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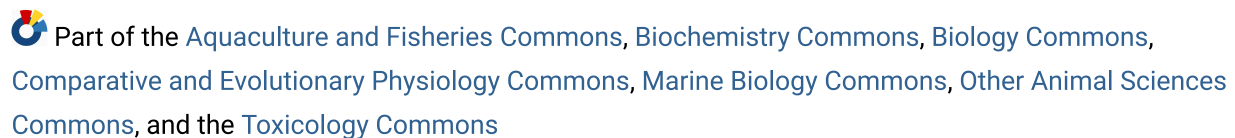

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Distribution and Elimination of 3-Trifluoromethyl-4-Nitrophenol (TFM) by Sea Lamprey (*Petromyzon marinus*) and Non-target, Rainbow Trout (*Oncorhynchus mykiss*) and Lake Sturgeon (*Acipenser fulvescens*)

by

Michael Le Clair

Honours Bachelor of Science, Wilfrid Laurier University, 2010

M.Sc. Thesis

Submitted to the Department of Biology in partial fulfillment of the requirements

for Masters of Science in Integrative Biology

Wilfrid Laurier University

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Abstract

The pesticide, 3-trifluoromethyl-4-nitrophenol (TFM), has been highly successful in the control of sea lamprey (*Petromyzon marinus*) populations in the Great Lakes. Treatments with TFM involve applying it to streams, where it targets larval sea lamprey which live burrowed in the stream substrate. While the toxic mechanism of TFM has been elucidated, and its effects on sea lamprey described, its effects on non-target fish species such as rainbow trout (*Oncorhynchus mykiss*) and lake sturgeon (*Acipenser fulvescens*) are not as well understood. The present work demonstrated that rainbow trout show a great capacity to detoxify the lampricide using glucuronidation, when exposed to TFM concentrations typically used in TFM applications, and with no adverse physiological effects. Larval sea lamprey, on the other hand, showed very little ability to detoxify TFM, and experienced pronounced reductions in glycogen concentration in the liver. In contrast to previous suggestions, lake sturgeon were able to biotransform TFM and generate TFM-glucuronide at levels that were similar to those observed in rainbow trout. However, they were exposed to a lower concentration of TFM, which does not rule out possible toxic effects of TFM at higher concentrations. In conclusion, this study has demonstrated that rainbow trout readily tolerate TFM at environmentally relevant concentrations, and that lake sturgeon are capable of TFM detoxification. This suggests that the greater sensitivity of lake sturgeon is a result of other factors such as body size, glycogen stores and/or possibly limitations in their capacity to use glucuronidation to detoxify TFM.

Acknowledgements

I would like to thank my supervisor, Dr. Michael P. Wilkie for his valuable advice and suggestions for my thesis. I would also like to thank my committee members, Dr. Deborah MacLatchy and Dr. Robin Slawson for their help and guidance. Additonally, I am also grateful to Jiangxiao Sun for all of her technical support and assistance when it came to any assays as well as troubleshooting the HPLC. I would also like to thank all members of the Wilkie, McGeer and Lee labs for their support. I would like to thank Dr. Jeff A. Bernardy for his assistance and guidance when it came to learning and troubleshooting the SPE and HPLC. Thanks are also due to Dr. Scott Ramsay for allowing me to use his lab for SPE processing. Lastly, I would like to thank my family and my friends for always being there and helping me whenever I called upon them.

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List of Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
EDTA	ethylenediaminetetraacetic acid
GLFC	Great Lakes Fisheries Commission
G-TFM	TFM-glucuronide
HPLC	High performance liquid chromatography
LC	Lethal Concentration
MS222	Tricaine methanesulfonate
PCr	phosphocreatine
Q	Succinate-Q Oxidoreductase (Complex II)
SPE	Solid phase extraction
TFM	3-trifluoromethyl-4-nitrophenol
UDP	uridine diphosphate

CHAPTER ONE

The Invasion of the Great Lakes by Sea Lamprey and the Possible Implications of Sea Lamprey Chemical Control Methods on Non-target Fish species

Background

The sea lamprey life cycle

The sea lamprey, *Petromyzon marinus* L., is a jawless vertebrate belonging to the superclass Agnatha. The multi-staged sea lamprey life cycle consists of a prolonged larval (ammocoete) phase, metamorphosis, the juvenile parasitic phase and the adult phase (Figure 1-1; O'Boyle and Beamish, 1977). The larval phase typically lasts 3-7 years, during which lamprey live as suspension feeding ammocoetes in the soft sediments of streams (Sutton and Bowen, 1994). During this period, the larval sea lamprey grow and build up large reserves of lipid (Lowe et al., 1973; Kao et al., 1997; O'Boyle and Beamish, 1977). These fuel stores become critical when the larval sea lamprey enter the non-trophic phase of metamorphosis, which lasts several months, and they mainly rely on their lipid stores to provide energy (Youson, 2003).

Metamorphosis is characterized by the development of eyes, changes in body colour and a reorganization of internal organs such as the liver and kidneys (Youson, 1979). The digestive and respiratory organs also undergo distinct changes including the development of an oral disc used for attaching to fish, and a rasping tongue which is used to penetrate the hide of fish so that the lamprey can ingest its blood. The gills are also re-organized from a unidirectionally ventilated respiratory organ to a tidally ventilated gill which enables the lamprey to breathe while attached to its host (Youson and Potter, 1979; Lewis, 1980; Wilkie, 2011).

Following metamorphosis, the sea lamprey travel downstream to large lakes, where they begin the parasitic phase of their life cycle. During this phase, the lamprey can remain attached to a fish for many days at a time, which often causes death of the host due to loss of blood, or infection of the wound(s) (Farmer et al., 1975; Bence et al., 2003). After 8-24 months spent as a

parasite, sea lamprey migrate up freshwater streams, undergo sexual maturation, spawn and die (Figure 1-1; Beamish and Potter, 1975; Bergstedt and Swink, 1995).

Invasion and control

The sea lamprey may in fact be native to Lake Ontario (Bryan et al., 2007; Waldman et al. 2004), but they were prevented from colonizing the other Great Lakes due to the presence of Niagara Falls. In 1919, the Welland Canal was widened and deepened to provide passage for larger ships, which allowed the sea lamprey to bypass Niagara Falls and enter Lake Erie from Lake Ontario (Great Lakes Fisheries Commission, 2000; O'Neal and Clapp, 2005). Within twenty years, the sea lamprey had invaded the remaining Great Lakes which, in combination with over-fishing, caused a dramatic reduction in fish populations (Figure 1-2; Great Lakes Fisheries Commission, 2000). Several endemic fish populations in the Great Lakes became extinct, including the longjaw cisco (*Coregonus alpenae* L.), the deepwater cisco (*Coregonus johannae* L.) and the blackfin cisco (*Coregonus nigripinnis* L.) (Miller et al., 1989). Other fish species that suffered heavy losses included economically important lake trout (*Salvelinus namaycush* W.) and walleye (*Sander vitreus* M.) populations (Nico and Fuller, 1999; O'Neal and Clapp, 2005).

In response to the sea lamprey invasion of the Great Lakes, Canada and the United States formed the Great Lakes Fishery Commission (GLFC) in 1955. The mandate of the GLFC was to protect and preserve the remaining Great Lakes fisheries by establishing means to eradicate sea lamprey (Great Lakes Fisheries Commission, 2000; McDonald and Kolar, 2007). Sea lamprey were not eradicated from the Great Lakes but several methods of sea lamprey population control were developed under the leadership of the GLFC.

Early methods of sea lamprey control included the use of mechanical or electric barriers to prevent adult lamprey from reaching their spawning grounds (McLaughlin et al., 2007). The use of electrical barriers were eventually discontinued because they did not reduce adult lamprey populations as greatly as expected and negatively impacted non-target fish populations that were also present (Lawrie 1970; Smith and Tibbles, 1980; McDonald and Kolar, 2007). Other techniques, such as chemically sterilizing male sea lamprey to prevent them from reproducing were tried, but were eventually discontinued (McLaughlin et al., 2007)

In the 1950s, it was decided the best way to control sea lamprey populations was to focus on using pesticides to kill larval sea lamprey, which lived burrowed in the substrate of streams. A further advantage of this approach was that it would be possible to eradicate several generations of sea lamprey at once due to the long time spent in their larval state (Applegate, 1950). With the failure of conventional methods to control the lamprey, scientists began the search for the pesticide that could control sea lamprey populations and prevent the further spread of sea lamprey.

The control of sea lamprey using TFM

Over 6000 chemicals were tested for their ability to selectively kill sea lamprey, but the compound 3-trifluoromethyl-4-nitrophenol (TFM) was the most effective (Hubert, 2003). TFM was found to be highly selective against larval sea lamprey even at low concentrations (Hubert, 2003). At concentrations that are lethal to larval sea lamprey, TFM normally has minimal deleterious effects to most juvenile or adult fish (Olson and Marking, 1973) because teleosts have a greater ability to detoxify the pesticide (Lech and Statham, 1975; Kane et al., 1993; Olson and Marking, 1973). The inability of larval sea lamprey to detoxify TFM via glucuronidation leads to greater build-up of TFM, and greater toxicity (Lech et al., 1974). Glucuronidation takes

place in the liver, where the enzyme UDP-glucuronyl transferase catalyzes the addition of glucuronyl acid to xenobiotic materials and endogenous compounds (Lech and Statham, 1975; Vue et al., 2002). This process allows the original substance to be converted to a more water-soluble form that can be eliminated via the kidney and digestive tract.

The mechanism of TFM toxicity

It has been shown that TFM acts by uncoupling oxidative phosphorylation in the mitochondria (Niblett and Ballantyne, 1976; Birceanu et al., 2011), the process normally used to generate adenosine triphosphate (ATP). ATP is the main energy currency used by different organisms to store and release the energy needed to power biological processes in the body. ATP is formed in the mitochondria from ADP and inorganic phosphate (P_i), in which the protein complexes comprising the electron-transport chain pump protons (H^+) from the mitochondrial matrix into the intermembrane space of the mitochondria. The proton pumping process leads to the generation of a proton motive force (electrochemical gradient) favoring the movement of H^+ from the intermembrane space back into the matrix. The flow of H^+ through another protein complex, ATP synthase, back into the matrix releases energy that is used to generate ATP from ADP and P_i.

TFM is a lipophilic weak acid, which is capable of passing through the mitochondrial membranes in its neutral but protonated state. As a result, TFM effectively binds protons on the acidic side of the membrane, diffuses across the membrane and releases the protons on the alkaline side, which causes the dissipation of the proton motive force between the intermembrane space and the mitochondrial matrix (Figure 1-3; Birceanu et al., 2011). With the disruption of the electrochemical H^+ gradient, oxidative phosphorylation is uncoupled, reducing the rate of ATP generation. Without the production of ATP, larval sea lamprey must depend on anaerobic

energy reserves, such as glycogen and phosphocreatine, to maintain their ATP supply. Once these stores are depleted, or are incapable of generating sufficient ATP, the animal's energy balance becomes compromised leading to homeostatic disturbances and eventual cell death (Birceanu et al., 2009, 2011).

The toxicity of TFM is directly linked to water pH (Marking and Olson, 1975; Hubert, 2003; Bills et al., 2003). At pH 6.5, the toxicity of TFM was found to be 45 times more toxic to larval sea lamprey than in water at pH 9.0 (Bills et al., 2003). This marked difference is believed to be due to the effect that pH has on the ionization state of TFM. Changes to pH alter the relative proportion of the water-soluble phenolate anion and the lipid soluble free phenol (Figure 1-4; Hubert, 2003). By examining the pKa of TFM, it can be determined to what extent the weak acid will dissociate into its conjugate base and hydrogen ion, affecting the ability of TFM to diffuse across membranes. With a pKa of 6.07, TFM in water with a pH of 8 would be present in its un-ionized form at 15%. At lower pH, rates of TFM uptake are thought to increase because the amount of the lipid permeable, soluble free phenol is greater leading to greater uptake across the gills (Figure 1-4; Hunn and Allen, 1974). However, few studies have examined how TFM uptake rate is affected by water pH. Additional factors affecting TFM uptake by sea lampreys and other fish species include the concentration of TFM and possibly the alkalinity of the water (McDonald and Kolar, 2007).

Despite its continued use for over fifty years, there is surprisingly little information on how effectively lamprey and different non-target fish species are able to eliminate TFM. Rainbow trout are known to use UDP-glucuronyl transferase to convert TFM to TFM-glucuronide in the liver (Figure 1-5; Hubert et al., 2001). This enzyme allows glucuronic acid to

bond to a xenobiotic material such as TFM through a glycosidic bond, resulting in a hydrophilic glucuronide which can be excreted in the urine or via the gastrointestinal tract (Lech and Statham, 1975) However, less is known about the degree to which lamprey are capable of conjugating TFM using UDP-glucuronyl transferase. A more thorough knowledge of TFM glucuronidation could help better determine why TFM is selective to lamprey. However, recent findings suggest that the capacity of larval sea lamprey to tolerate TFM is highest in the summer (Scholefield et al. 2008). One possible explanation for these findings is that sea lampreys may increase their capacity to detoxify TFM via glucuronidation. Thus, an improved understanding of how lampreys detoxify TFM could yield information that helps explain why the sensitivity of these animals varies seasonally.

Research Priorities and Objectives

Many studies have been performed to understand the effects of TFM on sea lamprey and many non-target fish species and invertebrates in the Great Lakes. However, there is a lack of information on the factors that influence TFM uptake, elimination and toxicity. The GLFC has therefore made it an important priority to understand more about the underlying mechanisms of TFM toxicity in lamprey and non-target fish species. Understanding the toxicity of TFM in lamprey and non-target fish species will allow for more accurate determinations of the most effective doses needed to treat lamprey infested streams in the Great Lakes. The overarching goal of my M.Sc thesis was to gain a better understanding of the factors that influence the uptake rates of TFM from the water, as well as the mechanism(s) of TFM detoxification in lamprey and non-target fish species. The objectives of this thesis were to:

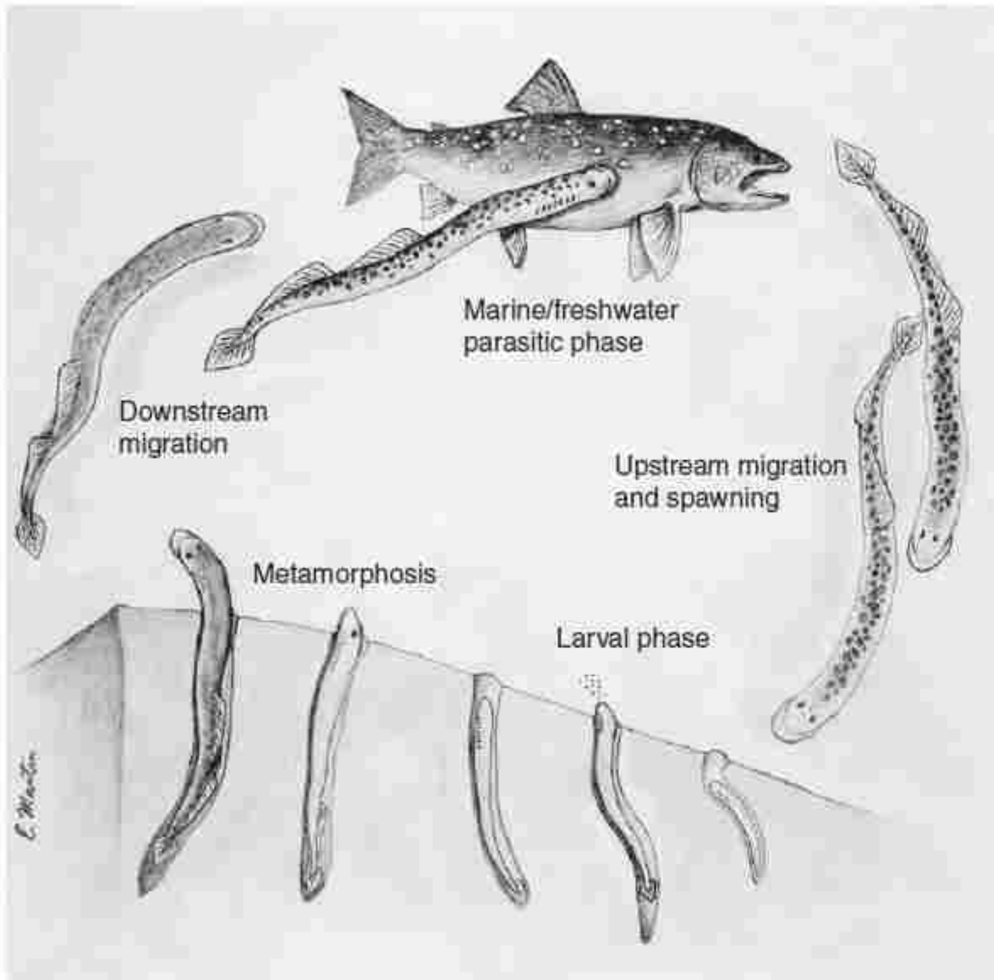
1. Establish a technique for quantifying TFM and TFM glucuronide in the whole bodies of larval sea lamprey, lake sturgeon and rainbow trout.
2. Determine if there are differences in the rates of uptake, distribution and elimination of TFM by larval sea lamprey compared to non-target rainbow trout and lake sturgeon.
3. Quantify the relative importance of glucuronidation in the detoxification of TFM by larval sea lamprey compared to non-target lake sturgeon.
4. Relate metabolic disturbances to the concentrations of TFM accumulated by larval sea lamprey, lake sturgeon and rainbow trout during exposure to the lampricide.

Relevance

The research conducted in this thesis revealed how the uptake, distribution and elimination of TFM in lamprey and non-target trout and sturgeon effectively detoxifies and eliminates TFM from the body. By learning more about the pharmacology of TFM in these fish species, this work could also lead to a better understanding of why fish experience adverse effects, and more rarely, non-target mortality following TFM treatment. Moreover, it could identify factors that explain why lamprey are able to resist TFM under certain conditions (e.g. changes in water pH, season), leading to residual lamprey that go on to complete metamorphosis and subsequently move into the Great Lakes where they parasitize commercial and game fish species at great economic costs. Ultimately, my work could lead to measures that allow TFM to be more effectively used, help protect non-target organisms and more effectively control lamprey populations.

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

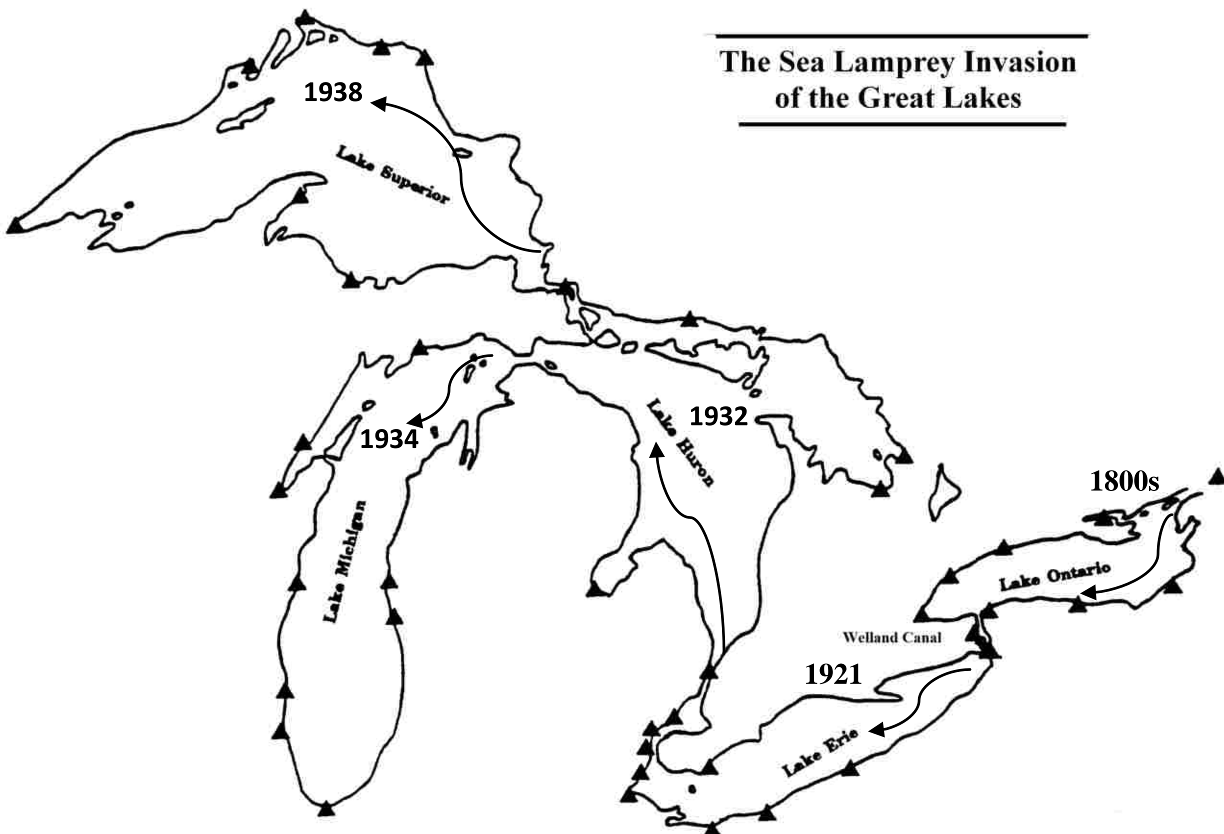
After spending 3-7 years in their larval state, sea lamprey undergo a complex metamorphosis that leads to their characteristic oral disc and rasping tongue; vitally important structures for its new lifestyle as a parasite. After 8-24 months in its parasitic phase, sea lamprey migrate upstream to spawn and die (with permission from Wilkie, 2011).



(Wilkie, 2011, with permission)

Figure 1-2: Suspected movement of sea lamprey through the Great Lakes.

Sea lamprey may have been native to Lake Ontario (Waldman et al., 2004; Bryan et al., 2005), but due to the presence of Niagara Falls, sea lampreys had no access to Lake Erie and the upper Great Lakes. Following the widening of the Welland Canal in 1919, sea lampreys gained access to Lake Erie, where they were first reported in 1921. By 1938 sea lampreys were reported to be in Lake Superior, and the other Great Lakes (Great Lakes Fisheries Commission, 2000).



Adapted from Jafvert and Rogers (1990)

Figure 1-3: Oxidative Phosphorylation

Oxidative phosphorylation is the process that produces ATP by passing electrons between specialized protein complexes (Complex I, II, III, IV) embedded in the inner mitochondrial membrane. The energy released from the electrons flowing through the electron transport chain is used by the protein complexes (Complex I, III, IV) to transport protons (H^+) across the inner mitochondrial membrane from the mitochondrial matrix into the inter-membrane space. This creates an electrochemical gradient across the membrane (proton motive force) favouring the movement of H^+ back into the matrix, which is normally prevented by the inner mitochondrial membrane. The inward flow of H^+ is restricted to the protein complex, ATP synthase, through which the protons flow back across the membrane and into the matrix, which releases the energy needed to power ATP formation from ADP and Pi. TFM is believed to interfere with this process by acting as a protonophore, which has the effect of non-specifically transporting H^+ across the inner membrane, which reduces the proton-motive force and the formation of ATP (Birceanu et al., 2011). The final electron acceptor in the process is O_2, which combines with H^+ to form water.

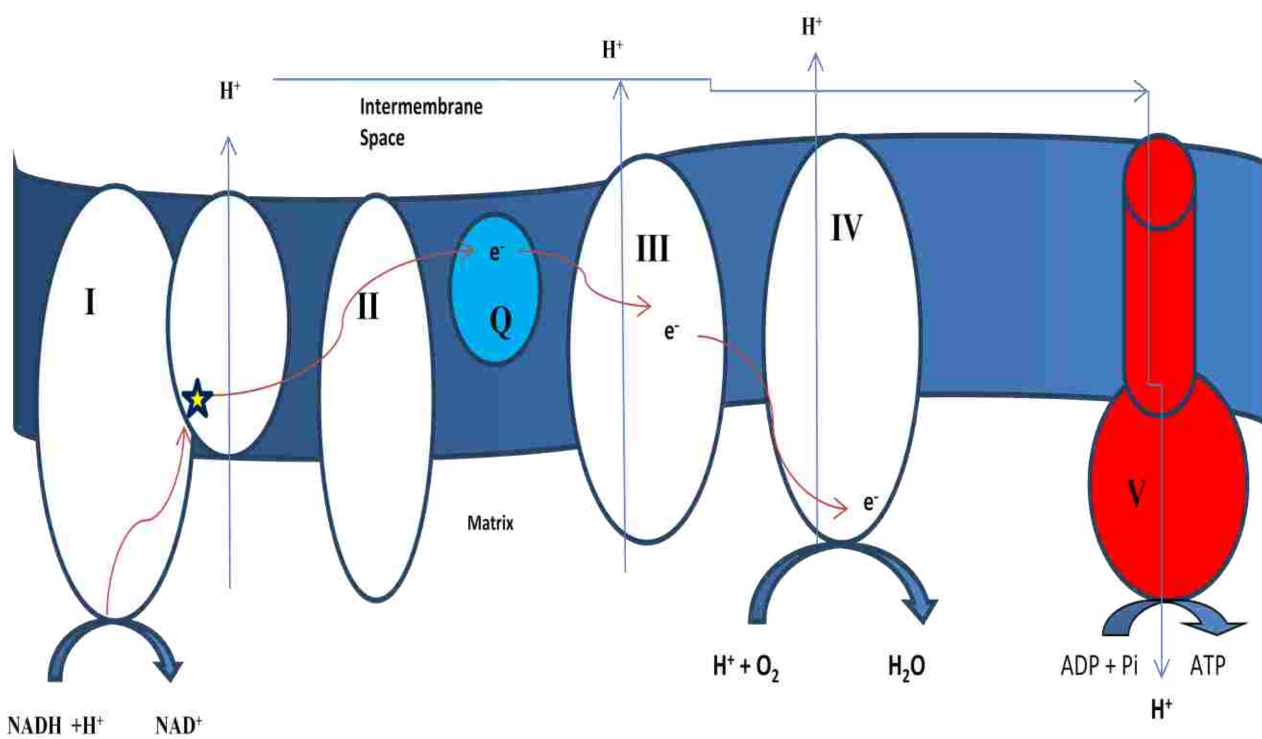

H+
H+
H+
Intermembrane
Space
I
II
III
IV
V
Q
e-
e-
e-
Matrix
NADH +H+
NAD+
H+ + O2
H2O
ADP + Pi
ATP
H+

Figure 1-4: Structure and dissociation equilibrium of TFM (pK_a = 6.07).

The un-ionized, phenolic form of TFM is on the left, which is more lipophilic and easily crosses the gills passively down a favorable water-to-blood gradient. Once inside, TFM acts as a protonophore, inhibiting the formation of ATP by the mitochondria (Birceanu et al., 2009).

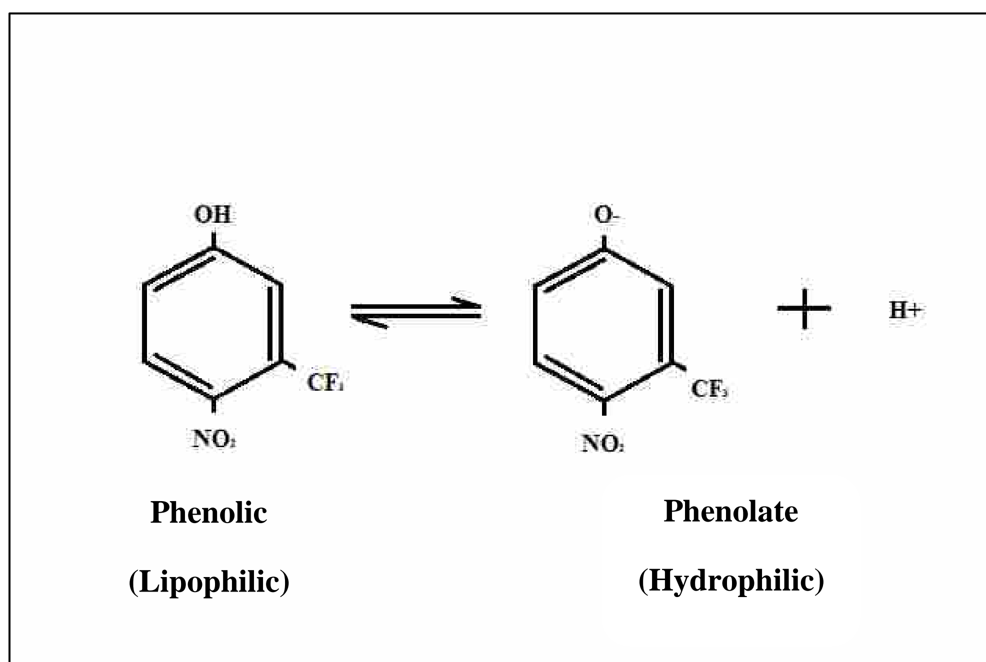
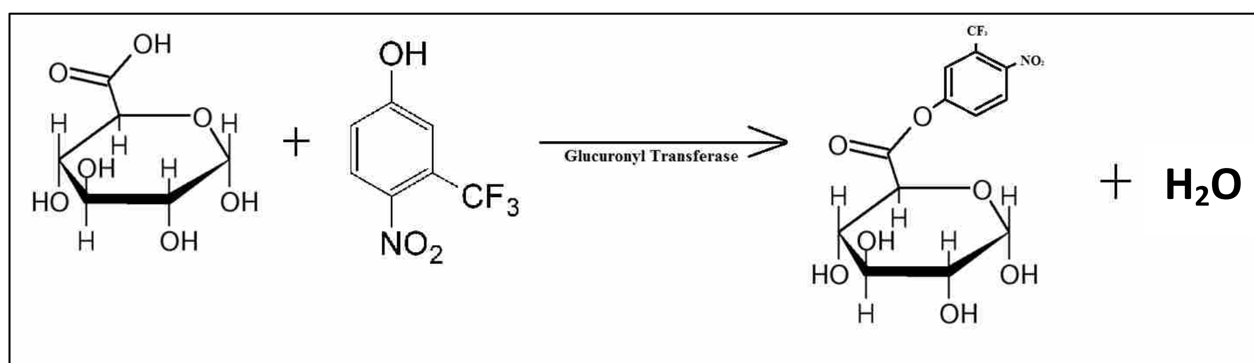

OH
CF3
NO2
O-
CF3
NO2
H+
Phenolic
(Lipophilic)
Phenolate
(Hydrophilic)

Figure 1-5: Process of glucuronidation.

UDP-glucuronyl transferase attaches glucuronyl acid to TFM through a glycosidic bond resulting in a TFM-glucuronide and water. This process takes place in the liver, and facilitates the excretion of the lampricide by making the xenobiotic material more hydrophilic (Voet et al., 2006).



(Voet et al., 2006)

CHAPTER TWO

The Resilience of a Great Lakes Non-target Fish, the Rainbow Trout (*Oncorhynchus mykiss*) to Lampricide Exposure

Introduction

In the early 20th century, the invasion of the Great Lakes by parasitic sea lamprey led to the collapse or near collapse of numerous sport, commercial and culturally significant fisheries (Miller et al., 1989; Nico and Fuller, 1999; Great Lakes Fisheries Commission, 2000). To combat this threat, the Great Lakes Fisheries Commission (GLFC) was created by Canada and the United States to establish strategies that could be implemented to eliminate sea lamprey from the Great Lakes (Great Lakes Fisheries Commission, 2000; McDonald and Kolar, 2007). After testing 6000 potential chemical control methods, the phenolic compound, 3-trifluoromethyl-4-nitrophenol (TFM) was found to selectively kill larval sea lamprey while having minimal effects on non-target fish species (Hubert 2003).

By uncoupling oxidative phosphorylation in the mitochondria, TFM interferes with the production of ATP, the main energy currency in the body (Niblett and Ballantyne, 1976; Birceanu et al., 2011). Without ATP as a viable energy store, animals must rely on glycolysis and on-board reserves of phosphocreatine to survive. As these anaerobic stores are depleted, homeostatic disturbances arise leading to death (Wilkie et al., 2007; Birceanu et al., 2009, 2011).

In order to combat the toxic effects that TFM has on the body, most non-target fish species have the ability to detoxify TFM through the process of glucuronidation in which TFM-glucuronide is formed from UDP-glucuronide via UDP-glucuronyl transferase (Olson and Marking, 1973; Lech and Statham, 1975; Kane et al., 1993). The resulting TFM-glucuronide is much more water soluble, which enables it to be excreted via the intestinal tract or the urine (Clarke et al., 1991). It is thought that larval sea lamprey have little capacity to perform glucuronidation compared to other fish species such as the rainbow trout (Birceanu et al., 2011). However, recent evidence that larval sea lamprey are more tolerant to TFM later in the summer

(Scholefield et al., 2008) suggests that they may have a greater capacity to detoxify TFM than previously thought. In addition, little information is available about how quickly sea lamprey accumulate TFM compared to other fish species.

Despite their greater tolerance to TFM, however, we still know little about how quickly TFM is cleared from the body of rainbow trout following routine treatments with TFM, or if routine TFM applications in the field have any adverse physiological effects. Accordingly, the primary purpose of this study was to compare the rates of TFM accumulation and elimination of rainbow trout to those of larval sea lamprey. In the present study, rainbow trout were exposed to the LC100 of TFM for larval sea lamprey, which approximates concentrations they could encounter during actual field treatments with the lampricide. Typically, sea lamprey control personnel apply TFM to rivers and streams at 1.2-1.3 times the LC100 (median lethal concentration, MLC) of TFM (Bills et al., 2003; McDonald and Kolar, 2007).

It was predicted that rainbow trout would not only efficiently biotransform and eliminate TFM when exposed to the LC100 of TFM, but that their capacity to form TFM-glucuronide would increase as the exposure proceeded. Based on recently published findings (Birceanu et al., 2011), it was also predicted that there would be some impact of TFM on tissue energy reserves in trout exposed for several hours exposure to TFM. Accordingly, we measured brain and liver glycogen, ATP and phosphocreatine (PCr) throughout and following the TFM exposure period. Similar experiments using sea lamprey were conducted to determine if they had any capacity to detoxify TFM during exposure to the lampricide.

Method and Materials

Experimental animals and set-up

Larval sea lamprey (N = 70; mass = 1.37 g ± 1 SEM) were provided by the Hammond Bay Biological Station (Millersburg, MI) following capture by U.S. Fish and Wildlife Service personnel using pulsed-DC electrofishers in the summer of 2011. All fish were experimented on over the summer of 2011. Animals were shipped to Wilfrid Laurier University in bags placed in 45-60 L coolers placed in ice containing well-oxygenated water and then transferred to 110-L tanks receiving continuously aerated well water (pH ~ 8.0; titratable alkalinity ~ 200 mg $CaCO_3$ l^{-1}; hardness ~ 450 mg $CaCO_3$ l^{-1}; temperature ~ 10-13°C; Birceanu et al., 2009) in a flow-through manner under a 12 h light : 12 h dark photoperiod. The holding tanks were lined with sand (5-7 cm deep) to serve as a burrowing substrate for the larvae, which were fed a weekly slurry of baker's yeast (1 g yeast per animal; Wilkie et al., 1999).

Fingerling rainbow trout (N = 60; mass = 25.4 g ± 1 SEM) were purchased from a commercial hatchery, Rainbow Springs Hatchery (Thamesford, ON) and transported to Wilfrid Laurier University in 10 L bags placed in 40 L coolers with well-aerated water. At the university, animals were held in the same aerated well water as larval sea lamprey, and held under a 12 h dark: 12 h light photoperiod. The fish were fed standard 3 mm fish feed three times a week until satiated (Omega Sea, LLC, Painesville, OH).

Prior to experimentation, animals were fasted 72 h to minimize the effects that ammonia excretion could have on water ammonia concentrations during static exposures to TFM. Mean length and weights with standard deviation and range were provided in Table 2-1. All experiments followed Canadian Council on Animal Care guidelines and were approved by the Wilfrid Laurier University Animal Care Committee.

Experimental protocol

Fish were exposed to 3-trifluoromethyl-4-nitrophenol (TFM) prepared from powdered TFM from Sigma-Aldrich (N27802) dissolved in deionized water. Precision TFM standards were provided courtesy of the Fisheries and Oceans Canada, Sea Lamprey Control Centre (Sault Ste. Marie, ON). Unless otherwise noted, all other chemical compounds, reagents and supplies were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO).

Larval sea lamprey exposure to the 12 h LC100 TFM

Prior to all experiments, larval sea lamprey were transferred individually into 1 L round chambers receiving aerated well water under flow through conditions. They were left overnight in order to acclimatize to their surroundings. Each container was lined with approximately 2 g of diffuse aquarium cotton to mimic the burrowing substrate of larval sea lamprey and to calm the animals (Wilkie et al., 1999). Every container housed four larval sea lamprey. The next morning, water flow was cut off to each chamber and sufficient amounts of TFM were added to each container to yield a nominal TFM concentration equivalent to the 12 h LC100 (nominal concentration = 7.6mg/L; Birceanu et al., 2009) of larval sea lamprey in Wilfrid Laurier University well water (Birceanu et al., 2011, 2013). The fish were exposed to this concentration as opposed to 1.2 to 1.3 times the 9 h LC100, which is typically used in the field (Bills et al., 2003) because higher TFM concentrations would have resulted in higher mortality over too short a time course, which would have compromised efforts to quantify patterns of TFM uptake and physiological disturbances in the lamprey and trout (Hubert 2003; McDonald and Kolar, 2007). The static TFM exposures lasted 3, 6, 9 and 12 h; tissue samples (muscle, liver, brain) were collected and snap-frozen in liquid N_2 at each time period and saved for subsequent metabolite and TFM analysis. A second group of larval sea lamprey were exposed to a similar

concentration of TFM overnight for 12 h, and then placed in clean TFM-free well water, and tissues were collected after 3, 6 and 24 h of post-TFM recovery after being euthanized with an overdose of tricaine methanesulfonate (1.5 g L^{-1} buffered with 3.0 g L^{-1} $NaHCO_3$). After tissues were collected and snap-frozen in liquid nitrogen, they were then stored at -80°C to prevent degradation of metabolites or tissue until processed further for the respective quantification of metabolite and TFM concentrations using spectrophotometric assays and high performance liquid chromatography (e.g. Wang et al., 1994; Hubert et al., 2001).

Rainbow trout exposure to the 12 h LC100 TFM

Prior to all experiments, fingerling rainbow trout were transferred into large 5 L individual rectangular holding chambers receiving aerated well water under flow through conditions and left overnight to acclimate to their surroundings. Each fish was compartmentalized into holding containers individually. The following morning, water flow was cut off to each chamber and sufficient amounts of TFM were added to each container to yield a nominal TFM concentration equivalent to the 12 h LC100 of larval sea lamprey (nominal concentration = 7.6mg/L). Due to size constraints, 32 trout were experimented at a time, with 4 animals per exposure period. This was performed twice, one immediately after the completion of the other, in order to account for all experimented fish. Static TFM exposures lasted 3, 6, 9 and 12 h and an additional 3 groups were exposed to TFM for 12 h and then were placed in clean, flowing TFM-free well water and sampled after 3, 6 and 24 h of depuration. For each sample period, sample size was 8, including a control group that was not exposed to TFM. Additionally, of these 8 control animals, 4 were sampled at the beginning of experimentation, and 4 at its conclusion. As for larval sea lamprey, the fish were euthanized with an overdose of tricaine methanesulfonate (0.5 g L^{-1}, buffered with 1.0 g L^{-1} $NaHCO_3$), followed by the collection of

blood and tissues, which were stored at -80°C and saved for later quantification of TFM, TFM-glucuronide, glycogen, lactate, ATP and PCr.

To confirm that target TFM concentrations were established, and that TFM was completely eliminated from the chambers during depuration, water samples were collected at 0.5 h of TFM exposure, and 0.5 h following introduction into TFM-free water. The TFM concentrations in the water samples were then immediately quantified using a Molecular Devices Spectramax 190 plate spectrophotometer at 395 nm, using Standard Operating Procedures for water TFM quantification and TFM standards provided by the Sea Lamprey Control Center, Sault Ste. Marie, Ontario (Instrument Operating Procedure, IOP012.3).

Tissue collection procedure

Tissue collection procedures were adapted from Birceanu et al. (2009). After the sea lamprey or rainbow trout were euthanized, they were blotted dry and their mass measured to the nearest 0.01 g, and length measured to the nearest mm. The livers of larval sea lamprey were collected after a longitudinal cut was made along the ventral side of each animal running posteriorly from the last branchiopore to the cloaca which exposed the liver, and other viscera. The liver was then subsequently removed and frozen in liquid nitrogen. The remaining carcass (mainly muscle and cartilaginous skeleton) was snap-frozen in liquid nitrogen for muscle metabolites and TFM quantification.

Rainbow trout were anesthetized with 0.5 g L^{-1} tricaine methanesulfonate buffered with 1 g $NaHCO_3$ and were then blotted dry and their mass and length recorded. The brain was then collected by making a transverse cut across the skull, followed by two longitudinal cuts to open the brain case. The brain was then carefully removed using a probe and fine tweezers, and snap-

frozen in liquid nitrogen. A longitudinal incision was then made down the mid-line of the fish to expose the liver which was then collected, snap-frozen in liquid N_2 and stored at -80^oC. A filet of muscle was also cut from the dorso-lateral surface of the animal, and stored in the same manner.

Tissue processing and metabolite analysis

For TFM analysis, the carcasses of the sea lamprey or muscle from rainbow trout were ground to a fine powder under liquid N_2 using a liquid N_2-cooled mortar and pestle. Remnants of skin and skeleton were removed, such that only muscle was remaining. Approximately 300 mg of powder was then used for TFM analysis using solid phase extraction (SPE) and high performance liquid chromatography (HPLC).

For energy stores and metabolite analysis, brain (trout only) or liver were added directly to ice cold 8% perchloric acid (PCA) containing 1 mM ethylenediaminetetraacetic acid (EDTA), and homogenized on ice using a handheld, Kontes motorized Pellet Pestel (Kimble Chase, Vineland, NJ, USA). The solution was then vortexed and chilled on ice for 10 minutes, and the extract split into two subsamples; the first was used for glycogen analysis, and the second for ATP and PCr (trout only) and lactate quantification. Subsample one was neutralized by adding 3 M K_2CO_3 and frozen in liquid N_2 and stored at -80^oC until processed for glycogen quantification. Subsample two was centrifuged for 5 minutes at 5^oC at 10,000 x *g*, the supernatant drawn off and then neutralized by adding 0.5 volumes of 2 M KOH, and then centrifuged again for 5 minutes at 10,000 x *g* and then stored at -80^oC until analyzed.

For glycogen analysis, 100 µL of supernatant was drawn off of subsample one and mixed with 120 µL of 2M acetate buffer. It was then allowed to sit on ice and a subsample (50 µL) was taken for background glucose. The remaining sample had amyloglucosidase added to convert

glycogen in the sample to free glucose during incubation in a hot water bath at 37°C. The reaction was terminated with 70% PCA and then the resulting solution neutralized with 3M K_2CO_3 and then stored at -80°C until analyzed for glucose.

Determination of tissue energy stores and metabolites

The concentrations of ATP and PCr in the trout liver were analyzed enzymatically using hexokinase, glucose-6-phosphate dehydrogenase, and creatine kinase (Bergmeyer 1983) using a microplate spectrophotometer (Spectramax 190; Molecular Devices, Sunnyvale, California) at 340 nm. Lactate in the brain and liver homogenates was analyzed using lactate dehydrogenase and quantified at 340 nm using the plate spectrophotometer. Glycogen, expressed in μmol glucosyl units per g wet weight, was analyzed using hexokinase and quantified at 340 nm using the microplate spectrophotometer.

Isolation of TFM and TFM-glucuronide

Ground muscle tissue was processed for TFM and TFM-glucuronide quantification by solid phase extraction (SPE) and HPLC as described by Hubert et al. (2001). Briefly, the TFM was extracted following the addition of 12mL of 80% methanol to the powder. This slurry was vigorously mixed using a tabletop shaker and then centrifuged at 10,000 x *g*. The supernatant was drawn off and transferred to a separate test tube, which was then eluted with methanol (100%) through a C18 OH 500mg SPE cartridge (14-388-13, Fisher Scientific, Ottawa, ON) covered with a layer of glass beads to prevent solid detritus from passing through. The eluent was then aspirated using a nitrogen evaporator to remove the methanol, followed by the addition of water and then NaOH to increase pH to approximately pH 9.5, converting the TFM to its ionized state. The resulting slurry was passed through styrenedivinylbenzene (SDB-XC; 0-51115-08793-6, 3M, St. Paul, MN) cartridges using 5% methanol, adjusted using water corrected

to pH 9.5 using 1 N NaOH. The methanol was then evaporated off, and the pH adjusted to pH 4 using 12 N HCl to convert TFM to its un-ionized state. This solution was then eluted through a C18 OH extraction column (14-388-13, Fisher Scientific, Ottawa, ON) which retained both TFM and its glucuronide conjugate. The glucuronide was then collected by eluting the column with 24.6 mmol L^{-1} acetate buffer and 75% methanol at a pH of 4, and the TFM by eluting the colulmn with 75% methanol. After aspirating each solution under N_2 to remove the methanol, the TFM-glucuronide solution was then incubated for 18 h in phosphate buffer containing β-glucuronidase to convert the TFM-glucuronide into its native TFM and glucuronyl acid constituents. The extract was then applied to a C18 OH 500mg column (14-388-13, Fisher Scientific, Ottawa, ON) and eluted using 75% methanol. The TFM extracts were then applied to a C18 OH 500 mg column, and eluted using 75% methanol, the methanol subsequently aspirated using N_2 evaporation, and the TFM concentrates resuspended in 2 mL of water and transferred to 2 mL vials for subsequent TFM quantification using HPLC.

Each sample vial (2 mL) was placed in an autosampler (Prostar 410 Autosampler, Varian Corporation, Palo Alto, CA), connected to a solvent delivery module (Prostar 230, Varian Corporation, Palo Alto, CA) and UV detector (Prostar 310, Varian Corporation, Palo Alto, CA) and exactly 200μL of sample injected onto a C18 reverse phase HPLC column (Kinetex 2.6μm XB-C18 100 Å 30 x 3.0 mm; Phenomenex, Torrance, CA) at room temperature, using a mobile phase comprised of 17% acetonitrile and 83% 20mM sodium borate buffer, pH 8.50. The flow rate was 0.5 mL min^{-1} for 9 minutes per sample, and the TFM peak was detected at a wavelength of 400nm with a retention time of 6 minutes. The areas under the TFM peak were then integrated using Varian Star Chromatography Workstation System Control software, version 5.51, and the concentrations determined using TFM standards prepared using analytical grade

TFM (SigmaAldrich, Product No. N27802, Sigma-Aldrich Chemical Co., St. Louis, MO) dissolved in reverse osmosis H_2O. All TFM concentrations were expressed in nmol g^{-1} wet weight.

Statistical analysis

All data are presented as the means ± the standard error of the mean (SE). Differences in tissue metabolite and energy stores were analyzed using one-way analysis of variance (ANOVA), and when significant variation was observed, differences between the means were quantified using the Tukey-Kramer post hoc test at the $P < 0.05$ level. When the assumptions for ANOVA were not satisfied, a nonparametric ANOVA followed by Dunn's test by ranks was used where applicable. All statistical analyses were done using GraphPad Prism (GraphPad Software Inc., La Jolla, California).

To determine the half-life of TFM, concentrations of TFM in live animals were plotted in a scatter plot and linear regression used to calculate the line of best fit. This was used to produce the rate constant (k) for TFM elimination using the following expression ($k = -2.303 \times m$) (Voet et al., 2006). The elimination half life (T ½) was equal to $T_{1/2} = 0.693 / k$.

Results

TFM and TFM glucuronide concentration in rainbow trout and larval sea lamprey muscle tissue

During TFM exposure and the post-exposure period, 78% of the larval sea lamprey died (Table 2-1). Nevertheless, both survivors and dead larval sea lamprey were sampled. No rainbow trout died due to TFM exposure during both the exposure and post-exposure experiments. Average TFM concentrations in the water were 8.3 mg L^{-1} ± 0.16 for larval sea lamprey and approximately 7.5 mg L^{-1} ± 0.08 for rainbow trout (Table 2-1).

High performance liquid chromatography (HPLC) revealed a retention time for TFM of 5.9 ± 0.6 minutes in both rainbow trout and in larval sea lamprey muscle, with no evidence of interfering peaks (Figure 2-1). Percent recovery of TFM consistently remained near 60%, based on the analysis of control ground muscle samples spiked with 104 ng mL^{-1} TFM (20.8 ng g^{-1} tissue fortification level) (Hubert et al., 2001).

The TFM concentration in fingerling rainbow trout muscle increased to 15 nmol g^{-1} wet tissue after 3 h of exposure, and stayed significantly different from control values throughout the exposure, and then stabilized at 20 nmol g^{-1} wet tissue for the remainder of the exposure period (Figure 2-2A). The accumulation of TFM-glucuronide lagged behind that of TFM, steadily increasing from approximately 10 nmol g^{-1} wet tissue before peaking near 25 nmol g^{-1} wet tissue after 9 h (Figure 2-2A).

Recovery in TFM-free water was accompanied by precipitous declines in both the TFM and TFM-glucuronide concentrations, each of which was completely eliminated from the body of the trout within 3 h (Figure 2-2B).

Not surprisingly, the situation was markedly different in larval sea lamprey in which the TFM concentrations approached 50 nmol g^{-1} wet tissue after only 3 h of exposure to an identical concentration of TFM as the rainbow trout (Figure 2-2B). The concentrations of TFM fluctuated between 30 and 50 nmol g^{-1} wet tissue throughout the 12 h exposure period. Unlike in the rainbow trout, only traces of TFM-glucuronide were detected during the 12 h TFM exposure period (Figure 2-2B).

The clearance of TFM was also much slower in the sea lamprey during the post-TFM exposure recovery period. After 24 h, the TFM concentrations in the carcass of sea lamprey were still approximately 5 nmol g^{-1} wet tissue. The concentrations of TFM-glucuronide remained just above levels of detection in the sea lamprey for the first 6 h of recovery, but were completely eliminated by 24 h (Figure 2-2B).

Energy stores and metabolite concentrations in fingerling rainbow trout brain and liver tissue

Exposure of the rainbow trout to TFM resulted in no change in brain glycogen concentrations, which were not significantly different from the baseline control values of 1.8 μmol g^{-1} wet tissue (Figure 2-3A). This coincided with no change in the concentrations of lactate in the brain tissue, which were stable near 6 μmol g^{-1} wet tissue (Figure 2-3B).

The situation was different in the liver, where significant decreases in liver glycogen concentration were observed after 12 h of TFM exposure, declining almost 60% from concentrations of 16 μmol g^{-1} wet tissue in control fish, to approximately 6 μmol g^{-1} wet tissue in fish exposed to TFM (Figure 2-4A). However, liver lactate concentrations remained stable near 6 μmol g^{-1} wet tissue during the TFM exposure and recovery periods (Figure 2-4B).

The declines in liver glycogen were accompanied by similar 50% reductions in liver ATP concentration, which decreased from approximately 1.8 µmol g^{-1} wet tissue in control animals to approximately 1 µmol g^{-1} wet tissue after 9 h, where it remained during the TFM exposure and recovery periods (Figure 2-5A). There were no significant changes in liver PCr concentration, however, which fluctuated between 5-7 µmol g^{-1} wet tissue during the experiment (Figure 2-5B).

Concentrations of glycogen and lactate in larval sea lamprey liver tissue

The disturbances to liver glycogen and lactate concentration were far more pronounced in the larval sea lamprey. After 3 h, liver glycogen concentration had decreased by 80%, from approximately 5 µmol g^{-1} wet tissue in the non-TFM exposed control animals (Figure 2-6A) and remained near these levels through the TFM exposure and recovery period (Figure 2-6A). Coinciding increases in the concentrations of lactate were also observed, which increased by two- to three-fold from 6 µmol g^{-1} wet tissue, to more than 15 µmol g^{-1} wet tissue by 12 h (Figure 2-6B). Liver lactate concentrations remained elevated throughout the full 24 h recovery period (Figure 2-6B).

Discussion

Metabolic effects of sub-lethal TFM exposure on non-target rainbow trout

The current study provides evidence that exposure to TFM, at concentrations comparable to those used in field applications of the lampricide, does cause metabolic disturbances in rainbow trout, but such disturbances are modest and likely readily corrected upon return to TFM-free water. In this study, rainbow trout were exposed to a concentration of TFM that was equivalent to the 12 h LC100 of larval sea lamprey (median lethal concentration) in order to assess metabolic response to a dose similar to that administered in the field. Sea lamprey control agents typically apply TFM at slightly higher concentrations than those used in the present study, approximately 1.2-1.3 times the LC100 of sea lamprey (MLC) (Bills et al., 2003; McDonald and Kolar, 2007). Because these values are well below concentrations of TFM known to cause mortality in salmonids (Bills et al., 2003; Birceanu et al., 2014), it is likely that the extent of the metabolic disturbance and TFM loading in the fish would have been comparable to field application levels, even if the trout had been exposed to these slightly higher concentrations of TFM. The minimal homeostatic disturbances seen through most of the TFM exposure are clearly related to the rainbow trout's ability to maintain steady state internal concentrations of TFM between 15-20 nmol g^{-1} wet tissue through the process of glucuronidation. These values of TFM were about 65-70% lower than TFM concentrations measured in the sea lamprey exposed to nearly identical water TFM concentrations, at which 78% of the sea lamprey eventually died as a result of the exposure. Of these mortalities, all post-exposure sea lamprey died and most of them during exposure. Those that survived until sampling likely survived due to being sampled at 3 h, the earliest sampling period.

The resilience of the rainbow trout to TFM, despite its persistent presence in the muscle tissue for 12 h exposure, was due to their ability to rapidly detoxify and remove TFM from the body through the process of glucuronidation (Lech and Statham, 1975). The role that glucuronidation plays in detoxifying TFM is further supported by studies in which the UDPGT enzyme inhibitor salicylamide was injected into trout exposed to TFM, leading to decreased TFM-glucuronide formation and a reduced capacity to tolerate TFM (Lech and Statham, 1975). Additionally, rainbow trout injected with salicylamide had a reduced capacity to tolerate the poison (Lech and Statham, 1975).

The lag in TFM-glucuronide accumulation compared to internal TFM concentrations may have been due to the well established "latency of glucuronidation", which may be related to the luminal location of both the active site for UDP-glucuronyltransferase and UDP glucuronic acid within the endoplasmic reticulum, an organelle in cells responsible for the folding and transporting of proteins (ER; Meech and Mackenzie, 1997). Although substrates for glucuronidation such as TFM are lipophilic, and would readily cross the ER membrane, UDP glucuronic acid movement into the ER lumen is transporter dependent, which likely limits the rate of glucuronidation (Muraoka et al., 2007). It might also have been because UDPGT activity was further induced in the face of the TFM burden, gradually increasing its activity over the first few hours of exposure, due to either allosteric factors or increased quantity of enzyme, but these hypotheses remain to be tested in the trout. This may also explain why it took approximately 6 h before TFM concentrations stabilized in the trout. Measurement of UDPGT enzyme activity and immunoblotting studies could allow researchers to localize and quantify the amount of enzyme in the hepatocytes in response to TFM (Clarke et al., 1991).

A characteristic feature of exposure to toxic concentrations of TFM is a decline in brain and liver glycogen in both sea lamprey and rainbow trout which likely contributes to death by causing profound hypoglycaemia (Birceanu et al., 2009, 2014; Clifford et al., 2012). However, glycogen concentrations in the brain did not drop markedly in the rainbow trout, and the decreases in liver concentrations of glycogen, while significant, were not observed until 12 h. Thus, glucose supply to the nervous system was not compromised.

It is likely that the trout would have been able to rapidly replenish their glycogen stores in the wild. Both liver and whole body glycogen levels are sensitive to fasting, but are rapidly replenished following feeding (Scarabello et al., 1991; Polakof et al., 2007). Indeed, the progressive decline in liver glycogen with TFM exposure in the present study also matched declines reported following 7 days of fasting in comparably sized rainbow trout (Polakof et al., 2007). Thus, in wild fish species transiently exposed to TFM, it is likely that the liver glycogen stores would likely be rapidly restored as the fish resumed feeding following treatments. The lack of any increase in liver or brain lactate suggests that there was likely no reliance on anaerobic glycolysis to maintain ATP supply during TFM exposure.

The decline in liver glycogen with TFM exposure was probably due to the greater physiological demands faced by the trout with TFM exposure including the need to use ATP-dependent pathways to detoxify TFM in the liver using glucuronidation (Clarke et al., 1991). The formation of TFM-glucuronide, and other conjugated xenobiotics, depends upon a steady supply of UDP-glucuronic acid, which is generated from glucose-1-phosphate arising from glycogenolysis (Banhegyi et al., 1988; Mulder, 1992). Faced with a steady influx of TFM into its body, the trout likely had to increase its rates of TFM-glucuronide production, which would

have increased demands for UDP-glucuronic acid and therefore glycogen over the 12 h exposure period. This would have been compounded by elevated steady state levels of TFM, which would have likely interfered with ATP production through its inhibitory effects on oxidative phosphorylation, which Birceanu et al. (2011) recently demonstrated interfered with this process in a dose-dependent manner. In combination, these effects would have limited ATP supply to the liver, and likely impaired glycogenesis via ATP dependent pathways. In other words, because glycogen supply could no longer match demand, glycogen concentrations gradually dropped markedly by 12 h. Similar gradual declines in liver ATP and glycogen concentrations were reported in rat liver slices incubated *in vitro* with paracetamol (Acetaminophen) for 20 h as it was undergoing biotransformation to paracetamol glucuronide (Evdokimova et al., 2001).

Increased respiratory demands for oxygen and greater ATP consumption due to greater work by the branchial musculature may have also further compounded any mismatch between ATP supply and demand. Birceanu et al. (2011) demonstrated that the rates of oxygen consumption by isolated trout liver mitochondria increased, suggesting that TFM could lead to overall increases in O_2 demand and perhaps in ventilation in rainbow trout. Indeed, increases in respiratory amplitude and vasodilation in the gills and liver were observed in post-metamorphic sea lamprey in which the gills were perfused with TFM, although the data set was somewhat limited (Agris, 1967). Thus, in addition to limiting in ATP supply, TFM exposure may also cause increased ATP demand in lamprey, and perhaps non-target fish such as trout, leading to increased reliance on liver glycogen reserves. Clearly, further studies are needed to determine how sub-lethal concentrations of TFM influence oxygen consumption and energy demand in the rainbow trout and other non-target fish species.

Detoxification and elimination of TFM by rainbow trout and sea lamprey

The difference in accumulation of TFM between larval sea lamprey and rainbow trout was pronounced. The marked 3-fold greater TFM accumulation in the sea lamprey compared to the trout was no doubt due to the ability of rainbow trout to efficiently detoxify TFM through its biotransformation to TFM-glucuronide, which allowed the fish to excrete it via the urine or gastrointestinal tract (Hunn and Allen, 1974; Schultz et al., 1979). Indeed, concentrations of TFM-glucuronide appeared to reach a steady state after approximately 9 h, when the concentration of TFM was matched by TFM-glucuronide. In contrast, the larval sea lamprey biotransformed virtually none of the TFM, with only traces of TFM-glucuronide appearing in the carcass during the TFM exposure period. With no efficient method of eliminating TFM from the body, the very high TFM concentrations were sustained in the larval sea lamprey throughout the TFM-exposure period.

The rapid elimination of lampricide by the rainbow trout following its recovery in TFM-free water was likely a combination of elimination of the more water soluble TFM-glucuronide via the gastrointestinal tract and urine (Hunn and Allen, 1974; Schultz et al., 1979), and likely via passive diffusion of un-ionized TFM across the gills. Based on a blood pH of 7.8, the amount of un-ionized TFM in the blood of rainbow trout would have been less than 2%, based on a pK for TFM of 6.07 (McDonald and Kolar, 2007). Thus. there would have been an outward directed diffusion gradient for un-ionized (phenolic) TFM across the gills following the introduction of the trout into clean water. However, this would not be a very efficient method of elimination on its own due to the small percentage of TFM found in its un-ionized form in the blood, where the bulk of TFM would remain in its more hydrophilic, ionized form (Hunn and Allen, 1975; McDonald and Kolar, 2007). Thus, the elimination of the more hydrophilic TFM-

glucuronide via the urine/feces likely explains the very short half-life of 1.8 h for TFM elimination, and almost complete clearance of TFM and TFM-metabolites by the rainbow trout following TFM exposure (Figure 2-7).

In contrast, the relatively slow elimination of TFM by larval sea lamprey was due to their inability to biotransform the TFM to its more water soluble glucuronide form, which would greatly reduce the contribution that the renal or gastrointestinal tracts make to TFM elimination following treatment. This inability of the sea lamprey to form TFM-glucuronide is consistent with the earlier findings of Lech and Stathan (1975), who reported that the survival of larval sea lamprey to TFM was unaffected following the administration of the glucuronyl transferase inhibitor, salicylamide. These findings also suggest that the larval sea lamprey are probably incapable of inducing UDPGT enzyme during TFM exposure. Similarly, Kane et al. (1994) reported that the activity of UDP-glucuronyl transferase in sea lamprey liver was very low compared to a number of teleosts including bluegill (*Lepomis macrochirus*). This lack of UDP-glucuronyl transferase activity in the liver could be related to the limited role that the larval sea lamprey liver plays in regulating a variety of physiological processes including the regulation of glucose homeostasis (O'Boyle and Beamish, 1977; Rovainen 1970) and in the production of nitrogenous wastes, in which other organs including the muscle and intestine play an important role (Wilkie et al., 2006). Indeed, the meninges of the brain in sea lamprey appear to be most important for controlling the supply of glucose to the nervous system in lamprey (Rovainen 1970; Foster et al., 1993).

Despite the lack of TFM glucuronidation capacity, sea lamprey were able to clear TFM from the body during recovery in TFM-free water. However, the elimination of TFM by the

lamprey was likely restricted to passive diffusion of un-ionized TFM across the gills (Clifford et al., 2012), which would help explain the 4-fold greater $T_{1/2}$ elimination for TFM seen in the sea lamprey compared to the rainbow trout (Figure 2-7).

Implications for TFM applications and non-target fish species in the field

The metabolic disturbances noted in fingerling rainbow trout were mainly limited to reductions in liver glycogen stores, and were of similar magnitude to observations made in fasted fish (Polakof et al., 2007). Moreover, the fish were able to recover from TFM exposure and eliminate the complete TFM burden with the first few hours of depuration. These findings suggest wild trout exposed to TFM during field applications of the lampricide, at concentrations near the MLC for sea lamprey, would readily tolerate and recover within a few hours of treatment. Any reductions in liver glycogen reserves, due to increased metabolic demand and/or biotransformation of TFM, would likely be replenished as the fish resumed feeding. Additionally, there were no fatalities during exposure indicating that these relatively modest metabolic disturbances were completely reversible. Although further studies on other non-target fish species would be prudent, other non-target teleosts also have a relatively high capacity to detoxify TFM using glucuronidation (Kane et al., 1994). Thus, it would be reasonable to suggest that similar, sub-lethal disturbances involving reversible reductions in liver glycogen would occur in those fish species following TFM treatments. Larval sea lamprey, on the other hand, experience marked reductions in not only liver glycogen stores, but also brain glycogen stores, which is a notable characteristic of TFM toxicity and unless allowed to recover in TFM-free water, are unable to survive TFM exposure (Birceanu et al., 2009; Clifford et al., 2012).

The marked differences in the TFM-elimination half-life along with determinations of liver glycogen could be used as index of how to predict how sensitive other non-target fish

species are to TFM. This could be achieved by exposing a non-target fish to environmentally relevant concentrations of TFM for periods approximating the TFM treatment period (9-12 h) and then measuring the TFM in the tissues immediately after, and during a post-exposure period. Using this information, the toxic and lethal internal thresholds for TFM could be determined, and the elimination half-life documented for different fish species. With such knowledge, the relative sensitivity of different species could be catalogued, and TFM treatments could be modified (e.g. changes in the length and/or application dose of TFM). This approach could reduce the need to perform more complicated toxicity tests. Thus, similar studies on the toxicity and elimination of not only TFM from fish species, but other toxicants could provide an alternate non-lethal end-point for predicting how sensitive different fish species are to TFM and other xenobiotics.

Tables and Figures

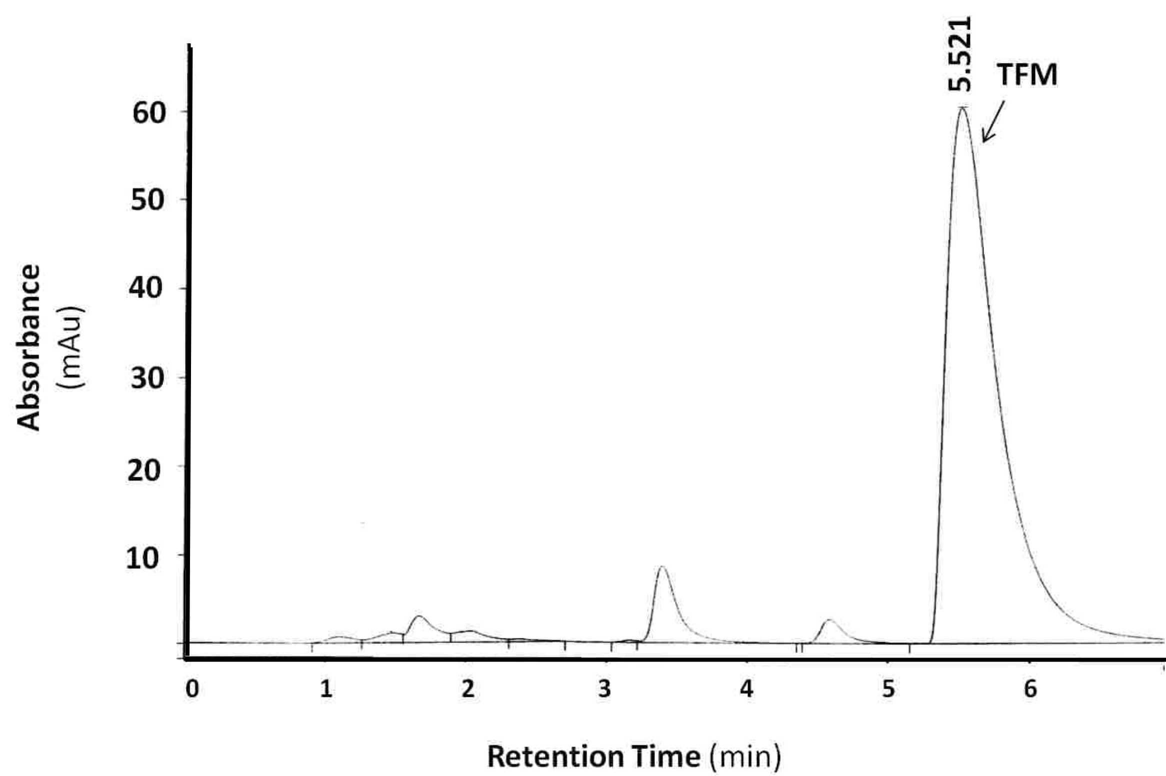
Table 2-1 – Fish mortalities during and after exposure to TFM, average TFM concentration and average length and weight of fingerling rainbow trout and larval sea lamprey following 12 h exposure to TFM.

	Total Mortalities (Brackets indicate post-exposure)	Average TFM Concentration (mg L^{-1})	Average Length (mm) ± SD (Range)	Average Weight (g) ± SD (Range)
Fingerling Rainbow Trout	0 (0)	7.5	129.3 ± 17.8 (168-100)	25.4 ± 25.4 (50.6-12.8)
Larval Sea Lamprey	50 (30)	8.3	100.9 ± 10.5 (124-70)	1.37 ± 0.5 (3.5-0.4)

Figure 2-1 – Quantification of TFM in rainbow trout and larval sea lamprey using HPLC.

Representative HPLC chromatograms displaying TFM peak retention time and shape in (A) fingerling rainbow trout and (B) larval sea lamprey exposed to respective measured TFM concentrations of 7.5 ± 0.08 mg L^{-1} and 8.3 ± 0.16 mg L^{-1}, which are near the LC100 of TFM of larval sea lamprey.

A)



B)

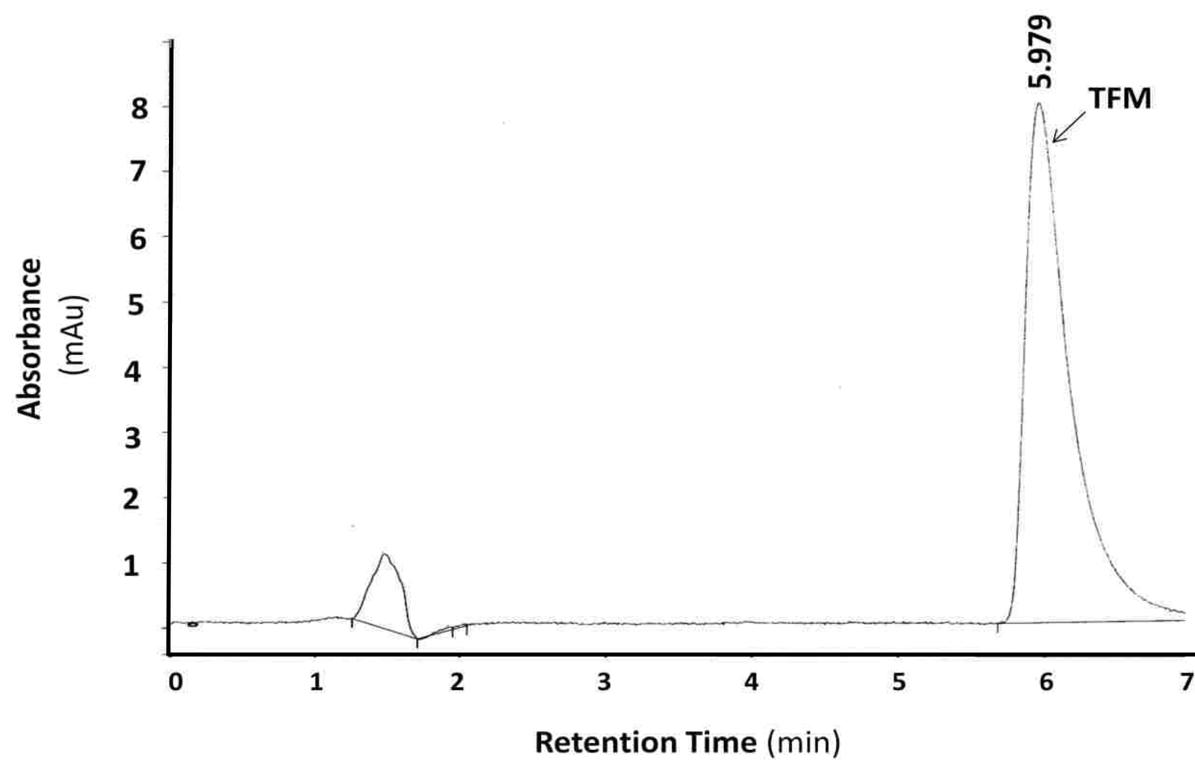
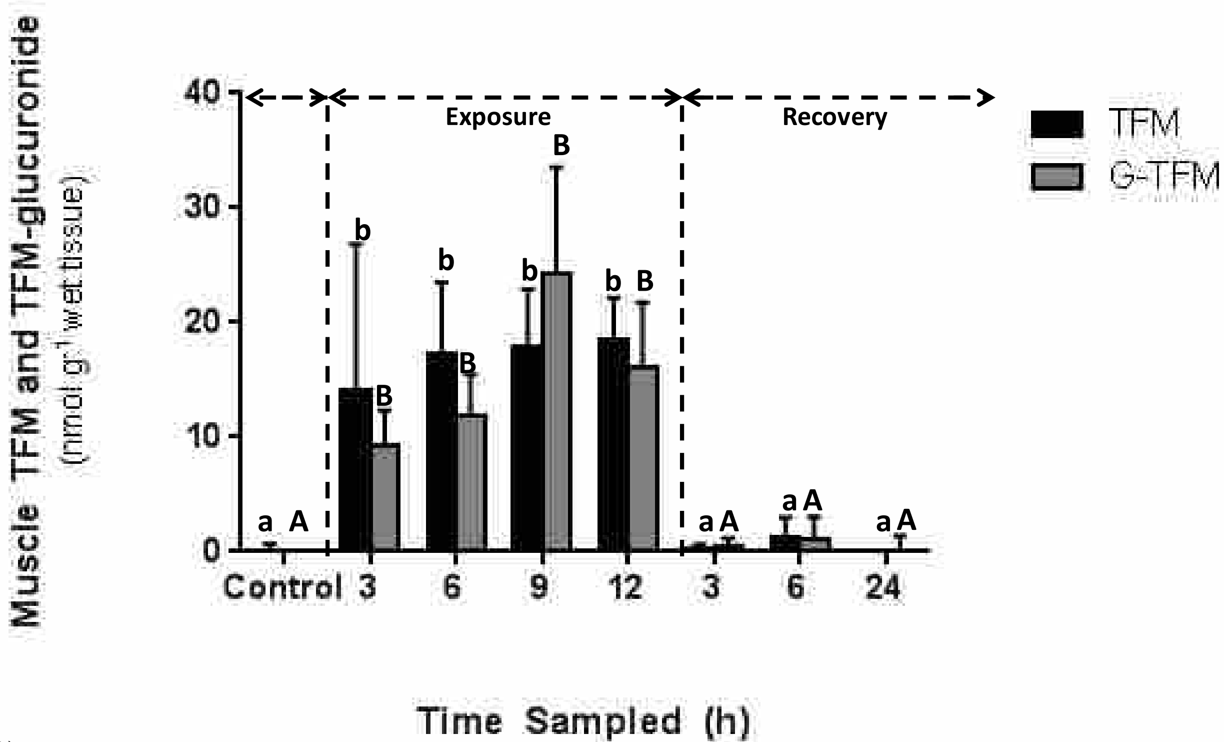


Figure 2-2 – TFM and TFM-Glucuronide accumulation in fingerling rainbow trout muscle tissue and larval sea lamprey carcass.

Concentrations of TFM and TFM-glucuronide in resting (A) fingerling rainbow trout and (B) larval sea lamprey exposed for 12 h to the 12 h LC100 concentration of TFM (7.6mg L^{-1}) of larval sea lamprey, and following recovery in TFM-free water. Darkly shaded bars denote concentrations of TFM, and lightly shaded bars concentrations of TFM-glucuronide. Data are presented as the mean +1 SEM. Bars sharing the same letter are not significantly different from one another. N = 8 for all sample groups. Bars sharing the same upper case letter denote treatments in which the measured TFM concentrations in each group are not significantly different from one another. Bars sharing the same lower case letter represent treatments in which the TFM-glucuronide concentrations are not significantly different ($P<0.05$).

A)



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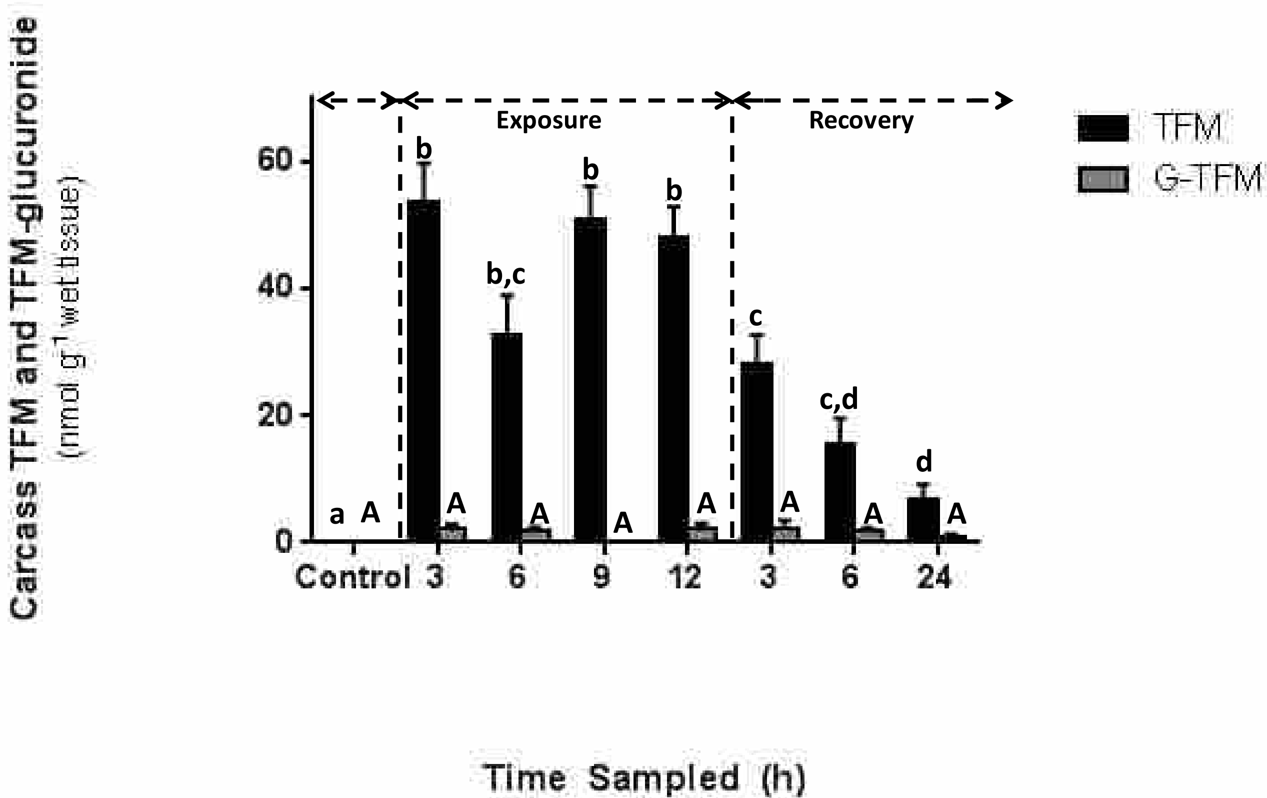
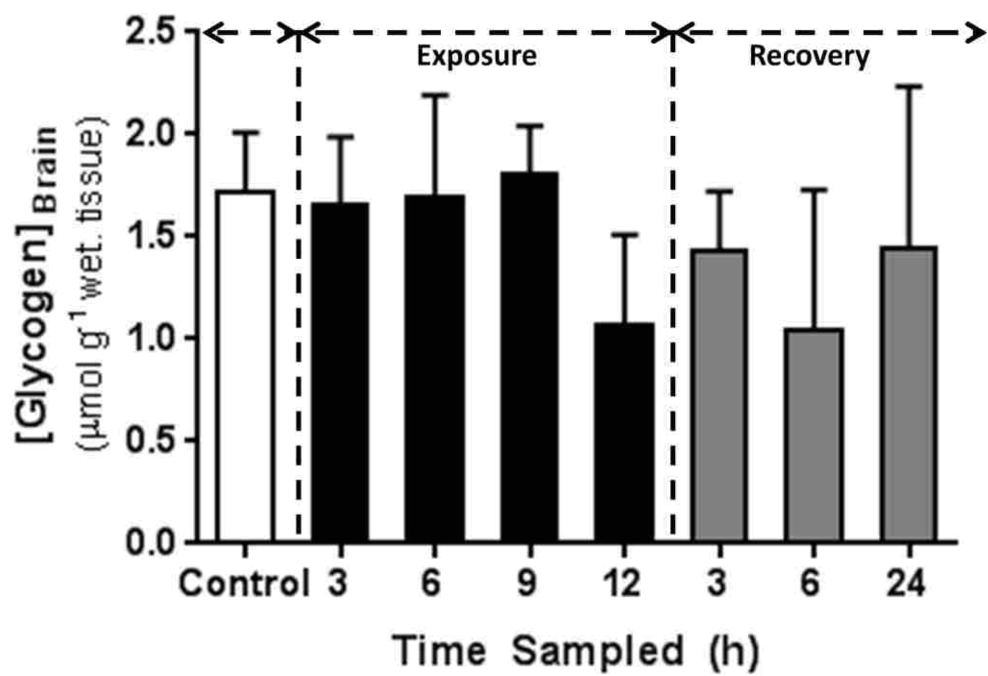


Figure 2-3 – Effects of TFM on glycogen and lactate concentrations in rainbow trout brain tissue.

Concentrations in brain tissue of (A) glycogen and (B) lactate in resting fingerling rainbow trout exposed to the 12 h LC100 of TFM for 12 h (7.6mg L^{-1}), and during recovery in TFM-free water for up to 24 h. Data presented as the mean +1 SEM. No significant changes were observed.

A)



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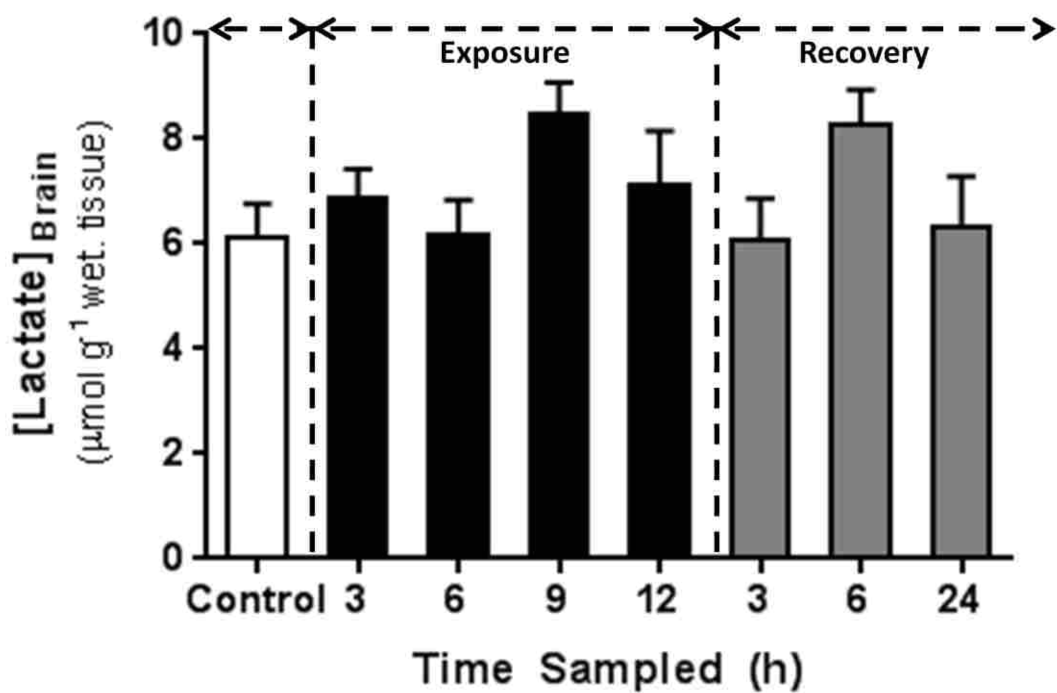
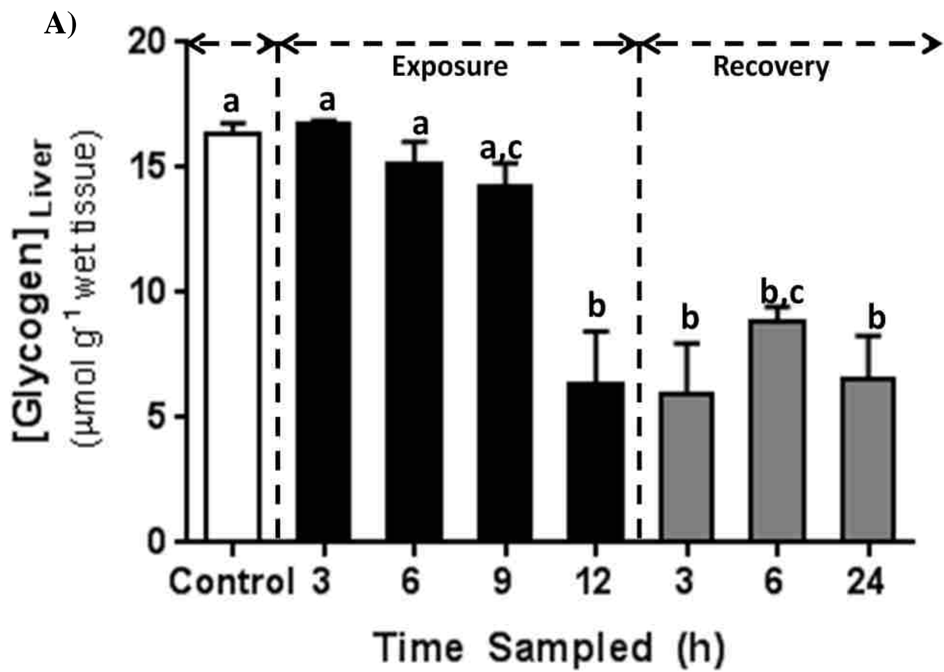


Figure 2-4 – Effects of TFM on liver glycogen and lactate concentrations in rainbow trout.

Concentrations in liver tissue of (A) glycogen and (B) lactate in resting fingerling rainbow trout exposed to the 12 h LC100 of TFM for 12 h (7.6mg L^{-1}), and during recovery in TFM-free water for up to 24 h. Data presented as the mean + 1 SEM. Bars sharing the same lower case letter represent treatments in which the glycogen concentrations are not significantly different ($P<0.05$). N=8 for all sample groups.

A)



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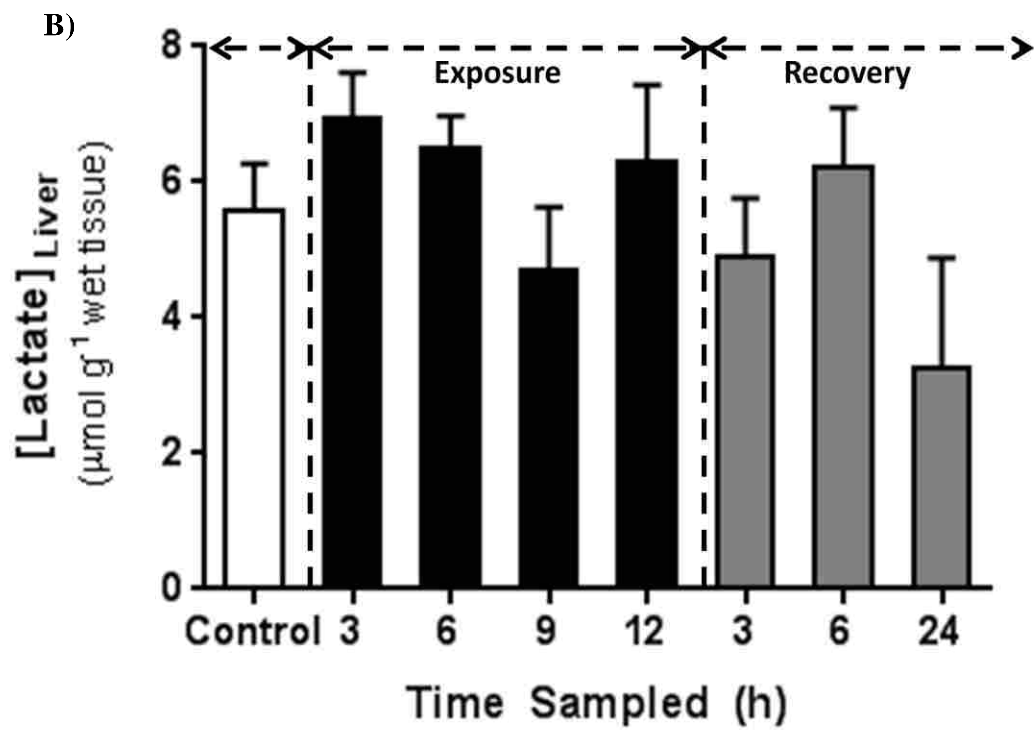
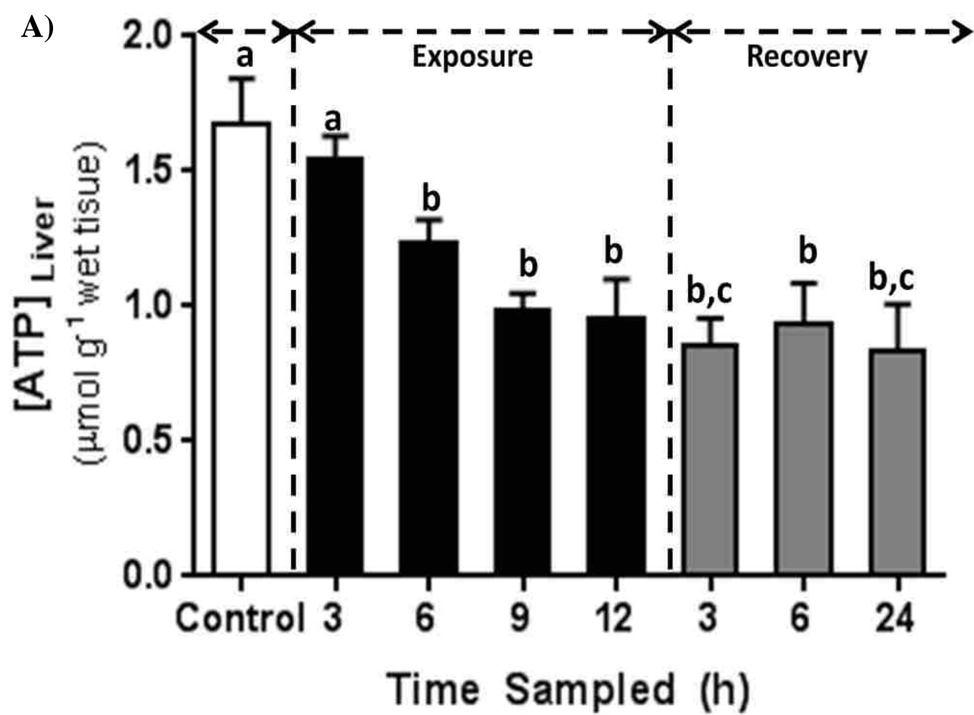


Figure 2-5 – Effects of TFM on liver ATP and PCr concentrations in rainbow trout.

Concentrations in liver tissue of (A) ATP and (B) PCr in resting fingerling rainbow trout exposed to the 12 h LC100 of TFM for 12 h (7.6mg L^{-1}), and during recovery in TFM-free water for up to 24 h. Data are expressed as the mean + 1 SEM. Bars sharing the same lower case letter represent treatments in which the ATP concentrations are not significantly different ($P<0.05$). N=8 for all sample groups.

A)



B)

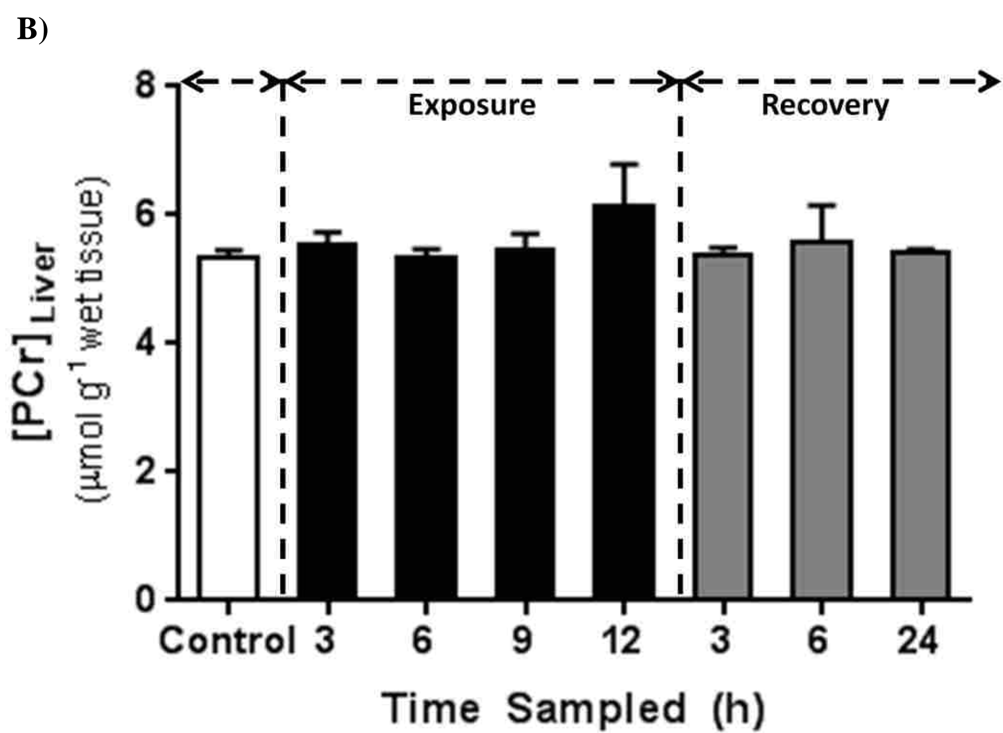
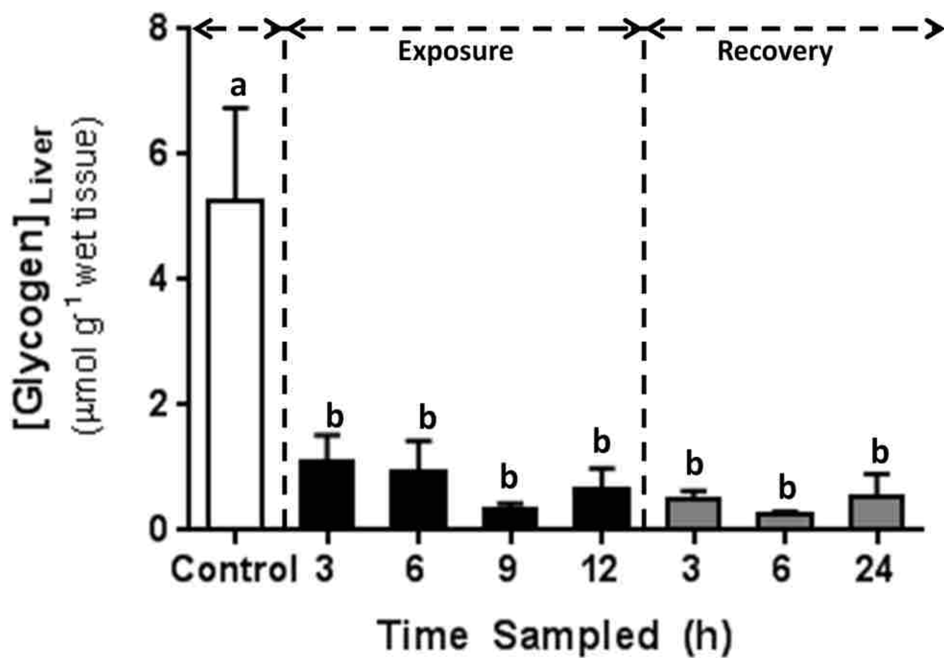


Figure 2-6 – Effects of TFM on liver glycogen and lactate concentrations in larval sea lamprey.

Concentrations in liver tissue of (A) glycogen and (B) lactate in resting larval sea lamprey exposed to the 12 h LC100 of TFM for 12 h (7.6mg L^{-1}), and during recovery in TFM-free water for up to 24 h. Data presented as the mean +1 SEM. Bars sharing the same lower case letter represent treatments in which the glycogen or liver concentrations are not significantly different ($P<0.05$). N=8 for all sample groups.

A)



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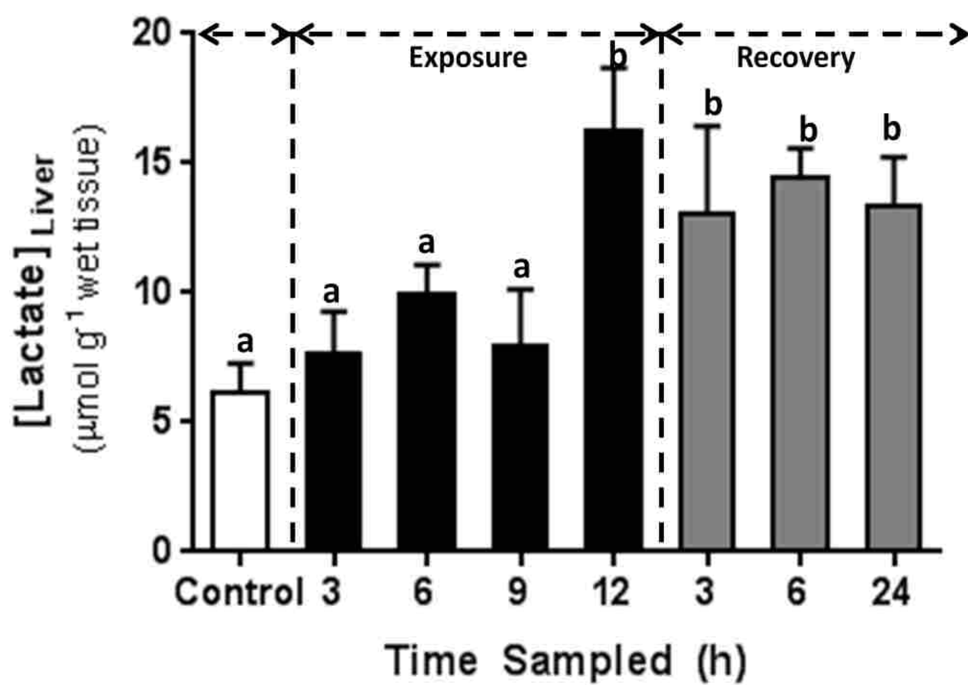
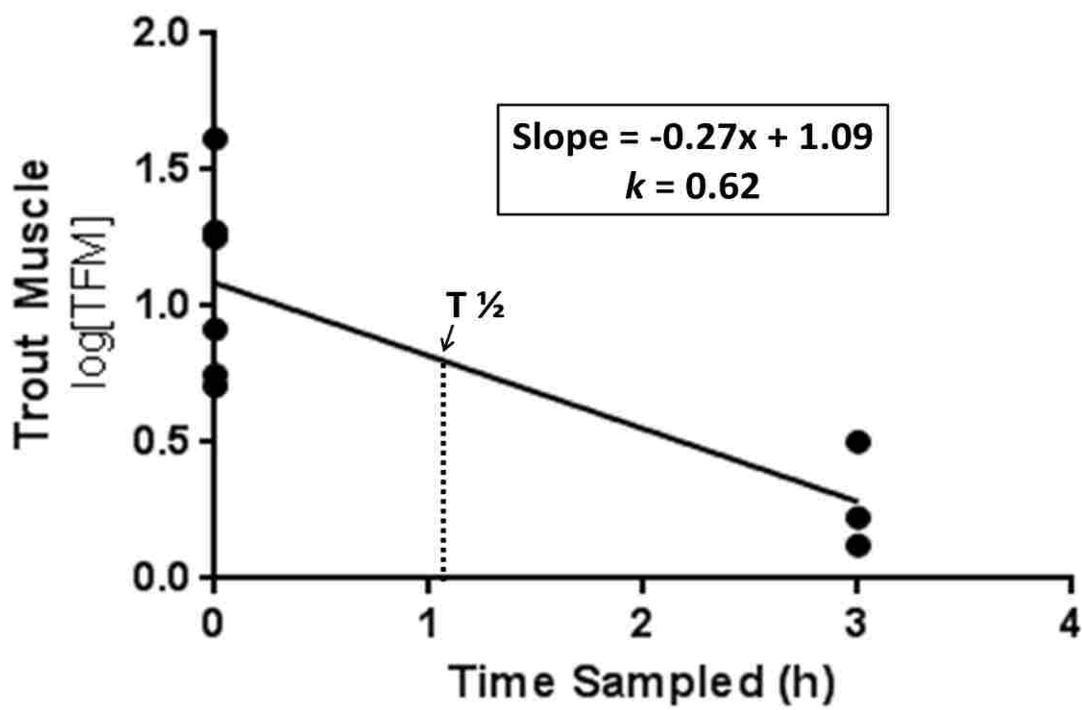


Figure 2-7 – Half-life of TFM in fingerling rainbow trout and larval sea lamprey muscle tissue and carcass.

Semi-log plot denoting the changes in the concentrations of muscle TFM concentrations in the fish muscle tissue of (A) fingerling rainbow trout and (B) the carcass of larval sea lamprey following re-introduction into TFM-free water. Fingerling rainbow trout had eliminated all TFM by 3 h of recovery, whereas residual TFM still remained in the muscle of larval sea lamprey following 24 h of recovery. Linear regression was used to calculate the line of best fit, which yielded a rate constant (k) for TFM elimination as calculated using the following expression ($k = -2.303 \times m$) (Voet et al., 2006). The elimination half life ($T_{1/2}$) equals ($T_{1/2} = 0.693 / k$) (Voet et al., 2006).

A)



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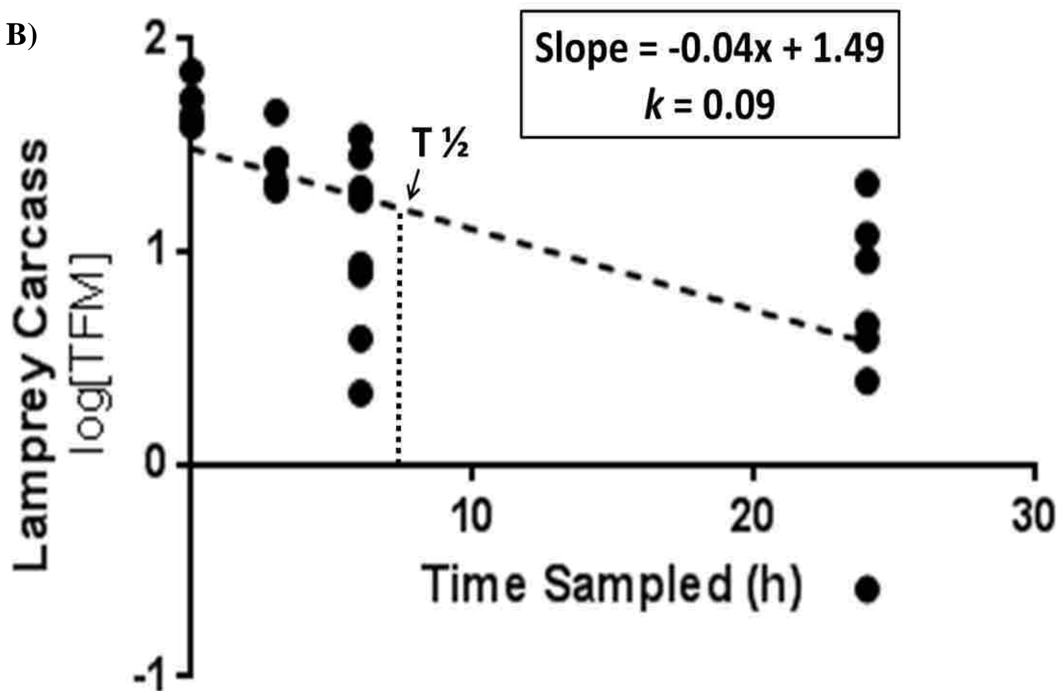


CHAPTER THREE

The Lake Sturgeon (*Acipenser fulvescens*) Detoxifies 3-Trifluoromethyl-4-Nitrophenol (TFM) Using Glucuronidation

Introduction

The pesticide, 3-trifluoromethyl-4-nitrophenol (TFM) is used to selectively control larval sea lamprey populations, and normally has minimal impact on non-target fish species (Applegate, 1961; Hubert et al., 2003; Boogaard et al., 2003; McDonald and Kolar, 2007; see also Chapter 2). The greater resilience of most non-target fish species such as the rainbow trout (*Oncorhynchuss mykiss*) to TFM is due to their ability to readily detoxify it by the process of glucuronidation, which prevents internal TFM from reaching toxic levels inside the animal during typical lampricide treatments (Lech and Statham 1975; Kane et al., 1994; Birceanu et al., 2014; Chapter 2). Larval sea lamprey, on the other hand, appear to have a much lower capacity to generate appreciable amounts of TFM-glucuronide, which allows whole body TFM to rapidly rise to toxic concentrations that can be several-fold greater than seen in the rainbow trout exposed to similar amounts of TFM (Lech and Statham, 1975; Kane et al., 1994; Chapter 2). At these much higher concentrations of TFM in the body, there are much greater disruptions to oxidative phosphorylation in the mitochondria, and a greater impairment of ATP production (Birceanu et al., 2011), which results in homeostatic disturbances and death due in part to a depletion of glycogen reserves that starve the nervous system of glucose (Wilkie et al., 2007; Birceanu et al., 2009; Clifford et al., 2012).

Kane et al. (1993) reported that glucuronidation capacity was variable in fish species, due to differences in the affinity (K_m) and maximum velocity (V_{max}) of the enzyme UDP-glucuronyltransferase (also known as UDP-glucuronosyltransferase; Testa and Kramer, 2008), which catalyzes the transfer of UDP glucuronic acid to lipophilic molecules including phenols (Clarke et al., 1991; Testa and Kramer, 2008). As a result, compounds such as steroid hormones (e.g. glucocorticoids), lipophilic xenobiotics, and phenols such as TFM are converted into more

water-soluble glucuronide conjugates that can be more easily excreted via the urine or in the bile via the gastrointestinal tract (Clarke et al., 1991; Testa and Kramer, 2008). According to the analysis of Kane et al. (1994), sea lamprey (in the parasitic phase) had the lowest TFM biotransforming efficiency, calculated by determining the V_{max}:K_m ratio, followed by channel catfish (*Ictalurus punctatus*), rainbow trout and bluegill (*Lepomis macrochirus*).

An inability to use glucuronidation to detoxify TFM may explain why non-target fish species are particularly sensitive to TFM at certain life stages (McDonald and Kolar, 2007). Of particular note, is the lake sturgeon (*Acipenser fulvescens*), which is thought to be more sensitive to TFM during its fingerling stages when it is still less than 100 mm in length (Boogaard et al., 2003). The lake sturgeon is an ecologically important fish that is threatened, extirpated, endangered or of special concern in 12 different US states and in the province of Ontario (Peterson et al., 2007). Thus, their sensitivity to TFM is an important concern to fisheries managers and to sea lamprey control agents, who rely on the use of TFM to control sea lamprey populations in order to protect the 7.5 billion dollar (US) Great Lakes fishery (GLFC, 2011).

Despite suggestions that sturgeon in their early life stages may lack the ability to detoxify TFM using glucuronidation, there is no published evidence identifying the root cause for the greater sensitivity of juvenile lake sturgeon to TFM. There is limited evidence to suggest that members of the sturgeon family (Family: Acipinseridae) have a limited capacity to use glucuronidation to biotransform xenobiotics (Singer and Ballantyne, 2004) including phenolic compounds, but this question has not been examined in lake sturgeon exposed to TFM.

To determine if juvenile lake sturgeon have the capacity to detoxify TFM using glucruonidation, the fish were exposed to the pesticide (12 h LC50 of TFM to larval sea

lamprey) and the concentrations of TFM and TFM-glucuronide were using high performance liquid chromatography (HPLC) following solid phase extraction of the TFM from ground whole body extracts of lake sturgeon sampled at regular intervals during TFM exposure.

Because a target of TFM is the mitochondria of the cells, in which TFM interferes with ATP production by uncoupling oxidative phosphorylation (Niblett and Ballantyne, 1976; Birceanu et al, 2011), measures of the physiological impact of TFM on the glycogen reserves and lactate were also quantified to characterize the sub-lethal effects of TFM on lake sturgeon.

Methods and Materials

Experimental animals and setup

Fingerling lake sturgeon (*Acipenser fulvescens*) (N=64) were provided courtesy of L. O'Connor, Sea Lamprey Control Centre, Fisheries and Oceans Canada (Sault Ste. Marie, ON). The lake sturgeon were shipped to Wilfrid Laurier University in 10 L bags placed in 40 L coolers containing well-oxygenated water, and then held in a 700 L Living Stream (Frigid Units, Inc., Akron, OH) receiving aerated well water (pH ~ 8.0; titratable alkalinity ~ 200 mg $CaCO_3$ 1^{-1}; hardness ~ 450 mg $CaCO_3$ 1^{-1}; temperature ~ 10-13°C; Birceanu et al., 2009) in a flow-through manner under a 12 h light : 12 h dark photoperiod. The fish were fed daily to satiation with commercially available, frozen blood worms (San Francisco Bay Brand, Inc., Newark, CA) that were immediately thawed in approximately 500 mL of well water, and added to the holding tank during feeding.

Prior to TFM exposure, the lake sturgeon were fasted for 72 h to minimize the effects that ammonia excretion and defecation could have on water quality. All experiments followed

Canadian Council on Animal Care guidelines, and were approved by the Wilfrid Laurier University Animal Care Committee.

Experimental protocol

The fingerling lake sturgeon were exposed to 3-trifluoromethyl-4-nitrophenol (TFM) prepared from powdered TFM (product no. N27802; Sigma Aldrich Chemical Company, St. Louis, MO) after being dissolved in deionized water. Precision TFM standards, provided courtesy of the Sea Lamprey Control Centre, were used to measure TFM exposure concentrations in the water. Unless otherwise noted, all chemical reagents and enzymes were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO).

Fingerling lake sturgeon exposure to the 12 h LC50 TFM of larval sea lamprey

Fingerling sturgeon (N=64) were transferred in groups of 4 into 5 L, covered, rectangular containers supplied with aerated well water under flow through conditions, and left overnight in order to acclimate to their surroundings. The following morning, water flow was cut-off to each chamber, and sufficient TFM was added to each container to yield a TFM concentration of 5.4 mg L^{-1}, which is equivalent to the 12 h LC50 of TFM to larval sea lamprey in Wilfrid Laurier University well water (5.4mg L^{-1}; Birceanu et al., 2009). Fish were exposed to this concentration of TFM, rather than concentrations used to treat larval sea lamprey infested streams in the field (1.2-1.3 times the LC100; Bills et al., 2003; McDonald and Kolar, 2007) because higher concentrations could have resulted in death and compromised efforts to quantify the uptake and elimination kinetics of TFM by the lake sturgeon.

Subsets (N=8 per group) of the fingerling sturgeon were exposed to TFM for 3, 6, 9 and 12 h, or permitted to recover from a 12 h TFM exposure for 3, 6 and 24 h (N = 64 total) in static, well aerated well water. Two additional subsets of 8 control animals that were not exposed to

TFM, were sampled at the beginning and end of experiments. At each sample time, length (93 mm ± 9.5), weight (3 g ± 1.05) and time of death (due to TFM or sampling) were recorded followed by collection of tissues (brain, liver, and carcass).

To confirm that the target water TFM concentrations had been established, or that TFM had been totally rinsed from the containers during the post-TFM recovery period, TFM was quantified in water samples that were collected at 0.5 h of TFM exposure and after 0.5 h of recovery in TFM-free water. Measurements were made on water samples without modification using a Molecular Devices Spectamax 190 plate spectrophotometer at 395 nm using Standard Operating Procedures provided by the Sea Lamprey Control Center, Sault Ste. Marie (Instrument Operating Procedure, IOP012.3).

Tissue collection procedure

Tissue collection procedures were completed as described in Chapter 2 for rainbow trout. Briefly, 0.5 g L^{-1} tricaine methanesulfonate (Syndel, Port Aberni, B.C.), buffered with 1 g L^{-1} sodium bicarbonate was used to anesthetize the fingerling sturgeon which were then removed from the anesthetic bath, blotted dry, followed by determinations of mass and length. The brain was collected by slicing laterally through the head, just above the eyes to expose the brain, which was then removed from the brain case using a pair of fine tweezers, and then placed in a chilled 0.5 mL centrifuge tube and snap-frozen using liquid nitrogen. The liver was collected by making another lateral incision posterior to the gills, followed by a longitudinal incision down the midline of the fish to expose the organ. The remaining carcass (mainly muscle, minus viscera) was then snap-frozen in liquid nitrogen for quantification of TFM and muscle metabolites. The brain, liver and carcass were stored at -80°C until analyzed.

Tissue processing and metabolite analysis

Due to the limited amount of brain tissue we were able to collect, analysis was restricted to determining phosphocreatine and lactate concentrations, two sensitive indicators of increased reliance on anaerobic metabolism (Hochachka 1992). Similarly, liver was analyzed for glycogen and lactate, to determine if the fish mobilized glycogen during the TFM exposure period. The tissue processing techniques were adapted from protocols used by Wang et al. (1994), Wilkie et al. (2001) and Birceanu et al. (2009), and completed exactly as described in Chapter 2.

Statistical analysis

All data in the Results are presented as the mean + SEM. Differences in tissue metabolite and energy stores were analyzed using one-way analysis of variance (ANOVA), and when significant variation was observed, differences between the means were quantified using the Tukey-Kramer post hoc test at the 0.05 level. When the assumptions for ANOVA were not satisfied, a nonparametric ANOVA followed by Dunn's test by ranks was used where applicable. All statistical analyses were done using SigmaPlot 11 (Systat Software, San Jose, California).

To determine the half-life of TFM, concentrations of TFM in live animals was plotted in a scatter plot and linear regression used to calculate the line of best fit. This was used to produce the rate constant (k) for TFM elimination using the following expression ($k = -2.303 \times m$) (Voet et al., 2006). The elimination half life ($T_{1/2}$) was equal to $T_{1/2} = 0.693 / k$.

Results

TFM and TFM-glucuronide concentration in fingerling lake sturgeon muscle tissue

There were no mortalities observed during the TFM exposure, but one mortality occurred during the TFM depuration period. The average TFM exposed to the fingerling lake sturgeon was 6.2 mg L^{-1}.

High performance liquid chromatography (HPLC) revealed a retention time for TFM of 5.9 +/- 0.6 minutes in fingerling lake sturgeon carcass, with no evidence of interfering peaks (Figure 3-1). The percent recovery of TFM consistently remained 60%, based on the analysis of control ground carcass samples spiked with 104 ng mL^{-1} TFM (20.8 ng g^{-1} tissue fortification level) (Hubert et al., 2001).

The TFM concentration in fingerling lake sturgeon muscle increased to 10 nmol g^{-1} wet tissue after 6 h of TFM exposure, but dropped to less than half this value by 9 h, before approaching 10 nmol g^{-1} wet tissue again after 12 h (Figure 3-2). Concentrations remained significantly elevated at this level for the remainder of the TFM exposure period, until 6 h of post-exposure in TFM-free water. TFM-glucuronide increased markedly as time of exposure increased and exceeded the concentration of TFM by 2.5 fold at 9 h of exposure (Figure 3-2). However, there was a marked, significant decline in TFM-glucuronide between 9 and 12 h, during which time TFM concentrations increased and exceeded TFM-glucuronide by 3-fold. During the post-exposure period, concentrations of TFM-glucuronide decreased 80% by 3 h of exposure, and by 6 h of recovery all of the TFM-glucuronide had been completely cleared from the body along with all traces of TFM.

Energy stores and metabolite concentrations in fingerling lake sturgeon brain tissue

Exposure of fingerling lake sturgeon to TFM had no statistically significant effect on brain PCr concentration. However, following 3 h of post-TFM recovery, concentrations of PCr increased 100% compared to controls and remained near 10 nmol g^{-1} wet tissue concentrations for the remainder of the recovery period (Figure 3-3). In contrast, concentrations of lactate did not significantly differ from control values throughout TFM exposure or the post-exposure recovery period (Figure 3-4).

Concentrations of glycogen and lactate in fingerling lake sturgeon liver tissue

Liver glycogen stores were highly variable, and did not significantly change throughout the TFM exposure and the post-TFM recovery periods (Figure 3-5A). Similarly, lactate concentrations did not significantly increase relative to the control values of 2 μmol g^{-1} wet tissue. Notably, there was an 80% decrease in liver lactate concentration at 24 h of the post-exposure recovery period (Figure 3-5B).

Discussion

Fingerling lake sturgeon use glucuronidation to biotransform TFM

The present study demonstrates that sturgeon equal to or less than 100 mm have the ability to biotransform TFM via the process of glucuronidation during prolonged (12 h) exposure to TFM. Exposing the sturgeon to the 12 h LC50 of larval sea lamprey, rather than the LC100, which is closer to concentrations used in field experiments, was meant to ensure that a large portion of fish survived exposure to the lampricide until sampling. All of the fish survived, suggesting that this group of fish were more tolerant to TFM than anticipated. However, in a preliminary experiment in which 10 fish were exposed to the LC100 for larval sea lamprey, all the fish died within a few hours. Thus, sensitivity of the sturgeon to TFM approached that of larval sea lamprey, as had been reported previously (Boogaard et al., 2003).

Earlier studies suggested that members of the sturgeon family only expressed low levels of the enzyme UDP-glucuronyl transferase in their livers (Singer and Ballantyne, 2004). However, more recent work on the white sturgeon (*Acipenser transmontanus*) suggests that glucuronidation, along with biotransformation by sulfation, takes on added importance when the fish are exposed to xenobiotics, including phenolic compounds similar to TFM (TenBrook et al., 2005). Thus, the present results indicate that the greater susceptibility of fingerling sturgeon to TFM when the fish are equal to or less than 100 mm is not due to an inability to biotransform TFM to TFM-glucuronide, at least when exposed to concentrations at or near the 12 h LC50 of TFM of larval sea lamprey in the same water.

It should be pointed out that while there was significant TFM-glucuronide accumulation by 9 h, the levels of this TFM conjugate dropped markedly by 12 h, and were accompanied by a simultaneous rise in TFM in the body (Figure 3-2). A possible explanation for this observation

was that glucuronidation capacity was reduced due to limitations in ATP supply later in the exposure period due to TFM's uncoupling effects on oxidative phosphorylation (Niblett and Ballantyne, 1976; Birceanu et al., 2011). Indeed, there were significant amounts of parent TFM in the body of the lake sturgeon throughout the entire TFM exposure (Figure 3-2). The glucuronidation process requires adequate ATP and glucose or glycogen (via glucose-1-phosphate formation) stores to synthesize the main substrate of the glucuronidation, UDP-glucuronic acid via the UDP-glucose dehydrogenase enzyme (Ritter, 2000; Evdokimova et al., 2001):

$$\text{UDP-glucose} + 2\text{NAD}^{+} \rightarrow \text{UDP-glucuronic acid} + 2\text{NADH} + 2\text{H}^{+}$$

Because the formation of UDP-glucose requires UTP, which is generated when UDP combines with ATP (Voet et al., 2006), glucuronidation is an energy consuming process that depends upon an adequate supply of ATP. If the TFM restricts ATP supply, it is possible that the sudden drop in TFM-glucuronide observed at 12 h of the exposure period was due to limitations in ATP supply leading to limited UTP, UDP-glucose and ultimately reduced UDP-glucuronic acid supply, which would have limited the ability of the lake sturgeon to glucuronidate TFM. However, accurate measurements of ATP, UTP, UDP-glucose and UDP-glucuronic acid are required to test this hypothesis.

Limitations in glucuronidation capacity could also be a reflection of the small body size of the sturgeon used in this study, which averaged 93 mm and 3 g. There are several factors that may have contributed to this increased susceptibility such as a higher uptake rate of TFM due to a higher metabolic rate. By taking in TFM at a more rapid pace, fingerling lake sturgeon may be unable to detoxify the lampricide efficiently. Due to their smaller size and earlier life stage,

fingerling lake sturgeon may also have a less developed liver which could hinder their ability to detoxify TFM following prolonged exposure to the lampricide.

Despite similar sensitivities to TFM, the present study clearly indicates that fingerling lake sturgeon have a capacity much higher than larval sea lamprey to utilize glucuronidation to detoxify TFM and eliminate it via the urine and/or gastrointestinal tract after being placed in TFM-free water. The half-life elimination rate for TFM in lake sturgeon was approximately 2.5 h (Figure 3-6), which was about 3-4 fold faster than observed in larval sea lamprey (Figure 2-7B). However, the capacity of the lake sturgeon to use glucuronidation was only about 50% of that observed in rainbow trout (Figure 2-7A). Since larger sturgeon (>100mm) are more tolerant of TFM, it is possible that the $T_{1/2}$ for TFM elimination decreases as they grow and develop. Studies are therefore needed to determine if the glucuronidation capacity of the lake sturgeon increases with age, and to determine if this explains the apparent increases in sturgeon tolerance to TFM with size.

Effects of TFM on energy stores in the sturgeon

There was little evidence that PCr or lactate homeostasis was altered in the lake sturgeon brain during exposure to TFM. Thus, TFM did not likely adversely affect ATP supply to this critical organ during exposure. Unfortunately, attempts to measure brain glycogen concentrations were unsuccessful due to the small size of the brains (≤ 9 mg). However, in several studies on larval sea lamprey, decreases in brain glycogen concentration due to TFM were accompanied by simultaneous decreases in brain phosphocreatine concentration and elevated lactate concentration, which is consistent with a greater reliance on anaerobic metabolism (glycogen, high energy phosphagens) to make up for shortfalls in oxidative ATP production in the mitochondria due to TFM (Birceanu et al., 2009, 2014; Clifford et al., 2012).

In contrast, there was a non-significant trend towards declining glycogen concentrations in the liver, as TFM exposure continued up to 9 h. This suggests that the fish may have been unable to provide adequate amounts of glucose to other parts of the body, so glycogen may have been mobilized to make up the deficit as the liver is an important organ for maintaining glucose homeostasis (Shanghavi and Weber, 1999; Moyes and Schulte, 2006). Furthermore, Lankford et al. (2005) reported that chronically stressed green sturgeon experienced a 50% decrease in liver glycogen levels similar to the fingerlings exposed to TFM in the present study.

Smaller lake sturgeon (<100 mm) might also be more sensitive to TFM because they have lower mass specific energy stores. I am not aware of any studies relating body mass to energy reserves in lake sturgeon, but Ferguson et al. (1993) reported that ATP, PCr and glycogen reserves all scaled in a positive fashion with body mass in rainbow trout. Due to their smaller size, sturgeon fingerlings may have smaller mass specific energy reservoirs, helping to explain their reduced capacity to detoxify TFM.

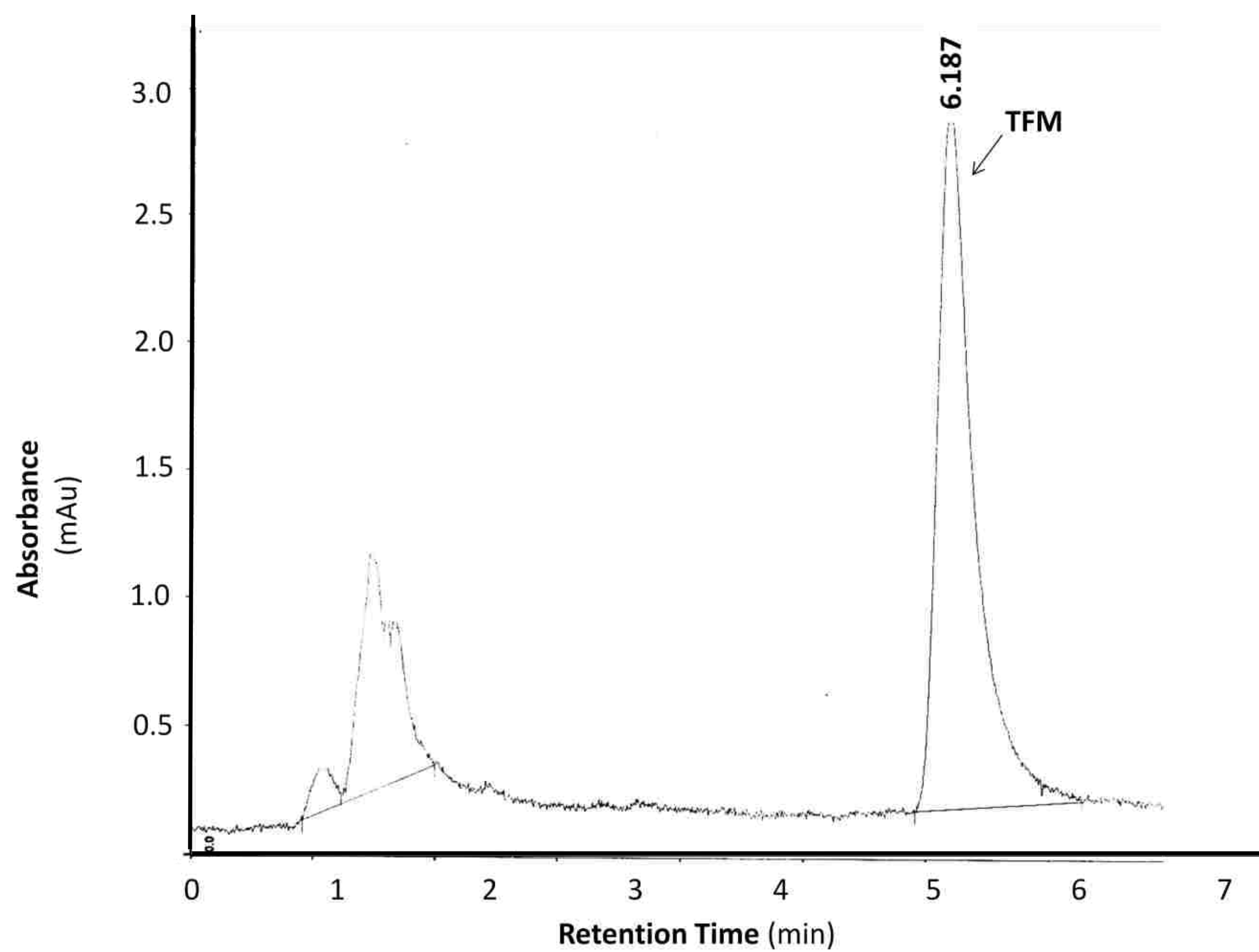
Implications for TFM application in the field

In the wild, fingerling lake sturgeon could be exposed to much higher concentrations of TFM than those used in the present study, which were based on the 12 h LC50 of TFM to larval sea lamprey. Thus, mortalities might be expected to be higher in sturgeon less than 100 mm in length. Small sturgeon could also have proportionally lower energy reserves, which could potentially compromise TFM survival. It is apparent that fingerling lake sturgeon sensitivity is directly related to the size of the fish (Boogaard et al., 2003) with escalating resistance after 100 mm in lengths. Therefore, it would be prudent to consider the size and life stage of lake sturgeon in waters being treated with TFM prior to lampricide application. In situations where vulnerable lake sturgeon are present, TFM applications could be timed to avoid exposing fingerling lake

sturgeon to the lampricide when they are at their most vulnerable (less than 100 mm in length), which is generally within 3 months of hatching (Boogaard et al., 2003). Finally, future studies should be done to determine if fingerling lake sturgeon are able to withstand low doses of TFM for prolonged periods (e.g. 24 h). Under such conditions, the lake sturgeon's capacity to glucuronidate TFM would result in the maintenance of relatively stable steady-state levels of TFM in the fish that are below the toxic threshold for the lampricide. Because larval sea lamprey are unable to detoxify TFM using glucuronidation, more prolonged exposure to lower concentrations of TFM would still result in lethal build-ups of the lampricide in the body, but the lampricide application period would need to be more prolonged.

Figure 3-1 – Quantification of TFM in fingerling lake sturgeon carcass using HPLC.

Representative HPLC chromatogram displaying TFM peak retention time and shape in fingerling lake sturgeon exposed to a measured TFM concentration of 6.2 ± mg L^{-1} and which is near the LC50 of TFM in larval sea lamprey.



6.187
TFM
Absorbance (mAu)
3.0
2.5
2.0
1.5
1.0
0.5
0
1
2
3
4
5
6
7
Retention Time (min)

Figure 3-2 – TFM and TFM-glucuronide accumulation in carcasses of fingerling lake sturgeon.

Concentrations of TFM and TFM-glucuronide in the carcass of resting fingerling lake sturgeon exposed to the 12 h LC50 of TFM of larval sea lamprey, and following their recovery in TFM-free water. Darkly shaded bars denote concentrations of TFM, and lightly shaded bars concentrations of TFM-glucuronide. Data are presented as the mean +1 SEM. Bars sharing a similar letter are not significantly different from one another ($P<0.05$). Lowercase letters refer to TFM concentrations while uppercase letters refer to TFM-glucuronide. N = 8 for all sample groups.

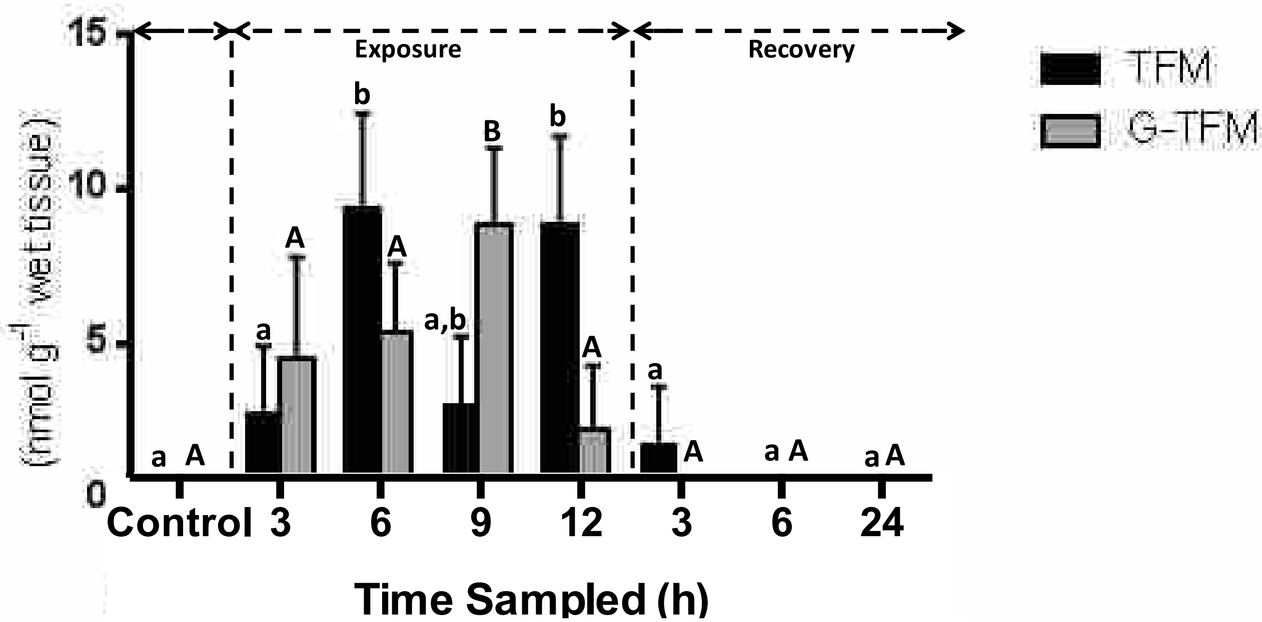
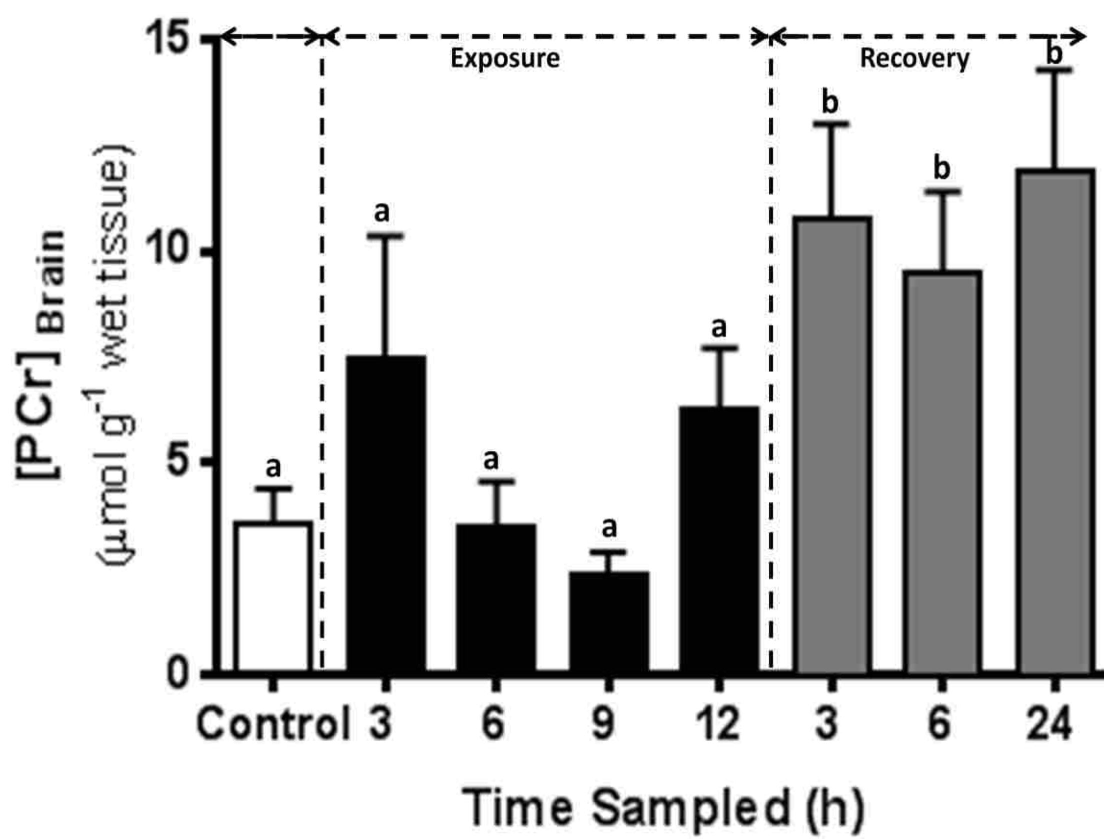

Carcass TFM and TFM-glucuronide
(nmol g^{-1} wet tissue)
Exposure
Recovery
TFM
G-TFM
Control
3
6
9
12
3
6
24
Time Sampled (h)

Figure 3-3 – Effects of TFM on brain PCr concentration in fingerling lake sturgeon.

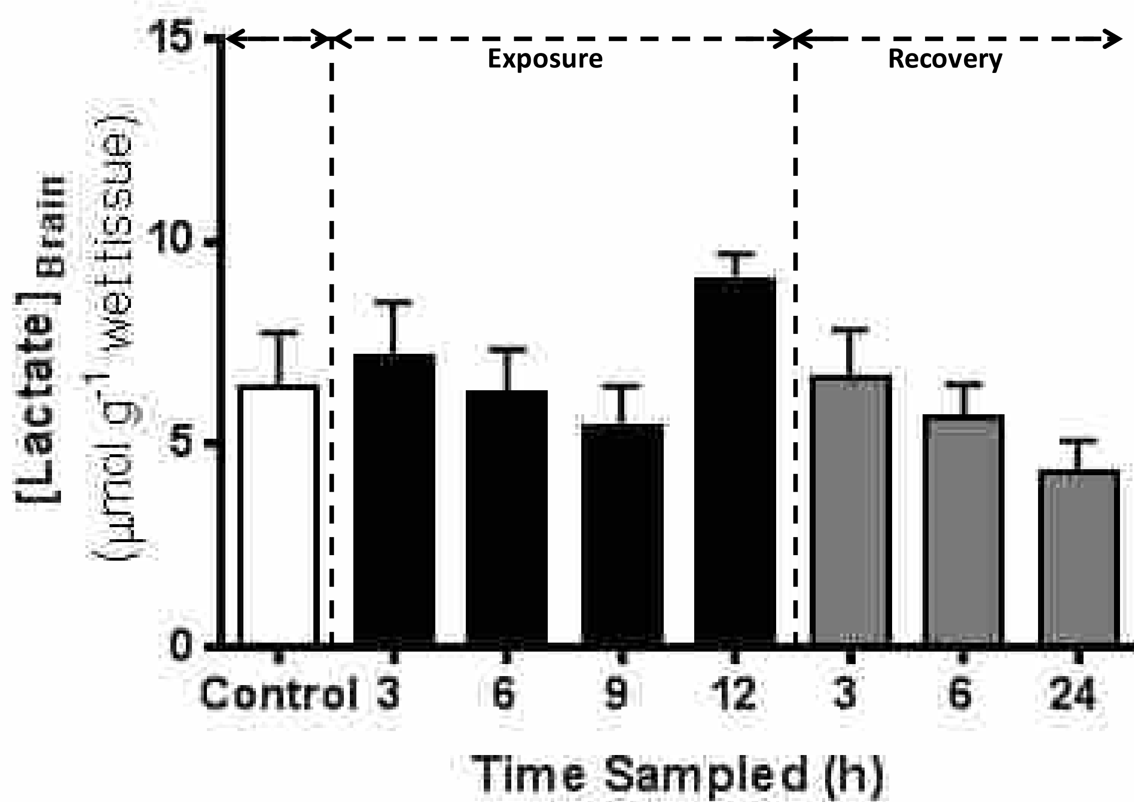
Concentration of phosphocreatine (PCr) in the brain of resting fingerling lake sturgeon exposed to the 12 h LC50 of TFM (5.4mg L^{-1}), and during recovery in TFM-free water for up to 24 h. Data are expressed as the mean + 1 SEM. Bars sharing the same letter are not significantly different ($P<0.05$). N=8 for all sample groups.



Exposure
Recovery
[PCr] Brain
(µmol g-1 wet tissue)
15
10
5
0
a
a
a
a
a
b
b
b
Control 3
6
9
12
3
6
24
Time Sampled (h)

Figure 3-4 – Effects of TFM on brain lactate concentration in fingerling lake sturgeon.

Concentration of lactate in the brain of resting fingerling lake sturgeon exposed to the 12 h LC50 of TFM for larval sea lamprey for 12 h (5.4mg L^{-1}), and during recovery in TFM-free water for 24 h. Data are expressed as the mean + 1 SEM. Bars sharing the same letter are not significantly different ($P<0.05$). $N=8$ for all sample groups.

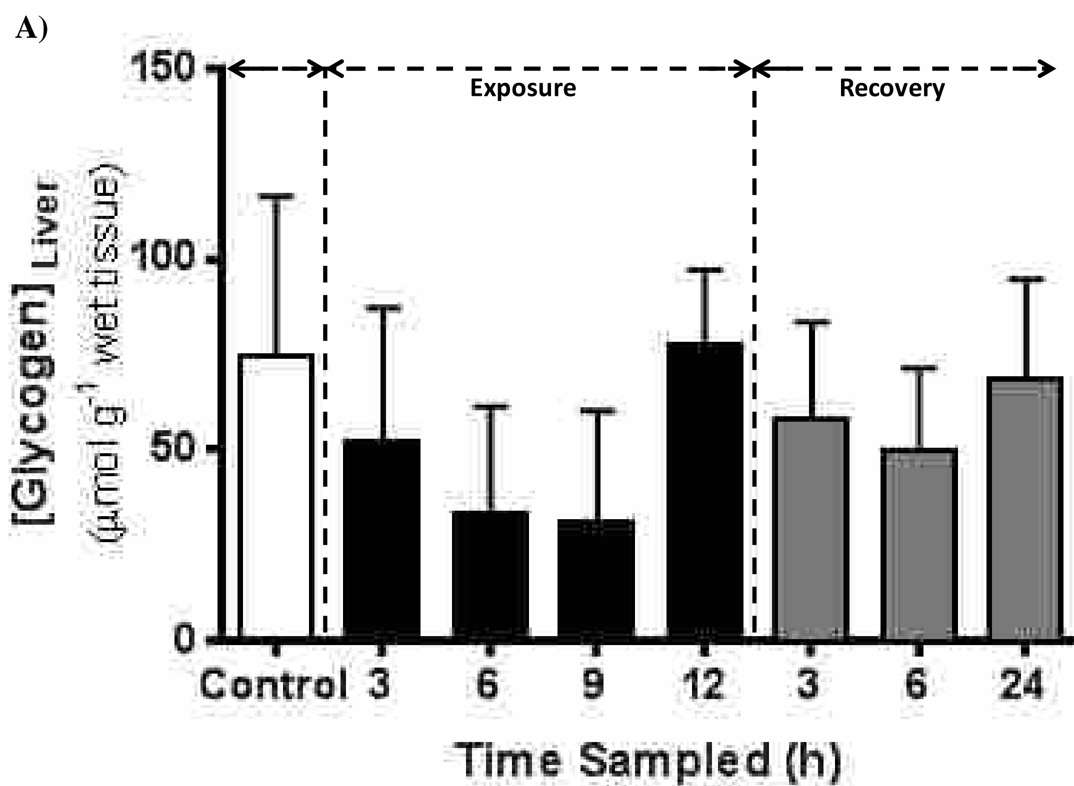


15
10
5
0
Exposure
Recovery
[Lactate] Brain
(µmol g-1 wet tissue)
Control 3
6
9
12
3
6
24
Time Sampled (h)

Figure 3-5 – Effects of TFM on liver glycogen and lactate concentration in fingerling lake sturgeon.

Concentration of (A) glycogen and (B) lactate in liver tissue of resting fingerling lake sturgeon exposed to the TFM 12 h LC50 of larval sea lamprey for 12 h (5.4mg L^{-1}), and during recovery in TFM-free water for up to 24 h. Data are presented as the mean + 1 SEM. Bars sharing the same letter are not significantly different ($P<0.05$). N = 8 for all sample groups.

A)



B)

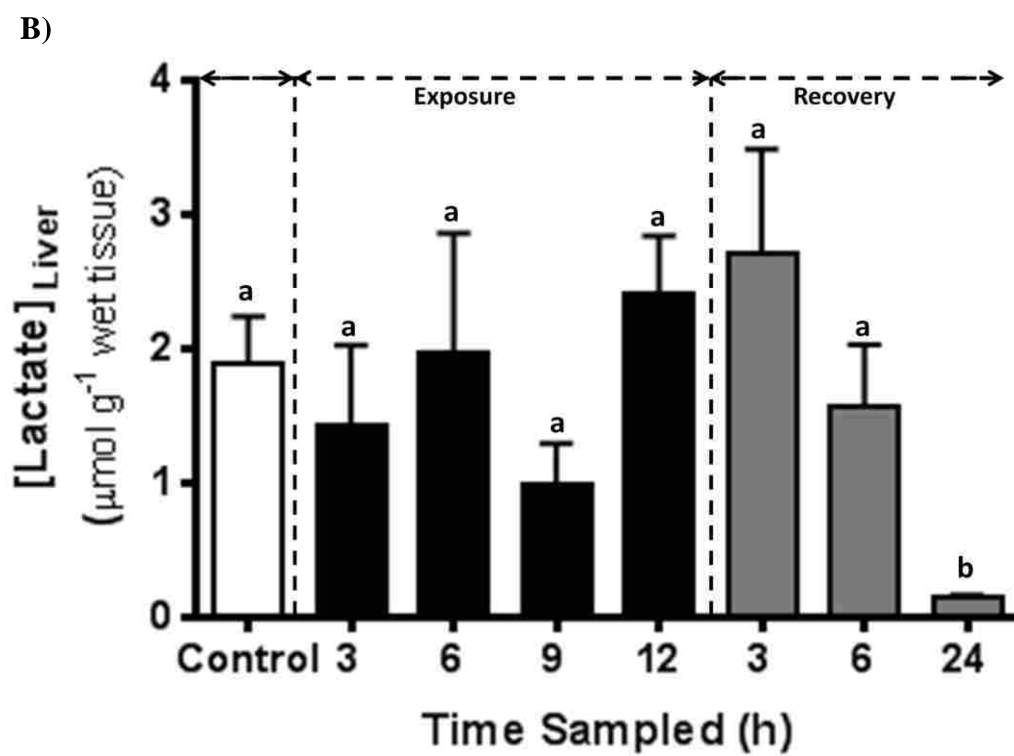
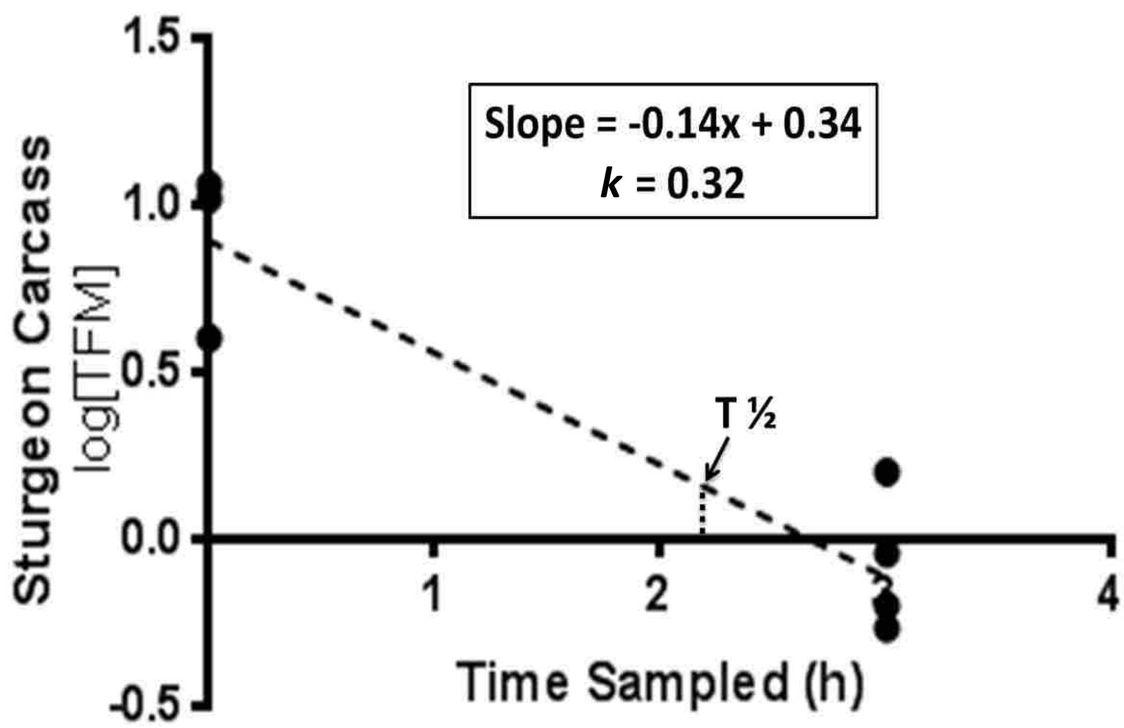


Figure 3-6 – Half-life of TFM in fingerling lake sturgeon carcass

Semi-log plot denoting the changes in the concentration of TFM in the carcass of fingerling lake sturgeon following re-introduction into TFM-free water. Fingerling lake sturgeon had cleared TFM from the body by 3 h of recovery. Linear regression was used to calculate the line of best fit, from which the rate constant (*k*) for TFM elimination was calculated using the following expression (k = -2.303 x *m*) (Voet et al., 2006). The elimination half life (T ½ equals 0.693 / *k* .


Slope = -0.14x + 0.34
k = 0.32
Sturgeon Carcass log[TFM]
1.5
1.0
0.5
0.0
-0.5
T ½
1
2
4
Time Sampled (h)

CHAPTER FOUR

An Integrative Approach to Understanding the Non-Target Effects of TFM in Rainbow Trout (*Oncorhynchus mykiss*) and Lake Sturgeon (*Acipenser fulvescens*)

An integrative model to explain TFM uptake and elimination by sea lamprey, rainbow trout and lake sturgeon

The lampricide, 3-trifluoromethyl-4-nitrophenol (TFM), exerts its toxic effects in rainbow trout and likely other non-target organisms by interfering with mitochondrial ATP production (Applegate et al., 1961; Birceanu et al., 2009). Due to its ability to act as a protonophore, TFM is thought to break down the proton gradient between the intermembrane space and the mitochondrial matrix. This gradient is established by protein complexes I, III and IV of the electron transport chain, which pump protons from the mitochondrial matrix into the intermembrane space, which establishes and maintains this proton-motive forces across the inner mitochondrial membrane (Birceanu et al., 2011). As a result, fewer protons pass through ATP synthase, resulting in a reduction in ATP synthesis (Figure 1-3) (Niblett and Ballantyne, 1976; Birceanu et al., 2011). The consequence of lower ATP production rates is a greater reliance on anaerobic respiration characterized by a decrease in tissue glycogen stores and an increase in lactic acid as a byproduct.

While the uncoupling effects of TFM on oxidative phosphorylation and therefore ATP supply are likely similar between different species (Birceanu et al., 2011, 2014), there appears to be wide variation in the ability of different organisms to detoxify TFM as demonstrated by Kane et al. (1993) and my own thesis. Glucuronidation is a major pathway utilized to detoxify TFM and eliminate it from the body via the kidneys or gastrointestinal tract (Lech and Statham, 1975; Hubert et al., 2001; Vue et al., 2002). Glucuronidation is mediated by the enzyme UDP-glucuronyl transferase (UGT), which biotransforms TFM to TFM-glucuronide by adding a glucuronic acid functional group to the molecule. In its glucuronidated form, TFM is more hydrophilic and easier to excrete (Lech and Costrini, 1972; Lech and Statham, 1975; Kane et al.,

1994). Lamprey are thought to have a low capacity to perform glucuronidation which is believed to explain their greater susceptibility to TFM (Lech and Statham, 1975; Kane et al., 1994). The present study supports this conclusion, but it also suggests that sea lamprey may have a limited ability to form TFM-glucuronide, which was detected in trace levels in their tissues, but at levels much lower than measured in the rainbow trout or the lake sturgeon. This limited ability to biotransform TFM is not sufficient, however, to allow the lamprey to eliminate TFM from its body when exposed to high external TFM concentrations during lampricide treatments. Under such conditions, TFM likely enters the body down water-to-blood TFM gradients by passive diffusion, and the only means available to excrete TFM is if the gradient is reversed, such as would occur if the animals were transferred to TFM-free water or if there were an increase in water pH (Figure 4-1).

An increase in water pH would alter the speciation of TFM leading to more ionized TFM compared to the more lipophilic un-ionized (phenolic) TFM (Hunn and Allen, 1974; McDonald and Kolar, 2008). Because TFM is likely taken up in its un-ionized state, an increase in pH would therefore lower rates of TFM accumulation, and decrease the effectiveness of lampricide treatments if not corrected by corresponding increases in TFM application rates. It is these dynamics that can complicate TFM applications, which is why it is critical that sea lamprey control personnel require an integrative knowledge of not only the toxicology of TFM, but an understanding of water geochemistry, limnology and ecology.

Changes in water pH may occur over the course of a few hours due to high rates of photosynthesis by algae and macrophytes (aquatic plants) which may occur when abundant nutrients and/or sunlight are available. As the plants and algae consume CO_2 during

photosynthesis, the removal of this weak acid causes the pH of the environment to rise. With a higher pH, TFM becomes less toxic due to more of the lampricide being present in its ionized and less lipophilic form (Figure 4-2). As discussed by Hunn and Allen (1974), TFM is a compound whose movement across the gills is influenced by the pH of the water to which the fish is exposed (Figure 4-1). This likely explains why the amounts of TFM needed to eradicate larval sea lamprey increases with water pH. Future experiments should use the HPLC methods described here and other approaches to further determine how TFM uptake, distribution and elimination by lamprey are influenced by such changes in environmental pH.

As expected, rainbow trout were capable of glucuronidation to a much greater degree than larval sea lamprey, eliminating TFM from the body almost immediately following exposure with minimal effects on energy stores in the body. The more rapid elimination of TFM from the body of trout was likely a combination of passive diffusion of un-ionized TFM across the gills down favorable blood-water gradients and the clearance of glucuronidated TFM via the gut and/or urine. As a freshwater fish, the trout have very high urine flow rates (Edwards and Marshall, 2013), which would augment TFM-glucuronide clearance during and following TFM exposure.

Unlike the rainbow trout, there has been speculation that sturgeon have a limited ability to detoxify phenolic compounds using glucuronidation (Singer and Ballantyne, 2004), which may explain the greater vulnerability of lake sturgeon to TFM, particularly those less than 100 mm in length (Boogaard, 2003; McDonald and Kolar, 2007). At this life stage, lake sturgeon have a 12-h LC50 for TFM that is comparable to that of larval sea lamprey (Boogaard et al., 2003). The present thesis, however, suggests that this greater vulnerability to TFM in juvenile

lake sturgeon is not mainly the result of a low capacity to glucuronidate TFM because substantial amounts of TFM-glucuronide were detected in the muscle of lake sturgeon during TFM exposure. The decrease in TFM-glucuronide at 12 h, however, could imply that other factors such as energy (ATP) supply limited their ability to use glucuronidation to detoxify TFM in the later stages of exposure. Indeed, glucuronidation requires adequate ATP and glucose or glycogen (via glucose-1-phosphate formation) stores in order to synthesize the main substrate of glucuronidation, UDP-glucuronic acid, via the UDP-glucose dehydrogenase enzyme (Ritter, 2000; Evdokimova et al., 2001).

How to modify TFM application regimens to protect lake sturgeon but still suppress sea lamprey populations

Despite using similar mechanisms of TFM detoxification, the lake sturgeon, at least those less than 100 mm, does not exhibit the same hardiness to TFM as the rainbow trout. This suggests additional factors are involved in the susceptibility of the fish to TFM such as body size, life stage and/or glycogen supply. However, in the present study, the lake sturgeon were exposed to a TFM concentration near the LC50 for larval sea lamprey, which is less than they might encounter in the wild during an actual TFM treatment (1.2 to 1.3 times the LC100; McDonald and Kolar, 2007). Thus, further changes to TFM application procedures should be considered to minimize the risk of TFM exposure to lake sturgeon if they are less than 100 mm in size. One possibility to consider might be to considering lowering the concentrations of TFM to which sea lamprey are exposed, but extending the exposure time (e.g. to 24 h) in waters containing lake sturgeon. Because the sturgeon are able to detoxify TFM via glucuronidation, exposure to lower concentrations of TFM would prevent internal TFM concentrations from rising to levels that would overwhelm their capacity to biotransform TFM. This would be

analogous to the administration of medication to humans or animals, in which great care is taken to ensure that the blood concentrations of the drug that result do not exceed the body's capacity to detoxify the agent. However, TFM would gradually build-up in the body of the sea lamprey, and eventually reach fatal concentrations due to their inability to form appreciable amounts of TFM-glucuronide. With such an approach, sensitive non-target species such as lake sturgeon would be protected from the adverse effects of TFM, while treatments would still effectively control sea lamprey populations.

Conclusions

This thesis used an integrative approach to demonstrate how TFM is handled by sea lamprey and non-target rainbow trout and lake sturgeon. Although TFM builds up quickly in the body, both rainbow trout and lake sturgeon showed a greater capacity to detoxify the lampricide as opposed to sea lamprey. These observations support the premise that these non-target fish species would readily recover from TFM exposure in the field. By learning more about how detoxification of TFM affects the vulnerability of non-target fish species to TFM, my thesis has provided the GLFC with a better understanding of why specific fish species are susceptible to this lampricide.

Figure 4-1 – Schematic diagram of the uptake, handling and elimination by TFM in non-target fish species

The main target of TFM is the mitochondria of cells, where the TFM interferes with oxidative phosphorylation. TFM enters the fish by crossing the gills in its lipophilic, un-ionized state past the gills where it acts as a protonophore in the mitochondria to interfere with the formation of ATP. In its ionized form, TFM is less lipophilic and tends not to passively diffuse back across the gills to the water. In the body, TFM is detoxified by combining TFM with glucuronic acid in a reaction catalyzed by UDP-glucuronyltransferase. The more water soluble TFM-glucuronide is then eliminated via the kidneys or gastrointestinal tract.

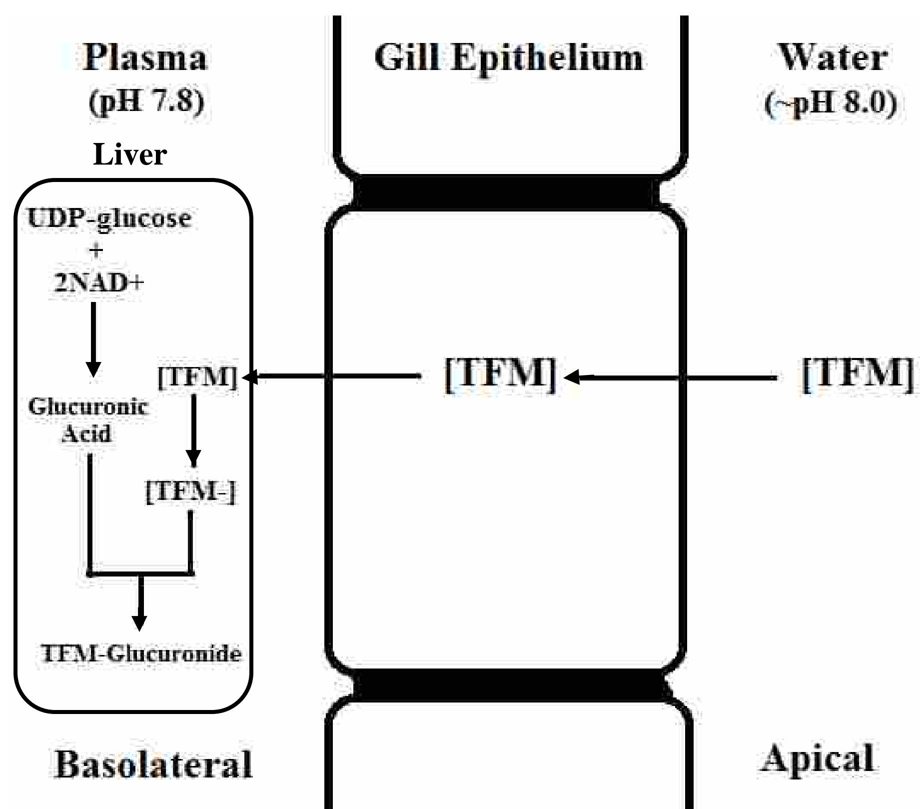
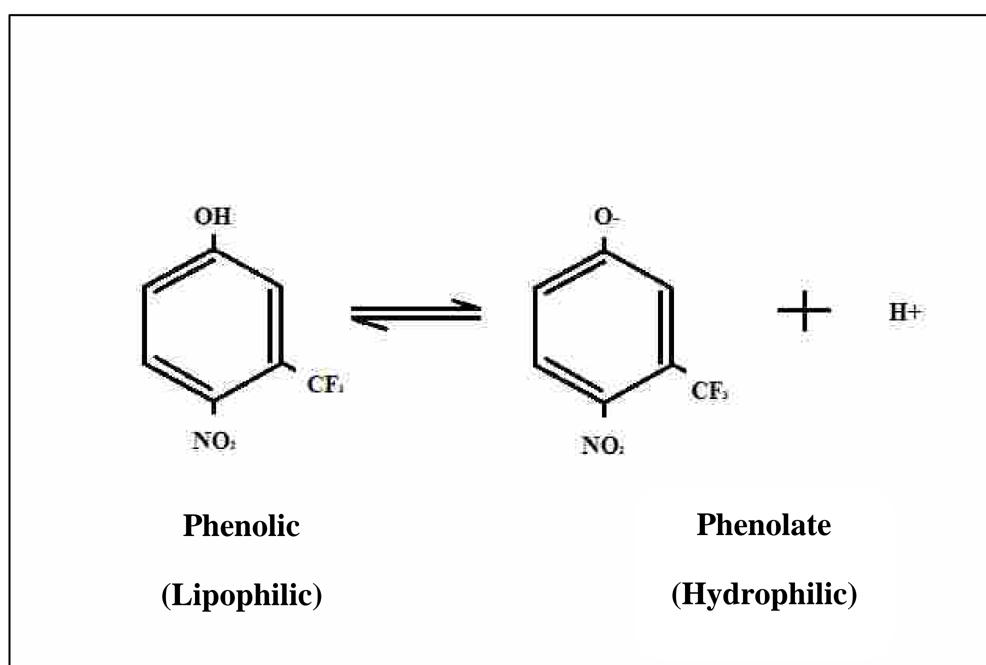

Plasma
(pH 7.8)
Gill Epithelium
Water
(~pH 8.0)
Liver
UDP-glucose
+
2NAD+
Glucuronic
Acid
[TFM]
[TFM-]
TFM-Glucuronide
[TFM]
[TFM]
Basolateral
Apical

Figure 4-2 – Schematic diagram demonstrating effect of pH on TFM speciation, pKa = 6.07

The un-ionized form of TFM on the left is more lipophilic and more prominent than the ionized form in basic conditions. In blood, with a pH of 7.8, over 98% of TFM will be in its less diffusible, ionized form.


OH
CF3
NO2
O-
CF3
NO2
H+
Phenolic
(Lipophilic)
Phenolate
(Hydrophilic)

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