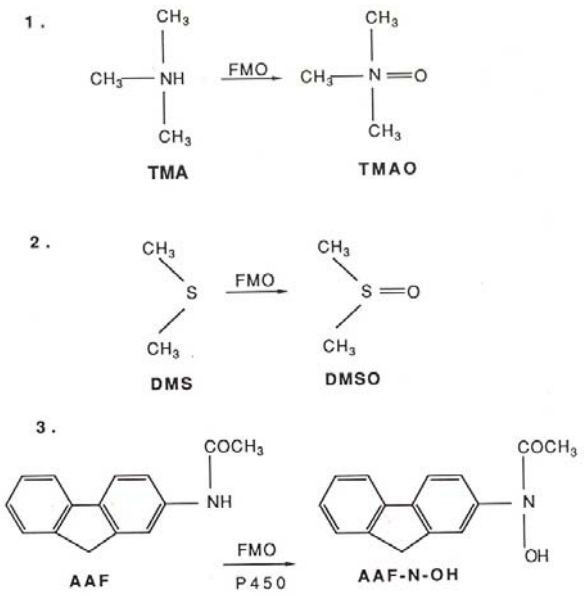
The catalytic cycle of FMOs generates initially a peroxy complex (enzyme-FAD-OOH) previous to substrate binding. This complex is transformed yielding hydrogen peroxide if no substrate is available for an FMO-mediated reaction. FMOs are catalysts of a 4-electron reduction of dyoxygen with 2 electrons from a reduced nicotinamide cofactor and other 2 electrons derived from the substrate. The enzyme forms a Michaelis complex with NADPH allowing a rapid reduction of the flavin that is bound to the enzyme. As such, the energy needed to perform the biotransformation reaction is present before the substrate is encountered by the peroxyflavin complex (Poulsen and Ziegler, 1995; Schlenk, 1998). Another important and distinctive aspect of FMO-catalyzed biotransformation is its second order rate that is not saturable by substrate (Poulsen and Ziegler, 1995). Figure 2 shows three representative reactions catalyzed by FMO.

Different tests are available for the *in vitro* evaluation of FMO activity. The most used assays for FMO quantification are reported by Ziegler and Pettit (1964) and Dixit and Roche (1984). The former is a spectrophotometric procedure that quantifies the N-oxygenation of N,N-dimethylaniline (DMA). The latter is based on the spectrophotometric quantification of methimazole oxide. Cashman and Hanzlik (1981) used thiobenzamide in a photometric assay to quantify FMO specific sulfo-oxidation. Cavagnaro et al. (1981) tested FMO activity by means of the NADPH oxidation rate using methimazole as substrate. A more recent report by Athena-Guo and Ziegler (1991) uses the oxidation of thiocholine to quantify FMO activity.

*Changes in Expression due to Physiological and Environmental Factors*

Different factors associated with species differences, changes in salinity and exposure to toxicants are cited as sources of variation in FMO expression.

Figure 2. FMO-mediated oxidations in (1) trimethylamine (TMA), (2) dimethylsulfoxide (DMS) and (3) N-acetylaminofluorene (AAF) (Taken from George, 1994)



Channel catfish (*Ictalurus punctatus*) exposed to aldicarb, a carbamate insecticide, showed a greater resistance to the deleterious effects of the pesticide as compared to rainbow trout (*Oncorhynchus mykiss*) (Perkins and Schlenk, 2000). This response is attributed to a lower rate of bioactivation in the former species. A correlation with previous studies in rainbow trout (Schlenk and Buhler, 1991) and channel catfish (Schlenk et al., 1993), confirmed that the main reason for such differences in susceptibility is given by the lack of FMO activity in channel catfish. Although catfish lack FMO activity, P450-mediated oxidation helps to biotransform the carbamate insecticide. However, the effectiveness for this biotransformation reaction is significantly better in trout through both FMO and P450 reactions, confirming that fish that have high levels of FMO are more susceptible to thioether pesticides than fish lacking FMO (Schlenk, 1995).

Other physico-chemical variables have been studied in terms of their effects on FMO expression in fish. Larsen and Schlenk (2002) reported a 50% increase in FMO activity in low temperature-acclimated rainbow trout (2-3 $^{o}$C) as compared to fish maintained at 10 $^{o}$C. Changes in FMO due to higher salinity and urea are cited too. Rainbow trout that were treated with cortisol and urea, via an aortic infusion, showed an increase in gill and liver FMO activities (El-Alfy et al., 2002).

Sexual differences in the expression of FMOs that were found in rats and mice (Falls et al., 1997) have also been observed in Japanese medaka. El-Alfy and Schlenk (2002) found that estrogen significantly reduced FMO1-like expression in male gills and female livers. On the other hand, testosterone significantly reduced the expression of FMO1-like protein in male livers and gills whereas the expression in females was not

affected at all. This suggests the presence of an additional FMO isoform that has not yet been identified.

## PHASE II BIOTRANSFORMATION REACTIONS IN FISH

This section presents general aspects about three of the most studied phase II reactions in fish: UDP-glucuronosyltransferase-, sulfotransferase- and glutathione-S-transferase-mediated reactions. Compared with these reactions, there have been very few reports on other phase II reactions in fish such as glucosidation, aminoacid conjugation, acetylation, etc. (James, 1987).

### UDP-glucuronosyltransferase-mediated Conjugation

Glucuronidation is one of the major phase II pathways in fish and other species. Although it is recognized as an important phase II reaction in fish, much more work has to be done to fully characterize this type of reaction in piscine species. Both endogenous (e.g., bilirubin, steroids, thyroid hormones, etc.) and exogenous compounds (e.g., PAHs, PCBs, chlorophenols, pesticides, etc.) are conjugated with glucuronic acid via UDP-glucuronosyltransferases (UDPGTs) (EC 2.4.1.17) (George, 1994).

Glucuronidation of endogenous compounds is important for the elimination of end products of heme catabolism (i.e., bilirubin) (Kasper and Henton, 1980), and excretion of inactivated steroids of testicular origin that, after conjugation with

glucuronic acid, have pheromone-type functions in salmon (Gracjer and Idler, 1963), rainbow trout (Hews and Kime, 1978), carp (Kime, 1980), and channel catfish (Schoonen et al., 1987). Glucuronides of 17β-estradiol in rainbow trout (Förlin and Haux, 1985) and conjugates of 17α,20β-dihydroxyprogesterone in female goldfish (Van der Kraak et al., 1989) are also cited as endogenous compounds that undergo glucoronide-based conjugation. Thyroid hormones are inactivated through glucuronide-type conjugates increasing their water solubility and thus facilitating excretion through the bile and urine (Finnson and Eales, 1997).

Different types of structurally diverse xenobiotics are conjugated by UDPGTs. Among them antimicrobials, pesticides, and toxicants of significant relevance have been reported in the literature. Chloramphenicol (Craverdi et al., 1985) and oxalinic acid (Ueno et al., 1985) are mentioned as substrates for glucuronic acid in fish. Some pyrethroids (Glickman et al., 1981), organophosphates (Takimoto et al., 1987), carbamates (Statham et al., 1975), and phenols (Lech, 1973; Kane et al., 1994) are excreted as glucuronic acid conjugates. Aflatoxicol B1 glucuronide, one of the metabolites of aflatoxin B1, was reported by Loveland et al. (1984) in rainbow trout exposed to this hepatocarcinogen. Other important environmental contaminants that are excreted as glucuronic acid conjugates are PAHs (Varanassi et al., 1984), phthalate ester plasticisers (Stalling et al., 1973) and chlorophenols (Oikari et al., 1988).

Although phase II reactions are generally considered beneficial for the biotransforming organism, a number of glucuronide conjugates are known to generate adverse reactions or toxic effects in mammals. Two types of these bioactive

glucuronides are N-O glucuronides of hydroxamic acids and acyl glucuronides of carboxylic acids. The former are reactive toward cellular nucleophiles such as methionine, tryptophan and guanosine. The latter have relevance in therapeutics given that many prescribed drugs like non-steroidal antinflammatories (NSAIDs) and other carboxylic-base therapeutants are suspected to generate toxic responses in patients due to the reaction of the corresponding glucuronic acid conjugates with cellular nucleophilic macromolecules (e.g., proteins, DNA). The UDPGTs isoforms that are responsible for the synthesis of undesirable glucuronides are reported in rats and humans (Spahn-Langguth and Benet, 1992; Ritter, 2000). No reports of these toxic reactions due to glucuronide-type conjugates have been noted in fish species.

In addition to the presence of particular isoforms responsible for UDPGT-mediated adverse reactions, the lack of some isoforms may lead to undesirable effects too. Diseases that are characterized by hyperbilirubinemia due to poor glucuronic acid-based conjugation in mammals happen due to genetic defects in *UT1A1*, one of the genes that encodes for a UDPGT family in humans (Burchell et al., 2000).

***Gene Characterization***

Recent developments achieved by the Washington University-Zebrafish Genome Resources Project indicate the presence of multiple UDPGT genes in fish (George and Taylor, 2002). These findings are based on analysis of DNA sequences and confirm preliminary studies done in plaice (*Pleuronectes platessa*) (Clarke et al., 1988; 1992). Ten different UDPGTs genes have been identified from nucleotide sequence analysis in zebrafish. Three of these, ZF5, ZF6, and ZF7, are very similar to the ones found in

the subfamily 1B of humans. ZF9 and ZF10 are more related to the human GT2 family. ZF1, ZF2, ZF8 seem to have an earlier divergence based on cladistic studies. ZF3 and ZF8b were found to be too short to be aligned with the corresponding exon for analysis (George and Taylor, 2002).

### *Cell and Tissue Distribution*

UDPGTs are located in the endoplasmic reticulum of cells in liver, kidney, intestine, skin, gills, etc. UDPGT activity may differ depending on the substrate and the organ evaluated. Although liver is the organ with the highest UDPGT activity toward phenols in species such as trout and plaice (*Pleuronectes platessa*), other organs appear to be more active in other species (George, 1994). As an example of such variations, branchial UDPGT activity is higher in vendace (*Coregonus albula*) and roach (*Rutilus rutilis*) in comparison to other organs (James, 1987). Intestinal UDPGT activity has been investigated in fish species too. Van den Hurk and James (2000) studied the role of glucuronidation and sulfation in the intestinal mucosa of channel catfish when exposed to benzo[a]pyrene-7,8-dihydrodiol. A very poor phase II-mediated intestinal conjugation of this metabolite was suggested to explain the high systemic availability of the compound when was ingested or produced by phase I-mediated reactions.

### *Catalytic Activity*

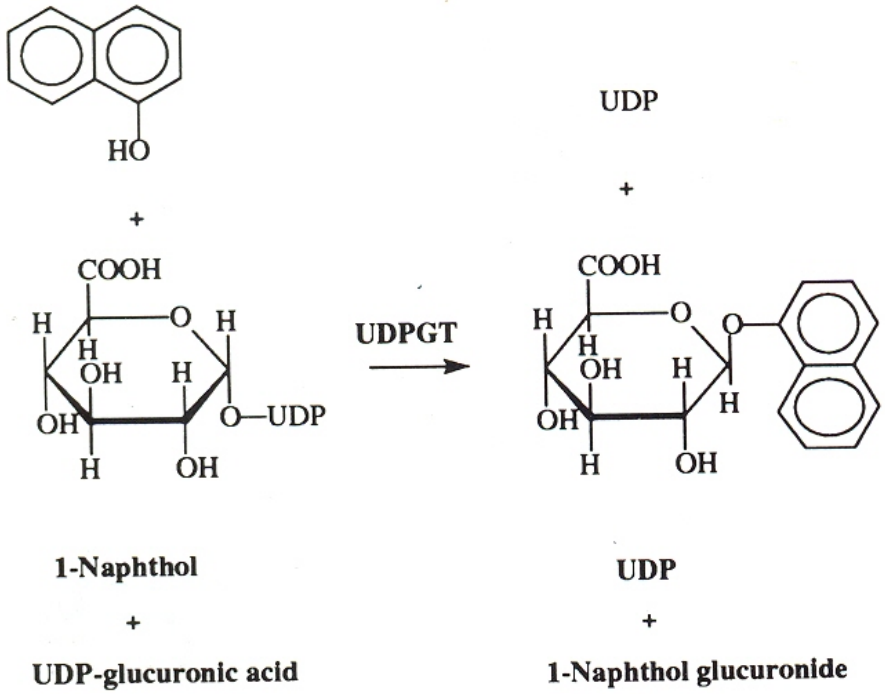
Substrates that are subject to glucuronidation include compounds that have functional groups like aliphatic alcohols, phenols, carboxylic acids, primary and secondary

aliphatic and aromatic amines and sulfhydryl groups. Nucleophilic heteroatoms such as O, N and S that are rich in electrons are the specific site for the reaction (Parkinson, 2001). Glucuronidation of 1-naphthol by UDPGT is presented in Figure 3 as an example of this reaction.

The UDPGT-mediated reaction requires the cofactor UDP-glucuronic acid, a high-energy, water-soluble molecule that is synthesized from glucose-1-phosphate (Parkinson, 2001). Substrates that are conjugated via UDPGTs may undergo hydrolysis by action of β-glucuronidases, a group of hydrolytic enzymes that are present in the intestinal microflora. β-glucuronidases are responsible for the release of the aglycone part of the conjugate and further reabsorption via enterohepatic recirculation which delays the excretion of xenobiotics.

*In vitro* assays that are used to determine UDPGT activity utilize different types of substrates. Many of the reports cite the use of phenol-type compounds like 4-nitrophenol (Winsness, 1969; Burchell and Coughtrie, 1989), and 3-trifluoromethyl-4-nitrophenol (TFM) (Lech and Statham, 1975; Kane at al., 1994). These phenol-type compounds have the ability to form conjugates that are quantified by colorimetric reactions in the visible range of the spectrum (e.g. $\lambda$=395). Other type of substrates like 4-methylumbelliferone have also been used to detect UDPGT activity based on lower fluorescence emission once the substrate is conjugated (e.g. excitation $\lambda$ = 323 nm and emission $\lambda$= 450 nm) (Schell and James, 1989).

Figure 3. Glucuronidation of 1-naphthol by UDP-glucuronosyltransferase (Taken from George, 1994).




*In vitro* evaluation of UDPGT activity exhibits a characteristic latency that may be overcome by pretreatment of microsomes with detergents. This latency is reported to be lower in fish microsomes as compared to mammalian ones (George, 1994). Triton X-100 (Castrén and Oikari, 1983, Ankley and Agosin, 1987) and Brij 56 (Finnson and Eales, 1997) are among the detergents used for facilitating substrate interaction with UDPGT within the microsomal membranes. Working with fish microsomes, Ankley and Agosin (1987) found that 0.1% Triton X-100 was the optimal concentration for increasing 4- and 5-fold UDPGT activities in bluegill and catfish, respectively. Although 0.2% of the detergent increased the activity as compared to 0.1%, the reaction was not linear in the first 20 minutes. It is suggested that Triton X-100 in concentrations greater than 0.1% may exert an inhibitory effect on UDPGT activity. As for UDPGT activity towards thyroxine and 3,5,3'-triiodothyronine, Brij 56

optimized the T3 and T4 glucuronidation activity in trout when used at 0.0125% (Finnson and Eales, 1997). Both, Triton X-100 and Brij 56 are nonionic, non-denaturating detergents that do not affect native interactions and structures of UDPGT proteins.

Physical treatment such as repeated freezing and thawing cycles (Castrén and Oikari, 1983; Schell and James, 1989) and sonication (Winsnes, 1969) are also suggested as an efficient way to disrupt microsomal membranes and enhance UDPGT activity *in vitro*. Freezing and thawing may increase UDPGT activity as much as 36% (Menon and Haberman, 1970; cited by Schell and James, 1989).

Castrén and Oikari (1983) reported optimal pH conditions for liver UDPGT activity in rainbow trout. Reaction rates in this species had no significant changes in a range between 7.0 and 7.8. As for incubation temperature, a range of 18 $^{o}$C to 24 $^{o}$C was considered optimum for the *in vitro* assay. A report in vendace (*Coregonus albula*) showed that UDPGT activity increased with temperatures up to 40 $^{o}$C (Castrén and Oikari, 1983). It is interesting to mention that although poikilotherms live in waters that are between 5 $^{o}$C and 30 $^{o}$C, the optimum temperature for UDPGT *in vitro* assays may go up to 35 $^{o}$C or 40 $^{o}$C, as was reported in the case of vendace. Differences among piscine species with regard to optimal conditions for UDPGT activity may be present although not many reports make full description of these variations.

### *Changes in Expression Associated with Physiological and Environmental Factors*

Different studies have analyzed the changes in the expression of UDPGT activity associated with physiological or environmental factors. Exposure to particular types of food components and/or toxicants as well as seasonal changes may be responsible for induction or inhibition of UDPGT activity.

Investigations that have compared different piscine species have shed some light on similarities and differences in UDPGT expression among fishes. When bluegill and channel catfish UDPGT activities towards 4-methylumbelliferone (4-MU) were compared, Ankley and Agosin (1987) found very similar results in both species (3.0 ± 0.8 and 2.5 ± 0.8 nmols of conjugated substrate / min / mg protein, respectively). A study by Ianelli et al. (1994) that included several fish species and substrates indicated greater differences in UDPGT activity among them. In this report, rainbow trout is cited as the species with the highest UDPGT-mediated conjugation of 4-MU, a common substrate used to evaluate UDPGT activity. On the other hand, when 2-hydroxybiphenyl was used as substrate, guppy had 10-fold greater activity than carp, zebrafish and trout. Another study indicated that plaice, a benthic feeding marine flatfish that is more frequently exposed to sediments, had the highest 4-nitrophenol UDPGT-mediated conjugation in any fish studied so far (George, 1994). Differences in UDPGT-based conjugation of toxicants have been used to explain variations in susceptibility among fish species. 3-trifluromethyl-4-nitrophenol (TFM) conjugation by UDPGT was compared among bluegill, rainbow trout, channel catfish and sea lamprey. The lower conjugation capacity in sea lamprey, compared to the other

species examined, explains the highest susceptibility of this species to the lampricide TFM (Kane et al., 1994).

Other physiological and environmental factors have been implicated in altered UDPGT activity. These include handling stress, starvation and variation in activity due to seasonal changes. Anderson et al. (1985) reported no effects on p-nitrophenol hepatic conjugation via UDPGT activity in juveniles of rainbow trout that were subject to starvation during 6 and 12 weeks. Another study by Blom et al. (2000) tested the effects on handling stress and food deprivation in several phase I-II biotransformation reactions in rainbow trout. In this report, food deprivation was the main factor that increased hepatic UDPGT activity. However, other reports showed that food deprivation either decreased (Vigano et al., 1993) or did not alter (Andersson et al., 1985a,b) UDPGT activity in this species. As a result, inconsistent variations have been found testing UDPGT changes in activity after experimenting with different physiological or environmental variables in fish.

Induction of UDPGT activity is reported in both mammalian and fish species. Diet and exposure to environmental pollutants are the most cited sources of induction. Induction studies of UDPGT and other phase II enzymes in mammals have been mainly planned for the identification of nutritional components that may increase phase II-type enzymatic activity and reduce the incidence of deleterious effects of pollutants in the environment. Compounds such as allyl sulphides and alkyl sulphides derived from *Allium* species (e.g., onion) are known to increase phase II enzymes activities such as UDPGT and glutathion-S-transferase in rats (Teysser et al., 2001). Epidemiological studies indicated that consumption of representatives of the genus

*Allium* reduces the risk of stomach, colon and breast cancer (Teysser et al., 2001). Other studies indicate that the consumption of green tea stimulated UDPGT activity towards p-nitrophenol in rats (51.8%) as compared to controls that drank water (Embola et al., 2002). This significant increase in UDPGT activity and hence conjugation of aromatic compounds has been attributed to the presence of polyphenols in the dried leaf and has been linked to the suppressive effects of green tea in the initiation, promotion and progression stages of cancer (Embola et al., 2002).

Studies on UDPGT induction in fish are mainly oriented to link the effects of environmental pollutants on UDPGT expression. These studies correlate the presence of particular waterborne or foodborne contaminants and the induction or inhibition effects on UDPGT activity.

Castrén and Oikari (1987) studied the effects of different types of waterborne toxicants on UDPGT activity in 1-2 year-old rainbow trout. The slimicide N-methyldithiocarbamate was found to significantly increase UDPGT activity in sublethally-exposed trout. In the same study, a lower UDPGT activity was found after exposing the fish to mixtures of pentachlorophenol and 2,4,6-trichlorophenol, two compounds regularly found in effluents from the pulp and paper industry.

Juvenile common carp (*Cyprinus carpio*) were injected intraperitoneally (40 mg / K body weight) with 3-methylcholanthrene, a PAH commonly used as an inducer of phase I-II reactions. An increase in UDPGT activity was found in trunk kidney (5.8-fold) followed by spleen (2.2-fold) as compared to controls. Liver induction was lower than the ones found in the other organs. This study is of particular interest given

that there are not many reports on induction of UDPGT activity in extrahepatic tissue (Taysse et al., 1998).

Rainbow trout juveniles that were exposed to effluents from pulp and paper industry (0.01% final concentration) showed no changes in UDPGT activity when compared to controls after 8 days. These findings do not preclude that higher concentrations and greater exposure times may exert an inducing effect on UDPGT activity (Sturm et al., 1999).

**Sulfotransferase-mediated Conjugations**

Sulfation is a secondary phase II reaction that conjugates most of the substrates that are conjugated by UDPGTs as well. This reaction is catalyzed by sulfotransferases (ST) (EC 2.8.2.2, EC 2.8.2.4), a group of cytosolic enzymes that have lower capacity than UDPGTs but a higher affinity for the substrates (Parkinson, 2001).

Some studies have identified ST activity towards thyroid hormones in plaice (Osborn and Simpson, 1969) and rainbow trout (Finnson and Eales, 1998). ST-mediated conjugation of thyroid hormones in rats is preferential toward active triiodothyronine ($T_3$), followed by inactive triiodothyronine ($rT_3$) and thyroxine ($T_4$). In contrast, trout have higher ST-mediated conjugation toward $rT_3$ (Finnson and Eales, 1998).

There is an increasing interest for determining the effects of STs on bioactivation of xenobiotics. As mentioned before for UDPGTs, STs are implicated in undesirable reactions that determine the activation of promutagens such as benzylic and allylic

alcohols, aromatic hydroxylamines and secondary nitroalkanes (Glatt, 2000). These studies have been done using human STs  but not fish.

### *Gene Characterizacion*

Gene characterization of STs for different animal species is based on what has been found in humans. Five types of STs are coded by different genes (HAST1 through HAST5) (Brix et al., 1998; Coughtrie et al., 1998). All of the human STs share more than 90% homology in the amino acid sequence but vary significantly in the substrate preferences (Brix et al., 1998). As a result, STs have been classified in three different families: phenol, hydroxysteroid and flavonol STs (Wood et al., 1994). On the other hand, very little is known about different ST isoforms in fish species. Preliminary reports indicate that, unlike mammalian STs, rainbow trout STs seem to be just a single form of the phenol-type (Finnson and Eales, 1998).

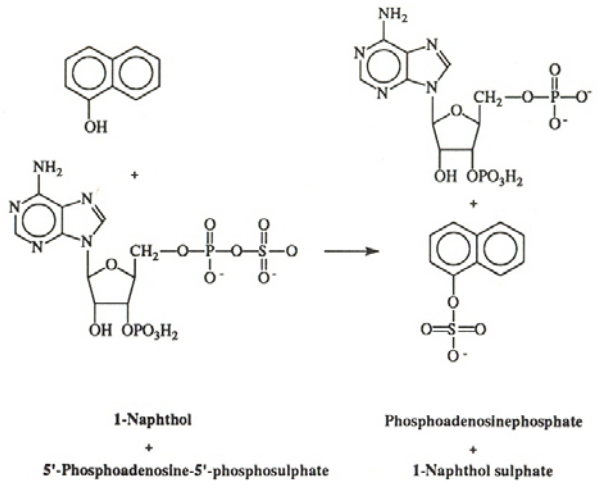
## Catalytic Activity

STs conjugate hydroxyl groups, aliphatic alcohols, aromatic amines and hydroxylamines (George, 1994). These are basically the same substrates that are conjugated by UDPGTs. However, the concentration of the phase I metabolite that undergoes a further phase II conjugation seems to determine whether a sulfate- or glucuronic-mediated conjugation takes place. Generally speaking, at low xenobiotic concentrations sulfate conjugates prevail, whereas the glucuronic conjugation increases as the phase I metabolite concentration is higher (Parkinson, 2001).

STs require the cofactor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) that donates a $SO_3^-$ group to the xenobiotic (Figure 4). PAPS is formed from ATP and inorganic sulfate by two enzymatic activities: ATP sulforylase and APS kinase (Coughtrie et al., 1998). The major source of sulfate that is required for the synthesis of PAPS comes from cysteine which is in limited concentration within the cytosol (Duffel and Jakoby, 1981; Yang et al., 1998b). Sulfation is relatively expensive pathway in terms of energy costs since two ATP molecules are used for the synthesis of one molecule of PAPS (Coughtrie et al., 1998).

ST activities are reported in diverse fish species using waterborne phenol substrates. Among these species are goldfish (*Carassius auratus*), guppy (*Poecilia reticulata*), minnow (*Phoxinus phoxinus*), roach (*Rutilus rutilis*), carp (*Cyprinus carpio*) and tench (*Tinca tinca*). However, substrates that are usually utilized to evaluate ST activity in mammals (e.g., acetaminophen, 7-ethoxycoumarin, pentachlorophenol) are poorly metabolized in rainbow trout (George, 1994).

Figure 4. Sulfation of 1-naphthol by arylsulfotransferase (Taken from George, 1994).



***Changes in the expression due to physiological or environmental factors***

Fewer investigations have dealt with the physiological and environmental factors that affect sulfotransferase expression in fish as compared to other phase I or II biotransformation reactions. An interesting report by Ohkimoto and collaborators (2003) indicates that environmental estrogens like bisphenol A, 4-n-octylphenol exert concentration-dependent inhibition of the sulfonation reactions towards 17-β-estradiol in zebrafish. This may have serious effects on conjugation of the endogenous estrogens as this reaction is inhibited by the presence of exoestrogens.

**Glutathione-S-transferase-mediated Conjugation**

Glutathione-S-transferases (GSTs) (EC 2.5.1.18) are a multigene family of dimeric, polyfunctional enzymes that primarily catalyze the conjugation between electrophilic compounds and the tripeptide glutathione (GSH). GSTs have a wide distribution from bacteria to vertebrates (Stenersen et al., 1987) and have been classified in 7 different classes: mu (μ), pi (π), alpha (α), theta (θ), sigma (σ), kappa (κ), and xi (ξ).This classification is based on substrate specificity, immunological cross-reactivity and protein sequences (Tomarev et al., 1993, Pemble et al., 1996).

In addition to the GSH-mediated conjugation, GSTs play roles in the biosynthesis of vasoactive metabolites of arachidonic acid, isomerization of steroids, intracellular transport of heme, bilirubin and steroid hormones, and Se-independent GSH peroxidase activity toward organic peroxides (George, 1994). GSTs are also part of the oxidative stress defense system. It is considered that GSTs may play a more

important role than glutathione peroxidase (GPx) activiy in fish as compared to mammals. GSTs are more abundant than GPxs in fish liver (Stephensen et al., 2002).

***Gene Expression***

Isolation and cloning of GSTs genes are reported in some fish species. Gallagher et al. (1998) reported isolation and cloning from English sole (*Pleuronectes vetulus*) and starry flounder (*Platichthys stellatus*) livers. These sequences showed ~ 95% identity when compared with that of GST-A in plaice (*Pleuronectes platessa*). In other studies, a 471 nucleotide GST-like cDNA was isolated from largemouth bass (*Micropterus salmoides*) (Henson et al., 2000). When attempts were made to compare the expression of this gene in marine species with other freshwater species, a faint band of similar size cDNA was recognized in brown bullhead (*Ictalurus nebulosus*) liver but not in channel catfish (*Ictalurus punctatus*). Based on these findings, several authors concluded that the presence of GST-A in largemouth bass, as well as in the other marine species that are phylogenetically distant, suggests and important and conserved function of GST-A in fish (Henson et al., 2000).

Hepatic GSTs have been purified and partially characterized in piscine species such as rainbow trout (*Oncorhynchus mykiss*), carp (*Cyprinus carpio*), plaice (*Pleuronectes platessa*), Atlantic salmon (*Salmo salar*) (George, 1994), and channel catfish (Gallagher et al., 1996). Nóvoa-Valiñas et al. (2002) isolated 5 different GST isoforms from Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*). The GST forms in channel catfish showed moderate immunological affinity for rat α and π class isozymes and very limited cross-reactivity to the rat μ class. On the other hand,

the forms in Atlantic salmon and brown trout were related to the π class, which is considered the most common in piscine species (George, 1994).

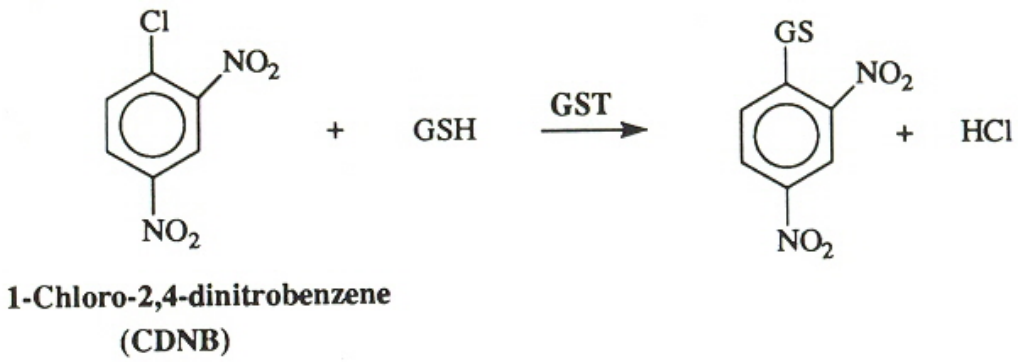
### *Cell and Tissue Distribution*

In both fish and mammals, these enzymes are at high, milimolar concentrations in the cytosolic fraction of the cell (Gallagher et al., 1996). Minor amounts of GSTs may also be found in the microsomal fraction. GSTs are mainly found in the liver. However, there are reports on GST activity from gills, kidney, intestinal ceca and olfactory epithelium in rainbow trout (Pérez-López et al., 2000). Van Veld et al. (1991) reported higher GST expression in killifish intestine than in the liver.

### *Catalytic Activity*

The range of substrates catalyzed by fish GSTs is less diverse than those catalyzed by rodents (George, 1994). GST activities are usually assessed using reference substrates such as 1-chloro-2,4-dinitrobenzene (CDNB) (Figure 5). This activity reflects the integration of the majority of GSTs isozymes allowing general comparisons between species. However, GST-CDNB conjugation does not necessarily reflect specific GST conjugation capabilities toward biologically or environmentally relevant toxicants (Gallagher et al., 1996). All of the fish species examined so far have shown activity toward CDNB as a model substrate (George, 1994).

Figure 5. Glutathion-S-transferase-catalyzed conjugation of 1-chloro-2,4-dinitrobenzene with glutathione (GSH) (Taken from George, 1994)




**1-Chloro-2,4-dinitrobenzene (CDNB)**

Another substrate that has been used to evaluate GST activity is 4-hydroxynon-2-enal (4HNE), a mutagenic and cytotoxic α-β-unsaturated aldehyde that is produced during oxidative stress reactions (Dickinson and Forman, 2002; Pham et al., 2002). Largemouth bass (*Micropterus salmoides*) showed a higher GST-mediated conjugation rate towards 4HNE than those elicited by mammals and other aquatic species (Pham et al., 2002). This type of reaction is of great importance in fish given the high levels of polyunsaturated fatty acids that piscine species have as compared to other animal groups. However, very little is known about 4HNE conjugation capabilities by other fish species. Pham et al. (2002) reported 415 ± 83, 415 ± 45 and 187 ± 26 nanomols/min/mg protein of GST-4HNE conjugate in male largemouth bass, female largemouth bass and male brown bullhead, respectively. The high protective role of GSTs in largemouth bass has been attributed to the fact that this species is rich in polyunsaturated fatty acids and hence very susceptible to induced oxidative stress and lipid peroxydation.

The role of GSTs in conjugation of other important contaminants and toxicants has not been fully investigated in fishes. Gallagher et al. (1996) found that although channel catfish catalyzed the GST-mediated conjugation of (±)-anti-benzo[a]pyrene-trans-7,8-dyhydrodiol-9,10-epoxide (BPDE), a highly mutagenic and carcinogenic metabolite that is derived from benzo[a]pyrene phase I metabolism; no conjugation through GST takes place towards aflatoxin B1-8,9-epoxide, the carcinogenic product obtained after phase I-mediated reaction of this *Aspergillus flavus* toxin (Hayes et al., 1991).

***Changes in Expression due to Physiological and Environmental Factors***

Some of the studies on GST characterization have considered physiological and environmental factors that affect GST expression in fish. Differences among species as well as between males and females in GST activity are cited in the literature (Gallagher et al., 2001). Although a variety of fish species examined regarding GSTs activity respond to model substrates such as CDNB (1-chloro-2,4-dinitrobenzene), differences among them are significant (George, 1994). Channel catfish (Gallagher et al., 1996) and largemouth bass (Pham et al., 2002) are among the species with the highest GST responses. In fact, the particular resistance that catfish showed when exposed to electrophilic compounds such as benzo[a]pyrene epoxides is partially attributed to its efficient GST-conjugation capabilities (Gallagher et al., 1996).

Interestingly, while there are reports on induction of GST activity due to exposure to contaminants, others indicate the opposite effect. Van Veld et al. (1991) found a significant induction of intestinal GST killifish activity in the heavily, PAH-

contaminated Elizabeth River. In the same study, when fish were exposed under lab-controlled conditions to dietary β-naphthoflavone (e.g., PAH-type of inducer), the intestinal GST activity was induced while the hepatic form was not affected. On the other hand, Gallagher et al. (2001) report that contaminants in Lake Apopka (mainly organochlorine-type) were responsible for a significant decline in GST activity in females as compared to counterparts from a reference site.

## SUMMARY

Phase I-II biotransformation reactions in fish species are of paramount importance to understand metabolism of both endogenous and exogenous compounds. This chapter reviewed the most studied phase I-II reactions in fish species. A great deal of information is constantly generated in this field, allowing a better understanding of the molecular and biochemical aspects of these reactions in fish. Rainbow trout, channel catfish and killifish are among the fish species in which more reports have been made in this field. However, due to promising perspectives in aquaculture, species such as tilapia, striped bass, hybrid striped bass, etc. are being reported more frequently in this regard. This review was intended to serve as a good discussion and comparison tool for the following research chapters that deal with biotransformation reactions in 8 different finfish species of importance in aquaculture in the U.S. and the world.

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# Chapter II

## Kinetics of phase I and II biotransformation reactions in aquacultured finfish

**Abstract**

Microsomes and cytosolic fraction from livers of market-size specimens (n = 3 – 8) of channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), tilapia (*Oreochromis sp*.), largemouth bass (*Micropterus salmoides*), striped bass (*Morone saxatilis*), hybrid striped bass (*Morone saxatilis* x *Morone chrysops*), and bluegill (*Lepomis machrochirus*) were used to study the kinetics of phase I (ECOD, EROD, PROD, BROD) and phase II (UDP-glucuronosyltransferase (UDPGT)-, sulfotransferase (ST)- and glutathione-S-transferase (GST)- mediated) biotransformation reactions. Maximal velocity ($V_{max}$), binding affinity ($K_m$) and catalytic efficiency ($V_{max}/K_m$) were used as endpoints for comparison. The highest catalytic efficiency for ECOD activity was performed by channel catfish and Atlantic salmon, followed by rainbow trout and tilapia. The lowest ECOD enzymatic efficiency was achieved by hybrid striped bass microsomes. EROD kinetics showed that Atlantic salmon had a higher catalytic efficiency than rainbow trout, channel catfish, tilapia and largemouth bass. Hybrid striped bass, striped bass and bluegill had negligible EROD activity when a 1 uM substrate concentration was tested for these species, indicating the lowest constitutive EROD activity of all the species used in this study. For both ECOD and EROD kinetics, maximum velocities were within the range of values that have previously been reported in non-induced fish suggesting that fish species used in this study had a baseline profile. None of the fish species showed either PROD or BROD activities when a 5 uM substrate concentration was tested for both alkoxycoumarins. As for phase II biotransformation reactions, the highest GST catalytic efficiency was performed again by channel catfish, tilapia, rainbow trout and Atlantic salmon. These values were significantly different from the lowest $V_{max}/K_m$ ratios showed by bluegill,

largemouth bass, striped bass and hybrid striped bass. UDPGT and ST catalytic efficiencies showed more similarities among all the species than the other phase I and phase II reactions. Rainbow trout presented the highest catalytic UDPGT efficiency of all but it was quite similar to the ones presented by tilapia, channel catfish, Atlantic salmon, largemouth bass and bluegill. Striped bass and its hybrid had the lowest UDPGT catalytic efficiencies of all. ST-mediated conjugation had no statistically significant differences when its catalytic efficiency was compared among the eight species. In summary, this study indicated some differences among fish species in terms of biotransformation reactions. Tilapia, channel catfish, Atlantic salmon and rainbow trout had the highest biotranforming capabilities when some phase I and II probes were tested. Striped bass, hybrid striped bass and bluegill were low metabolizers of the same probes and largemouth bass shared some capabilities with the first group and some low metabolic capabilities with the latter group.

**Keywords**: biotransformation, phase I-II reactions, aquacultured fish

**Background**

Disease outbreaks, associated with serious economic losses, are common in the aquaculture industry, particularly in intensive culture systems (Georgiadis et al., 2001). However, very few therapeutic drugs are currently approved for use in aquacultured species in the U.S.. The approval process of drugs is time-consuming and quite expensive. The US FDA estimates that between 20 and 40 million US dollars are needed for granting approvals for one drug in one species (FDA, 2006).

One alternative that has been suggested by the FDA and other research groups (Gingerich et al., 1998) is to speed up this process by testing whether or not fish species may be grouped based on similarities of drug metabolic profiles, residue depletion patterns in edible tissues, and other factors related with drug usage. If feasible, the *crop grouping* hypothesis could suggest the use of surrogate species as good representatives of specific groups of fish. Therefore, crop grouping could circumvent prolonged and costly investigations on each species for approval purposes. However, before any sanction could be made it in this regard, a body of sound, scientifically-supported information is needed.

*In vitro* metabolism of drugs in fish is required to provide scientific evidence linking similarities between therapeutics in piscine species. The purpose of this study was to compare the kinetics of specific phase I-II biotranformation reactions among 8 different finfish species of importance in aquaculture: channel catfish **(***Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*), tilapia (*Oreochromis sp*.), largemouth bass (*Micropterus salmoides*), striped bass (*Morone saxatilis*), hybrid striped bass (*Morone saxatilis* x *Morone chrysops*), and

bluegill (*Lepomis machrochirus*). These biotransformation profiles may help to better understand capabilities, similarities and differences of aquacultured fish species to metabolize drugs and other xenobiotics.

The model substrates 7-ethoxycoumarin (7-EC), 7-ethoxyresorufin (7-ER), 7-pentoxyresorufin (7-PR), 7-benzyloxyresorufin (7-BR), resorufin and 1-chloro-2,4-dinitrobenzene (CDNB) were used to investigate the kinetics of phase I and II reactions in fish livers that were processed to obtain the microsomal and cytosolic fractions enriched with the enzymatic systems that perform these reactions.

**Materials and methods**

Fish handling

Healthy, market-size specimens (n=8) of 8 fish species were obtained from commercial farms. After capture, the fish were transported to the Aquatic Pathobiology Laboratory at the University of Maryland (College Park) where they were sacrificed upon arrival by cervical transection. The species and sex distribution (male: female) used for this study were channel catfish (*Ictalurus punctatus*) (7m:1f) , rainbow trout (*Oncorhynchus mykiss*)( 8m), Atlantic salmon (*Salmo salar*) (4m:4f), tilapia (*Oreochromis sp.*)(8m), largemouth bass (*Micropterus salmoides*)(4m:4f), striped bass (*Morone saxatilis*)(8m), hybrid striped bass (*Morone saxatilis* x *Morone chrysops*)(2m:6f), and bluegill (*Lepomis machrochirus*)(4m:4f). Atlantic salmon individuals were acclimated at the Center for Veterinary Medicine (FDA - Laurel, MD) for several months before the tissue was harvested.

Microsomes and cytosolic fraction preparation

Microsomes and cytosol were prepared according to Vodicnik et al. (1981). Livers were weighed individually and kept on ice-cold KCl buffer (1.15%) (J.T. Baker – 3040-01). Livers were minced coarsely with scissors and rinsed as many times as needed until looking clear without blood residues. After discarding the last KCl rinsing, 4 volumes of ice-cold 0.25 M sucrose (ICN Biomedicals – 821271) were added (e.g. 1 g liver = 4 ml 0.25 M sucrose). The minced livers in sucrose were transferred to a pre-chilled glass homogenizer and homogenized by 6 full strokes. The homogenizer was kept on ice during this process. The sample was then transferred to an ice-cold, high-speed centrifuge tube and spun at 8,000 x g for 20 minutes at 4$^{o}$C (Biofuge 22 R – Heraeus Instruments). The supernatant was spun at 100,000 x g for 60 minutes at 4$^{o}$C (Beckman Ultracentrifuge XL-80). The tubes were removed to ice and the supernatant (cytosolic fraction) was aliquoted into cryotubes. The microsomal pellets were resuspended with 1 ml of SET buffer (pH=7.4) per gram of wet liver with a spatula. The resuspended microsomal fraction was transferred to a glass homogenizer and homogenized with 6 full strokes followed by aliquoting into cryotubes. Both cytosolic and microsomal fraction cryotubes were stored at –80$^{o}$C until performing the assays. Protein was measured using the BCA protein assay kit (Pierce - 23227) based on the colorimetric reaction with bicinchoninic acid.

The frozen microsomal and cytosolic preparations to be used for the phase I and II assays were all used within 3 months after processing. This was planned to prevent potential enzymatic degradation due to different storage time before conducting the *in vitro* assays. Nevertheless, a set of rainbow trout (n=6) microsomal and cytosolic

preparations were tested for glutathione-S-tranferase (GST) and ethoxyresorufin-*O*-deethylation (EROD) activities over a six-month period to evaluate and report possible changes in enzymatic activity associated with storage at -80˚C.

Phase I assays

Ethoxycoumarin-*O*-dealkylation (ECOD) kinetics (Modified from Haasch et al., 1994 and Schlenk et al., 2000). Reaction mixtures consisted of 50 uL of buffer Tris-HCl (100 mM, pH=7.4), 25 uL of microsomal fraction accounting for 100 ug of protein, 10 uL of 7-ethoxycoumarin (7-EC) (Sigma E1379) (20-100 uM range) and 25 uL of NADPH tetrasodium salt (0.1 mM within reaction mixture) (Calbiochem® 481973). Blanks consisted of reaction mixture with boiled microsomes. The rate of ECOD activity was monitored after 10 minutes of reaction (linearity of the reaction rate was confirmed in preliminary experiments) by reading the relative fluorescence units (RFU) of the 7-hydroxycoumarin (7-HC) produced by the reaction (excitacion = 380 nm, emission = 460 nm) in a microplate absorbance-fluorescence reader (TECAN, Genios ™, Austria). A 7-HC (Sigma U7626) calibration curve was used for the quantification of the reaction rate.

Ethoxyresorufin-*O*-deethylation (EROD) kinetics (modified from Eggens and Galgani, 1992 and Haasch et al., 1994). Reaction mixtures consisted of 50 uL of buffer Tris-HCl (100 mM, pH=7.4), 25 uL of microsomal fraction accounting for 100 µg of protein, 10 µL of 7-ethoyxresorufin (7-ER) (Sigma E3763) (range 0-2 µM) and 25 µL of NADPH (1 mM, tetrasodium salt) (Calbiochem® 481973). Blanks consisted of reaction mixtures with boiled microsomes. The rate of EROD activity was

monitored after 10 minutes of reaction (linearity of the reaction rate was confirmed in preliminary experiments) by reading the relative fluorescence units (RFU) of the resorufin produced by the reaction (excitation = 510 nm, emission = 580 nm) in a microplate absorbance-fluorescence reader (TECAN, Genios $^{TM}$, Austria). A resorufin calibration curve (0 - 0.5 uM) was used for the quantification of the reaction rate.

Pentoxyresorufin- (PROD) and benzyloxyresorufin-*O*-dealkylation (BROD) activities. PROD and BROD activities were assayed following the same protocol as for EROD. A 5 uM substrate concentration was tested as the initial concentration point. Phenobarbital-induced rat microsomes (R1078 – Xenotech, LLC) were used as a positive control to compare such activities.

Phase II assays

Glutathione-S-transferase kinetics. Glutathione-S-transferase (GST) activity was determined by the method of Habig and collaborators (1974). Reaction mixtures consisted of 165 uL of 100 mM Tris-HCl buffer (pH=7.4), 7 uL of 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma C 6396) (0.1-2.0 mM range), 3.5 uL of 60 mM L-glutathione reduced form (Sigma G 6529) and 10 ug of cytosolic protein. Blanks consisted of reaction mixtures with exception of the cytosolic fraction. The rate of CDNB conjugation with GSH was monitored after pipetting the cytosolic fraction to the reaction mixture and determining changes in absorbance ($\lambda$ = 340 nm) after 5 minutes at room temperature. Absorbance readings were obtained using a microplate fluorescence-absorbance reader (TECAN, Genios $^{TM}$, Austria). For final calculations a molar absorption coefficient ($\epsilon$=9.6 $mM^{-1}cm^{-1}$) was used adjusting the path length to

the corresponding 96-well plate volume (Styrene microtiter® S25-291-01, ThermoLabsystems, MA).

UDP-glucuronosyltransferase kinetics. UDP-glucuronosyltransferase (UDPGT) activity was measured by a method modified from Schell and James (1989). Reaction mixtures consisted of 50 uL of phosphate buffer (pH= 7.4) that contained Triton X-100 (Sigma X-100) at 0.1 % and $MgCl_2$ (Sigma M-0250) at 10 mM, 100 ug of microsomal protein, 25 uL of uridin 5'-diphosphoglucuronic acid trisodium salt (UDPGA) (Sigma U-6751) and 10 uL of resorufin (Aldrich 23,015-4) (1-50 uM range). Blanks consisted of the whole reaction mixture except for the UDPGA. 96-well microplates (Styrene microtiter® S25-291-01, ThermoLabsystems, MA) were used to hold the reaction components. After mixing the buffer mixture containing Triton and $MgCl_2$ with the microsomes and the UDPGA, the microplates were left at $4^oC$ during 20 minutes. 10 uL of each resorufin concentration were added to blanks and samples and a RFU reading was done at this point (time 0 of reaction). A second reading was performed after 10 minutes to estimate the reaction rate based on the difference between RFU in the blanks and the samples. Preliminary experiments showed the linearity of the reaction during the first 20 minutes. Samples were processed in triplicate. RFU values in the samples were expected to be lower than those in the blanks due to the UDPGA-mediated conjugation of resorufin.

Sulfotransferase kinetics. Sulfotransferase (ST) activity was measured by a method modified from Schell & James (1989) and Beckmann (1991). Reaction mixtures consisted of 50 uL of phosphate buffer (pH= 7.4), 100 ug of cytosolic protein, 10 uL of resorufin (Aldrich 23,015-4) (0.5-20 uM range) and 25 uL of adenosine 3'-

phosphate 5'-phosphosulfate lithium salt (PAPS) (Sigma A-1651). Blanks consisted of the whole reaction mixture except for the PAPS. 96-well microplates (Styrene microtiter® S25-291-01, ThermoLabsystems, MA) were used to hold the reaction components. After mixing the buffer and the cytosol, 10 uL of each resorufin concentration were added to blanks and samples and a RFU reading was done at this point (time 0 of reaction). 25 uL of PAPS were pipetted into the sample wells to start the reaction (25 uL of $DDH_20$ were pipetted into the blanks). A second reading was performed after 10 minutes to estimate the reaction rate based on the difference between RFU in the blanks and the samples. Preliminary experiments showed the linearity of the reaction during at least the first 10 minutes. Samples were processed in triplicate. RFU values in the samples were expected to be lower than those in the blanks due to the SULT-mediated conjugation of resorufin.

Calculation of kinetics parameters

Results on velocity rates for all of the phase I-II enzymes were graphed as Michaelis-Menten (velocity vs. [substrate]) and Lineweaver-Burk (1/velocity vs 1/[substrate]) plots. The kinetics parameters maximal velocity ($V_{max}$), Michaelis-Menten constant ($K_m$) and $V_{max}/K_m$ ratio were derived by regression of data from the double-reciprocal plots (Piszkiewicz, 1977; Cornish-Bowden, 1995).

Statistical analysis

Results for $V_{max}$, $K_m$ and $V_{max}/K_m$ kinetics parameters are reported as means +/- standard errors. Data sets were analyzed for normality (Shapiro-Wilcoxon test) and homogeneity of variances (Barlett's test). When necessary, log transformations were

calculated to comply with parametric statistical assumptions. Data being both normal and homogeneous were compared using one-way ANOVA test (comparison among species) followed by Tukey's mean separation test. SAS (Statistical Analysis Software) program was used for the data processing. Statistical significance was set at a significance level of $\alpha = 0.05$.

**Results**

Phase I assays

*ECOD and EROD kinetics*. Results for $V_{max}$ and $K_m$ values of ECOD and EROD kinetics are presented in Table 1 and 2, respectively. $V_{max}/K_m$ ratios are depicted in Figure 1 and 2. Michaelis-Menten and Lineweaver plots for each species are shown in Appendix 1 and 2.

Channel catfish had the highest ECOD $V_{max}$ of all the species followed by Atlantic salmon, rainbow trout, hybrid striped bass and tilapia. This value was significantly different ($p < 0.05$) from all the others. $K_m$s were quite similar among Atlantic salmon, rainbow trout, channel catfish and tilapia. Hybrid striped bass $K_m$ was the highest value of all, being significantly different from the others ($p < 0.05$). $V_{max}/K_m$ ratios indicated that as far as ECOD activity is concerned, channel catfish and Atlantic salmon were the most efficient species of all. Rainbow trout and tilapia had lower $V_{max}/K_m$ ratios and hybrid striped bass had the lowest $V_{max}/K_m$ ratio showing the lowest ECOD efficiency of all the tested species. Bluegill hepatic microsomes did not present any response to 7-EC despite the different substrate concentrations that were

tested. ECOD kinetics in striped bass and largemouth bass were not performed due to insufficient microsomal sample.

EROD kinetics parameters are shown for five of the eight species examined in this study. Striped bass, hybrid striped bass and bluegill had very low velocity values (Table 2) when 1uM substrate concentracion (e.g 7-ER) was tested. Higher and lower substrate concentration values did not reveal changes in the velocity rate for this probe. As a result, Table 2 shows velocities at only 1 uM substrate concentration for these 3 species. Atlantic salmon and tilapia were the species with the highest $V_{max}$ of all, followed by channel catfish, rainbow trout and largemouth bass. As far as $K_m$ is concerned, channel catfish and tilapia had the lowest values, showing statistically significant difference ($p < 0.05$) from those obtained in largemouth bass, rainbow trout and Atlantic salmon. The highest $V_{max}/K_m$ ratio of this reaction was obtained by Atlantic salmon. This value was significantly different ($p < 0.05$) from the ones in tilapia, rainbow trout, largemouth bass and channel catfish.

*PROD and BROD kinetics*. None of the fish species showed either PROD or BROD activities when a 5 uM substrate concentration was tested for both alkoxycoumarins. Phenobarbial-induced rat microsomes that were used as positive controls, showed both PROD (23 pmols/ min / mg protein) and BROD (183 pmols / min / mg protein) activities.

Phase II assays

*Glutathione-S-transferase kinectics*. Results for $V_{max}$ , $K_m$ and $V_{max}$ / $K_m$ ratios of glutathione-S-transferase (GST) kinetics are summarized in Table 3 and Figure 3. Michaelis-Menten and Lineweaver plots for each species are shown in Appendix 3.

The highest GST capacity in terms of maximum velocity ($V_{max}$ ) of the reaction was obtained for tilapia and Atlantic salmon, followed by rainbow trout, channel catfish and largemouth bass. The lowest and significantly different $V_{max}$ values ($p < 0.05$) were found in striped bass, hybrid striped bass and bluegill. As far as enzyme affinity for the substrate, channel catfish and striped bass showed the lowest values (e.g. higher affinities) followed by bluegill, tilapia, and hybrid striped bass. The highest $K_m$ values (e.g. lowest GST affinities) were found in largemouth bass, rainbow trout and Atlantic salmon. $V_{max}$ / $K_m$ ratios indicate that channel catfish and tilapia were the species that had the most efficient GST activity towards the substrate. Atlantic salmon and rainbow trout had lower ratios while striped bass, largemouth bass, bluegill and hybrid striped bass had the lowest GST efficiencies of all.

*UDP-glucuronosyltransferase kinetics*. Results for $V_{max}$ , $K_m$ and $V_{max}$ / $K_m$ ratios of UDP-glucuronosyltransferase (UDPGT) kinetics are summarized in Table 4 and Figure 4. Compiled Michaelis-Menten and Lineweaver plots for all the animals that were tested in each species are presented in Appendix 4.

Rainbow trout and Atlantic salmon had the highest UDPGT $V_{max}$ values of all species examined in the present work. On the other hand, channel catfish microsomes did not

have a UDPGT-based conjugation activity towards resorufin. $K_m$ values were quite similar among the 8 species tested and no statistically significant differences were found among them. As for $V_{max}$ / $K_m$ ratios, rainbow trout and Atlantic salmon had once again the highest values indicating the highest UDPGT efficiencies of all species. Bluegill, tilapia and largemouth bass had lower ratios than Atlantic salmon and rainbow trout. Striped bass and its hybrid had the lowest efficiency ratios for this reaction.

*Sulfotransferase kinetics*. Results for $V_{max}$ , $K_m$ and $V_{max}$ / $K_m$ ratios of sulfotransferase (ST) kinetics are summarized in Table 5 and Figure 5. Compiled Michaelis-Menten and Lineweaver plots for all the animals that were tested in each species are presented in Appendix 5.

Tilapia and channel catfish had the highest conjugation velocities followed by Atlantic salmon, rainbow trout and largemouth bass. Bluegill, hybrid striped bass and striped bass had again the lowest $V_{max}$ value of all the species. $K_m$s were very similar among all the species with the exception of striped bass which had the lowest values of all. $V_{max}/K_m$ ratios for ST activity indicated that Atlantic salmon had the best enzymatic efficiency followed by rainbow trout, channel catfish, tilapia, striped bass and largemouth bass. Bluegill and hybrid striped bass had the lowest ratios of all. $V_{max}$ values for ST activity in all the species were significantly lower than those found in UDPGT-based conjugation of resorufin which indicates lower capacity for the conjugation of the substrate. On the other hand, $K_m$ values showed that ST had higher affinity for the substrate as compared to the ones found in the UDPGT-based conjugation.

A summary of the catalytic efficiencies ($V_{max}/K_m$) for all of the phase I and II enzymatic reactions and the 8 species can be seen in Figure 6.

*Changes in EROD and GST activities due to storage*. The evaluation of changes for the 6 months storage time for EROD and GST activities showed that for the former reaction, the samples that were processed after 6 months had a significant lower activity (18.0 ± 2.1 pmols resorufin / minute / mg protein) than those processed immediately after the microsomal harvesting (30.3 ± 4.8). The samples that were tested 3 months after the microsomal harvesting had quite similar results (29.7 ± 4.0) to the one assayed immediately after the microsomes were obtained from the specimens. As for the GST activity (nmoles CDNB / minute / mg protein), no significant changes were obtained in either of the time points: After harvesting = 539 ± 27; 3 months = 596 ± 25; and 6 months = 534 ± 16. These average values were obtained after performing the assays with microsomes and cytosols from 6 different rainbow trout specimens (Figures 7 and 8).

**Discussion**

The present study was intended to compare the kinetics of phase I-II biotransformation reactions among eight different fish species assuming as the null hypothesis that no significant changes were present in the kinetics of the biotransformation reactions among them. Maximum velocity ($V_{max}$), Michaelis-Menten constant ($K_m$) and $V_{max}/K_m$ ratio, an indicator of enzymatic efficiency, were taken into account for such comparisons. Comparisons between our results and other studies was relatively difficult in some cases given that most of the investigations

have dealt with catalytic activities at saturating conditions of substrates instead of full kinetics of the reactions working a wider range of substrate concentrations.

Phase I reactions

*ECOD and EROD kinetics*. ECOD has been studied in mammals as a marker activity of CYP1A1, CYP1A2 CYP2B1, CYP2E1, and CYP2E6 (Ryan & Levin, 1990; Yun et al., 1991; Yamazaki et al., 1996). In fish, the catalysts of 7-EC are still unknown; nevertheless, this reaction is apparently catalyzed in rainbow trout by the CYP1A forms since treatment by CYP1A inducers enhances the formation of 7-hydroxycoumarin (7-HC) (Cravedi et al., 1998). Among the inducers of ECOD activity in fish species are Aroclor 1254 (a PCBs mixture) (Elcombe & Lech, 1979) and β-naphthoflavone (Haasch et al., 1994) in rainbow trout; 3-methylcholanthrene and polychlorinated biphenyls in tilapia (*niloticus x aureus*) (Ueng et al., 1992; Ueng & Ueng, 1995) and phenobarbital (Ueng et al., 1992) as well as PAHs and PCBs in carp (Machala et al., 1997). Most of these compounds have also been found to be inducers of EROD activity. On the other hand, there is substantial ECOD activity in non-CYP1A induced catfish, suggesting and additional constitutive role although the isozyme responsible for this activity has not been identified (Perkins, 1999; Perkins et al., 2000).

In the present study, channel catfish had the highest ECOD velocity of all species as well as the maximum catalytic efficiency. This velocity doubled the ones found for Atlantic salmon, rainbow trout, tilapia, and hybrid striped bass. Despite the statistically significant differences that were found among these 5 species with regard

to kinetics parameters ($V_{max}$ and $V_{max}/K_m$ ratio, particularly), the values were quite similar to those that have been found in non-induced fish in other studies. $V_{max}$ found in channel catfish (108.0 ± 6.9 pmols 7-HC/min/mg protein) was quite similar to the value found by Perkins (1999) (109.0 ± 3.0 pmols 7-HC/min/mg protein). ECOD activity for rainbow trout (42.1 pmols / min / mg protein) was lower than the one reported by Kleinow et al. (1990) in the same species (80 pmols/ min/mg protein). The $V_{max}$ values found for tilapia (32.0 ± 0.9 pmols 7-HC/min/mg protein) is below the ones reported in other studies. Ueng et al. (1992) and Ueng & Ueng (1995) found 143.0 ± 27.0 and 311.0 ± 38.0 pmols 7-HC/min/mg protein for ECOD activity in non-induced tilapias weighing 200-300 grams. These values are between 2 and 5-fold higher than the corresponding ones in the present work. These investigators performed ECOD assays at 37$^{o}$C while our incubations were performed at room temperature (20-21$^{o}$C). This factor may partially explain such differences between the studies. Despite these differences, ECOD activity in tilapia was considerably lower than the values obtained by other authors in 3-methylcholantrene-induced tilapias (433 ± 84 and 890 ± 65), and Aroclor 1254-induced tilapias (351 ± 49 and 543 ± 41) (Ueng et al., 1992; Ueng & Ueng, 1995; respectively). Reports for ECOD activities in other fish species different from rainbow trout, tilapia and channel catfish are scarce. This makes difficult to compare the results found in the present work with other investigations.

EROD activity has been studied in over 150 fish species covering more than 10 taxonomic families. This activity is mainly examined to determine its suitability as a biomarker of pollution. However, basal activities may vary considerably both among and within species (Whyte et al., 2000). For example, EROD in common carp

(*Cyprinus carp*io) has been examined in non-induced fish showing great variability in the basal activity: 4,600 pmols/min/mg protein (Ahokas et al., 1994), 600 pmols/min/mg protein (Curtis et al., 1993), and 15-85 pmols/min/mg protein (Machala et al., 1997). Other species such as mummichog (*Fundulus heteroclitus*) seems to have consistently high basal EROD activity which makes more difficult to determine a likely induction exerted by xenobiotics (Whyte et al., 2000).

In the present study, EROD $V_{max}$ values were quite similar among the species. Despite statistically significant differences among some of them, all values were below 100 pmols/min/mg protein. Hybrid striped bass, striped bass and bluegill showed the lowest velocities of all species when 1uM substrate was tested. No further activity was detected when higher substrate concentrations were examined in these three species.

Changes in phase I biotransformation reactions profiles due to the usage of drugs in aquacultured species have been reported. Interestingly, both ECOD (78 to 85%) and EROD (85 to 86 %) activities were significantly inhibited by enrofloxacin, a fluoroquinole antibiotic, after *in vivo* treatment in sea bass (*Dicentrarchus labrax*) (Vaccaro et al., 2003). Our research also found (chapter 4), the inducing effects on EROD activity (2.2 – 2.6 fold) due to *in vivo* treatment of channel catfish with albendazole at therapeutic dosage. This suggests that knowing baseline alkoxyresorufins and alkoxycoumarins activities in fish species may help to predict effects due to usage of drugs in aquacultured finfish species.

Bluegill specimens in our study showed that liver tissue contained notable amounts of encysted digenean trematodes. This type of parasite commonly infects in wild fish (Noga, 2000). Effects on phase I reactions due to bacterial pathogens, their immunostimulants (e.g. lipopoysacharides, LPS) and inflammatory cytokines in carp (*Cyprinus carpio*) have been cited in recent reports (Marionnet et al., 2006). Although this infestation was not related to bacterial pathogens, it is worth considering likely effects on phase I and phase II reactions due to this parasitic load. As it is cited in the following sections, phase II reactions were also reduced in bluegill as compared to other species.

*PROD and BROD kinetics*. The present results indicate that none of the fish species used in this study was reactive to either of these two alkoxyphenoxazones. Previous studies (Haasch et al., 1994) reported that sham-injected rainbow trout had no PROD activity and negligible BROD activity (1.1 pmols resorufin/min/mg protein). Despite the fact that no P450 isoform(s) has been identified in fish as responsible for PROD and/or BROD activities, inducing effects such as the ones exerted by isosafrole, β-NF and dexametasone in rainbow trout (Haasch et al., 1994) may help to differentiate between the expected baseline values, such as the ones found in our study, and a possible induction after exposure to contaminants or drugs.

Phase II reactions

*Glutathion-S-transferase kinetics*. GST activity is an indicator of conjugation of electrophilic compounds with glutathione (GSH), a cytosolic tripeptide that reaches milimolar concentrations within the cell. These electrophilic compounds that are

conjugated by GSH may exert adverse changes on macromolecules (e.g. proteins, lipids) within the cell structure. GSTs are an important part of the oxidative stress defense system playing a significant role in the biotransformation system (Stephensen et al., 2002). In the present study, GST kinetics was evaluated using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. This probe reflects the integration of different GSTs isozymes activities allowing general comparisons among species. All the fish species examined so far have shown activity towards CDNB as a model substrate (George, 1994).

Maximum velocity capacities ($V_{max}$) for CDNB conjugation were significantly different among the species (Table 3). The conjugating velocity varied in the following order: tilapia, Atlantic salmon > rainbow trout > channel catfish, largemouth bass > striped bass, hybrid striped bass, and bluegill. $K_m$s (binding affinities) were also significantly different among the species. This defined significant differences in catalytic efficiency ($V_{max}/K_m$) ratios. Interestingly, species such as Atlantic salmon that had one of the highest $V_{max}$ of all showed the highest $K_m$, too. On the other hand, channel catfish showed the lowest $K_m$ of all, which determined the highest catalytic efficiency of all the species as far as CDNB conjugation is concerned. Catalytic efficiencies ratios for CDNB conjugation were as follows: channel catfish, tilapia > rainbow trout , Atlantic salmon > largemouth bass, striped bass, hybrid striped bass, and bluegill (Figure 3).

Channel catfish and tilapia are among the species with the highest GST-mediated conjugation of CDNB. In channel catfish, several investigations have reported high

CDNB conjugation velocities ranging from 1325 (Gallagher et al., 1996) to 1500 nmols/min/mg protein (Gallagher et al., 1992). Ankley & Agosin (1987) reported 827 nmols CDNB/min/mg protein in channel catfish. These values are higher than the maximum velocities found in the present study (657 ± 39 nmols/min/mg protein). Incubations in the studies by Gallagher and collaborators were done at 30$^{o}$C while ours were performed at room temperature (20 $^{o}$C - 21$^{o}$C). However, other investigations that we performed in laboratory-acclimated channel catfish (chapter 3) showed a significantly higher CDNB conjugation (1972 nmols/min/mg protein) than the farm-raised specimens used in the present study. Both groups of fish were from the same source. Further discussion about differences in enzyme kinetics of farm-raised and laboratory-acclimated fish is presented in chapter 3.

Pathiratne and George (1996) reported high GST activity in tilapia (*Oreochromis niloticus*) (1900 ± 120 nmols/min/mg protein) using CDNB as a substrate. In our study, tilapia had the highest GST $V_{max}$ of all the species (1508 ± 70 nmols/min/mg protein) and the second highest catalytic efficiency ($V_{max}/K_m$) after channel catfish. GST activity has been considered of great importance in detoxification of electrophilic xenobiotics (Shailaja & D'Silva, 2003). Some authors have reported that a particular resistance to the exposure of carcinogenic compounds such as PAH's, PCB's, and other environmental hepatocarcinogens in channel catfish may result from the notable GST activity found in this species (Gallagher et al., 1996). Tilapia has also been reported as a good bioindicator of exposure to electrophilic compounds based on its GST–based conjugation responses (Gadagbui et al., 1996; Shailaja & D'Silva, 2003).

Rainbow trout, largemouth bass, Atlantic salmon and bluegill are also among the fish species in which CDNB conjugation through GST activity has been investigated. Generally, reports on these species indicate lower conjugating activities as compared to the ones found in channel catfish and tilapia. In rainbow trout, these reports have shown activity in a relatively wide range: 700 ± 80 nmols/min/mg protein (Pérez-López et al., 2000), 348 ± 17 nmols/min/mg protein (Laurén, 1989), and 183 ± 17 nmols/min/mg protein (Otto & Moon, 1996). Our study showed a $V_{max}$ value of 929 ± 65 (nmols/min/mg potein) which is higher than the one found by Pérez-López and collaborators. These studies worked with saturating conditions for the substrate and did not examine a range of substrate concentrations to determine kinetics parameters. Largemouth bass showed in the present study a $V_{max}$ value of 589 ± 52 nmols/min/mg protein which is lower than the one reported by Gallagher and collaborators (2000) in a full kinetic study (843 – 1154 nmols/min/mg protein). GST activity has been of particular interest in this species given the high risk of exposure to pollutants that are detoxified through GSTs and its higher order predatory habits (Gallagher et al., 2000; Pham et al., 2002). Largemouth bass showed in the study by Pham and collaborators (2002) one of the highest, GST-mediated 4-hydroxynonenal (4HNE) conjugation which was a good indicator of protection against peroxidation of polyunsaturated fatty acids in the liver. This activity was significantly higher than the ones reported in other mammalian and aquatic species. Despite the significant conjugation of 4HNE via GST activity, largemouth bass had in the present study a lower capacity to conjugate CDNB than tilapia, channel catfish, and rainbow trout. As far as Atlantic salmon is concerned, a report in immature (~ 60 g in weigh) juveniles found a CDNB conjugating activity of 290 ± 8 nmols/min/mg protein (Nóvoa-Valiñas et al., 2002). CDNB conjugation kinetics was studied in our research using Atlantic salmon ranging

from 492 to 1000 g. Maximum velocity in Atlantic salmon was one of the highest of all the species (1349 ± 107 nmols/min/mg potein) but so was enzyme affinity (0.5 ± 0.10 mM), yielding a lower catalytic efficiency (2816 ± 329) than channel catfish and tilapia. This value was not significantly different from the one found in rainbow trout (2260 ± 200), the other salmonid used in our research.

*UDP-glucuronosyltransferase kinetics*. Glucuronidation is one of the major phase II pathways in fish and other species. Both endogenous (e.g., bilirubin, steroids, thyroid hormones, etc.) and exogenous compounds (e.g., PCBs, pesticides, drugs, etc.) are conjugated with glucuronic acid via UDP-glucuronosyltransferases (UDPGTs) (EC 2.4.1.17) (George, 1994). Among the drugs and pesticides that are conjugated via UDPGTs are chloramphenicol (Craverdi et al., 1985), oxalinic acid (Ueno et al., 1985), pyrethroids (Glickman et al., 1981), organophosphates (Takimoto et al., 1987), carbamates (Statham et al., 1975), and phenols (Lech, 1973; Kane et al., 1994). Aflatoxicol B1 glucuronide, one of the metabolites of aflatoxin B1, is also formed in rainbow trout exposed to this hepatocarcinogen (Loveland et al., 1984). Other important environmental contaminants that are excreted as glucuronic acid conjugates are PAHs (Varanassi et al., 1984), phthalate ester plasticisers (Stalling et al., 1973) and chlorophenols (Oikari et al., 1988).

Standardized *in vitro* assays to determine UDPGT activity utilize different types of substrates. Many of the reports cite the use of phenol-type compounds like 4-nitrophenol (Winsness, 1969; Burchell and Coughtrie, 1989), and 3-trifluoromethyl-4-nitrophenol (TFM) (Lech and Statham, 1975; Kane at al., 1994). These phenol-type compounds have the ability to form conjugates that are quantified by colorimetric

reactions in the visible range of the spectrum (e.g. λ=395). Other type of substrates like 4-methylumbelliferone have also been used to detect UDPGT activity based on lower fluorescence emission once the substrate is conjugated (e.g. excitation λ = 323 nm; emission λ= 450 nm) (Schell and James, 1989). Resorufin, a metabolite obtained after the phase I-mediated biotransformation of 7-ethoxyresorufin, was used in the present study as substrate to examine both UDPGT and ST kinetics.

Kinetics of UDPGT activity in the present study showed lower variability among the species as compared to the phase I reactions and the GST-mediated conjugation. After combining $V_{max}$ and $K_m$ in a single parameter ($V_{max}/K_m$), the results indicate that with the exception of striped bass, largemouth bass and hybrid striped bass, the other species had quite similar results. The fact that 7-ethoxyresorufin has not been used extensively as catalytic probe to evaluate glucuronidation reactions makes difficult the comparison with other studies.

In the past, differences in UDPGT–based conjugation have helped to understand distinct sensitivities among fish species to pollutants or drugs that may be acting upon fish species. Kane et al. (1994) found that the lower $V_{max}/K_m$ ratio that showed sea lamprey (*Petromyzon marinus*) as compared to channel catfish, rainbow trout and bluegill helped to explain a high sensitivity to 3-trifluoromethyl-4-nitrophenol (TFM), due to lower UDPGT-based conjugation for further excretion of this lampricide metabolites.

*Sulfotransferase kinetics.* Sulfation is a secondary phase II reaction that conjugates most of the substrates that are conjugated by UDPGTs as well. This reaction is

catalyzed by sulfotransferases (ST) (EC 2.8.2.2, EC 2.8.2.4), a group of cytosolic enzymes that have lower capacity than UDPGTs but a higher affinity for the substrates (Parkinson, 2001). Some studies have identified ST activity towards thyroid hormones in plaice (Osborn and Simpson, 1969) and rainbow trout (Finnson and Eales, 1998). ST-mediated conjugation of thyroid hormones in rats is preferential toward active triiodothyronine ($T_3$), followed by inactive triiodothyronine ($rT_3$) and thyroxine ($T_4$). In contrast, trout have higher ST-mediated conjugation toward $rT_3$ (Finnson and Eales, 1998).

ST activities are reported in diverse fish species using waterborne phenol substrates. Among these species are goldfish (*Carassius auratus*), guppy (*Poecilia reticulata*), minnow (*Phoxinus phoxinus*), roach (*Rutilus rutilis*), carp (*Cyprinus carpio*) and tench (*Tinca tinca*). However, substrates that are usually utilized to evaluate ST activity in mammals (e.g., acetaminophen, 7-ethoxycoumarin, pentachlorophenol) are poorly metabolized in rainbow trout (George, 1994). Although ST-mediated conjugation has been considered non-inducible, induction of sulfotransferases in channel catfish after 3-MC treatment was reported by Gaworecki and collaborators (2004). The effects of environmental estrogens and other endocrine disrupters on ST-mediated conjugation have been studied lately showing additional implications of these pollutants on biotransformation capabilities (Nishiyama et al., 2002; Kirk et al., 2003; Ohkimoto et al., 2003).

ST-mediated conjugation in the present work corroborated the fact that sulfotransferases are considered lower capacity/higher affinity enzymes in comparison to glucuronosyltransferases. $V_{max}$ values for ST in all the species were several times lower than the corresponding values for UDPGT. On the other hand, the low $K_m$s

indicated the higher binding affinities of ST in comparison to the high values for UDPGT (lower affinities). This has been implicated in the role that each of these two biotransformation pathways play based on the substrate concentration that the enzymatic systems have to deal with within the cell. Interestingly, channel catfish microsomes were not reactive to 7-ethoxyresorufin for UDPGCT-based conjugation. However, cytosolic fractions of the same specimens responded to sulfotransferase conjugation.

ST-mediated conjugation of 7-ethoxyresorufin was one of the catalytic assays with the lowest variation among the species studied in this investigation. $V_{max}/K_m$ ratios did not show significant differences amongst them. However, $V_{max}$ values were significantly higher for rainbow trout, tilapia, channel catfish, Atlantic salmon and largemouth bass. Striped bass, hybrid striped bass and bluegill had the lowest ST biotransformation capacity. In the end, catalytic efficiencies were quite similar among the species due to higher binding affinities in the three species that had the lowest capacities ($V_{max}$).

**Conclusions**

Based on the catalytic probes that were used in this study to evaluate some phase I and II biotransformation reactions, the 8 fish species that were investigated exhibited differences among them. Channel catfish, tilapia, rainbow trout and Atlantic salmon had the maximum capacities and the highest catalytic efficiencies for most of these catalytic probes. Hybrid striped bass, striped bass and bluegill displayed the lowest

efficiencies. Largemouth bass was in between the two suggested groups, showing higher capabilities in some tests and lower in others.

In some cases, species that displayed low biotransformation capabilities (e.g. low $V_{max}$), had similar catalytic efficiencies than the ones with highest maximun velocities due to a higher binding affinity (e.g. low $K_m$) of their enzymatic systems. This was particularly evident for the UDPGT and ST reactions.

Major differences found in this study among the species with regard to GST-based biotransformation may help to understand their particular susceptibility or resistance to electrophilic compounds that exert deleterious effects on macromolecules within the cell. Channel catfish and tilapia exhibited the highest capacity and efficiency in GST-mediated conjugation. Other species (e.g. striped bass, hybrid striped bass, and bluegill) had very poor GST-based conjugation of CDNB.

Novel fluorescent catalytic probes that help to evaluate CYP3A phase I activity are needed to circumvent complicated and expensive assays that are regularly used (e.g. steroids biotransformation) to evaluate the activity of this CYP450 subfamily.This is crucial for a better understanding of drug metabolism in fish and other species.

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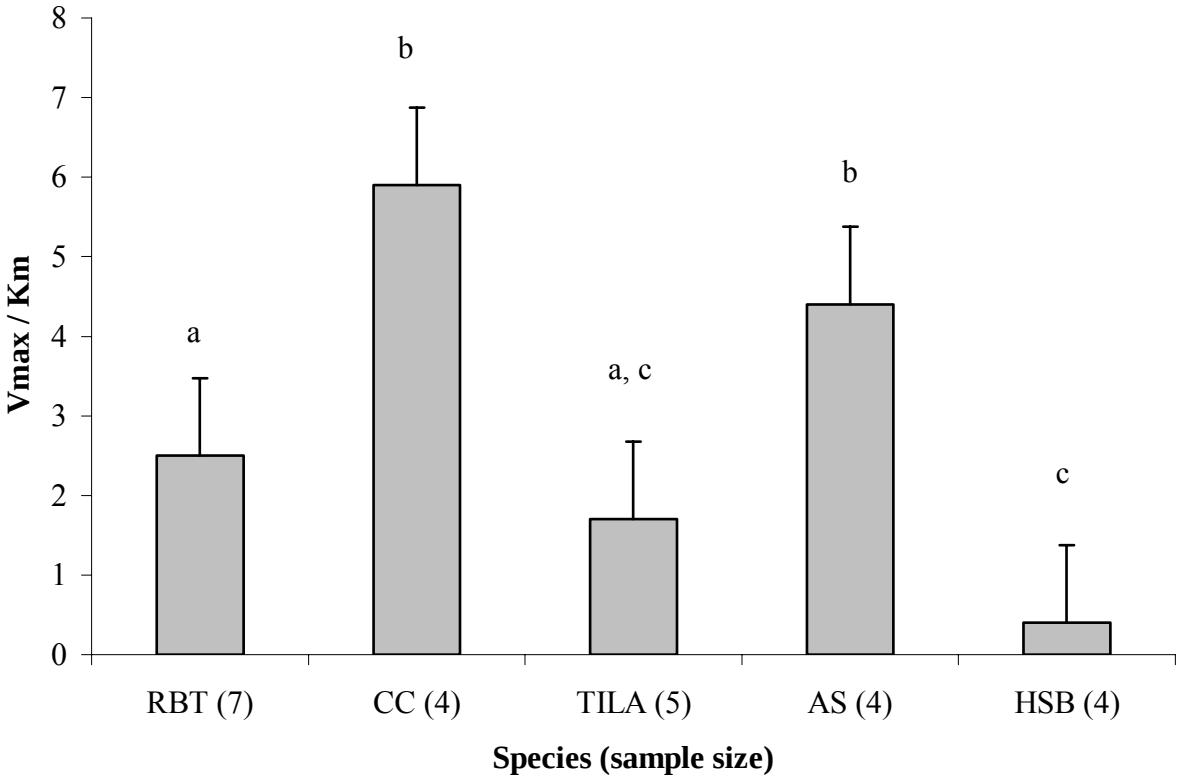
Table 1. $V_{max}$ and $K_m$ values (means ± SEM) for ECOD kinetics in farm-raised fish (Different letters in the same column denote statistically significant difference among the species, $p < 0.05$)

| **Species** (sample size) | **$V_{max}$** (pmols 7-hydroxycoumarin/min/mg protein) | **$K_m$** (μM) |
|---|---|---|
| Channel catfish (4) | 108.0 ± 7.0 [a] | 19.0 ± 1.2 [a] |
| Atlantic salmon (4) | 53.0 ± 1.8 [b] | 13.0 ± 1.4 [a] |
| Rainbow trout (7) | 42.0 ± 2.3 [b,c,d] | 19.0 ± 2.5 [a] |
| Hybrid striped bass (4) | 36.0 ± 1.8 [c,d] | 120.0 ± 39.0 [b] |
| Tilapia (5) | 32.0 ± 0.9 [d] | 20.3 ± 3.4 [a] |
| † Bluegill (7) | - | - |

† Microsomes did not show any activity towards 7EC.

ECOD kinetics in striped bass and largemouth bass were not performed due to insufficient microsomal sample.

Figure 1. $V_{max}/K_m$ ratio (means ± SEM) for ECOD kinetics in farm-raised fish (Different letters denote statistically significant difference among the species, $p < 0.05$)




RBT = rainbow trout, CC = channel catfish, TILA = tilapia, AS = Atlantic salmon, HSB = hybrid striped bass

Bluegill microsomes: no response to 7-EC

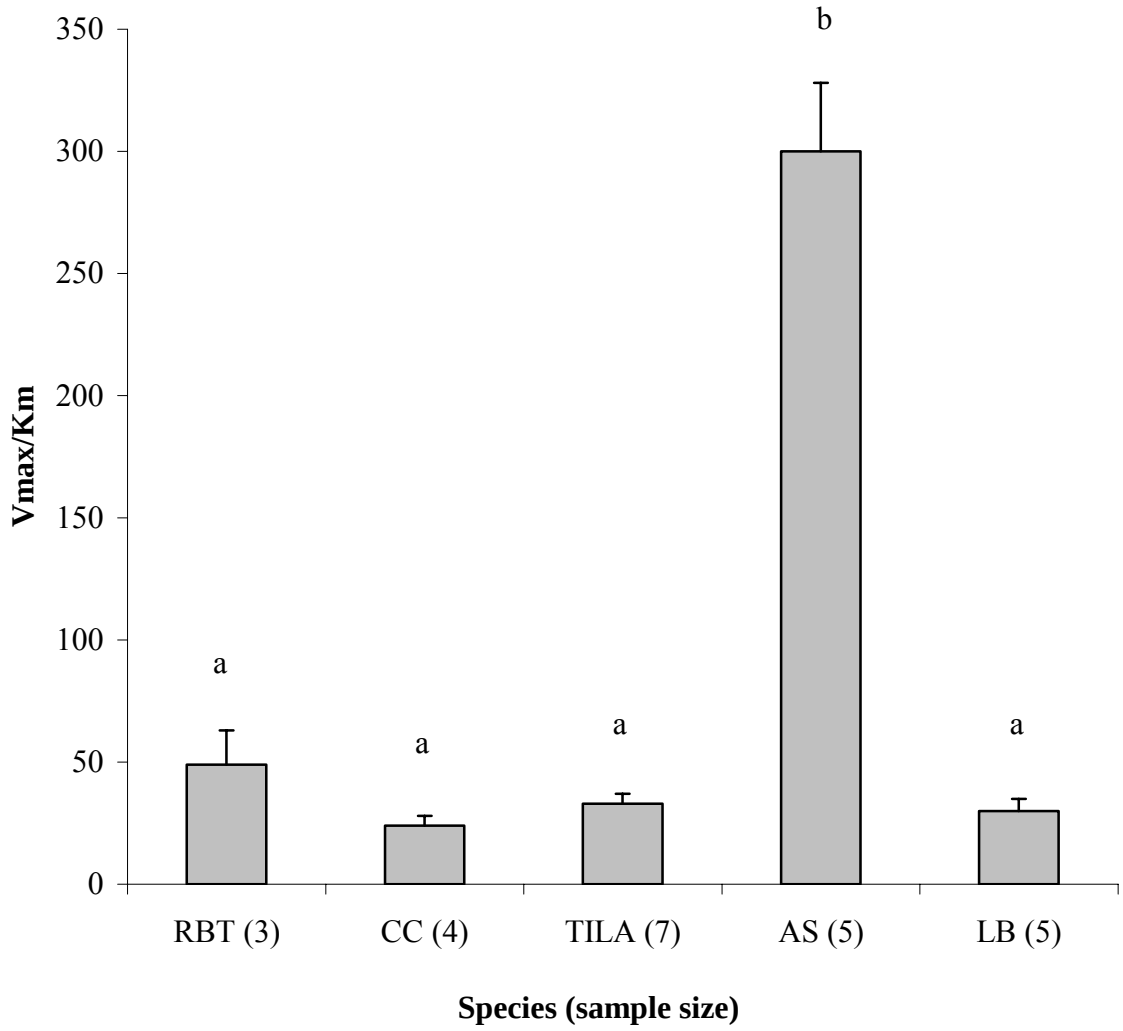
Striped bass and largemouth bass: insufficient sample to process ECOD activity.

Table 2. $V_{max}$ and $K_m$ values (means ± SEM) for EROD kinetics in farm-raised fish (Different letters in the same column denote statistically significant difference among the species, $p < 0.05$)

| **Species** (sample size) | **$V_{max}$** (pmols resorufin/min/mg protein) | **$K_m$** (μM) |
|---|---|---|
| Rainbow trout (3) | 28.0 ± 8.0 [a] | 0.6 ± 0.07 [a, b] |
| Channel catfish (4) | 39.0 ± 7.0 [a, b] | 1.8 ± 0.50 [a] |
| Tilapia (7) | 74.0 ± 15.0 [b] | 2.1 ± 0.30 [a] |
| Atlantic salmon (5) | 66.0 ± 7.0 [b] | 0.2 ± 0.02 [b] |
| Largemouth bass (5) | 27.0 ± 8.0 [a] | 0.9 ± 0.10 [a, b] |
| † Striped bass (4) | 4.0 ± 1.0 | - |
| † Hybrid striped bass (5) | 2.8 ± 0.3 | - |
| † Bluegill (6) | 3.7 ± 1.0 | - |

† Enzyme activity is reported at 1 uM substrate concentration only.

Figure 2. $V_{max}/K_m$ ratio (means ± SEM) for EROD kinetics in farm-raised fish (Different letters denote statistically significant difference among the species, $p < 0.05$)

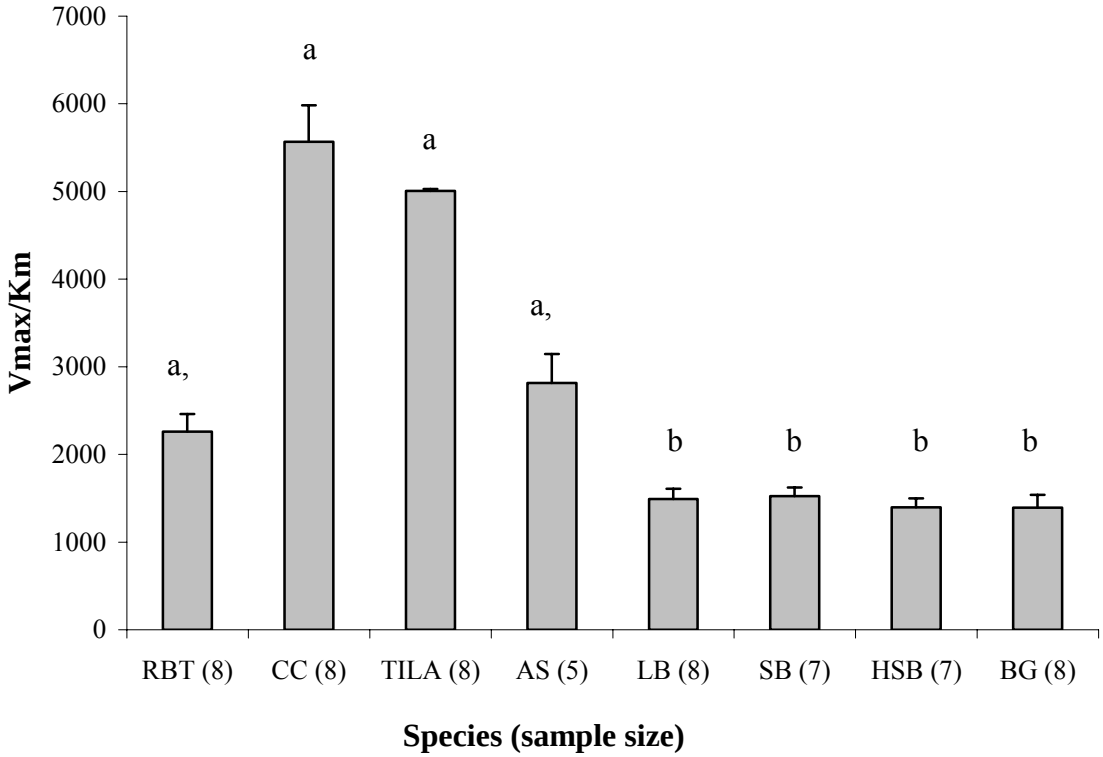



RBT = rainbow trout, CC = channel catfish, TILA = tilapia, AS = Atlantic salmon, LB = largemouth bass

Table 3. $V_{max}$ and $K_m$ values (means ± SEM) for glutathione-S-transferase (GST) kinetics in farm-raised fish (Different letters in the same column denote statistically significant difference among the species, $p < 0.05$)

| **Species** (sample size) | **$V_{max}$** (nmols CDNB / min / mg protein) | **$K_m$** (mM) |
|---|---|---|
| Rainbow trout (8) | 929 ± 65 [a] | 0.4 ± 0.05 [a, b] |
| Channel catfish (8) | 657 ± 39 [c] | 0.1 ± 0.02 [d] |
| Tilapia (8) | 1508 ± 70 [b] | 0.3 ± 0.01 [a, b, c] |
| Atlantic salmon (5) | 1349 ± 107 [b] | 0.5 ± 0.10 [a] |
| Largemouth bass (8) | 589 ± 52 [c] | 0.4 ± 0.06 [a, b] |
| Striped bass (7) | 334 ± 30 [d] | 0.2 ± 0.02 [c, d] |
| Hybrid striped bass (8) | 471 ± 39 [c, d] | 0.4 ± 0.04 [a, b, c] |
| Bluegill (8) | 354 ± 26 [d] | 0.3 ± 0.04 [b, c] |

Figure 3. $V_{max}$ / $K_m$ ratio (means ± SEM) for glutathione-S-transferase kinetics in farm-raised fish (Different letters denote statistically significant difference among the species, $p < 0.05$)



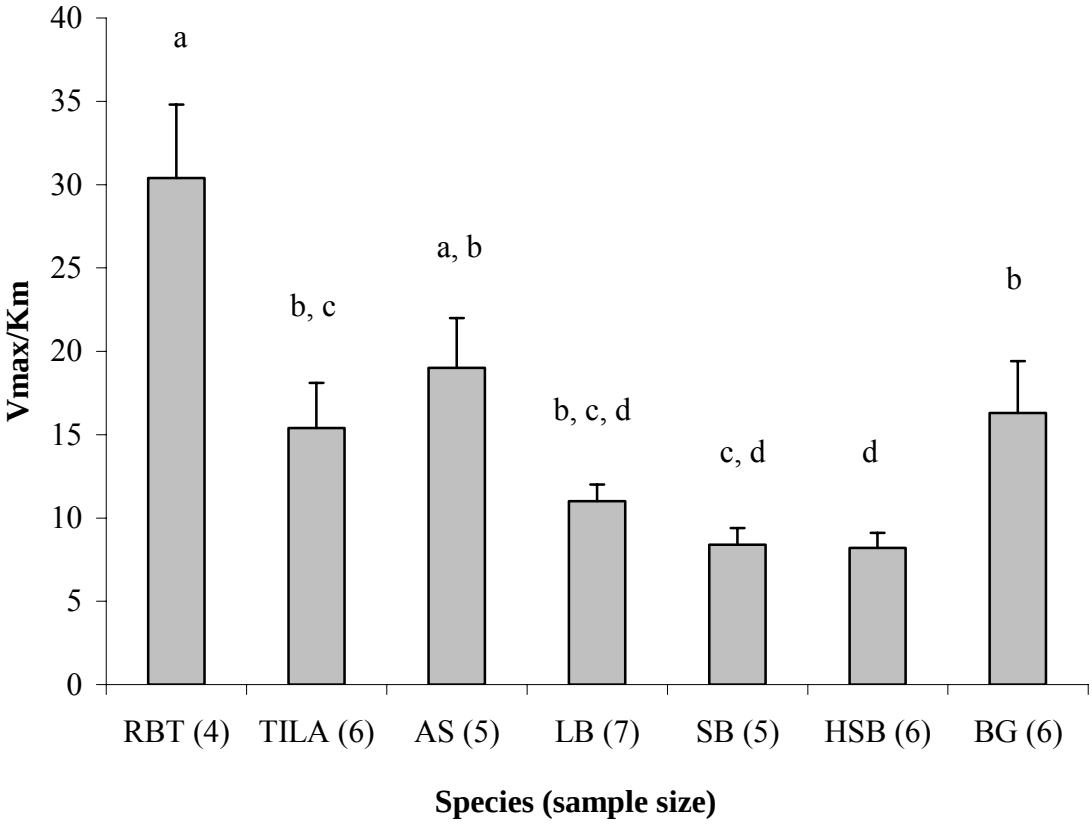

RBT = rainbow trout, CC = channel catfish, TILA = tilapia, AS = Atlantic salmon, LB = largemouth bass, SB = striped bass, HSB = hybrid striped bass, BG = bluegill

Table 4. $V_{max}$ and $K_m$ values (means ± SEM) for UDP-glucuronosyltransferase kinetics in farm-raised fish (Different letters in the same column denote statistically significant difference among the species, $p < 0.05$)

| **Species** (sample size) | **$V_{max}$** (pmols resorufin/ min / mg protein) | **$K_m$** (μM) |
|---|---|---|
| Rainbow trout (4) | 930 ± 258 [a] | 32.3 ± 8.7 [a] |
| † Channel catfish (5) | - | - |
| Tilapia (6) | 368 ± 89 [b] | 28.5 ± 8.7 [a] |
| Atlantic salmon (5) | 410 ± 86 [a, b] | 24.0 ± 6.0 [a] |
| Largemouth bass (7) | 273 ±16 [b] | 27.0 ± 3.0 [a] |
| Striped bass (5) | 231 ± 29 [b] | 29.0 ± 5.0 [a] |
| Hybrid striped bass (6) | 271 ± 37 [b] | 36.0 ± 7.0 [a] |
| Bluegill (6) | 263 ± 31 [b] | 17.5 ± 2.3 [a] |

† No UDPGT activity was detected using resorufin as substrate in channel catfish

Figure 4. $V_{max}/K_m$ ratio for UDP-glucuronosyltransferase kinetics in farm-raised fish (means ± SEM) (Different letters denote statistical significant difference among the species, $p < 0.05$)

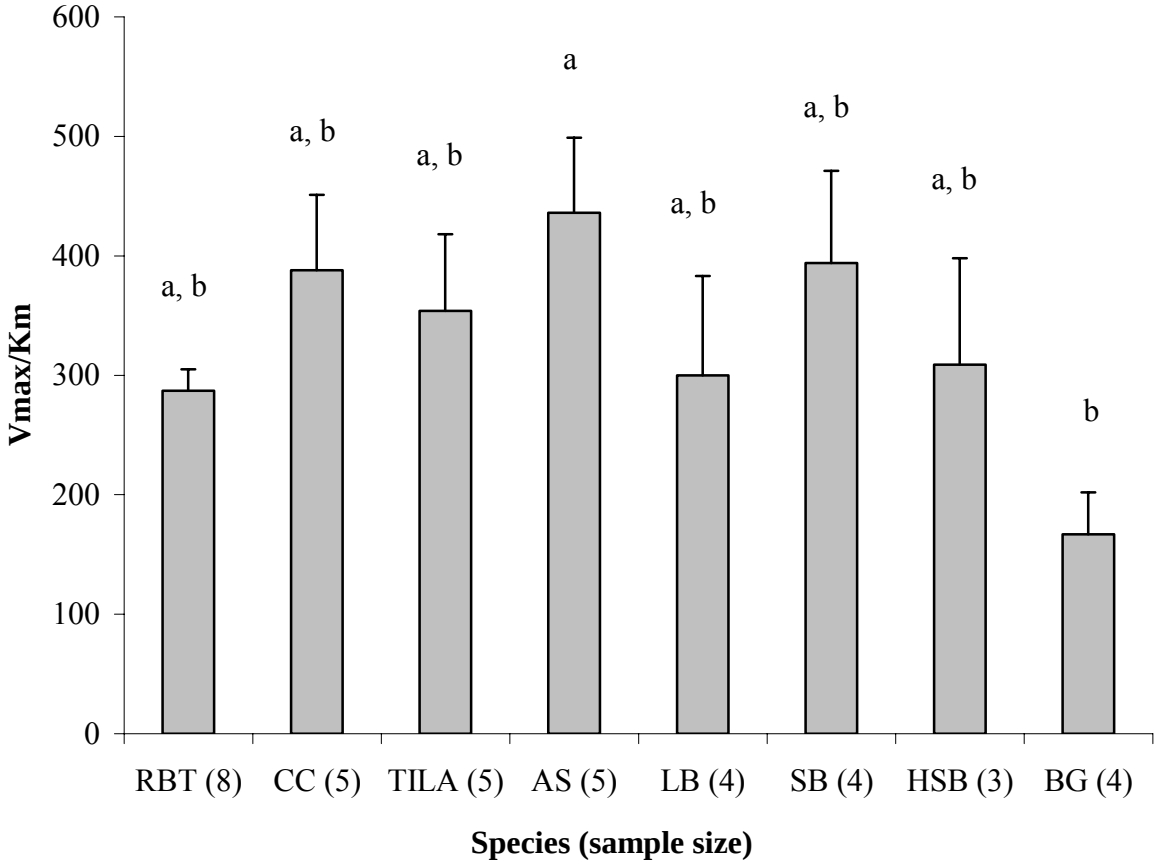



RBT = rainbow trout, TILA = tilapia, AS = Atlantic salmon, LB = largemouth bass
SB = striped bass, HSB = hybrid striped bass, BG = bluegill

Table 5. $V_{max}$ and $K_m$ values (means ± SEM) for sulfotransferase kinetics in farm-raised fish (Different letters in the same column denote statistically significant difference among the species, $p < 0.05$)

| **Species** (sample size) | **$V_{max}$** (pmols resorufin/ min / mg protein) | **$K_m$** (μM) |
|---|---|---|
| Rainbow trout (8) | 190 ± 20 [b, c, d] | 0.7 ± 0.1 [a] |
| Channel catfish (5) | 265 ± 27 [a, b] | 0.8 ± 0.1 [a] |
| Tilapia (5) | 328 ± 17 [a] | 1.0 ± 0.2 [a] |
| Atlantic salmon (5) | 215 ± 14 [b, c] | 0.5 ± 0.1 [a] |
| Largemouth bass (4) | 147 ±10 [c, d, e] | 0.6 ± 0.1 [a] |
| Striped bass (4) | 45 ± 5 [e] | 0.1 ± 0.03 [b] |
| Hybrid striped bass (3) | 46 ± 4 [e] | 0.3 ± 0.1 [a, b] |
| Bluegill (4) | 107 ± 23 [d, e] | 0.7 ± 0.1 [a] |

Figure 5. $V_{max}/K_m$ ratio (means ± SEM) for sulfotransferase kinetics in farm-raised fish (Different letters denote statistically significant difference among the species, $p < 0.05$)




RBT = rainbow trout, CC = channel catfish, TILA = tilapia, AS = Atlantic salmon, LB = largemouth bass, SB = striped bass, HSB = hybrid striped bass, BG = bluegill

Figure 6. Relative $V_{max}/K_m$ ratio (means ± SEM) for the phase I and II reactions tested in the eight species. Inter-species ratios are relative to each other and the Y-axis scale is provided for the purpose of species comparision only. Species shown include rainbow trout (RBT), channel catfish (CC), tilapia (TILA), Atlantic salmon (AS), largemouth bass (LB), striped bass (SB), hybrid striped bass (HSB) and bluegill (BG). Actual ratio values are presented in figures 1 through 5.

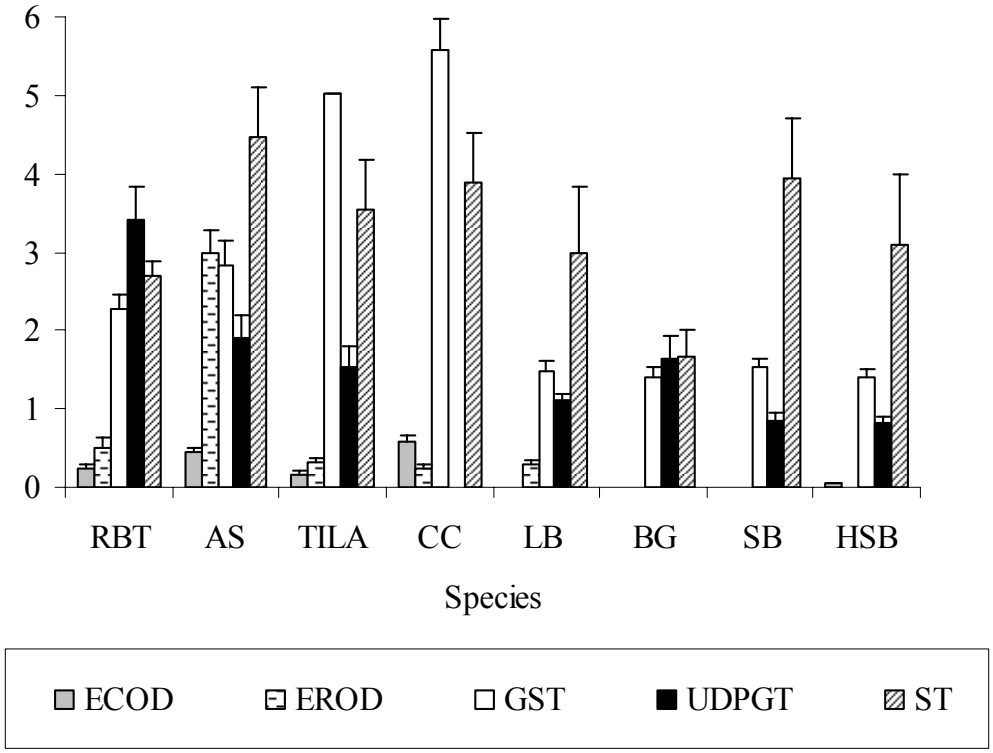


Figure 7. Changes in EROD activity in frozen rainbow trout microsomes (n=6) during the 6 months evaluation. Activity expressed as pmols resorufin/min/mg protein. Different letters in bars indicate significant differences among the treatments.

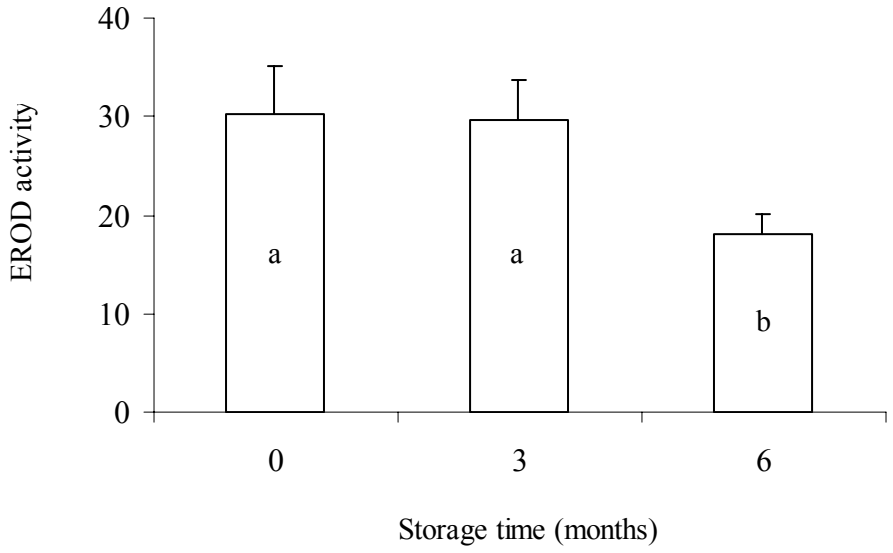
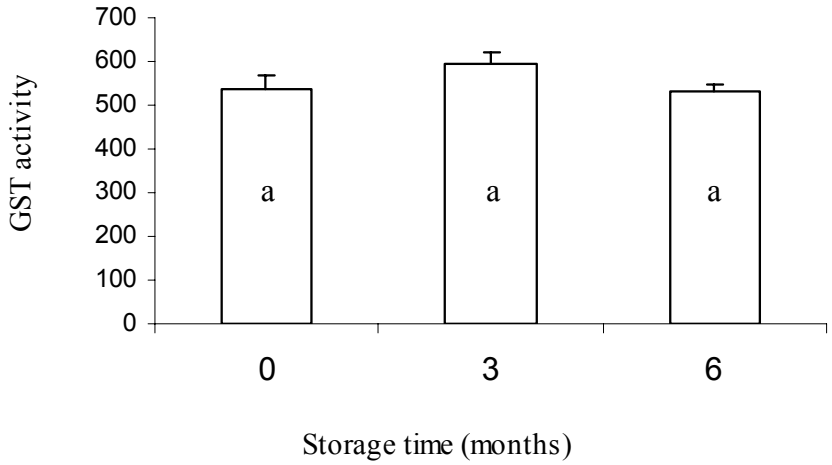
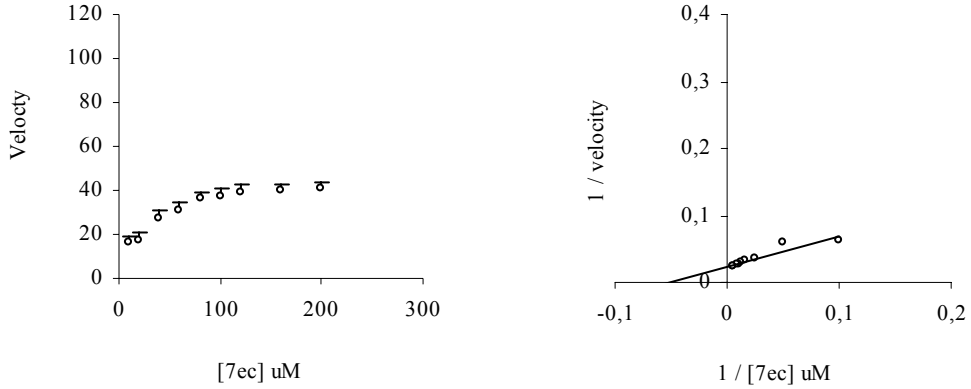


Figure 8. Changes in GST activity in frozen rainbow trout cytosols (n=6) during the 6 months evaluation. Activity expressed as nmols CDNB/min/mg protein. Different letters in bars indicate significant differences among the treatments.

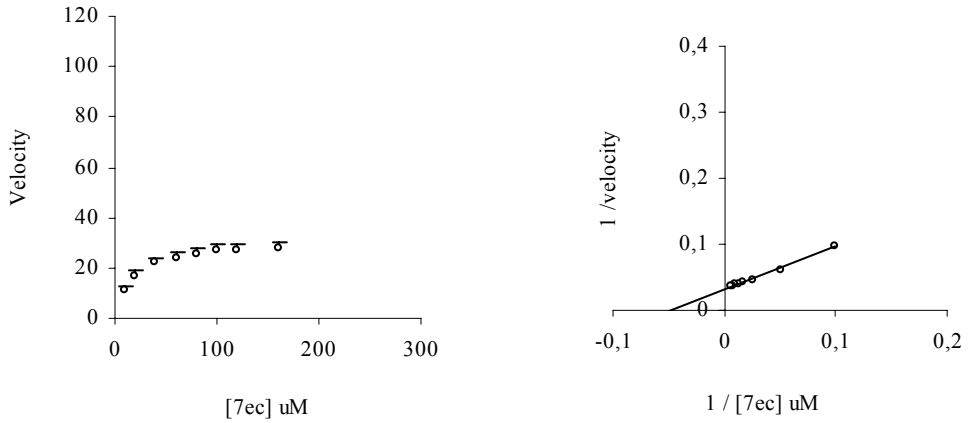


Appendix 1. Michaelis-Menten and double reciprocal plots for ECOD activity in farm-raised rainbow trout, tilapia, and channel catfish. Velocity expressed as pmols hydroxycoumarin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 4 to 7 fish per species.

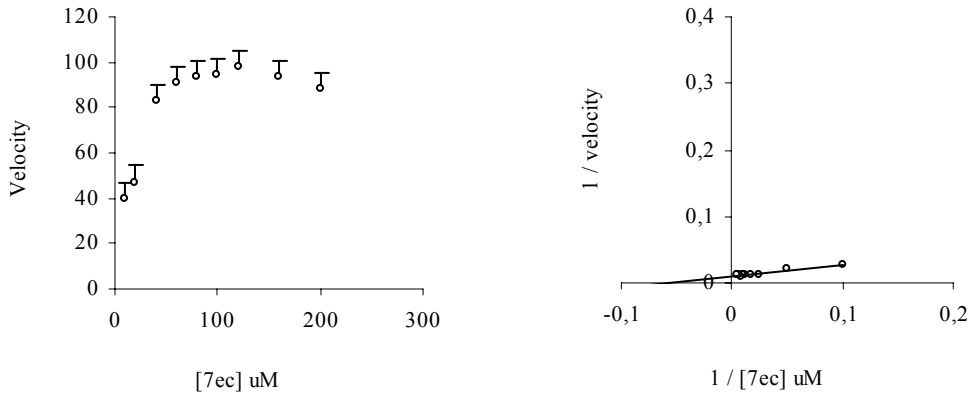
**Rainbow trout**




**Tilapia**

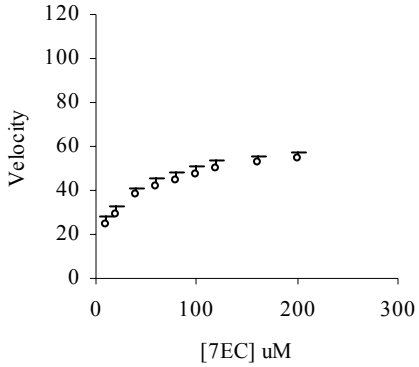


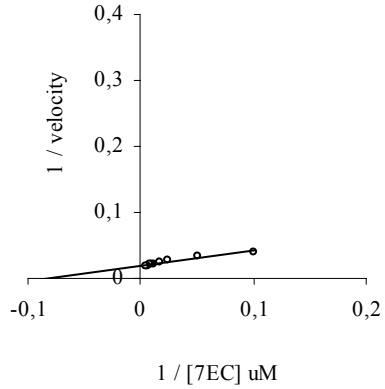

**Channel catfish**



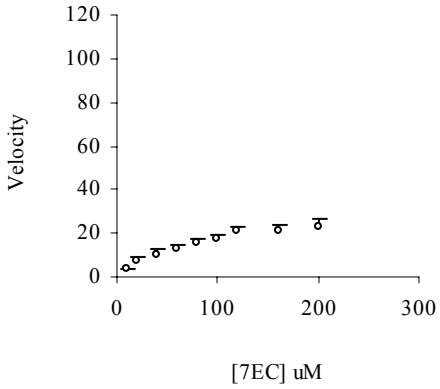
Appendix 1 (Cont). Michaelis-Menten and double reciprocal plots for ECOD activity in farm-raised Atlantic salmon and hybrid striped bass. Velocity expressed as pmols hydroxycoumarin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 4 fish per species.

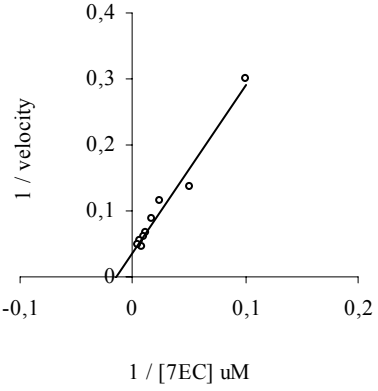
**Atlantic salmon**



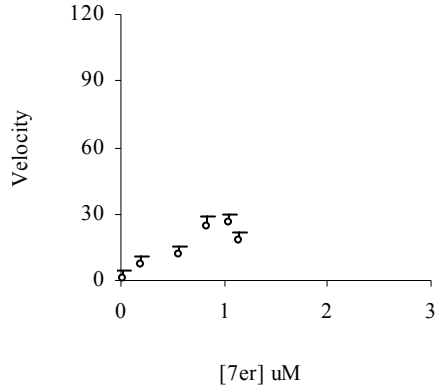

**Hybrid striped bass**

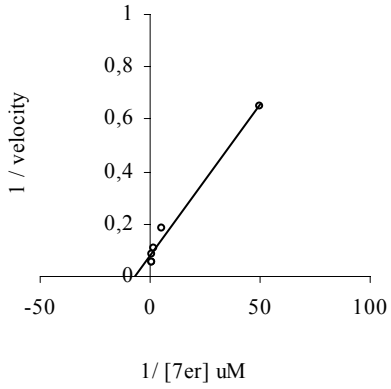


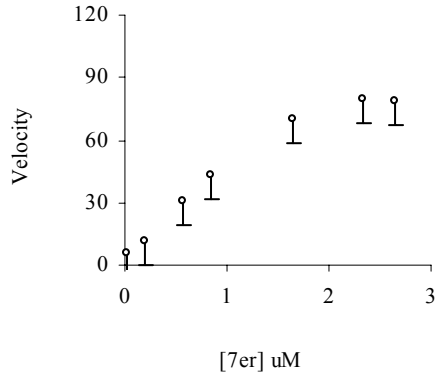
Appendix 2. Michaelis-Menten and double reciprocal plots for EROD activity in farm-raised rainbow trout, tilapia, and channel catfish. Velocity expressed as pmols resorufin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 3 to 7 fish per species.

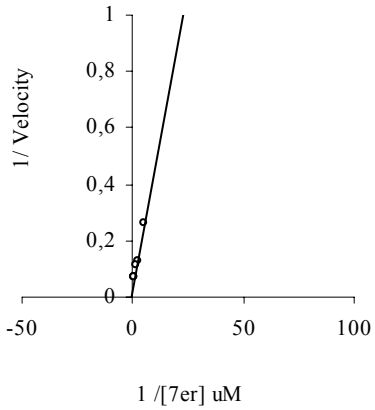
**Rainbow trout**



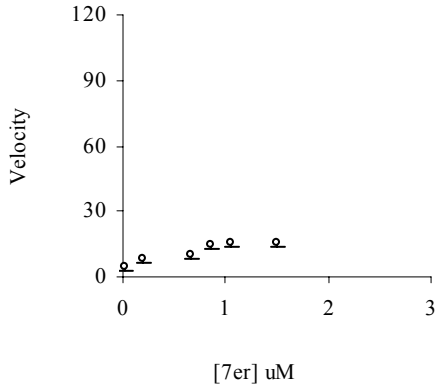



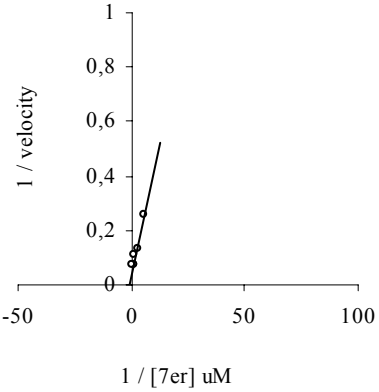

**Tilapia**



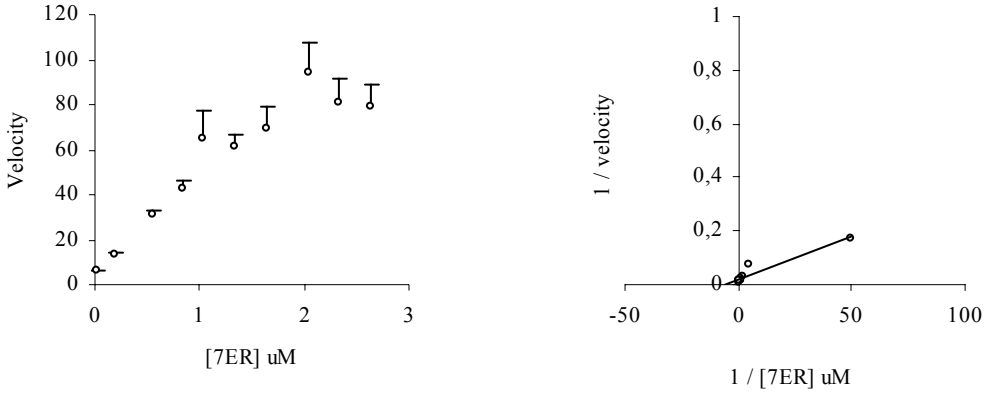

**Channel catfish**



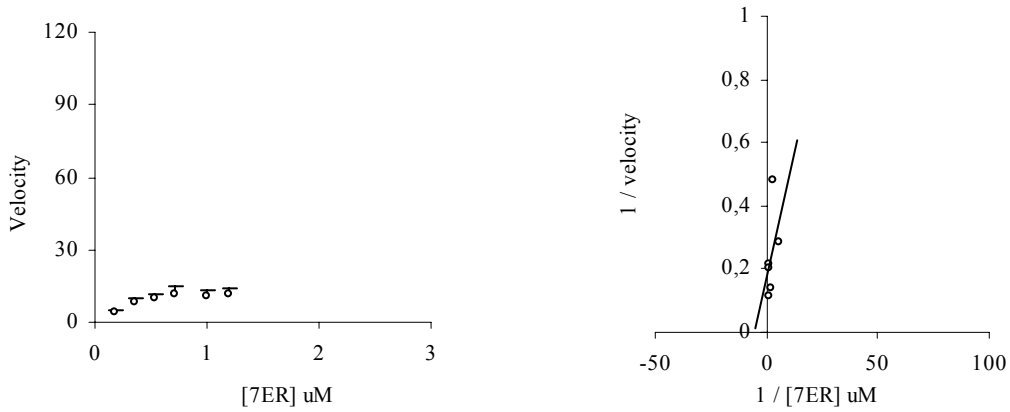



Appendix 2 (Cont.) Michaelis-Menten and double reciprocal plots for EROD activity in farm-raised Atlantic salmon and largemouth bass. Velocity expressed as pmols resorufin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 5 fish per species.

**Atlantic salmon**

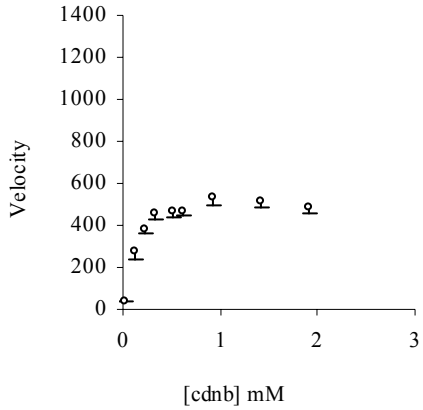


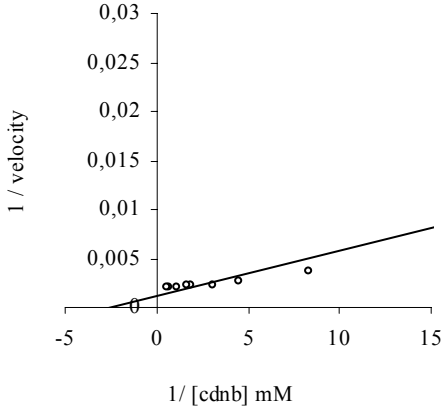

**Largemouth bass**



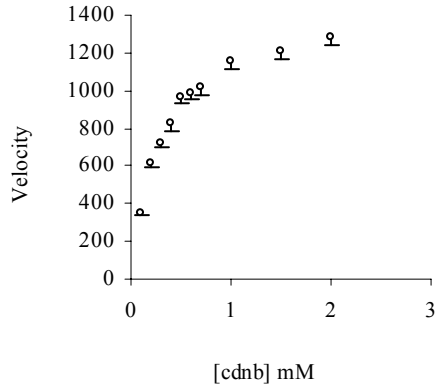
Appendix 3. Michaelis-Menten and double reciprocal plots for GST activity in farm-raised rainbow trout, tilapia, and channel catfish. Velocity expressed as nmols cdnb/min/mg protein and $K_m$ in mM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 8 fish per species.

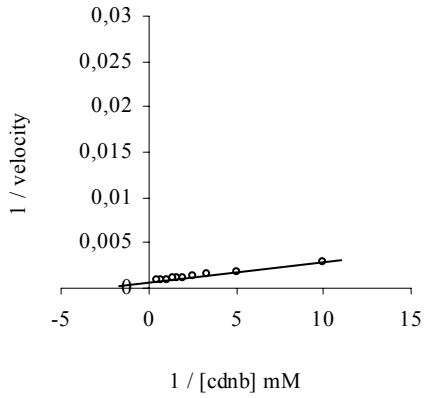
**Rainbow trout**



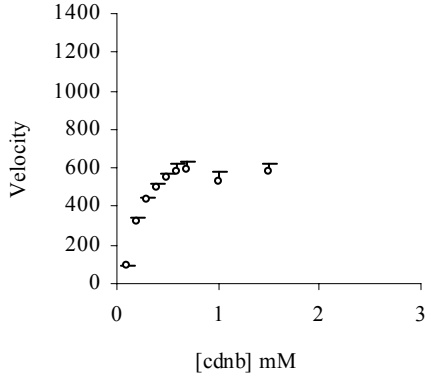



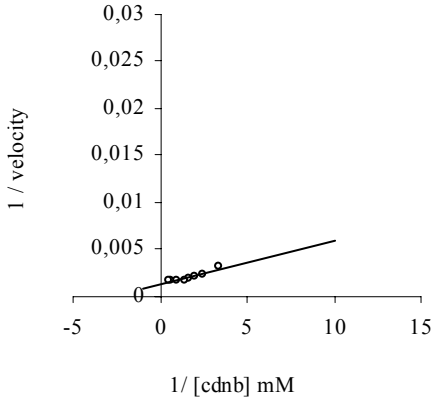

**Tilapia**



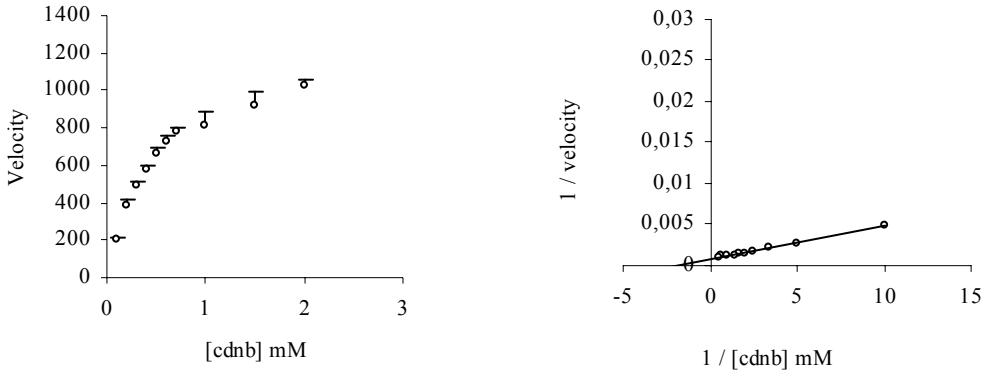

**Channel catfish**



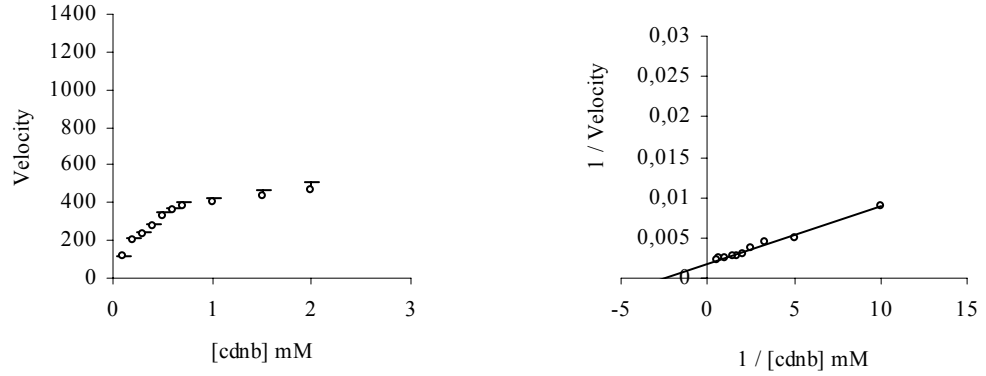



Appendix 3 (Cont.) Michaelis-Menten and double reciprocal plots for GST activity in farm-raised Atlantic salmon, largemouth bass, and striped bass. Velocity expressed as nmols cdnb/min/mg protein and $K_m$ in mM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 5 to 8 fish per species.

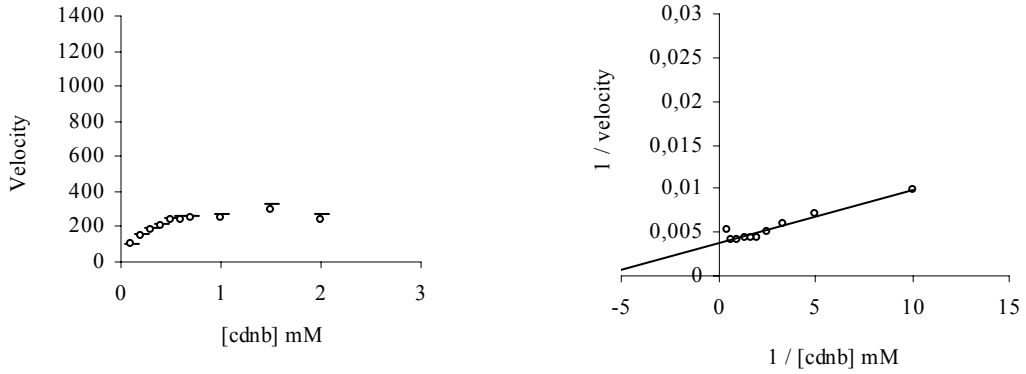
**Atlantic salmon**




**Largemouth bass**

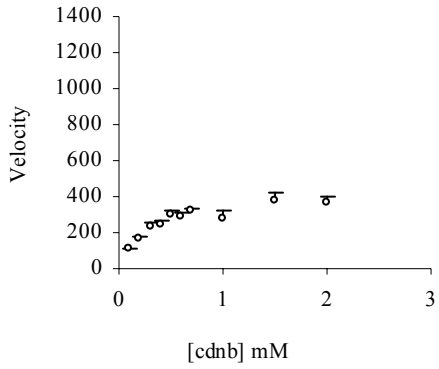


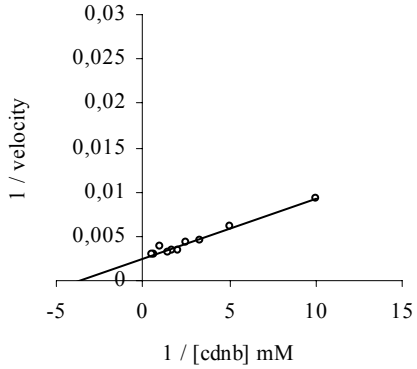

**Striped bass**



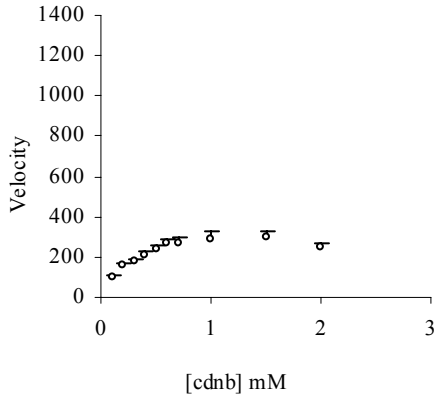
Appendix 3 (Cont.). Michaelis-Menten and double reciprocal plots for GST activity in farm-raised hybrid striped bass and bluegill. Velocity expressed as nmols cdnb/min/mg protein and $K_m$ in mM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 8 fish per species.

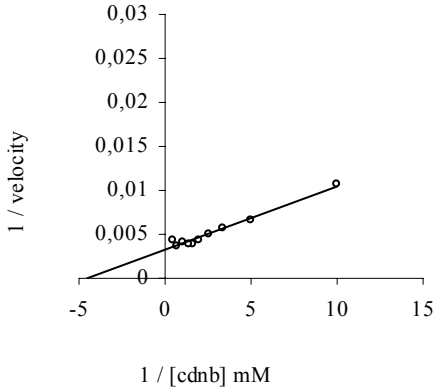
**Hybrid striped bass**



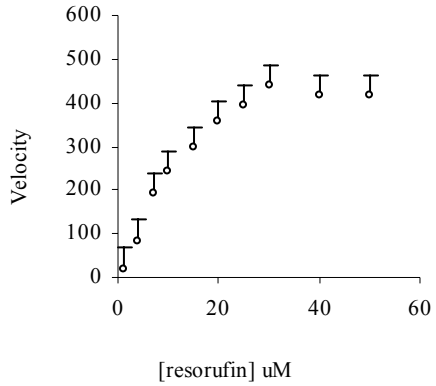

**Bluegill**

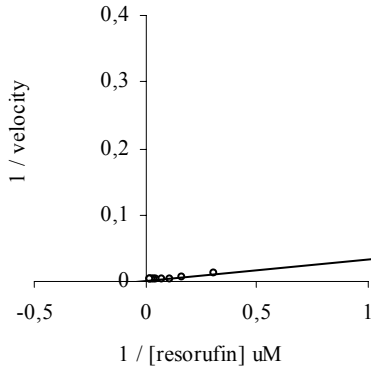


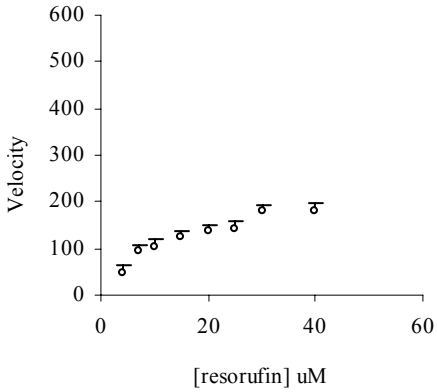
Appendix 4. Michaelis-Menten and double reciprocal plots for UDPGT activity in farm-raised rainbow trout, tilapia, and Atlantic salmon. Velocity expressed as pmols resorufin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 4 to 6 fish per species.

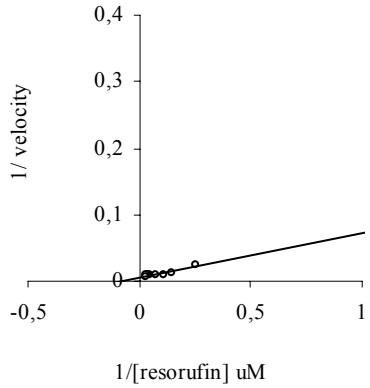
**Rainbow trout**



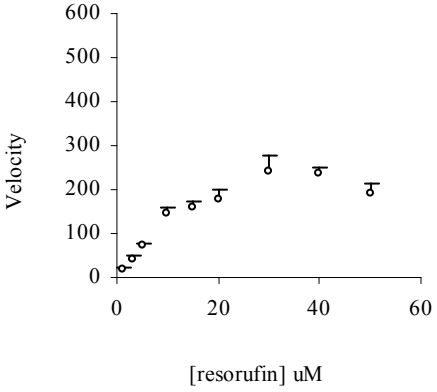



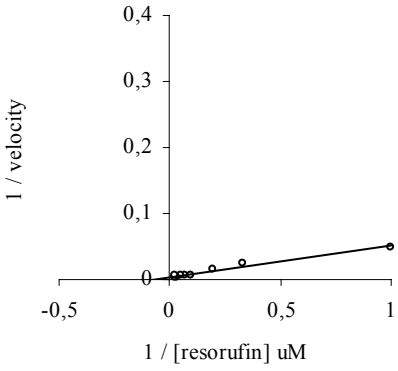

**Tilapia**



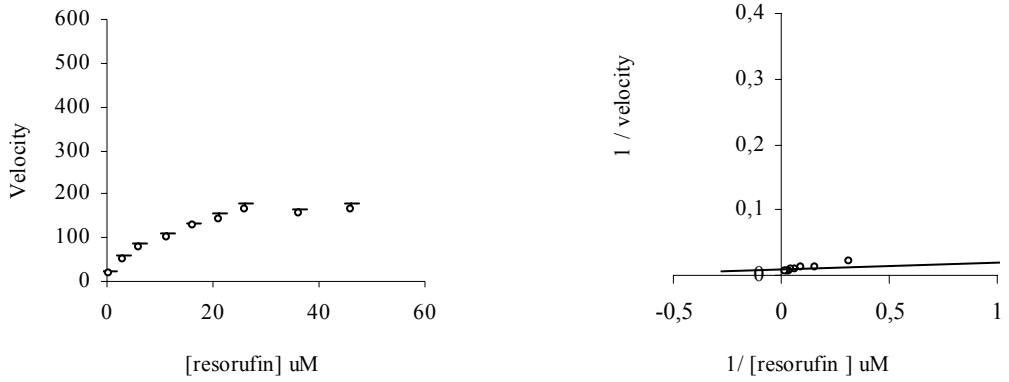

**Atlantic salmon**



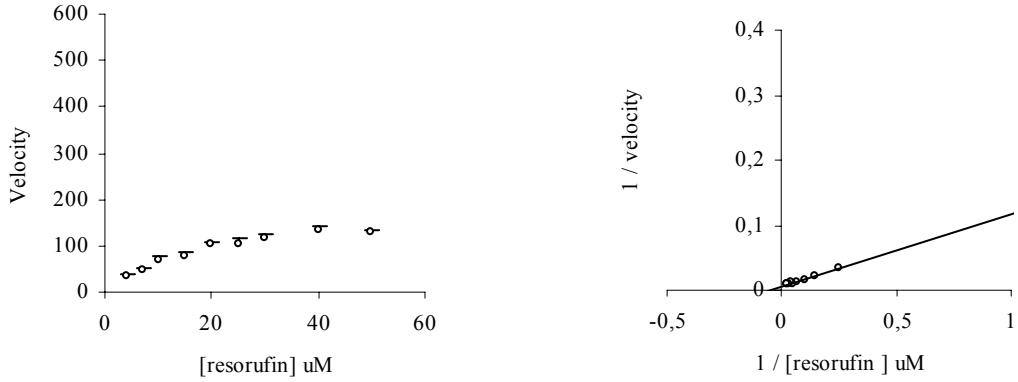



Appendix 4 (Cont.). Michaelis-Menten and double reciprocal plots for UDPGT activity in farm-raised largemouth bass, striped bass, and hybrid striped bass. Velocity expressed as pmols resorufin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 5 to 7 fish per species.

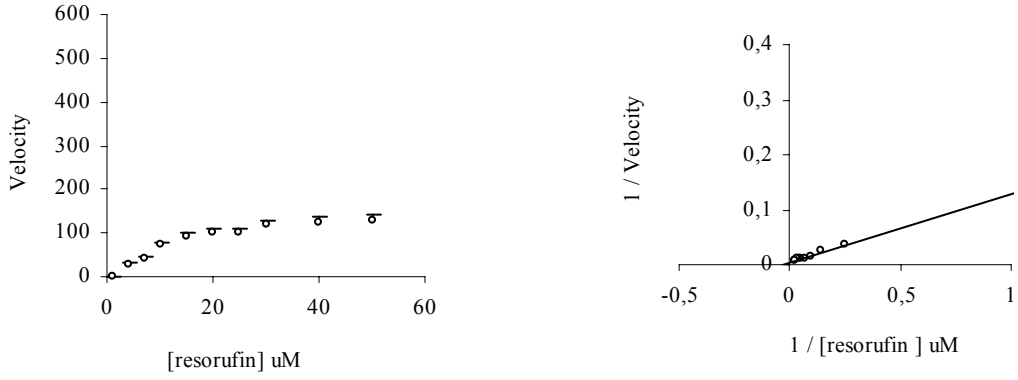
**Largemouth bass**




**Striped bass**

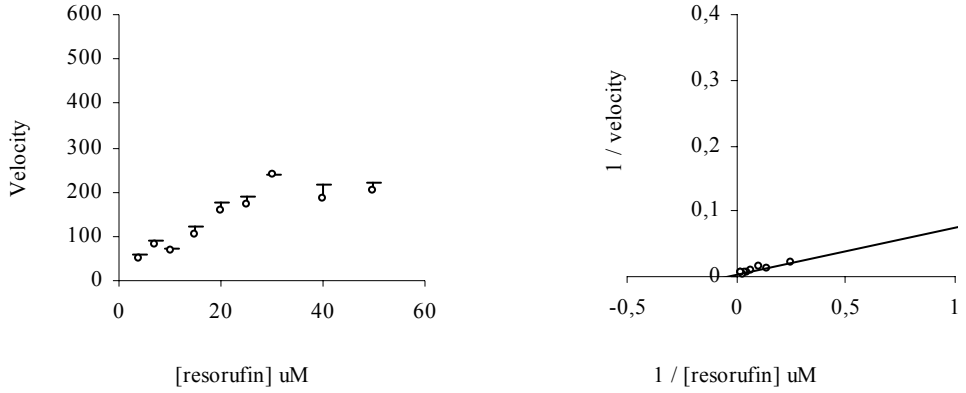



**Hybrid striped bass**



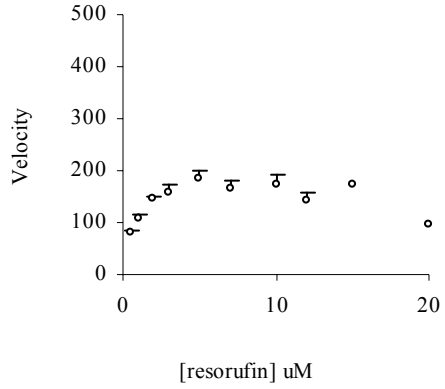
Appendix 4 (Cont.). Michaelis-Menten and double reciprocal plots for UDPGT activity in farm-raised bluegill. Velocity expressed as pmols resorufin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 6 fish.

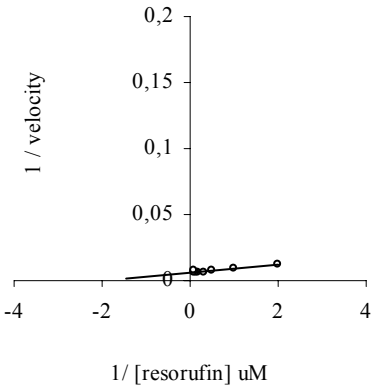
**Bluegill**



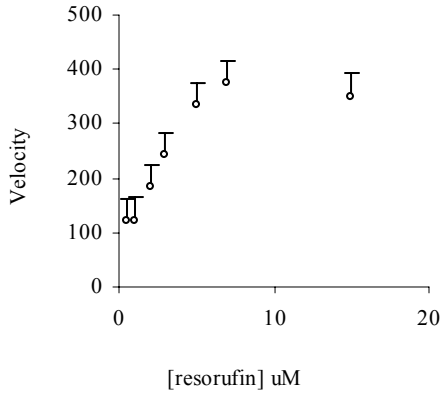
Appendix 5. Michaelis-Menten and double reciprocal plots for sulfotransferase activity in farm-raised rainbow trout, tilapia, and channel catfish. Velocity expressed as pmols resorufin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 5 to 8 fish per species.

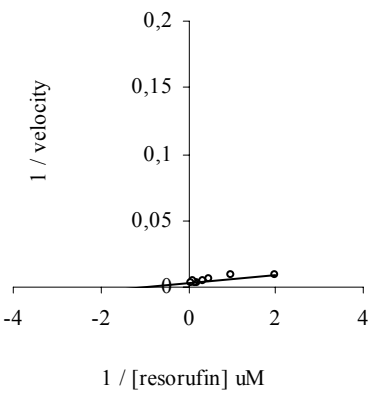
**Rainbow trout**



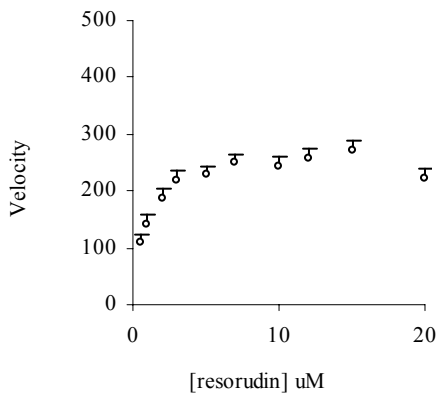



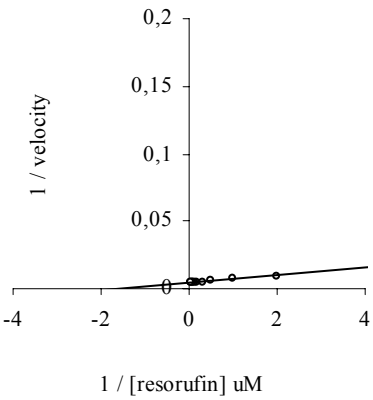

**Tilapia**



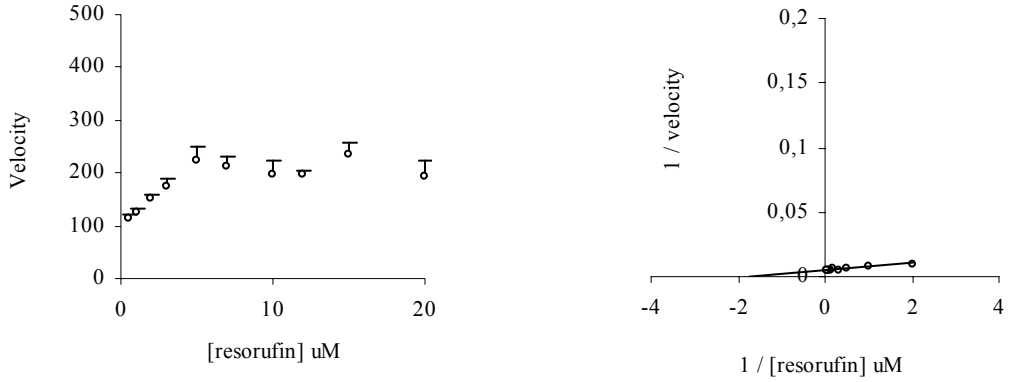

**Channel catfish**



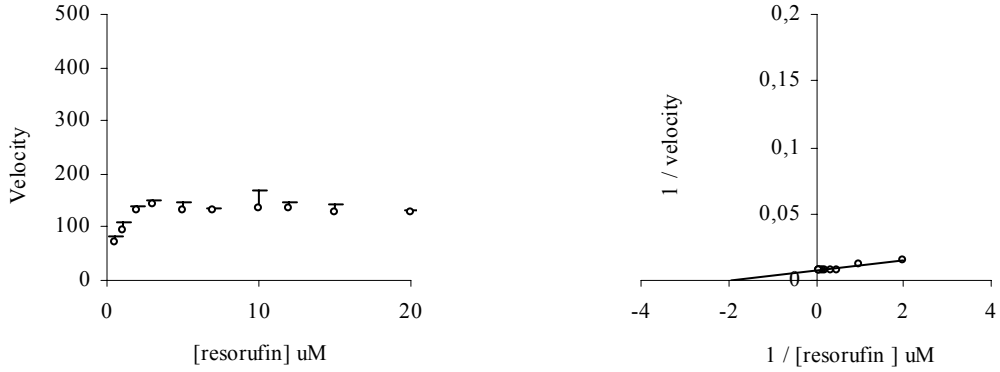



Appendix 5 (Cont.). Michaelis-Menten and double reciprocal plots for SULF activity in farm-raised Atlantic salmon, largemouth bass, and striped bass. Velocity expressed as pmols resorufin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 4 to 5 fish per species.

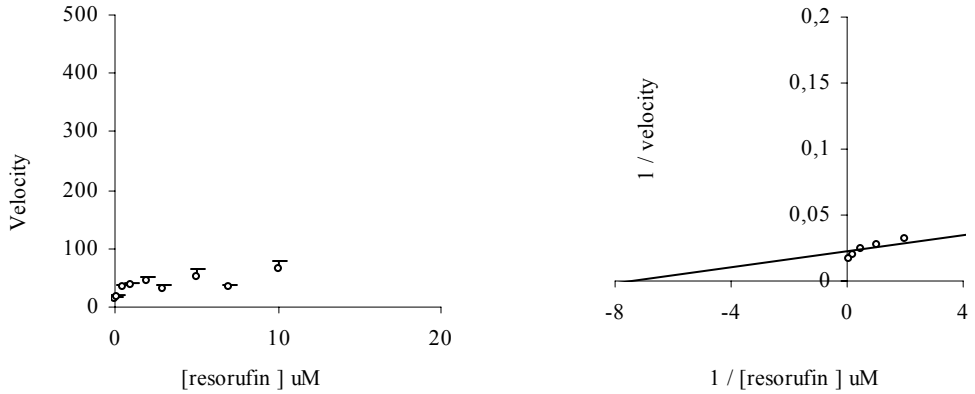
**Atlantic salmon**




**Largemouth bass**

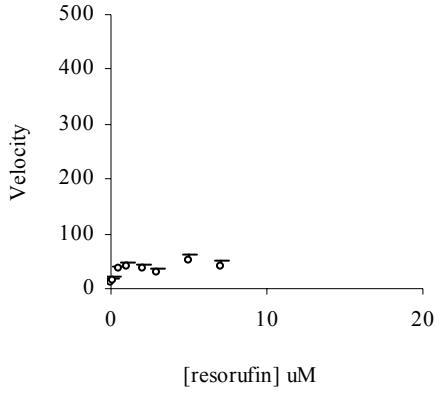


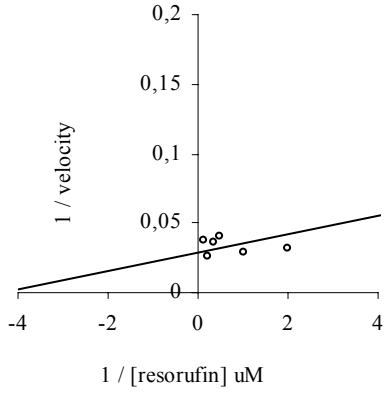

**Striped bass**



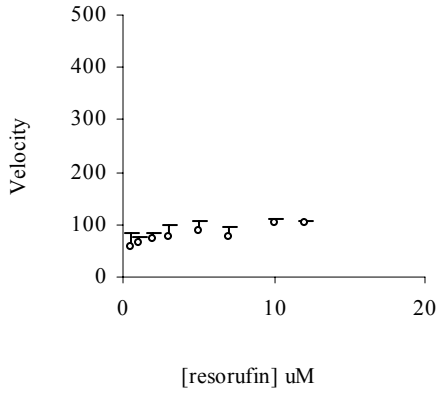
Appendix 5 (Cont.). Michaelis-Menten and double reciprocal plots for SULF activity in farm-raised hybrid striped bass and bluegill. Velocity expressed as pmols resorufin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 3 to 4 fish per species.

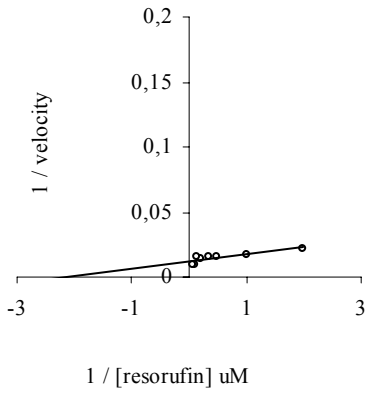
**Hybrid striped bass**




**Bluegill**



# Chapter III

## Phase I and II biotransformation kinetics in laboratory-acclimated and farm-raised tilapia (*Oreochromis sp.*), rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*)

**Abstract**

Hepatic microsomes and cytosolic fractions of laboratory-acclimated and farm-raised, market-size (n=3–8) tilapia (*Oreochromis sp*.), rainbow trout (*Oncorhynchus mykiss*), and channel catfish (*Ictalurus punctatus*), were used to compare phase I and phase II biotransformation kinetics. Specific phase I reactions included ethoxycoumarin- (ECOD), ethoxyresorufin- (EROD), pentoxyresorufin- (PROD), and benzyloxyresorufin-*O*-dealkylations (BROD); and phase II reactions included UDP-glucuronosyltransferase (UDPGT), sulfotransferase (ST) and glutathione-S-transferase (GST) activities. Maximal velocity ($V_{max}$), binding affinity ($K_m$) and catalytic efficiency ($V_{max}/K_m$) were used as endpoints for comparison between holding conditions and species. Farm-raised channel catfish had the highest ECOD $V_{max}$ compared to rainbow trout and tilapia. Tilapia and catfish showed significant differences for $V_{max}$ values. Significant differences in EROD $V_{max}$ were observed between laboratory-acclimated and farm-raised tilapia and channel catfish. $K_m$ was also significantly different between laboratory-acclimated and farm-raised fish in all these species. GST kinetics had significant differences between farm-raised and laboratory-acclimated fish in all the endpoints and species with the exception of $V_{max}$ values for tilapia. On the other hand, UDPGT-mediated conjugation was very similar between laboratory-acclimated and farm-raised specimens of tilapia and rainbow trout. Channel catfish microsomes were unresponsive to UDPGT-mediated resorufin conjugation. ST-mediated conjugation of the same substrate had lower capacity and higher affinity in all the species when compared to the UDPGT-based reaction. ST $V_{max}$ was significantly different between farm-raised and laboratory-acclimated channel catfish and tilapia. Significant changes for ST $K_m$ were present between farm-raised and laboratory-acclimated channel catfish. Farm-raised tilapia had a higher ST

catalytic efficiency (e.g., $V_{max}/K_m$) than the laboratory-acclimated counterparts. Despite the statistically significant differences that were found for some of the kinetics variables between laboratory-acclimated and farm-raised fish, biological implications due to these differences may be minor if we consider that such differences were within ranges that are cited as normal baseline values in other studies. Significant changes in phase I-II biotransformation reactions due to inducers (e.g., pollutants) are far greater than the values of this study that were determined in either laboratory-acclimated or farm-raised fish.

**Keywords**: phase I-II biotransformation, aquaculture, tilapia, rainbow trout, channel catfish.

## Background

Phase I and II biotransformations reactions are crucial for the metabolism of endogenous substrates and the detoxification of xenobiotics in fish and other species. Phase I reactions facilitate the biosynthesis or degradation of endogenous molecules such as steroids, fatty acids, and prostaglandins. These reactions are also responsible for the inactivation (e.g. detoxification) or activation of exogenous compounds from the environment. Phase II-reactions are mainly conjugation reactions of the phase I products or other parent compounds that are intended to facilitate the excretion of these metabolites by increasing their polarity. A variety of environmental and physiological factors that affect the expression of phase I and II reactions in fish have been studied and reported. Among these, seasonal-, sex-, diet-, and species-specific-related factors are considered sources of variation for the phase I and II catalytic expression of the biotransformation reactions.

The purpose of this study was to compare the kinetics of specific phase I and II biotranformation reactions between laboratory-acclimated and farm-raised specimens of three commercially important aquacultured species: tilapia, channel catfish and rainbow trout. Fish that are raised under intensive production systems may encounter stressful conditions that may eventually affect health status and productivity. The fish that were obtained from aquaculture facilities in the present work were raised in raceways (rainbow trout), ponds (channel catfish) and recirculating indoor tanks (tilapia). These are three of the most common systems used in aquaculture for fish grow out. Although these systems are designed for optimal conditions, each has disadvantages that may affect productivity due to stressful settings for the cultured

fish. However, production systems in aquaculture are expected to operate maintaining less stressful conditions than those that fish in the wild may encounter during starvation or severe changes in water temperature during season transitions. On the other hand, laboratory-acclimated fish are maintained keeping optimal and very stable conditions with regard to offered food, physico-chemical water variables, photoperiod, etc.

Alkoxycoumarins, alkoxyresorufins and 1-chloro-2,4-dinitrobenzene (CDNB), among other catalytic probes, have been used to examine P450-mediated and phase II biotransformation reactions in fish species. Not many studies have dealt with the kinetics of biotransformation reactions in fish species. The majority of these reports are focused on determining catalytic activity at a particular substrate concentration that is expected to reach saturation conditions for the enzymatic system. In this regard, comparisons between our study and other reports were sometimes limited to contrasting maximal velocities of our kinetics data and the velocity at a particular substrate concentration cited in other studies.

## Materials and methods

### Fish handling

Healthy, market-size specimens (n=8) of rainbow trout (*Oncorhynchus mykiss*), tilapia (*Oreochromis sp*.), and channel catfish (*Ictalurus punctatus)* were obtained from commercial farms. Farm-raised fish were transported overnight to the Aquatic Pathobiology Center (APC) at the University of Maryland (College Park, MD, USA) where they were sacrificed upon arrival by cervical transection. Our laboratory-

acclimated fish were maintained in locations under controlled photoperiod, diet regime and periodic changes of filtered water for at least 3 months prior to sacrifice and liver harvesting. All fish appeared healthy and in good condition before being used in the study.

Microsomes and cytosolic fraction preparation

Microsomes and cytosol were prepared according to Vodicnik et al. (1981). Livers were weighed individually and kept on ice-cold KCl buffer (1.15%) (J.T. Baker – 3040-01). Livers were minced coarsely with scissors and rinsed as many times as needed until looking clear without blood residues. After discarding the last KCl rinsing, 4 volumes of ice-cold 0.25 M sucrose (ICN Biomedicals – 821271) were added per gram of liver. The minced livers in sucrose were transferred to a pre-chilled glass homogenizer and homogenized by 6 full strokes. The homogenizer was kept on ice during this process. The sample was then transferred to an ice-cold, high-speed centrifuge tube and spun at 8,000 x g for 20 minutes at 4$^{o}$C (Biofuge 22 R – Heraeus Instruments). The supernatant was spun at 100,000 x g for 60 minutes at 4$^{o}$C (Beckman Ultracentrifuge XL-80). The tubes were removed to ice and the supernatant (cytosolic fraction) was aliquoted into cryotubes. The microsomal pellets were resuspended with 1 ml of SET buffer (pH=7.4) per gram of wet liver with a spatula. The resuspended microsomal fraction was transferred to a glass homogenizer and homogenized with 6 full strokes followed by aliquoting into cryotubes. Both cytosolic and microsomal fraction cryotubes were stored at –80$^{o}$C until performing the assays. Protein was measured using the BCA protein assay kit (Pierce - 23227) based on the colorimetric reaction with bicinchoninic acid.

Phase I assays

*Ethoxycoumarin-O-dealkylation (ECOD) kinetics*. This procedure was modified from Haasch et al., 1994 and Schlenk et al., 2000. Reaction mixtures consisted of 50 uL of buffer Tris-HCl (100 mM, pH=7.4), 25 uL of microsomal fraction (100 ug of protein), 10 uL of 7-ethoxycoumarin (7EC) (Sigma E1379) (20-100 uM range) and 25 uL of NADPH tetrasodium salt (Calbiochem® 481973) to reach 0.1 mM final concentration. Blanks consisted of reaction mixture with boiled microsomes. The rate of ECOD activity was monitored after 10 minutes of reaction by reading the relative fluorescence units (RFU) of the umbelliferone (7-hydroxycoumarin, Sigma U7626) produced by the reaction (excitation = 380 nm, emission = 460 nm) in a microplate absorbance-fluorescence reader (TECAN, Austria). An umbelliferone calibration curve was used for the quantification of the reaction rate. The linearity of the reaction rate was confirmed in preliminary experiments.

*Ethoxyresorufin-O-deethylation (EROD) kinetics.* This protocol was modified from Eggens and Galgani, 1992 and Haasch et al., 1994. Reaction mixtures consisted of 50 uL of buffer Tris-HCl (100 mM, pH=7.4), 25 uL of microsomal fraction accounting for 100 µg of protein, 10 µL of 7-ethoyxresorufin (7ER) (Sigma E3763) (0-2 µM) and 25 µL of NADPH (1 mM, tetrasodium salt) (Calbiochem® 481973). Blanks consisted of reaction mixtures with boiled microsomes. The rate of EROD activity was monitored after 10 minutes of reaction by reading the relative fluorescence units (RFU) of the resorufin produced by the reaction (excitation = 510 nm, emission = 580 nm) in a microplate absorbance-fluorescence reader (TECAN, Genios ™, Austria). A resorufin calibration curve (0 - 0.5 uM) was used for the quantification of the reaction rate. The linearity of the reaction rate was confirmed in preliminary experiments.

*Pentoxyresorufin- (PROD) and benzyloxyresorufin-O-dealkylation (BROD) activities*. PROD and BROD activities were assayed following the same protocol as for EROD. A 5 uM substrate concentration was tested as the initial concentration point. Phenobarbital-induced rat microsomes (R1078 – Xenotech, LLC) were used as a positive control to compare such activities.

Phase II assays

*Glutathione-S-transferase kinetics*. Glutathione-S-transferase (GST) activity was determined by the method of Habig et al. (1974). Reaction mixtures consisted of 165 uL of 100 mM Tris-HCl buffer (pH=7.4), 7 uL of 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma C 6396) (0.1-2.0 mM range), 3.5 uL of 60 mM reduced L-glutathione (Sigma G 6529) and 10 ug of cytosolic protein. Blanks consisted of full reaction mixtures with exception of the cytosol. The rate of CDNB conjugation with GSH was monitored after pippeting the cytosolic fraction to the reaction mixture and determining changes in absorbance ($\lambda$ = 340 nm) after 5 minutes at room temperature ($20^{o}$C). Absorbance readings were obtained using a microplate fluorescence-absorbance reader (TECAN $^{TM}$, Austria). For final calculations a molar absorption coefficient ($\epsilon$=9.6 $mM^{-1}cm^{-1}$) was used, adjusting the path length to the corresponding 96-well plate volume (Styrene microtiter® S25-291-01, ThermoLabsystems, MA).

*UDP-glucuronosyltransferase kinetics*. UDP-glucuronosyltransferase (UDPGT) activity was measured by a method modified from Schell and James (1989). Reaction mixtures consisted of 50 uL of phosphate buffer (pH= 7.4) that contained Triton X-100 (Sigma X-100) at 0.1 % and $MgCl_2$ (Sigma M-0250) at 10 mM, 100 ug of

microsomal protein, 25 uL of uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA) (Sigma U-6751) and 10 uL of resorufin (Aldrich 23,015-4) (1-50 uM). Blanks consisted of the whole reaction mixture except for the UDPGA. 96-well microplates (Styrene microtiter® S25-291-01, ThermoLabsystems, MA) were used to hold the reaction components. After mixing the buffer mixture containing Triton and $MgCl_2$ with the microsomes and the UDPGA, the microplates were left at $4^oC$ for a 20 minute pre-incubation. 10 uL of each resorufin concentration were then added to blanks and samples and a RFU reading was recorded at this point (time 0 of reaction). A second reading was performed after 10 minutes to estimate the reaction rate based on the difference between RFU in the blanks and the samples. Preliminary experiments showed the linearity of the reaction during the first 20 minutes. Samples were processed in triplicate. RFU values in the samples were expected to be lower than those in the blanks due to the UDPGA-mediated conjugation of resorufin.

*Sulfotransferase kinetics*. Sulfotransferase (ST) activity was measured by a method modified Schell & James (1989) and Beckmann (1991). Reaction mixtures consisted of 50 uL of phosphate buffer (pH= 7.4), 100 ug of cytosolic protein, 10 uL of resorufin (Aldrich 23,015-4) (0.5-20 uM) and 25 uL of adenosine 3'-phosphate 5'-phosphosulfate lithium salt (PAPS) (Sigma A-1651). Blanks consisted of the whole reaction mixture except for the PAPS. 96-well microplates (Styrene microtiter® S25-291-01, ThermoLabsystems, MA) were used to hold the reaction components. After mixing the buffer and the cytosol, 10 uL of each resorufin concentration were added to blanks and samples and a RFU reading was done at this point (time 0 of reaction). 25 uL of PAPS were pipetted into the sample wells to start the reaction (25 uL of $DDH_20$ were pipetted into the blanks). A second reading was performed after 10

minutes to estimate the reaction rate based on the difference between RFU in the blanks and the samples. Preliminary experiments showed the linearity of the reaction for at least 15 minutes. Samples were processed in triplicate. RFU values in the samples were expected to be lower than those in the blanks due to the ST-mediated conjugation of resorufin.

Calculation of kinetic parameters and statistical analyses

The maximal velocity ($V_{max}$), Michaelis-Menten constant ($K_m$) and $V_{max}/K_m$ ratio were derived by linear regression from Lineweaver-Burk (e.g., double-reciprocal) plots (Piszkiewicz, 1977; Cornish-Bowden, 1995). All results were reported as means +/- standard errors of the means (SEM). Data sets were analyzed for normality and homogeneity of variances. Data being both normal and homogeneous were compared using a two-sample T-test (comparison between laboratory-acclimated and farm-raised specimens of the same species) with a significance level set at $\alpha = 0.05$. SAS 8.2 ® (Statistical Analysis Software) program was run for this data processing.

**Results**

Phase I biotransformation reactions

$V_{max}$ , $K_m$ and $V_{max}/K_m$ values for ECOD and EROD kinetics are shown in Tables and Figures 1 and 2, respectively. Compiled data showing Michaelis-Menten and double reciprocal graphs for the individuals of each species are depicted in Appendix 1 and 2.

***ECOD kinetics.*** Significant differences in ECOD kinetics were found for $V_{max}$ values between laboratory-acclimated and farm-raised channel catfish and tilapia. $V_{max}/K_m$ was also significantly different between farm-raised and laboratory-acclimated tilapia. The rest of the parameters were quite similar between the two groups of fish in the three species (Table 1). Farm-raised channel catfish was the group with the highest $V_{max}$ of biotransformation (108 ± 6.9 pmols 7HC/min/mg protein) of all, while farm-raised tilapia was the group with the lowest biotransformation capacity (Table 1).

***EROD kinetics.*** Major differences for $V_{max}$, $K_m$ and $V_{max}/K_m$ values were present between laboratory-acclimated and farm-raised tilapia. Rainbow trout showed differences between the two groups for $K_m$ and $V_{max}/K_m$ while $V_{max}$ values were quite similar. In laboratory-acclimated channel catfish, the velocity of the reaction was very low and had negligible variations among the different 7-ER concentrations that were tested (0.18 – 1.50 μM). As a result, only the velocity at 1 uM 7-ER is reported for this group. Farm-raised catfish had a dose response allowing the calculation of the kinetics parameters.

***PROD / BROD kinetics.*** Neither laboratory-acclimated nor farm-raised fish of any of the three species showed PROD or BROD activities when a 5 μM substrate concentration pentoxyresorufin or benzyloxyresorufin was tested. Therefore, activity toward these two substrates will be reported as negligible for all species.

Phase II biotransformation reactions

GST, UDPGT and ST kinetics results are shown in Tables and Figures 3, 4 and 5, respectively. Compiled data showing Michaelis-Menten and double reciprocal graphs for the individuals of each species are depicted in Appendix 3, 4 and 5.

***GST kinetics.*** Results for $V_{max}$, $K_m$ and $V_{max}/K_m$ ratio were significantly different between laboratory-acclimated and farm-raised catfish and rainbow trout. Tilapia had very similar $V_{max}$ values between the two groups while the $K_m$ and $V_{max}/K_m$ ratio were significantly different. Laboratory-acclimated catfish was the group with the highest $V_{max}$ (1972 ± 125 nanomols CDNB / min / mg prot) while laboratory-acclimated rainbow trout had the lowest velocity of all (419 ± 32). Major differences between $V_{max}$ values were present in catfish (3-fold higher in laboratory-acclimated) and rainbow trout (2.2-fold higher in farm-raised).

***UDPGT kinetics.*** $V_{max}$, $K_m$ and $V_{max}/K_m$ ratio for UDPGT kinetics between laboratory-acclimated and farm-raised tilapia and rainbow trout had no significant differences. On the other hand, catfish microsomes did not respond to resorufin conjugation via UDPGT activity despite different protein levels were tested. Different Triton X-100 concentrations were also examined but none of them helped to detect any UDPGT activity towards resorufin in this species.

***ST kinetics.*** Significant differences in $V_{max}$ values for ST activity were present between laboratory-acclimated and farm-raised channel catfish and tilapia. In tilapia, the efficiency of the sulfoxidation reaction (e.g., $V_{max}/K_m$ ratio) towards resorufin was

significantly different between the two groups of fish. Both, laboratory-acclimated and farm-raised rainbow trout had quite similar $V_{max}$ values. As for $K_m$, with the exception of laboratory-acclimated channel catfish (0.1 μM), all the other groups had binding affinities that ranged between 0.6 and 1.0 μM. Catalytic efficiencies were significantly different between laboratory-acclimated and farm-raised tilapia. The other two species had no major differences between the two groups of fish.

## Discussion

The present study was intended to compare the kinetics of phase I and II biotransformation reactions between farm-raised and laboratory-acclimated fish assuming as the null hypothesis that no significant changes were present in the biotransformation reactions between the two groups of fish for each species.

### Phase I biotransformation reactions

***ECOD kinetics.*** ECOD has been studied in mammals as a marker activity of CYP1A1, CYP1A2 CYP2B1, CYP2E1, and CYP2E6 (Ryan & Levin, 1990; Yun et al., 1991; Yamazaki et al., 1996). In fish, the catalysts of 7EC are still unknown; nevertheless, this reaction is apparently catalyzed in rainbow trout by the CYP1A forms since treatment by CYP1A inducers enhances the formation of 7-hydroxycumarin (7HC) (Cravedi et al., 1998). Among the inducers of ECOD activity in fish species are Aroclor 1254 (a PCB mixture) (Elcombe & Lech, 1979) and β-naphthoflavone (Haasch et al., 1994) in rainbow trout, 3-methylcholanthrene and polychlorinated biphenyls in tilapia (*niloticus x aureus*) (Ueng et al., 1992; Ueng &

Ueng, 1995) and phenobarbital (Ueng et al., 1992) as well as PAHs and PCBs (Machala et al., 1997) in carp. Most of these compounds have also been found to be inducers of EROD activity. On the other hand, there is substantial ECOD activity in non-CYP1A induced catfish, suggesting additional constitutive role although the isozyme responsible for this activity has not been identified (Perkins, 1999; Perkins et al., 2000).

In the present study channel catfish had the highest ECOD activity of all the three species. $V_{max}$ values found in farm-raised (108.0 ± 6.9 pmols 7-HC/min/mg protein) was quite similar to the activity found by Perkins (1999) (109.0 ± 3.0 pmols 7-HC/min/mg protein) and almost doubled the values found in the laboratory-acclimated specimens (59.2 ± 7.3).

The $V_{max}$ values found for laboratory-acclimated and farm raised-tilapia (66.3 ± 5.1 and 32.0 ± 0.9 pmols 7-HC/min/mg protein, respectively) are below the ones reported in other studies. Ueng et al. (1992) and Ueng & Ueng (1995) found 143.0 ± 27.0 and 311.0 ± 38.0 pmols 7-HC/min/mg protein for ECOD activity in non-induced tilapias weighing 200-300 grams. These values are between 2 and 5-fold higher than the ones in the present work. These authors worked ECOD activity at 37$^{o}$C while our incubations were performed at room temperature (20-21$^{o}$C). This factor may partially explain such differences between the studies. Despite the differences found between farm-raised and laboratory-acclimated tilapias, both sets of values are considerably lower than the ECOD values obtained from 3-methylcholantrene-induced tilapias (433 ± 84 and 890 ± 65), and PCB mixture (Aroclor 1254)-induced tilapias (351 ± 49 and 543 ± 41) (Ueng et al., 1992; Ueng & Ueng, 1995; respectively).

ECOD kinetic parameters had no differences between farm-raised and laboratory-acclimated rainbow trout (Table 1). ECOD values in the present study for both farm-raised (42.1 pmols / min / mg protein) and laboratory-acclimated rainbow trout (50.3 pmols / min / mg protein) were lower than those found by Kleinow et al. (1990) in the same species (80 pmols/ min/mg protein).

$K_m$ values were quite similar between farm-raised and laboratory-acclimated fish of the three species (Table 1). This indicates that the binding affinity for the substrate was very similar among the two different groups of fish for each species. Catalytic efficiency ($V_{max}/K_m$) was statistically different only between farm-raised (1.7 ± 0.3) and laboratory-acclimated (5.9 ± 0.7) tilapia (Figure 2).

***EROD kinetics*.** The response found in EROD activity for laboratory-acclimated catfish suggests a very low constitutive CYP1A1 activity in this particular group as compared to their farm-raised counterparts. In addition to the low catalytic activity, when a range of 7-ER concentrations from 0.18 to 1.5 μM was tested, no changes were seen in the velocity of the reaction. EROD activity as low as the one found in our study (6.6 pmols/min/mg protein) has been reported in non-induced catfish by Ankley et al. (1987) (6.8 to 24.2 pmols/min/mg protein), and Murphy & Gooch (1997) (14-41 pmols/min/mg protein). The $V_{max}$ found in the farm-raised catfish (39 ± 7 pmols/min/mg protein) is also within the range of the activity reported in these studies. In the same report by Murphy and Gooch (1997), EROD activity was compared in laboratory-acclimated, wild-captured- and benzo[a]pyrene-induced-catfish. EROD activity in the wild fish (Back River), 76 pmols resorufin/min/mg protein, was 3.5-fold higher than the one reported for the laboratory-acclimated fish

(14-41 pmols resorufin.min/mg protein). The BaP-induced fish had a significantly higher EROD activity (~ 140 pmols resorufin/min/mg protein) than the laboratory-acclimated fish. In our study, despite the differences between the laboratory-acclimated (6.6 pmols resorufin/min/mg protein) and the farm-raised catfish (39 pmols/min/mg protein), both groups had similar EROD activities to the ones reported in non-induced catfish in other investigations. Differences in baseline EROD activity and after induction have been reported between male and female fish (Elksus et al., 1992 cited by Arinç et al., 2000; Whyte et al., 2000). Stegeman and Hahn (1994) report that EROD activity is higher in reproductively active and inactive rainbow trout males, and reproductively inactive females, when compared to reproductively active females. The authors concluded that there seems to be a direct correlation between CYP1A suppression and high 17β-estradiol levels in spawning females. In the present study, both the laboratory-acclimated and the farm-raised cattfish had 1 female and 3 males each. No differences in EROD activity linked to gender could be used to explain the high average value for the farm-raised specimens.

Rainbow trout showed quite similar $V_{max}$ values in both laboratory-acclimated and farm-raised fish. These values are also quite similar to the ones cited by Haasch et al. (1994) in non-induced rainbow trout microsomes tested at 1 uM substrate concentration (18.2 pmols/min/mg protein). However, it is notable that rainbow trout is cited as one of the species with the most variable baseline EROD enzymatic activity (Whyte et al., 2000). Although $V_{max}$ values in this species were very similar between laboratory-acclimated and farm-raised fish, enzyme affinity for the substrate ($K_m$) differed markedly between the two groups. In turn, the $V_{max}/K_m$ ratio was also

significantly affected. Gender distribution for these two groups was 7 males for laboratory-acclimated and 2 immature / 1 male for the farm-raised.

In tilapia, higher $V_{max}$ and $K_m$ values for EROD activity were found in the farm-raised tilapia as compared to the ones in the laboratory-acclimated fish. These results determined a significant difference in EROD catalytic efficiency. However, both parameters in farm-raised and laboratory-acclimated fish are within the ranges reported in different species and hybrids of non-induced tilapia. In other studies in which the same substrate concentrations were tested, Ueng and Ueng (1995) reported 38 ± 9 pmols resorufin/min/mg protein in *O. niloticus* x *aureus*, while EROD activity for *O. niloticus* was 80 ± 25 pmols/min/mg protein (Gadagbui et al., 1996), and for *O. mossambicus* was 40 ± 5 pmols/min/mg protein (Ueng et al., 1992). Despite similarities between these reports and our study, it is worth mentioning that while the former were of small size fish (40 ~ 130 grams of body weight, approximately), market-size fish (500 ~ 900 grams) were used in the present work.

*PROD / BROD kinetics*. PROD and BROD activities have been used to a lesser extent than other catalytic probes in fish. It is known that PROD and BROD are detectable in rainbow trout after induction with dexametasone and isosafrole, respectively (Haasch et al., 1994). Neither farm-raised nor laboratory-acclimated fish of any of the three species in the present work showed either PROD or BROD activities. Although the P450 isoforms responsible for PROD or BROD activities have not been identified in fish, their inducibility in fish may open alternatives for their use as biomarkers. For example, tilapia from contaminated waters in the Guandu River, Brazil, were found

to have higher PROD activity (23 ± 19 pmols resorufin/min/mg protein) as compared to non-detectable activity in reference-site fish (Parente et al., 2004).

Phase II biotransformation reactions

*GST kinetics*. With the exception of $V_{max}$ values in tilapia, all the other kinetics parameters showed significant differences between laboratory-acclimated and farm-raised in the three species used in this work. Nutritional status, dietary regimes, and water temperature are among the factors that have been considered as possible sources of variation in GST expression in different studies using fish.

In a study by Morrow et al. (2004), rainbow trout that were offered different protein (35 – 55%) and lipid (8 - 18%) levels in isocaloric diets, did not show any change in GST activity among the different treatments. Food deprivation has also been tested as a cause of change in GST activity. Blom et al. (2000) found a significant reduction in rainbow trout GST activity after 3 and 7 weeks of food deprivation. Andersson et al. (1985) reported the same effect after 6 weeks of food deprivation. Neither farm-raised nor laboratory-acclimated fish in our study were subjected to prolonged starvation periods. Farm-raised fish were food-deprived only during the time of delivery to our facilities.

Ethoxyquin, a common antioxidant used in animal feed, has been reported as an inducer of GST-CDNB conjugation (1.6-fold as compared to controls) in brown bullhead (*Ictalurus nebulosus*) (Henson et al., 2001). Ethoxyquin and other antioxidants like butylated hydroxyanisole are considered to be more effective

inducers of GST expression than the A*h* receptor agonists (Henson et al., 2001). On the other hand, ethoxyquin is also implicated with lowering P450 protein and cytochrome b5 contents in rainbow trout that were fed diets that contained 5.6 mM of the antioxidant (Eisele et al., 1983). We did not perform a proximate analysis or specific inclusion or deprivation of particular elements in the farm-raised or laboratory-acclimated fish diets. Although, diets offered to laboratory-acclimated and farm-raised fish were commercial-type, different components may have been present in formulations affecting the expression of phase I-II biotransformation reactions.

Water temperature changes have been associated with effects on GST activity in fish. George et al. (1990) found that GST activity was inversely correlated with water temperature in immature plaice (*Pleuronectes platessa*). Conversely, Ronisz et al. (1999) found a positive correlation between water temperature and GST activity in juvenile rainbow trout. Of the three species used in this work, farm-raised rainbow trout and channel catfish specimens were obtained from outdoors facilities during early spring time. Channel catfish were shipped from a farm located in Missouri where mild temperatures usually prevail during this time of the year. Thus, it is possible that these fish were acclimated to lower temperatures than their laboratory-acclimated counterparts (20 to 22 $^{o}$C) in our facilities. The farm-raised rainbow trout that were maintained outdoors in raceways at an aquaculture farm in Maryland were most likely acclimated to quite similar temperatures compared to the fish acclimated in the lab (13-16 $^{o}$C). Interestingly, differences between farm-raised and laboratory-acclimated kinetics are evident in our study despite having a similar acclimation temperature.

The stress conditions that our farm-raised fish underwent prior to liver harvesting (e.g., short-time food deprivation, transportation stress, etc.) were minor as compared to those reported in protocols that were tested in other works previously cited. In fact, for some of these reports, differences in GST activities have been found only when the modified feeding regime, feed composition or water temperature was maintained for long periods (e.g., weeks) during the experimental time. GST regulation and expression under different physiological conditions is not fully understood in fish.

*UDPGT kinetics*. Kinetics parameters for UDPGT using resorufin as a phase II substrate showed no variation between laboratory-acclimated and farm-raised tilapia and rainbow trout. Very similar $V_{max}$, $K_m$ and $V_{max}/K_m$ values were found in both groups of fish for these two species. On the other hand, channel catfish microsomes did not show UDPGT activity towards resorufin. Despite testing different conditions (e.g. protein levels, detergent concentration) for the *in vitro* UDPGT assay with channel catfish microsomes, no response was obtained in this species. Different degrees of response among fish species are cited in the literature. Plaice (*Pleuronectes platessa*) is one of the piscine species with the highest UDPGT-mediated conjugation capabilities using different substrates (George, 1994). This species has been found to have even higher UDPGT conjugation than mammals. However, we are not aware of other studies where lack of response to resorufin as a phase II substrate is cited for channel catfish. As it was noted in Chapter 2, channel catfish was the only species out of 8 different fish species that did not conjugate resorufin through UDPGT activity in the present study. We have no explanation for this lack of response to resorufin in channel catfish UDPGT.

Changes in the expression of UDPGT activity due to environmental or physiological factors have been partially studied in fish. Parameters such as water temperature, salinity level, feed availability and composition, as well as handling stress, have been analyzed to determine their possible implications on UDPGT activity in fish. For example, analyzing the effect of water temperature changes due to seasonality on UDPGT activity, immature plaice (*Pleuronectes platessa*) was reported to have higher activity during the spring compared to the lowest levels in summer (George et al., 1990). Koivusaari et al. (1981) found slightly decreasing liver UDPGT activity in rainbow trout during the fall. As for water salinity, Nagayama et al. (1968), cited by Clarke et al. (1991), did not find any effects on UDPGT activity due to acclimation of rainbow trout and tilapia to salt water.

Prolonged starvation was considered by Blom et al. (2000) as a source of UDPGT activity variation in rainbow trout. These authors found that after 3 and 7 weeks of food deprivation, fish had 150% and 200% higher liver UDPGT activities, respectively, than the controls. Conversely, Andersson et al. (1985) did not report any significant change in UDPGT activity in rainbow trout that was subject to food deprivation for 6 and 12 weeks. A reduced UDPGT activity towards p-nitrophenol has been found in mammals after starvation (Mandl et al., 1995). Prior to liver processing, our farm-raised fish experienced food deprivation only during the time spent for transportation to the lab facilities (i.e., < 24h).

Handling stress has been referenced as a source of change in UDPGT activity in fish. Juvenile RBT that were chronically stressed by chasing them with a net for 5 minute-periods during 7 days, had an increased UDPGT activity compared to controls (Blom

and Förlin, 1997). In our study, farm-raised fish were shipped overnight (e.g., catfish) or brought directly from the farm (e.g., tilapia, rainbow trout) a few hours before processing the livers. No other source of distress was evident prior to liver processing. UDPGT kinetics parameters had the lowest variation between farm-raised and laboratory-acclimated fish of all the phase I-II biotransformation reactions that were tested in this study. Both tilapia and rainbow trout specimens were all males.

*ST kinetics*. UDPGT and ST kinetics were evaluated using resorufin as a substrate in the present study. To the best of our knowledge, no previous studies in fish using resorufin as a phase II substrate for conjugation have been reported in the literature. ST has not been fully studied in fish despite some suggestions about its important role in detoxification and internal homeostasis. For example, studies on hepatic and intestinal ST activity in catfish (Tong and James, 2000; van den Hurk et al., 2002) and zebrafish (Ohkimoto et al., 2004), after exposure to PCBs and phytoestrogens, respectively, suggest important roles of this phase II biotransformation system in estrogen-like effects of contaminants due to deleterious effects on ST-mediated conjugation reactions.

Greater differences occurred between farm-raised and laboratory-acclimated fish in ST kinetics parameters when compared to the UDPGT results in the present work. $V_{max}$ values were significantly different between laboratory-acclimated and farm-raised channel catfish and tilapia. Channel catfish was the only species with significant changes between the two groups for enzyme binding affinity ($K_m$). $V_{max}/K_m$ ratios were significantly different in tilapia but not in the other two species.

ST activity has been regarded as a low capacity-high affinity reaction. In mammals, it is reported that at low concentrations of phenolic compounds, ST-mediated reactions prevail over UDPGT-based conjugations (George, 1994). Our results using resorufin as a substrate indicate the same trend. $V_{max}$ values were always significantly lower in ST reactions than in the corresponding UDPGT conjugations. As for enzyme binding affinity, ST reactions had also much lower $K_m$ values (e.g., higher affinity) than those found in the UDPGT-mediated reactions.

*Gender-related differences between farm-raised and laboratory-acclimated tilapia, channel catfish and rainbow trout*. Gender and hormonal profiles are among the physiological variables that may affect biotransformation reactions within species. Reduced expression of microsomal isoforms such as CYP2M1 (LMC1), CYP2K1 and CYP3A27 (LMC5) has been found in estradiol-treated salmonid species such as brook trout (*Salvelinus fontinalis*) (Pajor et al., 1990) and rainbow trout (*Oncorhynchus mykiss*) (Vodicnik &Lech, 1983). Different responses in EROD activity have also been attributed to steroids in black bream (*Acanthopagrus butcheri*) females compared to males (Webb et al., 2005). The mechanism that determines the estrogen derived down-regulation in P450 expression seems to involve the estrogen receptor. This receptor is part of the nuclear receptor superfamily (MacKay etal., 1996).

Androgens are also implicated with significant reduced hepatic metabolism through P450 isoforms. Administration of 11-ketotestosterone in particular was responsible for decreased hepatic microsomal biotransformation of B[a]P and p-nitroanisole in rainbow trout (Förlin & Hansson, 1982).

Due to the random sampling that we applied in this study for both the farm-raised and the laboratory-acclimated fish, our animals did not show an even sample size for gender. In some cases the differences observed in the kinetics between the laboratory-acclimated and the farm-raised fish happened despite having the same gender in the two groups (e.g., ECOD catfish, ECOD and EROD tilapia, SULF tilapia). Based on data collected from this study, any differences observed within species (e.g. GST in rainbow trout) cannot be associated with possible gender effect.

It is important to mention that not only gender *per se* should be considered as the source of variation in phase I and II biotransformation reactions in fish. The hormonal fluctuations during a reproductive cycle (e.g.. estrogen levels) are also a key factor that may impose gender-based differences on phase I and II activities. Females can show high phase I constitutive activity when low estradiol levels are present in reproductively inactive phases of their cycles, while the activity is significantly reduced during pre-spawning season (Scott & Sumpter, 1983). The similarities between genders in some of the phase I and II reactions in this study could be attributed to the hormonal profiles of the specimens associated with the reproductive maturation stage at the time of sampling. Changes between farm-raised and laboratory-acclimated fish in the present study should then be explained based on other physiological or environmental factors.

**Conclusions**

Despite statistical differences between farm-raised and laboratory-acclimated fish, ECOD and EROD $V_{max}$ values were within the range found in non-induced fish in

other studies. These statistical differences may have little or no biological relevance considering that the values are far from the ones reported in fish that have been exposed to contaminants or other sources of inducers. Neither farm-raised nor laboratory-acclimated fish of any of the three species showed PROD and / or BROD activities.

UDPGT activity was the phase II biotransformation reaction with the least amount of variation between farm-raised and laboratory-acclimated tilapia and rainbow trout. Channel catfish microsomes were unresponsive to resorufin as a UDPGT-conjugating substrate. ST activity showed variability between farm-raised and laboratory-acclimated fish for some of the kinetics parameters and was characterized by a lower capacity and higher enzymatic binding affinity as compared to UDPGT.

GST kinetics between farm-raised and laboratory-acclimated fish had the highest variability between the two groups of fish. No specific factors that we are aware of could be attributed to explain such variations between laboratory-acclimated and farm-raised fish for GST-based conjugation.

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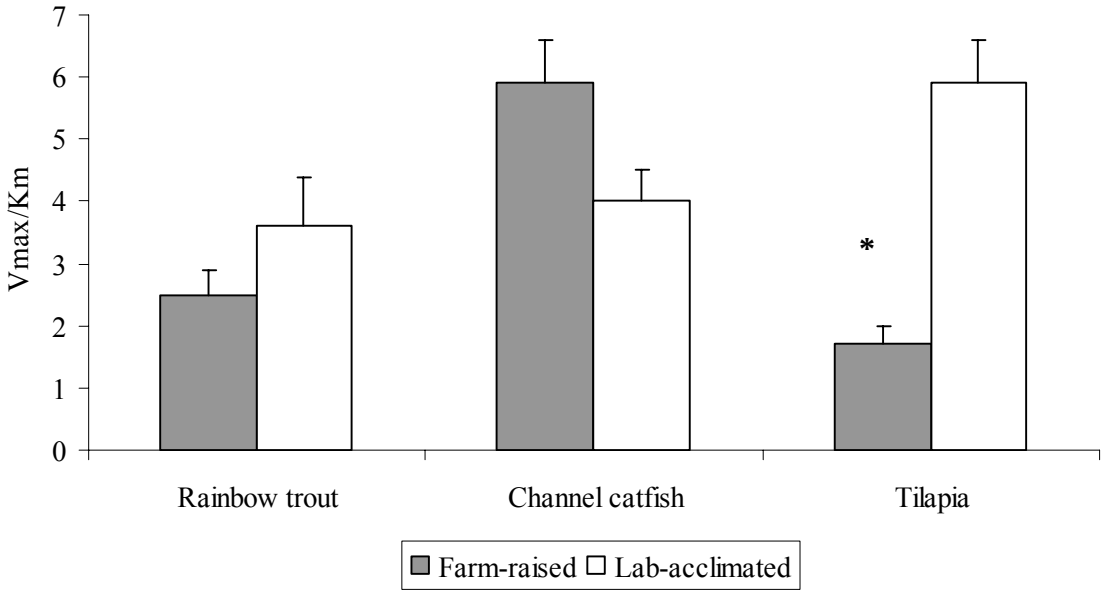
Table 1. $V_{max}$ and $K_m$ values (means ± SEM) for ECOD activity in farm-raised and laboratory-acclimated rainbow trout, channel catfish and tilapia

| **Species** (sample size) (gender distribution) | **$V_{max}$** (pmols 7-hydroxycoumarin/min/mg protein) | **$K_m$** (μM) |
|---|---|---|
| F-Channel catfish (4) (3M:1F) | 108.0 ± 7.0 | 19.0 ± 1.2 |
| L-Channel catfish (6) (3M:3F) | 59.2 ± 7.3 * | 15.2 ± 1.3 |
| F-Rainbow trout (7) (F) | 42.0 ± 2.3 | 19.0 ± 2.5 |
| L-Rainbow trout (6) (M) | 50.3 ± 3.8 | 17.9 ± 4.3 |
| F-Tilapia (5) (M) | 32.0 ± 0.9 | 20.3 ± 3.4 |
| L-Tilapia (6) (M) | 66.3 ± 5.1 * | 11.7 ± 0.6 |

F: farm-raised, L: laboratory-acclimated

* asterisk in the same column denotes statistically significant difference between farm-raised and laboratory-acclimated fish of the same species ($p < 0.05$)

Figure 1. $V_{max}/K_m$ ratio (means ± SEM) for ECOD kinetics in farm-raised vs. laboratory-acclimated rainbow trout, channel catfish and tilapia (n = 4 - 8 per species)




* Statistically significant difference ($p < 0.05$) between farm-raised and laboratory-acclimated fish of the same species.

Table 2. $V_{max}$ and $K_m$ values (means ± SEM) for EROD activity in farm-raised and laboratory-acclimated rainbow trout, channel catfish and tilapia

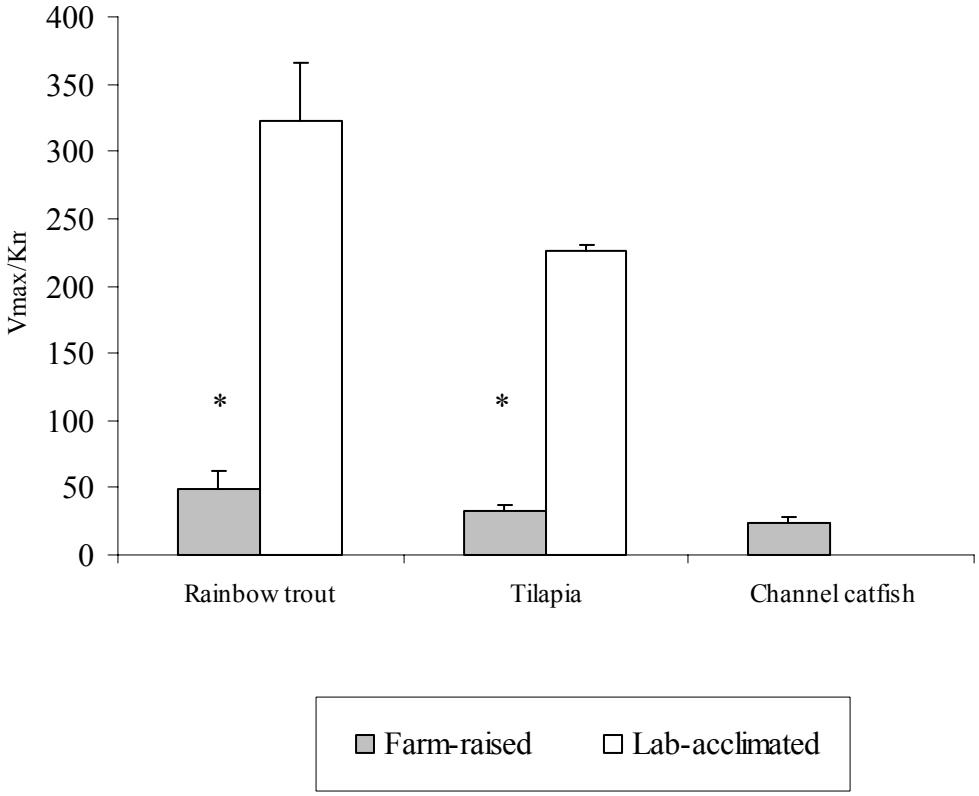
| **Species** (sample size) (gender distribution) | **$V_{max}$** (pmols resorufin/min/mg protein) | **$K_m$** (μM) |
|---|---|---|
| F-Channel catfish (4) (3M:1F) | 39.0 ± 7.0 | 1.8 ± 0.5 |
| † L-Channel catfish (4) (3M:1F) | 6.6 ± 1.6 | - |
| F-Rainbow trout (3) (F) | 28.0 ± 8.0 | 0.6 ± 0.07 |
| L- Rainbow trout (7) (M) | 30.0 ± 5.0 | 0.1 ± 0.01 * |
| F-Tilapia (7) (M) | 74.0 ± 15.0 | 2.1 ± 0.3 |
| L-Tilapia (8) (M) | 32.0 ± 2.0 * | 0.2 ± 0.04 * |

F: farm-raised, L: laboratory-acclimated

† Velocity is reported at 1 uM substrate concentration (due to very low activity neither $V_{max}$ or $K_m$ values are reported)

* asterisk in the same column denotes statistically significant difference between farm-raised and laboratory-acclimated fish of the same species ($p < 0.05$)

Figure 2. $V_{max}/K_m$ ratio (means ± SEM) for EROD kinetics in farm-raised vs. laboratory-acclimated rainbow trout, channel catfish and tilapia (n = 3 - 8 per species)




* Statistically significant difference ($p < 0.05$) between farm-raised and laboratory-acclimated fish of the same species.

Lack of response to different 7-ER concentrations in L-CC did not allow the calculation of $V_{max}/K_m$ ratio for this group.

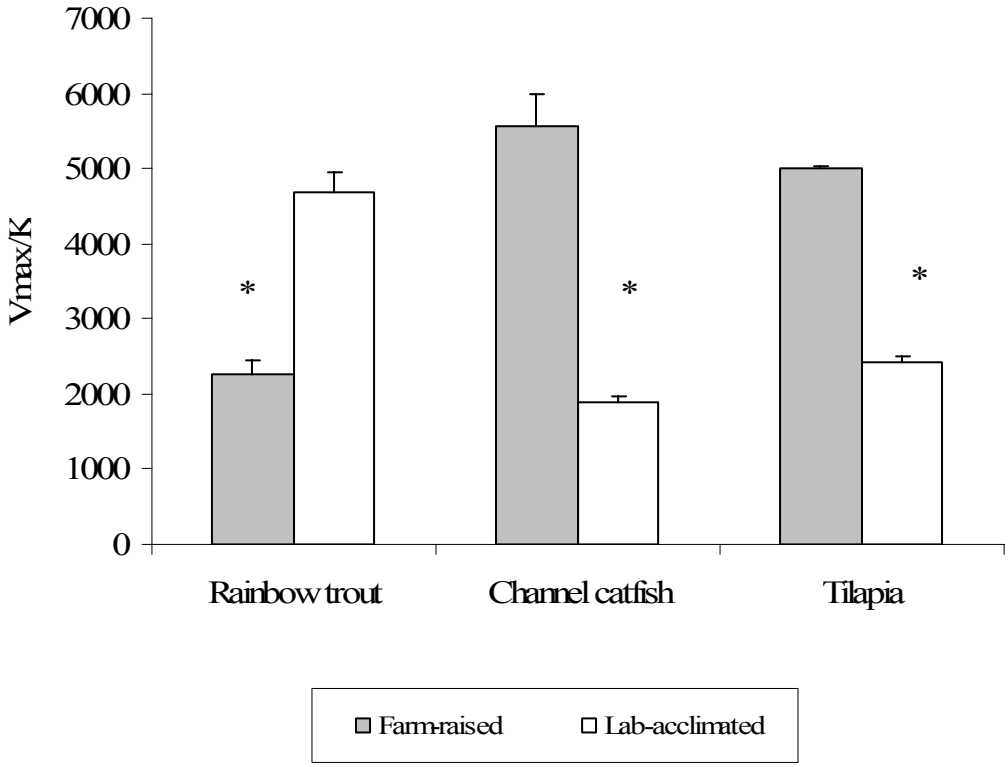
Table 3. $V_{max}$ and $K_m$ values (means ± SEM) for glutathione-S-transferase kinetics in farm-raised and laboratory-acclimated rainbow trout, channel catfish and tilapia

| **Species** (sample size) | **$V_{max}$** (nmols CDNB/min/mg protein) | **$K_m$** (mM) |
|---|---|---|
| F-Channel catfish (8) (7M:1F) | 657 ± 39 | 0.1 ± 0.02 |
| L-Channel catfish (6) (3M:3F) | 1972 ± 125 * | 1.1 ± 0.10 * |
| F-Rainbow trout (8) (F) | 929 ± 65 | 0.4 ± 0.05 |
| L-Rainbow trout (7) (M) | 419 ± 32 * | 0.1 ± 0.01 * |
| F-Tilapia (8) (M) | 1508 ± 70 | 0.3 ± 0.01 |
| L-Tilapia (7) (M) | 1474 ± 109 | 0.6 ± 0.05 * |

F: farm-raised, L: laboratory-acclimated

* asterisk in the same column denotes statistically significant difference between farm-raised and laboratory-acclimated fish of the same species ($p < 0.05$)

Figure 3. $V_{max}/K_m$ ratio (means ± SEM) for glutathione-s-transferase kinetics in farm-raised vs. laboratory-acclimated rainbow trout, channel catfish and tilapia (n = 6 - 8 per species)




*: Differences ($p < 0.05$) between farm-raised and laboratory-acclimated fish of the same species

Table 4. $V_{max}$ and $K_m$ values (means ± SEM) for UDP-glucuronosyltransferase activity in farm-raised and laboratory-acclimated rainbow trout, channel catfish and tilapia.

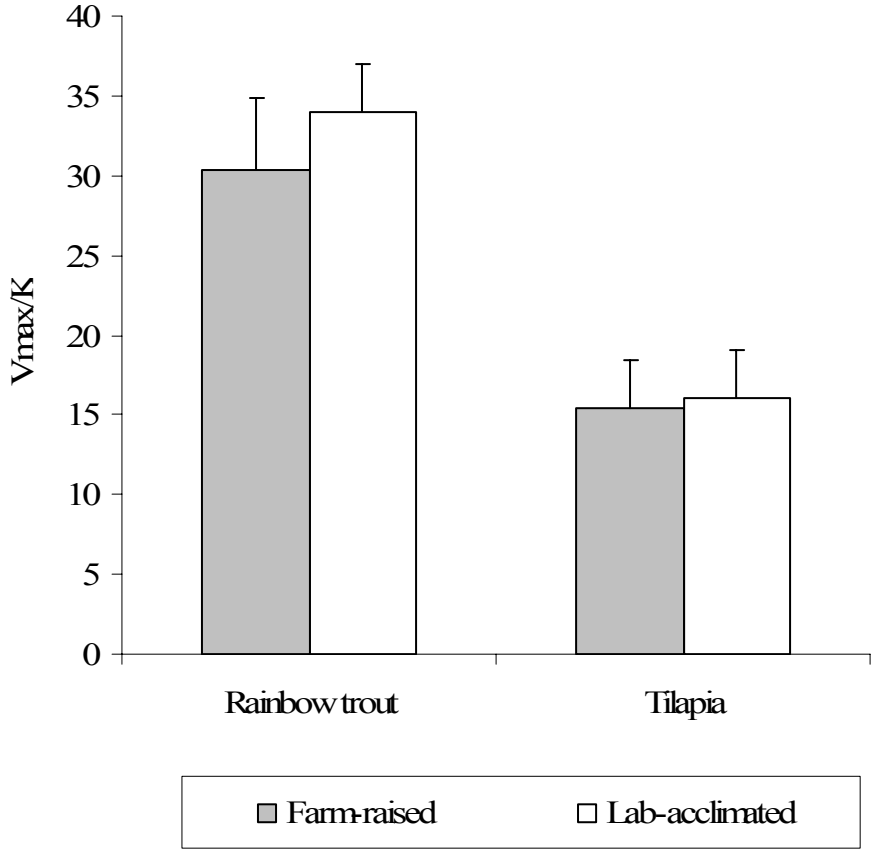
| **Species** (sample size) (gender distribution) | **$V_{max}$** (pmols resorufin/min/mg protein) | **$K_m$** (μM) |
|---|---|---|
| † F-Channel catfish (5) (4M:1F) | - | - |
| † L-Channel catfish (6) (3M:3F) | - | - |
| F-Rainbow trout (4) (F) | 930 ± 258 | 32.3 ± 8.7 |
| L-Rainbow trout (8) (M) | 834 ± 208 | 25.0 ± 6.0 |
| F-Tilapia (6) (M) | 368 ± 89 | 28.5 ± 8.7 |
| L-Tilapia (6) (M) | 400 ± 88 | 29.0 ± 9.0 |

F: farm-raised, L: laboratory-acclimated

† No UDPGT activity was detected using resorufin as substrate in channel catfish

* asterisk in the same column denotes statistically significant difference between farm-raised and laboratory-acclimated fish of the same species ($p < 0.05$)

Figure 4. $V_{max}/K_m$ ratio (means ± SEM) for UDP-glucuronosyltransferase kinetics in farm-raised vs. laboratory-acclimated fish (n = 4 - 8 per species)




***** Statistically significant difference ($p < 0.05$) between farm-raised and laboratory-acclimated fish of the same species

No UDPGT activity was detected using resorufin as substrate in channel catfish

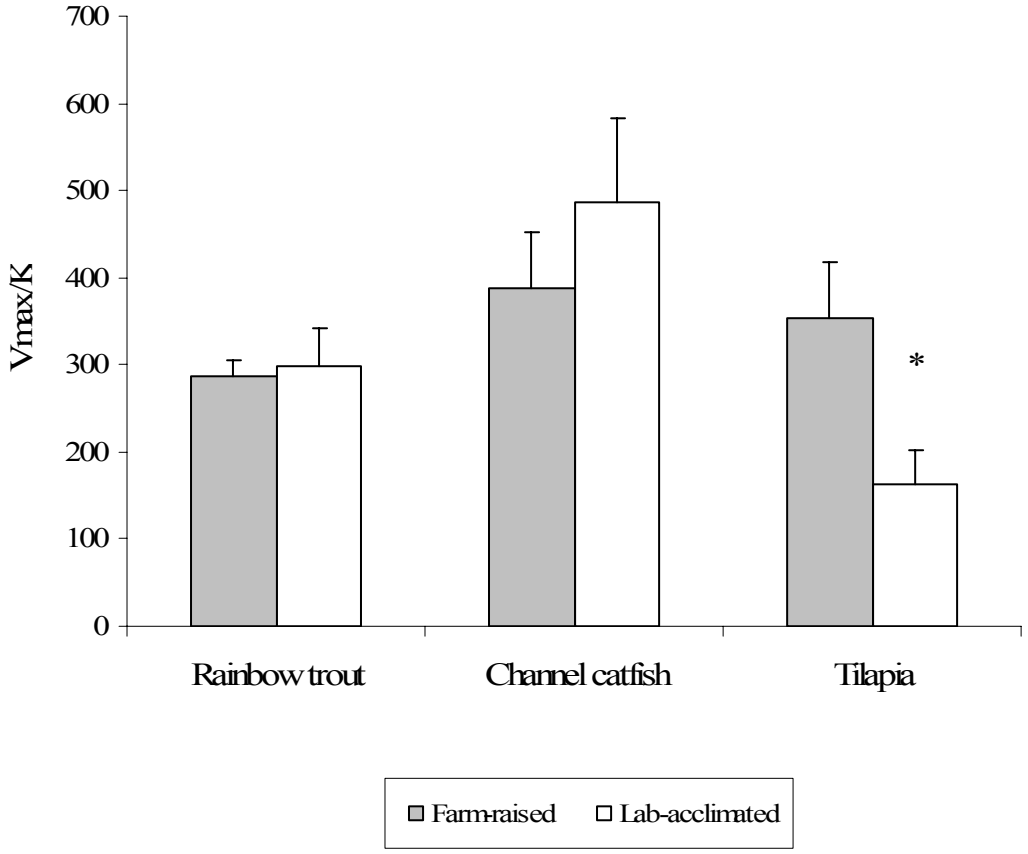
Table 5. $V_{max}$ and $K_m$ values (means ± SEM) for sulfotransferase activity in farm-raised and laboratory-acclimated rainbow trout, channel catfish and tilapia

| **Species** (sample size) | **$V_{max}$** (pmols resorufin / min /mg protein) | **$K_m$** (μM) |
|---|---|---|
| F-Channel catfish (5) (4M:1F) | 265 ± 27 | 0.8 ± 0.1 |
| L-Channel catfish (3) (2F:1M) | 49 ± 10 * | 0.1 ± 0.0 * |
| F-Rainbow trout (8) (F) | 190 ± 20 | 0.7 ± 0.1 |
| L-Rainbow trout (8) (M) | 239 ± 19 | 0.9 ± 0.1 |
| F-Tilapia (5) (M) | 328 ± 17 | 1.0 ± 0.2 |
| L-Tilapia (5) (M) | 86 ± 9 * | 0.6 ± 0.1 |

F: farm-raised, L: laboratory-acclimated

* asterisk in the same column denotes statistically significant difference between farm-raised and laboratory-acclimated fish of the same species ($p < 0.05$)

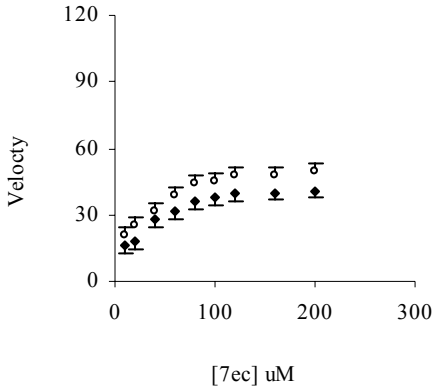
Figure 5. $V_{max}/K_m$ ratio (means ± SEM) for sulfotransferase kinetics in farm-raised vs. laboratory-acclimated fish (n = 3 - 8 per species)

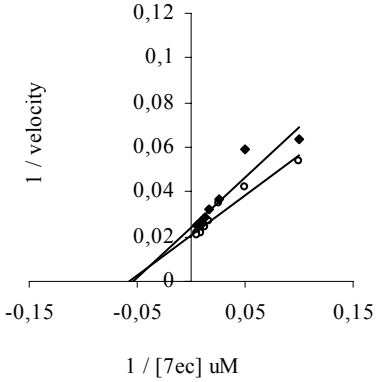



***** Statistically significant difference ($p < 0.05$) between farm-raised and laboratory-acclimated fish of the same species

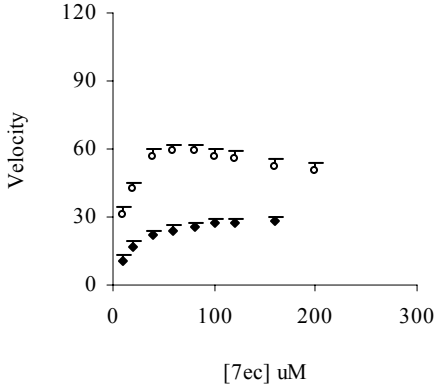
Appendix 1. Michaelis-Menten and double reciprocal plots for ECOD activity in farm-raised (◆) and laboratory-acclimated (○) rainbow trout, tilapia and channel catfish. Velocity expressed as pmols hydroxycoumarin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 6 to 8 fish per species.

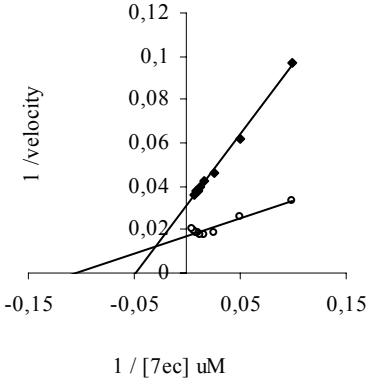
**Rainbow trout**



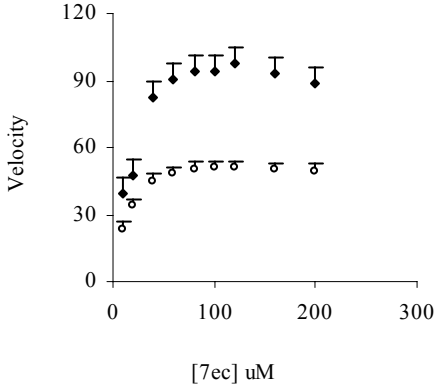



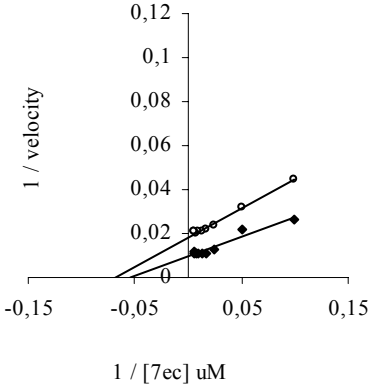

**Tilapia**



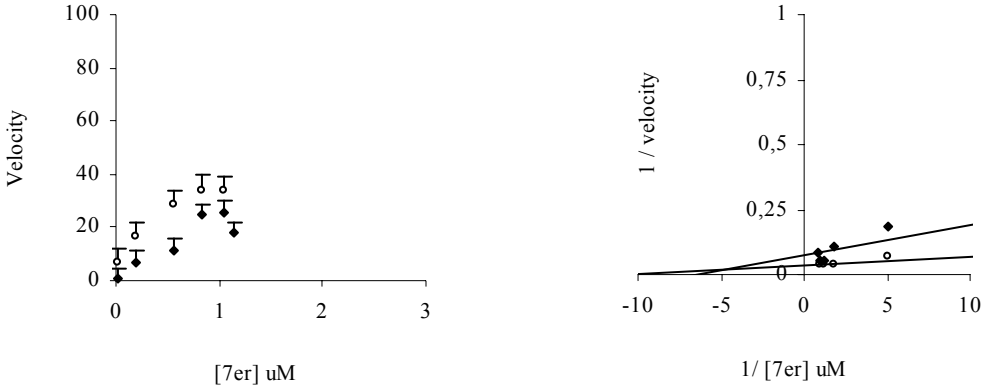

**Channel catfish**



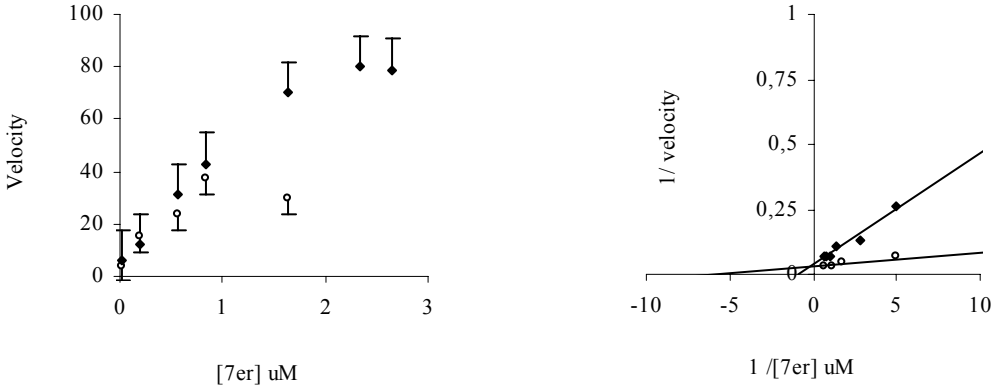



Appendix 2. Michaelis-Menten and double reciprocal plots for EROD activity in farm-raised (◆) and laboratory-acclimated (○) rainbow trout, tilapia and channel catfish. Velocity expressed as pmols resorufin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 3 to 8 fish per species.

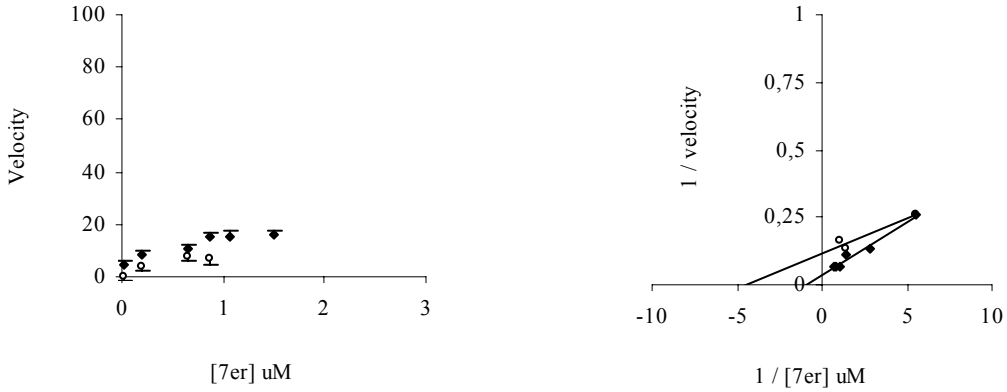
**Rainbow trout**




**Tilapia**

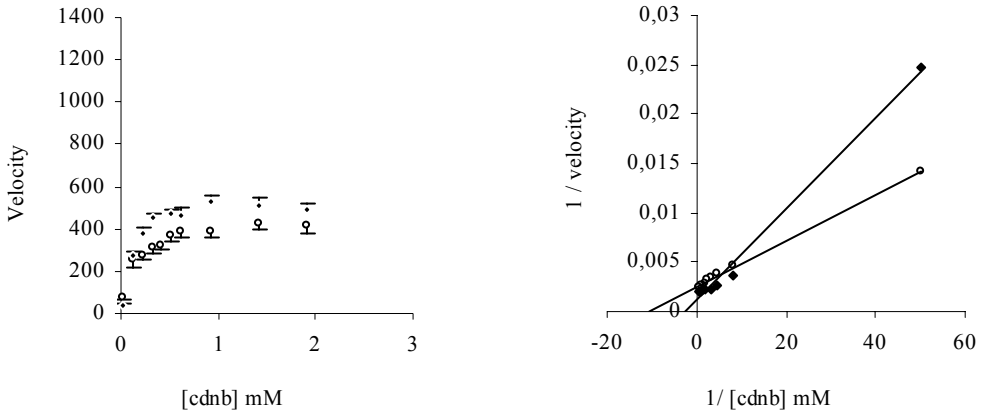



**Channel catfish**

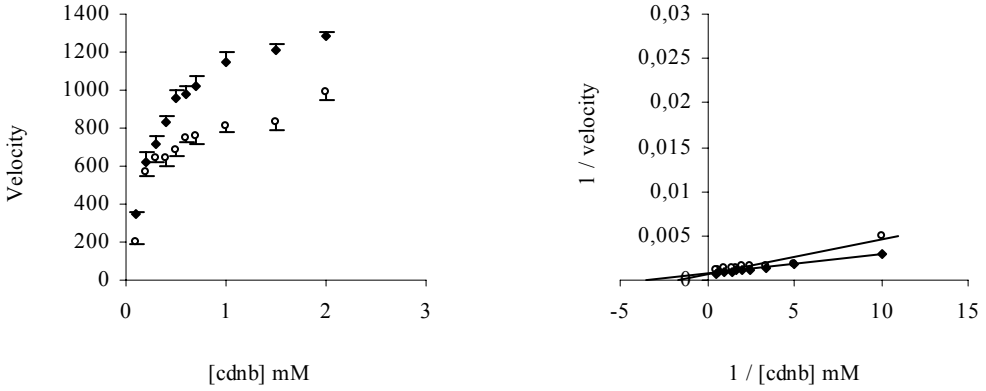


Appendix 3. Michaelis-Menten and double reciprocal plots for GST activity in farm-raised (◆) and laboratory-acclimated (○) rainbow trout, tilapia and channel catfish. Velocity expressed as nmols cdnb/min/mg protein and $K_m$ in mM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 3 to 8 fish per species.

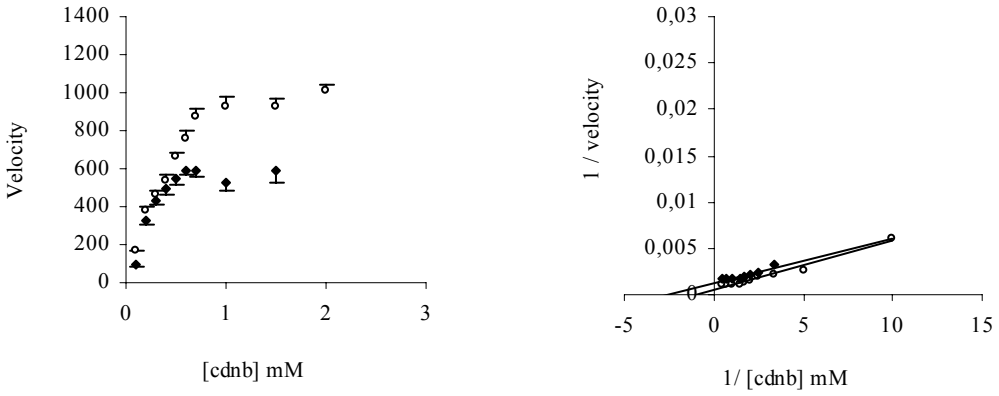
**Rainbow trout**




**Tilapia**

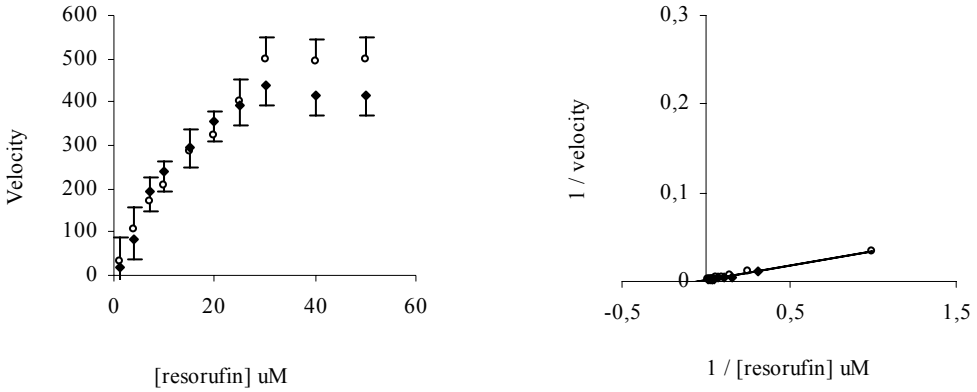



**Channel catfish**

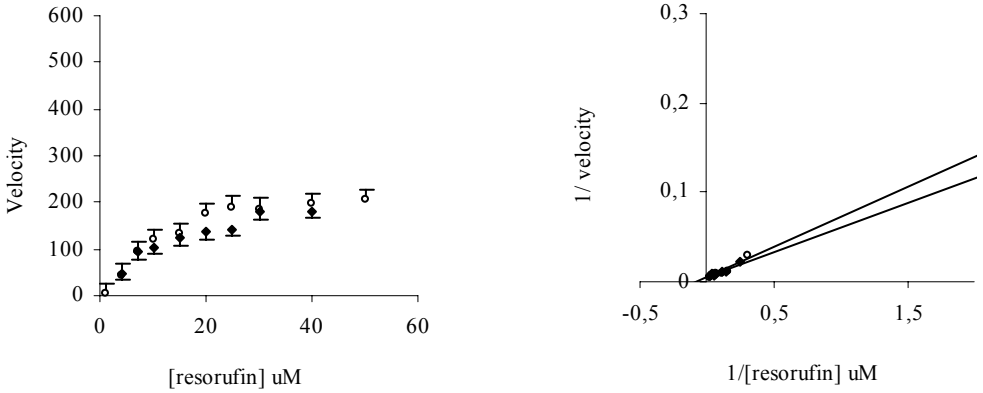


Appendix 4. Michaelis-Menten and double reciprocal plots for UDPGT activity in farm-raised (◆) and laboratory-acclimated (○) rainbow trout and tilapia. Velocity expressed as pmols resorufin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 4 to 8 fish per species.

**Rainbow trout**

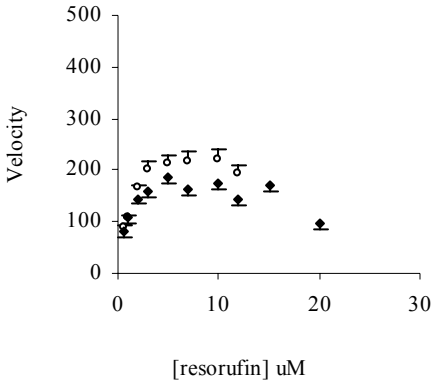


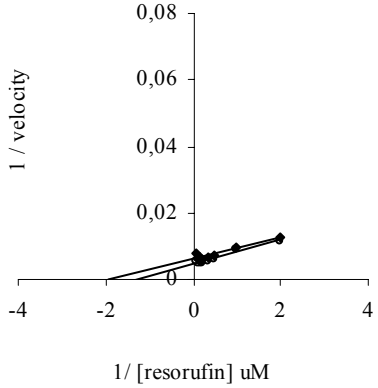

**Tilapia**



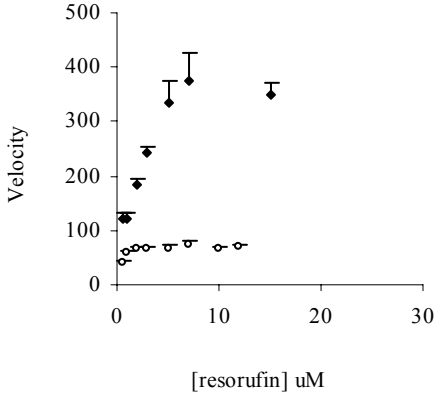
Appendix 5. Michaelis-Menten and double reciprocal plots for SULF activity in farm-raised (◆) and laboratory-acclimated (○) rainbow trout, tilapia and channel catfish. Velocity expressed as pmols resorufin/min/mg protein and $K_m$ in uM. Each data point in Michaelis-Menten plot indicates mean ± S.E. from 3 to 8 fish per species.

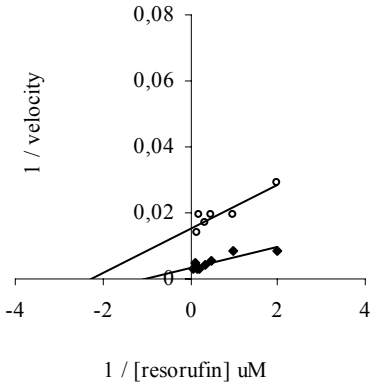
**Rainbow trout**



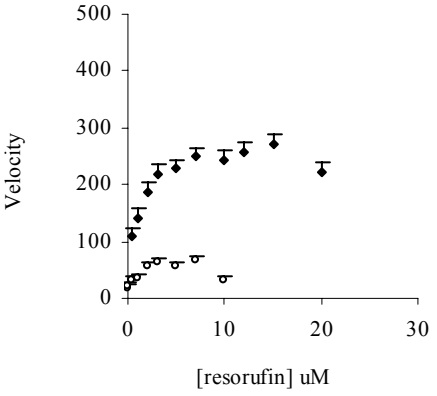



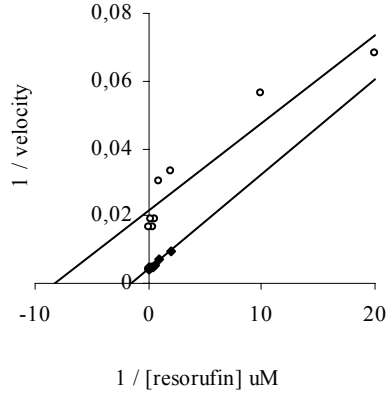

**Tilapia**




**Channel catfish**



# Chapter IV

## *In vitro* kinetics of hepatic albendazole sulfoxidation in channel catfish (*Ictalurus punctatus*), tilapia (*Oreochromis sp*.) and rainbow trout (*Oncorhynchus mykiss*): Inducing effects on EROD activity in ABZ-dosed channel catfish.

**Abstract**

Liver microsomes from market-size (n=6) rainbow trout, channel catfish and tilapia were used to investigate *in vitro* biotransformation kinetics of albendazole (ABZ). ABZ was transformed to a single metabolite, ABZ sulfoxide (ABZ-SO). Catfish displayed the highest maximal velocity ($V_{max}$ = 264.0 ± 58.6 pmols ABZ-SO/min/mg protein) followed by tilapia (112.3 ± 8.2) and rainbow trout (73.3 ± 10.3). $V_{max}$ in catfish was significantly different ($p < 0.05$) from the other two species. Michaelis-Menten constant ($K_m$) values (μM) varied significantly among the species: rainbow trout (3.9 ± 0.5), tilapia (9.2 ± 1.7) and catfish (22.0 ± 3.2). However, $V_{max}/K_m$ ratios showed no difference among the three species, making them equally efficient performing this phase I biotransformation reaction. In a second series of experiments, channel catfish (n=6 per treatment) were dosed *in vivo* with gel-food containing ABZ (10 mg / K , P.O.). Fish were sacrificed at 24, 48, 72 and 120 hours after dosage. Control fish were fed ABZ-free feed. Induction of ethoxyresorufin-o-deethylase (EROD) activity was significant ($p<0.05$) in all ABZ-dosed treatments as compared to controls. EROD induction was higher in males and immature fish than in females. No pentoxyresorufin- (PROD), benzyloxyresorufin-*O*-dealkylation (BROD) or glutathione-S-transferase (GST) induction was found in the ABZ-treated fish when compared to controls. Additional *in vitro* assays with catfish and rainbow trout microsomes showed lower EROD activity due to the presence of ABZ in the incubation mixture. On the other hand, 7-ethoxyresorufin (7-ER) did not alter the rate of ABZ sulfoxidation when both substrates were part of the incubation mixture.

**Keywords**: fish, albendazole (ABZ), biotransformation.

## Background

Albendazole (ABZ, [5-(propylthio)-1H-benzimidazol-2-yl]-carbamate), is a broad spectrum anthelmintic used for the treatment of liver flukes, tapeworms, and lung and gastrointestinal round worms in human (Cook, 1990; Ottesen et al., 1999) and veterinary medicine (Campbell, 1990).

The oxidative bioactivation of ABZ yields a phase I metabolite, ABZ-sulfoxide (ABZ-SO), and is a critical step for the expression of the anthelmintic activity. A second sulphoxidation produces albendazole sulphone (ABZ-$SO_2$), a pharmacologically inactive metabolite. A third biologically inactive metabolite, ABZ aminosulfone (ABZ-$_2NH_2SO_2$), is produced upon the hydrolysis of ABZ-$SO_2$ (Gottschall et al., 1990).

ABZ biotransformation metabolites have been studied in mice (Douch and Buchanan, 1979), pigs (Souhaili- El Amri et al., 1987), dogs (Delatour et al., 1991a), rats (Moroni et al., 1995), rabbits (Li et al., 1995), sheep (Cristòfol et al., 1998; Chiap et al., 2000), goats (Delatour et al., 1991b), cattle (Lanusse and Prichard, 1992), chicken (Csiko et al., 1996), humans (Rawden et al., 2000) and helminth parasites (Solana et al., 2001). Residue depletion of ABZ and its main metabolites after oral administration in rainbow trout, tilapia and Atlantic salmon has been studied by Shaikh et al. (2003 a, b).

ABZ sulfoxidation is mainly achieved by first pass metabolism in intestine (Villaverde et al., 1995; Redondo et al., 1999) and liver (Galtier et al., 1986; Virkel et

al., 2000). The metabolic pathways that are involved in the ABZ biotransformation are cytochrome P450 oxidases and flavin-containing oxidases (FMO) (Gottschall et al., 1990). ABZ has a chiral center which determines the formation of ABZ (-) sulphoxide after P450 catalytic activity or ABZ (+) sulphoxide after FMO-mediated sulfoxidation (Delatour et al., 1991a,b; Moroni et al., 1995). Specifically, CYP3A4 is directly responsible for the sulphoxidation of the parent compound in rats (Moroni et al., 1995), humans (Rawden et al., 2000) and presumably other animal species. Further sulphoxidation to generate the sulphone metabolite appears to be mediated by CYP1A (Souhaili-El Amri et al., 1988b). Contributions of each pathway to ABZ metabolism have been detected using different approaches including: 1) Inhibition of CYP reductase using an antiserum, 2) thermal partial-inactivation of FMO activity ($45^{o}$ x 15 min, $37^{o}$ x 60 min, $45^{o}$ x 3 min -inhibition of FMO with little effect on CYP), and 3) use of selective chemical inhibitors (Rawden et al., 2000). Inhibitors that have been used for determining contributions of human FMO and CYP in ABZ metabolism include ketoconazole (32-37% reduction of CYP3A4 activity), ritonavir (34-42% inhibition of CYP3A4 activity), methimazole (FMO reduction of 28 to 49%) and thioacetamide (FMO: 32-35%). Additive inhibition with ketoconazole and methimazole led to 69 ± 8 % loss of activity (Rawden et al., 2000).

ABZ and other benzimidazoles have been linked to inducing effects of phase I-II biotransformation enzymes. ABZ is reported to induce CYP1A-mediated activity (e.g., EROD) and protein content in rats (Souhaili-El Amri et al., 1988a; Asteinza et al., 2000; Baliharová et al., 2003), and in human microsomes and human hepatoma cell (HepG2) cultures (Rolin et al., 1989). In addition to higher EROD activity, an increase in CYP1A1 and CYP1A2 mRNA expression was found in HepG2 cells when

exposed to ABZ (Bapiro et al., 2002). Induction of S9 fraction activity, specifically EROD, MROD, PROD and BROD activities, and protein contents of rat livers after intraperitoneal ABZ injection is reported by Escobar-García et al. (2001). Besides CYP1A induction, ABZ induces to a lesser extent CYP2A6, CYP2E1 (Souhaili-ElAmri et al., 1988a), CYP2B1 and CYP2B2 (Asteinza et al., 2000) and CYP3A4 (Souhaili-ElAmri et al., 1988a; Asteinza et al., 2000) in rats. ABZ has also been reported to induce phase II biotransforming enzymes including glucuronosyltransferase activity in humans (Rolin et al., 1989) and glutathione-s-transferase in mouse serum and muscle (Derda et al., 2003).

Disease outbreaks, associated with serious economic losses, are common in the aquaculture industry, particularly in intensive culture systems (Georgiadis et al., 2001). However, very few therapeutic drugs are currently approved for use in aquacultured species in the U.S. The approval process of drugs is time-consuming and quite expensive. The US FDA estimates that between 20 and 40 million US dollars are needed for granting approvals for one drug in one species (FDA, 2006). Economic incentives in other areas of the pharmaceutical business and rigorous policies with regard to safety for the consumer, the environment and the aquacultured species themselves, make lengthy and the costly process of obtaining new approvals of needed drugs.

Species-grouping and study design. One alternative that has been suggested by the FDA and other research groups (Gingerich et al., 1998) would be projected to speed up this process by testing whether or not fish species may be grouped based on similarities of drug metabolic profiles, residue depletion patterns in edible tissues, and

other factors related with drug usage. If feasible, the *fish grouping* hypothesis could suggest the use of surrogate species as good representatives of specific groups of fish. Therefore, fish grouping could circumvent prolonged and costly investigations on each species for approval purposes. However, before using such an approach to make regulatory decisions, a body of sound, scientifically-supported information is needed.

*In vitro* metabolism of drugs in fish is required to provide scientific evidence linking similarities in metabolism among piscine species. This study investigated *in vitro* kinetics of ABZ hepatic biotransformation in three aquaculturally-relevant finfish species: tilapia, channel catfish and rainbow trout. A second series of experiments examined the induction of phase I (EROD, PROD, BROD) and phase II (GST) activities after ABZ dosage (10 mg/K P.O.) in channel catfish sacrificed 24, 48, 72 and 120 h post-treatment. A third set of experiments examined the interrelation between ABZ and EROD activity as well as likely changes in ABZ sulfoxidation patterns due to the presence of 7-ethoxyresorufin.

## Materials and methods

### Fish handling

Healthy, market-size specimens (n=6) of tilapia, channel catfish and rainbow trout were obtained from commercial sources. After capture, the fish were transported to the Aquatic Pathobiology Laboratory at the University of Maryland (College Park, MD) and the US Food and Drug Administration Agency (FDA) (Laurel, MD) where they were maintained under controlled conditions for at least 3 months prior to sacrifice and liver harvesting. For the *in vivo* exposure to ABZ, the fish were weighed

and transferred to individual 80 L flow-through glass tanks (water temperature: 25 ± 2 $^{o}$C). The fish were acclimated for 3 to 5 days and denied food for 2 days before the ABZ dosing. The ABZ was weighed into gel capsules and administered to fish via stomach tube with manual restraint. ABZ was given at a dose of 10 mg / Kg of body weight (Shaikh et al., 2006).

Microsomal and cytosolic fractions preparation

Microsomes and cytosol were prepared according to Vodicnik et al. (1981). All operations were performed at 4 $^{o}$C. Livers were weighed individually and minced with scissors in 1.15% ice-cold KCl buffer (J.T. Baker – 3040-01). This same buffer was used to rinse the mincate until getting rid of all blood residues. After discarding the last KCl rinsing, 4 volumes of 0.25 M sucrose (ICN Biomedicals – 821271) were added. The minced livers in sucrose were transferred to a glass homogenizer and homogenized by 6 full strokes. The sample was then transferred to a high-speed centrifuge tube and spun at 8,000 x g for 20 minutes (Biofuge 22 R – Heraeus Instruments). The supernatant was spun at 100,000 x g for 60 minutes (Beckman Ultracentrifuge XL-80). The tubes were removed to ice and the supernatant (cytosolic fraction) was aliquoted into cryotubes. The microsomal pellets were resuspended with 1 ml of SET buffer (pH=7.4) per gram of wet liver with a spatula. The resuspended microsomal fraction was transferred to a glass homogenizer and homogenized with 6 full strokes followed by aliquoting into cryotubes. Cytosolic and microsomal fractions were stored at –80$^{o}$C for no longer than 3 months until performing the assays. Protein was measured using the BCA protein assay kit (Pierce - 23227) based on the colorimetric reaction with bicinchoninic acid.

*In vitro* ABZ incubations

Microsomal fractions from channel catfish, tilapia and rainbow trout specimens were used for ABZ *in vitro* metabolism according to a modified method from Rawden et al. (2000). In an eppendorf centrifugation tube, phosphate buffer (0.1 M), microsomes (100 μg protein), ABZ (1 - 30 μM) (Sigma A-4673) and NADPH (1 mM, tetrasodium salt, Calbiochem 481973) were pipetted for a total 200 μL total reaction volume. After 40 minutes of incubation at room temperature on a shaker (Orbit – Lab-Line, USA), 200 uL of ice-cold acetonitrile were added to stop the reaction. The tubes were spun at 5,000 rpm for 20 minutes in a refrigerated high-speed centrifuge (Biofuge 22R – Heraeaus Instruments). Supernatants were stored at –80$^{o}$C until HPLC metabolite analysis. Preliminary experiments were done to determine linearity of the metabolic reaction with regard to incubation time and protein content.

HPLC analysis

ABZ metabolites obtained after *in vitro* incubation of microsomal fractions were analyzed according to Shaikh at al (2003b). The liquid chromatographic (LC) system consisted of a Hewlett-Packard Model 1050 (Palo Alto, CA) with a quaternary pump, autosampler and an Agilent series 1100 fluorescence detector (290 nm and 330 nm excitation and emission wavelengths, respectively). Analytical and guard columns were 5-Fm Luna C18 and ODS C18, respectively. An isocratic mobile phase consisted of acetonitrile/methanol/0.05 M ammonium acetate buffer (pH=5) in a ratio of 17:8:75. This mobile phase was used for the analysis of ABZ metabolites. Reference standards (1-10 mg) of ABZ, ABZ-SO, ABZ-$SO_2$ and ABZ-$_2NH_2SO_2$,

were prepared to obtain a range of calibration standards according to the level of quantification. Quantification of ABZ-SO from the *in vitro* incubation medium was performed based on calibration curves obtained with standards.

Calculation of ABZ sulfoxidation kinetics parameters

Maximal velocity ($V_{max}$), Michaelis-Menten constant ($K_m$) and $V_{max}/K_m$ were calculated by linear regression after obtaining double-reciprocal, Lineweaver-Burk plots.

EROD, PROD, BROD and GST assays

*Ethoxyresorufin-O-deethylation (EROD)* (Assays were conducted, with modification, based on Eggens and Galgani, 1992, and Haasch et al., 1994). Reaction mixtures consisted of 50 uL of Tris-HCl buffer (100 mM, pH=7.4), 25 uL of microsomal fraction accounting for 100 μg of protein, 10 μL of 7-ethoyxresorufin (7-ER) (Sigma E3763) (1 uM final concentration) and 25 μL of NADPH (1 mM tetrasodium salt, Calbiochem 481973). Blanks consisted of reaction mixtures with boiled microsomes. The rate of EROD activity was determined after 10 minutes of reaction. This time point was sufficient to detect metabolites and was within the linear response of the reaction overtime. Reaction was quantified by reading the fluorescence units of resorufin (excitation = 510 nm, emission = 580 nm) in a microplate absorbance-fluorescence reader (Genios $^{TM}$ - TECAN, Austria). A resorufin calibration curve (0 - 0.5 μM) was used for the quantification of the reaction rate.

*Pentoxyresorufin- (PROD) and benzyloxyresorufin-O-dealkylation (BROD) activities.* PROD and BROD activities were assayed following the same protocol as for EROD. A 5 μM substrate concentration was tested. Phenobarbital-induced rat microsomes (R1078 – Xenotech, LLC) (Lenexa, Kansas) served as positive controls to compare activities.

*Glutathione-S-transferase (GST) activity*. GST activity was determined by the method of Habig et al. (1974). Reaction mixtures consisted of 165 μL of 100 mM Tris-HCl buffer (pH=7.4), 7 μL of 1-chloro-2,4-dinitrobenzene (CDNB, Sigma C 6396) (1 mM final concentration), 3.5 μL of 60 mM reduced L-glutathione (Sigma G 6529) and 10 μg of cytosolic protein. Blanks consisted of reaction mixtures with exception of the cytosolic fraction. The rate of CDNB conjugation with GSH was evaluated after 5 minutes of reaction determining changes in absorbance ($\lambda$ = 340 nm) at room temperature. Absorbance readings were obtained using a microplate fluorescence-absorbance reader (Genios $^{TM}$ - TECAN, Austria). The molar absorption coefficient for CDNB ($\epsilon$=9.6 $mM^{-1}cm^{-1}$) was used for final calculations after adjusting the path length to the corresponding 96-well plate volume (Styrene microtiter® S25-291-01, ThermoLabsystems, MA).

*Co-incubation effects on EROD and ABZ metabolism in rainbow trout and channel catfish*. Microsomal fractions of both species were used to evaluate changes in EROD activity (1 μM 7ER) when three different ABZ concentrations (0.25, 0.5 and 1.0 μM) were included in the incubation mixture. EROD activity was performed as described previously in this manuscript. A second set of experiments tested for effects on ABZ sulfoxidation (1 μM ABZ in rainbow trout and 2 μM ABZ in channel catfish) when

three different 7-ER concentrations (0.25, 0.5 and 1.0 μM) were part of the incubation mixture. ABZ metabolites were analyzed following the aforementioned HPLC method.

Statistical analysis

Results from albendazole *in vitro* metabolism and phase I-II biotransformation reactions are reported as means +/- standard errors. Data sets were analyzed for normality (Shapiro-Wilcoxon test) and homogeneity of variances (Barlett's test). Log transformations were calculated for some variables to comply with statistical assumptions. Data being both normal and homogeneous were compared using a one-way ANOVA test (comparison among species) followed by Tukey's mean separation test using SAS (Statistical Analysis Software). Statistical significance was set at a significance level of $\alpha = 0.05$.

**Results**

*ABZ sulfoxidation kinetics*. ABZ was transformed by hepatic microsomes to a single metabolite, ABZ sulfoxide (ABZ-SO) in the three species. This ABZ sulfoxidation reaction was NADPH-dependent as no ABZ-SO was detected in controls (no NADPH in incubation mixture). Other metabolites such as ABZ-$SO_2$ and ABZ-$_2NH_2SO_2$ that have been reported in residue depletion studies with fish (Shaikh et al., 2003b) were not detected. Michaelis-Menten and double-reciprocal plots of the sulfoxidation reaction kinetics for each species are shown in Figures 1 to 5. Apparent maximum velocity ($V_{max}$), Michaelis-Menten constant ($K_m$) and $V_{max}/K_m$ values for this reaction

are presented in Table 1. Channel catfish had higher $V_{max}$ (264.0 ± 58.6 pmols ABZ-SO/min/mg protein) as compared to tilapia (112.3 ± 8.2) and rainbow trout (73.3 ± 10.3). $K_m$ values (μM) varied significantly ($p < 0.05$) among the species: rainbow trout (3.9 ± 0.5), tilapia (9.2 ± 1.7) and catfish (22.0 ± 3.2). These results indicate that rainbow trout had the highest binding affinity for the substrate. Statistical analysis of the $V_{max}/K_m$ ratios showed no difference among the three species: catfish (12.3 ± 1.9), tilapia (13.6 ± 1.7) and rainbow trout (19.2 ± 2.6).

*Changes in EROD, PROD, BROD and GST activities after ABZ treatment in channel catfish*. A significant induction (2.2 – 2.6 fold) on EROD activity was found in all the ABZ-dosed time points compared with controls (Figure 6). Induction in EROD activity was significantly greater in immature and male fish (23 ± 2 pmols resorufin/min/mg protein) as compared with females (15 ± 3 pmols resorufin/min/mg protein). Neither control fish nor ABZ-dosed fish showed PROD or BROD activities in the microsomal fraction. No induction of GST activity was found in either of the ABZ treatments as compared to control values (Figure 7). Interestingly, GST activity was lower in 120-h treatment when compared with control values.

*Changes in EROD activity due to ABZ co-incubation and changes in ABZ sulfoxidation rate due to 7-ER*. Channel catfish and rainbow trout EROD activity was significantly decreased by ABZ at 0.5 and 1.0 μM in rainbow trout and catfish microsomes (Figure 8). On the other hand, the rate of ABZ sulfoxidation was not altered by the presence of 7-ER at any concentration in either of these two species.

## Discussion

*ABZ sulfoxidation kinetics*. The present study compared hepatic *in vitro* ABZ sulfoxidation in rainbow trout, tilapia and channel catfish. *In vivo*, ABZ undergoes negligible phase II-type of biotransformation reactions and there is no sequential conjugation after the phase I oxidation. As a result, microsomes are considered a good model for ABZ *in vitro* metabolism (Wrighton et al., 1995). In the present work, the microsomal fractions of all three species transformed ABZ to ABZ-SO. No other metabolites such as ABZ-$SO_2$ or ABZ-$_2NH_2SO_2$, that are reported in residue depletion studies in fish (Shaikh et al., 2003b), were detectable. The absence of inactive metabolites could be due to limited oxidation of ABZ-SO to ABZ-$SO_2$ in the liver or notably faster metabolism of ABZ to ABZ-SO not allowing the detection of the sulfone or the aminosulfone during the incubation period tested. Higher rates of secondary sulfoxidations may occur in other organs (e.g., kidney) that were not evaluated in the present study. Nevertheless, these data are consistent with previous studies in other animal species. When microsomal fractions of sheep (Galtier et al., 1986), rat (Fargetton et al., 1986), pig (Souhaili El Amri et al., 1987), and human (Rawden et al., 2000), were incubated with ABZ as the parent compound, ABZ-SO was the only detected metabolite. Further analysis using ABZ-SO as the parent compound may help to understand whether or not the reaction occurs in a significant rate in the liver.

Channel catfish had the highest $V_{max}$ for the ABZ sulfoxidation of all three species. $V_{max}$ values in the fish species studied in this work were lower than those obtained for rat (590 pmols/min/mg protein) (Fargetton et al., 1986), pig (580 pmols/min/mg

protein) (Souhaili El Amri et al., 1987) and human microsomes (457 pmols/min/mg protein) (Rawden et al., 2000) in other studies. High capacity to perform biotransformation reactions is of particular interest when high substrate concentrations at the biotransformation site are likely to occur. In residue depletion studies Shaikh et al. (2003b, 2006) found that rainbow trout and tilapia cleared ABZ in muscle by 12 hours after ABZ ingestion while channel catfish did it after 8 hours (Shaikh et al., 2006). Of note is that we found in the present study that channel catfish had the highest ABZ sulfoxidation rate of all three species that correlates with the shortest depletion time for ABZ in muscle found by Shaikh and collaborators. ABZ-SO depletion in channel catfish was also the shortest (8 h) as compared with the ones found in tilapia (48 h) and rainbow trout (48 h) (Shaikh et al., 2003b). These authors found the longest retention time in muscle for ABZ (24 h) an ABZ-SO (96 h) in Atlantic salmon. Although we did not include Atlantic salmon in the present study, a low *in vitro* ABZ sulfoxidation rate may be expected if the same correlation found in the other three species is maintained.

The binding affinity ($K_m$) for ABZ sulfoxidation in channel catfish indicates that this species had the lowest binding affinity (e.g., highest $K_m$ value) of the three species. Tilapia and rainbow trout showed greater binding affinities for ABZ. The three fish species studied in the present work had higher binding affinities than those reported for rat (53.6 μM) (Fargetton et al., 1986) and pig microsomes (41.7 μM) (Souhaili El Amri et al., 1987). $K_m$ in rainbow trout was even lower, although quite similar to the value reported by Rawden et al. (2000) in human microsomes (4.6 ± 0.8). Fish with high biotransformation capacity may cope with extreme exposure concentrations better than those with low maximal velocities (Gallagher et al., 2000). However, for

the purpose of drug metabolism, the concentrations that more likely are present at the site of biotransformation are in a lower range than the $K_m$ value (e.g. [S] < $K_m$).

$V_{max}/K_m$ is a pure measure of enzyme activity and is not influenced by other physiological factors of liver clearance. This parameter is used as the basis for the extrapolation of *in vitro* data to the *in vivo* situation (Houston, 1994). Estimation of *in vivo* drug clearance is based on application of models that account for non-enzymatic, physiological factors as well as the $V_{max}/K_m$ estimate. The *venous equilibrium* model, one of the most used approaches for the prediction of *in vivo* clearance, combines the *in vitro* clearance value with the hepatic blood flow and the binding of the drug within the blood matrix, mainly with plasma proteins, as the main physiological factors that affect *in vivo* clearance (Wilkinson, 1987). When $V_{max}$ and $K_m$ values were combined in the present work to analyze the overall enzyme efficiency of the sulfoxidation process, the three species had very similar results. Consistent with previous studies in our laboratory, the high $V_{max}$ value found in catfish was accompanied by a high $K_m$ (e.g., low binding affinity). A similar but inverse relationship occurred in rainbow trout (low capacity along with low $K_m$ –high affinity-). Tilapia results were in between the ranges found for catfish and trout. In the end, the enzyme efficiency estimate ($V_{max}/K_m$) showed that the three species had quite similar capabilities to activate ABZ through the sulfoxidation process.

*Alkoxyresorufins and glutathione-S-transferase induction after ABZ treatment in channel catfish*. ABZ and other benzimidazoles have been linked to inducing effects of phase I-II biotransformation enzymes. ABZ is reported to induce CYP1A-mediated activity (e.g., EROD) and protein content in rats (Souhaili-ElAmri et al., 1988a;

Asteinza et al., 2000; Baliharová et al., 2003), human microsomes and hepatoma cell cultures (Rolin et al., 1989). Induction of glutathione-S-transferase (GST) activity in mouse serum and muscle (Derda et al., 2003) is also reported after ABZ treatment. Although most of the studies on cytochrome P450 induction due to ABZ treatment are intended to look for likely effects in humans with chronic parasitosis after prolonged treatments (e.g., months) with ABZ, we were interested in investigating whether or not ABZ could induce CYP1A and GST activities in fish after a therapeutic regime (single dose of 10 mg/K P.O.). Other cytochrome P450-mediated reactions (e.g., PROD and BROD), for which no isoforms responsible for their biotransformation have been identified in fish, were also investigated. Due to logistics, this part of the study was done only with channel catfish. All of the time points after the ABZ dosage (24-, 48-, 72- and 120-hours) showed significant induction of EROD activity, a good indicator of CYP1A expression (Whitlock et al., 1999). EROD activity at all of the exposure time points was significantly different ($p < 0.05$) from the one found in the control (ABZ-free feed). EROD activity has been used as a good indicator of CYP1A induction in fish (Whyte et al., 2000) and other species (Whitlock et al., 1999). Some of the CYP1A chemical inducers are organic contaminants that may be present in the water column, in sediments or in the food. Among these compounds are polyaromatic hydrocarbons (PAHs), coplanar polychlorinated biphenils (PCBs), polychlorinated dibenzo-p-dioxins (TCDD), dibenzofurans (PCDD/PCDF), and other halogenated compounds present in some pesticides and herbicides. CYP1A plays a critical role in the activation of chemical carcinogens leading to the formation of reactive, genotoxic metabolites (Goksøyr, 1995). For this reason, the induction of CYP1A1 protein has been widely used as a biomarker of pollution in many different species. EROD activity induction due to *in vivo* ABZ treatment in a fish species had

not been previously reported. In the present work, ABZ exposure evoked higher EROD activity in dosed-catfish (between 2.2 and 2.6 fold) than in controls. Haasch et al. (1994) found 66.3- and 38.8-fold induction in EROD activity after rainbow trout were treated with isosafrole and β-naphtoflavone, respectively. TCDD is reported to induce EROD activity up to 200-fold in some fish species (Whyte et al., 2000). The induction found in our study was seen in all the time point treatments. Reports on EROD induction within the first 48 hours after treatment with inducers are found in the literature. The extent of the EROD induction may depend on how easily the inducer is metabolized. PAHs, as an example of easily metabolized inducers, elicited increases in EROD activity in sea bass (*Dicentrarchus labrax*) after 24 h, followed by dramatic decline after one week (Lemaire et al., 1992). Effects due to halogenated inducers (e.g., TCDD) are reported to persist for several weeks in fish (Whyte et al., 2000). Although our exposure protocol only covered 120 hours, and the response was more typical of an easily-metabolized compound, it is worth considering likely implications of the EROD inducing effect in ABZ-treated fish in the future.

The expression at the transcriptional level of the *CYP1A* gene is mediated through a ligand-dependent transcription factor located in the cytoplasm and known as the arylhydrocarbon receptor (AhR). Some of the organic contaminants previously mentioned (PAHs, PCBs, TCDD, etc) are recognized as AhR ligands. TCDD is the most potent known AhR ligand. However, endogenous substrates such as bilirubin and biliverdin have also been described as AhR ligands. Regarding TCDD, authors suggest that AhR acts "promiscuously" as a transcription factor given that it modulates the expression of a battery of genes in addition to the *CYP1A* gene (Whitlock, 1999). This non–discriminatory feature of AhR could be suggested as an

explanation for the inducing effects of ABZ in the EROD activity found in the present work.

Interestingly, upon further analysis of our EROD induction data, we found that the effect was more significant in both males and immature fish than when females received the medicated feed. Differences in EROD activity and responses to inducing agents between male and female fish have been reported (Elksus et al., 1992 cited by Arinç et al., 2000; Whyte et al., 2000). Stegeman and Hahn (1994) reported that EROD activity is higher in reproductively active and inactive males, and reproductively inactive females, when compared to reproductively active females. There seems to be a direct correlation between CYP1A suppression and high 17β-estradiol levels in spawning females. In our study channel catfish specimens were classified as males, immature or females based on gonadal morphology and not on any type of sexual steroid profile. Although the biological relevance of the differences between the two groups remains unclear in our study, the data is consistent with those obtained by others.

Pentoxyresorufin- (PROD) and benzyloxyresorufin-*O*-dealkylation (BROD) have been used, among other alkoxyresorufins, to characterize responses of cytochrome P450 isoforms in mammals (Burke et al., 1985) and fish (Haasch et al., 1994). Induction of S9 fraction activity, specifically EROD, MROD, PROD and BROD activities, and protein concentration of rat livers after intraperitoneal ABZ injection were reported by Escobar-García et al. (2001). However, neither PROD nor BROD activities were detected in either controls nor ABZ-dosed fish in the present work. PROD and BROD induction has been reported in rainbow trout after intraperitoneal

injection of inducing agents such as isosafrole and dexamethasone (Haasch et al., 1994), but the P450 isoforms involved in such biotransformation reactions have not been identified in fish. In the same study by Haasch et al. (1994), non-induced rainbow trout had negligible baseline PROD/BROD activities. Although PROD/BROD inductions were reported in rats after ABZ treatment, it is not uncommon to find discrepancies between inducers and the target P450 isoforms when different animal species are compared. This makes the extrapolation of results between species difficult.

Glutathione-S-transferase (GST) activity was significantly induced in mouse serum and muscle after ABZ treatment (Derda et al., 2003). In the present work, we found that GST did not change at 24-, 48-, or 72-hours after ABZ dosage treatments, when compared to controls. On the contrary, GST activity in the 120-hour treatment was significantly lower than the one found in the control group. We have no clear explanation for this particular reduction in GST activity in this group.

*Effects of ABZ co-incubation on EROD activity and changes in ABZ sulfoxidation rate associated with co-incubation with 7-ER*. The ABZ sulfoxidation reaction yielding ABZ-SO has been identified as a P450- (CYP3A) and FMO-mediated reaction in various species (Moroni et al., 1995). In addition to these two enzymatic pathways, a second sulfoxidation mediated by a P450 isoform, CYP1A, generates ABZ-$SO_2$ (Souhaili-ElAmry et al., 1988b). There are no studies that have identified the isoforms involved in ABZ biotransformation in fish to date. Although we did not plan a thorough phenotyping study to discern specific biotransformation pathways and isoforms involved in ABZ biotransformation in fish, an *in vitro* incubation system

using channel catfish and tilapia microsomes was tested to determine effects of 7-ER (EROD activity substrate) on ABZ sulfoxidation rates. Effects on EROD activity due to ABZ were also studied in these two species. The purpose of this experiment was to investigate any role that CYP1A could have had during the ABZ sulfoxidation reactions in fish given what is known in other species (Souhaili-ElAmri et al., 1988b). The ABZ sulfoxidation rate remained unaltered despite the presence of 7-ER in the incubation mixture. This suggests that CYP1A does not play a significant role in the sulfoxidation of ABZ. On the other hand, ABZ significantly decreased the rate of EROD activity in both trout and channel catfish microsomes. Although we do not have a substantiated explanation for this finding, we could speculate that the lower EROD activity was due to either a chemical interaction between ABZ and 7-ER or due to effects on the CYP1A protein that impaired its catalytic activity. ABZ binds avidly to tubulin, the cytoskeletal protein, leading to toxic effects in parasites. ABZ could cause some inhibitory effect on CYP1A leading to a reduced EROD activity.

**Conclusions**

The *in vitro* incubation system used in this study provided a good indicator of the rate of ABZ sulfoxidation but not the production of the sulfone or aminosulfone. There were significant differences in $V_{max}$ and $K_m$ in ABZ sulfoxidation kinetics among tilapia, channel catfish and rainbow trout. Of importance, $V_{max}$ values in tilapia, channel catfish and rainbow trout correlated with muscle residue depletion times found by Shaikh and collaborators (2003b, 2006). $V_{max}/K_m$ ratios showed no differences among the three species investigated.

ABZ appears to be a weak inducer of EROD activity in channel catfish when compared to the effect exerted by organic pollutants as cited in other studies. This effect was more intense in males and immature catfish than in females. *In vitro* incubations combining 7-ER and ABZ suggested that ABZ sulfoxidation is not performed by CYP1A isoforms. On the other hand, ABZ inhibited EROD activity.

Further research in ABZ metabolism in fish should be directed to discern isoforms involved in the biotransformation pathways as well as the identification of the role that other organs such as the intestine may play in ABZ first pass metabolism.

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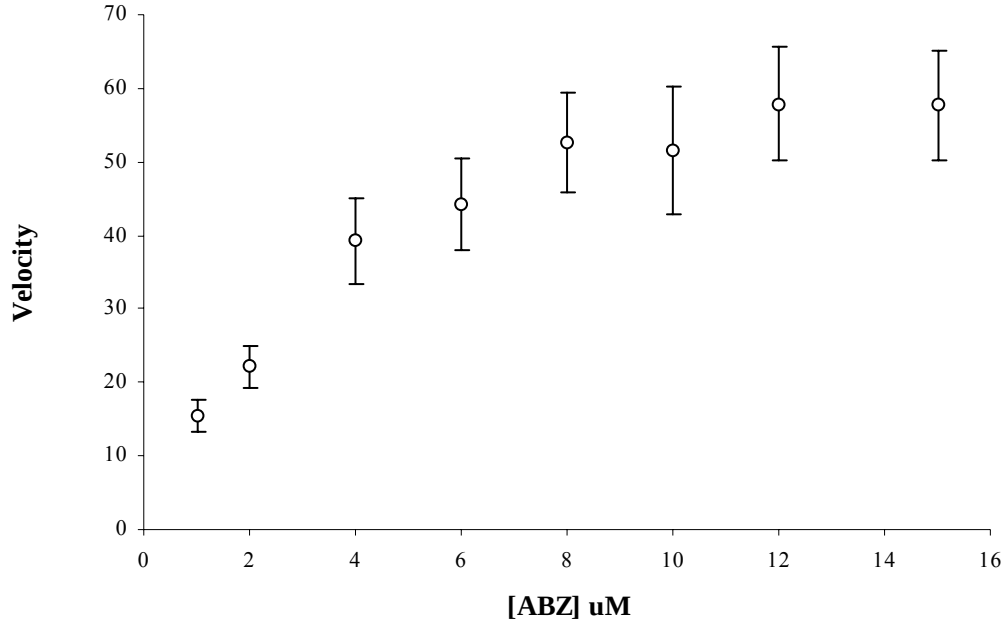
Figure 1. a. Michaelis-Menten plot of ABZ sulfoxidation by rainbow trout hepatic microsomes (n=6) (means ± SEM). Velocity expressed in pmols ABZ-SO/minute/mg protein. b. Lineweaver-Burk (double-reciprocal) plot.

a.




b.

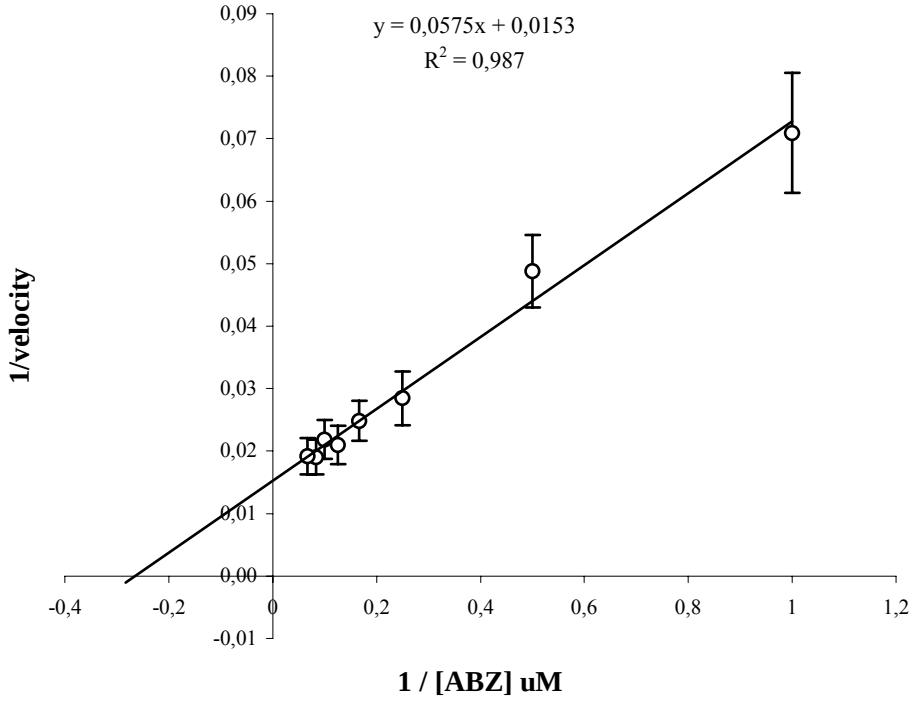
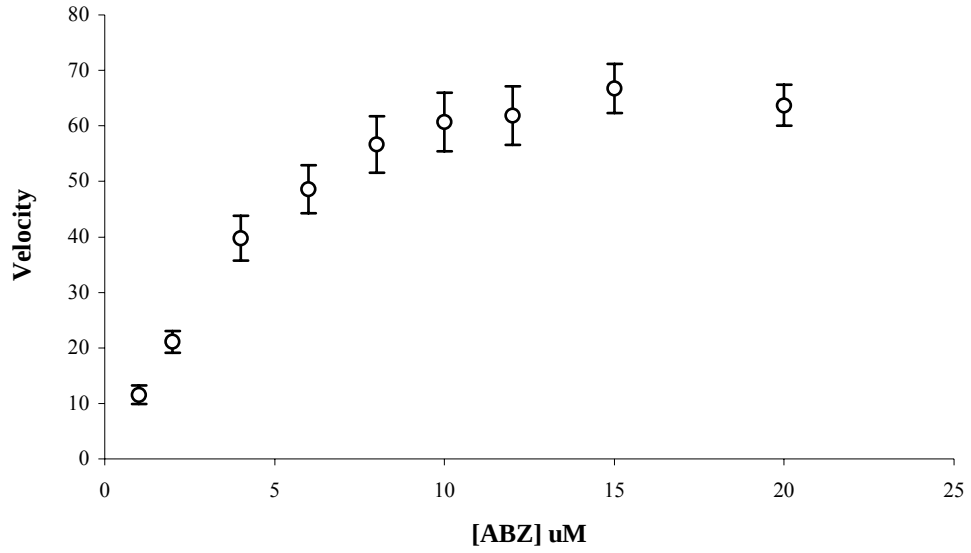


Figure 2. a. Michaelis-Menten plot of ABZ sulfoxidation by tilapia hepatic microsomes (n=6) (means ± SEM). Velocity expressed in pmols ABZ-SO/minute/mg protein. b. Lineweaver-Burk (double-reciprocal) plot.

a.

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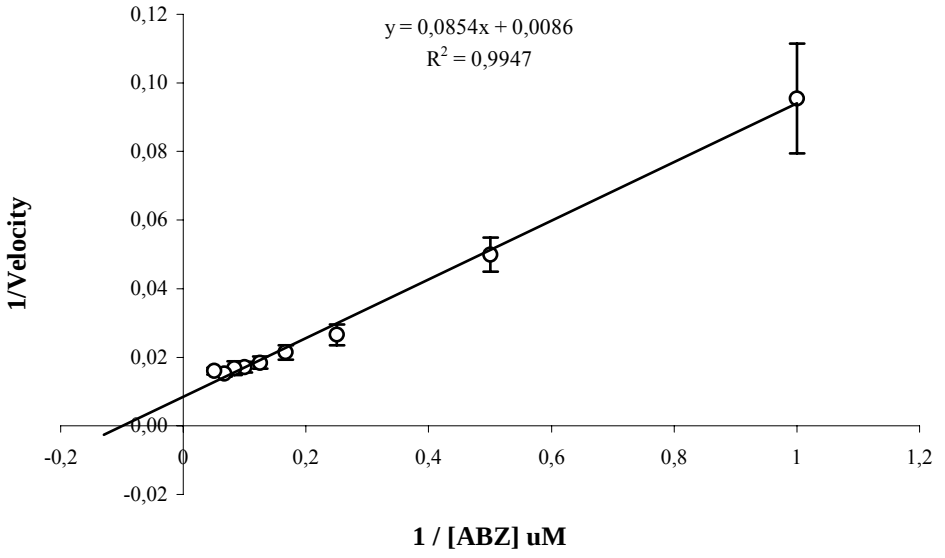
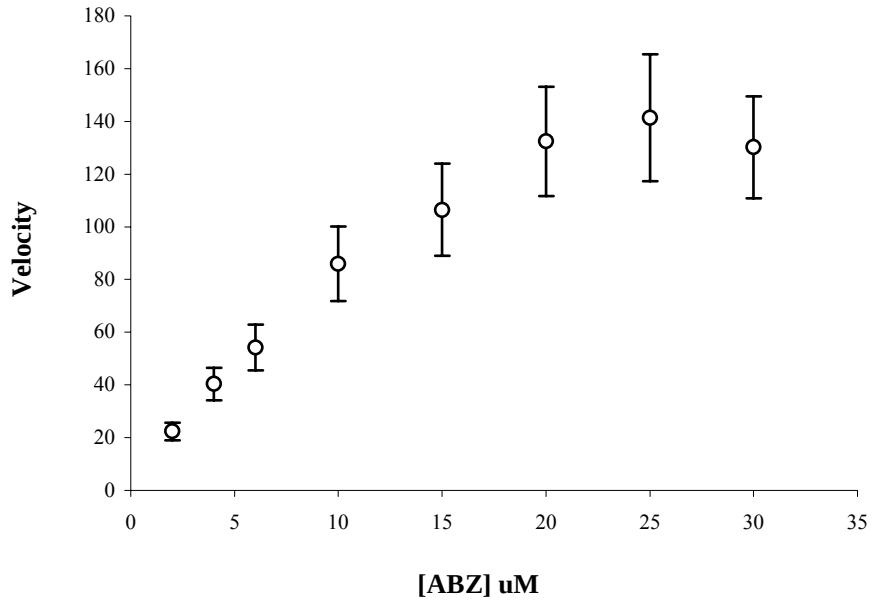


Figure 3. a. Michaelis-Menten plot of ABZ sulfoxidation by channel catfish hepatic microsomes (n=6) (means ± SEM). Velocity expressed in pmols ABZ-SO / minute / mg protein. b. Lineweaver-Burk (double-reciprocal) plot.

a.

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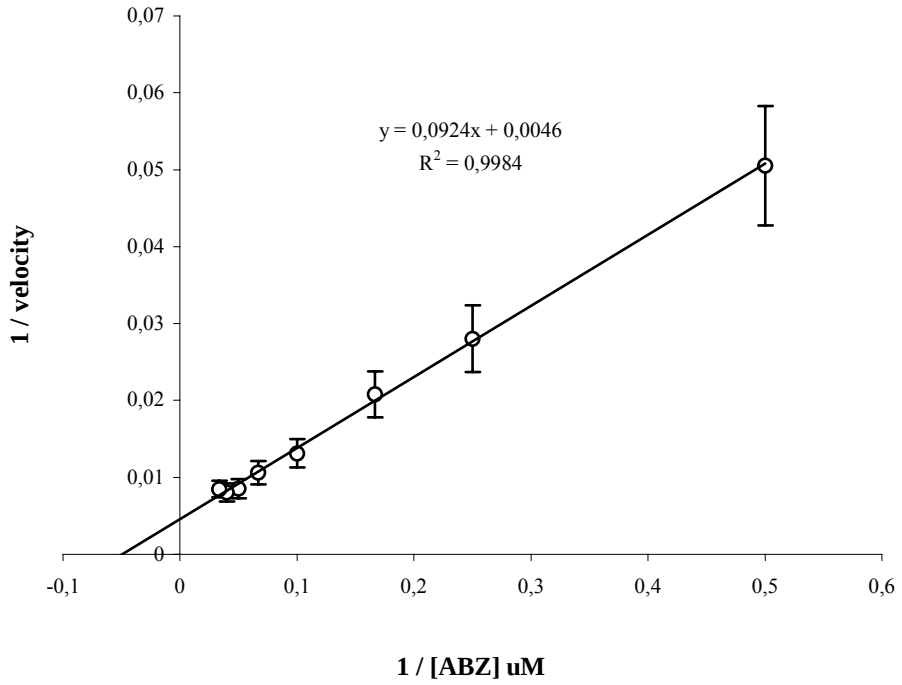


Figure 4. Michaelis-Menten plot of ABZ sulfoxidation by rainbow trout, tilapia and channel catfish hepatic microsomes (n=6). Velocity expressed in pmols ABZ-SO / minute / mg protein.

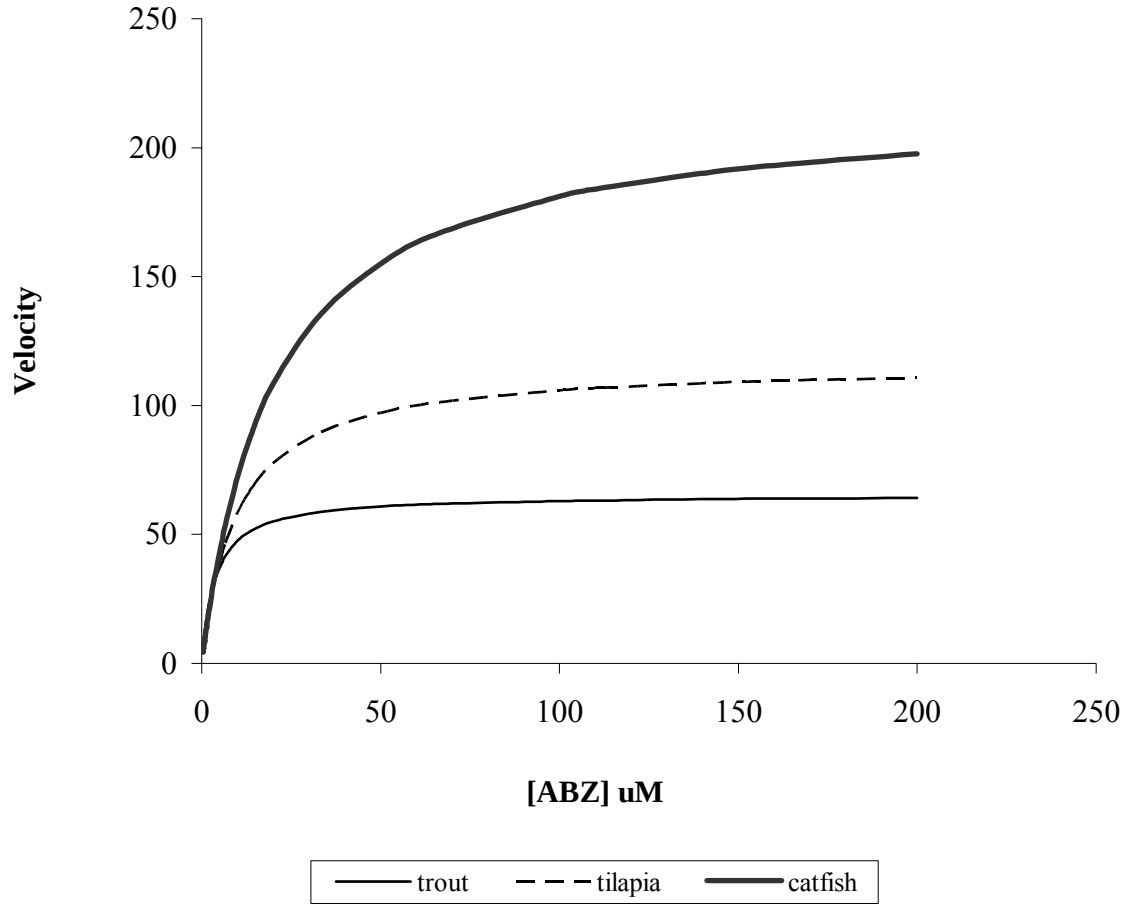


Figure 5. Double reciprocal plot of ABZ sulfoxidation by rainbow trout, tilapia and channel catfish hepatic microsomes (n=6).

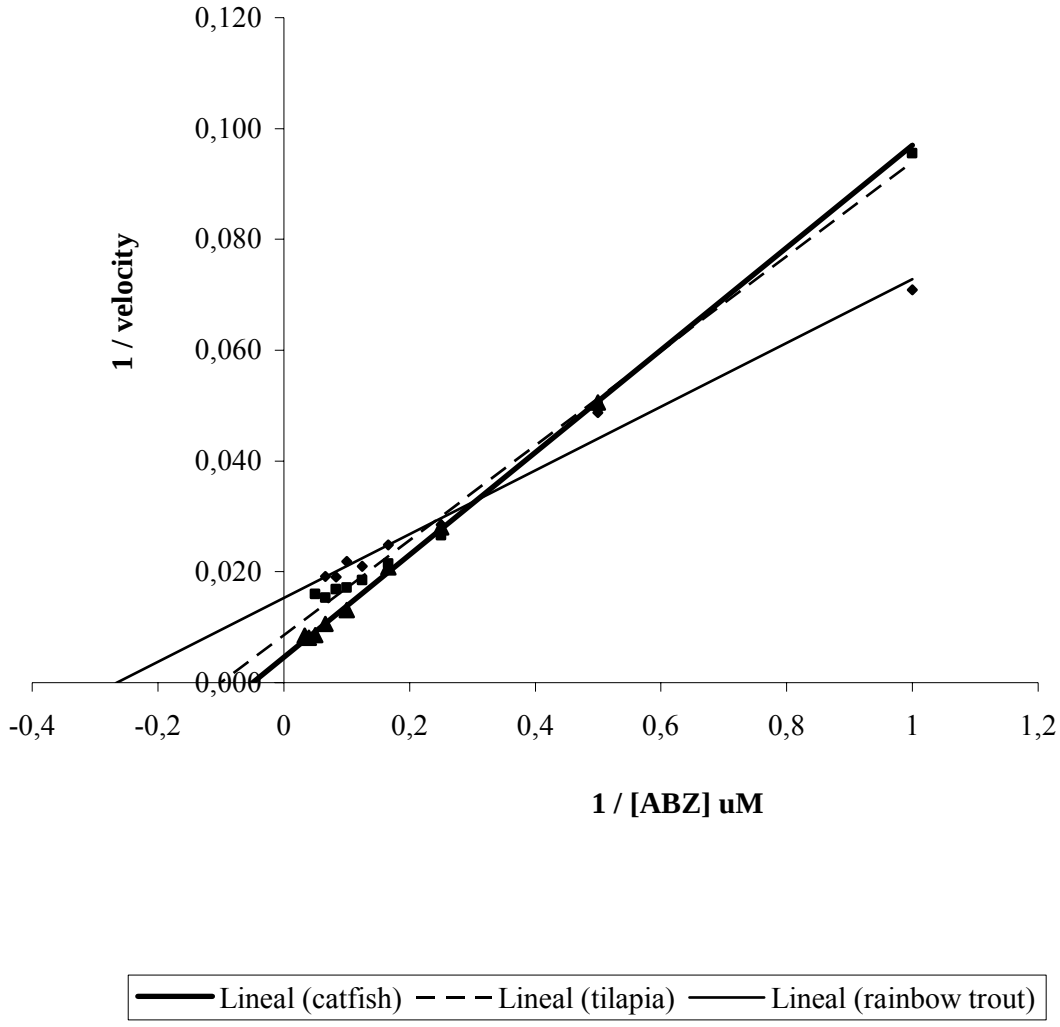


Figure 6. EROD activity (pmols resorufin/minute/mg protein) of hepatic microsomes from ABZ-dosed channel catfish (n=6) (means ± SEM). Different letters on bars denote statistically significant difference ($p < 0.05$).

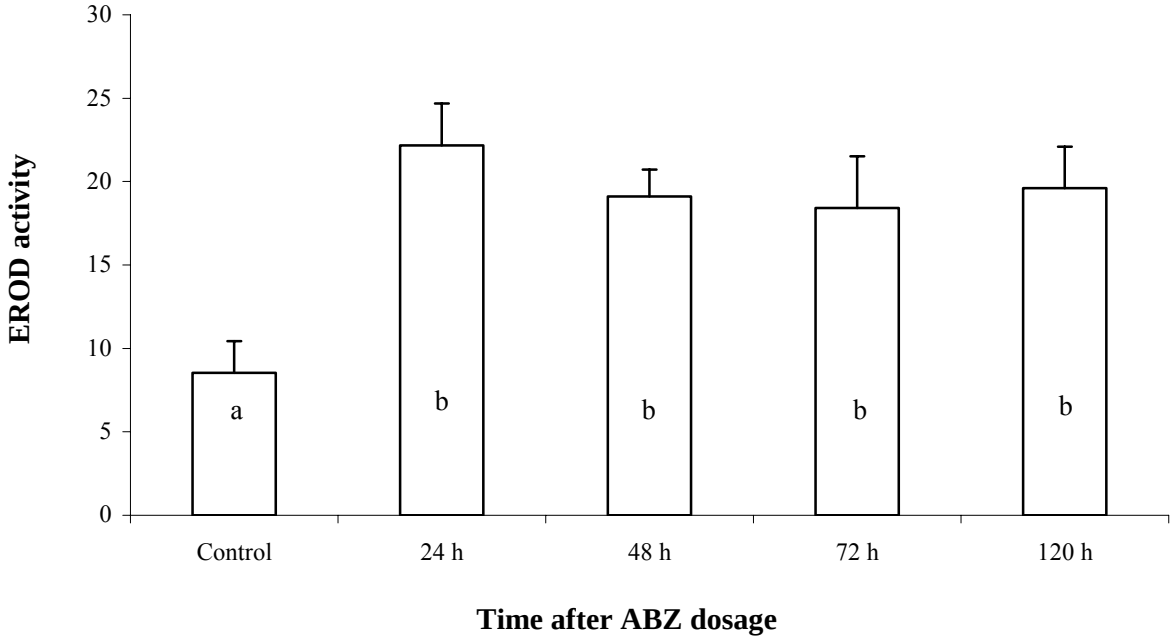


Table 1. $V_{max}$, $K_m$ and $V_{max}/K_m$ values for *in vitro* ABZ sulfoxidation of catfish, tilapia and trout hepatic microsomes (means ± SEM) (different letters in the same column denote significant differences among the species, $p<0.05$).

| **Species** (n=6) | **$V_{max}$** (pmols ABZ-SO/min/mg protein) | **$K_m$** (μM) | **$V_{max}/K_m$** |
|---|---|---|---|
| Catfish | 264.0 ± 58.6 [a] | 22.0 ± 3.2 [a] | 12.3 ± 1.9 [a] |
| Tilapia | 112.3 ± 8.2 [b] | 9.2 ± 1.7 [b] | 13.6 ± 1.7 [a] |
| Rainbow trout | 73.3 ± 10.3 [b] | 3.9 ± 0.5 [c] | 19.2 ± 2.6 [a] |

Figure 7. Glutathione-S-transferase (GST) activity of hepatic cytosolic fraction from ABZ-dosed channel catfish (n=6) (means ± SEM). Velocity expressed in nmols CDNB conjugate/minute/mg protein. Different letters on bars denote statistically significant difference ($p < 0.05$). Controls were offered ABZ-free feed.

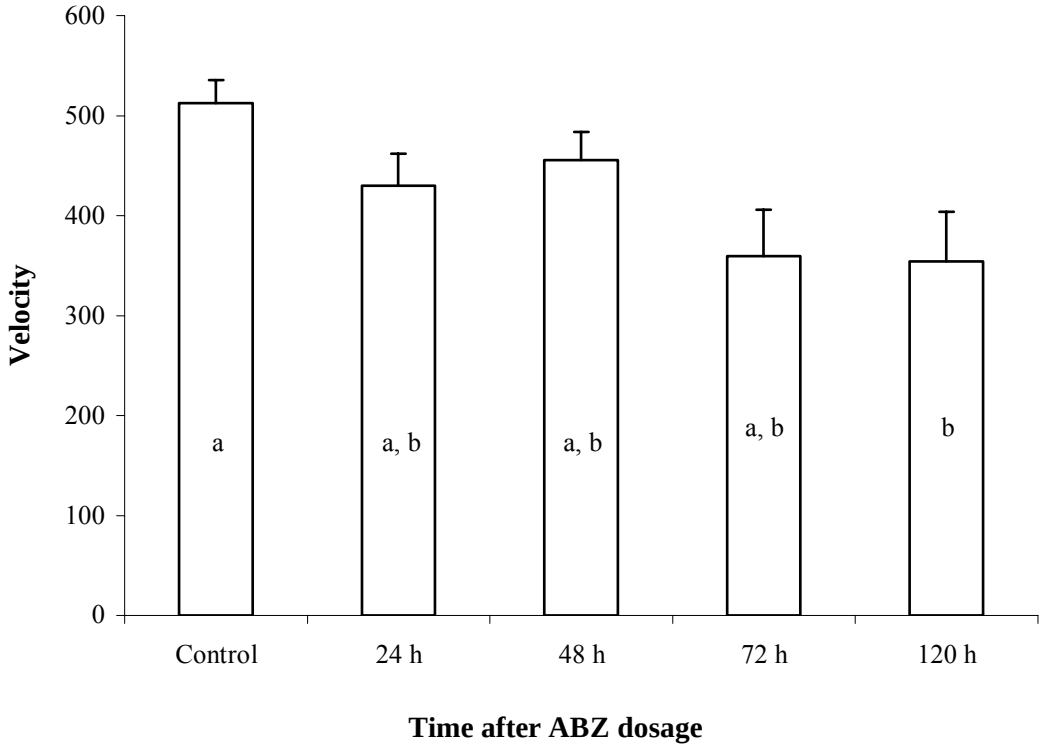
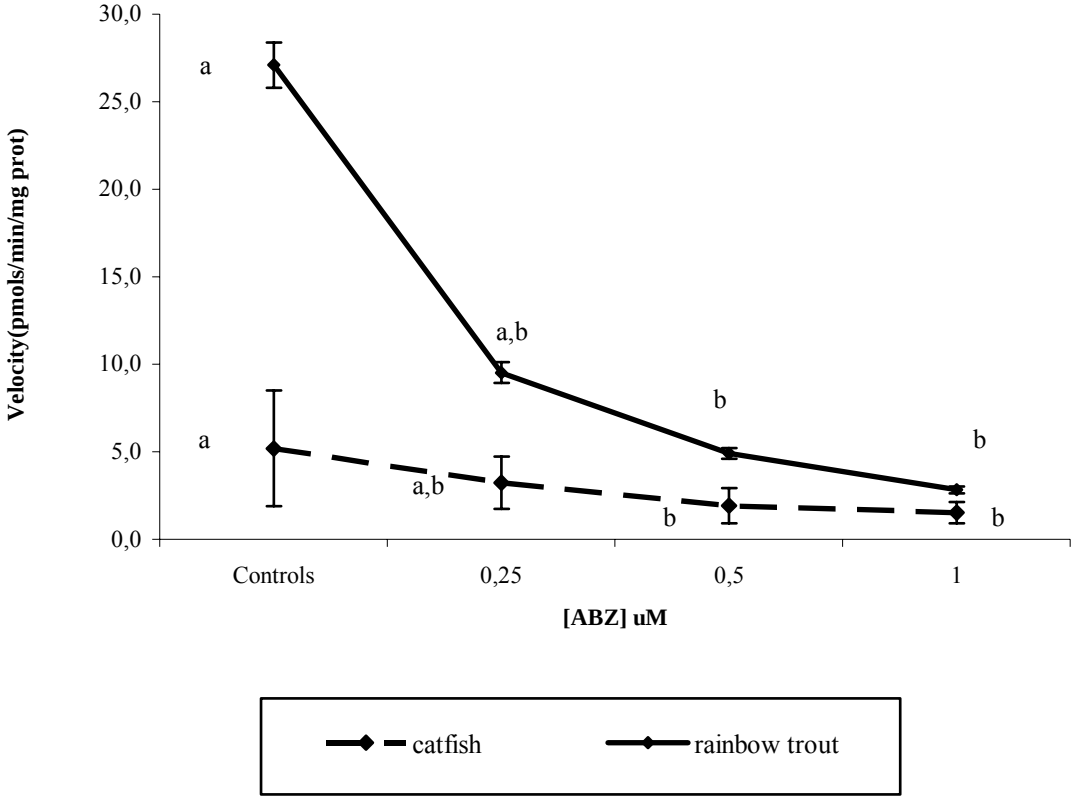


Figure 8. Changes in EROD velocity (pmols resorufin/min/mg protein) due to ABZ in channel catfish (n=6) and rainbow trout (n=8) (means ± SEM) (different letters on bars indicate significant differences within each species, $p < 0.05$). [ABZ] = 1 μM.



# Chapter V

# Final discussion and conclusions

The *crop grouping* hypothesis has been tested in the past in biological sciences. In agriculture, for instance, this concept has been applied to evaluate the residue chemistry of pesticides on plant crops. In this way, specific plants have been chosen to exemplify the responses of the group in which the species were grouped (i.e. leaf- , root- , grain-crops). In aquaculture, given the constraints of drugs availability, this hypothesis has been suggested as a way to circumvent significant amounts of money and time that would be needed to grant approvals for new therapeutic principles. In short, if surrogate fish species could be identified, they could be used as models of what would happen in the others that belong to the same group. Different variables such as phylogeny, habitats, temperatures in which the species are raised, metabolic rates, etc. have been proposed to test the *grouping* hypothesis.

In this research, 8 finfish species that belong to different phylogenetic and taxonomic groups, that have different metabolic rates as well as diverse habitats in which they thrive, were selected to test the similarities and dissimilarities of their phase I and II biotransformation capabilities using microsomal and cytosolic fractions of the liver. Three different research objectives were accomplished: 1) a comparison among the 8 species using model substrates to determine the kinetics of phase I and II biotransformation reactions (chapter II), 2) In chapter III, farm-raised and laboratory-acclimated specimens of channel catfish, rainbow trout and tilapia were compared to determine similarities and differences in biotransformation reactions, and 3) the same latter three species were tested to compare *in vitro* hepatic metabolism of albendazole, a specific therapeutic principle that undergoes metabolic biotransformation mainly through hepatic phase I reactions (chapter IV).

The analysis of chapter II biotransformation capabilities suggests that some of the species displayed higher biotransformation capabilities than others. For most of the seven phase I and II reactions that were tested; rainbow trout, tilapia, channel catfish and Atlantic salmon had better enzymatic efficiencies than those showed by striped bass, hybrid striped bass and bluegill. Largemouth bass shared some enzymatic capabilities with one group or the other. Although these proposed groups ("more efficient and less efficient metabolizers") were determined mainly through statistical analysis of central tendency parameters, some of the results are consistent with previous research reports that have demonstrated how susceptibility or resistance to different types of xenobiotics are explained based on particular capabilities in biotransformation of the species. For example, high glutathione-S-transferase activity that has been reported in channel catfish and tilapia as the explanation for their particular resistance to some electrophilic (i.e. carcinogenic) compounds was corroborated in the present work.

Interestingly, both groups of "more efficient" and "less efficient" metabolizers consist of species that have quite diverse taxonomy, thermal preference and evolutionary trends. For instance, rainbow trout, channel catfish and tilapia which had similar biotransformation capabilities in the present work, comprise very diverse characteristics in terms of these variables. Some preliminary approaches to test the *grouping* hypothesis have considered the use of representatives of different taxomomic groups, phylogeny trends and metabolic rates to assure a fair comparison among the species.

Coincidently, the "more efficient" metabolizers found in this study happened to be the more important species in terms of the revenue that they bring to the U.S. aquaculture market. Some authors have indicated that when trying to determine the variables and the species to be considered in the *grouping* studies, the perception of future or present economic importance should also be taken into account besides being "phylogenetically correct" for inclusion.

It has been suggested that besides the inter-specific differences, intra-specific factors such as size, age, gender, sexual maturity, or different holding conditions may contribute to variability in the *grouping* studies. Chapter III of the present study compared the kinetics of tilapia, rainbow trout and channel catfish from aquaculture systems and laboratory-acclimated conditions. Holding conditions may be quite different between these two systems. Particularly, water quality is a factor that changes considerably due to intensive conditions (i.e. high densities) that are set to reach the production goals in commercial aquaculture. On the other hand, laboratory-holding conditions represent almost an "ideal" situation for the animals (i.e. periodic water changes, no organic matter accumulation, low stock densities, etc). Despite the statistically significant differences that were found in this work for some of the kinetics variables between laboratory-acclimated and farm-raised fish, biological implications due to these differences may be minor if we consider that such differences were within ranges that are cited as normal baseline values in other studies. Significant changes in phase I-II biotransformation reactions due to inducers (e.g., pollutants) are far greater that the values that were found in either laboratory-acclimated or farm-raised fish in the present study.

In chapters II and III, the kinetics of some phase I and II biotransformation reactions was tested using model substrates of which metabolites structure and analytical procedures for detection are well known. In chapter IV, an antiparasitic drug (i.e. albendazole) was tested to investigate *in vitro* hepatic metabolism in three species. These three species were classified as "more efficient" metabolizers based on the results found in chapters II and III of the present study. Unfortunatelly, due to logistic reasons, one of those categorized as "poor metabolizers" (i.e. striped bass, bluegill) could not be included for comparison purposes. Although significant differences in $V_{max}$ and $K_m$ values were found among the three species, when these two parameters were combined to determine the catalytic efficacy of the phase I enzymes ($V_{max}/K_m$), all of the species were equally efficient transforming the parent compound. These three species were found to have very similar catalytic efficiencies for the phase I reactions that were tested in chapter II. The analysis of different kinetic parameters (i.e. $V_{max}$, $K_m$, $V_{max}/K_m$) is of great importance to determine the enzymatic performance of the species. Channel catfish had the highest $V_{max}$ value of the albendazole sulfoxidation reaction of the three species and yet the poorest enzymatic binding affinity. Conversely, rainbow trout displayed a low capacity (i.e. low $V_{max}$) but a high binding affinity. In the end, both species had the same catalytic efficiency as a result of the balance found when the two parameters are combined within a single one ($V_{max}/K_m$). Previous studies by Shaikh and collaborators have reported a shortest depletion time for ABZ-SO in muscle of channel catfish which is more consistent with the highest $V_{max}$ sulfoxidation rate that we found for this species. This is, the more velocity the species has, the more phase I metabolite is produced to complete the biotransformation process to facilitate muscle depletion.

Another interesting feature of this part of the study was to corroborate that some drugs may alter phase I-II biotransformation reactions due to induction of specific isoforms. ABZ was a good inducer of EROD activity in channel catfish dosed *in vivo* with the anthelmintic (10 mg/K). This is of particular interest given that under certain circumstances, the administration of this drug may affect the metabolism of other xenobioticos according to what is known about the environmental pollutants that are transformed by CYP1A.

The results found in the present study showed how enzymatic reactions are accomplished when specific substrates are included within *in vitro* systems of enriched microsomal and cytosolic fractions. However, results of biotransformation reactions may considerably vary during *in vivo* exposures. If two species happen to have quite similar efficiencies in their *in vitro* biotransformation reactions; differences in physiological factors like cardiac output, blood flow rate to organs such as liver and affinity of drugs for plasma proteins, among other factors, will determine the actual amount of substrate (i.e. drug) that is reaching the organ where the biotransformation reactions take place. The *in vitro* results that were found in the present work are a good tool to determine further modeling studies that put together all the variables that may serve to suggest final groups after considering all possible factors.

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