PHARMACOKINETIC STUDIES AND TISSUE RESIDUE ANALYSIS OF OXYTETRACYCLINE IN SUMMER FLOUNDER (*PARALICHTHYS DENTATUS*) MAINTAINED AT DIFFERENT PRODUCTION SALINITIES AND STATES OF HEALTH

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ABSTRACT

PHARMACOKINETIC STUDIES AND TISSUE RESIDUE ANALYSIS OF OXYTETRACYCLINE IN SUMMER FLOUNDER (*PARALICHTHYS DENTATUS*) MAINTAINED AT DIFFERENT PRODUCTION SALINITIES AND STATES OF HEALTH

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Summer flounder, *Paralichthys dentatus*, culture is becoming increasingly popular in the United States because of high market prices and consumer demand. In addition, flounder is a marine fish species that can tolerate a wide range of salinities, allowing for inland intensive fish culture. Oxytetracycline (OTC) is one of two available FDA-approved antibiotics for use in foodfish in the United States. Oxytetracycline was chosen for these studies because it is excreted primarily unchanged through the urine and the absorption, distribution and elimination of this drug may be influenced by environmental and physiological conditions. Four experiments were conducted to investigate: 1) pharmacokinetic parameters of oxytetracycline (50 mg/kg) following intravascular (IV), intraperitoneal (IP), intramuscular (IM) and per os (PO) administration in summer flounder maintained at 28 ppt salinity and 20^{o}C; 2) pharmacokinetic parameters of OTC (50 mg/kg) following IM and PO administration in summer flounder maintained at three different salinity levels of 0 ppt, 15 ppt and 32 ppt and the physiological adjustments summer flounder make to acclimate to environmental salinity; 3) OTC retention times in muscle tissue from summer flounder maintained at three different salinity levels (0 ppt, 15 ppt, 32 ppt) and treated with a single 50 mg/kg OTC dose via IM and PO administration; and 4) pharmacokinetic parameters of OTC (50 mg/kg) following IM and PO administration in clinically healthy and clinically diseased summer flounder maintained at 28 ppt and 20^{o}C. Oxytetracycline plasma concentrations were determined

using high performance liquid chromatography (HPLC) and analyzed using a non-compartmental pharmacokinetic model for all routes of drug administration.

Statistical comparisons were not made between the different routes of OTC exposure, but results from experiment one indicated that IV administration of OTC resulted in the largest area under the curve (AUC) value (8147.9 µg•h/ml) and the highest maximum plasma concentration (C_{max}) of 1173.2 µg/ml OTC at 5 min post-injection. Intramuscular injections of OTC resulted in prolonged total body elimination half-life (T ½) of 301.3 h and high fish-to-fish variability (0.6). Per os administration resulted in low C_{max} (0.2 µg/ml OTC) and poor systemic bioavailability (0.2 %).

Results from experiment two demonstrated that when OTC is administered IM AUC estimates are significantly ($p<0.05$) lower in summer flounder held at 0 ppt (1684.8 µg•h/ml) than fish maintained at 15 ppt or 32 ppt salinity (2067.8 µg•h/ml and 2241.3 µg•h/ml, respectively). Although not significantly different from other salinity treatments, time to maximum plasma concentration (T_{max}) was longer in fish held at 15 ppt and 32 ppt (312 h and 168 h, respectively) compared to cohorts in freshwater (0.5 h) and C_{max} values were higher in animals held at 15 ppt and 32 ppt (8.4 µg/ml OTC and 9.2 µg/ml OTC, respectively) than freshwater fish (4.9 µg/ml OTC) when OTC was administered via IM injection. No significant differences were detected in any of the pharmacokinetic parameters following PO dosing of OTC, however, the AUC estimates were lower in the 32 ppt acclimated fish (127.7 µg•h/ml) than in the 0 ppt or 15 ppt acclimated fish (190.2 µg•h/ml and 180.7 µg•h/ml, respectively). In addition, the T ½ was longer in the higher salinity groups (278.1 h and 266.0 h, respectively) than in the freshwater fish group (256.9 h). Physiological adjustments made by summer flounder including plasma and urine osmolality, urine flow rate and urine character, gill chloride cell size and density, and Na^+ - K^+ ATPase activity demonstrated trends that suggested physiological differences among the salinity groups. Plasma and urine osmolalities were typically significantly ($p<0.05$) higher in fish maintained at 32 ppt salinity than at the lower salinity treatments. In addition, urine flow rates were generally significantly ($p<0.05$) greater in freshwater adapted fish (0.13 - 0.21 ml of urine/kg/hour) in

comparison to fish in the salinity treatments of 15 ppt and 32 ppt (0.06 - 0.12 ml of urine/kg/hour and 0.09 – 0.11 ml of urine/kg/hour, respectively). Changes in gill chloride cell size and density and enzyme activity of Na^+ - K^+ ATPase revealed no significant differences between the salinity treatments but summer flounder in saltwater had numerically larger and more chloride cells than summer flounder in freshwater, but enzyme activity was greater in freshwater acclimated summer flounder compared to fish in seawater.

Experiment three results revealed similar OTC muscle tissue pharmacokinetic parameters in summer flounder following IM injection. However, there were significant differences ($p<0.05$) in the AUC parameters of the plasma and muscle OTC concentrations between fish maintained at different salinities following IM OTC treatment. These effects may be the result of a "depot" effect in the muscle tissue or may be related to the reduced solubility of OTC in the muscle tissue of marine fish. A single PO dose administration of OTC at 50 mg/kg did not result in plasma or tissue concentrations higher than the FDA tissue tolerance limit of 2 ppm.

Results of the fourth experiment demonstrated that following IM OTC administration healthy fish had significantly ($p<0.05$) higher AUC (4700.6 µg•h/ml) values than diseased cohorts (2576.2 µg•h/ml). Maximum plasma concentrations were also higher in the healthy fish than in the diseased fish, although values were not significantly different (23.4 µg OTC/ml and 20.2 µg OTC/ml, respectively for healthy and diseased fish). Additionally, in diseased fish, the mean resident time (MRT) (293.7 h) and T ½ (203.5 h) parameters were longer compared to parameters in healthy fish (253.2 h and 175.4 h, respectively), although values were not significantly different. No significant differences were detected in any of the pharmacokinetic parameters following PO OTC administration, however, healthy fish achieved higher maximum plasma OTC concentrations (1.0 µg OTC/ml) than diseased fish (0.7 µg OTC/ml). Fish-to-fish variation was greater in diseased animals than in healthy regardless of route of drug administration.

The results of these experiments indicated that OTC pharmacokinetic parameters are influenced by route of drug administration, environmental salinity and fish health status. These factors must be considered by veterinarians and governmental regulators when developing treatment regimens for summer flounder.

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DEDICATION

This dissertation is dedicated to my parents, Francis and Florence Hughes.

With love and appreciation

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My hope is this dissertation will make at least a modest contribution to the world of aquaculture and aquatic animal medicine.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
AUC	area under the curve
AUMC	area under the moment curve
BW	brackish water
C	Celsius
C_{max}	maximum plasma concentration
Ca^{2+}	calcium
$CaCO_3$	calcium carbonate
CC	chloride cell
CD	circular dichroism
Cl	clearance
Cl^-	chloride
cm	centimeters
d	days
DO	dissolved oxygen
EAEMP	European Agency for the Evaluation of Medicinal Products
F	systemic bioavailability
FDA	Food and Drug Administration
FW	freshwater
g	gram
GFR	glomerular filtration rate
h	hour
HPLC	High performance liquid chromatography
IM	intramuscular
INAD	Investigational New Animal Drug
IP	intraperitoneal
IV	intravascular
K^+	potassium
kg	kilogram

kHz	kilohertz
l	liter
Mg^{2+}	magnesium
mg	milligram
MIC	minimum inhibitory concentration
min	minute
ml	milliliter
mm	millimeter
mOD	milli-optical density
mMol	millimole
mOsmol	milli-osmolality
MRT	mean resident time
MTL	maximum tolerance limit
MW	molecular weight
µg	microgram
µm	micrometers
µmole	micromole
N	number of observations
Na^{+}	sodium
ND	not determined
NDR	no data reported
nmole	nanomole
nm	nanometers
OTC	oxytetracycline
P	probability
PO	per os
PPB	plasma protein binding
ppm	part per million
ppt	parts per thousand
SD	standard deviation
SE	standard error

sec	seconds
SW	seawater
T_{max}	time of maximum concentration
T ½	total body elimination half-life
U.S.	United States
V_d	volume of distribution
WDT	withdrawal time
x g	times the force of gravity

INTRODUCTION

Summer flounder, *Paralichthys dentatus*, culture is becoming increasingly popular in the United States, but it is not free of challenges. One of the challenges is to economically produce a disease-free and drug-free seafood product. To achieve this goal, research must be conducted to determine the absorption, distribution and excretion of drugs approved for use in aquaculture. Oxytetracycline (OTC) is one of the FDA approved chemotherapeutics for treating specific bacterial diseases in catfish, salmonids and lobster. Extra-label veterinary prescriptions or investigational new animal drug (INAD) permits are required to use OTC in any other fish species. Oxytetracycline was selected for this study because its distribution and excretion may be influenced by physiological adaptations fish make in response to changes in environmental conditions (i.e. salinity and temperature). Summer flounder tolerate a wide range of salinities and adapt by changing drinking behavior, gill cellular components and enzyme activity, glomerular filtration rate (GFR), and plasma and urine osmolality. Since euryhaline fish, like summer flounder, are able to make the necessary physiological adjustments, fish farmers may be able to produce this flounder species more economically at lower salinities in recirculating aquaculture systems. The potential impact of these environmental changes on drug metabolism must be identified to ensure that safe wholesome seafood products reach consumers. Past research has shown that different fish species have varying OTC withdrawal times and to date, comparison of drug withdrawal times in the same fish species maintained at different environmental salinities has not been reported.

In addition, fish farmers often must also combat bacterial diseases in their cultured fish populations. Intensive aquaculture practices and management increase the odds of bacterial infection in cultured fish. Fish infected with a bacterial pathogen such as *Vibrio anguillarum*, may have lesions involving internal organs, such as the kidney, that may be an integral part of drug excretion. Most pharmacokinetic trials are conducted in healthy subjects, which may overestimate drug absorption and underestimate elimination times. In order to ensure low drug residues, research must be conducted in fish with differing physiological status, i.e. healthy versus diseased.

CHAPTER 1

LITERATURE REVIEW

1.1: MARICULTURE

Over the past two decades, aquaculture has been heralded as one of the fastest growing facets of the United States agriculture industry and of the world food economy (Lee and Ostrowski, 2001; Naylor *et al*., 2001; Francis-Floyd, 1993). The high importance of aquaculture-supplied edible seafood products gains its stature by ensuring a consistent source of seafood that is vital "for the nutritional and financial health of a large segment of the world's population" (Tidwell and Allan, 2002). Rapidly increasing human populations in countries such as Asia, Africa and South America drive the demand for foodfish, as an inexpensive high protein food source (Tidwell and Allan, 2002). To date, foodfish production in the U.S. has primarily focused on freshwater catfish, trout and, to a lesser degree, the Atlantic salmon (Lee and Ostrowski, 2001). However, with statistics showing that approximately 61% of 283 marine fish stocks are over-harvested, the production of marine fish species is an opportunity for financial gain in supplying high quality, safe and wholesome marine aquaculture products for domestic and global markets (Lee and Ostrowski, 2001).

Mariculture, the production of marine aquatic species, in the U.S. is gaining popularity not only for stock enhancement but also for the production of marine species for food (Lee and Ostrowski, 2001). Flatfish species are excellent candidates for aquaculture production because of their high market value and consumer demand as well as their tolerance for different environmental salinity conditions. The euryhaline status of many of these species allows economically feasible inland intensive culture of these fishes. Economically, the commercialization of flounder aquaculture is of primary interest due to high consumer demand and high market prices. In 1996, dockside price was approximately $2.00/pound with a store value of $5.00/pound, and in 2002 flounder store prices ranged from $1.50 - $4.99 per pound in the winter season and from $2.50 to $5.95 per pound in the summer season (Dumas and Horton, 2002). In addition to the foodfish market, the popularity of flounder is high for recreational and sport fishing (Burke *et al*., 1999). Commercial-scale production of flounder, particularly summer flounder, is

growing in the northeastern United States where hormone-induced ovulation of captive brood stocks has been successful (Watanabe *et al*., 1998a).

The summer flounder, *Paralichthys dentatus*, is a left-sided flounder, meaning after metamorphosis both eyes are on the left side of this flatfish species. The summer flounder naturally inhabits coastal waters along the east coast of the United States from Maine to Florida (Bengtson and Nardi, 2000). Within the past decade, interest in the commercial production of summer flounder has grown in the United States. Wild stocks of flounder species have significantly declined over the past 50 years as a result of increasing numbers of flounder fishermen and the introduction of new harvesting techniques that have led to over-fishing through unsustainably high harvests (Bengtson and Nardi, 2000). Environmental stresses have also threatened flounder species (Waters, 1996; Nash and Novotny, 1995). As a result of these factors, the yearly landings of flounder has declined from 185 million pounds to 53 million pounds from 1984 to 1994 (Waters, 1996).

Culture of summer flounder is relatively new in this country. The U.S. government has only been funding research for development of aquaculture for the summer flounder since 1990 (Bengtson and Nardi, 2000; Bengston, 1999). Models for the intensive culture of this flatfish species have been developed based on commercial intensive production of the related Japanese flounder, *Paralichthys olivaceus* (Burke *et al*., 1999). The Japanese flounder and turbot (*Scophthalmus maximus*), both left-sided flatfish, are successfully cultured in Asia and Europe, respectively (Bengtson and Nardi, 2000). The first attempts to spawn and raise summer flounder in the U.S. occurred in 1970, however, because of high natural population stocks and the infancy of mariculture in the U.S. at that time, the culture of this fish was considered unnecessary and research interests subsided. Recently, with successful models for marine fish aquaculture systems and declines in natural stocks of summer flounder, serious commercial production of this flounder species began again in 1996 (Bengtson and Nardi, 2000).

Concurrent to the investigations of the summer flounder life cycle, development, nutritional, reproductive and husbandry requirements, are studies directed towards maximizing production potential (Tucker, 1998). In order to maximize production efficiency, especially of a saltwater species because the additional production cost of purchasing salts, husbandry techniques and health management must be optimized. Most facilities rearing saltwater fish cannot risk pumping natural seawater from oceans or estuaries because of the high cost involved in testing and treating that water for toxins and pathogens. In addition, commercially available salts required to make artificial seawater are a considerable cost factor for facilities raising these animals (cost of salt solution is estimated to be $\$53/m^3$ of seawater (Zucker and Anderson, 1999)). Summer flounder are a euryhaline fish species, which may be able to tolerate a wide range of salinities. Thus, production facilities may be able to maintain summer flounder stocks at salinities lower than seawater with success and save money. Preliminary studies in our laboratory have shown that summer flounder can be slowly acclimated to 0 ppt salt and maintained long term in freshwater with high hardness levels (>250 mg/l $CaCO_3$) (Hughes, unpublished). For summer flounder producers to remain economically viable, means of decreasing the cost of marine fish production must be investigated. One way to do this is to alleviate the cost of formulating full strength seawater. If summer flounder can be commercially reared at lower salinities with equal success as cohorts in seawater, producers will save significantly on production costs.

1.2: USE OF ANTIBIOTICS IN FISH CULTURE IN THE UNITED STATES

To maximize production, intensive fish culture facilities often maintain fish at high stocking densities and aim to maximize growth by high feed intake. High stocking densities and the resulting poor water quality typically lead to compromised fish health, disease and mortality. Infectious diseases are one of the most common causes of population and economic losses in commercial aquaculture (Alderman, 2000; Park *et al*., 1994). As a result, chemotherapeutics are frequently involved in the treatment of bacterial diseases in cultured fishes (Plumb, 1999).

Currently in the United States, there are only two antibiotics available and approved by the Food and Drug Administration (FDA) for use in foodfish: oxytetracycline (OTC, Terramycin for Fish®) and Romet®. The FDA-approved dose of OTC in feed is 55 – 83 mg OTC/kg of body weight for 10 d. Recommended doses for injectable formulations of OTC are 25 – 50 mg OTC/ kg of body weight (Piper *et al*, 1982). The FDA has established a tolerance limit of 2 ppm OTC in the raw edible portions of salmonids, catfish and lobster and requires a 21-day withholding period for the teleosts and a 30-day period for lobster. Currently, OTC requires an extra-label veterinary prescription for use in summer flounder because a complete pharmacokinetic study has not been conducted and published nor has an Investigational New Animal Drug (INAD) been approved by the FDA. The 1994 Animal Medicinal Use Control Act gave approval to licensed veterinarians for the use of compounds extra-label. However, this act did not approve drugs for extra-label use that were administered through medicated feed (Jensen and Greenlees, 1997). Typically, in the intensive culture of foodfish, the only route feasible is through the use of medicated feeds because of population size (Stoffregen *et al*., 1996; Xu and Rogers, 1994a). This restriction reinforces the need for more research to determine appropriate therapeutic doses, regimens and withdrawal times.

Van Dresser and Wilcke (1989) reported that OTC was one of the four most common antibiotic residues found in animal tissues. Approved withdrawal periods for FDA-approved drugs used in food animals are only legal for the specified species, dose, route and dosage regimen (Riviere and Sundlof, 2001). Currently, there is no published data to support a 21 day OTC withdrawal period for summer flounder following OTC therapy. Therefore, the prescribing veterinarian should recommend to the producer a longer withholding period ensuring a consumer-safe edible product. Additional variables in fish husbandry which may impact drug kinetics, especially withdrawal times, include water temperature and salinity (Treves-Brown, 2000). In addition, veterinarians should be aware that when treating diseased animals or populations, drug distribution and or elimination may be altered such that residues may persist past officially recommended withdrawal times (Riviere and Sundlof, 2001). Drug residues in products entering the human food chain may lead to bacterial resistance and other potential consumer health

threats, such as allergic reactions (Du *et al*., 1997; Smith *et al*., 1994). In most farmed fish species, the primary edible portions are muscle and skin; so residue studies are typically limited to these tissues. Du *et al*. (1997) reported that common cooking procedures of OTC-treated channel catfish (*Ictalurus punctatus*) fillets did not completely degrade the drug. Oxytetracycline is commonly used in human medicine such that indiscriminate exposure through contaminated meat products or water could lead to increased OTC resistance in human and animal pathogens (Aitcheson *et al*., 2000). Therefore, although costly, it is necessary to perform drug specific pharmacokinetic research in any fish species that has potential for commercial foodfish production.

In addition to the safety concerns of drug residues tainting fillets intended for human consumption, inappropriate and over-use of antibiotics has increased the incidence of drug resistant bacteria both *in vivo* and in the aquatic environment. Since OTC has been widely used in aquaculture, for many years, bacterial resistance to this drug is reported from both pathogenic bacteria within the host and bacteria found in the aquatic environment (Nonaka and Suzuki, 2002; DePaola, 1995; DePaola *et al*., 1995). Numerous animal studies have investigated bacterial resistance to OTC following trial administration of medicated feeds. DePaola (1995) found that subtherapeutic exposure levels of OTC to channel catfish may have had a significant effect on the microflora in the catfish gastrointestinal tract and rearing water. Prevalence of OTC resistant bacteria in aquarium water was below 40% prior to feeding OTC contaminated feed, but was 100% immediately following the feeding period. Plumb *et al*. (1995) found that approximately 16% of six common bacterial pathogens of channel catfish were resistant to OTC. Environmental bacterial resistance to OTC may be enhanced by the prolonged persistence of OTC in sediment. The half-life for OTC in sediments under fish farms ranged between 9 and 419 d depending on temperature, oxygen and water currents (Aitcheson *et al*., 2000; Doi and Stoskopf, 2000; Herwig and Gray, 1997; Björklund *et al*., 1990; Samuelsen, 1989). Contamination of sediment with OTC is likely associated with the accumulation of uneaten medicated feed particles and fish feces. Because OTC is poorly absorbed from the intestinal tracts of fish, fecal material deposited under pens or cages may contain high levels of the drug (Kerry *et al*., 1996; Lunestad *et al*., 1995;

Pouliquen *et al*., 1993; Björklund *et al*., 1990). Further, Rigos *et al*. (1999) and Xu and Rogers (1994b) found that almost half of the administered dose of OTC in pre-medicated oil-coated feed is lost through leaching to the aquatic environment. Herwig and Gray (1997) also found substantial increases in antibacterial resistance in sediments following medicated feed therapy. Oxytetracycline medicated feed is not highly palatable to fish, decreased feed intake may result in more medicated feed remaining in the environment and also may lead to subtherapeutic levels in fish populations (Hustvedt *et al*., 1991). These instances of low level drug exposure increase the potential for bacterial resistance in the targeted pathogen and in other organisms in the animal and in the environment.

1.3: OXYTETRACYCLINE

Oxytetracycline (460.40 MW) is a natural tetracycline compound that is derived from the fungus, *Streptomyces rimosus*. Tetracycline compounds are broad-spectrum antibiotics that are bacteriostatic by inhibiting bacterial protein synthesis at the 30S ribosome. Tetracyclines are 4 ring amphoteric compounds with side chain substitutions made for multiple drug compounds and activity (Figure 1-1). Tetracyclines are highly lipophilic, which allows them to widely distribute in the body (high V_d). They also have a low octanol: water coefficient indicating hydrophilicity. In mammals, tetracyclines are typically well absorbed from the gastrointestinal tract of fasted animals with systemic bioavailabilities of OTC ranging between 60 - 80% (Plumb, 1995); however, absorption rates are variable between species, drug formulations and chelation status. Protein binding affinity varies among the different tetracycline drugs but generally protein binding is moderate (20 - 40%). Bioavailability of OTC in fish, both fresh and saltwater fish, ranges from 0.6 - 80% (Haug and Hals, 2000; Doi *et al*., 1998; Elema *et al*., 1996; Björklund and Bylund, 1991; Black *et al*., 1991; Rogstad *et al*., 1991; Cravedi *et al*., 1987; Grondel *et al*. 1987). These drugs are renally excreted (60%) primarily unchanged: indicative of minimal metabolism/biotransformation. Renal excretion occurs primarily by glomerular filtration, but may occur by tubular secretion (Riviere and Spoo, 2001; Treves-Brown, 2000; Plumb, 1995). Oxytetracycline has a low risk of toxicosis and diffuses into most body fluids and tissues (Doi *et al*., 1998). It is particularly effective

against Gram-negative bacteria such as *Vibrio* sp., *Aeromonas* sp. and *Pseudomonas* sp. which are common pathogens isolated from marine fishes.

Despite the many therapeutic qualities of OTC, the use of OTC in fish also has several disadvantages. One primary problem that has developed with the widespread use of this drug is bacterial resistance as discussed in the previous section. Through R-plasmid mediated bacterial resistance, many fish pathogens, such as *Aeromonas* sp., are now resistant to OTC therapy (Treves-Brown, 2000; Smith *et al*., 1994). Bacterial resistance to OTC may occur by three mechanisms which are: 1) decreased intracellular OTC concentrations because of plasmid-borne transporters pumping drug out and decreased cellular permeability, 2) production of proteins that interfere with the binding to ribosomes and 3) enzyme inactivation (Huber 1988). A second concern is the low bioavailability of OTC in fish species (Treves-Brown, 2000). One cause of the limited bioavailability of OTC in fish is its affinity to bind to plasma proteins. The binding of drug to plasma proteins influences the concentration of active drug in the plasma and its distribution in the tissues (Björklund and Bylund, 1991). Different fish species may have variable OTC plasma protein binding capacity. For example, Uno (1996) reported that healthy ayu (*Plecoglossus altivelis*) had an OTC binding capacity of 68%; Björklund and Bylund (1991) determined that rainbow trout (*Oncorhynchus mykiss*) plasma protein binding capacity for OTC was about 55%, whereas channel catfish plasma protein binding capacity was 72%.

A major concern when using OTC in marine fish systems is that tetracyclines readily chelate with divalent and trivalent cations (i.e. magnesium, calcium and iron), which decrease both their absorption and efficacy (Tongaree *et al*., 1999; Machado *et al*., 1995; Lunestad and Goksøyr, 1990). Chelation is described as the "holding of a hydrogen or a metal atom between two atoms of a single molecule" (Morrison and Boyd, 1992). This ability to bind metal cations is most strongly pronounced in the hydrophilic tetracycline compounds, like tetracycline and oxytetracycline (Lunestad and Goksøyr, 1990). When drugs are undissociated (i.e. uncharged) they readily cross biological lipid membranes. Metal chelated, or bound OTC molecules, have a different charge and conformation than

their unchelated forms, which may explain the reduced lipid solubility and absorption of the complexed form. Circular dichroism (CD) studies demonstrated that metal ion complexation with OTC produced changes in the OTC molecular conformation and stoichiometry. The difference in CD spectra revealed that Mg^{2+} and Ca^{2+} complexation yielded different OTC configurations for the two metal chelates with Mg^{2+} causing the greatest affect (Tongaree *et al*., 1999). Tissue concentrations of OTC in rainbow trout held in seawater were 30% of the concentrations found in freshwater cohorts (Lunestad and Goksøyr, 1990). In addition, studies using tetracycline found that drug plasma distribution was reduced because Mg^{2+} and Ca^{2+} complexes with OTC decreased the drugs diffusion through erythrocyte membranes (Lunestad and Goksøyr, 1990). Previous research reviewed by Lunestad and Goksøyr (1990) revealed that at pH 8, commonly the pH of saltwater aquatic systems and that of intestinal fluid in marine fish (Wilson *et al*., 2002), the complex formation between OTC and cations is 1:1. When OTC is complexed with these cations (seawater of 35 ppt salinity typically contains about 54 mMol Mg^{2+} and 10 mMol Ca^{2+}), the antibacterial efficacy of the drug is reduced (Treves-Brown, 2000; Lunestad and Goksøyr, 1990; Berthon *et al*., 1983).

Barnes *et al*. (1995) found that minimum inhibitory concentrations (MIC) increased 40-60 fold when *Aeromonas salmonicida* was grown on marine-based agar (i.e. Bacto Marine Agar) compared to non-marine based agar (i.e. tryptone soy agar). This interaction between OTC and di- and tri-valent cations is particularly problematic in saltwater fish because they actively drink seawater, which may lead to the chelation of OTC associated with any medicated feed present in the gastrointestinal tract. In addition, Carroll *et al*. (1994) demonstrated that flounder showed an increase in drinking rates when water temperatures were $20^{o}C$ or greater. Also, saltwater fish typically have higher plasma and urine osmolalities that may act to inactivate this antibiotic *in vivo*. This evidence suggests that OTC may not be an effective oral therapeutic choice to treat diseased saltwater fish. However, OTC is one of only two FDA-approved chemotherapeutics available to fish farmers and the only FDA-approved route of therapeutic antibiotic drug exposure in fish is through medicated feeds.

1.4: PHARMACOKINETIC STUDIES

Veterinarians must be equipped with an adequate knowledge base to treat and prevent devastating stock losses. To do this, pharmacokinetic studies must be conducted using the specific drug and fish species of interest. Pharmacokinetics is defined by Riviere (1997) as "the use of mathematical models to quantitate drug concentrations in an animal." Complete pharmacokinetic studies are used to provide information such as treatment dosages, appropriate treatment schedules, and safe withdrawal times. Appropriate withdrawal periods should be based on firm knowledge of the pharmacokinetic properties of the drug in the species of interest (Horsberg, 1994). Numerous pharmacokinetic studies have been conducted in fish species; however, review of this literature reveals extremely divergent results, even when studies involve the same fish species and the same drug. These variations may be a consequence of differences in experimental designs, endogenous factors (i.e. size, physiological and health status), and environmental exposures. In addition, variation between individuals may contribute to the divergent results, if experimental animals cannot be resampled during the course of the trial. Given these factors, the data that is generated from these studies is normally only valid for the species and specific conditions under which the study was conducted (Horsberg, 1994).

Most teleost pharmacokinetic studies are carried out as single dose drug exposures, where the drug is administered intravascularly (IV), intraperitoneally (IP), intramuscularly (IM) and per os (PO) (Horsberg, 1994; Stoskopf, 1988). Blood and tissue samples are then collected at pre-determined time intervals post-exposure to establish a drug concentration versus time curve. From these studies many pharmacokinetic parameters can be estimated, such as: the absorption rate constant (k_a), maximum serum concentration (C_{max}), time to maximum serum concentration (T_{max}), the area under the curve (AUC), total bioavailability (F), the apparent volume of distribution (V_d), the total body clearance (Cl), the elimination rate constant (k_{el}), half life (T ½) for absorption (extravascular administration), distribution and elimination (Horsberg, 1994). Pharmacokinetic studies may be designed as either population-based or individual-based compartmental

investigations (Horsberg, 1994; Powers, 1993). Population-based studies, or "single individual-single sample", acquire samples from multiple individuals at each time point, and then use the mean values at the different time points to calculate the pharmacokinetic properties. Individual-based studies, or "single individual-multiple sample", require repeated blood sampling from specific individuals at different points during the trial (Horsberg, 1994; Powers, 1993). It is difficult in pharmacokinetic studies to predict what compartmental models may be derived from the drug trials. Compartmentalization of drug distribution in fish is influenced by species of fish, route of drug administration, drug and drug formulation, experimental design and environmental factors (i.e. water temperature) (Uno, 1996). For instance, IV administration of OTC has been analyzed as a one, two or three compartmental model depending on the fish species involved and environmental conditions (Uno, 1996).

The application of compartments in pharmacokinetic data analysis is a method to model data and to derive pharmacokinetic parameters. In compartmental models, the body is viewed as having a number of "equilibrium compartments" where each compartment represents, mathematically not necessarily anatomically or physiologically, a specific body area or tissue. Within the regions, rates of drug diffusion and elimination are similar (Riviere, 1997). Compartmental models assume that drug elimination takes place from a central compartment and that the drug distribution and elimination rate constants obey first-order kinetics (Brown, 2001). Noncompartmental models are gaining popularity because they have the flexibility to estimate the same pharmacokinetic parameters (Vd, Cl and T½) as compartmental models while maintaining physiological relevance (Brown, 2001; Riviere, 1997). An advantage of noncompartmental approaches is that no assumptions are required as to the rates or manner in which a drug is distributed or eliminated from the body (Martinez, 1998). In addition, these physiologically significant parameters are readily compatible to computer and graphic techniques (Riviere, 1997). The biggest difference between compartmental and noncompartmental analysis is the limitation of the noncompartmental models to estimate drug localization within the body or how long the drug resides in the body (Brown, 2001; Riviere, 1997). The T ½ parameter estimated from noncompartmental analysis is similar

to calculating the whole body half-life of the drug in the body rather than a half-life calculated from the terminal slope (Riviere, 1997). In noncompartmental analysis, also referred to as statistical moment analysis or SHAM (slopes, heights, areas, moments), a mean residence time (MRT,τ) is calculated and represents the mean time required for a drug molecule to transit through the body (Martinez, 1998). The half-life for noncompartmental analysis is then derived by the equation:

$$T\ ½ = 0.693\ (MRT) = (0.693) \bullet Vd_{ss}/Cl_b$$

where Vd_{ss} represents the volume of distribution at steady state and Cl_b represents drug clearance. Mean residence time is calculated by the equation:

$$MRT = AUMC/AUC = Vd_{ss}/Cl_b$$

where AUMC represents the area under the moment curve. The AUC represents the area under the curve. The MRT parameter represents the time at which 63.2% of the administered drug has been eliminated from the body (Riviere, 1999). The apparent volume of distribution at steady state, Vd_{ss}, is calculated by:

$$Vd_{ss} = (Dose \times AUMC)/ AUC^2$$

And total body clearance is calculated by:

$$Cl_b = Dose/AUC$$

The area under the curve, AUC, is the estimated mathematical area under the plasma concentration-time curve from time 0 to infinity (Riviere, 1997). The AUC may be estimated a number of ways including the trapezoidal method which may be implemented by many computer programs. The AUC is a measure of the extent of drug exposure (Martinez, 1998) and is considered the zero moment in the statistical moment method of noncompartmental pharmacokinetic analyses. The first moment is the area under the

moment curve, AUMC, and is the area under the plasma concentration-time versus time curve. The areas are calculated from time of drug dosing (t_0) to the final concentration (C_n) measured at time (t_n):

$$AUC_n = \int_{t0}^{tn} C_i dt$$

$$AUMC_n = \int_{t0}^{tn} tC_i dt$$

Drug systemic bioavailability (F) is calculated by:

$$F = AUC_{route} \bullet Dose_{iv} / AUC_{iv} \bullet Dose_{route}$$

Additional pharmacokinetic parameters that can be derived from the plasma concentration-time curves are the C_{max} and T_{max}. The C_{max} is the peak moment in time when plasma drug concentration neither increases nor decreases. The T_{max} is the specific point in time when the rate of drug input into the blood is equal to the rate of drug loss, the C_{max} (Notari, 1987).

In the past, most OTC pharmacokinetic studies have been conducted in freshwater fishes (Table 1-1) such as: rainbow trout (*Oncorhynchus mykiss*) (Namdari *et al*., 1999; Uno *et al*., 1997; Black *et al*., 1991; Björklund and Bylund, 1991; Rogstad *et al*., 1991; Björklund and Bylund, 1990; Grondel *et al*., 1989; Norlander *et al*., 1987; Salte and Liestøl, 1983), tench (*Tinca tinca*) (Reja *et al*., 1996), pacu (*Colossoma brachypomum*) (Doi *et al*., 1998), common carp (*Cyprinus carpio*) (Grondel *et al*., 1987), African catfish (*Clarias gariepinus*) (Grondel *et al*., 1989), channel catfish (*Ictalurus punctatus*) (Fribourgh *et al*., 1969a; Fribourgh *et al*., 1969b), arctic char (Haug and Hals, 2000), ayu (*Plecoglossus altivelis*) (Uno, 1996) and yellow perch (*Perca flavescens*) (Bowden, 2001). Only a limited number of OTC pharmacokinetic studies (Table 1-1) have been carried out in marine fish and most of these studies have been limited to salmonid species, such as: Atlantic salmon (*Salmo salar*) (Namdari *et al*., 1998; Elema *et al*., 1996; Björklund and Bylund, 1990; Bruno, 1989), chinook salmon (*Oncorhynchus*

tshawytscha) (Namdari *et al*., 1999; Abedini *et al*., 1998; Namdari *et al*., 1998; Namdari *et al*., 1996), coho salmon (*Oncorhynchus kisutch*) (Namdari *et al*., 1996) and sockeye salmon (*Oncorhynchus nerka*) (Strasdine and McBride, 1979). Malvisi *et al*. (1996) conducted an oral dose study of OTC distribution and residue depletion in sea bass (*Sparus aurata*) and sea bream (*Dicentrarchus labrax*) and, more recently, Rigos *et al*. (2003) investigated the pharmacokinetics of OTC in gilthead sea bream and Rigos *et al*. (2002) conducted a study in OTC pharmacokinetics in sea bass held at two different temperatures. Two studies (Namdari *et al*., 1999; Abedini *et al*., 1998) have been conducted comparing OTC pharmacokinetics in a freshwater salmonid, rainbow trout, and a saltwater salmonid, chinook salmon. These comparative studies found that between these two salmonids species, OTC elimination half-life, volume of distribution, and bioavailabilities were remarkably similar. However, these studies were performed at relatively low water temperatures (range of 10-11^{o}C) where OTC pharmacodynamics may be altered. Studies comparing OTC pharmacokinetic parameters in fish maintained at two different water temperatures (Rigos *et al*., 2002; Namdari *et al*., 1998; Namdari *et al*., 1996) demonstrated that fish at the higher water temperature had faster OTC elimination half-life. To date, no comparative studies have been conducted at water temperatures of 20-22^{o}C while comparing OTC pharmacokinetics in the same species at different salinity levels.

1.5: PHYSIOLOGY RELATED TO OSMOREGULATION OF EURYHALINE FISH

Euryhaline fish species are able to adapt to different salinities via several distinct physiological changes that occur in the gill, gut and kidney. Previous research investigating the impact of environmental salinity in fish has primarily been conducted in salmonid fishes due to their natural smoltification process in their life cycle. There is limited information available on the effects of salinity on the growth or the physiological adjustments of juvenile and/or adult summer flounder. However, recent studies have found that reduced salinities do not have a detrimental effect on survival, growth or development in summer flounder larvae (Specker *et al*., 1999; Watanabe *et al*., 1999;

Watanabe *et al*., 1998b). Bengtson (1999) reports that juvenile summer flounder grown in recirculating aquaculture systems grew equally well at salinities of 10 ppt, 20 ppt, and 30 ppt. A closely related species, the southern flounder (*Paralichthys lethostigma*), has also been successfully reared in salinities ranging from freshwater (0 ppt) to seawater (35 ppt) (Benetii, 2000).

The composition of fish blood and tissues is typically not the same as the dissolved materials in either fresh or seawater, thus creating an osmotic gradient between the external environment and the blood of the fish. In general, plasma and urine osmolality are higher in marine fish species than in freshwater fishes (Jobling, 1995). The plasma osmolality (Table 1-2) of euryhaline fish has been documented to show only transitory changes when salinity levels are altered, indicating that these fishes are able to adjust rapidly to environmental salinity differences (Jobling, 1995; Madsen *et al*., 1994; Verbost, 1994). Goswami *et al*. (1983) found that in the catfish, *Heteropneustes fossilis*, plasma osmolarity increased from 279 mOsmol to 348 mOsmol in fish held in seawater of 10 ppt to 30 ppt, respectively. In addition, Goswami *et al*. (1983) saw increases in urine and plasma osmolarity across three salinity (10 ppt, 25 ppt, 30 ppt) groups (98 mOsmol/l, 266 mOsmol/l and 313 mOsmol/l, respectively for urine and 279 mOsmol/l, 313 mOsmol/l and 348 mOsmol/l for plasma, respectively) and decreases in urine flow rate (4.0 ml/h/kg, 2.6 ml/h/kg and 1.3 ml/h/kg, respectively). Jensen *et al*. (1998) saw similar trends in the sea bass, *Dicentrarchus labrax;* Plante *et al*. (2002) in the winter flounder, *Pseudopleuronectes americanus*; and Sampaio and Bianchini (2002) in another flounder species, *Paralichthys orbignyanus*.

The primary barrier between the dissolved substances in the blood and the aquatic environment is a thin layer of epithelium covering the gill and skin (Wedemeyer, 1996). Located in gill epithelium are chloride cells, which are mitochondria-rich cells found at the base of the gill filaments in most fish species. Their primary function is thought to be ionoregulation, an important aspect of osmoregulation in both marine and freshwater fishes. Environmental salinity influences how the gill chloride cells will function. In freshwater fish, the chloride cells actively transport sodium (Na^+) and chloride (Cl^-) ions

from the water into the blood. These cells help maintain homeostasis by transporting monovalent ions from the water into the blood to replace ions lost by diffusion through the gills and in the copious urine that is produced (Wedemeyer, 1996; Jobling, 1995). In marine fish, the direction of active transport is reversed. The body fluid of a marine teleost is more dilute than the surrounding medium and ions tend to diffuse through the gill epithelium into the blood, which imposes a salt (Na^+, Cl^-) load on the fish. Chloride cells aid in the excretion of this excess salt (Varsamos *et al*., 2002; Hartl *et al*., 2001; Jobling, 1995). The mechanism by which the chloride cells function is directly related to the activity of a sodium – potassium (Na^+- K^+) ATPase enzyme. Histochemical studies indicate that this enzyme activity is primarily located on the baso-lateral membranes of the chloride cells. These membranes become hypertrophied in seawater adapted fish. Foskett *et al*. (1983) found that gill chloride cell hypertrophy is associated with an increase of the baso-lateral membrane tubular system and is directly related to the increased activity of gill Na^+- K^+ ATPase. Specific activity of Na^+- K^+ ATPase in the organs of freshwater and saltwater fishes appears to be proportional to the level of sodium transport demanded by the environment and the species (Jampol, 1970). Jobling (1995) presented findings that confirmed the involvement of Na^+- K^+ ATPase in ion transport over the fish gill: 1) the greater the external salinity, the greater the sodium efflux over the gill tissue, and 2) Na^+ - K^+ ATPase activity in the tissues of euryhaline species was greater in the tissues of animals adapted to seawater than those in freshwater. Studies have been conducted to evaluate the gill chloride cell size, number and Na^+- K^+ ATPase activity when fish were transferred from freshwater to saltwater or vice versa (Weng *et al*., 2002; Wilson *et al*., 2002; Mancera and McCormick, 2000). Madsen *et al*. (1994) found that in striped bass (*Morone saxatilis*) the gill chloride cell size increased by 16% in freshwater reared fish exposed to seawater for 21 d. Yoshikawa *et al*. (1993) also found that chloride cell size was significantly greater in saltwater adapted long-jawed mudsuckers (*Gillichthys mirabilis*) than in freshwater cohorts. However, Brown (1992) found that the total chloride cell densities in sea trout, *Cynoscion nebulosus*, did not change between fish held in seawater or freshwater. McCormick *et al*. (1989) found that gill Na^+- K^+ ATPase activity was positively correlated with environmental salinity. Enzyme activity was 2.5- and 5- fold higher in Atlantic salmon (*Salmo salar*) smolts

acclimated to 10 and 30 ppt over freshwater (0 ppt) cohorts. However, Varsamos *et al*. (2002) and Stagg and Shuttleworth (1982) did find that in certain marine euryhaline fish species such as the European flounder (*Platichthys flesus*) that freshwater acclimation resulted in increased or similar enzyme activity compared to saltwater acclimated cohorts. Similarly, Lasserre (1971) reported that in the marine fish species, the thick-lipped mullet (*Crenimugil labrosus)* and sea bass (*Dicentrarchus labrax),* gill enzyme activity increased in freshwater adapted cohorts. Ultrastructural studies confirm that chloride cells of fish adapted to seawater have a characteristic morphology and location in the gill epithelium (Shikano and Fujio, 1998; Jobling, 1995; Jürss and Bastrop, 1995). King and Hossler (1991) completed an ultrastructural examination of striped bass gill arches and found that changes in ultrastructure and chloride efflux occurred within 3 h after transfer from freshwater to seawater. Although chloride cell morphology and function may not directly affect drug metabolism, these changes indicate altered physiology and confirm differences between freshwater and saltwater adapted fish.

The amount of urine produced by a fish is determined by the amount of blood filtered by the renal glomeruli (glomerular filtration rate or GFR) and the number of glomeruli present. Nishimura and Imai (1982) stated that urine flow and GFR are linearly related suggesting that one parameter may be used to estimate the other. Elger *et al*. (1987) confirmed this finding in winter flounder (*Pseudopleuronectes americanus*) by injecting fish with polyfructosan as a glomerular filtration marker. In the freshwater fish, the osmotic pressure is a result of diffusion of water into blood, resulting in high GFR and copious urine production. In marine teleosts, water has a tendency to move from the fish to the aquatic environment, thus dehydrating the fish. To conserve water, marine fish drink water and actively absorb monovalent ions, such as Na^+, in the intestine (later to be excreted via the chloride cells in the gill), which allows water to passively follow. Thus, marine fish have a lower GFR and urine production than freshwater fish. The GFR is approximately 5 ml/kg/hr in freshwater fish and approximately 0.5 ml/kg/hr in marine fish (Jobling, 1995). This difference in GFR will most likely impact excretion rates of drugs like OTC that are excreted through the kidney.

The physiological adaptations that permit these fish to adequately adjust to different salinity levels may also affect the absorption and excretion of OTC. Alterations in environmental, plasma and urine ion concentrations may impact OTC availability and metabolism. Oxytetracycline binds readily with ions such as calcium and magnesium and becomes less available and inactivated by these ions found at higher levels in saltwater and in the plasma and urine of marine fish, therefore leaving less active drug for reaching therapeutic levels. It may be that fish held in higher salinities require higher treatment dosages and longer withholding periods. In addition, the difference in glomerular filtration rate between freshwater and saltwater maintained fish might significantly alter OTC excretion. Because the GFR of freshwater fish is about 10 times that of saltwater fish, it is expected that the renally excreted OTC will be more rapidly cleared from fish in low salinity water. Conversely, it is proposed that the lower GFR in the marine fish will prolong the predicted half-life of the drug.

The difference in gill Na^+- K^+ ATPase activity between the low and high salinity fish indicates a primary physiological salinity adaptation. Although OTC metabolism is not directly influenced by this enzyme activity, plasma osmolality is a result of this enzyme's function. Veterinarians need to be aware of these potential differences in drug metabolism and excretion in order to make valid and correct inferences about drug doses, treatment protocols and withdrawal times.

1.6: PHARMACOKINETICS IN DISEASED ANIMALS

The majority of teleost pharmacokinetic studies have been conducted in healthy animals. However, only diseased animals are typically treated with antibiotics and the assumption is that the pharmacokinetic properties between healthy and sick individuals will be similar (Riviere and Sundlof, 2000; Uno, 1996). This assumption may be erroneous, especially if a disease process (i.e. bacterial infection) changes drug half-life by either increasing the volume of distribution (i.e. altered blood flow to tissues) or decreasing drug clearance (i.e. kidney disease) (Riviere and Sundlof, 2001). Another consideration when treating diseased animals with antibiotics, like OTC, is that when renal clearance is

impaired by disease, the elimination of OTC is reduced potentially leading to tetracycline toxicosis (Riviere and Spoo, 2001). In fish, only two reports have been published where the pharmacokinetics of OTC were compared between healthy and diseased subjects. Bruno (1989) observed that Atlantic salmon (*Salmo salar*) infected with *Aeromonas* sp. had higher OTC levels than healthy cohorts 8 weeks post-injection. Uno (1996) also demonstrated significant differences in OTC absorption after oral administration between healthy ayu (*Plecoglossus altivelis*) and *Vibrio*-infected ayu. Fish infected with the bacteria had lower maximum serum and tissue concentrations than healthy fish. In addition, the bioavailability of OTC was reduced by 60% in the diseased animals and the AUC was approximately half that of healthy fish for muscle, liver and kidney tissues. These studies imply that OTC pharmacokinetics and withdrawal times for diseased fish may not be the same as for healthy individuals.

In intensive marine aquaculture, there are several bacterial species that are ubiquitous in the marine environment and also may cause disease when the fish host becomes susceptible to infection. Like mammals, when fish become stressed their immune function is compromised leaving them vulnerable to disease-causing organisms. In intensive fish culture practices, the physiological and environmental demands made of the fish are increased. When problems arise, such as system failures leading to adverse water quality indices, stressed fish may succumb to infections caused by pathogens that under normal circumstances they may be able to overcome by adequate immune function and protection. During the course of this research, fish were unintentionally subjected to water temperatures for several days that approached their upper lethal temperature threshold (>28^{o}C) (Liewes, 1984) because of failure of the facility air conditioning unit. Shortly following this period of high water temperatures, a percentage of fish within the population starting showing gross clinical signs of bacterial septicemia; gross signs included emaciation, oral masses, head swelling, skin ulcerations, swollen coeloms, exophthalmia and lethargy. The primary bacteria isolated from fish with clinical signs of disease were *Vibrio* and *Mycobacterium* sp. Although there are no FDA-approved chemotherapeutics for the treatment of mycobacteriosis in foodfish, OTC, though effective against sensitive *Vibrio* sp. infections, is not approved for use in summer

flounder (Lower and Poet, 2001). Cox and Rainnie (1991) infected fingerling Atlantic salmon (*Salmo salar*) with *V. anguillarum* by bath immersion and intraperitoneal injection and then treated the infected animals with OTC for 10 d and found that mortalities were reduced in infected fish following OTC therapy.

Vibriosis is one of the most common disease syndromes in marine aquaculture (Park *et al*., 1994). *Vibrio* species are commonly found as part of the natural microflora in marine and estuarine environments. Therefore, intensive culture systems using natural sea or brackish water are potentially at risk for exposure to this pathogen. There are at least nine *Vibrio* species that are potential aquatic animal pathogens, with *Vibrio anguillarum* being the most widespread of these bacterial species (Park *et al*., 1994).

Vibrio spp. are Gram-negative, polar flagellated curved rods that are presumably transmitted in a saltwater fish population via fish to fish contact. The exact pathogenesis of these organisms in fish is not yet known, but it is assumed to be similar to that of other Gram-negative bacteria (i.e. endotoxin production). Rasmussen and Larsen (1987) determined that some of the outer membrane proteins of *V. anguillarum* were responsible for the virulence of this organism. Additional research has demonstrated that mucus from the skin, gills or intestine of rainbow trout (*Oncorhynchus mykiss* L.) was a strong attractant for *V. anguillarum* (Larsen *et al*., 2001). External clinical signs of infection include erosive skin lesions, fin hemorrhage and necrosis. Internal lesions include intestinal inflammation, hemorrhage of internal organs, hypertrophy of the spleen and kidney and liquefaction of these internal organs (Bullock, 1999; Sano and Fukuda, 1987; Umbreit and Tripp, 1975; Levin *et al*., 1972). In the winter flounder (*Pseudopleuronectes americanus*), Levin *et al*. (1972) described the microscopic lesions of the kidney as focal interstitial and tubular necrosis. Bacteria may be isolated from coelomic fluid, liver, kidney, and intestine (Umbreit and Tripp, 1975). Laurencin and Germon (1987) cultured *V. anguillarum* from the anterior kidney of rainbow trout (*Salmo gairdneri* R.) three days following a 24 hour bath exposure at 10^5 bacteria per ml.

Mycobacteriosis is another common bacterial disease of both fresh and saltwater fish caused by *Mycobacterium* spp. These are Gram-positive, acid-fast positive bacilli. *Mycobacterium marinum, M. fortuitum, and M. chelonae* are historically the most common *Mycobacterium* sp. isolated from fishes. Recently, another species, *M. chesapeakei,* has been identified from wild striped bass in the Chesapeake Bay (Heckert *et al*., 2001). Mycobacteriosis is a chronic progressive disease that may or may not present with gross external signs of disease. Common external clinical signs in most fish may include lethargy, anorexia, emaciation and skin ulcerations. Internal findings may include granulomatous inflammation and granulomas in target tissues such as kidney, liver, spleen or heart. Typically, the teleost response to *Mycobacterium* sp. infection is the formation of multiple discrete granulomas with numerous intracellular bacteria (Bruno *et al*., 1998; Colorni *et al*., 1998; Austin and Austin, 1993). However, in summer flounder, the granulomatous inflammatory response is typically observed without the formation of discrete granulomas (Hughes *et al*., 2002a; Hughes *et al*., 2002b). Grossly, mycobacteriosis in summer flounder may present as large masses on the mandible and head, operculum and in the retro-bulbar space. Internally, these areas have a significant infiltration of epithelioid macrophages that obliterate normal tissue architecture and within these areas of inflammation there are numerous extracellular acid-fast organisms. In the liver, spleen and kidney tissue, there is similar effacing granulomatous inflammation but with fewer organisms. This tissue response to *Mycobacterium* spp. by the summer flounder is unusual for teleost fish. The initial granulomatous response to *Mycobacterium* spp. by fish may be marked and obliterate the normal tissue architecture; however, over time (4-6 weeks), the granulomatous inflammation generally organizes into discrete granulomas. In the summer flounder, it does not appear that organized granulomas form in response to *Mycobacterium* spp. infection (Hughes *et al*., 2002a; Hughes *et al*., 2002b).

Both vibriosis and mycobacteriosis are bacterial diseases that may target the fish kidney and lead to pathologic lesions in the kidney as well as in other organs. Because OTC is primarily excreted unchanged through the urine, it is possible that when disease affects

the kidney that blood flow and glomerular filtration rates are altered when compared to rates in unaffected fish.

1.7: RESEARCH STATEMENT

The summer flounder is a good candidate species for marine aquaculture in the United States because of good market prices and their ability to tolerate wide salinity ranges. Their culture at lower salinities will permit inland production, thus minimizing the amount of salt required will reduce production cost overhead, thereby making flounder grow-out more profitable. These practices can also be extended and applied to other marine fish that are euryhaline. Oxytetracycline was selected for use in this project because it is one of two FDA-approved chemotherapeutics available in the U.S. and because it is excreted primarily unchanged through the urine. Because of the physiological adjustments made by fish to adapt to either freshwater or saltwater, urine volume and character may be significantly different and may impact the rate of drug elimination for drugs excreted primarily through the urine. Oxytetracycline has poor bioavailability in fish when given orally and the absorption rate and efficacy is decreased when it is chelated with di and tri-valent cations. However, OTC is still a common drug used by fish farmers.

The first objective of this research was to describe the pharmacokinetic parameters of OTC in summer flounder maintained at standard environmental culture conditions; 28 ppt and 20^{o}C. It was hypothesized that summer flounder would absorb and eliminate OTC similar to other saltwater fish, such as chinook salmon, Atlantic salmon or sea bass.

The second objective was to investigate specific physiological adaptations of summer flounder to changes in environmental salinity concentrations (0 ppt, 15 ppt, 32 ppt) and to determine if these physiological alterations impacted the pharmacokinetic parameters of OTC. The hypothesis was that the physiological changes associated with alterations in environmental salinity levels would alter OTC absorption and excretion in summer

flounder such that fish held in freshwater would have higher drug absorption and faster drug elimination than fish held in brackish or seawater environments.

The third objective was to determine muscle tissue residue retention time of OTC administered to summer flounder held in different salinity environments (0 ppt, 15 ppt, 32 ppt). The hypothesis was that summer flounder held in freshwater would have shorter withdrawal times than fish in brackish or seawater environments.

The fourth objective was to compare OTC pharmacokinetic parameters in healthy summer flounder to those of summer flounder demonstrating clinical signs of a mixed bacterial infection of *Vibrio* spp. and *Mycobacterium* spp. It was hypothesized that the pharmacokinetic parameters of OTC would be different between healthy summer flounder and summer flounder with clinical signs of disease.

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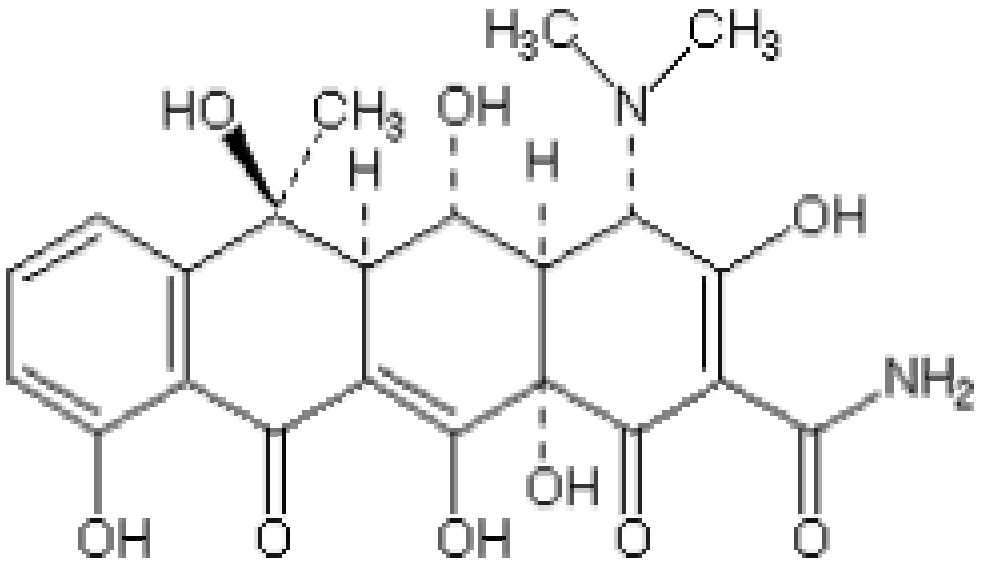

Figure 1-1. Chemical structure of oxytetracycline (OTC).

Table 1-1. Summary of pharmacokinetic parameters[1] of oxytetracycline (OTC) in various fish species. Table is sorted by salinity level, route of drug administration and water temperature. (Continued on next two pages)

Species	Salinity[2]	Route[3]	Water Temp (^{o}C)	Dose (mg/kg)	AUC (ug•h/ml)	T 1/2 $_{\alpha}$ (h)	T 1/2 $_{\beta}$ (h)	T 1/2 $_{\gamma}$ (h)	Cl (ml/kg/h)	Vd (l/kg)	F (%)	Tmax (h)	Cmax (ug/ml)	MRT	Reference
Rainbow trout	FW	IA	11	50	7781.19	0.74	18.95	NDR	6.43	0.87	NDR	NDR	NDR	NDR	Abedini et al., 1998
Tench	FW	IM	12	100	6093	NDR	NDR	NDR	NDR	NDR	NDR	6.4	99.7	121.2	Reja et al., 1996
Yellow Perch	FW	IC	18	50	134	NDR	28	NDR	NDR	NDR	NDR	0.08	32	NDR	Bowden, 2001
Yellow Perch	FW	IM	18	50	2659	NDR	124	NDR	NDR	NDR	NDR	4	29	NDR	Bowden, 2001
Carp	FW	IM	20	60	NDR	NDR	NDR	78.6	NDR	2.1	80	14	56.8	NDR	Grondel et al., 1987
Red Pacu	FW	IM	23	5	343	NDR	62.65	NDR	NDR	NDR	NDR	NDR	NDR	NDR	Doi et al., 1998
Yellow Perch	FW	IP	18	50	1718	NDR	112	NDR	NDR	NDR	NDR	2	32	NDR	Bowden, 2001
Artic charr	FW	IV	17	10	1591.4	1.5	16.5	NDR	6.54	2.57	NDR	NDR	NDR	301.2	Haug and Hals, 2000
Artic charr	FW	IV	17	20	3321.0	1.8	12.2	NDR	6.27	2.90	NDR	NDR	NDR	357.1	Haug and Hals, 2000
Rainbow trout	FW	IV	10	5	196.9	5.9	81.5	NDR	25.4	2.988	8	NDR	NDR	NDR	Black et al., 1991
African catfish	FW	IV	12	60	5369	5.2	80.3	NDR	NDR	1.33	NDR	NDR	NDR	NDR	Grondel et al., 1989
African catfish	FW	IV	12	60	3759	0.6	6.3	89.5	NDR	2.1	NDR	NDR	NDR	NDR	Grondel et al., 1989
Rainbow trout	FW	IV	15	50	2554	0.549	51.7	NDR	19.6	1.46	NDR	NDR	NDR	NDR	Uno et al., 1997
Rainbow trout	FW	IV	16	20	1129	1.528	60.3	NDR	16.2	1.39	5.6	NDR	NDR	79.3	Bjorklund and Bylund, 1991
Rainbow trout	FW	IV	16	20	1222	NDR	74.7	NDR	NDR	NDR	NDR	NDR	NDR	NDR	Bjorklund and Bylund, 1991
Ayu	FW	IV	18	25	1439	0.969	52.1	NDR	17.4	1.31	NDR	NDR	NDR	NDR	Uno, 1996
Carp	FW	IV	20	60	5862	3.5	50.8	139.8	0.17	2.1	NDR	NDR	NDR	NDR	Grondel et al., 1987
Red Pacu	FW	IV	23	5	688.89	NDR	50.97	NDR	0.121	543.11	49.8	NDR	NDR	NDR	Doi et al., 1998

Species	Salinity[2]	Route[3]	Water Temp (°C)	Dose (mg/kg)	AUC (ug•h/ml)	T 1/2 α (h)	T 1/2 β (h)	T 1/2 γ (h)	Cl (ml/kg/h)	Vd (l/kg)	F (%)	Tmax (h)	Cmax (ug/ml)	MRT	Reference
Rainbow trout	FW	PO	5	75	NDR	NDR	NDR	NDR	NDR	NDR	NDR	24	3.2	NDR	Bjorklund and Bylund, 1990
Rainbow trout	FW	PO	7	150	322	NDR	278.4	NDR	NDR	NDR	2.6	72	NDR	NDR	Rogstad et al., 1991
Rainbow trout	FW	PO	10	75	NDR	NDR	NDR	NDR	NDR	NDR	NDR	12	5.3	NDR	Bjorklund and Bylund, 1990
Rainbow trout	FW	PO	11	50	2884.34	40.03	479.43	NDR	NDR	NDR	NDR	18.17	5.77	NDR	Abedini et al., 1998
Rainbow trout	FW	PO	15	100	32.1	NDR	23	NDR	NDR	NDR	NDR	NDR	NDR	50.3	Uno et al., 1992
Amago salmon	FW	PO	15	100	58.7	NDR	16	NDR	NDR	NDR	NDR	NDR	NDR	24.6	Uno et al., 1992
Rainbow trout	FW	PO	16	75	258	NDR	74.9	NDR	NDR	NDR	NDR	12	2	NDR	Bjorklund and Bylund, 1991
Rainbow trout	FW	PO	16	75	NDR	NDR	NDR	NDR	NDR	NDR	NDR	1	2.1	NDR	Bjorklund and Bylund, 1990
Artic charr	FW	PO	17	50	341.9	NDR	367.0	NDR	NDR	NDR	4.2	30.3	1.51	NDR	Haug and Hals, 2000
Artic charr	FW	PO	17	100	1188.1	NDR	444.2	NDR	NDR	NDR	7.3	17.8	3.93	NDR	Haug and Hals, 2000
Yellow Perch	FW	PO	18	50	383	NDR	50	NDR	NDR	NDR	NDR	15	6	NDR	Bowden, 2001
Carp	FW	PO	20	60	NDR	NDR	NDR	NDR	NDR	NDR	0.6	14-20	0.07-0.28	NDR	Grondel et al., 1987

Species	Salinity[2]	Route[3]	Water Temp (°C)	Dose (mg/kg)	AUC (ug•h/ml)	T 1/2 α (h)	T1/2 β (h)	T 1/2 γ (h)	Cl (ml/kg/h)	Vd (l/kg)	F (%)	Tmax (h)	Cmax (ug/ml)	MRT	Reference
Chinook salmon	SW	IA	11	50	7126.79	0.62	6.79	NDR	7.02	0.89	NDR	NDR	NDR	NDR	Abedini et al., 1998
Atlantic salmon	SW	IV	7	20	929.3	NDR	NDR	NDR	NDR	NDR	NDR	NDR	NDR	NDR	Elema et al., 1996
Sea bass	SW	IV	13.5	40	494.26	0.98	69	NDR	73.5	5.62	NDR	NDR	NDR	71.58	Rigos et al., 2001
Sea bass	SW	IV	22	40	529.35	0.192	9.65	NDR	68.7	2.59	NDR	NDR	NDR	37.7	Rigos et al., 2001
Atlantic salmon	SW	PO	7	50	45.1	NDR	NDR	NDR	NDR	NDR	1.94	12	0.42	NDR	Elma et al., 1996
Chinook salmon	SW	PO	11	50	1947.59	72.51	428.19	NDR	NDR	NDR	NDR	17.88	5.32	NDR	Abedini et al., 1998

[1]Pharmacokinetic parameter abbreviations: AUC: area under the plasma concentration-time curve; T ½α: absorption half-life; T ½β: elimination half-life (2 compartmental model); T ½γ: elimination half-life (three compartmental model); Cl: total body clearance of the drug; Vd: the volume of distribution; F: absolute systemic bioavailability of the drug; Tmax: time of the maximum drug concentration within the body; Cmax: maximum drug concentration within the body; MRT: mean residence time of OTC; NDR: no data reported.

[2]Salinity level: FW = 0 ppt – 5 ppt; SW = > 15 ppt.

[3]Route of drug administration: IA: intrarterial; IC: intracardiac; IM: intramuscular; IV: intravascular; PO: per os.

Table 1-2. Summary of plasma and urine osmolalities, glomerular filtration rate (GFR) and urine flow rate in several euryhaline fish species.

Fish Species	Salinity[1]	Water Temp. (^{o}C)	Plasma Osmolality (mOsmol/l)	Urine Osmolality (mOsmol/l)	GFR (ml/kg/hr)	Urine Flow (ml/kg/hr)	Reference
Paralichthys lethostigma (Southern flounder)	SW	20	319	318	1.41	0.314	Hickman, 1968a
	SW	23	309	275	1.672	0.204	
Paralichthys lethostigma (Southern flounder)	SW	20	303.5	295.3	NDR	<0.2	Hickman, 1968b
	SW	20	318	304	NDR	>0.2	
Paralichthys orbignyanus	SW	22	320	NDR	NDR	NDR	Sampaio and Bianchini, 2002
	FW	22	216	NDR	NDR	NDR	
Pleuronectes flesus (European flounder)	SW	5	364	NDR	NDR	NDR	Lange and Fugelli, 1965
	FW	2	304	NDR	NDR	NDR	
Gasterosteus aculeatus (Stickleback)	SW	25	340	NDR	NDR	NDR	Lange and Fugelli, 1965
	FW	25	290	NDR	NDR	NDR	
Dicentrarchus labrax (Sea bass)	FW	25	240	NDR	NDR	NDR	Jensen *et al*., 1998
	FW	25	325	NDR	NDR	NDR	
	BW	25	325	NDR	NDR	NDR	
	SW	25	325	NDR	NDR	NDR	
	SW	25	350	NDR	NDR	NDR	
	SW	25	355	NDR	NDR	NDR	
Heteropneustes fossilis (Catfish)	FW	25	269	55	NDR	6.9	Goswami *et al*., 1983
	BW	25	279	98	NDR	4	
	SW	25	313	266	NDR	2.6	
	SW	25	348	313	NDR	1.3	
Pseudopleuronectes americanus (Winter flounder)	SW	10	334	NDR	NDR	NDR	Plante *et al*., 2002
Pseudopleuronectes americanus (Winter flounder)	SW	15	316	319	0.61	0.11	Elger *et al*., 1987
	FW	15	272	213	1.58	0.21	

[1]Salinity levels: FW = freshwater (0-5 ppt); BW = brackish water (10-20 ppt); SW = seawater (> 20 ppt). NDR = no data reported.

CHAPTER 2

PHARMACOKINETICS OF OXYTETRACYCLINE IN SUMMER FLOUNDER, *PARALICHTHYS DENTATUS*

Prepared for submission to the *Journal of Veterinary Pharmacology and Therapeutics*

2.1: ABSTRACT

The pharmacokinetic parameters of oxytetracycline (OTC) following a single 50 mg/kg dose via intravascular (IV), intraperitoneal (IP), intramuscular (IM) and per os (PO) administration were investigated in the summer flounder, *Paralichthys dentatus*, maintained at 28 ppt salinity and 20^{o}C in recirculating aquaculture systems. Oxytetracycline plasma concentrations were determined using high performance liquid chromatography (HPLC) and analyzed using a non-compartmental pharmacokinetic model. No statistical comparisons were made between the parameters for the different routes of OTC treatment, but IV administration resulted in the largest area under the curve (AUC) value (8147.9 µg•h/ml) and the highest C_{max} of 1173.2 µg/ml at 5 min post-injection. Intramuscular injections resulted in prolonged total body elimination (T ½) rate of 301.3 h and high fish-to-fish variability (0.6). Oral administration resulted in low plasma concentrations (0.2 µg/ml) and poor systemic bioavailability (0.2%).

Keywords: pharmacokinetics, oxytetracycline, flounder, *Paralichthys*

2.2: INTRODUCTION

Oxytetracycline (OTC) is one of two FDA-approved antibiotics available in the United States for use in foodfish. Currently, OTC is labeled specifically for use in channel catfish (*Ictalurus punctatus*), salmonids and lobster for the treatment of specific bacterial diseases. Other foodfish species may be treated with OTC for bacterial diseases, but either an extra-label veterinary prescription or site investigational new animal drug (INAD) permit is required. Although numerous pharmacokinetic studies have been conducted in fish using OTC, the majority of research has been conducted in freshwater species (Bowden, 2002; Haug and Hals, 2000; Namdari *et al*., 1999; Doi *et al*., 1998; Uno *et al*., 1997; Reja *et al*., 1996; Uno, 1996; Black *et al*., 1991; Björklund and Bylund, 1991; Rogstad *et al*., 1991; Björklund and Bylund, 1990; Grondel *et al*., 1989; Grondel *et al*., 1987; Norlander *et al*., 1987; Salte and Liestøl, 1983; Fribourgh *et al*., 1969a; Fribourgh *et al*., 1969b). Only a limited number of OTC pharmacokinetic studies have been carried out in marine fish and most of these studies have been limited to salmonid species, such as: Atlantic salmon (*Salmo salar*) (Namdari *et al*., 1998; Elema *et al*., 1996; Björklund and Bylund, 1990; Bruno, 1989), chinook salmon (*Oncorhynchus tshawytscha*) (Namdari *et al*., 1999; Abedini *et al*., 1998; Namdari *et al*., 1998; Namdari *et al*., 1996), coho salmon (*Oncorhynchus kisutch*) (Namdari *et al*., 1996) and sockeye salmon (*Oncorhynchus nerka*) (Strasdine and McBride, 1979). Malvisi *et al*. (1996) conducted an oral dose study of OTC distribution and residue depletion in sea bass (*Sparus aurata*) and sea bream (*Dicentrarchus labrax*). More recently, Rigos *et al*. (2002) conducted a study of OTC pharmacokinetics in sea bass held at two different water temperatures. Extrapolated doses and drug withholding periods are often used when data is not available for an untested species of interest (Doi *et al*., 1998). However, extrapolation can be particularly risky because there can be high variability in drug pharmacokinetics in fish. Pharmacokinetic parameters may vary by fish species, environmental conditions and drug formulation.

Summer flounder, *Paralichthys dentatus*, is a flatfish species of emerging interest in the United States because of high market prices and consumer demand (Dumas and Horton, 2002). Culture of summer flounder and other flounder species like southern flounder (*Paralichthys lethostigma*) is increasingly popular in the eastern United States where methods to induce spawning have been successful. The purpose of this study was to determine the pharmacokinetics of OTC in summer flounder following different routes of drug administration. The pharmacokinetic parameters derived from this study provide information on OTC absorption, distribution and elimination in a marine flatfish species maintained under standard culture conditions.

2.3: MATERIALS AND METHODS

2.3.1: FISH HUSBANDRY

Two hundred and sixty-four healthy juvenile (<30 cm, 150±22 g) summer flounder (GreatBay Aquafarms, Portsmouth, NH) were arbitrarily divided equally into eight 568 L recirculating aquaculture systems. Two 568 L fiberglass rectangular tanks shared a common sump, pump and biological filter, making 4 identical systems. Water in each tank was continuously passed through activated carbon to bind free OTC in the water column. Each of these four systems was arbitrarily designated as IV (intravascular), IP (intraperitoneal), IM (intramuscular), or PO (per os) based on route of OTC administration. The different routes of drug exposure were conducted concurrently. Water quality indices (dissolved oxygen (DO), ammonia, nitrites, nitrates, salinity, temperature, and pH) were monitored daily. Water quality indices were regarded as optimal when values were within these limits: temperature: 19-21^{o}C (YSI 85 model 85/10, Aquatic Eco-Systems, Apopka, FL); salinity: 28 (±1) ppt (YSI 85 model 85/10, Aquatic Eco-Systems, Apopka, FL); pH: 7.8-8.2 (Sension1 pH meter, HACH, Loveland, CO); ammonia: <0.2 mg/L; nitrite: <10 mg/L; nitrate: <50 mg/L; and, DO: 6.0-8.0 mg/L (YSI 85 model 85/10, Aquatic Eco-Systems, Apopka, FL). Ammonia, nitrites and nitrates were measured with a spectrophotometer (DR2010 spectrometer, HACH, Loveland, CO). Salinity adjustments were made by adding synthetic sea salt (Forty

Fathoms Crystal Sea Salt, Marine Enterprises International, Inc., Baltimore, MD). The pH of the systems was maintained by adding sodium bicarbonate when the pH dropped below the desired range. Fish were fed a commercial floating diet formulated specifically for summer flounder (Shur-Gain, Nova Scotia, Canada; protein: 50%, fat: 15%; 6.5 mm pellets). Fish were fasted 24 h prior to sampling and 24 h following OTC exposure.

Fish were anesthetized with buffered MS-222 (100 mg/L, tricaine methanesulfonate, Sigma Chemical Co., St. Louis, MO) for all routes of OTC administration, blood collection and tagging. All experimental fish were individually tagged with a t-bar anchor tag (Floy Tag, Inc., Seattle, WA) in the dorsal musculature on the visual side of the fish. Although anesthesia may alter certain blood parameters and other physiological and biochemical functions, there is no evidence that it interferes with OTC pharmacokinetic properties (Horsberg, 1994).

2.3.2: ROUTES OF DRUG ADMINISTRATION

Oxytetracycline (Bio-Mycin 200; 200 mg/ml oxytetracycline; Boehringer Ingelheim Vetmedica Inc., St. Joseph, MO) was administered as a single dose of 50 mg/kg to anesthetized fish for all routes (Piper *et al*., 1982). Intravascular injections were given using a 100 µl Hamilton syringe with a 25-gauge needle in the caudal tail vessels and blood samples were withdrawn from a site 3-5 cm caudal to the site of drug injection. For intraperitoneal drug administration, fish were held in a head-down manner and drug was injected using a 100 µl Hamilton syringe with a 25-gauge needle into the caudal coelomic cavity. Intramuscular injections were given using a 100 µl Hamilton syringe with a 25-gauge needle in the dorsal musculature between the lateral line and dorsal fin on the eyed side of the fish. At the site of parenteral drug administration slight pressure was applied for 10 sec to minimize OTC reflux from injection site. Oral drug exposure was administered via stomach gavage using a curved stainless steel 20-gauge 3" gavage tube (Popper and Sons, Inc, New Hyde Park, NY) and 100 µl Hamilton syringe. Gavage placement in the stomach was confirmed manually.

2.3.3: SAMPLING TECHNIQUES

Blood collection times following IV and IP OTC administration were divided into 3 groups each consisting of 11 bleeding times:

1 0, 5, 10, 20, 40 min and 1.5, 3, 6, 12, 18, 24 h

2 48, 72, 120, 168, 216, 264, 312, 360, 408, 456, 504 h

3 552, 600, 648, 696, 744, 792, 840, 888, 936, 984, 1032 h

Blood collection times following IM and PO OTC exposure were divided into 3 groups each consisting of 11 bleeding times:

1 0, 15, 30 min, 1, 2, 4, 8, 16, 24, 48, 72 h

2 120, 168, 216, 264, 312, 360, 408, 456, 504, 552, 600 h

3 648, 696, 744, 792, 840, 888, 936, 984, 1032, 1080, 1128 h

The bleeding times of the respective groups (IV and IP, or IM and PO) were selected based on literature information and results from previous experiments of OTC dosing in summer flounder. The three time frames were designed such that each fish was bled at least once during different phases of drug movement through the body (i.e. absorption, distribution and elimination). Accordingly, six fish were bled at every specified time interval with each fish being bled three times over the entire time of the trial, once in each time group (1, 2, 3). The bleeding schedule of individual fish was pre-determined so that at least 48 h elapsed before any one fish was resampled. The IV and IP routes had a greater concentration of bleeding times during the first 24 h because it was predicted that drug absorption, distribution and elimination would occur more quickly following these routes of OTC exposure compared to the IM or PO routes. Samples were collected for the IM and PO routes to 1128 h (47 d) in comparison to the IV and IP routes which were collected to 1032 h (43 d) because it was predicted that tissue and plasma OTC levels would be detectable for a longer time period following the IM or PO routes.

2.3.4: BLOOD COLLECTION AND PLASMA STORAGE

Approximately 0.3 ml of blood was withdrawn from the caudal tail vessels at each bleeding time. No more than 1.0 ml of blood volume was taken from a single fish during the entire course of the experiment. The blood sample was placed immediately into a plasma separator tube containing lithium heparin (Microtainer, Becton Dickinson, Fisher Scientific, Pittsburgh, PA), mixed by inversion several times and kept on ice until centrifugation. Samples were centrifuged (Centra GP8R, International Equipment Company, Needham Heights, MA) at 3000 x g for 10 min at 12^{o}C. Plasma was stored at –80^{o} C until analysis.

2.3.5: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROCEDURE

Thawed plasma samples were filtered with a MPS micropartition device (Millipore, Beford, MA) equipped with a disposable YMT ultrafiltration membrane disc (3000 molecular weight cutoff, Amicon, Inc., Beverly, MA) and centrifuged at 14,000 x g for 40 min at 22^{o}C (Beckman Microfuge R centrifuge, Beckman Instruments, Inc., Palo Alto, CA). A sample of the ultrafiltrate (20 µl) was then injected directly onto a high-performance-liquid-chromatography (HPLC) column. A Hypersil 3 micron C-18, 150 mm x 4.6 mm ID (Phenomenex, Torrance, CA) analytical reversed phase column was used. The HPLC system consisted of a Beckman Coulter System Gold chromatography unit equipped with a manual sample injector (Beckman Coulter Model 7725i) and a 126 solvent delivery module (Beckman Coulter Instruments, Inc., Fullerton, CA). HPLC effluents were analyzed with a Beckman 166 variable wavelength detector set at 355 nm. The mobile phase (pH 3.3) was a 70:30 mixture of an aqueous mobile phase (0.01M oxalic acid and 0.03M octane sulfonic acid sodium salt) and an organic mobile phase (acetonitrile) (Meinertz *et al*., 1998). This mixture was kept in a sealed container to prevent evaporation of the acetonitrile and was maintained on a magnetic stirrer to prevent separation of the phases. The flow-rate was 1.5ml/min, with each sample run taking approximately 10 min. Data was processed by the Beckman Coulter Analytical Series System Gold data acquisition software (Karat 32, Beckman Coulter Instruments,

Inc., Fullerton, CA). Known standards of OTC ranging from 0.05 - 50.0 μg/ml were prepared to establish a regression line upon which the unknown OTC concentrations were calculated. The calibration regression curve was rejected if less than 0.995. The detection limit was determined by running OTC spiked flounder plasma to find the minimum detectable concentration. The detection limit of OTC in flounder plasma for this HPLC system was 0.05 μg OTC/ml (0.05 ppm). To verify consistent HPLC operation a known 2.5 μg/ml standard solution of OTC was routinely injected into the HPLC unit for evaluation. Plasma samples with OTC concentrations above the standard curve range were diluted 1:10 or 1:100 to fall within the linear range. Recovery of OTC was determined by comparing spiked filtered OTC flounder plasma samples and unfiltered spiked samples. Recovery of OTC from filtered flounder plasma was 95% (±3.4). Plasma OTC concentrations that were determined by HPLC anlaysis to be lower than 0.05 ppm were assigned a value of zero because values lower than the limit of detection could not be accurately differentiated from zero.

2.3.6: DATA ANALYSIS

The raw plasma OTC concentration data were log-transformed to stabilize variances. Log-means were calculated and a MIXED effects model with fish as a random variable was used to estimate between fish variance across all times, given as the intraclass correlation coefficient (SAS Systems, version 8.2, SAS Institute, Inc., Cary, NC). Log-transformed data was exponentiated to corresponding geometric means in the original units. Using the geometric means, a non-compartmental model was used to estimate the area under the concentration-time curve (AUC) and the area under the moment curve (AUMC) of OTC in summer flounder plasma using the trapezoidal method for all routes of OTC administration. Additional pharmacokinetic parameters were estimated using the derived AUC and AUMC predicated values:

$$MRT = AUMC/AUC$$

$$Vd_{ss} = (Dose \bullet AUMC)/AUC^2$$

$$Cl_b = Dose/AUC$$

$T ½ = 0.693 \bullet MRT$

$F = AUC_{route} \bullet Dose_{iv} / AUC_{iv} \bullet Dose_{route}$

Where MRT is the mean residence time of OTC, Vd_{ss} is the volume of distribution at steady state, Cl_b is the total body clearance, T ½ is the total body elimination half-life and F is the bioavailability calculated for the multiple drug administration routes using the intravascular (IV) data.

To include all variation not associated with time and to give conservative estimates, a second partitioning of variation was performed with fish variation left in the model. A bootstrap randomization procedure using MULTTEST was used to estimate the 95% confidence intervals of the pharmacokinetic parameters (Cole, 1999; Riviere, 1999).

2.4: RESULTS

The semi-logarithmic plots of plasma concentration-time profiles of OTC for each route of drug exposure are shown in Figure 2-1 and the pharmacokinetic parameter estimates are summarized in Table 2-1. In Figure 2-1 each plotted point represents the mean of six fish, such that the mean is not an actual HPLC reading, thus, explaining why values may go below the limit of detection. The plasma concentration-time profiles demonstrate different characteristic curves depending on the route of OTC drug administration. The IV graph shows immediate maximum drug concentration in the plasma followed by declining plasma concentrations associated with rapid drug distribution and elimination. The IP graph shows a similar trend as the IV graph, but demonstrates a delayed time to maximum drug concentration presumably because of the greater time required for drug absorption from the peritoneal cavity. Both the IM and PO graphs depict a longer absorption phase compared to the IV and IP routes.

No statistical comparisons were made between the different routes of OTC administration, but the AUC was largest for the IV route of OTC administration and was smallest in the PO exposure (8147.9 µg•h/ml and 17.9 µg•h/ml, respectively). The MRT and total body elimination half-life was longest in the IM route of OTC administration

(434.9 h and 301.3 h, respectively). In addition, T_{max} and fish-to-fish variation was highest in the IM dosed fish (168 h and 0.6, respectively). In the IV treated group, time to maximum OTC concentration *in vivo* was shortest and maximum plasma concentration was highest (0.083 h and 1173.2 µg/ml, respectively). When OTC was administered PO, systemic bioavailability (0.2 %) was markedly reduced whereas IP and IM routes of OTC administration had bioavailabilities of 59% and 49%, respectively.

2.5: DISCUSSION

This is the first report of plasma pharmacokinetic parameters for a marine flatfish species following a single dose of OTC administered via IV, IP, IM and PO routes. The AUC estimate (8147.9 µg/h/ml) of OTC in summer flounder plasma following IV administration was similar to the AUC reported for the marine chinook salmon (7126.8 µg/h/ml) and freshwater rainbow trout (7781.2 µg/h/ml) following a single IV dose OTC at 50 mg/kg (Abedini *et al*., 1998). However, these AUC estimates were higher than what is reported for other IV dosages of OTC or other routes of OTC administration (Haug and Hals, 2000; Doi *et al*., 1998; Uno *et al*., 1997; Reja *et al*., 1996; Björklund and Bylund, 1991; Grondel *et al*., 1989; Grondel *et al*., 1987). This may be an effect of drug dose, route, environmental conditions or data analysis. The smaller AUC values in the plasma of the summer flounder following extravascular methods of administration (IP, IM and PO) indicated that these routes were not as efficient routes of drug delivery. The PO route was especially poor with an AUC of 17.9 µg/h/ml. This value is also lower than what is reported for other marine fish species treated orally with OTC. The saltwater yellowtail, *Seriola quinquerodiata*, had an AUC of 32.1 µg/h/ml following PO OTC at 100 mg/kg (Uno *et al*., 1992). These low PO AUC estimates indicated that OTC is not well absorbed from the intestinal tracts of these saltwater fish.

The total body elimination half-lives (T ½) for all administration routes reported here for the summer flounder were longer than what has been observed in other marine teleosts maintained at 20^{o}C. This may be a result of the non-compartmental analysis of the data which generates total body elimination rather than elimination from the terminal phase of

the pharmacokinetic curve. In the summer flounder, the T½ following OTC via IM administration was longer (301.3 h) than any other route. This may be the result of a "depot" effect in the muscle tissue. In addition, the time to T_{max} in the plasma for the IM route was longer compared to the other routes of OTC dosing in the summer flounder. Both of these prolonged parameters suggested that OTC may be residing longer in the muscle tissue resulting in long elimination half-life and time to maximum plasma concentrations as well as high fish-to-fish variation. In a preliminary dosing study, where summer flounder were treated with the same IM 50 mg/kg OTC dose there was a significant amount of drug in the vicinity of the IM injection 8 h post-injection (Hughes, 2003 unpublished). In this preliminary study, OTC in the muscle tissue was observed as long as 900 h post-injection.

The systemic bioavailability of OTC was extremely poor following oral gavage (0.2%). This may be a result of the reduced solubility and absorption of OTC following chelation with cations found in seawater (i.e. magnesium and calcium). When OTC is complexed with these cations (seawater of 35 ppt typically contains about 54 mMol Mg^{++} and 10 mMol Ca^{++}), the antibacterial efficacy of the drug is reduced (Treves-Brown, 2000; Lunestad and Goksøyr, 1990; Berthon *et al*., 1983). Chelated OTC molecules have a different charge than their unchelated counterparts, which may explain the reduced lipid solubility and absorption of the complex-bound form. Tissue concentrations of OTC in rainbow trout held in seawater were 30% of the concentrations found in freshwater cohorts (Lunestad and Goksøyr, 1990). Drug plasma distribution was reduced because of the Mg^{2+} and Ca^{2+} complexes, which act to decrease the drugs diffusion through erythrocyte membranes (Lunestad and Goksøyr, 1990). In addition, previous research reviewed by Lunestad and Goksøyr (1990) revealed that at pH 8, commonly the pH of saltwater aquatic systems (Wilson *et al*., 2002), the complex formation between OTC and cations is 1:1. This interaction becomes particularly problematic in saltwater fish since they must actively ingest seawater to maintain hydration. Therefore, OTC as an oral bolus or in medicated feed is in direct contact with seawater cations in the stomach and intestine of the fish potentially reducing its solubility and ultimately reducing the drugs absorption resulting in low plasma concentrations.

Although the IV and IP routes of OTC administration in summer flounder appeared to give the highest plasma drug concentrations in the shortest amount of time with the highest systemic bioavailability and the smallest degree of fish-to-fish variation, these administration routes are not practical in large population fish culture. The results from this study indicated that IM and PO dosing of OTC may be inappropriate for this flatfish species maintained under these conditions at 28ppt and 20^{o}C. A single IM OTC injection resulted in a long total body elimination half-life, a prolonged time to T_{max} and high fish-to-fish variation. The prolonged elimination half-life of OTC following IM injection impacted the predicted onset of drug steady-state, which would be longer in this administration route than compared to the other administration routes with shorter half-lives. Although not an FDA-approved method of treating any foodfish species, the IM route of administration may be used by veterinarians to treat valuable diseased broodstock not intended for human consumption. Therefore, these IM OTC pharmacokinetic characteristics of a long elimination half-life and high fish-to-fish variability should be considered before initiating a treatment regimen. Oral dosing of OTC at 50 mg/kg in summer flounder resulted in extremely poor absorption and systemic bioavailability. These characteristics make it unlikely that plasma or tissue levels of OTC will achieve bacteriostatic concentrations.

2.6: ACKNOWLEDGMENTS

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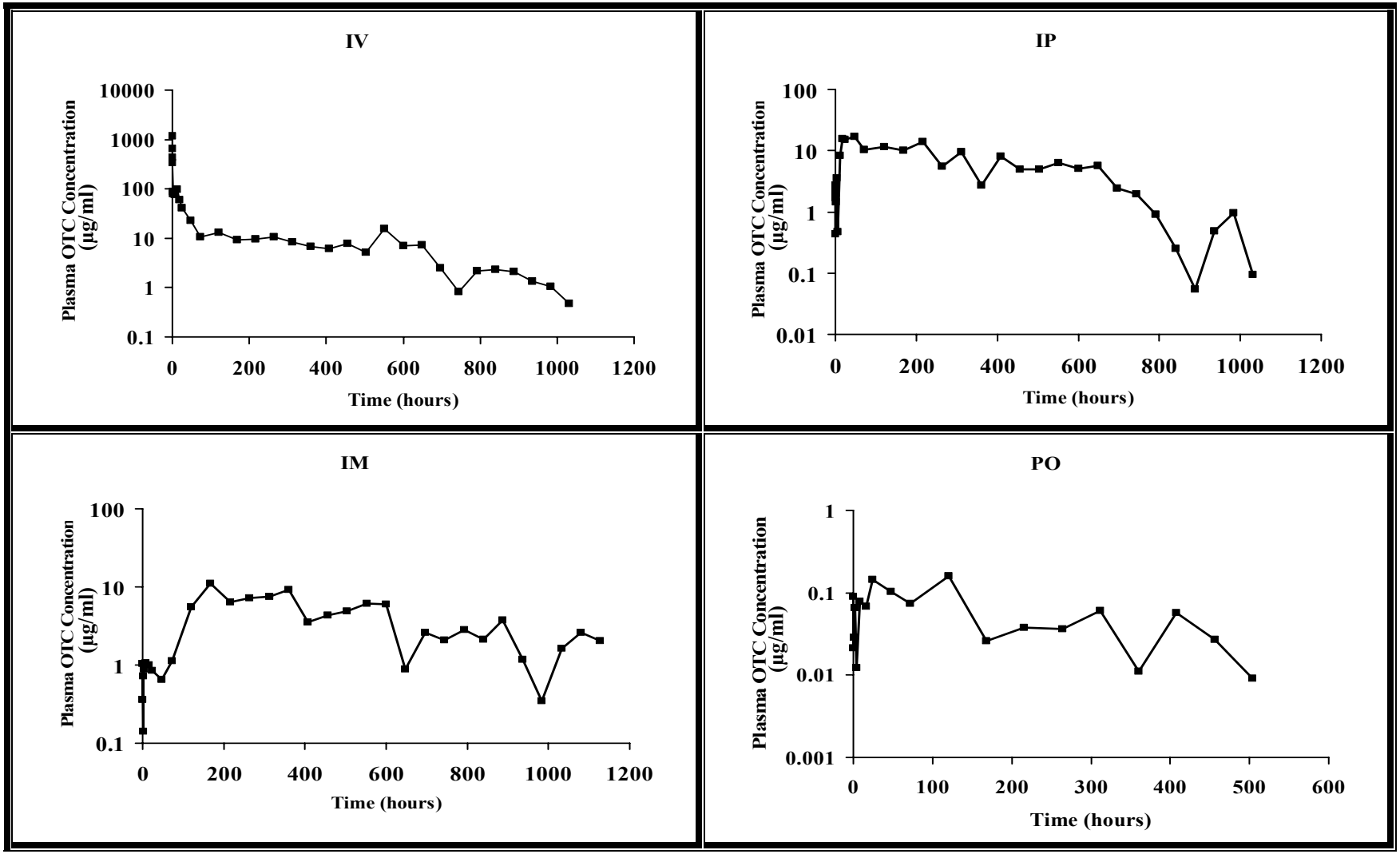

Figure 2-1. Semi-logarithmic plots of plasma concentration-time profiles of oxytetracycline (50 mg/kg) after intravascular (IV), intraperitoneal (IP), intramuscular (IM), and per os (PO) administration to summer flounder, *Paralichthys dentatus,* maintained at 28 ppt and 20°C. Note the different concentration scales along the y-axis for each route of OTC administration. Each point represents the mean of six fish.

Table 2-1. Pharmacokinetic parameters[1] of oxytetracycline (50 mg/kg) after intravascular (IV), intraperitoneal (IP), intramuscular (IM), and per os (PO) administration to summer flounder, *Paralichthys dentatus*, maintained at 28 ppt and 20°C.

Route of OTC Administration	AUC (µg•h/ml)	MRT (h)	Cl_b (ml/min/kg)	Vd_{ss} (l/kg)	T ½ (h)	T_{max} (h)	C_{max} (µg/ml)	F (%)	Fish-Fish Variation
IV	8147.9 [7499.4, 9386.9][2]	264.4 [238.7, 289.5]	0.01 [0.005, 0.015]	1.6 [1.3, 1.8]	183.2 [165.4, 200.6]	0.08	1173.2		0.0
IP	4820.7 [4225.1, 5940.0]	304.27 [276.6, 332.2]	ND	ND	210.86 [191.7, 230.2]	48	17.0	59	0.2
IM	4025.3 [3579.4, 4749.5]	434.9 [405.6, 464.3]	ND	ND	301.3 [281.1, 321.7]	168	11.1	49	0.6
PO	17.9 [12.4, 26.1]	196.7 [141.1, 258.7]	ND	ND	136.3 [97.8, 179.3]	120	0.2	0.2	0.0

[1]Pharmacokinetic parameter abbreviations; AUC: area under the plasma concentration-time curve after a single dose of OTC at 50 mg/kg; MRT: mean residence time of OTC in summer flounder following a single dose of OTC (50 mg/kg); Cl_b: total body clearance of the drug; Vd_{ss}: the volume of distribution at steady state; T ½ : total body elimination half-life; T_{max}: time of the maximum drug concentration within the body; C_{max}: maximum drug concentration within the body; F: absolute systemic bioavailability of the drug; fish-fish variation: intraclass correlation coefficient of residuals; ND: estimates not determined because absorption is not complete.

[2] Values in brackets are the 95% confidence limits as determined through bootstrap procedure.

CHAPTER 3

IMPACT OF ENVIRONMENTAL SALINITY AND THE ASSOCIATED PHYSIOLOGICAL ALTERATIONS OF GILL AND URINE CHARACTERISTICS ON THE PHARMACOKINETIC PARAMETERS OF OXYTETRACYCLINE ADMINISTERED TO SUMMER FLOUNDER, *PARALICHTHYS DENTATUS*, MAINTAINED AT THREE SALINITY LEVELS

Prepared for submission to *Aquaculture*

3.1: ABSTRACT

The impact of environmental salinity and the associated physiological alterations of the gill and urine characteristics on the pharmacokinetic parameters of oxytetracycline (OTC) in summer flounder, *Paralichthys dentatus*, maintained at three salinity levels (0 ppt, 15 ppt and 32 ppt) were determined. A single OTC dose of 50 mg/kg was administered via intramuscular (IM) injection or per os (PO) gavage. Significant differences ($p<0.05$) in the area under the curve (AUC) values were observed in flounder given IM injections of OTC. The AUC parameter was largest for fish held in 32 ppt (2241.3 µg•h/ml) compared to values from fish maintained in 15 ppt or 0 ppt (2067.8 µg•h/ml and 1684.8 µg•h/ml, respectively). Corresponding to the elevated AUC parameters in the 15 ppt and 32 ppt maintained flounder, T_{max} and C_{max} parameters were also prolonged in these treatments (312 h and 8.4 µg OTC/ml, respectively for 15 ppt fish; 168 h and 9.2 µg OTC/ml, respectively for 32 ppt fish), although not significantly different, compared to values estimated from fish held at 0 ppt (0.5 h and 4.9 µg OTC/ml, respectively). No significant differences were detected following PO administration of OTC, however, AUC values were lower in the fish housed in 15 ppt and 32 ppt (180.7 µg•h/ml and 127.7 µg•h/ml, respectively) as were the C_{max} values (0.5 µg OTC/ml and 0.43 µg OTC/ml, respectively) compared to flounder held in 0 ppt (AUC = 190.2 µg•h/ml; C_{max} = 0.6 µg OTC/ml, respectively). Significant physiological alterations were detected in plasma and urine osmolalities as well as other urine characteristics such as urine volume, color, specific gravity, flow rate and OTC concentration. Non-significant numeric trends were noted in gill chloride cell size, density and enzyme function. Results indicate that environmental salinity does impact OTC absorption and distribution in summer flounder.

Keywords: Pharmacokinetics, oxytetracycline, *Paralichthys*, flounder, salinity

3.2: INTRODUCTION

Oxytetracycline (OTC) is one of two available FDA-approved antibiotics for use in foodfish in the United States. Currently OTC, administered through medicated feed, is only labeled for use in channel catfish (*Ictalurus punctatus*) and salmonids against specific bacterial pathogens. Extra-label veterinary prescriptions or an Investigational New Animal Drug (INAD) permit are required for the legal use of OTC in other foodfish species, other routes of administration or for other bacterial diseases. Despite these restrictions OTC is commonly used in aquaculture to treat bacterial diseases. Plumb *et al*. (1995) reported that 84% of six common bacterial pathogens isolated from catfish were susceptible to OTC.

Oxytetracycline is a broad-spectrum bacteriostatic antibiotic that is excreted primarily unchanged through the urine. In mammals, tetracyclines are typically well absorbed from the gastrointestinal tract of fasted animals with systemic bioavailabilities of OTC ranging between 60-80% (Plumb, 1995). However, in published reports of bioavailability of OTC in fish, both fresh and saltwater species, values ranged from 0.6-80% (Haug and Hals, 2000; Doi *et al*., 1998; Elema *et al*., 1996; Björklund and Bylund, 1991; Black *et al*., 1991; Rogstad *et al*., 1991; Cravedi *et al*., 1987; Grondel *et al*., 1987). This difference in range of systemic bioavailability may be related to dose, drug formulation, species differences or environmental conditions, such as salinity. It is known that OTC readily chelates with divalent cations such as Ca^{2+} and Mg^{2+}, which are common in seawater. Seawater of 35 ppt contains approximately 54 mMol Mg^{2+} and 10 mMol Ca^{2+} (Lunestad and Goksøyr, 1990). When OTC is chelated with cations its lipid solubility is reduced ultimately decreasing absorption and systemic bioavailability. Lunestad and Goksøyr (1990) reported that tissue concentrations of OTC in seawater dwelling rainbow trout are on average 30% less than concentrations found in freshwater reared trout.

The pharmacokinetics of OTC have been studied in numerous freshwater and marine teleosts (Bowden, 2002; Rigos *et al*., 2002; Haug and Hals, 2000; Namdari *et al*., 1999; Abedini *et al*., 1998; Doi *et al*., 1998; Namdari *et al*., 1998; Uno *et al*., 1997; Elema *et*

al., 1996; Malvisi *et al*., 1996; Namdari *et al*., 1996; Reja *et al*., 1996; Uno, 1996; Black *et al*., 1991; Björklund and Bylund, 1991; Rogstad *et al*., 1991; Björklund and Bylund, 1990; Bruno, 1989; Grondel *et al*., 1989; Grondel *et al*., 1987; Norlander *et al*., 1987; Salte and Liestøl, 1983; Strasdine and McBride, 1979; Fribourgh *et al*., 1969a; Fribourgh *et al*., 1969b); however, only two studies have compared the OTC pharmacokinetics between a freshwater-acclimated fish and a marine fish (Namdari *et al*., 1999; Abedini *et al*., 1998). The results from these two investigations revealed similar OTC pharmacokinetic parameters between the two groups of salmonids in each study. Both of these studies were conducted at water temperatures between 10-11^{o}C and compared fish only acclimated to freshwater (0 ppt) and saltwater (24 ppt).

Summer flounder, *Paralichthys dentatus*, are a euryhaline flatfish species. Recent studies have found that reduced salinities did not have a detrimental effect on survival, growth or development in summer flounder larvae (Specker *et al*., 1999; Watanabe *et al*., 1998; Watanabe *et al*., 1999). Bengtson (1999) reported that juvenile summer flounder grown in recirculating aquaculture systems grew equally well at salinities of 10 ppt, 20 ppt, and 30 ppt. In order for fish to survive at different salinities, certain physiological adjustments must be made. To maintain hydration, saltwater fish must actively ingest seawater. Consequently, these fish must be able to eliminate excess ions, such as Na^{+} and Cl^{-}. To achieve this, saltwater fish have highly active gill chloride cells, have a low urine flow rate and volume, and eliminate highly concentrated urine. Conversely, freshwater do not drink water since the osmotic difference is reversed. These fish must eliminate water to conserve internal osmolality. Freshwater fish have low to moderately active gill chloride cells, a high urine flow rate and volume, and eliminate very dilute urine. Additionally, plasma osmolality is also affected by environmental salinity. Goswami *et al*. (1983) found that in the catfish, *Heteropneustes fossilis*, plasma osmolarity increased from 279 mOsmol to 348 mOsmol in fish held in seawater of 10 ppt to 30 ppt, respectively. In addition, Goswami *et al*. (1983) saw increases in urine and plasma osmolarity across three salinity (10 ppt, 25 ppt, 30 ppt) groups (98 mOsmol/l, 266 mOsmol/l and 313 mOsmol/l, respectively for urine and 279 mOsmol/l, 313 mOsmol/l and 348 mOsmol/l for plasma, respectively) and decreases in urine flow rate (4.0 ml/h/kg,

2.6 ml/h/kg and 1.3 ml/h/kg, respectively). Jensen *et al*. (1998) saw similar trends in the sea bass, *Dicentrarchus labrax;* Plante *et al*. (2002) in the winter flounder, *Pseudopleuronectes americanus*; and Sampaio and Bianchini (2002) in another flounder species, *Paralichthys orbignyanus*. The physiological alterations of plasma and urine osmolality and urine flow rates may impact OTC pharmacokinetic parameters. The morphological and functional changes of the gill chloride cell will not impact OTC behavior but rather confirm the ability of summer flounder to tolerate a wide range of environmental salinity conditions.

Oxytetracycline is excreted primarily through the urine and its absorption is negatively affected by the presence of seawater cations, the purpose of this research was to investigate the impact of environmental salinity on OTC pharmacokinetic parameters following IM and PO administration in summer flounder. In addition, the physiological adjustments such as plasma and urine osmolality, urine flow rate, urine character, gill chloride cell size and density as well as gill Na^+- K^+ ATPase activity made by the summer flounder maintained at three salinity levels (0 ppt, 15 ppt, and 32 ppt) were examined.

3.3: MATERIALS AND METHODS

3.3.1: FISH HUSBANDRY

For each route of OTC administration (IM and PO), three hundred and six healthy juvenile (25 cm, 192 ± 41 g and 25 cm, 204 ± 38 g, respectively) summer flounder (GreatBay Aquafarms, Portsmouth, NH) were arbitrarily divided equally into six 568 L recirculating aquaculture systems. Two of the 568 L fiberglass rectangular tanks shared a common sump, pump and biological filter, making 3 identical systems. Water in each tank was continuously passed through activated carbon to bind free OTC in the water column. Each of these three systems was arbitrarily designated as freshwater (0 ppt), brackish water (15 ppt) or seawater (32 ppt). Fish were slowly acclimated to experimental salinity concentrations over time and were maintained at the desired salinity levels for at least 4 weeks prior to the start of each experiment. All three tanks received

the same route of OTC administration (IM or PO) such that the three salinity levels of each route were conducted simultaneously. Water quality indices (dissolved oxygen (DO), ammonia, nitrites, nitrates, salinity, temperature, and pH) were monitored daily. Water hardness, measured as total hardness (mg/L $CaCO_3$), was monitored bi-monthly. Water quality parameters were regarded as optimal when parameters were within these limits: temperature: 19-21^{o}C (YSI 85 model 85/10, Aquatic Eco-Systems, Apopka, FL); pH: 7.8-8.2 (Sension1 pH meter, HACH, Loveland, CO); ammonia: <0.2 mg/L; nitrite: <10 mg/L; nitrate: <50 mg/L; total hardness: > 200 mg/L $CaCO_3$; and, DO: 6.0-8.0 mg/L (YSI 85 model 85/10, Aquatic Eco-Systems, Apopka, FL). Ammonia, nitrites, nitrates and hardness were measured with a spectrophotometer (DR2010 spectrometer, HACH, Loveland, CO). Water salinity was measured using a digital membrane probe (YSI 85 model 85/10, Aquatic Eco-Systems, Apopka, FL) and confirmed with a temperature compensated salinity refractometer (Aquatic Eco-Systems, Apopka, FL). Salinity adjustments were made by adding synthetic sea salt (Forty Fathoms Crystal Sea Salt, Marine Enterprises International, Inc., Baltimore, MD). The pH of the systems was maintained by adding sodium bicarbonate when the pH dropped below the desired range. Fish were fed a commercial floating diet formulated specifically for summer flounder (Shur-Gain, Nova Scotia, Canada; protein: 50%, fat: 15%; 6.5 mm pellets). Fish were fasted 24 h prior to sampling and 24 h following OTC administration.

Fish were anesthetized with buffered MS-222 (100 mg/L, tricaine methanesulfonate, Sigma Chemical Co., St. Louis, MO) for both routes of OTC administration, blood collection and tagging. All experimental fish were individually tagged with a t-bar anchor tag (Floy Tag, Inc., Seattle, WA) in the dorsal musculature on the visual side of the fish. Although anesthesia may alter certain blood parameters and other physiological and biochemical functions, there is no evidence that it interferes with OTC pharmacokinetic properties (Horsberg, 1994).

3.3.2: ROUTES OF DRUG ADMINISTRATION

Oxytetracycline (Bio-Mycin 200; 200 mg/ml oxytetracycline; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) was administered as a single dose to anesthetized fish at a dose of 50 mg OTC/kg of body weight for both routes (Piper *et al*., 1982). Intramuscular injections were given using a 100 µl Hamilton syringe with a 25-gauge needle in the dorsal musculature between the lateral line and dorsal fin on the eyed side of the fish. At the site of IM drug administration slight pressure was applied for 10 sec to minimize OTC reflux from injection site. Oral OTC was administered via stomach gavage using a curved stainless steel 20-gauge 3" gavage tube (Popper and Sons, Inc, New Hyde Park, NY) and 100 µl Hamilton syringe. Gavage placement in the stomach was confirmed manually.

3.3.3: SAMPLE COLLECTION TIMES

Blood collection times following IM and PO OTC exposure were divided into 3 groups consisting of 11 bleeding times each:

1 0, 15, 30 min, 1, 2, 4, 8, 16, 24, 48, 72 h

2 120, 168, 216, 264, 312, 360, 408, 456, 504, 552, 600 h

3 648, 696, 744, 792, 840, 888, 936, 984, 1032, 1080, 1128 h

The bleeding times of the two routes (IM and PO) were selected based on literature information and results from previous experiments of OTC dosing in summer flounder. The three time frames were designed such that each fish was bled at least once during different phases of drug movement through the body (i.e. absorption, distribution and elimination). Accordingly, six fish were bled at every specified time interval with each fish being bled three times over the entire time of the trial, once in each time group (1, 2, 3). The bleeding schedule of individual fish was pre-determined so that at least 72 h elapsed before any one fish was resampled.

3.3.4: BIOLOGICAL SAMPLE COLLECTION AND HANDLING

3.3.4.1: BLOOD COLLECTION AND PLASMA STORAGE

Approximately 0.4 - 0.5 ml of blood was withdrawn from the caudal tail vessels at each bleeding time. No more than 1.5 ml of blood volume was taken from a single fish during the entire course of the experiment. The blood sample was placed immediately into plasma separator tubes containing lithium heparin (Microtainer, Becton Dickinson, Fisher Scientific, Pittsburgh, PA), mixed by inversion several times and kept on ice until centrifugation. Samples were centrifuged (Centra GP8R, International Equipment Company, Needham Heights, MA) at 3000 x g for 10 min at 12^{o}C. Plasma was stored at –80^{o} C until analysis.

3.3.4.2: URINE COLLECTION AND STORAGE

An indwelling urinary catheter was placed in six individually housed summer flounder from each salinity level for each route of OTC treatment. The fish were housed for 72 h in 76 L glass aquariums equipped with a charcoal filter and air source. Specifically tagged fish were removed from each system and acclimatized to the smaller tank environment for 48 h prior to the start of the experiment. These flounder had not been bled prior to placement in glass aquariums. At the completion of 72 h, fish were removed from the experimental population. A 41 cm 5 French (1.7 mm) polyethylene catheter (Tyco Healthcare Group LP, Mansfield, MA) was inserted into the urinary papilla of each fish and advanced for approximately 3 cm and anchored *in situ* with 4-0 silk suture. Catheters were capped-off with water-tight seals. Six fish from each salinity level were given OTC either by IM injections or oral gavage as described previously. Blood and urine were collected at 0 h (immediately after catheter placement and before OTC administration) and 72 h post-OTC injection. Urine was also collected at 24 and 48 h. Urine was collected by removing the catheter seal and aspirating the entire contents of the catheter with a syringe. Experiments for each route of OTC administration (IM and PO)

were conducted in duplicate. Blood was handled as previously described for plasma separation and storage.

3.3.4.3: GILL COLLECTION

For both the IM and PO routes of OTC administration, at sample collection times 0, 8, 552 and 1128 h, six fish from each salinity level were humanely euthanized by an over-dose of MS-222 followed by cervical separation. For chloride cell size and density determinations, chloride cells were specifically stained using Champy-Maillet's method (McCormick, 2002, personal communication; Hartl *et al*., 2001). Briefly, a group of 4-5 filaments from the second gill arch was excised below the septum and placed in Champy-Maillet's fixative (0.4 % osmium tetroxide, 50 mg/ml zinc powder and 25 g/ml metallic iodine, OZI) for 18-24 h. This fixation-coloration process with OZI reduces osmic acid to osmium and cellular reactivity specifically targets gill chloride cells because of reactivity with phospholipids in the tubular system of the chloride cell (Schreiber and Specker, 1999; Niebauer *et al*., 1969). Following fixation, gill arches were rinsed with deionized water three times and dehydrated through a series of increasing alcohol concentrations, cleared with xylene, embedded in paraffin wax and sectioned at 5 μm. Mounted tissues were then examined using light microscopy. Chloride cells were intensely black.

For NA^{+}- K^{+} ATPase activity, excised gill filaments from the first gill arch were placed in SEI buffer (250 mM sucrose, 10 mM Na_2 EDTA and 50 mM imidazole, pH 7.3), frozen immediately on dry ice and maintained at -80 ^{o} C until assayed.

3.3.5: PLASMA AND URINE OSMOLALITY

Osmolality of plasma and urine samples were determined on a freezing-point depression osmometer (2430E Multi-Osmette, Precision Systems, Inc., Natick, MA). Prior to analysis, thawed urine samples were centrifuged for 10 min at 3000 x g (Centra GP8R, International Equipment Company, Needham Heights, MA) to settle suspended

particulate matter. The osmometer was calibrated daily with commercially available known standards ranging from 0 to 3000 mOsmol/l. Standard calibration curves were rejected if the correlation coefficient was less than 0.9995. Plasma and urine samples were run in duplicate.

3.3.6: GILL CHLORIDE CELL SIZE AND DENSITY

For gill chloride cell density estimates, the proportion of the gill membrane containing chloride cells was first estimated by determining the proportion of fields ($0.38mm^2$) that contained one or more chloride cells (always greater than 80%) (McCormick, 1990). Five fields containing chloride cells were selected at random and positively staining cells were counted. The count was then multiplied by the proportion of fields to yield a value for cell density - expressed as cells per mm^2. Gill chloride cell size was estimated by selecting five random fields in which ten positively staining cells from that field were measured in micrometers at 400 x magnification.

3.3.7: GILL NA^+- K^+ ATPase ACTIVITY

Enzyme activity was measured using the technique of McCormick (1993) and McCormick and Bern (1989), which modified the protocol for 96-well microplates. For enzyme activity analysis, thawed gill filaments were homogenized in 85 µl SEI buffer supplemented with 0.1% Na deoxycholate. The homogenate was then centrifuged at 5000 x g for 30 sec to remove any insoluble material. An assay mixture (Solution A) containing 50 mM imidazole (pH 7.5), 4.6 U/ml lactate dehydrogenase, 5.1 U/ml pyruvate kinase, 2.8 mM phosphoenolpyruvate, 0.22 mM NADH, 0.7 mM ATP was made just prior to assay. Assay Solution B was a duplicate of Solution A but additionally contained 0.5 mM ouabain. A salt solution was also prepared containing imidazole (50 mM), NaCl (189 mM), $MgCl_2$ (10.5 mM) and KCl (42 mM) (Sigma Chemical Company, St. Louis, MI). The salt solution was mixed with Solutions A and B separately in a 3:1 ratio of salt to the respective solutions. With the microplate kept on ice, 10 µl of each homogenate sample was added to quadruplicate wells. Solution A-plus-salt mixture (200

µl) was added to two wells per sample and Solution B-plus-salt mixture (200 µl) was added to the other two wells per sample. The plate was then read on a temperature controlled plate reader using a kinetic program which measured well activity every 10 sec for 10 min at 340 nm (Thermomax, Molecular Devices Corp., Menlo Park, CA). The linear rate in each pair of wells was determined and Na^+- K^+ ATPase activity was calculated as the difference in ATP hydrolysis in the absence and presence of ouabain, expressed as µmole ADP per milligram of protein per hour. Protein content of the homogenate was measured using a BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard. Prior to each assay run, a standard curve was obtained from 0 – 20 nmol ADP/well with the acceptable slope of the curve ranging from 17-19 mOD/nmole ADP/ well.

3.3.8: URINE FLOW AND CHARACTER EVALUATION

Urine color, volume and specific gravity were determined immediately following urine collection. Urine color was subjectively evaluated and color was scored as (Fig. 3-1):

Clear	1
Slight yellow	2
Yellow	3
Bright Yellow	4

Urine specific gravity was determined using a veterinary refractometer (Reichert Analytical Instruments, Inc., Depew, NY). Urine flow was calculated by dividing the urine volume over a 24 hour period by the fish weight.

3.3.9: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROCEDURE

Thawed plasma and urine samples were filtered with a MPS micropartition device (Millipore, Beford, MA) equipped with a disposable YMT ultrafiltration membrane disc (3000 molecular weight cutoff, Amicon, Inc., Beverly, MA) and centrifuged at 14,000 x g for 40 min at 22^{o}C (Beckman Microfuge R centrifuge, Beckman Instruments, Inc., Palo Alto, CA). A sample of the ultrafiltrate (20 μl) was then injected directly onto a high-performance-liquid-chromatography (HPLC) column. A Hypersil 3 micron C-18, 150 mm x 4.6 mm ID (Phenomenex, Torrance, CA) analytical reversed phase column was used. The HPLC system consisted of a Beckman Coulter System Gold chromatography unit equipped with a manual sample injector (Beckman Coulter Model 7725i) and a 126 solvent delivery module (Beckman Coulter Instruments, Inc., Fullerton, CA). HPLC effluents were analyzed with a Beckman 166 variable wavelength detector set at 355 nm. The mobile phase (pH 3.3) was a 70:30 mixture of an aqueous mobile phase (0.01M oxalic acid and 0.03M octane sulfonic acid sodium salt) and an organic mobile phase (acetonitrile) (Meinertz *et al*., 1998). This mixture was kept in a sealed container to prevent evaporation of the acetonitrile and was maintained on a magnetic stirrer to prevent separation of the phases. The flow-rate was 1.5ml/min, with each sample run taking approximately 10 min. Data was processed by the Beckman Coulter Analytical Series System Gold data acquisition software (Karat 32, Beckman Coulter Instruments, Inc., Fullerton, CA). Known standards of OTC ranging from 0.05 - 50.0 μg/ml were prepared in order to establish a regression line upon which the unknown OTC concentrations were calculated. The calibration regression curve was rejected if it was less than 0.995. The detection limit was determined by running OTC spiked flounder plasma to find the minimum detectable concentration. The detection limit of OTC in flounder plasma for this HPLC system was 0.05 μg OTC/ml. To verify consistent HPLC operation a known 2.5 μg/ml standard solution of OTC was periodically injected into the HPLC unit for evaluation. Recovery of OTC was determined by comparing spiked filtered OTC flounder plasma samples and unfiltered spiked samples. Recovery of OTC from filtered flounder plasma was 95% (±3.4). Plasma OTC concentrations that were determined by HPLC anlaysis to be lower than 0.05 ppm were assigned a value of zero

because values lower than the limit of detection could not be accurately differentiated from zero.

3.3.10: PLASMA PROTEIN BINDING

Plasma from fish acclimated to each salinity level but not exposed to OTC was used for determining OTC plasma protein binding. The binding capacity of OTC to plasma proteins was determined by ultrafiltration in 1.5 ml centrifuge tubes, using Millipore Ultrafree MC filters (Nihon Millipore Ltd., Tokyo, Japan) with a 10,000 nominal molecular weight cut-of limit. Oxytetracycline-free plasma was spiked with OTC at 5 μg/ml. The total drug concentration and free drug fraction, ultrafiltrates of plasma samples, were determined by HPLC as described previously. The quantity of bound OTC was calculated as the difference between total and free components. Drug bound to the filter was determined by ultrafiltering 5 μg/ml of OTC solution and comparing the drug concentration in the filtrates and the unfiltered sample (Uno, 1996; Björklund and Bylund, 1991). Plasma protein content of the homogenate was measured using a BCA protein assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard. The recovery of OTC in the ultrafiltration procedure was 97.8%.

3.3.11: DATA ANALYSIS

The raw plasma OTC concentration data were log-transformed to stabilize variances. Model adequacy was assessed using standardized residual plots for plasma and urine osmolality as well as other urine characteristics. Log-means of the OTC plasma data were calculated and a MIXED effects model with fish as a random variable was used to estimate between fish variance across all times, given as the intraclass correlation coefficient (SAS Systems, version 8.2, SAS Institute, Inc., Cary, NC). Log-transformed data was exponentiated to corresponding geometric means in the original units. Using the geometric means, a non-compartmental model was used to estimate the area under the concentration-time curve (AUC) and the area under the moment curve (AUMC) of OTC in summer flounder plasma using the trapezoidal method for both routes of OTC

administration. Additional pharmacokinetic parameters were estimated using the derived AUC and AUMC:

$$MRT = AUMC/AUC$$
$$T\ ½ = 0.693 \bullet MRT$$

Where MRT is the mean residence time of OTC and T ½ is the total body elimination half-life.

To include all variation not associated with time, to give conservative estimates, a second partitioning of variation was performed with fish variation left in the model. A bootstrap randomization procedure using MULTTEST was used to estimate the confidence intervals of the pharmacokinetic parameters (Cole, 1999; Riviere, 1999). A multiple comparison test statistic, *z*, was used with a Bonferroni correction procedure to detect significant differences between salinity treatment comparisons.

3.4: RESULTS

3.4.1: PHARMACOKINETIC PARAMETERS

The plasma concentration-time profiles of OTC after IM and PO administration in summer flounder maintained at three different salinity levels are shown in Fig. 3-2 and Fig. 3-3, respectively. In Figures 3-2 and 3-3 each plotted point represents the mean of six fish, such that the mean is not an actual HPLC reading, thus, explaining why values may go below the limit of detection. The pharmacokinetic parameters are summarized in Table 3-1. Graphic plots of data demonstrated that summer flounder maintained at different salinities have very similar pharmacokinetic curves following IM and PO administration of a single 50 mg/kg dose of OTC. In Figures 3-2 and 3-3 there are physiologically unexplainable peaks and troughs, especially during the first 400 h. These results may be best explained by chromatography technique. Following IM OTC injections, the AUC parameters for fish in the three salinity treatments were significantly different ($p < 0.05$). The AUC parameters appeared to be positively correlated with

environmental salinity following IM dosing of OTC. The fish maintained in freshwater had the smallest AUC (1684.8 µg•h/ml), whereas the brackish water and seawater housed fish had significantly higher AUC parameters of 2067.8 µg•h/ml and 2241.3 µg•h/ml, respectively. The larger AUC parameter observed in the seawater treatment group was a combined result of the higher C_{max} recorded in this group, and it may also be noted from Fig. 3-2 that at times 24, 72, 168, 408, 648, 799 and 1080 h the marine (32 ppt) fish had higher plasma concentrations than the other two groups. Although not significantly different from other salinity groups, the C_{max} following IM injection in the summer flounder in seawater was higher (9.2 µg/ml) compared to the other salinity treatments (4.9 µg/ml and 8.4µg/ml, respectively). These factors contributed to increase the overall AUC value. For the PO route of OTC administration no significant differences could be detected between the salinity treatments for any of the pharmacokinetic parameters. However, the AUC values for the PO route of OTC exposure appeared to be negatively correlated with environmental salinity. The AUC parameters in the 0 ppt, 15 ppt and 32 ppt were 190.2 µg•h/ml, 180.7 µg•h/ml, and 127.7 µg•h/ml, respectively. The fish held at 0 ppt had a higher C_{max} (0.6 µg/ml) than fish maintained in either of the higher salinity levels (0.5 and 0.4 µg/ml, respectively). A comparison of the C_{max} values following IM and PO OTC administration demonstrated that plasma concentrations reached significantly higher levels overall following IM injection. However, IM dosing of OTC resulted in higher fish-to-fish variation compared to PO treatment.

There was a non-significant numerical trend of the MRT and T ½ following IM injection of OTC in freshwater flounder (422.6 h and 292.9 h, respectively) which were longer than these same parameters in fish maintained in brackish (15 ppt) or seawater (32 ppt) (429.0 h and 297.3 h ; 415.4 h and 287.9 h, respectively). Following PO administration, the MRT and T ½ were longer in the brackish water and seawater maintained fish (401.3 h and 278.1 h; 383.8 h and 266.0 h, respectively) than in the freshwater acclimated fish (370.7 h and 256.9 h, respectively), although these parameters were not significantly different between the salinity treatments.

3.4.2: GILL CHLORIDE CELL SIZE AND DENSITY AND ENYZME ACTIVITY

Although no significant difference were observed between the three salinity treatments, there was a numeric trend for gill chloride cells in the freshwater fish to be fewer in number and smaller in size. Gills collected from fish in the IM trial group revealed that fish in the 32 ppt salinity environment had the largest chloride cell size (118.59 ± 29.83) and the 15 ppt salinity treatment had the highest chloride cell density (7.80 ± 1.08) (Table 3-2; Fig. 3-4 and 3-5). In the freshwater acclimated summer flounder, there was a higher prevalence of chloride cells distributed on the distal lamellar surface as opposed to fish in higher salinities where chloride cells were found to primarily reside in the basal inter-lamellar space (Fig. 3-6). Because of a processing error of gills collected from the PO trial, staining of the chloride cells was not successful from these fish.

No significant differences were detected between gill Na^+ - K^+ ATPase enzyme activity between flounder maintained at 0 ppt, 15 ppt and 32 ppt (Table 3-3 and Fig. 3-7). However, trends in both the IM and PO OTC administration trials revealed an increase in enzyme activity in freshwater acclimated flounder compared to seawater acclimated cohorts.

3.4.3: PLASMA AND URINE OSMOLALITY

Plasma, urine and tank water osmolality values are presented in Table 3-4 and in Fig. 3-8. In both the IM and PO routes of OTC administration, fish maintained in freshwater had the lowest plasma and urine osmolalities. Plasma and urine osmolality were directly correlated with tank salinity. Plasma osmolality ranged from 300.00 to 338.25 mOsmol/l in the IM trial and from 269.50 to 323.83 mOsmol/l in the PO trial. Urine osmolality ranged from 64.08 to 329.29 mOsmol/l in the IM and from 79.19 mOsmol/l to 332.53 mOsmol/l in the PO trial. Urine osmolality was significantly different ($p<0.05$) between the 0 ppt fish and the fish maintained at 15 ppt and 32 ppt salinities in the IM OTC treated group and urine osmolality was significantly different from the 0 ppt and the 32

ppt PO OTC treated fish. In addition, plasma osmolality was significantly different between the 0 ppt fish and the 15 ppt and 32 ppt fish in the PO trial.

3.4.4: URINE CHARACTERISTICS

Urine characteristics (i.e. urine volume, urine color and urine specific gravity) are given in Tables 3-5. Urine volume was typically higher in freshwater fish than fish maintained at higher salinities. At 24 and 48 hour collection times, urine volume was significantly ($p<0.05$) greater in the 0 ppt fish compared to the 32 ppt fish in the IM trial. In the PO OTC dosed fish, this was also true at collection times 24 and 72 h. Urine was a significantly ($p<0.05$) darker yellow color in the 32 ppt fish than in the 0 ppt fish at all time points (Fig. 3-1). Urine specific gravity in the IM treated fish, was significantly different ($p<0.05$) between all three salinity treatments at all three collection times. In the PO trial, urine specific gravity was significantly different between the 0 ppt and 15 ppt groups and the 0 ppt and 32 ppt groups, but no statistical difference was detected between the 15 ppt and 32 ppt salinity treatments.

Table 3-6 summarizes urine flow rate, urine OTC concentrations and corresponding plasma OTC concentrations. Urine flow rate was higher at all three collection times in the freshwater fish compared to fish in the other salinity treatments for both the IM and PO administration routes. Typically, urine flow rates ranged between 0.13 to 0.31 ml/kg/hr for the 0 ppt fish, between 0.05 to 0.12 ml/kg/hr for the 15 ppt treatment and 0.07 to 0.11 ml/kg/hr for 32 ppt treatments.

Although there were no significant differences ($p>0.05$) between the salinity treatments for plasma OTC concentrations, there were significant differences ($p<0.05$) in urine OTC levels. For both the IM route, urine OTC concentrations were higher in the 15 ppt and 32 ppt held fish at all collection times. In the IM trial, urine OTC levels ranged from15.98 µg/ml to 17.71 µg/ml for the 0 ppt fish, between 64.35 µg/ml to111.53 µg/ml for the 15 ppt fish and between 52.37µg/ml to 113.86 µg/ml for the 32 ppt fish. In the PO trial, urine OTC levels ranged from 0.49 µg/ml to 1.35 µg/ml for the 0 ppt fish, between 5.54

µg/ml to 12.00 µg/ml for the 15 ppt fish and between 96.38 µg/ml to 232.46 µg/ml for the 32 ppt fish.

3.4.5: PLASMA PROTEIN BINDING

Results of plasma protein binding (PPB) determinations (Table 3-7) revealed that plasma protein binding was significantly different ($p<0.05$) between the salinity treatments. In the IM trial, PPB was significantly different between all three salinity treatments, where PPB ranged from 24.3 %, 33.2% to 54.6 % for the 0 ppt, 15 ppt and 32 ppt treatments, respectively. Following PO OTC, PPB significant differences were detected between the 32 ppt treatment and the 15 ppt and 0 ppt treatments. The PPB range for the PO trial was from 34.2 %, 32.1% to 60.4% for the 0 ppt, 15 ppt and 32 ppt treatments, respectively.

3.5: DISCUSSION

Environmental salinity had a limited effect on OTC pharmacokinetic parameters following both IM and PO administration. The effect of salinity may be a result of chelation with cations such as Ca^{2+} and Mg^{2+} found in seawater or the physiological alterations flounder undergo in response to different levels of salinity, such as changes in plasma and urine osmolality and urine flow rate. When OTC is bound with divalent cations, the charge on the OTC molecule is altered, thus, reducing its lipid solubility and ultimately its systemic absorption and bioavailability. Results from this study show that OTC absorption from the gastrointestinal tract following PO drug administration in summer flounder was limited and was negatively correlated with environmental salinity. The C_{max} values for the PO trial were 5-12% of those determined for the IM trial. For fish receiving OTC orally, the high salinity group had lower AUC values than fish maintained in freshwater conditions. This finding in conjunction with the smaller C_{max} value for the 32 ppt fish suggested that OTC was not being as well absorbed in marine fish as by fish in freshwater. Fish held in seawater must ingest water to maintain hydration, such that the gastrointestinal tract environment of these fish is similar to their marine environment (Lunestad and Goksøyr, 1990) permitting orally dosed OTC to be in

direct contact with Ca^{2+} and Mg^{2+} cations. In addition, the pH of the gut fluid of marine fish mimics the pH of the marine environment, typically between 7.8 and 8.2, allowing for a 1:1 complex ratio between OTC and the cations present in the seawater (Lunestad and Goksøyr, 1990). Furthermore in the PO trial, fish maintained in salinities above 15 ppt had significantly higher plasma and urine osmolalities compared to the fish in freshwater, which possibly influenced the MRT and T ½ by altering drug behavior in the plasma and glomerular filtration.

In fish receiving IM injections of OTC, salinity affected the extent of drug absorption, which was reflected in the significantly different AUC values among the three salinity treatments. The AUC values were highest in the seawater maintained fish, which was the opposite effect of what was observed during the PO trial. In addition, although not significantly different, the MRT and T ½ were shorter in the 32 ppt fish compared to the other salinity groups. These findings were unexpected since it was hypothesized that fish in freshwater would have a faster elimination rate because of lower plasma and urine osmolalities and higher urine flow rates. Hence, it was expected that OTC total body elimination would be faster in freshwater acclimated summer flounder. However, since the drug was deposited directly in the muscle tissue, tissue-binding and drug retention in this space may have affected the pharmacokinetic parameters. Riviere (1999) describes that the estimation of MRT may be problematic when a fraction of the administered dose spends time in another space besides the plasma before being eliminated. Although it appeared that OTC could distribute from muscle tissue to plasma, the prolonged time to T_{max} in both the 15 ppt and 32 ppt treatments (312 h and 168 h, respectively) compared to the freshwater group (0.5 h) suggested that there was an effect of salinity following IM injection. In addition, results from a preliminary dosing study, where summer flounder were treated with a similar IM 50 mg/kg OTC dose, it was observed that there was still a significant amount of drug in the vicinity of the IM injection site 900 h post-injection as determined by fluorescence (Hughes, unpublished). Furthermore, in comparison of OTC following IM and PO administration, there was a higher degree of inter fish-to-fish variation following IM injections compared to PO treated fish confirming that drug

movement within the body was variable from one fish to the next. These factors should be considered when evaluating and interpreting results from the IM trial.

The physiological adjustments the summer flounder made in response to environmental salinity conditions were characteristic for fish acclimated to those salinities. Plasma and urine osmolality were typically significantly higher in the fish acclimated to the higher salinity groups compared to fish at 0 ppt. In addition, other urine characteristics were also affected by salinity, such as urine flow rate, volume, color and specific gravity. Fish in freshwater had higher urine flow rates and eliminated a less concentrated urine. Following these physiological changes, OTC concentrations in the urine were higher per ml of urine for the 15 ppt and 32 ppt maintained fish suggesting that the fish were concentrating urine and OTC molecules prior to elimination. The higher OTC urine concentrations in the 32 ppt fish was believed to be a result of physiological urine concentration by the fish.

The differences observed in the plasma protein binding affinity of OTC were unexpected. Summer flounder maintained in 32 ppt salinity environments had significantly higher plasma protein binding affinity for OTC than flounder in brackish and freshwater. It was hypothesized that differences in blood chemistry, such as blood pH, albumin levels or osmolality may be responsible for these differences. However, further research is required to definitively determine what parameter is responsible for the differences.

Alterations in gill chloride cell size and density were minimal. There was a non-significant numeric trend for the fish maintained in the 15 ppt and 32 ppt treatments to have higher chloride cell densities and larger chloride cells, suggesting that summer flounder are well adapted to differences in environmental salinity. In some fish species, a transfer from seawater to freshwater may result in a significant decrease of chloride cell number and subsequent loss of osmoregulatory balance. However, the slight alterations of the morphological changes observed in this experiment using summer flounder indicated that the summer flounder is a strongly euryhaline species and maintains chloride cell size and function. Varsamos *et al*. (2002) documented an increase in

chloride cell number in sea bass, *Dicentrarchus labrax*, when fish were transferred to both freshwater and doubly concentrated seawater (70 ppt). The difference in chloride cell distribution in the gill tissue between fish in fresh and saltwater is well documented (Varsamos *et al*., 2002; Hartl *et al*., 2001; Uchida *et al*., 1996) and confirms the view that lamellar chloride cells degenerate during seawater adaptation (Perry, 1997; Uchida *et al*., 1996; Foskett and Scheffey, 1982). In the present study using summer flounder, there was a trend for $Na^+ - K^+$ ATPase activity to be increased in the freshwater acclimated fish compared to seawater acclimated cohorts. In stenohaline fish, environmental salinity changes result in decreased enzyme in freshwater and increased activity in saltwater. However, Varsamos *et al*. (2002) and Stagg and Shuttleworth (1982) did find that in certain marine euryhaline fish species such as the European flounder (*Platichthys flesus*) that freshwater acclimation resulted in increased or similar enzyme activity levels compared to seawater acclimated cohorts. Lasserre (1971) found similar findings in marine teleosts such as the thick-lipped mullet (*Crenimugil labrosus)* and the sea bass (*Dicentrarchus labrax)* where gill enzyme activity increased in freshwater adapted cohorts of the same species. These findings distinguish euryhaline fish species from stenohaline species since there is no loss of osmoregulatory ability in the euryhaline fishes.

In summary, environmental salinity minimally impacted OTC pharmacokinetic parameters. Although summer flounder adapted to different saline environments by altering plasma and urine characteristics, the primary impact on OTC behavior in saltwater maintained fish was the binding of OTC to cations present in seawater. This relationship between OTC bioavailability and environmental salinity should be considered when treating summer flounder maintained in seawater.

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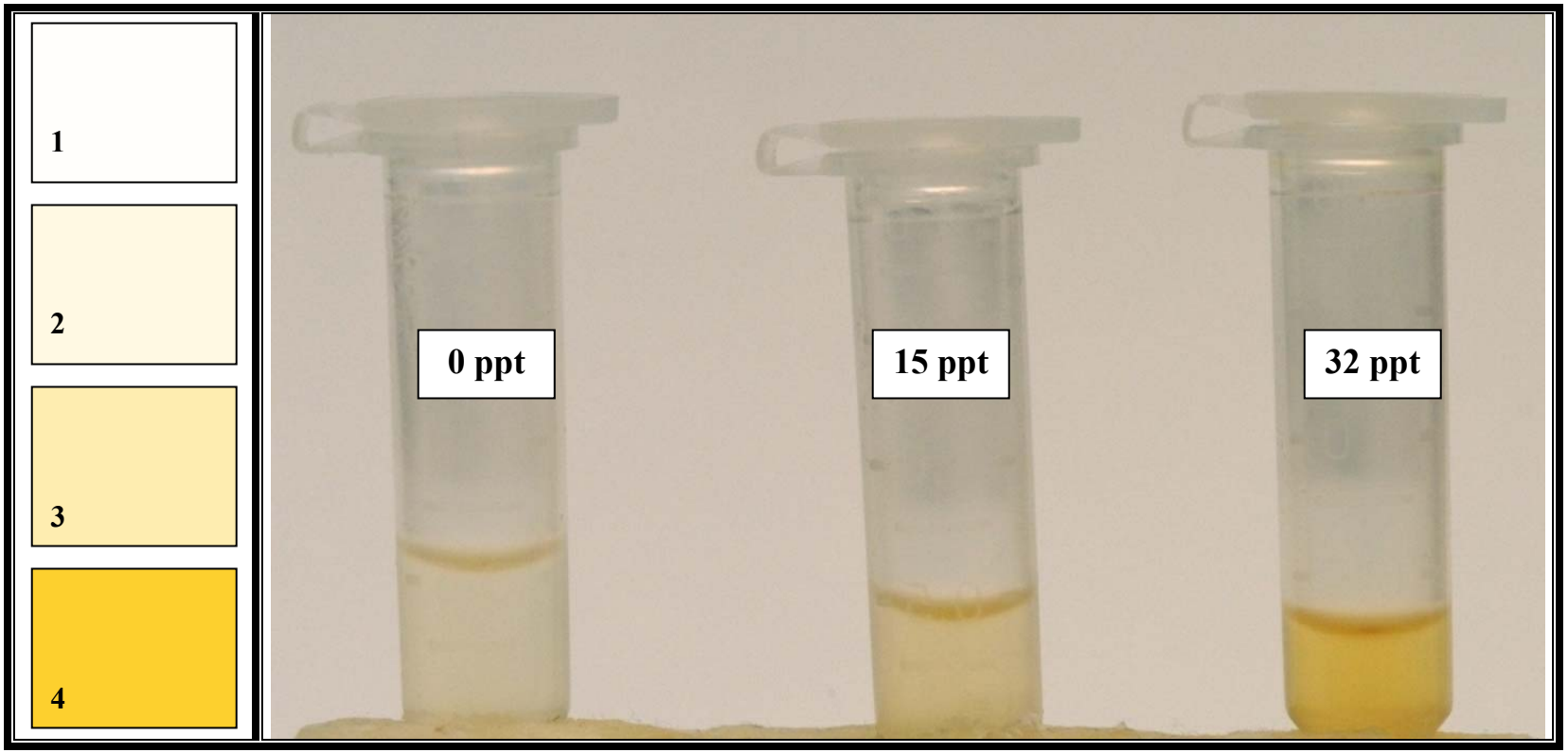

Figure 3-1. Urine samples collected from summer flounder*, Paralichthys dentatus*, fitted with indwelling urinary catheters and maintained at three different salinity levels, 0 ppt, 15 ppt, and 32 ppt. Color scale to left of picture 1=clear; 2=slight yellow; 3=yellow and 4=bright yellow.

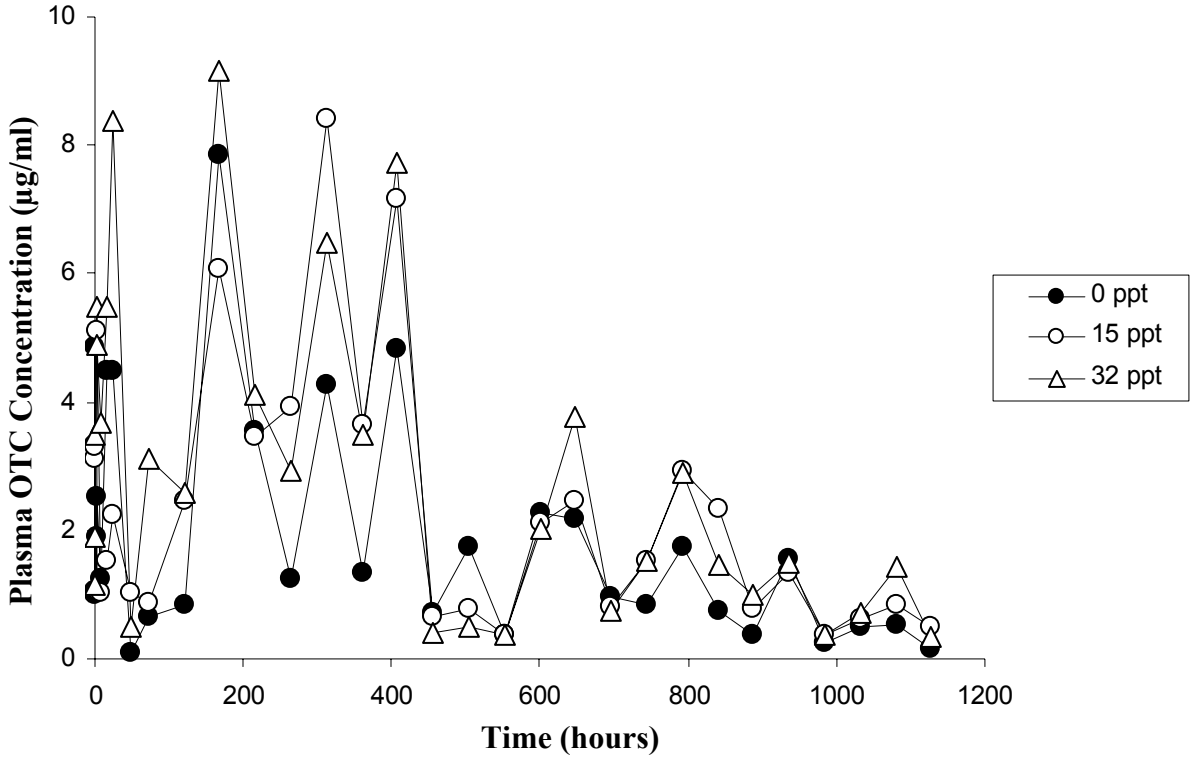

Figure 3-2. Plasma concentration-time profile of oxytetracycline (50 mg/kg) administered intramuscularly (IM) to summer flounder, *Paralichthys dentatus*, maintained at three salinity levels: freshwater (0 ppt), brackish water (15 ppt), and seawater (32 ppt). Each point represents the mean of six fish.

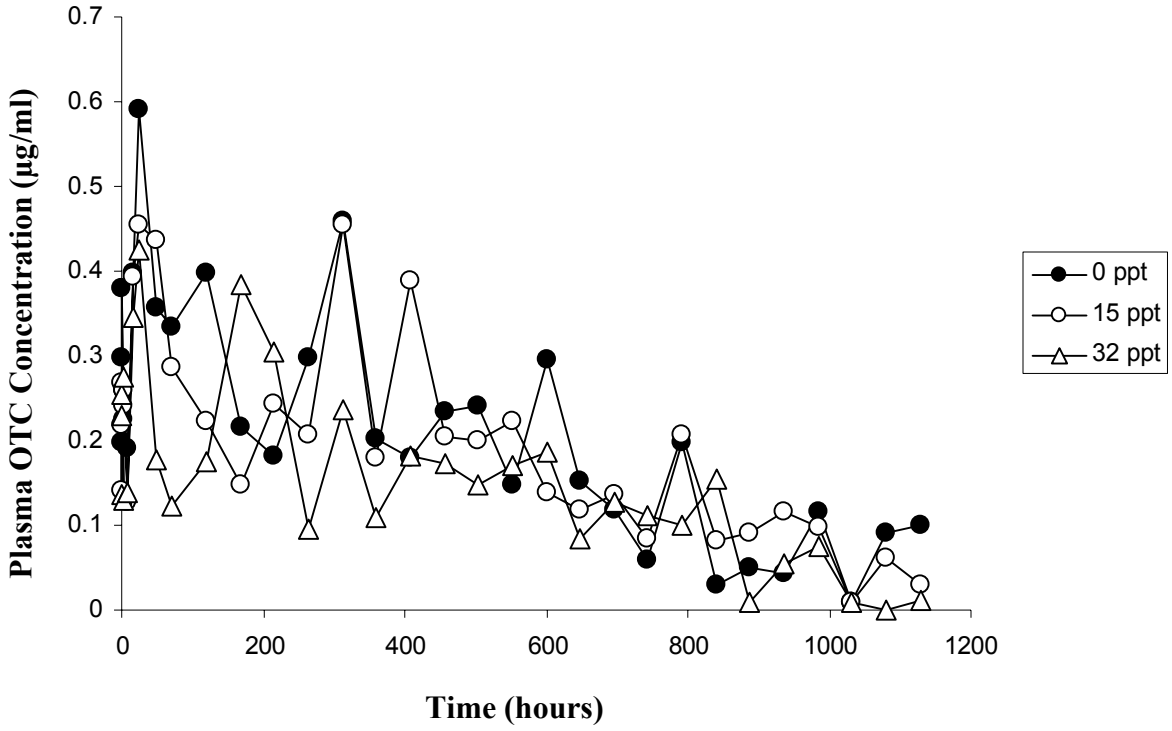

Figure 3-3. Plasma concentration-time profile of oxytetracycline (50 mg/kg) administered orally (PO) to summer flounder, *Paralichthys dentatus*, maintained at three salinity levels: freshwater (0 ppt), brackish water (15 ppt), and seawater (32 ppt). Each point represents the mean of six fish.

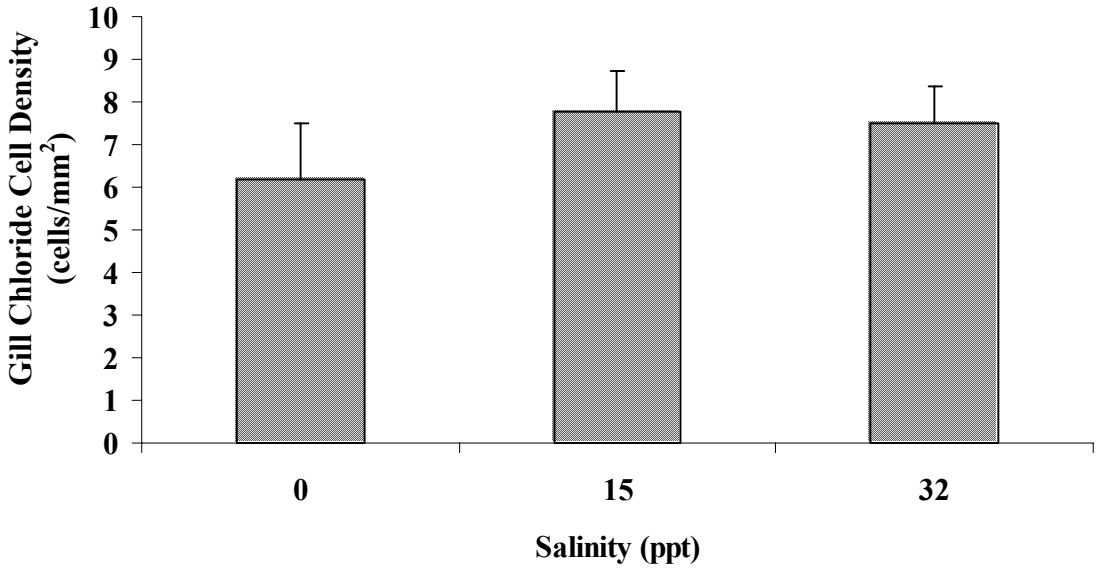

Figure 3-4. Effect of environmental salinity on gill chloride cell density in summer flounder, *Paralichthys dentatus*, maintained at three salinity levels (0 ppt, 15 ppt, 32 ppt) and 1128 hours post-intramuscular (IM) injection of oxytetracycline (50 mg/kg). Data represent the mean of 6 observations ± SD.

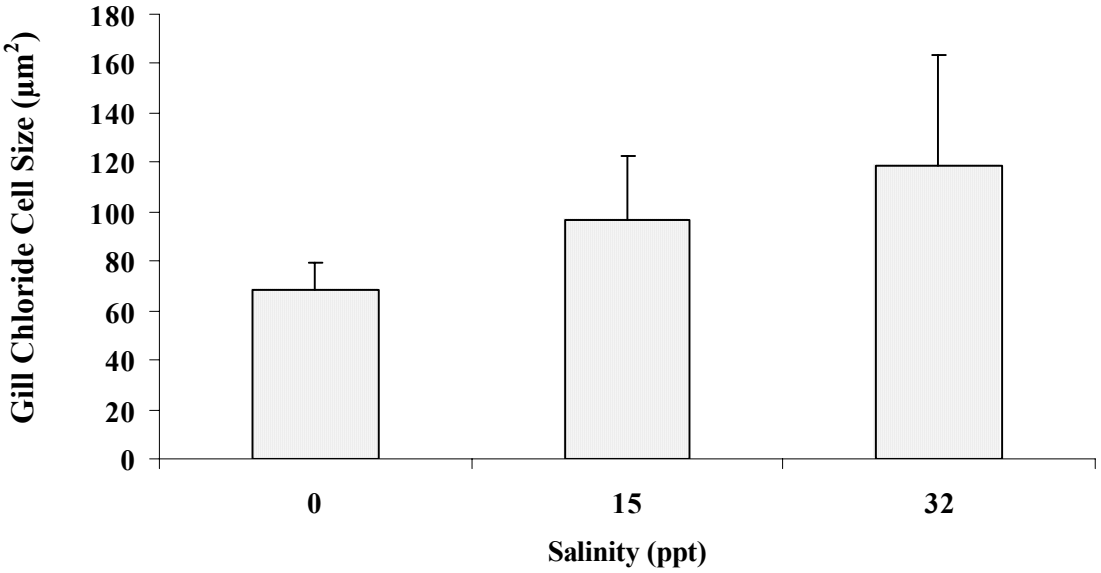

Figure 3-5. Effect of environmental salinity on gill chloride cell size in summer flounder, *Paralichthys dentatus*, maintained at three salinity levels (0 ppt, 15 ppt, 32 ppt) and 1128 hours post-intramuscular (IM) injection of oxytetracycline (50 mg/kg). Data represent the mean of 6 observations ± SD.

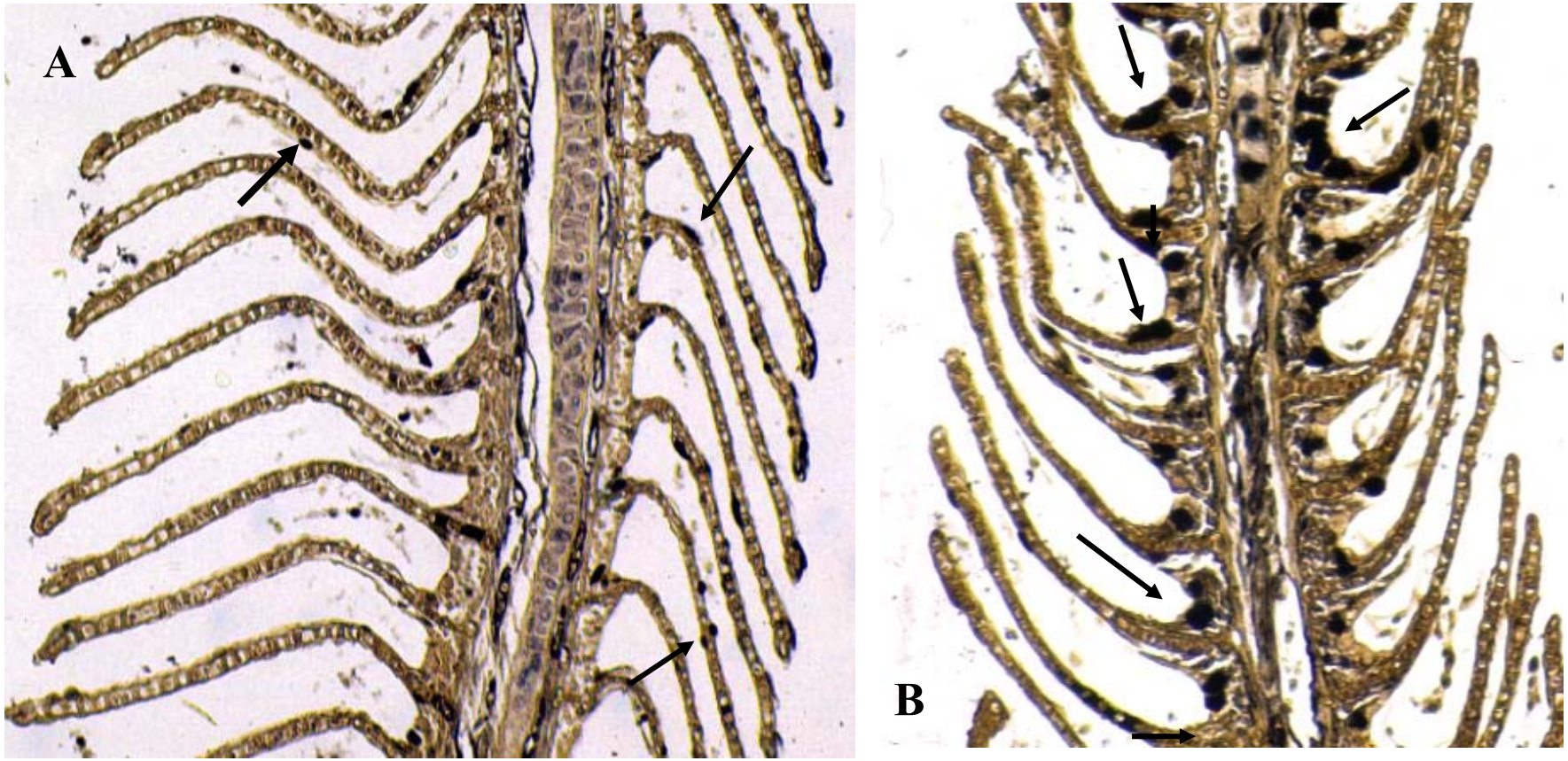

Figure 3-6. Photomicrographs of gill filaments from summer flounder, *Paralichthys dentatus*, maintained at different salinities showing gill chloride cells. Gill chloride cells specifically stained with Champy-Maillet's fixative. A. Gill from flounder maintained at 0 ppt (60 x). Note distribution of lamellar chloride cells (arrows). B. Gill from flounder maintained at 32 ppt (66 x). Note size and distribution of chloride cells in the interlamellar space (arrow).

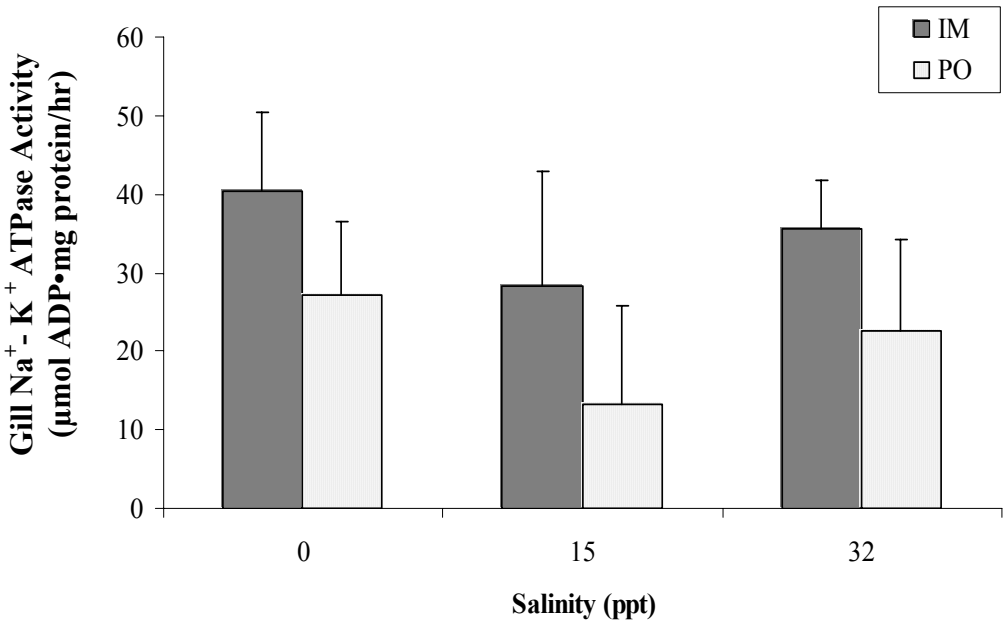

Figure 3-7. Gill Na^{+}- K^{+} ATPase activity in the gills of summer flounder, *Paralichthys dentatus*, maintained at three salinity levels 0 ppt (freshwater), 15 ppt (brackish water) and 32 ppt (seawater). Salinity acclimated fish were administered oxytetracycline (OTC, 50 mg/kg) via IM or PO routes and gills were collected 1128 hours post-OTC administration. (Error bars represent standard deviation).

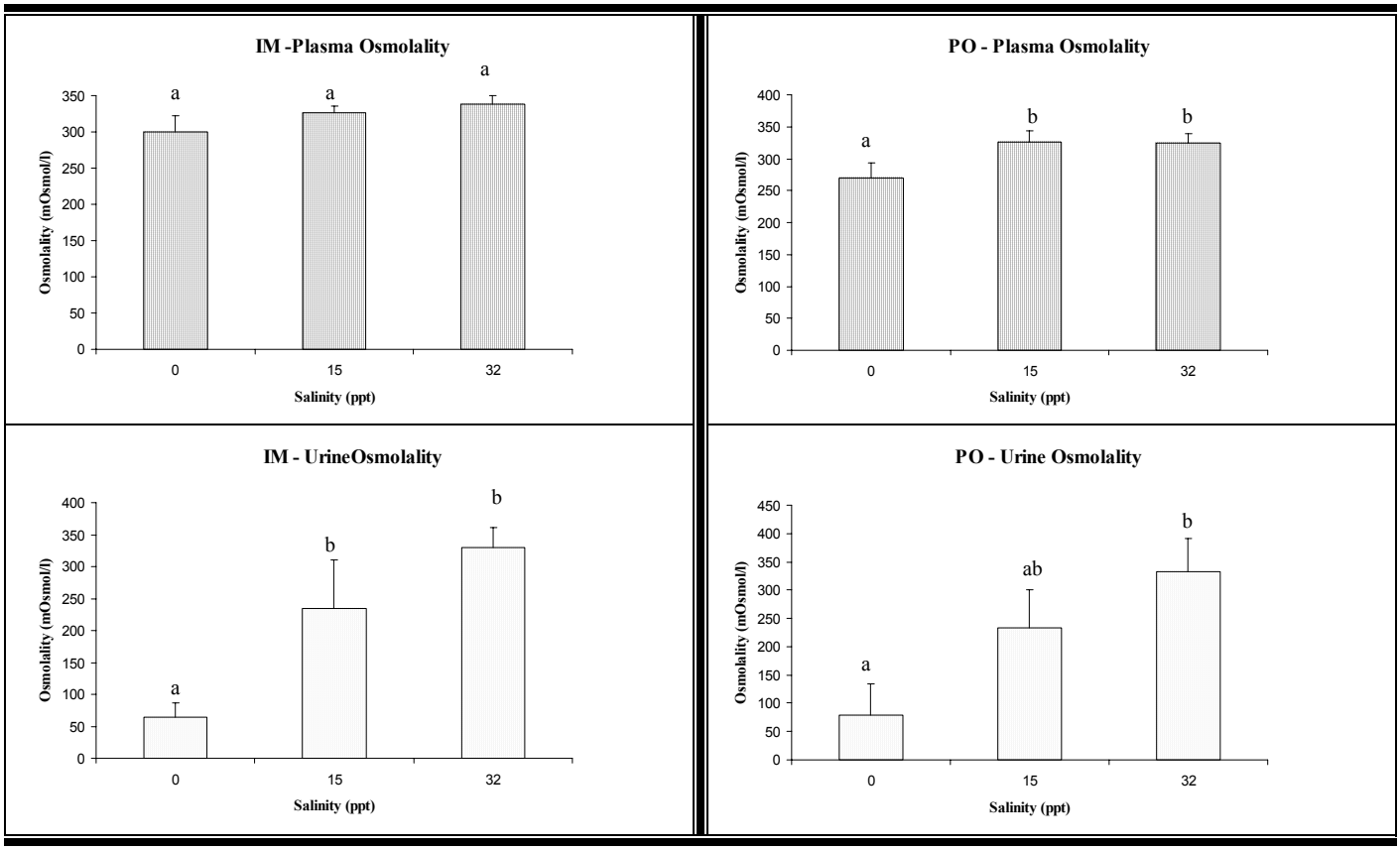

Figure 3-8. Differences in plasma and urine osmolality in summer flounder, *Paralichthys dentatus*, maintained at three environmental salinity levels. (Bar columns within a route of OTC administration (IM, PO) denoted by a different letter are significantly different at $p < 0.05$, using a *z* multiple comparison test statistic with a Bonferroni correction; error bars represent standard deviation).

Table 3-1. Pharmacokinetic parameters[1] of oxytetracycline (OTC, 50 mg/kg) administered intramuscularly (IM) or orally (PO) to summer flounder, *Paralichthys dentatus,* maintained at three salinity levels freshwater (0 ppt), brackish water (15 ppt) and seawater (32 ppt).

Route of OTC Administration	Salinity (ppt)	AUC (µg•h/ml)	MRT (h)	T ½ (h)	T_{max} (h)	C_{max} (µg/ml)	Fish-to-Fish Variation
IM	0	1684.8[a2] [1452.3, 2092.1][3]	422.6 [386.6, 460.0]	292.9 [267.9, 318.8]	0.5	4.9	0.6
	15	2067.8[b] [1762.3, 2588.4]	429.0 [393.0, 468.2]	297.3 [272.4, 324.5]	312	8.4	0.6
	32	2241.3[c] [1967.6, 2748.5]	415.4 [375.8, 456.9]	287.9 [260.4, 316.6]	168	9.2	0.5
PO	0	190.2 [172.8, 219.2]	370.7 [344.0, 399.4]	256.9 [238.4, 276.8]	24	0.6	0.2
	15	180.7 [161.7, 209.6]	401.3 [374.9, 430.5]	278.1 [259.8, 298.3]	24	0.5	0.3
	32	127.7 [113.3, 152.6]	383.8 [350.6, 420.7]	266.0 [243.0, 291.6]	24	0.4	0.3

[1]Pharmacokinetic parameter abbreviations; AUC: area under the plasma concentration-time curve after a single dose of OTC at 50 mg/kg; MRT: mean residence time of OTC in summer flounder following a single dose of OTC (50 mg/kg); T ½: total body elimination half-life; T_{max}: time of the maximum drug concentration within the body; C_{max}: maximum drug concentration within the body; fish-fish variation: intraclass correlation coefficient.

[2]Values within a column and within a route of OTC administration (IM, PO) denoted by a different letter are significantly different at $p<0.017$, using a *z* multiple comparison test statistic with a Bonferroni correction.

[3] Bracketed values represent the 95% confidence limits obtained by the bootstrap procedure.

Table 3-2. Gill chloride cell size and density in summer flounder, *Paralichthys dentatus*, maintained at three salinity levels (0 ppt, 15 ppt, 32 ppt) 1128 hours following intramuscular (IM) injection of oxytetracycline (OTC, 50 mg/kg).

Route of OTC Administration	Salinity (ppt)	Gill Chloride Cell Size (μm^2)	Gill Chloride Cell Density (cells/mm^2)
IM	0	68.03 ± 11.63 [1]	6.20 ± 1.28
	15	96.83 ± 26.09	7.80 ± 0.91
	32	118.59 ± 44.93	7.52 ± 0.84
PO		ND	

[1]Data are mean values (n=6) ± SD. No significant differences were detected between the salinity treatments. ND=not determined.

Table 3-3. Gill Na^+ - K^+ ATPase activity in summer flounder, *Paralichthys dentatus*, maintained at three salinity levels (0 ppt, 15 ppt, 32 ppt) 1128 hours following intramuscular (IM) injection or oral gavage (PO) of oxytetracycline (OTC, 50 mg/kg).

Route of OTC Administration	Salinity (ppt)	Gill Na^+ - K^+ ATPase Activity (µmol ADP•mg protein/hr)
IM	0	40.36 ± 9.96[1]
	15	28.32 ± 14.61
	32	35.55 ± 6.30
PO	0	27.12 ± 9.36
	15	13.28 ± 12.43
	32	22.53 ± 11.75

[1]Data are mean values (n=6) ± SD. No significant differences were detected between salinity treatments.

Table 3-4. Plasma and urine osmolalities of summer flounder, *Paralichthys dentatus*, maintained at three salinity levels (0 ppt, 15 ppt and 32 ppt). Fish were administered oxytetracycline (OTC, 50 mg/kg) by intramuscular injection (IM) or oral gavage (PO).

Route of OTC Administration	Salinity (ppt)	Plasma Osmolality (mOsmol/l)	Urine Osmolality (mOsmol/l)
IM	0	300.00 ± 22.27[a 1]	64.08 ± 22.53[a]
	15	325.79 ± 9.39[a]	233.61 ± 76.13[b]
	32	338.25 ± 11.32[a]	329.29 ± 32.25[b]
PO	0	269.50 ± 23.14[a]	79.19 ± 54.95[a]
	15	326.19 ± 18.06[b]	233.65 ± 66.60[ab]
	32	323.83 ± 15.61[b]	332.53 ± 58.90[b]

[1]Data are mean values (n=6) ± SD. Values denoted by a different letter within a route of OTC administration are significantly different ($p<0.05$).

Table 3-5. Urine volume, color and specific gravity measured at 24, 48 and 72 hours post-urinary catheter placement and OTC administration (50 mg/kg) via intramuscular (IM) or per os (PO) in summer flounder, *Paralichthys dentatus*, fitted with indwelling urinary catheters and maintained in individual aquariums at three salinity levels of 0 ppt, 15 ppt and 32 ppt.

Route of OTC Administration	Salinity (ppt)	Urine Characteristics								
		Urine Volume (ml)			Urine Color[2]			Urine Specific Gravity		
		Time (hours)			Time (hours)			Time (hours)		
		24	48	72	24	48	72	24	48	72
IM	0	1.54 [a1]	1.45 [a]	0.98 [a]	2.43 [a]	2.15 [a]	2.22 [a]	1.001 [a]	1.001 [a]	1.001 [a]
	15	0.81 [ab]	0.40 [b]	0.66 [a]	3.38 [ab]	3.33 [b]	3.24 [b]	1.006 [b]	1.007 [b]	1.008 [b]
	32	0.59 [b]	0.73 [b]	0.53 [a]	3.58 [b]	3.17 [b]	3.39 [b]	1.011 [c]	1.010 [c]	1.015 [c]
PO	0	1.95 [a]	0.91 [a]	0.65 [a]	1.67 [a]	1.41 [a]	1.43 [a]	1.001 [a]	1.001 [a]	1.002 [a]
	15	1.16 [b]	0.67 [a]	0.53 [b]	2.85 [b]	3.58 [b]	3.38 [b]	1.009 [b]	1.009 [b]	1.012 [b]
	32	1.61 [b]	0.36 [a]	0.41 [b]	2.89 [b]	2.78 [b]	3.35 [b]	1.011 [b]	1.012 [b]	1.012 [b]

[1]Means (n=6) within a column and within an OTC route of administration (IM, PO) with no letter in common are significantly different as determined by a Bonferroni multiple comparison correction procedure ($p<0.05$).

[2] Urine color was subjectively evaluated based on intensity of yellow color of the urine sample. Colors were assigned values such that: clear (no color) = 1; slight yellow color = 2; light yellow = 3 and dark yellow = 4.

Table 3-6. Urine flow rate and urine oxytetracycline (OTC) concentrations measured at 24, 48 and 72 hours post-urinary catheter placement and OTC administration (50 mg/kg) via intramuscular (IM) and per os (PO) in summer flounder, *Paralichthys dentatus*, fitted with indwelling urinary catheters and maintained in individual aquariums at three salinity levels (0 ppt, 15 ppt and 32 ppt). Corresponding 72 hour plasma OTC concentrations are also given.

Route of OTC Administration	Salinity (ppt)	Urine Characteristics						Plasma Characteristics
		Urine Flow Rate (ml/kg/hr)			Urine OTC (µg/ml)			Plasma OTC (µg/ml)
		Time (hours)			Time (hours)			Time (hours)
		24	48	72	24	48	72	72
IM	0	0.21 [a1]	0.19 [a]	0.13 [a]	17.71 [a]	16.71 [a]	15.98 [a]	11.83 [a]
	15	0.12 [ab]	0.06 [b]	0.09 [a]	64.35 [b]	94.21 [b]	111.53 [b]	15.48 [a]
	32	0.09 [b]	0.11 [ab]	0.09 [a]	52.37 [ab]	84.87 [b]	113.86 [b]	11.42 [a]
PO	0	0.31 [a]	0.16 [a]	0.23 [a]	0.70 [a]	0.49 [a]	1.35 [a]	0.42 [a]
	15	0.12 [b]	0.09 [a]	0.05 [b]	5.54 [a]	7.19 [ab]	12.00 [a]	0.45 [a]
	32	0.09 [b]	0.08 [a]	0.07 [b]	232.46 [b]	96.38 [b]	105.18 [a]	0.77 [a]

[1]Means (n=6) within a column and within an OTC route of administration (IM, PO) with no letter in common are significantly different as determined by a Bonferroni multiple comparison correction procedure ($p<0.05$).

Table 3-7. Plasma protein binding of oxytetracycline (OTC, 50 mg/kg) administered intramuscularly (IM) or orally (PO) to summer flounder, *Paralichthys dentatus*, maintained at three salinity levels (0 ppt, 15 ppt and 32 ppt).

Route of OTC Administration	Salinity (ppt)	Plasma Protein Binding (%)
IM	0	24.3 ± 2.0 [a 1]
	15	33.2 ± 1.9 [b]
	32	54.6 ± 1.9 [c]
PO	0	34.2 ± 2.8[a]
	15	32.1 ± 2.6 [a]
	32	60.4 ± 1.6[b]

[1]Data are mean values (n=6) ± SD. Values denoted by a different letter within a route of OTC administration are significantly different ($p<0.05$).

CHAPTER 4

A PRELIMINARY STUDY OF OXYTETRACYCLINE RETENTION TIMES IN MUSCLE TISSUE FROM SUMMER FLOUNDER, *PARALICHTHYS DENTATUS*, MAINTAINED AT THREE DIFFERENT ENVIRONMENTAL SALINITY LEVELS

Prepared for submission to *Aquaculture*

4.1: ABSTRACT

Summer flounder, *Paralichthys dentatus*, maintained at 0 ppt, 15 ppt, and 32 ppt were given a single dose of 50 mg/kg OTC via intramuscular injections (IM) or oral gavage (PO). Using a non-compartmental model, plasma pharmacokinetic parameters estimated 552 – 1128 h following IM OTC administration indicated that summer flounder held at 32 ppt still had significantly higher ($p<0.05$) OTC plasma concentrations than fish maintained at the lower salinity treatments. The AUC for the 32 ppt summer flounder was 826.3 µg•h/ml, whereas AUC parameters for the 0 ppt and 15 ppt treatments were 665.2 µg•h/ml and 810.1 µg•h/ml, respectively. The IM muscle tissue AUC estimate for the 32 ppt maintained fish was significantly lower compared to the other salinity treatments suggesting that OTC deposition and distribution may be affected by environmental salinity. This may be due to chelation of OTC with cations such as Mg^{2+} and Ca^{2+} and/or alterations in muscle water content and plasma osmolality. Although not significantly different from the other salinity treatments, in both the plasma and muscle tissue samples the IM 32 ppt treatment had the longest total body elimination half-life (T½). Per os administration of a single 50 mg/kg OTC dose failed to result in OTC plasma or muscle tissue concentrations greater than 0.2 µg/ml or 0.3 µg/g, respectively.

Keywords: flounder, *Paralichthys*, oxytetracycline, pharmacokinetics, residue

4.2: INTRODUCTION

Currently in the United States there are only two antibiotics available and approved by the Food and Drug Administration (FDA) for use in foodfish. Oxytetracycline (OTC, Terramycin for Fish®) is one of these approved antibiotics. The FDA has established a tolerance limit of 2 ppm OTC in the raw edible portions of salmonids, catfish and lobster and legal use of OTC requires a 21-day withholding period for these teleosts and a 30-day period for the lobster. Currently, OTC is an extra-label veterinary prescription for summer flounder, *Paralichthys dentatus*, in part because no complete pharmacokinetic research in this fish species has been conducted and published nor has an Investigational New Animal Drug (INAD) been approved by the FDA for general use in summer flounder. The 1994 Animal Medicinal Use Control Act gave approval to licensed veterinarians for the use of compounds as extra-label. However, this act did not approve drugs for extra-label use that were administered through medicated feed (Jensen and Greenlees, 1997). Typically, in the intensive culture of foodfish the only route feasible is through the use of medicated feeds because of population size (Stoffregen *et al*., 1996; Xu and Rogers, 1994a). Because of this, studies determining withdrawal times in marine foodfish species other than salmonids are needed. Although not FDA-approved, IM injections are often given to valuable broodstock that are not intended for human consumption.

Van Dresser and Wilcke (1989) found that OTC was one of the four most common antibiotic residues found in animal tissues. Withdrawal periods for FDA approved drugs used in food animals are only valid for the specified species, dose, route and dosage regimen (Riviere and Sundlof, 2001). The withdrawal time for OTC in catfish and salmonids is 21 d, but there is no data yet to confirm that this would be an appropriate withdrawal time for summer flounder or other marine fish species. Therefore, the prescribing veterinarian must recommend to the producer a longer withholding period ensuring a consumer-safe wholesome product. Additional variables in fish husbandry which may impact drug kinetics, especially withdrawal times, include water temperature and salinity (Treves-Brown, 2000). Drug residues in products entering the human food

chain may lead to bacterial resistance and other potential health threats to the consumer, such as allergic reactions by hypersensitive individuals (Du *et al*., 1997; Smith *et al*., 1994). Du *et al*. (1997) reported that common cooking procedures of OTC-treated channel catfish (*Ictalurus punctatus*) fillets did not completely degrade the drug. Therefore, although costly, it is necessary to perform drug specific pharmacokinetic research in any fish species that has potential for commercial foodfish production, such as the summer flounder.

This project is a preliminary investigation into the OTC muscle retention times in summer flounder maintained at three environmental salinity levels. Data from this research is regarded as preliminary because of the limited sample size (42 total fish per route of administration with six fish at each sampling time) and high variability such that the 95% confidence limits were extremely wide and little confidence could be placed in the estimated withdrawal times for each salinity level. However, this data does suggest that environmental salinity does impact OTC pharmacodynamics in summer flounder.

4.3: MATERIALS AND METHODS

4.3.1: FISH HUSBANDRY

For each route of OTC administration (IM and PO), forty-two healthy juvenile (25 cm, 192 ± 41 g and 25 cm, 204 ± 38 g, respectively) summer flounder (GreatBay Aquafarms, Portsmouth, N.H.) were arbitrarily divided equally into six 568 L recirculating aquaculture systems. Two of the 568 L fiberglass rectangular tanks shared a common sump, pump and biological filter, making 3 identical systems. Water in each tank was continuously passed through activated carbon to bind free OTC in the water column. Each system was arbitrarily designated as freshwater (0 ppt), brackish water (15 ppt) or seawater (32 ppt). Fish were slowly acclimated to experimental salinity concentrations over time and were maintained at the desired salinity levels for at least 4 weeks prior to the start of each experiment. All three tanks received the same route of OTC administration (IM or PO) such that the three salinity levels of each route of OTC administration were conducted simultaneously. Water quality indices (dissolved oxygen

(DO), ammonia, nitrites, nitrates, salinity, temperature, and pH) were monitored daily. Water hardness, measured as total hardness (mg/l $CaCO_3$), was monitored bi-monthly. Water quality parameters were regarded as optimal when parameters were within these limits: temperature: 19-21^{o}C (YSI 85 model 85/10, Aquatic Eco-Systems, Apopka, FL); pH: 7.8-8.2 (Sension1 pH meter, HACH, Loveland, CO); ammonia: <0.2 mg/L; nitrite: <10 mg/L; nitrate: <50 mg/L; total hardness: > 200 mg/L $CaCO_3$; and, DO: 6.0-8.0 mg/L (YSI 85 model 85/10, Aquatic Eco-Systems, Apopka, FL). Ammonia, nitrites, nitrates and hardness were measured with a spectrophotometer (DR2010 spectrometer, HACH, Loveland, CO). Water salinity was measured using a digital membrane probe (YSI 85 model 85/10, Aquatic Eco-Systems, Apopka, FL) and confirmed with a temperature compensated salinity refractometer (Aquatic Eco-Systems, Apopka, FL). Salinity adjustments were made by adding synthetic sea salt (Forty Fathoms Crystal Sea Salt, Marine Enterprises International, Inc., Baltimore, MD). The pH of the systems was maintained by adding sodium bicarbonate when the pH dropped below the desired range. Fish were fed a commercial floating diet formulated specifically for summer flounder (Shur-Gain, Nova Scotia, Canada; protein: 50%, fat: 15%; 6.5 mm pellets). Fish were fasted 24 h prior to sampling and 24 h following OTC administration.

Fish were anesthetized with buffered MS-222 (100 mg/L, tricaine methanesulfonate, Sigma Chemical Co., St. Louis, MO) for both routes of OTC administration, blood collection and tagging. All experimental fish were individually tagged with a t-bar anchor tag (Floy Tag, Inc., Seattle, WA) in the dorsal musculature on the visual side of the fish. Although anesthesia may alter certain blood parameters and other physiological and biochemical functions, there is no evidence that it interferes with OTC pharmacokinetic properties (Horsberg, 1994).

4.3.2: ROUTES OF DRUG ADMINISTRATION

Oxytetracycline (Bio-Mycin 200; 200 mg/ml oxytetracycline; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) was administered as a single dose to anesthetized fish at a dose of 50 mg OTC/kg of body weight for both routes (Piper *et al*., 1982). Intramuscular injections were given using a 100 µl Hamilton syringe with a 25-gauge needle in the dorsal musculature between the lateral line and dorsal fin on the eyed side

of the fish. At the site of IM drug administration slight pressure was applied for 10 sec to minimize reflux from injection site. Oral OTC was administered via stomach gavage using a curved stainless steel 20-gauge 3" gavage tube (Popper and Sons, Inc, New Hyde Park, NY) and 100 µl Hamilton syringe. Gavage placement in the stomach was confirmed manually.

4.3.3: SAMPLE COLLECTION TIMES

Blood and muscle tissue samples were collected at 552, 648, 744, 840, 936, 1032, and 1128 h following IM and PO OTC administration. The sample collection times for the two routes (IM and PO) were selected based on literature information and results from previous experiments of OTC dosing in summer flounder. The muscle collection times were designed such that at least one of the initial collection times would contain a sample with a drug residue concentration above 2 ppm OTC, the FDA tolerance limit for OTC in fish fillets (Oriani, 1999). Six fish were bled and euthanized by MS-222 overdose followed by cervical separation at every specified time interval.

4.3.4: BIOLOGICAL SAMPLE COLLECTION AND HANDLING

4.3.4.1: BLOOD COLLECTION, PROCESSING AND PLASMA STORAGE

Approximately 0.4 - 0.5 ml of blood was withdrawn from the caudal tail vessels at each bleeding time. No more than 1.5 ml of blood volume was taken from a single fish during the entire course of the experiment. The blood sample was placed immediately into plasma separator tubes containing lithium heparin (Microtainer, Becton Dickinson, Fisher Scientific, Pittsburgh, PA), mixed by inversion several times and kept on ice until centrifugation. Samples were centrifuged (Centra GP8R, International Equipment Company, Needham Heights, MA) at 3000 x g for 10 min at 12^{o}C. Plasma was stored at -80^{o} C until analysis.

4.3.4.2: MUSCLE TISSUE COLLECTION, PROCESSING AND STORAGE

Dorsal muscle fillets were removed from euthanized flounder and stored at -80^{o} C until analysis. For high performance liquid chromatography (HPLC) analysis, approximately 1 g of thawed muscle tissue was homogenized (PowerGen 700, Fisher Scientific, Suwanee, GA) in 4 ml of oxalic acid/acetonitrile/octanesulfonic acid (HPLC buffer) then sonicated for 10 sec at 20 kHz (Sonicator Cell Disruptor, Model W-225R, Heat Systems Ultrasonics, Inc., Plainview, NY) and centrifuged (Model Centra 7R IEC, International Equipment Co., Needham Heights, MA) at 2000 x g for 15 min. The resulting supernatant was collected and stored at –80^{o}C for later HPLC analysis.

4.3.5: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROCEDURE

Thawed plasma and extracts of muscle samples were filtered with a MPS micropartition device (Millipore, Beford, MA) equipped with a disposable YMT ultrafiltration membrane disc (3000 molecular weight cutoff, Amicon, Inc., Beverly, MA) and centrifuged at 14,000 x g for 40 min at 22^{o}C (Beckman Microfuge R centrifuge, Beckman Instruments, Inc., Palo Alto, CA). A sample of the ultrafiltrate (20 μl) was then injected directly onto a HPLC column. A Hypersil 3 micron C-18, 150 mm x 4.6 mm ID (Phenomenex, Torrance, CA) analytical reversed phase column was used. The HPLC system consisted of a Beckman Coulter System Gold chromatography unit equipped with a manual sample injector (Beckman Coulter Model 7725i) and a 126 solvent delivery module (Beckman Coulter Instruments, Inc., Fullerton, CA). HPLC effluents were analyzed with a Beckman 166 variable wavelength detector set at 355 nm. The mobile phase (pH 3.3) was a 70:30 mixture of an aqueous mobile phase (0.01M oxalic acid and 0.03M octane sulfonic acid sodium salt) and an organic mobile phase (acetonitrile) (Meinertz *et al*., 1998). This mixture was kept in a sealed container to prevent evaporation of the acetonitrile and was maintained on a magnetic stirrer to prevent separation of the phases. The flow-rate was 1.5ml/min, with each sample run taking approximately 10 min. Data was processed by the Beckman Coulter Analytical Series System Gold data acquisition software (Karat 32, Beckman Coulter Instruments, Inc.,

Fullerton, CA). Known standards of OTC ranging from 0.05 - 50.0 μg/ml were prepared in order to establish a regression line upon which the unknown OTC concentrations were calculated. The calibration regression curve was rejected if less than 0.995. The detection limit was determined by running OTC spiked flounder plasma and muscle filtrates to find the minimum detectable concentrations. The detection limit of OTC in flounder plasma for this HPLC system was 0.05 μg OTC/ml (0.05 ppm OTC) and was 0.2 μg OTC/g for the muscle. To verify consistent HPLC operation a known 2.5 μg/ml standard solution of OTC was periodically injected into the HPLC unit for evaluation. Recovery of OTC was determined by comparing spiked filtered OTC flounder plasma and muscle ultrafiltrate samples and unfiltered spiked samples. Recovery of OTC from filtered flounder plasma was 95% (±3.4) and from muscle tissue was 89% (±6.3). Plasma and muscle OTC concentrations that were determined by HPLC anlaysis to be lower than the respective detection limits were assigned a value of zero because values lower than the limit of detection could not be accurately differentiated from zero.

4.3.6: DATA ANALYSIS

The raw plasma and muscle OTC concentration data were log-transformed to stabilize variances. Log-means were calculated and a MIXED effects model with fish as a random variable was used to estimate between fish variance across all times (SAS Systems, version 8.2, SAS Institute, Inc., Cary, NC). Log-transformed data was exponentiated to corresponding geometric means in the original units. Using the geometric means, a non-compartmental model was used to estimate the area under the concentration-time curve (AUC) and the area under the moment curve (AUMC) of OTC in summer flounder plasma using the trapezoidal method for both routes of OTC administration. Additional pharmacokinetic parameters were estimated using the derived AUC and AUMC:

$$MRT = AUMC/AUC$$

$$T\,½ = 0.693 \bullet MRT$$

Where MRT is the mean residence time of OTC and T ½ is the total body elimination half-life.

To include all variation not associated with time, to give conservative estimates, a second partitioning of variation was performed with fish variation left in the model. A bootstrap randomization procedure using MULTTEST was used to estimate the confidence intervals of the pharmacokinetic parameters (Cole, 1999; Riviere, 1999). A multiple comparison test statistic, *z*, was used with a Bonferroni correction procedure to detect significant differences between salinity treatment comparisons.

4.4: RESULTS

The plasma and muscle concentration-time profiles following IM and PO OTC administration are given in Fig. 4-1 and Fig. 4-2, respectively. In Figures 4-1 and 4-2 each plotted point represents the mean of six fish, such that the mean is not an actual HPLC reading, thus, explaining why values may go below the limit of detection. A summary of the estimated pharmacokinetic parameters are given in Table 4-1 and Table 4-2 for plasma and muscle data from 552-1128 h post OTC administration. The IM plasma data demonstrated similar plasma concentrations 936 h post-injection for all three salinities. The muscle data also revealed similarity between the three salinity groups, especially 744 h post-injection. However, the intersection of the maximum tolerance limit (MTL), 2 ppm OTC, and the muscle concentrations for each salinity treatment cross at different times. Although all three treatments bisect the MTL before 744 h post-injection, it appeared that the summer flounder maintained at 15 ppt had higher OTC residue in the muscle the longest amount of time post-injection. The fish held at 32 ppt had lower muscle OTC levels than the other treatments for the majority of sample times. The plasma and muscle OTC concentrations following PO administration showed similar trends between the three salinity groups, however, muscle tissue OTC levels never achieved drug concentrations above 0.35 μg OTC/g.

The estimated plasma AUC values measured between 552 - 1128 h post OTC administration for both the IM and PO groups showed that in the higher salinity treatments the AUC values were greater than in the freshwater treatment. Following IM administration, the difference in the plasma AUC parameter was significantly different

($p<0.05$) between all three treatments. In addition for the IM group, there was a non-significant numeric trend, for the MRT and T ½ to be longer in the 15 ppt and 32 ppt fish than for summer flounder held at 0 ppt.

In the muscle tissue (Table 4-2), the AUC following IM administration was significantly different ($p<0.05$) for all three salinity levels. The 15 ppt group had the highest AUC value (619.2 µg•h/ml) followed by the 0 ppt group (504.6 µg•h/ml) and then the 32 ppt (466.9 µg•h/ml). Similar to numeric trend observations in the plasma, the MRT and T ½ were longer in the muscle tissue from the 32 ppt maintained summer flounder (727.6 h and 504.2 h, respectively).

4.5: DISCUSSION

This study indicated that environmental salinity impacted OTC pharmacokinetic parameters following a single IM dose of 50 mg/kg OTC. It is well known that OTC complexation with divalent cations reduces the drugs solubility and bacterial efficacy (Treves-Brown, 2000; Lunestad and Goksøyr, 1990; Berthon *et al*., 1983). There were significant differences in the AUC parameter in both plasma and muscle tissue estimates between the three salinity treatments 552 – 1128 h post OTC IM injection. In the plasma, the AUC estimate was highest for the high salinity treatment and lowest for the freshwater treatment demonstrating that there was higher OTC plasma concentrations 552 h post IM injection in summer flounder held in seawater compared to cohorts in freshwater. This suggested that elimination of OTC may be slower in fish held in brackish or seawater and this was confirmed by the longer MRT and T ½ time of the 32 ppt treatment. The T_{max} and C_{max} also support this observation where C_{max} in the 32 ppt fish was achieved 96 h later than the lower salinity groups. In the muscle tissue pharmacokinetic parameters, the AUC for the high salinity group was lower than the other two groups. A possible explanation of this finding may be that IM drug deposition of OTC behaves differently in different salinities. In addition, the muscle C_{max} value for the 32 ppt was lower than expected, but this may be a function of OTC deposition in the muscle of the seawater maintained flounder, such that the drug bolus does not distribute

as well in comparison to the other groups. Freshwater fish have higher muscle water content (Martínez-Álvarez, 2002) than saltwater fish, which may affect muscle blood flow, drug solubility and drug distribution. The AUC value was lower in the 32 ppt flounder because OTC injected into the muscle of these fish may have reduced solubility because of interaction with cations such as Ca^{2+} and Mg^{2+} that may be found in higher concentrations in the muscle tissue and plasma of saltwater fish.

Traditional methods for determining withdrawal time (WDT) require the sampling of 15-20 fish per sample collection time to overcome high inter-fish variation (Oriani, 1999). Traditionally, the results of tissue depletion studies are used to statistically calculate the upper bound of the 99^{th} percentile tolerance limit with a 95% confidence interval (Oriani, 1999; Riviere, 1999). However, in this summer flounder experiment, because the sample size was limited to six fish per sample time, the confidence limits were extremely wide suggesting low confidence in the estimates. So, an alternative subjective method proposed by The European Agency for the Evaluation of Medicinal Products (EAEMP, 1996) was implemented. In the EAEMP method, a safety time-span of 25% is added to the time when the tissue concentration drops below the MTL. In this study, following a single IM injection of 50 mg/kg OTC, the 0 ppt summer flounder had an EAEMP-estimated withdrawal time of 34 d, the 15 ppt fish had a 37 d WDT and the 32 ppt fish had a 29 day WDT (Table 4-3).

These results are subjective and have not been derived using approved FDA methods, but the times derived from the EAEMP alternative method suggests that the 21 day WDT approved for channel catfish and salmonids is not appropriate for summer flounder following IM OTC treatment. Although there is an increase in the estimated WDT from the 0 ppt fish to the 15 ppt, the 32 ppt maintained fish have a shorter alternative WDT. When OTC was deposited in the muscle of these marine fish, the solubility of OTC may be negatively affected by cations present in higher concentrations in the muscle and plasma of saltwater acclimated fish and therefore drug distribution is altered. Figure 4-3 illustrates the OTC deposition within the muscle tissue 8 h post-IM injection.

Interestingly, oral dosing of a single OTC treatment at 50 mg/kg did not elicit plasma or muscle OTC concentrations above 0.35 μg/ml. It is speculated that absorption from the gastrointestinal tract was reduced because of poor systemic bioavailability of OTC in teleosts following oral treatment and was potentially further reduced because of a decreased lipid solubility of OTC associate with chelation by cations present in the tank water. Therefore, withdrawal times could not be determined from this data and extrapolation was not appropriate in this case. Further studies are required to accurately determine the WDT of OTC in summer flounder and to determine the effect of environmental salinity on OTC tissue retention times in the summer flounder.

4.6: ACKNOWLEDGMENTS

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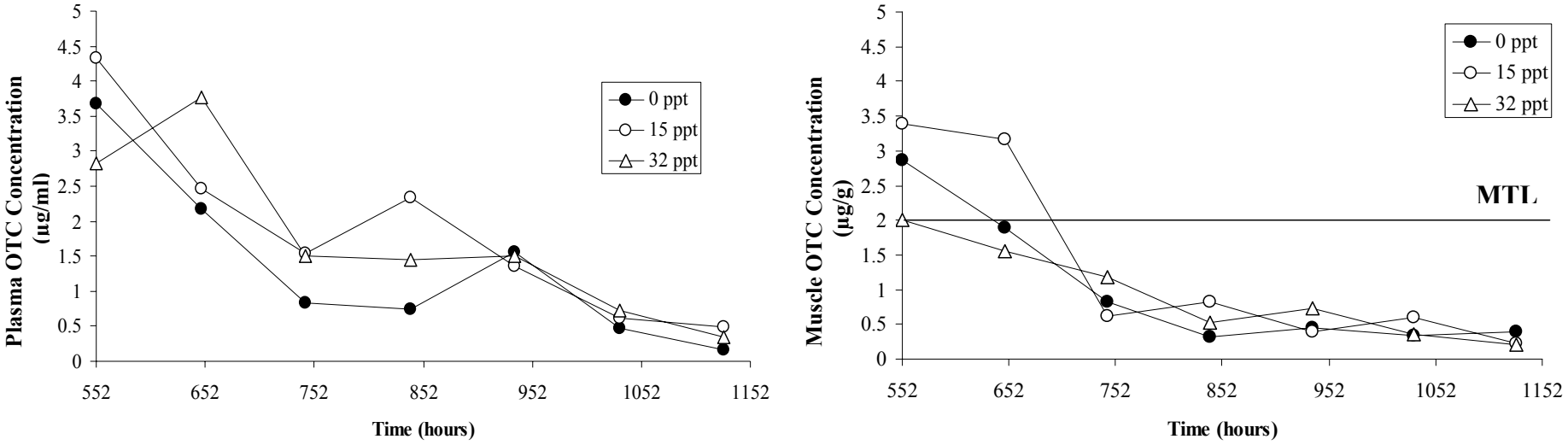

Figure 4-1. Plasma and muscle tissue concentration-time profiles of oxytetracycline (OTC, 50 mg/kg) starting 552 hours post-administration following intramuscularly (IM) injection to summer flounder, *Paralichthys dentatus*, maintained at three salinity levels: freshwater (0 ppt), brackish water (15 ppt), and seawater (32 ppt). Each point represents the mean of six fish. Black line on muscle figure represents the FDA maximum tolerance limit (MTL) of 2 ppm OTC in fish muscle tissues.

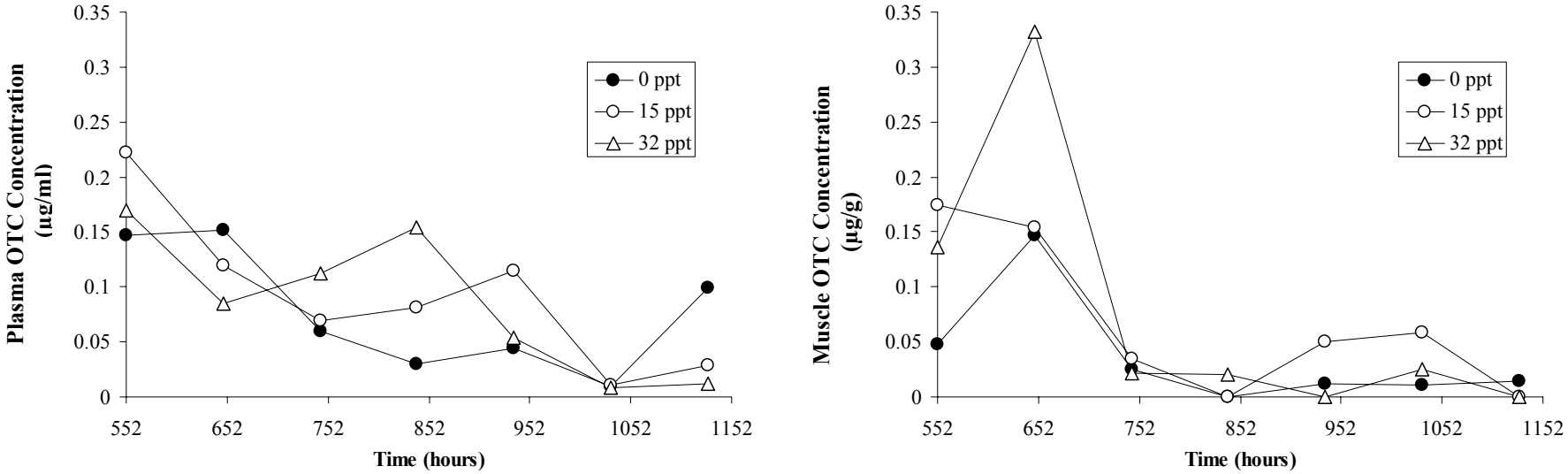

Figure 4-2. Plasma and muscle tissue concentration-time profiles of oxytetracycline (OTC, 50 mg/kg) starting 552 hours post-administration following per os (PO) gavage to summer flounder, *Paralichthys dentatus*, maintained at three salinity levels: freshwater (0 ppt), brackish water (15 ppt), and seawater (32 ppt). Each point represents the mean of six fish.

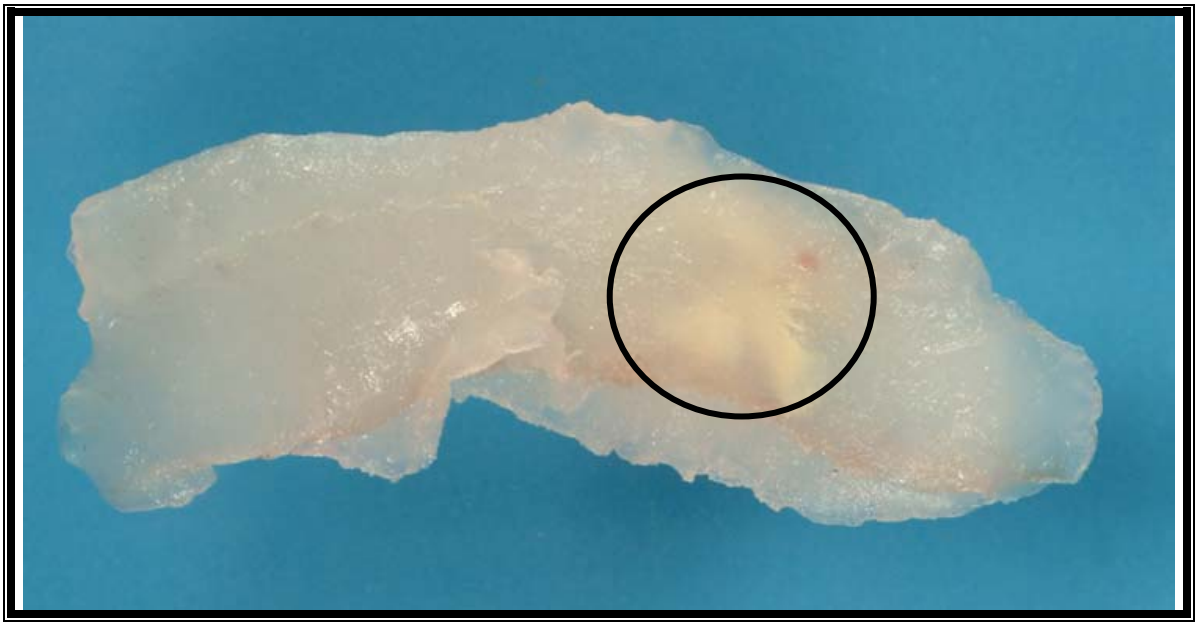

Figure 4-3. Portion of muscle from summer flounder, *Paralichthys dentatus*, 8 hours post oxytetracycline (50 mg/kg) intramuscular (IM) injection. Circle denotes area of drug deposition typical at site of IM injection.

Table 4-1. Pharmacokinetic parameters[1] of oxytetracycline (OTC, 50 mg/kg) in plasma 552-1128 hours following intramuscular (IM) or per os (PO) administration to summer flounder, *Paralichthys dentatus*, maintained at three different salinity levels (0 ppt, 15 ppt and 32 ppt).

Route of OTC Administration	Salinity (ppt)	Plasma					
		AUC (µg•h/ml)	MRT (h)	T ½ (h)	T_{max} (h)	C_{max} (µg/ml)	Fish-to-Fish Variation
IM	0	665.2[a2] [553.3, 811.7]	739.7 [711.5,767.8]	512.6 [493.1, 532.1]	552	3.7	0.5
	15	810.1 [b] [613.0, 1092.1]	740.5 [703.9, 778.5]	513.1 [487.8, 539.5]	552	4.3	0.5
	32	826.3 [c] [667.7,1033.6]	754.9 [727.5, 783.3]	523.2 [504.1, 542.8]	648	3.8	0.4
PO	0	32.5 [22.9, 44.9]	739.1 [693.6, 789.8]	512.2 [480.6, 547.3]	648	0.2	0.2
	15	39.5 [28.4, 53.5]	741.7 [698.9, 789.1]	514.0 [484.4, 546.9]	552	0.2	0.3
	32	32.8 [21.9, 46.7]	738.0 [689.0, 790.5]	511.4 [477.5, 547.8]	552	0.2	0.3

[1]Pharmacokinetic parameter abbreviations; AUC: area under the plasma concentration-time curve after a single dose of OTC at 50 mg/kg; MRT: mean residence time of OTC in summer flounder following a single dose of OTC (50 mg/kg); T ½: total body elimination half-life; T_{max}: time of the maximum drug concentration within the body; C_{max}: maximum drug concentration within the body; fish-fish variation: intraclass correlation coefficient.

[2]Values within a column and within a route of OTC administration (IM, PO) denoted by a different letter are significantly different at $p<0.05$, using a *z* multiple comparison test statistic with a Bonferroni correction. Bracketed values represent the 95% confidence limits obtained by the bootstrap procedure.

Table 4-2. Pharmacokinetic parameters[1] of oxytetracycline (OTC, 50 mg/kg) in muscle fillets 552-1128 hours following intramuscular (IM) or per os (PO) administration to summer flounder, *Paralichthys dentatus*, maintained at three different salinity levels (0 ppt, 15 ppt and 32 ppt).

Route of OTC Administration	Salinity (ppt)	Muscle Tissue					
		AUC (µg•h/ml)	MRT (h)	T ½ (h)	T_{max} (h)	C_{max} (µg/ml)	Fish-to-Fish Variation
IM	0	504.6[a2] [387.6 667.2	711.1 [686.3, 738.5]	492.8 [475.6, 511.8]	552	2.9	0.3
	15	619.2[b] [427.7, 871.4]	691.1 [660.8, 723.5]	479.0 [458.0, 501.4]	552	3.4	0.4
	32	466.9[c] [370.8, 591.4]	727.6 [702.3, 754.9]	504.2 [486.7, 523.1]	552	2.0	0.3
PO	0	17.1 [10.2, 25.5]	691.6 [637.4, 750.6]	479.3 [441.7, 520.2]	648	0.2	0.3
	15	23.7 [13.3, 36.6]	731.3 [656.9, 809.3]	506.8 [455.2, 560.9]	552	0.2	0.1
	32	31.9 [21.0, 44.4]	662.6 [623.1, 707.6]	459.2 [431.8, 490.4]	648	0.3	0.3

[1]Pharmacokinetic parameter abbreviations; AUC: area under the plasma concentration-time curve after a single dose of OTC at 50mg/kg; MRT: mean residence time of OTC in summer flounder following a single dose of OTC (50 mg/kg); T ½: total body elimination half-life; T_{max}: time of the maximum drug concentration within the body; C_{max}: maximum drug concentration within the body; fish-fish variation: intraclass correlation coefficient.

[2]Values within a column and within a route of OTC administration (IM, PO) denoted by a different letter are significantly different at $p<0.05$, using a *z* multiple comparison test statistic with a Bonferroni correction. Bracketed values represent the 95% confidence limits obtained by the bootstrap procedure.

Table 4-3. Withdrawal time estimates in summer flounder, *Paralichthys dentatus*, muscle tissue following intramuscular injection of oxytetracycline (50 mg/kg). Estimates were derived using the European Agency for the Evaluation of Medicinal Products (EAEMP) alternative method.

Salinity (ppt)	Time (days) when muscle concentration is 2 ppm	25% of previous column (days)[1]	EAEMP Estimated Withdrawal Time
0	27.00	6.75	33.75
15	29.25	7.31	36.56
32	23.00	5.75	28.75

[1] Calculated by multiplying days from "estimated time when muscle concentration is 2 ppm" times 0.25.

CHAPTER 5

PHARMACOKINETIC PARAMETERS OF OXYTETRACYCLINE IN HEALTHY AND DISEASED SUMMER FLOUNDER, *PARALICHTHYS DENTATUS*

Prepared for submission to the *Journal of Fish Diseases*

5.1: ABSTRACT

Pharmacokinetic parameters of oxytetracycline (OTC) following a single intramuscular (IM) or per os (PO) dose of 50 mg/kg OTC were compared between healthy and diseased summer flounder, *Paralichthys dentatus*, maintained at 20°C at 28 ppt salinity. Non-compartmental model analysis was used to estimate pharmacokinetic parameters for both routes of OTC exposure. Following IM OTC administration, healthy fish had significantlu higher area under the curve (AUC) (4700.6 µg•h/ml) and C_{max} (23.4 µg OTC/ml) values than diseased cohorts (2576.2 µg•h/ml and 20.2 µg OTC/ml, respectively). Although not significantly different, the mean resident time (MRT) (293.7 h) and total body elimination half-life (T ½) (203.5 h) in diseased fish were longer than in healthy fish (253.2 h and 175.4 h, respectively). No significant differences were detected in the parameters following PO OTC administration, but healthy fish achieved a higher mean maximum plasma OTC concentration (1.0 µg OTC/ml) than diseased fish (0.7 µg OTC/ml). Fish-to-fish variation was greater in diseased animals than in healthy regardless of route of drug administration. Additionally, histopathology confirmed that the clinically diseased fish had moderate to severe granulomatous inflammation of renal tissue, whereas clinically healthy fish had only minimal pathologic changes. Thus, observed lesions in diseased fish had an impact on OTC absorption and elimination.

Keywords: *Paralichthys*, flounder, oxytetracycline, pharmacokinetics, disease

5.2: INTRODUCTION

In intensive fish production, bacterial disease outbreaks are common sequelae to poor animal husbandry. When fish and aquaculture systems are inadequately managed or equipment failure occurs, fish become stressed leading to compromised immune function and potentially resulting in disease and death. Many bacterial pathogens are ubiquitous in the aquatic environment and do not cause disease until the fish host becomes susceptible. Examples of ubiquitous bacteria in the marine environment include Gram-negative *Vibrio* spp. and Gram-positive *Mycobacterium* spp. These groups of bacterial pathogens are known to cause high morbidity and mortality in cultured marine flatfish species (Hughes *et al*., 2002a; Hughes *et al*., 2002b; Mulcahy, 2002; Grisez *et al*., 1996; Olsson *et al*., 1996; Kusuda and Salati, 1993; Watkins *et al.*, 1981).

Antibiotics are often used in fish production to treat fish with bacterial infections. However, in the United States, there are only two available FDA-approved antibiotics for use against specific bacterial diseases in channel catfish (*Ictalurus punctatus*) and salmonids. Use of these antibiotics in other fish species or for other bacterial diseases requires an extra-label veterinary prescription. Oxytetracycline (OTC) is one of the two approved antimicrobials for use in catfish and salmonids in the United States. Oxytetracycline is a broad spectrum bacteriostatic antibiotic that is effective against many Gram-negative bacteria such as *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp., which are common marine fish pathogens. There is concern, in both human and veterinary medicine, about the increasing incidence of bacterial resistance to antibiotics. To date, there is no published information on OTC pharmacokinetics in flounder. Therefore, treatment regimens are based on data from other fish species which may not be appropriate for flounder.

Although there have been numerous studies conducted investigating the pharmacokinetics of OTC in other freshwater and marine fish species (Bowden, 2002; Rigos *et al*., 2002; Haug and Hals, 2000; Namdari *et al*., 1999; Abedini *et al*., 1998; Doi *et al*., 1998; Namdari *et al*., 1998; Uno *et al*., 1997; Elema *et al*., 1996; Malvisi *et al*.,

1996; Namdari *et al*., 1996; Reja *et al*., 1996; Black *et al*., 1991; Björklund and Bylund, 1991; Rogstad *et al*., 1991; Björklund and Bylund, 1990; Bruno, 1989; Grondel *et al*., 1989; Grondel *et al*., 1987; Norlander *et al*., 1987; Salte and Liestøl, 1983; Strasdine and McBride, 1979; Fribourgh *et al*., 1969a; Fribourgh *et al*., 1969b), these studies have almost all used healthy fish subjects. However, in practice, diseased animals are treated with antibiotics with the assumption that pharmacokinetic properties between healthy and sick individuals will be similar (Riviere and Sundlof, 2001; Uno, 1996). This assumption may be erroneous especially if a disease process (i.e. bacterial infection) changes drug half-life by either increasing the volume of distribution (i.e. altered blood flow to tissues) or decreasing drug clearance (i.e. kidney disease) (Riviere and Sundlof, 2001). In fish, only two reports have been published in which the pharmacokinetics of OTC were compared between healthy and diseased subjects (Bruno, 1989; Uno, 1996).

The present study investigated the pharmacokinetic parameters of OTC following per os (PO) and intramuscular (IM) administration in clinically healthy and clinically diseased summer flounder, *Paralichthys dentatus*. The diseased animals exhibited clinical signs and were diagnosed with a mixed bacterial infection following non-intentional exposure of the whole population to water temperatures above 25^{o}C.

5.3: MATERIALS AND METHODS

.3.1: FISH HUSBANDRY

A population of summer flounder (177 g ± 34, 24 cm ± 1.5) (GreatBay Aquafarms, Portsmouth, NH) was maintained in four 2650 liter rectangular fiberglass tanks. Water quality indices (dissolved oxygen (DO), ammonia, nitrites, nitrates, salinity, temperature, and pH) were monitored daily. Water quality indices were regarded as optimal when parameters were within these limits: temperature: 19-21^{o}C (YSI 85 model 85/10, Aquatic Eco-Systems, Apopka, FL); salinity: 28 (±1) ppt (YSI 85 model 85/10, Aquatic Eco-Systems, Apopka, FL); pH: 7.8-8.2 (Sension1 pH meter, HACH, Loveland, CO); ammonia: <0.2 mg/L; nitrite: <10 mg/L; nitrate: <50 mg/L; and, DO: 6.0-8.0 mg/L (YSI

85 model 85/10, Aquatic Eco-Systems, Apopka, FL). Ammonia, nitrites and nitrates were measured with a spectrophotometer (DR2010 spectrometer, HACH, Loveland, CO). Salinity adjustments were made by adding synthetic sea salt (Forty Fathoms Crystal Sea Salt, Marine Enterprises International, Inc., Baltimore, MD). The pH of the systems was maintained by adding sodium bicarbonate when the pH dropped below the desired range. Fish were fed a commercial floating diet formulated specifically for summer flounder (Shur-Gain, Nova Scotia, Canada; protein: 50%, fat: 15%; 6.5 mm pellets). Fish were fasted 24 h prior to sampling and 24 h following OTC administration.

A subpopulation of summer flounder was unintentionally exposed to elevated water temperatures (>25^{o}C) for several days following a facility air conditioning failure while fish were being routinely maintained. Ambient room temperature was hot enough to elevate tank water temperatures from the desired range of 19-21^{o}C. Flounder began to show clinical signs of disease approximately 10-14 d following the water temperature spike. Approximately 60% of the flounder population (ca. 400 individuals) exhibited external signs of disease. The most common clinical signs of disease were emaciation, raised ulcerated skin lesions, oral masses, exophthalmia, head swelling, coelomic swelling, and hemorrhage at the base of the fins and increased mortality rates (Fig. 5-1; Hughes *et al*., 2002a). Flounder were divided into groups of healthy fish and those fish showing clinical signs of disease. Based on the presentation of clinical signs and the diagnostic results from mortalities and healthy cohorts, it was determined that the population had a mixed bacterial infection. Although approximately 40% of the population did not have gross signs of disease, it was assumed that all animals in the facility had been equally exposed to the pathogenic bacteria following the elevated water temperature stress. Fish that did not have external clinical signs of disease were designated as healthy, however, all fish had been exposed to the pathogenic bacteria and were possibly sub-clinically infected. Based on the prevalence and severity of external clinical signs, it was assumed that animals that did not show outward signs of infection had resisted the infection, overcome the infection or that changes associated with the disease were minimal and not grossly visible. In addition, selection of healthy versus

diseased fish was based primarily on the clinical signs that may be more commonly associated with one bacterial disease rather than a mixed bacterial infection.

For the healthy fish versus the diseased fish experiment, tank design consisted of two pairs of 568 liter fiberglass rectangular tanks. Each pair of tanks shared a common sump, pump and biological filter. Water in each tank was continuously passed through activated carbon to bind free OTC in the water column. One tank from each pair was arbitrarily designated to house the clinically healthy fish and the other tank to house the diseased fish. One paired system was then arbitrarily designated as IM (intramuscular) and the other designated as PO (per os) based on route of drug administration. The different routes of drug exposure were conducted concurrently. Each system housed 84 flounder (i.e. 42 clinically healthy flounder in one tank and 42 diseased flounder in the other tank). Water quality parameters and feeding regimes throughout the experiment were the same as previously described.

Fish were anesthetized with buffered MS-222 (100 mg/L, tricaine methanesulfonate, Sigma Chemical Co., St. Louis, MO) for both routes of OTC administration, blood collection and tagging. All experimental fish were individually tagged with a t-bar anchor tag (Floy Tag, Inc., Seattle, WA) in the dorsal musculature on the visual side of the fish. Although anesthesia may alter certain blood parameters and other physiological and biochemical functions, there is no evidence that it interferes with OTC pharmacokinetic properties (Horsberg, 1994).

5.3.2: ROUTES OF DRUG ADMINISTRATION

Oxytetracycline (Bio-Mycin 200; 200 mg/ml oxytetracycline; Boehringer Ingelheim Vetmedica, Inc., St. Joseph, MO) was administered as a single dose of 50 mg OTC/kg of body weight to anesthetized fish (Piper *et al*., 1982). Intramuscular injections were given using a 100 µl Hamilton syringe with a 25-gauge needle in the dorsal musculature on the eyed side of the fish with slight pressure being applied to the site of injection for 10 sec post-injection to minimize reflux from injection site. Oral drug exposure was administered via stomach gavage using a curved stainless steel 20-gauge 3” gavage tube

(Popper and Sons, Inc, New Hyde Park, NY) and 100 µl Hamilton syringe. Gavage placement in the stomach was confirmed manually.

5.3.3: SAMPLING TECHNIQUES

Blood collection sampling times following IM and PO OTC exposure were divided into 3 groups consisting of 7 bleeding times each:

1 15, 30 min, 1, 2, 6, 12, 24 h

2 48, 72, 120, 168, 216, 264, 312 h

3 360, 432, 504, 576, 648, 720, 792 h

These bleeding times were selected based on results from previous experiments of dosing OTC in summer flounder via the IM and PO routes. The three groups were designed such that each fish was bled at least once during different phases of drug movement through the body (i.e. absorption, distribution and elimination). Accordingly, six fish were bled at every specified time interval with each fish being bled three times over the entire time of the trial, once in each time group (1, 2, and 3). The bleeding schedule of individual fish was pre-determined so that at least 48 h elapsed before any individual fish was resampled.

5.3.4: BLOOD COLLECTION AND PLASMA STORAGE

Approximately 0.3 ml of blood was withdrawn from the caudal tail vessels of sampled fish at each bleeding time. No more than 1.0 ml of blood volume was taken from any one fish during the entire course of the experiment. The blood sample was placed immediately into plasma separator tubes containing lithium heparin (Microtainer, Becton Dickinson, Fisher Scientific, Pittsburgh, PA), mixed by inversion several times and kept on ice until centrifugation. Samples were centrifuged (Centra GP8R, International Equipment Company, Needham Heights, MA) at 3000 x g for 10 min at 12^{o}C. Plasma was stored at –80^{o}C until analysis.

5.3.5: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY PROCEDURE

After thawing, plasma samples were filtered with a MPS micropartition device (Millipore, Bedford, MA) equipped with a disposable YMT ultrafiltration membrane disc (3000 molecular weight cutoff, Amicon, Inc., Beverly, MA) and centrifuged at 14,000 x g for 40 min at 22^{o}C (Beckman Microfuge R centrifuge, Beckman Instruments, Inc., Palo Alto, CA). A sample of the ultrafiltrate (20 µl) was then injected directly onto a high-performance-liquid-chromatography (HPLC) column. A Hypersil 3 micron C-18, 150 mm x 4.6 mm ID (Phenomenex, Torrance, CA) analytical reversed phase column was used. The HPLC system consisted of a Beckman Coulter System Gold chromatography unit equipped with a manual sample injector (Beckman Coulter Model 7725i) and a 126 solvent delivery module (Beckman Coulter Instruments, Inc., Fullerton, CA). HPLC effluents were analyzed with a Beckman 166 variable wavelength detector set at 355 nm. The mobile phase (pH 3.3) was a 70:30 mixture of an aqueous mobile phase (0.01M oxalic acid and 0.03M octane sulfonic acid sodium salt) and an organic mobile phase (acetonitrile) (Meinertz *et al*., 1998). This mixture was kept in a sealed container to prevent evaporation of the acetonitrile and was maintained on a magnetic stirrer to prevent separation of the phases. The flow-rate was 1.5 ml/min, with each sample run taking approximately 10 min. Data was processed by the Beckman Coulter Analytical Series System Gold data acquisition software (Karat 32, Beckman Coulter Instruments, Inc., Fullerton, CA). Known standards of OTC ranging from 0.05 - 50.0 µg/ml were prepared in order to establish a regression line upon which the unknown OTC concentrations were calculated. The calibration regression curve was rejected if less than 0.995. The detection limit was determined by running OTC spiked flounder plasma to find the minimum detectable concentration. The detection limit of OTC in flounder plasma for this HPLC system was 0.05 µg OTC/ml (0.05 ppm OTC). To verify consistent HPLC operation, a known 2.5 µg/ml standard solution of OTC was periodically injected into the HPLC unit for evaluation. Recovery of OTC was determined by comparing spiked filtered OTC flounder plasma samples and unfiltered spiked samples. Recovery of OTC from filtered flounder plasma was 95% (±3.4). Plasma OTC concentrations that were determined by HPLC anlaysis to be lower than the

detection limit were assigned a value of zero, because values lower than the limit of detection could not be accurately differentiated from zero.

5.3.6: BACTERIOLOGY

At the conclusion of the study (792 h), all fish in each tank system (IM and PO) were humanely euthanized by an overdose of MS-222 followed by cervical separation. The posterior kidney of all fish was cultured using Mini-Tip Culturettes (Becton Dickinson Microbiology Systems, Cockeysville, MD). In addition, external lesions such as oral masses or head masses were also cultured. Bacterial specimens were swabbed onto tryptone soy agar (TSA) plates supplemented with 2% NaCl and Lowenstein-Jensen slants. Cultures were incubated at 25^{o}C. Swab samples of bacterial specimens from external lesions were smeared on glass microscope slides and allowed to air dry. Slides were then heat fixed and stained with either Gram stain (BACTO Gram stain kit, DIFCO Laboratories, Detroit, MI) or Kinyouns (cold) acid fast stain (TB stain kit, Becton Dickinson, Sparks, MD).

Upon identification of bacterial isolates, minimum inhibitory concentrations (MIC) were determined using the broth dilution method (Ferraro *et al*., 2000). Briefly, PBS was inoculated with the bacteria to a 0.5 McFarland using a Klett meter (Klett-Summerson photoelectric colorimeter, Klett Manufacturing Co., Inc., NY, NY). Serial dilutions were then prepared using Mueller-Hinton broth (MHB). Subsamples (10 µl) from the dilutions were plated on 2% NaCl TSA to confirm bacterial growth. Oxytetracycline concentrations were prepared in MHB and ranged from 0 µg OTC /ml to 200 µg OTC/ml. *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) were used as quality control bacteria for the assay.

5.3.7: HISTOLOGY

Samples of tissues (external lesions, posterior kidney, liver, spleen and heart) from clinically affected flounder were collected and preserved in 10% neutral buffered formalin for routine histological examination. Similar organ samples were also collected from healthy individuals for comparison. Tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (Luna, 1968). In addition, special stains of Brown-Hopps and Ziehl-Neelson were applied to selected tissue sections.

5.3.8: DATA ANALYSIS

The raw plasma OTC concentration data were log-transformed to stabilize variances. Log-means were calculated and a MIXED effects model with fish as a random variable was used to estimate between fish variance across all times (SAS Systems, version 8.2, SAS Institute Inc., Cary, NC). Log-transformed data was exponentiated to corresponding geometric means in the original units. Using the geometric means, a non-compartmental model was used to estimate the area under the concentration-time curve (AUC) and the area under the moment curve (AUMC) of OTC in summer flounder plasma using the trapezoidal method for both routes of OTC administration. Additional pharmacokinetic parameters were estimated using the derived AUC and AUMC:

$$MRT = AUMC/AUC$$

$$T\,½ = 0.693 \bullet MRT$$

Where MRT is the mean residence time of OTC and T ½ is the total body elimination half-life.

To include all variation not associated with time, to give conservative estimates, a second partitioning of variation was performed with fish variation left in the model. A bootstrap randomization procedure using MULTTEST was used to estimate the confidence intervals of the pharmacokinetic parameters (Cole, 1999; Riviere, 1999). A multiple

comparison test statistic, *z*, was used with a Bonferroni correction procedure to detect significant differences between the healthy and diseased groups of fish.

5.4: RESULTS

5.4.1: PHARMACOKINETIC PARAMETERS

The plasma concentration-time profiles of OTC following IM and PO administration in the clinically healthy fish and clinically diseased fish are shown in Fig. 5-2 and Fig. 5-3. In Figures 5-2 and 5-3 each plotted point represents the mean of six fish, such that the mean is not an actual HPLC reading, thus, explaining why values may go below the limit of detection. The pharmacokinetic parameter estimates are summarized in Table 5-1. The plasma concentration-time profiles graphically demonstrate the similarity of OTC profiles in healthy and diseased summer flounder. The IM graph showed that the healthy individuals had plasma concentrations slightly higher than diseased cohorts for the first 360 h. This result for the IM group was also reflected in Table 5-1 where the AUC for the healthy group was significantly higher ($p<0.05$) than the AUC of diseased fish (4700.6 μg•h/ml and 2576.2 μg•h/ml, respectively). Although not significantly different, the MRT was also longer in the diseased group compared to the healthy group (293.7 h and 253.2 h, respectively). Furthermore, the C_{max} for the healthy fish (23.4 μg/ml) was higher than for the diseased animals (20.2 μg/ml). The total body elimination half-life was longer in the fish with clinical signs of disease compared to the fish without signs of disease (203.5 h and 175.4 h, respectively). Fish-to-fish variation was also greater in the diseased fish than in the healthy fish group (0.24 and 0.16, respectively).

The plasma concentration-time graph of the PO OTC treated fish revealed that the main difference between the healthy and diseased fish was that C_{max} was higher in the healthy group of fish, although this finding was not statistically significant. As seen in Table 5-1 with C_{max} values for the healthy group at 1.0 μg/ml and 0. 7 μg/ml for the diseased group. As in the IM groups, fish-to-fish variation was higher in the diseased group compared to the healthy group (0.41 and 0.29, respectively).

5.4.2: BACTERIOLOGY

The predominant bacterial isolates from cultures of healthy and diseased summer flounder are listed in Table 5-2. Approximately 80% of the entire experimental subpopulation, IM and PO treatment groups, were positive for bacterial growth cultured from the posterior kidney. Approximately 95% of the diseased fish from both the IM and PO diseased group were positive for bacterial growth, whereas approximately 65% of the healthy fish from both the IM and PO group were positive for bacterial growth despite the absence of gross external lesions. *Vibrio anguillarum* and *V. alginolyticus* were the most common bacterial isolates cultured from the posterior kidney and *Edwardsiella sp.* was less commonly isolated. Impression smears from external lesions such as oral masses, opercular masses, head and eye swellings stained with Kinyouns (cold) acid-fast stain revealed a high density of extracellular acid-fast organisms in these lesions (Fig. 5-4). Cultures from these sites as well as some cultures from the posterior kidney of diseased fish were positive for raised yellow colonies on Lowenstein-Jensen slants. From previous studies, it was determined that similar bacterial isolates from lesions such as these had greater than 80% homology to *Mycobacterium marinum* as determined by gas chromatography and PCR (Heckert and Baya, personal communication).
Minimum inhibitory concentration (MIC) values are listed in Table 5-2. *Vibrio anguillarum* and *V. alginolyticus* had MIC values of 0.31 µg/ml OTC and *Edwardsiella* sp. had a MIC value of 0.625 µg/ml OTC. The MIC for *Mycobacterium* spp. could not be determined because this bacterial isolate would not grow in the MHB. The MIC values for the quality control organisms, *Staphylococcus aureus* and *Escherichia coli,* were within the reported ranges (0.12 -1.0 ppm tetracycline and 0.5-2.0 ppm tetracycline, respectively) for these bacterial species tested against tetracycline drug compounds.

5.4.3: HISTOLOGY

Histological preparations from tissues of healthy and diseased fish revealed that fish without external signs of infection had no or minimal tissue changes. Minor tissue changes included renal tubule dilation and nephrocalcinosis. These minor tissue changes

were present in fish examined from both groups of healthy and diseased fish. Fish that demonstrated clinical signs of disease had lesions that most commonly involved severe effacing granulomatous inflammation consisting of epithelioid macrophages that disrupted, and in some cases obliterated, normal tissue architecture (Fig. 5-5). Lesions were related but not limited to areas where external lesions were noted. For example, in fish with exophthalmia, granulomatous inflammation was noted in the area of the affected eye, whereas changes in other tissues such as the kidney or liver were only sometimes present. Special stains, Brown-Hopps and Ziehl-Neelson, of affected tissue revealed that acid-fast positive bacteria were present in higher numbers in lesions of the eye and head region. However, the acid-fast positive bacterial density was much lower in tissues such as the heart, liver or kidney.

5.5: DISCUSSION

5.5.1: PHARMACOKINETIC PARAMETERS

Summer flounder in this study were divided into groups of healthy and diseased fish based on the presence of clinical signs. Although multiple bacterial organisms were cultured from these flounder, the predominant outward clinical signs were related to a *Mycobacterium* sp. infection. This is the first report of plasma pharmacokinetic parameters comparing healthy and diseased marine flatfish. To date, there have only been two published studies in fish where the pharmacokinetics of OTC was compared between healthy and diseased subjects. Bruno (1989) observed that Atlantic salmon (*Salmo salar*) infected with *Aeromonas* sp. had higher OTC levels than healthy cohorts 8 weeks post-injection. Uno (1996) also demonstrated significant differences in OTC absorption after oral administration between healthy ayu (*Plecoglossus altivelis*) and *Vibrio* infected ayu. Infected fish had lower maximum serum and tissue concentrations than healthy fish. In addition, the bioavailability of OTC was reduced by 60% in the diseased ayu and the AUC was approximately half that of healthy ayu for muscle, liver and kidney tissues (Uno, 1996).

The results of this experiment conducted in summer flounder demonstrated that although the pharmacokinetic curves of the plasma concentration-time OTC profiles following both IM and PO drug administration appeared very similar, there are certain pharmacokinetic parameters that were different between healthy and diseased flounder. For IM dosed fish, the AUC was significantly higher for the healthy individuals compared to diseased fish. This may indicate that drug concentration was greater in healthy fish because blood flow and tissue function were not compromised by disease. When fish are sick, blood flow dynamics and tissue function may be altered such that drug absorption and distribution are significantly changed compared to healthy fish. Uno (1996) speculated that lower serum AUC values in *Vibrio* infected ayu compared to healthy ayu were related to reduced absorptive capacity of the intestine because of damage related to the bacterium or its toxin. There was a numeric trenn in the summer flounder, for the MRT and total body elimination half-life to be longer in the diseased fish than in the healthy fish. These prolonged times suggested that drug retention and elimination were delayed as a result of the physiological status of the diseased fish. These parameters indicated that OTC may persist longer in sick individuals than healthy fish. Similar to the findings of Uno (1996), diseased summer flounder had lower maximum plasma concentrations than did healthy flounder. Fish-to-fish variation was also greater in diseased fish than healthy individuals, which indicates that drug behavior may be more variable in a population of sick fish than in a population of healthy fish.

Pharmacokinetic parameters from orally dosed OTC flounder revealed that diseased fish had higher AUC values than the healthy fish and that diseased fish had shorter MRT and total body elimination half-life parameters, although these findings were not statistically significant. These findings are in contrast to what was observed in the IM treatment groups. However, in the orally treated fish, as with the IM dosed group, the C_{max} in comparison to the healthy fish indicated that the diseased fish achieved lower plasma drug concentrations. Similar to the trend in the IM treated fish, fish-to-fish variation was greater in the diseased fish group following PO exposure. These findings suggested that following orally administered OTC, a physiologically compromised fish will not achieve plasma OTC levels as high as healthy cohorts. These findings supported the conclusion

that special considerations are needed when establishing a clinical dosage regimen for clinically diseased fish regardless of drug administration route.

5.5.2: BACTERIOLOGY AND HISTOLOGY

Vibriosis is one of the most common disease syndromes in marine aquaculture (Park *et al*., 1994). Numerous species of *Vibrio* are commonly found as part of the natural microflora in marine and estuarine environments. There are at least nine reported *Vibrio* species that are potential aquatic animal pathogens, with *Vibrio anguillarum* being the most widespread of these bacterial species (Park *et al*., 1994). *Vibrio* bacteria are Gram-negative, polar-flagellated, curved rods that are presumably transmitted in a saltwater fish population via fish to fish contact. The exact pathogenesis of these *Vibrio* organisms in fish is not yet known, but it is assumed the pathogenesis is similar to other Gram-negative bacteria (i.e. endotoxin production). External lesions of infection include skin lesions and fin hemorrhage and necrosis. Internal lesions include intestinal inflammation, hemorrhage of organs, hypertrophy of the spleen and kidney and necrosis of these organs (Bullock, 1999; Sano and Fukuda, 1987; Umbreit and Tripp, 1975; Levin *et al*., 1972). In the winter flounder (*Pseudopleuronectes americanus*), Levin *et al*. (1972) described the microscopic lesions of vibriosis in the kidney as focal interstitial and tubular necrosis. Bacteria may be isolated from coelomic fluid, liver, kidney, and intestine (Umbreit and Tripp, 1975).

Mycobacteriosis is another common bacterial disease of saltwater fish caused by *Mycobacterium* spp. (Chinabut, 1998; Wolf and Smith, 1999; Austin and Austin, 1993). These are Gram-positive, acid-fast positive bacilli. *Mycobacterium marinum, M. fortuitum, and M. chelonae* are historically the most common *Mycobacterium* isolates reported from fishes. Mycobacteriosis is a chronic progressive disease that may or may not present with gross external signs of disease. Common external clinical signs in most fish may include lethargy, anorexia, emaciation and skin ulcerations. Internal findings may include granulomatous inflammation and granulomas in target tissues such as kidney, liver, spleen or heart. Typically, the teleost response to *Mycobacterium* sp.

infections is the formation of multiple discrete granulomas with numerous intracellular bacteria (Wolf and Smith, 1999; Bruno *et al*., 1998; Colorni *et al*., 1998). However, in summer flounder, a generalized granulomatous inflammatory response is typically observed without the formation of discrete granulomas (Hughes *et al*., 2002a; Hughes *et al*., 2002b). Grossly, mycobacteriosis in summer flounder may present as large masses on the mandible and head, operculum and in the retro-bulbar space. Internally, these areas have a significant infiltration of epithelioid macrophages that obliterate normal tissue architecture and within these areas of inflammation there is a high prevalence of extracellular acid-fast organisms. In the liver, spleen and kidney tissue of the summer flounder, similar effacing granulomatous inflammation is observed but with fewer organisms present. This tissue response to *Mycobacterium* spp. by the summer flounder is unusual for fish.

These two groups of bacteria, *Vibrio* spp. and *Mycobacterium* spp., may cause severe lesions in infected fish. A common target tissue in the teleost for these pathogens is the teleost posterior kidney with infections leading to significantly altered renal function and blood flow to this organ. Since OTC is eliminated primarily unchanged through the urine, there is potential that when the renal tissue and blood flow is altered by disease the pharmacokinetic parameters of OTC will be impacted.

In this study, summer flounder infected with a mixed bacterial infection, predominantly *Vibrio* spp. and *Mycobacterium* spp., had altered OTC pharmacokinetic parameters when compared to healthy cohorts. In diseased fish, OTC persisted longer than in healthy fish following IM administration, indicating that withdrawal times may be longer when treating diseased fish via IM injections. Since OTC is primarily excreted unchanged through the urine, any lesions that affect the kidney could impact OTC elimination. In addition, following both IM and PO routes of OTC administration the fish-to-fish variability was higher in the diseased subpopulation of flounder, suggesting that when treating a sick population of fish the pharmacokinetics (absorption, distribution and elimination) of OTC will be more variable between individuals. Therefore, these factors

must be considered when treating diseased summer flounder and recommending withholding times.

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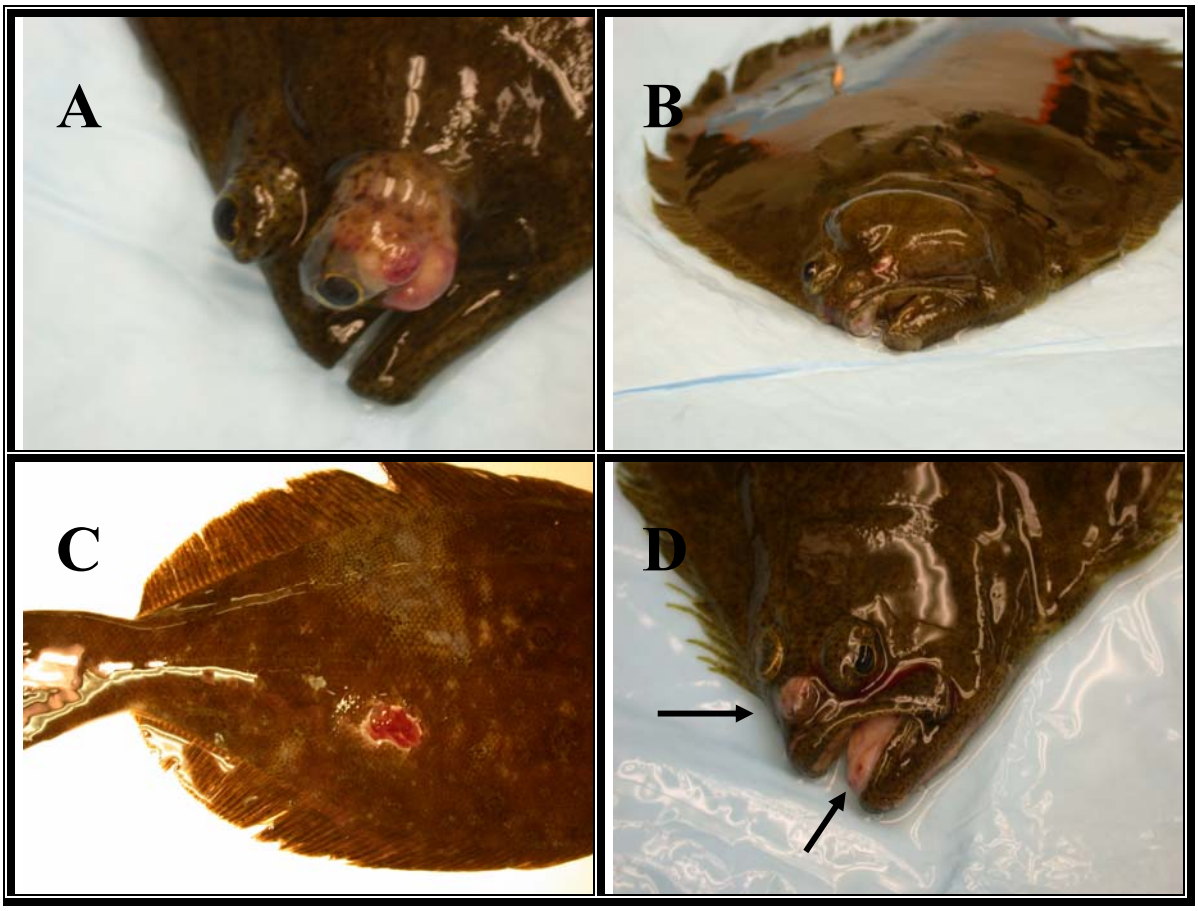

Figure 5-1. Clinical presentations of diseased summer flounder, *Paralichthys dentatus*, following a period of elevated water temperatures (> 25^{o}C). A mixed population of *Vibrio* spp., *Edwardsiella* sp. and *Mycobacterium* spp. was isolated from the fish population. A. Summer flounder with unilateral exophthalmia due to proliferative tissue in the retrobulbar region. B. Summer flounder with head swelling. C. Ulcerative skin lesion on summer flounder, and D. Masses on mandible and rostrum (arrows) of summer flounder.

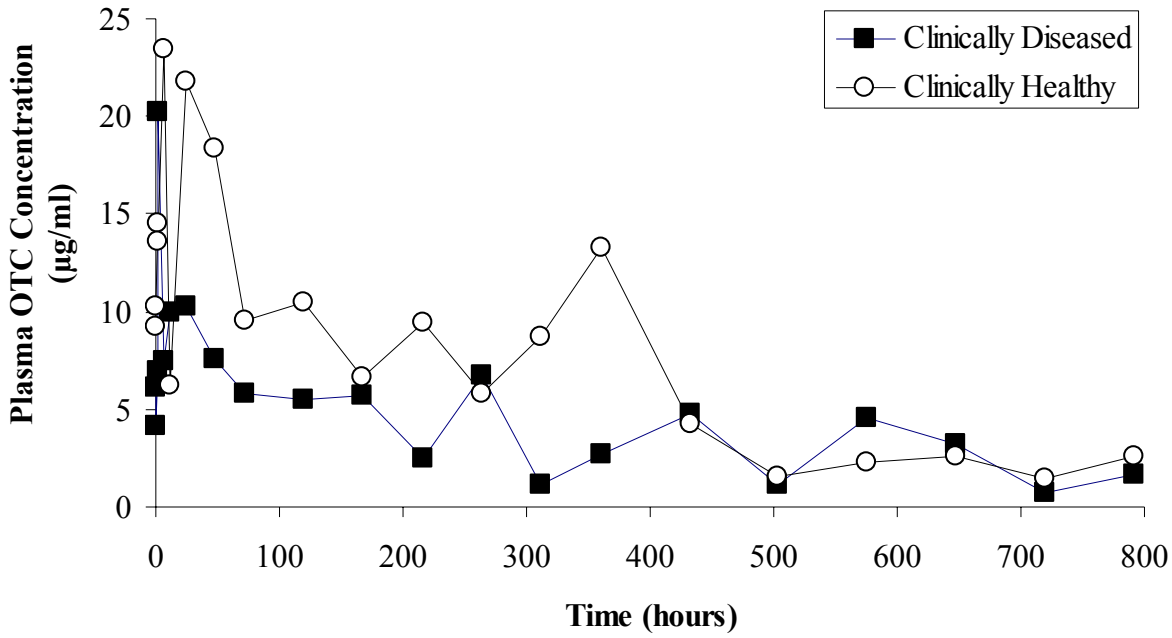

Figure 5-2. Plasma concentration-time profile of oxytetracycline (50 mg/kg) following intramuscular (IM) administration to healthy and diseased summer flounder, *Paralichthys dentatus*. Each point represents the mean of 6 fish.

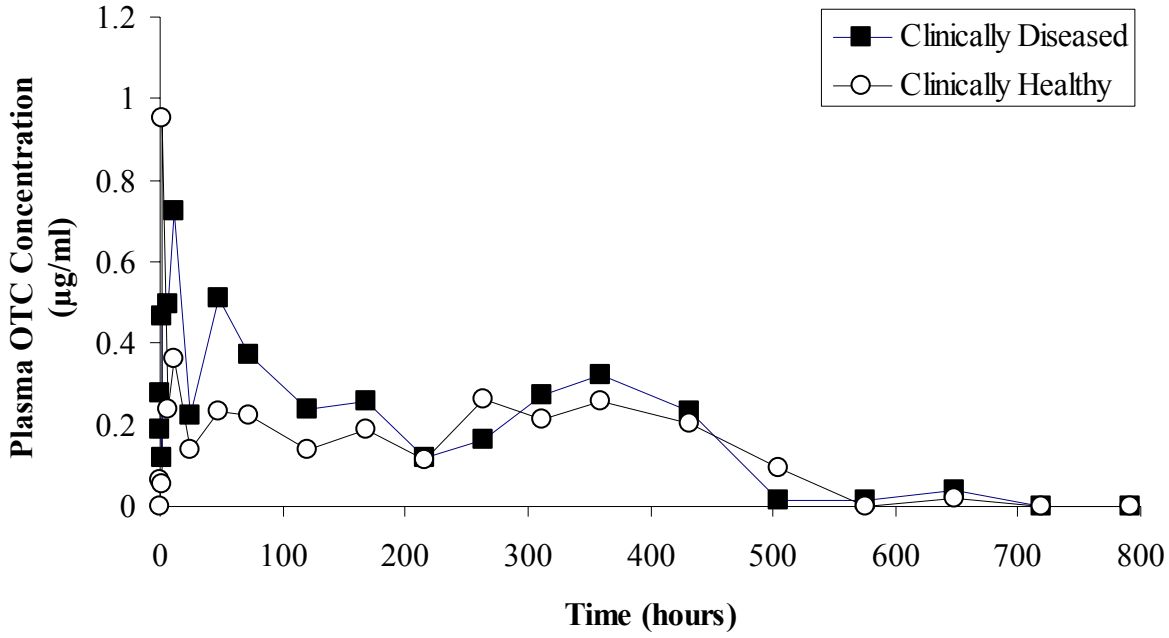

Figure 5-3. Plasma concentration-time profile of oxytetracycline (50 mg/kg) following per os (PO) administration to healthy and diseased summer flounder, *Paralichthys dentatus*. Each point represents the mean of 6 fish.

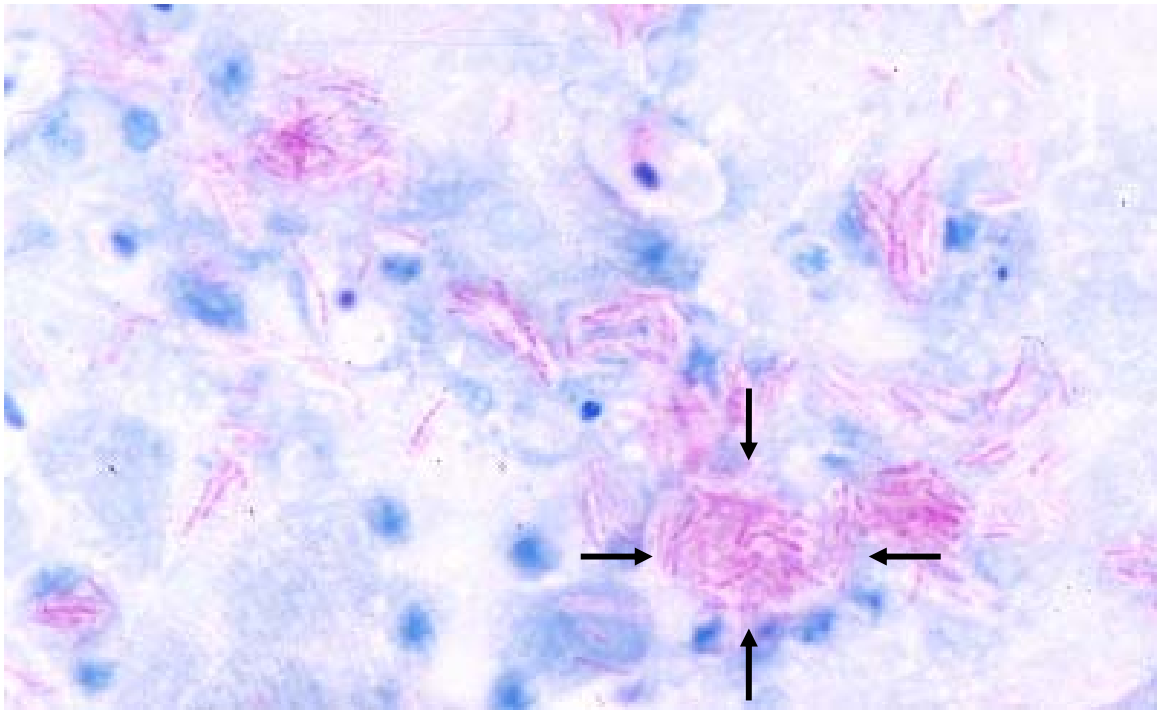

Figure 5-4. Impression smear of oral mass from diseased summer flounder, *Paralichthys dentatus,* stained with Kinyouns (cold) acid-fast stain. Arrows indicate a cluster of acid-fast positive *Mycobacterium* spp. organisms.

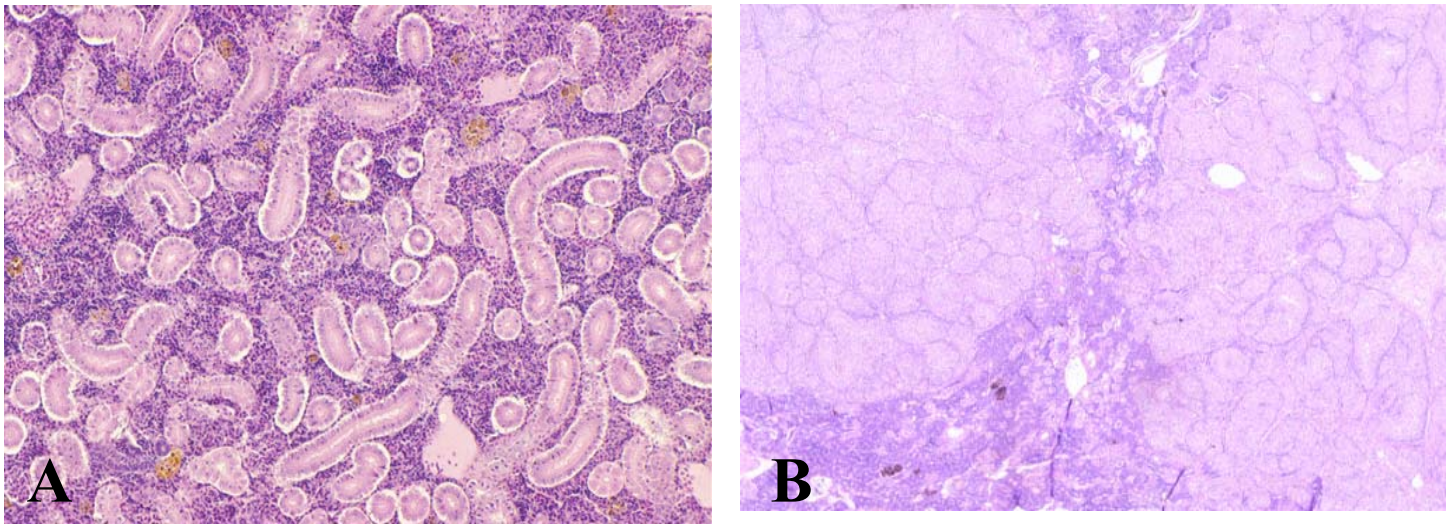

Figure 5-5. Posterior kidney from summer flounder, *Paralichthys dentatus* (H & E stain). A. Normal posterior kidney from healthy fish demonstrating typical tubule density and parenchymal architecture (200 x). B. Posterior kidney from diseased fish with infiltrating granulomatous inflammation that obliterates normal renal architecture. Renal tubule density is significantly diminished (66 x).

Table 5-1. Pharmacokinetic parameters[1] of oxytetracycline (50 mg/kg) following intramuscular (IM) or per os (PO) administration in healthy versus clinically diseased summer flounder, *Paralichthys dentatus*, maintained at 28 ppt and 20°C.

Route of OTC Administration	Clinical Signs of Disease (Yes/No)	AUC (µg•h/ml)	MRT (h)	T ½ (h)	T_{max} (h)	C_{max} (µg/ml)	Fish-to-Fish Variation
IM	No	4700.6[a2] [4262.0, 5337.0][3]	253.2 [236.2, 269.9]	175.4 [163.7, 187.0]	6	23.4	0.16
	Yes	2576.2[b] [2305.7, 2998.5]	293.7 [267.9, 321.9]	203.5 [185.6, 223.1]	2	20.2	0.24
PO	No	83.5 [71.3, 103.9]	253.5 [223.0, 294.9]	175.7 [154.5, 204.4]	2	1.0	0.29
	Yes	110 [89.7, 148.5]	229.6 [192.7, 284.3]	159.1 [133.5, 197.0]	12	0.7	0.41

[1]Pharmacokinetic parameter abbreviations; AUC: area under the plasma concentration-time curve after a single dose of OTC at 50 mg/kg; MRT: mean residence time of OTC in summer flounder following a single dose of OTC (50 mg/kg); T ½: total body elimination half-life; Tmax: time of the maximum drug concentration within the body; Cmax: maximum drug concentration within the body; fish-fish variation: intraclass correlation coefficient.

[2]Values with a column and within a route of OTC administration (IM, PO) denoted by a different letter are significantly different at p=0.025, using a *z* multiple comparison test statistic with a Bonferroni correction.

[3] Values in brackets are the 95% confidence limits as determined by the bootstrap procedure.

Table 5-2. Minimum inhibitory concentrations (MIC) of oxytetracycline (OTC) for bacterial isolates cultured from the posterior kidney of healthy and diseased summer flounder, *Paralichthys dentatus*.

Bacterial Isolate	**MIC (µg/ml OTC)**
Vibrio anguillarum	0.31
Vibrio alginolyticus	0.31
Edwardsiella sp.	0.625
Mycobacterium spp.	ND[1]
Staphylococcus aureus	0.625[2]
Escherichia coli	0.625[3]

[1] MIC value for *Mycobacterium* spp. could not be determined (ND) because bacteria would not grow in appropriate media for MIC determination.
[2]Reported quality control range for ATCC *S. aureus* to tetracyclines: 0.12-1.0 TC µg/ml.
[3]Reported quality control range for ATCC *E. coli* to tetracyclines: 0.5-2.0 TC µg/ml.

CHAPTER 6

SUMMARY / CONCLUSIONS

Summer flounder, *Paralichthys dentatus*, culture has become increasingly popular in the United States, because of high market prices and consumer demand. In addition, flounder are a marine flatfish species that can tolerate a wide range of salinities, allowing for intensive inland fish culture. Since these fish can thrive in lower salinity environments, as opposed to full-strength seawater, inland flounder producers can rear these animals in diluted artificial seawater, thereby saving production overhead by reducing the cost of salt purchases. As for any production scheme, intensive rearing conditions such as high densities and frequent handling, result in elevated stress and compromised immune function potentially leading to disease and death. Bacterial diseases are one of the leading causes of stock loss in aquaculture, hence antibiotic therapy is a common practice. However, currently in the United States there are only two available FDA-approved antibiotics for use in channel catfish (*Ictalurus punctatus*) and salmonids against specific bacterial diseases. Use of these approved antibiotics at other doses or routes of administration, in other species or for other bacterial diseases requires an extra-label veterinary prescription by a veterinarian or an Investigational New Animal Drug (INAD) permit. The use of antibiotics in foodfish production is restricted to prevent the contamination of seafood products by drug residues. However, to date there is no published data regarding the use of either of the two antibiotics in summer flounder. Without the appropriate pharmacokinetic information, veterinarians must extrapolate treatment regimens and withdrawal times based on drug behavior in other fish species, which is risky.

Oxytetracycline (OTC) is one of two available FDA-approved antibiotics for use in foodfish as formulated in medicated feed. Oxytetracycline was chosen for these studies because it is excreted primarily unchanged through the urine and the absorption, distribution and elimination of this drug may be influenced by environmental and physiological conditions. Oxytetracycline readily chelates with di- and trivalent cations that reduce the drugs lipid solubility and antibacterial efficacy. In seawater, Ca^{2+} and Mg^{2+} cations are present in high concentrations. Hence, in saltwater fish, OTC absorption and behavior may be different compared to when administered to fish in freshwater. In addition, when fish are acclimated to freshwater, plasma and urine

osmolality are reduced compared to fish at seawater, urine flow is increased and urine character is also changed. Fish acclimated to seawater, have higher plasma and urine osmolalities as well as reduced urine flow compared to freshwater fish. In this study, OTC pharmacokinetics was investigated in summer flounder maintained at standard culture parameters (28 ppt and 20^{o}C), as well as, three different salinity levels (0 ppt, 15 ppt and 32 ppt) and different health status (healthy and diseased).

In the first experiment, OTC was administered to summer flounder maintained at 28 ppt and 20^{o}C via four routes of OTC administration: intravascular (IV), intraperitoneal (IP), intramuscular (IM) and per os (PO). Although no statistical comparisons were made between the four routes of drug administration, the IM and PO routes had some disadvantages compared to the IV and IP routes. Intramuscular injection resulted in long total body elimination half-life (T ½) and high fish-to-fish variation. Possible explanations for these findings include prolong drug deposition at the site of injection. When drug is deposited IM it is assumed that the drug will slowly diffuse into the general circulation by diffusion across vessels perfusing the muscle. However, if OTC binds with cations (either by exposure to environmental seawater or muscle electrolyte concentration) diffusion may be altered and highly variable. Oxytetracycline appeared to reside longer in muscle tissue prolonging mean resident time (MRT) and T ½. Furthermore, IM injections of OTC may cause localized tissue responses such as edema and necrosis, which may lead to altered vascularization and tissue fibrosis that may ultimately affect drug behavior at this site. Per os OTC administration resulted in low plasma concentrations and very low systemic bioavailability. These findings are also related to OTC chelation with cations. Fish in seawater actively ingest environmental seawater to maintain hydration, therefore OTC particles in the gastrointestinal tract are essentially in modified seawater allowing for 1:1 chelation of OTC to divalent cations. Thus, OTC absorption is reduced resulting in low plasma concentrations and systemic bioavailability.

Oxytetracycline administered IV resulted in similar AUC and V_d values to those of chinook salmon. Other pharmacokinetic parameters such as MRT and T ½ were longer

than what has been reported in the literature for other marine fish species, however, this may be a result of drug dose, drug formulation or pharmacokinetic model analysis. Additionally, the other routes of OTC exposure resulted in varying parameter values in comparison to other fish species. Therefore, the hypothesis that summer flounder would absorb and eliminate OTC similar to other marine fish is not wholly supported.

In the second experiment, summer flounder were maintained at three environmental salinity levels 0 ppt (freshwater), 15 ppt (brackish water) and 32 ppt (seawater). Oxytetracycline was administered to acclimated summer flounder via IM and PO routes. The PO route was selected based on FDA restrictions where currently the only approved route of medicating foodfish is via medicated feed, whereas IM administration was selected for situations where valuable broodstock may require immediate drug therapy, provided these animals are not intended for human consumption. Results from this study revealed that salinity minimally impacted OTC pharmacokinetic parameters following IM and PO administration. Fish maintained to 15 ppt and 32 ppt seawater had significantly larger AUC values and prolonged times to time of maximum plasma drug concentration (T_{max}) compared to fish held at 0 ppt following IM injections. This finding may be attributed to OTC residing in muscle tissue longer in the seawater-acclimated fish, and associated with low muscle moisture content or higher muscle ionic concentrations, resulting in biased MRT and T ½ parameters. In addition, similar to the results of the first experiment, IM injections of OTC resulted in high fish-to-fish variability. Following PO OTC administration, OTC absorption was limited especially in the 32 ppt maintained flounder. This indicated that OTC is poorly absorbed from the gastrointestinal tract of flounder in general but also demonstrated that increased environmental salinity further decreases the absorption of OTC. This is likely related to OTC chelation with cations present in seawater. In this experiment, the physiological adaptations summer flounder make in response to environmental salinity were also investigated. Plasma and urine osmolality were typically significantly increased in the saltwater maintained fish compared to freshwater fish. In addition, urine characteristics, such as urine color, urine specific gravity and urine OTC concentration were all increased in the 32 ppt fish compared to fish at 0 ppt. Conversely, urine volume and urine flow

rates were decreased in the high salinity fish compared to the fish at 0 ppt. Gill chloride cell size and density as well as enzyme function indicated trends that suggested the summer flounder are strongly euryhaline and are well adapted to make physiological adjustments for survival in environmental salinities ranging from 0 – 32 ppt.

The hypothesis that the physiological alterations associated with environmental salinity would alter OTC absorption and elimination was not supported. Although some significant differences were detected among the AUC values following IM injections and numeric trends were observed for the other parameters, the physiological alterations merely confirm that summer flounder adapt well to different saline environments but do not significantly affect OTC pharmacokinetics. The differences and trends observed in the pharmacokinetics appeared to be more affected by the interaction of OTC and cations present in the seawater.

The third experiment evaluated muscle retention of OTC in summer flounder administered OTC via IM and PO routes while maintained at three salinity levels (0, 15 and 32 ppt). Data for this study was collected 552 – 1128 h post OTC administration. Although the results were preliminary findings, plasma and muscle OTC concentrations revealed that environmental salinity may have affected muscle residue levels of OTC. Similar to previous experiments, there was a significant increase across salinity levels in plasma AUC values following IM OTC administration. However, data from muscle tissue reveals a smaller AUC parameter in the 32 ppt maintained fish. This may indicate that tissue distribution is affected by salinity, which may be explained by reduced OTC solubility by OTC chelation with cations *in vivo* (i.e. muscle and plasma). The muscle maximum OTC tissue concentration (C_{max}) was lower in the 32 ppt fish suggesting that tissue distribution following absorption may be hindered by plasma and muscle changes associated with salinity of the tank water. Using an alternative method to determine withdrawal times (WDT), the WDT for all three salinity groups were greater than the 21-day recommended withholding time for salmon and catfish following OTC therapy. However, the hypothesis that summer flounder would have shorter WDT in freshwater compared to fish maintained in brackish or seawater was not supported. Per os

administration of a single 50 mg/kg OTC treatment in the summer flounder failed to produce plasma or muscle tissue OTC concentrations above the 2 ppm OTC tolerance limit set by the FDA. Thus, the WDT determined in this study for summer flounder were based on IM OTC treatment, which is currently not an acceptable route for antibiotic therapy in foodfish in the United States.

The fourth experiment compared OTC pharmacokinetic parameters in healthy and diseased summer flounder. Following a water temperature spike near lethal temperature levels for the summer flounder, about half of the population exhibited clinical signs of disease. Clinically diseased fish were emaciated and had head and eye masses, coelomic swellings and skin erosions. Groups of fish were treated with a single dose of 50 mg/kg OTC via IM or PO routes. Results revealed lower C_{max} values in the diseased fish compared to the clinically healthy fish. The AUC value was lower in IM treated diseased fish, whereas the MRT and T ½ were longer compared to parameters estimated for healthy fish. Differences between the groups of healthy and diseases flounder were more difficult to determine following PO administration because plasma OTC concentrations were low. The hypothesis that OTC pharmacokinetics would be different between healthy and diseased summer flounder was supported.

In conclusion, OTC absorption and elimination parameters in the summer flounder were affected by environmental salinity levels. However, the differences in OTC pharmacokinetic parameters between fish maintained at three different salinity levels appears to be related to the interaction of OTC with cations present in greater concentrations in the seawater rather than as a result of physiological adaptations flounder make in response to tank water salinity. Although the physiological changes indicated that flounder adapt well to a wide range of salinities, the overall alterations do not appear to significantly affect OTC absorption. On the other hand, changes in plasma and urine osmolality may affect OTC distribution *in vivo*. Oxytetracycline administered PO was not well absorbed across the gastrointestinal tract of summer flounder maintained at salinities of 0 ppt, 15 ppt or 32 ppt. However, OTC absorption was even less following PO administration to summer flounder held in seawater. For these reasons, OTC is not an

effective choice for treating bacterial diseases in summer flounder using PO administration of 50 mg/kg dose. Further experiments are required to determine if increased dosage and frequency of administration will improve OTC absorption in the summer flounder.

APPENDIX 1

CHAPTER 2 RAW DATA

APPENDIX 2

CHAPTER 3 RAW DATA

APPENDIX 3

CHAPTER 4 RAW DATA

APPENDIX 4

CHAPTER 5 RAW DATA

VITA

Kathleen was born in the metropolitan area of Washington, D.C. and grew up in Melbourne, Australia and Washington, D.C. She graduated (cum laude) from Sweet Briar College, Sweet Briar, VA with a major in English/Creative Writing and a minor in Biology. Following graduation from Sweet Briar College, she pursued post-graduate studies in Organic Chemistry, Biochemistry, Physics and Calculus at Sweet Briar College and The American University, Washington, D.C. She received a Masters degree in Animal Science at the University of Maryland College Park, MD where her thesis investigated the efficacy of phytase on striped bass diets composed primarily of plant protein products. Kathleen was then accepted to the dual degree program (DVM/Ph.D.) at the Virginia-Maryland Regional College of Veterinary Medicine (VMRCVM) where she obtained her degree of veterinary medicine in 1999. She then continued the pursuit of her Ph.D. degree in the VMRCVM Aquatic Medicine Program. Her dissertation research focused on the pharmacokinetic parameters of oxytetracycline in summer flounder maintained at different production salinity levels and health status.