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The Influence of Abiotic Factors on the Uptake and Elimination of 3-Trifluoromethyl-4-Nitrophenol by Larval Sea Lamprey (Petromyzon marinus)

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The Influence of Abiotic Factors on the Uptake and Elimination of 3-Trifluoromethyl-4-Nitrophenol by Larval Sea Lamprey (*Petromyzon marinus*)

by

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Abstract

The back bone of sea lamprey (*Petromyzon marinus*) control in the Great Lakes is the use of the piscicide, 3-trifluoromethyl-4-nitrophenol (TFM), which is applied to streams containing larval sea lampreys. However, TFM effectiveness can be influenced by abiotic factors such as pH and temperature, which fluctuate daily and seasonally. The objectives of this thesis were to evaluate the influence of pH, temperature, and season on the toxicity, uptake, and elimination of TFM by larval sea lamprey. Radio-labeled TFM (^{14}C-TFM) was used to determine how TFM uptake rates varied at different water pHs or temperatures during exposure to TFM at the 12 h LC_{50} (4.6 mg L^{-1}) or 12 h LC_{99} (7.6 mg L^{-1}) of larval lamprey. These experiments indicated that TFM uptake rates were 4-5.5 fold greater at pH 6.5 compared to pH 9.0 and whole body TFM accumulation was also greater at pH 6.5. These differences were likely because a greater proportion of the total TFM (sum of ionized plus un-ionized TFM) concentration was in its more lipophilic, un-ionized (phenolic) form at pH 6.5 compared to pH 9.0. Uptake was also greater at 22 °C compared to 6 °C, which was likely caused by increased gill ventilation due to greater metabolic rates at warm temperatures. The effects of pH and temperature on TFM excretion were then tested by injecting lamprey with 100 nmol g^{-1} ^{14}C-TFM and measuring its appearance in TFM-free water over 24 h. In contrast to uptake, elimination rates were 1.3-1.9 fold greater at pH 9.0 than at pH 6.5 during 2-4 h of depuration in TFM-free water. Yet, temperature had no effect on the elimination rates. Season affected the sensitivity of lamprey to TFM, where the 12 h LC_{50} for lamprey was 2.5 fold greater in summer than in spring. Yet, season did not influence whole body TFM concentrations in lamprey that experienced mortality during TFM exposure, averaging between 40-50

nmol g^{-1} wet weight. However, the internal TFM burden was approximately 2.5 fold greater in these fish compared to those that survived. Differences in TFM detoxification capacity may have explained these findings, but whole body concentrations of the metabolite, TFM-glucuronide, were below detection. Neither was mRNA abundance of the enzyme UDP-glucuronosyltransferase (UGT) influenced by season or temperature. TFM applications during spring and fall could increase TFM efficiency when larval sea lampreys are less tolerant to TFM. Applying TFM to large streams in spring or fall should also be considered because it would result in reduced concentrations of TFM needed to control larval sea lamprey populations, resulting in more economical treatments, while still protecting Great Lakes fisheries from sea lamprey parasitism/predation.

I dedicate this research in loving memory of my grandfather, Robert M. Swanson, who taught me how to fish at a young age.

Co-Authorship

Work present in this thesis was completed with the cooperation of Alexandra Muhametsafina and Dr. Oana Birceanu. Seasonal and temperature toxicity experiments of larval sea lamprey exposed to 3-trifluoromethyl-4-nitrophenol (TFM) were conducted jointly with Alexandra Muhametsafina as a part of her M.Sc. thesis and these experiments were done at the Hammond Bay Biological Station, US-Geological Survey, Millersburg, MI, USA. Analyses of mRNA abundance of the enzyme uridine-diphosphate-glucuronosyltransferase were conducted with the assistance of Dr. Oana Birceanu at Wilfrid Laurier University. High performance liquid chromatography analyses of lamprey tissues were conducted by Leslie Bragg in the lab of Dr. Mark Servos at the University of Waterloo, Waterloo, Ontario. I conducted all other experiments and analysis presented in this thesis.

Acknowledgments

First, I would like to thank my supervisor, Dr. Michael Wilkie for his invaluable advice, knowledge, and patience. I am thankful for the support that my committee members, Dr. Tristan A.F. Long and Dr. James McGeer have given me throughout the completion of this thesis. Much gratitude goes to Karen Slaght, Dr. Nicholas Johnson, Dr. Michael Hansen, and the staff at Hammond Bay Biological Station, U.S.-Geological Survey, Millersburg, MI, USA, for the use of their facility to conduct experiments. My sincere thanks also goes to Dr. Scott M. Ramsay and Dr. Deborah MacLatchy for the use of their lab space. Method development for radioactive TFM experiments were done with the help of a labmate, Laura Tessier. I would like to thank the Great Lakes Fisheries Commission and The Brule River Sportsmen's Club for their financial support while completing this thesis. I would like to thank my fellow lab mates for the years of assistance with assays and experiments, support, and laughter. Lastly, I would like to thank my family and friends for their constant support, patience, and compassion throughout this thesis.

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Chapter 1:

Introduction and Literature Review

Sea Lamprey Life Cycle

The invasion of the Great Lakes by parasitic sea lampreys (*Petromyzon marinus*) in the early 20th century decimated its fisheries, and profoundly altered its ecosystem at numerous levels (Great Lakes Fishery Commission 2011). Sea lampreys typically spend the first 3-7 years of their life as larval sea lampreys (ammocoetes), during which they live burrowed in the substrate of streams (Beamish and Potter 1975; Youson 1980). During this stage they are suspension feeders, ingesting detritus, algae, diatoms, and protozoans (Moore and Mallatt 1980; Sutton and Bowen 1994). Once a larval sea lamprey has accumulated sufficient lipid reserves it undergoes metamorphosis, which takes several months (Lowe et al. 1973; O'Boyle and Beamish 1977; Potter et al. 1978; Moore and Mallatt 1980; Youson et al. 1993). However, many other factors besides lipid reserves influence when larval sea lampreys go through metamorphosis (Youson 2003), including stream temperature (Purvis 1980; Young et al. 1990), population density (Manion and Smith 1978; Morman 1987), weight, length, and sex (Potter et al. 1978; Holmes and Youson 1994). Metamorphosis is characterized by complex structural and physiological changes including the development of eyes, a buccal disk with teeth, and a complex restructuring of the intestine, liver, and kidneys (Youson 1980; Sidon and Youson 1983). After metamorphosis juvenile sea lampreys migrate to the ocean in the case of anadromous populations or to one of the Great Lakes in the case of the landlocked populations (Beamish and Potter 1975; Wilkie 2011). Juvenile sea lampreys are parasitic, feeding on the blood of teleosts and other fishes by using their newly developed oral disk and rasping tongue (Figure 1-1; Farmer et al. 1975; Farmer 1980; Araujo et al. 2013; Silva et al. 2013; Silva et al. 2014). The gills are also restructured during metamorphosis,

switching from unidirectional mode of gill ventilation in larval sea lampreys to tidal ventilation, allowing the sea lamprey to irrigate the gills while attached to fish during feeding or to hard, rocky substrate (Lewis 1980; Rovainen 1996).

A sea lamprey will consume the blood of its host for approximately 12-18 months, before migrating back to freshwater streams, where they spawn and then die (Applegate 1950). Sea lampreys do not home to their natal streams, but do use multiple sensory cues to guide their migration and to spawn. Such cues include pheromones secreted by larval sea lamprey and adult male lampreys (which attract spawning sea lampreys to appropriate habitat), photoperiod, water temperature, and stream discharge (Applegate and Smith 1950; Li et al. 1995, 2002; Bjerselius et al. 2000; Binder and McDonald 2008; Johnson and Li 2010; Binder et al. 2010; Johnson et al. 2012, 2015; Chung-Davidson et al. 2013). The ability of sea lamprey to use some (many) of these cues likely explains why they were able to successfully invade and establish populations in the Great Lakes in the early 20th century.

Sea Lamprey Invasion

Sea lamprey entered Lake Erie from Lake Ontario after modifications were made to the Welland Canal during the late 1800's and early 1900s, which allowed them to circumvent Niagara Falls. From that point onward, they were able to launch their invasion of the Upper Great Lakes, where they devastated commercial, recreational, and culturally significant fisheries (Eshenroder & Burnham-Curtis 1999; Eshenroder 1992; GLFC 2011). It remains disputed whether or not sea lampreys are in fact native to Lake Ontario. Comparisons of DNA markers in both the Great Lakes population and the Atlantic ocean populations of sea lampreys to geological timeframes suggest that there

are two distinct populations (Waldman et al. 2004, 2009; Bryan et al. 2005; Eshenroder 2009, 2014). Furthermore, during metamorphosis there is variation in the development of mitochondrial rich cells (MRCs), chloride cells, and salt water tolerance between the anadromous (sea run) and land-locked populations of sea lampreys (Potter et al. 1978; Bartels and Potter 2004; Reis-Santos et al. 2008; Zydlewski and Wilkie 2013). However, the lack of any historical references to sea lampreys prior to the mid-late 1800s in Lake Ontario, and the absence of sea lamprey in any adjacent lakes argues against the presence of an indigenous sea lamprey population in Lake Ontario (Eshenroder 2009). There is no question, however, that sea lamprey have been and continue to be a destructive invasive species in the remaining Great Lakes, in Lake Champlain and in the Finger Lakes in the US Northeast (Heinrich et al. 2003; Eshenroder 2009, 2014; Great Lakes Fishery Commission 2011).

By the mid-1940s and 1950s, sea lamprey populations in all of the Great Lakes had grown exponentially (Applegate 1950; Smith et al. 1974; Heinrich et al. 1980; Smith and Tibbles 1980). Combined with increases in commercial fishing harvest, increased sea lamprey populations led to the collapse of commercial, recreational, and culturally significant fisheries in the Great Lakes (Applegate 1950; Smith and Tibbles 1980; Eshenroder 1992). These fisheries have rebounded since the collapse due to a comprehensive sea lamprey control program introduced in the late 1950s, but fish populations still remain threatened by sea lampreys (Adair and Sullivan 2013; Siefkes et al. 2013). Preferred host species include lake trout (*Salvelinus namaycush*), lake whitefish (*Coregonus clupeaformis*), and other top predators, which are often killed due to direct blood loss or secondary infections (Farmer et al. 1975; Smith and Tibbles 1980). Indeed,

the amount of blood consumed by a juvenile sea lamprey may range from 3-30% of their body weight per day (Farmer et al. 1975), resulting in mortality rates of up to 60% (Swink 2003).

Sea Lamprey Control

Control of sea lamprey populations started in the mid-1940s with the use of physical and electrical barriers to prevent the spawning adult sea lamprey from migrating to desirable spawning grounds (Applegate 1950). These methods of control were not effective at reducing the populations and alternative methods were sought. In the late 1950s and early 1960s, chemical control methods were pursued, and a chemical was discovered, 3-trifluoromethyl-4-nitrophenol (TFM), which was selectively toxic to sea lamprey (Applegate et al. 1961; Applegate and King, Jr. 1962). The use of TFM since its discovery has helped greatly reduce sea lamprey populations in the Great Lakes (Siefkes et al. 2013).

Currently, the sea lamprey control program relies on the following methods of control: operation of low-head barrier dams to prevent up-stream migration and spawning of adult sea lampreys (Lavis et al. 2003), the trapping of upstream migrating sea lampreys to reduce the number of spawning adults (Mullett et al. 2003; Li et al. 2007; Siefkes et al. 2013), and the use of the lampricides TFM and 5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide (niclosamide; Applegate et al. 1961, Howell et al. 1964). Lampricide treatments are the most efficient control method, and are considered the backbone of sea lamprey control (Siefkes et al. 2013). However, lampricide treatment protocols could still be improved to reduce financial costs (McDonald and Kolar 2007), residual sea lamprey populations (Johnson et al. 2014; Swink and Johnson 2014), and minimize adverse

effects on non-target organisms (Boogaard et al. 2003). While most non-target organisms are more tolerant of TFM than sea lampreys, adverse effects to non-target organisms remain an ongoing concern of the sea lamprey control program.

TFM Treatments

Lamprey infested streams are treated every two to three years, with selection based on Empirical Stream Treatment Ranking (ESTR) models (Hansen et al. 2003). With such models, streams are selected based on the number of resident larval sea lamprey expected to metamorphose the following year. Streams with the most favorable costs to benefit ratio are usually treated (Hansen et al. 2003; Slade et al. 2003). The likelihood of metamorphosis is based on calculating the condition factor [CF= $(\text{Mass}/(\text{Length}^3)) \times 10^6$; Holmes and Youson 1994]. Streams with a high proportion of animals with a CF of 1.5, and minimum length of 120 mm and mass of 3.0 g, are considered most likely to have large numbers of metamorphosing animals the following year, and are selected for treatment (McDonald and Kolar 2007).

Factors that can influence the effectiveness of TFM treatments are larval sea lamprey abundances, year class and size structure, and water chemistry and stream discharge (Hansen et al. 2003). The dose of TFM applied is based on the mean lethal concentration (MLC) of TFM necessary to kill 99.9% of larval sea lamprey over 9 h. Typically, concentrations used in treatments range from 1.2-1.5 times the MLC (McDonald and Kolar 2007). Because the MLC is significantly affected by differences in water pH and alkalinity, the MLC for each population to be treated with TFM may be determined from stream side toxicity tests, or tables that summarize lab toxicity tests under a wide range of water pHs and alkalinities (Figure 1-2; Bills et al. 2003). However,

other factors such as temperature or season, also appear to affect TFM treatment effectiveness (Scholefield et al. 2008).

Areas for Improvement

Treatment of sea lamprey infested streams with TFM is approximately 90% effective, but juvenile sea lamprey populations have increased since the early 2000s in all five of the Great Lakes (Adair and Sullivan 2013). Wounding rates on lake trout have also recently been above target levels and have drawn concerns (Adair and Sullivan 2013). Increases in sea lamprey abundance could be due to one or more of the following: (1) increased reproduction of sea lampreys upstream of deteriorating barrier dams, (2) reduced lampricide control efforts throughout the 2000s, (3) increased survival of juvenile sea lampreys due to changes in the fish-community, and (4) greater abundances of residual larval sea lampreys (Adair and Sullivan 2013; Siefkes et al. 2013; Johnson et al. 2014). One way to address these challenges is to improve lampricide treatment effectiveness. To achieve this goal, both Adair and Sullivan (2013) and Siefkes et al. (2013) emphasize the need for further research on the modes of uptake and excretion of TFM, and how external factors could influence TFM toxicity. Increased abundance of residual larval sea lampreys could be caused by sub-optimal TFM treatments, which currently may not consider the full effect of water chemistry or seasonal differences in TFM sensitivity (e.g. Bills et al. 2003; Scholefield et al. 2008). By improving our understanding of how these factors influence TFM efficacy, it may be possible to modify treatment regimens to lower sea lamprey populations from their current levels, and reduce the impacts of sea lamprey on lake trout and other fisheries.

The Role of Seasonality in TFM Toxicity

Applegate et al. (1961) observed that larval sea lamprey were most susceptible to TFM during late fall, winter, and early spring, with greatest tolerance to TFM in mid- to late summer. Even though Applegate et al. (1961) emphasized that TFM treatments would be the least cost effective during summer months, for practical reasons (e.g. availability of staff, access to streams, more predictable weather) TFM treatments occur during July and August. Scholefield et al. (2008) confirmed that tolerance of larval sea lampreys to TFM varied with season, with low tolerance during the spring months and greater tolerance in mid-summer. They suggested seasonal variation in pH, temperature, feeding activity, habitat, photoperiods, nutritional level, and/or lipid content potentially explained their observations (Scholefield et al. 2008). As previously noted, pH-alkalinity models are used to ensure that the dose of TFM used kills the majority larval sea lamprey, but models do not consider seasonal changes in TFM toxicity. Nor does the pH-alkalinity model consider how factors such as temperature, nutritional status, or the health of the animals affect TFM sensitivity. Therefore there is a need to better understand the physico-chemical and biological factors that result in seasonal variation in the sensitivity of sea lampreys to TFM.

Determination of the Effects of Water Chemistry on the Uptake and Excretion of TFM

It is known that the toxicity of TFM is influenced by the pH of the treatment stream due to the chemical nature of TFM(Kanayama 1963; Hunn and Allen 1974; Bills et al. 2003; Hubert 2003). TFM is a weak acid, with a pK_a of 6.07, the pH at which 50 % of the TFM is in its un-ionized state (phenolic form) and 50 % is in its ionized form (phenolate ion; Hunn and Allen 1974; Hubert 2003). Under low pH conditions, there is

more un-ionized TFM which is thought to be more toxic than ionized TFM because it is more lipid soluble and can presumably easily cross the gills and enter the blood stream (Smith et al. 1960; Applegate 1961; Hubert 2003; McDonald and Kolar 2007).

A practical implication of the pH dependency of TFM is that treatment effectiveness could be altered due to diurnal changes in water pH (Odum 1956; McDonald and Kolar 2007). Diurnal changes in pH can vary between 0.1 to 1.0 units or more, due to photosynthesis and aerobic respiration by algae, phytoplankton and aquatic vegetation (Wetzel 1983). Additionally, stream pH could vary seasonally due to agricultural runoff, ground water sources, and land use practices (Hynes 1970;Tiedemann et al. 1988; Campbell and Doeg 1989; Poudel et al. 2013;). Stream pH within the Great Lakes Basin will also vary between streams due to geomorphological features and precipitation events. By developing a better understanding of how water pH influences TFM toxicity, it may be possible to improve the effectiveness of TFM treatments. Thus, one goal of my M.Sc. thesis was to determine how variation in water pH influences TFM uptake and excretion by larval sea lamprey.

In addition to variations in pH, stream temperature also varies seasonally, which could influence TFM toxicity (Allan 1995; Scholefield et al. 2008). Stream temperature affects fish metabolism since they are poikilotherms (Gehrke and Fielder 1988). Since metabolic rates are elevated under warm temperatures, respiration, movement, food consumption, digestion and detoxification will all occur at increased rates (Hill et al. 2008). The mode of uptake of TFM is thought to occur by passive diffusion across the gills (Hunn and Allen 1974). Under warmer temperatures respiration increases which could result in an increase in the uptake rates of TFM during treatments due to increased

irrigation of the gill with TFM-laden water (O'Boyle and Beamish 1977; Lewis 1980). Additionally, TFM excretion rates in surviving fish could increase following TFM exposure at warmer temperatures because TFM detoxification pathways could be up-regulated, and/or the outward diffusion of TFM across the gills elevated (Lewis 1980; Koivusaari et al. 1981; Koivusaari 1983; Hänninen et al. 1984). Thus, another goal of my thesis was to determine how the excretion of TFM by larval sea lamprey is influenced by different temperatures.

Variation in TFM Detoxification Capacity

To determine if there are differences in the TFM detoxification capacity of larval sea lamprey at different times of the year, the possibility of changes in glucuronidation capacity were examined. In non-target fishes, TFM is detoxified by the process of glucuronidation (Lech and Costrini 1972; Lech and Statham 1975; Kane et al. 1994), a Phase II detoxification pathway used in the biotransformation and elimination of xenobiotic compounds that have poor water solubility (Dutton 1980). This biotransformation process makes xenobiotic compounds more water soluble, which makes it easier to excrete the substance via the bile or urine. This is achieved by conjugating a UDP-glucuronic acid (UDPGA) molecule to the xenobiotic compound, usually within the liver, but it can also occur in the kidneys, gills, intestine, and heart (Dutton 1980; Timbrell 2009). Glucuronidation is facilitated by the enzyme uridine-diphosphate-glucuronosyltransferase (UGT) in the rough endoplasmic reticulum (RER) of the cell (Dutton 1980). Limiting factors in glucuronidation are the amounts of UGT and/or UDPGA. Unfortunately, most studies of glucuronidation in fish have been directed towards understanding how fish deal with organic pollutants instead of

pesticides. This is due to the industrial release of organic pollutants into freshwater or marine environments (Clarke et al. 1991; Leaver et al. 2007).

Glucuronidation of TFM has been observed in rainbow trout (*Oncorhynchus mykiss*), channel catfish (*Ictalurus punctatus*), and bluegill (*Lepomis macrochirus*; Lech and Costrini 1972; Lech 1974; Lech and Statham 1975; Kane et al. 1994; Vue et al. 2002). In contrast, the capacity of sea lamprey to use glucuronidation appears to be limited (Lech and Costrini 1972; Kane et al. 1994), which likely explains their greater sensitivity to TFM. However, seasonal variation in the capacity of sea lamprey to glucuronidate TFM has not yet been investigated. Thus, a final goal of my thesis was to determine if the greater tolerance of sea lamprey to TFM in the summer was due to a greater capacity to detoxify TFM using glucuronidation during the summer months.

Research Objectives

Although the effect of water chemistry on TFM toxicity has been thoroughly studied (Kanayama 1963; Hunn and Allen 1974; Dawson et al. 1975; Marking and Olson 1975; Kawatski and Bittner 1975; Seelye et al. 1988; Bills et al. 2003; McDonald and Kolar 2007), how water chemistry specifically influences the uptake and excretion of TFM is unknown. Accordingly, the overarching goal of my thesis was to determine the influence that different water chemistries and season have on the uptake, detoxification, and elimination of TFM by larval sea lamprey. To achieve this goal, the objectives of my thesis were to:

I. Determine how differences in water pH influence the rates of uptake and elimination of TFM by larval sea lamprey (Chapter 2).

II. Ascertain how warmer water temperatures affect rates of TFM uptake and elimination in larval sea lamprey, and determine if this contributes to their greater TFM tolerance in the summer (Chapter 3).

III. Characterize how the capacity of larval sea lampreys to detoxify TFM using glucuronidation changes with season and to determine if this contributes to their greater TFM tolerance during the summer months (Chapter 3).

To address these objectives, radio-labeled TFM (^{14}C-TFM) was used to measure the rates of TFM uptake and clearance when larval sea lampreys were exposed to TFM under different pH and temperature regimens. To determine if the capacity of sea lamprey to detoxify TFM changed with season, high performance liquid chromatography (HPLC) was used to measure TFM and TFM-glucuronide concentrations in sea lamprey exposed to TFM at different times of the year. In addition, the mRNA abundance of UGT in larval sea lampreys was measured, using quantitative polymerase chain reaction (QPCR), in spring, summer, and fall and at different temperatures.

Figure 1-1. Life cycle of a sea lamprey (*Petromyzon marinus*).

A) Larval phase sea lamprey live burrowed into the substrate of tributaries flowing into the Great Lakes. B) After 3-7 years they undergo a complex metamorphosis that leads to the development of an oral disk and rasping tongue which is used to consume the blood of its host. They feed and live in this parasitic, juvenile phase for 12-18 months, before C) migrating upstream, to spawn and then die (Wilkie 2011).

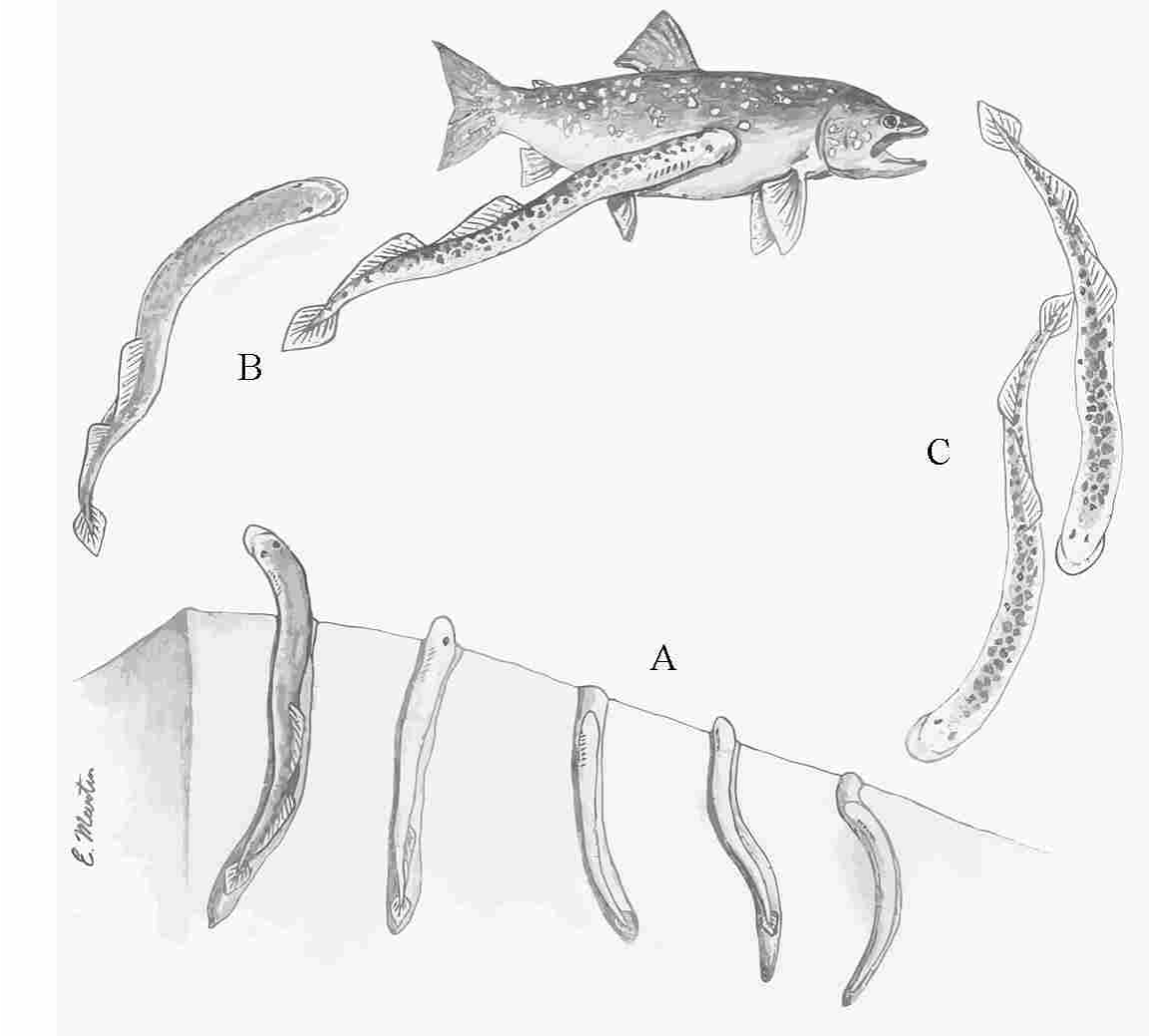


Figure 1-2. Chemical structure of 3-trifluoromethyl-4-nitrophenol (TFM) and its dissociation equilibria.

3-trifluoromethyl-4-nitrophenol is a organic weak acid, with a pK_a of 6.07, which is the pH where 50 % of the total TFM is in its un-ionized (phenolic) form and 50 % is in its ionized (phenolate ion) form. At acidic pH a greater proportion of TFM is in its un-ionized form, compared to alkaline pH(Hunn and Allen 1974; Hubert 2003).

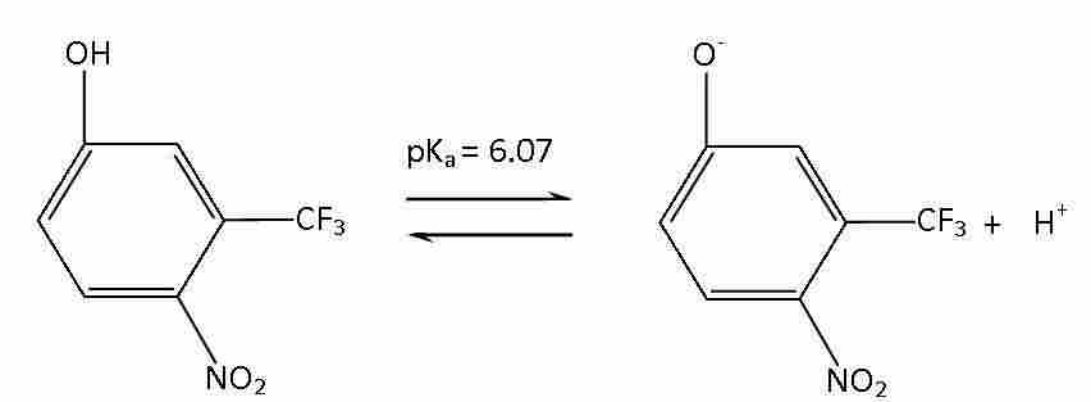
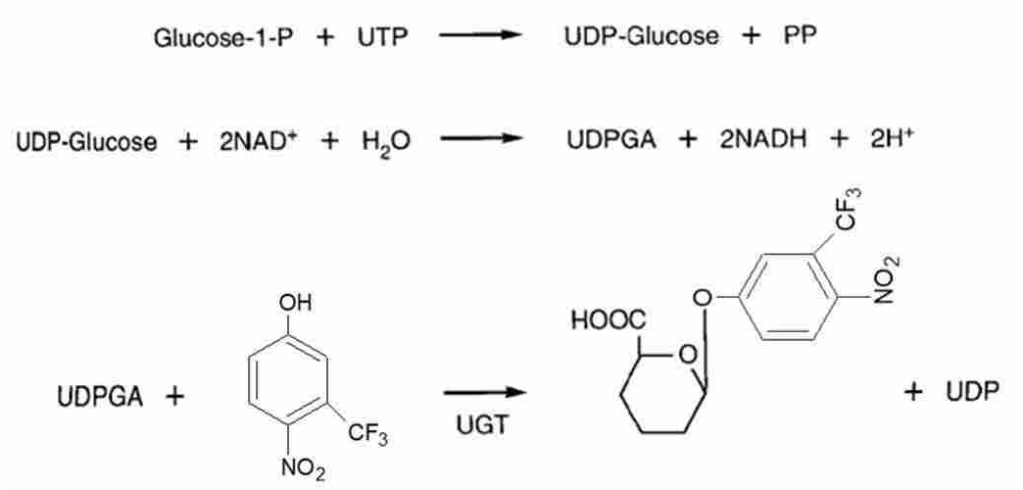

OH
CF3
NO2
pKa = 6.07
O-
CF3 + H+
NO2

Figure 1-3. The detoxification of 3-trifluoromethyl-4-nitrophenol (TFM) through glucuronidation.

The substrate, UDP-glucuronic acid (UDPGA), is generated from glucose-1-phosphate, an intermediate of glycogenolysis. Glucose-1-phosphate then reacts with uridine-triphosphate (UTP) to form UDP-glucose and pyrophosphate (PP). UDP-glucose is then hydrolyzed to form UDPGA. The enzyme, uridine-diphosphate-glucuronosyltransferase (UGT) facilitates the attachment of glucuronic acid to organic, poorly soluble molecules such as TFM, to form TFM-glucuronide. The glucuronidated molecule is more hydrophilic and easier to excrete via the gastrointestinal tract and/or via renal pathways (Dutton 1980; Timbrell 2009).


Glucose-1-P + UTP → UDP-Glucose + PP
UDP-Glucose + 2NAD+ + H2O → UDPGA + 2NADH + 2H+
UDPGA +
OH
CF3
NO2
UGT
HOOC
O
O
CF3
NO2
+ UDP

Chapter 2:

The Effects of Water pH on Uptake and Elimination of 3-Trifluoromethyl-4-Nitrophenol (TFM) by Larval Sea Lamprey

Introduction

The piscicide, 3-trifluoromethyl-4-nitrophenol (TFM) has been used to control invasive sea lamprey (*Petromyzon marinus*) populations in the Great Lakes since the early 1960s (Applegate et al. 1961; Hubert 2003; McDonald and Kolar 2007). Applied at regular intervals to nursery streams and rivers containing larval sea lampreys, TFM specifically targets the animals in their burrows, where they live as relatively sedentary, suspension feeders (Beamish and Potter 1975; Moore and Mallatt 1980; Sutton and Bowen 1994). 3-Trifluoromethyl-4-nitrophenol uncouples oxidative phosphorylation (Niblett and Ballantyne 1976; Birceanu et al. 2011) which interferes with mitochondrial ATP production leading to a depletion of energy reserves, particularly glycogen and phosphocreatine or phosphoarginine, and eventually causing death (Viant et al. 2001; Wilkie et al. 2007a; Birceanu et al. 2009; Clifford et al. 2012). The specificity of TFM is related to the relative inability of larval sea lampreys to detoxify TFM using glucuronidation (Lech and Statham 1975; Kane et al. 1994). In non-target fishes, this conjugation reaction adds a glucuronic acid functional group to TFM making it easier to excrete via the gastrointestinal or urinary tract (Dutton and Montgomery 1958; Dutton 1980; Clarke et al. 1991).

The toxicity of TFM is also influenced by a variety of biotic and abiotic factors. Factors such as life stage (Henry et al. 2015), season (Scholefield et al. 2008), and water chemistry (Bills et al. 2003) can markedly influence TFM toxicity and the effectiveness of TFM treatments. Ineffective treatments can lead to residual larval sea lampreys that can survive TFM exposure, and subsequently complete metamorphosis (McDonald and Kolar 2007; Hansen and Jones 2009). Water pH is the most important chemical factor to

influence TFM toxicity, because changes in pH can affect the amount of un-ionized and ionized TFM, pK_a =6.07 (Kanayama 1963; Hunn and Allen 1974; Hayton and Stehly 1983; Bills et al. 2003). In its un-ionized (phenolic) form, TFM is more toxic than ionized (phenolate ion) TFM because it is more lipophilic and thought to cross the gills more readily (Hunn and Allen 1974; Hayton and Stehly 1983). However, this hypothesis has not been directly tested in larval sea lampreys. The toxicity of TFM to larval sea lampreys does in fact increase with reductions in water pH (Bills et al. 2003), which was thought to be the result of a greater proportion of phenolic TFM existing at lower pHs (Figure 2-1). Indeed, Hunn and Allen (1974) demonstrated that TFM accumulation was greater in rainbow trout (*Oncorhynchus mykiss*) at more acidic pHs, but they did not test the effects of TFM on larval sea lamprey, the target of TFM treatments. Currently, there is no evidence that rates of TFM uptake are in fact greater in larval sea lampreys at lower pHs. This question is not just of academic importance but one of management importance because the pH of streams in the Great Lakes varies widely (McDonald and Kolar 2007). Streams also experience diurnal swings in water pH due to photosynthesis by plants, algae and cyanobacteria and the respiratory processes of aquatic organisms (Odum 1956; Cummins 1974; Wetzel 1983). By altering TFM speciation, such changes in water pH could markedly affect TFM treatment effectiveness by altering rates of TFM uptake (Bills et al. 2003; McDonald and Kolar 2007).

Changes in water pH could also affect rates of TFM elimination because of the sea lampreys' limited capacity to use glucuronidation to detoxify TFM (Lech and Statham 1975; Kane et al. 1994). In its phenolic (un-ionized) form, TFM is likely eliminated via passive diffusion down its concentration gradient across the gills. Differences in the gill

microenvironment pH, in which gill boundary layer pH is lower than bulk water pH due to CO_2 and metabolic acid excretion at the gill (Playle and Wood 1989), could also alter the speciation of TFM, leading to differences in the inward gradients favouring un-ionized TFM uptake and electrochemical gradients favouring inward ionized TFM uptake. Increases in external water pH could also result in a reversal of the TFM gradient, due to more ionized TFM persisting, which could generate an outward gradient of un-ionized TFM that would lead to passive TFM diffusion out of the animal, by trapping the TFM in the water in its ionized form. This would lower treatment effectiveness and increase the likelihood of survival of sea lampreys when the treatment stream's pH is more alkaline.

A primary goal of this study was to use ^{14}C-TFM to measure rates of TFM uptake in larval sea lampreys acclimated to acidic (pH 6.5), moderate (pH 7.8), and alkaline pH (pH 9.0). To test the hypothesis that TFM elimination does in fact take place via passive diffusion across the gills, and that TFM excretion is augmented by increases in external water pH, larval sea lampreys were injected with ^{14}C-TFM, and the rates of TFM clearance were also measured at different pHs.

Method and Materials

Experimental Animals and Set-up

Great Lakes' strain larval sea lampreys were captured by pulsed-DC electrofishing (ABP-2 Electrofisher, Electrofishing Systems, LLC, Madison, WI, USA) from streams and tributaries of Lake Huron or Michigan. These lampreys were acquired from the Hammond Bay Biological Station, United States Geological Survey, Millersburg, MI, USA, in 2014 and 2015. The larvae were housed in 110 L fiberglass aquaria at 12 h dark: 12 h light intervals in the aquatics facility at Wilfrid Laurier University. Tanks were lined with 3-5 cm of sand to provide the animals with burrowing substrate. The tanks continuously received aerated well water (pH 7.9 ± 0.4; hardness = 460 mg L^{-1} as $CaCO_3$; dissolved oxygen ≥ 80 % saturation; T = 13 ± 2 °C) at a flow rate of approximately 500 mL min^{-1}, and the animals were fed a slurry of Baker's yeast (1 g of yeast per lamprey) once per week. All experiments were approved by the Wilfrid Laurier Animal Care Committee, and followed guidelines and principles of the Canadian Council of Animal Care (CCAC).

Experimental Procedures

Acclimation to Different Water pHs

One week prior to experimentation, larval sea lampreys were acclimated to a desired pH of 6.5, 7.8 or 9.0 ± 0.2 in a glass aquaria (Volume = 40 L), with the bottom lined with 3-5 cm of sand to provide burrowing substrate. The pH was maintained using a Radiometer PHM82 pH meter connected to a TTT80 autotirator (Radiometer Copenhagen, Denmark) which regulated the drop-wise addition of 0.5 M HCl or 0.5 M

KOH into the aquarium water using a solenoid valve (P/N: 01540-02, Cole Parmer, IL, USA). One day prior to experimentation, the larvae were transferred in groups of 4 to darkened, plastic, 1 L containers which continuously received water of the appropriate pH at a rate of 100-200 mL min^{-1}, and were left to acclimate overnight. The containers were positioned in a recirculating system (150 L), also equipped with a pH-stat control system, in which the pH-corrected water drained from a head tank (Volume = 50 L) into each container via a flow-splitter at approximately 100-200 mL min^{-1}, before draining into a lower reservoir (Volume = 50 L) from which the water was returned to the head tank using a submersible pump. Immediately prior to each series of experiments, water flow was cut-off to the holding containers for measurement of TFM uptake or excretion using ^{14}C-TFM (see below).

Experimental Protocols

Rates of TFM Uptake

Two series of experiments were completed to measure rates of TFM uptake. In the first series, larval sea lamprey (n=12; 0.9 g ± 0.02 ; 90 mm ± 0.44) were exposed to the 12 h LC_{50} of TFM (LC_{50} = 4.6 mg L^{-1} TFM) which was previously determined at circumneutral pH (pH ~ 8.0; Birceanu et al. 2009). In these experiments, rates of TFM uptake were measured using ^{14}C-TFM over 3 h. In the second series, TFM uptake was measured in larval sea lamprey (n=12; 1.1 g ± 0.01; 90 mm ± 0.35) exposed to a concentration of TFM that was equivalent to the 12 h LC_{99} of TFM (LC_{99} = 7.6 mg L^{-1}; also measured at circumneutral pH), but over 1 h. Acclimation and exposure water quality parameters are reported in Table 2-1 for both exposures. Prior to each TFM uptake experiment, water flow to each container was shut off, and the container volume

adjusted to 750 mL. This was then immediately followed by the addition of 2.5 µCi of ^{14}C-TFM, from a 1.0 mCi stock (^{14}C-TFM provided courtesy of Dr. T. Hubert, Upper Midwest Environmental Sciences Center, US-Geological Survey, LaCrosse, WI, USA; DuPont/New England Nuclear, DE, USA) to each container, followed by sufficient non-radioactive (cold) field grade TFM (35% active ingredient dissolved in isopropanol; provided courtesy of Department of Fisheries and Ocean, Canada; Clariant SFC GMBH WERK, Griesheim, Germany) to yield nominal concentrations of either 4.6 mg L^{-1} or 7.6 mg L^{-1}, depending upon the experiment. The containers were then covered, and left for 10 min to allow the non-radioactive and radioactive TFM to thoroughly mix. Water samples (10 mL) were subsequently taken at 0 and 1 h for series two experiments, plus an additional sample at 3 h for series one experiments. The pH of each container was monitored hourly using a handheld pH meter and electrode (pH 11 meter, Oakton Instruments, IL, USA) and when necessary, adjusted to the desired pH using 0.1 M HCl or 0.1 M KOH using a Pasteur pipette. Following each experiment the tail of the lamprey was lightly pinched using forceps to confirm survival. After which, the animals were euthanized with an overdose of tricaine methanesulfonate (1.5 g L^{-1}; MS-222; Syndel Labs, Port Alberni, BC, Canada) buffered with 3.0 g L^{-1} $NaHCO_3$. The animals were then thoroughly rinsed in non-radioactive water of the same chemistry and pH for 30 s, followed by 50 mg L^{-1} non-radioactive TFM for 30 s to remove any surface bound radioactive TFM, before a final rinse in non-radioactive water (~30 s). Each whole animal was then transferred to a 15 mL polypropylene, centrifuge tubes (05-527-90, Corning Incorporated-Life Sciences, NY, USA) for subsequent digestion in 10 mL of 1N HNO_3 and processing prior to measuring whole animal radioactivity.

Rates of TFM Excretion

After the overnight acclimation period (12 h) in their experimental chambers, measurements of TFM excretion were measured in each group of pH acclimated sea lampreys (n=8 each pH; 1.0 g ± 0.02;100 mm ± 0.27) at the appropriate pH (pH = 6.5, 7.8 or 9.0 ± 0.2). The larval sea lamprey were first anaesthetized one at a time using 0.2 g L^{-1} of MS-222 buffered with 0.4 g L^{-1} $NaHCO_3$, followed by the intraperitoneal (IP) injection of 100 nmol TFM g^{-1} fish containing 0.005 µCi ^{14}C-TFM g^{-1} fish in sea lamprey saline (100mM NaCl, 5mM KCl, 2mM $CaCl_2$, 2mM $MgSO_4$, 3.5mM NaH_2PO_4, and 5.5mM Glucose, pH 7.4). The total volume of ^{14}C-TFM-labeled solution injected was equivalent to 2 % of each larval sea lamprey's mass. The mean specific activity (MSA) of the injection solution was 98.7 counts per min (CPM) $nmol^{-1}$. The injection site was inspected for leakage of the solution, before returning the lamprey to its container. Immediately after returning the animal to its container, a water sample (10 mL) was collected to obtain background measurements of radioactivity, and the animals left for 1 h to allow the ^{14}C-TFM-labeled solution to uniformly distribute throughout the body. Water samples (10 mL) were then collected for determination of TFM excretion rates from 0-4 h (water samples collected at 0, 1, 2, 4 h), 4-12 h (4, 6, 8, 10, 12 h), and 24 h. After each flux measurement period (0-4 h, 4-12, 24 h) the water in the container was replaced with fresh non-radioactive water which was at the same pH (6.5, 7.8 or 9.0), before initiating the next measurement. Water samples were refrigerated until processed for measurement of ^{14}C-radioactivity and total TFM concentrations. The pH of each container was monitored every hour for 12 h, and was adjusted to the desired pH as described above. Twenty-four h post injection, the lampreys were pinched on the tail to determine

survivorship and subsequently euthanized using 1.5 g L^{-1} MS-222 buffered with 3.0 g L^{-1} $NaHCO_3$. The lamprey were then removed from their containers, thoroughly rinsed in non-radioactive water for 30 s, followed by 50 mg L^{-1} non-radioactive TFM for 30 s to remove any surface bound radioactive TFM, before a final rinse in non-radioactive water for 30 s. Lamprey were subsequently digested and measured for whole body radioactivity as previously described.

Analytical Methods

Whole Body Beta-Radioactivity Measurements

Lamprey were digested at 60 °C for 48 h (Blewett et al. 2013), vortexed at regular intervals to ensure thorough digestion of the carcass, and then centrifuged for 5 min at 1228 x *g* to separate the supernatant from digested carcass (IEC Medilite 12,Thermo Electron Corporation, MA, USA). Aliquots of supernatant (2 mL) were then removed and added to 7 mL scintillation vials in duplicate, followed by 4 mL of Ultima Gold™ AB (PerkinElmer, MA, USA) organic scintillation cocktail, vortexed, and left overnight in the dark to minimize chemiluminescence prior to quantifying whole body beta radiation. Water samples (2 mL) were mixed with 4 mL of Optiphase Hisafe 2 (PerkinElmer, MA, USA) scintillation cocktail, vortexed, and also left in the dark for 12 h prior to determining beta radioactivity. A scintillation counter (LS6500, Beckman Coutler, Brea, CA, USA) was used to determine the beta counts per min (CPM) of tissue and water samples. Precision standards (0, 4, 8, 12 mg L^{-1} TFM, prepared by Department of Fisheries and Oceans, Canada) were used to spectrophotometrically determine the non-radioactive TFM concentration in water samples using a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, England, UK) at a wavelength of 395 nm according to

the Standard Operating Procedures of the Department of Fisheries and Oceans, Sea Lamprey Control Centre, Sault Ste. Marie, Ontario, Canada (IOP: 012.4).

Calculations

Uptake

TFM uptake rates (nmol g^{-1} h^{-1}) were calculated using equation 1 (Blewett et al. 2014):

$$\text{TFM Uptake Rate} = \frac{\text{CPM}_{\text{Lamprey}}}{\text{MSA} \times \Delta\text{T}} \quad (1)$$

where $CPM_{lamprey}$ is the counts per min (CPM g^{-1}) tissue in each larval sea lamprey, MSA is the mean specific activity of ^{14}C-TFM (CPM nmol TFM^{-1}) after a 3 h exposure to the 12 h LC_{50} or a 1 h exposure at the 12 h LC_{99}, and ΔT is the duration of exposure to TFM (h). Internal TFM concentrations were determined by taking the $CPM_{lamprey}$ (CPM g^{-1}) and dividing it by MSA (CPM $nmol^{-1}$ TFM) using equation 2.

$$\text{TFM Burden} = \frac{\text{CPM}_{\text{Lamprey}}}{\text{MSA}} \quad (2)$$

Excretion

TFM-efflux rates were determined by calculating the difference in water radioactivity at the beginning and end of a sampling period. This data was then used to determine the TFM-efflux rate ($J^{14C\text{-}TFM}$) in CPM g^{-1} h^{-1}(Wilkie et al. 2007b) using equation 3:

$$J^{14_{C-TFM}} = \frac{\text{CPM}_i - \text{CPM}_f}{\text{T} \times \text{M}} \quad (3)$$

where CPM_i and CPM_f denote the initial and final CPM in the water for a given flux period, T is the flux period duration (h), and M is the mass of the lamprey (g). After $J^{14C\text{-}TFM}$ was calculated, the TFM clearance rate (nmol g^{-1} h^{-1}; Wilkie et al. 2007) was calculated using equation 4:

$$\text{TFM Clearance} = \frac{J^{14\text{C-TFM}}}{\text{MSA}} \quad (4)$$

where MSA is the mean specific activity (CPM g^{-1} nmol TFM^{-1}) of TFM injected into the larval sea lamprey.

Percent excreted was determined by first calculating the amount of TFM remaining (TFM burden) as described using equation 2 (above)-and subtracting this value from the amount of TFM injected (TFM_{intial}; nmol g^{-1}) and then dividing by the concentration of TFM injected (TFM_{intial}; nmol g^{-1}) as described in equation 5 below:

$$\text{Percent Excreted} = \frac{\text{TFM}_{\text{intial}} - \text{TFM}_{\text{Final}}}{\text{TFM}_{\text{intial}}} \times 100 \quad (5)$$

^{14}C-TFM standards (0, 0.001, 0.005, 0.01, 0.05, and 0.1 µCi mL^{-1} ^{14}C-TFM) were prepared and used to determine beta counter efficiency (94.7 %). Digested non-radioactive lamprey were also spiked with increasing ^{14}C-TFM concentrations and compared to spiked water samples to determine quench in the tissue digests. No quenching was observed.

Statistical Analysis

All data are presented as the mean ± 1 standard error of the mean (SEM). Excretion rates are all reported as the absolute values. The rates of TFM uptake, excretion, and percent TFM excreted were analyzed using one-way analyses of variance (ANOVA) followed by a Tukey Honest Significant Difference post-hoc tests when data were normally distributed and homoscedastic. When assumptions were not met, even after data transformation (Log_{10} or power transformations), a Kruskal-Wallis rank sum test followed by a Dunn's multiple comparison test was used. Linear regression analyses were used to evaluate the relationship between uptake rates and the concentration of

either un-ionized or ionized TFM at different pHs. For all statistical tests, the level of significance was set at $P < 0.05$. The Statistical analyses and figures were produced using R version 3.1.3, RStudio version 0.98.1103, and ggplot2, ISBN: 978-0-387-98140-6.

Results

Effect of pH on Rates of TFM Uptake

The TFM uptake rate for larval sea lampreys exposed to a nominal TFM concentration of 4.6 mg TFM L^{-1} (measured TFM= 4.16 mg TFM L^{-1}) for 3 h was 18.0 nmol g^{-1} h^{-1} (SEM ± 1.9) at pH 6.5 (measured pH = 6.87), compared to rates of 15.9 nmol g^{-1} h^{-1} (SEM ± 0.7) at pH 7.8 (measured pH = 8.06), and 3.5 nmol g^{-1} h^{-1} (SEM ± 0.2) at pH 9.0 (measured pH = 8.74; Figure 2-2, A). The TFM Uptake rates observed at pH 6.5 were significantly greater than rates observed at pH 9.0 ($P \leq 0.001$; Kruskall Wallis, Dunn's Multiple Comparisons test) and rates observed at pH 7.8 were greater than rates observed at pH 9.0 ($P \leq 0.001$). A similar trend was observed in larval sea lamprey exposed to the higher nominal TFM concentration of 7.6 mg L^{-1} (measured TFM= 7.03 mg TFM L^{-1}) for 1 h, where TFM uptake rates were 49.5 nmol g^{-1} h^{-1} (SEM ± 3.1) at the lowest pH (measured pH of 6.85), compared to 46.7 nmol g^{-1} h^{-1} (SEM ± 4.9) at pH 7.8 (measured pH = 7.8), and 12.6 nmol g^{-1} h^{-1} at pH 9.0 (SEM ± 1.0; measured pH = 8.82; Figure 2-2, B). Whereas the uptake rates observed at pH 6.5 were significantly greater than rates observed at pH 9.0 ($P \leq 0.001$) and rates observed at pH 7.8 were signficantly greater than rates observed at pH 9.0 ($P \leq 0.001$; 1-way ANOVA; Tukey HSD).

The internal TFM burden for sea lampreys exposed to a nominal TFM concentration of 4.6 mg TFM L^{-1} (measured TFM= 4.16 mg TFM L^{-1}) for 3 h was 51.8 nmol g^{-1} (SEM ± 6.3) at pH 6.5 (measured pH = 6.87), compared to 48.5 nmol g^{-1} (SEM ±1.9) at pH 7.8 (measured pH = 8.06), and 9.9 nmol g^{-1} (SEM ± 1.1) at pH 9.0 (measured pH = 8.74; Figure 2-2, C). The internal TFM burdens at pH 6.5 were determined significantly greater than TFM burdens observed at pH 9.0 ($P \leq 0.001$;

Kruskal-Wallis test) and internal TFM burden observed at pH 7.8 were significantly greater than TFM burdens observed at pH 9.0 ($P \leq 0.001$).

The internal TFM burden for larval sea lampreys exposed to the higher nominal TFM concentration of 7.6 mg L^{-1} (measured TFM= 7.03 mg TFM L^{-1}), was 49.4 nmol g^{-1} (SEM ± 3.2) at the lowest pH (measured pH = 6.85), compared to 47.9 nmol g^{-1} (SEM ± 6.6) at pH 7.8 (measured pH = 7.82), and 12.8 nmol g^{-1} at pH 9.0 (SEM ± 1.3; measured pH = 8.82; Figure 2-2, C). The internal TFM burdens at pH 6.5 were determined to be greater than TFM burdens observed at pH 9.0 ($P \leq 0.001$; one-way ANOVA, Tukey HSD) and internal TFM burden observed at pH 7.8 were greater than TFM burdens observed at pH 9.0 ($P \leq 0.001$).

Predicted TFM-OH and TFM-O^- concentrations were calculated at each pH using the Henderson Hasselbalch equation. Linear regressions were then used to evaluate the relationship between uptake rates of TFM by larval sea lamprey exposed to the 12 h-LC_{99} for 1 h to predicted TFM-OH and TFM-O^- concentrations at their respective pHs. This analysis indicated a positive relationship between uptake rates and TFM-OH, as pH increased ($P \leq 0.001$; Figure 2-3, A). In contrast, linear regression indicated a negative relationship between uptake rates and TFM-O^-, with an alkaline pH resulting in lower uptake at proportionally greater external concentrations of TFM-O^- ($P \leq 0.05$; Figure 2-3, B).

Effect of pH on Rates of TFM Excretion

Initial excretion rates (2 h) for lampreys injected with 85 nmol g^{-1} TFM were 6.2 nmol g^{-1} h^{-1} at a nominal pH of 6.5 (measured pH = 6.43; SEM ± 1.1), 6.9 nmol g^{-1} h^{-1} at

a nominal pH of 7.8 (measured pH = 8.12;SEM ± 1.2), and 10.7 nmol g^{-1} h^{-1} at a nominal pH of 9.0 (measured pH = 8.96; SEM ± 1.6). Excretion rates 4 h post injection for lampreys injected with 85 nmol g^{-1} TFM were 4.0 nmol g^{-1} h^{-1} at a nominal pH of 6.5 (measured pH = 6.43; SEM ± 1.5), 5.8 nmol g^{-1} h^{-1} at a nominal pH of 7.8 (measured pH = 8.12; SEM ± 1.2), and 7.8 nmol g^{-1} h^{-1} at a nominal pH of 9.0 (measured pH = 8.96; SEM ± 1.3).Excretion rates measured over 2 h and 4 h of the depuration period at pH 9 were greater than rates observed at pH 6.5 (Figure 2-4A; $P \leq 0.05$; one-way ANOVA, Tukey-Kramer HSD), Beyond 4 h, excretion rates were not significantly different between pHs at 6, 8, 12 and 24 h. The total percentage of TFM excreted by larval sea lampreys over 24 h at three pHs was approximately 95% and was not significantly influenced by pH (Figure 2-4, B).

Discussion

The Effect of Water pH on TFM Uptake

Significantly lower rates of TFM uptake at an alkaline pH (pH 9.0) compared to an acidic pH (pH 6.5) were consistent with the hypothesis that the majority of TFM is taken up in its un-ionized form (phenolic). However, at pH 7.8 rates of TFM uptake were not statically different to rates observed at pH 6.5. This unexpected observation might be explained by the chemistry of the gill microenvironment which can be very different from the bulk water chemistry. Playle and Wood (1989) showed that the expired water pH was lower than that of the bulk water in rainbow trout acclimated to water pHs that were greater than pH 6.0 due to a combination of CO_2 and H^+excretion across the gills. Thus, acidification of water adjacent to the gill could have resulted in greater amounts of un-ionized TFM in the gill microenvironment than predicted based on calculations made using the more alkaline bulk water, and therefore explain the similar rates of TFM uptake at pH 7.8 compared to pH 6.5 (Figure 2-4, A). Playle and Wood (1989) also demonstrated that the amount of acidification was less when the inspired (bulk water) pH was closer to pH 6.5. Thus, as the bulk water pH decreased in this case, there could have been less change (acidification) in water pH at pH 6.5 compared to when the bulk water pH was pH 7.8. As a result, less additional un-ionized TFM would have arisen at a bulk water pH of 6.5 compared to pH 7.8 for a given concentration of TFM. However, other factors including water buffering capacity (Playle and Wood 1989), which is very high in Laurier well water (hardness = 460 mg L^{-1} as $CaCO_3$) and the effects of pH on gill permeability (McDonald 1983; Erickson et al. 2006) could influence the rates of uptake of TFM, and should be considered in future studies. The divided flux chambers used by

Mallat and Stinson (1990), separated the gills from the rest of the body and using similar approaches by Playle and Wood (1989), expired gill water pH could be measured, which reflects gill boundary layer pH. By using different water pHs and chemistries, it should be possible to test how pH and water chemistry effect the gill microenvironment potentially altering the uptake of TFM by lamprey. Additionally, sites and mechanisms of TFM uptake and elimination in larval sea lamprey could be determined using these divided flux chambers and ^{14}C-TFM.

The greater rates of TFM uptake reported here at pH 6.5 compared to pH 9.0, supports earlier suggestions that at lower pHs, the greater sensitivity of sea lampreys to a given concentration of TFM is because a greater proportion of TFM is in its un-ionized form leading to more rapid accumulation of TFM (Hunn and Allen 1974). Assuming that TFM has a pKa of 6.07 (Hubert 2003), the proportion of TFM in its un-ionized (phenolic) species and in its ionized (phenolate) form was calculated using the Henderson-Hasselbalch equation (McDonald and Kolar 2007). At pH 9.0 there would have been a decrease in the proportion of un-ionized TFM compared to the ionized form of TFM (Table 2-3; Hunn and Allen 1974; Hayton and Stehly 1983; Bills et al. 2003; McDonald and Kolar 2007). In its un-ionized form, TFM is more lipophilic compared to ionized, charged form of TFM which would lead to greater rates of TFM uptake and accumulation (Figure 2-2). Hunn and Allen (1974) demonstrated that as pH increases, TFM accumulation decreased in rainbow trout, which they suggested was the result a greater proportion of un-ionized TFM in the water. This also likely explains why greater amounts of TFM are required to cause death at more alkaline pHs in acute toxicity tests in the lab, and to effectively control sea lamprey populations in the field (Bills et al. 2003). Internal

TFM burdens also varied with pH with acidic pHs resulting in a greater TFM burden compared to alkaline pHs. Hunn and Allan (1974) observed similar patterns of accumulation of TFM in channel catfish (*Ictalurus punctatu*) at pH 6, 7, 8, and 9, and found TFM concentrations to be 15.50, 7.25, 1.60, and 0.14 nmol g^{-1}, at each respective pH. These concentrations were all less than results found in larval sea lamprey by the present study, but the trend is consistent between the two studies with more TFM accumulating in fish at acidic pHs compared to alkaline pHs.

As the rates of TFM uptake decreased with reductions in the concentration of un-ionized TFM at higher pH, there were corresponding reductions in the inwardly directed gradients for un-ionized TFM. However, at alkaline pH, where concentrations of the un-ionized TFM would be low and the ionized form of TFM very high > 99.8 % (Table 2-3), TFM was still taken-up by the lamprey. Further, linear regression showed no positive relationship between TFM uptake and the amount of predicted ionized TFM (Figure 2-3). This suggests that the uptake of ionized TFM is limited with TFM uptake primarily being in its un-ionized form. This is likely because the tight junctions between adjacent pavement cells and ionocytes (mitochondria rich cells) are very tight in freshwater fishes, including lampreys (Evans et al. 2005), which would preclude inward diffusion of ionized TFM. A tight junction is where two epithelia cells are tightly joined by transmembrane proteins embedded in each cell's plasma membrane creating an almost impermeable barrier to ion movement (Hill et al. 2008). In the gill epithelia of fishes, including lamprey,they also function as a barrier between the exterior and interior environments (Bartels and Potter 2004; Evans et al. 2005). Tight junctions can be leakier in some environments, however. For instance, some ions, such as Na^{+}, can passively

move across the gill epithelia via leaky tight junctions, but only in marine environments as part of the Na^+ excretory process (Bartels and Potter 2004; Evans et al. 2005). Nevertheless, future studies specifically evaluating whether tight junctions are facilitating the uptake and excretion of the ionized form of TFM are needed.

The diffusability of different substances across the phospholipid bilayer of epithelial cells is influenced not only by charge, (e.g. phenolate vs phenolic form of TFM) but by its size and lipid solubility (Cronin and Livingstone 2004). The size and structure of TFM is very similar to 2,4-dinitrophenol (2,4 DNP) which is known to easily diffuse across the gill membrane (McKim and Goeden 1982; McKim et al. 1987). The more lipid soluble a substance , the greater its diffusability across gill epithelial tissue (Hughes 1980). This can be determined by the Log_{10} of the octanol:water partition coefficient (K_{OW}), which is described as the ratio between the concentrations of the compound of interest when dissolved in polar and non-polar solution (Cronin and Livingstone 2004). Compounds that have higher Log_{10} K_{OW} values (>3.0) are considered to be more hydrophobic and diffuse more readily across the gill epithelia (Saarikoski et al. 1986; Mckim and Erickson 1991; Howe et al. 1994; Cronin and Livingstone 2004). With a predicted $Log_{10}(K_{OW})$ value of 2.77 (Mckim and Erickson 1991; Cronin and Livingstone 2004), TFM has a high lipid solubility which suggest it should easily diffuse across epithelial membranes. At alkaline bulk water pHs, where less un-ionized TFM will persist, the properties of K_{OW} are less pronounced due to lower concentrations of un-ionized TFM (Nichols et al. 2015). Under more acidic conditions where more phenolic TFM persist, properties associated with K_{OW} are more pronounced making TFM more easily diffusible across epithelial membrane.

The Effect of Water pH on TFM Excretion

Initial excretion rates at an alkaline pH were elevated compared to more acidic pH. Hence, the hypothesis that larval sea lamprey would have greater excretion rates of TFM at an alkaline pH was partially accepted. Whether TFM is primarily being excreted across the gills or the gastrointestinal is unknown. However, the gastrointestinal excretion of TFM is thought to rely on the phase II detoxification process, glucuronidation, which has been shown to be very limited in sea lamprey (Lech and Costrini 1972; Lech and Statham 1975; Kane et al. 1994). However, we are aware of no studies on the role that the renal excretion of TFM and its metabolites plays in the elimination of TFM. Nor is there any data on what role biliary excretion plays in TFM elimination, or whether or not TFM is biotransformed to another product in the liver (Lech and Costrini 1972). Potential candidates of biotransformation in the liver include, glucuronidation or sulfation of TFM (Hubert et al. 2005). Indeed, sea lamprey do express the genes for glucuronidation (discussed in Chapter 3), but whether or not the genes are expressed strongly enough to produce sufficient mRNA to produce sufficient amounts the enzyme of UDP-glucuronosyltransferase is questionable.

If TFM is crossing the gills, its excretion could be influenced by a combination of factors including free-TFM concentrations in the blood, boundary layer pH, and bulk water pH (Figure 2-4, A; Hunn and Allen 1974; Playle and Wood 1989). Each of these factors would influence the blood-water TFM-OH diffusion gradient. In the blood (pH ~7.85; Boutilier et al. 1993) TFM would primarily persist as the ionized, phenolate ion form due to its relatively lower pK_a near 6.07, rather than its un-ionized phenolic form which would more easily diffuse across the gill (Clifford et al. 2012). Escher et al. (2011)

discusses how the blood plasma-water partition coefficients of hydrophobic compounds, which are based on the K_{OW}, bind to blood plasma proteins and lipids once they have been transferred across the gill membrane. Escher et al. (2011) further discuss that since the majority of the hydrophobic compound in its ionized state binds to blood plasma lipids and proteins, passive diffusion down the diffusion gradient across the gills would be difficult. Even at physiological pH, however, a small but significant portion of TFM will be in this un-ionized form, which could diffuse across the gill into the water down favorable gradients following a lampricide treatment, when TFM is no longer present or present in very low concentrations. In this respect, the water could act as a "sink" into which surviving sea lamprey could unload the lampricide.

At more alkaline (pH 9.0) or circumneutral pH (pH 7.8), we predicted that this un-ionized TFM would be trapped as ionized TFM in the gill microenvironment, contributing to the generation of a larger gradient for TFM excretion into TFM free water (Figure 2-4, A). At a more acidic pH (e.g. pH 6.5), we predicted that the outward gradient favoring un-ionized TFM should have been lower, resulting in lower TFM excretion rates. Indeed, this was observed over the first 4 hours in TFM free water, but beyond this time, pH had no significant effect on the rates of TFM excretion.

Unexpectedly, TFM excretion rates were comparable beyond 4 h, suggesting that the differences in water pH have only had a transiently effect on TFM elimination. But, this could be important if TFM treatments were interrupted due to mechanical breakdowns, sudden weather events, or other adverse events. Under such conditions, lamprey might be likely to excrete a greater proportion of their TFM body burden, which could allow them to partially recover from treatment and increase the possibility of their

survival if sufficient TFM was not used when the treatment resumes. Under most circumstances, however, this seems unlikely. Nevertheless, it is clear that sea lampreys are able to rapidly clear TFM from their bodies. Indeed, by 24 h of depuration, more than 95 % of the loaded TFM was eliminated by sea lamprey at acidic, moderate and high pH (Figure 2-4, B).

Implications for Sea Lamprey Control

The present study demonstrates that differences in water pH can markedly influence rates of TFM uptake, with much less pronounced effects on TFM excretion. Variation in the rates of TFM uptake due to changes in pH could therefore compromise TFM effectiveness at higher pH, or threaten non-target species at lower pH. Since TFM treatments are done over 12 h, both photosynthesis and aerobic respiration can influence the pH of the stream causing stream pH to vary by up to 2-3 units in some cases depending upon factors such as bicarbonate buffer capacity, temperature and overall productivity (Odum 1956; Vannote et al. 1980; Wetzel 1983). More eutrophic streams with an abundance of macrophytes, algae, and/or cyanobacteria will be more prone to such changes compared to less productive streams (Wetzel 1983). Such fluctuations in pH, even relatively small changes, could markedly affect TFM uptake and excretion through pH–dependent changes in TFM speciation, and ultimate affect toxicity. Because pH is on a log scale, even a slight shift in pH due to photosynthesis that raises pH by 0.3, would increase the amount of ionized TFM by 3 fold, leading to lower TFM accumulation. Sea lamprey control agents closely monitor pH and adjust application rates accordingly (B. Stephenson, DFO, personal communication), but lags in making corrections could conceivably result in less TFM accumulation and extend the period

needed for TFM to accumulate to toxic levels. This could potentially result in greater numbers of residual larval sea lampreys that survive treatment and eventually undergo metamorphosis into parasitic juveniles that migrate downstream to the Great Lakes. Thus, if stream pH fluctuates it may be prudent in some cases to extend treatment times to ensure that TFM uptake is sufficient to cause death of the larval sea lampreys being targeted. Additional factors including geomorphological features, rain events, runoff from agricultural, and timber harvests could also affect water quality and increase the susceptibility of rivers and streams to markedly fluctuating pH resulting in inefficient TFM treatments (Hynes 1970; Tiedemann et al. 1988; Campbell and Doeg 1989; McDonald and Kolar 2007; Poudel et al. 2013).

Finally, the current pH-Alkalinity models are based on the MLC, the minimum lethal concentration of TFM needed to kill 99.9 % of sea lamprey during a treatment. By learning more about how pH influences the uptake and elimination of TFM by larval sea lamprey it may be possible to fine tune TFM application procedures, and models. Potential modifications could include adjusting the pH of an alkaline stream by temporarily bubbling CO_2 into the stream to reduce pH (McDonald and Kolar 2007). At more alkaline pHs, it may be possible to the extend the treatment time, rather than TFM concentration since uptake rates of TFM at alkaline pHs were low. An increased exposure period from 12 h to 24 h would potentially lead to increased accumulation of TFM, reducing the MLC needed for the infested stream. Ultimately, by improving our understanding of how TFM and pH interact, it will be possible to decrease the risk of residual sea lampreys in waters that are prone to fluctuations in pH over short periods of

time, and reduce the numbers of sea lampreys that complete metamorphosis and go on to plague Great Lakes' fisheries.

Table 2-1. Water quality parameters pH and temperature (°C) during a one week acclimation of larval sea lamprey prior to measurement of TFM uptake and elimination at three different pHs (6.5, 7.8, and 9.0 pH) .

Data presented as the mean ± SEM.

TFM Uptake Experiments

	Acclimation				Exposure			
	LC_{50}		LC_{99}		LC_{50}		LC_{99}	
Nominal pH	pH	Temperature (°C)	pH	Temperature (°C)	pH	Temperature (°C)	pH	Temperature (°C)
6.5	6.52 ± 0.04	20.6 ± 0.13	6.52 ± 0.04	20.6 ± 0.13	6.87 ± 0.14	17.7 ± 0.05	6.85 ± 0.18	17.6 ± 0.07
7.8	8.25 ± 0.02	19.1 ± 0.07	7.79 ± 0.02	20.4 ± 0.58	8.06 ± 0.03	18.3 ± 0.05	7.82 ± 0.15	17.3 ±0.06
9.0	8.85 ± 0.06	20.7 ± 0.19	8.85 ± 0.06	20.7 ± 0.19	8.74 ± 0.07	17.4 ± 0.50	8.82 ± 0.06	17.2 ± 0.17

TFM Excretion Experiments

	Acclimation		Experiment	
Nominal pH	pH	Temperature (°C)	pH	Temperature (°C)
6.5	6.68 ± 0.09	19.9 ± 0.27	6.43 ± 0.03	19.6 ± 0.13
7.8	8.25 ± 0.02	19.1 ± 0.07	8.12 ± 0.03	16.9 ± 0.07
9.0	8.85 ± 0.06	20.7 ± 0.19	8.96 ± 0.04	18.8 ± 0.19

Table 2-2. Distribution, speciation, diffusion gradients and Nernst potential calculations for different species of 3-trifluoromethyl-4-nitrophenol (TFM) at different pHs (6.5, 7.8, and 9.0 pH) following the determination of TFM uptake rates.

Data calculated for larval sea lamprey exposed to 4.6 mg L^{-1}, which is the 12 h LC_{50} for TFM in hard, Wilfrid Laurier well water. TFM-OH gradients with positive values denote an inwardly directed gradient favoring TFM uptake. Negative Nernst Potential values denote inward gradients for the ionized form of TFM ($TFM\text{-}O^-$).

	Blood	Water (pH 6.5)	Water (pH 7.8)	Water (pH 9.0)
pH	7.85[a]	6.87	8.06	8.74
Total TFM (nmol/mL; LC_{50})	0.038[b]	19.85	19.36	21.10
TFM-OH (nmol/mL)[c]	0.0062	2.71	0.196	0.0045
$TFM\text{-}O^-$ (nmol/mL)[d]	0.03738	17.14	19.165	21.05
% TFM-OH	1.63	13.68	1.02	0.21
% $TFM\text{-}O^-$	96.70	86.32	98.98	99.79
Chemical Gradient[e]		2.704	0.190	-0.002
Nernst Potential (mV)[f]		-153.22	-156.01	-158.35

[a]Arterial blood pH measured in adult sea lampreys (Boutilier et al. 1993). [b]Whole body TFM burdens calculated by dividing the counts per minute of radiation in each fish (CPM g^{-1} fish) by the mean specific activity (MSA) of TFM in the water. [c]$[TFM\text{-}OH] = [TFM]_{total}/[1 + \text{antilog}(pH - pK_a\ TFM])$, where pH is blood or bulk water pH and the pK_a of TFM = 6.07 (Hunn and Allen 1974). [d]$[TFM\text{-}O^-] = [TFM]_{total} - [TFM\text{-}OH]$. [e]Chemical gradient calculated by $[TFM]_{total}$-$[TFM]_{blood}$.

[f]Nerst potential = $(RT/zF)\ln[TFM]o/[TFM]i$, where R is the Ideal Gas Constant, T is temperature (˚K), F is the Faraday constant, and z is the valence of ionized TFM (minus one), and [TFM] is the concentration outside (o) and inside the (i) the animal (e.g. Wilkie and Wood 1995).

Figure 2-1. Chemical structure of 3-trifluoromethyl-4-nitrophenol (TFM) and its dissociation equilibria.

Because TFM is a weak acid with a pK_a of 6.07, there is a higher proportion of total TFM in its un-ionized phenolic form at lower pH compared to more alkaline pHs, where the ionized (phenolate) form predominates (Hunn and Allen 1974; Hayton and Stehly 1983).

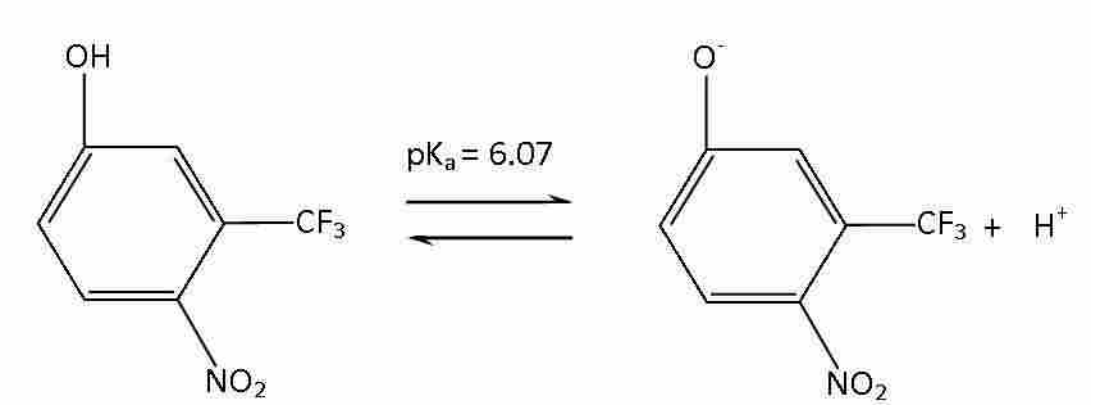

OH
CF3
NO2
pKa = 6.07
O-
CF3 + H+
NO2

Figure 2-2. Effects of pH on the rate of 3-trifluoromethyl-4-nitrophenol uptake.

Changes in uptake rates of 3-trifluoromethyl-4-nitrophenol (TFM) during exposure to (A) 4.6 mg L^{-1} for 3 h, (B) 7.6 mg L^{-1} for 1 h, and (C) internal burden of TFM in larval sea lamprey at three different pHs (6.5, 7.8, and 9.0 pH). Data are presented as the mean + SEM (N = 9-12 fish at each pH). Letters denote statistical significance in the rates of TFM uptake at each pH with uptake rates at the LC_{50} ($P \leq 0.001$) and (B) LC_{99} ($P \leq 0.001$) being significant. Internal TFM burdens at different pHs for both the LC_{50} and LC_{99} were significant (LC_{50}, $P \leq 0.001$; LC_{99}, $P \leq 0.001$).

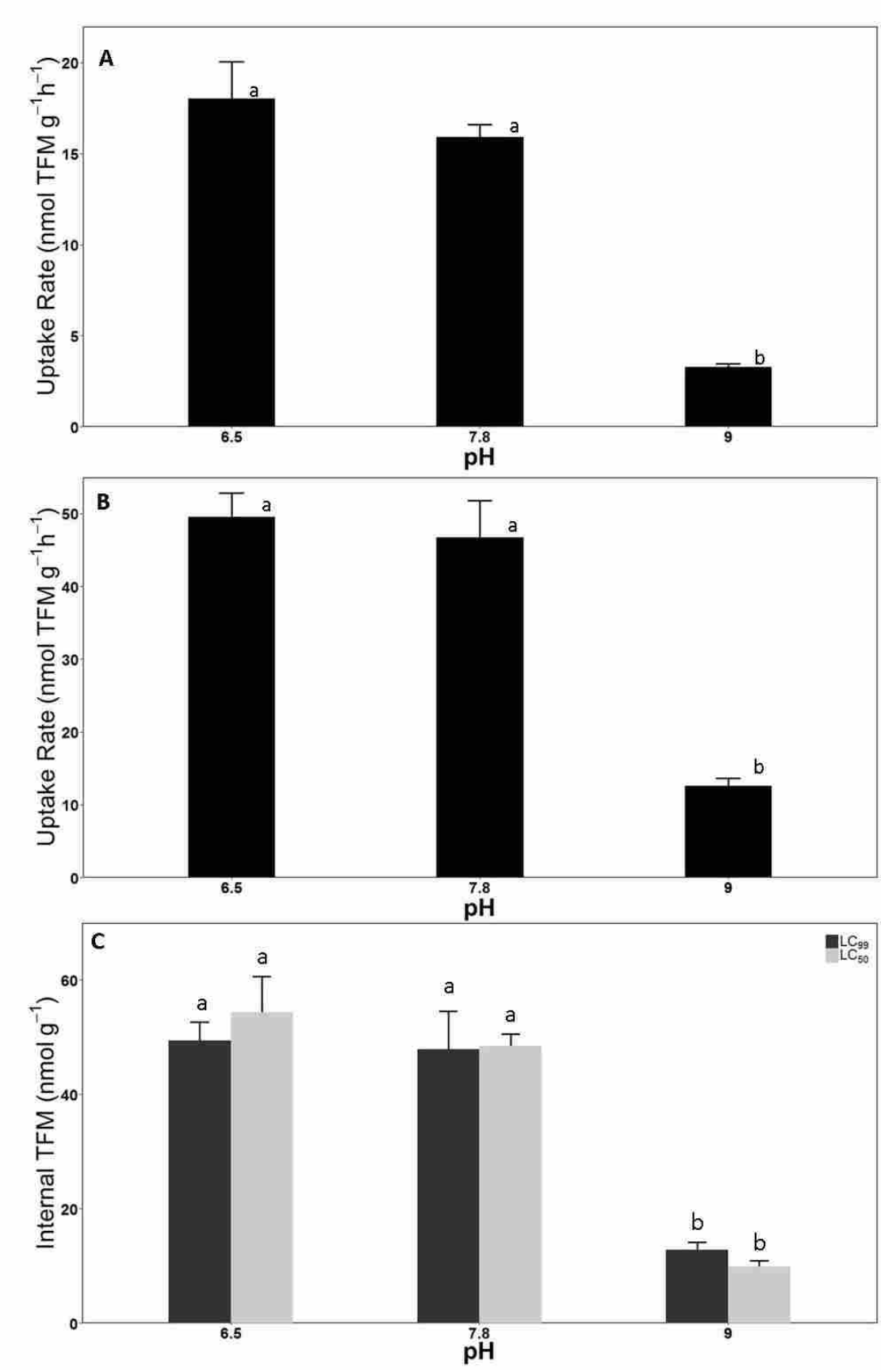

A
Uptake Rate (nmol TFM g^{-1}h^{-1})
0
5
10
15
20
a
a
b
6.5
7.8
9
pH
B
Uptake Rate (nmol TFM g^{-1}h^{-1})
0
10
20
30
40
50
a
a
b
6.5
7.8
9
pH
C
Internal TFM (nmol g^{-1})
0
20
40
60
a
a
a
a
b
b
LC$_{99}$
LC$_{50}$
6.5
7.8
9
pH

Figure 2-3. Effects of pH on the speciation and the rate of 3-trifluoromethyl-4-nitrophenol uptake.

Uptake rates of 3-trifluoromethyl-4-nitrophenol (TFM) for lamprey exposed to 7.6 mg L^{-1} for 1 h against external concentrations of (A) TFM-OH and (B) $TFM\text{-}O^-$ at three different pHs (6.5, 7.8, and 9.0 pH). Linear regressions and correlation coefficients (r^2) were used to determine the possibility that larval lamprey are taking up ionized TFM. Rates observed at 6.5, 7.8 and 9.0 are resented by a dark square, grey circle, and light grey triangle, respectively.

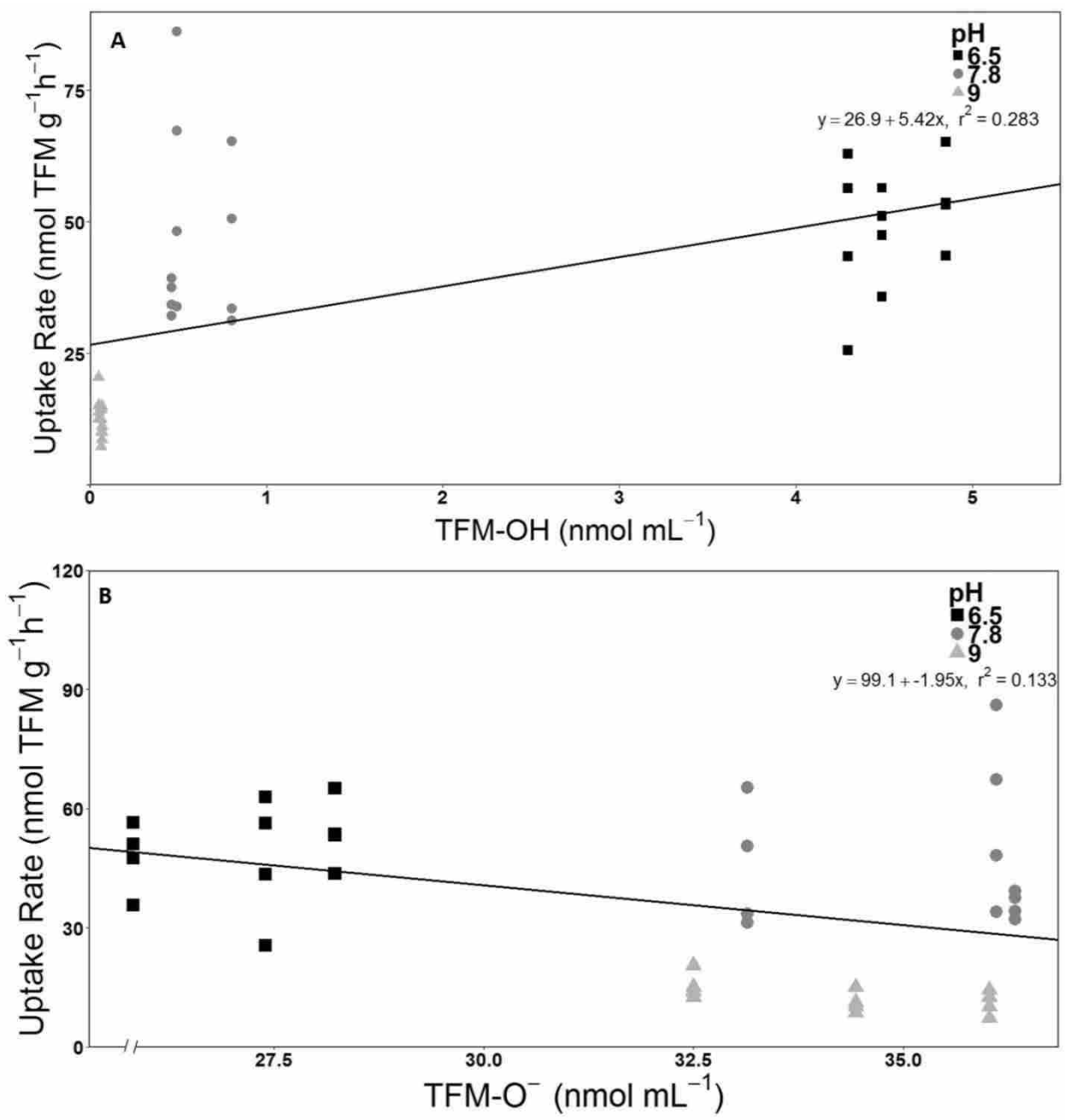

A
pH
6.5
7.8
9
y = 26.9 + 5.42x, r² = 0.283
Uptake Rate (nmol TFM g⁻¹h⁻¹)
TFM-OH (nmol mL⁻¹)
0
25
50
75
0
1
2
3
4
5
B
pH
6.5
7.8
9
y = 99.1 + -1.95x, r² = 0.133
Uptake Rate (nmol TFM g⁻¹h⁻¹)
TFM-O⁻ (nmol mL⁻¹)
0
30
60
90
120
27.5
30.0
32.5
35.0

Figure 2-4. Effects of pH on excretion rates of 3-trifluoromethyl-4-nitrophenol. Changes in (A) excretion rates of 3-trifluoromethyl-4-nitrophenol (TFM) and (B) total percentage of TFM excreted over 24 h by larval sea lamprey at different pHs (6.5, 7.8, and 9.0 pH). Data for (A) is presented as the absolute value of the mean + SEM, and data for (B) is presented as the mean + SEM (N =8 per treatment). Letters denote significant differences between the TFM excretion rates at each time period. Bulk water pH did not have a significant effect on the (B) total percentage of TFM excreted over 24 h.

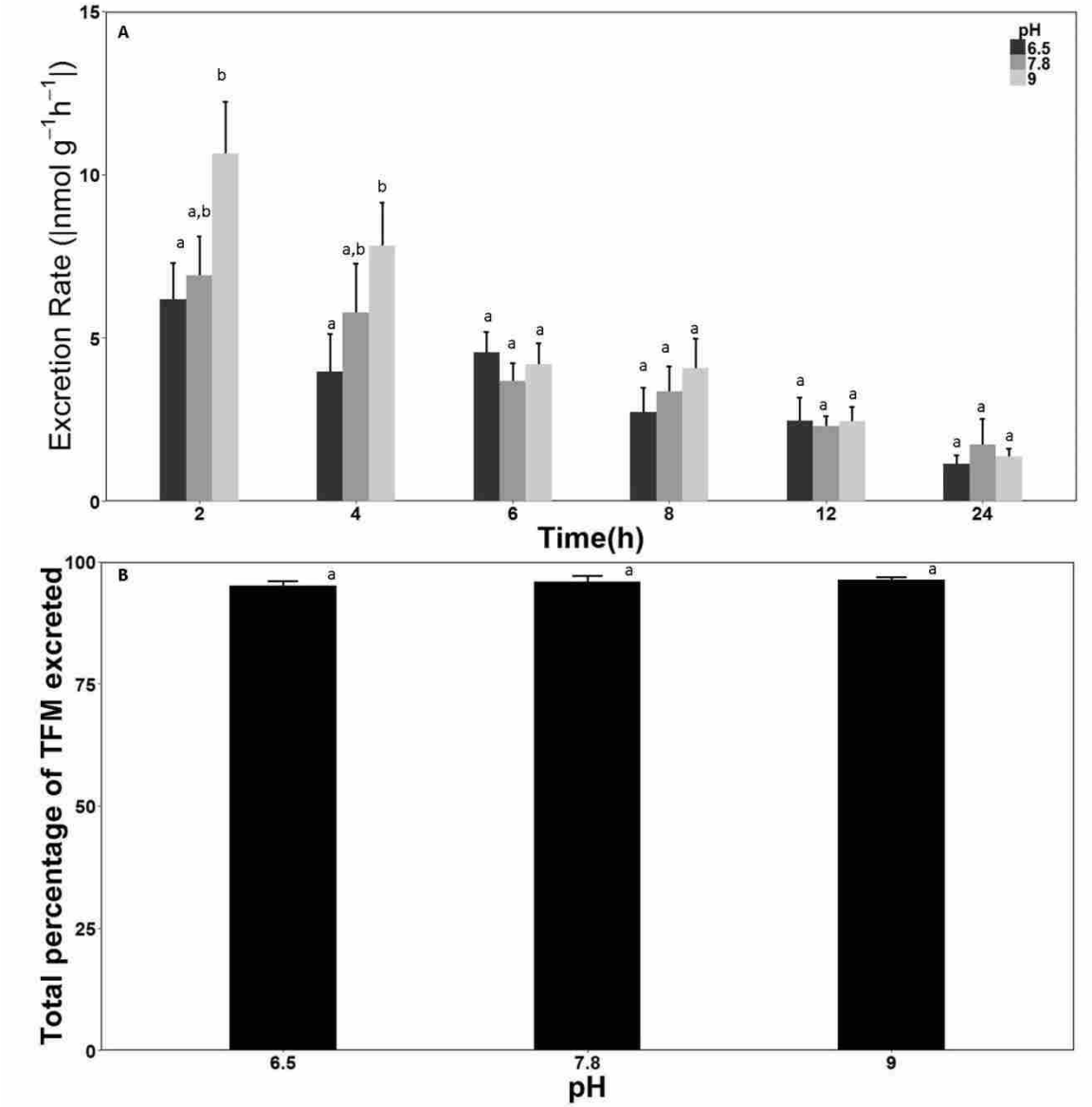
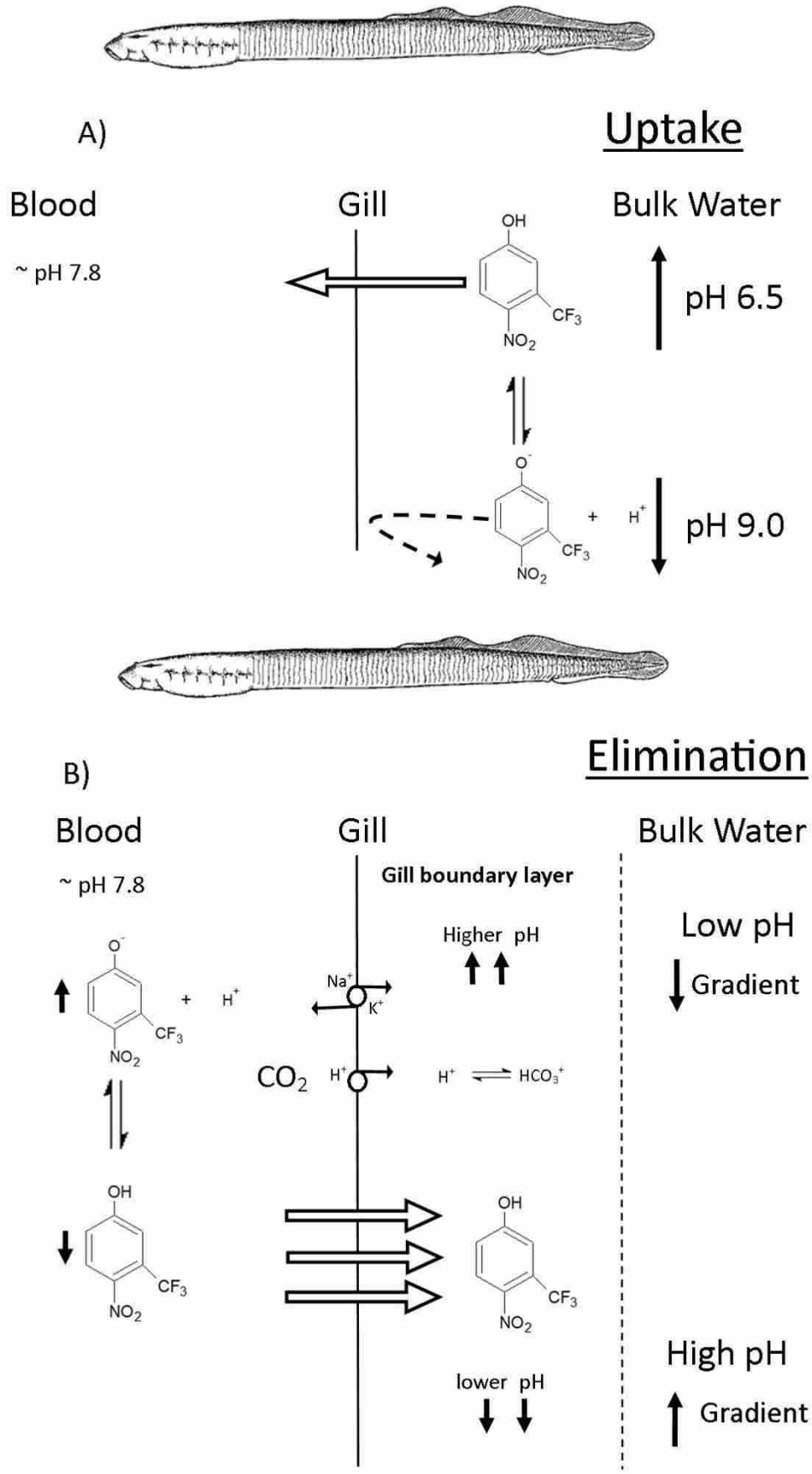

A
pH
6.5
7.8
9
Excretion Rate (|nmol g^{-1}h^{-1}|)
15
10
5
0
a
a,b
b
a
a,b
b
a
a
a
a
a
a
a
a
a
a
a
a
2
4
6
8
12
24
Time(h)
B
Total percentage of TFM excreted
100
75
50
25
0
a
a
a
6.5
7.8
9
pH

Figure 2-5. Model of uptake and elimination of 3-trifluoromethyl-4-nitrophenol. A) Influence of water pH on the amounts of ionized and un-ionized of TFM in the blood and water, and its influence on inwardly directed TFM diffusion gradients. TFM is thought to primarily diffuse across the gill as un-ionized TFM. At a low pH, there is more un-ionized TFM (phenolic) at a given total TFM concentration, where Total TFM = ionized (phenolate ion) + un-ionized (phenolic) species of TFM. As a result, there is a larger inwardly directed un-ionized TFM gradient at lower pH and a given TFM concentration. At a higher pH more TFM is ionized, reducing the inward gradient of TFM. Events in the boundary layer of the gill, which is generally more acidic than the bulk water, should be considered when calculating TFM diffusion gradients in future studies. Solid arrows for both (A) and (B) demonstrate un-ionized TFM diffusing across the gill membrane while the dotted arrow indicates the inability of the majority of ionized TFM to diffuse across the gill membrane. B) The influence of external pH on the excretion of TFM across the gills. The lower boundary layer pH could result in a build-up of un-ionized TFM in this region, which would impair TFM excretion in more acidic or circumneutral pH water. At high pH, however, less TFM would be in its un-ionized form resulting in greater rates of TFM unloading. An upward arrow in the blood indicates an increase of ionized TFM in the blood while a downward arrow next to un-ionized TFM indicates a decrease of un-ionized TFM.


A)
Uptake
Blood
~ pH 7.8
Gill
Bulk Water
pH 6.5
pH 9.0
B)
Elimination
Blood
~ pH 7.8
Gill
Gill boundary layer
Bulk Water
Higher pH
Low pH
Gradient
CO2
lower pH
High pH
Gradient

Chapter 3:

Factors Influencing 3-Trifluoromethyl-4-Nitropheonl (TFM) Toxicity in Larval Sea Lamprey

Introduction

The discovery of 3-trifluoromethyl-4-nitrophenol (TFM) as a selective piscicide to larval sea lampreys in their natal streams in the early 1960s significantly suppressed invasive sea lamprey (*Petromyzon marinus*) populations in the Great Lakes, and remains the primary method of sea lamprey control (Lawrie 1970; Hubert 2003; McDonald and Kolar 2007; Siefkes et al. 2013). The toxicity of TFM is due to the impairment of mitochondrial ATP production through the uncoupling of oxidative phosphorylation (Niblett and Ballantyne 1976; Birceanu et al. 2011). Limited ATP production forces the lamprey to rely on anaerobic energy reserves, such as glucose and glycogen, leading to a mismatch between energy supply and demand, eventually causing death (Wilkie et al. 2007a; Birceanu et al. 2009, 2011). Currently, TFM is approximately 90 % effective when used to control sea lamprey populations in the Great Lakes (Adair and Sullivan 2013), but the reason why some larvae survive TFM treatments is currently unclear.

Recent field observation have shown that the sensitivity of larval sea lamprey to TFM exposure varies with season, with the LC_{50} of larval sea lamprey being low in May, and 2 to 3-fold higher by late July and early August (Scholefield et al. 2008). This change in tolerance may explain why the effectiveness of TFM treatments decreases in mid- to late summer. Scholefield et al. (2008) postulated that changes in larval sea lampreys' condition factor, along with changes in abiotic factors, such as water temperature, pH and alkalinity, may explain these changes in TFM sensitivity.

The phase II detoxification pathway, glucuronidation, has been heavily studied within vertebrates and is one possible pathway used to detoxify phenolic based compounds such as TFM (Dutton and Montgomery 1958; Lech 1973, 1974; Dutton 1980;

Kane et al. 1994; Timbrell 2009). Glucuronidation is the process of a toxic compound being catalyzed by the enzyme uridine-diphosphate-glucuronosyltransferase (UGT; Dutton 1980), in the presence of the co-substrate uridine-diphosphate-glucuronic acid (UDPGA; Riddick 1998, Timbrell 2009; Figure 3-1). Once the UDPGA molecule is attached to the functional group of a compound, the polarity of that compound changes, making it more hydrophilic, thus facilitating its excretion (Dutton 1980; Timbrell 2009).

Rainbow trout (*Oncorhynchus mykiss*) have a high capacity to detoxify TFM using UGT compared to juvenile and adult sea lamprey (Lech 1974; Clarke et al. 1991; Kane et al. 1994). Furthermore, the activity of UGT has been shown to be lower in spring, increase in the summer, and decrease again in the fall, following seasonal temperature trends (Koivusaari et al. 1981; Koivusaari 1983; Hänninen et al. 1984). Thus, it remains possible that seasonal and/or temperature induced changes in UGT activity may explain why higher concentrations of TFM are needed to treat lampricide infested streams during the summer (Scholefield et al. 2008).

This study tested the hypothesis that season and temperature influence TFM toxicity in larval sea lamprey. To this end, larval sea lampreys were exposed to a range of TFM concentrations at four seasonal time points (May, June, August, and October) to determine if TFM toxicity and whole body TFM and TFM-glucuronide burden change seasonally with TFM exposure. Furthermore, liver UGT mRNA abundance was analyzed in control animals, to determine the potential impact of season and temperature on UGT gene expression and the lamprey's corresponding TFM detoxification capacity.

Changes in temperature also likely influence the rates of uptake and elimination of xenobiotic compounds such as phenol in fish (Patra et al. 2015). However, direct investigations have yet to occur on the effect of temperature on the uptake and elimination rates of TFM by lamprey. As temperatures increase, metabolic rates and gill ventilation increase in fish to meet the increase in metabolic demand (Patra et al. 2009). However, increased temperature can result in greater accumulation of the molecule (Black et al. 1991) and/or enhanced elimination through passive diffusion across the gills (Hunn and Allen 1974). This would result in less effective treatments, leading to residual larval sea lampreys which subsequently complete metamorphosis and go on to parasitize economically and culturally significant fish populations in the Great Lakes (McDonald and Kolar 2007). A second goal of the study was to therefore evaluate how temperature affects rates of TFM uptake and elimination in larval sea lamprey. To this end, ^{14}C-TFM was used to measure rates of TFM uptake in larval sea lampreys across three temperatures (6, 13, and 22 °C). To determine the effects of temperature on lampricide elimination rates, larval sea lampreys were injected with 100 nmol g^{-1} ^{14}C-TFM and the amount of radioactive TFM eliminated in the water was measured over 24 h.

Methods and Materials

Experimental Animals

All experiments were approved by the Wilfrid Laurier University Animal Care Committee and followed the Canadian Council of Animal Care guidelines. Larval sea lampreys were captured by pulsed-DC electrofishing (ABP-2 Electrofisher, Electrofishing Systems, LLC, Madison, WI, USA) from tributaries of Lake Huron and Lake Michigan by United States Fish and Wildlife Service personnel (Ludington, MI USA) and transported to the Hammond Bay Biological Station (HBBS; United States Geological Survey, Millersburg, MI, USA). Animals were maintained by the HBBS personnel until the start of the experiments or until they were shipped to Wilfrid Laurier University.

Holding and Acclimation

Experiment 1 – Effects of Season and Temperature on TFM Toxicity

To determine the effects of season and temperature on TFM toxicity, experiments were performed in two different years, 2013 and 2014. For the experiments conducted in 2013, larvae (0.6 g ± 0.014 ; 70 mm ± 0.68) were collected in May, June, August, and October and were housed in 40 L glass aquaria (n=100 per aquarium) for 5 days prior to experimentation, to allow them to acclimate to the experimental conditions. Glass aquaria were filled with approximately 3-5 cm of sand to provide the animals with burrowing substrate. Filtered, aerated Lake Huron water (pH 7.8 ± 0.4; hardness = 150 mg L^{-1} as $CaCO_3$; dissolved oxygen ≥ 80 % saturation) at ambient stream temperatures, and corresponding to the season the fish were collected, was supplied to the tanks at a flow

rate of approximately 500 mL min^{-1}. Water quality conditions of each exposure are described in Table 3-1.

For the experiments conducted in 2014, Great Lakes' strain larval sea lampreys (0.85 g ± 0.029; 85 mm ± 0.96) were collected from the Au Sable River, MI USA, in June, and were housed at HBBS in three 200 L plastic tanks (n=250 animals per tanks), filled with sand as described above, and received aerated Lake Huron water at a flow rate of 500 mL min^{-1}. Prior to experiments, the animals were acclimated to three different temperatures: 6, 12 and 22 ˚C for two weeks in the same water, which was heated or chilled using an in-house heat exchange system.

Experiment 2 – Effects of Temperature on Rates of TFM Uptake and Elimination

Great Lakes' strain larval sea lampreys were captured in spring 2015 and housed at HBBS, before being transported to Wilfrid Laurier University in bags partially-filled with oxygen-saturated water and contained in a 70 L cooler. No mortalities were observed upon arrival at Wilfrid Laurier University, where the larvae (250 animals per aquarium) were initially housed in 110 L fiberglass aquaria, lined with 5 cm of sand receiving well water (500 mL min^{-1}; pH 7.8 ± 0.4; hardness = 460 mg L^{-1} as $CaCO_3$; dissolved oxygen ≥ 80 % saturation; T = 13 ± 2 °C) at a rate of 1 L min^{-1}. Larvae were fed a slurry of Baker's yeast (1 g yeast/lamprey) once a week, but food was withheld one week prior to experiments. All animals were kept under a 12 h dark: 12 h light photoperiod. Fish were acclimated to 13 °C (ambient), or at 6 °C by cooling the water with a ½ HP-aquarium chiller (MFG# 36063, Coralife, Franklin, WI, USA), or at 22 °C using a 150 W submersible heater (MFG# 3616090, Eheim Jager, Wüstenrot – Finsterrot, Germany) for a minimum of one week prior to experimentation.

Experimental Design

Experiment 1 – Effects of Season and Temperature on TFM Toxicity and Detoxification Capacity

Preliminary Toxicity Tests

In 2013, larval sea lampreys (n = 10 per concentration) were exposed to 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3, 4, 5 mg TFM L^{-1} for 24 h in May, June, August, and October to narrow down the range of the 12 h TFM LC_{50} and $LC_{99.9}$. Field grade TFM (35% active ingredient dissolved in isopropanol; provided courtesy of HBBS, US-Geological Survey; Clariant SFC GMBH WERK, Griesheim, Germany) was used for exposures. Water samples were collected at 0, 12, and 24 h. Glass aquaria (18 L) were bathed in temperature controlled water reflecting ambient stream temperature found in May, June, August, and October. Each aquarium was filled with 16 L of well aerated Lake Huron water. Survivorship was monitored each hour for the first 12 h and again at 24 h. Dead fish were immediately removed and snap frozen in liquid N_2 for later determination of whole body TFM and TFM-glucuronide burden via high performance liquid chromatography (HPLC). Lampreys that survived the 24 h exposure were euthanized with an overdose of tricaine methanesulfonate (1.5 g L^{-1} buffered with 3.0 g L^{-1} of $NaHCO_3$; MS-222; Syndel Labs, Port Alberni, BC, Canada), snap frozen in liquid nitrogen, and stored -80ºC until processed for further analysis (TFM quantification).

Toxicity Tests

In 2013, larval sea lampreys (n=15 per concentration; 0.6 g ± 0.03; 70 mm ± 0.94) were exposed in triplicate to the following nominal TFM concentrations: May-0, 0.5, 0.8,

1.0, 1.2, 1.5, and 2.0 mg L^{-1} TFM, June-0, 1.25, 1.75, 2.25, 2.50, 2.75, and 3.0 mg L^{-1} TFM, August-0, 2.0, 3.0, 3.75, 4.50, 4.75, and 5.0 mg L^{-1} TFM, and October-0, 1.0, 1.3, 1.6, 1.8, 2.0, and 2.3 mg L^{-1} TFM. Each aquarium was filled with 30 L of Lake Huron water, with temperature matching the ambient stream temperatures found in May (T = 6 °C), June (T = 20 °C) , August (T = 24 °C), and October (T = 11 °C) of 2013. Survivorship was determined each hour for 12 h of TFM exposure by lightly pinching each lamprey's tail with tweezers, and dead animals were removed and snap-frozen with liquid N_2 for further analysis. Lampreys that survived TFM exposure over the next 12 h (24 h in total) were euthanized using MS-222, snap-frozen with liquid N_2, and then stored -80 °C.

Liver Detoxification Capacity

To determine whether there were differences in TFM detoxification capacity with season or temperature, livers were collected from larval sea lamprey not exposed to TFM in the spring, summer, or fall (2013 experiments) and following acclimation to nominal temperatures of 6, 12, and 22 °C (2014 experiments). One day prior to tissue sampling, the larvae were placed in 1 L darkened plastic containers (N = 12 containers; n=4 larvae per container) receiving Lake Huron water at a rate of ~150 mL min^{-1}. Cotton (2 g per container) was added as artificial burrowing substrate to calm the animals. In both experiments water temperature was controlled using either a chiller/heater to temperatures reflective of ambient stream temperatures in which they animals were captured, or to the target temperature (6, 12, and 22 °C). Water quality conditions are described in Table 3-1.

Prior to sampling, the water level in each container was reduced to 750 mL and an anesthetic dose of MS-222 (0.5 g L^{-1}; buffered with 1.0 g L^{-1} of $NaHCO_3$), followed by a lethal dose of MS-222 (1.5 g L^{-1}; buffered with 3.0 g L^{-1} of $NaHCO_3$) was used. The liver was then quickly removed from the body and snap frozen in liquid N_2. Liver tissues were then transported to Wilfrid Laurier University on dry ice for analysis.

Experiment 2 – Effects of Temperature on Rates of TFM Uptake and Elimination

Acclimation to Different Water Temperatures

One week prior to experiments, larval sea lampreys were removed from their holding tanks at Wilfrid Laurier and placed in a 40 L aquarium (N = 60 per aquarium), lined with 3-5 cm of sand to provide burrowing substrate. Water temperature was controlled via a chiller or a heater to the desired temperatures of 6, 13, 22 °C ± 0.2, as previously described. One day prior to the experiment, the larvae were transferred in groups of 4 to darkened, plastic, 1 L containers (9 containers) which continuously received water of the appropriate temperature at a rate of 100-200 mL min^{-1}, and left to acclimate overnight. Each container was bathed in temperature controlled water throughout the entire exposure period.

Rates of TFM Uptake and Excretion

After the one week acclimation to their respective temperatures (6, 13, and 22 ˚C), larval sea lamprey (n=12 per time point; 1.6 g ± 0.06 ; 100 mm ± 1.2) were exposed to 7.6 mg L^{-1} TFM, which is the 12 h TFM LC_{99} previously determined in our laboratory using Wilfrid Laurier University well water (Birceanu et al. 2009). The exposure lasted for 0.5, 1, or 2 h and TFM uptake rates were measured using ^{14}C-TFM exactly as

described in Chapter 2. The water quality for these experiments is reported in Table 3-2. The temperature of each container was monitored every hour and it never fluctuated more than 1 °C. Following the flux measurement period, the animals were euthanized with an overdose of MS-222 (1.5 g L^{-1}; buffered with 3.0 g L^{-1} $NaHCO_3$), and processed for measurement of whole animal radioactivity as described in Chapter 2.

Larval sea lamprey (n=8 per temperature; 1.7 g ± 0.07 ; 100 mm ± 1.3) used for measurements of TFM excretion were acclimated to either 6, 13, or 22 ˚C for one week, and then placed in individual 100 mL, darkened glass containers 12 h prior to the beginning of the experiment. The following morning rates of TFM were measured in each sea lamprey following the administration of an intraperitoneal (IP) injection of 100 nmol TFM g^{-1} fish containing 0.005 µCi ^{14}C-TFM g^{-1} in physiological saline (100mM NaCl, 5mM KCl, 2mM $CaCl_2$, 2mM $MgSO_4$, 3.5 mM NaH_2PO_4, and 5.5mM Glucose, pH 7.4) as described in Chapter 2. The temperature of each container was monitored hourly for 12 h and after 24 h, following which the sea lamprey were euthanized and processed for whole body determination of radioactivity as described in Chapter 2. All larvae survived the experiment.

Analytical Techniques

Water TFM Analysis

Water samples were analyzed for TFM concentration spectrophotometrically using either a Genesys 6 spectrophotometer (at HBBS; Thermo Electron Corporation, MA USA) or NovaSpec II (Pharmacia Biotech, Cambridge, England UK), and precision standards (0, 0.5, 1.0, 2.0, 3.0, 5.0 and 7.0 mg L^{-1} TFM) at a wavelength of 395 nm

according to the Department of Fisheries and Oceans, Sea Lamprey Control Centre Standard Operating Procedures (IOP: 012.4).

Solid Phase Extraction and HPLC Quantification of TFM and TFM Glucuronide

Whole body TFM and TFM-glucuronide concentrations were quantified by using methods adapted from Hubert et al. (2001). All solutions were made with HPLC grade chemicals and filtered reverse osmosis water (0.20 µm Millipore filters, Millipore, ME, USA). Prior to extraction, a sub-set of control samples were spiked with 100 ng mL^{-1} TFM (Sigma Aldrich, St. Louis, MO, USA) to determine percent recovery during processing.

Briefly, whole bodies were ground under liquid N_2 and approximately 300 mg of tissue was collected. Next, 4 mL of 80 % methanol was applied to the sample, which was shaken for 10 min (MaxQ 2000 orbital shaker, Thermo Fisher Scientific Inc, MA USA) and centrifuged for 10 min at 1228 × *g* (IEC Medilite 12, Thermo Electron Corporation, MA, USA). The supernatant was then removed and placed in a clean tube for further processing. The process was repeated two more times with the final volume being evaporated to ~8 mL using a nitrogen gas evaporator (N-EVAP Analytical Evaporator, Organomotion, MA, USA), in a 55 °C water bath. Approximately 1 cm of high-density glass filter beads (Empore Filter Aid 400, 3M, St. Paul, MN, USA) were then placed on top of a solid phase extraction column (SPE; Bond Elute LRC-C18 OH, 500 mg, Agilent Technologies, Santa Clara, CA, USA), which was then conditioned with 10 mL of 100 % methanol, followed by 10 mL of 70 % methanol. The samples were applied to the columns and the methanol in the captured effluents evaporated as described above. The pH of each sample was adjusted to 9.5 ± 0.2 using 10 N NaOH. A second set of SPE

cartridges (Empore SDB-XC 10mm diameter/6 mL volume, 3M, St. Paul, MN, US) were conditioned first with 10 mL of 100 % methanol, followed by 10 mL of pH 9.5 water. Samples were applied to columns and the resulting effluent was captured in clean tube, evaporated as described above, and the pH adjusted to 4.0 ± 0.2 using 12.1 N HCl. Samples were passed over a third SPE column (Bond Elute LRC-C18 OH, 500 mg, Agilent Technologies, Santa Clara, CA, USA) that was conditioned with 10 mL of 100 % methanol, followed by 10 mL of 24.6 mM acetate buffer (pH 4.0 ± 0.2). During the third SPE, the columns were first eluted with 12 mL of 60 % 24.6 mM acetate buffer: methanol (pH 4.0 ± 0.2) to remove TFM-glucuronide, and the eluent was collected in a clean glass tube. The same column was then eluted with 6 mL 75 % methanol to remove the TFM, and the eluent was collected in a second clean glass tube. The tubes containing TFM were evaporated to 1 mL using the evaporation methods described above and 1 mL of 40 mM sodium borate buffer (pH 8.5 ± 0.2) was added to the sample, resulting in a final volume of 2 mL. Analysis of whole body TFM concentrations was determined using HPLC (see below).

The tubes containing TFM-glucuronide were treated with a 1 mL solution of β-glucuronidase (1,644,000 units g^{-1}; from bovine liver; Sigma Aldrich, MO, USA) dissolved in 400 mM potassium phosphate buffer (pH 6.8 ± 0.2). Samples were then incubated in a water bath (35 °C) for 18 h, to allow the enzyme to digest TFM-glucuronide to TFM, after which the reaction was stopped by the addition of 12.1 N HCl. These samples were passed over a SPE column (Bond Elute LRC-C18 OH, 500 mg, Agilent Technologies, Santa Clara, CA, USA), previously conditioned with 10 mL of 100 % methanol, followed by 10 mL 4.92 mM acetate buffer (pH 4.0 ± 0.2). The columns

were then eluted with 6 mL of 75% methanol, and the methanol eluent evaporated off using the methods described above to 1 mL, to which 1 mL of 40 mM sodium borate buffer (pH 8.5 ± 0.2) was added, resulting in a final volume of 2 mL, which was then analyzed for TFM content using HPLC. The TFM glucuronide concentration in the fish, was considered to be equivalent to the TFM concentration measured in the B-glucuronidase treated sample using HPLC.

Whole body TFM concentrations were measured in the whole body homogenates using HPLC and TFM standards (0.015, 0.05, 0.25, 0.5, 1.5, and 5.0 µg mL^{-1} TFM), prepared from analytical grade TFM (Sigma Aldrich, St. Louis, MO, USA) in 20 mM sodium borate buffer, pH 8.5 ± 0.2) according to Hubert et al. (2001).

Internal TFM and TFM-glucuronide concentrations were determined using a Varian HPLC set-up comprised of a Varian ProStar 410 auto-sampler, ProStar 230 solvent delivery module, and Prostar 310 UV-vis detector (Varian, Inc., Palo Alto, CA, USA), and fitted with a reverse phase HPLC column (Kinetex 2.6 µm XB-C18 100A 100 x 3.00 mm; Phenomenex Inc., CA, USA). Samples were injected (0.1 mL) via a mobile phase comprised of 83 % 20 mM sodium borate buffer (pH 8.5 ± 0.2) and 17 % acetonitrile, which yielded a TFM retention time of 4.66 min. The chromatographic data generated was subsequently completed using Varian Star 5.51 software (Varian, Inc., Palo Alto, CA, USA). Percent recovery of TFM was determined in control sea lamprey samples that were spiked with 100 ng mL^{-1} and was 58 ± 9 %.

RNA Extraction and Quantification

Larval sea lamprey liver samples from both 2013 and 2014 were kept at -80 °C prior to mRNA extraction by Dr. O. Birceanu (Department of Biology, Wilfrid Laurier), who also conducted the subsequent cDNA isolation, primer synthesis and real-time quantitative polymerase chain reaction procedures (see below). The mRNA was first extracted from livers (n=6), which were weighed on dry ice prior to homogenization. Total mRNA extraction was conducted using RiboZol Reagent (RiboZol™ RNA Extraction Reagent, AMRESCO LCC, OH, USA), by following the manufacturer's protocol. Briefly, livers were sonicated on ice, in 250 µL RiboZol™, after which they were allowed to sit at room temperature for 5 min. Next, 50 µL chloroform was added and the samples were shaken by hand for 15 s and allowed to sit at room temperature for 2-3 min. Samples were then centrifuged for 15 min at 12,000 × *g* at 2-8 °C, and the upper aqueous phase containing RNA and was transferred to a new centrifuge tube. To precipitate the RNA, 125 µL of molecular grade isopropyl alcohol (Sigma-Aldrich, MO, USA) was added and allowed to sit for 15 min at room temperature. Samples were then centrifuged at 12,000 × *g* for 10 min at 2-8 °C. The supernatant was poured off and the RNA pellet was washed two times and with 250 µL of 70 % ethanol. Samples were centrifuged at 7,500 × *g* for 5 min at 2-8 °C and the supernatant was poured off, being careful not to remove the pellet. After centrifugation all the ethanol was removed from the tube and samples were left open for 10 min to evaporate any remaining ethanol. Pellets were then dissolved with RNase-free water (water, sterile, nuclease-free, AMRESCO LCC, OH, USA) and incubated for 10 min at 55 °C, to dissolve all the RNA. Samples were then immediately chilled on ice and Total RNA was quantified using a

Nanodrop8000 Spectrophotometer (Thermo Scientific, DE, USA). RNA quality was assessed using the 260/280 ratio, which was 1.8-2.0 for all samples.

cDNA Synthesis

Prior to first strand cDNA synthesis, 1.0 µg RNA was treated with DNase I (1.0 µL; Sigma-Aldrich, MO, USA) in $MgCl_2$ buffer (1.0 µL) for 15 min, at room temperature. RNase-free water was then added to bring the volume to 10.0 µL. The reaction was incubated at 37 ºC for 30 min, after which it was terminated by the addition of 1.0 µL 25 mmol l^{-1} EDTA stop solution and incubated at 70 ºC for 10 min. First strand cDNA synthesis was performed by following the manufacturer's instructions (Fermentas, Burlington, ON, CA). Briefly, a master mix was prepared containing the following ingredients per sample: 2.0 µL 10X RT buffer, 0.8 µL 25X dNTP Mix (100 mM), 2.0 µL 10X RT Random Primers, 1.0 µL Mulstiscribe Reverse Transcriptase and 4.2 µL RNase-free water. Next, 10 µL of the master mix were added to each sample and gently mixed by pipetting. The tubes were then placed in a thermocyler (C1000 Thermal Cycler, Bio-Rad, Hercules, CA, USA) and the following temperature cycle was used: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min, after which the tubes were stored at -80 °C for later analysis.

Primer Synthesis and Efficiency, and Real-Time Quantitative Polymerase Chain Reaction

Primers for UGT were designed using Integrated DNA Technologies, PrimerQuest® software. The zebrafish (*Danio rerio*) UGT1ab1 sequence (accession number NM_213422.2) was aligned with each of the two UGT genes annotated in sea lamprey (Ensembl transcript scaffold GL478009:1:108690:1 and GL479521:1:33375:1). Primers were designed based on a highly conserved DNA sequence between the three

genes. Therefore, the mRNA abundance of the UGT gene in the present study does not distinguish between the two UGT isoforms present in the sea lamprey (Smith et al. 2013). The forward (CACACAAGAGCTACAAGGAGAA) and reverse (TCACAAACTCCACCCAGAAC) primers yielded a 102 base pair product.

Transcript levels were analyzed using the 2× concentrated PerfecTa® SYBR® Green FastMix® reaction cocktail (Quanta Biosciences, Gaithersburg, MD, USA) in a 15 µL reaction. All standards were assessed in triplicate, while the samples were assessed in duplicate, in a C1000 Touch Thermal Cycler with CFX96^{TM} Real-Time system (Bio-Rad, Hercules, CA, USA). The reaction mixture, per sample, was: 14 µL RNase-free water, 16 µL of PerfecTa® SYBR® Green FastMix® reaction cocktail, 1 µL primer pair mixture and 1 µL cDNA. The primer pair mixture was derived by diluting each primer 10 times, for a final concentration of 10 µmol L^{-1} for the reverse and forward primers.

To determine primer efficiency, 1 µL cDNA was pooled from each sample and the cDNA pool was serially diluted 1, 4, 16, 64, 256 and 1024 times in RNase-free water to establish a relative standard curve. The standard curve was run on a temperature gradient (60, 58.7, 55.2 and 52 °C), to determine the annealing temperature at which the primers were most efficient. Primer efficiency was then calculated by determining the slope of the standard curve at each temperature and using the ThermoFisher Scientific qPCR efficiency calculator (http://www.thermofisher.com/ca/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html). The annealing temperature for the sea lamprey UGT was determined to be 60 °C, which was subsequently used for the analysis. The PCR running conditions were: 94 °C for 2 min,

95 °C for 30 s, 60 °C for 30 s. These conditions were repeated for 40 cycles, followed by 72 °C 10 min. A melt curve analysis was done starting at 60 °C and increasing to 95 °C in increments of 0.5 °C every 10 s. Copy number of each gene was determined by using the relative standard curve at 60 °C. All samples were assayed for UGT and normalized to total RNA.

Whole Body and Water Sample Processing for Determination of TFM Rates of Uptake and Excretion

For both uptake and excretion experiments, larval sea lampreys were digested in 10 mL of 1N HNO_3 in polypropylene centrifuge tubes at 60 °C for 48 h, and vortexed to ensure thorough mixing and digestion of the tissue slurry. Samples were then centrifuged for 5 min at 1228 × *g* (Medilite 12, Thermo Electron Corporation, MA, USA). Aliquots of supernatant (2 mL) were then removed and added to 7 mL scintillation vials in duplicate, followed by 4 mL of Ultima Gold™ AB (PerkinElmer, MA, USA) organic scintillation cocktail, vortexed, and left overnight in the dark to minimize chemiluminescence prior to quantifying whole body beta radiation. Aliquots of water samples (2mL) were mixed with 4 mL of Optiphase Hisafe 2 (PerkinElmer, MA, USA) scintillation cocktail, vortexed, and also left in the dark for 12 h prior to determining beta radioactivity. A beta counter (LS6500, Beckman Coutler, Brea, CA, USA) was used to determine the beta CPM of tissue and water samples.

Calculations

Uptake

TFM uptake rates (nmol g^{-1} h^{-1}) were calculated using equation 1 (Blewett et al. 2014):

$$\text{TFM Uptake Rate} = \frac{\text{CPM}_{\text{Lamprey}}}{\text{MSA} \times \Delta T} \quad (1)$$

where $CPM_{lamprey}$ is the counts per min (CPM) g^{-1} tissue in each larval sea lamprey, W is the mass of the fish (g), MSA is the mean specific activity of ^{14}C-TFM in water (CPM $nmol^{-1}$ TFM) after 1 h of exposure to the 12 h-LC_{99}, and ΔT is the duration of exposure to TFM (h). Internal TFM concentrations were determined by taking the $CPM_{lamprey}$ (CPM g^{-1}) and dividing it by MSA (CPM $nmol^{-1}$ TFM) using equation 2.

$$\text{TFM Burden} = \frac{\text{CPM}_{\text{Lamprey}}}{\text{MSA}} \quad (2)$$

Excretion

The TFM-efflux rate ($J^{14C\text{-}TFM}$) in CPM g^{-1} h^{-1}(Wilkie et al. 2007b) was calculated using equation 3:

$$J^{14C-TFM} = \frac{\text{CPM}_i - \text{CPM}_f}{T \times M} \quad (3)$$

where CPM_i and CPM_f denote the initial and final CPM in water for a given flux period, T is the flux period duration (h), and M is the mass of the lamprey (g). After $J^{14C\text{-}TFM}$ was calculated, the TFM clearance rate (nmol g^{-1} h^{-1}; Wilkie et al. 2007) was calculated using equation 4:

$$\text{TFM Clearance} = \frac{J^{14C-TFM}}{\text{MSA}} \quad (4)$$

where MSA is the mean specific activity (CPM g^{-1} $nmol^{-1}$ TFM) of TFM injected into the larval sea lamprey.

Percent excreted was determined by first calculating the amount of TFM remaining (TFM burden) as described using equation 2 (above) and subtracting this value from the amount

of TFM injected (TFM_{intial}; nmol g^{-1}) and then dividing by the concentration of TFM injected (TFM_{intial}; nmol g^{-1}) as described in equation 5 below:

$$\text{Percent Excreted} = \frac{TFM_{intial} - TFM_{Final}}{TFM_{intial}} \times 100 \qquad (5)$$

The efficiency of the beta counter (94.7 %) was determined by measuring the CPM of ^{14}C-TFM standards (0, 0.001, 0.005, 0.01, 0.05, and 0.1 µCi mL^{-1} ^{14}C-TFM) relative the known disintegrations per minute (DPM) based on the amount of ^{14}C-TFM used for the experiment (µCi). Digested non-radioactive lamprey were also spiked with increasing ^{14}C-TFM and compared to spiked water samples to determine quench in the tissue digests. No quenching was observed.

Statistical Analysis

All data are presented as the mean ± 1 standard error of the mean (SEM). Probit analysis, linear regression, and maximum likelihood estimates were used to calculate the LC_{50} using Comprehensive Environmental Toxicity Information System software (CETIS, Tidepool Scientific Software, McKinleyville, CA, USA). The 95% confidence intervals (CI) were used to determine if differences in the LC_{50s} were observed between each time point (Wheeler et al. 2006). Whenever data did not meet the assumptions of the analyses of variances (ANOVA), it was either log_{10} transformed or power transformed. Only non-transformed data are shown. If the log or power-transformation did not meet the assumptions of the ANOVA, then a Kruskal-Wallis rank sum test, followed by Dunn's was used. For all statistical tests, the level of significance was set at a P value < 0.05. Statistical analysis and figures were produced using R version 3.1.3, RStudio version 0.98.1103, and ggplot2, ISBN: 978-0-387-98140-6.

Results

Toxicity Tests and Whole Body TFM and TFM-Glucuronide Burden

Each toxicity test was conducted at ambient stream temperatures in 2013, under the conditions described in Table 3-1. The 12 h LC_{50} of TFM was determined along with their 95% confidence intervals (CI) in May, June, August, and October. In May, the 12 h LC_{50} was 1.18 mg L^{-1} (95% CI= 1.15 - 1.23), while in June, it was approximately 2-fold higher, and was calculated to be 2.55 mg L^{-1} (95% CI= 2.41 - 2.69). In August, a TFM concentration approximately 2.7-fold higher than in May [3.15 mg L^{-1} (95% CI= 3.04 - 3.26)] was needed to elicit 50% mortality over 12 h, while in October, the 12 h LC_{50} decreased closer to the levels observed for the May animals, measuring 1.64 mg L^{-1} (95% CI= 1.59 - 1.69; Figure 3-2).

The whole body TFM burden and TFM-glucuronide concentration were the same in fish that suffered mortality following exposure to the 12 h LC_{50} during May, June, August and October, ranging from 20.0 to 72.7 nmol TFM g^{-1} wet weight and from 0.1 to 4.3 nmol TFM-glucuronide g^{-1} wet weight, respectively (Figure 3-3, A). However, significant differences in the whole body TFM burden between survivors and mortalities were observed ($P < 0.05$; Kruskal-Wallis test, Dunn's post-test). In those lamprey that experienced mortality following exposure to 2.0 mg L^{-1} TFM, the whole body TFM burden was 2.5-fold greater than those that survived at the same exposure concentration ($P \leq 0.05$; Figure 3-3, A). However, TFM-glucuronide was below detection in both survivors and mortalities.

Seasonal and Temperature Effects on UGT mRNA Abundance

Liver mass was not influenced by season (Figure 3-4, A), but total RNA was 1.4 fold higher in May than in August (Figure 3-4, B; $P \leq 0.05$; Kruskal-Wallis, Dunn's post-test). Uridine-diphosphate-glucuronosyltransferase mRNA abundance (UGT μg^{-1} RNA) was not influenced by season (Figure 3-4, C).

In contrast with season, acclimation temperature significantly affectedliver mass, with mass being its highest at 6 °C, but 40 % and 60 % lower at 12 °C and 22 °C, respectively (Figure 3-5A; $P \leq 0.01$one-way ANOVA, Tukey-Kramer HSD). Total RNA and UGT mRNA abundance (UGT μg^{-1} RNA) were not, however, influenced by the different temperature acclimation regimens (Figure 3-5, B & C).

Effects of Water Temperature on Rates of TFM Uptake

The TFM uptake rate for larval sea lampreys exposed to a nominal TFM concentration of 7.6 mg TFM L^{-1} (measured TFM= 7.64 mg TFM L^{-1}) for 0.5 h significantly increased in a step-wise manner, from approximately 20 nmol g^{-1} h^{-1} to 45 nmol g^{-1} h^{-1} as the water temperature increased from 6 °C to 13 °C and then 22 ˚C (Figure 3-6, A). However at 6 °C no significant differences in TFM uptake rates were observed among the different exposure times of 0.5, 1, and 2 h. In fact, at 22 °C, TFM uptake rates were significantly reduced, by 40-50 %, when the TFM exposure period was 1 and 2 h, compared to 0.5 h (Figure 3-6, A two-way ANOVA).

The internal TFM burden accumulated by the larval sea lampreys following TFM exposure at 6 °C was determined using a two-way ANOVA to be strongly related to time of exposure, steadily increasing as the exposure time increased from 0.5 h, to 1 h and 2 h

($P \leq 0.05$; Figure. 3-6, B). Although no change in TFM burden was observed between 0.5 h and 1 h at 13 °C, internal TFM concentrations were more than 3-fold greater than at the lower temperatures following exposure at 22 °C (Figure. 3-6, B). Notably, there was no relationship between TFM accumulation and time of exposure at 22 °C, during which whole body TFM fluctuated around 20-25 nmol g^{-1} (Figure 3-6, B).

Effects of Water Temperature on TFM Excretion

Acclimation temperature had no effect on excretion rates with rates being consistent at each time point (Figure 3-7, A). Nor was the total percentage of TFM excreted by larval sea lampreys over 24 h influenced by temperature (Figure 3-7, B).

Discussion

Effects of Season on the Tolerance of Larval Sea Lamprey to TFM

The toxicity of TFM is greater in early spring and mid-fall compared to late summer, as suggested by the lower 12 h LC_{50} value recorded in May and October compared to August. Similarly, Scholefield et al. (2008) reported that TFM toxicity was highest in the spring compared to late summer. Increased detoxification capacity of larval sea lamprey did not appear to play a role in the fish's higher TFM tolerance in the summer, as the whole body TFM-glucuronide levels did not change from May to October (Figure 3-3, B). In addition, fish did not to take up more TFM later in the summer season compared to early spring, as whole body TFM levels remained unchanged (Figure 3-3, A) in dead larvae exposed to the respective TFM LC_{50} of that season. However, whole body TFM burden was less in fish that survived exposure to 2.0 mg L^{-1} TFM compared to fish that died (Figure 3-3, B) suggesting that more TFM tolerant fish were either able to limit TFM accumulation and/or eliminate it via excretion or metabolic processes. Although, there could have been post-mortem TFM accumulation, this seems unlikely because the animals were quickly removed and snap frozen in liquid N_2 at death Kane et al. (1994) also demonstrated that sea lamprey have a limited capacity to use glucuronidation, which is significantly lower than that of non-target fishes, such as rainbow trout, channel catfish (*Ictalurus punctatus*), and bluegill (*Lepomis macrochirus*). However, the present study demonstrated that fish that survived exposure to TFM for 24 h at a concentration of 2.0 mg TFM L^{-1} could still be using glucuronidation to partially detoxify and eliminate TFM due to lower internal TFM burdens found in surviving fish compared to dead fish.

It is possible that temperature alone plays a role in TFM toxicity, as the reported river temperature for 2013 tracked the increases in the TFM LC_{50} values. Metabolic rate also increases with temperature, which enhances the ability of fishes to take-up and eliminate chemicals (Hooper et al. 2013). Increased temperatures lead to higher ventilation rates resulting in increased uptake rates via the gills, thus increasing the toxicity of a chemical (Ford et al. 2004; Patra et al. 2009). However, higher temperatures have also shown to lead to increased excretion rates in fish, which results in a decrease in the chemical's toxicity (Koivusaari et al. 1981; Maruya et al. 2005; Noyes et al. 2009). Recent work has shown that temperature influences the toxicity of phenols in freshwater fish, with higher temperatures leading to increased toxicity (Patra et al. 2015). However, this remains to be investigated in sea lamprey exposed to TFM, as other abiotic factors, such as water pH (Chapter 2) and daylight, or their combination, could have an effect on TFM tolerance (van Genderen et al. 2005; Paterson et al. 2007; Hooper et al. 2013; Nahrgang et al. 2013).

Effects of Season and Temperature the Detoxification of TFM via Glucuronidation

The abundance of UGT mRNA did not vary with season which did not support the hypothesis that seasons have an effect on mRNA abundance of UGT. Furthermore, temperature did not have an effect on UGT mRNA abundance. This further suggests that variation in glucuronidation capacity does not explain the seasonal variation in TFM sensitivity in larval sea lamprey. However, glucuronidation cannot be fully dismissed because seasonal differences in protein abundance and enzyme activities have not yet been addressed. Seasonal changes in UGT activity have been observed in rainbow trout and common carp (*Cyprinus carpio*) with both species exhibiting decreased UGT activity

during winter months and elevated activity during summer months (Koivusaari et al. 1981; Koivusaari 1983; Curtis et al. 1990; Daidoji et al. 2006). An additional factor involved in glucuronidation is diurnal changes in UGT, where UGT activity has been shown in *Drosphila* to be at its highest at 20:00 and its lowest by 16:00 (Hooven et al. 2009). Diurnal changes in lamprey UGT activity could be a factor in the detoxification of TFM that has yet to be incorporated in experimentation evaluating glucuronidation. It seems less likely that glucuronidation capacity changes with season in sea lamprey because the activity of UGT is very low within the liver (Lech 1974; Lech and Statham 1975; Kane et al. 1994). Moreover, Le Clair (2014) demonstrated that during (12 h exposure) and after (24 h recovery) exposure to TFM, rainbow trout were able to clear TFM using glucuronidation while larval sea lampreys were unable to eliminate TFM. However, this earlier and present study determined whole body TFM and TFM-glucuronide concentrations, not TFM and TFM-glucuronide concentrations in either the bile or liver specifically. Since bile and liver (~1 % liver mass body mass^{-1}) are only a small percentage of the whole body volume, internal TFM and TFM-glucuronide concentrations could be underestimated. Additionally, it should be noted that UGT has only been examined in adult, not larval, sea lamprey due to the much larger liver in migrating sea lampreys (Kane et al. 1994). However, the liver in the adult stage is unlike that of the larval sea lamprey due to the accumulation of bile salts that arise from the loss of bile ducts following metamorphosis (Youson 2003), which could impair enzyme activities by altering protein structure.

Notably, the larval sea lampreys' wet liver mass was influenced by colder temperatures. Potential increases in lipid content of the liver at colder temperatures could

explain some of the differences in TFM sensitivity observed (Dawson and Grimm 1980). Livers that have greater lipid content tend to have lower amounts of glycogen (Shuter et al. 2012). Since UDPGA is reliant on the breakdown of glycogen (Dutton 1980) and TFM causes the animal to consume glycogen during TFM exposure (Birceanu et al. 2009), livers with lower glycogen reserves would have a limited ability to use glucuronidation (Dutton 1980; Mandl et al. 1995; Ioannides 2002; Timbrell 2009). Decreases in the LC_{50s} observed in May and October could be related to changes in lipid:glycogen ratios within the liver. If an organism is starved, by surviving a harsh winter, for example, glycogen and lipid stores will be depleted, resulting in the organism allocating its resources to basic metabolic functions that are needed for survival (Beamish 1964; Lowe et al. 1973; Mogensen and Post 2012; Shuter et al. 2012). As previously suggested by Wilkie et al. (2007a) and Birceanu et al. (2009, 2011), TFM targets glycogen stores, making a lamprey that has survived a harsh winter, and thus has lower liver glycogen levels, more susceptible to TFM treatments. This could explain the observed changes in the LC_{50s} among May, June, and August, found by the present study and by Scholefield et al. (2008). However, this does not explain the sudden drop in the LC_{50} observed in October since body condition, including glycogen reserves, would be greater. The reduced LC_{50} in October is more likely due to other factorssuch as colder temperature which would reduce metabolic rates, possibly impairing the lamprey's ability to eliminate/metabolise TFM. .

Effects of Temperature on the Uptake and Excretion of TFM

At 22 °C, TFM uptake rates were greater at 0.5 h compared to 6 °C, where uptake rates remained unchanged, irrespective of the duration of the exposure. This observation

was consistent with the hypothesis that uptake rates at a warm temperature would be greater than rates at a cold temperature. At 6 °C, internal TFM burdens at 6 °C were greater after 2 h exposure to TFM compared to 22 °C where internal TFM burdens remained unchanged, irrespective of the duration of the exposure (Figure 3-6, B). This suggests that at warmer temperatures, the uptake, and elimination of TFM is elevated resulting in increased survivorship at warmer temperatures.

The primary mode of uptake of TFM is thought to be passive diffusion across the gills (Hunn and Allen 1974). At warmer water temperatures chemical reactions occur at a faster rate resulting in an increase in metabolic demand causing the fish to increase gill ventilation rates (Moffit and Crawshaw 1983; Gehrke and Fielder 1988). As ventilation rates are increased, the amount of inspired water is greater, leading to higher rates of toxicant uptake (Patra et al. 2009). The greater initial (0.5 h) uptake rates observed at 13 and 22 °C are therefore likely explained by increases in ventilation rates. Additionally temperature could influence gill permeability with warmer water resulting in increased permeability (Isaia 1979; Robertson and Hazel 1999). If uptake of TFM occurs by passive diffusion of the phenolic form of TFM (Hunn and Allen 1974), then an increase in TFM uptake rates would be expected.

In the current study, temperature had no effect on excretion rates of TFM by larval sea lamprey when injected with radioactive TFM and left in TFM-free water. This was inconsistent with the hypothesis that warmer temperatures would increase TFM excretion. Seasonality and temperature still have an effect on the overall toxicity of TFM, but greater elimination via passive diffusion at warmer temperatures does not seem to explain the sea lamprey's greater TFM tolerance under warmer conditions.

Implications for Sea Lamprey Control

Larval sea lampreys were less susceptible to TFM in the summer compared to the spring and fall, with glucuronidation having a limited role, if any, in the observed increase in tolerance. The decrease in the LC_{50} in October was an unexpected finding since lampreys are thought to have fed throughout the summer building lipid and glycogen stores which would be expected to increase TFM tolerance. Moreover, temperature in October was 11 °C, which would presumably lower their metabolic rates and lower TFM uptake, leading to greater survival. Nevertheless, the present findings clearly demonstrate that TFM tolerance is greatest in mid- to late-summer. This suggest that re-scheduling TFM treatments to earlier or later in the year might be beneficial for large streams or rivers to decrease the total amount of TFM required and to reduce the risk of residual sea lampreys that survive treatments, complete metamorphosis, and subsequently parasitize economically and culturally significant fish in the Great Lakes.

Table 3-1. Water quality parameters [% dissolved oxygen (DO), temperature (°C), and pH] and morphometrics of larval sea lamprey exposed to a range of 3-trifluoromethyl-4-nitrophenol (TFM) concentrations in 2013 and not exposed to TFM in 2014.

Data presented as the mean ± SEM.

Exposed to TFM

2013						
Season	% DO	Temperature (°C)	pH	n	Length (mm)	Mass (g)
May (6 °C)	106.5 ± 0.35	5.6 ± 0.06	7.6 ± 0.12	328	70 ± 1.14	0.80 ± 0.04
June (20 °C)	89.8 ± 0.53	20.4 ± 0.16	7.9 ± 0.19	335	70 ± 1.08	0.70 ± 0.03
August (23 °C)	88.5 ± 0.59	23.5 ± 0.05	8.3 ± 0.01	360	60± 0.74	0.50 ± 0.01
October (11 °C)	99.9 ± 0.60	11.6 ± 0.14	8.3 ± 0.01	270	70 ± 1.02	0.60 ± 0.02

Not Exposed to TFM

2014						
Temperature (°C)	Measured Temperature (°C)	% DO	pH	n	Length (mm)	Mass (g)
6	7.8 ± 0.18	93.2 ± 2.57	8.11 ± 0.03	47	90 ± 1.85	0.9 ± 0.05
12	11.2 ± 0.09	94.6 ± 1.97	8.29 ± 0.04	49	90 ± 1.84	0.9 ± 0.06
22	22.1 ± 0.19	90.1 ± 0.97	8.14 ± 0.05	46	80 ± 1.13	0.8 ± 0.11

Table 3-2. Water quality parameters pH and temperature (°C) during a one week acclimation of larval sea lamprey prior to measurements of TFM uptake and elimination at three different temperatures (6, 13, and 22 °C).

Data presented as the mean ± SEM.

TFM Uptake Experiments

	Acclimation		Exposure	
Nominal Temperature (°C)	pH	Temperature (°C)	pH	Temperature (°C)
6	8.14 ± 0.02	6.9 ± 0.20	7.94 ± 0.04	7.9 ± 0.11
13	7.79 ± 0.01	14.1 ± 0.15	7.87 ± 0.03	13.3 ± 0.18
22	8.15 ± 0.03	19.9 ± 0.12	8.04 ± 0.03	19.2 ± 0.21

TFM Excretion Experiments

	Acclimation		Exposure	
Nominal Temperature (°C)	pH	Temperature (°C)	pH	Temperature (°C)
6	8.14 ± 0.02	6.9 ± 0.05	7.92 ± 0.04	7.6 ± 0.04
13	7.79 ± 0.05	14.1 ± 0.03	7.59 ± 0.07	13.4 ± 0.06
22	8.15 ± 0.09	19.9 ± 0.02	7.98 ± 0.04	20.3 ± 0.07

Figure 3-1. The detoxification of 3-trifluoromethyl-4-nitrophenol (TFM) through glucuronidation.

The enzyme, uridine-diphosphate-glucuronosyltransferase (UGT) facilitates the attachment of UDP-glucuronic acid (UDPGA), to organic, poorly soluble molecules such as TFM, to form TFM-glucuronide. The substrate, UDPGA is generated from glucose-1-phosphate, an intermediate of glycogenolysis. Glucose-1-phosphate then reacts with uridine -triphosphate (UTP) to form UDP-glucose and pyrophosphate (PP). UDP-glucose is then hydrolyzed to form UDPGA. Because the glucuronidated molecule is more hydrophilic, it is easier to excrete via the gastrointestinal tract and/or via renal pathways (Dutton 1980; Timbrell 2009).

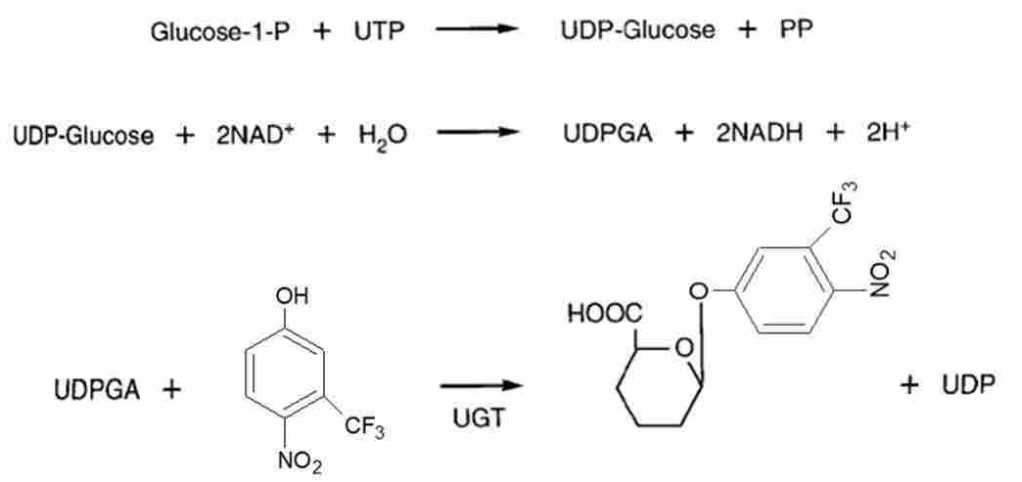

Glucose-1-P + UTP
UDP-Glucose + PP
UDP-Glucose + 2NAD+ + H2O
UDPGA + 2NADH + 2H+
UDPGA +
OH
CF3
NO2
UGT
HOOC
O
O
CF3
NO2
+ UDP

Figure 3-2. Seasonal differences in the toxicity of 3-trifluoromethyl-4-nitrophenol (TFM) to larval sea lamprey.

Changes in the 12 h LC_{50} and ambient water temperature of larval sea lamprey collected from the Au Sable River, MI, USA, in May, June, August, and October 2013. Data expressed as the 12 h LC_{50} plus the 95 % confidence interval (CI). Statistical significance was determined by evaluating if confidence intervals overlapped (Wheeler et al. 2006). The inset denotes changes in daily stream temperatures (°C), for the Au Sable River, MI, USA, from May to October of 2013. Data courtesy of survey station 04137500 of the US-Geological Survey, Michigan Water Grayling Field Office, Grayling, MI, USA.

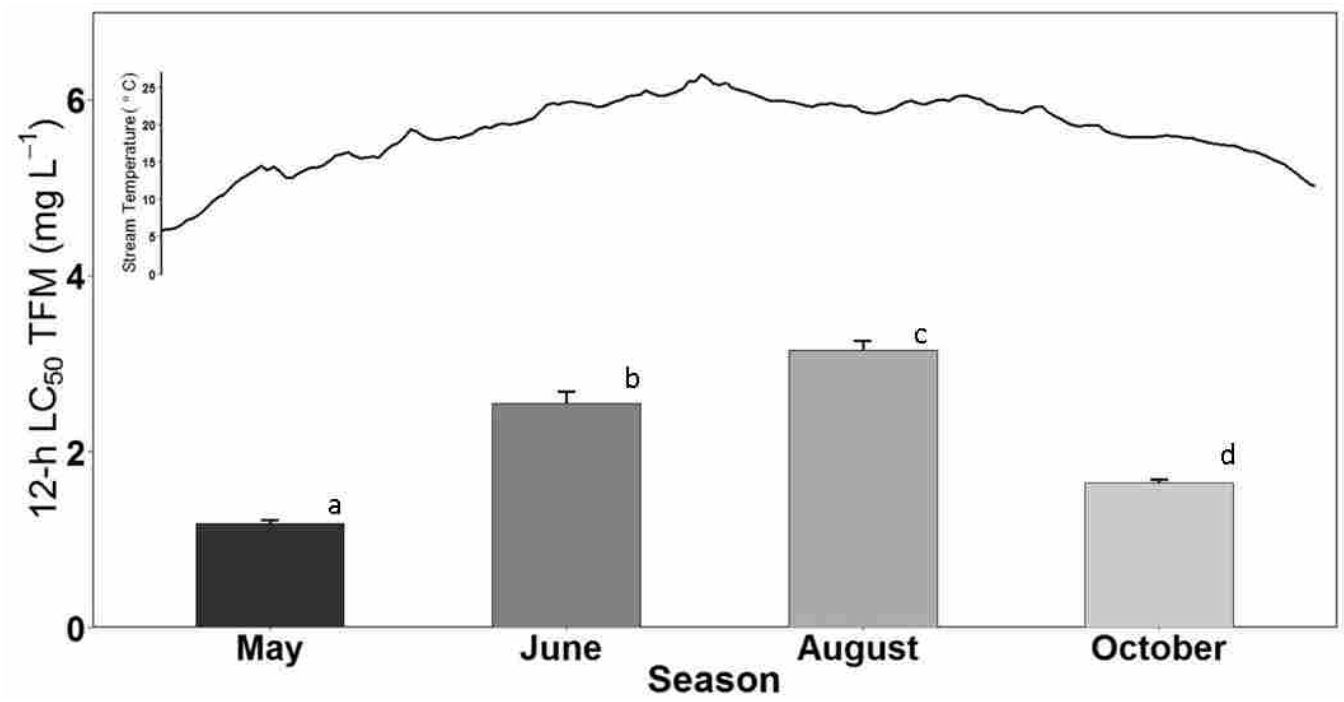

12-h LC50 TFM (mg L−1)
Stream Temperature (°C)
0
5
10
15
20
25
0
2
4
6
a
b
c
d
May
June
August
October
Season

Figure 3-3. The concentrations of 3-trifluoromethyl-4-nitrophenol (TFM) and TFM-glucuronide in larval sea lamprey following TFM exposure during different seasons. Effects of TFM exposure (12 h LC_{50}; 2.0 mg L^{-1}) on (A) the concentrations of TFM and TFM-glucuronide in larval sea lamprey collected from the Au Sable River, in May, June, August, and October, in 2013, and (B) the concentration of TFM in the whole body sea lamprey that either survived or experienced mortality during exposure to 2.0 mg L^{-1} TFM regardless of season. Data are presented as the mean + SEM (n = 5-9 fish) and data points sharing the same letter are not statistically different.

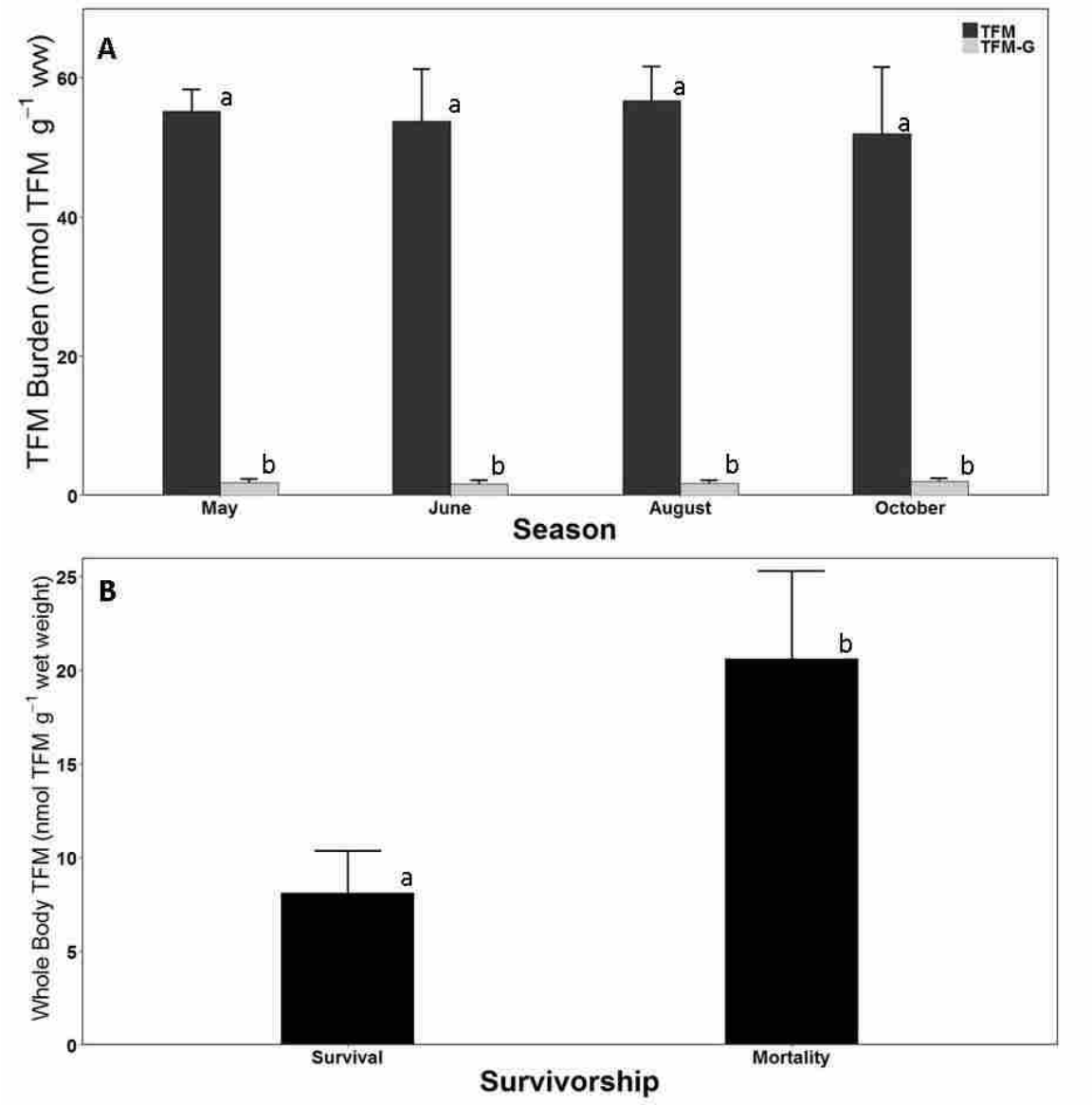

A
TFM
TFM-G
TFM Burden (nmol TFM g^{-1} ww)
0
20
40
60
a
b
a
b
a
b
a
b
May
June
August
October
Season
B
Whole Body TFM (nmol TFM g^{-1} wet weight)
0
5
10
15
20
25
a
b
Survival
Mortality
Survivorship

Figure 3-4. Effects of season on liver mass, RNA content, and mRNA abundance of uridine-diphosphate-glucuronosyltransferase (UGT).

Changes in liver tissue (A) mass, (B) RNA content, and (C) mRNA abundance of uridine-diphosphate-glucuronosyltransferase (UGT) in larval sea lamprey at four seasonal time points (May, June, August, and October). Data are presented as the mean + SEM (n = 6). Data points sharing the same letter are not statistically different.

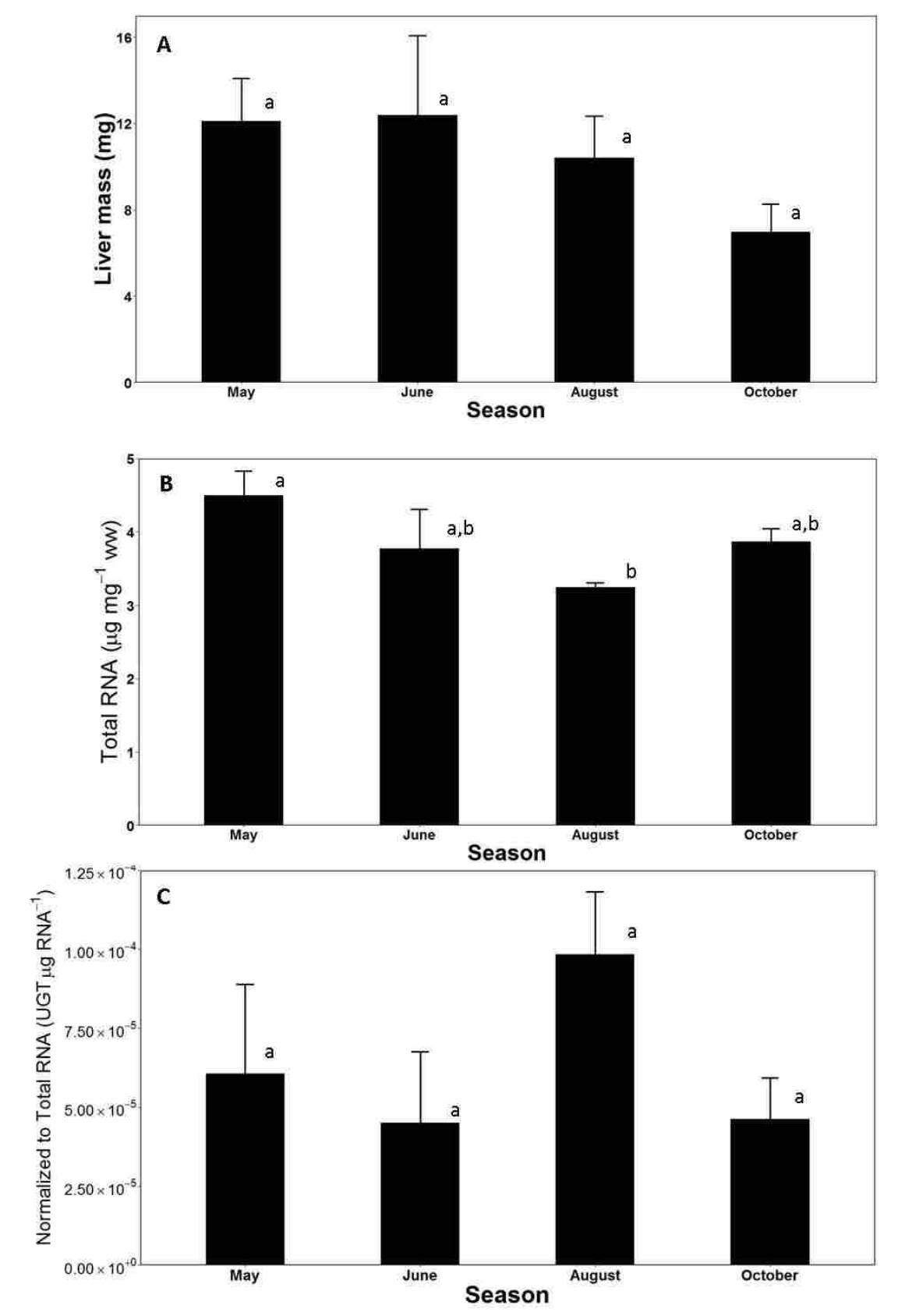

A
Liver mass (mg)
16
12
8
4
0
a
a
a
a
May
June
August
October
Season
B
Total RNA (µg mg^{-1} ww)
5
4
3
2
1
0
a
a,b
b
a,b
May
June
August
October
Season
C
Normalized to Total RNA (UGT µg RNA^{-1})
1.25 × 10^{-4}
1.00 × 10^{-4}
7.50 × 10^{-5}
5.00 × 10^{-5}
2.50 × 10^{-5}
0.00 × 10^{+0}
a
a
a
a
May
June
August
October
Season

Figure 3-5. Effects of temperature on liver mass, RNA content, and mRNA abundance of uridine-diphosphate-glucuronosyltransferase (UGT).

Changes in liver tissue (A) mass, (B) RNA content, and (C) mRNA abundance of uridine-diphosphate-glucuronosyltransferase (UGT) in larval sea lamprey at three temperatures (6, 12, and 22 °C). Data are presented as the mean + SEM (n= 6). Data points sharing the same letter are not statistically different.

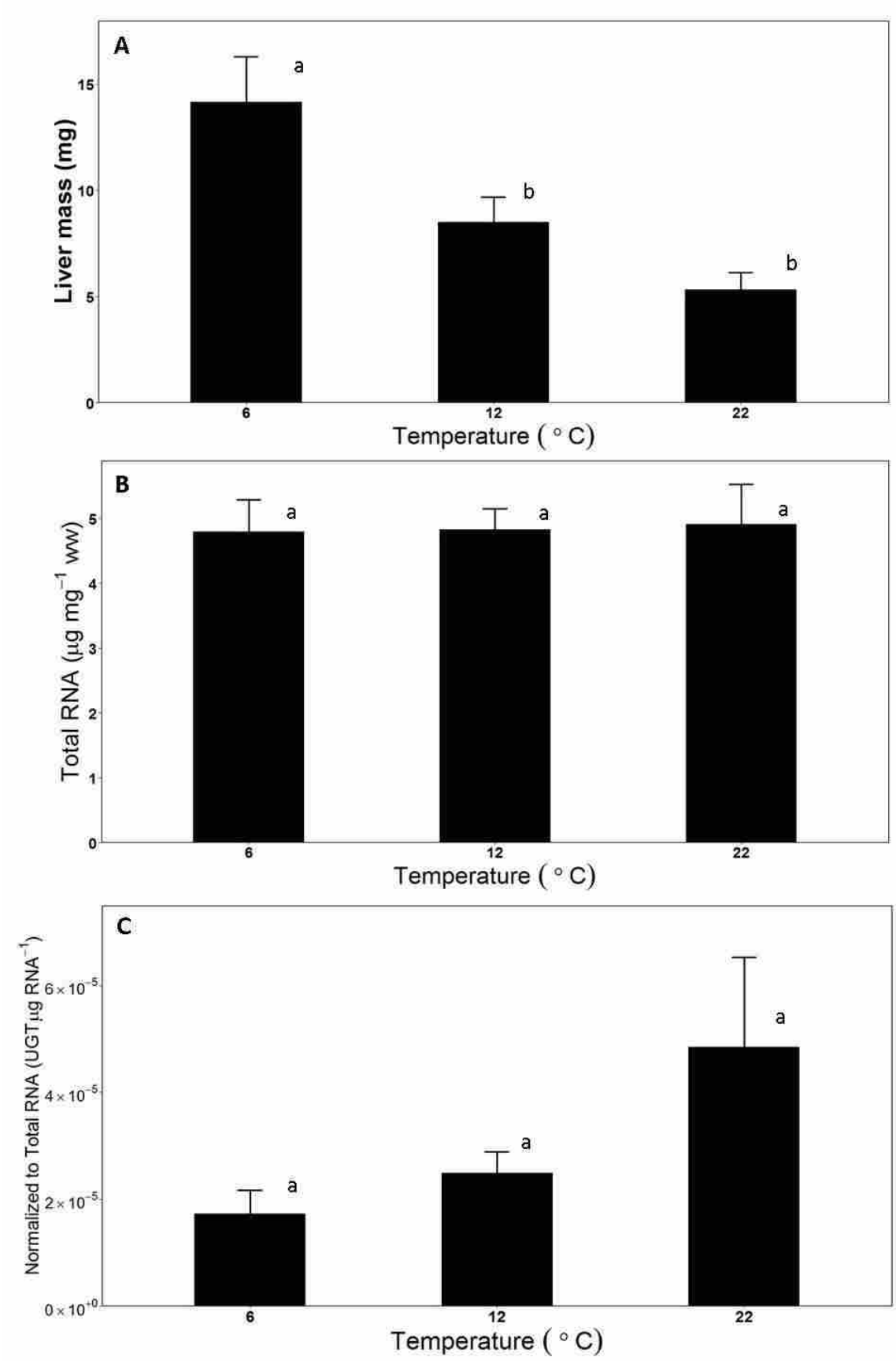

A
Liver mass (mg)
0
5
10
15
a
b
b
6
12
22
Temperature (° C)
B
Total RNA (µg mg^{-1} ww)
0
1
2
3
4
5
a
a
a
6
12
22
Temperature (° C)
C
Normalized to Total RNA (UGT µg RNA^{-1})
0×10^{-0}
2×10^{-5}
4×10^{-5}
6×10^{-5}
a
a
a
6
12
22
Temperature (° C)

Figure 3-6. Effects of temperature and time on uptake rates of 3-trifluoromethyl-4-nitrophenol.

Changes in (A) uptake rates and (B) internal burden of 3-trifluoromethyl-4-nitrophenol (TFM) at different temperatures (6,13, and 22 °C) and at 0.5 h (black bars), 1 h (medium grey), and 2 h (light grey) for larval sea lamprey exposed to the 12 h LC_{99} of TFM, 7.6 mg L^{-1}. Data are presented as the mean+ SEM (n = 11-12). Letters denote a temperature effect on TFM uptake rates of TFM, while the asterisk (*) denotes a time effect within one temperature.

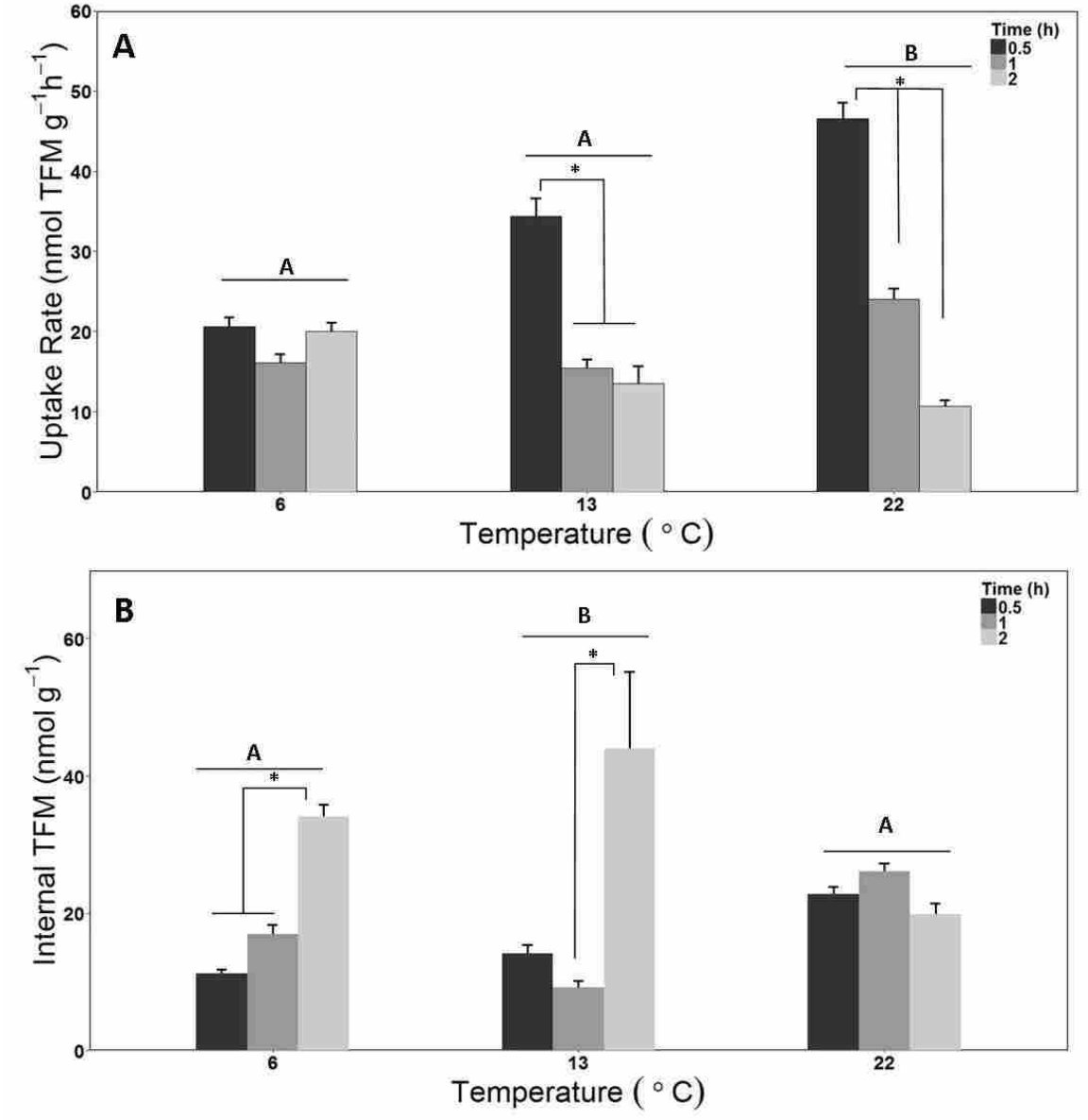
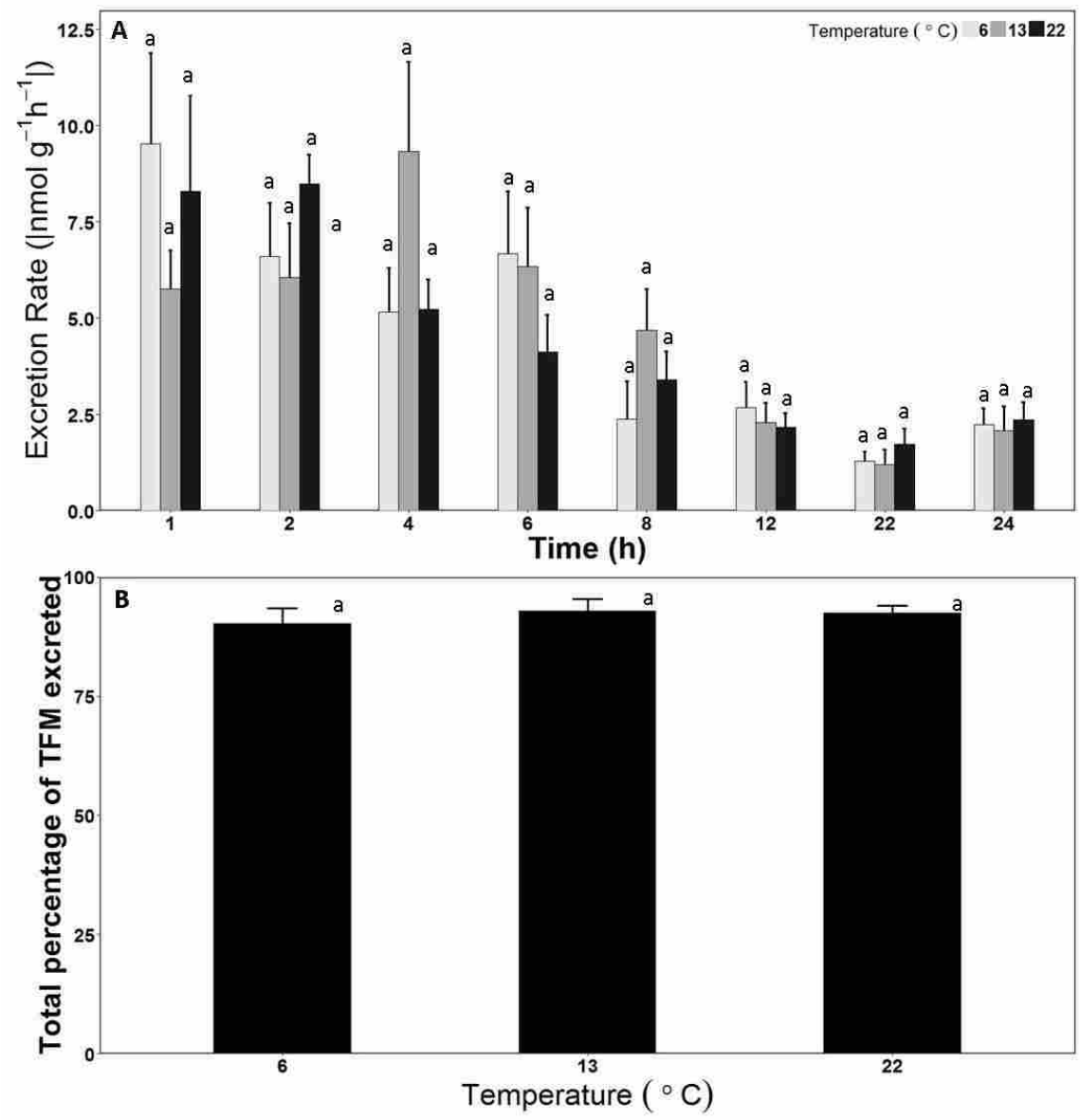

A
Time (h)
0.5
1
2
Uptake Rate (nmol TFM g^{-1}h^{-1})
60
50
40
30
20
10
0
A
A
B
*
*
6
13
22
Temperature (° C)
B
Time (h)
0.5
1
2
Internal TFM (nmol g^{-1})
60
40
20
0
A
B
A
*
*
6
13
22
Temperature (° C)

Figure 3-7. Effects of temperature on excretion rates of 3-trifluoromethyl-4-nitrophenol by larval sea lamprey.

Changes in (A) excretion rates of 3-trifluoromethyl-4-nitrophenol (TFM) and (B) total percent excreted over 24 h by larval sea lamprey at different temperatures (6 °C-light bars, 13 °C-medium bars, and 22 °C- dark bars). Data for (A) is presented as the absolute value of the mean + SEM, while data for (B) is presented as the mean + SEM (n=8). Data points sharing the same letter are not statistically different.


A
Temperature (° C) 6 13 22
Excretion Rate (|nmol g^{-1}h^{-1}|)
12.5
10.0
7.5
5.0
2.5
0.0
1
2
4
6
8
12
22
24
Time (h)
B
Total percentage of TFM excreted
100
75
50
25
0
6
13
22
Temperature (° C)

Chapter 4:

An Integrated Model of Abiotic Factors Influencing the Toxicity of 3-Trifluoromethyl-4-Nitrophenol (TFM) in Larval Sea Lamprey (*Petromyzon marinus*)

Introduction

The piscicide, 3-trifluoromethyl-4-nitrophenol (TFM) has been used for over 60 years to control invasive sea lamprey populations in the Great Lakes (Great Lakes Fishery Commission 2011), but there remains a need to better understand how TFM works and how it can be used more effectively (McDonald and Kolar 2007; Adair and Sullivan 2013). It is well known that the TFM sensitivity is strongly influenced by abiotic variables such as water pH (Bills et al. 2003; McDonald and Kolar 2007), temperature (Marking and Bills 1976), and season (Scholefield et al. 2008), but the underlying reasons for this are poorly understood. The present thesis sheds considerable light on the underlying mechanisms that result in variations in TFM sensitivity due to variation in pH, season, and temperature. Below, I propose how this knowledge can be used to develop more effective methods of TFM application in the integrated pest management of sea lamprey.

Integrated Pest Management of the Sea Lamprey in the Great Lakes

Invasive sea lamprey became a threat to the Great Lakes in 1900s, resulting in the collapse of major fisheries within the Great Lakes by the 1940s and 1950s (Siefkes et al. 2013). By treating infested streams with TFM every 2 to 4 years, sea lamprey populations have been greatly reduced leading to the restoration of some of these major fisheries (McDonald and Kolar 2007; Siefkes et al. 2013). Additionally, barriers and traps are used to prevent migrating adult sea lamprey from reaching desirable spawning habitat. By gaining a greater understanding of TFM toxicity, changes in TFM usage can be made resulting in the improvement and development of other control methods besides TFM. By

integrating present and new control methods, control of sea lamprey populations might be improved.

Effect of pH on TFM Speciation, Uptake, and Elimination

The chemical property, pH, strongly influences TFM toxicity, $pK_a = 6.07$ (Hubert 2003; McDonald and Kolar 2007), and the present study demonstrates that this results in higher rates of TFM uptake in more acidic water compared to alkaline pH. This is caused by more un-ionized, phenolic TFM being present at an acidic pH, compared to alkaline pH, where the ionized, phenolate form of TFM predominates. Moreover, the total amount of TFM accumulated in the larval sea lamprey was 3.75-5.50 fold greater at low and immediate pH (pH 6.5 and 7.8) compared to more alkaline pH (pH 9.0; Figure 2-2, C). This data, along with linear regression analyse (Figure 2-3) supports the hypothesis that the method of TFM uptake is by passive diffusion of un-ionized TFM across the gills (Figure 4-1). The greater the amount of un-ionized TFM in the water, compared to the blood, the greater the inwardly directed un-ionized TFM gradient, and the greater the rate of TFM uptake. Furthermore, pH stimulated excretion rates over the first 2-4 h of depuration in in TFM-free water at pH 9.0 compared to pH 6.5. Thus, the underlying basis for adjusting TFM application rates according to pH (Bills et al. 2003) is that TFM uptake rates are higher in acidic versus more alkaline pH and initial excretion rates are greater at a more alkaline pH than acidic pH.

The speciation of TFM might also be strongly influenced by events occurring in the gill microenvironment, which would reflect water pH at the gill-water interface and be more acidic than the bulk water due to CO_2 and metabolic acid excretion at the gill (Figure 4-1, A; Playle and Wood 1989). There is little evidence that TFM is entering the

fish in its ionized, phenolate form even at alkaline pH. Greater ionized TFM at alkaline pHs could result in a greater potential for some ionized TFM to enter the blood, due to high inwardly directed electrochemical gradients for TFM. However, as demonstrated in Chapter 2, there was no relationship between rates of TFM uptake and ionized TFM (Figure 2-3). Other weak organic acids similar to TFM such as 2,4,-dinitrophenol, are also influenced by bulk water pH (Erickson and McKim 1990; Howe et al. 1994; Erickson et al. 2006). Depending on the pK_a of the weak organic acid, acidic pHs facilitate the uptake of these similar compounds by causing more of the un-ionized form of the compound to be present which can easily diffuse across the gill membrane into the fish. Alkaline bulk pHs can assist the offloading of weak organic acids by influencing the gill microenvironment allowing for a larger gradient of the un-ionized form of the compound to exist (Erickson et al. 2006).

Influence of Season on TFM Toxicity in Larval Sea Lamprey

Scholefield et al. (2008) demonstrated that during May, larval sea lamprey tolerance to TFM was low when compared to July and August. They postulated that different factors, including changes in, temperature, nutritional status, and pH, could influence TFM sensitivity. Seasonal changes in larval sea lamprey tolerance to TFM have similarly been shown in the present study. Temperature could be the primary factor to explain why season influences TFM toxicity. Temperature influenced the rate of TFM uptake with warmer temperatures resulting in greater initial rates (0.5 h) of TFM uptake (Figure 3-6). At higher temperatures, gill ventilation rates will be greater due to an increase in metabolic rate (Figure 4-2). An interesting observation was that TFM accumulated in fish acclimated to a colder temperature (6 °C) when the fish was exposed

to TFM for longer periods (2 h). However, this did not hold true for fish acclimated to warmer temperatures (Figures 3-6 and 4-2). This suggests that TFM may be detoxified more effectively in warmer versus colder waters. However, temperature had no effect on excretion rates of TFM, suggesting that TFM might be metabolized more efficiently within the animal. Recent studies have shown that similar compounds, such as, 2,4, dinitrophenol, are metabolized in a similar manner in some fishes (Patra et al. 2015). Patra et al. (2015) reported that temperature had similar effects on the uptake and elimination of endosulfan, chlorpyrifos, and phenol. They observed that at warmer temperatures, metabolic rates increased resulting in increased ventilation rates leading to greater uptake rates of endosulfan and chlorpyrifos (Patra et al. 2009). However this does not mean the toxicity of the compound will increase, decrease, or stay the same as temperature can also affect elimination process, specifically the metabolism of the toxic compound(s), as suggested here for TFM.

Implications for the Integrated Pest Management of Sea Lamprey

Applications of TFM are the primary method of control for sea lamprey populations in the Great Lakes (McDonald and Kolar 2007). Even though TFM treatments are usually 90% effective (Johnson et al. 2014), improvements to treatments will assist in reducing financial costs associated with treatments, the possibility of residual larval sea lamprey that survive treatment, and minimize adverse effects to non-target species. Financial costs associated with TFM treatments can be reduced by modifying treatment protocols and schedules to better reflect the influences of season, pH, and temperature on TFM toxicity. Observations from the present study will therefore

assist TFM treatment mangers in determining more optimal treatment protocols and schedules based on the effects that season, temperature, and pH have on TFM toxicity.

A potential modification to increase TFM effectiveness when a sea lamprey infested stream is more alkaline would be to temporally bubble CO_2 into the water to reduce the pH of the stream (McDonald and Kolar 2007). However, the effects of temporally reducing the pH in an alkaline stream on the flora and fauna will need to be taken into account as this could have an additional negative effect to non-target organisms. It may also be advisable to alter TFM treatment schedules in response to differences in the populations of larval sea lampreys. For instance, TFM could be conserved if rivers with larger populations of lamprey were treated ineither spring or fall when lampreys are more susceptible to the lampricide. Lastly a final modification to TFM treatment protocols would reduce the overall concentration of TFM used to treat the stream but extend the duration of treatment from 12 h to 24 h. By improving the suppression of sea lamprey populations in the Great Lakes through modifications to TFM treatments based on the present study's findings, TFM treatments potentially could be more economical, environmental, and socially beneficial.

Future Directions and Conclusion

Further investigations should address the combined effect of temperature and pH on uptake and excretion rates of TFM. Understanding the combined effect of temperature and pH on the toxicity of TFM would allow for managers to make better field decisions allowing for more efficient treatments. Studies targeting the effects pH have on the gill microenvironment resulting in changes in the uptake or offloading of TFM are also needed. Furthermore, investigation into the effects of other seasonal factors such as

daylight, nutritional status and food availability, and weather patterns, could also improve our understanding of how the sensitivity of lampreys to TFM varies with season. Lastly, more focused investigations into glucuronidation or other detoxification pathways, are needed to better understand how sea lamprey potentially detoxify TFM, beginning with a better understanding of how the kinetics of the enzyme UDP-glucuronosyltransferase change with season and life stage in sea lampreys. Glucuronidation of TFM by larval sea lamprey cannot yet be fully dismissed as a contributing factor to the observed seasonal changes in TFM tolerance, since the concentrations of both TFM and TFM-glucuronide in the whole body may not reflect concentrations in the liver and the bile, where most TFM-glucuronide would be found.

Figure 4-1. Model of uptake and elimination of 3-trifluoromethyl-4-nitrophenol at different pHs.

(A) TFM is thought to primarily diffuse across the gill as un-ionized TFM. At a low pH, there is more un-ionized TFM (phenolic) at a given total TFM concentration, where Total TFM = ionized (phenolate ion) + un-ionized (phenolic) TFM. As a result, there is a larger inwardly directed un-ionized TFM gradient at lower pH at a given TFM concentration. At higher pH more TFM is ionized, reducing the inward gradient of TFM. Events in the boundary layer of the gill, which is generally more acidic than the bulk water, also need to be considered when calculating TFM diffusion gradients, and should be addressed in future studies. Solid arrows in both (A) and (B) indicate un-ionized TFM moving across the gill membrane while the dotted arrow indicates ionized TFM inability to move across the gill membrane. (B) The influence of external pH on the excretion of TFM across the gills following TFM exposure in "clean" TFM-free water. At lower pH, rates of TFM excretion are generally lower than observed in more alkaline water. The lower boundary layer pH could result in a build-up of un-ionized TFM in this region, which would impair TFM excretion in more acidic or circumneutral pH water. At high pH, however, less TFM would be in its un-ionized form resulting in greater rates of TFM unloading. An upward facing arrow next to ionized TFM in the blood indicates greater ionized TFM at blood pH, while a downward arrow indicates lower of un-ionized TFM at a typical blood pH.

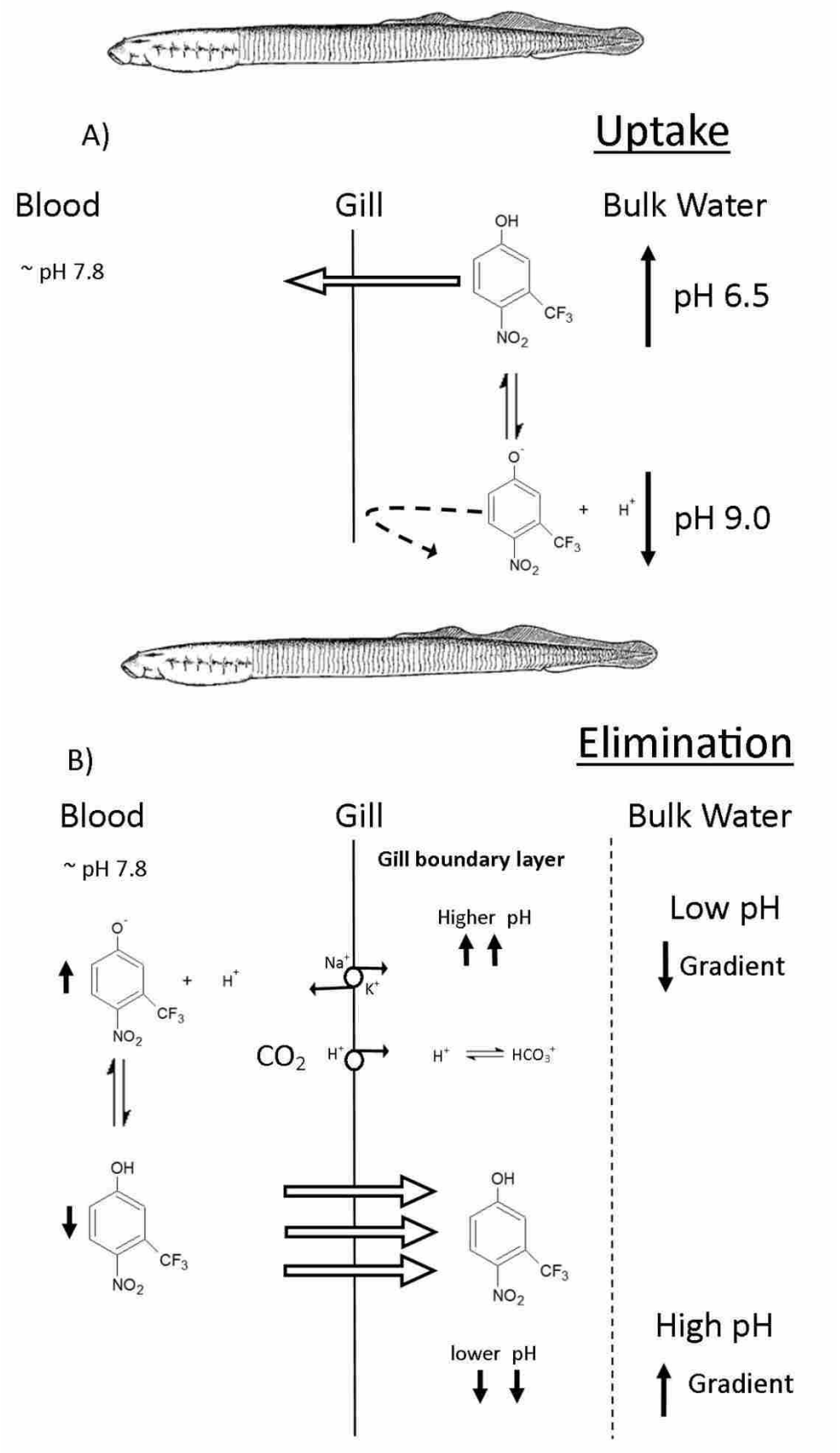
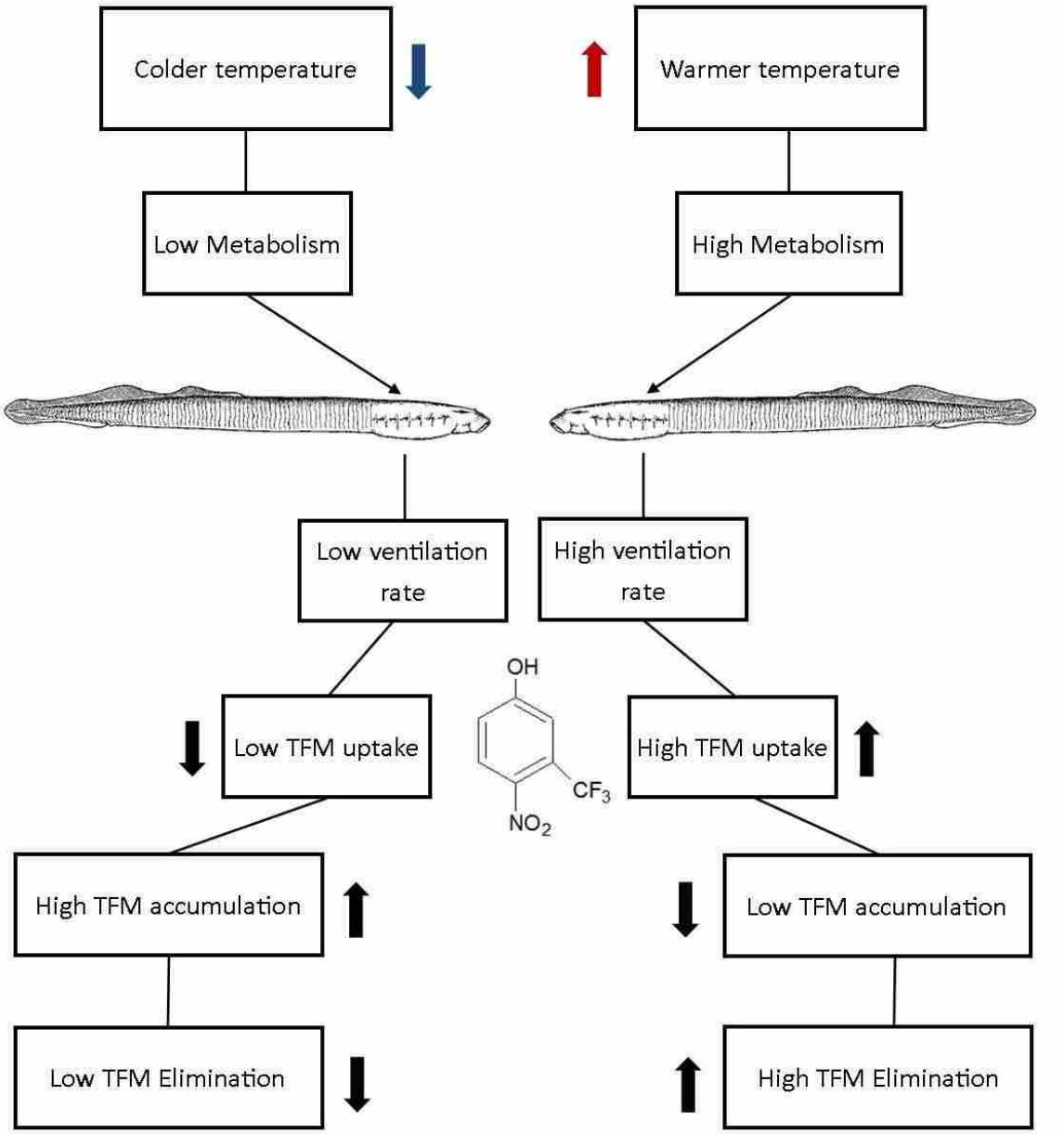

A)
Uptake
Blood
~ pH 7.8
Gill
Bulk Water
pH 6.5
pH 9.0
B)
Elimination
Blood
~ pH 7.8
Gill
Gill boundary layer
Bulk Water
Higher pH
Low pH
Gradient
CO2
lower pH
High pH
Gradient

Figure 4-2. Model of uptake and elimination of 3-trifluoromethyl-4-nitrophenol at different temperatures by larval sea lampreys.

Colder temperatures decreases metabolic rate in larval sea lamprey resulting in decreased rates of gill ventilation. As a result, rates of TFM are lower, as well as rates of TFM elimination (excretion) across the gills. TFM elimination may also be slowed due to impaired detoxification in the liver via mechanisms that have not yet been identified. At warmer temperatures, metabolic rate is higher resulting in increased gill ventilation and greater TFM uptake, but also more rapid TFM elimination through enhanced excretion of TFM and/or improved detoxification in the liver. Due to these differences, the tolerance of larval sea lamprey to TFM is greater at warmer temperatures.

Influence of Temperature on Uptake and Elimination



Appendix A

List of Abbreviation

^{14}C-TFM	Carbon radioisotope Labeled 3-trifluoromethyl-4-nitrophenol
ATP	Adenosine triphosphate
cDNA	Copy DNA
GLFC	Great Lakes Fisheries Commission
HBBS	Hammond Bay Biological Station
HPLC	High performance liquid chromatography
LC	Lethal Concentration
mRNA	Messenger RNA
qPCR	Real-Time Quantitative Polymerase Chain Reaction
RER	Rough endoplasmic reticulum
SPE	Solid phase extraction
TFM	3-trifluoromethyl-4-nitrophenol

TFM-G	TFM-glucuronide
UDPGA	Uridine-diphosphate-glucuronic acid
UGT	Uridine-diphosphate-glucuronosyltransferase

References

Adair, R., and Sullivan, P. 2013. Sea lamprey control in the Great Lakes, 2012: annual report to the Great Lakes Fishery Commission. Great Lakes Fishery Commission, Ann Arbor, MI, USA.

Allan, J. D. 1995. Phyiscal factors of importance to the biota. Pages 45–82 Stream Ecology Structure and function of running waters, First edition. Chapman & Hall, London, UK.

Applegate, V. C. 1950. Natural history of the sea lamprey (*Petromyzon marinus*), in Michigan. US, Dept. of the Interior, Fish and Wildlife Service, 1950.

Applegate, V. C. 1961. Downstream movements of lampreys and fishes in the carp lake river, Michigan. Washington, Bureau of Commercial Fisheries, 1961.

Applegate, V. C., Howell, J. H., Moffett, J. W., Johnson, B. G. H., and Smith, M. A. 1961. Use of 3-trifluoromethyl-4-nitrophenol as a selective sea lamprey larvicide. Great Lakes Fishery Commission Technical Report 1:1–35.

Applegate, V. C., and King Jr., E. L. 1962. Comparative toxicity of 3-trifluormethyl-4-nitrophenol (TFM) to larval lamprey and eleven species of fishes. Transactions of the American Fisheries Society 91:342–345.

Applegate, V. C., and Smith, B. R. 1950. Sea lamprey spawning runs in the Great Lakes, 1950. U.S. Fish and Wildlife Service Special Scientific Report Fisheries. Washington: US Fish and Wildlife Service, 1950.

Araujo, M. J., Novais, D., Antunes, C., and Araújo, M. J. 2013. Record of a newly metamorphosed anadromous sea lamprey (*Petromyzon marinus* Linnaeus, 1758) feeding on a freshwater fish. Journal of Applied Ichthyology 29(6):1380–1381.

Bartels, H., and Potter, I. C. 2004. Cellular composition and ultrastructure of the gill epithelium of larval and adult lampreys: implications for osmoregulation in fresh and seawater. The Journal of Experimental Biology 207(Pt 20):3447–3462.

Beamish, F., and Potter, I. 1975. The biology of the anadromous sea lamprey (*Petromyzon marinus*) in New Brunswick. Journal of Zoology 177:57–72.

Beamish, F. W. H. 1964. Influence of starvation on standard and routine oxygen consumption. Transactions of the American Fisheries Society 93(1):103–107.

Bills, T. D., Boogaard, M. A., Johnson, D. A., Brege, D. C., Scholefield, R. J., Westman, R. W., and Stephens, B. E. 2003. Development of a pH/alkalinity treatment model

for applications of the lampricide TFM to streams tributary to the Great Lakes. Journal of Great Lakes Research 29:510–520.

Binder, T. R., and McDonald, D. G. 2008. The role of temperature in controlling diel activity in upstream migrant sea lampreys (*Petromyzon marinus*). Canadian Journal of Fisheries and Aquatic Sciences 65:1113–1121.

Binder, T. R., McLaughlin, R. L., and McDonald, D. G. 2010. Relative importance of water temperature, water level, and lunar cycle to migratory activity in spawning-phase sea lampreys in Lake Ontario. Transactions of the American Fisheries Society 139(3):700–712.

Birceanu, O., McClelland, G. B., Wang, Y. S., Brown, J. C. L., and Wilkie, M. P. 2011. The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) uncouples mitochondrial oxidative phosphorylation in both sea lamprey (*Petromyzon marinus*) and TFM-tolerant rainbow trout (*Oncorhynchus mykiss*). Comparative Biochemistry and Physiology - C Toxicology and Pharmacology 153(3):342–349.

Birceanu, O., McClelland, G. B., Wang, Y. S., and Wilkie, M. P. 2009. Failure of ATP supply to match ATP demand: the mechanism of toxicity of the lampricide, 3-trifluoromethyl-4-nitrophenol (TFM), used to control sea lamprey (*Petromyzon marinus*) populations in the Great Lakes. Aquatic Toxicology 94:265–274.

Bjerselius, R., Li, W., Teeter, J. H., Seelye, J. G., Johnsen, P. B., Maniak, P. J., Grant, G. C., Polkinghorne, C. N., and Sorensen, P. W. 2000. Direct behavioral evidence that unique bile acids released by larval sea lamprey (*Petromyzon marinus*) function as a migratory pheromone. Canadian Journal of Fisheries and Aquatic Sciences 57:557–569.

Black, M. C., Millsap, D. S., and Mccarthy, J. F. 1991. Effects of acute temperature on change and toxicant respiration uptake by rainbow trout, *Salmo gairdneri* (Richardson). Physiological Zoology 64(1):145–168.

Blewett, T. A., Chow, T. L., Maclatchy, D. L., and Wood, C. M. 2014. A species comparison of 17-α-ethynylestradiol uptake and tissue-specific distribution in six teleost fish. Comparative Biochemistry and Physiology - C Toxicology and Pharmacology 161(1):33–40.

Blewett, T., MacLatchy, D. L., and Wood, C. M. 2013. The effects of temperature and salinity on 17-α-ethynylestradiol uptake and its relationship to oxygen consumption in the model euryhaline teleost (*Fundulus heteroclitus*). Aquatic Toxicology 127:61–71.

Boogaard, M. A., Bills, T. D., and Johnson, D. A. 2003. Acute toxicity of TFM and a

TFM/niclosamide mixture to selected species of fish, including lake sturgeon (*Acipenser fulvescens*) and mudpuppies (*Necturus maculosus*), in laboratory and field exposures. Journal of Great Lakes Research 29(Supplement 1):529–541.

Boutilier, R. G., Ferguson, R. A., Henry, R. P., and Tufts, B. L. 1993. Exhaustive exercise in the sea lamprey (*Petromyzon marinus*): relationships between anaerobic metabolism and intracellular acid-base balance. Journal of Experimental Biology 88:71–88.

Bryan, M. B., Zalinski, D., Filcek, K. B., Libants, S., Li, W., and Scribner, K. T. 2005. Patterns of invasion and colonization of the sea lamprey (*Petromyzon marinus*) in North America as revealed by microsatellite genotypes. Molecular Ecology 14(12):3757–3773.

Campbell, I., and Doeg, T. 1989. Impact of timber harvesting and production on streams: a review. Marine and Freshwater Research 40(5):519-539.

Chung-Davidson, Y.-W., Priess, M. C., Yeh, C.-Y., Brant, C. O., Johnson, N. S., Li, K., Nanlohy, K. G., Bryan, M. B., Brown, C. T., Choi, J., and Li, W. 2013. A thermogenic secondary sexual character in male sea lamprey. The Journal of Experimental Biology 216:2702–2712.

Clarke, D. J., George, S. G., and Burchell, B. 1991. Glucuronidation in fish. Aquatic Toxicology 20:35–56.

Clifford, A. M., Henry, M., Bergstedt, R., McDonald, D. G., Smits, A. S., and Wilkie, M. P. 2012. Recovery of larval sea lampreys from short-term exposure to the pesticide 3-trifluoromethyl-4-nitrophenol: implications for sea lamprey control in the Great Lakes. Transactions of the American Fisheries Society 141(6):1697–1710.

Cronin, M. T. D., and Livingstone, D., editors. 2004. Predicting chemical toxicity and fate. CRC Press LLC, Boca Raton, Fl.

Cummins, W. 1974. Structure and function of stream ecosystems. BioScience 24(11):631–641.

Curtis, L. R., Fredrickson, L. K., and Carpenter, H. M. 1990. Biliary excretion appears rate limiting for hepatic elimination of benzo[*a*]pyrene by temperature-acclimated rainbow trout. Fundamental and Applied Toxicology 15(3):420–428.

Daidoji, T., Kaino, T., Iwano, H., Inoue, H., Kurihara, R., Hashimoto, S., and Yokota, H. 2006. Down regulation of bisphenol a glucuronidation in carp during the winter pre-breeding season. Aquatic Toxicology 77(4):386–392.

Dawson, A. S., and Grimm, A. S. 1980. Quantative seasonal change in the protein, lipid

and energy content of the carcass, ovaries and liver of adult female plaice, *Pleuronectes platessa*. Journal of Fish Biology (16):493–504.

Dawson, V. K., Cummings, K. B., and Gilderhuis, P. A. 1975. Efficacy of 3-trifluoromethyl-4-nitrophenol (TFM), 2',5-dicholoro-4'-nitrosalicylanilide (Bayer 73), and a 98:2 mixture as lampricides in laboratory studies.US Fish and Wildlife Service, Investigation in Fish Control, Report 78.

Dutton, G. J. 1980. Glucuronidation of drugs and other compounds. CRC Press, Inc., Boca Raton, FL, USA.

Dutton, G. J., and Montgomery, J. P. 1958. Glucuronide synthesis in fish and the influence of temperature. Page 17p Conference Proceedings of the Biochemical Society.

Erickson, R. J., and McKim, J. M. 1990. A model for exchange of organic chemicals at fish gills: flow and diffusion limitations. Aquatic Toxicology 18(4):175–197.

Erickson, R. J., McKim, J. M., Lien, G. J., Hoffman, A. D., and Batterman, S. L. 2006. Uptake and elimination of ionizable organic chemicals at fish gills: i. model formulation, parameterization, and behavior. Environmental Toxicology and Chemistry / SETAC 25(6):1512–1521.

Eshenroder, R. L. 1992. Decline of lake trout in Lake Huron. Transactions of the American Fisheries Society 121:548–550.

Eshenroder, R. L. 2009. Comment: mitochondrial DNA analysis indicates sea lampreys are indigenous to Lake Ontario. Transactions of the American Fisheries Society 138(5):1178–1189.

Eshenroder, R. L. 2014. The role of the Champlain canal and Erie canal as putative corridors for colonization of Lake Champlain and Lake Ontario by sea lampreys. Transactions of the American Fisheries Society 143(3):634–649.

Eshenroder, R. L., and Burnham-Curtis, M.K. 1999. Species succession and sustainablity of the Great Lakes fish community. Pages 145–184 *in* W. W. Taylor and C. Paola Ferreri, editors. Great Lakes Fisheries Policy and Managment: A Binational Perspective, First edition. Michigan State University Press, East Lansing.

Evans, D. H., Piermarini, P. M., and Choe, K. P. 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiological Review 85:97–177.

Farmer, G. J. 1980. Biology and physiology of feeding in adult lampreys. Canadian Journal of Fisheries and Aquatic Sciences 37:1751–1761.

Farmer, G. J., Beamish, F. W. H., and Robinson, G. A. 1975. Food-consumption of adult landlocked sea lamprey, *Petromyzon marinus*, L. Comparative Biochemistry and Physiology A-Physiology 50:753–757.

Ford, J., Tibbetts, I., and Carseldine, L. 2004. Ventilation rate and behavioural responses of two species of intertidal goby (Pisces: Gobiidae*)* at extremes of environmental temperature. Hydrobiologia 528:63–73.

Frau, F., Cidu, R., and Ardau, C. 2012. Short-term changes in water chemistry in the Baccu Locci stream (Sardinia, Italy) affected by past mining. Applied Geochemistry 27:1844–1853.

Gehrke, P. C., and Fielder, D. R. 1988. Effects of temperature and dissolved oxygen on heart rate, ventilation rate and oxygen consumption of spangled perch, *Leiopotherapon unicolor* (Günter 1859), (Percoidei, Teraponidae). Journal of Comparative Physiology B 157: 771-782.

van Genderen, E. J., Ryan, A. C., Tomasso, J. R., and Klaine, S. J. 2005. Evaluation of acute copper toxicity to larval fathead minnows (*Pimephales promelas*) in soft surface waters. Environmental Toxicology and Chemistry / SETAC 24(2):408–414.

Great Lakes Fishery Commission. 2011. Strategic vision of the Great Lakes Fishery Commission 2011-2020. Great Lakes Fishery Commission, Ann Arbor, MI.

Hänninen, O., Lindström-Seppä, P., Koivusaari, U., Väisänen, M., Julkunen, A., and Juvonen, R. 1984. Glucuronidation and glucosidation reactions in aquatic species in boreal regions. Biochemical Society Transactions 12(1):13–17.

Hansen, G. J. A., and Jones, M. L. 2009. Variation in larval sea lamprey demographics among Great Lakes tributaries: a mixed-effects model analysis of historical survey data. Journal of Great Lakes Research 35:591–602.

Hansen, M. J., Adams, J. V, Cuddy, D. W., Richards, J. M., Fodale, M. F., Larson, G. L., Ollila, D. J., Slade, J. W., Steeves, T. B., Young, R. J., and Zerrenner, A. 2003. Optimizing larval assessment to support sea lamprey control in the Great Lakes. Journal of Great Lakes Research 29:766–782.

Hayton, W. L., and Stehly, G. R. 1983. pH control of weak electrolyte toxicity to fish. Environmental Toxicology and Chemistry 2(3):325–328.

Heinrich, J. W., Mullett, K. M., Hansen, M. J., Adams, J. V, Klar, G. T., Johnson, D. A., Christie, G. C., and Young, R. J. 2003. Sea lamprey abundance and management in Lake Superior, 1957 to 1999. Journal of Great Lakes Research 29 (Supplement 1):566–583.

Heinrich, J. W., Weise, J. G., and Smith, B. R. 1980. Changes in biological characteristics of the sea lamprey (*Petromyzon marinus*) as related to lamprey abundance, prey abundance, and sea lamprey control. Canadian Journal of Fisheries and Aquatic Sciences 37:1861–1871.

Henry, M., Birceanu, O., Clifford, A. M., McClelland, G. B., Wang, Y. S., and Wilkie, M. P. 2015. Life stage dependent responses to the lampricide, 3-trifluoromethyl-4-nitrophenol (TFM), provide insight into glucose homeostasis and metabolism in the sea lamprey (*Petromyzon marinus*). Comparative Biochemistry and Physiology - C Toxicology and Pharmacology 169:35–45.

Hill, R. W., Wyse, G. A., and Anderson, M. 2008. Animal physiology, Second edition. Sinauer Associates, Inc., Sunderland, Massachusetts, USA.

Holmes, J. A., and Youson, J. H. 1994. Fall condition factor and temperature influence the incidence of metamorphosis in sea lamprey, *Petromyzon marinus*. Canadian Journal of Zoology 72:1134–1140.

Hooper, M. J., Ankley, G. T., Cristol, D. A., Maryoung, L. A., Noyes, P. D., and Pinkerton, K. E. 2013. Interactions between chemical and climate stressors: a role for mechanistic toxicology in assessing climate change risks. Environmental Toxicology and Chemistry 32(1):32–48.

Hooven, L. A., Sherman, K. A., Butcher, S., and Giebultowicz, J. M. 2009. Does the clock make the poison? circadian variation in response to pesticides. PloS one 4(7):e6469, 1-8.

Howe, G. E., Marking, L. L., Bills, T. D., Rach, J. J., and Mayer, F. L. 1994. Effects of water temperature and pH on toxicity of terbufos, trichlorfon, 4-nitrophenol, and 2,4,-dinitrophenol to the amphipod *Gammarus pseudolimnaeus* and rainbow trout (*Oncorynchus mykiss*). Environmental Toxicology and Chemistry 13(1):51-66.

Howell, J. H., King, Jr., E. L., Smith, A. J., and Hanson, L. H. 1964. Synergism of 5,2'dichloro-4'-nitro-salicylanilide and 3-trifluoromethyl-4-nitrophenol in a selective lamprey larvicide. Great Lakes Fishery Commission, Ann Arbor, MI, USA. Technical Report, 8.

Hubert, T. D. 2003. Environmental fate and effects of the lampricide TFM: a review. Journal of Great Lakes Research 29:456–474.

Hubert, T. D., Bernardy, J. A., Vue, C., Dawson, V. K., Boogaard, M. A., Schreier, T. M., and Gringerich, W. H. 2005. Residues of the lampricides 3-trifluoromethyl-4-nitrophenol and niclosamide in muscle tissue of rainbow trout. Journal of Agricultural and Food Chemistry 53:5342–5346.

Hughes, G. M. 1980. Functional morphlogy of fish gills. Pages 15–36 *in* B. Lahlou, editor. Epithelial transport in lower vertebrate. Cambridge University Press, London, UK.

Hunn, J. B., and Allen, J. L. 1974. Movement of drugs across the gills of fishes. Annual Review of Pharmacology and Toxicology 14:47–54.

Hynes, H. B. 1970. Chemical characteristics of flowing water. Pages 36–52 The ecology of running waters.

Ioannides, C., editor. 2002. Enzyme systems that metabolise drugs and other xenobiotics. John Wiley & Sons, LTD, Chichester, UK.

Isaia, J. 1979. Non-electrolyte permeablity of trout gills: effects of temperature and adrenaline. Journal of Physiology 286:361–373.

Johnson, N. S., and Li, W. 2010. Understanding behavioral responses of fish to pheromones in natural freshwater environments. Journal of Comparative Physiology A 196:701–711.

Johnson, N. S., Swink, W. D., Brenden, T. O., Slade, J. W., Steeves, T. B., Fodale, M. F., and Jones, M. L. 2014. Survival and metamorphosis of low-density populations of larval sea lampreys (*Petromyzon marinus*) in streams following lampricide treatment. Journal of Great Lakes Research 40(1):155–163.

Johnson, N. S., Tix, J. A., Hlina, B. L., Wagner, C. M., Siefkes, M. J., Wang, H., and Li, W. 2015. A sea lamprey (*Petromyzon marinus*) sex pheromone mixture increases trap catch relative to a single synthesized component in specific environments. Journal of Chemical Ecology 41(3):311–321.

Johnson, N. S., Yun, S. S., Buchinger, T. J., and Li, W. 2012. Multiple functions of a multi-component mating pheromone in sea lamprey *Petromyzon marinus*. Journal of Fish Biology 80:538–554.

Kanayama, R. K. 1963. The use alkalinity and conductivity measurments to estimate concentations of 3-trifluoromethyl-4-nitrophenol required for treating lamprey streams. Great Lakes Fishery Commission, Ann Arbor, MI, USA.Technical Report, 7.

Kane, A., Kahng, M., Reminschuessel, R., Nhamburo, P., and Lipsky, M. 1994. UDP-glucuronyltransferase kinetics for 3-trifluoromethyl-4-nitrophenol (TFM) in fish. Transactions of the American Fisheries Society 123:217–222.

Kawatski, J. A., and Bittner, M. A. 1975. Uptake, elimination, and biotransformation of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) by larvae of the aquatic

midge *Chironomus tentans*. Toxicology 4(2):183–194.

Koivusaari, U. 1983. Thermal acclimatization of hepatic polysubstrate monooxygenase and UDP-glucuronosyltransferase of mature rainbow trout (*Salmo gairdneri*). Journal of Experimental Zoology 227:35–42.

Koivusaari, U., Harri, M., and Hänninen, O. 1981. Seasonal variation of hepatic biotransformation in female and male rainbow trout (*Salmo gairdneri*). Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 70(2):149–157.

Lavis, D. S., Hallett, A., Koon, E. M., and McAuley, T. C. 2003. History of and advances in barriers as an alternative method to suppress sea lampreys in the Great Lakes. Journal of Great Lakes Research 29(Supplement 1):362–372.

Lawrie, A. H. 1970. The sea lamprey in the Great Lakes. Transactions of the American Fisheries Society 99(4):766–775.

Leaver, M. J., Wright, J., Hodgson, P., Boukouvala, E., and George, S. G. 2007. Piscine UDP-glucuronosyltransferase 1b. Aquatic Toxicology 84:356–365.

Lech, J. J. 1973. Isolation and identification of 3-trifluoromethyl-4-nitrophenyl glucuronide from bile of rainbow trout exposed to 3-trifluoromethyl-4-nitrophenol. Toxicology and Applied Pharmacology 24(1):114–124.

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

Lech, J. J., and Costrini, N. V. 1972. In vitro and in vivo metabolism of 3-trifluoromethyl-4-nitrophenol (TFM) in rainbow trout. Comparative and General Pharmacology 3:160–166.

Lech, J., and Statham, C. 1975. Role of glucuronide formation in the selective toxicity of 3-trifluoromethyl-4-nitrophenol (TFM) for the sea lamprey: comparative aspects of TFM uptake and conjugation. Toxicology and Applied Pharmacology 31:150–158.

Lewis, S. V. 1980. Respiration of lampreys. Canadian Journal of Fisheries and Aquatic Sciences 37:1711–1722.

Li, W., Scott, A. P., Siefkes, M. J., Yan, H., Liu, Q., Yun, S.-S., and Gage, D. A. 2002. Bile acid secreted by male sea lamprey that acts as a sex pheromone. Science 296:138–141.

Li, W., Sorensen, P. W., and Gallaher, D. D. 1995. The olfactory system of migratory adult sea lamprey (*Petromyzon marinus*) is specifically and acutely sensitive to unique bile acids released by conspecific larvae. The Journal of General Physiology

105(5):569–587.

Li, W., Twohey, M., Jones, M., and Wagner, M. 2007. Research to guide use of pheromones to control sea lamprey. Journal of Great Lakes Research 33:70–86.

Lowe, D. R., Beamish, F. W. H., and Potter, I. C. 1973. Changes in the proximate body composition of the landlocked sea lamprey *Petromyzon marinus* (L.) during larval life and metamorphosis. Journal of Fish Biology 5:673–682.

Mandl, J., Bánhegyi, G., Kalapos, M. P., and Garzó, T. 1995. Increased oxidation and decreased conjugation of drugs in the liver caused by starvation. altered metabolism of certain aromatic compounds and acetone. Chemico-Biological Interactions 96:87–101.

Manion, P. J., and Smith, B. R. 1978. Biology of larval and metamorphosing sea lampreys, *Petromyzon marinus*, of the 1960 year class in the big garlic river, Michigan, part II, 1966-1972. Great Lakes Fishery Commission, Ann Arbor, MI, USA. Technical Report 30:1–37.

Marking, L. L., and Bills, T. D. 1976. Toxicity of rotenone to fish in standardized laboratory tests. U.S. Fish and Wildlife Service, Investigations in Fish Control, Report 72.

Marking, L. L., and Olson, L. E. 1975. Toxicity of the lampricide 3-trifluoromethyl-4-nitrophenol (TFM) to non-target fish in static tests. U.S. Fish and Wildlife Service, Investigations in Fish Control, Report 60.

Maruya, K. A., Smalling, K. L., and Vetter, W. 2005. Temperature and congener structure affect the enantioselectivity of toxaphene elimination by fish. Environmental Science & Technology 39(11):3999–4004.

McDonald, D. G. 1983. The effects of H^+ upon the gills of freshwater fish. Canadian Journal of Zoology 61(4):691–703.

McDonald, D. G., and Kolar, C. S. 2007. Research to guide the use of lampricides for controlling sea lamprey. Journal of Great Lakes Research 33(2):20–34.

Mckim, J. M., and Erickson, R. J. 1991. Environmental impacts on the physiological mechanisms controlling xenobiotic transfer across fish gills. Physiological Zoology 64(1):39–67.

McKim, J. M., and Goeden, H. M. 1982. A direct measure of the uptake efficiency of a xenobiotic chemical across the gills of brook trout (*Salvelinus fontinalis*) under normoxic and hypoxic conditions. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 72(1):65–74.

McKim, J. M., Schmieder, P. K., Carlson, R. W., Hunt, E. P., and Niemi, G. J. 1987. Use of respiratory-cardiovascular responses of rainbow trout (*Salmo gairdneri*) in identifying acute toxicity syndromes in fish: part 1. pentachlorophenol, 2,4-dinitrophenol, tricaine methanesulfonate and 1-octanol. Environmental Toxicology and Chemistry 6(4):295–312.

Moffit, B. P., and Crawshaw, L. I. 1983. Effects of acute temperature changes on metabolsim, heart rate, and ventilation frequency in carp *Cyprinus carpio* L. Physiological Zoology 56(3):397–403.

Mogensen, S., and Post, J. R. 2012. Energy allocation strategy modifies growth-survival trade-offs in juvenile fish across ecological and environmental gradients. Oecologia 168(4):923–933.

Moore, J. W., and Mallatt, J. M. 1980. Feeding of larval lamprey. Canadian Journal of Fisheries and Aquatic Sciences 37:1658–1664.

Morman, R. H. 1987. Relationship of density to growth and metamorphosis of caged larval sea lampreys, *Petromyzon marinus* Linnaeus, in Michigan streams. Journal of Fish Biology 30(2):173–181.

Mullett, K. M., Heinrich, J. W., Adams, J. V., Young, R. J., Henson, M. P., McDonald, R. B., and Fodale, M. F. 2003. Estimating lake-wide abundance of spawning-phase sea lampreys (*Petromyzon marinus*) in the Great Lakes: extrapolating from sampled streams using regression models. Journal of Great Lakes Research 29(Supplement 1):240–252.

Nahrgang, J., Brooks, S. J., Evenset, A., Camus, L., Jonsson, M., Smith, T. J., Lukina, J., Frantzen, M., Giarratano, E., and Renaud, P. E. 2013. Seasonal variation in biomarkers in blue mussel (*Mytilus edulis*), icelandic scallop (*Chlamys islandica*) and atlantic cod (*Gadus morhua*)-implications for environmental monitoring in the Barents sea. Aquatic Toxicology 127:21–35.

Niblett, P. D., and Ballantyne, J. S. 1976. Uncoupling of oxidative phosphorylation in rat liver mitochondria by the lamprey larvicide TFM (3-trifluoromethyl-4-nitrophenol). Pesticide Biochemistry and Physiology 6(4):363–366.

Nichols, J. W., Du, B., Berninger, J. P., Connors, K. A., Chambliss, C. K., Erickson, R. J., Hoffman, A. D., and Brooks, B. W. 2015. Observed and modeled effects of pH on bioconcentration of diphenhydramine, a weakly basic pharmaceutical, in fathead minnows. Environmental Toxicology and Chemistry 34:1425–1435.

Noyes, P. D., McElwee, M. K., Miller, H. D., Clark, B. W., Van Tiem, L. A., Walcott, K. C., Erwin, K. N., and Levin, E. D. 2009. The toxicology of climate change:

environmental contaminants in a warming world. Environment International 35(6):971–986.

O'Boyle, R. N., and Beamish, F. W. H. 1977. Growth and intermediary metabolism of larval and metamorphosing stages of landlocked sea lamprey, *Petromyzon marinus*. Eniviornmental Biology of Fishes 2(2):103–120.

Odum, H. T. 1956. Primary production in flowing waters. Limnology and Oceanography 1(2):102–117.

Paterson, G., Drouillard, K. G., and Haffner, G. D. 2007. PCB elimination by yellow perch (*Perca flavescens*) during an annual temperature cycle. Environmental Science & Technology 41(3):824–829.

Patra, R. W., Chapman, J. C., Lim, R. P., Gehrke, P. C., and Sunderam, R. M. 2009. Effects of temperature on ventilatory behavior of fish exposed to sublethal concentrations of endosulfan and chlorpyrifos. Environmental Toxicology and Chemistry / SETAC 28(10):2182–2190.

Patra, R. W., Chapman, J. C., Lim, R. P., Gehrke, P. C., and Sunderam, R. M. 2015. Interactions between water temperature and contaminant toxicity to freshwater fish. Environmental Toxicology and Chemistry 34(8):1809–1817.

Playle, R. C., and Wood, C. M. 1989. Water chemistry changes in the gill micro-environment of rainbow trout: experimental observations and theory. Journal of Comparative Physiology B 159(5):527–537.

Potter, I. C., Wright, G. M., and Youson, J. H. 1978. Metamorphosis in the anadromous sea lamprey, *Petromyzon marinus* L. Canadian Journal of Zoology 56:561–570.

Poudel, D. D., Lee, T., Srinivasan, R., Abbaspour, K., and Jeong, C. Y. 2013. Assessment of seasonal and spatial variation of surface water quality, identification of factors associated with water quality variability, and the modeling of critical nonpoint source pollution areas in an agricultural watershed. Journal of Soil and Water Conservation 68:155–171.

Purvis, H. A. 1980. Effects of temperature on metamorphosis and the age and length at metamorphosis in sea lamprey (*Petromyzon marinus*) in the Great Lakes. Canadian Journal of Fisheries and Aquatic Sciences 37:1827–1834.

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

Riddick, D. 1998. Drug biotransformation. Pages 38–54 *in* H. Kalant and W. H. E.

Roschlau, editors. Priciples of Meidcal Pharmacology, Sixth edition. Oxford Univesity Press, New York, New York, USA.

Robertson, J. C., and Hazel, J. R. 1999. Influence of temperature and membrane lipid composition on the osmotic water permeablity of teleost gills. Physiological and Biochemical Zoology 72(5):623–632.

Rovainen, C. M. 1996. Feeding and breathing in lampreys. Brain, Behavior and Evolution 5:297–305.

Saarikoski, J., Lindström, R., Tyynelä, M., and Viluksela, M. 1986. Factors affecting the absorption of phenolics and carboxylic acids in the guppy (*Poecilia reticulata*). Ecotoxicology and Environmental Safety 11(2):158–173.

Scholefield, R. J., Slaght, K. S., and Stephens, B. E. 2008. Seasonal variation in sensitivity of larval sea lampreys to the lampricide 3-trifluoromethyl-4-nitrophenol. North American Journal of Fisheries Management 28(5):1609–1617.

Seelye, J. G., Johnson, D. A., Weise, J. G., and King, Jr., E.L. 1988. Guide for application rates of lampricides for control of sea lamprey ammocetes. Great Lakes Fishery Commission, Ann Arbor, MI, USA. Technical Report 52.

Shuter, B. J., Finstad, A. G., Helland, I. P., Zweimüller, I., and Hölker, F. 2012. The role of winter phenology in shaping the ecology of freshwater fish and their sensitivities to climate change. Aquatic Sciences 74(4):637–657.

Sidon, E. W., and Youson, J. H. 1983. Morphological changes in the liver of the sea lamprey, *Petromyzon marinus* L., during metamorphosis. ii. canalicular degeneration and transformation of the hepatocytes. Journal of Morphology 178(3):225–246.

Siefkes, M. J., Steeves, T. B., Sullivan, W. P., Twohey, M. B., and Li, W. 2013. Sea lamprey control: past,present, and future. Pages 651–704 *in* W. W. Taylor, A. J. Lynch, and N. J. Leonard, editors. Great Lakes Fisheries Policy and Managment: A Binational Perspective, Second edition. Michigan State University Press, East Lansing.

Silva, S., Araújo, M. J., Bao, M., Mucientes, G., and Cobo, F. 2014. The haematophagous feeding stage of anadromous populations of sea lamprey *Petromyzon marinus*: low host selectivity and wide range of habitats. Hydrobiologia 734(1):187–199.

Silva, S., Servia, M., Vieira-Lanero, R., Barca, S., and Cobo, F. 2013. Life cycle of the sea lamprey *Petromyzon marinus*: duration of and growth in the marine life stage. Aquatic Biology 18:59–62.

Slade, J., Adams, J., Christie, G., Cuddy, D. W., Fodale, M. F., Heinrich, J. W., Quinlin, H. R., Weise, J. G., Weisser, J. W., and Young, R. J. 2003. Techniques and methods for estimating abundance of larval and metamorphosed sea lampreys in Great Lakes tributaries, 1995 to 2001. Journal of Great Lakes Research 29(Supplement 1):137–151.

Smith, B. R., Tibbles, J. J., and Johnson, B. G. 1974. Control of the sea lampery (*Petromyzon marinus*) in Lake Superior, 1953-1970. Great Lakes Fishery Commission, Ann Arbor, MI, USA. Technical Report, 26.

Smith, B., and Tibbles, J. 1980. Sea lamprey (*Petromyzon marinus*) in Lakes Huron, Michigan, and Superior: history of invasion and control, 1936-78. Canadian Journal of Fisheries and Aquatic Sciences 37:1780–1801.

Smith, J. J., Kuraku, S., Holt, C., Sauka-Spengler, T., Jiang, N., Campbell, M. S., Yandell, M. D., Manousaki, T., Meyer, A., Bloom, O. E., Morgan, J. R., Buxbaum, J. D., Sachidanandam, R., Sims, C., Garruss, A. S., Cook, M., Krumlauf, R., Wiedemann, L. M., Sower, S. A., Decatur, W.A., Hall, J. A., Amemiya, C. T., Saha, N. R., Buckley, K. M., Rast, J. P., Das, S., Hirano, M., McCurley, N., Guo, P., Rohner, N., Tabin, C. J., Piccinelli, P., Elgar, G., Ruffier, M., Aken, B. L., Searle, S. M. J., Muffato, M., Pignatelli, M., Herrero, J., Jones, M., Brown, C. T., Chung-Davidson, Y-W., Nanlohy, K. G., Libants, S. V, Yeh, C.-Y., McCauley, D. W., Langeland, J. A., Pancer, Z., Fritzsch, B., de Jong, P. J., Zhu, B., Fulton, L. L., Theising, B., Flicek, P., Bronner, M. E., Warren, W. C., Clifton, S. W., Wilson, R. K., and Li, W. 2013. Sequencing of the sea lamprey (*Petromyzon marinus*) genome provides insights into vertebrate evolution. Nature Genetics 45(4):415–421, 421e1–e2.

Smith, M. A., Applegate, V. C., and Johnson, B. G. H. 1960. Colorimetric determination of halogenated nitrophenols added to streams as sea lamprey larvicides. Analytical Chemistry 32:1670–1675.

Sutton, T. M., and Bowen, S. H. 1994. Significance of organic detritus in the diet of larval lampreys in the Great Lakes basin. Canadian Journal of Fisheries and Aquatic Sciences 51(11):2380–2387.

Swink, W. D. 2003. Host selection and lethality of attacks by sea lampreys (*Petromyzon marinus*) in laboratory studies. Journal of Great Lakes Research 29:307–319.

Swink, W. D., and Johnson, N. S. 2014. Growth and survival of sea lampreys from metamorphosis to spawning in Lake Huron. Transactions of the American Fisheries Society 143(2):380–386.

Tiedemann, A. R., Quigley, T. M., and Anderson, T. D. 1988. Effects of timber harvest on stream chemistry and dissolved nutrient losses in northeast Oregon. Forest Science 34(2):344–358.

Timbrell, J. A. 2009. Principles of biochemical toxicology, Forth edition. Informa Healthcare USA, Inc., New York, New York, USA.

Vannote, R. L., Minshall, G. W., Cummins, K. W., Sedell, J. R., and Cushing, C. E. 1980. The river continuum concept. Canadian Journal of Fisheries and Aquatic Sciences 37(1):130–137.

Viant, M. R., Walton, J. H., and Tjeerdema, R. S. 2001. Comparative sublethal actions of 3-trifluoromethyl-4-nitrophenol in marine molluscs as measured by *in vivo* ^{31}P NMR. Pesticide Biochemistry and Physiology 71(1):40–47.

Vue, C., Bernardy, J. A., Hubert, T. D., Gingerich, W. H., and Stehly, G. R. 2002. Relatively rapid loss of lampricide residues from fillet tissue of fish after routine treatment. Journal of Agricultural and Food Chemistry 50:6786–6789.

Waldman, J., Daniels, R., Hickerson, M., and Wirgin, I. 2009. Mitochondrial DNA analysis indicates sea lampreys are indigenous to Lake Ontario: response to comment. Transactions of the American Fisheries Society 138(5):1190–1197.

Waldman, J. R., Grunwald, C., Roy, N. K., and Wirgin, I. I. 2004. Mitochondrial DNA analysis indicates sea lampreys are indigenous to Lake Ontario. Transactions of the American Fisheries Society 133(4):950–960.

Wetzel, R. G. 1983. Limnology, Second edition. Saunders College Publishing, Philadelphia, PA, USA.

Wheeler, M. W., Park, R. M., and Bailer, A. J. 2006. Comparing median lethal concentration values using confidence interval overlap or ratio tests. Environmental Toxicology and Chemistry / SETAC 25(5):1441–1444.

Wilkie, M. P. 2011. Lampreys: environmental physiology. Pages 1788–1799 Encyclopedia of Fish Physiology: From Genome to Environment.

Wilkie, M. P., Holmes, J. A., and Youson, J. H. 2007a. The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) interferes with intermediary metabolism and glucose homeostasis, but not with ion balance, in larval sea lamprey (*Petromyzon marinus*). Canadian Journal of Fisheries and Aquatic Sciences 64:1174–1182.

Wilkie, M. P., Morgan, T. P., Galvez, F., Smith, R. W., Kajimura, M., Ip, Y. K., and Wood, C. M. 2007b. The african lungfish (*Protopterus dolloi*): ionoregulation and osmoregulation in a fish out of water. Physiological and Biochemical Zoology

80(1):99–112.

Young, R. J., Kelso, J. R. M., and Weise, J. G. 1990. Occurrence, relative abundance, and size of landlocked sea lamprey (*Petromyzon marinus*) ammocoetes in relation to stream characteristics in the Great Lakes. Canadian Journal of Fisheries and Aquatic Sciences 47:1773–1778.

Youson, J. H. 1980. Morphology and physiology of lamprey metamorphosis. Canadian Journal of Fisheries and Aquatic Sciences 37:1687–1710.

Youson, J. H. 2003. The biology of metamorphosis in sea lampreys: endocrine, environmental, and physiological cues and events, and their potential application to lamprey control. Journal of Great Lakes Research 29(Supplement 1):26–49.

Youson, J., Holmes, J., Guchardi, J. A., Seelye, J. G., Beaver, R. E., Sower, S. A., and Beamish, F. 1993. Importance of condition factor and the influence of water temperature and photoperiod on metamorphosis of sea lamprey *Petromyzon* marinus. Canadian J ournal of Fisheries and Aquatic Sciences 50:2448–2456.

Zydlewski, J., and Wilkie, M. P. 2013. Freshwater to seawater transitions in migratory fishes. Pages 253–326 *in* S. D. McCormick, A. Farrell, and C. Brauner, editors. Fish Physiology: Euryhaline Fishes. Academic Press.