Piscine cytochromes P450 (CYP) and their response to antimicrobial drugs

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Abstract

Most research on the P450 (CYP) system in teleosts has been done on environmental factors that influence their induction. Less is known about CYP metabolism of aquaculture antibiotics. This paper outlines the impact of aquaculture antimicrobials on the piscine CYP system, with particular regard to interactions with tetracyclines, fluorokinolones, sulfonamides, berberine and chloramphenicol, as paradigms for current, potential and discontinued piscine antimicrobial drugs. It gives an overview of literature reports and advances in the field of biological significance of the CYP in fish. Emphasis has been placed on highlighting the most significant isoforms for biotransformation of drugs, and their drug response mechanisms. The challenge is to elucidate the differences in responses of CYP enzymes in different species to antimicrobial treatment since they may have relevance for the use of antimicrobials in aquaculture, especially since drug interactions with the fish CYP may alter their distribution, metabolism, and elimination. They can impact the metabolism of other drugs metabolized by the same system with an effect on the physiology of fish administered these antimicrobials. Also, they can affect the persistence of residues and the length of the withdrawal period. For food animals such as farmed fish, this knowledge is a fundamental biomedical goal.

Introduction

In farming of aquatic organisms, medications are needed to maintain animal health and manage fish populations. However, the development of therapeutic agents for the treatment of fish diseases involves long and complex processes. When compared with mammals, fish have some differences in pharmacokinetics that need to be considered. For example, differences in bioavailability of tetracycline, quinolones, and beta-lactam antimicrobials have been documented (Storey 2005). Oxytetracycline and amoxicillin are not well absorbed from the intestines of fish. The doses of these antimicrobials are generally 2 to 5 times that needed to produce therapeutic systemic concentrations in mammalian species (Burka *et al.* 1997). Also, for several antimicrobials, the cations present in seawater inhibit their absorption from the intestine, while their pharmacokinetics is affected by water temperature as well (Burka *et al.* 1997; Storey 2005). As in other animals, most pharmaceuticals are metabolized by CYP enzymes in fish, which makes them of critical importance both for detoxification or formation of toxic metabolites.

Drugs are usually lipophilic, which allows them to enter their site of action of target organs or tissues via cell membranes and exert their effect. As lipid soluble compounds, they are difficult to eliminate from the body. Metabolism, or biotransformation of these compounds into more polar, inactive metabolites, is generally an enzymatic process (Hildebrand *et al.* 1994; Bernhardt 2006). The CYP monooxygenases are a large superfamily of proteins present in most tissues. They are of central importance in detoxification or activation of a number of foreign hydrophobic compounds, including many therapeutic agents, chemical carcinogens and environmental pollutants (Nebert & Gonzales 1987). Many of these enzymes are inducible by the compounds they metabolize. This monooxygenase system perhaps has the widest-ranging spectrum of substrates,

overlapping those of most of the other metabolizing enzymes (Schenkman 1999; Guengerich 2004). They accept as substrate molecules as small as ethanol and as large as the polycyclic aromatic hydrocarbon (PAH), benzo(a)pyrene (BP) or the antibiotic erythromycin, that is, ranging in molecular weight (MW) from just about 40 to over a 1000 kDa (Guengerich 1996).

CYP isoforms are found in almost every phylum in which they have been sought (Heffernan & Winston 1997). Although they were originally discovered in mammalian hepatic microsomal preparations, they have subsequently been found in many organs and tissues of numerous other animals and in some plants, fungi and bacteria. CYP gene superfamily comprises over 8000 genes and pseudogenes distributed across a wide range of biological domains (Nelson 2009; Parente et al 2009). Already by 1998 there were more than 1000 CYP DNA sequences, and the problem of genetic nomenclature is becoming daunting (Nelson 1998; 2009).

A better understanding of the role of each CYP in drug metabolism and drug-induced toxicity is vital. At this time, however, most research on the CYP system in fish has been done on environmental factors that influence CYP induction. Much less is known about metabolism of aquaculture antibiotics by the CYP system (Snegaroff *et al.* 1989; Ishida 1992; Moutou *et al.* 1998; Vaccaro *et al.* 2003; Topic Popovic *et al.* 2007, 2012; Yu &Yang 2010; Zhou *et al.* 2011; Hu *et al.* 2012).

Knowledge of the multiplicity, function and regulation of CYP forms in non-mammalian and non-traditional species continues to grow in importance. Research on aquatic species monooxygenase systems expanded rapidly in the mid-1970s so that already by the late 1970s and 1980s several major reviews of microsomal CYP systems in aquatic species appeared (Bend & James 1978; Lech & Vodicnik 1984; Stegeman *et al.* 1984). Direct information regarding the identity of CYP forms in aquatic species is most abundant

for fish. The first CYPs purified from fish were from the elasmobranch little skate (*Raja erinacea*) (Bend *et al.* 1977). So far multiple CYP forms have been purified, partially purified or cloned from the freshwater and marine fish, mainly from rainbow trout (*Onchorhynchus mykiss*), perch (*Perca fluviatilis*), scup (*Stenotomus chrysops*), cod (*Gadus morhua*), tilapia (*Oreochromis niloticus*), zebrafish (*Danio rerio*), killifish (*Fundulus heteroclitus*), goldfish (*Carassius auratus*), stickleback (*Gasterosteus aculeatus*), medaka (*Oryzias latipes*) (Andersson & Förlin 1992; Stegeman 1995; Ueng & Ueng 1995; Gu *et al.* 2005; Luckenbach *et al.* 2005; Tseng *et al.* 2005; Goldstone & Stegeman 2008; Oh *et al.* 2009; Zanette *et al.* 2009; Goldstone *et al.* 2010; Hassanin *et al.* 2012).

The aim of this paper is to present a critical review of the impact of aquaculture antimicrobials on the piscine CYP system and to give an overview of recent literature reports and advances in the field of biological significance of the CYP enzymes in fish.

CYP system in fish

Although the research on mammalian CYPs continues to dominate the literature, there is a growing recognition of the biological significance of CYPs in fish. There are about 20 000 species of fish, representing nearly one-half of all known vertebrate species. These species comprise 200 families, 32 orders and three classes within the subphylum Vertebrata. They are extraordinary diverse, and inhabit virtually every niche within the world's fresh and marine waters (Stegeman 1989). They are also a direct food source for humans as well as conveyors of toxic chemicals to human beings (Sen & Arinc 1998).

Initially it was thought that fish lacked CYP-linked monooxygenases, but studies carried out in the late 1960s by Buhler and Rasmusson (1968) and Dewaide and Henderson (1968) showed that these enzymes were present in the livers of rainbow trout and other fish. Enzyme activities of fish hepatic microsomes were generally lower than in mammals and many fish CYPs had temperature optima of about 25ºC, explaining why they were not detected in the earlier studies where incubations at higher temperatures were employed. Subsequently, as observed in mammals, multiple CYP forms were discovered in fish, predominately localized in the liver, but also found in lower concentrations in other tissues, like kidney, gut, gall bladder, gonads, nervous tissue, endocrine cells, gills etc. (Andersson & Förlin 1992; Buhler 1995; Sarasquete & Segner 2000).

Fish hepatic microsomes exhibit typical reduced CO absorption spectra with a peak near 450 nm, and electron paramagnetic resonance characteristics with low-spin *g* values near 2.41, 2.25, and 1.91 typical for CYPs. Specific contents of CYPs in fish hepatic microsomes cluster between 0.2 and 0.5 nmol/mg. They range from less than 0.1 nmol/mg to nearly 2.0 nmol/mg microsomal protein. Such differences in content can occur within a single species, depending on strain, sex or chemical treatment (Andersson & Förlin 1992). Fish hepatic microsomes catalyze epoxidation, hydroxylation, dealkylation and oxidation reactions ascribed to CYP in other systems (Gillam 2007; Gillam & Hunter 2007). Substrates metabolized include many that are used to characterize CYPs in mammals, but activities found in teleosts might be catalyzed by CYP forms largely unrelated to the catalyst for the same activity in mammalian systems. Conversely, CYP forms that are structural homologues in teleosts and mammals could have different activities (Stegeman 1993). One of the major differences between mammalian and fish CYPs is that some fish species have much higher constitutive levels of aryl hydrocarbon hydroxylase (AHH) than mammalian counterparts (Lee *et al.* 1992). CYP1B1 was found to be the major isoform

involved in AHH activity in human lymphocytes (Toide et al. 2003). Goksøyr *et al.* (1987) described certain characteristic features of the fish xenobiotic metabolizing systems: generally lower specific activities compared to mammals, but high capability to activate procarcinogens to reactive intermediates; high responsiveness of monooxygenase activities to the polycyclic aromatic hydrocarbons (PAH) class of inducers (3-MC, *beta*-Naphthoflavone BNF, BP, etc.), but an apparent refractiveness to induction by the PB. In fish, CYPs have mostly been studied as catalysts for bioactivation of carcinogens and biomarkers of environmental contamination (Råbergh *et al.* 2000).

As in the mammalian system, multiple forms of CYPs belonging to the families of CYP1A, CYP1C, CYP2B, CYP2E, CYP2M, CYP2K, CYP2R, CYP2U, CYP3A, CYP11A, CYP17, CYP19, CYP26 are found in fish (Godard *et al*. 2005; Parente *et al*. 2009; Goldstone *et al.* 2010). Intensive research on fish CYPs rapidly reveals novel cytochromes, like cloning and characterization of CYP26 in zebrafish *Lythrypnus zebra* (Haque *et al.* 1998), CYP2N1 and CYP2N2 in killifish (Oleksiak *et al.* 2000), cloning of CYP1B1 in scup and plaice *Pleuronectes quadrituberculatus* (Godard *et al.* 2000; Leaver & George 2000), cloning, sequencing and tissue expression of CYP3A27 in rainbow trout (Lee *et al.* 1998), and many others. There are 54 CYP isoforms reported to be purified, partially purified, or cloned from aquatic species (Arinc & Sen 1999; Uno 2012). The number of fish species from which full-length coding regions of CYP1A genes have been sequenced has increased from four (rainbow trout, plaice, toadfish *Opsanus beta* and scup) to over a dozen, which include CYP1A sequences from tomcod *Microgadus tomcod*, butterflyfish *Chaetodon ocellatus*, sea bream *Sparus aurata*, sea bass *Dicentrarchus labrax*, Atlantic salmon *Salmo salar*, medaka *Oryzias latipes*, mummichog *Fundulus heteroclitus*, yellow catfish *Pelteobagrus fulvidraco*, crucian carp *hybridized Prussian carp* and killifish (Morrison *et al.* 1998; Uno, 2012). Zebrafish was found to have a total of 94

CYP genes, distributed among 18 gene families found also in mammals (Goldstone *et al.* 2010). In mammals, sex, diet and age are among the factors known to influence monooxygenase systems. Marked sex differences also occur in CYP activities in fish (Gray *et al.* 1991; Topic Popovic *et al.* 2007). Moreover, there are changes in activity associated with species, strain, season, water temperature and gonadal status of fish (Goksøyr & Förlin 1992; Husøy *et al.* 1994). Although no detailed study is available on the influence of immunomodulating factors on the fish biotransformation system (Marionnet *et al.* 1998; Reynaud & Deschaux 2006), it can generally be said that disease may influence CYP activity through interference with endocrine homeostasis, impairment of intermediary metabolism, release of toxins, changes in tissue morphology and prolonged changes in energy intake (Guengerich 1996). Also, drug interactions on fish health have received considerably less attention compared to that on human health. Chemical interactions can affect biomarker responses in situations of mixed exposure to complex mixtures of environmental pollutants and drugs, which can result in misinterpretation of biomarker data. In that sense, inhibition of catabolic CYP enzyme activities (*i.e.* CYP1A and CYP3A) are linked to adverse outcomes in fish such as bioaccumulation of procarcinogens and estrogens, which can lead to carcinogenesis or cause endocrine disruption (Celander 2011).

CYP1A1 and CYP1A2 in fish

Although several isoforms have been identified in fish, CYP1A has received the most attention as the major hydrocarbon-inducible CYP. Subfamily CYP1A proteins in fish have reduced CO maxima at 447 nm, are the primary catalysts for 7-ethoxyresorufin O-deethylase (EROD), ethoxycoumarin O-deethylase, and AHH, metabolize BP on the

benzo-ring, are strongly inhibited by α-naphthoflavone, are inducible by PAHs, and show reciprocal cross-reactivity with antibodies and to mammalian 1A1 (Stegeman 1993). Members of CYP1 family, including those belonging to CYP1A and 1B subfamilies, are known to play a prominent role in the activation of a number of environmentally-occurring procarcinogens and many drugs (Goldstone & Stegeman 2006; Goldstone *et al*. 2007; Parente et al. 2009). For many years, it had been assumed that fish express only one *CYP1A* gene product that has been characterized as a CYP1A1 protein (Råbergh *et al.* 2000). However, two genomic *CYP1A* clones were isolated in 1994 (Berndtson & Chen) from 3-MC treated rainbow trout and were characterized as *CYP1A1* and *CYP1A2* based on sequence homology, presence or absence of xenobiotic regulatory elements in 5' flanking regions, and significant differences in intron sequence. Also, there are distinctions in temporal patterns of AHH and EROD induction, and disparate results regarding multiple protein or mRNA products induced in fish by common 1A1 and/or 1A2 inducers (Goksøyr & Förlin 1992; Stegeman 1993). Differences have been reported in the catalytic and immunological properties of hepatic microsomes from BNF (inducer of CYP1A1 in mammals) and isosafrole (inducer of CYP1A2 in mammals) treated trout (Buhler 1995; Buhler & Wang-Buhler 1998; Stegeman 1995). It is well established that the distinct difference between cytochromes CYP1A1 and 1A2 resides in their spin state. While CYP1A1 is a low spin heme protein, CYP1A2 exists as a high spin heme protein (Arinc & Sen 1999). Although *CYP1A* gene subfamily evolved early during vertebrate evolution and seems to be highly conserved across vertebrate taxa (Goldstone *et al.* 2007), constitutive levels and inducibility of CYP1A protein and catalytic activity exhibit a rather large variability between fish species and populations (Parente et al. 2009). Also, the position of the ancestral *CYP1A* locus remains a question: opposing orientations of the pufferfish and

frog *CYP1A* genes pose a conundrum with regard to determining whether *CYP1A1* or *CYP1A2* resides in the ancestral *CYP1A* gene locus (Goldstone & Stegeman 2006).

CYP1A1 is localized in endothelial cells of heart, pillar cells of gills, kidney, hepatocytes, sinusoidal endothelium, and billiary epithelial cells of fish (Lester *et al.* 1993). Contrary to mammals, where CYP1A shows a heterogeneous distribution throughout the hepatic parenchyma, no zonation can be observed in fish liver (Sarasquete & Segner 2000). However, the levels of the CYP1A1 protein found in livers of rainbow trout are low in fish not exposed to PAHs or halogenated aromatic compounds (Buhler & Wang-Buhler 1998). Upon immunohistochemical examination of liver sections unexposed to such inducers, the CYP1A1 was barely detectable (Buhler 1995). Relative molecular mass of CYP1A1 cross-reacting protein bands in Western blots of hepatic microsomes, probed with an anti-fish antibody, varies among the teleost species, and ranges between 54 and 59 kDa. Also, a *CYP1A3* gene has been isolated and sequenced from the rainbow trout (Carvan *et al.* 1999). Four CYP type 1 family enzymes (CYP1B, CYP1C1, CYP1C2 and CYP1D) have been isolated from diverse fish species (Uno *et al.* 2012).

CYP3A4 in fish

CYP3A is a gene subfamily composed of multiple forms of CYP3A enzymes as characterized by immunochemistry, catalytic activities, and cDNA cloning and expression. CYP3A is inducible by steroidal chemicals and by a variety of compounds, including naturally-occurring and synthetic glucocorticoids and macrolide antibiotics (Quattrochi & Guzelian 2001). Husøy *et al.* (1994) demonstrated organ distribution and cellular localization of cod CYP3A-like isozymes in control and BNF treated cod, analyzed by

imunohistochemistry and showed its occurrence in liver hepatocytes, respiratory epithelial cells of gills, intestinal and caecal mucosal epithelium, epithelium of renal tubules and pancreatic acinar cells.

A strong similarity in the structure and catalytic function was found between trout and human CYP3A4 (Miranda *et al.* 1991), where polyclonal antibodies (IgG) generated against trout CYP LMC5 reacted strongly with CYP3A1 in dexamethasone-induced rat liver microsomes and with CYP3A4 in human hepatic microsomes in immunoblots. Buhler (1995) reports CYP3A4 isolation from livers of untreated trout, having a MW of 59 kDa, reduced CO maxima of 448 nm, and significant activity for the 6β-hydroxylation of testosterone and progesterone and for the N-demethylation of benzphetamine. Upon Western blot analysis, concentrations of CYP3A4 from the livers of trout were found to be 32 % higher in liver microsomes from sexually mature males than in females (Buhler & Wang-Buhler, 1998).

Control of CYP3A expression is thought to be mediated by the Ah (aryl-hydrocarbon) receptor/ARNT pathway in zebrafish, whereas, the pregnane X receptor (PXR) is suggested to activate CYP3A expression in Atlantic salmon (Finn 2007; Uno *et al.* 2012). PXR regulates the expression of CYP3A isozymes, as well as other CYP isoforms, by binding as a heterodimer with the 9-*cis* retinoic acid receptor (Bainy 2007). PXR genes have been cloned from a variety of vertebrate species, including zebrafish (Bainy & Stegeman 2004). However, there is a lack of knowledge of how pharmaceuticals and other xenobiotics interact with CYP3A and PXR in fish. Wassmur *et al.* (2010) established that the rainbow trout PXR is less responsive to prototypical PXR agonists than its mammalian counterparts and that it also responds differently to glucocorticoid receptor agonists. Since there are also species differences, in order to understand how

pharmaceuticals affect biotransformation in fish, sites of interaction on biotransformation pathways need to be characterized.

Therapeutic agents and CYPs

In recent years a huge progress has been achieved in CYP research and better understanding of the role of each CYP in drug metabolism and drug-induced toxicity (Lu 1995; Cravedi 2002; Reynaud & Deschaux 2006; Li *et al.* 2008; Zanette *et al.* 2009; Goldstone *et al.* 2010; Uno *et al.* 2012). Drug response varies greatly across groups and individuals. This variability is due to many pharmacological factors. In phase I and phase II metabolism, this variability may be a reflection of enzyme inhibition, enzyme induction, and/or genetic differences and disease (Meyer 1994; Cozza & Armstrong 2001).

Impact of aquaculture antimicrobials on the fish CYP system

Many countries throughout the world, including Europe and the USA, have strictly regulated controls on use of veterinary medicines, particularly for use in food animal species, including fish. In the recent decades, the European legislation regarding veterinary pharmaceuticals has introduced restrictions of the use of medicines for the treatment of farm animals, and as a result, available veterinary medicinal products for fish are now authorized. The basic Directives of European legislation on veterinary medicinal products have been frequently and substantially amended over time. The main document regarding the availability of aquaculture medicines is the Regulation for the establishment of

maximum residue limits (CEC 1990; CR No 37/2010). Currently approved in the EU are amoxicillin, florfenicol, flumequine, oxolinic acid, oxytetracycline, sarafloxacin and sulfadiazine trimethoprim, while certain compounds, including chloramphenicol and the nitrofurans are specifically prohibited for use in food animals in Europe and in the USA.

In the USA, all drugs legally used in aquaculture must be approved by the FDA's (Food and Drug Administration) Center for Veterinary Medicine (FDA/CVM). The Center defines drug ingredients, manufacturers, species, routes of delivery, dose forms, withdrawal periods, tolerances, and uses by species, including dose rates and limitations. The most common route of delivery of these legal antibiotics to fish occurs through mixing with specially formulated feed. In the USA there are currently only three FDA-approved and available antibiotic drugs for use in fish. They are oxytetracycline, ormetoprim and sulfadimethoxine, and florfenicol. FDA will sometimes allow veterinarians to prescribe the use of medicated feed for fish species other than those listed on the label. For example, oxytetracycline medicated feed approved for use in catfish, may be prescribed off label for hybrid striped bass by a licensed veterinarian (Durborow & Francis-Floyd 1996).

Despite strict regulations, except *via* medicated feed, fish may get exposed to antimicrobial drugs *via* aquatic environment. Discharge from sewage treatment plants has been identified as their primary source (Lindberg *et al.* 2005; Smith *et al.* 2012). Numerous pharmaceuticals and other environmental contaminants are metabolized by CYP enzymes in fish, and therefore CYP enzymes are of critical importance both for detoxification and formation of toxic metabolites (Dorne *et al.* 2007; Beijer 2010). Although generally effect levels for pharmaceuticals are higher than those found in the environment (Corcoran *et al.* 2010), the risks to wild fish populations have not been thoroughly characterized, and there has been a lack of consideration given to the likely chronic nature of exposures, or the potential mixture effects.

Interactions with tetracyclines

Environmental exposure to oxytetracycline (OTC) and sulfathiazole was proven to indirectly increase the catalytic activity of aromatase, potentially through transcriptional level modulation (Ji *et al.* 2010). It also resulted in greater expression of *CYP17*, *CYP19*, or *3βHSD2*, which play crucial role in steroidogenic pathways. Topic Popovic *et al.* (2012) determined responses to OTC treatment and investigated whether antibodies to known mammalian CYP forms (CYP1A1 and CYP3A4) would react with their respective CYP isoforms in fish (hybrid striped bass (*Morone saxatilis* male x *Morone chrysops* female), channel catfish (*Ictalurus punctatus*), and Nile tilapia). Additionally, expression of these CYPs as well as the activity of CYP1A2 and CYP3A4 was also assessed. Both goat anti-rat CP1A1 and rabbit anti-human CYP3A4 showed good cross-reactivity with the three species tested in this study. Although some antibodies only recognize proteins from species closely related to the sources of the immunogen (Al-Arabi & Goksøyr 2002), the antibodies used in this work recognized epitopes from the divergent groups examined. Considerable non-specific binding was displayed, which was expected regarding the source of the primary antibodies (Topic Popovic *et al.* 2007). Immunological cross-reactivity has previously been demonstrated between fish CYP3A and both rat CYP3A1 and human CYP3A4 (Miranda *et al.* 1991). Also, clear, putative, OTC-related responses were found in liver and the hepatic phase I xenobiotic metabolizing enzyme system of the three different fish species in this observational study. This observational approach demonstrated species differences both in control activities and in the timing and extent of hepatic responses to OTC. Further, resorufin benzyl ether (BzRes) was proven to be a

334 better substrate than benzyloxy-4-trifluoromethylcoumarin (BFC) for monitoring CYP3A
335 activities in fish. The data of the study indicated that antibiotics should also be evaluated in
336 the target species under conditions of use. The variable effects in the response among
337 different fish species to antibiotics in this work is a valid reason to critically use assays
338 developed for mammals for evaluating drug response in fish. Moreover, the unique
339 responses of CYP enzymes in different fish species to OTC treatment may have relevance
340 for the use of other antibiotics in aquaculture.
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343 **Interactions with fluorokinolones**
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345 The first indications that CYP1A were involved in the metabolism of oxolinic acid were
346 described by Ishida (1992), who treated carp (*Cyprinus carpio*) with polychlorinated
347 biphenyl (PCB), a CYP 1A inducer, before the treatment with oxolinic acid. CYP1A was
348 also responsible for difloxacin (DIF) metabolism in crucian carp liver (Fu *et al.* 2011) and
349 its *N*-demethylation in Chinese idle (*Ctenopharyngodon idellus*) kidney (Yu & Yang 2009;
350 Yu *et al.* 2010). After DIF treatment, CYP1A expression gradually decreased and was
351 significantly lower than the control. CYP1A1 transcript level was down-regulated by DIF,
352 suggesting that DIF is both the substrate and inhibitor of CYP1A.
353 Enrofloxacin (EF) was assayed for the effect on induction of CYP1A mRNA in
354 Atlantic tomcod (Williams *et al.* 1997). Pretranslational suppression of CYP1A induction
355 was found in spawning females. EF exhibited potent inhibition on the CYP1A-related
356 EROD activity as well as CYP1A expressions at both protein an mRNA levels in crucian
357 carp (Hu *et al.* 2012). In sea bass, EF was proven able to provoke the inactivation of the
358 CYP3A enzyme (Vaccaro *et al.* 2003). A single dose of i.p. injection of 3 mg/kg of EF or

multi doses of 1 mg/kg were sufficient to cause a generalized depression of CYP activities in sea bass. EF was an effective inactivator of the erythromycin *N*-demethylase activity (Vaccaro *et al.* 2003; Hu *et al.* 2012), while immunoblot analysis showed a strong decrease of staining intensity of a protein band immunoreactive with anti CYP3A27. Therefore, it can be postulated that fluorokinolone-antibiotics such as EF in sea bass have an adverse reaction with the CYP enzymes and hence a strong potential to cause long-lasting interactions. Compared to OTC, oxolinic acid was better absorbed and faster excreted from the treated fish (Björklund *et al.* 1991).

However, significant differences were noted in *O*-dealkylation and Western blotting responses between compounds of fluorokinolonic drug class when rainbow trout were fed medicated diets with oxolinic acid and flumequine (Moutou *et al.* 1998). Both oxolinic acid and flumequine effects were related to the CYP1A subfamily. The induction of day 10 post-treatment with oxolinic acid was confirmed by immunoblotting, while the lack of evidence for CYP1A induction after flumequine administration suggests that flumequine caused an activation of CYP1A, probably by protein stabilization. Also, the effects of oxolinic acid on *O*-dealkylation were delayed and longer-lasting compared with those of flumequine (Moutou *et al.* 1998). Such differences in CYP response post treatment can be explained with differences in the pharmacokinetics of oxolinic acid and flumequine in rainbow trout.

Interactions with sulfonamides

The antibacterial drug sulfamethoxazole did not markedly inhibit gill EROD activity of three-spined sticklebacks (*Gasterosteus aculeatus*) in the work of Beijer *et al.* (2010),

384 although inhibition of EROD activity by sulfamethoxazole has been reported in fish
385 hepatocytes *in vitro* (Laville *et al.* 2004). Combination ormetoprim-sulfadimethoxine
386 incorporated in diet was administered to summer flounder (*Paralichtys dentatus*) in study
387 by Topic Popovic *et al.* (2007). Immunoblots of pre-treatment and treated summer flounder
388 hepatic microsomes probed with goat anti-rat CYP1A1 antibodies exhibited specific bands
389 in the region of 65-70 kDa. Dealkylation of the hepatic CYP1A2 marker 3-cyano-
390 ethoxycoumarin was significantly increased relative to pre-treatment fish through 10 days
391 and returned to pre-treatment levels by post-treatment day 21. When identical samples
392 were tested for BFC and BzRes activities, males and females yielded different curves. BFC
393 activity was markedly higher in males, but females had higher BzRes activity, with both
394 substrates specific for CYP3A4 enzymes. In this work (Topic Popovic *et al.* 2007) authors
395 demonstrated the usefulness of mammalian antibodies for summer flounder CYP Western
396 blotting and quantified CYP1A2 and 3A4 metabolism by modifying kits developed for
397 mammalian microsomes, which could facilitate future work on piscine hepatic
398 microsomes. As an observational study with no concurrent, untreated fish at the post-
399 treatment sampling times, inferences were limited to comparisons with pre-treatment fish
400 and over sampling times. While CYP1A1 and CYP3A4 enzyme activities exhibited sharp
401 increases and decreases through post-treatment day 10, activities for both isozymes
402 returned to pre-treatment levels by post-treatment day 21. Considering the age of the
403 summer flounder used and the relatively short observational period, it is unlikely that the
404 extent of fluctuations seen in enzyme activity, 50 to 100 percent, was due to normal,
405 possibly confounding physiological factors. Further, these temporal perturbations in CYP
406 enzyme activity correlate strongly with the presence of potentiated sulfonamide residues
407 found in summer flounder (Kosoff *et al.* 2007). Thus, as has been demonstrated repeatedly
408 in mammalian systems, therapeutic compounds that are not substrates of phase I oxidative

409 enzymes have the potential for interfering with the biotransformation of other therapeutic
410 compounds or environmental chemicals. For food animals such as the summer flounder,
411 the implications of this prospect impact the withdrawal time of additional therapeutics or
412 the presence of environmental contaminants in edible tissues.
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415 **Interactions with berberine and chloramphenicol**
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417 Berberine, a quaternary isoquinoline alkaloid, could enhance the serum bactericidal
418 activity in fish by activating the complement system, giving it a potential for prevention or
419 treatment of fish diseases (Ji *et al.* 2012), and has long been considered as an antibiotic
420 candidate for controlling systemic bacterial infections in fish, especially in synergistic
421 action with enrofloxacin (Zhang *et al*, 2009). In the work of Zhou *et al.* (2011) berberine
422 acted as a potent inhibitor of crucian carp CYP1A, reducing not only CYP1A mRNA
423 expression in a dose-dependent manner, but also directly inhibiting this enzyme
424 competitively. Also, high berberine doses inhibited CYP3A through the downregulation of
425 its expression at the both mRNA and protein level. The authors compared the identity of
426 this crucian carp CYP1A isoform with those of human at the amino acid level and found
427 that it had a stronger identity with human CYP1A2 than CYP1A1. Hence, the mechanisms
428 underlying the effects of berberine on crucian carp CYP1A isoform and human CYP1A
429 expressions may be quite different.
430 Chloramphenicol (CP), a broad spectrum antibiotic, has previously been used for
431 treatment of fish bacterial diseases, particularly furunculosis and diseases caused by
432 pseudomonads (Snieszko 1954). In trout liver cells the biotransformation of labeled CP,
433 CP-glucuronide, was found to be the major metabolite, along with the CP-base, CP-alcohol

and CP-oxamic acid, while the metabolic rate of the 3H-CP reached 0.2 nmol/hr/106 trout hepatocytes (Cravedi & Baradat 1991). Snegaroff *et al.* (1989) measured interactions between chloramphenicol and monooxygenases by measuring aldrin epoxidase (AE) and found that the AE activities in trout liver were severely inhibited under higher concentrations, but not inhibited by chloramphenicol under concentrations below a certain threshold. Chloramphenicol was also shown to have an inhibitory effect on the hepatic EROD activities in rainbow trout, both *in vivo* and *in vitro* (Snegaroff *et al.* 1989), however, it had very little or no inhibitory effect on trout lauric acid (ω-1)-hydroxylase and dimethylbenz[*a*]anthracene hydroxylase activities (Miranda *et al.* 1998). Understanding the inhibition mechanism would minimize the selection of drugs that could either inhibit or destroy CYPs. Decreased CYP levels could result in unacceptably high plasma drug level and long drug half-life.

Conclusion

There is an array of antimicrobial drugs evaluated for the effects on CYPs in fish. While the majority of these compounds are regularly detected in the aquatic environment from human-use and agriculture, some are utilized in aquaculture for treatment of fish diseases. However, drug interaction studies are lacking outside of mammalian species and not enough is known about the effects of aquaculture antibiotics on the piscine CYP system. Although fish CYPs are likely inducible by mammalian inducers, the specific isoforms responsive to such inductions are still unclear, and information on fish CYP induction potential and catalytic activity is often inferred from mammalian systems, assuming similar patterns. Since both CYP1A1 and CYP3A4 have become important markers of chemical

exposure in many species including fish, perturbations of the activities of these enzymes may have consequences in the metabolism of antimicrobial drugs, especially CYP3A4, arguably the most important enzyme involved in the metabolism of xenobiotics.

Antimicrobial drugs must be evaluated extensively in the target organism. Drug interactions with the fish CYPs may alter their distribution and metabolism, as well as elimination. They can impact the metabolism of other drugs and xenobiotics metabolized by the same system with an effect on the physiology of fish administered these antimicrobials. The differences in responses of CYP enzymes in different fish species to antimicrobial treatment may have relevance for the use of antimicrobials in aquaculture. The variable effects in the response among different fish species to antimicrobial drugs is a valid reason to critically use assays developed for mammals for evaluating drug response in various fishes. Also, the use of CYP antibodies across phyla can be of value if caution is exercised in the interpretation. Substrates developed for use in mammalian systems have been assayed in fish, and are useful for CYP-mediated functional comparison between mammalian and fish species. Standardized protocols for drug impact on fish CYPs will facilitate those studies and standards will be necessary for the future application on teleosts.

Since the metabolism of aquaculture antimicrobials by the CYP enzymes could also determine the metabolism of other xenobiotics and endogenous compounds, along with persistence of residues and the length of the withdrawal period before fish being apt for human consumption, and taking into consideration other challenges and concerns regarding drug approval/administration/dosing/metabolism, impact of antimicrobial drugs on piscine CYPs requires further in-depth studies, equal to their mammalian counterparts.

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