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The Influence of Water Chemistry and Gill Physiology on the Uptake of the Lampricide TFM by Lake Sturgeon (Acipenser fulvescens)

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The Influence of Water Chemistry and Gill Physiology on the Uptake of the Lampricide

TFM by Lake Sturgeon (*Acipenser fulvescens)*

By

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Abstract

Application of 3-trifluoromethyl-4-nitrophenol (TFM) to control invasive sea lamprey (*Petromyzon marinus*) within the Laurentian Great Lakes seldom causes non-target mortality. However, under certain conditions, TFM can harm species such as the lake sturgeon (*Acipenser fulvescens*). Lake sturgeon less than 100mm in length are particularly vulnerable to TFM-induced mortality, and are more sensitive to TFM toxicity with increasing water alkalinity as compared to sea lamprey. The objectives of this study were to evaluate the influence of pH and alkalinity on the uptake of TFM by juvenile sturgeon using radio-labeled TFM (^{14}C-TFM). An additional objective was to resolve why younger (YOY; young of the year) lake sturgeon were more vulnerable to TFM than older (1+; 1 year or older) animals under the same conditions. Inverse relationships were observed between the rates of TFM uptake with water pH. These pH effects support the hypothesis that greater TFM toxicity at low pH is likely a direct result of increasing concentrations of the un-ionized, more lipophilic form of TFM at lower pH, leading to greater rates of uptake via passive diffusion across the gills. Uptake of TFM was also reduced as water alkalinity increased from low (50 CaCO$_3$ L^{-1}) to moderate alkalinity (150 mg CaCO$_3$ L^{-1}), but further reductions in TFM uptake were negligible at higher alkalinities. The reductions in TFM uptake between low and moderate alkalinity were likely due to a higher capacity of the water to buffer acidic equivalents (H$^+$ and CO$_2$) excreted across the gill, resulting in less acidification of the gill microenvironment and therefore the formation of less un-ionized TFM. Measurements of Na$^+$/K$^+$-ATPase and V-ATPase activity, as well as western blotting and immunohistochemical staining, demonstrated that TFM had no adverse effects on the ionoregulatory machinery of the gills. Regardless of water chemistry conditions, the rates of TFM uptake were greatest in the YOY sturgeon than in the 1+ fish, likely due to the higher mass specific metabolic rates of the smaller fish. In conclusion, the inverse relationship between body size and TFM uptake contributes to the greater sensitivity of YOY sturgeon to TFM. Alkalinity is also protective against TFM toxicity, but the protective effects of alkalinity are negligible in waters of high alkalinity. To minimize the risk of non-target mortality in lake sturgeon, it would be prudent to conduct treatments in the fall when sturgeon are larger and have lower rates of TFM uptake. Water chemistry also has pronounced effects on TFM uptake by lake sturgeon, and

should be considered prior to TFM applications to protect them from the adverse of effects of TFM, without compromising sea lamprey control efforts.

Acknowledgements

Table of Contents

List of Tables

List of Figures & Illustrations

Chapter 1

Introduction and Literature Review

Sea Lamprey Biology

The sea lamprey (*Petromyzon marinus*; Linnaeus 1758) is a jawless fish of the order Petromyzontiformes, and is distributed within the Eastern and Western North Atlantic Ocean and within many rivers and lakes of the associated ocean basins (Figure 1-1; Renaud, 2011). Although sea lampreys are anadromous fish, there are landlocked, freshwater populations within the Laurentian Great Lakes and Lake Champlain (McDonald and Kolar, 2007; Renaud, 2011; Eshenroder, 2014). They spend the first 3-7-years of their life as larvae (ammocoetes) burrowed in the sediment of rivers and streams, filter-feeding on detritus and diatoms (Figure 1-2). Once they accumulate sufficient lipid stores, they undergo a prolonged-metamorphosis (several months) into juvenile sea lamprey (Beamish and Potter, 1975; Youson, 2003). This metamorphosing period is characterized by complex morphological and physiological changes, including the development of an oral sucker reinforced by an annular-ring of cartilage (buccal-disk; Farmer, 1980; Youson, 1980), eyes (Osorio and Retaux, 2008), and a restructuring of the kidneys, liver and gill (Sidon and Youson, 1983; Bartels and Potter, 2004). These juveniles migrate downstream to larger bodies of water where they feed by parasitizing larger fish. Lamprey attach to their prey with an oral disk and teeth, while utilizing a rasping tongue and anti-coagulant secretions to puncture a hole and feed on the blood of their hosts (Farmer, 1980). After 1-2 years, adult lamprey migrate upstream to spawn and die (Beamish and Potter, 1975; Silva et al., 2013; Youson, 2003). Sea lamprey do not selectively return to their natal streams, but they do utilize temperature and stream discharge cues to gauge stream suitability, as well as pheromones to identify spawning habitat (Applegate and Smith, 1950; Li et al., 2002; Wagner et al., 2006; Binder and McDonald, 2008; Johnson et al., 2015; Wagner et al., 2016). During

reproduction, females deposit tens-of-thousands of eggs, which hatch within 10-13 days (Scott and Crossman, 1973; Potter, 1980; Kircheis, 2004).

Sea Lamprey Invasion and the History of Control in the Laurentian Great Lakes

Whether sea lamprey naturally colonized Lake Ontario following the last ice age (Waldman et al. 2004; Bryan et al., 2007), or were introduced via artificial canals (Eshenroder, 2014) is disputed. It is clear, however, that the sea lamprey's access to the remaining Great Lakes was obstructed by Niagara Falls until modifications to the Welland Canal in the late 19th and early 20th centuries allowed their passage into Lake Erie. Within 20 years, sea lamprey were in each of the Great Lakes, which combined with overfishing, resulted in the drastic declines in fish species with commercial, recreational, and cultural significance (Smith and Tibbles, 1980; Coble et al., 1990; Eshenroder and Burnham-Curtis, 1999; Hansen, 1999). A single juvenile (parasitic life-stage) is capable of killing nearly 21 kg of fish (Kitchell and Breck, 1980), and selectively feeds on larger host fishes such as lake trout ((*Salvenlinus namaycush*; Swink, 2003; Hansen et al., 2016). Lake trout were extirpated from the lower Great Lakes (Lakes Michigan, Erie and Ontario) and their populations significantly reduced from the upper Great Lakes during the 1940's and 1950's (Figure 1-3; Lakes Huron and Superior; Berst and Spangler, 1973; Lawrie, 1978; Coble at al., 1990; Hansen, 1999; Eshenroder and Amatangelo, 2005; GLFC, 2008; Muir et al., 2013). This lead to alewife (*Alosa psuedoharengus*) population explosions and die-offs (Brown, 1972; O'Gorman et al., 2013) that polluted shorelines and ruined local economies dependent on fishing and tourism (Scott and Crossman, 1973; Tanner and Tody, 2002). The loss of the economic staple that is provided by commercial, recreational and tribal fisheries for Canada and the U.S.A. could not be ignored. Today, the current economic output of Great Lakes'

fisheries is valued at greater than $7 billion annually for the U.S.A. alone (Southwick and Associates, 2012). In 1955 the federal governments of Canada and the U.S.A. founded the Great Lakes Fisheries Commission by treaty, giving them the responsibility of reducing sea lamprey populations and rehabilitating the Great Lakes ecosystem (GLFC, 1955; Siefkes, 2017).

Control efforts began with the implementation of 21 mechanical or screen-type weirs and one electrical screen within sea lamprey infested streams of Lakes Huron, Michigan and Superior (Applegate and Smith 1950; Applegate et al., 1952). These barriers were expensive to build and operate, and allowed for the upstream migration and spawning of sea lamprey during ice break-up and floods (Smith and Tibbles, 1980). The development of electrical current barriers was shown to operate at lower costs than physical barriers, and was more efficient at preventing the upstream migration of spawning sea lamprey as it allowed for the downstream movement of ice, flood waters and debris. However, electrical barriers were not considered effective in reducing sea lamprey populations and were reported to also cause substantial mortality of non-target fishes (Smith and Tibbles, 1980; McDonald and Kolar, 2007; McLaughlin et al., 2007). In 1951, research was funded to develop piscicides that could be applied in streams to target the ammocoetes burrowed in the substrate (Applegate, 1950; Applegate et al., 1961). Chemical screening was conducted extensively until seven years later when the lamprey selective compound, 3-trifluoromethyl-4-nitrophenol (TFM) was discovered (Applegate et al., 1961). This compound was later proven to be highly toxic to sea lamprey due to their reduced ability to detoxify the compound as compared to non-target fishes (Lech and Statham, 1975; Howell et al., 1980; Kane et al., 1993). TFM was successful in controlling sea lamprey infestations from streams without having major effects on non-target fish and was later used as a lampricide (sea

lamprey piscicide) in a highly regulated chemical control program within all the Great Lakes by 1986 (Applegate et al., 1961; McDonald and Kolar, 2007).

Although TFM was found to reduce the number of sea lamprey within the Great Lakes, large volumes were required to complete treatments, and so an alternative lampricide was researched to reduce the costs of the control program (Howell et al., 1964). Another agent, niclosamide (5,2'-dichloro-4'-nitro-salicylanilide; also known as Bayluscide®), was found to increase the toxicity of TFM without significantly affecting its selectivity to sea lamprey when used in combination (TFM to Bayluscide® ratio of 98:2 to 99.5:0.5; Howell et al., 1964; Dawson, 2003). To further reduce the costs of control efforts, niclosamide was developed into a granular form (Granular Bayluscide®, or GB) which would slowly dissolve in water to allow for bottom release of the compound (Howell et al., 1980). This development allowed niclosamide to be applied to the bottom of rapid- or slow-moving waters where TFM would be less effective (Dawson, 1980; McDonald and Kolar, 2007). The use of lampricides was very successful, reducing the catches of spawning sea lamprey by 92% in Lake Superior by the year 1978 (Smith and Tibbles, 1980).

One of the milestones for sea lamprey management in 1992 was to incorporate further research into suppressing sea lamprey populations to target levels, while also reducing the cost of the program. Emphasis was placed on reducing the reliance on lampricides by 50% in the year 2000, and developing more efficient methods of population assessment and control in lentic areas (GLFC, 1992). It was found that ammocoetes become irritated by Granular Bayluscide® exposure, stimulating them to leave their burrows (McDonald and Kolar, 2007). This allowed for

a feasible and more efficient method of treating and surveying larval populations in lentic environments, such as the mouth of a river (GLFC, 2008; Dawson, 2003). Research and development of selective in-stream barriers resulted in the engineering of low-head structures based on the different mode of swimming and poor jumping capacity of the sea lamprey (Beamish, 1978; Youngs, 1979; Reinhardt et al., 2009; Almeida and Quintella, 2013), as well as the timings of seasonal migrations (Hunn and Youngs, 1980). These barriers would not be feasible for every stream, however, as they can also block non-target fishes with little to no jumping ability that are of great importance to the Great Lake's ecosystem (Hunn and Youngs, 1980; Dodd et al., 2003).

In the 1970s, P,P-bis(1-aziridinyl)-N-methylphosphinothioic amide (Bisazir) was found to damage genetic material in the sperm of male sea lampreys without reducing their ability to fertilize eggs. This led to its later experimental use as a sea lamprey sterilant in Lake Superior from 1987 to 1997, and the St. Marys River from 1991-2011 (Chang et al., 1970; Hanson and Manion, 1978; Hansen 1990; Kaye et al., 2003). This program has since been halted as a method of population control in the St. Marys River due to difficulty in determining its ability to reduce lamprey populations. Additionally, the sterile release method would only target one generation of sea lamprey, whereas advances in the application of Bayluscide® had made it an efficient method at removing many generations of lamprey from the river, with a clear and immediate relationship detected between treatments and larval population estimates the next year (Bravener and Twohey, 2016).

In 1995, the Lampricide Control Task Force was created, which improved the efficiency of treatments by minimizing lampricide use and impacts on stream ecosystems, yet maximizing the numbers of sea lamprey killed. Additionally, the Task Force was responsible for defining lampricide control options for near- and long-term stream selection and target setting (Klar and Young, 2004). Subsequently, the number of streams treated were reduced and treated at lower discharges, a pH/alkalinity model was produced to predict more accurately the minimum lethal concentrations (12 h $LC_{99.9}$; MLC; Bills et al., 2003) required for treatment, and single block treatments were conducted for large dendritic streams, instead of multiple different treatments (Berge et al., 2003). By the year 1999, the amount of TFM applied annually had been reduced by approximately 36% (Brege et al., 2003). To date, further reductions are not likely not possible without compromising treatment effectiveness (GLFC, 2008).

3-Trifluoromethyl-4-Nitrophenol (TFM) Ecological Fate and Effect

Sea lamprey populations are primarily controlled within the Great Lakes via the application of TFM (McDonald and Kolar, 2007). TFM is a solid at room temperature, has a low vapor pressure (Hubert, 2003), is sparingly soluble in water and highly soluble in most organic solvents with a $Log_{10}(K_{OW})$ value of 2.77 (McKim and Erickson, 1991; Cronin and Livingston, 2004; Applegate et al., 1961). TFM is detoxified by non-target fishes via the process of glucuronidation (Figure 1-4), which makes compounds more water-soluble, and therefore easier to excrete via the urine or feces. Glucuronidation occurs in the liver, where the enzyme UDP-glucuronyl transferase catalyzes the addition of glucuronic acid to xenobiotic materials and endogenous compounds (Clark et al., 1991, Lech and Statham, 1975; Vue et al., 2002). Sea lamprey on the other hand, have low levels of this enzyme, which explains their greater

sensitivity to TFM (Kane et al., 1994; Bills et al., 2003; Lech and Statham, 1975; Bussy et al., 2018a).

The mode of toxic action for TFM has been attributed to the uncoupling of oxidative phosphorylation within the mitochondria (Niblett and Ballantyne, 1976; Birceanu et al., 2011), which leads to impaired ATP production. This causes fish to metabolize glycogen and phosphocreatine stores due to impaired oxidative ATP production by the mitochondria. When these anaerobic energy reserves are insufficient to meet the ATP demands of the body, mortality ensues (Wilkie et al., 2007; Birceanu et al., 2009, 2014). The mode of toxic action of TFM appears to be the same in both the sea lamprey and the non-target rainbow trout (*Oncorhynchus mykiss*) when they are exposed to toxic concentrations of the compound (Birceanu et al., 2011, 2014). Although TFM is selective to sea lamprey, and non-target mortality is seldom observed during lampricide treatments, non-target exposure is unavoidable. For this reason, the GLFC strives to continually find new or improved methods of sea lamprey control, while trying to minimize adverse effects on non-target fish species (GLFC, 2011).

Lake Sturgeon Biology and Species of Concern

The lake sturgeon (*Acipenser* fulvescens) is endemic to the Central U.S.A, the Great Lakes and the Hudson Bay drainages of Canada (Harkness and Dymond, 1961; Scott and Crossman, 1973) and is currently a species of concern as they are listed as endangered or threatened within Canada and the U.S.A. (Auer, 1999). Prior to European settlement of North America, lake sturgeon were abundant within the Great Lakes, however, overfishing and restricted movement due to dams and navigation locks built in the 1800s and early 1900s,

populations were decimated (Thuemler, 1985; Auer, 1999). Modern conservation and restoration efforts are also complicated by ongoing anthropogenic disturbances including the destruction of historic spawning grounds and critical down-stream habitats (Auer, 1996; Wilson and McKinley, 2004). Additionally, the length of time required for sexual maturation and associated low rates of recruitment affect the ability of this species to re-establish itself within the Great Lakes (Harkness and Dymond, 1961; Thuemler, 1988; Peterson et al., 2007).

Lake sturgeon belong to the Acipenseridae, which are a phylogenetically ancient family of fishes whose body structure and life history have changed little over the last 400-million years (Nelson, 2006). Sturgeon are cartilaginous, benthic fishes, with five lateral rows of ossified-scutes (bony plates) that develop with age (Peterson, 2007). Barbels are located above the mouth for sensing food, and the mouth is protrusible, inferior and detached from the skull (Vecsei and Peterson, 2006; Peterson, 2007). Their gills resemble those of teleost fish, but one difference is the presence of a spiracle, and a dorso-ventrally-located opening on the gill cover (operculum). It is believed this slit allows for the irrigation of their gills during feeding when the buccal cavity is occluded (Burggren, 1978), the respiratory movement of water would likely occur in a "quasitidal" fashion (Figure 1-5; Burggren et al., 1979).

Boogaard et al. (2003) completed the first size-dependent toxicity experiment for juvenile lake sturgeon, a species that was previously shown to be sensitive to TFM toxicity by Johnson et al. (1999). Boogaard et al. (2003) determined that lake sturgeon swim-up fry and juveniles (< 100 mm; young of the year; YOY) had TFM LC_{50} values that were near or less than that of sea lamprey making them vulnerable to TFM toxicity (Figure 1-6). Concern for the mortality of

YOY sturgeon lead to the development of the "sturgeon protocol" for applications of TFM in U.S. tributaries that were known to contain juvenile lake sturgeon (Adair and Sullivan, 2009). This protocol was meant to ensure that sea lamprey control treatments did not surpass 1x the MLC for TFM within streams, or 1.2x the MLC of a TFM and 1% niclosamide mixture, and the treatment of streams would only occur after August 1, to allow juvenile sturgeon to grow to greater than 100 mm in length (Adair and Sullivan, 2009). However, the introduction of this protocol resulted in greater wounding rates among native fishes and increased spawning numbers of larval sea lamprey within the upper Great Lakes (Slade, 2012; Sullivan et al., 2013). Additionally, greater survivorship of larval sea lamprey following TFM treatments in lake sturgeon producing streams was observed when exposures were conducted in late September, as opposed to earlier in the season (Scholefield et al., 2008). This resulted in some streams being treated more often to deal with high larval densities, increasing the amount of TFM required (Boogaard et al., 2011). As a result, the "sturgeon protocol" was discontinued.

Lake sturgeon sensitivity to TFM remains a problem in the Great Lakes. During a TFM treatment in 2014 on the Muskegon River, 31 YOY sturgeon were found dead (O'Connor et al., 2017), which was 2-fold greater than the total number of sturgeon mortalities recorded in the history of sea lamprey control (13 total prior mortalities recorded; Johnson et al., 1999; O'Connor et al., 2017). Further, lamprey attacks on lake sturgeon conducted within laboratory tests have been shown to reduce growth and condition factor, as well as cause lethality of the host species (Patrick et al., 2009; Sepulveda et al., 2013), and attacks have been predicted to reduce population abundance and reproductive potential of lake sturgeon populations in the Great Lakes (Sutton et al., 2004).

The reason for the lake sturgeon's greater TFM sensitivity in these early life stages has not yet been elucidated, but it may be due to greater oxygen consumption rates in the smaller fish resulting in greater uptake of TFM (Goolish, 1991; Tessier et al., 2018), or simply be due to a relative inability to detoxify TFM in the first year of life. The greater sensitivity of lake sturgeon to TFM in waters of high alkalinity is less clear. It may be due to the use of quasitidal flow during respiration (Burrgen 1978), which could alter patterns of gas exchange as compared to a uni-directional buccal-opercular flow, which is typically used by teleost fishes (Figure 1-5). The recirculation of water within the opercular chamber may allow for the accumulation of acidic by-products (such as H^+ or CO_2) excreted across the gills, creating a more acidic medium at the site of TFM exposure. This would increase the likelihood of the phenolic form of TFM being present, which is more lipophilic and passively crosses the gills (Smith et al., 1960; Applegate et al., 1961; Hunn and Allen, 1974; McDonald and Kolar, 2007; Hlina et al., 2017), increasing the overall uptake of TFM.

Freshwater Osmoregulation and Gill Physiology

Freshwater fishes are hyperosmotic to their environment and need to actively compensate for diffusive ion loss and osmotic gain of water (Evans et al., 2005; Marshall and Grosell, 2006). The gills are the primary organ of ion uptake and prevent ionic imbalances by actively pumping ions from fresh water via mitochondrion-rich cells (MR cells; ionocytes). These cells are located within the gill epithelium and are specialized ionoregulatory cells that contain an array of ion transport proteins (Evans et al., 2005; Hwang et al., 2011). Additionally, the diet of fresh water fish is a major component of osmoregulation, with the intestine functioning in ion-uptake

(Marshall and Grosell, 2006). The kidney is also important, as it reduces the loss of ions excreted in the urine by tubular reabsorption (Evans and Claiborne, 2005; Marshall and Grosell, 2006).

Two major ion-transport proteins found within MR cells of fish gills are Na^+/K^+-ATPase and vacuolar type H^+-ATPase (V-ATPase; Figure 1-8). These proteins utilize ATP to actively transport ions across epithelial membranes to create electrochemical gradients for desired ion uptake (Evans et al., 2005; Evans and Claiborne, 2005). The Na^+/K^+-ATPase is an electrogenic pump located on basolateral membranes of MR cells, and pumps two K^+ ions into the cell, in exchange for pumping three Na^+ ions into the blood (Blanco and Mercer, 1998), and is one of the largest consumers of ATP (Skou, 1957). The V-ATPase is a vacuole type electrogenic pump located on either the apical or basolateral membranes of MR cells, where it pumps H^+ across the epithelial membrane, into the external water. This creates an electrochemical gradient for Na^+ uptake via an acid-sensing ion channel (ASIC; Bartels and Potter, 2004; Evans et al., 2005; Dymowska et al., 2014). Recently, there has been evidence to support the presence of Na^+/H^+ exchanger within MR cells that have apically localized V-ATPase, which is proposed to be involved in Na^+ uptake (Evans, 2011). The Cl^-/HCO_3^- exchanger is present on the apical surface of MR cells where localized acidification within apical microvilli by V-ATPase lowers HCO_3^- activity, driving Cl^- uptake. Basolateral-located Cl^- annion channels allow for the transfer of Cl^- into the blood, likely a cystic fibrosis transmembrane conductance regulator (CFTR) channel (Goss et al., 1998; Wilson et al., 2000; Marshall, 2002; Evans, 2011; Wilkie, 2011). Piermarini et al (2002) has demonstrated the presence of pendrin, an anion exchanger, within apical membranes of MR cells with basolateral V-ATPase presence in the Atlantic stingray. It is proposed that H^+ extrusion by V-ATPase creates intracellular bicarbonate accumulation, which is

used to create favorable HCO_3^- gradients for Cl^-/HCO_3^- exchange (Piermarini et al., 2002; Evans, 2011).

The functional surface area of the basolateral membranes of MR cells are generally amplified by extensive infolding resulting in a highly complex tubular system, which is also the site of expression for Na^+/K^+-ATPase. Although most fishes have this tubular system, it is not present within the freshwater MR cells of sea lamprey (Wilson and Laurent, 2002; Evans et al., 2005). Additionally, the apical membrane of MR cells is also amplified by a collection of tubules and vesicles (tubulovesicular system), which is distinct from the tubular system (Wilson and Laurent, 2002). MR cells cover approximately 10% of the gill epithelium, whereas the other 90% is covered by pavement cells (Evans et al., 2005). Pavement cells (PVCs) do not have as high a density of mitochondria as MR cells and are thought to be involved in mostly passive transport (Laurent and Dunel, 1980), although some freshwater fish PVCs have been found to be rich in V-ATPase (Galvez et al., 2002). MR cells play a key role in the majority of physiological processes and are often located on the afferent edge of gill filaments and interlamellar regions (Evans et al., 2005). There are two subtypes of MR cells (Figure 1-8) for freshwater fish, peanut lectin – insensitive (PNA$^-$) and penut lectin – sensitive (PNA$^+$). PNA$^-$ are ultrastructurally similar to pavement cells as they have greater V-ATPase activity, and acid stimulated, phenamil- and bafilomycin sensitive Na^+ uptake channels (Galvez et al., 2002; Reid et al., 2003). The PNA$^+$ cells have greater amounts of Na^+/K^+-ATPase, and approximately half the amount of V-ATPase as PNA$^-$ cells (Glavez et al., 2002; Reid et al., 2003; Marshall and Grosell, 2006). Carbonic anhydrase catalyzes intracellular CO_2 hydration to H^+ and HCO_3^- to be used by ion pumps for electrochemical gradients (Gilmour et al., 2007).

Apart from being the main respiratory organ for fish, the gills are the main organ for acid-base regulation (90%) and can correct acid-base disturbances within hours (Evans et al., 2005). For example, during metabolic alkalosis or acidosis, the gills regulate the flux of H^+ or HCO_3^- to and from their environment to correct for the acid-base equilibrium imbalance (Goss et al., 1992). Fish can be faced with acid-base perturbations caused by external sources such as changes in water pH, salinity or temperature, as well as internal sources including lactic acid build-up from exercise or hypercapnia (Heisler, 1989; Evans 2005). Perry and Wood (1984) demonstrated that the acclimation of rainbow trout to low Ca^+ conditions (50 µequiv L^{-1}) resulted in an immediate reduction in Na^+ and Cl^- levels within the first 24 h, however, the fish had recovered to normal Na^+ and Cl^+ efflux by day 7. Additionally, the regulation of Na^+/K^+-ATPase has been shown to be under endocrine control (McCormick, 2001; Evans, 2002), with cortisol treatments shown to increase the number of branchial MR cells in freshwater salmonids (Laurent and Perry, 1990; Perry et al., 1992; McCormick, 2001). In freshwater tilapia (*Oreochromis mossambicus*), cortisol has been shown to increase Na^+/K^+-ATPase activity and associated increases in the expression of the protein (Dang et al., 2000). As well, exposure of eels (*Anguilla rostrate* and *A. japonica*) to exogenous cortisol has been shown to increase Na^+/K^+-ATPase activity (Epstein et al., 1971; Kamiya, 1972; Forrest et al., 1973). Cortisol is the major corticosteroid in fish, and functions as both a glucocorticoid (stress hormones) and a mineralocorticoid (control electrolyte balance; Hazon and Balment, 1998; Greenwood et al., 2003).

It is possible that under the stressors induced by TFM, due to its effect as an uncoupler of oxidation phosphorylation (Birceanu et al., 2011), that it might influence the activity, expression or distribution of ion-transport proteins on the gill epithelium of sturgeon. Additionally, the influence of alkalinity on gill ionocytes, the major ion and acid base regulating cells, is not known. It is also unknown whether TFM interferes with ATP-dependent ion and acid-base regulation processes in the gills of lake sturgeon. Changes in the expression of these proteins with different alkalinity exposures and/or TFM could indicate potential influences on gill functional processes. With sturgeon populations at only 1-2% of their historic population levels, the sensitivity to TFM for this already threatened species is of major concern for future lampricide treatments, and restoration of the Great Lake's sturgeon populations.

Research Objectives and Hypotheses

The observed sensitivities of juvenile sturgeon below 100 mm and the reduced protective effect of alkalinity make this species a concern for continued lampricide control operations (Boogaard et al., 2003; O'Connor et al., 2017). However, the reason for their greater TFM sensitivity is unknown. One of the goals of this study was to determine how TFM accumulation in the juvenile sturgeon is influenced by water pH, alkalinity and body size. Another goal of this study was to determine whether TFM exposure influenced the distribution and physiology of MR cells at the gills, and if water alkalinity acclimations influenced these MR cells, and possibly predisposed sturgeon to TFM toxicity.

To achieve these goals, the objectives of my thesis were to:

I.   Determine the influence of alkalinity and pH on the uptake of TFM for young of the year (YOY) and one year old (1+) sturgeon.

II.   Assess the influence of different alkalinity acclimations on the activity, expression and distribution of Na^+/K^+- ATPase and V-type H^+-ATPase (V-ATPase) proteins present in MR cells of the gill epithelium of juvenile lake sturgeon.

To address the first objective, uptake rates of TFM were measured using the radio-tracer ^{14}C-TFM during exposures in low, moderate and high alkalinity waters (50, 150 and 250 mg L^{-1}, respectively) and pH (pH 6.5, 8.0 and 9.0), in different size groups (< 100 mm, > 130 mm in length) of juvenile sturgeon. It was predicted that TFM uptake rates would be reduced in waters of higher alkalinity, due to a reduced ability for sturgeon to acidify the gill micro-environment in this more highly buffered medium. It was also hypothesized that TFM uptake would be inversely related to water pH, due to differences in the proportion of the phenolic (un-ionized) compound at different water pHs. Additionally, it was hypothesized that YOY sturgeon would have greater rates of TFM uptake as compared to 1+ sturgeon, which would likely result in greater rates of accumulation, and thus would correlate with the greater sensitivity of this smaller size class to TFM toxicity (Boogarrd et al, 2003).

To address the second objective, juvenile lake sturgeon were acclimated to set water alkalinities for a minimum of one week prior to exposure to the sea lamprey MLC (12 h $LC_{99.9}$) of TFM. Sturgeon were sampled at regular time intervals of 0, 3, 6 and 9 h, followed by the excision and processing of their gills for the measurement of Na^+/K^+-ATPase and V-ATPase

activity, expression and distribution. The potential for TFM exposure and alkalinity acclimations to affect the activity, expression and distribution of important ionoregulatory proteins within the gills of juvenile sturgeon was assessed.

Figure 1-1. The native range of the sea lamprey.

Sea lamprey are native to the both the western and eastern sides of the North Atlantic Ocean (areas shaded in black), but they are an invasive species within the Laurentian Great Lakes (areas shaded in grey). Image taken from Almeida and Quintell, (2013).
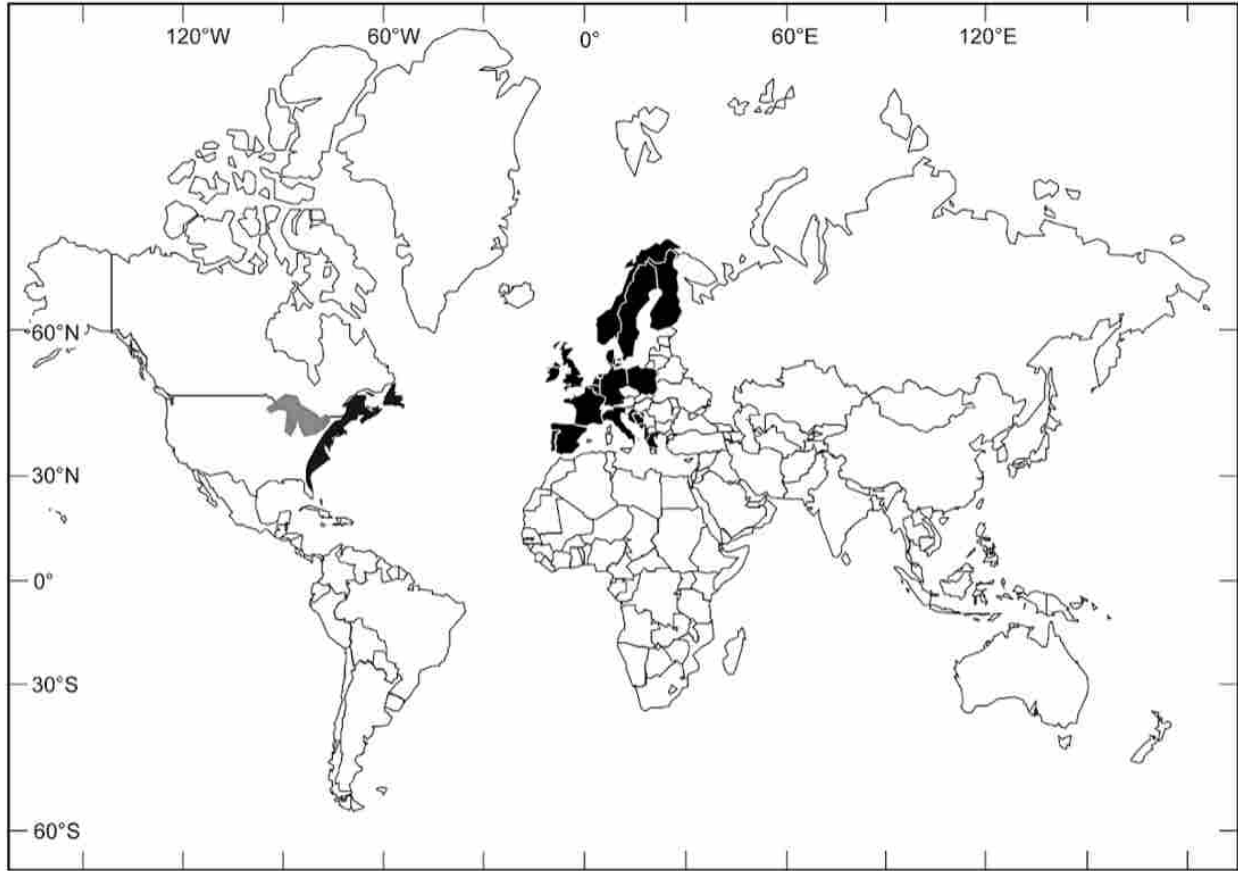
Figure 1-2. Life cycle of a Sea Lamprey (*Petromyzon marinus*).

Hatched larval lamprey (ammocoetes) filter feed and burrow into the sediments of rivers and streams for 3-7 years. Following metamorphosis, the landlocked juvenile sea lamprey migrates downstream to the Great Lakes, where it feeds parasitically on teleost fishes before sexually maturing. Mature adults migrate upstream where they spawn and then die. Drawing made by Emily Martin.

Marine Parasitic
Juvenile Phase

Juvenile Downstream
Migration

Adult Freshwater
Spawning Phase

Metamorphosis

Larval (Ammocoete)
Phase
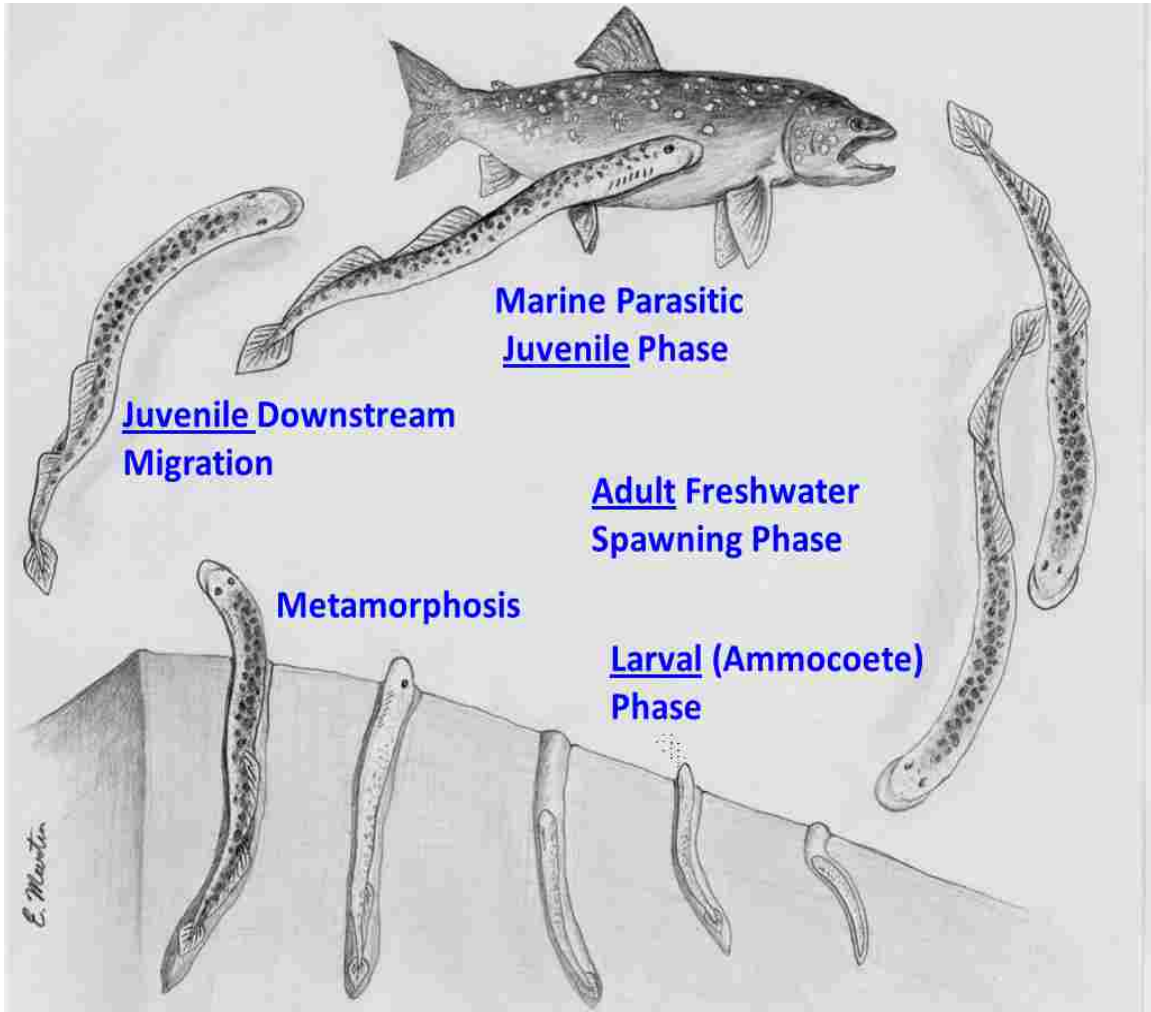
Figure 1-3. Estimated abundance of adult sea lamprey and lake trout in Lake Superior.

The estimated population abundance for adult sea lamprey (solid black line) and the relative abundance of lake trout (gray shading; fish/km/net night) in the waters of Lake Superior. The dashed line represents an estimation on the sea lamprey within Lake Superior from the year they were first discovered (1938), to when sea lamprey abundance was first modeled from trap catch data (1957). The vertical bar represents the first year lampricide treatments were conducted (1959). Figure taken from Siefkes (2017).
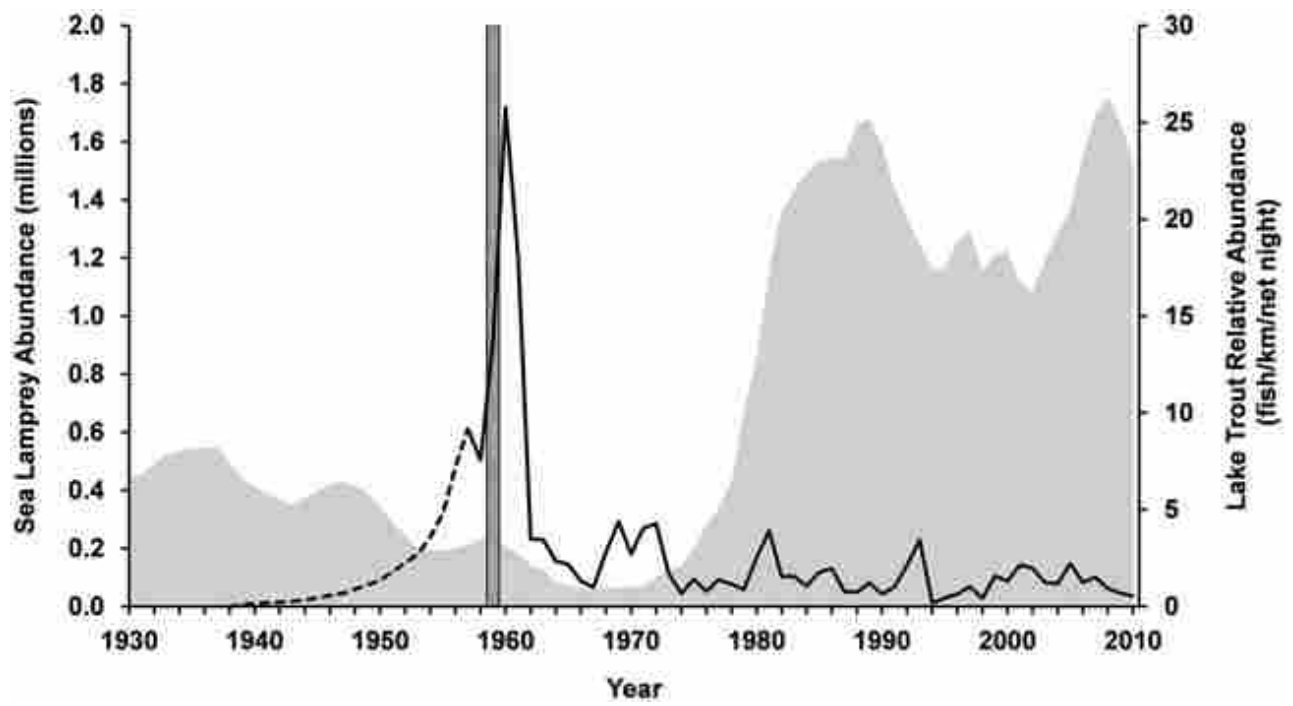
Figure 1-4. Process of glucuronidation.

UDP-glucuronyl transferase (UDPGT) is a hepatic endoplasmic reticulum membrane-bound protein, responsible for TFM detoxification. It attaches glucuronic acid to TFM through a glycosidic bond, resulting in TFM-glucuronide. This process takes place in the liver, and makes the compound more hydrophilic, so it can be removed from the body via urination and defecation (Lech and Statham,1975; Howell et al., 1980; Kane et al., 1993).

UDPGA + TFM → (UDP-glucuronyl transferase) → TFM-glucuronide + UDP

TFM   TFM-glucuronide
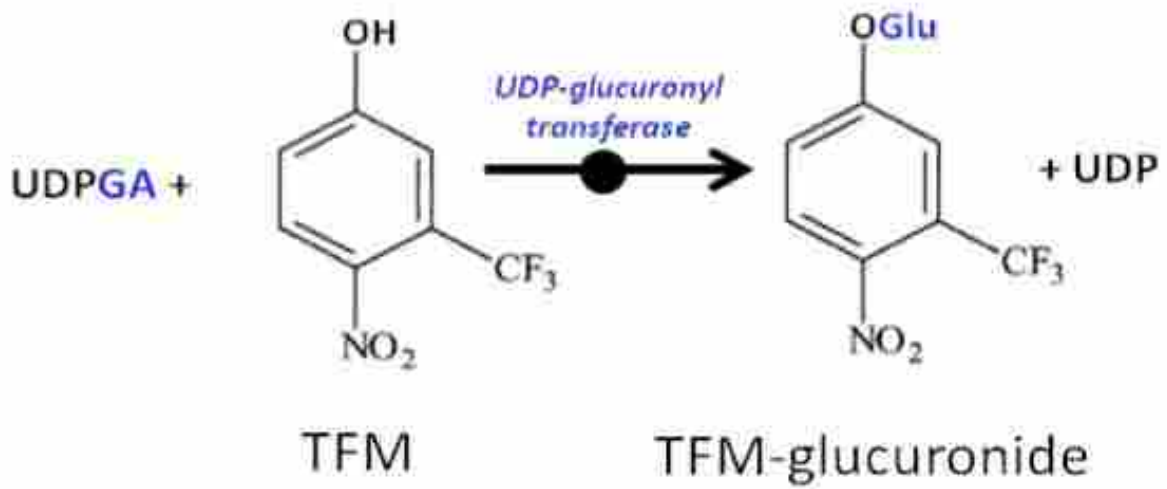
Figure 1-5. Gill ventilation in teleost fish (A) and sturgeon (B & C).

Teleost fish and sturgeon typically utilize buccal-opercular pressure gradients (A and B, respectively) to create a unidirectional flow of water from the buccal cavity, across the gill sieve, and exiting via the operculum via the opercular valve. Sturgeon can also utilize quasitidal flow (Burggren et al., 1979) to irrigate the gills when the buccal cavity is unavailable, such as feeding or foraging at the bottom of water columns. Quasitidal flow draws water into the opercular chamber through the dorso-lateral opercular slit when the mouth is closed or occluded, the opercular valve is closed, and the buccal cavity drops to create pressure. As the buccal cavity arises, the mouth remains closed or occluded, the opercular valve opens to allow water to exit. During this process, water crosses the gill sieve dorsally, in a posterior direction, before crossing the ventral portion of the gill sieve in an anterior-posterior direction and exiting. Drawing made by Dr. Michael P. Wilkie.

A

Buccal Opercular

Mouth · Operculum

Gill Sieve

Teleosts

B

O. Slit

Mouth Closed

Buccal-Operc

C

"Quasitidal"

Lake Sturgeon

27

Figure 1-6. The sensitivity of lake sturgeon to TFM.

Shown is the log scale of the 12 h LC_{50} (the concentration required for 50% mortality during a 12 h exposure) of TFM for sturgeon (●) and sea lamprey (◇), respective to their total length. The toxicity of TFM to lake sturgeon is inversely related to their body size. The 12 h LC_{50} of juvenile sturgeon below 10cm is below or near the 12 h LC_{50} for larval sea lamprey of a similar size range. Modified from McDonald and Kolar (2007).
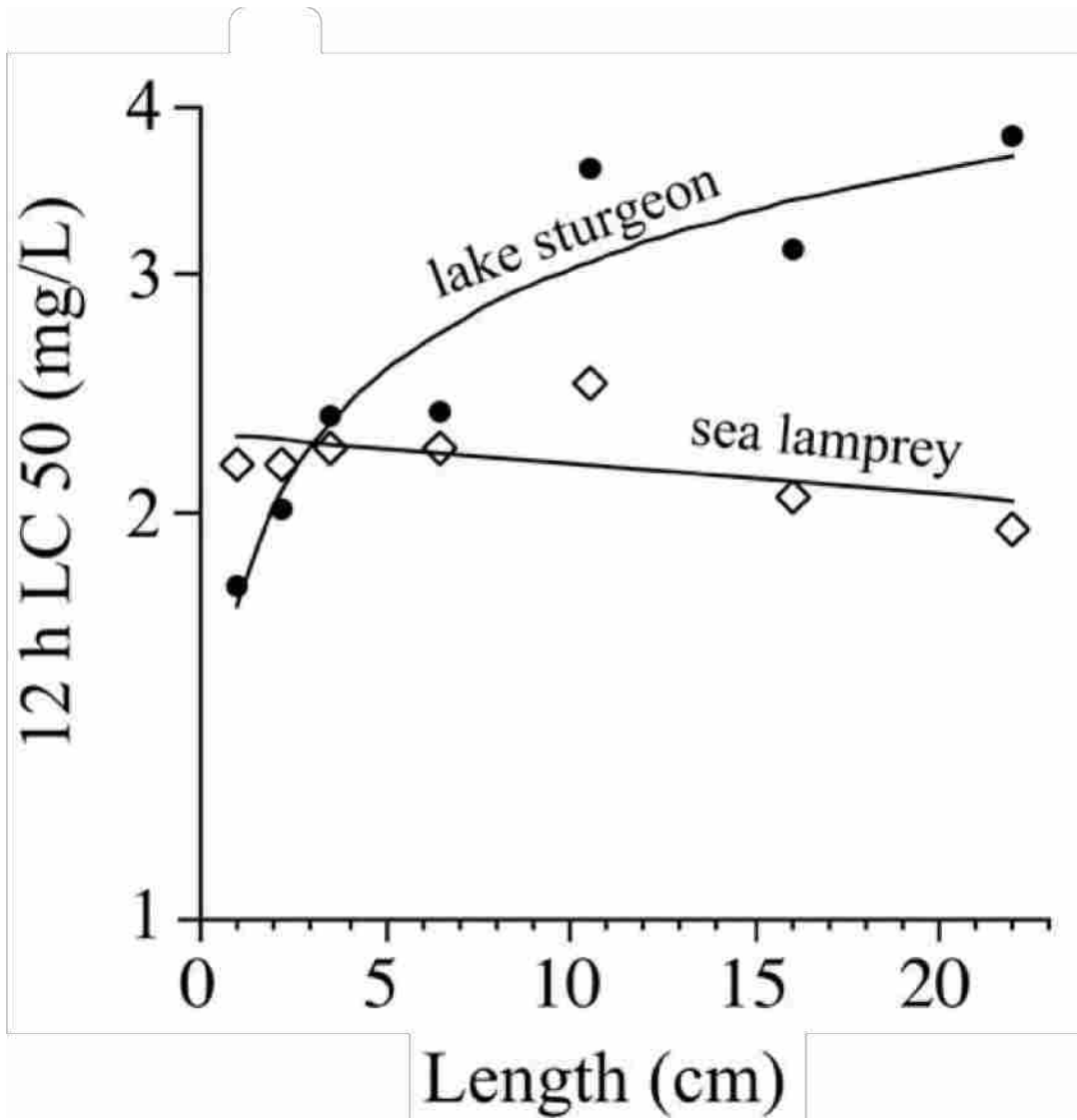
Figure 1-7. Structure and dissociation equilibrium of TFM (pK$_a$ = 6.07).

The un-ionized, phenolic form of TFM is on the left, which is more lipophilic and easily crosses the gills passively down a favorable water-to-blood gradient (Birceanu et al., 2009). In a more acidic medium, greater concentrations of the phenolic form exist. The ionized form of TFM is shown on the right, which exists in greater concentrations in more alkaline mediums
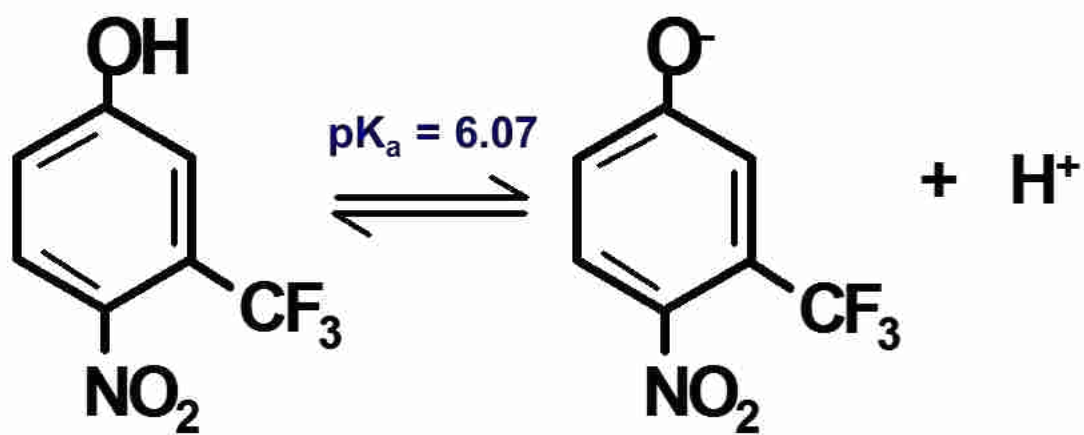
Figure 1-8. Working model of osmoregulation for freshwater fish gills.

The current working model for ionic exchangers, channels and pumps mediating the uptake of Na^+ and Cl^- by the fish gill epithelium in freshwater or seawater. Two mitochondrial rich cells, PNA^- and PNA^+, are shown. NKA: Na^+/K^+-ATPase, V-ATPase: vacuolar type proton ATPase, NHE: Na^+,H^+ exchanger, Rhcg: rhesus glycoprotein, SLC4/SLC26: Cl^-/HCO_3^- exchanger, NCC: Na^+ and Cl^- cotransporter, NBC: Na^+ and HCO_3^- cotransporter, and CLC: Cl^- channel. Circles represent active transport, requiring ATP. The actual distribution and nomenclature of the specific cells are debated and may be species specific. Modified from Evans (2011).
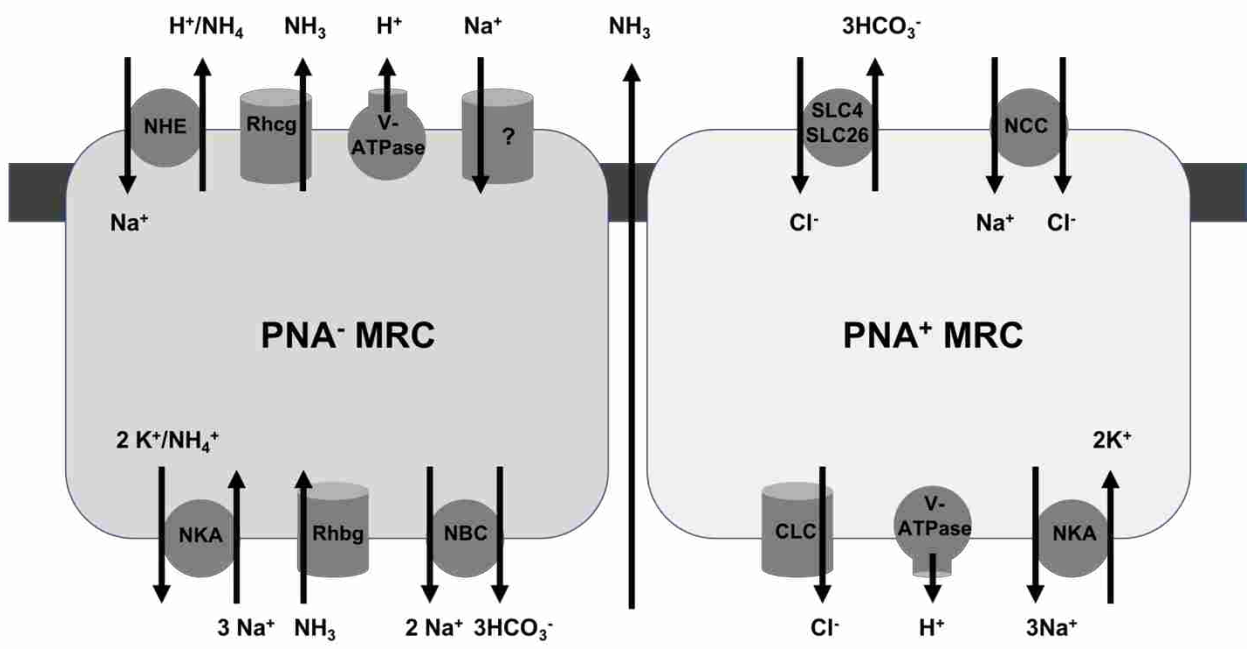
Chapter 2

The Effects of Water pH and Alkalinity on the Uptake of 3-Trifluoromethyl-4-Nitrophenol

(TFM) in Juvenile Lake Sturgeon

Introduction

The lake sturgeon (*Acipenser fulvescens*) is endemic to the Central United States, the Great Lakes and the Hudson Bay drainages of Canada (Harkness and Dymond, 1961; Scott and Crossman, 1973). They were once abundant within their natural ranges, but due to overfishing and restricted movement from dams and navigation locks built in the1800s and early 1900s, populations were decimated (Thuemler, 1988; Auer, 1999). Currently, few healthy populations of lake sturgeon exist, in part due to the time required for sexual maturation (approximately 15-20 years), the associated low rates of recruitment, and further habitat destruction (Harkness and Dymond, 1961; Thuemler, 1988; Peterson et al., 2007). Conservation and restoration efforts have been hampered by ongoing anthropogenic disturbances including the destruction of historic spawning grounds and critical down-stream habitats (Auer, 1996; Wilson and McKinley, 2004).

Within the Laurentian Great Lakes, lake sturgeon have been identified in 57 different rivers during sea lamprey control operations, with historical evidence of lake sturgeon presence in 40 additional tributaries. Of these streams, 72 are known to have had an infestation of the larval sea lamprey (*Petromyzon marinus*), an invasive species which has plagued the Great Lakes since the early 20th century (O'Connor et al., 2017). Larval sea lamprey spend this life stage burrowed in the substrate of streams as filter feeding ammocoetes, before undergoing a profound metamorphosis into parasitic juveniles that parasitize/prey on teleost fishes after migrating into the Great Lakes (Farmer, 1980; Youson, 2003). The presence of sea lamprey in the Great Lakes subsequently led to the decimation of economically important fisheries for both Canada and the United States, and since then various methods of control have been implemented to reduce their populations (Applegate, 1950; Smith et al., 1974; Heinrich et al., 1980; Smith and Tibbles, 1980;

Great Lakes Fisheries Commission, 2011). The most efficient sea lamprey control method to date has been the use of lampricides (selective sea lamprey piscicides) such as 3-trifluoromethyl-4-nitrophenol (TFM; McDonald and Kolar, 2007). Recently, however, there has been growing concern over the influence that this compound might have on non-target fish, including the lake sturgeon (Christie and Goddard, 2003).

Lake sturgeon are particularly sensitive to TFM when below a length of 10cm (Boogaard et al., 2003; McDonald and Kolar, 2007). However, they are also believed to be more vulnerable to TFM toxicity in waters of higher alkalinity, in contrast to observations that have been made in other fish species exposed to TFM in waters of higher alkalinity (Bills et al. 2003; Pratt et al. 2013; O'Conner et al., 2017). The reason for the lake sturgeon's greater TFM sensitivity in these early life stages has not yet been elucidated. One possibility is that due to their small size, fingerling lake sturgeon would have greater metabolic rates resulting in greater uptake of TFM. Indeed, Tessier et al. (2018) demonstrated that like other fishes, rates of oxygen uptake by larval sea lamprey scaled inversely with body size in an allometric fashion (Goolish, 1991), as well as TFM uptake. A second possibility is that smaller, presumably younger, sturgeon lack a sufficient capacity to detoxify TFM in the first year of life, making them more vulnerable to TFM than in later life stages.

The greater sensitivity of lake sturgeon to TFM in waters of high alkalinity is less clear. It may be due to the use of quasitidal flow during respiration (Burggren, 1978), which could alter patterns of gas exchange as compared to a unidirectional buccal-opercular flow, which is typically used by most fish. Quasitidal flow draws water into the opercular chamber through the

dorso-lateral opercular slit when the mouth is closed or occluded, the opercular valve is closed, and the buccal cavity drops to create pressure. As the buccal cavity rises, the mouth remains closed or occluded, and the opercular valve opens to allow water to exit. During this process, water crosses the gill sieve dorsally, in a posterior direction, before crossing the ventral portion of the gi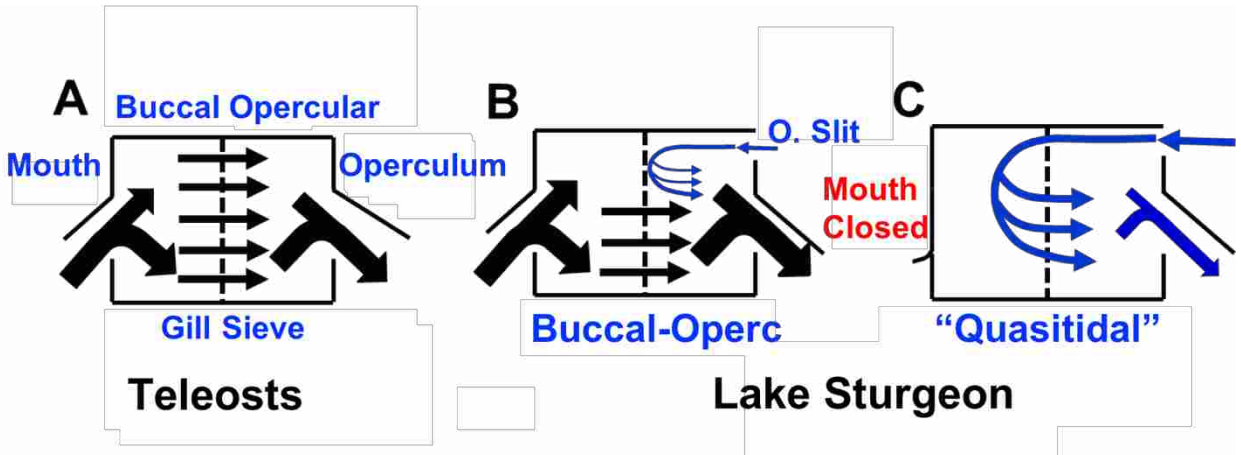ll sieve in an anterior-posterior direction and exiting (Burrgen, 1978). The recirculation of water within the operculum may allow for the accumulation of acidic by-products (such as H^+ or CO_2) from acid-base regulation and respiration at the gills, creating a more acidic medium at the site of TFM uptake. With a lower gill micro-environment pH, a greater proportion of the phenolic form of TFM would be present, which is more lipophilic and would pass across the gill epithelia with greater ease, increasing the overall uptake of TFM (Figure 2-1; Applegate et al., 1961; Hunn and Allen, 1974; McDonald and Kolar, 2007; Hlina et al., 2017).

Increased water alkalinity reduces the uptake of TFM and other chlorinated phenols by rainbow trout (*Oncorhynchus mykiss*; Hunn and Allen 1974; Erickson et al., 2006a,b), likely because at higher alkalinity the buffering capacity of the water would be higher which would decrease the amount of acidification that takes place in the gill microenvironment, effectively increasing the pH in that region (Erickson et al., 2006a,b). As a result, the proportion of un-ionized TFM in the gill microenvironment would be lower, resulting in lower TFM uptake and toxicity. Indeed, alkalinity has been shown to reduce the toxicity of TFM for sea lamprey and most non-target fish (Bills et al, 2003), but the protective effects of higher alkalinity have been questioned in juvenile lake sturgeon (Pratt et al., 2003, O'Connor et al., 2017). To resolve this issue, a better understanding of how pH and alkalinity influence the uptake of TFM in juvenile sturgeon is required.

The goal of this study was to determine how variation in water pH and alkalinity influences TFM accumulation by juvenile sturgeon. Accordingly, the radio-tracer ^{14}C-TFM was used to measure rates of TFM uptake in waters of low, moderate and high alkalinity (50, 150 and 250 mg $CaCO_3$ L^{-1}, respectively) and pH (pH 6.5, 8.0 and 9.0) in young of the year (YOY; < 100 mm in length), and older (1+; > 130 mm in length) lake sturgeon. It was predicted that rates of TFM uptake would be reduced in higher alkalinity water, due to a reduced ability of the sturgeon to acidify the gill micro-environment in this more highly buffered medium. It was also hypothesized that TFM uptake would be inversely related to water pH, due to differences in the proportion of the phenolic (un-ionized) compound at different water pHs. Finally, based on the known allometric relationships between body size and metabolic rate in sturgeon (Allen and Cech, 2007; Svendsen et al., 2014), I tested the hypothesis that TFM uptake rates would be greater in the YOY versus the larger 1+ animals.

Materials and Methods

Experimental Animals and Set-up

Two age groups of lake sturgeon, young of the year (YOY; N=150; length < 100 mm, with a mean of 76 ± 1 mm; mass 1.4 ± 0.1 g) and greater than 1 year old (1+; N=150; length > 115 mm with a mean of 136 ± 1 mm; mass $= 8.5 \pm 0.01$ g) were reared from the eyed-egg stage at the Alma Research Station (University of Guelph, Alma, ON, Canada). The eggs were collected from wild fish in the spring of 2016, and were fertilized at the Sustainable Sturgeon Culture Centre, Elmo, ON (Courtesy of Joe Hunter, Rainy River First Nation) and transported by plane to Pearson International Airport, Toronto, ON, and then to the Alma Research Station, Elora, ON. Prior to experimentation, the juvenile sturgeon were transported from the research station to Wilfrid Laurier University, Waterloo, ON in the fall of 2016 (YOY), and the summer of 2017 (1+) and housed for 2 weeks in an approximately 600 L G-Hab system (Pentair Aquatic Eco-Systems, Apopka, FL, U.S.A.), filled with recirculating dechlorinated City of Waterloo tap water (pH ~ 8.0; titratable alkalinity ~ 250 mg $CaCO_3$ L^{-1}; hardness ~ 350 mg $CaCO_3$ L^{-1}; temperature ~ 14-16 °C). The water replacement rate was 10% of the total volume per day, and the system was equipped with biological, mechanical and UV filtration.

Fish were housed in the dark and fed daily with commercially-available blood worms (larval Chironimids; San Francisco Bay Brand, Inc., Newark, CA, U.S.A.) at 2% of their body mass. All fish were fasted for 72 h prior to experiments to minimize fouling of the water due to defecation and reduce the accumulation of excreted ammonia (Wood, 2001). All experiments and fish husbandry followed the Canadian Council of Animal Care guidelines, and were approved by the Wilfrid Laurier University Animal Care Committee.

Experimental Procedures

Acclimation to Different Water Alkalinity and pH

Subsets of both age groups of lake sturgeon were chronically exposed to water of a set alkalinity and pH for a minimum of one week prior to TFM exposures (Table 2-1). Acclimation to the appropriate alkalinities or pH was carried out using 37 L glass aquaria (N=25 per aquaria), contained within a flow-through system containing reconstituted water of the appropriate chemistry and temperature. Water was produced daily in 400 L batches following the methods set out by the American Public Health Association (APHA; 1989) and the American Society of Testing Materials (ASTM, 2000). $CaSO_4$, KCl and $MgSO_4$ were added to reverse osmosis water to maintain the same water hardness (101.1 ± 2.6 mg/L $CaCO_3$) in experiments, and $NaHCO_3$ was added to achieve desired alkalinities. Diluted HCl (0.5 – 2 N) and NaOH (0.5 – 2 N) were added to reach desired pH. Each aquarium received sufficient aeration to ensure dissolved oxygen (D.O.) was never less than 90% saturation (D.O. = 93.8 ± 0.4%), and each possessed activated carbon and biological filtration systems. Water composition was confirmed twice per day for alkalinity and pH, respectively using commercial kits (Hach, Alkalinity Test Kit, Model AL-AP, Hach Canada, Mississauga, ON) and a handheld pH meter (pH 11 meter, Oakton Instruments, IL, U.S.A.). Ca^{2+} and Mg^{2+} were measured using flame atomic absorption spectroscopy (AAS, PinAAcle 900T, Perkin Elmer, Waltham, MA, U.S.A.)

Experimental Protocols

Effects of pH and Alkalinity on TFM Uptake

Rates of TFM uptake were measured using ^{14}C-TFM, based on methods described by Blewett et al. (2013) and by Tessier et al. (2018). One day prior to each set of experiments, lake

sturgeon (N=10) were transferred to darkened, rectangular flux-chambers (dimensions = 6 x 10 x 14 cm ; volume = 750 mL; N = 1 sturgeon per container) receiving water of the appropriate alkalinity or pH (Table 2-2). The next morning, water flow was cut-off to each container, the volume adjusted to exactly 750 mL, followed by the addition of ^{14}C-TFM ("hot" TFM; provided courtesy of T. Hubert, Upper Midwest Environmental Sciences Center, U-S Geological Survey, LaCrosse, WI, U.S.A.; DuPont/New England Nuclear, Delaware, U.S.A.) and non-radioactive TFM ("cold" TFM; 35% active ingredient in isopropanol; provided courtesy of the Deparment of Fisheries and Oceans Canada; Clariant SFC GMBH WERK, Griesheim, Germany) to meet target concentrations of 2.5, 5.0 or 10 mg L^{-1} of TFM (both hot and cold), with a mean specific activity (MSA) of 110.8 ± 0.3 CPM $nmol^{-1}$ TFM. For the sturgeon acclimated to low pH (~ 6.5), however, nominal TFM concentrations were 1.0, 2.5 and 5.0 mg L^{-1} to prevent lethal exposure, because 10 mg L^{1} would be 20-fold greater than the 12 h $LC_{99.9}$ for juvenile sea lamprey (0.5 mg L^{-1}) in similar water pH (6.5) and alkalinity (150 mg $CaCO_3$ L^{-1}; Bills et al., 2003), and likely lethal to the sturgeon. The nominal concentration range for TFM of $1.0 - 10$ mg L^{-1} was selected to best represent the span of concentrations that are used during TFM applications to streams in the Great Lakes (Bills et al., 2003; McDonald and Kolar, 2007). Three different concentrations of TFM (low, medium and high) for each treatment were used to determine if TFM uptake was dose dependent under the different water chemistry conditions. Following a 15 min mixing period, water samples were collected at 0 h and 1 h for determination of cold TFM concentration, and ^{14}C-TFM radioactivity, to determine the MSA of TFM during the experiment. Preliminary experiments had shown that a 1 h exposure prevented TFM-induced mortality at higher TFM concentrations, and that this was the optimum exposure time for sufficient ^{14}C-TFM to accumulate in the fish. Immediately following the 1 h water sample, the fish were euthanized

with an overdose of tricaine methanesulfonate (1.5 g L^{-1} buffered in 3.0 g L^{-1} NaHCO$_3$; TMS; Syndel Labs, Port Alberni, BC, Canada) buffered with NaHCO$_3$ (3.0 g L^{-1}), and the whole bodies were removed and washed in concentrated, non-radioactive ("cold") TFM (10 mg L^{-1}) to remove residual radioactive ("hot") TFM from the body surface, followed by a rinse in deionized water. Measurements of body mass and the standard length of each fish were then measured, before transferring each animal to individual, 50 mL polypropylene centrifuge tubes (Conical Centrifuge Tubes, Corning Falcon™, NY, USA), followed by subsequent processing for whole body radioactivity measurements.

Effects of Excretion on the Rates of TFM Uptake

Despite the relatively short ^{14}C-TFM exposure period, it was necessary to determine if simultaneous clearance of ^{14}C-TFM labelled TFM or its metabolites by the fish resulted in possible underestimates of TFM uptake rates. Accordingly, the fish were treated with a known inhibitor of the process of glucuronidation, salicylamide, as glucuronidation is thought to be the primary method of TFM detoxification and clearance in non-target fish (Lech 1974; Lech and Statham 1975) and lake sturgeon (LeClair 2014, Bussy et al., 2018a). These sturgeon (length = 96.3 ± 0.8 mm; mass = 3.1 ± 0.1g) were acclimated for one week to moderate pH (8.14 ± 0.03) and alkalinity (150 ± 1 mg CaCO$_3$ L^{-1}), before N=10 sturgeon were exposed to 25 mg L^{-1} salicylamide for 2 h prior to measuring TFM uptake using the methods described above (^{14}C labelled TFM exposure concentration = 8.9 mg L^{-1}). TFM uptake was also measured in a group of simultaneous control fish (N=10) that were acclimated and exposed to TFM under the same conditions, without being exposed to salicylamide. It was predicted that if TFM uptake measures were underestimated due to excretion of ^{14}C-TFM via the gastrointestinal tract or renal routes,

then TFM uptake rates would be higher following salicylamide treatment. Passive diffusion of TFM across the gills would not be expected, as there would be a large inwardly directed TFM gradient during the first hour of TFM exposure.

Analytical Methods

Whole Body Beta-Radioactivity Measurements

Immediately after rinsing the animals in "cold" TFM and deionized water, the carcasses (whole bodies) of each lake sturgeon were digested in ten times their body mass of 1 mol L^{-1} HNO_3 within individual 50 mL centrifuge tubes, for 2 d at 60 °C. The digested samples were intermittently vortexed during the digestion process, followed by centrifugation for five minutes at 1228 x g when the process was complete. At this time, 2 mL of the resulting supernatant was added to 4 mL of Ultima Gold ™ AB organic scintillation cocktail (PerkinElmer, MA, U.S.A.; in duplicate), and left overnight in the dark to minimize chemiluminescence, prior to measuring the beta radioactivity on a scintillation counter (LC 6500 Multi-Purpose Scintillation Counter, Beckman Coulter, CA, USA). Rates of uptake were calculated according to Tessier and Wilkie (2018) using the following equation:

$$\text{TFM Uptake Rate} = CPM_{sturgeon}/(MSA \times \Delta T) \tag{1}$$

where uptake is measured in nmol g^{-1} h^{-1}, CPM is the total whole-body radioactivity in counts per minute of ^{14}C-TFM g^{-1} body mass, MSA is the specific activity of the water samples (CPM $nmol^{-1}$ of TFM), and ΔT is the time of exposure (h).

Statistical Analyses

All data are presented as the mean ± 1 standard error of the mean (SEM). Data were assessed for normality using the Shapiro-Wilk test, and for homogeneity of variance using the Levene test, where appropriate. Rates of uptake based on water alkalinity or pH, and TFM concentration were analyzed using a two-way ANOVA, followed by a Tukey Honest Significant Difference post-hoc test where appropriate. When the data did not meet the normality and homoscedasticity assumptions for ANOVA following $\log_{10}$ or power transformations, a Kruskal-Wallis rank sum test followed by a Dunn's multiple comparison test were used. A student's t-test was used to analyze the rates of TFM uptake between YOY and 1+ sturgeon for each exposure treatment. For all statistical tests, the significance was set at the $P < 0.05$ level, and all statistical analyses were completed using SPSS 14.0 (SPSS, Inc., Chicago, IL, U.S.A.).

Results

Effects of Salicylamide Treatment on the Measured Rates of TFM Uptake

Juvenile sturgeon exposed to salicylamide prior to ^{14}C-TFM exposure had an uptake rate of 11.75 ± 0.43 nmol g^{-1} h^{-1} measured over 1 hour which was not significantly different (P = 0.707) from the rate of uptake observed for control fish without salicylamide exposure, which was 10.93 ± 0.62 nmol g^{-1} h^{-1} (data not shown).

Effects of pH on Rates of TFM Uptake for YOY and 1+ Lake Sturgeon

Water pH had a pronounced effect on rates of TFM uptake in both YOY and 1+ lake sturgeon. In YOY sturgeon, TFM uptake at all concentrations of TFM tested was greatest at low pH (measured pH = 6.50 ± 0.02) and significantly greater than the corresponding measurements made at moderate pH (measured pH = 8.19 ± 0.01; P $\leq$ 0.001) and at high pH (measured pH = 9.03 ± 0.01; P $\leq$ 0.001; Figure 2-2A). The greatest rate of uptake for the YOY sturgeon was 59.5 ± 3.28 nmol g^{-1} h^{1}, observed at the highest TFM concentration (4.6 ± 0.1 mg L^{-1}) in low pH water, which was 15-fold greater than the rate of uptake observed for the high pH treatment (4.0 ± 0.3 nmol g^{-1} h^{-1}; pH 9.03 ± 0.02; P $\leq$ 0.001), at a similar TFM concentration. It was also notable, that TFM uptake was dose dependent, increasing with TFM concentration in the moderate and high pH acclimated fish (P $\leq$ 0.009; Figure 2-2A). Although TFM uptake increased with exposure concentration between 0.8 and 2.2 mg L^{-1} in the low pH acclimated fish (P $\leq$ 0.001), the differences measured between 2.2 and 4.6 mg L^{-1} TFM were not significantly different (P = 0.961; Figure 2-2A).

Similar trends were observed in 1+ lake sturgeon, in which TFM uptake rates were greatest in the fish acclimated to low pH (measured pH = 6.53 ± 0.01), with the greatest rate of uptake (18.50 ± 1.11 nmol g^{-1} h^{-1}) measured when the fish were exposed to a TFM concentration of 5.2 mg L^{-1}. Rates were significantly lower in the fish acclimated to moderate (measured pH = 8.19 ± 0.01; P ≤ 0.001) and high pH (measured pH = 8.97 ± 0.01; P ≤ 0.001), with TFM uptake rates that were about 50-90% lower than in the low pH condition (Figure 2-2B). TFM uptake was also dose dependent in this group, significantly increasing with TFM concentration at three pHs (P ≤ 0.035; Figure 2-2B). Compared to the smaller, YOY sturgeon, the rates of TFM uptake measured at moderate and high pH were 50-75% lower in the larger, 1+ animals (P ≤ 0.001; Figure 2-2A,B).

Effects of Alkalinity on the Rates of TFM Uptake for YOY and 1+ Lake Sturgeon

The rate of TFM uptake was also markedly influenced by alkalinity, and more so in the YOY lake sturgeon than in the 1+ animals. Again, TFM uptake was dose dependent, increasing with TFM concentration at all three alkalinities tested, but the response was most pronounced in the fish acclimated to low alkalinity, where TFM uptake significantly increased with each step-up in TFM concentration (P ≤ 0.012; Figure 2-3A); at moderate and high alkalinity rates of uptake were only significantly different between lowest and highest TFM concentrations examined at each alkalinity (P ≤ 0.001; Figure 2-3A). The rates of uptake between the lowest and highest TFM concentrations was not statistically significant in the moderate alkalinity (P = 0.084) and high alkalinity (P = 1.000) treatments.

As with the YOY sturgeon, uptake rates were highest in 1+ animals exposed to TFM in waters of low alkalinity (measured alkalinity = 52 ± 1) at a TFM concentration of 9.1 ± 0.2 mg L^{-1}. This rate of uptake was 10-fold higher than the rates observed at a similar TFM concentration in waters of high alkalinity (measured alkalinity = 252 ± 1 mg CaCO$_3$ L^{-1}; P ≤ 0.001) in which the uptake rates averaged 1.61 ± 0.14 nmol g^{-1} h^{-1} (Figure 2-3B). TFM uptake was also dose dependent in the 1+ lake sturgeon, at all three alkalinities tested (P ≤ 0.016; Figure 2-3B). However, it should be emphasized that even at the highest exposure concentration of TFM (9.4 ± 0.1 mg L^{-1}), TFM uptake rates in high alkalinity water were very low, just above the levels of detection, as compared to low and moderate alkalinity treatment groups (Figure 2-3 B). At all three alkalinities, the rates of TFM uptake were substantially lower, by 40-80% at comparable TFM concentrations, in the larger 1+ animals (P ≤ 0.001; Figure 2-3A,B).

Discussion:

Effects of Age-Class on Rates of TFM Uptake and Sensitivity

The sensitivity of lake sturgeon to TFM is of growing concern in the Great Lakes, particularly for YOY fish that are less than 100 mm in length (Boogaard et al. 2003; McDonald and Kolar 2007; O'Connor et al., 2017). The present study demonstrates that the greater TFM sensitivity of these fish compared to larger, 1+ sturgeon, may be related to their much higher rates of TFM uptake. This is probably a consequence of the much smaller masses of the YOY sturgeon compared to their larger, 1+ counterparts. Like numerous other fish species, rates of oxygen consumption are inversely proportional to body mass in sturgeon, with scaling co-efficients in lake sturgeon and green sturgeon (*Acipenser medirostris*) falling between 0.9-1.0 (Allen and Cech, 2007; Svendsen et al., 2014). Thus, the higher rates of TFM uptake in the smaller YOY lake sturgeon were likely a result of higher rates of gill ventilation due to greater oxygen demands. Tessier et al. (2018) recently demonstrated that TFM uptake by larval sea lamprey was correlated with routine rates of oxygen consumption, which also scaled inversely with body size. Future experiments should use similar approaches to examine the possible relationships between TFM uptake and oxygen consumption in the lake sturgeon, to shed more light on how life stage dependent differences in TFM sensitivity are related to respiratory demands.

The present findings suggest that the greater sensitivity of YOY compared to 1+ lake sturgeon to TFM is clearly related to greater rates of TFM uptake by the smaller, younger fish, but this may not be the only contributing factor. Although lake sturgeon are known to clear TFM following glucuronidation (LeClair, 2014), salicylamide-exposed sturgeon did not have rates of

TFM uptake that were significantly different than controls, suggesting that clearance did not influence the rates of uptake measured during the TFM exposures. However, the greater sensitivity of YOY sturgeon to TFM could be related to a lower, relative capacity to detoxify TFM. Sturgeon have been shown to detoxify TFM using Phase II biotransformation, which includes the conjugation of TFM to TFM-glucuronide using glucuronidation (LeClair, 2014; Bussy et al., 2018a), as observed in sea lamprey, channel catfish (*Ictalurus punctatus*), bluegill (*Lepomis macrochirus*) and rainbow trout (Olson and Marking, 1973; Lech, 1974; Kane et al., 1994; Lech and Statham, 1975; Bussy et al. 2018a,b). However, the capacity of sturgeon to utilize uridine diphosphate glucuronyltransferase (UDPGT) as compared to TFM tolerant species, such as the rainbow trout or bluegill (Kane et al., 1994), is not known. Nor is much known about how life stage affects Phase II detoxification pathways in lake sturgeon.

In many humans and rats, drug detoxification capacities in the liver lag behind many other developmental processes in neonates, infants and juveniles (children; Saghir et al., 2012), but less is known about ontogeny and xenobiotic metabolism in fish. Kane et al., (1993), studying why the sensitivity of bullfrog (*Lithobates catesbeianus*) larvae (pre-metamorphic tadpoles) to TFM was greater than in adult frogs, examined the enzyme kinetics of UDPGT in response to TFM using liver microsomes from each life stage. They noted that the respective Vmax (maximal enzyme activity) of UDPGT was less in larvae (pre-metamorphic tadpoles) compared to adult frogs, but that the Km (inverse of UDPGT affinity for TFM) was less in the larvae. As a result, the Vmax/Km ratio, an index of enzyme efficiency, was similar in both groups, suggesting that the relative detoxification capacity of the two life-stages was the same. Similar UDPGT kinetics studies, combined with gene expression studies, in developing lake

sturgeon could prove to be of value in determining whether or not the greater vulnerability of YOY sturgeon is also influenced by differences in their capacity to detoxify and excrete TFM.

The Effect of pH on TFM Uptake by Juvenile Lake Sturgeon

As expected, TFM uptake was inversely related to water pH in both YOY and 1+ animals, and also to alkalinity. The reductions in TFM uptake with increasing pH, observed in both YOY and 1+ animals, were likely a direct result of pH-dependent effects on the speciation of TFM. With a pK_a of approximately 6.07 (Hubert, 2003), the amount of TFM in its un-ionized (phenolic) form and its ionized (phenolate ion) form, changes with water pH (Hunn and Allen, 1974; McDonald and Kolar, 2007; Hlina et al., 2017). As a weak acid, a greater proportion of TFM is in its un-ionized, more lipophilic form at lower pHs than at higher pHs (Figure 2-1), leading to greater rates of uptake via passive diffusion across the gills (Hunn and Allen, 1974).

Significantly greater rates of TFM uptake for juvenile sturgeon exposed to TFM in water of lower pH (6.5) compared to more alkaline pH (9.0) support the original hypothesis that TFM is primarily taken-up in its un-ionized (phenolic) form (Hunn and Allen, 1974; McDonald and Kolar, 2007; Hlina et al., 2017). This finding is consistent with well-established observations that the toxicity of TFM is greater in lamprey and non-target fishes, such as sturgeon, at lower pH (Marking and Olson, 1975; Bills et al., 2003; Boogaard et al., 2003).

Hlina et al. (2017), using ^{14}C-TFM to determine the rates of TFM uptake in larval sea lamprey, calculated the respective amounts of TFM present in its un-ionized (phenolic; TFM-OH) and ionized (TFM-O$^-$) states at different pHs using the Henderson-Hasselbalch equation.

The concentration of TFM (4.2 mg L^{-1}) and the pH of the exposure mediums (6.86, 7.96 and 8.78) were comparable to those used in the current study (nominal TFM concentration of 5.0 mg L^{-1}; pH of exposure mediums ~ 6.5, 8.0 and 9.0). It was determined that at the lower water pH of 6.86, 13.7% of the total TFM present in the medium was in its un-ionized state ([TFM OH] = 2.71 nmol TFM mL^{-1}; [Total TFM] = 19.85 nmol TFM mL^{-1}), whereas in the higher water pH of 8.74, the amount of TFM in its un-ionized state was more than 98% lower ([TFM OH] = 0.05 nmol TFM mL^{-1}; [Total TFM] = 21.10 nmol TFM mL^{-1}). Because TFM uptake continued, despite the near absence of un-ionized TFM in the water, they argued that at least some TFM is likely taken-up in the ionized form as well, contrary to prior assumptions suggesting that TFM is taken up only in its un-ionized state (Hunn and Allen, 1974; Marking and Hogan, 1967). However, they failed to address events that might be taking place in the microenvironment of the gills, where the acidification of water crossing the gills could increase the proportion of un-ionized TFM and enhance the uptake of TFM at higher water pH. Thus, calculations of TFM speciation based on bulk-water pH measurements may actually underestimate the bioavailability of TFM, by underestimating the actual concentration of un-ionized TFM in the gill microenvironment.

A mechanistic model produced by Erickson et al. (2006a) demonstrated that excretory products from the gills (H^+, CO_2) affect the relative abundance of chlorinated phenols in their neutral and ionized states in the gill microenvironment, as compared to that of the bulk water. This finding was further explained in a companion study (Erickson et al., 2006b), in which they assessed the influence of exposure-water pH on the uptake of nine weakly acidic, chlorinated phenols, and a nonionizable control (1,2,4-trichlorobenzene), by rainbow trout. The results of the

model were tested by increasing the alkalinity of the exposure-medium to increase HCO_3^- and CO_3^{2-} buffering at the gills. The addition of buffer reduced the pH change in expired *vs.* respired water from 1.1 to 0.4 units and also resulted in lower rates of uptake for the phenols tested (Erickson et al., 2006b), corresponding to model predictions (Erickson et al., 2005). However, reductions observed in the rates of uptake of the nine phenols due to increased pH were less than what would be predicted based on the modeled changes in speciation alone, suggesting that ionized compounds may also be taken-up across the gills (Erickson et al., 2006). One possible explanation for this finding is that ionized phenols, including TFM, may be transferred via ion transport proteins located on the gills. Organic anion transporters, such as Mrp2 protein (multi-drug resistance-associated protein 2), could be a candidate, with genes identified in the gills, liver and other tissues of larval and adult sea lamprey, where it is thought to be an anion transporter in bile salt homeostasis (Cai et al., 2013).

Effects of Water Alkalinity on TFM Uptake

Water alkalinity also inversely affected rates of TFM uptake, but the effects were far more pronounced in the YOY lake sturgeon than in the 1+ animals. In the YOY animals, an increase in alkalinity from approximately 50 to 150 mg $CaCO_3$ L^{-1} resulted in marked reductions in TFM uptake, with absolute decreases ranging from approximately 32-40 nmol $g^{-1}h^{-1}$ at the highest TFM concentration (nominal = 10 mg L^{-1}), whereas the absolute decreases in TFM uptake were only one tenth to one quarter of these values in the 1+ animals. That the step from moderate to high alkalinity water only reduced TFM uptake slightly suggests that any additional interactions affecting TFM uptake and likely TFM toxicity, would be limited above 150 mg $CaCO_3$ L^{-1}.

The present findings suggest that alkalinity indirectly effects TFM speciation by altering the buffering capacity of the water. At lower alkalinity, the bicarbonate buffering capacity of the water is much less than at higher alkalinity. This can indirectly affect TFM speciation by directly influencing how much the water crossing the gills is acidified by H^+ excretion via V-ATPase proteins (proton pumps) known to be present in fish gills (Lin and Randall, 1995; Evans et al., 2005) and by the hydration of CO_2 expired across the gills (Wright et al., 1986; Conley and Mallatt, 1988; Rahim et al., 1988; Playle and Wood, 1989; Erickson et al., 2006a). In lower alkalinity water, H^+ and CO_2 excretion will tend to acidify this expired water more, leading to a lower water pH in the gill microenvironment, and therefore converting more TFM to its un-ionized form. In contrast, at higher alkalinities, the acidification will be less pronounced due to the higher buffering capacity of the water, resulting in less un-ionized TFM and lower uptake. In smaller, YOY fishes with higher metabolic rates, the amount of acidification of the gill microenvironment may be more pronounced due to higher mass specific H^+ and CO_2 excretion rates, leading to much higher TFM uptake at low alkalinity. However, when these processes are buffered at higher alkalinities it is likely less acidification results, leading to less TFM uptake. Thus, the hypothesis of Hlina et al. (2017; above) that at least some TFM uptake occurs in its ionized form could have some merit.

The present experiments are in agreement with previous studies, in which higher alkalinity resulted in lower levels of uptake for various chlorinated phenols by rainbow trout (Erickson et al., 2006b). Erickson et al. (2006b) reported that increased water alkalinity caused a reduction in the difference between the pH of inspired and expired water at the gill epithelium,

which likely decreased the proportion of the un-ionized state of the chlorinated phenols studied, reducing passive diffusion through the gills. Similar processes likely explain why the toxicity of TFM, a fluorinated phenol, is also strongly correlated with changes in pH ($pK_a = 6.07$; Hubert, 2003; Figure 2-1), due to the greater presence of the un-ionized, phenolic TFM in more acidic mediums, as compared to the phenolate form at higher pH (Bills et al., 2003; McDonald and Kolar, 2007).

Relevance to Sea Lamprey Control

The present study provides evidence that the greater sensitivity to TFM of YOY lake sturgeon less than 100 mm in length (Boogaard et al., 2003) results from the higher mass specific rates of TFM uptake compared to their older (1+) and larger counterparts. Moreover, water chemistry significantly impacts the rates at which lake sturgeon take up TFM, with the highest rates of uptake in lower pH waters, as previously reported in rainbow trout, catfish and sea lamprey (Hunn and Allen, 1974; Hlina, 2017), with the greatest toxicity taking place under these conditions (Boogaard et al., 2003; Bills et al., 2003; McDonald and Kolar, 2007; O'Connor et al., 2017).

The effects of alkalinity are not as clear-cut, however, with increases from low (50 mg L^{-1} CaCO$_3$) to moderate alkalinities (~ 150 mg L^{-1} as CaCO$_3$) resulting in marked reductions in TFM uptake, but further increases in alkalinity having little effect on TFM uptake. At first glance, these findings appear to be at odds with those reported by O'Connor et al. (2017), who reported that the lake sturgeon experienced increased mortality as alkalinity increased when exposed to the 1.4 times the MLC of TFM to sea lamprey. However, it should be noted that the

lake sturgeon in that study were in fact exposed to 1.4 times the MLC of sea lamprey as calculated from the pH-alkalinity tables used to determine TFM application amounts in the field (Bills et al., 2003). In other words, the lake sturgeon were exposed to higher concentrations of TFM as alkalinity increased. According, to the pH-alkalinity-TFM toxicity tables published by Bills et al. (2003), the MLC of lamprey increases with alkalinity at pH 8.1 (Bills et al., 2003). In lake sturgeon, TFM toxicity also decreases with alkalinity, but only in low to moderate levels (< 85 mg L^{-1} as $CaCO_3$ at pH 7.7); further increases in alkalinity have little effect on TFM sensitivity (Johnson et al., 1999). Thus, it is proposed that as alkalinity increases to levels beyond a certain threshold, that the sensitivity of lamprey to TFM continues to decrease, but not that of lake sturgeon, increasing the sturgeon's vulnerability to the lampricide. This is supported by the present results, which demonstrated that further reductions in TFM uptake were either absent or minimal, when alkalinity was increased from 150 and 250 mg L^{-1} $CaCO_3$.

The present findings could have important implications for reducing or eliminating the possible influence of sea lamprey control efforts on lake sturgeon population restoration efforts in the Great Lakes. Lake sturgeon fecundity is low and sexual maturation occurs in females between the ages of 18-27, with females spawning every 4-9 years (Harkness and Dymond, 1961; Fortin et al., 1993; Peterson et al., 2007). Thus, reducing the mortality of juvenile lake sturgeon could enhance sturgeon recovery efforts within the Great Lakes (Sutton et al, 2004; Velez-Espino and Koops, 2008). Although reducing the concentration of TFM during applications or waiting for a TFM-tolerable size allow for the survival of lake sturgeon juveniles, damage to fisheries caused by increased numbers of parasitic sea lamprey could be an adverse effect of reducing TFM application concentrations or delaying treatments. Further, lamprey

attacks on lake sturgeon conducted within laboratory tests have been shown to reduce growth and condition factors, as well as cause lethality (Patrick et al., 2009; Sepulveda et al., 2013), and attacks have been predicted to reduce population abundance and reproductive potential of lake sturgeon populations in the Great Lakes (Sutton et al., 2004).

An understanding of the implications that water chemistry and life stage have on the rates of TFM uptake by lake sturgeon, however, could provide information useful for modifying lampricide application protocols. For example, recent work by Birceanu (2017; unpublished) has shown that exposure of larval lamprey and juvenile lake sturgeon to lower TFM concentrations (24 h $LC_{99.9}$, instead of 12 h $LC_{99.9}$) for a longer duration (24 h instead of 9 h) significantly reduces sturgeon mortality, while maintaining 100% mortality of sea lamprey. The size dependent effects on uptake observed in the present study suggest that this approach would be best utilized when sturgeon are greater than 130 mm, when the rates of TFM uptake are comparably less than YOY sturgeon, and the protective effect of alkalinity is more apparent. Thus, for field applications, this "long and low" regimen would yield the best results in the late Fall, when sturgeon within a river are a sufficient size to withstand TFM exposure, or have already migrated to lower reaches of the stream or into the larger lakes where TFM concentrations would be diluted (Johnson et al., 1999).

Table 2-1. Measured water quality indices for Young of Year (YOY) and 1+ lake sturgeon during one week acclimations to set experimental treatment conditions.

Data presented as the mean ± standard error of the mean (SEM).

Age	Experimental Treatment	Alkalinity (mg CaCO$_3$ L^{-1})	pH	Temperature (°C)
YOY	Low Alkalinity	57 ± 1	8.00 ± 0.01	14.2 ± 0.2
YOY	Medium Alkalinity	152 ± 1	8.18 ± 0.02	14.3 ± 0.2
YOY	High Alkalinity	251 ± 1	8.37 ± 0.01	14.2 ± 0.1
YOY	Low pH	145 ± 2	6.57 ± 0.06	14.1 ± 0.3
YOY	Medium pH	152 ± 1	8.18 ± 0.02	14.3 ± 0.2
YOY	High pH	152 ± 1	8.95 ± 0.04	14.8 ± 0.2
1+	Low Alkalinity	51 ± 1	8.00 ± 0.01	14.8 ± 0.2
1+	Medium Alkalinity	152 ± 1	8.23 ± 0.03	14.7 ± 0.2
1+	High Alkalinity	250 ± 1	8.40 ± 0.01	14.2 ± 0.3
1+	Low pH	150 ± 1	6.51 ± 0.02	14.9 ± 0.3
1+	Medium pH	152 ± 1	8.23 ± 0.03	14.7 ± 0.2
1+	High pH	151 ± 1	8.91 ± 0.06	14.8 ± 0.1

Table 2-2. Measured water quality indices and size of young of the year (YOY) and one-year old (1+) lake sturgeon used to measure rates of TFM uptake.

Data presented as the mean ± standard error of the mean (SEM).

Age	Experimental Treatment	Alkalinity (mg CaCO$_3$ L^{-1})	pH	TFM (mg L^{-1})	Length (mm)	Mass (g)
YOY	Low Alkalinity	60	7.97 ± 0.00	2.5 ± 0.1	74.4 ± 1.3	1.1 ± 0.1
YOY	Low Alkalinity	60	7.99 ± 0.01	5.6 ± 0.2	73.2 ± 1.7	1.1 ± 0.1
YOY	Low Alkalinity	60	8.00 ± 0.00	10.4 ± 0.2	70.4 ± 1.7	1.0 ± 0.1
YOY	Medium Alkalinity	153	8.21 ± 0.02	3.6 ± 0.1	73.7 ± 2.7	1.4 ± 0.1
YOY	Medium Alkalinity	153	8.21 ± 0.01	6.7 ± 0.2	72.7 ± 1.1	1.3 ± 0.1
YOY	Medium Alkalinity	153	8.15 ± 0.03	13.5 ± 0.2	74.0 ± 1.7	1.5 ± 0.1
YOY	High Alkalinity	250	8.36 ± 0.00	2.7 ± 0.1	74.4 ± 1.3	1.3 ± 0.1
YOY	High Alkalinity	250	8.37 ± 0.00	5.9 ± 0.2	73.2 ± 1.7	1.0 ± 0.1
YOY	High Alkalinity	250	8.39 ± 0.00	10.7 ± 0.2	70.4 ± 1.7	1.0 ± 0.1
YOY	Low pH	152	6.49 ± 0.02	0.8 ± 0.0	79.9 ± 2.3	1.5 ± 0.1
YOY	Low pH	152	6.49 ± 0.02	2.2 ± 0.2	81.0 ± 2.8	1.8 ± 0.2
YOY	Low pH	152	6.50 ± 0.02	4.6 ± 0.1	81.0 ± 1.7	1.9 ± 0.1
YOY	High pH	152	9.05 ± 0.02	2.4 ± 0.2	81.2 ± 2.1	1.6 ± 0.1
YOY	High pH	152	9.03 ± 0.02	4.7 ± 0.1	82.1 ± 2.6	1.6 ± 0.2
YOY	High pH	152	9.00 ± 0.01	9.0 ± 0.6	79.5 ± 2.1	1.5 ± 0.1
1+	Low Alkalinity	52	8.01 ± 0.01	2.3 ± 0.1	137.1 ± 3.7	8.5 ± 0.6
1+	Low Alkalinity	52	8.00 ± 0.01	3.9 ± 0.1	134.4 ± 4.6	8.7 ± 0.9
1+	Low Alkalinity	52	8.00 ± 0.01	9.1 ± 0.2	143.6 ± 3.9	9.9 ± 0.6
1+	Medium pH	150	8.21 ± 0.01	2.5 ± 0.0	133.4 ± 1.3	7.6 ± 0.3
1+	Medium pH	150	8.17 ± 0.01	4.9 ± 0.0	132.3 ± 1.7	7.7 ± 0.2
1+	Medium pH	150	8.18 ± 0.00	9.9 ± 0.0	131.1 ± 1.7	7.7 ± 0.2
1+	High Alkalinity	255	8.39 ± 0.01	2.5 ± 0.0	140.3 ± 4.6	9.9 ± 1.0
1+	High Alkalinity	255	8.37 ± 0.02	5.1 ± 0.0	140 ± 3.7	9.3 ± 0.6
1+	High Alkalinity	255	8.41 ± 0.01	9.9 ± 0.0	133.8 ± 5.0	9.1 ± 1.2
1+	Low pH	153	6.55 ± 0.01	1.4 ± 0.00	140.7 ± 2.3	8.2 ± 0.3
1+	Low pH	153	6.54 ± 0.01	2.5 ± 0.00	136.0 ± 2.3	8.3 ± 0.4
1+	Low pH	153	6.50 ± 0.01	5.2 ± 0.00	135.5 ± 2.9	8.2 ± 0.4
1+	High pH	153	8.96 ± 0.01	2.5 ± 0.0	138.8 ± 3.1	8.3 ± 0.4
1+	High pH	153	8.98 ± 0.01	4.8 ± 0.0	135.4 ± 2.7	8.1 ± 0.2
1+	High pH	153	8.97 ± 0.01	10.0 ± 0.1	134.2 ± 2.8	8.2 ± 0.4

Figure 2-1. Structure and dissociation equilibrium of TFM (pK$_a$ = 6.07).

The un-ionized, phenolic form of TFM is on the left, which is more lipophilic and easily crosses the gills passively down a favorable water-to-blood gradient (Birceanu et al., 2009). The ionized, phenolate form of TFM is on the right, which as an anion would not be able to passively cross the gills (Hunn and Allen, 1974). In a more acidic medium (pH < 6.07), greater concentrations of the phenolic form exist, whereas in a more alkaline medium (pH > 6.07), greater concentrations of the phenolate form exist.
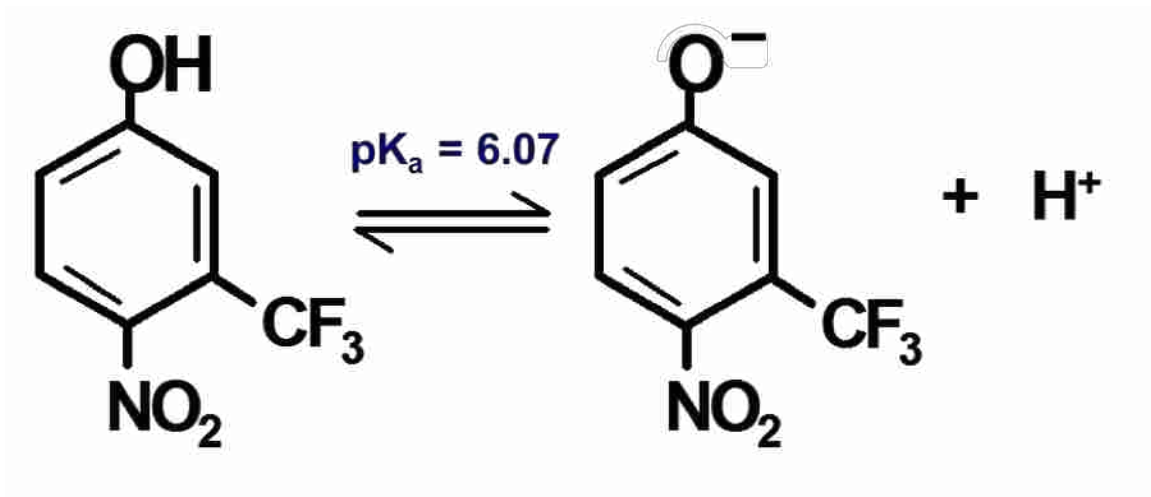
Figure 2-2. Effects of water pH and 3-trifluoromethyl-4-nitrophenol (TFM) concentrations on the rate of TFM uptake in young of the year (YOY) and one year old (1+) lake sturgeon. The rates of TFM uptake (nmol g^{-1} h^{-1}) for YOY lake sturgeon (A; ~9 month) and 1+ lake sturgeon (B; ~15 months) at different pH (actual values in Table 2-1) and TFM concentrations (mg L^{-1}). Uptake rates are reported as the mean (N = 7-10 sturgeon) ± 1 SEM. Upper case letters denote statistically significant differences among the rates of TFM uptake amongst the different pH treatments ($P < 0.05$). Measurements sharing the same lower-case letter within a given pH treatment, are not statistically significant from one another ($P < 0.05$).
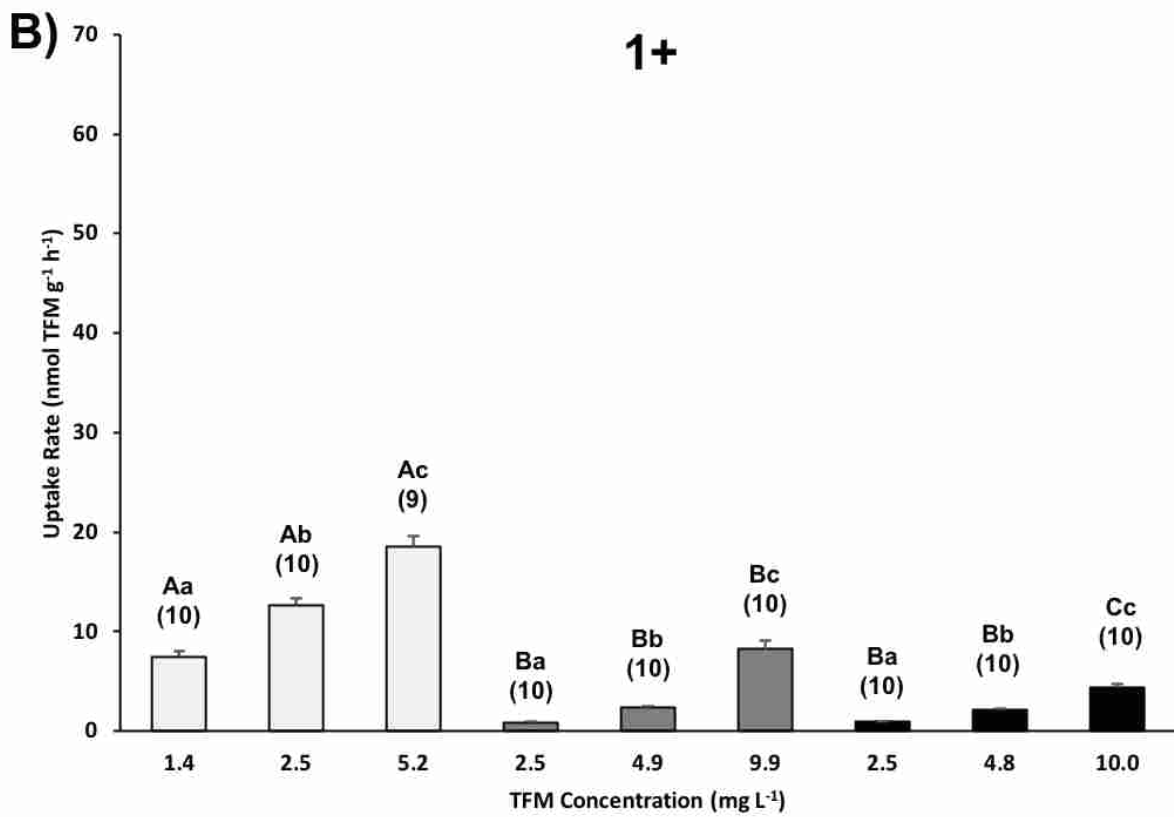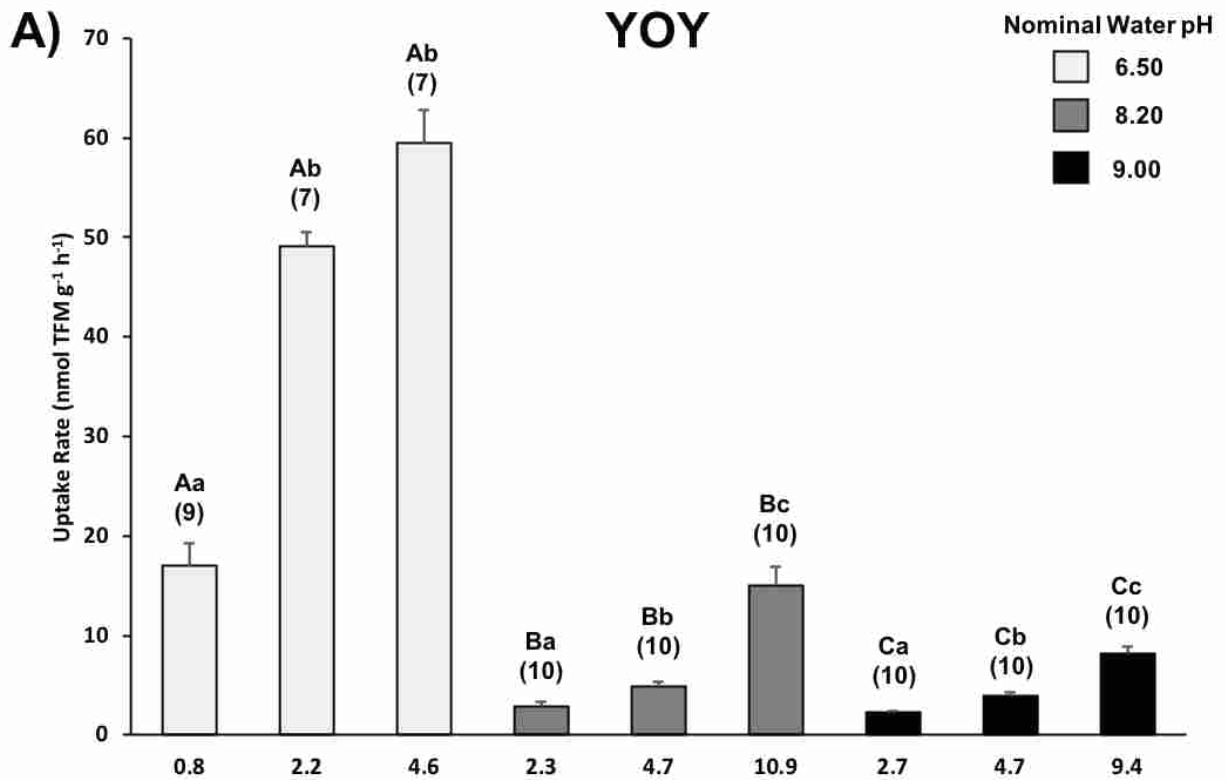
Figure 2-3. Effects of water alkalinity and 3-trifluoromethyl-4-nitrophenol (TFM) concentrations on the rate of TFM uptake in young of the year (YOY) and one-year old (1+) lake sturgeon.

The rates of TFM uptake (nmol g^{-1} h^{-1}) for YOY lake sturgeon (A; ~9 months) and 1+ lake sturgeon (B; ~15 months) at varying alkalinity (mg L^{-1} $CaCO_3$) and TFM concentrations (mg L^{-1}). Uptake rates are reported as the mean (N = 8-10 sturgeon) ± 1 SEM. Upper case letters denote statistically significant differences among the rates of TFM uptake amongst the different alkalinity treatments ($P < 0.05$). Measurements sharing the same lower-case letter within a given alkalinity treatment, are not statistically significant from one another ($P < 0.05$).
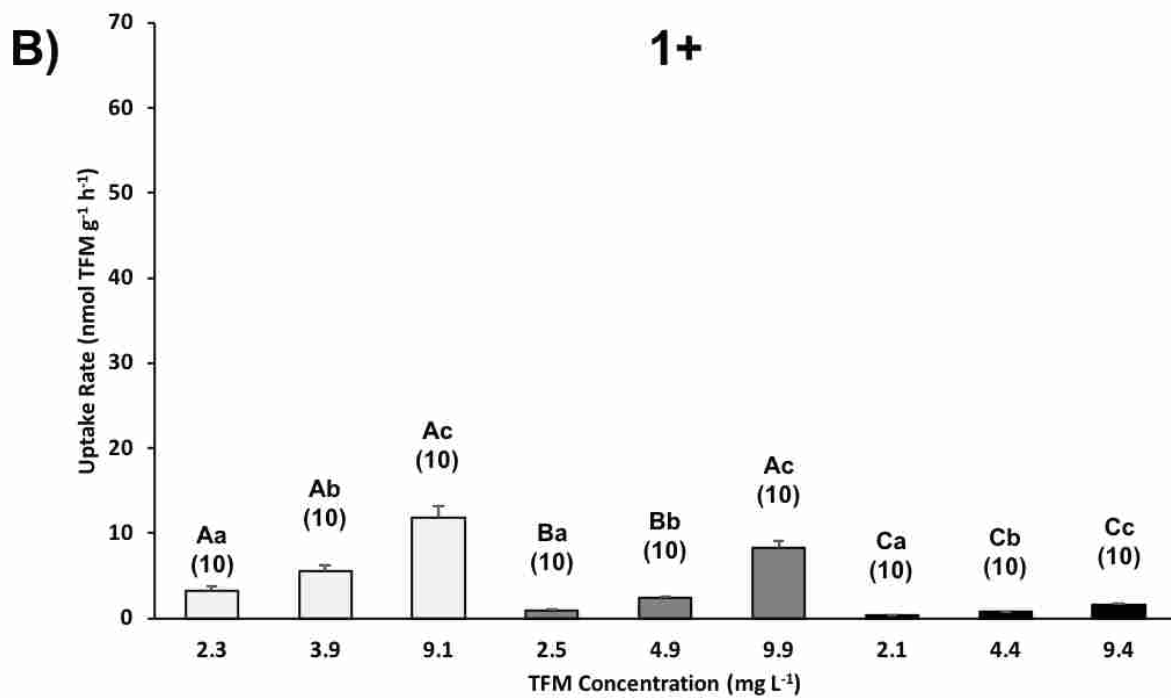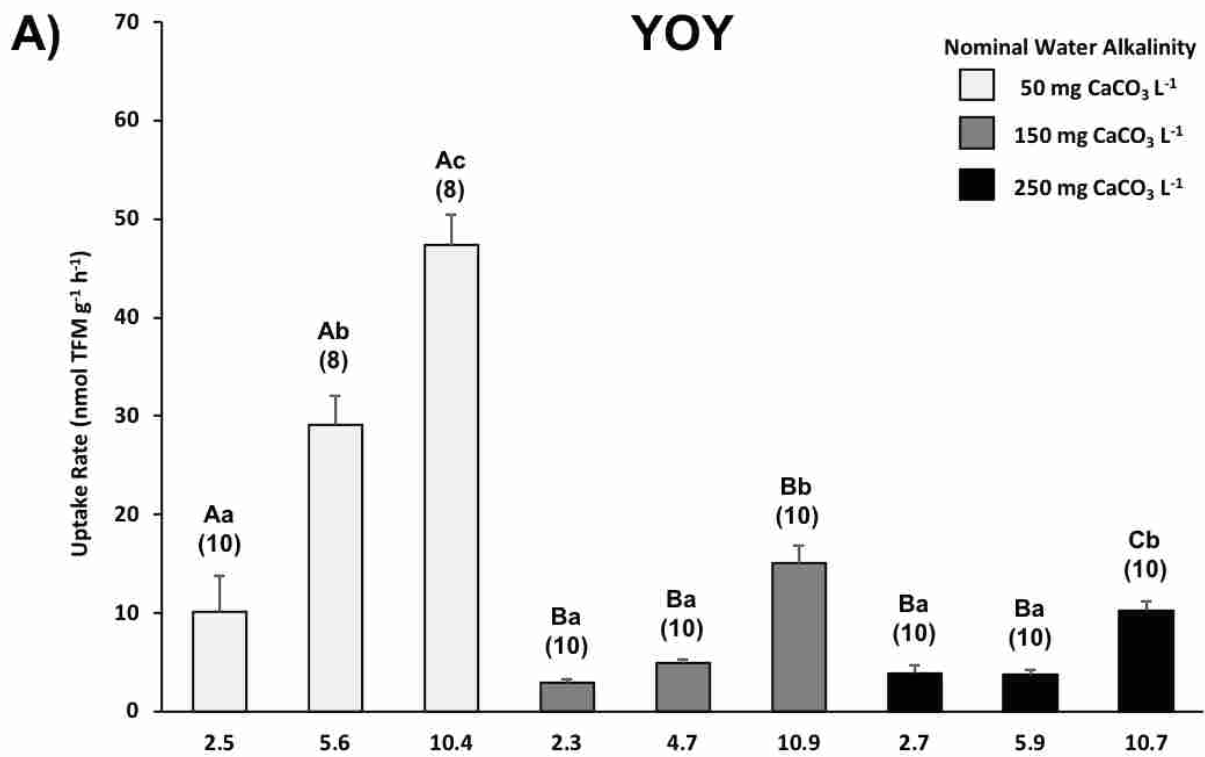
Chapter 3:

The Effects of 3-Trifluoromethyl-4-Nitrophenol on the Structure and Physiology of

Juvenile Lake Sturgeon Gills

Introduction

In the early 20th century, the invasive sea lamprey (*Petromyzon marinus*) gained access to the upper Laurentian Great Lakes, contributing to the decimation of economically-important fisheries to both Canada and the U.S.A. (Thuemler, 1988; Auer, 1999). Of the methods of population control used since the introduction of sea lamprey to the Great Lakes, the most widely used, and most efficient method, is the application of the lamprey-selective pesticide 3-trifluoromethyl-4-nitrophenol (TFM) to streams and tributaries where larval lamprey burrow (Applegate et al., 1961; Howell et al., 1964; McDonald and Kolar, 2007). Although non-target mortality is rare during sea lamprey control applications of TFM, some species of fish have shown sensitivity to the pesticide at concentrations routinely applied within streams (1.3-1.5 times the $LC_{99.9}$ for sea lamprey; Bills et al., 2003; Boogaard et al., 2003). The lake sturgeon (*Acipenser fulvescens*) is a species of particular concern to sea lamprey control efforts, due to its observed sensitivity to TFM when below the size of 100 mm (Johnson et al., 1999; Boogaard et al, 2003; O'Connor et al., 2017). Additionally, the protective effect of increased water alkalinity on the toxicity of TFM, as seen in other fish (Bills et al., 2003), is reduced in the lake sturgeon, demonstrated through empirical evidence and predictive modeling by O'Connor et al. (2017). This model showed a decrease in juvenile sturgeon survivorship with increases in water alkalinity and absolute TFM concentration. The physiological explanation for these observed sensitivities to TFM for the juvenile sturgeon below 100 mm, and in waters of higher alkalinity, have not been fully addressed. However, since lake sturgeon population levels remain depressed at 1-2% of their historic levels (Hay-Chmielewski and Whelan, 1997), it is imperative to better understand why juvenile lake sturgeon are more sensitive to TFM, and how their susceptibility is influenced by water chemistry.

TFM has been shown to uncouple oxidative phosphorylation in the mitochondria, creating a deficit in ATP supply that does not match demand (Birceanu et al., 2011). This creates a dependence on glycolysis and phosphocreatine to meet ATP demands, leading to the depletion of glycogen reserves in the brain and liver, followed by death (Wilkie et al., 2007; Birceanu et al., 2009; Clifford et al., 2012). It is predicted that a reduction in ATP supply at the gills would likely starve ion-pumps located in the gill epithelium that are crucial for maintaining ionic-gradients, leading to disturbances in ionic homeostasis. A study by Mallatt (1987), demonstrated that TFM exposure resulted in damage to MR cells (mitochondrion-rich cells; ionocytes) of gills in sea lamprey. Observable morphological changes to MR cells included cell rounding, enlargement of mitochondrial profiles, vacuolization of the cytoplasm and the widening of intercellular spaces (Mallatt, 1987). These MR cells mediate the active transepithelial movements of Na^+ and Cl^- uptake across the gill and are essential for osmoregulation and maintaining intracellular homeostasis (Wilkie et al. 1998; Randall et al., 1972; Bartels and Potter, 2004; Reis-Santos et al., 2008). MR cells also play a role in acid-base balance by transporting protons (H^+) and HCO_3^- out of the cell to generate electrical gradients that drive passive Na^+ influx through an epithelial Na^+ channel (Figure 1-8; Lin et al., 1994; Bury and Wood, 1999; Wilson et al, 2000; Kumai and Perry, 2012; Dymowska et al., 2012). Vacuolar type proton ATPase (V-ATPase) located on the basolateral membrane is thought to actively pump H^+ outside the MR cell to create an electrochemical gradient for the uptake of Cl^- via the Cl^-/HCO_3^- exchanger (Evans, 2011). V-ATPase on the apical membrane use ATP to pump H^+ out of the cell to create gradient gradients for Na^+ influx through the epithelial Na^+ channel (Lin and Randall, 1995; Evans et al., 2005). Na^+ is then taken up into the blood following transport into MR cells,

via an ouabain-sensitive basolaterally located Na^+/K^+-ATPase (Bartels et al., 1998; Marshall 2002; Wilkie, 2011). TFM is suspected of interfering with ATP production (Niblett and Ballantyne 1976; Birceanu et al. 2009, 2011), but to date it is not clear if TFM interferes with ATP-dependent ion regulation processes in the gills. The goal of this study was to determine whether TFM exposure influenced the distribution and physiology of MR cells of the gills, and if water alkalinity influenced these MR cells to possibly predispose sturgeon to TFM toxicity.

Accordingly, juvenile lake sturgeon were acclimated to set water alkalinities for a minimum of one week prior to exposure to the sea lamprey MLC (12 h $LC_{99.9}$) of TFM. Sturgeon were sampled at regular time intervals during exposure to TFM for 0, 3, 6 and 9 h, followed by the excision and processing of their gills for the measurement of Na^+/K^+-ATPase and V-ATPase activity, expression and distribution. The potential for TFM exposure and alkalinity acclimations to influence the structure and physiology of MR cells was then assessed.

Materials and Methods

Experimental Animals and Set-up

Lake sturgeon were shipped by plane from the University of Manitoba (Winnipeg, MB, Canada) to Wilfrid Laurier University (Waterloo, ON, Canada). They were then held for two weeks in an aerated 680 L re-circulating G-Hab system (Pentair Aquatic Eco-Systems, Apopka, FL, U.S.A.) and running on a 48 h automatic water-exchange, receiving dechlorinated city water (pH ~ 8.0; titratable alkalinity ~ 300 mg $CaCO_3$ L^{-1}; hardness ~ 290 $CaCO_3$ L^{-1}; temperature ~ 14-16 °C). Fish were housed under a 12 h light: 12 h dark photoperiod and fed daily with commercially-available blood worms (San Francisco Bay Brand, Inc., Newark, CA) at 2% of their body mass. All fish were starved for 72 h prior to experiments to reduce ammonia accumulation from the metabolism of proteins and amino acids (Wood, 2001). All experiments and fish husbandry followed Canadian Council of Animal Care guidelines and were approved by the Wilfrid Laurier University Animal Care Committee.

Experimental Procedures

Acclimation to Different Water Alkalinities

Sturgeon (N=96; length 118 ± 1.9 mm; mass 4.4 ± 0.2 g) were moved to three 37 L glass aquariums and acclimated to set water alkalinity and pH (Table 3-1) for a minimum of one week with activated carbon and biological filtration systems, and 50% water changes every other day. Aquariums received reconstituted waters prepared following the methods set out by the American Public Health Association (1989). $CaSO_4$, KCl and $MgSO_4$ were used to control water hardness (101.1 ± 2.6 mg $CaCO_3$ L^{-1}) and $NaHCO_3$ to control desired alkalinities. Ca^{2+} and Mg^{2+} were measured to determine water hardness using flame atomic absorption spectroscopy (AAS,

PinAAcle 900T, Perkin Elmer, Waltham, MA, U.S.A.). Water composition was confirmed twice per day during the acclimations, with alkalinity and pH measured using commercial kits (Hach, Alkalinity Test Kit, Model AL-AP) and a handheld pH meter (pH 11 meter, Oakton Instruments, IL, U.S.A.).

TFM Exposure and Gill Tissue Preparation

One day prior to each set of experiments, lake sturgeon (N=10) were transferred to darkened flux-chambers (volume = 750 mL; N=1 sturgeon per container) receiving water on flow-through at the desired alkalinity (Table 3-1). These fish were left to acclimate in their containers for 12 h prior to TFM exposure. Lake sturgeon were exposed to control conditions, as well as TFM (35% active ingredient in isopropanol; provided courtesy of the Department of Fisheries and Oceans Canada; Clariant SFC GMBH WERK, Griesheim, Germany) for 3, 6 and 9 h at the 12 h MLC for sea lamprey ($LC_{99.9}$; Bills et al., 2003) of TFM corresponding to the water alkalinity and pH (Table 3-1). At each sampling period (including controls) sturgeon were euthanized with an overdose of tricaine methanesulfonate (1.5 g L^{-1} buffered in 3.0 g L^{-1} $NaHCO_3$; TMS; Syndel Labs, Port Alberni, BC, Canada), followed by the excision of the left half of the branchial basket (gills), which was then snap frozen in liquid nitrogen and stored at -80 °C for the Na^+/K^+-ATPase, V-ATPase and total ATPase activities, as well as Western blotting. The right half of the branchial basket was also excised, but instead was fixed in 3% paraformaldehyde (PFA)/phosphate-buffered saline at 4 °C, then dehydrated in an ethanol series (1 h in 70%, 95% and three times in 100%), cleared using xylene (3 x 1 h), before being infiltrated and embedded in paraffin wax (Richard-Allan Scientific, Kalamazoo, MI, USA).

Sections (5 μm) of gill tissue were prepared and transferred to 3-aminoprpyl-triethoxysilane

(APS) coated microscope slides for later immunohistochemistry analysis.

Analytical Techniques

Na^+/K^+-ATPase and V-ATPase Activity Assay

Following the method of McCormick (1993), Na^+/K^+-ATPase and V-ATPase activity

was measured using a kinetic microassay. Tissues were homogenized in SEI buffer (250 mM

sucrose, 10 mM Na_2EDTA, 50 mM imidazole) with sodium deoxycholate added to 0.1% volume

of SEI buffer, using a Precellys 24 bead homogenizer (Bertin; 2 x 15s with a 30s pause) and

centrifuged (12,000 x g for 30s, 4 °C). The supernatant was decanted and used for the ATPase

activity assay and immunoblotting experiments. Ten μL of supernatant was transferred to 96-

well microplates, with the addition of 200 μL of assay mixture. Three assay mixtures (AM) were

utilized in triplicate. The first (AM A) contained 50 mM imidazole buffer, 2 mM

phosphoenolpyruvate, 0.16 mM nicotinamide adenine dinucleotide (NADH), 0.5 mM adenosine

triphosphate (ATP), 2.86 U mL^{-1} lactic dehydrogenase, and 3.57 U mL^{-1} pyruvate kinase. The

second (AM B) contained AM A with 0.5 mM of ouabain, used to inhibit Na^+/K^+-ATPase

activity. The third contained AM B with 10 μM of bafilomycin A1 (LC labs, Boston, MA,

U.S.A.), used to inhibit V-ATPase activity. The microplates were then mixed at 1000 RPM for 1

minute and then read on a microplate spectrophotometer (Epoch 2, BioTek Instruments, Inc.,

VT, U.S.A.) using a kinetic assay, reading at 340 nm every 47 s, for 15 min. Standard curves

were produced for ADP to determine the amount of ATP (nmol) converted to ADP. Total protein

concentrations were calculated using a BCA Protein Assay (G-Biosciences, St. Louis, MI,

U.S.A.). Both ADP and total protein assays were used to calculate the activity of Na^+/K^+-

ATPase, V-ATPase and total ATPase as micromoles of adenosine diphosphate (ADP) per milligram of protein per hour.

Antibodies

The commercial, well validated antibodies used for this study were chosen based on prior studies involving sturgeon, as well as teleost fishes (Witters et al., 1996; Wilson and Laurent, 2002, Wilson et al., 2007; Reis-Santos et al., 2008; Sorenson, 2015). Na^+/K^+-ATPase was detected using the α5 mouse monoclonal antibody, developed by Douglas Fambrough (Johns Hopkins University; Takeysau, 1998), for immunoblotting and immunofluorescence microscopy. The α5 mouse monoclonal antibody was obtained as culture supernatant from Developmental Studies Hybridoma Bank, University of Iowa under contract N01-HD-7-3263 from National Institute for Child Health and Human Development. V-ATPase was detected using the B2 antibody, a rabbit polyclonal anti-peptide antibody (Wilson et al., 2007) for both immunoblotting and immunofluorescence microscopy. A mouse tubulin monoclonal antibody (12g10) was used for a reference protein during Western blotting (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, U.S.A., under contract N01-HD-7-3263 from the National Institute of Child Health and Human Development). For Western blotting analyses, secondary antibodies included (anti-mouse or anti-rabbit) horseradish peroxidase conjugated secondary antibody (Novus Biologicals, Littleton, CA, U.S.A.). For immunohistochemistry, secondary antibodies included goat anti-rabbit Alexa Fluor 555 and goat anti-mouse Alexa Fluor 488 conjugates (Life Technologies, Carlsbad, CA, U.S.A.). For immunohistochemistry, J3 (equivalently diluted isotyped culture supernatant) was used was used as a negative control (Davids Biotechnologie GmbH, Ragensburg, Germany).

Western Immunoblotting

Gill protein expression was determined using the methods set out by Wilson et al. (2007), using SDS-PAGE (polyacrylamide gel electrophoresis) and Western immunoblotting. The remaining homogenate (250 µL) from the ATPase activity assay was mixed with an equal volume of 2 x Laemmili's buffer, heated (70 °C) for 10 min, and then stored at 4 °C. Before loading onto the gels, protein concentrations were determined using a BCA Protein Assay (G-Biosciences, St. Louis, MO, USA), and adjusted to 1 µg mL^{-1}, vortexed, and centrifuged (12,000 x g for 5 min). Homogenized gill-tissue (20 µg) was loaded into 1.5 mm thick discontinuous gels (8% T resolving gel, 4% T stacking gel). Gels were run for 15 min at 75 V, then 1 hour at 150 V, using a Bio-Rad MiniPROTEAN® Tetra cell system (Bio-Rad, Hercules, CA). Gels were transferred to Immu-Blot® PVDF membranes (pore size 0.2 µm; Bio-Rad), in a Hoefer TE22 transfer cell using Towbin's buffer (Holliston, MA, U.S.A.). Membranes were washed in TTBS (Tris-buffered saline with 0.05% Tween-20) and transfer was confirmed with Ponceau S. staining (0.5% in 1% acetic acid). Following another TTBS rinse, membranes were blocked with 5% skim-milk-powder solution (12 h, room temperature). Membranes were incubated with primary antibodies in 1% BSA/TTBS, with 0.05% sodium azide as preservative (1 h, room temperature). Membranes were rinsed in TTBS, then incubated in secondary antibodies in TTBS (1 h, room temperature), and then rinsed in TTBS. Enhanced Chemiluminescence (ECL) Western Blotting Detection Solution (Clarity™ Western blotting substrate, Bio-Rad) was added to the membranes, which were then imaged using the Azure c300 imaging system (Azure Biosystems Inc., Dublin, CA, U.S.A.). Quantification of bands was done using Multi Gauge

Software (V3.1 Fujifilm, Tokyo, Japan). The QL/pixel^2 was subtracted from the grayscale pixel value of 65,535 and normalized to the tubulin control.

Immunofluorescence

The immuno-staining process followed a protocol modified from Wilson (2007). Slides were air dried at 60 °C before being dewaxed through a series of xylene baths and rehydrated with subsequent ethanol baths and tap water. Antigen retrieval was performed using 0.05% citraconic anhydride (pH 7.3) in a boiling water bath for 30 min (Namimatsu et al., 2005), and a 1% sodium dodecyl sulfate (SDS) in PBS soak (Brown et al., 1996). Samples were rinsed 3 x in deionized water following each antigen retrieval step, then sections were circled with a hydrophobic barrier (PAP pen, Sigma-Aldrich), and then blocked with a 5% normal goat serum/BLOK solution (Millipore, Temecula, USA) for 20 min. Sections were then incubated with a combination of mouse and rabbit primary antibodies, diluted 1:100 and 1:500, respectively, for 12 h at 4 °C in a humidity chamber. Slides were then rinsed in 0.05% Tween-20 in PBS (TPBS), pH 7.4, for 5, 10 and 15 min in Coplin jars, and then incubated with the conjugated secondary antibodies diluted 1:500 in BLOK solution for 1 h at 37 °C. Samples were then rinsed in TPBS for 5 min, stained with DAPI, diluted 1:50,000 in TPBS, for 10 min and rinsed again in TPBS for 15 min. Glycerol-PBS (1:1) was used to mount coverslips. These slides were analyzed using fluorescent microscopy (DM5500, Leica) to assess the presence of ionocytes in gill filaments.

Statistical Analyses

All data are presented as the mean ± 1 standard error of the mean (SEM). Data were assessed for normality using the Shapiro-Wilk test, and for homoscedasticity using the Levene test. Data was normalized using a log10 transformation where necessary. The influence of alkalinity and TFM exposures on the activity and expression of Na^+,K^+-ATPase and V-ATPase was assessed using a one-way analysis of variance (ANOVA). No post-hoc tests were applicable. For all statistical tests, the significance was set at the $P < 0.05$ level, and all tests were completed using SPSS 14.0 (SPSS, Inc., Chicago, IL, U.S.A.).

Results

Na$^+$/ K$^+$-ATPase and V-ATPase Activity Assay

TFM and alkalinity acclimations did not have a significant effect on the ion transporter activity in the gills of juvenile sturgeon (Figures 3-1). An ANOVA showed no significant differences in the activity of Na$^+$/K$^+$-ATPase following TFM exposure (P = 0.478) or alkalinity acclimations (P = 0.504), with values averaging 2.74 ± 0.66 µmol ADP mg protein^{-1} h^{-1}, with a range of 1.11-5.13 µmol ADP mg protein^{-1} h^{-1}. Additionally, there were no significant differences found in the activity of V-ATPase, with values averaging 0.61 ± 0.10 µmol ADP mg protein^{-1} h^{-1} following TFM exposure (P = 0.493) and different alkalinity acclimations (P = 0.641), with a range of 0.28-1.52 µmol ADP mg protein^{-1} h^{-1}. Total ATPase activity was also not significantly affected by TFM exposure (P = 0.494) or alkalinity acclimations (P = 0.528), with activity averaging 20.58 ± 3.20 µmol ADP mg protein^{-1} h^{-1}, with a range of 12.05-31.06 µmol ADP mg protein^{-1} h^{-1}. The power of these analyses was reported at 0.403.

Western Immunoblotting

For the western blot analyses, Na$^+$/K$^+$-ATPase α5 antibody strongly immunoreacted with a band at approximately 100kDa, the proposed size of the α-subunit of the transporter. Similarly, the B2/BvA1 antibody for V-ATPase immunoreacted with a pair of bands at approximately 56 kDa, corresponding to the predicted molecular mass of the V-ATPase B subunit. Na$^+$/K$^+$-ATPase Western blot analyses (Figure 3-2) showed no significant changes in expression from the different TFM exposures (P = 0.421) or based on alkalinity acclimation (P $\geq$ 0.420). V-ATPase western blot analyses also showed no significant changes in expression from the different TFM

exposures (P = 0.825) or based on alkalinity acclimations (P ≥ 0.534). The power of these

analyses was reported at 0.531.

Immunofluorescence

Distinct Na^+/K^+-ATPase or V-ATPase immunoreactive cells were identified in the

branchial epithelial cells of the filament and the lamella of the lake sturgeon (Figure 3-3, 3-4).

Staining of the cytoplasm of the epithelial cells closely associated with MRCs was observed for

both Na^+/K^+-ATPase and V-ATPase fluorescent stains. Na^+/K^+-ATPase staining was restricted to

the basolateral membrane or to the cytoplasm. V-ATPase staining generally had a cytoplasmic

distribution, however, staining was also limited to the apical region in some cases. There were no

notable differences in ionocyte distribution within the gill epithelium of lake sturgeon following

different alkalinity acclimations (Figure 3-4).

Discussion

Immunofluorescent Staining

The strong distinction between MR cells having either Na^+/K^+-ATPase or V-ATPase localization indicates the presence of two separate MR cells within the gills of juvenile lake sturgeon. Additionally, the cytoplasmic staining of Na^+/K^+-ATPase or V-ATPase is likely representative of the presence of the extensive tubular system of the basolateral membrane, whereby these proteins are expressed (Wilson and Laurent, 2002; Evans, 2005). These results are supported by similar immunohistochemical staining patterns observed in white sturgeon (*Acipenser transmontanus*) by Baker et al. (2009). However, a small subpopulation of MR cells with both Na^+/K^+-ATPase or V-ATPase localization was observed in white sturgeon, which was not observed in the lake sturgeon of the present study.

Influence of Alkalinity and TFM on Gill Ionocytes

Juvenile lake sturgeon are currently listed as a threatened species within Canada and the U.S.A., and concern has been raised over their observed sensitivities to TFM during sea lamprey control treatments (Christie and Goddard, 2003; Peterson et al., 2007). Lake sturgeon below the length of 100 mm have been shown to have 12 h LC_{50} values that are less than the $LC_{99.9}$ concentrations of TFM used during sea lamprey control operations, which usually occur at 1.3 – 1.5 times the minimum lethal concentration (MLC; $LC_{99.9}$ of sea lamprey) to account for attenuation via dilution of TFM as it moves downstream (Boogaard et al., 2003; Christie and Goddard, 2003; McDonald and Kolar, 2007; O'Connor et al., 2017). Additionally, the protective mechanism on TFM toxicity that has been associated with increasing alkalinity for other fish (Bills et al., 2003) is reduced in juvenile lake sturgeon, as predicative-modeling has shown that

survivorship is negatively correlated with increasing water alkalinity (O'Conner et al., 2017). Prior studies have provided evidence to suggest that TFM can cause structural damage and potentially interfere with the gills of rainbow trout and sea lamprey (Christie and Battle, 1963; Mallat et al. 1987; 1994). Additionally, the mode of toxic action for TFM has been attributed to the uncoupling of oxidative phosphorylation within mitochondria (Birceanu, 2011). Thus, it was predicted that the shortage of ATP supply during TFM exposure would likely starve the energy demanding ion-pumps within the gills that are crucial for maintaining ionic homeostasis. The results of this study indicate that the exposure of juvenile sea lamprey to TFM, at concentrations around the MLC used during sea lamprey control operations, had no effect on gill MR cell ATPase activity, expression or distribution. It was also predicted that acclimations to different water alkalinity would influence the structure of MR cells in the gills of sturgeon, which may predispose the fish to TFM toxicity. However, this study demonstrates that acclimating juvenile lake sturgeon to low, medium and high water-alkalinities did not have a significant influence on the activity, expression or distribution of gill Na^+/K^+-ATPase and V-ATPase.

A similar study by Sorensen (2015) demonstrated that total ATPase activity and Na^+/K^+-ATPase activity was 2-3 fold and 4-fold greater, respectively, in soft water (~ 40 mg $CaCO_3$ L^{-1}) vs hard water (~ 450 mg $CaCO_3$ L^{-1}) acclimated larval sea lamprey and rainbow trout. These results provided support to the hypothesis that acclimations to soft water resulted in greater ATP demands at the gill. It is possible that the stressors induced by compensatory responses to increase the rates of Ca^{2+} and Mg^{2+} uptake may be responsible for the increases in ATPase activity. However, no significant increases in the activity of Na^+/K^+-ATPase or total ATPase activity was observed in the current study, suggesting that the external media concentration of

HCO$_3^-$ and CO$_3^{2-}$, does not significantly influence the overall ATP demands of lake sturgeon MR cells.

Additionally, the study by Sorensen (2015) demonstrated that exposing sea lamprey to their TFM 12 h LC$_{50,}$ and rainbow trout to the determined sea lamprey TFM 12 h LC$_{99.9}$, had no effect on the expression and distribution of Na$^+$/K$^+$-ATPase and V-ATPase within the gills following four week acclimations to hard or soft water. There was no observed effect on the activity of Na$^+$/K$^+$-ATPase, V-ATPase and total ATPase for sea lamprey and Na$^+$/K$^+$-ATPase, V-ATPase for rainbow trout during these exposures. However, the trout had a 30-40% increase in total ATPase activity following 6 and 9 h of exposure in both hard and soft water acclimations. Although no changes in plasma ion concentrations of Na$^+$ and Cl$^+$ were found, measurements of ^{24}Na$^+$ flux for trout in hard water showed a 3-fold increase in the loss of Na$^+$ in the first 3 h of TFM exposure, which restored to control levels within 6 and 9 h of exposure. No significant differences in flux of ^{24}Na$^+$ was observed for trout in soft water. A study by Birceanu et al. (2014) also showed Na$^+$ loss in rainbow trout exposed to their determined LC$_{50}$, with no change in the activity of Na$^+$/K$^+$-ATPase. It was proposed that the increased total ATPase activity observed in the gills of rainbow trout in the Sorenson (2015) study was due to a compensatory response induced by the increase in O$_2$ consumption following TFM exposure, leading to increased gill perfusion and surface area, elevating Na$^+$ losses. This would suggest that a similar compensatory response is reduced or absent in the sea lamprey and juvenile lake sturgeon, as no differences in total ATPase activity were found following exposure to TFM in sea lamprey during the studies by Sorensen (2015) and Birceanu et al. (2009), or for juvenile lake sturgeon during the current study. This was supported with other studies by Sorensen (2015) where

juvenile lake sturgeon (3.0 ± 1.1 SD g, 93 ± 9.5 SD mm) were acclimated for four weeks to hard water and exposed to the sea lamprey 12 h LC_{50}. For those sturgeon, no effect was observed on the activity, expression or distribution of Na^+/K^+-ATPase and V-ATPase in the gills.

Implications for Future Research

This study demonstrates that the sensitivity of juvenile sturgeon and the proposed reductions in the protective mechanism of alkalinity for this fish during TFM applications (O'Connor et al., 2017) are not due to changes the gill activity, expression and distribution of Na^+/K^+-ATPase and V-ATPase within MR cell at different water alkalinities. Additionally, TFM exposure did not appear to affect the distribution and physiology of MR cells in the gills at concentrations equivalent to the minimum lethal concentration (MLC; $LC_{99.9}$ for sea lamprey), which are the concentrations routinely applied in the field during sea lamprey control operations (Bills et al., 2003; McDonald and Kolar, 2007). It should be noted, however, that the length of the fishes used during the current study were approximately 118 mm in length (length 118 ± 1.9 mm), which is greater than those lengths of swim up fry and fingerlings (< 100 mm in length) determined to be the more susceptible to TFM toxicity than most teleost fishes (Boogaard et al., 2003). Lake sturgeon sac fry and fingerlings (> 125 mm in length) in the Boogaard et al. (2003) study were shown to have high tolerance to TFM toxicity, so there may be potential for TFM to cause greater disturbances in MR cells within the gills of sturgeon of a smaller size, which would not have been present in the gills of sturgeon in this study. Additionally, a study by Fu et al. (2018) demonstrated no significant differences in the age of transition for Na^+ uptake (via Na^+/K^+-ATPase) from the integument to the gills of larval rainbow trout in soft- vs hard-water, suggests a lack of plasticity in gill ionoregulatory development in the early life-stage of this fish.

It may be possible that the lack of plasticity in the ionoregulatory development of sturgeon in its early swim-up fry and fingerling (< 100 mm in length) life-stages may put greater strain on ionoregulatory processes when in waters of high or low alkalinity, which may predispose the sturgeon to TFM toxicity. Thus, studies on the development of ion-transport proteins in the early life stages of sturgeon are recommended to determine the influence of water alkalinity on ionic homeostasis. Additionally, the activity, expression and distribution of Na^+/K^+-ATPase and V-ATPase within the gills of juvenile sturgeon was found to not be affected by water alkalinity or TFM exposure, however, there are other transporters which are also important for ionic regulation in fishes.

Further studies should also address the effects of alkalinity and TFM exposure on the activity, expression and distribution of pendrin and Cl^-/HCO_3^-, which are anion exchangers localized within the apical membrane of branchial MR cells involved in Cl^- uptake and acid-base regulation (Katoh et al., 2003; Tresguerres et al., 2006; Piermarini et al., 2002; Evans, 2011). MR cells with Cl^-/HCO_3^- exchangers present have had V-ATPase localized to the basolateral membrane, where basolateral proton extrusion is thought to provide intracellular HCO_3^- to drive the apical exchange with Cl^- (Evans, 2011). Greater concentrations of HCO_3^- in higher alkalinity waters may therefore influence the degree of HCO_3^- excretion required for Cl^- exchange at the gills in sturgeon, and thus may affect the ATP demands and transport proteins required for ionic regulation. Additionally, Na^+/H^+ exchangers should also be assessed, as they have been shown to be present on the apical surface of cells containing a high density of basolaterally located Na^+/K^+-ATPases, as noted in the gills of sturgeon in the current study (Edwards et al., 1999; Wilson et al., 2000; Hirata et al., 2003). Should juvenile sturgeon have a limited ability to

regulate ion-flux with external media at younger developmental stages, they may be more susceptible to TFM induced reductions in ATP supply within the gills, putting additional strain on the ionoregulatory capacity under these conditions.

An explanation for the reduced protective mechanism on TFM toxicity for juvenile lake sturgeon in waters of higher alkalinity remains unresolved. However, the observed sensitivity of this fish when below 100 mm can be attributed to greater rates of TFM uptake, which was demonstrated earlier in this report (Chapter 2). The reason for the greater rates of TFM uptake in sturgeon less than 100 mm is yet to be determined, but is most likely influenced by the inverse relationship between gill ventilation rates and body size (Goolish, 1991). Additionally, the greater sensitivity of YOY sturgeon to TFM could be related to a lower, relative capacity to detoxify TFM. Sturgeon have been shown to detoxify TFM using Phase II biotransformation, which includes the conjugation of TFM to TFM-glucuronide using glucuronidation (LeClair, 2014; Bussy et al., 2018a). The capacity of YOY and 1+ lake sturgeon to utilize uridine diphosphate glucuronyl transferase (UDPGT) should be assessed to determine whether or not their greater vulnerability to TFM toxicity is due to differences in their ability to detoxify and excrete the lampricide.

Table 3-1. Water chemistry with acclimation to different water alkalinities.

Water alkalinity (mg $CaCO_3$ L^{-1}), pH and temperature (°C) data collected during (A) acclimation to different water alkalinities, and (B) during the exposure of lake sturgeon to TFM for 0, 3, 6 or 9 h. Lower case letters denote statistical significance between alkalinity treatments. Data is presented as the mean ± SEM.

A. One Week Acclimation

Alkalinity Experiment	Alkalinity (mg $CaCO_3$ L^{-1})	pH	Temperature (°C)
Low	52 ± 1	8.07 ± 0.02	13.6 ± 0.1
Medium	148 ± 2	8.43 ± 0.02	13.6 ± 0.1
High	250 ± 3	8.77 ± 0.02	13.6 ± 0.1

B. Exposure to TFM

Alkalinity Experiment	Alkalinity (mg $CaCO_3$ L^{-1})	pH	Temperature (°C)	TFM (mg L^{-1})
Low	52 ± 1	8.00 ± 0.01	12.8 ± 0.1	1.4 ± 0.1
Medium	150 ± 1	8.36 ± 0.01	13.9 ± 0.1	4.7 ± 0.1
High	250 ± 1	8.74 ± 0.01	13.8 ± 0.1	9.7 ± 0.1

Figure 3-1. Effects of TFM exposure on the activity of gill ion-transporters in juvenile lake sturgeon.

Vacuolar H^+-ATPase (V-ATPase; white bars) Na^+/K^+-ATPase (grey bars) and total ATPase (black bars) activity of juvenile lake sturgeon (*Acipenser fulvescens*) with no TFM exposure (control), or following 3, 6 or 9 h of TFM exposure. TFM concentrations were determined from the pH/alkalinity tables used to determine the sea lamprey minimum lethal concentration (MLC) of TFM (Bills et al., 2003). Numbers within brackets indicate the number of sturgeon within each sample group. No statistical differences were determined among TFM exposure durations, or alkalinity groups (denoted by the similar letters). Data are expressed as the mean $\pm$ SEM.
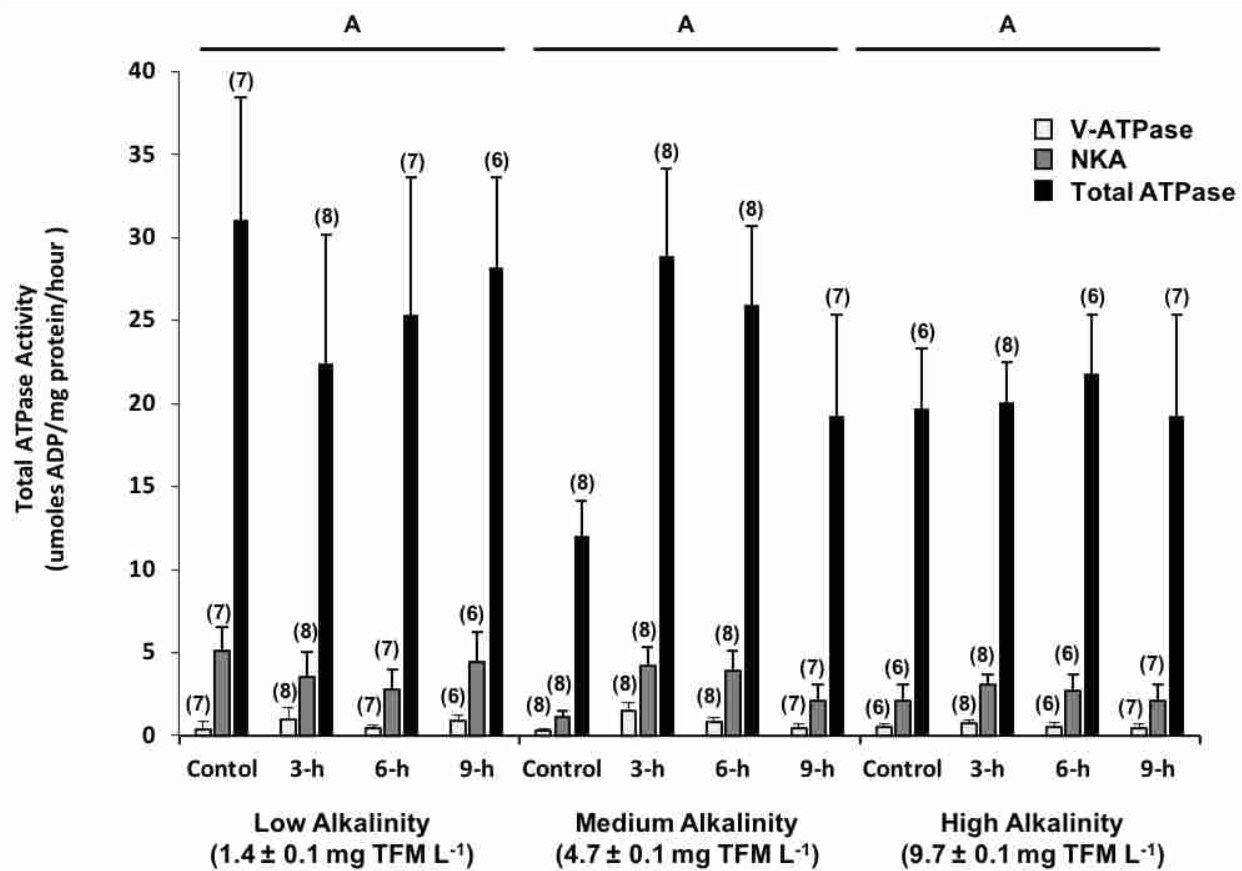
Figure 3-2. Effects of TFM exposure on the expression of gill ion-transporters in juvenile lake sturgeon.

Vacuolar H^+-ATPase (V-ATPase; white bars), Na^+/K^+-ATPase (NKA; grey bars) and total ATPase (black bars) expression are shown for juvenile lake sturgeon (*Acipenser fulvescens*) with no TFM exposure (control), or following 3, 6 or 9 h of TFM exposure. Additionally, representative immunoblots of the gills are shown above their respective bars, with Na^+/K^+-ATPase strongly immunoreactive with a band at approximately 100kDa, and V-ATPase strongly immunoreactive with a band at approximately 56 kDa. TFM concentrations were determined from the pH/alkalinity tables used to determine the sea lamprey minimum lethal concentration (MLC) of TFM (Bills et al., 2003). Numbers within brackets indicate the number of sturgeon within each sample group. No statistical differences were determined between TFM exposure durations, or alkalinity groups (denoted by similar letters). Data are expressed as the mean ± SEM.
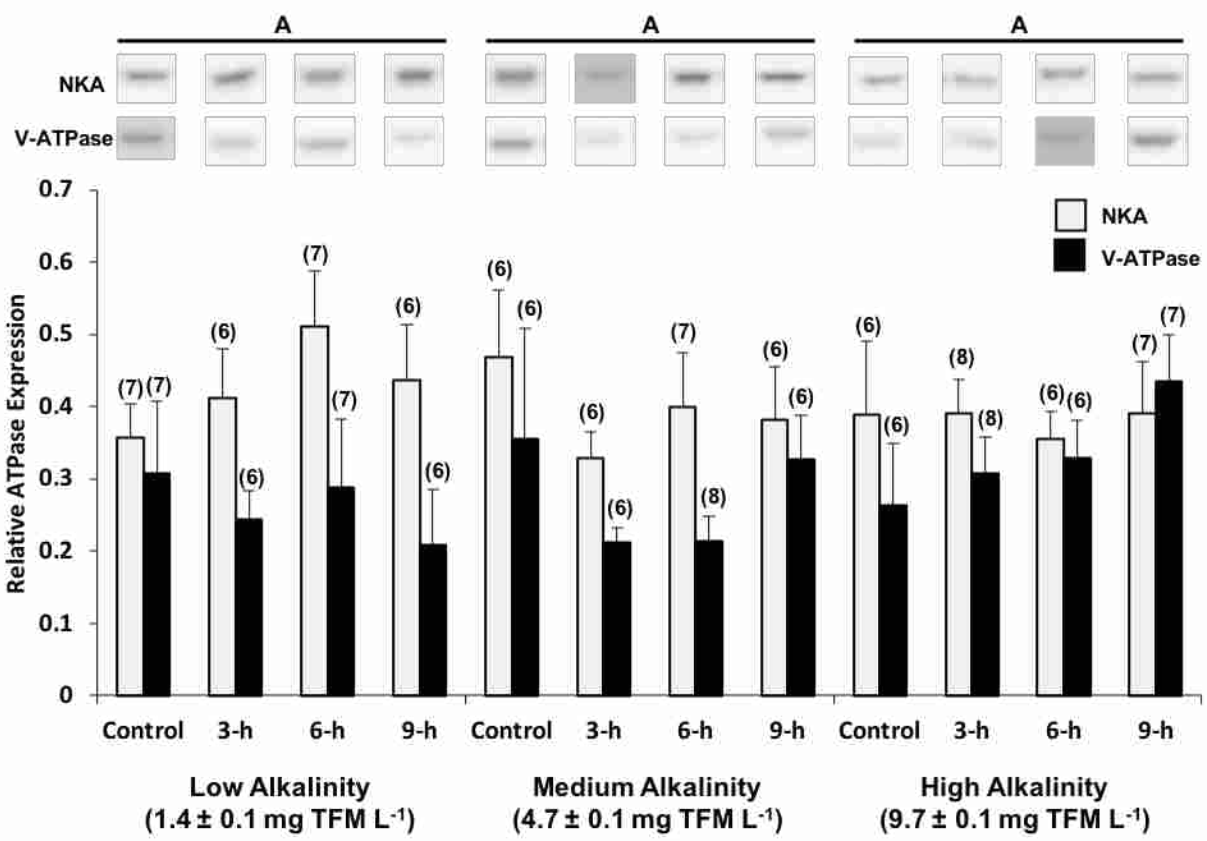
Figure 3-3. Immunohistochemistry of juvenile lake sturgeon gill.

A sagittal section of juvenile lake sturgeon gill, close up of secondary lamellae. Green staining (a; Alexa Flour 568) is H^+-ATPase (V-ATPase), magenta (b; Alexa Flour 488) is vacuolar Na^+/K^+-ATPase, grey (c) is the phase contrast image, blue (d) is DAPI, and the final merged image (e). The white arrow head indicates a V-ATPase stained mitochondrion-rich (MR) cell, the speckled arrow head indicates a Na^+/K^+-ATPase stained MR cell, the white arrow indicates the lamellae and the speckled arrow indicates the filament. Scale bar is 50μm.
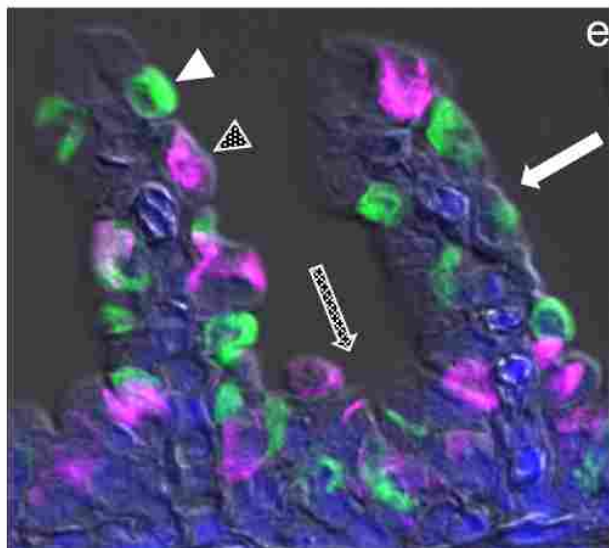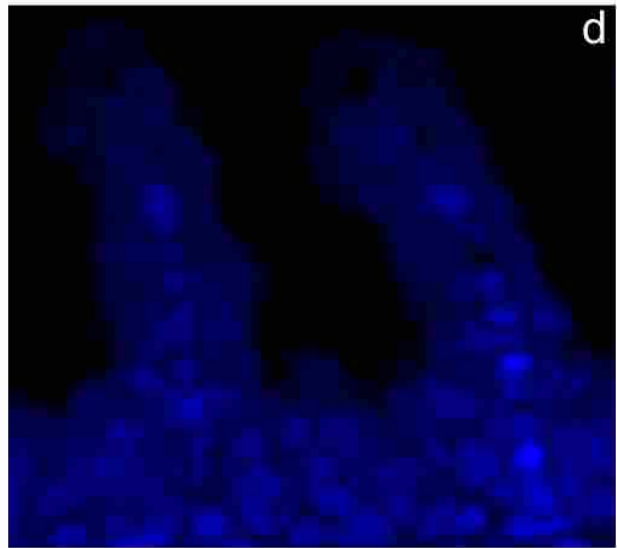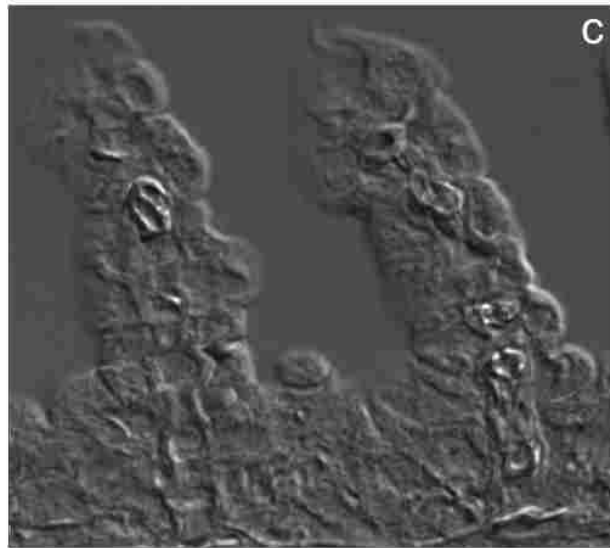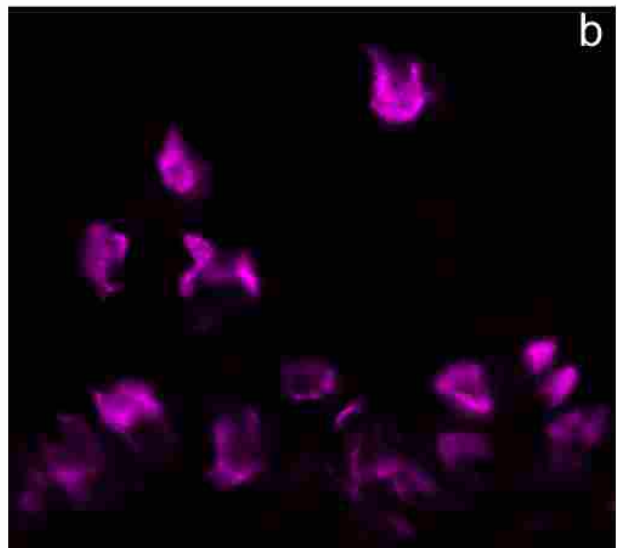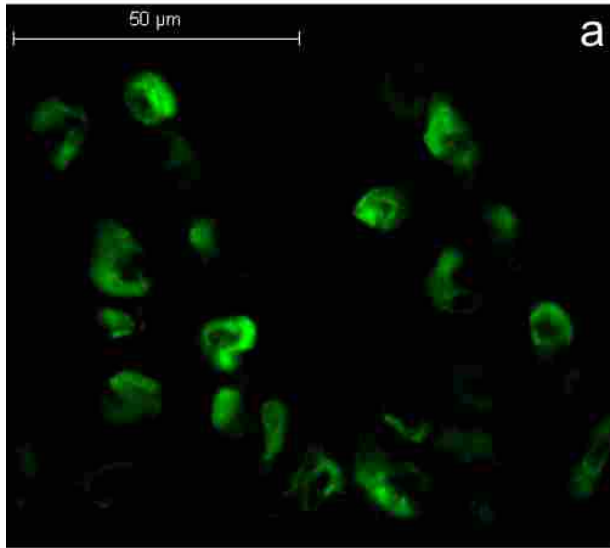
Figure 3-4. Immunohistochemistry of juvenile lake sturgeon gill.

A sagittal section of juvenile lake sturgeon (*Acipenser fulvescens*) gill. Green staining (Alexa Flour 568) is H^+-ATPase (V-ATPase), magenta (Alexa Flour 488) is vacuolar Na^+/K^+-ATPase, grey is the phase contrast image. Low alkalinity acclimated sturgeon are represented with the letter L, moderate alkalinity the letter M, and high alkalinity the letter H. Three separate sturgeon (N = 3) are shown for each alkalinity acclimation, denoted by the number of apostrophes ('). Scale bar is 50μm.
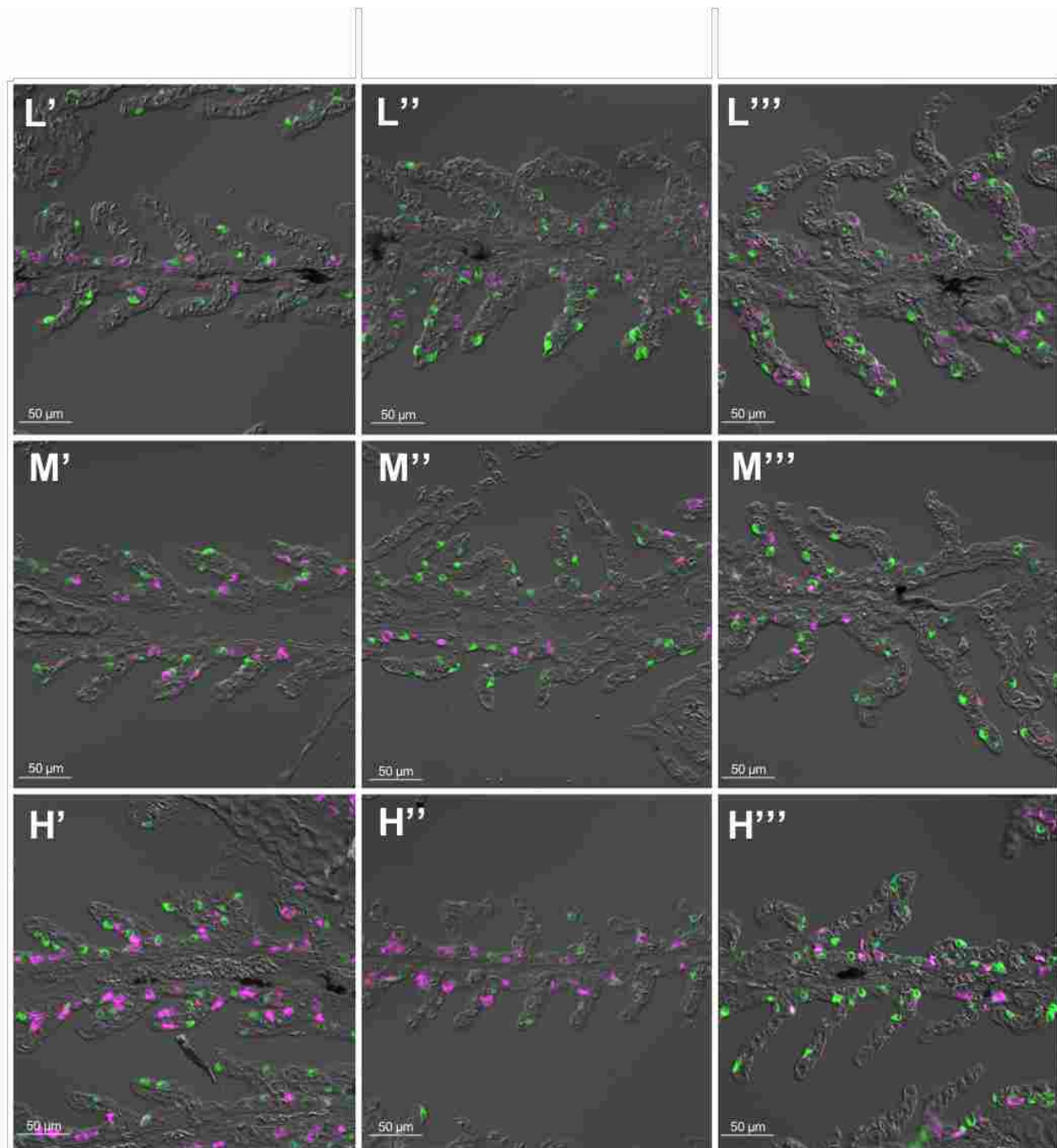
Chapter 4

An Integrated Model Describing the Influence of Water Alkalinity on the Rate of Uptake of

3-Trifluoromethyl-4-Nitrophenol (TFM) by Juvenile Lake Sturgeon

Introduction

Sea lamprey control within the Laurentian Great Lakes has relied on the application of 3-trifluoromethyl-4-nitrophenol (TFM) for over 60 years (Howell et al., 1980; GLFC, 2008). However, much research needs to be done to improve lampricide treatment effectiveness, reduce the impacts on non-targets species, and improve the understanding of the toxic effects of this nitro phenolic compound (McDonald and Kolar, 2007). Lake sturgeon are currently a threatened species within Canada and the U.S.A. (Auer, 1999; COSEWIC, 2006), and they have been shown to be most sensitive to TFM in their young of the year (YOY, < 100 mm) life stage (Johnson et al., 1999; Boogaard et al., 2003). Additionally, a recent study by O'Connor et al. (2017) demonstrated a reduced protective effect of alkalinity for juvenile lake sturgeon, as compared to sea lamprey. Concerns over the effects of TFM on this species have resulted in alterations in the protocols for applying the piscicide, which led to greater survival of larval lamprey during treatments (Scholefield et al., 2008; Boogaard et al., 2011). The gills are thought to be the main route of uptake for TFM (Hunn and Allen, 1974), and previous experiments found evidence of morphological damage to the gills of sea lamprey following TFM exposure (Mallatt, 1987). The gills are also the main source of ionoregulation for fish (Hunn and Allen, 1974), therefore, it was hypothesized that TFM may interfere with ionoregulatory processes within mitochondrion-rich (MR) cells, and alkalinity might influence the distribution and physiology of these cells, predisposing the sturgeon to TFM toxicity. It was the overarching goal of this thesis to use an integrative approach to determine the influence of water pH and alkalinity on the rates of TFM uptake in juvenile sturgeon at the sensitive YOY stage (< 100 mm), and the more TFM tolerant 1+ stage (> 130 mm). Additionally, this project aimed to determine the influence of

alkalinity and TFM on the distribution and physiology of Na^+/K^+-ATPase and vacuolar type H^+-ATPase (V-ATPase), two key ionoregulatory proteins within the gills of juvenile lake sturgeon. Below, I explain how research has been crucial in the history of sea lamprey control, and how my study can be used to better understand the influence of TFM on non-target species of concern. I developed a model to explain the influence of alkalinity on the uptake of TFM at the gills, and subsequently its influence on toxicity. Additionally, I propose how my findings can contribute to the development of more effective methods of TFM application, and what areas of study are required to further develop the efficacy and efficiency of sea lamprey control operations.

The Role of Research in the Development of Sea Lamprey Control

Since the invasion of the Great Lakes by the sea lamprey, emphasis on restoring the Great Lakes ecosystem and economy have been a major drive in the successful rehabilitation of its fisheries (GLFC, 1955; GLFC, 2008; Siefkes, 2017). The continued efforts by the Canadian and American governments have allowed for integrative research and development of methods for invasive species control, which continue to become more efficient at removing sea lamprey from the Great Lakes, reducing the cost of control operations, and reducing the impacts on non-target species (GLFC, 2008; Siefkes, 2017). The understanding of the biology, ecology, biochemistry and physiology of the sea lamprey, as well as non-target species, have been key in providing the foundation upon which new methods of control can be developed and implemented. The discovery of the selective compound TFM was an innovation founded through toxicological research, and was implemented based on knowledge of the reproductive life-cycle and migratory patterns of the sea lamprey (Applegate, 1950; Applegate et al., 1961). Further toxicological

studies on non-target species and the influence of abiotic factors, such as the dilution of

lampricides flowing downstream, pH and alkalinity, on the toxicity of this compound to fish

allowed for a more accurate determination of the concentrations of TFM required during a

treatment, and increased precision when attempting to achieve those concentrations (Boogaard et

al., 2003; Christie and Goddard, 2003; McDonald and Kolar, 2007; Siefkes, 2018). Physiological

studies have determined the toxic mode of action for TFM in sea lamprey and provide an

understanding of why the compound is selectively toxic (Wilkie et al., 2007; Birceanu et al.,

2009, 2011). Chemosensory cues, genome sequencing, gene knockdown and sterilization

techniques are all currently being researched as substitutes to TFM applications, which would

reduce the total TFM required via sea lamprey control operations (GLFC, 2008; Siefkes, 2017).

The evolution of control tactics requires ongoing research to allow changes to existing protocols

to improve the efficiency of sea lamprey control operations. The findings of this research push

forward the current understandings of the physiological processes that occur within a non-target

species of concern, the lake sturgeon, and provide information that can be used to develop future

control techniques.

Influence of pH, Alkalinity and Body Size on TFM Toxicity for Juvenile Lake Sturgeon

It has been hypothesized that the uptake of TFM is dependent upon the presence of TFM

in its un-ionized, phenolic form (Hunn and Allen, 1974; McDonald and Kolar, 2007; Hlina et al.,

2017). The findings of my study have shown that juvenile sturgeon exposed to TFM in lower

water pH have significantly greater rates of TFM uptake than juvenile sturgeon in waters of

greater pH (Figure 2-2), which support this hypothesis. The observed sensitivities to TFM for

fish in more acidic mediums (Hunn and Allen, 1974; Marking and Olsen, 1975; Bills et al.,

2003) can be attributed to the greater rates of uptake that were observed in waters of lower pH

for juvenile sturgeon, and sea lamprey (Hlina et al., 2017), as a result of greater proportions of

TFM in its un-ionized (phenolic) state, which is more lipophilic.

Alkalinity has been shown to have a protective effect on TFM toxicity for sea lamprey

and brown trout (Bills et al., 2003). However, the recent modeling of in-stream toxicity tests by

O'Connor et al. (2017) demonstrated a reduced protective effect on TFM toxicity for juvenile

lake sturgeon in higher alkalinity waters, when compared to the protective effect for sea lamprey

(based on minimum lethal concentration data from Bills et al., 2003). The explanation for this

relationship with alkalinity was unknown, but can now be attributed to the limited effects that

increasing alkalinity has on suppressing rates of TFM uptake for YOY sturgeon in waters of

higher alkalinity (> 150 mg $CaCO_3$ L^{-1}; Figure 2-3). Sturgeon greater than 130 mm (1+) had

significantly decreasing rates of TFM uptake with increasing alkalinity (> 150 mg $CaCO_3$ L^{-1}),

which suggests that the effects of increasing alkalinity are less for these larger lake sturgeon. The

greater observed sensitivities to TFM for sturgeon of smaller body size (< 100 mm; Boogaard et

al., 2003) can be explained by the several-fold greater rates of TFM uptake observed in YOY

sturgeon (< 100 mm) of the present study, as compared to 1+ sturgeon (> 130 mm). Similar

findings were reported in sea lamprey, in which TFM uptake rates were correlated with routine

rates oxygen consumption and inversely proportional to body size (Tessier et al., 2018).

Below, I propose a model that explains the protective effect of alkalinity is due to the

greater buffering capacity of higher alkalinity waters. I propose that in such waters, acidification

of the gill microenvironment by H^+ and CO_2 excretion is lower at higher alkalinity, decreasing

the amount of lipophilic un-ionized TFM (Figure 4-1). The gills are known to be a main point of acid/base regulation for fish, and the excretion of H^+ and CO_2 occur via epithelial V-ATPase proteins and diffusion, respectively (Wright et al., 1986; Conley and Mallatt, 1988; Rahim et al., 1988; Playle and Wood, 1989; Lin and Randall, 1995; Evans, 2005; Erickson et al. 2006A,B). In a study conducted by Erickson et al. (2006), changes in the pH of expired *vs* inspired waters for rainbow trout were greater in waters of lower alkalinity and correlated with greater rates of chlorinated phenol uptake. This supports the findings of the current study, where waters of higher alkalinity resulted in lower rates of TFM uptake in juvenile sturgeon, which was likely a result of a greater HCO_3^- buffering capacity, and lower water alkalinity resulted in greater rates of TFM uptake.

Influence of Alkalinity and TFM Exposure on the Gills of Juvenile Lake Sturgeon

The present study was able to demonstrate that lake sturgeon readily acclimate to varying water alkalinities (50 to 250 mg $CaCO_3$ L^{-1}), as demonstrated by the absence of any differences in the activity, expression and distribution of Na^+/K^+-ATPase and vacuolar-type H^+-ATPase (V-ATPase) within the mitochondrion-rich (MR) cells of juvenile lake sturgeon gills. Additionally, exposure to TFM at sea lamprey minimum lethal concentrations (MLC; 12 h $LC_{99.9}$; Bills et al., 2003) did not have an influence on the activity, expression or distribution of these ion-transport proteins. Thus, the reduced protection observed for juvenile lake sturgeon exposed to TFM in waters of higher alkalinity (O'Connor et al., 2017) was not caused by changes in the activity, expression or distribution of Na^+/K^+-ATPase and V-ATPase ion-transporters in the gill. Additionally, TFM exposure does not have an effect on the activity, expression or distribution of these ATPase proteins within the gill of juvenile lake sturgeon.

Future Directions and Conclusions

Despite the 90% reductions in sea lamprey abundance within the Laurentian Great Lakes since their peak abundance in the mid 20th century, the GLFC strives to increase the efficiency of treatments by reducing the amounts of lampricides required, and reducing the influence of these treatments on non-target species (Pearce et al., 1980; Smith and Tibbles, 1980; Heinrich et al., GLFC, 1992, 2000; Larson et al., 2003; Morse et al., 2003; Lavis et al., 2003; GLFC, 2008, 2011). The presence of TFM in its un-ionized and ionized state in exposure water has been hypothesized to influence the bioavailability of TFM and thus its toxicity, but further investigation of the influence of the gill microenvironment on the speciation of TFM is required. Hlina et al. (2017) determined that at the lower water pH of 6.86, 13.7% of the total TFM present in the medium was in its un-ionized state ([TFM OH] = 2.71 nmol TFM mL^{-1}; [Total TFM] = 19.85 nmol TFM mL^{-1}), whereas at a higher water pH of 8.74, the amount of TFM in its un-ionized state was more than 98% lower ([TFM OH] = 0.05 nmol TFM mL^{-1}; [Total TFM] = 21.10 nmol TFM mL^{-1}). Although un-ionized TFM was nearly absent from the bulk water, TFM was found to still be taken up, thus the acidification of water crossing the gills could increase the proportion of un-ionized TFM and enhance the uptake of TFM in higher water pH. Thus, calculations of TFM speciation based on bulk-water pH measurements may actually underestimate the bioavailability of TFM, by underestimating the actual concentration of un-ionized TFM in the gill microenvironment. Future investigations should also address the potential for the uptake of TFM in its ionized state via anion transport proteins located on the gills to better explain the differences between the rates of TFM uptake and the rates of speciation of TFM in its ionized or un-ionized state. Organic anion transporters, such as Mrp2 protein

(multi-drug resistance-associated protein 2), could be candidates. Indeed, the genes have been identified in the gills, liver and other tissues of larval and adult sea lamprey, and it is thought to be involved in bile salt homeostasis (Cai et al., 2013). The Mrp2 protein has been located in the brains of killifish (*Fundulus heteroclitus*) and spiny dogfish shark (*Squalus acanthias*; Miller et al., 2002) and in the liver of the little skate (*Raja erinacea*; Rebbeor et al., 2000), but has not yet been localized within the gills of fish other than the sea lamprey (Evans et al., 2005).

Additionally, future studies should also examine the potential life stage dependent differences in TFM sensitivity for lake sturgeon by measuring oxygen consumption to assess respiratory and metabolic demands, and how this might correlate with TFM uptake. Uridine diphosphate glucuronyl transferase (UDPGT) kinetics studies combined with gene expression studies in developing lake sturgeon could provide evidence of detoxification capacities and whether or not they correlate with observed sensitivities for the YOY sturgeon. Indeed, little is known about the capacity for, and the life-stage effects on Phase II detoxification pathways within juvenile lake sturgeon. The degree to which YOY juvenile sturgeon can detoxify TFM should be assessed and compared to more tolerant 1+ sturgeon, as well as TFM sensitive non-target species (channel catfish; *Ictalurus punctatus*), or TFM tolerant non-target fishes (bluegill; *Lepomis macrochirus*); and rainbow trout (*Oncorhynchus mykiss*), which are known to detoxify via glucuronidation (Olson and Marking, 1973; Lech, 1974; Kane et al., 1994; Lech and Statham, 1975; Bussy et al. 2018a,b). It is possible the YOY sturgeon's limited capacity to detoxify TFM could make it more sensitive to TFM toxicity, and the limited, or sufficient capacity for other non-targets to detoxify TFM could explain their known sensitivity, or tolerance (Boogard et al., 2003).

During a recent study by Birceanu (2017, unpublished) it was demonstrated that decreasing the concentration of TFM during treatments (24 h $LC_{99.9}$ instead of 12 h $LC_{99.9}$) and increasing the duration (24 h instead of 9 h) of TFM applications have resulted in 100% mortality of sea lamprey, and significantly reduced sturgeon mortality. This method of application might be developed further utilizing the rates of TFM uptake found for juvenile lake sturgeon in the current study, as well as the rates of uptake at similar pH and alkalinity treatments for sea lamprey. It was shown in this study that 1+ sturgeon have significantly lower rates of TFM uptake as compared to those YOY sturgeon, and sturgeon are known to detoxify TFM via glucuronidation (LeClair, 2015; Bussy et al., 2018a). Thus the "long and low" could be more selective for the 1+ sturgeon by reducing the accumulation of TFM within the body, decreasing the need to detoxify the lampricide. In this manner, a new protocol for the application of TFM could be developed to determine the least amount of TFM required for reaching 100% mortality of sea lamprey during "long and low" conditions, to maximize the efficacy of sea lamprey control, and to reduce or eliminate sturgeon mortality. By lowering the concentration of TFM applied and reducing the need for more frequent treatments in lake sturgeon producing streams (Boogaard et al., 2011), this method could have the potential to not only reduce the effects of TFM on lake sturgeon, but also other non-target species that co-exist in streams infested with larval sea lamprey.

Figure 4-1: Proposed model of the influence of water alkalinity on the rate of 3-trifluoromethyl-4-nitrophenol (TFM) uptake in juvenile lake sturgeon.

There is an inverse relationship between the rate of 3-trifluoromethyl-4-nitrophenol (TFM) uptake and the alkalinity of an exposure medium for juvenile lake sturgeon. TFM is thought to primarily diffuse across the epithelial membranes of the gills when in its un-ionized (phenolic) state, allowing it to be taken up into the blood of the exposed fish. In waters of higher alkalinity, there would be higher concentrations of bicarbonate (HCO_3^-) available to buffer the excretions of acidic compounds (CO_2, H^+) at the gills, raising the pH of the gill boundary layer (Erickson et al., 2006). HCO_3^- in the blood is converted to CO_2 via carbonic anhydrase, which can diffuse across the epithelial membrane of the gills (Bone and Moore, 2008), where it is hydrated with or without carbonic anhydrase within the mucus at the gill-water boundary layer (Wright et al., 1986; Conley and Mallatt, 1988; Rahim et al., 1988; Playle and Wood 1989; Erickson et al. 2006A). Vacuolar type H^+-ATPase (V-ATPase) actively pumps protons across the epithelial membrane to create electro-chemical gradients that favor Na^+ influx across the apical epithelium. Waters of greater pH would have less TFM present in its un-ionized state, which would result in lower rates of TFM uptake, as compared to waters of lower alkalinity, which would subsequently result in lower water pH around the gills. The gradient-colour change (from lighter to dark shade of blue) in the gill-water boundary layer is representative of the reduction of this layer due to the buffering of acidic excretions by water HCO_3^-, reducing the degree of acidification on the bulk-water.
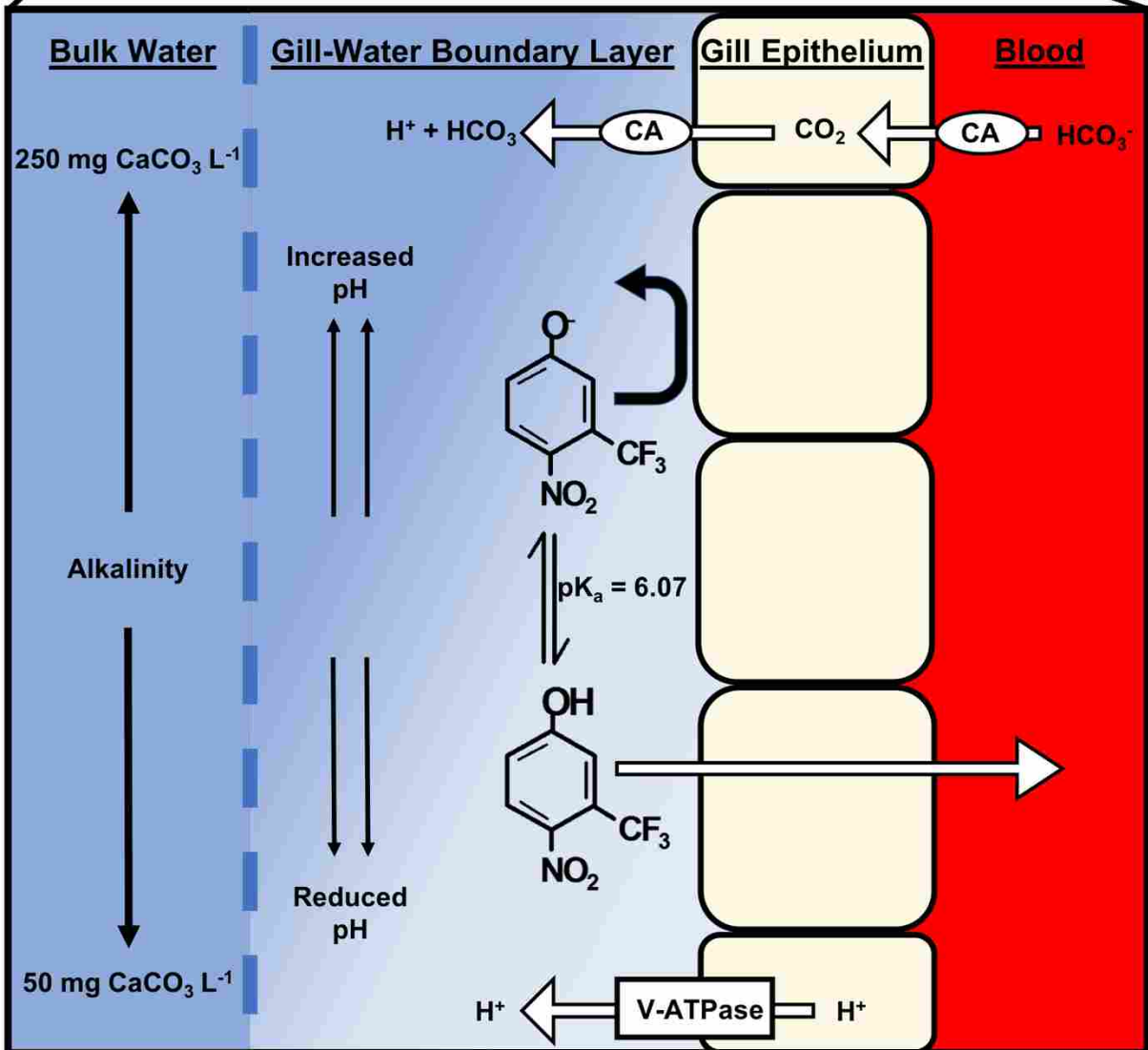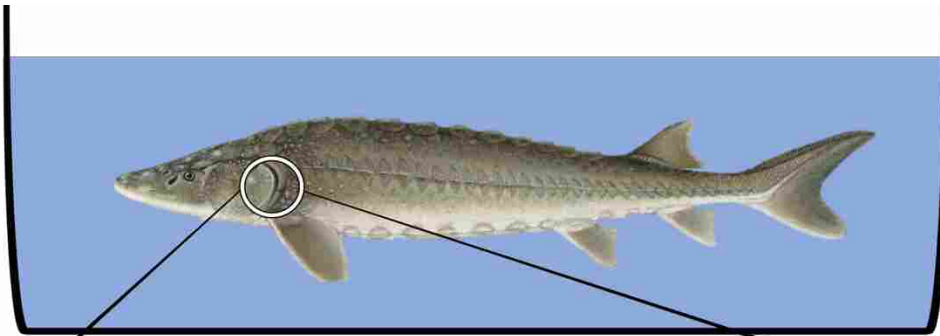
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