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THE EFFECTS OF LAMPRICIDE 3-TRIFLUOROMETHYL-4-NITROPHENOL TOXICITY ON THE GILLS OF LARVAL SEA LAMPREY AND NON-TARGET RAINBOW TROUT AND LAKE STURGEON

Lisa A. Sorensen
Wilfrid Laurier University, lisa.sorensen16@gmail.com

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by

Lisa A. Sorensen

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Abstract

The pesticide, 3-trifluoromethyl-4-nitrophenol (TFM), is widely used in the Great Lakes to control invasive sea lampreys (*Petromyzon marinus*) populations, but much about its sub-lethal effects remains unknown. A better understanding of its toxicity is needed to improve TFM effectiveness and to protect non-target organisms from its potential adverse effects. The objectives of this thesis were to test the following two hypotheses: (1) impairment of mitochondrial ATP production by TFM interferes with ATP-dependent ion-uptake by fish, leading to altered electrolyte balance, and (2) perturbations of gill function by TFM are exacerbated in soft, ion poor water (SW; 40 mg $CaCO_3$ L^{-1}). Accordingly, larval lamprey, juvenile rainbow trout (*Oncorhynchus mykiss*), and juvenile sturgeon (*Acipenser fulvescens*) were exposed to TFM concentrations toxic to larval sea lamprey for 12 h. In lamprey, the toxicity of TFM was 10-fold greater in SW compared to hard water (HW; 450 mg $CaCO_3$ L^{-1}). The activity and expression of Na^+/K^+-ATPase (NKA) and H^+-ATPase (V-ATPase) remained unchanged during TFM exposure in both HW and SW. However a 28% decrease in plasma Na^+ in HW and a 10% decrease in plasma Cl^- in SW was found in sea lamprey during recovery from TFM exposure. In trout, TFM led to initial Na^+ losses, which was compensated by increases in NKA and Total ATPase activity by 6 h in both HW and SW. These initial Na^+ loses may be due to an "osmo-respiratory compromise", where TFM-induced increases in O_2 consumption may have lead to increased gill-perfusion and surface area, elevating ion-losses. There was no effect on enzyme activity or expression in sturgeon exposed to TFM in HW, but plasma Na^+ dropped by 20% with a 22% increase in plasma Cl^- following recovery. No significant effects of TFM on gill structure were observed in all three species. The greater sensitivity of lamprey in SW versus HW can partially be explained by greater susceptibility to ionic disturbances, but other factors are also likely involved. It is concluded that the effects

of TFM on gill-mediated ion exchange are minimal and do not translate into substantive disturbances to internal electrolyte balance. However, there is a potential that these effects may persist post-TFM exposure.

Co-Authorship

Work presented in this thesis was completed with the cooperation of Michael Le Clair, Dr. Jonathan M. Wilson, Malcolm Glennie, and Rozalba Luka. Both hard water juvenile rainbow trout and lake sturgeon exposure experiments were conducted jointly at Wilfrid Laurier University with Michael Le Clair as apart of his M.Sc. thesis. Preparation of immunohistochemistry and light microscopy and the imaging of immunohistochemistry slides were conducted by Jonathan M. Wilson at the Centro Interdiscplinar de Investigação Marinha e Ambiental (CIIMAR) in Porto, Portugal. Light microscopy imaging was conducted with the assistance of Malcolm Glennie and Rozalba Luka. I conducted and analysed all other work presented in this thesis.

Acknowledgements

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CHAPTER 1:

Introduction and Literature Review

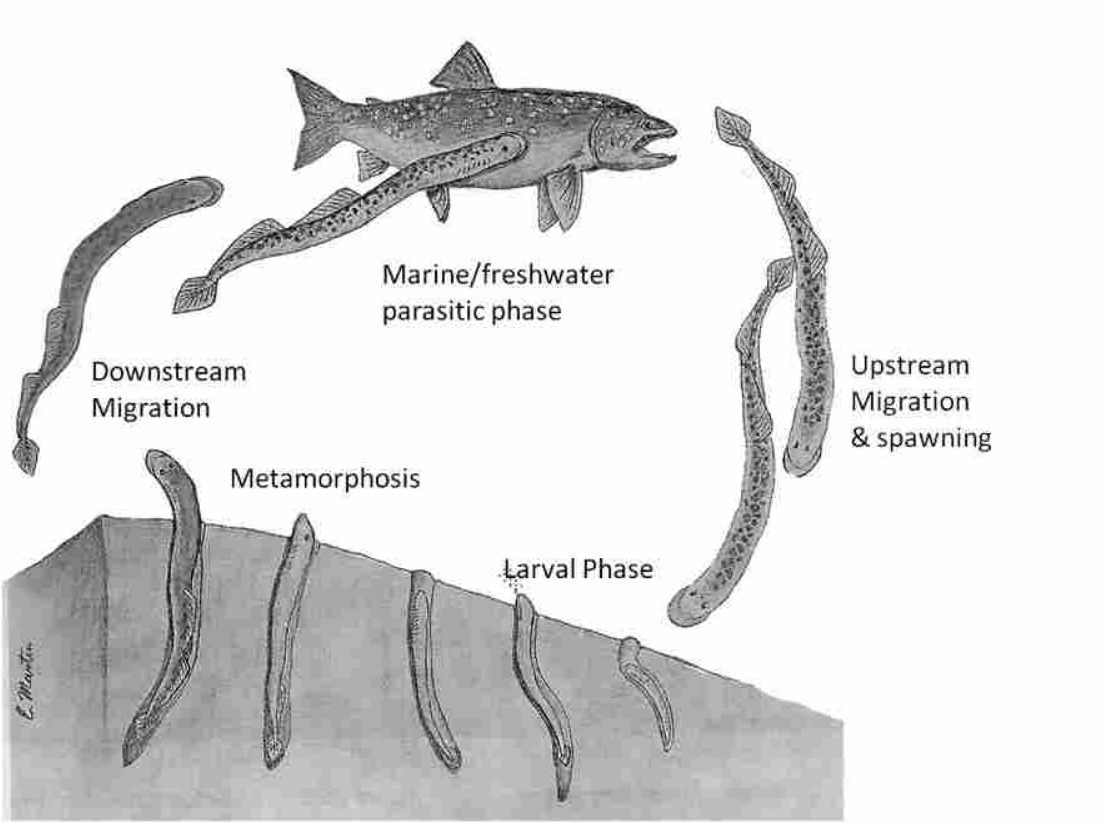
1. The Biology and Life Cycle of the Sea Lamprey *(Petromyzon marinus*)

Sea lampreys (*Petromyzon marinus*) are an invasive species that inhabits the cool waters of North American lakes and rivers (Youson, 1980). These fish have a distinctive naked, slimy, eel-like appearance with seven-gill openings located behind the eyes (Osório and Rétaux, 2008). There are two populations of sea lamprey, an andromous population that migrates to sea during its juvenile parasitic phase, and a landlocked population, which is restricted to the freshwaters of the Great Lakes (Bryan *et al.,* 2005; Eshenroder, 2009).

The complex life cycle of the sea lamprey begins with a 3-7 years filter-feeding larval stage, in which the larvae (also known as ammocoetes) live burrowed within the soft substrates of slow moving streams (Beamish, 1980; Youson, 1980). At the end of this stage, they then enter a non-feeding period of metamorphosis, which is characterized by distinct morphological changes including the development of eyes (Osório and Rétaux, 2008), an oral sucker reinforced by an annular-ring of cartilage, and numerous sharp denticles or teeth (Farmer, 1980; Youson, 1980). The gills are also reorganized, switching from a unidirectionally to a tidally ventilated gill (Rovainen and Schieber, 1975; Youson, 1980; Bartels and Potter, 2004). In late fall or early spring, newly metamorphosed sea lamprey migrate downstream to lakes or marine environments where they feed on the blood of teleost fishes (Lowe *et al.,* 1973; Beamish, 1979; Farmer, 1980), but also elasmobranchs in marine environments (Wilkie *et al.*, 2004; Gallant *et al.,* 2006). Lampreys attach to their prey with their oral disc, and in combination use their rasping tongue, teeth, and through the secretion of a saliva containing anti-coagulates and cytolytic enzymes, they ingest and digest the blood of their hosts (Farmer, 1980). Following a 12-24 month year feeding period, adult lampreys migrate upstream, spawn, and die (Figure 1.1; Beamish, 1980; Bergstedt and Swink, 1995).

Figure 1.1 Sea Lamprey Life Cycle.

The life cycle of the parasitic sea lamprey begins with a prolonged sedentary filter-feeding larval stage where the larvae are known as ammocoetes. During a three-to-four month non-feeding phase, larval lampreys metamorphose into parasitic juveniles, which migrate downstream to freshwater lakes or marine environments where they proceed to feed on host fishes for 16-20 months. Following sexual maturation, lampreys migrate upstream to freshwater rivers and streams where they spawn and die (Wilkie, 2011, with permission).


Marine/freshwater
parasitic phase
Downstream
Migration
Upstream
Migration
& spawning
Metamorphosis
Larval Phase

2. History and Control of Larval Sea Lampreys in the Great Lakes

The origin of sea lampreys in the Great Lakes continues to be debated. It still remains unclear whether sea lampreys are native to Lake Ontario and Lake Champlain (Waldman *et al.,* 2004, 2006; Bryan *et al.,* 2005) or whether lamprey were established in Lake Ontario via the Erie Canal (Smith, 1995) or introduced by other means such as an unintentional introduction or extreme weather events (Eshenroder, 2009). The movement of sea lampreys into the remaining Great Lakes however, is thought to have been facilitated by the widening and deepening of the Welland Canal in 1913 (Eshenroder and Burnham-Curtis, 1999; McDonald and Kolar, 2007).

Lamprey parasitism along with overfishing led to massive declines in economically important fish populations, including the collapse of Lake Trout (*Salvelinus namaycush*), and Whitefish (*Coregonus* spp.) populations; and the extinction of commercially important *Coregonus alpenae*, *C. johannae*, and *C. nigripin*nis in the Great Lakes (Renaud, 1997; Smith and Tibbles, 1980; McDonald and Kolar, 2007). In 1955, concern over the continued population decline in Canadian and American fisheries led to the establishment of the Great Lakes Fishery Commission (GLFC). The GLFC's principle goals were to eradicate sea lamprey populations (McDonald and Kolar, 2002). Initial control efforts used mechanical and electrical barriers to prevent adults from migrating to spawning areas upstream, but this initial effort was abandoned due to its limited success in controlling lamprey populations and mortality in non-target fishes (Smith and Tibbles, 1980). In 1958, the halogenated phenol, 3-trifluoromethyl-4-nitrophenol (TFM), was discovered and demonstrated to selectively target larval sea lamprey (Applegate *et al.,* 1961; Smith and Tibbles, 1980; Christie and Goddard, 2003). Subsequent use of TFM in the 1960's significantly reduced sea lamprey populations, as indicated by greatly reduced catches of spawning sea lampreys, and decreases in the rates of mortality in fishes due to lamprey predation/parasitism (Christie and Goddard, 2003).

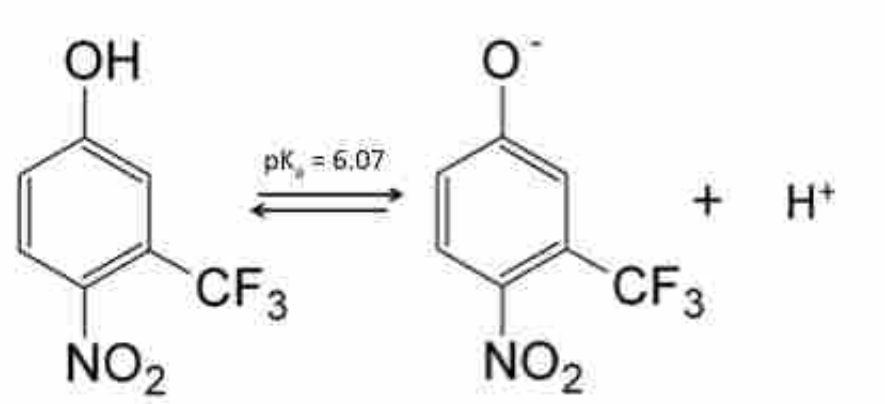
TFM is applied alone or in conjunction with a molluscicide chemical known as niclosamide, which increases the sensitivity of larval sea lampreys to the treatment (Christie and Goddard, 2003; McDonald and Kolar, 2007). Importantly, TFM naturally photo-degrades with a half-life of approximately 3 days in natural stream waters (Carey and Fox, 1981). The toxicity of TFM is reduced with increasing pH, alkalinity, and conductivity of the treatment water. For this reason, doses of TFM are determined using the pH/alkalinity model, which relates the minimal lethal concentration (MLC) of TFM, or the concentration of TFM necessary to kill 99.9% of the population during a 9 h exposure (Bills *et al.,* 2003). TFM is normally applied to treatment streams at 1 to 1.5 times the MLC either as TFM or a TFM/niclosamide mixture (McDonald and Kolar, 2006). Other methods of sea lamprey control include the use of traps (Smith and Tibbles, 1980), and barriers to block upstream migration of adult lampreys (McLaughlin *et al.,* 2007), and experimental techniques including the use of pheromones to attract migrating sea lampreys to traps (Li *et al*. 2007). However, TFM still remains the principal tool used to manage sea lamprey populations in the Great Lakes (McDonald and Kolar, 2007).

3. 3-Trifluoromethyl-4-nitrophenol: Mechanism of Action

The toxicity of TFM varies with pH because the ratio of un-ionized to ionized TFM increase in more acidic water (Figure 1.2). At lower pH, the lipophillic un-ionized free-phenol form of TFM readily crosses the lipid bilayers of the gill epithelium and enters the blood stream (Hunn and Allen, 1974). Work using isolated mitochondria has demonstrated that TFM likely causes metabolic arrest by interfering with ATP production in the body via the uncoupling of oxidative phosphorylation in the mitochondria (Niblett and Ballantyne, 1976; Birceanu *et al.,* 2010), which generates most of the adenosine triphosphate (ATP)

Figure 1.2 Chemical structure of 3-trifluoromethyl-4-nitrophenol (TFM) and its dissociation equilibria.

TFM is a weak acid with a pKa of 6.07. At lower pHs, a greater proportion of TFM is in the lipid soluble un-ionized form, which may lead to greater TFM uptake rates and account for TFM's greater toxicity in more acidic waters (McDonald and Kolar, 2006).


OH
pK$_a$ = 6.07
O$^-$
+
H$^+$
CF$_3$
NO$_2$
CF$_3$
NO$_2$

needed to power biological processes in the body (Figure 3). In contrast to the conversion of glucose to pyruvate via glycolysis, ATP production via the oxidation of glucose to CO_2 and H_2O by oxidative phosphorylation is approximately 18-times more efficient than anaerobic glycolysis, generating a theoretical yield of 36 ATP (Berner *et al.,* 2007; Semenza, 2011). In oxidative phosphorylation, the synthesis of ATP is coupled to the transfer of electrons from NADH or $FADH_2$ produced via glycolysis, the citric acid cycle, and/or the beta-oxidation fatty acids to O_2, resulting in the generation of a proton gradient across the inner-mitochondrial membrane (Berg *et al.,* 2002). Electron transfer is facilitated through protein complexes (Complex I,III, IV), which are located in the inner-mitochondrial membrane (Berg *et al.,* 2002, Nelson and Cox, 2008). As electrons move between these protein complexes, protons are pumped out of the mitochondrial matrix into the inter-membrane space (Nelson and Cox, 2008). The uneven distribution of protons across the inner-mitochondrial membrane generates a pH gradient and transmembrane electrical potential called the proton-motive force (PMF) that favours the flow of protons back into the mitochondrial matrix. As the protons move down the PMF through ATP-synthase, the energy that is released is harnessed to phosphorylate ADP to ATP (Figure 1.3; Berg *et al.,* 2002; Wallace and Starkov, 2000).

Recently, Birceanu *et al.* (2011) demonstrated that TFM disrupts oxidative phosphorylation by increasing the permeability of the inner mitochondrial membrane to protons, ultimately dissipating the PMF needed to drive ATP synthesis (Figure 1.4; Applegate *et al.,* 1966; Birceanu *et al.,* 2011). Interference with ATP production forces larval lamprey to rely on phosphocreatine (PCr) and anaerobic glycolysis to meet their basal energy demands (Birceanu *et al.,* 2009). This results in a reduction of PCr in the brain, but more importantly glycogen, which is the main oxidative fuel used to drive ATP synthesis in the brain (Rovainen *et al.,* 1969; Birceanu *et al.,* 2009). Death ultimately occurs in larval lamprey when fuel stores are depleted and metabolic wastes build up in the body, particularly in the

Figure 1.3 Summary of oxidative phosphorlyation.

The reduction of NADH to NAD^+ by Complex I results in the release of two electrons and the transfer of H^+ from the matrix into the inter-membrane space. Reduction of $FADH_2$ by Complex II also releases electrons, which along with those generated at Complex I, are transferred to ubiquinone (Q). The reduced ubiquinone serves as an electron carrier and passes electrons to Complex III, which are then passed to cytochrome c (Cyt c), a mitochondrial intermembrane electron carrier. Transfer of electrons through Complexes I and III also results in the transfer of 4 H^+ from the mitochondrial matrix into the intermembrane space. The cytochrome c moves electrons to Complex IV, which are then transferred to oxygen, the final electron acceptor, leading to the generation of water. As a result of this process, 2 more protons are simultaneously pumped via Complex IV into the inter-membrane space from the mitochondrial matrix. Proton transfer results in the accumulation of protons within the inter-membrane space and an electrochemical gradient that is directed into the mitochondrial matrix across inner mitochondrial membrane, referred to as the proton motive force (PMF). The inner mitochondrial membrane is normally impermeable to H^+. Accordingly, H^+ movements down the PMF are restricted to the ATP synthase (Complex V), which releases the energy needed to convert ADP to ATP through the process of phosphorylation. Adapted from Nelson and Cox (2008).

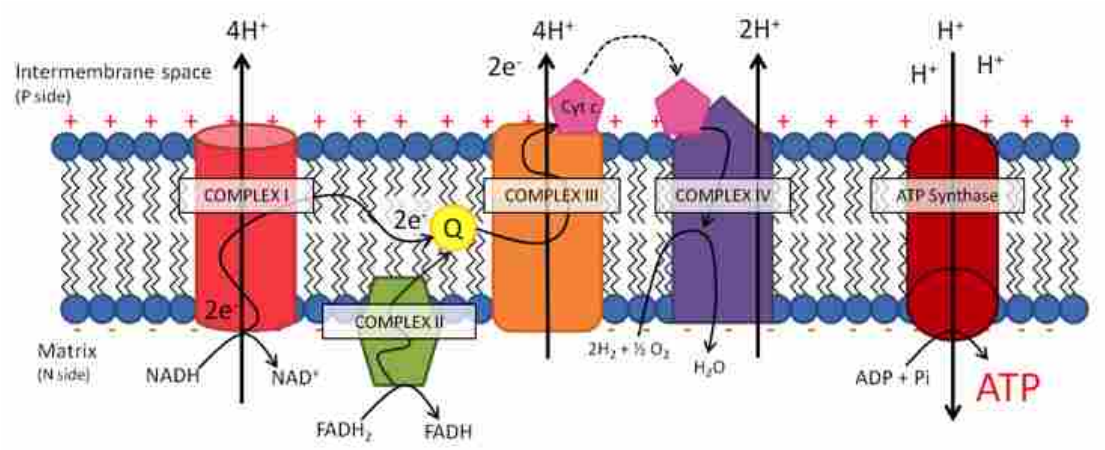
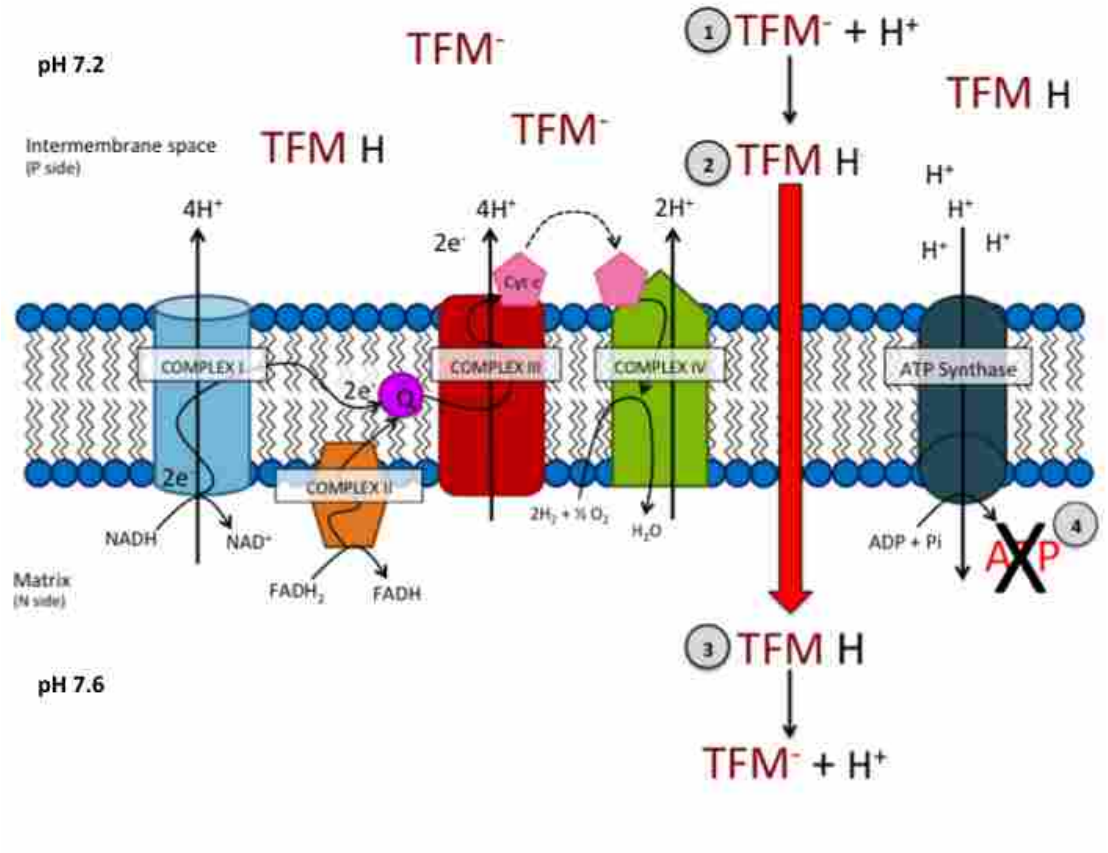

4H+
4H+
2H+
H+
H+
H+
2e-
Intermembrane space
(P side)
Cyt c
COMPLEX I
COMPLEX III
COMPLEX IV
ATP Synthase
2e-
Q
2e-
COMPLEX II
Matrix
(N side)
NADH
NAD+
2H2 + ½ O2
H2O
ADP + Pi
ATP
FADH2
FADH

Figure 1.4 Proposed model of oxidative phosphorylation uncoupling by 3-trifluoromethyl-4-nitrophenol (TFM).

Within the intermembrane space (1) the anionic form of TFM binds with positively charged protons, which are pumped across the inner mitochondrial membrane (IMM) by protein complexes I, III, and IV, producing (2) un-ionized TFM. Un-ionized TFM then diffuses across the IMM into the matrix and dissociates back to H^+ and ionized TFM (TFM^-), releasing protons back into the matrix (3). The redistribution of protons about the IMM dissipates the proton-motive force (PMF) necessary to drive ATP-synthesis, uncoupling oxidative phosphorylation and impairing ATP production via this pathway (4; Niblett and Ballantyne, 1976; Wallace and Starkov, 2000; Birceanu *et al.,* 2011).


pH 7.2
TFM⁻
1 TFM⁻ + H⁺
TFM H
Intermembrane space
(P side)
TFM H
TFM⁻
2 TFM H
H⁺
H⁺
H⁺
H⁺
4H⁺
4H⁺
2e⁻
2H⁺
Cyt c
COMPLEX I
2e⁻
Q
COMPLEX III
COMPLEX IV
ATP Synthase
2e⁻
COMPLEX II
NADH
NAD⁺
FADH₂
FADH
2H₂ + ½ O₂
H₂O
ADP + Pi
4
ATP
Matrix
(N side)
3 TFM H
pH 7.6
TFM⁻ + H⁺

brain (Wilkie *et al.,* 2007; Birceanu *et al.,* 2009; Clifford *et al.,* 2012). Similar reductions in brain PCr and glycogen were also observed in rainbow trout exposed to acutely lethal concentrations of the lampricide (Birceanu *et al.,* 2014).

Fish detoxify and metabolize TFM through glucuronidation, in which a membrane-bound enzyme within the hepatic endoplasmic reticulum, UDP-glucuronyltransferase (UDPGT), conjugates the hydroxyl group of TFM with α-D-glucuronic acid to form β-D-glucuronide (Figure 1.5; Lech and Statham, 1975; Howell *et al.,* 1980; Kane *et al.,* 1993). Once conjugated with UDP-glucuronic acid, TFM can be readily excreted in the bile and eliminated via defecation (Lech and Costrini, 1972) or via the urine (Allen and Hunn, 1977). The greater sensitivity of sea lampreys to TFM compared to non-target fishes is thought to be due to lower expression of UDPGT and correspondingly lower rates of TFM-glucuronidation, ultimately resulting in higher rates of TFM accumulation and higher concentrations of free TFM in the blood and tissues than in other fishes following TFM exposure (Lech and Costrini, 1972; Lech and Staham, 1975; Kane *et al.*, 1993). However, insufficient glucuronyl-transferases activities in some species of fishes can also make them sensitive to TFM.

4. Non-target Fishes and Sensitivity to TFM

Concern over TFM use arose with the identification of a number of TFM sensitive non-target animals including juvenile members of the catfish family Ictaluridae (black bullhead *Ictalurus melas*), channel catfish (*Ictalurus punctatus*), juvenile lake sturgeon (*Acipenser fulvescens*), and mudpuppies (*Necturus maculosus*; Table 1.1; Boogaard *et al.,* 2003). The TFM sensitivity of these animals was characterized by LC50's that were at or below two-times the MLC of larval sea lamprey (Boogaard *et al.,* 2003). There is particular concern about the high sensitivity of lake sturgeon to TFM, whose continual population declines are a

Figure 1.5 Glucuronidation of TFM.

UDP-glucuronyltransferase (UDPGT), a hepatic endoplasmic reticulum membrane-bound protein, facilitates the detoxification and elimination of TFM. UDPGT conjugates the hydroxyl group of TFM with α-D-glucuronic acid (UDPGA) to form β-D-glucuronide (TFM-glucuronide), which is readily excreted through urination and defecation (Lech and Statham, 1975; Howell *et al.,* 1980; Kane *et al.,* 1993).

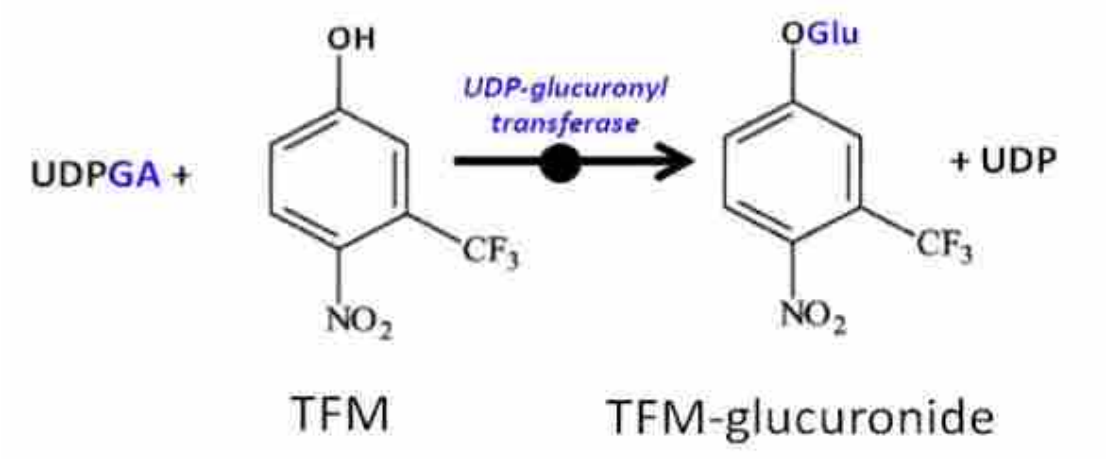

UDPGA +
OH
UDP-glucuronyl transferase
OGlu
+ UDP
CF3
NO2
CF3
NO2
TFM
TFM-glucuronide

Table 1.1 TFM acute toxicity data.

Comparison of acutely lethal concentrations of TFM to kill 50% of the population (LC_{50}) for juvenile Lake Sturgeon, Rainbow Trout, Lake Whitefish, and Channel Catfish and comparison to Sea Lamprey minimal lethal concentration (MLC; $LC_{99.9}$) used for lampricidal treatments under different water quality conditions as summarized by Boogaard *et al.* (2003).

Species	**pH**	**Alkalinity**	**LC_{50} (mg/L)**	**Lower Limit (mg/L)**	**Upper Limit (mg/L)**	**Sea Lamprey MLC (mg/L)**	**Toxic Ratio**
Lake Sturgeon	7.8	102	3.51	3.22	3.82	1.8	1.95
Lake Sturgeon	7.9	102	3.72	3.17	4.37	2.1	1.77
Rainbow Trout	7.9	120	8.40	7.93	8.89	2.3	3.95
Rainbow Trout	7.9	129	10.2	8.75	11.9	2.4	4.25
Lake Whitefish	8.0	120	4.31	3.89	4.78	2.6	1.66
Channel Catfish	8.1	112	4.70	4.23	5.22	2.8	1.68

major concern in many Great Lakes and North American freshwater ecosystems (Species at Risk Public Registry, 2014). Lake sturgeon are large, cartilaginous, potamodromous fish endemic to the Central United States, Great Lakes, and the Hudson Bay drainages of Canada (Peterson *et al.,* 2007). Historically, lake sturgeon were one of the most abundant fishes in the Great Lakes, however populations have significantly declined and/or been extirpated in the lower reaches of of Lake Ontario, Lake Erie, and Lake Michigan (Ferguson and Duckworth, 1997) with populations as low as 1% of their former numbers in Lake Michigan (Hay-Chmielewski and Whelan, 1997). Lake sturgeon population declines were due to over-harvest of the fish in the 19th and 20th centuries and habitat degradation. However, population declines were further exacerbated by the lake sturgeon's low rate of recruitment due to their low fecundity and slow rates of sexual maturation. Females become sexually mature between 18-27 years old and males between 12-15 years old, with females spawning only every 4-9 years and males every 1-3 years (Harkness and Dymond, 1961; Fortin *et al.,* 1993; Peterson *et al.,* 2007).

Lake sturgeon are listed as endangered in Illinois, Indiana, Ohio, and Pennsylvania, threatened in Michigan and New York, and of special concern in Minnesota and Wisconsin (Heinrich *et al.,* 2003; GLFC, 2009). In Ontario, the lake sturgeon is provincially listed as threatened (Species at Risk Public Registry, 2014). Despite efforts that have been made to improve water quality and habitat, lake sturgeon populations remain suppressed (Sutton *et al.,* 2003).

As demonstrated by the Boogaard *et al.* (2003) study, lake sturgeon exhibit sensitivity to TFM near levels used for lampricide treatments when less than 12 centimeters in length (Johnson *et al.,* 1999). For this reason, efforts are made to restrict TFM treatments to periods when sturgeon exceed this minimum size. However, this has proven difficult in many rivers, because lake sturgeon may still be near this threshold size for several months following hatch (Heinrich *et al.,* 2003). For instance, field studies found lake sturgeon ranging in size from

approximately 8–23 cm in length in some streams throughout August. Previously, lampricide treatments were reduced in some rivers, including the Bad, Ontonagon and Sturgeon Rivers in Michigan to protect juvenile lake sturgeon (Heinrich *et al.,* 2003), but this practice was abandoned due to concerns about the effectiveness of using lower concentrations of TFM to treat these rivers for sea lamprey (B. Stevens, Department of Fisheries and Oceans, *personal communication*, September 14, 2014). Nevertheless, there remains a need to better understand how lake sturgeon respond to TFM, and to determine the factors that make them more sensitive to TFM than other non-target fishes.

5. Sub-lethal Effects of TFM and the Gill as a Potential Target of TFM

In addition to sturgeon, much research has been conducted to identify other non-target species that are sensitive to TFM exposure (<2 X MLC). However, the sub-lethal effects of TFM have been largely overlooked (McDonald and Kolar, 2007). Brown trout (*Salmo trutta*) and snails (*Goniobasi liuescen*) exposed to TFM have lowered survivability when exposed to a second stressor (Cairns *et al.,* 1976). Thus, it is possible that the ability of even "TFM-tolerate" fishes to control homeostasis or to resist other stressors could be compromised by TFM treatments. As such, a better understanding of the sub-lethal effects of TFM on fishes could have important implications for the long-term viability of fisheries in the Great Lakes.

A likely target of TFM is the gills, which is thought to be the main route of TFM uptake and likely vulnerable to TFM toxicity (Hunn and Allen, 1974). Evidence for a direct toxicological effect on the gills by TFM has been demonstrated in larval lamprey, in which TFM causes morphological changes in the ionocytes of the gill following TFM exposure (Christie and Battle, 1963; Mallatt, 1987; Mallatt *et al.,* 1994). Specific damage to ionoctyes has lead to the hypothesis that disruption of ion homeostasis may directly contribute to TFM-

mortality in larval lamprey (Mallatt *et al.,* 1994; 1995). Therefore, there remains a need to determine whether or not TFM impairs ion regulation in fish during TFM exposure.

In fresh water, fish are hyperosmotic to their environment, leading to a loss of ions as ions move down their electrochemical gradients (Evans *et al.,* 2005). Fish prevent such ionic imbalances by actively transporting ions from fresh water via ATP-dependent transport proteins located in the gills, and through the re-uptake of ions from the blood as it is filtered through the kidneys (Evans and Claiborne, 2005). Ion balance is therefore reliant on ATP-supply. When ion-balance is not maintained – whether due to decreased energy supply or other physiological disturbances – blood pressure rises and plasma blood volume drops, leading to circulatory failure, and eventually, circulatory collapse (Milligan and Wood, 1982).

The mitochondrial rich cells (MRCs; aka. Ionocytes, chloride cells) and the pavement cells (PVCs) within the epithelium of the gill filaments and lamellae mediated ionoregulation (Figure 1.6; Evans and Claiborne, 2005). Na^+ uptake in lampreys and teleosts is proposed to occur in the MRCs via apical epithelial Na^+ channels (eNac) and vacuolar (V type) H^+-ATPase (V-ATPase) pumps and ouabin-sensitive basolateral located Na^+,K^+-ATPases (NKA; Figure 1.7; Bartels *et al.,* 1998; Marshall, 2002; Bartels and Potter, 2004; Wilkie, 2011). Evidence for a Cl^-/HCO_3^- exchanger suggests that it is present on the apical surface of MCRs and PVCs in some species, where localized acidification within the apical microvilli by H^+-ATPase lowers HCO_3^- activity necessary to drive Cl^- uptake. Cl^- is then transferred into the blood via basolaterally located anion channels, likely a CFTR channel (Figure 1.7; Goss *et al.,* 1998; Wilson *et al.,* 2000; Marshall, 2002; Wilkie, 2011). Ca^{2+} enters the MRCs via an apical Ca^{2+} channel and is passed to the blood via a basolateral Ca^{2+}-ATPase or Na^+, Ca^{2+} exchanger (Figure 1.7; Flik and Verbost, 1993; Flik *et al.* 1996). Two functional subtypes of MRCs have been identified in teleosts: Peanut agglutinin positive (PNA+) and peanut agglutinin negative (PNA-; Goss *et al*., 2001). Goss and colleagues showed that both cell

Figure 1.6 The structural arrangement of the gills.

Gill arrangement in (a,b) larval sea lamprey and (c,d) teleost fish. Positions of gill filaments differ between species and their corresponding arrangement. The arrangement of cartilaginous gill in filter feeding larval lamprey results in the unidirectional movement of water, trapping food particles in the mucous of the pharynx and exchanging oxygen in the gill filament and lamellae before it passes out through the branchiopore (a,b). Images adapted from Mallatt (1979; 1981). Gill arch arrangement in teleost results in the unidirectional movement of water from the buccopharynx through the gill filaments and out (c,d). Adapted from Wilson and Laurent (2002).

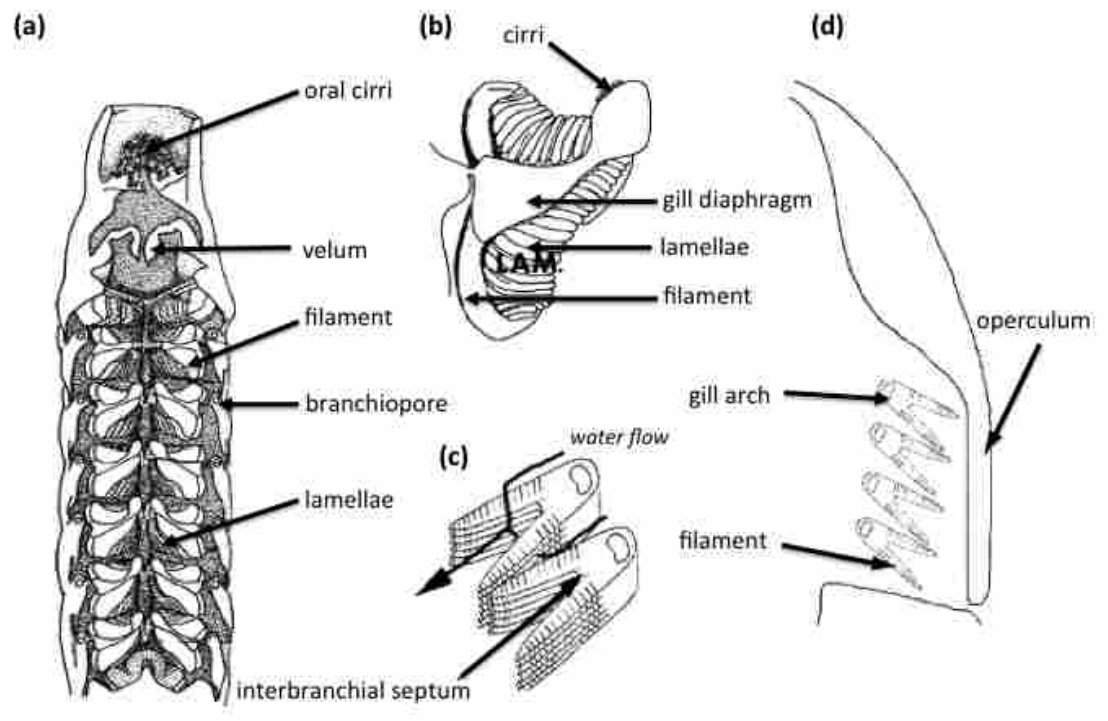
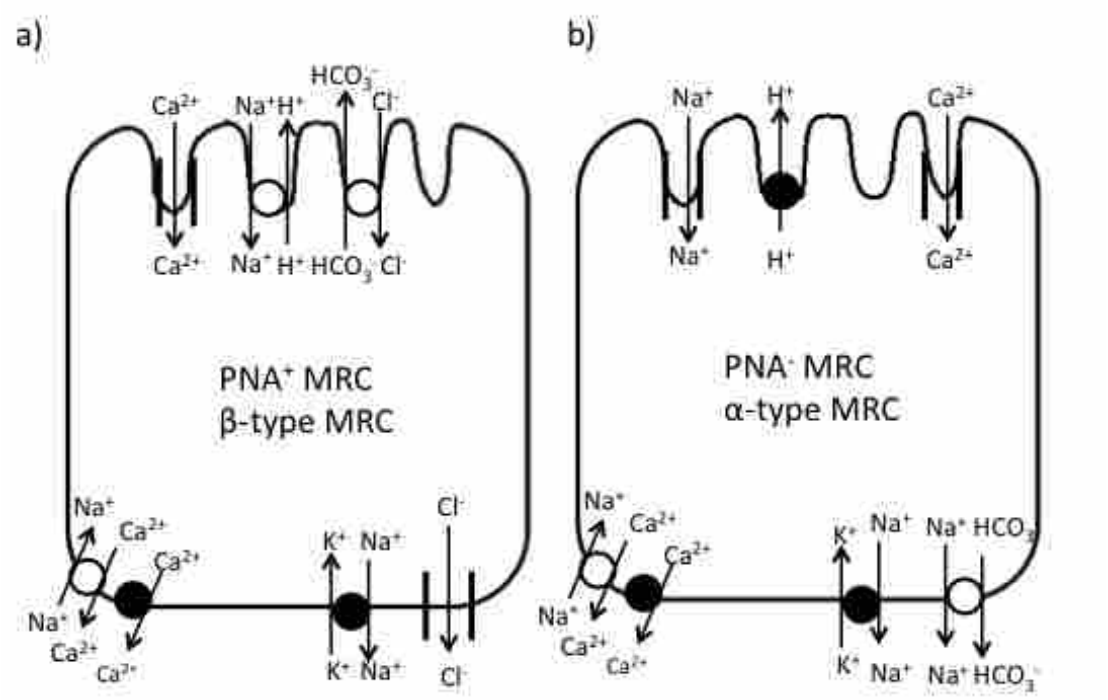

(a)
oral cirri
velum
filament
branchiopore
lamellae
interbranchial septum
(b)
cirri
gill diaphragm
lamellae
filament
L.A.M.
(c)
water flow
(d)
operculum
gill arch
filament

Figure 1.7 Ion uptake model in the gill epithelium of freshwater fish.

This model depicts the uptake of ions (Na^+, Ca^{2+}, and Cl^-) from the fresh water environment to the blood via ATPase pumps (solid circles), co-transporters and exchangers (open circles), and passive diffusion (parallel lines) in β – and α–sub-type mitochondrial rich cells (MRC) of the gill epithelium. Arrows depict the movement of ions through MRCs. Adapted from Marshall (2002) and Dymowska *et al.* (2012).


a)
b)
PNA+ MRC
β-type MRC
PNA- MRC
α-type MRC

types demonstrate high expression of NKA, however PNA^+ cells are characterized by low V-ATPase levels and possess no phenamil-sensitive Na^+-transport and are therefore proposed to be involved in base-excertion. Peanut lectin insensitive (PNA^-) are ultrastructurally similar to pavement cells in that they have been found to express both high levels of V-ATPase activity and acid-stimulated, phenamil- and bafilomycin- sensitive Na^+ uptake (Goss *et al*., 2001; Galvez *et al*., 2002; Reid *et al*., 2003).

The involvement of the PVCs in ion regulation is thought to be minimal, as PVCs possess few mitochondria and ultimately have a lower capacity to power ion-uptake processes (Evans and Claiborne, 2006). However, the presence of apical V-type proton ATPases and amiloride-sensitive eNac Na^+ channels in rainbow trout PVCs (Lin *et al.,* 1994; Sullivan *et al.,* 1995; Fenwick 1999; Wilson *et al.,* 2000) and Na^+-H^+ antiporters in euryhaline teleost PVCs (Wilson *et al.,* 2000; Marshall, 2002) suggest that the PVCs may play a role in ion uptake in fresh water. TFM may therefore impact the function of both these cell types in fish, due to the reliance on ATP for transporter activity.

The lamprey gill epithelium possess three classifications of MRCs: ammocoete MR cells (AMRs) found in ammocoetes, intercalated MR (IMRs) cells found between PVCs in both ammocoetes and post-metamorphic lamprey, and chloride cells (CC) which appear only after metamorphosis and vanish in upstream migrants (Wilson and Laurent, 2002; Zydlewski and Wilkie, 2012). IMRs are thought to be the primary site of ion regulation in larval lamprey as morphological alterations and increased surface area occur in these cells when exposed to altered water chemistry. These changes are not observed in AMRs (Bartels *et al.,* 1998).

Both PVC and ionocyte ultrastructure, density and surface areas change in response to environmental stressors including water hardness, ion content, pH, oxygen starvation and hypercapnia in fishes (for review see: Nilsson *et al.,* 2012). However, very little work has been done to elucidate how ionocytes, chloride cells and PVCs respond to similar

disturbances in lampreys, let alone how TFM alters the structure or function of these cells in larval sea lampreys.

7. Non-target Sub-lethal Effects of TFM on the Gills and the Influence of Water Quality

Mallatt *et al.* (1994; 1995) reported that trout ionocytes were not affected in the same manner as those found in larval sea lampreys when exposed to treatment doses of TFM. This suggests that trout may not experience disruption to ion homeostasis as a result of TFM exposure. However, previous studies in our lab have demonstrated no disturbances to plasma ion balance in larval sea lampreys after TFM exposure (Wilkie *et al.,* 2007; Birceanu *et al.,* 2009), except for sub-lethal interference with Na^+ uptake by rainbow trout (Birceanu, 2009). Due to TFM's adverse effects on oxidative phosphorylation, one possible explanation for this observation is that limited ATP supply resulted in lower rates of Na^+ uptake during TFM exposure. However, this hypothesis has not yet been tested in larval sea lamprey, trout or other non-target fishes

Waters with low ion content may exacerbate the sub-lethal effects of TFM due to an increased reliance on ATP mediated ion-transport to maintain osmotic balance (Leino *et al.,* 1987; Boisen *et al.,* 2003; Craig *et al.,* 2007). For instance, previous toxicity studies have found that greater water hardness decreases TFM toxicity to fish (Olson and Marking, 1973; 1975; Bills and Marking, 1976).

Many teleosts respond to ion-poor water by increasing their abundance of MRCs, which ultimately increases their capacity to actively transport ions across the gills (Leino *et al.,* 1987; Greco *et al.,* 1996). Because active transport requires the use of ATPases, TFM interference with ATP production in the gills could result in more severe ion disturbances in TFM treated soft waters compared to harder waters because the active transport of ions is limited by both availability of ions and ATP. Changes in the size and/or direction of ionic

gradients between extracellular fluid and water have been found to have a direct effect on Na^+,K^+-ATPase activity in juvenile coho salmon (*Oncorhynchus kisutch*, Morgan and Iwama, 1998); tilapia (*O. mossambicus*; Kültz *et al.,* 1992), euryhaline killifish (*Fundulus heteroclitus L*; Towle *et al.,* 1977), and pupfish (*Cyprinodon salinus L.;* Stuenkel and Hillyard, 1980), with activity lowest in iso-osmotic environments where this gradient is minimal.

8. Research Objectives and Hypothesis

The relationship between water quality and the toxicological effects of TFM exposure on the gills of larval sea lampreys and non-target fishes has been largely overlooked. However, it is important to examine how TFM affects gills in different water qualities to determine how normal compensatory adjustments in the gill epithelium to ion-poor water may be impaired, and whether these impairments persist once TFM is removed from the system. Water hardness in the upper Great Lakes ranges between 40 to 80 mg $CaCO_3$ L^{-1} (Upper Lakes Reference Group, 1977). However, Ontario lakes and streams have been found to have a very wide range in water hardness, ranging from levels from 2 to 1803 mg L^{-1}, with an average between 40 and 200 mg $CaCO_3$ L^{-1} (Ontario Ministry of the Environment, 1974). Therefore, there is a potential for there to be variation in TFM-sensitivity based on treatment locations. Furthermore, TFM-induced damage to the gills has been well researched but the sub-cellular mechanism for damage to this organ, whether in larval lamprey, rainbow trout, lake sturgeon or other fishes has not yet been resolved. Accordingly, the overarching goal of my research was to better characterize how TFM exposure disturbs ion homeostasis in larval sea lampreys, and two non-target species, the rainbow trout and the lake sturgeon. A major focus of my work was to determine if TFM interferes with ATP-dependent pathways in the gill epithelium resulting in a disruption of gill

function (mainly ion-homeostasis), and whether these effects were exacerbated in ion-poor soft water.

To identify the sub lethal effects of TFM on the gills in hard and soft water, I used an integrative approach employing biochemical and molecular analyses to examine gill-damage and ion disturbances in larval lamprey, rainbow trout (*O. mykiss*), and lake sturgeon (*A. fulvescens*). The physiology of the rainbow trout is very well characterized, and its tolerance to TFM makes it a useful model to use for understanding the toxic mechanisms of TFM action. Conversely, juvenile lake sturgeons were used as a TFM-sensitive model because they have demonstrated sensitivity to TFM at the fingerling stage (Boogaard *et al.,* 2003).

Using lamprey, trout, and sturgeon I tested three hypotheses:

I. Ionic disturbances and altered gill morphology and function contribute to fish mortality in larval lamprey but not in rainbow trout when exposed to TFM in hard waters (Chapter 2).

II. The greater sensitivity of juvenile lake sturgeon to TFM is due to impaired ion-homeostasis during TFM exposure and their lower capacity to detoxify TFM compared to rainbow trout (Chapter 3).

III. Ionic-disturbances and gill damage in both rainbow trout and larval sea lampreys will be greater in ion-poor soft water due to greater dependence on ATP-dependent-pathways to maintain ion homeostasis (Chapter 4).

Using similar methodology, these hypotheses were tested by exposing fish to TFM (either the LC_{50} or $LC_{99.9}$) for different time-intervals with a subset of fish permitted to recover from TFM exposure for 24 h prior to sampling the blood and gill tissue from the fishes. The blood of each fish was analyzed for ion concentrations, and the expression and abundance of ion transporters (NKA and V-ATPase), determined using western blotting and biochemical assays. Gross gill morphology was also examined using light microscopy to determine if TFM caused gill damage. Ion-tracer experiments were also conducted to determine if limited supplies of ATP during TFM exposure impaired Na^+-uptake in fish exposed to TFM in waters of different ion composition.

The ultimate goal of my thesis was to help generate a better understanding of the toxic mode of action of TFM and to better explain the differences in species-specific sensitivity to TFM. As a result of this work, it is hoped that sea lamprey control personnel and government agencies will have better understanding about why the sensitivity of different fishes to TFM varies and to better protect sensitive fishes from the possible effects of lampricide treatments

CHAPTER TWO:

The Effect of the Lampricide, 3-Trifluoromethyl-4-nitrophenol (TFM), on Gill Structure and Function in Juvenile Rainbow Trout and Larval Lamprey

1. Introduction

The pesticide, 3-trifluoromethyl-nitrophenol (TFM), is commonly used to eradicate larval sea lampreys (*Petromyzon marinus*) in streams draining into the Great Lakes (Christie and Goddard, 2003; McDonald and Kolar, 2007). Sea lampreys are an invasive species in the Great Lakes, which as juveniles parasitize commercial, sports, and cultural significant fishes by feeding on their blood using their characteristic oral disc and rasping tongue (Lowe *et al.,* 1973; Beamish *et al*, 1979; Farmer, 1980). During its 1-2 year parasitic phase, a single juvenile lamprey can kill 40 or more pounds of fish (Smith, 1968). Indeed, sea lampreys, along with overfishing, were thought to be primarily responsible for the near collapse of lake trout, and whitefish (*Coregonus* spp.) fisheries in the 1950s (Renaud, 1997; Smith and Tibbles, 1980; McDonald and Kolar, 2007).

In 1955, concern over the continued decline of fisheries in the Great Lakes prompted Canada and the United States to establish the Great Lakes Fishery Commission (GLFC), an inter-governmental organization that aimed to eradicate and minimize further sea lamprey predation of fishes (McDonald and Kolar, 2007). This led to the subsequent development of TFM as a pesticide that could be applied to lamprey nursery streams to kill multiple generations of sea lamprey. After TFM treatments were initiated in the 1960s and 1970s, the mortality rates of commercial and recreational fish populations due to lamprey predation were greatly reduced (Applegate *et al.,* 1961; Smith and Tibbles, 1980; Christie and Goddard, 2003). However, despite its continued success, little is still known about the effects of TFM on the physiology of non-target fish species (McDonald and Kolar, 2002).

TFM is normally applied to treatment streams at 1 to 1.5 times the $LC_{99.9}$ of larval sea lamprey for 9 h, which is also known as the minimum lethal concentration (MLC; McDonald and Kolar, 2007). It is applied as either TFM or as a TFM/niclosamide mixture streams at concentrations that are normally significantly lower than those known to be toxic to non-

target teleost fishes, which are generally approximately 3-5 times more tolerant to TFM than larval sea lampreys (Bills *et al.,* 2003; Boogaard *et al.,* 2003).

The mode of action of TFM is by interference with ATP production in the mitochondria, where TFM uncouples oxidative phosphorylation (Niblett and Ballantyne, 1976; Birceanu *et al.,* 2011). Interference with ATP production results in a reduction of glycogen stores in the brain, which leds to death (Wilkie *et al.,* 2007; Birceanu *et al.,* 2009; Clifford *et al.,* 2012). Similar reductions in brain glycogen have been observed in rainbow trout, when exposed to acutely lethal concentrations of TFM (Birceanu *et al.,* 2014).

Variation in the sensitivity to TFM between sea lamprey and non-target fishes is primarily due to differences in each species' capacity to metabolize TFM via the hepatic endoplasmic reticulum membrane-bound enzyme UDP-glucuronyltransferase (UDPGT). This enzyme conjugates the hydroxyl group of TFM with α-D-glucuronic acid to form β-D-glucuronide (Lech and Statham, 1975; Howell *et al.,* 1980; Kane *et al.,* 1993), a soluble molecule that is readily eliminated via renal pathways or the gastrointestinal tract (Lech and Costrini, 1972; Clarke *et al.,* 1991). Sea lampreys have lower expression of UDPGT and a lower rate of TFM-glucuronidation, which results in higher rates of TFM accumulation and higher concentrations of free TFM in the blood and tissues than in non-target fish species exposed to comparable concentrations of TFM (Lech and Costrini, 1972; Lech and Staham, 1975; Birceanu *et al.,* 2014; Le Clair, 2014). However, TFM has been found to effect a number of juvenile fishes populations, including members of the catfish family Ictaluridae (black bullhead *Ictalurus melas*), channel catfish (*Ictalurus punctatus*), juvenile lake sturgeon (*Acipenser fulvescens*), and mudpuppies (*Necturus maculosus*), with mortalities occurring at or below 2 times the MLC for larval sea lamprey (Boogaard *et al.,* 2003).

The primary site of TFM uptake is thought to be the gill (Christie and Battle, 1963; Youson and Freeman, 1976). The lipophillic un-ionized free-phenol form of TFM is predicted to readily cross the lipid bilayers of the gill epithelium and enter the blood stream

(Hunn and Allen, 1974). Evidence for a direct toxicological effect on the gills by TFM was shown in larval lamprey, in which TFM caused morphological changes in the ionocytes of the gill following TFM exposure (Christie and Battle, 1963; Mallatt, 1987; Mallatt *et al.,* 1994). Specific damage to ionoctyes (also called mitochondria rich cells or MRCs) has led to the hypothesis that disruption of ion homeostasis may directly contribute to TFM-induced mortality in larval lamprey (Mallatt *et al.,* 1994; 1995).

Sodium uptake in lampreys and teleosts is proposed to occur in the MRCs via apical epithelial Na^+ channels (eNac) and vacuolar (V-type) H^+-ATPase pumps (V-ATPase) and a ouabin-sensitive basolaterally located Na^+,K^+-ATPase (NKA; Bartels *et al.,* 1998; Marshall, 2002; Bartels and Potter, 2004; Wilkie, 2011). Accordingly, ATP-deficiencies may starve these transporters of energy, and subsequently impair ion-homeostasis during TFM exposure in non-target fish species. However, conflicting evidence has been generated to support this hypothesis. Trout ionocytes were not found to exhibit the same damage as those of larval sea lampreys with TFM exposure, suggesting that trout do not experience disruptions to ion homeostasis as a result of TFM exposure (Mallatt *et al.* 1985; 1994), while other studies have found no ion disturbances in larval lamprey after TFM exposure (Wilkie *et al.,* 2007; Birceanu *et al.,* 2009) but sub-lethal interference with Na^+ uptake in trout gills (Birceanu *et. al.*, 2014).

To clarify whether or not TFM influences ion-homeostasis in larval sea lamprey and rainbow trout, light microscopy was used to determine if TFM caused structural damage to the gills. Immunohistochemistry and western blotting, along enzyme activity measurements, were used to determine if TFM interfered with NKA and V-ATPase function. Unidirectional fluxes of Na^+ were also measured across the gills of larval sea lamprey and rainbow trout to better assess if TFM exposure interfered with ion uptake and/or increased branchial ion permeability. A key aspect of this study was that these processes were examined using TFM

exposure concentrations near those commonly used during typical TFM-treatments in the Great Lakes.

2. Methods and Materials

2.1 Animals and Experimental Holding

Larval sea lamprey (*P. marinus*; 1.7 ± 0.1 g, 109.8 ± 1.6 mm, *N*=72) were collected from northern Michigan streams by US Fish and Wildlife Service personnel using pulsed DC fishing, and shipped overnight in coolers containing two 20 l plastic bags filled with O_2 saturated water to Wilfrid Laurier University (Waterloo, ON). The animals were then transferred to a 60 l tank continuously receiving aerated well water (pH ~ 7.8, 10-13°C, titratable alkalinity ~ 200 mg $CaCO_3$ L^{-1}, water hardness ~ 450 mg $CaCO_3$ L^{-1}, 80-100% dissolved O_2). Fingerling rainbow trout (13.0 ± 0.9 g, 101.7 ± 2.7 mm, *N*=96) were purchased from Rainbow Springs Trout Farm (Thamesford, ON, Canada) and were held in 120 L tanks also receiving Wilfrid Laurier well water. All fish were held under a 12 h light: 12 h dark photoperiod. To provide burrowing substrate for the larval sea lamprey, the bottoms of the tanks were lined with fine silica sand (~4-5 cm deep). The larvae were fed once weekly with a slurry of baker's yeast (2 g of yeast per animal; Grain Harvest, Waterloo, ON) and trout were fed three times a week with 3.0-point fine floating pellets (fed to satiated; 3.0 Corey Feed Mills, Elmira, ON). Animals were held in the lab for a minimum of 4 weeks prior to experimentation, and the trout were starved for 48 h prior to experiments. All experiments and fish housing were approved by the Wilfrid Laurier Animal Care Committee and followed the Canadian Council of Animal Care guidelines.

2.2 Experimental Protocols

2.2.1 Sensitivity of Gills to TFM exposure

Larval sea lamprey (1.7 ± 0.1 g, 109.8 ± 1.6 mm, *N*=72) were acclimated overnight in individual 450 ml containers in a 120 L flow-through system continually receiving aerated Wilfrid Laurier well water. Diffuse aquarium cotton (2 g) was added to each experimental container to minimize stress to the fish and to limit changes in metabolic rate and ion balance due to excessive activity (Wilkie *et al.,* 1999). The next day, water flow to each container was shut off and the sea lamprey were exposed to their nominal TFM LC_{50} [4.6 mg L^{-1}; Birceanu *et al.,* 2009 (measured 4.70 ± 0.08 mg L^{-1})] and sampled under control conditions (no TFM) or after 3, 6, or 9 h of TFM exposure, or after a 24 h recovery period following 9 h TFM exposure. Rainbow trout (13.0 ± 0.9 g, 101.7 ± 2.7 mm; *N*=72) were exposed to TFM in the same system but to the nominal TFM $LC_{99.9}$ for larval sea lamprey [7.6 mg L^{-1}; Birceanu *et al.,* 2009 (measured 7.31 ± 0.08 mg L^{-1})] and no aquarium cotton was added. The fish were sampled following 0, 3, 6, and 9 h of TFM exposure, and following a 24 h post-TFM exposure recovery period. All exposures were done in the dark as larval sea lampreys are negatively phototactic (Beamish, 1980) and TFM is photosensitive (Carey & Fox, 1981). Blood was collected in both species by severing the tail behind the caudal fin and collecting the blood into saline heparinized capillary tubes. The blood was then centrifuged at 10,000 x *g* for 5 minutes, and the plasma was drawn off and frozen for later determination of plasma ion levels. Gill tissue (gills 2 and 3 for rainbow trout and 0.5 mm cross-sections of whole gill basket sections for larval lamprey) were also collected from both species, and the tissue was either flash frozen in liquid nitrogen and saved for determination of NKA and V-ATPase activity and expression, or fixed in 1:20 volume of gill to 10% buffered formalin solution (pH 7.4) for 24 h, then rinsed 3 times with 1X Phosphate Buffer Solution (pH 7.5), and stored in 70% ethanol at 4°C. Prior to storage in ethanol, trout gills were additionally decalcified

with a 0.6 mM sodium citrate and 19.6 mM formic acid solution. Gill tissue was prepared for light microscopy and immunohistochemistry analyses by fixing tissues in 20% DMSO/methanol at –20°C for 48 h followed by storage in 100% methanol at –20°C and processing for paraffin embedding (type 6; Richard-Allan Scientific, Kalamazoo, MI, USA). Following paraffin embedding, 5 µm sections were collected onto 3-aminopropyltriethoxysilane-coated slides (Sigma Aldrich; St. Louis, MO).

Concentrations of TFM were confirmed by spectrophotometry using a THERMOmax microplate spectrophotometer at a wavelength of 396 nm (SpectraMax 190, Molecular Devices, CA) and TFM standards provided by the Fisheries and Oceans Canada Sea Lamprey Control Center (Sault Ste Marie, Ontario).

2.2.2 TFM-Induced Disturbances to Unidirectional Na^+-Movements

Gill-mediated ion-uptake in trout was measured using ^{24}Na and well-established methodologies (Morris and Bull, 1970; Wilkie *et al.,* 1998). Briefly, 12 h prior to experimentation, juvenile rainbow trout (13.3 ± 0.8 g, 111.8 ± 2.3 mm; *N*=12) were placed in 760 ml containers continuously receiving water at a rate of 50 ml min^{-1} (pH ~ 7.8, 10-13°C, hardness ~ 450 mg $CaCO_3$, 80-100% dissolved O_2). The rates of Na^+ influx, net flux and efflux were determined under control conditions, and following 3, 6, or 9 h of TFM exposure. At the beginning of each flux measurement period, the volume of the containers were adjusted to 400 ml and 2 µCi $^{24}Na^+$ (dissolved in 0.01 N HNO_3) was added and allowed to mix for 10 minutes, prior to collecting the initial water sample at time 0. Water samples (10 ml) were collected at regular intervals from each chamber. Control unidirectional fluxes were conducted the day before TFM exposure, during which time water samples were collected at 0, 2 and 4 h of the flux measurement period, after which the containers were flushed with well water and placed back on flow-through overnight. The following morning, flow was once again shut off to each container and the volumes adjusted to 400 ml, TFM was

added to each chamber [7.6 mg L^{-1} (measured 8.67 ± 0.06 mg L^{-1})], followed by the addition of $^{24}Na^{+}$ radioisotope (2.5 μCi $^{24}Na^{+}$; McMaster University Nuclear Reactor, Hamilton, Ontario). After a 10-minutes mixing period, 10 ml water samples were taken from each chamber at 0, 3, 6, and 9 h TFM exposure. Following each flux period, duplicate 4 ml water samples were analyzed for total radioactivity using a 1480 Automatic Wallacl82 Wizard Gamma Counter with the WIZARD2 Automatic Gamma Counter software (PerkinElmer, MA, United States of America). After the second flux period, the experimental containers were once again flushed with well water and the fish were put back on flow-through overnight. The following day, fish were euthanized with an overdose of 1:2 parts tricaine methanesulfonate (1.0 g L^{-1} trout):sodium bicarbonate, weighed, and sampled. The concentrations of non-radioactive (cold) Na^{+} in the water samples were determined once $^{24}Na^{+}$ radioactivity was exhausted (approximately two weeks) using atomic absorption spectroscopy (SpectrAA 880, N_2 gas; Varian,171 Mississauga, ON), and concentrations of TFM were confirmed using the microplate spectrophotometer as described above.

2.3 Analytical Techniques

2.3.1 Concentrations of Na^{+} and Cl^{-}

Plasma and water Na^{+} concentrations were measured and quantified by atomic absorption spectroscopy on plasma samples diluted with e-pure to fall within the linear range of the calibration curve, and undiluted water samples (SpectrAA 880, N_2 gas; Varian 171, Mississauga, ON). Plasma chloride concentrations were analyzed using a chloridometer and standard operating procedures (926 Chloride Analyzer, Sherwood Scientific, Cambridge, UK).

2.3.2 Na^+, K^+-ATPase and Vacuolar H^+-ATPase Activity

The activity of gill NKA and V-ATPase was determined using a modified enzyme kinetic microplate assay developed by McCormick (1993). In this assay, ouabain-sensitive activity (NKA) and bafilomycin-sensitive activity (V-ATPase) were coupled to the formation of ADP and NADH, via conversion by lactic-dehydogenase and pyruvate kinase enzymes and measured as a decrease in absorbance per unit time. Approximately 40 mg of gill tissue was weighed and thawed in 400 µl SEI buffer (150 mM sucrose, 10 mM sodium ethylenediaminetetraacetic acid, 50 mM imidazole, pH 7.3), followed by the addition of 100 µl of SEID (0.1% sodium deoxycholic acid in SEI). The tissues were then homogenized for 30 seconds with a motorized pestle (Gerresheimer Kimble Kontes LLC, Düsseldorf, Germany), and the homogenate was centrifuged at 10,000 x *g* for 3 minutes. The reaction media was prepared with 50 mM imidazole, 2.8 mM phosphoenolpyruvate, 0.22 mM reduced nicotinamide adenine dinucleotide, 0.7 mM adenosine triphosphate with 4 U mL^{-1} lactic dehydrogenase (Calbiochem, 427217, Gibbstown, NJ) and 5 U mL^{-1} pyruvate kinase (Sigma Aldrich, P1506-5kU, St. Louis, MO). Due to the low activity of NKA and V-ATPase in the sea lamprey gill, 2.5 mM ATP was added to the reaction media. To measure NKA and V-ATPase, NKA was inhibited by the addition of 1.0 mM ouabain and V-ATPase by the addition of 1.0 mM ouabain and 10 µM bafilomycin A1 (LC Laboratories, 88899-55-2, Woburn, MA) dissolved in dimethyl sulfoxide (DMSO) to two sub-sets of reaction media. Since DMSO changed the absorbance of the reaction media, 14.2 µM DMSO was also added to NKA and total ATPase reaction media. Following centrifugation, 5 µl of the supernatant was added to each well for trout and 10 µl of supernatant for lamprey in quadruplicate for total ATPase, NKA, and V-ATPase. Exactly 50 µl of salt solution [50 mM imidazole, 189 mM NaCl, 10.5 mM $MgCl_2 \bullet H_2O$, 42 mM KCl (84 mM KCl for non-salmonids)] and 150 µl of reaction media for total ATPase, NKA, and V-ATPase activity determination were then added to each well. Activity was determined by measuring the absorbance of each well every

30 seconds for 20 minutes for trout and 10 seconds for 10 minutes for lamprey at 25°C on the 96-well microplate spectrophotometer (SpectraMax 190, Molecular Devices, CA). The activity of NKA was measured as the difference between total ATPase activity and NKA-inhibited activity. V-ATPase activity was measured as the difference between NKA-inhibited activity and NKA- and V-ATPase-inhibited activity. An ADP standard curve was utilized to determine the amount of ATP (nmol) converted to ADP. The specific activity for NKA and V-ATPase was then expressed per mg protein, which was determined in each sample analyzed using the Bradford Protein Assay (Bradford, 1976) with bovine serum albumin (BSA) standard (ALB001-250, Bioshop, Burlington, ON).

Remaining gill supernatant (~200 ul) from the activity assay was then diluted with an equal volume of 2 x Laemmli's buffer (0.125 M Tris-HCl pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate, 0.01% bromophenol blue, and 100 mM dithiothreitol), heated to 70°C for 10 minutes, and stored at -80°C for future SDS-Page electrophoresis and western blot analysis.

2.3.3. Antibodies

The antibodies chosen in this study were based on prior successful use on teleost fish and lamprey species (Witters *et al.,* 1996; Wilson & Laurent, 2002) and followed the protocol outlined by Reis-Santos *et al.* (2004). The NKA transporter was detected using the panspecific α5 mouse monoclonal antibody developed by Douglas Fambrough (Johns Hopkins University; Takeyasu, 1988) and αRbNKA affinity-purified anti-peptide rabbit polyclonal antibody (Ura *et al.,* 1996; Wilson *et al.,* 2007). Both of these antibodies specifically bind to the α-subunit of NKA. The affinity-purified rabbit anti-peptide polyclonal B2/BvA1 antibody was used to detect vacuolar proton ATPase (V-ATPase), which demonstrated immunoreactivity with a conserved region of the B1 and B2 subunit isoform of V-ATPase of the eel (*Anguilla anguilla*; Wilson *et al.,* 2007). Both αRbNKA and B2/BvA1 antibodies were provided by Dr. Jonathan Wilson (Centro Interdisciplinar de Investigação

Marinhae, Ambienta, Portugal). α5, αRbNKA, and B2/BvA1 were used for immunoblotting and α5/αRbNKA was used for immunofluorescence.

2.3.4. SDS Page and Western Blot Analysis

The abundance of NKA and V-ATPase transporter was determined using SDS-Page electrophoresis followed by western blot analysis. Prior to loading, samples used for NKA and V-ATPase activity (Methods and Materials 3.2.3) were thawed, and diluted to 0.5 µg/µl total protein concentration (trout) and 0.4 µg/µl total protein concentration (lamprey) with 1X Laemmli's buffer. After vortexing, the samples were heated to 70°C for 10 minutes, and centrifuged for 5 minutes at 10,000 x *g*. Exactly 40 µl of sample was then loaded onto 1.5 mm thick mini polyacrylamide gels (8%T resolving gel and 4% stacking gel) and run in duplicate with Precision Plus Protein Western C Standards protein markers (#161-0376 BioRad, Hercules, CA). The gels were run at 75 V for 15 minutes using a Biorad Mini-PROTEAN® Tetra cell system (BioRad, Hercules, CA). The voltage was then increased to 150 V and the gels run for 1 h. Following electrophoresis, gels were equilibrated in 0.25 mM Tris 19.20 mM glycine 2.46 M methanol 1.04 mM SDS transfer buffer and wet-transferred to polyvinylidene fluoride membranes (BioTrace PVDF, 0.45 µm, Pall Corporation, Pensacola, FL) for 1 hour at 100 V using the Biorad Mini Trans-Blot® Cell system (BioRad, Hercules, CA). The membranes were then rinsed in TTBS (0.05% Tween-20 in Tris-buffered saline, pH 7.4) for 5 minutes and stained with Ponceau S (Ponceau S: 0.1% (x/v) stain in 1% (v/v) acetic acid; Bioshop, Burlington, ON), and gently mixed for 1 h on a shaker. The membranes were then destained in deionized water (e-pure water) to confirm the transfer-efficiency to the PVDF membrane. After the membranes were rinsed with TTBS, membranes were then blocked for 1 h in a 5% dry milk in 1 X TTBS solution. The membranes were then rinsed 3 times in 1 X TTBS for 5 minutes and incubated with either α5/αRbNKA (α subunit of NKA) or B2/BvA1 (β subunit of V-ATPase) antibodies diluted 1:1000 in 1%BSA/0.05% sodium

azide/1X TTBS overnight at 4°C on a shaker. The following morning, antibodies were recovered, and the membranes were rinsed in 1X TTBS prior to incubation with peroxidase-conjugated secondary goat anti-rabbit or anti-mouse Ig antibody and Precision Protein™ StrepTactin-HRP conjugate (#161-0381; BioRad, Hercules, CA) diluted 1:20,000 in 1X TTBS for 1 h. After which, the membranes were once again rinsed with 1X TTBS prior to detection with Clarity Western ECL Substrate (BioRad, Hercules, CA) and imaged on Molecular Imager® VersaDoc™ MP Imaging System (BioRad, Hercules, CA). The intensity of the bands were then semi-quantified using SigmaScan Pro Software Version 1.0 (Systat Software Inc., San Jose, CA). The average of the controls was used to calculate relative density for comparisons. The absence of the primary antibody in the dilution buffer was used as a no primary control.

2.3.5. Immunohistochemistry

Sections were air dried, dewaxed in Clear Rite (Richard-Allan Scientific; Kalamazoo, MI), and circled with a hydrophobic barrier (ImmunoPen, Sigma Aldrich; St. Louis, MO). After rehydrating the sections in a 5% goal serum 0.1% BSA/TPBS (0.05%Tween020/PBS, pH 7.4) solution for 20 minutes, the slides were incubated with α5. The slides were then rinsed for 5, 10, and 15 minutes with TPBS in Coplin jars and incubated for 1 h at 37°C with secondary antibodies goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594-conjgate (Invitrogen, Carlsbad, CA) diluted to 1:400 in BSA/TPBS. Following incubation, slides were once again rinsed for 5, 10, and 15 minutes in TPBS and a 10% Mowiol, 40% glycerol, 0.1% DABCO, 0.1 M Tris (pH 8.5) solution was used to mount the coverslips. Slides were viewed with a Leica DM6000 B wide field epifluorescence microscope with a digital camera (DFC340FX, Leica Microsystems, Wetzlar, Germany). The absence of a primary antibody in the dilution buffer in the immunohistochemistry protocol was used as null controls. The substitution of the primary antibody with normal mouse serum or rabbit for

the α5 antibodies was used as negative control. In all controls, there was no visible background staining found in the gill epithelium. In order to correct for differences in tissue cross sectional area, slides were stained with DAPI to use total nuclei area as a proxy for tissue area. Each section was imaged at three different fields and analyzed for NKA immunofluorescence and DAPI nuclear-staining at the Centro Interdiscplinar de Investigação Marinha e Ambiental (CIIMAR) in Porto, Portugal. The analysis of these images was conducted at Wilfrid Laurier University and total NKA fluorescence was then measured using the SigmaScan Pro Software Version 1.0 (Systat Software Inc., San Jose, CA). Total NKA fluorescence was calculated by measuring NKA average fluorescence intensity for each image (three images per fish) correcting for differences in tissue area using DAPI staining.

2.3.6. Light Microscopy

A subset of prepared slides were used for light microscopy and stained using a standard haematoxylin and eosin (H&E) staining protocol in order to examine gill structural changes at the Centro Interdiscplinar de Investigação Marinha e Ambiental (CIIMAR) in Porto, Portugal. Gill sections were examined on a Jenaval light microscope fitted with Zeiss Axiocam MRC 5 camera and images were obtained using the Zen 2011 Blue Edition software (Carl Zeiss MicroImaging Gmbh, Germany) at Wilfrid Laurier University. Each gill section was imaged at two different fields with four fish analyzed per treatment group. Structural damaged by TFM was rated on a 1 to 6 scale according to Kiemer and Black (1997) and Thomassen (1993). Briefly, Grade 1 referred to gill tissue with healthy pseudo stratified epithelial cells and few mucous cells on the gill lamella. Grade 2 gills exhibited minimal signs of hyperplasia or clubbing at the distal ends of the lamella and minor hypertrophy to the mucous cell hyperplasia with no contour disturbance. Grade 2 properties along with increased visible folding and wrinkling of surface epithelial cells was defined as Grade 3. Grade 4 gills presented extensive hyperplasia, fusion of the lamellae, and/or

necrosis, and increased lifting and/or desquamation of the epithelium over a large area was defined as Grade 5. The final stage, grade 6, was represented by total necrosis and lamellae destroyed and fish were dead or dying.

2.4. Calculations and Statistics

2.4.1 Determination of Na^+ Uptake

Rates of Na^+ influx ($J_{in}^{Na^+}$) were based on reductions in water radioactivity during each flux sample period, the container volume, and the mass of the fish using the following formula (Wood, 1998):

$$J_{in}^{Na^+} = \frac{(CPM_i - CPM_f)}{MSA} \bullet \frac{V}{W \times T},$$

where CPM_i and CPM_f are the radioactive counts of the isotope in the water at the start and end of each flux period (counts min^{-1} mL^{-1}), *MSA* is the mean specific activity of $^{24}Na^+$ during the flux period (counts min^{-1} μmoL^{-1}), *V* is the container volume (ml), *W* is the mass of the fish (g), and *T* is the time elapsed between the start and finish of the flux period (h).

Estimates of $J_{net}^{Na^+}$ were calculated using the following equation:

$$J_{net}^{Na^+} = \frac{(X_i - X_f)}{M \times T}$$

where X_i and X_f are the 'cold' (non-radioactive) Na^+ concentrations in the water at the start and finish of each flux period (nmol mL^{-1}), and *V*, *W*, and *T* are the same as previously stated above. Outward movements of Na^+ ($J_{out}^{Na^+}$) were calculated from the difference between $J_{net}^{Na^+}$ and $J_{in}^{Na^+}$:

$$J_{out}^{Na^+} = J_{net}^{Na^+} - J_{in}^{Na^+}$$

2.4.2 Statistics

Data were presented as the mean ± 1 standard error of the mean (SEM). All comparisons were determined using Prism 6 Version 1.0 (GraphPad Software Inc, La Jolla, CA). TFM exposure and Post-TFM Recovery were treated as separated experiments. Therefore, significance between controls and TFM-exposure groups were determined using one-way analysis of variance (ANOVA) and a Holm-Sidak post-test was used when significant variability was observed between sample groups at the $p<0.05$ level. When the conditions for homogeneity of variance were not met, a nonparametric ANOVA test was used for comparisons between control and TFM-exposure groups followed by Dunn's post-test. Controls and 24 h recovery groups were compared with an unpaired T-test or an unpaired T-test with Welch correction, where appropriate.

3. Results

3.1 Ion Balance and Unidirectional Na^+ Movements Following TFM Exposure in Rainbow Trout

3.1.1 Plasma Concentrations

Exposure of juvenile rainbow trout to the $LC_{99.9}$ of TFM for larval sea lamprey did not significantly alter the respective plasma ion concentrations of Na^+ or Cl^-, which were maintained near control concentrations of 138 ± 1.0 mM and 113 ± 1.0 mM for the duration of the exposure and following the 24 h recovery period (Figure 2.1).

3.1.2 Na^+-Uptake and Loss Using Radiotracer $^{24}Na^+$

Similarly, TFM exposure did not impair $J_{in}^{Na^+}$, which was not significantly different from the control rates of 414.7 ± 30.4 nmol g^{-1} h^{-1} (Figure 2.2). The rates of $J_{out}^{Na^+}$ were

Figure 2.1 Effects of TFM on plasma ion balance in juvenile rainbow trout.

Concentrations of plasma Na^+ and Cl^- in resting juvenile trout *(Oncorhynchus mykiss*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h $LC_{99.9}$ concentration (7.6 mg L^{-1}) of larval sea lamprey and following 24 h recovery in TFM-free water. Open bars denote Cl^- concentrations and solid bars concentrations of Na^+. Data are expressed as the mean +1 SEM (*N*; $p<0.05$).

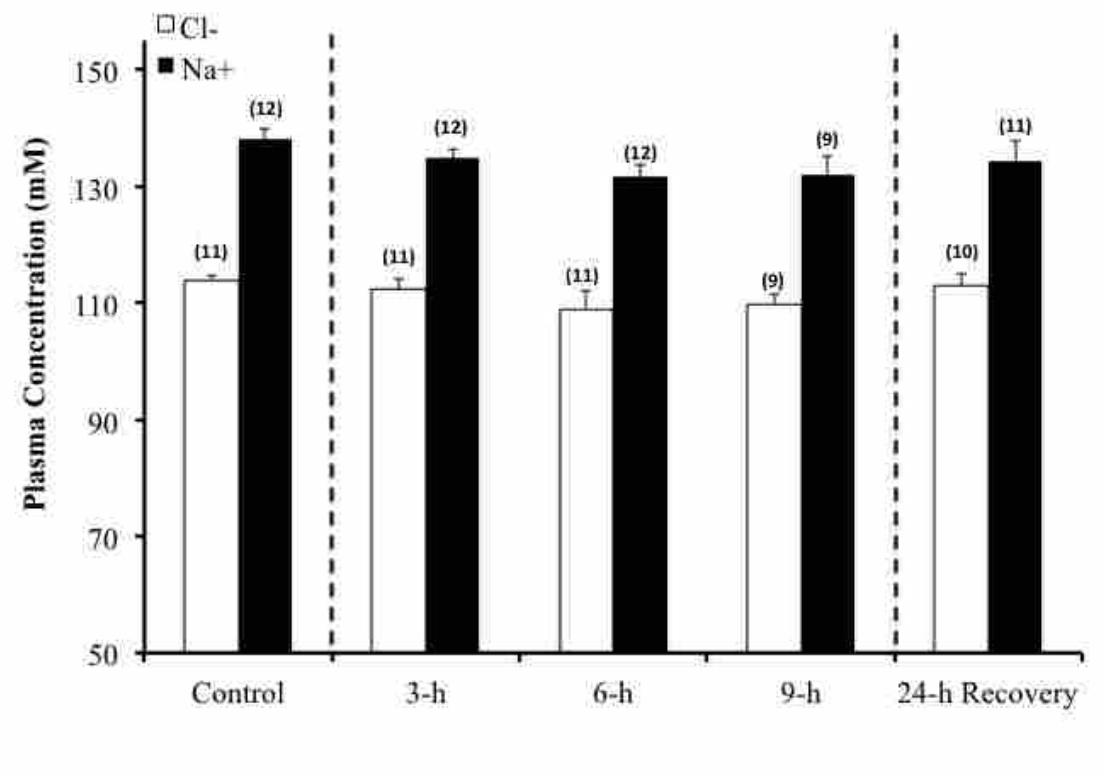
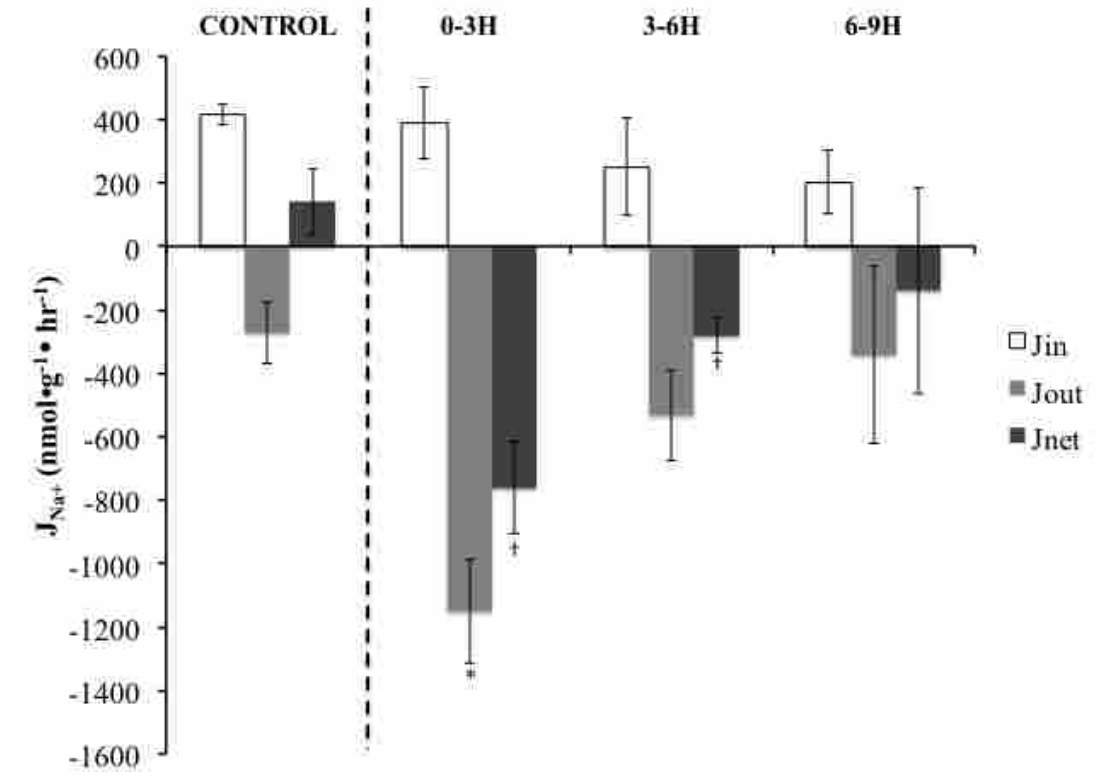

□Cl-
■Na+
Plasma Concentration (mM)
150
130
110
90
70
50
(11)
(12)
(11)
(12)
(11)
(12)
(9)
(9)
(10)
(11)
Control
3-h
6-h
9-h
24-h Recovery

Figure 2.2 Unidirectional movement of sodium in juvenile rainbow trout during TFM exposure.

Radiotracer movement of $^{24}Na^{+}$ (2.5 μCi) across the gills of resting juvenile rainbow trout *(Oncorhynchus mykiss*) measured during a 4-h control flux (*N*=12) and during exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h $LC_{99.9}$ concentration (7.6 mg L^{-1}) between 0-3 h, 3-6 h, and 6-9 h (*N*=8). Open bars indicate influx rates, gray bars efflux rates, and solid bars net-flux rates of sodium. Data are expressed as the mean +1 SEM. * Indicates a significant difference between control and exposure groups for $J_{out}^{Na^+}$ and † indicates a significant difference between control and exposure groups for $J_{net}^{Na^+}$ ($p<0.05$).


CONTROL
0-3H
3-6H
6-9H
600
400
200
0
-200
-400
-600
-800
-1000
-1200
-1400
-1600
J_{Na^+} (nmol•g^{-1}• hr^{-1})
Jin
Jout
Jnet
*
†
‡

however, 3-fold greater than the control rates of -271.2 ± 97.8 nmol g^{-1} h^{-1} at 3 h of TFM exposure, resulting, in net Na^+ losses of -761.74 ± 143.46 nmol g^{-1} h^{-1} (Figure 2.2). This increased diffusive Na^+ losses and net Na^+ loss was not maintained and by 6 h and 9 h the respective rates of $J_{out}^{Na^+}$ and $J_{net}^{Na^+}$ were restored to control levels (Figure 2.2).

3.2 Ion balance Following TFM Exposure in Larval Lamprey

As in rainbow trout, plasma Cl^- concentrations were not significantly different from the control values of 80 mmol L^{-1} throughout the duration of TFM exposure and the 24 h recovery period. In contrast, plasma Na^+ concentration followed a downward trend from 6 to 9 h of TFM exposure, and by 24 h of recovery, TFM was 28% lower than control values (Figure 2.3).

3.3 Na^+,K^+-ATPase and Vacuolar H^+-ATPase Activity and Expression

3.3.1 Juvenile Rainbow Trout

Similar to plasma ion concentrations, TFM had minimal effects on ion transporter activity and expression in the gills of juvenile rainbow trout. NKA activity underwent a 30 % increase at 6 h of TFM exposure, but otherwise fluctuated around the control rates of 0.74 ± 0.10 μmol ADP mg $protein^{-1}$ h^{-1} for the most of the experiment and the 24 h recovery period (Figure 2.4). V-ATPase activity did not change either, during the exposure or recovery. Total ATPase activity was also unchanged during the experiment, with an average activity of 3.98 μmol ADP mg $protein^{-1}$ h^{-1}. During recovery, however, there was a 50 % increase in total ATPase activity from 3.00 ± 0.41 μmol ADP mg $protein^{-1}$ h^{-1} in the control fish to 4.50 ±0.54 μmol ADP mg $protein^{-1}$ h^{-1} (Figure 2.4).

The αRbNKA antibody was found to immunoreact with a band located at approximately 100 kD, the proposed size of the α-subunit of the NKA transporter. A separate

Figure 2.3 Effects of TFM on plasma ion balance in larval lamprey.

Concentrations of plasma Na^+ and Cl^- in resting larval lamprey (*Petromyzon marinus*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h LC_{50} concentration (4.6 mg L^{-1}) of larval sea lamprey and following 24 h recovery in TFM-free water. Open bars denote Cl^- concentrations and solid bars concentrations of Na^+. Data are expressed as the mean +1 SEM (*N*). † Indicates significant difference found between control concentrations of Na^+ and 24 h recovery levels ($p<0.05$).

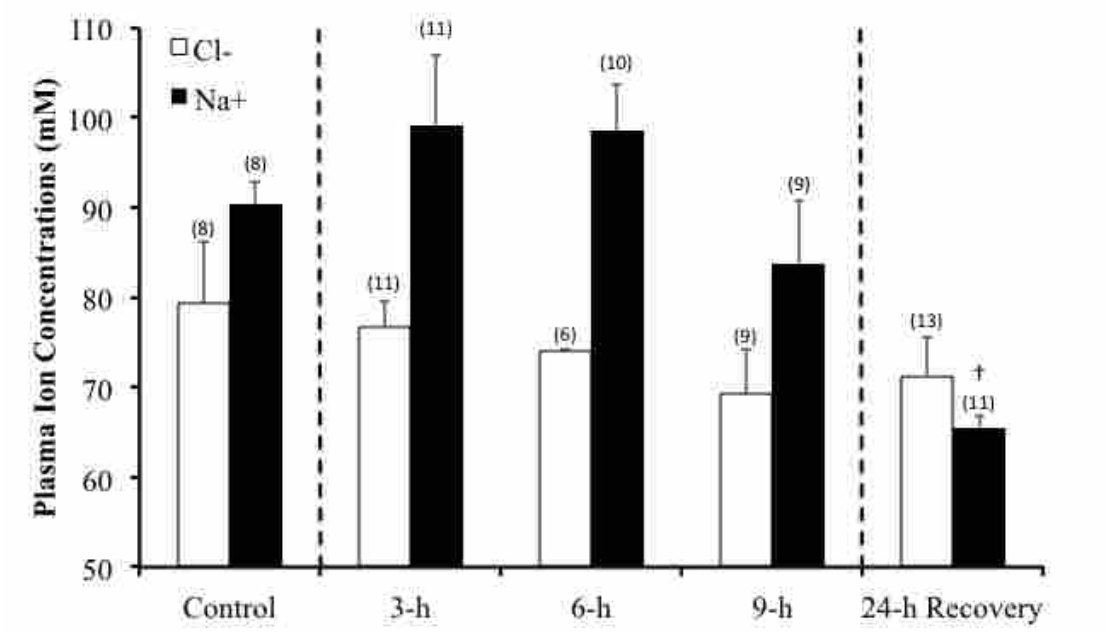

Plasma Ion Concentrations (mM)
110
100
90
80
70
60
50
Cl-
Na+
(8)
(8)
(11)
(11)
(10)
(6)
(9)
(9)
(13)
†
(11)
Control
3-h
6-h
9-h
24-h Recovery

Figure 2.4 Effects of TFM on gill ion-transporter activity in juvenile rainbow trout.

Na^+,K^+-ATPase (NKA), vacuolar H^+-ATPase (V-ATPase), and total ATPase activity of resting juvenile trout (*Oncorhynchus mykiss*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h $LC_{99.9}$ concentration (7.6 mg L^{-1}) of larval sea lamprey and following 24 h recovery in TFM-free water. Open bars denote NKA activity, gray bars V-ATPase activity, and solid bars Total ATPase activity. Data are expressed as the mean +1 SEM (*N*). * Indicates a significant difference found between the control and TFM exposure groups; † Indicates a significant difference found between control and 24 h recovery ($p<0.05$).

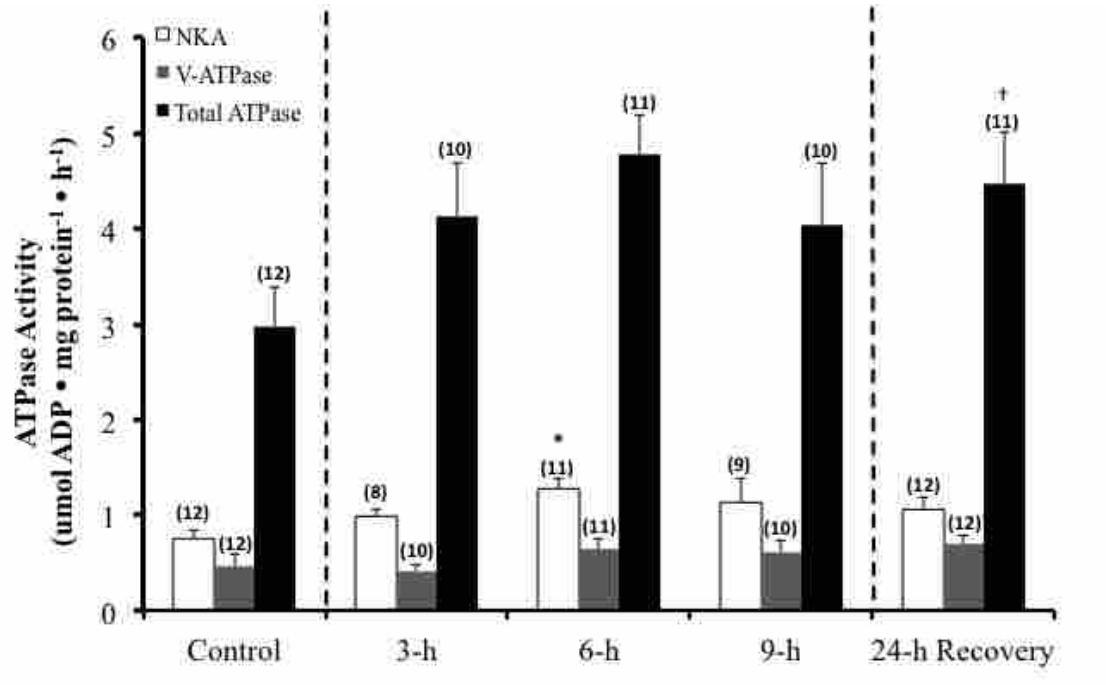
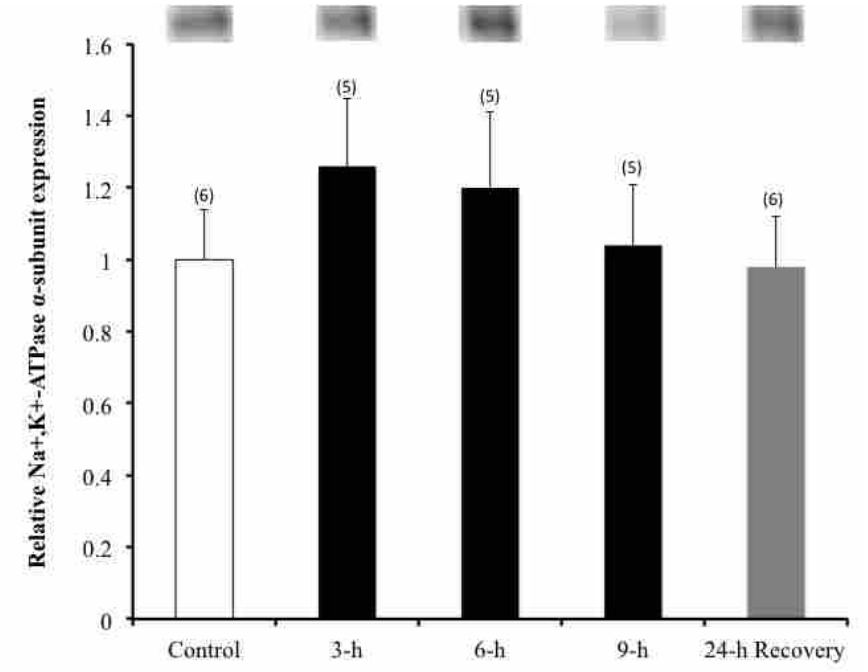

NKA
V-ATPase
Total ATPase
ATPase Activity
(umol ADP • mg protein-1 • h-1)
0
1
2
3
4
5
6
(12)
(12)
(12)
(8)
(10)
(10)
*
(11)
(11)
(11)
(9)
(10)
(10)
(12)
(12)
†
(11)
Control
3-h
6-h
9-h
24-h Recovery

Figure 2.5 Effects of TFM on gill ion-transporter expression in juvenile rainbow trout.

NKA α-subunit expression in resting juvenile trout (*Oncorhynchus mykiss*), during exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h $LC_{99.9}$ concentration (7.6 mg L^{-1}) of larval sea lamprey, and following 24 h recovery in TFM-free water. Data are expressed as the mean +1 SEM (*N*; $p<0.05$). Representative immunoblots of gill probed with the NKA α-subunit antibody αRbNKA are shown above their respective bars taken from the same blots.


Relative Na+,K+-ATPase α-subunit expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
(6)
(5)
(5)
(5)
(6)
Control
3-h
6-h
9-h
24-h Recovery

series of Western Blotting experiments was conducted to ensure banding at 100 kD for NKA was not due to false positives by the secondary antibody. As such, a no-positive controls procedure was conducted for NKA and a single molecular weight band was seen at 75 kD (data not shown). Western blot analysis revealed that the increase in NKA activity at 6 h was not due to increased quantities of NKA protein, which remained unchanged through the duration of the experiment (Figure 2.5).

3.3.2 Larval Lamprey

In larval lamprey, ion-transporter activity was not affected by exposure to TFM. There were no changes in NKA or Total ATPase activity during TFM exposure or following the recovery period (Figure 2.6). The activity of V-ATPase remained below levels of detection (data not shown). Like trout, α5 was found to immunoreact with a band located at approximately 100 kD and the B2/BvA1 antibody yielded a pair of bands located at approximately 56 kD, along with several other higher and lower molecular weight proteins in larval lamprey. Similar to trout, there were no changes in the expression of the NKA α-subunit or of the V-ATPase B subunit as a result of TFM exposure or during the recovery period (Figure 2.7 a and b). Again, a separate series of Western Blotting experiments was conducted to ensure banding at 100 kD for NKA and 56 kD for V-ATPase was not due to false positives via the secondary antibody, and like trout, a single molecular weight band was seen at 75 kD for both antibodies (data not shown).

3.4 Na^+,K^+-ATPase (NKA) Distribution in the Rainbow Trout Gill

NKA was found to localize to the basolateral membrane of branchial epithelia cells of the gill filament and proximal regions of the lamellae in the rainbow trout. These cells were mainly concentrated within the interlamellar spaces primarily on the gill filament, but reactivity was stronger on the afferent side of the gill filament. Additional weaker

Figure 2.6 Effects of TFM on gill ion-transporter activity in larval lamprey.

Na^+,K^+-ATPase (NKA) and total ATPase activity of resting larval lamprey (*Petromyzon marinus*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h LC_{50} concentration (4.6 mg L^{-1}) of larval sea lamprey and following 24 h recovery in TFM-free water. Open bars denote NKA activity and solid bars Total ATPase activity. Data are expressed as the mean +1 SEM (*N*; $p<0.05$).

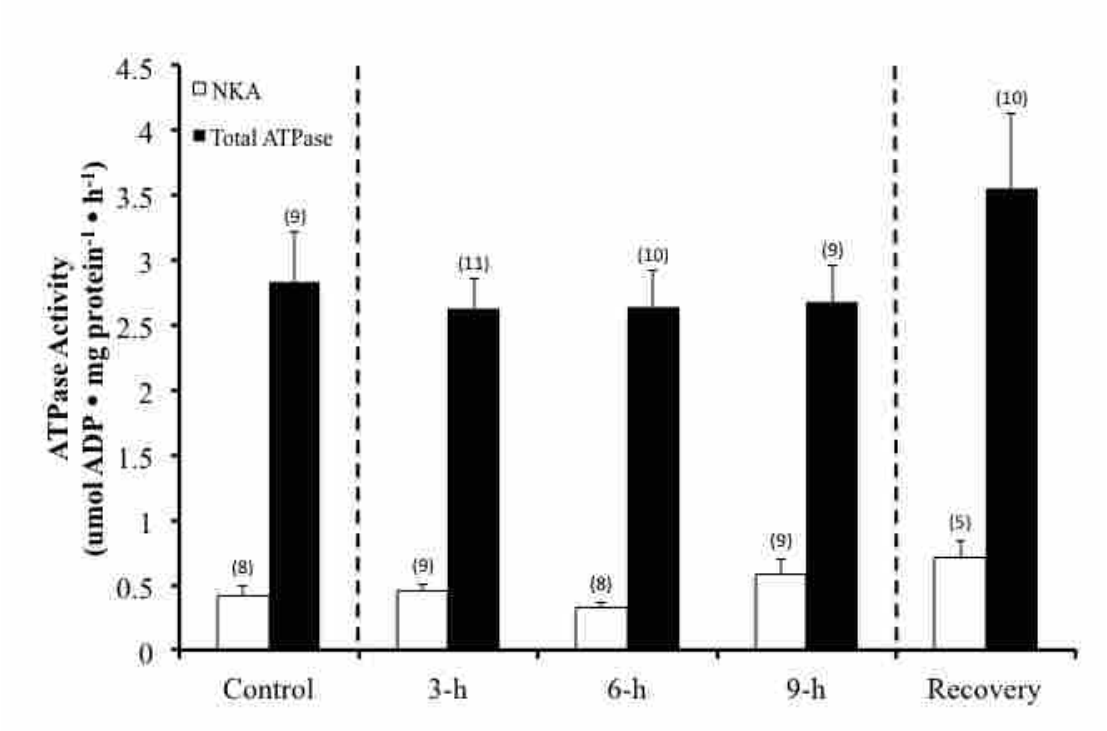
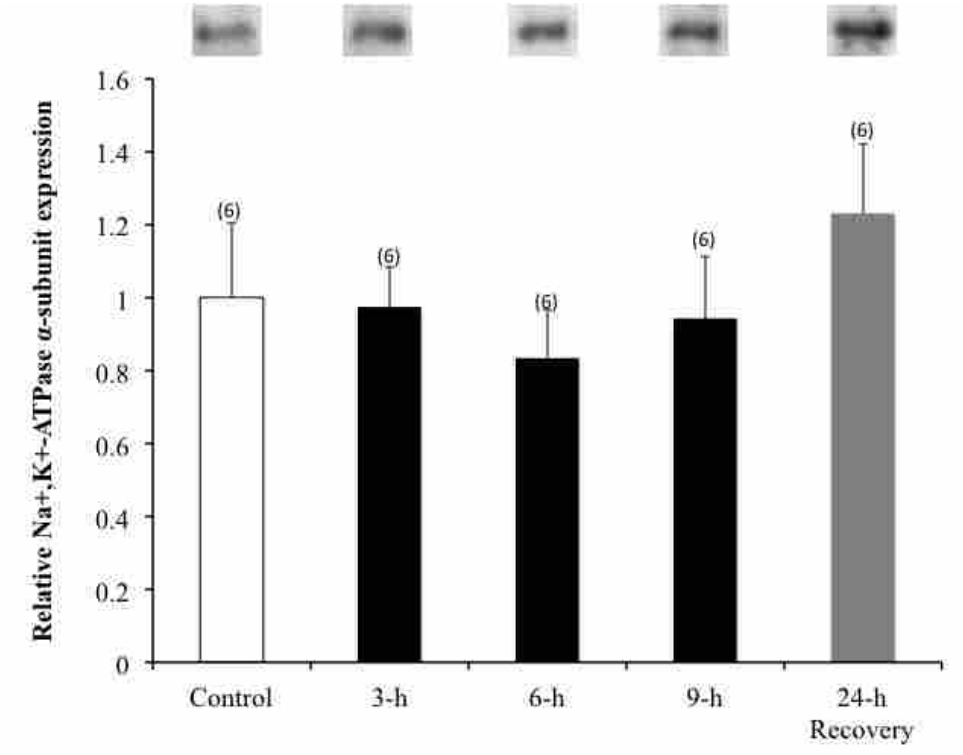

NKA
Total ATPase
ATPase Activity
(umol ADP • mg protein-1 • h-1)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
(8)
(9)
(9)
(11)
(8)
(10)
(9)
(9)
(5)
(10)
Control
3-h
6-h
9-h
Recovery

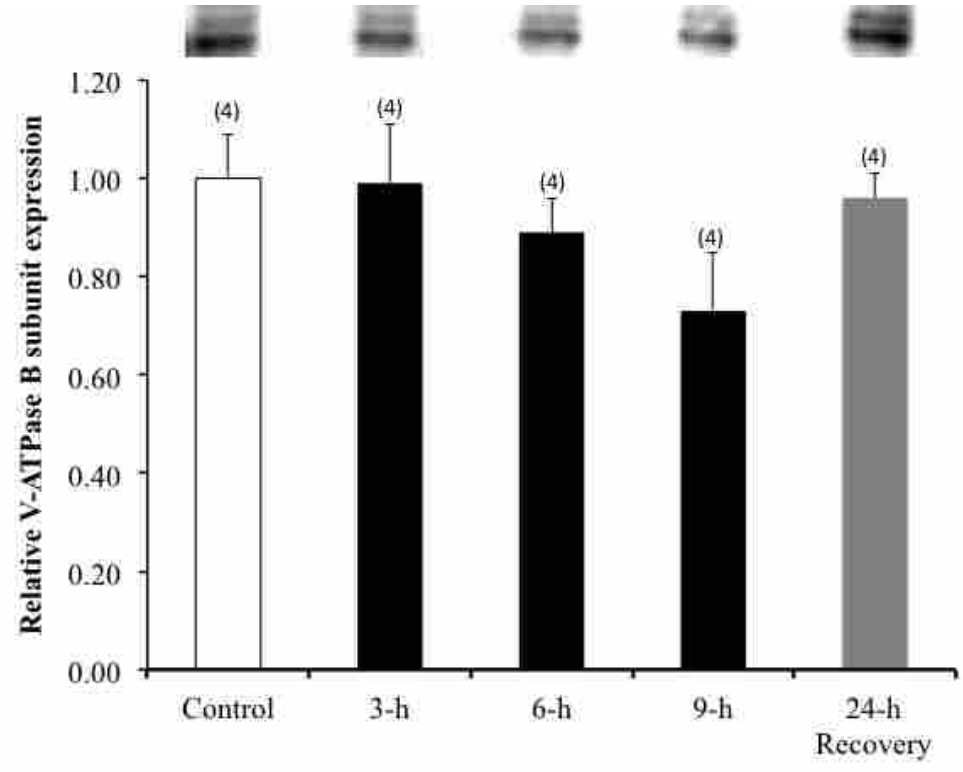
Figure 2.7 Effects of TFM on gill ion-transporter expression in larval lamprey.

(a) NKA α-subunit and (b) V-ATPase B-subunit expression in resting larval lamprey (*Petromyzon marinus*), during exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h LC_{50} concentration (4.6 mg L^{-1}) of larval sea lamprey, and following 24 h recovery in TFM-free water. Data are expressed as the mean +1 SEM (*N*; $p<0.05$). Representative immunoblots of gill probed with the NKA α-subunit antibodies α5 and V-ATPase B-subunit antibody B2/BvA1 are shown above their respective bars taken from the same blots.

a)


Relative Na+,K+-ATPase α-subunit expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
(6)
(6)
(6)
(6)
(6)
Control
3-h
6-h
9-h
24-h Recovery

b)


Relative V-ATPase B subunit expression
1.20
1.00
0.80
0.60
0.40
0.20
0.00
(4)
(4)
(4)
(4)
(4)
Control
3-h
6-h
9-h
24-h Recovery

cytoplasmic staining was noted in some cells in close association with MRCs. There were no notable differences between control gills or gills exposed to 7.6 mg L^{-1} TFM for 9 h (Figure 2.8). Global fluorescence analysis of the gill filaments found no significant differences between either the control or 9 h TFM treatment group (Figure 2.9), as was found with the western blot analysis (Figure 2.5).

3.5 Light Microscopy

3.5.1 Juvenile Rainbow Trout

H&E stained slides demonstrated no evidence of histological damage in the gill tissue of fish exposed to the TFM $LC_{99.9}$ of larval lamprey (7.6 mg L^{-1}). Both filamental and lamellar cells and tissues were found to be healthy and did not visually differ from control gill tissue. Gill tissue exhibited healthy pseudo stratified epithelial cells and few mucous cells on the gill lamella. There was no evidence of fusion of the lamellae, epithelial lifting, necrosis, or hyperplasia or clubbing of the lamellar tips. All sections exhibited characteristics of grade 1 gills as detailed in section 2.3.6 of Materials and Methods (Figure 2.10).

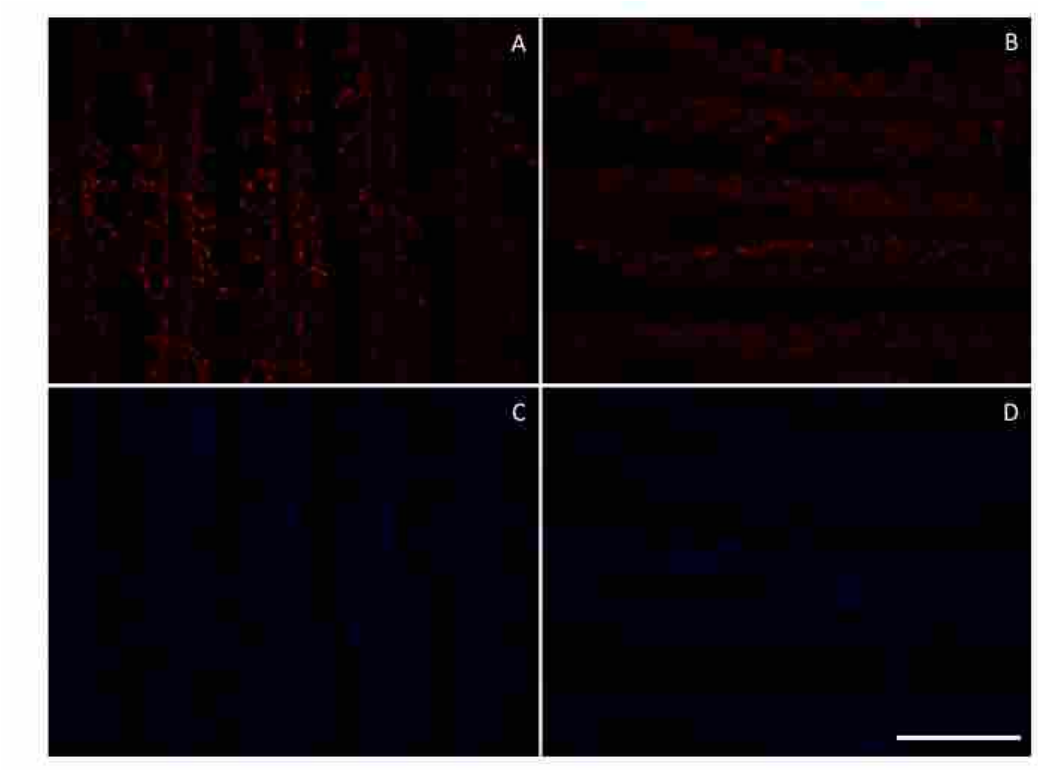
3.5.2 Larval Sea Lamprey

Larval sea lamprey exposed to TFM also exhibited no obvious signs of gill tissue damage following exposure to TFM. Both the filament and lamellar respiratory structures appeared healthy and did not visually differ from control gill tissue, exhibiting the characteristics of grade 1 gills. There was no evidence of hypertrophy of the gill epithelium or clubbing of the lamellae. Epithelial tissue was also found to exhibit similar healthy pseudo stratified epithelial cells and few mucous cells on the gill lamella as trout. However, the volume of inter-lamellar cell mass was qualitatively found to be more pronounced in control group, and decreases in the volume of this cell-mass were noted in 2 of the 4 fish exposed to TFM for either 6 h or 9 h (Figure 2.11).

Figure 2.8 Immunolocalization and relative immunofluorescence of Na^+,K^+-ATPase (NKA) transporter in TFM exposed gills of juvenile rainbow trout.

Immunolocalization of NKA α subunit at (a) 1600 X and (b) 400 X magnification. Within each each panel, α5 labelled primary antibody was used to detect the NKA α subunit (A,B), while DAPI nuclear staining was used to localize cells (C,D). Arrowheads indicate NKA basolateral labeling of mitochondrial-rich cells (MRCs) in control juvenile rainbow trout (*Oncorrhyncus mykiss* A,C) and during exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h LC_{50} concentration (7.6 mg L^{-1}; B,D) for 9 h.

a)



b)

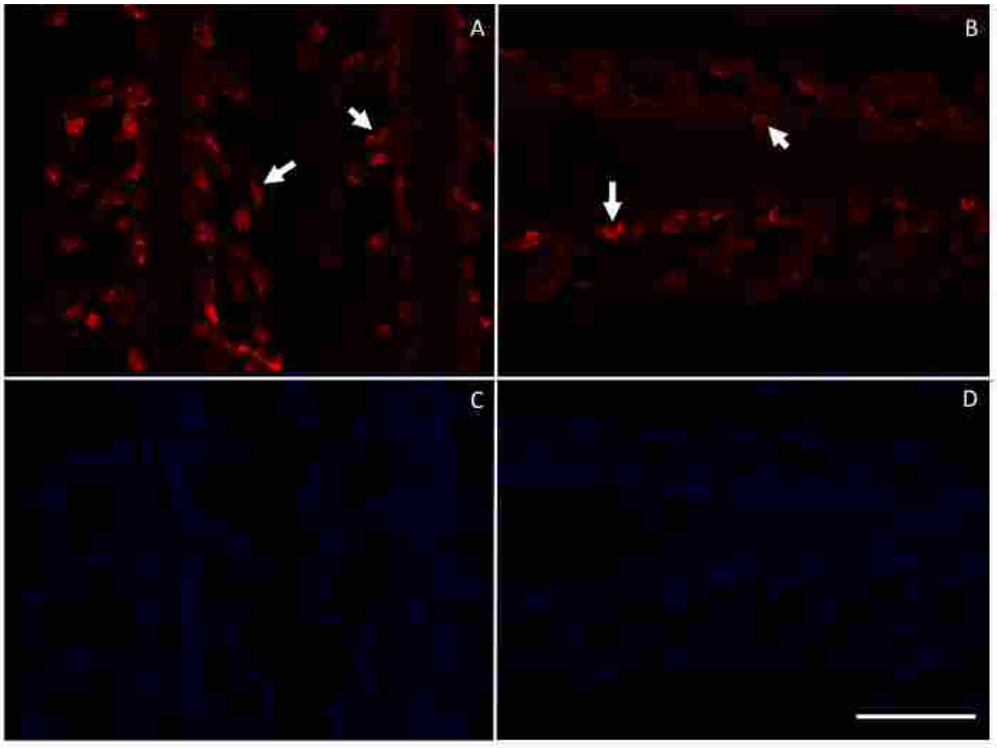


Figure 2.9 Immunolocalization and relative immunofluorescence of Na^+,K^+-ATPase (NKA) transporter in TFM exposed gills of juvenile rainbow trout.

Relative immunofluorescence of gill transporter expression in control juvenile rainbow trout (*Oncorrhyncus mykiss*) and during exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h LC_{50} concentration (7.6 mg L^{-1}) for 9 h. Data are expressed as the mean +1 SEM (*N*; $p<0.05$).

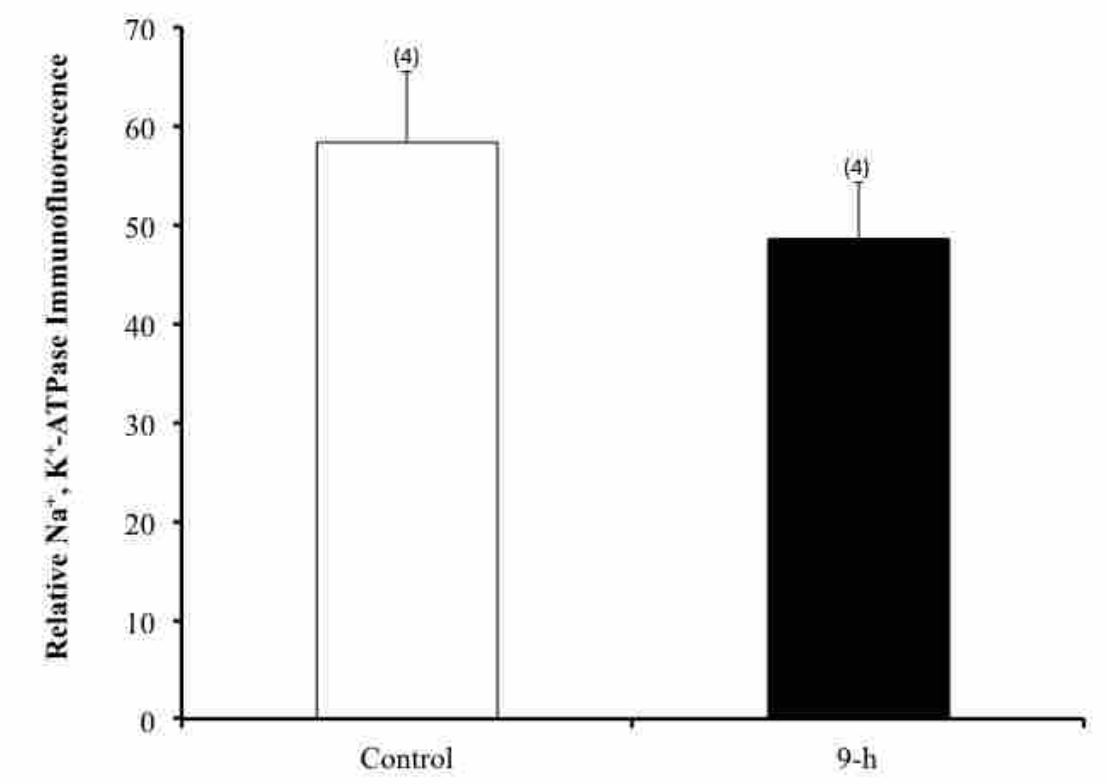

Relative Na+, K+-ATPase Immunofluorescence
70
60
50
40
30
20
10
0
(4)
(4)
Control
9-h

Figure 2.10 Effects of TFM on gill structure of juvenile rainbow trout.

Haematoxylin and eosin stained trout gills of (a) control fish and fish exposed to $LC_{99.9}$ of TFM for larval lamprey (7.6 mg L^{-1}) for (b) 3 h, (c) 6 h and (d) 9 h. Gill sections were of 4–5 μm thickness. Scale bar, 50 μm.

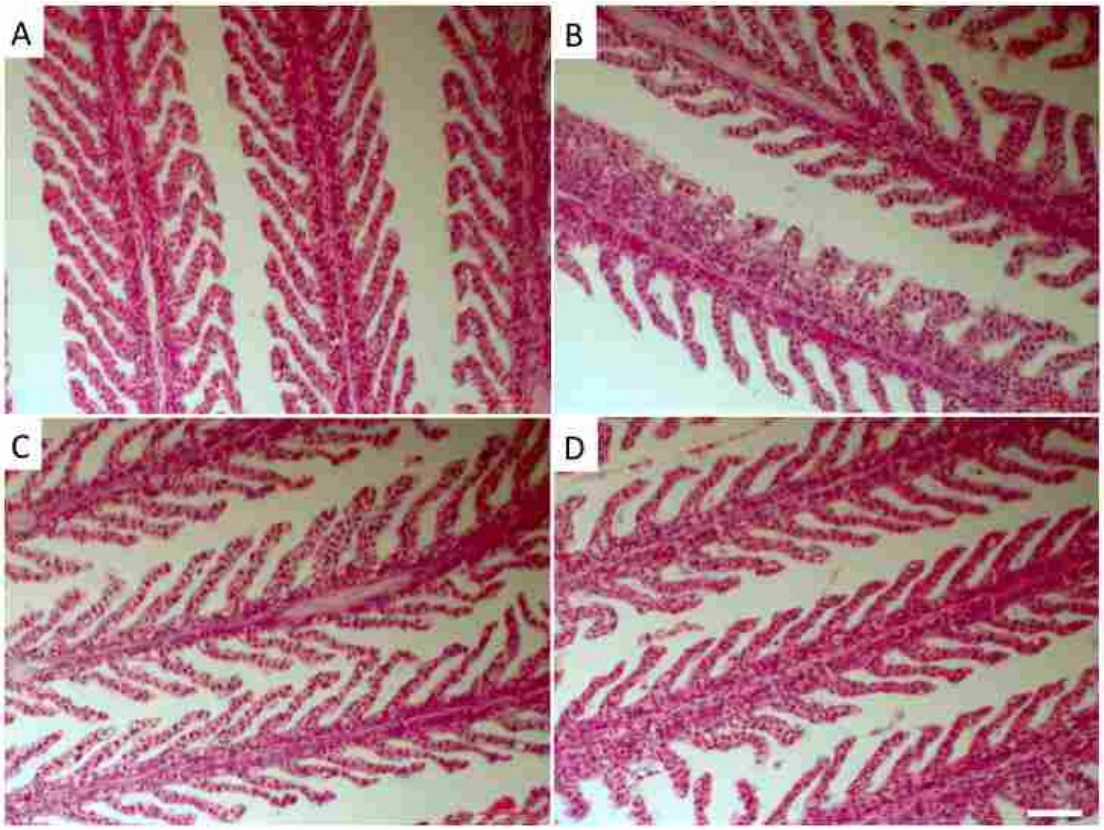
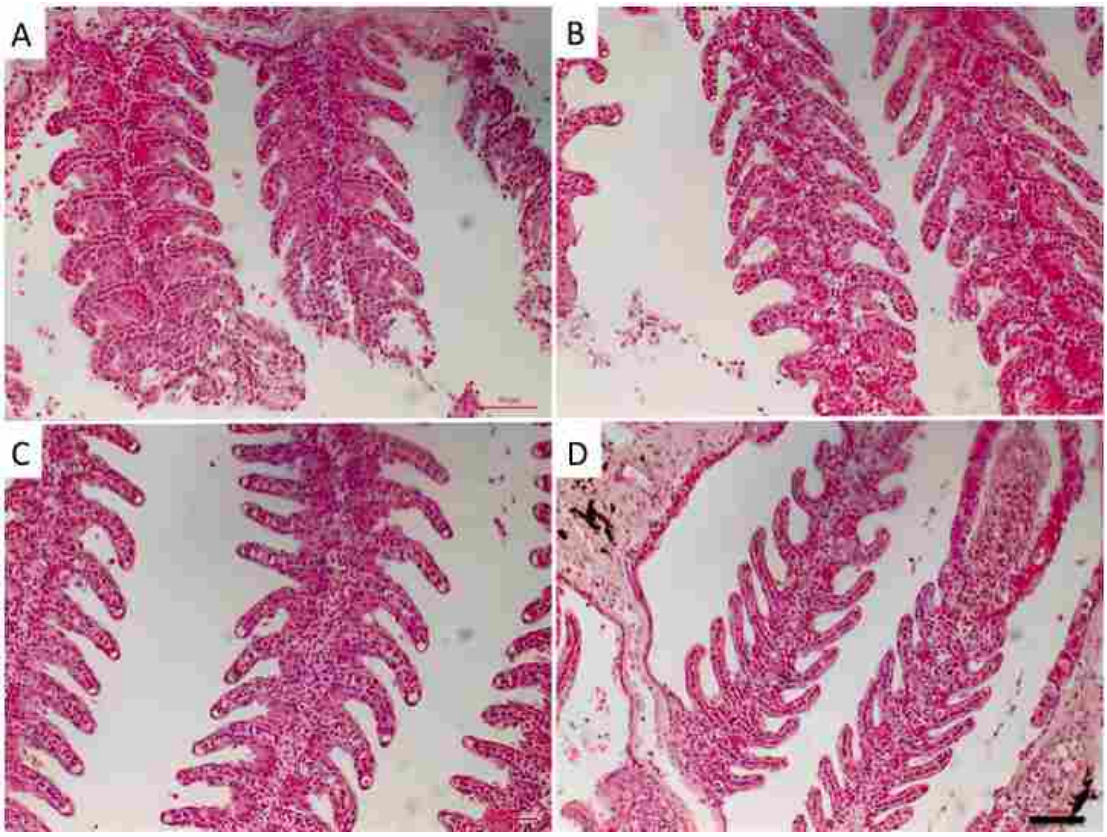

A
B
C
D

Figure 2.11 Effects of TFM on gill structure of larval lamprey.

Haematoxylin and eosin stained lamprey gills of (a) control fish and fish exposed to LC_{50} of TFM for larval lamprey (4.6 mg L^{-1}) for (b) 3 h, (c) 6 h and (d) 9 h. Gill sections were of 4–5 μm thickness. Scale bar, 50 μm.


A
B
C
D

4. Discussion

4.1 The Effect of TFM on Ionoregulation

Exposure to TFM did not have any notable adverse effects on gill function or ionoregulation in the rainbow trout or in the larval sea lamprey. Because TFM interferes with mitochondrial ATP production in these fishes (Birceanu *et al*. 2011), it was predicted that both the NKA and V-ATPases would become starved of ATP thereby leading to reductions in plasma Na^+ and impaired metabolic H^+ extrusion. Our results did not support this hypothesis. Rather it appears that TFM only had minor transient effects on ion regulation in lamprey and rainbow trout, despite the decreases in ATP supply that are known to result from TFM exposure (Wilkie *et al.,* 2007; Birceanu *et al.,* 2009; 2011; 2014)

The 12 h $LC_{99.9}$ (7.6 mg L^{-1}) of larval sea lamprey represents a concentration that approaches levels to which these animals or non-target fishes could be exposed during a typical lampricide treatment. Typically, sea lamprey control agents apply TFM at 1.2-1.3 times the minimal lethal concentration (MLC = 12 h $LC_{99.9}$) of TFM (McDonald and Kolar, 2003; Christie *et al.,* 2003), well below lethal levels for trout (Boogaard *et al*., 2003; Birceanu *et al*., 2014). However, TFM exposure initially resulted in greater Na^+ losses, possibly through increases in either blood flow to the gills and/or increases in branchial permeability due to the TFM itself. Nevertheless, the fish were able to readily recover from this loss by 9 h through reductions in $J_{out}^{Na^+}$, which were likely brought about through decreases in branchial ion permeability. This net loss of Na^+ did not physiologically impact plasma ion concentrations, as plasma Na^+ remained unchanged for the duration of the experiment (Figure 2.1). Indeed, the total net Na^+ loss in the trout during TFM exposure was 3.54 mmol/kg, which is only 10% of the total exchangeable Na^+ pool (35 mmol/kg; Grosell *et al.,* 2002) and unlikely to have significant adverse affects. During exposure to toxic

concentrations of copper, Grosell *et al*. (2002) suggested that mortality is generally seen in fishes when the total exchangeable Na^+ pool is reduced by 30 %.

A transent increase in NKA activity at 6 h likely played a role in compensating for the initial Na^+ losses observed, which returned to control values by 9 h TFM exposure (Figure 2.4 and 2.5). NKA has 2-subunits, and is located on the basolateral membrane of MRCs (Karnaky *et al.,* 1976; McCormick, 1990). The catalytic α-subunit binds ATP and Na^+, and has an extracellular K^+-binding site, which binds ouabain, a well-known antagonist of the transporter. A smaller β subunit is glycosylated and functions to direct the complex to the plasma membrane (Marshall, 2002). Changes in NKA activity was not found to be correlated with increased abundance of the α-subunit (Figure 2.5; 2.8; 2.9). Therefore, the changes observed in NKA activity at 6 h were likely due to post-transcriptional activation. NKA is believed to be post-transcriptionally activated via protein kinase A and cAMP (Tipsmark and Madsen, 2001), resulting in increased enzyme activity within a few hours in salmonids following transfer to sea water (Mancera and McCormick, 2000; Tipsmark and Madsen, 2001). Increases in NKA activity have also been reported to occur as rapidly as 1 h in killifish (Towle *et al.,* 1977). Longer-term increases in NKA activity are primarily due to protein synthesis (Mancera and McCormick, 2000). This study suggests that the changes in NKA activity that were observed during TFM exposure in the rainbow trout were likely a compensatory reaction in response to a decreased availability in ATP supply in the fish and due in part to the Na^+ losses seen earlier on in the exposure. The significant rise in total ATPase activity after TFM-exposure (Figure 2.4) indicates that while the effects of TFM were minimal on individual transporters, collectively, there was a compensatory response characterized by an increase in ATPase-mediated transport that likely arose due a TFM-induced deficiency in ATP-supply.

One possible explanation for initial loss of Na^+ observed in this study is that a decrease in ATP-availability due to the uncoupling effects of TFM on oxidative

phosphorylation could have stimulated the hypoxic response in sea lampreys and non-target fishes, despite an abundance of oxygen in the blood and tissues. Adenosine is produced locally and rapidly via the breakdown of ATP and is believed to serve as an endogenous signal during hypoxia, with a 1% drop in ATP resulting in a 900% increase in adenosine (Bickler and Buck, 2007). When ATP concentrations decrease, adenosine concentrations accumulate stimulating vasodilation in cardiac and respiratory tissues, and oxygen uptake pathways (Collis and Hourani, 1993). For instance, only 58% of the vasculature in the secondary lamellae of resting rainbow trout are perfused with blood (Booth, 1978). When oxygen demands increase, the vascular surface area increases in order to meet the greater metabolic demands (Booth, 1979). However, there are consequences for increasing the functional surface area of the gills, mainly ion loss increases as the gills become more permeable for oxygen-uptake. This is known as the 'osmoregulatory compromise' (Nilsson, 1986; Gonzalez and McDonald, 1992). As a result of increased perfusion of the gill vasculature during exercise, an increased hydrostatic pressure is created by greater blood flow leading to an increase in ion loss (Randall *et al.,* 1972; Wood and Randall, 1973; Gonzalez and McDonald, 1992). Thus initial increases in Na^+ loss during exposure were likely a result of increases in the functional surface area of the gill to promote oxygen uptake, and ultimately ATP production. While whole animal MO_2 rates were not measured in this experiment, previous *in vivo* and *in vitro* experiments have found increased rates of O_2 consumption during TFM exposure (Smith and King, 1969 as cited by Kawatski and McDonald 1974; Kawatski *et al.,* 1974). Subsequent reductions in branchial permeability due to changes gill ultrastructure and/or reductions in blood flow could have resulted in the restoration of $J_{in}^{Na^+}$ to pre-exposure levels. However, measurements of branchial perfusion, oxygen consumption, and unidirectional ion movements are needed to test this hypothesis.

In larval lamprey exposed to the 12 h LC_{50} (4.6 mg L^{-1}), there was a steady decline of plasma Na^+ concentration during the exposure period, which persisted through 24 h of recovery in TFM-free water (Figure 2.3). However, there were no reductions or compensatory increases in either V-ATPase expression (Figure 2.7b) or NKA activity and/or expression (Figure 2.6 and 2.7a) during TFM exposure or the 24 h recovery period. Similar findings were reported by Birceanu *et al.* (2009), who observed no changes in NKA activity, tissue or plasma ions, or Na^+ uptake in larval sea lamprey during exposure to its TFM 12 h LC_{50} (4.6 mg L^{-1}; Birceanu *et al.,* 2009). However, the effects of TFM on the gills may persist in larval sea lampreys after TFM exposure due to ongoing impairment of ATP synthesis and/or acid-base disturbances. Indeed, persistent reductions in liver glycogen and ATP were reported in larval lamprey following short-term exposure (6 h) to the 12 h $LC_{99.9}$ of TFM, which did not return to control concentrations until after 24 h recovery (Clifford *et al.,* 2012).

It is important to note, that enzyme activity assays are measured under optimal conditions, which would make it difficult to determine if a lack of ATP supply impaired ion uptake in larval sea lamprey. Thus, impairments to ATP supply, leading to decreased NKA activity and less Na^+ uptake across the basolateral membrane, could have been sufficient to slow rates of Na^+ uptake resulting in decreased plasma Na^+. The slower recovery and more persistent ionic disturbances experienced by the lamprey compared to the trout was likely due to the slower TFM clearance from the sea lamprey body. Unlike the trout, which can rapidly clear TFM from its body within 1 h of post-TFM recovery (Le Clair, 2014), sea lampreys have a low capacity to conjugate TFM to the more water soluble TFM-glucuronide (Lech and Statham, 1975; Kane *et al.,* 1993; Hubert *et al.,* 2005) which can readily be cleared via the bile or renal systems (Hunn and Allen 1975; Schultz *et al.,* 1979; Birceanu *et al.,* 2014; Le Clair, 2014). As a result, the sea lamprey likely suffered from the "run-on" effects of TFM, even after TFM exposure ceased, which could include an impaired ability to correct post-TFM exposure ionic disturbances.

The most plausible route for TFM removal in larval lampreys is the gill. TFM is a weak acid with a pKa of 6.07 (Hubert *et al.,* 2003), and exists as the ionized phenolate form ($TFM\text{-}O^-$) or the un-ionized (TFM-OH) phenol form. At lower pH, the lipophillic un-ionized free-phenol form predominates, which readily crosses the lipid bilayers of the gill epithelium and enters the blood stream (Hunn and Allen 1974; Tessier *et al.,* unpublished findings). The physiological pH of blood is approximately 7.85 (Boutilier *et al*. 1993). At this pH, TFM exists primarily in its phenolate form (McDonald and Kolar 2007) and therefore outward diffusion gradients from the gills would be unfavourable if TFM was still present in the water (Hunn and Allen, 1975). However, TFM elimination would still remain slow during the depuration period in TFM-free water because most of the TFM would be present in the blood as ionized TFM-O- at physiological pH, not the more diffusible un-ionized form of TFM. As a result, ATP supply could still be reduced leading to persistent decreases in plasma Na^+ concentration during the recovery period in TFM-free water.

4.2 The Effect of TFM on Acid-Base Balance

While there were only minor effects of TFM on ion regulation in rainbow trout and sea lamprey, it still remains unclear if TFM affects acid-base homeostasis. Fish prevent acid-base disturbances via physiochemical buffering mechanisms and the transport of acid/base equivalents across the gill using ion exchange proteins. The bicarbonate buffering capacity of fishes is low compared to terrestrial animals since metabolically generated CO_2 readily passes through the gills in well-aerated water, leading to not only a lower blood pCO_2, but also lower total CO_2 and HCO_3^- in fish (Boutilier *et al.,* 1984; Tufts *et al.,* 1988; Evans *et al.,* 2005). As a result, the bicarbonate buffering capacity in the blood of fishes is very low compared to terrestrial vertebrates, forcing them to rely on the exchange of acid-base equivalents with their environment for acid-base regulation. Approximately, 90% of net acid-base transfers occur at the gills and involves the secretion of endogenous acid (H^+ and/or NH^+_4) and base

(HCO_3^- and/or OH^-) with the apical absorption of Na^+ and Cl^-, respectively, independent of one another (Evans *et al.,* 2005).

The V-ATPase has been demonstrated to play a key role in correcting hypercapnic acidosis leading to the proposal that acid secretion mechanisms are electrically linked via an apical V-ATPase transporter to Na^+-absorption through a ENaC-transporter channel in MRCs (Lin and Randall, 1993; Lin *et al.,* 1994), or within the pavement cells (Goss *et al.* 1992; Sullivan *et al.,* 1995; Fenwick 1999; Wilson *et al.,* 2000). In the present study there were no changes in V-ATPase activity or abundance in either fish during TFM exposure or plasma Na^+ and Cl^-. At first glance, this might imply that neither the trout nor the sea lamprey experienced TFM-induced acid-base disturbances. However, the post-TFM exposure reductions in plasma Na^+ and maintenence of plasma Cl^- may be suggestive of a post-TFM exposure alkalosis in lamprey, which is indicative of a sustained compensatory metabolic alkalosis during the period of TFM exposure when TFM is known cause lactate accumulation, which is normally associated with the simultaneous development of a metabolic acidosis (Wilkie *et al.,* 2007; Birceanu *et al.,* 2009; 2014). However, direct measurements of blood and tissue acid-base status are needed to confirm this hypothesis.

4.3 The Effect of TFM on Gill Histology

The lack of notable changes in gill histology following TFM exposure in both larval lamprey and rainbow trout suggests that the damage incurred by the gills was minimal (Figure 2.10; 2.11). Similar to Mallatt *et al*. (1994,1995), we found no evidence that the gills of rainbow trout were damaged by TFM exposure and only minimal changes to gill histology in larval lamprey were observed in response to treatment concentrations. Prior studies had shown that toxic exposure to TFM causes dilation of the vascular beds and edema within the connective tissue between the respiratory epithelium and vascular stasis with an increased number of erythrocytes of the arterioles and capillaries in both trout and lamprey at 3 and 6

mg L^{-1} TFM (Christie and Battle, 1963). However, we found no evidence of vascular edema in the present sea lamprey or rainbow trout following TFM exposure. In larval sea lamprey exposed to the 9 h $LC_{99.9}$ of TFM, scanning and transmission electron microscopy revealed that there were increases in the intercellular spaces in the epithelium and rounding of the MRCs (Mallatt *et al.,* 1994). This ultrastructural examination also revealed a disappearance of microvilli, increased vacuolization in the cytoplasm, an enlargement of the nuclei, and alterations to the mitochondrial cristae of these cells in larval lamprey, leading to the suggestion that TFM damages the MRCs (Mallatt *et al.,* 1994). However, the present and earlier studies (Birceanu *et al.* 2009; 2014) do not support this hypothesis.

Differences in either water quality and/or the TFM doses used could explain the differences between the present study and the earlier work of Christie and Battle (1963) and Mallatt *et al.* 1994). In our study, we examined the LC_{50} for larval lamprey with a water hardness of 450 mg L^{-1} $CaCO_3$. Water quality was not reported in the work of Christie and Battle (1963). However, Mallatt *et al.* (1984;1994) exposed larval to their TFM $LC_{99.9}$ in much softer water than used here, approximately 130 mg L^{-1} $CaCO_3$. It is well known that the toxicity of TFM increases with decreasing pH and alkalinity (Bills *et al.* 2003), but a few studies have suggested that TFM toxicity is also increased in soft waters in developing rainbow trout (Olson and Marking, 1973) and in aquatic midges (Kawatski and Bittner, 1975). Perhaps under these conditions, and/or in waters of lower pH or alkalinity, gill damage is more pronounced with TFM exposure.

Although this study did not note any structural damage to the gills, one interesting observation during light micrscopy analysis was a decrease in interlamellar cell mass (ILCM) during prolonged exposure to TFM in the larval sea lamprey. This was not seen in control animals where there was a pronounced interlamellar cell mass (ILCM) between adjacent lamellae in the gills of larval sea lamprey examined (Figure 2.11). This prelimary observation may suggest that in response to decreased ATP-availability due to TFM-exposure, the larval

sea lamprey could decrease their interlamellar cells to increase the lamellar surface area for branchial gas exchange during TFM exposure. Similar gill remodeling has been noted in crucian carp (*Carassius carassius*) and in goldfish (*Carassius auratus*) with environmental hypoxia and increased temperature (Sollid *et al*., 2003). During both short-term and chronic exposure to hypoxia, crucian carp have been found to reduce ILCM at a temperature dependent rate, with higher temperatures (20°C) and hypoxia resulting in the almost complete loss of the interlaemellar cell mass (Sollid *et al.,* 2003; Sollid *et al.,* 2005a; Sollid *et al.,* 2005b; Sollid *et al.,* 2006). Long-term exposure to copper (300 μg L^{-1} in the ambient water) was also found to increase the ILCM in Crucian carp, but also corresponded to a greater tolerance to high copper concentrations (Schjolden *et al.,* 2007). Gill remodeling is not unique to hypoxia in Crucian carp. Similar increases in ILCM have been found for aluminum in juvenile brook trout (Mueller *et al.,* 1991) and zinc in rainbow trout (Lappivaara *et al.,* 1995).

Nilsson *et al*. (2012) has suggested that the widespread occurrence of the interlamellar cell mass may be a key mechanism for reducing the uptake of toxic substances. Lampreys are a phylogenically ancient species that spend the majority of their time buried in the soft sediment of streambeds as larvae (Beamish, 1980; Youson, 1980) where periodic fluctuation in oxygen availability is likely, and where they could be exposed to numerous toxic substances and subject to pronounced variations in water temperature. It is possible that this change in ILCM may be an acclimatory response to fluctuations in oxygen availability akin to crucian carp and may also serve an important role in counteracting environmental insults, as well as TFM exposure.

4.4 Conclusions and Future Directions

Despite its well known effects on oxidative phosphorylation and ATP supply, TFM had minimal effects on gill function and ionoregulation in larval sea lampreys exposed to

lethal TFM concentrations, and in rainbow trout exposed to doses of TFM that they would likely encounter during field applications of the lampricide. Moreover, juvenile rainbow trout were remarkably resilient to TFM exposure, and this tolerance to TFM seems to be due to their superior ability to detoxify TFM via the liver UDP glucuronyl-transferase (Birceanu *et al.,* 2014).

While the effect of TFM on lamprey is more pronounced, disturbances to ion balance were relatively minor. It is important to note, however, that the sea lamprey in the present study were exposed to the LC_{50} of TFM, not 1.2-1.3 times the $LC_{99.9}$, which is typically used for field applications of the lampricide (McDonald and Kolar, 2007). For the purpose of our experiment, the LC_{50} was chosen to gain a better resolution of how TFM impacts sea lamprey osmoregulation without introducing confounding artifacts following mortality. Thus, at higher concentrations, the physiological impact of TFM on the gills could be greater. Indeed, the declines in plasma Na^+ concentration that were observed suggest that at higher doses of TFM, ionoregulatory disturbances could be more pronounced in larval sea lampreys. While minor, the present experiments demonstrated that there is the potential for TFM-sensitive species to experience gill function impairments when exposed to treatment doses of TFM. Because sensitivity to TFM has been found to be specie-specific and age-dependent (Olson and Marking, 1973; Boogaard *et al.,* 2003), efforts should be made to identity and to better characterize how gill mediated ion exchange and acid-base regulation in TFM sensitive-species are affected by TFM exposure at environmentally relevant concentrations.

CHAPTER THREE:

The Effect of Lampricide, 3-Trifluoromethyl-4-nitrophenol, on Protected Juvenile Lake Sturgeon

1. Introduction

The lake sturgeon, *Acipenser fulvescens*, is one of the mostly widely distributed fish species in North America and is endemic to three key watersheds – the Laurentian Great Lakes, Hudson-James Bay, and the Mississippi River – and five provinces in Canada – Alberta, Saskatchewan, Manitoba, Ontario, and Quebec (Harkness and Dymond, 1961; Scott and Crossman, 1973; Houston, 1987; Peterson *et al.,* 2007). However, current populations have significantly declined and/or been extirpated in the lower Laurentian Great Lakes including Lake Ontario, Lake Erie, and Lake Michigan (Ferguson and Duckworth, 1997), with populations only at 1% of their former numbers in Lake Michigan (Hay-Chmielewski and Whelan, 1997). Lake sturgeon are presently listed as endangered in Illinois, Indiana, Ohio, and Pennsylvania, threatened in Michigan, New York, Canada, and of special concern in Minnesota and Wisconsin (Heinrich *et al.,* 2003; Species at Risk Public Registry, 2014). The decline of Lake Sturgeon populations are thought to be primarily due to low fecundity, delayed sexual maturation, low rates of recruitment, commercial over-harvest, and habitat degradation (Harkness and Dymond, 1961; Peterson *et al.,* 2007). They are particularly susceptible to anthropogenic disturbances (Sutton *et al.,* 2003). Despite efforts made to improve water quality, the recovery of lake sturgeon populations has not yet been achieved in any of the Great Lakes (Sutton *et al.,* 2003).

Concerns have been raised that efforts to restore lake sturgeon populations may be confounded by the need to use the pesticide, 3-trifluoromethyl-4-nitrophenol (TFM), to control invasive sea lamprey (*Petromyzon marinus*) populations in the Great Lakes (Christie and Goddard, 2003; McDonald and Kolar, 2007). This chemical has been applied to larval sea lamprey nursery streams for sixty years, which has helped to control parasitic sea lamprey populations in the Great Lakes, and allowed the re-establishment of many commercial, recreational, and culturally significant fisheries (McDonald and Kolar, 2007). However, some

populations of fishes and amphibians, including the lake sturgeon and mudpuppy (*Necturus maculosus*), are sensitive to relatively low doses of TFM (Boogaard *et al.,* 2003). Juvenile lake surgeons are particularly vulnerable to TFM when they are less than 100 mm in length (Johnson *et al*., 1999; Boogaard *et al.,* 2003). With lake sturgeon populations continuing to decline (DeHaan *et al.,* 2006), a better understanding of how TFM adversely affects lake sturgeon physiology could make it possible to predict how sturgeon populations are potentially affected by TFM, and if necessary, to modify the dose and/or timing of TFM treatments when lake sturgeon are present.

The mode of action of TFM is impaired ATP production, which takes place via the uncoupling of oxidative phosphorylation in the mitochondria (Niblett and Ballantyne, 1976; Birceanu *et al.,* 2011). As a result, the supply of ATP does not match demand, leading to significant reductions in high-energy phosphagens such as phosphocreatine in sea lamprey and rainbow trout (*Oncorhynchus mykiss*) and phosphoarginine in molluscs (Viant *et al.,* 2001; Wilkie *et* al, 2007; Birceanu *et al.,* 2009; Clifford *et al*., 2012). Glycogen reserves are also often reduced, particularly in the brain due to increased reliance on anaerobic glycolysis to generate ATP (Birceanu *et al.,* 2009; Clifford *et al*., 2012).

Related to these metabolic disturbances, is the possibility that TFM could also directly impact ATP-dependent transport processes related to acid-base and ionoregulation (Mallatt, 1985; Mallatt *et al.,* 1994; 1995). In larval sea lamprey, TFM was reported to cause morphological changes to gill ultrastructure, particularly the mitochondrial rich cells (MRCs), but not in more tolerant fish such as the rainbow trout (Christie and Battle, 1963; Mallatt *et al.,* 1994; 1995). The MRCs are intricately involved in maintaining acid-base and ion balance via apical Na^+/H^+ exchange (NHE) or a bafilomycin-sensitive apical vacuolar proton ATPase transporter protein (V-ATPase), which serves to transport protons out of the cell, generating a electrical gradient that drives passive Na^+ influx through an epithelial Na^+ channel (ENaC; Lin *et al.,* 1994; Bury and Wood, 1999; Fenwick *et al.,* 1999; Wilson *et al.,* 2000; Kumai and

Perry, 2012). Once transported into the MRC, Na^+ is then taken up into the blood via an ouabain-sensitive basolateral located Na^+, K^+-ATPase (NKA; Bartels *et al.,* 1998; Marshall, 2002; Bartels and Potter, 2004; Wilkie, 2011). The overarching goal of the present study was to determine if TFM impaired ion balance in the lake sturgeon due to interference with ATP-production, which reduces ATP-supply necessary to power the NKA and/or V-ATPases in the gill.

In the present study, sub-sets of juvenile lake sturgeon were exposed to a sub-lethal dose of TFM (12 h LC_{50}) for 9 h, and sampled at regular time intervals (3, 6, 9 h), or allowed to recover from TFM in clean, TFM-free water 24 h prior to sampling. At each sample period, blood and gill tissue was collected and processed for the measurement of plasma ion levels, NKA and V-ATPase activity and quantity. Potential damage to the gills by TFM was evaluated by examining the gross morphology of the gills following TFM exposure.

2. Methods and Materials

2.1 Animals and Experimentation

2.1.1 Animal Holding and Experimentation

Juvenile lake sturgeon (*A. fulvescens*; 3.0 ± 1.1 SD g, 93 ± 9.5 SD mm, *N*=120) were provided courtesy of L. O'Connor, Sea lamprey Control Centre, Fisheries and Oceans Canada (DFO), Sault Ste. Marie, ON. The fish were transported to Wilfrid Laurier University in aerated coolers, and then transferred to 500 L living streams receiving Wilfrid Laurier well-water on flow-through basis (pH ~ 7.8, 10-13°C, water hardness ~ 450 mg $CaCO_3$, 80-100% dissolved O_2) in a carbon-filtration system. Fish were held under a 12 h light: 12 h dark photoperiod and fed daily to satiation with frozen bloodworms (Hikari Bio-Pure, Hikari, Sales USA Inc, CA, USA). The fish were acclimated to lab conditions for a minimum of 4 weeks and starved for 48 h immediately prior to experiments. All experiments and fish

housing were approved by the Wilfrid Laurier Animal Care Committee and followed Canadian Council of Animal Care guidelines.

2.1.2 Sensitivity of the Gills to TFM exposure

Fish were acclimated overnight in well aerated, 450 ml experimental buckets, continuously receiving aerated, Wilfrid Laurier well water. The next day, water flow was shut off and the juvenile lake sturgeon (*N*=40) were exposed to the nominal 12 h TFM LC_{50} for larval lamprey (4.6 mg L^{-1}; Birceanu *et al.,* 2009) and sampled following 0, 3, 6, and 9 h of TFM exposure, and following a 24 h recovery period in clean TFM-free water. The TFM used was prepared from a powdered stock (N27802; Sigma Aldrich, St. Louis, MO), which was dissolved in e-pure water prior to application to the experimental containers. Since TFM is photosensitive, all exposures were conducted in the dark (Carey and Fox, 1981). Following each sample point, fish were euthanized in a solution of 1 part tricaine methanesulfonate (MS222) : 2 parts sodium bicarbonate solution (0.5 g MS222 anaesthetizing dose and 1.0 g MS222 killing dose). Blood was then sampled by caudal severance posterior to the urogenital opening and collected into heparinized capillary tubes, and centrifuged for 5 minutes at 10,000 x *g*. The plasma was then collected, flash frozen in liquid nitrogen, and stored at -80°C for later plasma ion analysis. The gills (arches 2 and 3) were excised from each side of the fish. The gills (left side) were flash frozen in liquid nitrogen and stored at -80ºC until processed for measurements of NKA and V-ATPase transporter activity and quantity. The gill tissue from the right side was fixed in 1:20 volume of gill to 10% buffered formalin solution (pH 7.4) for 24 h at 4°C, rinsed with 1X Phosphate Buffer Solution (pH 7.5), and stored in 70% ethanol at 4°C.

Exposure concentrations of TFM were measured spectrophotometrically, using precision standards provided by DFO, on a SpectraMax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 396 nm.

2.2 Analytical Techniques

2.2.1 Plasma Na^+ and Cl^-

Plasma Na^+ concentrations were measured and quantified by atomic absorption spectrometry (SpectrAA 880, N_2 gas; Varian, 171, Mississauga, ON). Concentrations of plasma Cl^- were determined colorimetrically using the mercuric thiocyanate assay on the plate spectrophotometer (Zall *et al.* 1956).

2.2.2 Na^+,K^+-ATPase and Vacuolar H^+-ATPase Activity

Branchial NKA and V-ATPase activity were measured using a modified enzyme kinetic microplate assay (McCormick, 1993; Reis-Santos *et al.,* 2004). Activity measurements were made as written in Chapter 2 for trout (Methods and Materials 2.3.2). Briefly, frozen gill tissue (~40 mg) was thawed in 400 µl SEI buffer and homogenized with a motorized pestle following the addition of 100 µl of SEID (0.1% sodium deoxycholic acid in SEI). Samples were then centrifuged at 10,000 x *g* for 3 minutes and 5 µl of the supernatant was pipetted into a 96-well microplate in quadruplicate for total ATPase, NKA, and V-ATPase. Each well of the microplate contained 50 µl of the salt solution and 150 µl of the assay mixture (with 1.0 mM ouabain added to both the NKA and V-ATPases assay mixture and 10 µM bafilomycin A1 V-ATPase assay mixture), which was then analzed on a 96-well microplate spectrophotometer at 25°C and changes in absorbence were measured every 30 seconds for 20 minutes with SOFTmax software (SpectraMax 190, Molecular Devices, CA). Activity due to NKA was measured as the difference between total ATPase activity and ouabain-inhibited activity. Similarly, V-ATPase was measured as the difference between ouabain-inhibited activity and ouabain- and bafilomycin-inhibited activity. The amount of ATP (nmol) consumed by the ATPases was determined using an ADP standard curve and total protein concentration of the supernatant was measured using the Bradford Protein Assay with a bovine serum albumin (BSA) standard (ALB001-250; Bioshop, Burlington, ON) to

calculate activity. The remaining supernatant from the activity assay (~200 µl) was then diluted with an equal volume of 2X Laemmli's buffer, heated to 70°C for 10 minutes in a water bath, and stored at -80°C for future SDS-Page analysis.

2.2.3. Antibodies

As was described in Chapter 2 (Material and Methods 2.3.3), the pan-specific α5 mouse monoclonal antibody purchased from the Developmental Studies Hybridoma Bank at the University of Iowa and developed by Douglas Fambrough was used to detect NKA (Johns Hopkins University; Takeyasu, 1988). The affinity-purified rabbit anti-peptide polyclonal B2/BvA1 antibody was also used to detect V-ATPase, which was donated by J. Wilson at Centro Interdisciplinar de Investigação Marinhae, Ambienta (CIIMAR; Porto, Portugal). Both antibodies have previously demonstrated specific immunoreactivity with their corresponding transporters in fish (Witters *et al.,* 1996; Wilson and Laurent, 2002; *et. al.*, 2004; Reis-Santos *et al.,* 2004).

2.2.4. SDS Page and Western Immunoblotting

SDS-Page and Western Immunoblotting was used to analyze NKA and V-ATPase transporter expression and followed the protocol outlined by Reis-Santos *et al.* (2004) as is written in Chapter 2 (Material and Methods 2.3.4). Briefly, samples previously diluted in Laemmli's buffer during the enzyme activity assay were thawed and further diluted to a final total protein concentration of 0.35 µg/µl with 1X Laemmli's buffer. Samples were then vortexed, heated to 70°C for 10 minutes, and centrifuged for 5 minutes at 10,000 rpm. After centrifugation, 40 µl of sample was loaded onto 1.5 mm thick mini polyacrylamide gels (8%T resolving gel and 4% stacking gel) in duplicate with Precision Plus Protein Western C Standards protein markers (#161-0376; BioRad, Hercules, CA) and run at 150V for 1 h using a Biorad Mini-PROTEAN® Tetra cell system (BioRad, Hercules, CA). Once completed, the gels were equilibrated in transfer buffer, followed by wet-transfer to PVDF membranes using

the Biorad Mini Trans-Blot® Cell system for 1 h at 100 V (BioRad, Hercules, CA). After transferring proteins to the membranes, the membranes were rinsed in 1X TTBS and Ponceau S staining was used to confirm transfer-efficiency to PVDF membranes. Membranes were then blocked for 1 h with 5% dry milk 1X TTBS, rinsed in 1X TTBS, and incubated overnight on a shaker with either α5 or B2/BvA1 antibodies (diluted 1:1000 in 1%BSA/0.05% sodium azide/1X TTBS) at 4°C. The next day, membranes were rinsed with 1X TTBS and incubated with peroxidase-conjugated secondary goat anti-rabbit or anti-mouse Ig antibody and Precision Protein™ StrepTactin-HRP conjugate diluted 1:20,000 in 1X TTBS for 1 h. Following incubation, membranes were washed with 1X TTBS and bands were detected with Clarity Western ECL Substrate (BioRad, Hercules, CA). Densitometry of the bands were semi quantified using SigmaScan Pro Software Version 1.0 (Systat Software Inc., San Jose, CA). Relative density was calculated by comparisons to the average of the controls. The absence of the primary antibody in the dilution buffer was used as a no primary control.

2.2.5. Light Microscopy

Changes to gill structure due to TFM exposure was assessed using light microscopy. Gill tissue was prepared for light microscopy by fixing tissues in 20% DMSO/methanol at –20°C for 48 h followed by storage in 100% methanol at –20°C and processing for paraffin embedding (type 6; Richard-Allan Scientific, Kalamazoo, MI, USA). Following paraffin embedding, 5 μm sections were collected onto 3-aminopropyltriethoxysilane-coated slides (Sigma Aldrich; St. Louis, MO) and subsequently stained using a standard haematoxylin and eosin (H&E) staining protocol. Slides were then examined using a Jenaval light microscope fitted with Zeiss Axiocam MRC 5 camera and imaged with the Zen 2011 Blue Edition software (Carl Zeiss MicroImaging Gmbh, Germany). Slide and staining preparation was conducting by Jonathan M. Wilson at CIIMAR (Porto, Portugal) and both imaging and

analysis was done at Wilfrid Laurier University. Damage to the gills was then graded on according to Kiemer and Black (1997) on a 1 to 6 scale with the following criteria:

Grade 1: Healthy pseudo-stratified epithelial cells with few mucous cells on the gill lamella

Grade 2: Minor hyperplasia or clubbing of gill lamella on the distal ends with minimal hypertrophy of the mucous cells and no contour disturbance.

Grade 3: Minor to moderate hyperplasia or clubbing of gill lamella at the distal ends and hypertrophy of the mucous cells with increased visible folding and/or wrinkling of the surface epithelial cells

Grade 4: Extensive hyperplasia of epithelial and mucous cells, fusion of the lamellae, and/or necrosis.

Grade 5: Appearance of desquamation and/or lifting of epithelium over a large area

Grade 6: Total necrosis of the gill epithelium, complete loss of lamella structure, and death of fish.

2.3 Statistical Analysis

Data were presented as the mean ± 1 standard error of the mean (SEM). All comparisons were unpaired and statistical significance was determined using Prism 6 Version 1.0 (GraphPad Software Inc, La Jolla, CA). TFM exposure and Post-TFM Recovery were treated as separated experiments. Therefore, significance between controls and TFM-exposure groups were determined using one-way analysis of variance (ANOVA), followed by a Holm-Sidak post-test when significant variability was observed between sample groups at the $p<0.05$ level. When the conditions for homogeneity of variance were not met, a nonparametric ANOVA test was used for comparisons between control and TFM-exposure groups followed by Dunn's post-test. An unpaired T-test, using Welch correction when

necessary, was used for comparisons between the control and post-TFM exposure recovery group.

3. Results

3.1 Plasma Ion Concentrations

Plasma concentrations of Na^+ were not significantly altered during TFM exposure, remaining stable near 134.6 ± 10.1 mM. After depuration in "clean" TFM-free water, plasma Na^+ values dropped to 20 % below control measurements (Figure 3.1). No changes in plasma Cl^- were observed during TFM exposure, which fluctuated around 127.2 ± 7.0 mM. After 24 h recovery in TFM-free water, however, there was a marked 22% increase in plasma Cl^- concentration at which time it approached 160 mM. It was also noteworthy, that the plasma Cl^- exceeded plasma Na^+ by approximately 40 % at this time (Figure 3.1).

3.2 Na^+,K^+-ATPase and V-ATPase Activity and Expression

NKA activity and Total ATPase activity were not found to significantly change with TFM exposure or recovery (Figure 3.2). The activity of NKA did not significantly deviate from a control level of 2.64 ± 0.64 μmol ADP mg $protein^{-1}$ h^{-1}. Nor was total ATPase activity significantly affected, remaining near its control rate of 6.01 ± 1.08 μmol ADP mg $protein^{-1}$ h^{-1} (Figure 3.2). Similarly, V-ATPase activity was also unchanged remaining at a control rate of 0.92 ± 0.12 μmol ADP mg $protein^{-1}$ h^{-1} (Figure 3.2).

The no-primary control for both α5 and B2/BvA1 protocols revealed a single band at the 75 kd range. The α5 antibody immunoreacted with the proposed size of the α-subunit of the NKA transporter at approximately 100 kd. Similarly, the B2/BvA1 antibody immunoreacted with a pair of bands located at approximately 56 kd range, in addition to other higher molecular weight proteins. Western blotting revealed that there were no

Figure 3.1 Effects of TFM on plasma ion balance in juvenile lake sturgeon.

Concentrations of plasma Na^+ (shaded bars) and Cl^- (open bars) in resting juvenile lake sturgeon (*Acipenser fulvescens*) following exposure to 4.6 mg L^{-1} of 3-trifluoromethyl-4-nitrophenol (TFM) and following 24 h depuration in TFM-free water. Data are expressed as the mean +1 SEM (*N*). * Indicates a significant difference between control and 24 h recovery Cl^- concentrations ($p<0.05$); † Indicates a significant difference between control and 24 h Na^+ concentrations ($p<0.05$)

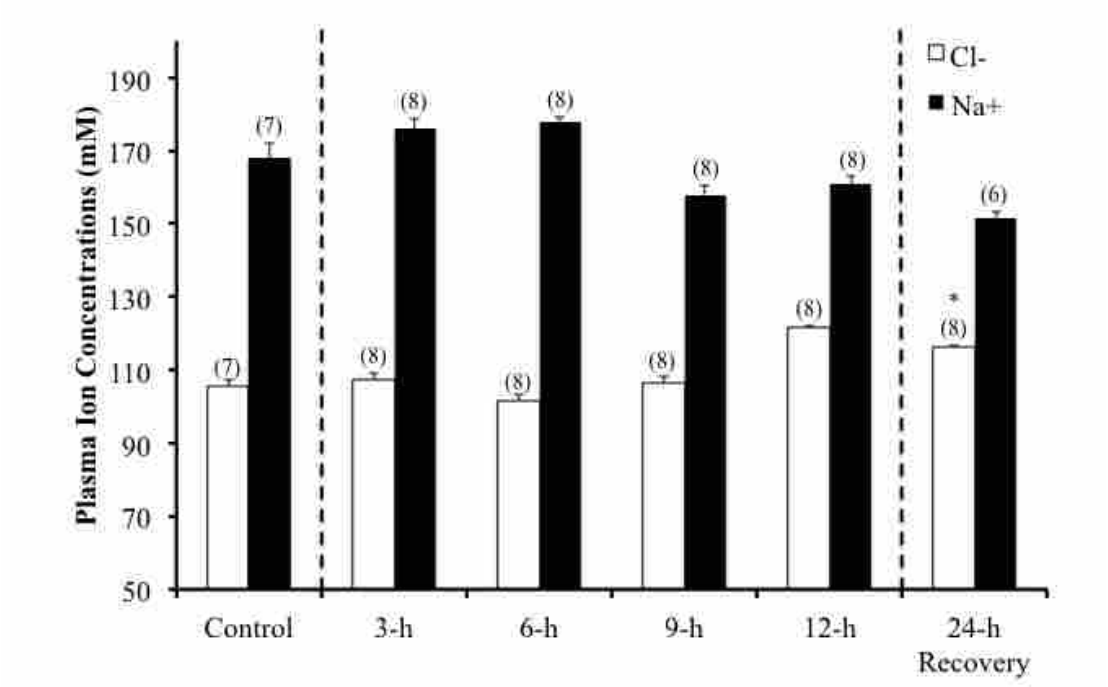
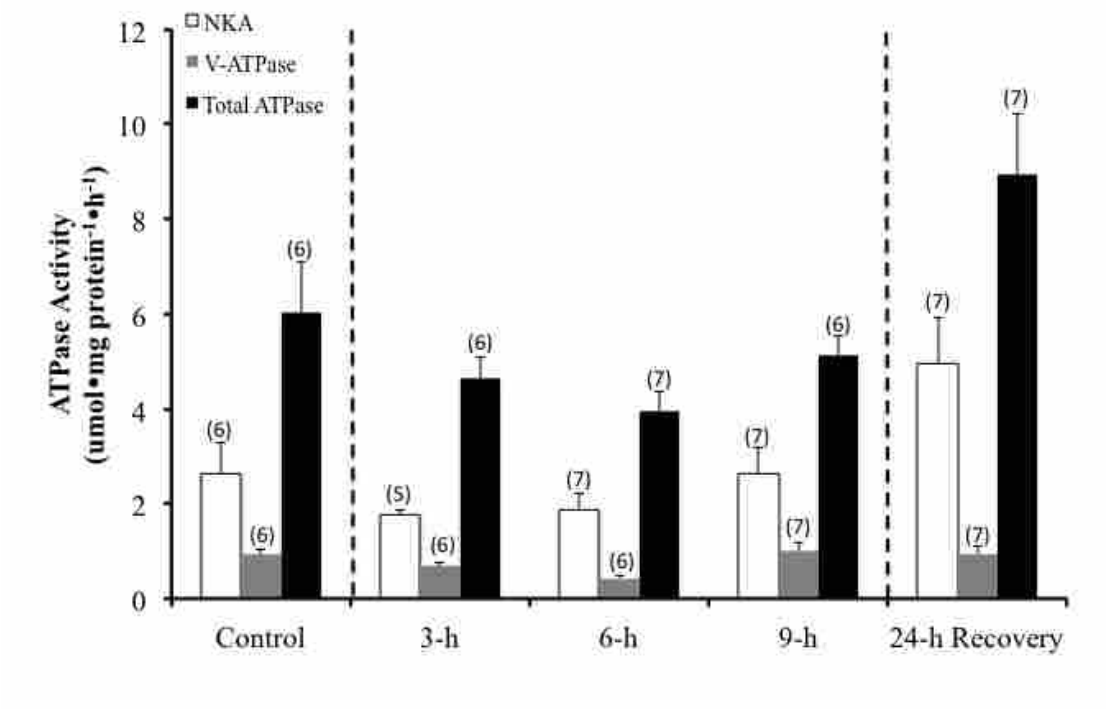

Plasma Ion Concentrations (mM)
190
170
150
130
110
90
70
50
Cl-
Na+
(7)
(7)
(8)
(8)
(8)
(8)
(8)
(8)
(8)
(8)
(6)
*
Control
3-h
6-h
9-h
12-h
24-h
Recovery

Figure 3.2 Effects of TFM on gill ion-transporter activity in lake sturgeon.

Na^+,K^+-ATPase (NKA; open bars), vacuolar H^+-ATPase (V-ATPase; gray bars), and total ATPase (dark bars) activity of resting lake sturgeon (*Acipenser fulvescens*) following exposure to 4.6 mg L^{-1} 3-trifluoromethyl-4-nitrophenol (TFM) and following 24 h depuration in TFM-free water. Data are expressed as the mean +1 SEM (*N*).


NKA
V-ATPase
Total ATPase
ATPase Activity
(umol•mg protein-1•h-1)
12
10
8
6
4
2
0
(6)
(6)
(6)
(5)
(6)
(6)
(7)
(6)
(7)
(7)
(6)
(7)
(7)
(7)
(7)
Control
3-h
6-h
9-h
24-h Recovery

significant changes in the abundance of either NKA or V-ATPase in the gills during or after TFM exposure (Figure 3.3a and b).

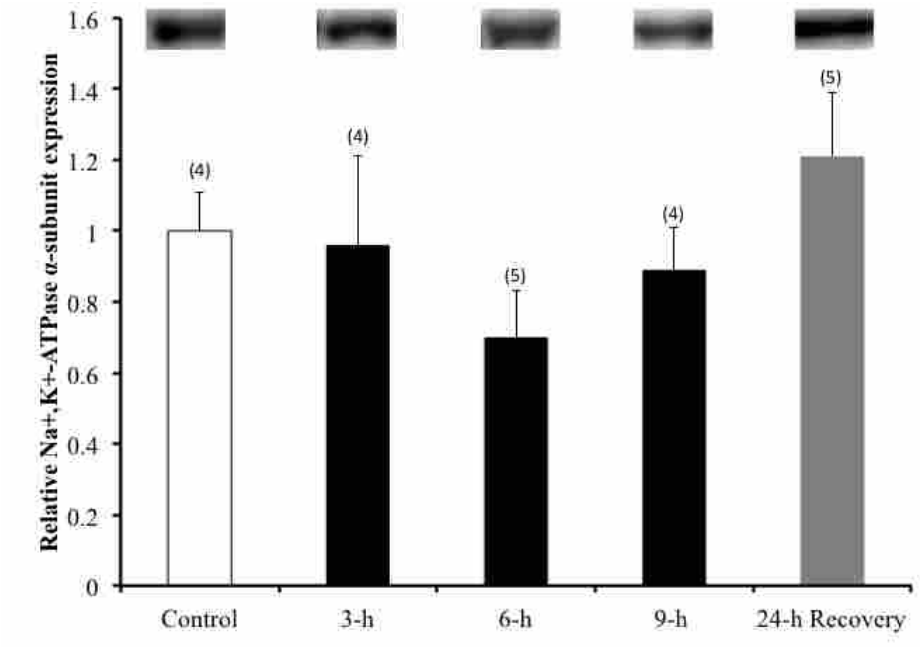
3.3 Gill Structure

Hemotoxilyn and eosin stained slides showed no significant evidence of histological disturbance in the gill tissue of fish exposed to the TFM LC_{50} of larval sea lamprey (4.6 mg L^{-1}). Using the criteria of Kiemer and Black (1997; See section 3.2.7 of the Material and Methods), the gills of most fish exposed to TFM displayed grade 1 characteristics, as the both filament and lamellae did not visually differ from control gill tissue in gills, showing no signs of necrosis, epithelial lifting or clubbing. However, 1 of 4 fish exposed to TFM for 9 h exhibited grade 2 characteristics, with notable hypertrophy at the distal tips of lamellae and minor clubbing (Figure 3.4)

Figure 3.3 Effects of TFM on gill ion-transporter expression in juvenile lake sturgeon.

(a) Na^+,K^+-ATPase (NKA) α-subunit and (b) vacuolar H^+-ATPase (V-ATPase) B-subunit expression in resting juvenile lake sturgeon (*Acipenser fulvescens*) during exposure to 4.6 mg L^{-1} 3-trifluoromethyl-4-nitrophenol (TFM), and following 24-h recovery in TFM-free water. Data are expressed as the mean +1 SEM (*N*). Representative immunoblots of gills probed with the NKA α-subunit antibodies α5 and V-ATPase B-subunit antibody B2/BvA1 are shown above their respective bars taken from the same blots.

(a)


Relative Na+,K+-ATPase α-subunit expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
(4)
(4)
(5)
(4)
(5)
Control
3-h
6-h
9-h
24-h Recovery

(b)

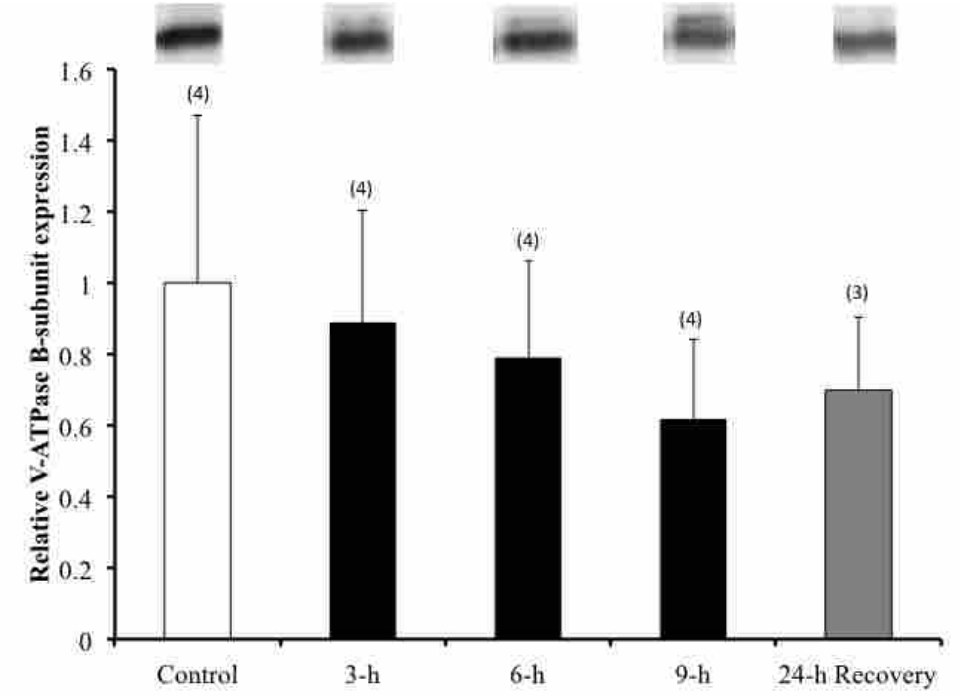
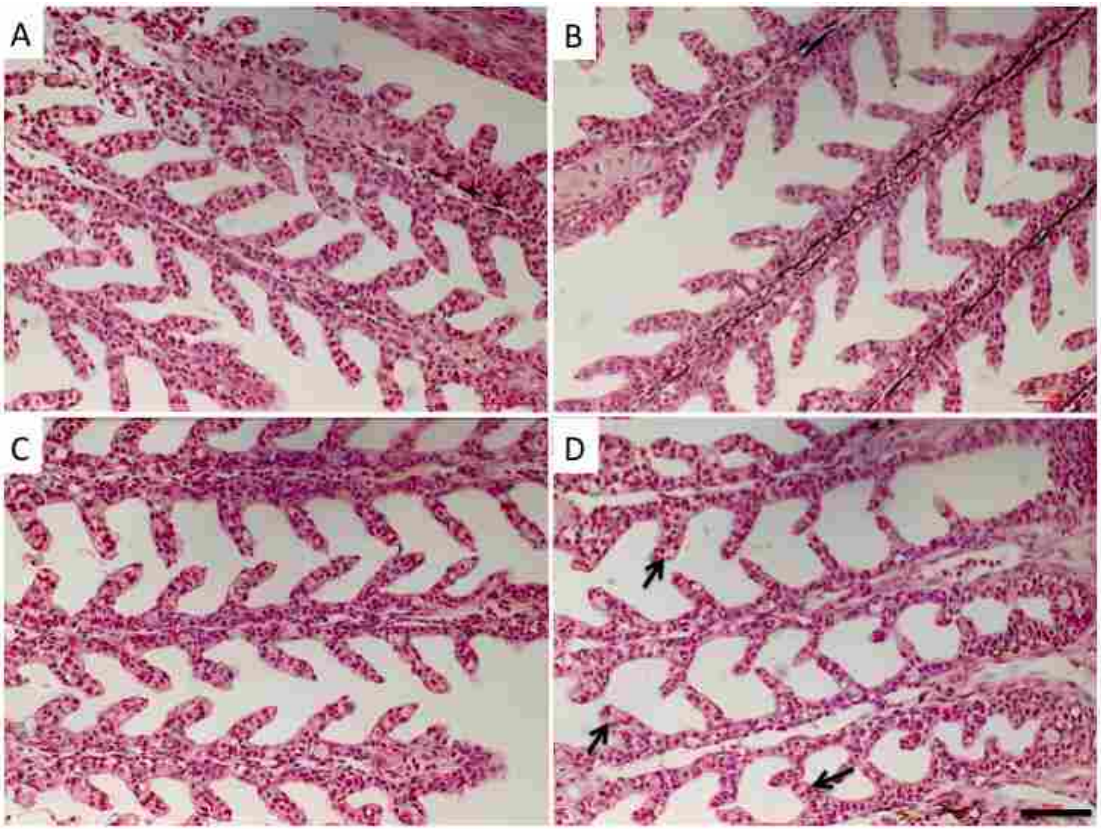

Relative V-ATPase B-subunit expression
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
(4)
(4)
(4)
(4)
(3)
Control
3-h
6-h
9-h
24-h Recovery

Figure 3.4 Effects of TFM on gill structure of juvenile lake sturgeon.

Haematoxylin and eosin stained lake sturgeon gills filaments and lamellae of (a) control (0 h) fish and fish exposed to the LC_{50} of TFM for larval lamprey (4.6 mg L^{-1}) for (b) 3 h, (c) 6 h and (d) 9 h. Arrows indicate lamellae that exhibit signs of hypertrophy. Gill sections were of 4–5 μm thickness. Scale bar, 50 μm.


A
B
C
D

4. Discussion

4.1 The Effects of TFM on Gill Structure and Function

Juvenile lake sturgeons have a lower tolerance to TFM compared to other non-target species (Boogaard *et al.,* 2003). Sturgeon that are less than 100 mm in length are particularly vulnerable, and have a 12 h LC_{50} that falls near the $LC_{99.9}$ concentration of larval sea lamprey (Johnson *et al.,* 1999; Boogaard *et al.,* 2003). Because TFM is typically applied at 1.3-1.4 times the $LC_{99.9}$ [also referred to as the minimal lethal concentration (MLC; Bills *et al.,* 2003)], the TFM concentrations needed to eradicate larval sea lampreys may overlap with those known to cause mortality in some juvenile lake sturgeon (McDonald and Kolar 2007). Despite earlier evidence suggesting that TFM can potentially interfere with and cause structural damage to the gills of rainbow trout and sea lamprey (Christie and Battle, 1963; Mallat *et al.* 1985; 1994), the present study suggests that the TFM has few adverse effects on gill structure and function in juvenile lake sturgeon. Because TFM interferes with oxidative phosphorylation in the mitochondria (Niblett and Ballentyne 1976; Birceanu *et al.,* 2011), it was predicted that TFM exposure would starve ion pumps including the NKA and V^{+}-ATPases of ATP leading to disturbances to internal ion homeostasis. Previous work on larval sea lamprey had shown that exposure to TFM leads to morphological changes in MRC structure, such as cell rounding, enlargement of mitochondrial profiles, vacuolization of the cytoplasm, widening of the intercellular spaces, and necrosis of ion-uptake cells following exposure to the MLC of TFM (Mallatt *et al.,* 1985; 1994; 1995). These findings suggested that a disruption of ionoregulation may contribute to mortality in larval sea lamprey (Mallatt *et al.,* 1994) and other TFM-sensitive species. However, TFM was not found to have any physiologically relevant effects on gill function or structure in juvenile lake sturgeon, with the exception of some minor clubbing and hypertrophy of the distal tips of the lamellae observed in only 1 fish of the 9 h exposure group. It is possible that greater damage could be

observed at higher concentrations of TFM that are nearer to concentrations used in the field. Thus, further studies near 1 to 1.3 times the MLC of TFM are warranted to test this possibility in lake sturgeon.

Further support for the conclusion that moderate concentrations of TFM had few adverse effects on the gills was provided by measurement of plasma Na^+ or Cl^- concentrations, which did not change during TFM-exposure in juvenile lake sturgeon. A lack of effect of TFM on plasma Na^+ and Cl^- and whole body ion concentrations was also noted in larval lamprey when exposed to their 12 h LC_{50} of TFM (Chapter 2; Birceanu *et al.,* 2009; Wilkie *et al.,* 2007), suggesting that TFM has minimal effects on ionoregulation. This conclusion was further supported by the lake sturgeon's ability to sustain NKA and V-ATPase activity (Figure 3.2 and 3.3) in the face of TFM exposure, and the absence of major pathological alterations in gill structure (Figure 3.4). However, there was a significant decrease in plasma Na^+ concentrations and an increase in plasma Cl^- during the recovery in lake sturgeon (Figure 3.1). Like lake sturgeon, a similar drop in Na^+ concentration below Cl^- concentrations was found in the plasma of larval sea lamprey following 24 h of recovery after TFM exposure (Chapter 2), with no notable change in NKA activity or expression to explain the alterations in plasma ion concentrations. Such marked changes in plasma Na^+ and Cl^- are suggestive of an acid-base disturbance, likely incurred during and after TFM exposure.

Fish compensate for acid-base disturbances in two ways: (1) physiochemical buffering with bicarbonate and/or nonbicarbonate buffers and/or (2) the net transport of acid-base constituents from the fish with the surrounding media (Evans *et al.,* 2005). The means by which such acid-base control is achieved is via the MRCs and the pavement cells (PVCs). In general, Na^+ is taken-up from the water via an apical epithelial Na^+ channel (eNac) on the MRC, down the electrochemical gradients generated by V-ATPase pumps (V-ATPase) and the NKA. This arrangement, and/or electroneutral Na^+/H^+ (NHE) also results in the net excretion of acidic equivalents (Kumai and Perry, 2012). The movement of Cl^- and base

equivalents is facilitated by Cl^-/HCO_3^- exchangers present on the apical surface of MRCs and PVCs (Goss *et al.,* 1998; Wilson *et al.,* 2000; Marshall, 2002). Thus, differences in base or acidic equivalent flux can be achieved by controlling the net flux of Na^+ versus Cl^- across the gill, with increases in net Cl^- flux from the fish accompanying net H^+ flux, and increases in net Na^+ flux resulting in net base loss (Maetz, 1972; Cameron, 1976; Wood *et al.,* 1984; Wilkie *et al*., 1998, 2001). The marked increase in Cl^- and decreased concentration of Na^+ in the plasma after 24 h of recovery was therefore suggestive of increased net Na^+ over Cl^- loss, or a net HCO_3^- loss (equivalent of net H^+ gain) perhaps due the presence of a post-TFM exposure metabolic alkalosis (Goss *et al.,* 1995).

At first glance a metabolic alkalosis might seem counterintuitive, but it should be noted that TFM exposure is associated with increased rates of glycolysis, characterized by marked reductions in liver and brain glycogen stores and increased lactate during the period of TFM exposure (Birceanu *et al.,* 2009, 2014; Clifford *et al*., 2012; Le Clair 2014). Thus, it is highly likely that the fish underwent a pronounced metabolic acidosis during actual TFM exposure, followed by a compensatory metabolic alkalosis, which persisted during the post-TFM recovery period. The increase in net base excretion, as characterized by the greater anion gap between plasma Cl^- and Na^+ was likely due to greater post-TFM exposure clearance of the residual base load. Similar responses have been reported in alkalotic rainbow trout and bullhead (*Ictalurs punctatus)* during 6 h of recovery from hypercapnia and hyperoxia, in which there was an increase in the net uptake of Cl^- and drop in Na^+ uptake, which were correlated with decreases in MRC fractional surface area ($\mu m^2/mm^2 \times 10^{-3}$) under acidotic conditions and increased MRC fractional surface area during the period of post-hypercapnic or -hyperoxic alkalosis (Goss *et al.,* 1992; Goss and Perry, 1993; Goss *et al.,* 1994). Similar changes in net Cl^- vs Na^+ movements were also reported in white sturgeon (*Acipenser transmontanus;* Baker *et al.,* 2009). Although the sturgeons studied were too small to obtain blood for acid-base measurements, based on the likelihood of TFM-induced

acid-base disturbances, it was predicted that compensatory increases in gill V-ATPase activity would take place. Rainbow trout typically increase V-ATPase expression following hypocarbia exposure (Lin and Randall, 1993; Lin *et al.,* 1994; Sullivan *et al.,* 1995), but white sturgeon do not experience changes in NHE3 protein levels or either V-ATPase activity or V- ATPase B subunit expression in response to a respiratory acidosis (Baker *et al.,* 2009). This led Baker *et al.,* (2009) to suggest that NHE_3 and V-ATPase may only play a limited role in branchial pH compensation in hypocarbia exposure in the white sturgeon. This may also explain why, despite strong circumstantial evidence of acid-base disturbances, that changes in V-ATPase activity and expression was minimal during TFM exposure. While the kidneys may be involved in acid-excretion, it has been found that 92% of net H^+ efflux occurs in the gills and only 8% in the kidneys of rainbow trout following exhaustive exercise (Wood, 1988), 68 % in the gills : 32 % in the kidneys after NH_4C1 loading in catfish (Cameron and Kormanik, 1982), and 93% in the gills : 7% in the kidneys during hyperoxia in trout (Wood *et al.,* 1984), suggesting that the kidneys play only a minor role in acid-excretion. It is more likely that the present abundance of V-ATPases was sufficient to handle any increases in metabolic acid-load brought about by TFM exposure.

While there were only minimal effects of TFM on ion homeostasis in the sturgeon, ionoregulatory and acid-base disturbances could be more pronounced at higher concentrations of TFM due to a greater reliance on anaerobic glycolysis to make up the ATP shortfall, and associated increases in metabolic acid production. It would, therefore, be beneficial to measure changes in intracellular pH in key tissues such as the brain and muscle, and plasma pH during and after TFM exposure to better understand the impact of TFM on non-target species. Measurements of changes in O_2-consumption, CO_2 excretion, and net acid excretion during TFM-exposure and recovery would also shed more light on the acid-base consequences of TFM exposure in not only lake sturgeon but also other non-target fishes.

4.2 Gill Histology Following TFM Exposure

Despite suggestions that TFM targets the gills, and prior evidence of damage to gills in the TFM-sensitive larval sea lamprey (Mallat *et al.,* 1994; 1995), we found little evidence of TFM-induced pathological effects on the gills of sturgeon. Using the rating system by Kiemer and Black (1997), we found only minor hypertrophy to the gill lamella on the distal ends with no contour disturbance (grade 2 damage) following 9 h of TFM exposure in 1 of the 4 fish examined at that time (Figure 3.4). Damage was not observed in any of the other sample time points suggesting that TFM-induced damage to the gills is likely minimal and unlikely to cause fish distress during exposure to TFM. However, it is noteworthy that in the Mallatt *et al*. (1994) study, fish were exposed to the 9 h $LC_{99.9}$ for larval lamprey and sampled only once fish were moribund. In our study, the lake sturgeon were exposed to the 12 h LC_{50} of larval lamprey and therefore juvenile lake sturgeon may still demonstrate similar pathology when exposed to treatment concentrations of TFM. In lake sturgeon less than 100 mm in length, the LC_{50} is approximately 1.5X the LC_{50} of larval lamprey but changes with development (Boogaard *et al.,* 2003).

While no changes in gross gill morphology were observed in the present study, it should also be noted that changes to MRC structure, surface area or abundance would not be evident using haematoxylin and eosin staining. Either scanning electron or confocal microscopy would be required to tease out how the fine gill architecture of the lake sturgeon and other fishes respond to TFM exposure and during the post-TFM exposure recovery period.

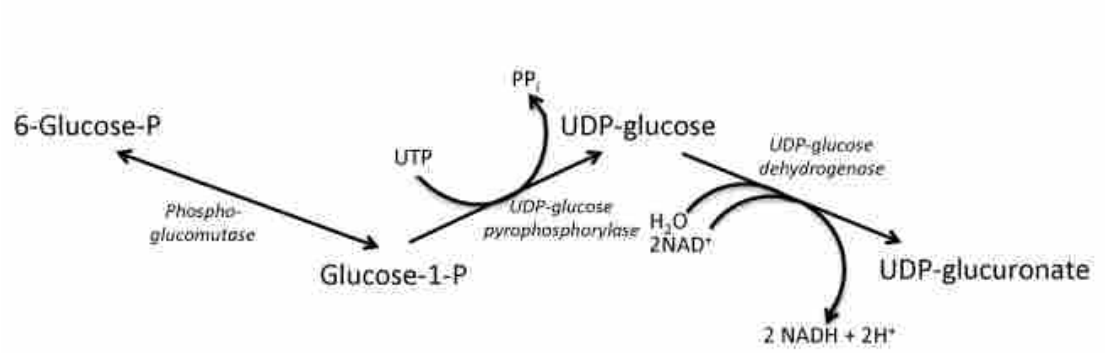
4.3 Factors Affecting the Sensitivity of the Lake Sturgeon to TFM

In the absence of any visible impairment of the gills, the physiological factors that might explain the sensitivity of lake sturgeon less than 100 mm to TFM is unclear. One possibility is that the greater susceptibility to TFM is due to a lower capacity of the lake

sturgeon to detoxify TFM using glucuronidation, as observed in larval sea lamprey. Larval lamprey possess a low capacity to detoxify TFM, characterized by levels of glucuronidated-TFM that are just above levels of detection in muscle tissue and blood, and a much higher ratio of free to conjugated TFM than observed in trout exposed to toxic concentrations of the lampricide (Lech and Statham, 1974; Le Clair, 2014). However, despite similar sensitivity to TFM, juvenile lake sturgeon were not only able to detoxify TFM via the UDPGT pathway, but following recovery, TFM was found to be completely absent from muscle tissue by 6 h of depuration in TFM-free water (Le Clair, 2014). Additionally, these rates of TFM clearance and TFM-glucuronide concentrations were similar to concentrations measured in rainbow trout exposed to their TFM LC_{50}, which are highly tolerant to TFM-exposure (Birceanu *et al.,* 2014). At these concentrations (11.0 mg L^{-1} TFM), parent-TFM peaked in trout muscle tissue at 3 h and steadily declined with concentrations not significantly different from the control by 12 h of exposure. Additionally, concentrations of glucuronidated-TFM surpassed parent-TFM by 6 h, reaching a peak concentration at 9 h (Birceanu *et al.,* 2014). However, unlike trout, there was significant drop in glucuronidated-TFM to control concentrations by 12 h of TFM exposure, while concentrations of parent-TFM remained high in sturgeon (Le Clair, 2014). This decline in glucuronidated-TFM could be due to a decreased ATP supply, which is required to synthesize UDP-glucuronic acid, the substrate for glucuronidation. Glucuronic acid arises from UDP-glucose, which is synthesized from glucose-1-phosphate and UTP and requires ATP (Figure 3.5; Wamelink *et al.,* 2009). It is therefore possible that at higher doses of TFM, more comparable to those used to treat lamprey-infested streams, that the detoxification pathways for TFM could become overwhelmed, resulting in toxicity and fish mortalities.

Figure 3.5 UDP-Glucuronic acid formation.

UDP-glucoronidateion arises from 6-glucose-phosphate (6-glucose-P), which is converted to glucose-1-P via the reversible transfer of the phosphate group at position 6' to 1'. UTP is then catalyzed to inorganic phosphate (PP_i) by UDP-glucose pyrophosphorylase to synthesize UDP-glucose, which is further catalyzed by UDP-glucose dehydrogenase to form glucuronic acid (or UDP-glucuronate) via the reduction of NAD^+ (Wamelink *et al.,* 2009).


6-Glucose-P
Phospho-
glucomutase
Glucose-1-P
UTP
PPi
UDP-glucose
UDP-glucose
pyrophosphorylase
UDP-glucose
dehydrogenase
H2O
2NAD+
UDP-glucuronate
2 NADH + 2H+

4.4 Implications for Future Research

The present study indicates that the lake sturgeon is able to withstand moderate doses of TFM. Exposure of sturgeon to concentrations of TFM that are toxic to larval sea lampreys (LC_{50}) had minimal effects on Na^+ and Cl^- homeostasis. However, post-TFM exposure increases in Cl^- concentration, accompanied by simultaneous declines in Na^+ suggested that the animals may be correcting for an alkalosis that likely arose to counterbalance metabolic acid production arising from prior TFM exposure. Nevertheless, it seems unlikely that acid-base disturbances would lead to death due to the resilience of sturgeon to tolerate sustained reductions in extracellular pH and intracellular pH in the heart, white muscle, brain, and liver (Baker *et al.,* 2009). It is, however, important to note that this present study only exposed juvenile fish to LC_{50} doses of TFM for larval lamprey. Therefore, there is a potential that TFM may cause greater disturbances in sturgeon and TFM-sensitive animals. Furthermore, while ionregulation does not appear to be a factor in contributing to mortality in these fish with exposure to TFM, the underlying cause remains unclear. Measurements of TFM glucuronidation during TFM exposure indicate that there is a potential that detoxification pathways may be overwhelmed late into exposures leading to non-target fish mortality in juvenile sturgeon (LeClair, 2014). Therefore studies should eventually be expanded to examine further how life stage influences the susceptibility of lake sturgeon to this lampricide, particularly in juveniles that are less than 100 mm in size, and larvae that are less than 35 mm (Johnson *et al.,* 1999; Boogaard *et al.,* 2003).

CHAPTER FOUR:

The Effect of 3-Trifluoromethyl-4-nitrophenol on Gill Structure and Function in Juvenile Rainbow Trout and Larval Lamprey Acclimated to Soft Water

1. Introduction

The sea lamprey, *Petromyzon marinus*, is an invasive species, which inhabits the Great Lakes and its tributaries (Youson, 1980). Along with overfishing, parasitism by juvenile sea lamprey decimated teleost fish populations in the Great Lakes, leading to the collapse, or near collapse, of lake trout (*Salvelinus namaycush*), whitefish (*Coregonus* spp.), and other fisheries in the 1950s (Renaud, 1997; Smith and Tibbles, 1980; McDonald and Kolar, 2007). In response to the crisis, a sea lamprey population control program was initiated, which included the use of the pesticide, 3-trifluoromethyl-4-nitrophenol (TFM), to specifically target burrow-dwelling larval sea lampreys in their nursery streams (Applegate *et al.,* 1961; Smith and Tibbles, 1980; Christie and Goddard, 2003; McDonald and Kolar, 2007). Since TFM treatments began in the 1960s, the use of this lampricide has led to significant reductions in sea lamprey populations in the Great Lakes, and corresponding reductions in teleost mortality (Christie and Goddard, 2003). In general, TFM is thought to have minimal adverse effects on non-target fishes (McDonald and Kolar, 2007). Because the effects of TFM are dose dependent, however, it is important to ensure the TFM concentrations do not approach toxic thresholds in non-target fishes by carefully monitoring not only TFM concentrations in the water, but water pH, which can profoundly influence TFM speciation and uptake by fishes (Hunn and Allen, 1974; Bills *et al.,* 2003; Boogaard *et al.,* 2003; McDonald and Kolar, 2007). However, the sensitivity of different fish species and aquatic vertebrates to TFM varies widely, with some species of juvenile fish being particularly vulnerable to the chemical. Members of the catfish family Ictaluridae such as black bullhead (*Ictalurus melas*) and channel catfish (*Ictalurus punctatus*), lake sturgeon (*Acipenser fulvescens*), stonecats (*Noturus flavus*), and madtoms (*Noturus stigmosus*) are particularly sensitive to TFM (Boogaard *et al.,* 2003). Amphibians such as mudpuppies (*Necturus maculosus*) also appear to be sensitive TFM (Boogaard *et al.,* 2003)**.** As a result, concerns

regarding the potential adverse effects that TFM treatment has on non-target vertebrate and invertebrate species has increased in recent years. Despite such concerns, the adverse sub-lethal and lethal effects of TFM and the underlying mechanisms of toxicity in non-target organisms remain largely unexamine.

Due the inhibitory effects of TFM on oxidative ATP-production by the mitochondria (Niblett and Ballantyne, 1976; Birceanu *et al.*, 2011, 2014; Clifford *et al.*, 2012), a number of ATP-dependent biological processes may be affected by TFM-exposure. A potential target of TFM is ionoregulation, which relies on active transport processes in the gills, kidneys and other organs of fishes (Evans *et al.*, 2005; Edwards and Marshall, 2013). Exposure to TFM TFM was been shown to inflict damage on the ionocytes (aka. mitochondrial rich cells; MRCs) of the lamprey gill (Mallatt, 1987; Mallatt *et al.,* 1994), the site of active ion uptake in freshwater fishes (Evans *et al.,* 2005). The active transport of sodium (Na^+) is mediated by these cells, using apically located epithelial Na^+ channels (eNac) through which Na^+ passes down electrochemical gradients that are generated the pumping of H^+ across the apical membrane using V-ATPase transporters, and the export of intracellular Na^+ into the plasma via basolaterally positioned ouabain-sensitive NKA transporters (Bartels *et al.,* 1998; Marshall, 2002; Bartels and Potter, 2004). Due to the ATP demands of this process, it is possible that impaired ATP production due to TFM exposure could ultimately interfere with Na^+ uptake, leading to compromised internal ion and osmotic balance (Mallatt *et al*., 1994)

While the effects of pH on TFM speciation and sensitivity in sea lamprey and non-targets is well established, less is known about the effects that TFM has on gill-mediated physiological processes in waters with low ion content and/or water hardness. In waters with low ion content, there could be a higher dependency on ATP consuming ion-transporters to maintain ion balance (Leino *et al.,* 1987; Boisen *et al.,* 2003; Craig *et al.,* 2007), which could make the fish more vulnerable to TFM-induced ionic disturbances. Indeed, it has been noted that toxicity of TFM to fish and invertebrates is greater in soft water, but the underlying

causes have not been elucidated (Olson and Marking, 1973; 1975; Marking and Olson, 1975; Bills and Marking, 1976).

The goal of the present study was to determine if the sensitivity of larval sea lampreys and rainbow trout to ionic disturbances and gill damage was greater in ion poor water (~ 40 mg $CaCO_3$ L^{-1}), which would presumably increase their dependency upon active ion transport to maintain internal ion balance. Accordingly, larval sea lamprey and rainbow tout were acclimated to ion-poor, soft water, and then exposed to the 12 h TFM LC_{50} and $LC_{99.9}$ of larval sea lamprey, respectively, and changes in plasma ion concentration were followed through the exposure. The activities and abundance of NKA and V-ATPase transporters in the gills were also examined to further assess if TFM compromised the ionoregulatory capacity of the gills during TFM exposure in soft, ion-poor water.

2. Methods and Materials

2.1 Experimental Animals and Set-up

2.1.1 Animal Collection, Holding and Soft Water Acclimation

Larval sea lampreys (*P. marinus*; 1.3 ± 0.1 g, 106.5 ± 1.4 mm, *N*=134) were collected by US Fish and Wildlife Service personnel and delivered to the Hammond Bay Biological Station (Millersburg, MI), where they were held in flowing, well-aerated Lake Huron water for several weeks. Sub-sets of the animals were then placed in plastic bags filled with oxygen saturated water, sealed and placed in a 70 L plastic cooler and shipped overnight to Wilfrid Laurier University in Waterloo, ON. Following their arrival, the animals were transferred to 70 L aquaria, containing approximately 5 cm of sand to provide burrowing substrate, and continuously receiving aerated dechlorinated City of Waterloo tap water (water hardness 214.8 mg $CaCO_3$ L^{-1}) at a rate of 0.5 L min^{-1}. Juvenile rainbow trout (*O. mykiss*; 53.8 ± 3.4 g, 168.9 ± 4.1 mm, *N*=120), purchased from Rainbow Springs Trout Hatchery (Thamesford,

ON, Canada), were placed in a 120 L tank receiving the same dechlorinated water at an approximate rate of 1.0 L min^{-1}. After a minimum of 1 week, the dechlorinated water was diluted in a step wise (25 % per week) manner using reverse osmosis water over a four week acclimation period until a final hardness of ~ 40 mg $CaCO_3$ L^{-1} was obtained (pH ~ 7.3, 10-13°C, 80-100% dissolved O_2, 381.2 μM Na^+). Both sets of fish were held in the continuously flowing soft-, ion-poor water for four weeks before experimentation. A second set of juvenile rainbow trout (29.5 ± 1.5 g, 142.0 ± 2.7 mm; *N*=26), also purchased from Rainbow Springs Trout Hatchery, were held in 120 L tanks receiving Wilfrid Laurier well water for approximately three months (pH ~ 7.8, 10-13°C, water hardness ~ 450 mg $CaCO_3$, 80-100% dissolved O_2) prior to soft-water acclimation and $^{24}Na^+$ radiotracer experiments. Juvenile rainbow trout were then placed in 60 L experimental containers in a recirculating system with 500 L of water fitted with a carbon filter on outflow to reduce water ammonia nitrite and nitrate concentrations, and acclimated to soft-water (final hardness ~ 48 mg L^{-1} $CaCO_3$; pH ~ 7.7; 10-13°C; 80-100% dissolved O_2; 494.94 μM Na^+) as described above. All fish were held under a 12 hour light: 12 hour dark photoperiod. The larval sea lamprey tank also contained 1 g of diffused aquarium cotton per animal to provide the animals with an artificial burrowing substrate (Wilkie *et al.,* 1998), which prevented excessive activity (thigmokinesis; Rovainen and Schieber, 1975). Juvenile trout were fed daily with 3.0-point fine floating pellets (fed to satiated; 3.0 Corey Feed Mills, Elmira, ON), and prior to experiments, were starved for 48 h. Sea lampreys were not fed during acclimation.

All experiments and fish housing were approved by the Wilfrid Laurier Animal Care Committee and followed Canadian Council of Animal Care guidelines.

2.1.2 3-Trifluoromethyl-4-nitrophenol (TFM)

Field formulation TFM (35% active ingredient dissolved in isopropanol; Clariant SFC GMBH WERK, Griesheim, Germany) was used for all the toxicity tests and

experiments, and was provided courtesy of the Sea Lamprey Control Centre, Fisheries and Oceans Canada (DFO, Sault Ste. Marie, ON). Concentrations of TFM in all experimental exposure media were confirmed using a THERMOmax microplate spectrophotometer and SOFTmax Pro software (SpectraMax 190, Molecular Devices, CA) with precision TFM standards provided by DFO.

2.2 Experimental Protocols

2.2.1 TFM Toxicity Test in Soft Water

A range finder experiment was conducted to determine TFM exposure concentrations in soft water. Larval sea lamprey (1.5 ± 0.1 g, 110.5 ± 1.3 mm, *N*=70) were exposed to the nominal TFM concentrations of 0.0, 0.2, 0.3 0.4, 0.5, 0.75, and 1.0 mg L^{-1} TFM in duplicate in 10 L of soft water using 20 L holding containers (buckets; *N* = 5 per container, *N* =10 total at each concentration) for 12 h (pH 7.06 ± 0.03, temperature 12-15°C, dissolved O_2 80-100% saturation, water hardness 42 mg L^{-1} $CaCO_3$, 369 μM Na^+). All exposures were completed in the dark because larval sea lampreys are negatively phototactic (Beamish, 1980). When animals were unresponsive to a tail-pinch using forceps, they were deemed to be dead, removed from the testing chamber, and the time of death recorded. The 12 h LC_{50} and $LC_{99.9}$ concentrations were then calculated using the Comprehensive Environmental Toxicity Information System (CETIS™) toxicity analysis software (v1.8.5.1; Tidepool Scientific Software; McKinleyville, CA).

2.2.2 Effects of TFM on Internal Electrolyte Balance and Gill Function

Larval sea lamprey were transferred to individual 450 ml experimental holding chambers positioned in a tray into which water drained from the head-tank of a 120 L recirculating system containing soft water (pH 7.06 ± 0.08, temperature 13-15°C, dissolved O_2 80-100%, water hardness 47.8 ± 1.8 mg $CaCO_3$ L^{-1}, 395.0 ± 0.09 μM Na^+) and allowed to

acclimate to the set-up overnight. Rainbow trout were similarly transferred to individual 750 ml chambers in the same 120 L^{-1} soft-water recirculation system (pH 7.2 ± 0.06, temperature 13-15°C, dissolved O_2 80-100%, water hardness 46.6 ± 2.3 mg $CaCO_3$ L^{-1}, 380 ± 0.11 µM Na^+) and also left to acclimate overnight. The following day, flow to the containers was cut-off, and the sea lamprey exposed to a nominal TFM concentration of 0.38 mg L^{-1} (measured 0.44 ± 0.01 mg L^{-1}), which was equivalent to their 12 h LC_{50} determined in the acute toxicity tests (section 3.2.1 above). Rainbow trout on the other hand, were exposed to a nominal TFM concentration of 0.80 mg L^{-1} (measured 1.10 ± 0.04 mg L^{-1}), which was equivalent to the 12 h $LC_{99.9}$ of TFM for the larval sea lamprey. Containers housing lamprey were supplied with 2 g of aquarium cotton, and exposures for both trout and lamprey were conducted in the dark.

At pre-selected time intervals, the sea lamprey and the rainbow trout were euthanized in a 1:2 tricaine methanesulfonate (MS222) : sodium bicarbonate solution (1.5 g MS222 for larval lamprey, and 1.0 g MS222 for juvenile trout). Sampling took place at 0, 3, 6, 9, and 12 h of TFM exposure and following a 24 h depuration period in TFM-free soft water. Following death, blood from the sea lamprey was sampled by making an incision through the heart, immediately behind the last branchiopore and the blood was collected in drop-wise fashion using a heparinized capillary tube. In the rainbow trout, blood was collected by caudal puncture using a 1 ml plastic syringe fitted with a 21-gauge needle and pre-rinsed with sodium-heparin (54 U mL^{-1}; H3393, Sigma-Aldrich; St. Louis, MO). For both sea lamprey and rainbow trout, the whole blood was then transferred to 0.5 ml microcentrifuge tube and centrifuged at 10,000 x *g*, the plasma drawn off, transferred to a 0.5 ml microcentrifuge tube, and then snap-frozen in liquid N_2 until analyzed for plasma ion concentration.

Gills were collected from larval sea lamprey by making multiple 0.5 mm transverse-sections along the length of the branchial region. Alternate sections were either snap-frozen in liquid N_2 and stored at -80ºC for later determination of gill NKA and V-ATPase transporter activity and expression or fixed in 20 volumes of 10% buffered formalin solution (pH 7.4) for

24 h at 4°C. In the rainbow trout, gill arches 2 and 3 were excised from the both sides of the fish but processed in an otherwise identical fashion. Following fixation, the gill tissue of both sea lamprey and rainbow trout was rinsed in 1X Phosphate Buffer Solution (PBS; pH 7.5) three times, and trout gills were decalcified with a 0.6 mmol L^{-1} sodium citrate and 19.6 mmol L^{-1} formic acid solution prior to storage in 70% ethanol. Both the fixed sea lamprey and rainbow trout gills were stored in 70% ethanol at 4°C, and then prepared for light microscopy and immunohistochemistry analyses by fixing tissues in a 20% DMSO/methanol solution at –20°C for 48 h followed by storage in 100% methanol at –20°C, and then paraffin embedding (type 6; Richard-Allan Scientific, Kalamazoo, MI, USA). Following paraffin embedding, 5 μm sections were collected onto 3-aminopropyltriethoxysilane-coated slides (Sigma Aldrich; St. Louis, MO), cover slipped, and then stored until light microscopy and immunohistochemistry analysis took place.

2.2.3 TFM-Induced Disturbances to Na^+-Uptake Using Radiotracer $^{24}Na^+$

The uptake of Na^+ by the gills was measured in trout using the radiotracer $^{24}Na^+$ and well-established methodologies (Morris and Bull, 1970; Wilkie *et al.,* 1998). Juvenile trout (29.5 ± 1.5 g, 142.0 ± 2.7 mm, *N*=12) acclimated to soft water were placed in 800 ml chambers positioned in the same 120 L recirculation system described above and left to acclimate overnight for 12 h. The next day, water flow to the chambers was shut off, and the volume of the container was adjusted to 750 ml, followed by the addition of 2 μCi of $^{24}Na^+$ (dissolved in 0.01 N HNO_3 and 2 ml of deionized water). Following a 10-minute mixing period, 10 ml water samples were then collected at 0, 2, and 4 h to measure the unidirectional Na^+ flux (influx, efflux, net flux) across the gills under control (TFM-free) conditions. Following the control flux measurement period, the containers were flushed three times with soft water, and flow was re-established to the containers. The next morning, water flow was shut-off again to each container and the volumes adjusted to 750 ml, but TFM was added to

each container [nominal concentration of 0.80 mg L^{-1}(measured 0.61 ± 2.7 mg L^{-1})], followed by 2 µCi of $^{24}Na^{+}$. After 10 minutes, water samples were collected at 0, 3, 6, and 9 h of TFM exposure. At the completion of the flux, the fish were then euthanized as described above, weighed, and plasma was collected for measurement of plasma Na^{+} concentration and $^{24}Na^{+}$ radioactivity, and calculation of the mean specific activity of $^{24}Na^{+}$ in the blood. Total radioactivity of the water and blood samples was measured using a 1480 Automatic Wallacl82 Wizard Gamma Counter with WIZARD2 Automatic Gamma Counter software (PerkinElmer, MA, United States of America) using 4 ml of water in duplicate for each sample period. The radioactivity of the plasma was measured in 50 µl of plasma diluted to 4 ml using deionized water. After $^{24}Na^{+}$ radioactivity was exhausted (approximately two weeks; half-life = 14.7 h) in the water samples, the non-radioactive ("cold") Na^{+} concentration was determined using atomic absorption spectroscopy (SpectrAA 880, N_2 gas; Varian,171 Mississauga, ON). The concentrations of TFM were measured as previously described.

2.3 Analytical Techniques

2.3.1 Plasma Ion Concentrations

Concentrations of plasma Na^{+} was determined using atomic absorption spectrometry (SpectrAA 880, N_2 gas; Varian,171 Mississauga, ON). Plasma Cl^{-} concentrations were measured by coulimetric titration using a chloridometer (926 Chloride Analyzer, Sherwood Scientific, Cambridge, UK).

2.3.2 Na^{+},K^{+}-ATPase and Vacuolar H^{+}-ATPase Activity

Gill NKA and V-ATPase activity was measured using a modified enzyme kinetic microplate assay (McCormick, 1993; Reis-Santos *et al.,* 2004) as described in Chapter 2 (Methods 2.3.2). Briefly, frozen gill tissue (~40 mg) was thawed in 400 µl SEI buffer, homogenized with a motorized pestle following the addition of 100 µl of SEID (0.1% sodium

deoxycholic acid in SEI), and samples were centrifuged at 10,000 x *g* for 3 minutes. Next, 5 µl and 10 µl of the supernatant was added to each well of a 96-microwell microplate for trout and lamprey, respectively, in quadruplicate for total ATPase, NKA, and V-ATPase. Each microwell contained 50 µl of the salt solution and 150 µl of the assay mixture (with 1.0 mM ouabain added to both the NKA and V-ATPases assay mixture and 10 µM bafilomycin A1 V-ATPase assay mixture) were added to the plate. The microplate was then run on a 96-well microplate spectrophotometer at 25°C (SpectraMax 190, Molecular Devices, CA). The NKA activity was calculated as the difference between total ATPase activity and NKA-inhibited activity, and V-ATPase as the difference between NKA-inhibited activity and NKA- and V-ATPase-inhibited activity. Standard curves for ADP were used to determine the amount of ATP (ηmol) converted to ADP. Total protein concentrations were calculated using the Bradford assay with bovine serum albumin (BSA) standards (ALB001-250, Bioshop, Burlington, ON). Both ADP and total protein assays were used to calculate activity and total ATPase, NKA, and V-ATPase activity measurements were expressed as $\mu mol \cdot ADP \cdot mg^{-1} \cdot protein \cdot h^{-1}$. The remaining supernatant from the activity assay (~200 µl) was then diluted with an equal volume of 2X Laemmli's buffer, heated to 70°C for 10 minutes in a water bath, and stored at -80°C for future SDS-Page analysis.

2.3.3 Antibodies

As described in Chapter 2 (Material and Methods 2.3.3), the α-subunit of NKA was detected using panspecific α5 mouse monoclonal antibody developed by D. Fambrough (Johns Hopkins University; Takeyasu, 1988) and used for lamprey and the αRbNKA affinity-purified anti-peptide rabbit polyclonal antibody (Ura *et al.,* 1996; Wilson *et al.,* 2007) for trout. The affinity-purified rabbit anti-peptide polyclonal B2/BvA1 antibody was also used to detect V-ATPase in both species. All antibodies were chosen based on prior successful use in fishes (Witters *et al.,* 1996; Wilson & Laurent, 2002; Chloe *et. al.*, 2004). Both αRbNKA and

B2/BvA1 antibodies were provided by Dr. Jonathan Wilson (Centro Interdisciplinar de Investigação Marinhae, Ambienta, Portugal).

2.3.4. SDS Page and Western Immunoblotting

SDS-Page and Western Immunoblotting was used to analyze NKA and V-ATPase transporter expression and followed the protocol outlined by Reis-Santos *et al.* (2004) as was written in Chapter 2 (Methods and Materials 2.3.4). Briefly, the gill homogenates diluted in 2 X Laemmli's from Methods and Materials 3.2.3 were thawed and further diluted to 0.4 µg/µl and 0.35 µg/µl total protein concentration with 1 X Laemmli's buffer in sea lamprey and trout, respectively. Samples were then vortexed, heated to 70°C for 10 minutes, and centrifuged for 5 minutes at 10,000 x *g* prior to loading 40 µl of sample onto 1.5 mm thick mini polyacrylamide gels (8%T resolving gel and 4% stacking gel). Samples were run in duplicate along with Precision Plus Protein Western C Standards protein marker at 150 V for 1 h using a Biorad Mini-PROTEAN® Tetra cell system (BioRad, Hercules, CA). Subsequently, the gels were washed in transfer buffer and wet-transferred to PVDF membranes for 1 h at 100 V using the Biorad Mini Trans-Blot® Cell system (BioRad, Hercules, CA). Following transfer, the membranes were equilibrated in 1X TTBS and then stained with Ponceau S stain to confirm protein transfer-efficiency to PVDF membranes. The membranes were then blocked with 5% dry milk in 1X TTBS for 1 h, and rinsed prior to incubation with either α5/αRbNKA or B2/BvA1 antibodies (diluted 1:1000 in 1%BSA/0.05% sodium azide/1X TTBS) overnight at 4°C. The next day, the membranes were washed with 1X TTBS prior to incubation with peroxidase-conjugated secondary goat anti-rabbit or anti-mouse Ig antibody and Precision Protein™ StrepTactin-HRP conjugate diluted 1:20,000 in 1X TTBS for 1 h at room temperature. Membranes were once again washed before detection for immunoreactivity with Clarity Western ECL Substrate (BioRad, Hercules, CA) and imaging on Molecular Imager® VersaDoc™ MP Imaging System (BioRad, Hercules, CA).

SigmaScan Pro Software Version 1.0 (Systat Software Inc., San Jose, CA) was used to semi-quantify the density of the respective bandscompared to the average of the controls. The absence of the primary antibody in the dilution buffer was used as a no primary control.

2.3.5 Immunohistochemistry

Immunohistochemistry was used to analyze expression and localization of NKA and followed the protocol provided in Chapter 2 (Methods and Materials 2.3.5). Briefly, the prepared 3-aminopropyltriethoxysilane-coated slides were air dried, dewaxed in Clear Rite, and circled using a hydrophobic barrier pen prior to rehydration in a 5% goal serum 0.1% BSA/TPBS (pH 7.4) solution for 20 minutes. Slides were then incubated with α5 or αRbNKA and rinsed with TPBS in Coplin jars and incubated for 1 h at 37°C with secondary antibodies goat anti-mouse Alexa Fluor 488 or goat anti-rabbit Alexa Fluor 594-conjgated diluted to 1:200 (Molecular Probes Inc, Eugene, OR, USA) in BSA/TPBS. Slides were washed with TPBS before a 10% Mowiol, 40% glycerol, 0.1% DABCO, 0.1 mol l^{-1} Tris (pH·8.5) solution was used to mount the coverslips and slides were then imaged using a Leica DM6000 B wide field epifluorescence microscope fitted with a digital camera (DFC340FX, Leica Microsystems, Wetzlar, Germany). Null controls and negative controls were conducted as previously described. In order to account for differences in cell mass between samples, slides were also stained with DAPI. Each slide was imaged at three different fields of view and analyzed for NKA immunofluorescence and DAPI nuclear-staining. All immunohistochemistry preparation and imaging was conducted at the Centro Interdiscplinar de Investigação Marinha e Ambiental (CIIMAR) in Ambienta, Portugal. Images were then analyzed at Wilfrid Laurier University and total NKA fluorescence was measured using the SigmaScan Pro Software Version 1.0 (Systat Software Inc., San Jose, CA). Total NKA fluorescence was calculated by comparing NKA average intensity against the total area of

fluorescence. Differences in cellular mass were accounted for by dividing the sum area stained by DAPI.

2.3.6 Light Microscopy

From the mounted slides prepared, subsets were stained with haematoxylin and eosin (H&E) using commonly practiced staining techniques to examine gill structural changes. Gill sections were then examined on a Jenaval light microscope fitted with Zeiss Axiocam MRC 5 camera and images were obtained using the Zen 2011 Blue Edition software (Carl Zeiss MicroImaging Gmbh, Germany) at Wilfrid Laurier University. Four fish were examined from each treatment group and three fields of view were imaged at both 100X and 400X. Damage to the gills by TFM was then rated on a scale of 1 to 6 using standard criteria established by Kiemer and Black (1997) and Thomassen (1993). Briefly, Grade 1 gills referred to tissue with healthy mono-layered epithelial cells and few mucousal cells on the gill lamella. Grade 2 gills exhibited minimal signs of hyperplasia or clubbing at the distal ends of the lamella and minor hypertrophy to the mucousal cell hyperplasia with no contour disturbance. Cells exhibing Grade 2 properties plus increased visible folding and wrinkling of surface epithelial cells were defined as Grade 3. Grade 4 gills presented extensive hyperplasia, fusion of the lamellae, and/or necrosis. Cells with these characteristics, plus increased lifting and/or desquamation of the epithelium over a large area were defined as Grade 5. The final stage, Grade 6, was represented by total necrosis and destruction of the lamellae in fish that were dead or dying. Grading of the gills was conducted blind to limit bias.

2.4 Calculations and Statistical Analysis

2.4.1 Determination of Na^+ Uptake

Rates of Na^+ influx ($J_{in}^{Na^+}$) were based on reductions in water radioactivity during each flux sample period, the container volume, and the mass of the fish using the following formula (Wood, 1998):

$$J_{in}^{Na^+} = \frac{(CPM_i - CPM_f)}{MSA} \frac{V}{W \times T},$$

where CPM_i and CPM_f are the radioactive counts of the isotope in the water at the start and end of each flux period (counts min^{-1} mL^{-1}), *MSA* is the mean specific activity of $^{24}Na^+$ during the flux period (counts min^{-1} μmoL^{-1}), *V* is the container volume (ml), *W* is the mass of the fish (g), and *T* is the time elapsed between the start and finish of the flux period (h).

Estimates of $J_{net}^{Na^+}$ were calculated using the following equation:

$$J_{net}^{Na^+} = \frac{(X_i - X_f)}{M \times T}$$

where X_i and X_f are the 'cold' (non-radioactive) Na^+ concentrations in the water at the start and finish of each flux period (nmol mL^{-1}), and *V*, *W*, and *T* are the same as previously stated above. Outward movements of Na^+ ($J_{out}^{Na^+}$) was calculated from the difference between $J_{net}^{Na^+}$ and $J_{in}^{Na^+}$:

$$J_{out}^{Na^+} = J_{net}^{Na^+} - J_{in}^{Na^+}$$

2.4.2 Statistics

Data were presented as the mean ± 1 standard error of the mean (SEM). All comparisons were unpaired tests and statistical significance was determined using Prism 6 Version 1.0 (GraphPad Software Inc, La Jolla, CA). TFM exposure and Post-TFM Recovery were treated as separated experiments. Therefore, significance between controls and TFM-exposure groups were determined using one-way analysis of variance (ANOVA) and a Holm-

Sidak post-test was used when significant variability was observed between sample groups at the $p<0.05$ level. When the conditions for homogeneity of variance were not met, a nonparametric ANOVA test was used for comparisons between control and TFM-exposure groups followed by Dunn's post-test. Controls and 24 h recovery groups were compared using an unpaired T-test or an unpaired T-test with Welch correction for comparisons between the control and recovery, as appropriate ($p <0.05$).

3. Results

3.1 Acute Toxicity of TFM in Soft Water

The larval sea lampreys exposed to TFM in soft water were found to have a 12 h LC_{50} of 0.39 mg L^{-1} (CI = 0.34 – 0.43 mg L^{-1}), and 12 h $LC_{99.9}$ of 0.80 mg L^{-1} (CI = 0.61-1.93 mg L^{-1} TFM (data not shown). Subsequently, all sea lamprey experiments were conducted at a nominal TFM concentration equal to the 12 h LC_{50} value, and the rainbow trout experiments were conducted using the 12 h $LC_{99.9}$ of larval sea lamprey.

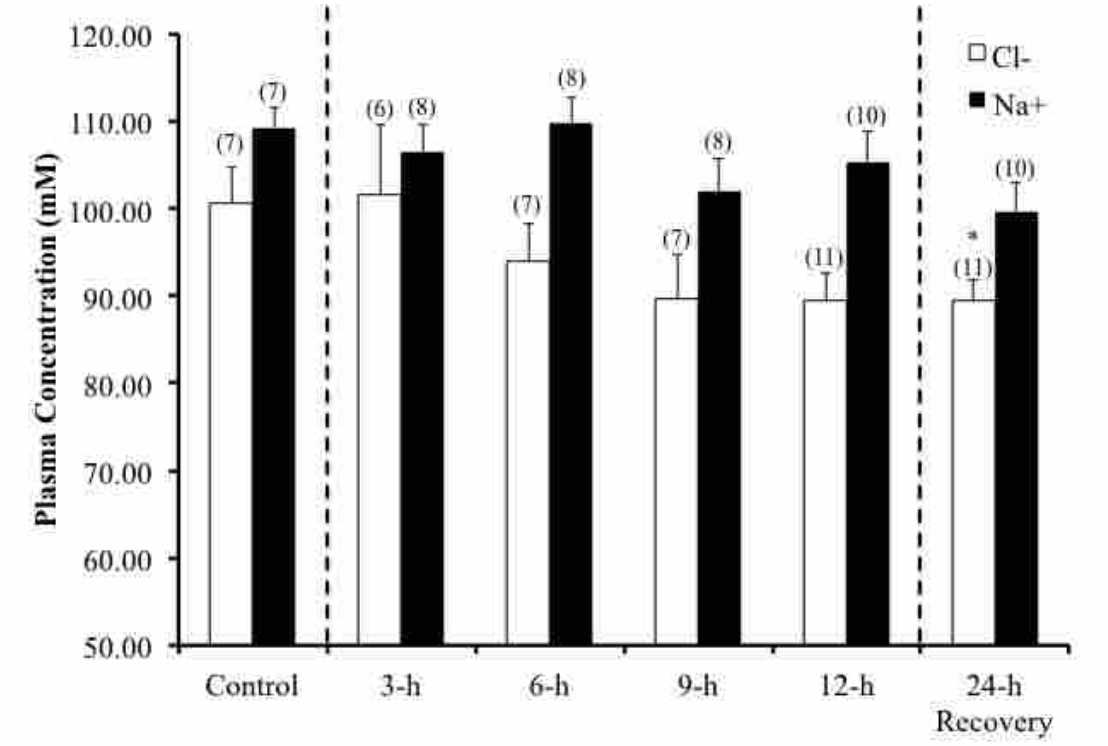
3.2 The Effects of TFM Exposure on Gill Function in Larval Sea Lamprey

3.2.1 Plasma Concentrations

TFM exposure had minor effects on plasma Na^+ homeostasis in larval lamprey, which was stable near control values of 109.9 ± 2.8 mM throughout the TFM exposure period and during the 24 h depuration period in TFM-free water. There was, however, an effect on plasma Cl^- concentrations, which decreased by almost 10 % during the TFM exposure period from control values of 100.57 ± 4.2 mM, where it remained through the 24 h depuration period (Figure 4.1).

Figure 4.1 Effects of TFM on plasma ion balance in larval lamprey.

Concentrations of plasma Na^+ and Cl^- in resting larval sea lamprey *(Petromyzon marinus*) during exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at its 12 h LC_{50} (0.38 mg L^{-1}) and following 24 h recovery in TFM-free water. Open bars denote Cl^- concentration and solid bars denote Na^+ concentration. Data are expressed as the mean +1 SEM (*N*). * Indicates a significant difference between controls and the 24 h recovery group ($p<0.05$).


Plasma Concentration (mM)
120.00
110.00
100.00
90.00
80.00
70.00
60.00
50.00
Cl-
Na+
(7)
(7)
(6)
(8)
(7)
(8)
(7)
(8)
(11)
(10)
(11)
(10)
*
Control
3-h
6-h
9-h
12-h
24-h Recovery

3.2.2 Na^+,K^+-ATPase and Vacuolar H^+-ATPase Activity and Expression

In larval sea lamprey, V-ATPase activities were below levels of detection and are therefore not reported. Nevertheless, measurements of total ATPase activity and NKA activity were completed indicating that there was a downward trend in ion-transporter capacity. There were no significant changes in Total ATPase activity from the control values of 8.9 ± 0.6 μmol ADP mg protein^{-1} h^{-1} after 3 h or NKA activity which remained near control values of 2.0 ± 0.3 μmol ADP mg protein^{-1} h^{-1} (Figure 4.2).

Western blot analysis using the α5 antibody for NKA yielded a single band located at approximately 100 kD, the proposed size of the α-subunit of the NKA transporter (Hwang and Lee, 2007). The B2/BvA1 antibody for V-ATPase immunoreacted with a pair of bands at approximately 56 kD along with a few higher and lower molecular weight bands as was found in the Reis-Santos *et al*. (2008) study. Semi-quantification of these bands found no changes in the protein expression levels of the NKA α-subunit or the V-ATPase B subunit between controls, the exposure groups, or the animals permitted to recover in TFM free water (Figure 4.3a; 4.3b). No false positives (bands) were found following the completion of the no primary antibody control experiments, however a band was observed at 75 kD for both NKA and V-ATPase.

3.3 The Effects of TFM Exposure on Gill Function in Rainbow Trout

3.3.1 Plasma Concentrations

Exposure of juvenile rainbow trout to the $LC_{99.9}$ of TFM for larval sea lamprey did not alter plasma Na^+, which did not significantly change from the control concentrations of 168.0 ± 4.0 mM. There were no significant reductions in plasma Cl^- from the control values during TFM exposure, with values fluctuating around the control measurement of 105.6 ± 1.7 mM. However, there was a significant 20 % increase in plasma Cl^- concentrations following 24 h depuration in TFM-free water (Figure 4.4).

Figure 4.2 Effects of TFM on gill ion-transporter activity in larval lamprey.

Differences in Na^+,K^+-ATPase (NKA) and total ATPase activity of resting larval sea lamprey (*Petromyzon marinus*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at its 12 h LC_{50} (0.38 mg L^{-1}) and following 24 h recovery in TFM-free water. Open bars denote NKA activity and solid bars Total ATPase activity. Data are expressed as the mean +1 SEM (*N*; $p<0.05$).

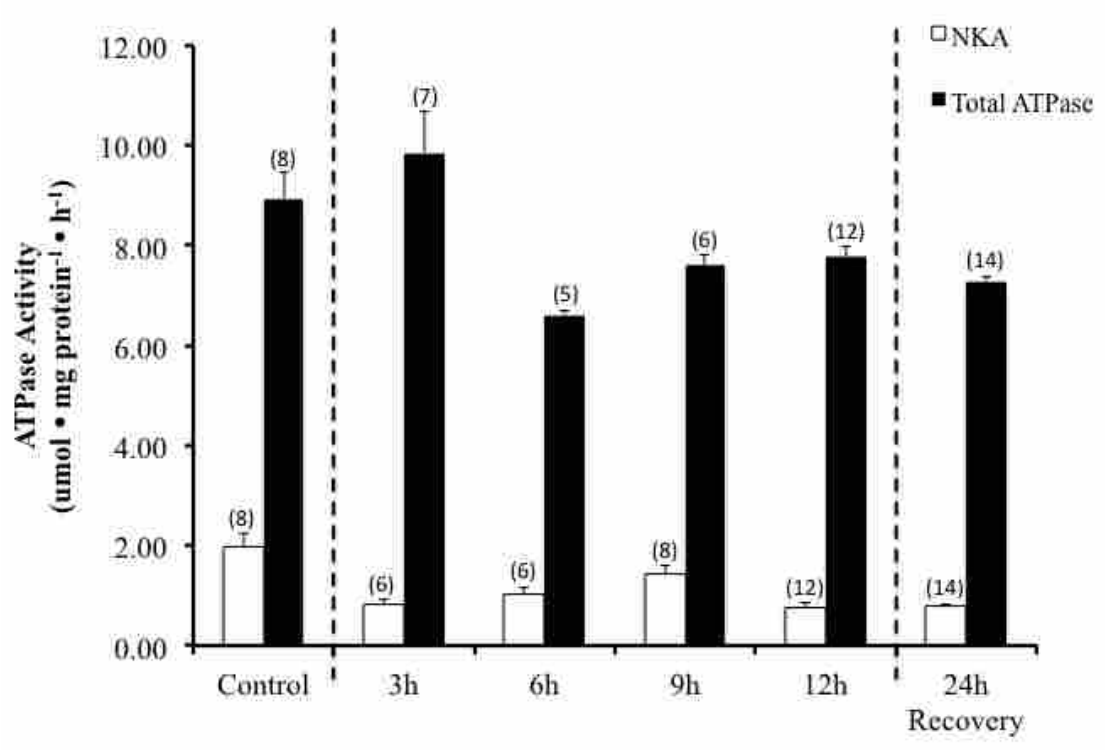
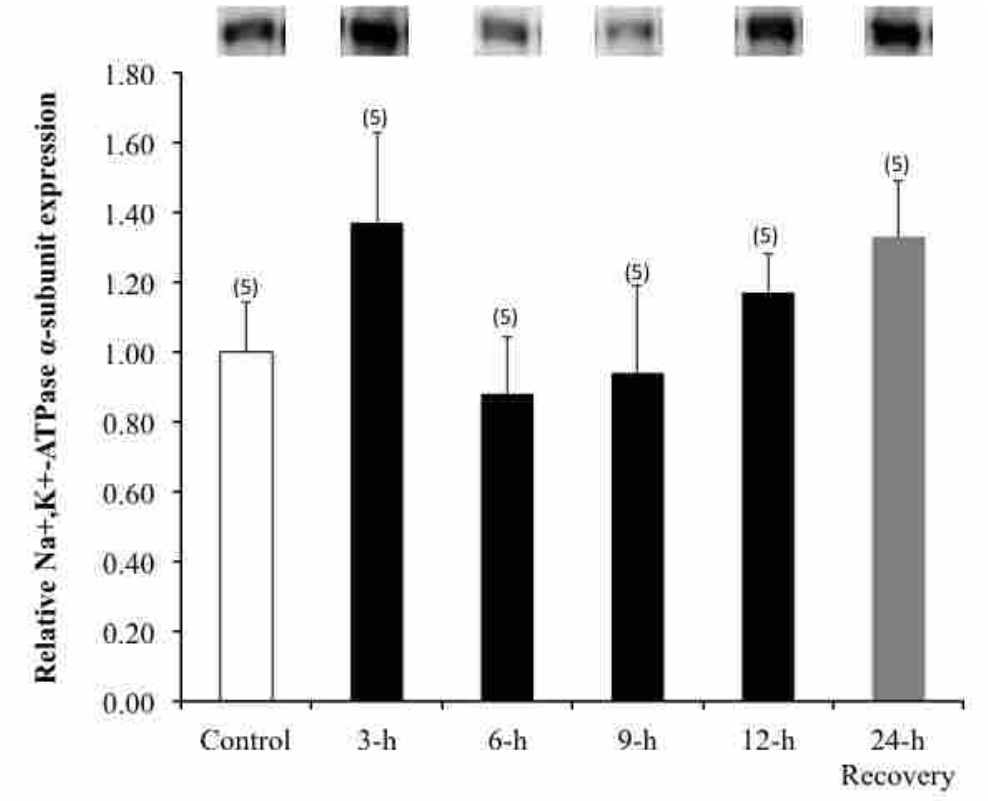

NKA
Total ATPase
ATPase Activity
(umol • mg protein-1 • h-1)
12.00
10.00
8.00
6.00
4.00
2.00
0.00
(8)
(8)
(6)
(7)
(6)
(5)
(8)
(6)
(12)
(12)
(14)
(14)
Control
3h
6h
9h
12h
24h
Recovery

Figure 4.3. Effects of TFM on gill ion-transporter expression in larval lamprey.

Differences in (a) Na^+,K^+-ATPase (NKA) α-subunit and (b) vacuolar H^+-ATPase (V-ATPase) B-subunit expression in resting larval lamprey (*Petromyzon marinus*), during exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h LC_{50} (0.38 mg L^{-1}) of larval sea lamprey, and following 24 h recovery in TFM-free water. Data are expressed as the mean +1 SEM (*N*; $p<0.05$). Representative immunoblots of gill probed with the NKA α-subunit antibodies α5 and V-ATPase B-subunit antibody B2/BvA1 are shown above their respective bars taken from the same blots.

(a)


Relative Na+,K+-ATPase α-subunit expression
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
(5)
Control
3-h
6-h
9-h
12-h
24-h Recovery

(b)

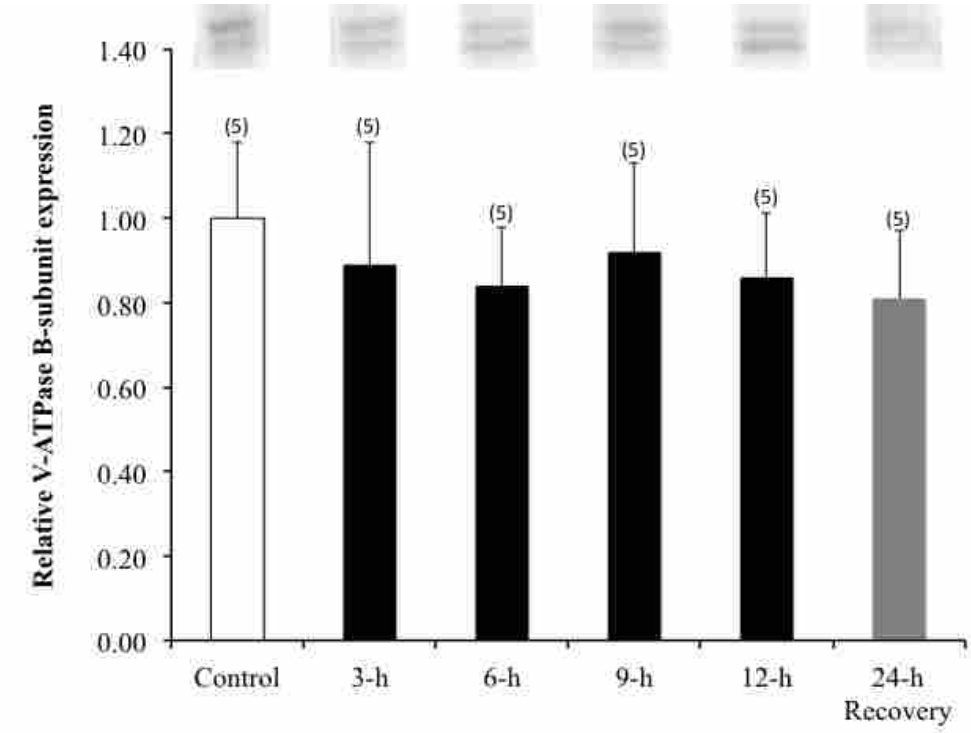
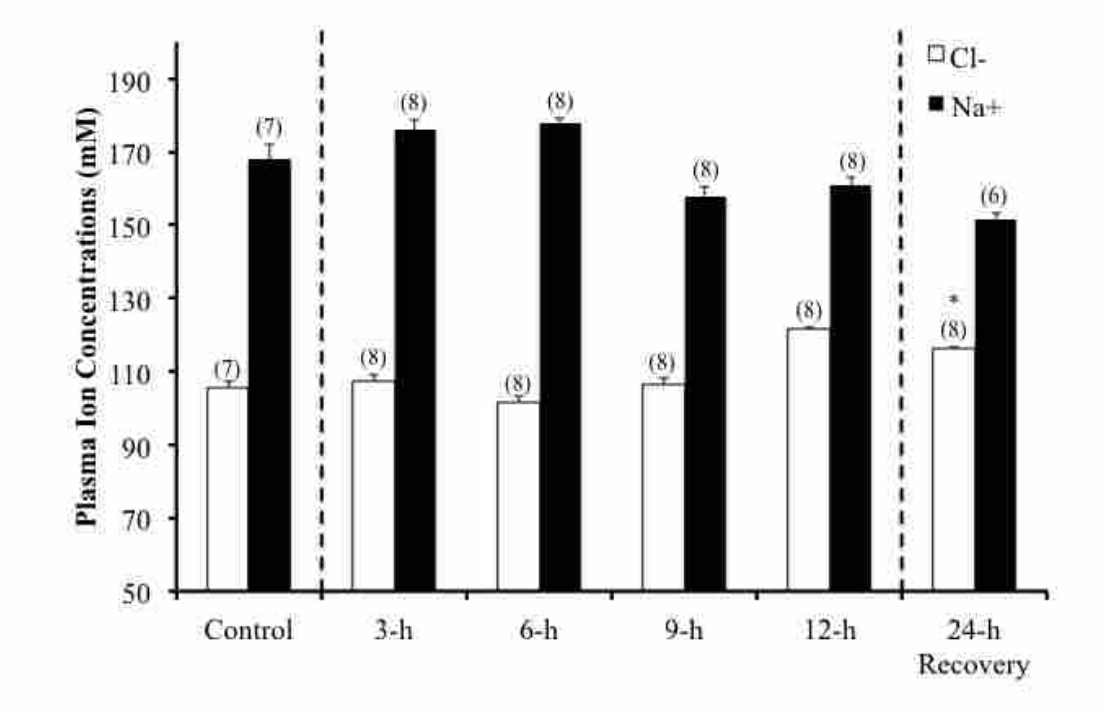

Relative V-ATPase B-subunit expression
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
(5)
Control
3-h
6-h
9-h
12-h
24-h Recovery

Figure 4.4 Effects of TFM on plasma ion balance in juvenile rainbow trout.

Concentrations of plasma Na^+ and Cl^- in resting juvenile rainbow trout *(Oncorhynchus mykiss*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h $LC_{99.9}$ (0.8 mg L^{-1}) of larval sea lamprey and following 24 h recovery in TFM-free water. Open bars denote Cl^- concentrations and solid bars the concentrations of Na^+. Data are expressed as the mean +1 SEM (*N*). * Indicates a significant difference between controls and the 24 h recovery group ($p<0.05$).


Plasma Ion Concentrations (mM)
190
170
150
130
110
90
70
50
Cl-
Na+
(7)
(7)
(8)
(8)
(8)
(8)
(8)
(8)
(8)
(8)
(8)
(6)
*
Control
3-h
6-h
9-h
12-h
24-h
Recovery

3.3.2 Na^+-Uptake and loss using radiotracer $^{24}Na^+$

Exposure to TFM had no effect on $J_{in}^{Na^+}$ which averaged 681.8 ± 51.6 nmol g^{-1} h^{-1} under control conditions. Similarly there were no significant changes on $J_{out}^{Na^+}$ or $J_{net}^{Na^+}$ under control conditions, which averaged -935.43 ± 332.69 nmol g^{-1} h^{-1} and -253.67 ± 295.13 nmol g^{-1} h^{-1}, respectively. Although not found to be significant, there appeared to be a net loss of Na^+ at 3-6, with a mean rate of -1783.90 ± 612.45 nmol g^{-1} h^{-1} during the first 3 h of TFM exposure. However, this net loss of Na^+ was corrected by 6-9 h, when $J_{net}^{Na^+}$ was once inwardly directed at a rate of to 542.45 ± 562.85 nmol g^{-1} h^{-1} (Figure 4.5).

3.3.3 Na^+,K^+-ATPase and Vacuolar H^+-ATPase Activity and Expression

Under control conditions, NKA activity averaged 3.31 ± 0.13 μmol ADP mg $protein^{-1}$ h^{-1}, and remained near these control levels during the TFM exposure and recovery periods (Figure 4.6). Like NKA, there were no changes in V-ATPase values from the control activity rate of 0.65 ± 0.03 μmol ADP mg $protein^{-1}$ h^{-1} (Figure 4.6). There was however, a 1.5-fold significant rise in total ATPase activity at 6 h and 9 h of TFM exposure from the control rate of 8.22 ± 0.47 μmol ADP mg $protein^{-1}$ h^{-1}, which was followed by a decline towards control rates thereafter (Figure 4.6).

In trout, the αRbNKA immunoreacted with a band located at approximately 100 kD. Western blot analysis of the NKA α-subunit revealed no significant differences for transporter expression in the gill (Figure 4.7). No false positives were found in the no-primary control blots, but a single band was noted at the 75 kD range, as reported in the sea lamprey above.

3.3.4 Na^+,K^+-ATPase (NKA) distribution in the rainbow trout gill

There was no visible background staining found in the gill epithelium for either

Figure 4.5 Unidirectional movements of Na^+ across the gills for juvenile rainbow trout during TFM exposure.

Unidirectional (influx, efflux, net flux) movements of Na^+ across the gills of resting juvenile rainbow trout *(Oncorhynchus mykiss*) measured during a 4 h control flux (*N*=12) and during exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h $LC_{99.9}$ (0.80 mg L^{-1}) of larval sea lamprey (*N*=11). Open bars indicate influx rates, lightly shaded bars efflux rates, and dark shaded bars net-flux rates of Na^+. Data are expressed as the mean +1 SEM (*N*; $p<0.05$)

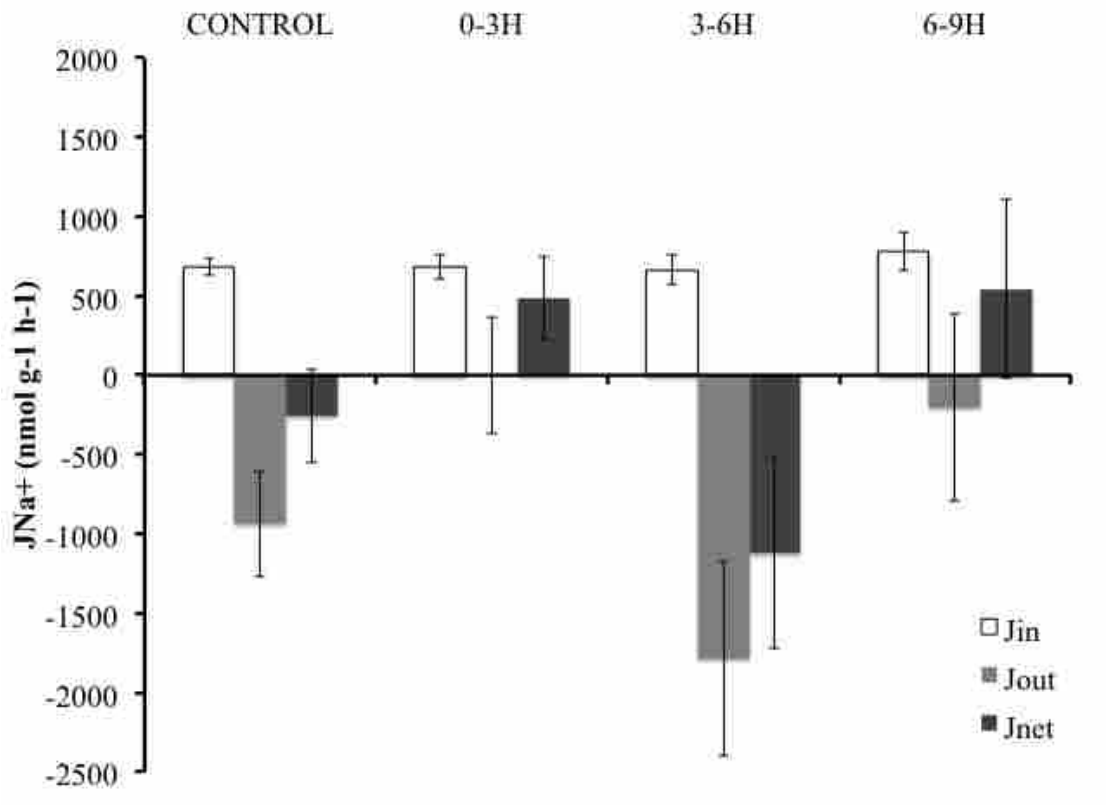

CONTROL
0-3H
3-6H
6-9H
2000
1500
1000
500
0
-500
-1000
-1500
-2000
-2500
JNa+ (nmol g-1 h-1)
Jin
Jout
Jnet

Figure 4.6 Effects of TFM on gill ion-transporter activity in juvenile rainbow trout.

Changes in the Na^+,K^+-ATPase (NKA), vacuolar H^+-ATPase (V-ATPase), and total ATPase activities of resting juvenile rainbow trout (*Oncorhynchus mykiss*) following exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h $LC_{99.9}$ (0.80 mg L^{-1}) of larval sea lamprey and following 24 h recovery in TFM-free water. Open bars denote NKA activity, gray bars V-ATPase activity, and solid bars Total ATPase activity. Data are expressed as the mean +1 SEM (*N*). * Indicates significant difference between TFM exposure groups and the control group ($p<0.05$).

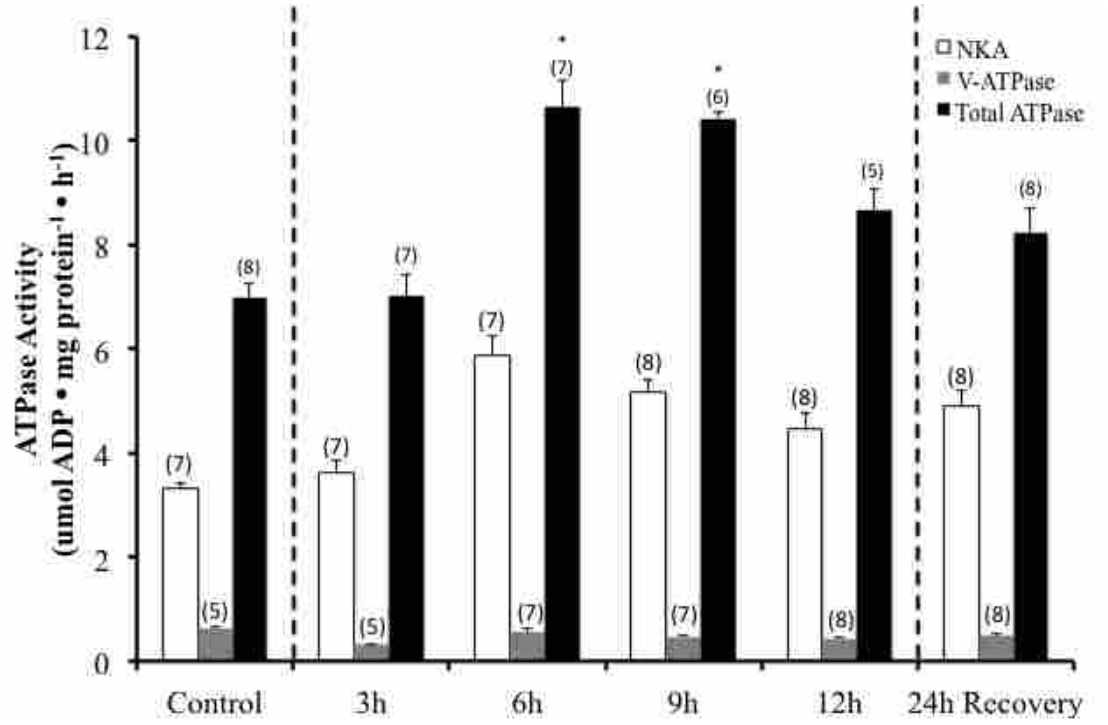
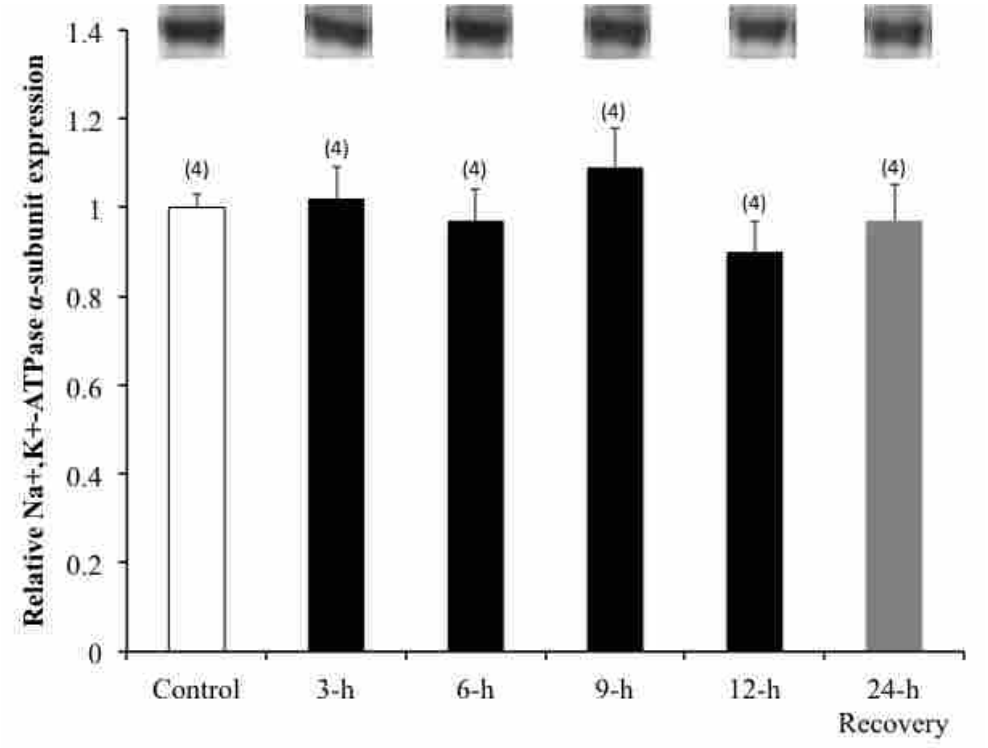

ATPase Activity
(umol ADP • mg protein-1 • h-1)
12
10
8
6
4
2
0
NKA
V-ATPase
Total ATPase
(7)
(5)
(8)
(7)
(5)
(7)
(7)
(7)
(8)
(6)
(7)
(8)
(8)
(5)
(8)
(8)
(8)
Control
3h
6h
9h
12h
24h Recovery

Figure 4.7 Effects of TFM on gill ion-transporter expression in juvenile rainbow trout.

Changes in NKA α-subunit expression in resting juvenile trout (*Oncorhynchus mykiss*) during exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h $LC_{99.9}$ concentration (0.80 mg L^{-1}) of larval sea lamprey, and following 24 h recovery in TFM-free water. Data are expressed as the mean +1 SEM (*N*; $p<0.05$). Representative immunoblots of gill probed with the NKA α-subunit antibodies αRbNKA are shown above the respective bars taken from the same blot.


Relative Na+,K+-ATPase α-subunit expression
1.4
1.2
1
0.8
0.6
0.4
0.2
0
(4)
(4)
(4)
(4)
(4)
(4)
Control
3-h
6-h
9-h
12-h
24-h Recovery

control. Immunohistochemical analysis revealed that the NKA was localized to the basolateral membrane of epithelia cells of the gill filament and proximal regions of the lamellae (Figure 4.8). These cells were mainly concentrated within the interlamellar spaces primarily on the gill filament. Reactivity was stronger on the afferent side of the gill filament, but cell staining was distributed on the either side. Additional weaker cytoplasmic staining was noted in some cells in close association with MRCs. There were no notable differences between control gills or gills exposed to 0.80 mg L^{-1} TFM for 12 h (A-E) or following 24 h of depuration in TFM free water (F; Figure 4.8) Global relative fluorescence analysis of the gill filaments found no significant differences between either the control, TFM treatment groups, or 24 h of recovery (Figure 4.9).

3.4 The Effects of TFM Exposure on Gill Structure in Rainbow Trout

H&E stained slides demonstrated only minor evidence of histological disturbance in the gill tissue of the rainbow trout exposed to the TFM $LC_{99.9}$ of larval lamprey (0.8 mg L^{-1}). Control gills were found to be healthy with mono-layered epithelial cells and few mucous cells on the secondary lamellae. Gills from the 6 h, 12 h, and 24 h recovery groups were not found to visually differ from the controls that were rated as Grade 1. Clubbing was noted on the tips of some of the secondary lamellae with minor hypertrophy in one fish at 3 h and in two fish at 9 h, which were correspondently designated grade 2 gills. All other gills sections examined were rated grade 1 and were considered to be healthy normal gills (Figure 4.10).

Figure 4.8 Immunolocalization of Na^+,K^+-ATPase (NKA) transporter in TFM exposed gills of juvenile rainbow trout.

Immunolocalization of NKA α subunit (red) at 0 h (control; A), 3 h (B), 6 h (C), 9 h (D), 12 h (E), and 12 h of TFM exposure followed by a 24 h recovery in TFM-free water (F). The corresponding DAPI-nuclear stained images are shown below each NKA labeled figure as blue-stained images.

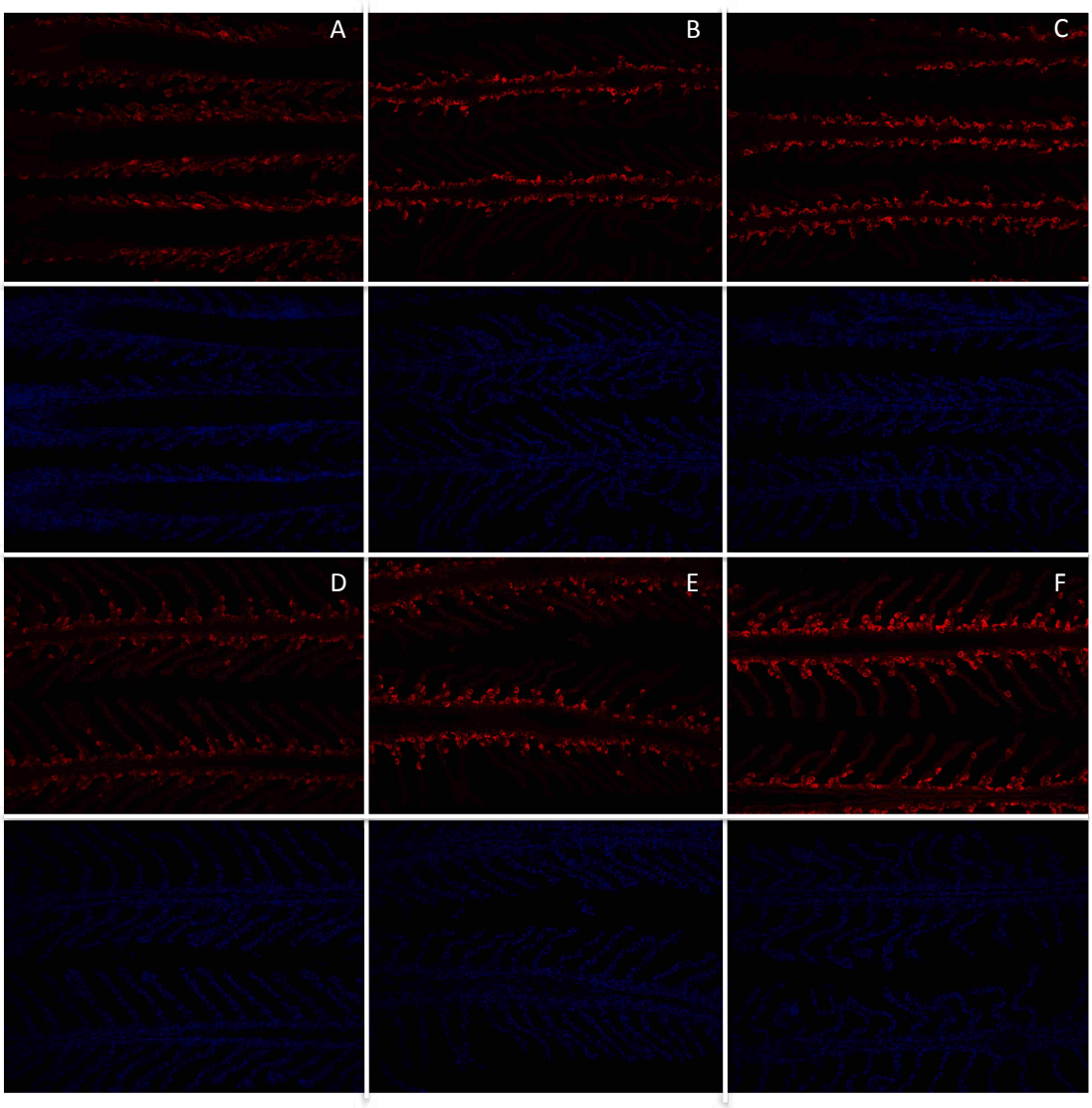

A
B
C
D
E
F

Figure 4.9 Relative immunofluorescence of Na^+,K^+-ATPase (NKA) transporter in TFM exposed gills of juvenile rainbow trout.

Relative immunofluorescence of gill NKA expression in control juvenile rainbow trout (*Oncorrhyncus mykiss*) and during exposure to 3-trifluoromethyl-4-nitrophenol (TFM) at the 12 h $LC_{99.9}$ concentration (0.80 mg L^{-1}) for 12 h and following 12 h exposure and 24 h recovery in TFM-free water. Data are expressed as the mean +1 SEM (*N*; $p<0.05$).

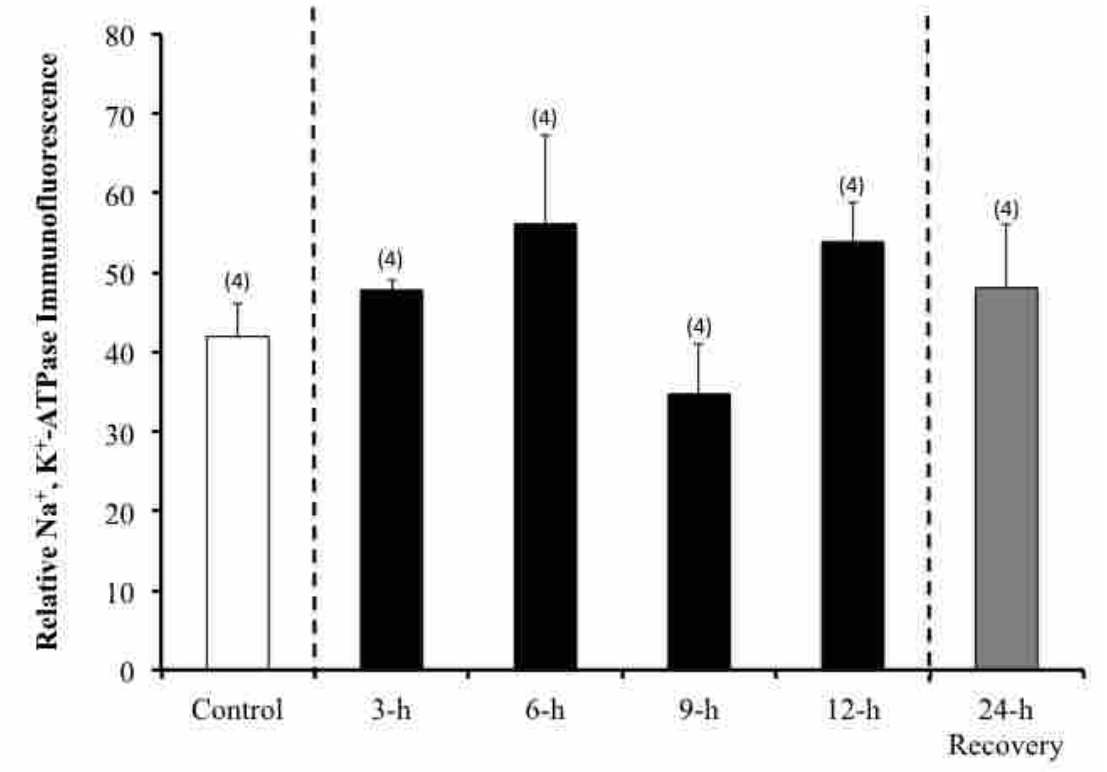
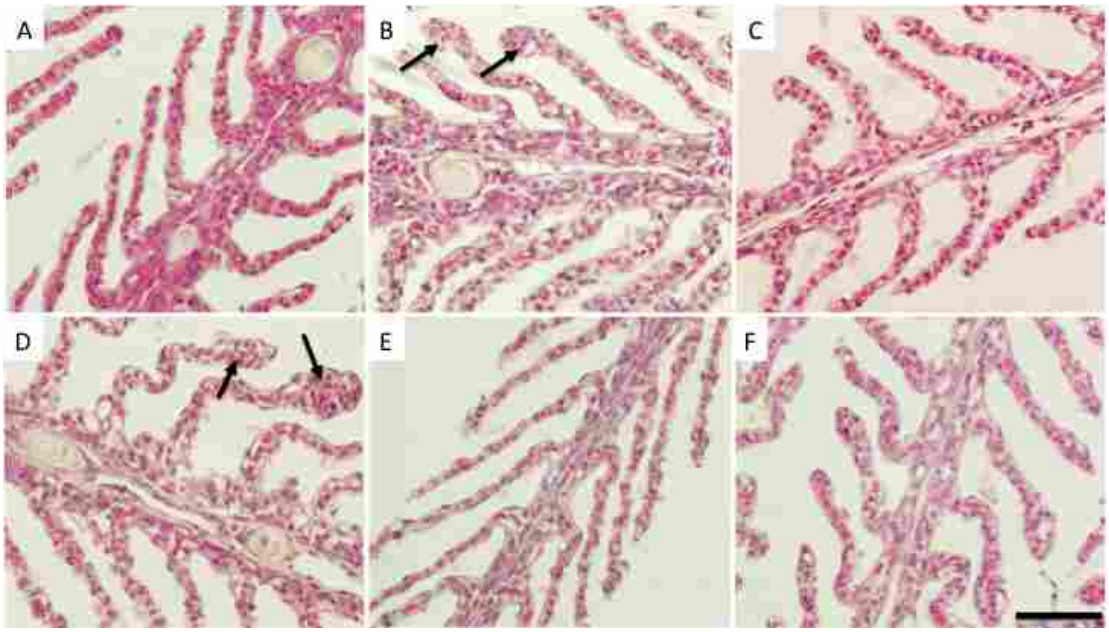

Relative Na+, K+-ATPase Immunofluorescence
80
70
60
50
40
30
20
10
0
(4)
(4)
(4)
(4)
(4)
(4)
Control
3-h
6-h
9-h
12-h
24-h
Recovery

Figure 4.10 Effects of TFM on gill structure of juvenile rainbow trout

Haematoxylin and eosin stained rainbow trout gills from (a) control fish and fish exposed to $LC_{99.9}$ of TFM for larval sea lamprey (0.8 mg L^{-1}) for (b) 3 h, (c) 6 h, (d) 9 h, (e) 12 h and (f) following 24 h recovery in TFM-free water. Gill sections were 4–5 μm thick. Arrows indicate hypertrophy of the lamellar tips. Images taken at 400X. Scale bar, 100 μm.


A
B
C
D
E
F

4. Discussion

4.1 The Effect of TFM on Gill Function and Structure

4.1.1 Differences in the Toxicity of TFM in Soft Water versus Hard Water

It is well established that water pH and alkalinity markedly influence TFM toxicity in sea lampreys and non-target fishes (Bills *et al.,* 2003; McDonald and Kolar, 2007). However, the effects that differences in water hardness have on TFM toxicity in sea lampreys and non-target fishes have received less attention. Indeed, water hardness varies markedly in the Great Lakes, ranging from very soft water (between 40 to 80 mg L^{-1} as $CaCO_3$) in many tributaries of Lake Superior (Upper Lakes Reference Group, 1977), to very hard water (up to 1803 mg L^{-1} as $CaCO_3$) in some river systems that feed or drain Lake Ontario (Ontario Ministry of the Environment, 1975). Olson and Marking (1973) reported that the sensitivity of larval sea lampreys to TFM was greater in soft compared to hard waters, but the underlying basis for such observations was not established and the interpretation of their results may have been complicated by differences in water pH.

The LC_{50} of TFM reported here for sea lamprey in soft water (0.4 mg L^{-1}) was approximately 1/10 that was previously reported in much harder (~450 mg as $CaCO_3$ L^{-1}) Wilfrid Laurier well water. It is important to note that the pH of the water in the present study (~ pH 7.1) was lower than the preceding studies on gill function in rainbow trout and sea lamprey conducted in hard water (~ pH 7.8; Chapter 2). The results of preliminary toxicity tests on the larval sea lamprey would reflect such differences in water pH, however, which markedly influences TFM speciation (Bills *et al.,* 2003; Clifford *et al.,* 2012). Indeed, the measured 12 h LC_{50} for TFM to sea lamprey in the pH 7.1, low alkalinity water of the present study was 0.4 mg L^{-1}, very close to the values reported in toxicity tables published by Bills *et al.* (2003). Nevertheless, the differences in water pH make it more difficult to determine if the

differences in water hardness and ion content also have contributed to the differences in TFM toxicity that were observed.

The uptake of TFM appears to be mainly, if not exclusively, in its un-ionized more lipophilic form as opposed to its ionized, more hydrophilic form (Hunn and Allen 1974; L. Tessier, A. Smits, and M.P. Wilkie, unpublished findings). Based on the assumption that TFM uptake takes place in this form, the toxicity of TFM can therefore be expressed as un-ionized TFM rather than total TFM. This would make it possible to correct for the differences in water pH between the two studies, and directly compare the toxicities of TFM in soft *versus* hard water. Accordingly, to determine if the observed differences in TFM toxicity were mainly due to pH-induced differences in TFM speciation, the proportion of total TFM in its in un-ionized form was calculated using the Henderson-Hasselbalch equation and the known pK_a of TFM (pKa = 6.07; Hubert, 2003). Based on these calculations the trout and sea lamprey in the previous hard water experiments were exposed to un-ionized concentrations of TFM of, 0.15 mg L^{-1} and 0.09 mg L^{-1}, respectively. In softwater, however, the concentrations of un-ionized TFM were in fact lower, at 0.08 mg L^{-1} and 0.04 mg L^{-1} (Table 4.1). Therefore, the difference in pH does not likely completely explain the greater TFM toxicity observed in soft water.

4.2 The Effect of TFM on Ionoregulation in Larval Sea Lamprey

Despite previous histological and ultrastructural studies on sea lamprey gills that have demonstrated there was significant damage to the MRCs in the branchial epithelium following TFM exposure (Mallatt *et al.,* 1985; 1994), the present results suggest that TFM had only minor effects on ionoregulation. Plasma Na^+ levels were near control values throughout the duration of the TFM exposure, and the reductions in plasma Cl^-, however, may have been more closely related to acid-base regulation rather than arising from impaired

Table 4.1 Calculated LC_{50} and $LC_{99.9}$ values for un-ionized ($TFM\text{-}O^-$), ionized (TFM-OH), and total 3-trifluoromethyl-4-nitrophenol (Total TFM) in hard and soft water.

The LC_{50} and $LC_{99.9}$ were calculated from total TFM concentrations, and converted to TFM-OH, and $TFM\text{-}O^-$ using the Henderson-Hasselbalch equation. Toxicity tests were conducted in hard water (total water hardness of ~450 mg $CaCO_3$ L^{-1}, pH 7.8; Chapter 2) and soft water (total water hardness of ~40 mg $CaCO_3$ L^{-1}, pH 7.1; present chapter).

		[TOTAL TFM] (mg L^{-1})	[TFM-OH][a] (mg L^{-1})	[$TFM\text{-}O^-$][b] (mg L^{-1})
HARDWATER:	$LC_{99.9}$	7.60	0.14	7.46
	LC_{50}	4.60	0.08	4.42
SOFT WATER:	$LC_{99.9}$	0.80	0.07	0.73
	LC_{50}	0.38	0.03	0.35

[a] $[TFM\text{-}OH] = [TFM]_{total}/[1 + antilog(pH - pKa_{TFM})]$, where pH is the experimental water pH and pKa_{TFM} is 6.07 (Hubert, 2003). The pKa_{TFM} denotes the negative log of the dissociation constant of TFM (or pKa of TFM).

[b] $[TFM\text{-}O^-] = [TFM]_{total} - [TFM\text{-}OH]$.

ATP supply to the ion transport machinery (Figure 4.1) as discussed below in 4.4. A similar lack of of ion-disturbances has also been reported in prior TFM-hardwater studies (Wilkie *et al*., 2007; Birceanu *et al*., 2009; Henry *et al*., 2011). Additionally, despite reports of TFM-induced damage to the MRCs in previous histological studies of the gill epithelium (Mallatt *et al.*, 1994; 1995), there were no significant changes in ion-transporter activity or protein expression levels were observed in response to TFM exposure in the present experiments (Figure 4.2; 4.3). Decreases in transporter activities have been found with sub-lethal exposure to alumimum, copper, and lead (Camargo *et al.,* 2009; Salgam, *et al.,* 2013; da Silvia and Martinez, 2014), which have been linked to loss or damage of MRCs in the lamellae and filamental epithelium in freshwater fishes (Dang *et al.,* 2000; Monette and McCormick, 2008; Carmargo *et al.,* 2009). The apparent lack of an effect on transporter activity or abundance suggests that sub-lethal exposure of TFM does not critically damage transporter machinery as previously suggested (Mallat *et al*., 1985; 1994).

4.3 The Effect of TFM on Ionoregulation in Rainbow Trout

Although the tolerance of salmonid fishes to TFM is 3-5 X higher compared to larval sea lamprey (Boogaard *et al.,* 2003), the mechanism of TFM toxicity in sea lampreys and rainbow trout results from impaired ATP production due to an uncoupling of oxidative phosphorylation (Birceanu *et al.,* 2009; Henry, 2011; Le Clair, 2014). Because TFM interferes with ATP production, it was predicted that it would interfere with ATP-dependent ion exchange processes in the gill to a greater degree in soft, ion poor waters where the energy demands for ion balance would be expected to be higher than in hard water, which has has a higher ion content. The 2-3 fold higher total ATPase activity and the 4-fold higher NKA activity in the control softwater *versus* hardwater acclimated larval sea lamprey and rainbow trout (Chapter 2, Table 4.2) support the hypothesis that acclimation to soft, ion-

Table 4.2 Comparison of gill ATPase transporter activity between hard and soft water. Changes in the Na^+,K^+-ATPase (NKA), vacuolar H^+-ATPase (V-ATPase), and total ATPase activities of resting juvenile rainbow trout (*Oncorhynchus mykiss*) acclimated to hard water (total water hardness of ~450 mg $CaCO_3$ L^{-1}, pH 7.8) and soft water (total water hardness of ~40 mg $CaCO_3$ L^{-1}, pH 7.1). Hard water data from Chapter 2. Data sharing the same letter represent mean values that are not significantly different from one another.

Water Type	**NKA**	**V-ATPase**	**Total ATPase**
		(umol ADP • mg protein^{-1} • h^{-1})	
Hard Water	0.74 ± 0.01[a]	0.46 ± 0.12[a]	2.94 ± 0.40[a]
Soft Water	3.31 ± 0.12[b]	0.63 ± 0.03[a]	7.00 ± 0.25[b]

poor water results in greater ATP demands at the gill. These greater ATP demands did not appear to result in more marked disturbances to ionoregulation in either fish species following exposure to toxic concentrations of TFM, however. The minimal effects that TFM had on the rates of Na^+ influx in the rainbow trout suggest that TFM exposure did not impair the ion uptake capacity of the fish (Figure 4.5). These findings are also similar to findings from previous TFM-toxicity studies conducted in hard and moderately hard waters (Birceanu *et al.,* 2009; 2014; Henry, 2011).

The appearance of an increase in Na^+-efflux ($J_{out}^{Na^+}$) rates at 6 h of TFM might be explained by elevated ventilation rates as a result of increase oxygen-consumption. Respiration is stimulated in isolated mitochondria during TFM exposure (Birceanu *et al.,* 2011). An increase in oxygen consumption was also reported in *in vivo* in larval sea lamprey (Smith and King, 1969 cited in Kawatski and McDonald 1974) and in midge *Chironomus tentans* (Kawatski *et al.,* 1974) during TFM exposure. Curiously, Smith and King (1969) did not observe an increase in O_2 consumption in rainbow trout exposed to TFM, but the TFM concentrations to which they were exposed were not reported (cited in Kawatski and McDonald 1974). Based on the balance of data available, it therefore seems reasonable to suggest that TFM likely stimulates oxygen consumption in a dose-dependent manner, as occurs during exercise. During vigorous exercise, the vasodilation of the gill vasculature has been found to coincide with increases in ion-loss, a process termed "osmoregulatory compromise" (Randall *et al.,* 1972; Wood and Randall, 1973; Gonzalez and McDonald, 1992). The vascular dilation reported in the gills in both TFM-exposed sea lamprey and trout exposed to lethal concentrations of TFM (Christie and Battle, 1963) also supports this suggestion. Measurements of TFM uptake and oxygen consumption are needed, however, to test this hypothesis, and to determine the time course of such responses.

There were no significant changes in either NKA or V-ATPase activity or transporter abundance during exposure to TFM (Figure 4.6 and 4.7). However, the 30-40 % increases in

total ATPase activity after 6 h in the rainbow trout was likely a compensatory response to increase their combined capacity to take-up Na^+, Ca^{2+}, Mg^{2+} from the water (McCormick, 1993) in response to decreased ATP supply during TFM exposure (Figure 4.6). Compensatory increases in NKA activity were reported during exposure of rainbow trout to their 12 h TFM LC_{50} in hardwater studies (Birceanu *et al.,* 2014).

Despite prior predictions that TFM would negatively impact ion-homeostasis due to ATP-dependency for protein function, these findings suggest that the existing levels of ion-regulatory proteins were sufficient to prevent marked disturbances to ion-balance despite decreases in ATP productions noted in other tissues in similar studies (Henry, 2011; Birceanu *et al.,* 2014; Le Clair, 2014). While natural soft waters usually have lower pH (pH 6-7; our experimental water was measured at 7 and higher), and low levels of Ca^{2+}, Na^+, and Cl^-, which limits the availability of Ca^{2+}, Na^+ and Cl^- for uptake permeability, we found trout to be extremely tolerant to these stressors when exposed to TFM.

The potential increases in total ATPase activity that were observed could have been achieved via a post-translational phosphorylation event mediated by protein kinase A and cAMP, which is reversible when the transporters are no longer needed (Tipsmark and Madsen, 2001). Quantitative measurements of the branchial NKA distribution and abundance and relative densities of NKA α-subunit expression support this theory as neither were found to change during TFM exposure (Figure 4.8; 4.9). Another possible explanation for adjustment in ion loss despite no significant changes being observed for transporter activity levels or expression is the possibility that there were adjustments in the ratio of α-subunit isoform expressed. NKA is a P-type ATPase that is made up of 2 (αβ) protein complex with four α (α1−4) and three β (β1−3) isoforms (Hwang and Lee, 2007). NKA α-isoforms (α1a and α1b) expression levels have been found to change in rainbow trout acclimated to 80% SW from FW, corresponding to an elevation in gill NKA activity (Richards *et al.,* 2003).

Replacing subunits with an isoform that has greater Na^+ or K^+ affinities may compensate for temporary changes in ion-balance without necessitating energy expenditure or increased enzyme expression.

4.4 Potential Acid-Base Disturbances

In sea lamprey, there is some preliminary evidence that TFM may cause acid-base-disturbances. Acid-base balance is managed by both the physiochemical buffering of acid/base equivalents with bicarbonate and/or nonbicarbonate buffers and the net transport of acid-base constituents between the fish and its environment (Evans *et al*., 2005). The movement of Na^+ is indirectly linked with acid-balance, as Na^+ is taken-up from the water via an electroneutral Na^+/H^+ exchanger (NHE) or an apical epithelial Na^+ channel (eNac) down an electrochemical generated by V-ATPases in exchange for H^+ (Kumai and Perry, 2012). Similarly, base equivalents are exchanged with Cl^- via Cl^-/HCO_3^- exchangers present on the apical surface of MRCs and PVCs (Goss *et al.,* 1998; Wilson *et al.,* 2000; Marshall, 2002). As a result, net increases in Cl^- flux from the fish lead to net H^+ efflux, and increases in net Na^+ efflux in net base loss (Maetz, 1972; Cameron, 1976; Wood *et al.,* 1984; Wilkie *et al.,* 1998, 2001).

The decline in plasma Cl^- concentrations in lamprey may be indicative of a compensatory alkalosis (Figure 4.1) likely incurred due to an increased reliance on anaerobic glycolysis, which is necessitated to meet ATP-demand in the face of impaired aerobic ATP production by the mitochondria (Niblett and Ballantyne, 1976; Birceanu *et al.* 2009, 2014; Clifford *et al.,* 2012). The reliance on anaerobic pathways leads to excess lactate production, and concordantly, metabolic H^+ production (Hochachka and Somero 2002). Increases in lactate are commonly observed during TFM exposure in lamprey (Wilkie *et al.,* 2007; Birceanu *et al.,* 2009; Henry, 2011; Clifford *et al.,* 2012). However more detailed measurements of blood pH, total CO_2 concentration, and acid-excretion have not yet been

made. The continual decline of plasma Cl^- concentrations into the recovery period suggests that lamprey may have been excreting acid well after the TFM exposure had ceased. However, despite dropping, plasma Cl^- concentrations are within range of those noted in other studies (Wilkie *et al.,* 2007; Birceanu *et al.,* 2009), and were not likely a factor in the greater mortality observed in soft compared to hard water.

The differences between the ionic disturbances observed in hard and soft water may also reflect differences in the availability of bicarbonate in the external water. Fishes living in soft water rely more on internal physiochemical buffering of metabolic acid with bicarbonate and/or non-bicarbonate buffers because the supply of environmental [HCO_3] for Cl^-/HCO_3^- exchange is limited. Due to this limitation, soft water acclimated fish compensate for respiratory acidosis much slower than fish acclimated to hard water (Janssen and Randall 1975; Börjeson, 1977; Perry *et al.,* 1987; Dimberg 1988; Vermette and Perry 1988; Hyde and Perry 1989; Borch *et al.,* 1993; Larsen and Jensen, 1997). A similar explanation may explain why TFM-exposed fish in soft water are more sensitive to TFM than those acclimated to hard water.

While trout have a greater means of tolerating the effects of TFM exposure via glucuronidation and eliminating the solubilized toxin via defecation and the renal system (Lech and Statham, 1975; Howell *et al.,* 1980; Clarke *et al.,* 1991; Kane *et al.,* 1993) increases in lactate concentrations suggests increased reliance on anaerobic pathways to maintain homeostasis (Birceanu *et al.,* 2009; 2014; Le Clair, 2014). While lactate concentrations have not been compared between hard and soft water for rainbow trout, it is known that TFM toxicity is greater in soft water (Olson and Marking, 1973). Additionally, while TFM does not appear to have a pronounced effect on ionoregulation, it remains unclear why TFM sensitivity increases in soft water. Because ATP-demands for maintaining ion balance is related to salinity (Morgan and Iwama, 1998), it is possible that energy-reserves for other homeostatic processes may be limited in softer waters. However, further

experimentation is required to investigate differences in the metabolic profile between soft- and hard-water acclimated trout and sea lamprey during TFM exposure.

The difference between the trout and the sea lamprey could also be related to the very different acid-base buffering capacities of the two fishes. Sea lampreys have a low bicarbonate and non-bicarbonate buffering capacity in their blood compared to teleost fishes such as the rainbow trout (Tufts *et al.,* 1989; Boutilier *et al.,* 1993). As a result, acid-base disturbances (metabolic acidosis) would be expected to be more pronounced in the sea lamprey than the trout if they were subjected to similar loads of internal TFM. Of course, the amount of internal TFM likely present in the trout was less than in the sea lamprey, due to the former's higher capacity to detoxify TFM via glucuronidation (Lech and Statham, 1974; Kane *et al.,* 1994; Birceanu *et al.,* 2014). This too, would result in less pronounced metabolic H^+ production, and less extreme disturbances to acid-base balance. Clearly, further studies are needed to more accurately compare and quantify how TFM affects pH homeostasis in lamprey.

4.5 The Effect of TFM on Gill Structure

Prior histological studies have demonstrated a direct impact of TFM on the gills of sea lamprey, causing edema, swelling of the vasculature of the gill epithelium, and specific damage to the microvilli and even cytolysis of the MRCs (Christie and Battle, 1963; Mallatt *et al.,* 1984; 1994). Similar MRC-specific damage was not found in rainbow trout (Mallatt *et al.,* 1994). However, mucus cell hyperplasia, vascular edema, and epithelial lifting has been noted in American brook lamprey (*Entosphenus lamottei*, Christie and Battle, 1963) leading to the hypothesis that TFM may interfere with gill function as a result of damage to MRCs (Mallatt *et al.,* 1994; 1995). Contrary to these studies, we only found evidence of minor damage to the secondary lamellae in one fish at 3 h and in two fish at 9 of TFM exposure, with clubbing and hypertrophy most noticeable at the distal ends of the lamella (Figure 4.9).

This tissue damage was not seen following 24 h of recovery from TFM in the trout (Figure 2.9), or in the trout exposed to TFM in hard water (Chapter 2). These alterations to gill morphology were not found to cause significant impairment to gill function in TFM-exposed trout, despite prior predictions that the larger outward electrochemical gradient of soft water would incur more significant damage to the MRCs due to higher dependency on ATP-dependent transporters to maintain ion balance. One explanation for these differences is that the Christie and Battle (1963) study sampled fish following TFM-induced mortality, when a cascade of events could have lead to post-mortem damage to the gills. As such, the present study, along with prior studies finding no significant changes to ion balance or transporter activity (Henry, 2011; Birceanu *et al.,* 2014) argue against any substantial TFM-mediated interference with gas exchange or physiological processes mediated by the gills of sea lampreys or trout following their exposure to TFM.

4.6 Implications for Lamprey Control and Future Directions

The application of TFM serves as the principle control method for invasive sea lamprey populations (McDonald and Kolar, 2003). However, despite its success in reducing sea lamprey populations and protecting sport and commercial fisheries from sea lamprey predation, the effects of TFM on non-target organisms and the roles that both abiotic and biotic factors play in the toxicity of TFM to non-target species has been a subject of increasing concern. While the selectively of TFM has been demonstrated and correlated to the ability of aquatic species to detoxify TFM via the liver UDP glucuronyl-transferase, species-specific differences in TFM-sensitivity have not been completely resolved. As the present study has showed, TFM appears to have minimal effects on gill function and ionoregulation in both sea lamprey and rainbow trout, but the effects on acid-base balance in TFM-sensitive sea lamprey and non-target fishes remain unexplored. However, it is important to note, that sea lamprey in the present study were exposed to the LC_{50} of TFM, not 1.2-1.3

times the $LC_{99.9}$, which is typically used for field applications of the lampricide (McDonald and Kolar, 2007). As such, the impact of TFM on gill function could be more pronounced in the field.

Because sensitivity to TFM has been found to be species-specific and age-dependent for lamprey, sturgeon, and teleost fish (Olson and Marking, 1973; Boogaard *et al.,* 2003; Henry, 2011), continued effort should be made to identify and protect TFM-sensitive species. Additionally, while minor, the greater sensitivity of sea lamprey and trout in soft water suggests that more research is necessitated to understand the role that water chemistry plays in TFM-toxicity. As such, lamprey infested streams should be surveyed for known sensitive-species prior to treatment, and whenever possible, consideration should be given to altering lampricide treatment regimens to times of the year when non-target species are more tolerant this pesticide.

CHAPTER FIVE:

An Integrative Model of TFM Toxicity on the Gills of Larval Sea Lampreys and Non-target Fishes

1. An Overview of the Integrated Response of the Fish Gill to TFM

For over 60 years, TFM has been successfully used to control invasive sea lamprey populations in the Great Lakes, but much about the toxic effects of this nitrophenolic compound still remains unknown, particularly how non-target animals may be effected (McDonald and Kolar, 2007). Because the gills are the main route of TFM-uptake (Hunn and Allen, 1974) and prior experiments found evidence of histological damage to the gills (Christie and Battle, 1963; Mallat *et al*., 1985; 1994), it was hypothesized that ATP-dependent functions of the gills are likely a target of TFM, leading to internal ionic disturbances (Mallat *et al*., 1994). As such, the overarching goal of my thesis was to use an integrative approach to better understand the effects that the lampricide had on ATP-dependent ionoregulatory processes in the gills of fishes. To address these goals I had two principle objectives: (1) to determine how lampricide treatments affect gill structure and function in larval sea lampreys and non-target species, and (2) to evaluate how environmental factors, such as water hardness, influence TFM toxicity on the gills.

Despite previous observations, my thesis demonstrated that the gills are remarkably resilient to the impacts of TFM on ATP-supply. Light microscopy revealed little evidence of damage to gill structure in all three species of fish. Moreover, there were only minor effects on ionoregulatory processes in sea lampreys, rainbow trout and in lake sturgeon. Water hardness, like pH and alkalinity (Bills *et al*., 2003), was also found to play an important role in TFM-sensitivity, with softer water resulting in greater lethality in fish, likely as a result of increased energy demands to maintain ion-balance against greater outwardly directed Na^+ and Cl^- electrochemical gradients (Morgan and Iwama 1998). Below, I present three models, which summarize the effects of TFM on larval sea lamprey, juvenile rainbow trout and lake sturgeon, discuss gaps that require further research, and explain how this research benefits the

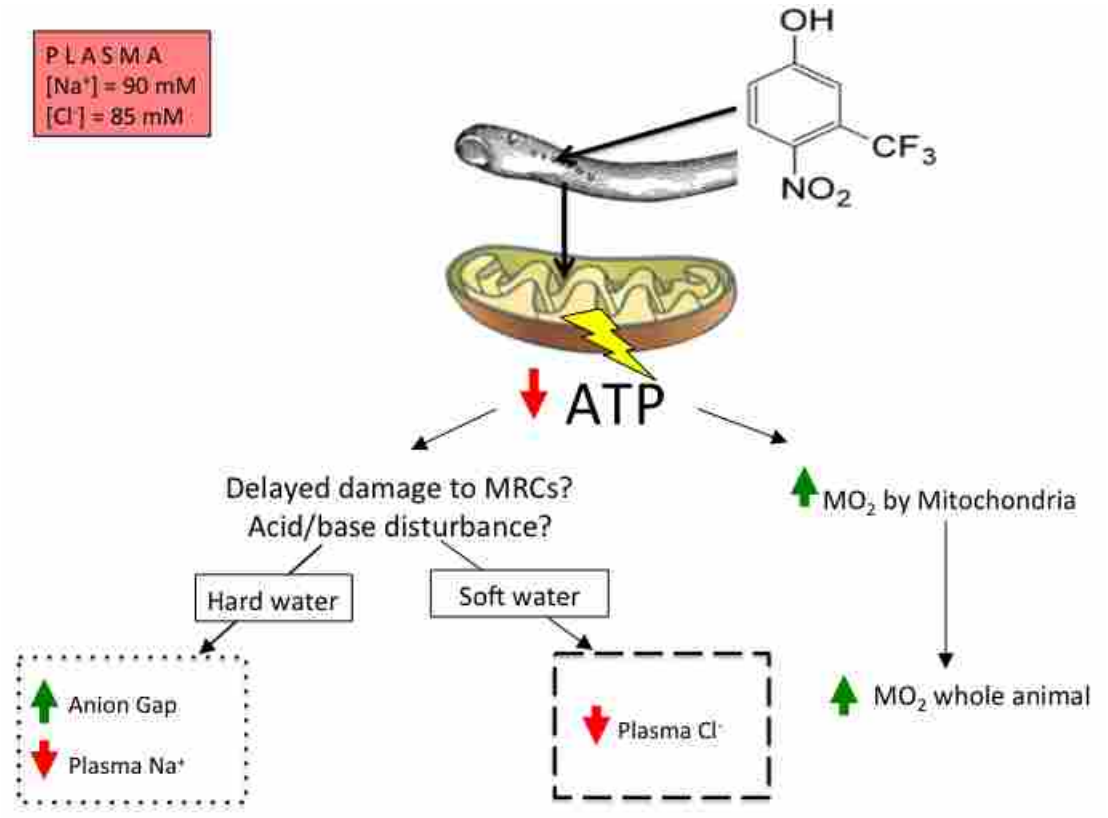
lamprey control program and integrate into the fundamental aspects of ecosystem management.

1.1 Model of TFM Toxicity in Larval Lamprey

In its' un-ionized state, the phenolic pesticide, 3-trifluoromethyl-4-nitrophenol, is proposed to enter the body of sea lamprey via the gills (Hunn and Allen, 1974; Youson and Freeman, 1976), in which it uncouples oxidative phosphorylation within the mitochondria (Niblett and Ballantyne, 1976; Birceanu *et al*., 2011). Lamprey are particularly vulnerable to TFM due to their inability to detoxify and solubilize TFM, which leads to greater TFM accumulation than in non-target fishes (Lech and Costrini, 1971; Lech, 1973; Lech and Statham, 1974; Le Clair, 2014). Due to the sea lamprey's minimal capacity to detoxify TFM, concentrations of the lampricide continue to rise in the blood and tissues, leading to starvation of the nervous tissue and ultimately, death (Wilkie *et al*., 2007; Birceanu *et al*., 2009). As such, there is a greater reliance on anaerobic pathways to meet ATP demands, leading to a depletion of anaerobic energy stores, glycogen and phosphocreatine, and a concordant increase in lactate in the brain and liver (Wilkie *et al*., 2007; Birceanu *et al*., 2009; Clifford *et al*., 2012; Henry *et al., Submitted*) and likely metabolic acid. This rise in intracellular H^+ would lead to an acidosis, which the lamprey may attempt to counterbalance retaining excess metabolic HCO_3, as evidenced by an anion gap characterized by an increase in plasma Cl^- and a decrease in Na^+ following 24 h depuration in TFM-free water (Figure 2.3). This response was not observed in soft-water (Figure 4.1). This could be a reflection of a decreased availability of bicarbonate in the external water (Jansen and Randall, 1975; Börjeson, 1977; Perry *et al.*, 1987; Dimberg, 1988; Vermette and Perry, 1988; Hyde and Perry, 1989; Borch *et al*., 1993; Larsen and Jensen, 1996). The above model is summarized in Figure 5.1.

Figure 5.1 Proposed integrative model of TFM toxicity on larval sea lamprey.

TFM enters the circulatory system of lamprey via the gills and disrupts ATP production in the mitochondria of cells within the body. This results in a decrease in ATP supply, depleting anaerobic energy stores, glycogen and PCr, and increasing lactate and H^+. Due to the sea lamprey's minimal capacity to detoxify TFM, concentrations continue to rise in the blood and tissues, which may lead to changes in the morphological structure of the mitochondrial rich cells (MRCs) of the gill epithelium and/or and acidosis in fish. In hard water, the increases in H^+ may be corrected via the physiochemical buffering of acid/base constituents in the water, leading to a rise in the anion gap and plasma Na^+ that carries over into recovery (dotted box). This correction is not seen in soft-water, where decreases in plasma Cl^- concentrations during recovery are observed which may be indicative of sustained post-TFM metabolic H+ excretion to correct a TFM-induced acidosis (dash box). The insufficiency of oxidative phosphorylation energy-supply pathway increases MO_2 of mitochondria, and results in a rise in whole body MO_2. Boxes indicate major findings from this thesis. Solid arrows indicated observed relationships.


PLASMA
[Na+] = 90 mM
[Cl-] = 85 mM
OH
CF3
NO2
ATP
Delayed damage to MRCs?
Acid/base disturbance?
MO2 by Mitochondria
Hard water
Soft water
Anion Gap
Plasma Na+
Plasma Cl-
MO2 whole animal

1.2 Model of TFM Toxicity in Juvenile Rainbow Trout

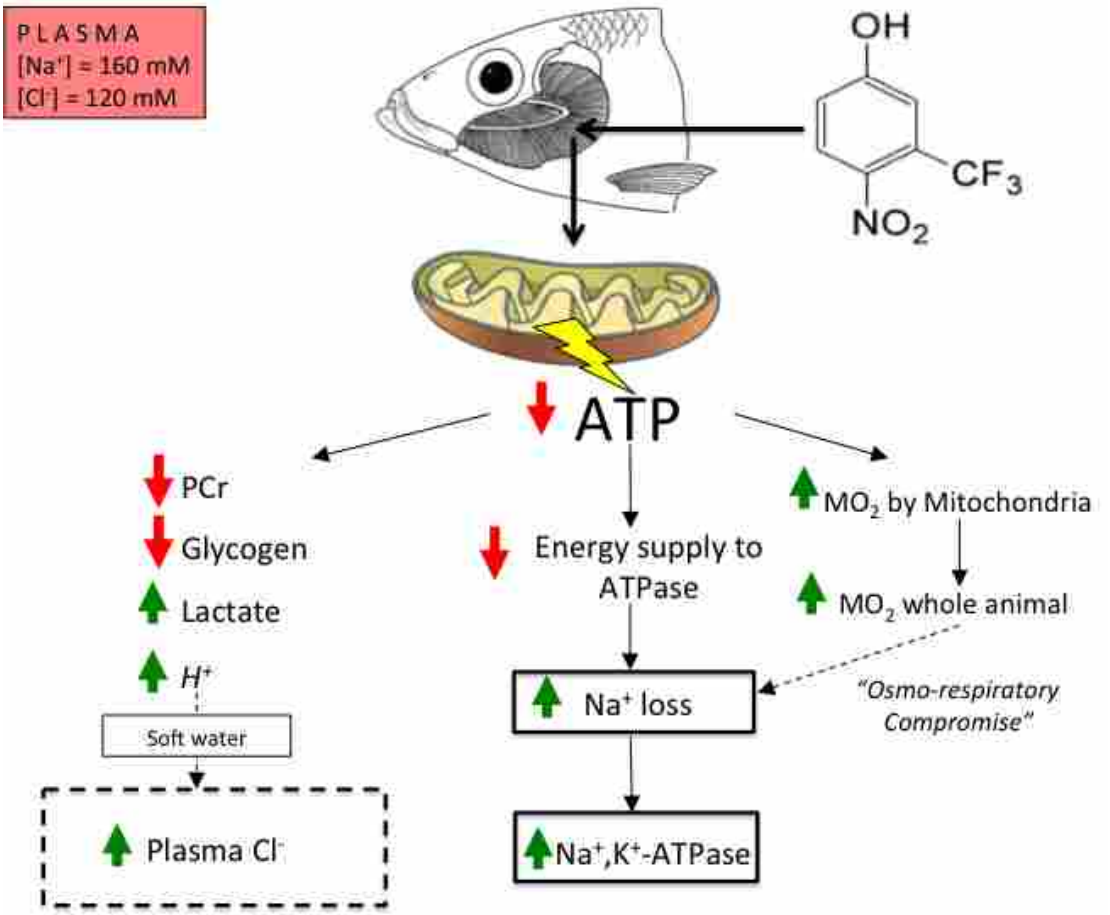
Like larval lamprey, TFM enters the circulatory system via the gills, and interferes with oxidative phosphorylation in the animal (Niblett and Ballantyne, 1976; Birceanu *et al.*, 2011). However, detoxification and elimination pathways help minimize the toxic build-ups of TFM within blood and tissues of trout (Birceanu *et al*., 2014; Le Clair 2014). Despite detoxification via glucuronidation, TFM still causes a mismatch between ATP supply and demand in the trout, forcing the the fish to rely more anaerobic energy stores, such as glycogen and phosphocreatine to generate ATP. This is particularly important within the brain, where glycogen concentrations dropped and lactate concentrations increased markedly (Birceanu *et al*., 2014: Le Clair, 2014), possibly leading to increased metabolic acid production (Hochachka and Somero, 2002). In both hard- and soft-water, decreased energy supply to the ATPase located in the MRCs of the gill epithelium may promote Na^+ loss during TFM exposure (Figure 2.2 and Figure 4.5). This increase in Na^+ efflux could potentially be exacerbated by increased whole body O_2-consumption due to stimulation of mitochondrial respiration by TFM (Niblett and Ballantyne, 1976; Birceanu *et al*., 2011). Such "osmo-respiratory" losses appear to be compensated by the activation and recruitment of branchial ATPase pumps (Figure 2.4 and 2.6). This ability to compensate for ion loss and the decreased availability of ATP may contribute to the rainbow trout's resistance against ionoregulatory disturbance and their greater TFM tolerance (Boogaard *et al*., 2003). This model is summarized in Figure 5.2.

1.3 Model of TFM Toxicity in Juvenile Sturgeon

Little about the physiological response of lake sturgeon to TFM is known; however, like lamprey, juvenile lake sturgeon are known to be more sensitive to TFM, with a LC_{50} falling within the range of the MLC that is used to treat lamprey-infested streams when

Figure 5.2 Proposed integrative model of TFM toxicity on rainbow trout.

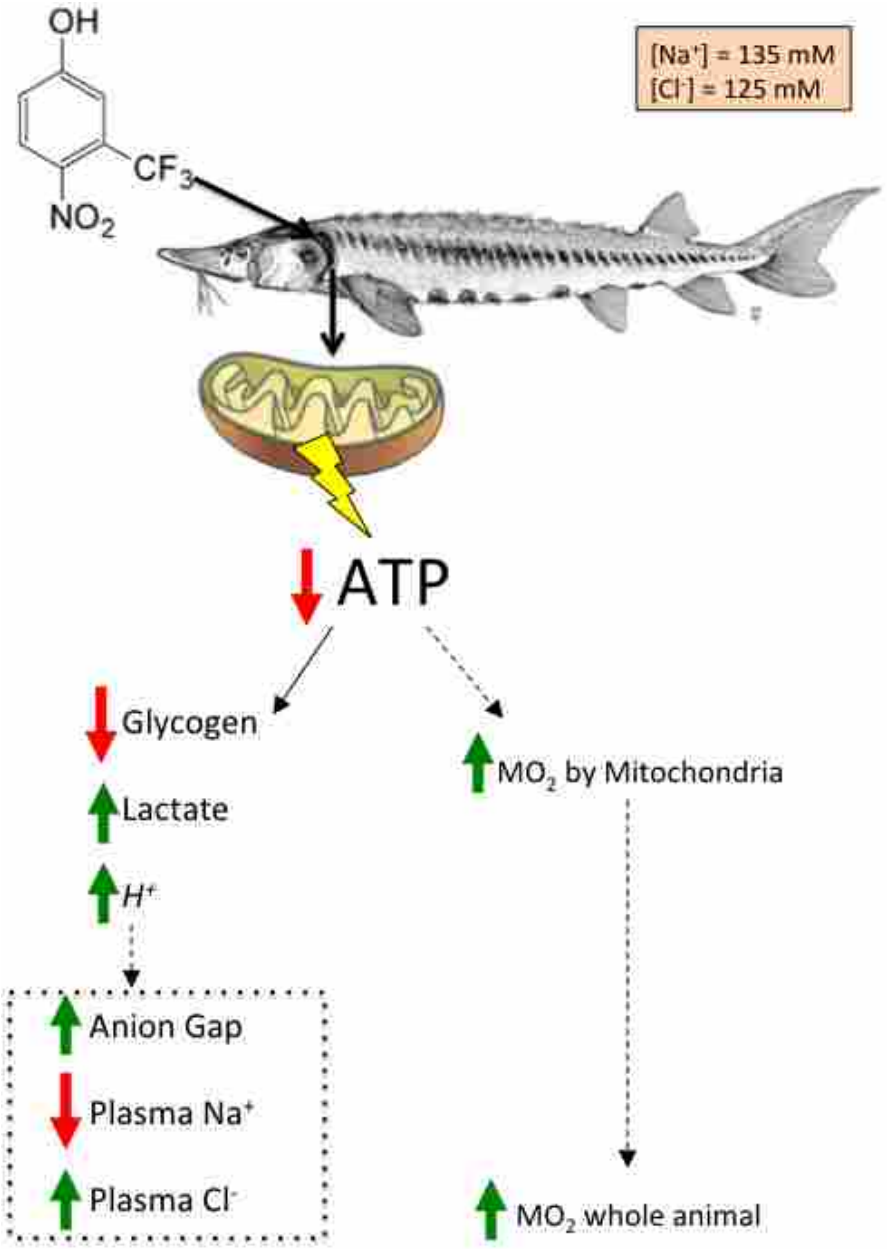
TFM enters the circulatory system of trout via the gills, and once in the animal it disrupts ATP production in the mitochondria. This results in a decrease in ATP supply, decreasing anaerobic energy stores, glycogen and PCr, and increasing lactate and H^+. Due to the lower buffering capacity of soft water, a greater acidosis may result due to a reduced ability to take-up HCO_3^- from the environment. Such an acidosis may be corrected via the physiochemical buffering of acid/base constituents, contributing the rise in plasma Cl^- that was observed, which was likely a reflection of a corresponding persistent alkalosis following depuration in TFM-free water (dashed box). This is not seen in hard water. Decreased energy supply to the ATPase located in the mitochondrial-rich cells (MRCs) of the gill epithelium may promote Na^+ loss during TFM exposure (solid box). Such losses could potentially be exacerbated by increased whole body O_2-consumption due to stimulation of mitochondrial respiration by TFM. These "osmo-respiratory compromise" Na^+ losses may have been compensated by the activation and recruitment of synthesized NKA activity increases during TFM exposure (solid box). Boxes indicate major findings from this thesis project. Solid arrows indicate observed relationships and dash arrows suspected relationships.


PLASMA
[Na+] = 160 mM
[Cl-] = 120 mM
OH
CF3
NO2
ATP
PCr
Glycogen
Lactate
H+
Soft water
Plasma Cl-
Energy supply to ATPase
MO2 by Mitochondria
MO2 whole animal
Na+ loss
"Osmo-respiratory Compromise"
Na+,K+-ATPase

less than 100 mm in length (Boogaard *et al*., 2003). It was predicted that the increased sensitivity was due to their decreased ability to detoxify TFM, akin to larval sea lamprey. But despite these predictions, Le Clair (2014) demonstrated that juvenile lake sturgeons are not only able to detoxify TFM via glucuronidation, but eliminate TFM. However, declines in glucuronidated-TFM, despite high concentrations of parent-TFM, were found late into the TFM-exposure, suggesting that the potential that the TFM detoxification pathways may become overwhelmed, as ATP-supply declines (Le Clair, 2014). There is some circumstantial evidence to suggest that TFM affects lake sturgeon similarly, leading to a decline in anaerobic energy stores, namely, declines glycogen concentration in the liver (Le Clair, 2014), and presumably similar rises in the anaerobic byproducts, lactate and H^+, which may drive down the intercellular pH. Similar to lamprey, it also appears that sturgeon attempt to correct the correspondingly metabolic acidosis, with an excess of metabolic HCO_3 uptakeas evidenced by increasing an anion gap caused by an increase in plasma Cl^- and a decrease in Na^+ following 24 h depuration in TFM-free water (Figure 3.1). While it has not been measured in sturgeon, it can be presumed based on numerous in *vivo* and in *vitro* studies, that TFM likely leads to similar increases in mitochondrial O_2 consumption and similarly, rises in whole body MO_2 (Figure 5.2; Smith and King, 1969 as cited by Kawatski and McDonald 1974; Kawatski *et al.,* 1974). Based on these findings, the reason for the increased sensitivity of juvenile lake sturgeon to TFM still remains unknown. However, it is important to note that both the Le Clair (2014) study and this thesis were conducted at the TFM LC_{50} of larval sea lamprey. It is therefore possible that at at higher doses of TFM, more comparable to those used to treat lamprey-infested streams, that the detoxification pathways for TFM may become overwhelmed, resulting in toxicity and fish mortalities and perhaps histological disturbances not seen at lower concentrations of TFM (Figure 5.3).

Figure 5.3 Proposed integrative model of TFM toxicity on juvenile lake sturgeon.

TFM enters the circulatory system of sturgeon via the gills, and disrupts ATP production in the mitochondria of the animal. This results in a decrease in ATP supply, depleting anaerobic energy stores, glycogen and PCr, and increasing lactate and H^+. The corresponding increases in H^+ is corrected via the physiochemical buffering of acid/base constituents in the water, leading to a rise in the anion gap that carries over into recovery (dashed box). Increased MO_2 by the mitochondria likely results in a rise in whole body MO_2. Boxes indicate major findings from this thesis project. Solid arrows indicate observed relationships and dash arrows suspected relationships.


OH
CF3
NO2
[Na+] = 135 mM
[Cl-] = 125 mM
ATP
Glycogen
Lactate
H+
Anion Gap
Plasma Na+
Plasma Cl-
MO2 by Mitochondria
MO2 whole animal

1.4 The Effect of Water Hardness

One of the key components of this study was investigating the factors that influence TFM sensitivity such as water hardness. It was predicted that softer waters would have a greater effect on ionoregulation due to increased energy demands to maintain ion-balance against blood-water electrochemical gradients that favour ion loss (Morgan and Iwama, 1998). Additionally, the lower pH (pH 6-7), buffering capacity (HCO_3^-), and concentrations of Ca^{2+}, Na^+, and Cl^-, which would limit the availability of these ions for ionoregulation in soft water (Morgan and Iwama, 1998), was predicted to further exacerbate the toxicity of TFM. Earlier studies demonstrated that the toxicity of TFM is greater in soft water (Olson and Marking, 1973; 1975; Sills and Allen, 1975; Bills and Marking, 1976) and in agreement with these studies, I noted that the lethality of TFM increased substantially in soft-water (~40 mg HCO_3^- L^{-1}) compared to hard water (~450 mg HCO_3^- L^{-1}), with LC_{50} and $LC_{99.9}$ values that were approximately $1/10^{th}$ the concentrations seen in hard water (Chapter 4). Furthermore, there is evidence that TFM did indeed lead to more pronounced ionic disturbances in soft-water, as supported by a greater number of minor histological disturbances to gill vasculature in trout during TFM exposure in soft water. These responses were not found in hard water experiments (Figure 2.6; Figure 2.9), suggesting that, like pH and alkalinity, the toxicity of TFM on fishes is directly influenced by water hardness. As such, like higher pH environments, hard water may provide protection against the potentially adverse effects of TFM.

2. Future Directions

Based on the findings of the thesis project, there is some preliminary evidence that TFM may lead to distrubances in acid-base balance as a result of increased reliance on anaerobic glycolysis. These disturbances appear to be exacerbated in soft ion-poor water,

where the physiochemical buffering would be limited. Therefore, future studies should address the hypothesis that increased reliance on anaerobic pathways during TFM exposure leads to acidosis in fish, and that such disturbances are more pronounced in softer water. In order to test these hypotheses, future studies should measure acid excretion (Wilkie *et al*., 2001), decreases in blood pH, and/or changes in total CO_2 to better quantify the extent of TFM-induced acid-base disturbances in sea lamprey and non-target fishes in hard and soft waters

Future experiments should also test the potential that TFM affects gas exchange, leading to morphological alterations in the gills of lamprey and non-target fish. Prior research has demonstrated that TFM exposure results in vasodilation of the gills (Christie and Battle, 1963) and greater oxygen consumption (Smith and King, 1969 as cited by Kawatski and McDonald 1974; Kawatski *et al.,* 1974; Niblett and Ballantyne, 1976; Birceanu *et al*., 2011). The possibility that a potential osmorespiratory compromise, in which increased oxygen consumption results in greater ion losses, takes place in sea lamprey and non-target fishes should also be investigated. Thus, whole animal MO_2 studies and additional quantitative measurements of the respiratory surface area and vasculature of the gill filaments need to be made in order to test the TFM-induced osmorespiratory compromise hypothesis and whether or not water chemistry influences these factors.

Lastly, it is also necessary to better understand the underlying mechanisms for lake sturgeon sensitivity to TFM. Because fish were only exposed to the LC_{50}, higher doses of TFM may be necessary to determine whether or not detoxification pathways are overwhelmed by decreased ATP supply during TFM exposure. Additionally, because like trout and lamprey, juvenile lake sturgeons have demonstrated similar decreases in TFM-sensitivity as both pH and alkalinity increases (Johnston *et al*., 1999), it would be beneficial to quantify both TFM and glucuronidated-TFM constituents under different water quality regimens

3. Implications for Sea Lamprey Control and Integrated Ecosystem Management in the Great Lakes and Conclusions.

Despite TFM's long term use to control sea lamprey populations in the Great Lakes, there are concerns about the effects of lampricide treatments on non-target organisms, the short-and-long term impacts of the release of pesticides into the environment, and the associated costs of lampricide use to the Great Lakes Fisheries Commission (GLFC; McDonald and Kolar, 2007). Controlling sea lampreys with TFM is important for ecosystem management in the Great Lakes because it helps protect economically and culturally important fishes, such as the lake trout, from sea lamprey predation/parasitism (McDonald and Kolar, 2007). On average, the GLFC spends 5 million dollars a year (US) to treat 50-60 streams in the Great Lakes basin with lampricide (Christie *et al*., 2003). By providing better framework models for the toxic mode of action of TFM on lamprey and non-target fish, biochemists may be able to develop more cost effective formulations of lampricide, which better target the physiology of lamprey, while reducing the impact on non-target species. While it is important to prevent fish mortality as a result of lamprey predation, understanding the potential adverse effects of TFM on fishes such as the trout and lake sturgeon is also important for aquatic risk assessment. In the case of the lake sturgeon, a better understanding of its response to TFM could be particularly important in for determining if juvenile lake sturgeon populations are potentially harmed, or not, by TFM treatments.

This thesis used an integrative approach to address concerns regarding the potential adverse effects of TFM on the gills of sea lampreys and non-target fishely, namely on gill structure and ionoregulation. By shedding greater light on the mechanisms of toxicity, this research has demonstrated that current treatment protocols are generally sufficient to protect non-target fishes from the potentially adverse effects of TFM on ion-regulation. However, the reason(s) for the greater sensitivity of lake sturgeon to TFM remains unclear and caution

should continue to be exercised when treating streams known to be inhabited by sturgeon under 100 mm in length.

APPENDIX A

ADP	Adenosine-diphosphate
ATP	Adenosine-triphosphate
CA	Carbonic Anhydrase
DMSO	Dimethyl sulfoxide
ILCM	Interlamellar cell mass
IMM	Inner-mitochondrial membrane
MRC	Mitochondrial-rich cell
NKA	Na^+,K^+-ATPase
PCr	Phosphocreatine
PMF	Proton-motive force
PVC	Pavement cell
TFM	3-Trifluoromethyl-4-nitrophenol
UDGTP	UDP-glucuronyltransferase
V-ATPase	Vacuolar (V type) H^+-ATPase

References

Allen JL and Hunn JB. (1977). Renal excretion in channel catfish following injection of quinaldine sulphate or 3-trifluoromethyl-4-nitrophenol. *Journal of Fish Biology* 10: 473-479

Applegate VH, Howell JH, Moffett JW, Johnson BGH, and Smith MA. (1961). Use of 3-trifluormethyl-4-nitrophenol as a selective sea lamprey larvicide. Great Lakes Fish. *Great Lakes Fishery Commission Technical Report No. 1*

Applegate, V.C., Johnson, B.G.H., and Smith, M.A. (1966). The relation between molecular structure and biological activity among mono-nitrophenols containing halogens. *Great Lakes Fishery Commission Technical Report* 11: 1-19.

Baker DW, Matey V, Huynh KT, Wilson JM, Morgan JD, and Brauner CJ. (2003). Complete intracellular pH protection during extracellular pH depression is associated with hypercarbia tolerance in white sturgeon, Acipenser transmontanus. *American Journal of Physiology: Regulatory Integrative and Comparative Physiology* 296: R1868-R1880

Bartels H, Potter IC, Pirlich K, and Mallatt J. (1998). Categorization of the mitochondria-rich cells in the gill epithelium of the freshwater phases in the life cycle of lampreys. *Cell and Tissue Research* 291(2) 337-349

Bartels H and Potter IC. (2004). Cellular composition and ultrastructure of the gill epithelium of larval and adult lampreys: implications for osmoregulation in fresh and seawater. *The Journal of Experimental Biology* 207(20): 3447-3262

Beamish F. (1979). Migration and spawning energetics of the anadromous sea lamprey, *Petromyzon marinus*. *Environmental Biology of Fishes* 4(1): 3-7

Beamish F. (1980). Biology of the North American Anadromous Sea Lamprey, *Petromyzon marinus*. *Canadian Journal of Fisheries and Aquatic Science* 37(11): 1924-1943

Bemis WE, Findeis EK, and Grande L. (1997). An overview of Acipenseriformes. *Environmental Biology of Fishes* 48: 25-71

Berg JM, Tymoczko JL, and Stryer L. (2002). Chapter 18 Oxidative Phosphorylation. In Biochemistry fifth ed. WH Freeman and Company, NY

Bergstedt RA and Swink WD. (1995). Seasonal growth and duration of the parasitic life stage of the landlocked sea lamprey *(Petromyzon marinus)*. Canadian Journal of Fisheries and Aquatic Sciences 52: 1257-1264

Berner RA, Vandenbrooks JM, and Ward PD. (2007). Oxygen and evolution. *Science* 316: 557-558

Bickler PE and Buck LT. (2007). Hypoxia tolerance in reptiles, amphibians, and fishes: life with variable oxygen availability. *Annual Review of Physiology* 69: 145-170.

Bills TD and Marking LL. (1976). Toxicity of 3-trifluoromethyl-4-nitrophenol (TFM), 2'5-dichloro-4'-nitrosalicylanilde (Bayer 73) and a 98:2 mixture to fingersings of seven fish species and to eggs and fry of coho salmon. *US Fish and Wildlife Service Investigation Journal* Fish Control 69: 1-9

Bills TD, Boogaard MA, Johnson DA, Brege DC, Scholefield RJ, Westman RW, and Stephens BE. (2003). Development of a pH/alkalinity treatment model for applications of the lampricide TFM to streams and tributary to the Great Lakes. *Journal of Great Lakes Research* 29(Suppl. 1): 510-520.

Birceanu, O. (2009). Mechanism(s) of 3-Trifluoromethyl-4-Nitrophenol (TFM) Toxicity in Sea Lamprey (*Petromyzon marinus*) & Rainbow Trout (*Oncorhynchus mykiss*). Theses and Dissertations (Comprehensive). Wilfrid Laurier University, Waterloo, ON. Paper 927.

Birceanu O, McClelland GB, Wang YS, and Wilkie MP. (2009). Failure of ATP supply to match ATP demand: The mechanism of toxicity of the lampricided, 3-trifluoromethyl-

4-nitrophenol (TFM), used to control sea lamprey (*Petromyzon marinus*) populations in the Great Lakes. *Aquatic Toxicology* 94: 265-274.

Birceanu O, McClelland GB, Wang YS, Brown JCL, and Wilkie MP. (2011). The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) uncouples mitochondrial oxidative phosphorylation in both sea lamprey (*Petromyzon marinus*) and TFM-tolerant rainbow trout (*Oncorhychus mykiss*). *Comparative Biochemistry Physiology Part C Toxicology & Pharmacology* 153(3): 342-349.

Birceanu O, Sorensen LA, Henry M, McClelland GB, Wang YS, and Wilkie MP. (2014). The effects of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) on fuel stores and ion balance in a non-target fish, the rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology, Part C 160:30-41.

Bleau H, Daniel C, Chevalier G, van Tra H, and Hontela A. (1996). Effects of acute exposure to mercury chloride and methyl mercury on plasma Cortisol, T3, T4, glucose and liver glycogen in rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 34: 221-235

Boisen AMZ, Amstrup J, Novak I, and Grosell M. (2003). Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*). *Biochimica et Biophysica Acta* 1618: 207-218

Boogaard MA, Bills TD, Johnson DA. (2003). Acute Toxicity of TFM and a TFM/Niclosamide Mixture to Selected Species of Fish, Including Lake Sturgeon (*Acipenser fulvescens*) and Mudpuppies (*Necturus maculosus*), in Laboratory and Field Exposures. *Journal of Great Lakes Research* 29(Suppl. 1): 529-541

Booth JH. (1978). The distribution of blood flow in the gills of fish: application of a new technique to rainbow trout (*Salmo gairdneri*). *Journal of Experimental Biology* 73: 119-130.

Booth JH. (1979). The effect of oxygen supply, epinephrine and acetylcholine on the distribution of blood flow in trout gills. *Journal of Experimental Biology* 83: 31-39

Borch K, Jensen FB, and Andersen BB. (1993). Cardiac activity, ventilation rate and acid-base regulation in rainbow trout exposed to hypoxia and combined hypoxia and hypercapnia. *Fish Physiology and Biochemistry* 12: 101-110.

Borjeson H. (1977). Effects of hypercapnia on the buffer capacity and haematological values in *Salmo salar (L.)*. *Journal of Fish Biology* 11: 133-142

Boutilier RG, Heming TA, and Iwama GK. (1984). Physiochemical parameters for use in fish respiratory physiology. In Fish Physiology, vol. X. Hoar WS and Randall DJ (Eds.) pp. 403-430. New York: Academic Press

Boutilier RG, Ferguson RA, Henry RP, and Tufts BL. (1993). Exhaustive exercise in the sea lamprey (*Petromyzon marinus*): relationship between anaerobic metabolism and intracellular acid-base-balance. *Journal of Experimental Biology* 178: 71-88.

Bryan MB, Zalinski D, Filcek B, Libants S, Li W, Scribner KT. (2005). Patterns of invasion and colonization of sea lamprey (*Petromyzon marinus*) in North America as revealed by microsatellite genotypes. *Molecular Ecology* 14: 3757-3773.

Bury NR and Wood CM. (1999). Mechanism of branchial silver uptake by rainbow trout is via the proton coupled Na+ channel. *American Journal of Physiology* 277: R1385-R1391.

Cairns JJ, Calhoun WF, McGinniss, and Strakd W. (1976). Aquatic organisms response to severe stress following acutely sublethal toxicant exposure. *Journal of the American Water Resources Association* 12(6): 1233-1243

Cameron JN. (1976). Branchial ion uptake in arctic grayling: resting values and effects of acid-base disturbance. *Journal of Experimental Biology* 64: 711-725

Cameron JN and Kormanik GA. (1982). The acid–base responses of gills and kidneys to infused acid and base loads in the channel catfish, *Ictalurus punctatus*. *Journal of Experimental Biology* 99: 143-160.

Camargo MMP, Fernandes MN, and Martinez CBR. (2009). How aluminium exposure promotes osmoregulatory disturbances in the neotropical freshwater fish *Prochilus lineatus*. *Aquatic Toxicology* 94: 40-46.

Carey JH and Fox ME. (1981). Photodegradation of the lampricide 3-trifluoromethyl-4-nitrophcnol (TFM); pathway of the direct photolysis in solution. *Journal of Great Lakes Research* 7: 234-241

Chang JCH, Wu SM, Tseng YC, Lee YC, Baba O, and Hwang PP. (2007). Regulation of glycogen metabolism in gills and liver of the euryhaline tilapia (*Oreochromis mossambicus*) during acclimation to seawater. *Journal of Experimental Biology* 210: 3494-3504

Christie GC, Adams JV, Steeves TB, Slade JW, Cuddy D, Fodale MF, Young RJ, Kuc M, and Jones ML. (2003). Selecting Great Lakes streams for lampricide treatment based on larval sea lamprey survey. *Journal of Great Lakes Research* 29: 152-160

Christie GC and Goddard CI. (2003). Sea Lamprey International Symposium (SLIS II): Advances in the Integrated Management of Sea Lamprey in the Great Lakes. *Journal of Great Lakes Research* 29: 1-14

Christie RM and Battle HI. (1963). Histological effects of 3-trifluoromethyl-4-nitrophenol (TFM) on larval lamprey and trout. *Canadian Journal of Zoology* 41: 51-61

Choe KP, O'Brien S, Evans D, Toop T, and Edwards S. (2004). Immunolocalization of Na+/K+-ATPase, carbonic anhydrase II, and vacuolar H+-ATPase in gills of freshwater adult lampreys, *Geotria australis*. *Journal of Experimental Zoology A*. 301: 654-665

Clarke DJ, George SG, and Burchell B. (1991). Glucuronidation in fish. *Aquatic Toxicology* 20: 35-56

Clifford AM, Henry M, Bergstedt R, McDonald DG, Smits AS, and Wilkie MP. (2012). Recovery of Larval Sea Lampreys from Short-Term Exposure to the Pesticide 3-

Trifluoromethyl-4-Nitrophenol: Implications for Sea Lamprey Control in the Great Lakes. *Transactions of the American Fisheries Society* 141: 1697-1710

Collis MG and Hourani SM. (1993). Adenosine receptor subtypes. *Trends in Pharmacology Science* 14: 360-366

Craig PM, Wood CM, and McClelland GB. (2007). Gill membrane remodeling with soft-water acclimation in zebrafish (*Danio rerio*). *Physiological Genomics* 30: 53-60

Dang Z, Lock RAC, Flick G, and Wendelaar Bonga SE. (2000). Na/K–ATPase immunoreactivity in branchial chloride cells of *Oreochromis mossambicus* exposed to cooper. *Journal of Experimental Biology* 151: 517-428

da Silvia AOF and Martinez CBB. (2014). Acute effects of cadmium on osmoregulation of the freshwater teleost *Prochilodus lineatus*: Enzymes activity and plasma ions. *Aquatic Toxicology* 156: 161-168

DeHaan PW, Libants SV, Elliott RF, and Scribner KT. (2006). Genetic Population Structure of Remnant Lake Sturgeon Populations in the Upper Great Lakes Basin. *Transactions of the American Fisheries Society* 135: 1478-1492

Dimberg K. (1988). High blood CO2 levels in rainbow trout ex- posed to hypercapnia in bicarbonate-rich hard freshwater - methodological verification. *Journal of Experimental Biology* 134: 463-466

Dymowska AK, Hwang PP, and Goss GG. (2012). Structure and function of ionocytes in the freshwater fish gill. *Respiratory Physiology & Neurobiology* 184: 282-292.

Eshenroder RL and MK Burnham-Curtis. (1999). Species succession and sustainability of the Great Lakes fish community. Pages 141–180 in W. W. Taylor and C. P. Ferreri, editors. Great Lakes fishery policy and management: a binational perspective. Michigan State University Press, East Lansing.

Eshenroder RL. (2009). Comment: mitochondrial DNA analysis indicates sea lampreys are indigenous to Lake Ontario. *Transactions of the American Fisheries Society* 138: 1178-1189

Evans DH and Claiborne JB. (2005). The Physiology of Fishes, third ed. CRC Press, NY, pp. 87-197.

Evans DH, Piermarini PM, and Choe KP. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological Review* 85: 97-177

Farmer GJ. (1980). Biology and physiology in adult lampreys. *Canadian Journal of Fisheries and Aquatic Science* 37: 1751-1761.

Fenwick JC, Wendelaar Bonga SE, and Flik G. (1999). In vivo bafilomycin-sensitive Na(+) uptake in young freshwater fish. *Journal of Experimental Biology* 202(24): 3659-3666

Ferguson M and Duckworth G. (1997). The status and distribution of lake sturgeon, *Acipenser fulvescens*, in the Canadian provinces of Manitoba, Ontario and Quebec: a genetic perspective. *Environmental Biology of Fishes* 48: 299-309

Flik G and Verbost PM. (1993). Calcium transport in fish gills and intestine. *Journal of Experimental Biology* 184: 17-29

Flik G, Klaren PHM, Schoenmakers TJM, Bijvelds MJC, Verbost PM, and Wendelaar Bonga SE. (1996). Cellular calcium transport in fish: Unique and universal mechanisms. *Physiological and Biochemical Zoology* 69(2): 402-417.

Fortin R, Mongeau J-R, DesJardins G, and Dumont P. (1993). Movements and biological statistics of the lake sturgeon (*Acipenser fulvescens*) populations from the St. Lawrence and Ottawa River system, Quebec. *Canadian Journal of Zoology* 71: 638-650

Gallant J, Harvey-Clark C, Myers RA, and Stokesbury MJW. (2006). Sea Lamprey Attached to a Greenland Shark in the St. Lawrence Estuary, Canada. *Northeastern Naturalist* 13(1): 35-38

Gonzalez RJ and McDonald DG. (1992). The relationship between oxygen consumption and ion loss in a freshwater fish. *Journal of Experimental Biology* 163: 317-332.

Goss GG and Wood CM. (1990). Na+ and Cl- uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout. 1. Responses to environmental hyperoxia. *Journal of Experimental Biology* 152: 521-547.

Goss GG, Laurent P, and Perry SF. (1992.) Evidence for a morphological component in acid-base regulation during environmental hypercapnia in the brown bullhead (*Ictalurus nebulosus*). *Cellular Tissue Research* 268: 539-552

Goss GG and Perry SF. (1993). Physiological and morphological regulation of acid–base status during hypercapnia in rainbow trout (*Oncorhynchus mykiss*). *Canadian Journal of Zoology* 71(8): 1673-1680

Goss GG, Laurent P, and Perry SF. (1994). Gill morphology during hypercapnia in brown bullhead (*Ictalurus nebulosus*): role of chloride cells and pavement cells in acid-base regulation. *Journal of Fish Biology* 45(5): 705-718

Goss GG, Perry SF, and Laurent P. (1995). 10 Ultrastructural and Morphometric Studies on Ion and Acid-Base Transport Processes in Freshwater Fish. *Fish Physiology* 14: 257–284

Goss GG, Perry SF, Fryer JN, and Laurent P. (1998). Gill morphology and acid-base regulation in freshwater fishes. *Comparative Biochemistry and Physiology A* 119(1): 107-115

Greco AM, Fenwick JC, and Perry SF. (1996). The effects of soft-water acclimation on gill structure in the rainbow trout *Oncorhynchus mykiss*. *Cell and Tissue Research* 285(1): 75-82

Grosell M, Nielsen C, and Bianchini A. (2002). Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. *Comparative Biochemistry and Physiology Part C* 133: 287-303

Harkness WJK and Dymond JR. (1961). The lake sturgeon. Ontario Department of Lands and Forests, Fish and Wildlife Branch, Toronto, 97 pp

Hay-Chmielewski EM and Whelan GE. (1997). State of Michigan lake sturgeon rehabilitation strategy. Michigan Department of Natural Resources, Fisheries Special Report 18, Lansing. Available: http://www.michigandnr.com/PUBLICATIONS/. (May 2012).

Heinrich JW, Mullett KM, Hansen MJ, Adams JV, Klar GT, Johnson DA, Christie GC, and Young RJ. (2003). Sea lamprey abundance and management in Lake Superior, 1957 to 1999. *Journal of Great Lakes Research* 29(Suppl. 1): 566-583

Henry, Matthew. (2011). Effects of 3-Trifluoromethyl-4-Nitrophenol (TFM) on Energy Stores and Gill Function in Sea Lamprey (*Petromyzon marinus*) and Rainbow Trout (*Oncorhynchus mykiss*). Theses and Dissertations (Comprehensive). Wilfrid Laurier University, Waterloo, ON, Paper 1014.

Houston JJ. (1987). Status of the lake sturgeon (*Acipenser fulvescens*) in Canada. *The Canadian Field-Naturalist* 101: 171-185.

Howell JH, Lech JJ, Allen JL. (1980). Development of Sea Lamprey (*Petromyzon marinus*) Larvicides. *Canadian Journal of Fisheries and Aquatic Science* 37(11): 2103-2107

Hubert TD. (2003). Environmental Fate and Effects of the Lampricide TFM: a Review. *Journal of Great Lakes Research* 29(Suppl. 1): 456-474

Hunn JB and Allen JL. (1974). Movement of drugs across the gills of fishes. *Annu Rev Pharmacology* 14(1): 47-54

Hwang PP and T. (2007). New insights into fish ion regulation and mitochondrion-rich cells. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology* 148(3): 479-497

Hyde DA and Perry SF. (1989). Differential approaches to blood acid-base regulation during exposure to prolonged hypercapnia in two freshwater teleost: The rainbow trout (*Salmo*

gairdneri) and the American eel (*Anguilla rostrata*). *Physiology and Biochemistry Zoology* 62: 1164-1186.

Janssen RG and Randall DJ. (1975). The effects of changes in pH and PCO_2 in blood and water on breathing in rainbow trout (*Salmo gairdneri*). *Respiratory Physiology* 25: 235-245.

Johnson DA, Weisser JW, and Bills TB. (1999). Sensitivity of lake sturgeon (*Acipenser fulvescens*) to the lampricide 3-trifluoromethyl-4-nitorpheenol (TFM) in field and laboratory exposures. *Great Lakes Fishery Commission Technical Report 62.* Available: http:// www.glfc.org/pubs/TechReports/ (May 2012).

Kane AS, Day WW, Reimschuessel I, and Lipsky MM. (1993). 3-Trifluoromethyl-4-nitrophenol (TFM) toxicity and hepatic microsomal UDP-glucoronyltransferase activity in larval and adult bullfrogs. *Aquatic Toxicology* 27: 51-60.

Kane AS, Kahng MW, and Reimschuessel R. (1994). UDP-glucuronyltransferase kinetics for 3-trifluoromethyl-4-nitrophenol (TFM) in fish. *Transactions of the American Fisheries Society* 123: 217-222.

Karnaky KJ, Kinter LB, Kinter WB, and Stirling CE. (1976). Teleost chloride cell. 2. Autoradiographic localization of gill Na+,K+-ATPase in killifish (*Fundulus heteroclitus*) adapted to low and high salinity environments. *Journal of Cellular Biology* 70: 157-177

Kawatski JA, Dawson VK, and Ruevers ML. (1974). Effect of TFM and Bayer 73 on in vivo oxygen consumption of the aquatic midge *Chironomus tentans*. *Transactions of the American Fisheries Society* 3: 551-556.

Kawatski JA and McDonald MJ. (1974). Effect of 3-trifluoromethyl-4-nitrophenol on *in vitro* tissue respiration of four species of fish with preliminary notes on its *in vitro* biotransformation. *Comparative and General Pharmacology* 5: 67-76

Kawatski JA and Bittner MA. (1975). Uptake, elimination, and biotransformation of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) by larvae of the aquatic midge *chironomus tentans*. *Toxicology* 4(2): 183-194.

Kiemer MCB and Black KD. (1997). The effects of hydrogen peroxide on the gill tissues of Atlantic salmon, *Salmo salar L*. *Aquaculture* 153: 181-189

Kültz D, Bastrop R, Jürss K, and Siebers D. (1992). Mitochondria-rich (MR) cells and the activities of Na+/K+-ATPase and carbonic anhydrase in the gill and opercular epithelium of *O. mossambicus* adapted to various salinities. *Comparative Biochemistry and Physiology* 102B: 293-301.

Kumai Y and Perry SF. (2012). Mechanisms and regulation of Na^+ uptake by freshwater fish Review. *Respiratory Physiology & Neurobiology* 184: 249-256

Lappivaara J, Nikinmaa M, and Tuurala H. (1995). Arterial oxygen-tension and the structure of the secondary lamellae of the gills in rainbow-trout (*Oncorhynchus mykiss*) after acute exposure to zinc and during recovery. *Aquatic Toxicology* 32: 321-331

Larsen BK and Jensen FB. (1997). Influence of ionic composition on acid-base regulation in rainbow trout (*Oncorhynchus mykiss*) exposed to environmental hypercapnia. *Fish Physiology and Biochemistry* 16(2): 157-170

Le Clair Michael W. (2014). Distribution and Elimination of 3-Trifluoromethyl-4-Nitrophenol (TFM) by Sea Lamprey (*Petromyzon marinus*) and Non-target, Rainbow Trout (*Oncorhynchus mykiss*) and Lake Sturgeon (*Acipenser fulvescens*). Theses and Dissertations (Comprehensive). Wilfrid Laurier University, Waterloo, ON, Paper 1632.

Lech JJ and Costrini NV. (1972). In vitro and in vivo metabolism of 3-trifluoromethyl-4-nitrophenol (TFM) in rainbow trout. *Comparative and General Pharmacology* 3(10): 160-166

Lech JJ. 1974. Glucuronide formation in rainbow trout - effect of salicylamide on the acute toxicity, conjugation and excretion of 3-trifluoromethyl-4-nitrophenol. *Biochemical Pharmacology* 23: 2403-2410.

Lech JJ and Statham CN. (1975). Role of glucuronide formation in the selective toxicity of 3-trifluoromethyl-4-nitrophenol (TFM) for the sea lamprey: comparative aspects of TFM uptake and conjugation in sea lamprey and rainbow trout. *Toxicology and Applied Pharmacology* 31(1): 150-158

Leino RL, McCormick JH, and Jensen KM. (1987). Changes in gill histology of fathead minnows and yellow perch transferred to soft water or acidified soft water with particular reference to chloride cells. *Cellular and Tissue Research* 250(2): 389-399

Li W, Twohey M, Jones M, and Wagner M. (2007). Research to Guide Use of Pheromones to Control Sea Lamprey. *Journal of Great Lakes Research* 33(Suppl. 2): 70-86

Lin H and Randall DJ. (1993). H+-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. *Journal of Experimental Biology* 180: 163-174.

Lin H, Pfeiffer D, Vogl A, Pan J, and Randall D. (1994). Immunolocalization of H^+-ATPase in the gill epithelial of rainbow trout. *Journal of Experimental Biology* 195(1): 169-183

Lowe DR, Beamish FWH, and Potter IC. (1973). Changes in the proximate body composition of landlocked sea lamprey *Petromyzon marinus (L.)* during larval life and metamorphosis. *Journal of Fish Biology* 5: 673-682.

Maetz J. (1972). Branchial sodium exchange and ammonia excretion in the goldfish *Carassius auratus*. Effects of ammonia loading and temperature changes. *Journal of Experimental Biology* 56: 601-620

Mallatt J. (1979). Surface Morphology and Functions of Pharyngeal Structures in the Larval Lamprey *Petromyzon marinus*. *Journal of Morphology* 162: 249-274.

Mallatt J. (1981). The suspension feeding mechanism of the larval lamprey *Petromyzon marinus*. *Journal of Zoology, London* 194: 103-142

Mallatt J. (1985). Ultrastructural effects of 3-trifluormethyl-4-nitrophenol on gills of the larval lamprey *Petromyzon marinus*. *Canadian Journal of Zoology* 63: 155-164

Mallatt J. (1987). Effects of TFM and Bayer 73 on gill ultrastructure of sea lamprey ammocoetes and rainbow trout fry. *Great Lakes Fishery Commission Project Completion Report*

Mallatt J, McCall RD, Bailey JF, and Seelye J. (1994). Effects of lampricides on the gill ultrastructure of larval sea lampreys and rainbow trout fry. *Canadian Journal of Zoology* 72: 1653-1664.

Mallatt J, Lampa SJ, Bailey JF, Evans MA, and Tate W. (1995). Quantitative ultrastructure of gill epithelial cells in the larval lamprey *Petromyzon marinus*. *Canadian Journal of Fisheries and Aquatic Science* 52(6): 1150-1164

Mancera JM and McCormick SD. (1999). Influence of cortisol, growth hormone, insulin-like growth factor I and 3,30, 5-triiodo-L-thyronine on hypo-osmoregulatory ability in the euryhaline teleost *Fundulus heteroclitus*. *Fish Physiology and Biochemistry* 21: 25-33.

Marking LL and Olson LE. (1975). Toxicity of the lampricide 3-trifluoromethyl-4-nitrophenol to nontarget fish in flow through tests. *US Fish and Wildlife Service Investigation and Fish Control* 62: 3-9

Marshall WS. (2002). Na^+, Cl^-, Ca^{2+}, and Zn^{2+} transport by fish gills: Retrospective review and prospective synthesis. *Journal of Experimental Zoology* 293: 264-283.

McCormick S.D and Bern HA. (1989). In vitro stimulation of Na^+,K^+-ATPase activity and ouabain binding by cortisol in coho salmon gill. *American Journal of Physiology* 256: R707-R715

McCormick SD. (1990). Fluorescent labelling of Na^+,K^+-ATPase in intact cells by use of a fluorescent derivative of ouabain: Salinity and teleost chloride cells. *Cell and Tissue Research* 260: 529-533

McCormick SD. (1993). Methods for non-lethal gill biopsy and measurement of Na,K-ATPase activity. *Canadian Journal of Fisheries and Aquatic Sciences* 50(3): 656-658

McDonald GD and Kolar CS. 2007. Research to Guide the Use of Lampricides for Controlling Sea Lamprey. *Journal of Great Lakes Research* 33(2): 20-34

McLaughlin RL, Hallett A, Pratt TC, O'Connor LM, and McDonald DG. (2007). Research to Guide Use of Barriers, Traps, and Fishways to Control Sea Lamprey. *Journal of Great Lakes Research* 33(Suppl. 2): 7-19

Milligan CL and Wood CM. (1982). Disturbances in haematology, fluid volume distribution and circulatory function associated with low environmental pH in the rainbow trout, *Salmo gairdener*. *Journal of Experimental Biology* 99: 397-415

Mommsen TP. (1984). Metabolism of the fish gill. In: Hoar, W.S., Randall, D.J. (Eds.), *Fish Physiology, Vol. 10*. Academic Press, New York, pp. 203-238.

Mommsen TP, Walsh PJ, and Moon T. (1985). Gluconeogenesis in and kidney of Atlantic salmon. *Molecular Physiology* 8: 89-100

Monette MY and McCormick SD. (2008). Impacts of short-term acid and aluminum exposure on Atlantic salmon (*Salmo salar*) physiology: a direct comparison of parr and smolts. *Aquatic Toxicology* 86: 216-226

Morgan JD and Iwama GK. (1998). Salinity effects on oxygen consumption, gill Na+,K+-ATPase and ion regulation in juvenile coho salmon. *Journal of Fish Biology* 53: 1110-1119

Morris R and Bull JM. (1970). Studies on fresh-water osmoregulation in the ammocoete larvae of *Lampetra planeri (Bloch)*. *Journal Experiment Biology* 52: 275-290

Mueller ME, Sanchez DA, Bergman HL, McDonald DG, Rhem RG, and Wood CM. (1991). Nature and time course of acclimation to aluminium in juvenile brook trout (*Salvelinus fontinalis*). II. Gill histology. *Canadian Journal of Fisheries and Aquatic Sciences* 48: 2016-2027

Nelson DL and Cox MM. (2008). Principles of Biochemistry Fifth Edition. W.H. Freeman and Company New York: New York.

Niblett PD and Ballantyne JS. (1975). Uncoupling of oxidative phosphorylation in rat liver mitochondria by the lamprey larvicide TFM (3-trifluoromethyl-4-nitrophenol). *Pesticide Biochemistry and Physiology* 6(4): 363-366.

Nilsson S. (1986). Control of gill blood flow. In Fish Physiology S. Nilsson and S. Holmgren (Eds.), pp. 87-101. London: Croom Helm.

Nilsson GE and Renshaw GMC. (2004). Hypoxic survival strategies in two fishes: extreme anoxia tolerance in the North European crucian carp and natural hypoxic preconditioning in a coral-reef shark. Review. *The Journal of Experimental Biology* 207: 3131-3139

Nilsson GE, Dymowskab A, and Stecyk JAW. (2012). New insights into the plasticity of gill structure. *Respiratory Physiology and Neurobiology* 184: 214-222

Olson LE and Marking LL. (1973). Toxicity of TFM (lampricide) to six early life stages of rainbow trout (*Salmo gairdneri*). *Journal of the Fisheries Research Board of Canada* 30: 1047-1052

Olson LE and Marking LL. (1975). Toxicity of four toxicants to green eggs of salmonids. *Progressive Fish Culturist* 37: 143-147.

Ontario Ministry of the Environment. (1974). Water quality data, Ontario lakes and streams 1974. Vol. IX. Water Resources Branch.

Osório J and Rétaux S. (2008). The lamprey in evolutionary studies. *Development Genes and Evolution* 218: 221-235.

Panserat S, Médale F, Brèque J, Plagnes-Juan E, and Kaushik S. (2000). Lack of significant long-term effect of dietary carbohy- drates on hepatic glucose-6-phosphate expression in rainbow trout (*Oncorhynchus mykiss*). *Journal of Nutritional Biochemistry* 11: 22-29

Perry SF, Malone S, and Ewing D. (1987). Hypercapnic acidosis in the rainbow trout, *Salmo gairdneri.* I. Branchial ionic fluxes and blood acid-base status. *Canadian Journal of Zoology* 65: 888-895.

Peterson DL, Vecsei P, and Jennings CA. (2007). Ecology and biology of the lake sturgeon: a synthesis of current knowledge of a threatened North American *Acipenseridae*. Reviews in *Fish Biology* and *Fisheries* 17: 59-76

Polakof S, Arjona FJ, Sangiao-Alvarellos S, Martin del Rio MP, Mancera JM, and Soengas JL. (2006). Food deprivation alters osmoregulatory and metabolic responses to salinity acclimation in gilthead sea bream *Sparus auratus*. *Journal of Comparative Physiology B* 176: 441-452.

Randall DJ, Baumgarten D, and Malyusz M. (1972). The relationship between gas and ion transfer across the gills of fishes. *Comparative Biochemistry and Physiology* A 41: 629-637

Reis-Santos P, McCormick SD, and Wilson JM. (2008). Ionoregulatory changes during metamorphosis and salinity exposure of juvenile sea lamprey (*Petromyzon marinus L*). *Journal of Experimental Biology* 211: 978-988

Renaud CB. (1997). Conservation status of Northern Hemisphere lampreys (Petromyzonitdae). *J Applied Ichthyology* 13: 143-148

Richards JG, Semple JW, Bystriansky JS, and Schulte PM. (2003). Na+/K+- ATPase a-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *Journal of Experimental Biology* 206: 4475–4486.

Rovainen CM, Lowry OH, and Passonneau JV. (1969). Levels of metabolites and production of glucose in the lamprey brain. *Journal of Neurochemistry* 16(10): 1451-1458.

Rovainen CM and Schieber MH. (1975). Ventilation of larval lampreys. *Journal of Comparative Physiology* 104: 185-203

Saglam D, Atli G, and Mustafa C. (2013). Investigations on the osmoregulation of freshwater fish (*Oreochromis niloticus*) following exposures to metals (Cd, Cu) in differing hardness. *Ecotoxicology and Environmental Safety* 92: 79-86

Sangiao-Alvarellos S, Arjona FJ, Martin del Rio MP, Miguez JM, Mancera JM, and Soengas JL. (2005). Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*. *Journal of Experimental Biology* 208: 4291-4304.

Schultz DP, Harman PD, and Luhning CW. (1979). Uptake, Metabolism, and Elimination of the Lampricide 3-Trifluoromet hyl-4-nitrophenol by Largemouth Bass (*Micropterus salmoides*). *Journal of Agriculture and Food Chemistry* 27(2): 328-331

Schjolden J, Sørensen J, Nilsson GE, and Poléo ABS. (2007). The toxicity of copper to crucian carp (*Carassius carassius*) in soft water. *Science of the Total Environment* 384: 239-251.

Scott WB and Crossman EJ. (1974). *Freshwater fishes of Canada*. Bull. 184, Fisheries Research Board Canada, Ottawa.

Semenza G. (2011). Hypoxia-inducible factor 1: Regulator of mitochondrial metabolism and mediator of ischemic preconditioning. *Biochimica et biophysica acta* 1813(7): 1263–1268

Singer TD and Ballantyne JS. (2004). Sturgeon and paddlefish metabolism. In: LeBreton GTO, Beamish FWH, McKinley RS (Eds.). *Sturgeons and Paddlefish of North America*. Kluwer Academic Publishers, Dordrecht, pp. 167-194.

Smith SH. (1995). Early changes in the fish community of Lake Ontario. *Great Lakes Fishery Commission, Technical Report 60*, Ann Arbor, Michigan.

Smith, SH. (1968). Species Succession and Fishery Exploitation in the Great Lakes. *Journal of the Fisheries Research Board of Canada* 25(4): 667-693

Smith BR and King Jr. EL. (1969). Lamprey control in the United States. *Great Lakes Fishery Commission Annual Report*: 34-49 Appendix C

Smith BR and Tibbles JJ. (1980). Sea Lamprey (*Petromyzon marinus*) in Lakes Huron, Michigan, and Superior: History of Invasion and Control, 1936-78. *Canadian Journal of Fisheries and Aquatic Science* 37: 1780-1801

Sollid, J, De Angelis P, Gundersen K, and Nilsson G.E. (2003). Hypoxia induces adaptive and reversible gross-morphological changes in crucian carp gills. *Journal of Experimental Biology* 206: 3667-3673.

Sollid J, Weber RE, and Nilsson GE. (2005a). Temperature alters the respiratory surface area of crucian carp Carassius carassius and goldfish *Carassius auratus*. *Journal of Experimental Biology* 208: 1109-1116.

Sollid J, Kjernsli A, De Angelis PM, Røhr ÅK, and Nilsson GE. (2005b). Cell proliferation and gill morphology in anoxic crucian carp. *American Journal of Physiology: Regulatory Integrative and Comparative Physiology* 289: R1196-R1201.

Sollid, J and Nilsson GE. (2006). Plasticity of respiratory structures—adaptive remod- eling of fish gills induced by ambient oxygen and temperature. *Respiratory Physiology and Neurobiology* 154: 241-251.

Sollid J, Rissanen E, Numminen H, Thorstensen T, Vuori KAM, Nikinmaa M, Nilsson and GE. (2006). HIF-1 and iNOS in crucian carp gills during hypoxia induced transformation. *Journal of Comparative Physiology B* 176: 359-369

Species at Risk Public Registry. 2014. Lake Sturgeon Great Lakes - Upper St. Lawrence populations. Retrieved from Species at Risk Public Registry, Government of Canada, website: http://www.sararegistry.gc.ca/species/speciesDetails_e.cfm?sid=842

Stuenkel EL and Hillyard SD. (1980). Effects of temperature and salinity on gill Na+,K+-ATPase activity in the pupfish, *Cyprinodon salinus*. *Comparative Biochemistry and Physiology* 67(A): 179-182

Sullivan G, Fryer J, and Perry S. (1995). Immunolocalization of proton pumps (H+-ATPase) in pavement cells of rainbow trout gill. *Journal of Experimental Biology* 198(Pt 12): 2619-2629.

Sutton TM, Johnson BL, Bills TD, and Kolar CS. (2003). Great Lakes Fishery Commission, Project Completion Report, Ann Arbor, Michigan. Available: http://www.glfc.org/research/reports/. (Sept 2011).

Takeyasu, K, Tamkun, MM, Renaud KJ, and Fambrough DM. (1988). Ouabain-sensitive (Na^+, K^+)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the a-subunit of an avian sodium pump. *Journal of Biology and Chemistry* 263: 4347-4354

Thomassen JM. (1993). Hydrogen peroxide as a delousing agent for Atlantic salmon. In: Boxhall, GA and Defaye D (Eds.). Pathogens of Wild and Farmed Fish. Sea Lice. Ellis Horwood Limited.

Tipsmark CK and Madsen SS. (2001). Rapid modulation of Na+/ K+ATPase activity in osmoregulatory tissues of a salmonid fish. *Journal of Experimental Biology* 204: 701-709.

Towle DW, Gilman ME, and Hempel JD. (1977). Rapid modualtion of gill Na+,K+-dependent ATPase activity during acclimation of the killifish Fundulus heteroclitus to salinity change. *Journal of Experimental Zoology* 202: 179-186

Tseng YC, Huang CJ, Chang JCH, Teng WY, Baba O, Fann MJ, and Hwang PP. (2007). Glycogen phosphorylase in glycogen-rich cells is involved in the energy supply for ion regulation in fish gill epithelia. *American journal of physiology. Regulatory, integrative and comparative physiology American Physiological Society* 293: R482-R491

Tseng YC and Hwang PP. (2008). Some insights into energy metabolism for osmoregulation in fish. Review. *Comparative Biochemistry and Physiology, Part C* 148: 419-429

Tufts BL, Ferguson RA, and Boutilier RG. (1988). In *vivo* and in *vitro* effects of adrenergic stimulation on chloride/bicarbonate exchange in rainbow trout erythrocytes. *Journal of Experimental Biology* 140: 301-312.

Upper Lakes Reference Group. (1977). The waters of Lake Huron and Lake Superior. Vol. 1. Summary and recommendations. Report to the International Joint Commission.

Ura K, Soyano K, Omoto N, Adachi S, and Yamauchi K. (1996). Localization of Na-K-ATPase in tissues of rabbit and teleosts using an antiserum directed against a partial sequence of the α - subunit. *Zoological Science* 13: 219-227

Vermette MG and Perry SF. (1988). Adrenergic involvement in blood oxygen transport and acid-base balance during hyper- capnic acidosis in the rainbow trout, *Salmo gairdneri. Journal of Comparative Physiology B* 158: 107-115.

Viant MR, Walton JH, and Tjeerdema W. (2001). Comparative sublethal actions of 3-trifluoromethyl-4-nitrophenol in marine molluscs as measured by in vivo 13PNMR. *Pesticide Biochemistry and Physiology* 71: 40-47

Vijayan MM and Moon TW. (1992). Acute handling stress alters hepatic glycogen metabolism in food-deprived rainbow trout (*Oncorhynchus mykiss*). *Canadian Journal of Fish and Aquatic Science* 49: 2260-2266

Waldman JR, Grunwald C, Roy NK, and Wirgin II. (2004). Mitochondrial DNA analysis indicates sea lampreys are indigenous to Lake Ontario. *Transactions of the American Fisheries Society* 133: 950-960.

Waldman JR, Grunwald C, Roy NK, and Wirgin II. (2006). Evaluation of the native status of sea lampreys in Lake Champlain based on mitochondrial sequencing analysis. *Transactions of the American Fisheries Society* 135: 1076-1085.

Wallace KB and Starkov AA. (2000). Mitochondrial targets of drug toxicity. *Annual Review of Pharmacology and Toxicology* 40: 353-388

Wamelink MMC, Struys EA, and Jacobs C. (2009). The biochemistry, metabolism and inherited defects of the pentose phosphate pathway: a review. *Journal of Inherited Metabolic Disease* 31(6): 703-717

Wilkie MP, Couturier J, and Tufts BL. (1998). Mechanisms of acid-base regulation in migrant sea lampreys (*Petromyzon marinus*) following exhaustive exercise. *Journal of Experimental Biology* 201(9): 1473-1482

Wilkie MP, Bradshaw PG, Joanis Vincent, Claude JF, and Swindell SL. (2001). Rapid Metabolic Recovery Following Vigorous Exercise in Burrow-Dwelling Larval Sea Lampreys (*Petromyzon marinus*). *Biology Faculty Publications. Paper 46. http://scholars.wlu.ca/biol_faculty/46*

Wilkie MP, Turnbull S, Bird J, Wang YS, Claude JF, and Youson JH. (2004). Lamprey parasitism of sharks and teleosts: high capacity urea excretion in an extant vertebrate relic. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* 138(4): 485-492.

Wilkie MP, Wang Y, Walsh PJ, Youson JH. (2007). The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) interferes with intermediary metabolism and glucose homeostasis, but not with ion balance, in larval sea lamprey (*Petromyzon marinus*). *Canadian Journal of Fisheries and Aquatic Science* 64(9): 1174-1179.

Wilkie MP. (2011). Lampreys: Environmental physiology. *Encyclopedia of Fish Physiology: From Genome to Environment* 3: 1788-1799.

Wilson JM, Laurent P, Tufts BL, Benos DJ, Donowitz M, Vogl AW, and Randall DJ. (2000). NaCl uptake by the branchial epithelium in freshwater teleost fish: An immunological approach to ion-transport protein localization. *Journal of Experimental Biology* 203: 2279-2296.

Wilson JM and Laurent P. (2002). Fish gill morphology: Inside out. *Journal of Experimental Zoology* 293: 192-213

Wilson JM, Leitão A, Gonçalves AF, Ferreira C, Reis-Santos P, Fonseca A-V, da Silva JM, José Antunes JC, Pereira-Wilson C, and Coimbra J. (2007). Modulation of branchial ion

transport protein expression by salinity in glass eels (*Anguilla anguilla L*). *Marine Biology* 151: 1633-1645

Witters H, Berckmans P, and Vangenechten C. (1996). Immunolocalization of Na+,K+-ATPase in the gill epithelium of rainbow trout, *Oncorhynchus mykiss*. *Cell and Tissue Research* 283: 461-468.

Wood CM and Randall DJ. (1973). The influence of swimming activity on sodium balance in the rainbow trout (*Salmo gairdneri*). *Journal of Comparative Physiology* 82: 207-233

Wood CM, Wheatly MG, and Höbe H. (1984). The mechanisms of acid-base and ionoregulation in the freshwater rainbow trout during environmental hyperoxia and subsequent normoxia. III. Branchial exchanges. *Respiratory Physiology* 55: 175-192

Wood CM. (1988). Acid-base and ionic exchanges at the gills and kidney after exhaustive exercise in rainbow trout. *Journal of Experimental Biology* 136: 461-481.

Youson JH and Freeman PA. (1976). Morphology of the Gills of Larval and Parasitic Adult Sea Lamprey, *Petromyzon marinus L. Journal of Morphology* 179: 73-104

Youson JH. (1980). Morphology and physiology of lamprey metamorphosis. Canadian Journal of *Canadian Journal of Fisheries and Aquatic Sciences* 37: 1687-1710.

Zall DM, Fisher MD, and Garner QM. (1956). Photometric determination of chloride in water. *Analytical Chemistry* 28: 1665-1678.

Zydlewski J and Wilkie MP. (2012). 6 - Freshwater to seawater transition in migratory fishes. *Fish Physiology* 32: 253-365