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Distribution and Stability of the Lampricide 3-Trifluoromethyl-4-Nitrophenol (TFM) in Non-Target Rainbow Trout (Oncorhynchus mykiss) and White Sucker (Catostomus commersonii)

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Distribution and Stability of the Lampricide 3-Trifluoromethyl-4-Nitrophenol (TFM) in Non-Target Rainbow Trout (*Oncorhynchus mykiss*) and White Sucker (*Catostomus commersonii*)

by

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Abstract

The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) is applied to tributaries of the Great Lakes to control invasive sea lampreys (*Petromyzon marinus*). Although TFM is selectively toxic to larval sea lampreys, non-target mortality can occur during lampricide treatments. It is important to know whether or not TFM played a role in the death of these fishes, and the most direct means to do this is using forensic science principles. The objectives of this study were to: (i) determine the acute toxicity of TFM to the non-target fishes, rainbow trout *(Oncorhynchus mykiss)* and white sucker *(Catostomus commersonii),* and how the lampricide is distributed in the blood, liver and white muscle of these fishes, (ii) establish concentrations in the blood and tissues that could cause death to non-target fishes, and (iii) ascertain the most appropriate methods of blood and tissue sample storage and preservation for the investigations of non-target mortality. TFM concentrations and relative amounts of TFM-metabolites were measured in the blood and tissues of the non-target fish using LC-MS/MS following exposure to their 9-h LC_{25} for 6 h. Data showed that rainbow trout had a 12-h LC_{50} of 13.3 mg l^{-1} compared to a value of 15.0 mg l^{-1} for white sucker and that TFM concentrations for both species were by far the greatest in the liver, which is well supplied with blood and also equipped with organic anion transporters which transport xenobiotics such as ionized TFM into hepatocytes. At lethal exposure concentrations, a “spill-over” effect was observed characterized by TFM concentrations that exceeded the detoxification capacity of the liver, leading to significantly increased concentrations in the blood and muscle, compared to those

measured at sub-lethal exposure concentrations. The testing of various storage methods on the stability of TFM and its metabolites in blood and tissues showed that while liver accumulated the greatest amount of TFM, it was also prone to large changes in both parent TFM and its metabolites at sub-optimal storage conditions for lengths of time greater than 1 h. Concentrations of TFM were most stable in muscle, likely due to its relative lack of detoxification enzymes and isolation from the microbes of the gastrointestinal tract. The present study demonstrates that, if possible, liver, white muscle, and whole blood should be collected from non-target fishes following unexplained mortality and although quick freezing using liquid N_2 or dry ice and longer-term storage at temperatures lower than -20˚C is optimal, keeping samples on ice or at room temperature for no longer than 1 h can yield lampricide measurements of reliable quality.

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Chapter 1: General Introduction

History of Sea lamprey (Petromyzon marinus) in the Great Lakes

The sea lamprey (*Petromyzon marinus*) is a jawless fish that spends the first 3-7 years of its life burrowed in the sediment of streams and rivers as filter-feeding larvae before metamorphosing into juvenile parasitic lampreys, which feed on the blood of teleost fish (Beamish and Potter, 1975; Potter, 1980; Youson, 2003). Metamorphosis involves physiological changes such as the development of eyes, a buccal funnel with teeth and a rasping tongue, and the re-organization of gills and internal organs (Youson, 1980). After metamorphosis the juvenile sea lamprey migrates downstream to larger bodies of water such as the ocean or the Great Lakes, in the case of anadromous or landlocked populations, respectively, where they begin their juvenile blood feeding parasitic stage (Beamish and Potter, 1975; Youson, 1980; Farmer et al., 1975). After 12-20 months in the parasitic phase, during which a single lamprey may kill up to 18 kg of lake trout (*Salvelinus nemaychus;* Swink, 2003), the adult sea lamprey then migrates back to freshwater streams to spawn and then die (Applegate, 1950; Beamish and Potter, 1975; Halliday, 1991).

Although sea lampreys are not native to the Great Lakes there is evidence of their presence in Lake Ontario in the 1800's (Radforth, 1944; Scott and Crossman, 1947; Lark, 1973). The invasion of Lake Erie is thought to have occurred in the 1920's following modifications to the Welland Canal, which allowed the lampreys to by-pass the natural barrier provided by Niagara Falls (Radforth, 1944; Christie, 1974). The invasion into the remaining Upper Great Lakes in the early 20th century, in combination with pollution and overfishing, lead to the decline of native teleost

fish populations such as the lake trout (Smith and Tibbles, 1980), triggering the collapse of commercial and recreational fisheries (Lawrie, 1970; Christie, 1974).

In 1946, the Great Lakes Sea Lamprey Committee was founded as a joint effort between the Canadian and American governments to develop measures to control sea lamprey populations in the Great Lakes (Lawrie, 1970; McDonald and Kolar, 2007). Initial control efforts, including electrical and physical barriers, were generally successful in preventing the upstream migration of spawning adult sea lamprey, but this also caused harm to non-target fish populations by interfering with their movements (Smith et al., 1974; Smith and Tibbles, 1980).

Lampricides

In the 1950s, after testing over 6,000 different chemicals, 3-trifluoromethyl-4-nitrophenol (TFM; Figure 1-1) was discovered to selectively target larval sea lampreys at concentrations that were much less toxic to most non-target organisms (Applegate et al., 1961; Hubert, 2003). Later it was discovered that when mixed with TFM, the molluscide niclosamide (aka Bayluscide; at 0.5-2% of the TFM concentration) significantly reduced the amount of TFM needed to treat a river or stream without reducing the selectivity to larval sea lamprey (Howell, 1964; Associate Committee on Scientific Criteria for Environmental Quality, 1985). Currently, an integrated sea lamprey control program overseen by the Great Lakes Fisheries Commission (GLFC) includes traps and dams to prevent adults from migrating upstream to spawn (Lavis et al., 2003), the release of sterile males to compete with fertile males (Twohey et al., 2003), and applications of the

lampricides to streams and rivers containing populations of larval sea lampreys (Applegate, 1961; McDonald and Kolar, 2007).

Many non-target fish species including rainbow trout *(Oncorhynchus mykiss)*, and white sucker *(Catostomus commersonii)* are found in the tributaries of the Great Lakes and can be exposed to the lampricides (Scott and Crossman, 1974; National Research Council of Canada, 1985). This poses a challenge for the sea lamprey control program to minimize negative effects on non-target organisms, while also minimizing residual lamprey populations that survive a lampricide treatment (Boogaard et al., 2003). The concentrations of TFM and niclosamide that are applied during sea lamprey control treatments are based on previous reports of the Minimum Lethal Concentration (MLC) required to kill 99.9% of larval sea lamprey during a 9 hour exposure (9-h $LC_{99.9}$; Brege et al., 2003), but are typically 1.2 to 1.5 times the MLC to reduce the risk of larval lampreys surviving the treatment (Bills et al., 2003; McDonald and Kolar, 2007). In the past, the concentration of TFM was adjusted to 1.0 times MLC when sensitive, non-target fish species such as larval lake sturgeon (*Acipenser fulvescens*) are present in the stream (Boogaard et al., 2003; McDonald and Kolar, 2007). But this practice was discontinued due to unacceptably high numbers of residual larval lamprey that survived treatment, which subsequently metamorphosed and migrated downstream to the Great Lakes (Sutton et al., 2003; Dobiesz et al., 2018)

TFM Toxicity and Metabolism

TFM exerts its toxic effects by uncoupling oxidative phosphorylation in mitochondria (Niblett and Ballantyne, 1976; Birceanu et al., 2011). A study by

Birceanu et al. (2011) concluded that TFM acts as a protonophore, effectively causing a decrease in the proton motive force, which is used to drive ATP production via ATP synthase in the inner mitochondrial membrane (Figure 1-2). The work built on previous studies suggesting that TFM caused a mismatch between adenosine triphosphate (ATP) supply and demand after it was found that glycogen and phosphocreatine levels in the brain and other tissues of larval sea lamprey dropped significantly after exposure to TFM (Wilkie et al., 2007; Birceanu et al., 2009). These effects are caused by the decline in ATP production that leads to greater reliance on anaerobic metabolism, which uses up energy molecule reserves like phosphocreatine and glycogen, possibly leading to neural starvation and death (Birceanu et al., 2009; Clifford et al., 2012; Henry et al., 2014).

Compared to non-target fish, larval sea lampreys are more sensitive to TFM because of their lower capacity to detoxify the lampricide using glucuronidation and sulfation (Lech and Statham, 1975; Kane et al. 1994). The hepatic enzymes uridine diphosphate glucuronsyltransferase (UDPGT) and phenol sulfotransferase (PST) catalyze the attachment of glucuronic acid (Lech and Costrini, 1972; Lech et al., 1973; Clark et al., 1991; Kane et al., 1994) and sulfonate (James, 1987; Bussy et al., 2017a), respectively to the lipophilic TFM, creating a more hydrophilic metabolite that is easier to excrete (Figure 1-3A, 1-3B). In contrast to sea lamprey, most non-target fishes have higher UDPGT activity and greater UDPGT affinity for TFM (Kane et al. 1994). Although the major metabolite of TFM detoxification is known to be the glucuronide conjugate (Lech and Costrini 1972; Lech et al. 1973; Kane et al. 1994),

the sulfate conjugate has also recently been identified in rainbow trout by Bussy et al. 2017b.

The toxicity of TFM is inversely related to water pH (LeMaire, 1961; Bills et al. 1988), and with a dissociation constant (pK_a) of approximately 6.07 (Smith et al. 1960; Hubert, 2003), its toxicity is roughly 5 times greater at pH 7 than at pH 8 (McDonald and Kolar, 2007). As water pH goes down, a greater proportion of TFM is in its lipid-soluble, un-ionized form making it easier for the TFM to diffuse down its concentration gradient across the gill epithelium, resulting in greater toxicity due to higher rates of accumulation (Figure 1-1; Hunn and Allen, 1974; Mcdonald and Kolar, 2007; Hlina, 2015).

Non-Target Mortality

The toxic effects of TFM occur when an animal's capacity to eliminate the lampricide is overwhelmed (Lech and Statham, 1975). Although most non-target fish can tolerate TFM concentrations up to five times higher than is required to kill larval sea lamprey (Boogaard et al., 2003), mortality can occur during lamprey control treatments after sudden, drastic drops in water pH (Bills et al. 2003) due to aerobic respiration by aquatic vegetation and algae, precipitation or agricultural runoff (Wetzel, 1983; Poudel et al., 2013). Accordingly, stream characteristics like water flow and pH are monitored during TFM treatments, and TFM application can be altered following changes in water pH. However, non-target mortality can also result from unrelated events such as contamination, disease, or natural environmental stressors such as high temperatures and/or oxygen deprivation due to high rates of plant respiration or the decomposition of decaying organic matter

following events such as algal blooms (Meyer and Barclay, 1990). In many cases, causes of non-target mortality following lampricide treatments can be determined by closely monitoring water chemistry and flow, but may go unnoticed due to time of day, location or weather conditions. When unexplained non-target fish mortality coincides with lampricide application, and the causes of the fish kill are unclear, forensic toxicology approaches could potentially be used to deduce the cause of death, but to date the information needed to conduct such investigations are lacking.

Current Investigation Protocols and Room for Improvement

Currently, there are three levels of impact that describe the severity of a non-target fish kill. The American Public Health Association (APHA) defines a minor kill as less than 100 fish found dead within 1.6 km, a moderate kill as 100-1000 fish found dead within 1.6 km, and a major kill as more than 1000 fish found dead within 1.6 km (as cited by The Great Lakes Fishery Commission, 2016). Investigations for minor fish kills include filling out collection forms for reporting a number of different water quality parameters and stream assessment characteristics, while in the case of a moderate or major fish kill when the cause of mortality is unexplained, an investigation must be carried out which will include the collection of water and biological samples for lampricide measurements. While the aim of the investigation is to determine the cause of the fish kill, gaps exist in how to collect, preserve and store samples during the pre-analytic phase (Butzbach, 2010) of the investigation that precedes sample processing and analysis. The overarching goals of my thesis were to (i) determine tissue concentrations of TFM that could cause death in non-target fishes, (ii) identify the key metabolites associated with TFM exposure, and

(iii) to determine the most appropriate methods of tissue collection, preservation and storage to be used in forensic investigations of unexplained fish kills during or following TFM applications.

The current protocols set in place by the U.S. Fish and Wildlife Service (FWS) and The Department of Fisheries and Oceans Canada (DFO) can be found in the Great Lakes Fishery Commission standard operating procedures (TOP:026.). Briefly, it includes the collection of blood, muscle and liver from 20 fish of each species of fish found dead and storage on ice or on dry ice if it is available, while samples are delivered to the Upper Midwest Environmental Sciences Centre (UMESC). While it is well known that for measurements of analytes in tissue samples, the best protocol is to keep tissues samples as cold as possible to prevent degradation and subsequent decomposition of analytes (Wang et al. 1994; Hubert et al. 2001; Butzbach, 2010), sea lamprey control agents in the field might not always have access to these resources. In cases where optimal storage is not possible, it is important to know how stable TFM and its metabolites will be in the tissues that are collected and how different storage conditions can affect the reliability of the measurements. After death, there are numerous biochemical processes that occur within tissues, which may have undesirable effects on the concentration of TFM and its metabolites. Enzymatic processes in tissues do not stop immediately after death, and enzymes responsible for metabolism of TFM and its metabolites could result in concentration changes (Butzbach, 2010). Further, all tissues contain different types and amounts of enzymes due to their specific roles in the bodies of the fish. For instance, liver is known to accumulate drugs due to its role in the detoxification of xenobiotics

(Anhalt et al. 1981; James, 1987; Clark et al. 1991; Skopp, 2010), therefore the presence of metabolic enzymes suggests this tissue will be more prone to decomposition than other tissues such as white muscle, leading to changes in concentration (Paterson, 1993; Skopp, 2004). Because TFM applications may take place in relatively remote locations, where sampling and storage conditions may be sub-optimal, it is important to know how different handling and storage conditions affect postmortem lampricide concentrations and to establish parameters about how long tissues are viable for relevant forensic toxicological analysis. Forensic science is defined as the use of scientific principles and techniques for the collection, examination, and analysis of evidence for legal investigations (Forensic science, 2018). Due to the potential legal implications of a large-scale fish kill, it is critical that tissue samples are handled and stored properly, and the chain of custody be followed in order to minimize the risk of unreliable measurements.

Thesis Objectives

My thesis will address the following objectives:

I. Determine the acute toxicity of TFM to the non-target fishes, rainbow trout and white sucker and determine the distribution of TFM and its metabolites in blood and tissues of these fish (Chapter 2).

II. Establish concentrations of TFM in blood and tissues that could cause death in rainbow trout and white sucker exposed to lethal concentrations (Chapter 2).

III. Ascertain what methods of blood and tissues sample preservation and storage are most appropriate for investigating incidents of non-target mortality in rainbow trout and white sucker (Chapter 3).

To determine the acute toxicity of TFM to rainbow trout and white sucker, I conducted acute toxicity experiments, which were then followed by a series of experiments in which both species were exposed to known concentrations of TFM, followed by quantification of how much TFM accumulated in the liver, muscle and blood of the fishes that survived the TFM exposure. Objective two was investigated by exposing both species to a range of sub-lethal and lethal concentrations of TFM followed by quantification of TFM in the liver, muscle and blood immediately following death or after 12 h of exposure. Lastly, objective three was determined by exposing both species to known concentrations of TFM and analyzing liver, muscle and blood samples for quantification of TFM following various storage and preservation methods for periods of one hour and one week.

Liver, muscle and blood samples of the fishes were analyzed at the Upper Midwest Environmental Sciences Centre (UMESC), in La Crosse, Wisconsin, U.S.A. for TFM concentrations, along with the relative amounts of the TFM metabolites, TFM-glucuronide and TFM-sulfate using LC-MS/MS.

Figure 1-1. Dissociation equilibrium of 3-trifluoromethyl-4-nitrophenol (TFM). TFM is a weak acid with a pK_a of 6.07. At a pH lower than 6.07, the equilibrium of TFM shifts towards the un-ionized phenolic form (left), while at a pH higher than 6.07, the equilibrium shifts towards the ionized, phenolate form (right; Hunn and Allen, 1974).

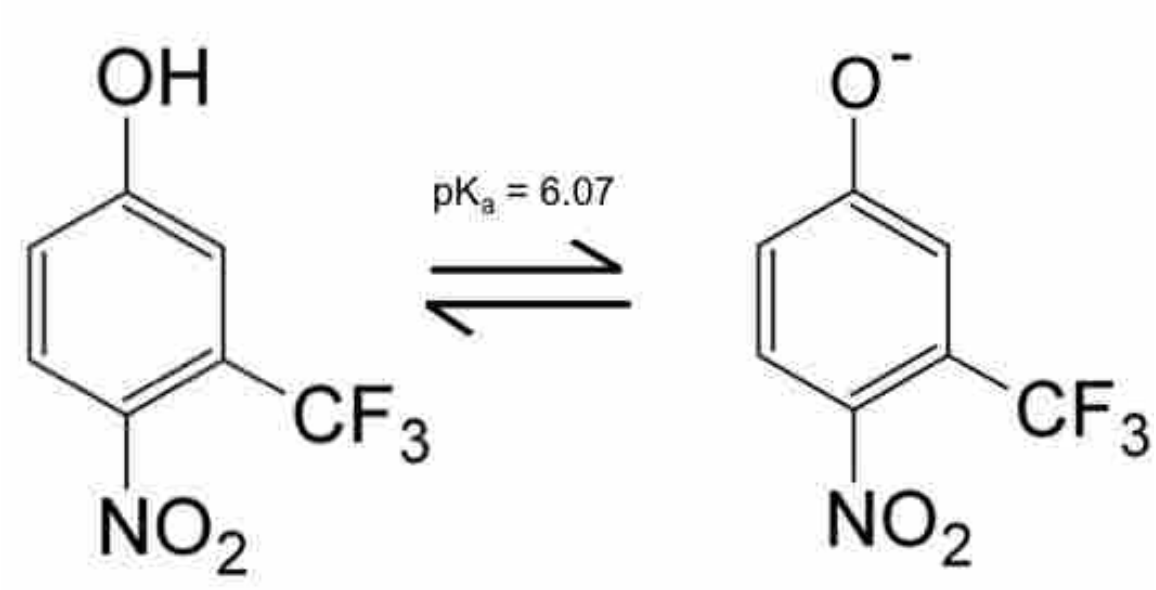

OH
pK$_a$ = 6.07
O$^-$
CF$_3$
NO$_2$
CF$_3$
NO$_2$

Figure 1-2. Mitochondrial oxidative phosphorylation. Simplified diagram of mitochondrial ATP production by oxidative phosphorylation. Protein complexes, I, III, and IV, located in the inner mitochondrial membrane, pump protons across the membrane using energy released from passing electrons through the electron transport chain (ETC). This creates a proton gradient (proton motive force) and allows protons to flow down the gradient, back into the matrix via ATP synthase, which harnesses the released energy by phosphorylating ADP into the high energy molecule ATP.

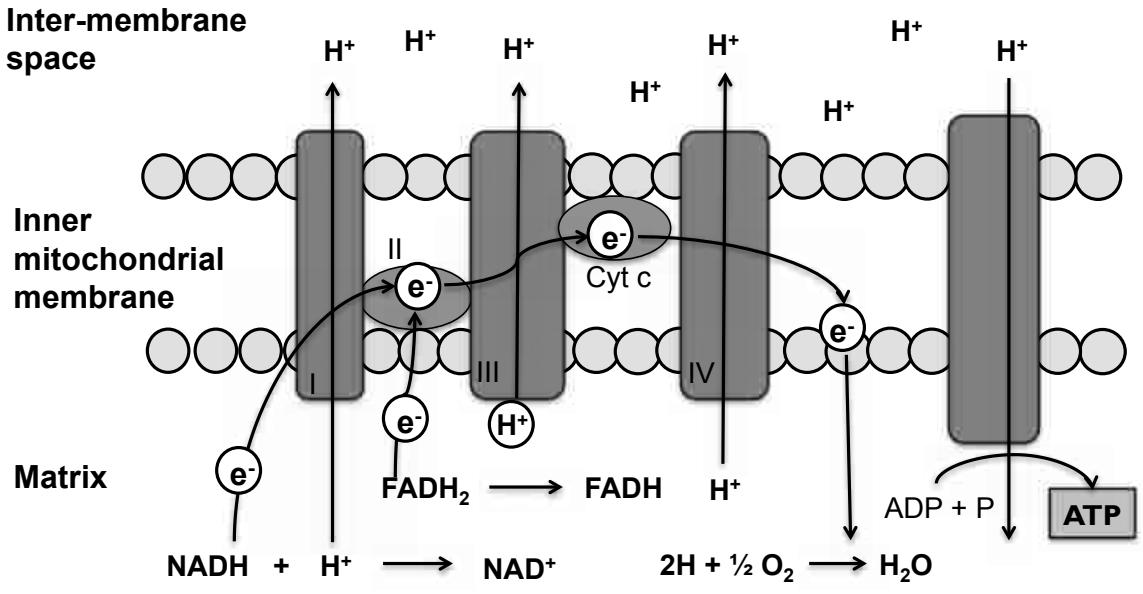
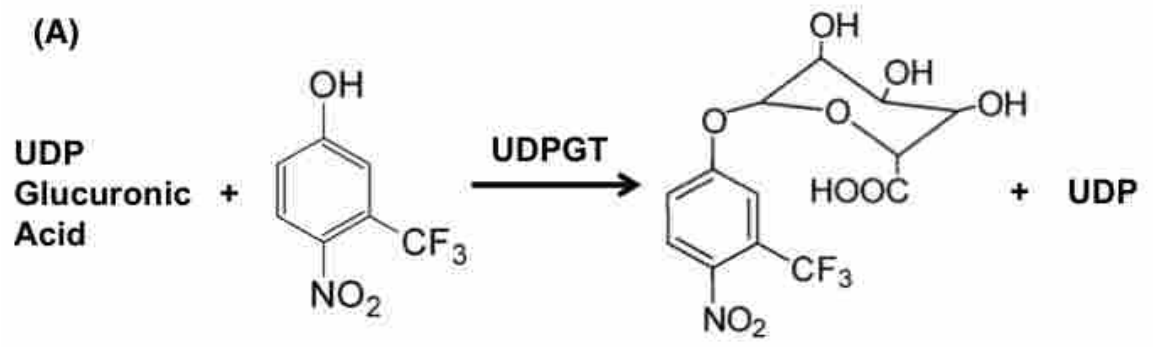

Inter-membrane space
Inner mitochondrial membrane
Matrix
H+
e-
Cyt c
II
I
III
IV
FADH2 → FADH
NADH + H+ → NAD+
2H + ½ O2 → H2O
ADP + P
ATP

Figure 1-3. Structure and detoxification of 3-trifluoromethyl-4-nitrophenol (TFM). Detoxification via (A) glucuronidation and (B) sulfation. Uridine diphosphate glucuronsyltransferase (UDPGT) catalyzes the addition of glucuronic acid from uridine diphosphate glucuronic acid (UDPGA) to TFM to form TFM-glucuronide (Clark et al. 1991). Phenol sulfotransferase (PST) catalyzes the addition of sulfate from adenosine 3'-phosphate 5'-phosphosulphate (PAPS) to TFM to form TFM-sulfate (Bussy et al. 2017b).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Glucuronic
Acid
+
OH
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NO2
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OH
OH
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O
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HOOC
CF3
NO2
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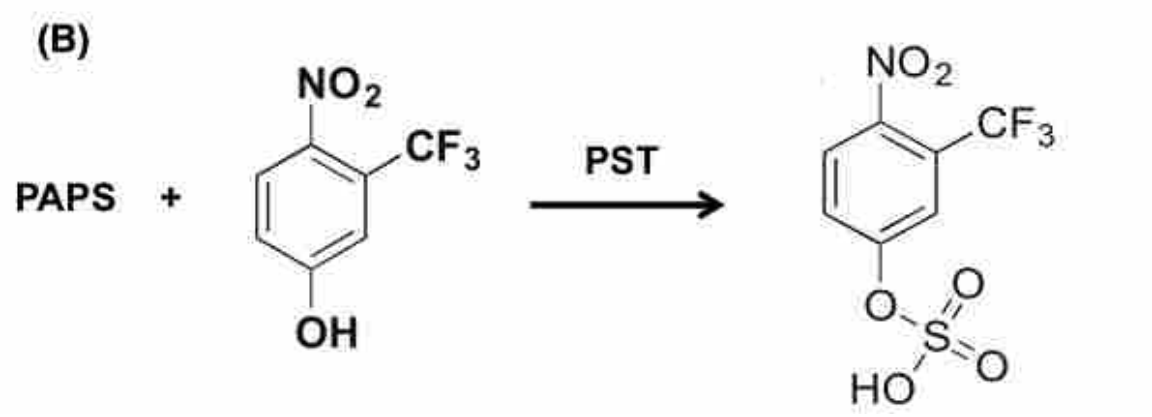
UDP


(B)
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OH
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NO2
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Chapter 2:

Toxicity, Distribution, and Detoxification of 3-Trifluoromethyl-4-Nitrophenol (TFM) in Two Non-Target Fish Species

Introduction

The piscicides 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide are applied to tributaries of the Great Lakes to control invasive and parasitic sea lampreys (*Petromyzon marinus;* Applegate et al., 1957; Applegate et al., 1961; Howell, 1964; Smith and Tibbles, 1980; Hubert, 2003; McDonald and Kolar, 2007). TFM selectively targets larval sea lampreys due to their lower capacity to detoxify the lampricide via phase two metabolic reactions including glucuronidation and sulfation, compared to non-target fishes (Lech, 1974; Lech and Statham, 1975; Kane et al., 1994; Bussy et al., 2017b). The hepatic enzymes uridine diphosphate glucuronsyltransferase (UDPGT) and phenol sulfotransferase (PST) facilitate the addition of glucuronic acid (Lech and Costrini, 1972; Lech et al., 1973; Kane et al., 1994) and sulfate (Bussy et al., 2017a), respectively, to the lipophilic TFM, making it more hydrophilic and easier to excrete via bile or urine. Yet, non-target mortality can occur if the bioavailablility of TFM increases as a result of changes in water pH or over-application of the lampricide (Boogaard et al., 2003; O'connor et al., 2017)

With a pKa of 6.07, the toxicity of TFM is inversely related to pH in both sea lamprey and non-target fishes, which increases TFM bioavailability by increasing the proportion of TFM that is present in its un-ionized (phenolic) form, compared to its ionized (phenolate) form (Hunn and Allen, 1974; McDonald and Kolar, 2007). In its un-ionized form, TFM is more lipophilic making it easier to be taken up via the gills, resulting in a greater rate of accumulation (Hunn and Allen, 1974; Hlina et al., 2017).

Although non-target fish can typically tolerate TFM concentrations 3-5 times higher than is required to kill larval sea lamprey (Boogaard et al., 2003), mortality can occur after sudden, decreases in water pH (Bills et al., 2003). This may occur as a result of precipitation, agricultural runoff, or and aerobic respiration by aquatic vegetation and algae (Wetzel, 1983; Poudel et al., 2013). For this reason, water pH and flows are continuously monitored during TFM treatments, and TFM application is adjusted in response to changes in water chemistry or pH. However, fish kills can also arise due to the release of contaminants into the waterways, disease, or natural environmental stressors related to high temperatures and/or oxygen deprivation resulting from high rates of plant respiration or the decomposition of decaying organic matter following events such as algal blooms (Meyer and Barclay, 1990). Because non-target mortality can arise from a variety of causes, there may be situations in which it is necessary to ascertain what, if any, contribution TFM may have made to the unexplained deaths of fishes.

The most direct means to ascertain if TFM contributed to unexpected incidences of mortality in fishes is to measure the concentrations of TFM and/or its metabolites in different tissues collected from the animals. To successfully employ this forensic toxicology approach, it will be important to develop reproducible and accurate methods of TFM and TFM-metabolite analysis, identify which tissues are the most reliable indices of TFM and/or TFM-metabolite concentration, and how best to collect, handle and preserve the tissues prior to analysis. It will also be important to identify appropriate model fish species that can be used for such studies.

Two fishes that could be exposed to TFM during lamprcide treatments are the rainbow trout (*Oncorhynchus mykiss*) and white sucker (*Catostomus commersonii*), which are both widespread in the Great Lakes basin (Scott and Crossman, 1973). The toxicity of TFM to rainbow trout is well defined (Lech and Costrini, 1972; Lech et al., 1973; Lech, 1974; Lech and Statham, 1975; Hunn and Allen, 1974; Birceanu et al., 2014), but less is known about how TFM affects white sucker. Both white sucker and rainbow trout non-target mortality have been recorded during and following TFM application (Smith and Tibbles, 1974; Rossi, 1999; Dahl and Mcdonald, 1980).

The goal of the present study was to characterize how TFM and its metabolites are distributed in different tissues of the rainbow trout and white sucker and to determine concentrations of TFM in the blood and tissues that could cause death to non-target fishes exposed to lethal concentrations. First, to determine the relative sensitivity of rainbow trout and white sucker to TFM, I conducted acute toxicity experiments comparing rainbow trout to white sucker. These experiments were followed by a series of experiments in which both species were exposed to known concentrations of TFM, followed by quantification of how much TFM accumulated in the liver, muscle and blood of the fishes. Rainbow trout were also exposed to a range of sub-lethal and lethal concentrations followed by quantification of TFM in the blood and tissues. The concentrations of TFM were measured using LC-MS/MS, along with the relative amounts of the TFM metabolites, TFM-glucuronide and TFM-sulfate.

Material and Methods

Experimental Animals and Holding

Rainbow trout were purchased from Rainbow Springs Trout Hatchery (Thamesford, Ontario) and white suckers were captured by seine netting from Canagagigue Creek, Elmira, Ontario. Prior to importing the white suckers into Laurier's fish holding facilities, the white suckers were treated for ectoparasites in a formalin bath (0.75% formalin; SOP I23 - Wilfrid Laurier Animal Care Committee). Both the trout and white sucker were held in separate 800 L holding tanks in Wilfrid Laurier's Centre for Cold Regions and Water Science, each tank receiving a mixture of reverse osmosis water and de-chlorinated, City of Waterloo tap water (pH ~ 8.0, alkalinity ~150 mg l^{-1} as $CaCO_3$, temperature ~15˚C). Rainbow trout were kept in a ~2000 L recirculating system, equipped with mechanical and UV filtration. The water supplied to white suckers was drained directly to waste to prevent mixing of water with that supplying other fish in the facility. Rainbow trout were fed with EWOS 3mm floating pellets and white suckers were fed a mixture of bloodworms and EWOS #1 micro crumble feed three times per week to satiation. Fish were held for at least two weeks but food was withheld for approximately 72 h before commencing experiments to minimize build-up of ammonia. Both groups of fish were held under a 12 h light and 12 h dark photoperiod. All fish husbandry and experiments were approved by the Wilfrid Laurier University Animal Care Committee and followed guidelines of the Canadian Council of Animal Care (CCAC).

Experimental Protocol

Series 1 - Determination of the Acute Toxicity of TFM to Rainbow Trout and White Sucker

The 12-h LC_{50}, the concentration of TFM required to kill 50% of the exposed animals over 12 hours, was determined in acute toxicity experiments. The TFM exposures were conducted in de-chlorinated tap water by exposing groups of rainbow trout (9.2 ± 0.3 g, 8.8 ± 0.1 cm) to a range of nominal TFM concentrations between 5 and 30 mg l^{-1}, while white sucker (22.1 ± 3.3 g, 10.6 ± 3.5 cm) were exposed to a range of TFM concentrations between 2 and 32 mg l^{-1}. Field formulation TFM (35% active ingredient dissolved in isopropanol; Clariant SFC GMBH WERK, Griesheim, Germany), provided courtesy of Fisheries and Oceans Canada (DFO), was used for all toxicity and TFM exposure experiments. The night before experiments, appropriate amounts of TFM were added to 12 L glass aquaria filled with aerated water. The TFM concentrations were verified immediately following TFM addition, and the next morning by spectrophotometry using a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, UK) at a wavelength of 395 nm following Standard Operating Procedures of the Department of Fisheries and Oceans, Sea Lamprey Control Centre, Sault Ste. Marie, Ontario (IOP: 012.4). Six trout or 5 white suckers were then placed into each aquarium. Rainbow trout were exposed to each test concentration in triplicate (N = 18 per concentration), but this was not possible for white sucker which comprised a single group of N = 5 fish at each test concentration. Water temperature in the aquaria was controlled by immersing them in a water bath, thermostatted to the appropriate

temperature using an in-line chiller (Coralife ¼ HP Aquarium Chiller, Franklin, WI) connected in series to a lower reservoir. Dissolved oxygen (DO; > 90 % saturation), temperature (rainbow trout = 17.3 ± 0.01˚C; white sucker = 11.7 ± 0.09˚C), pH (rainbow trout = 8.24 ± 0.01; white sucker = 8.15 ± 0.02) and alkalinity (rainbow trout = 231.4 ± 2.4 mg l^{-1} as $CaCO_3$; white sucker = 282.2 ± 6.8 mg l^{-1} as $CaCO_3$) were monitored throughout the toxicity trials.

Series 2 – Distribution of TFM and its Metabolites in Rainbow Trout and White Sucker

The goal of these experiments were to ascertain how TFM and its metabolites were distributed in different tissues of larger rainbow trout (N = 10, 199 ± 10 g, 24.8 ± 0.33 cm) and white sucker (N = 10, 99.1 ± 9.4 g, 19.49 ± 0.611 cm) following 6 h of exposure to their approximate 9-h LC_{25} of TFM, as determined for each species above. This value was chosen to ensure that the TFM concentration was sufficiently high to result in measurable accumulation of parent TFM and TFM-metabolites in different tissues, while minimizing the possibility of partial mortality during experiments. The 6 h exposure period also provided sufficient time for TFM accumulation. Birceanu et al. (2014) reported that in rainbow trout exposed to their 12-h LC_{50}, TFM concentration peaked after 3 – 6 h of exposure. Accordingly, an exposure period of 6 hours was chosen in the present study. It should be noted, however, that the goal of these experiments was not to determine toxicity, but to examine the distribution of TFM and its metabolites in a living fish. I therefore used the acute toxicity data from Series 1 as a guide to determine a suitable concentration (9-h LC_{25}) to which to expose the fish, without causing mortality over the 6 h exposure period. The experimental setup was identical for both species, with each

acclimated overnight to darkened, individual 4 L exposure chambers contained within a 200 L flow through system continuously receiving dechlorinated, city of Waterloo tap water at a flow rate of 0.5 liters per minute. Water chemistry and temperature differed slightly between the experiments (rainbow trout: water pH = 8.5 ± 0.002, T = 15.30 ± 0.05˚C, alkalinity = 255 mg l^{-1} as $CaCO_3$, DO > 90%; white sucker: water pH = 8.30 ± 0.02, T = 12.30 ± 0.06˚C, alkalinity = 339 mg l^{-1} as $CaCO_3$, DO > 90%). Prior to the addition of TFM, incoming water flow to the system was shut off to yield a closed re-circulating system in which a submersible pump was used to pump water from a lower reservoir (~ 70 L) to a head tank (~ 70 L), from which water drained into the individual exposure chambers of the fish. Based on the total volume of the system, sufficient TFM was added to achieve the desired TFM exposure. After 6 h of TFM exposure, all fish were euthanized with an overdose of tricaine methanesulfonate (MS222; 0.5 g l^{-1}, buffered with 1.0 g l^{-1} $NaHCO_3$) before collection of blood, liver and muscle which were snap-frozen in liquid N_2 and stored at -80˚C until processing for quantification of TFM, and characterization of the TFM metabolites, TFM-glucuronide and TFM-sulfate at the Upper Midwest Environmental Sciences Centre (UMESC), U.S. Geological Survey, La Crosse, Wisconsin, U.S.A. Whole blood was collected from the caudal vein/artery by "caudal puncture" using a sodium heparin coated syringe, with a sub-sample centrifuged at 10,000 x *g* for 3 minutes to separate plasma from the erythrocytes (red blood cells); all three fractions (red blood cells, plasma, whole blood) were snap-frozen and stored at -80˚C as described above for muscle and liver. Due to size constraints, only whole blood was collected from white suckers.

Series 3 - Dose-dependent Changes in the Distribution of TFM and its Metabolites in Rainbow Trout Tissues

To characterize how TFM concentrations in the tissues of rainbow trout varied with TFM dose, smaller rainbow trout (4.8 ± 0.2 g, 7.1 ± 0.1 cm) were exposed to a range of nominal TFM concentrations between 5 and 30 mg l^{-1} over 12 hours, which approximates a typical TFM treatment time. As described above, TFM was added to 12 L glass aquaria the night before the experiments and TFM concentrations were verified by spectrophotometry. The next morning, five trout were placed into each individual aquarium and the experiment was run in triplicate (N = 15 in total) at each concentration. The pH averaged 8.480 ± 0.004 and alkalinity averaged 255 mg l^{-1} $CaCO_3$ over the 12-h exposure. Tissues (blood, liver and muscle) were collected as described above, immediately following death or after 12 h of TFM exposure. Tissues were then stored at -80˚C until processing for quantification of TFM, and characterization of the TFM metabolites, TFM-glucuronide and TFM-sulfate.

Analytical Methods

Water TFM Measurements

Water TFM concentrations were measured spectrophotometrically using either a 96-well microplate spectrophotometer (Epoch 2, Biotek Instruments, USA) or a standard spectrophotometer (NovaSpec II), using 1.5 mL polystyrene cuvettes. Samples were measured at wavelength of 395 nm against precision standards as described above.

Liver, Muscle and Blood Sample Processing for LC-MS/MS

All blood and tissue samples were processed and analyzed at UMESC. Aliquots of tissue or blood were pre-weighed (50-100 mg) and transferred to a 2 ml polypropylene microcentrifuge tube, followed by the addition of 100 µl acetonitrile containing 1% formic acid to the tube. The contents were homogenized in a SPEX SamplePrep Geno/Grinder 2010 at 1200 strokes per minute for 90 seconds followed by the addition of 400 µL of the acetonitrile / 1% formic acid solution before vortexing the sample for 15 seconds. The samples were cooled at 4˚C for 20 minutes and then centrifuged for 10 minutes at 12000 x *g* using a Beckman Avanti 30 High Speed Compact centrifuge (Ramsay, Minnesota, U.S.A.), and the supernatant was transferred to Phree phospholipid removal 96-well plates (Phenomenex, Torrance, California, U.S.A.). The first aliquot was filtered through the Phree cartridge into a 2 ml reservoir well plate by centrifuging at 500 RCF for 5 minutes. The microcentrifuge tubes were then rinsed with 500 µl acetonitrile + 1% formic acid and then vortexed and centrifuged as described above. This aliquot was transferred to the Phree cartridge and then vacuum filtered through into the 2 ml reservoir well plate for 5 minutes before being centrifuged at 500 RCF for 10 minutes. The reservoir was then sealed and either immediately injected into the reversed phase liquid chromatography column (Phenomenex, Kinetex 1.7 µm Evo C18) interfaced with a quadrupole time of flight mass spectrometry system (Agilent Technologies, 1290 Infinity II LC and 6530 Accutate-Mass Q-TOF LC/MS system) for quantification of TFM and its metabolites. Unused samples were frozen at -80˚C for later analysis.

Quantitation of TFM and TFM-metabolites in Liver, Muscle and Blood Samples

The combination of liquid chromatography and mass spectrometry physically separates the analytes (TFM and its metabolites) and then accurately identifies the compounds using mass spectrometry. In the present study, an LC mobile phase with a gradient between solution A (900mL Epure, 100mL MeOH, 385mg ammonium acetate and 0.1% formic acid) and solution B (700mL MeOH and 300mL Isopropanol) was used for the separation of TFM and its metabolites through the liquid chromatography column over 4 minutes at a flow rate of 0.350 ml min^{-1}. The gradient was as follows (% of A: % of B; time in minutes): (0.0: 100; 0.0), (0.0: 100; 2.7), (80:20; 3.0) with column temperature set at 45 °C. The eluent was then pumped into the mass spectrometer source where it was broken down into ionized fragments and then accelerated through a vacuum by an electrical field for analysis based on the mass to charge ratio (*m/*z) of the ion fragment (Pitt, 2009). The time-of-flight analyzer measures the time it takes for the ion to reach the detector, which depends on the *m/z* ratio because heavier ions reach a lower velocity. The ion counting detector measures the signal from the ions and a chromatogram is produced with peaks areas relating to the intensity of signal from the ions, and thus the amount of ions in the sample (Guilhaus, 1995). By comparing the relative signal size, the mass/charge ratio vs time of flight against known TFM standards, the concentrations of parent compounds can be quantified. Due to the lack of standards for TFM metabolites TFM-glucuronide and TFM-sulfate, a standard curves could not be generated for these compounds, making it impossible to report

metabolite concentrations; therefore all measurements of metabolites were reported in relative peak areas as described by Bussy et al., (2017b).

Calculations and Statistical Analysis

Concentrations of TFM in tissues were determined by applying peak area counts of the sample to the standard curve equation produced by measuring the peak areas from standards of known concentration. A dilution factor taking into account the volume of the extraction solution and homogenized tissue was applied to the calculated concentration of the sample in ng ml^{-1}. The resulting concentration in ng g^{-1} was then converted into nmol g^{-1} TFM by dividing by the molecular weight of TFM (207.11 g mol^{-1}). Dilution factors were also applied to the peak areas of the metabolites TFM-glucuronide and TFM-sulfate. The extraction efficiency of TFM was calculated in different tissue matrices by spiking the different TFM-free tissues with a known amount of TFM (spike tissue) and performing the extraction as described above. Another sample was measured that had the same spike solution of know concentration but without the tissue (spike null). Extraction efficiency was then calculated using the equation below:

$$\text{Extraction Efficiency (\%)} = \frac{\text{Spike tissue (ng/g)}}{\text{Spike null (ng/g)}} \times 100\ \% \qquad (1)$$

A tissue specific extraction coefficient (EC; Table 2-1) was then applied to the raw TFM concentration measured in each sample in ng ml^{-1}, followed by the dilution factor (DF) then divided by the molecular weight of TFM to yield the true concentration of TFM in the tissue in nmol g^{-1} for muscle and liver or nmol ml^{-1} for blood via the following formula:

$$\text{Tissue [TFM]} = \frac{\text{Sample [TFM] X EC X DF}}{207.11} \quad (2)$$

Signal area measurements of TFM metabolites in all tissues were normalized to the highest signal area in liver samples and represented as a percentage.

Lethal concentrations (LC) of TFM were determined by probit analysis (Sprague, 1969) using R package 'ecotox' (Hlina, unpublished). Concentrations of TFM in tissues were analyzed using either a one-way analyses of variance (ANOVA) followed by a Tukey Honest Significant Difference post-hoc test when data were homoscedastic and normally distributed, or a modified students t-test. If these assumptions were not met (even after transformations; inverse, square root, Log_{10}), a Kruskal-Wallis rank sum test was performed followed by a Dunn's test of multiple comparisons was used. The level of significance was set at a P value ≤ 0.05. Statistical analysis and figures were produced using R version 3.4.2, RStudio version 1.1.383 (RStudio, 2016), and 'ggplot2' (Wickham, 2016). All data are presented as the mean ± 1 standard error of the mean (SEM).

Results

Series 1 - Determination of the Acute Toxicity of TFM

The 12-h LC_{50} to rainbow trout was of 13.3 mg l^{-1} (CI = 12.4 – 14.4; Figure 2-2A) and a 9-h LC_{25} of 13.6 mg l^{-1} (CI = 11.8 – 15.2). White sucker was slightly more tolerant to TFM with a 12-h LC_{50} of 15.0 mg l^{-1} (Figure 2-2B) and a 9-h LC_{25} of 18.9 mg l^{-1}. Due to no partial mortalities at 9 or 12 h, 95% confidence intervals could not be calculated for the white sucker acute toxicity test.

Series 2 - Distribution of TFM and its Metabolites in the Tissues of Rainbow Trout and White Sucker

TFM-glucuronide eluted from the LC column first at 1.32 min, followed by TFM-sulfate at 1.65 min and parent TFM at 2.04 minutes (Figure 2-1). Following exposure to respective concentrations of 12.9 ± 0.1 and 18.3 ± 0.1 mg l^{-1} TFM (~ their respective 9-h LC_{25}) for 6 h, the greatest TFM concentration was found in the liver for both rainbow trout and white sucker averaging at 67.5 ± 11.2 nmol g^{-1} and 210.5 ± 25.8 nmol g^{-1} wet weight, respectively (Figures 2-3A and 2-4A). Muscle contained much less TFM, however, averaging 3.8 ± 0.6 nmol g^{-1} wet weight in rainbow trout (Figure 2-3A) and 38.8 ± 5.4 nmol g^{-1} wet weight, in white sucker (Figure 2-4A). Interestingly, in the rainbow trout blood fractions, the concentration of TFM within red blood cells averaged 14.1 ± 1.6 nmol ml^{-1}, which was about 40 times more TFM than the plasma, which averaged 0.38 ± 0.1 nmol ml^{-1} ($P < 0.01$; Figure 2-3A). Notably, more TFM had accumulated in the whole blood of white

sucker compared to rainbow trout, in which the respective values were approximately 50.0 ± 5.6 nmol ml^{-1}, and 4.9 ± 0.5 nmol ml^{-1} (Figures 2-3A, 2-4A).

Like the parent compound, the relative amounts of TFM-glucuronide were highest in the liver for rainbow trout and white sucker, followed by whole blood and then muscle (Figures 2-3B, 2-4B). In contrast, relative TFM sulfate levels in whole blood were similar to those measured in liver for both species (Figures 2-2C, 2-3C). Interestingly, the relative amounts of TFM-glucuronide in rainbow trout were much higher in the plasma compared to red blood cells, opposite to the trend observed for parent TFM (Figure 2-3B), and TFM-sulfate was more or less evenly distributed between the plasma and red blood cells in rainbow trout (Figure 2-3C).

Series 3 - Dose-dependent Changes in the Distribution of TFM and its Metabolites in Rainbow Trout Tissues

The nominal TFM concentrations of 5, 10, 15, 20, 25 and 30 mg l^{-1} in the rainbow trout range finder were measured as 5.45, 11.34, 16.47, 21.89, 25.33 and 30.98 mg l^{-1}, respectively. At the three lowest concentrations of water TFM, liver TFM was more or less stable, fluctuating around 180 nmol g^{-1} wet weight. An abrupt, 70 % increase in liver TFM was then observed when the fish were exposed to 21.9 mg L^{-1} TFM, which began to cause partial mortality during the experiment, but did not result in a difference in liver TFM between fish that survived and fish that died. Tissue TFM then increased a further 70 % when it peaked at external TFM concentration of 25.3 mg L^{-1} (Figure 2-5A). White muscle exhibited a more gradual accumulation of TFM, which increased in a step-wise fashion before stabilizing near 17 nmol g^{-1} wet weight in fish that survived the higher exposure concentrations

(Figure 2-5B). Concentrations of TFM in muscle of fish that did not survive were significantly higher ($P < 0.01$), reaching about 28 nmol g^{-1} and plateauing near this concentration at the three highest exposure concentrations (Figure 2-5B). The accumulation of TFM in the whole blood matched the trend seen in the white muscle of fish exposed to TFM at the three lowest concentrations, during which the ratio of muscle:blood TFM was approximately 1:1. However, the increase from 16.5 to 21.8 mg L^{-1} TFM resulted in an abrupt 4-fold increase in whole blood TFM concentration of fish that did not survive, at which point it more or less stabilized between 70 and 90 nmol ml^{-1}, while blood concentrations of fish that survived 12 h remained unchanged from levels, 25-30 nmol ml^{-1}, found at water concentrations of 21.9 and 25.3 mg l^{-1} (Figure 2-5C).

The relative amounts of metabolites in the tissues of rainbow trout exposed to various TFM concentrations generally increased as exposure TFM increased, resulting in highest relative amounts at the middle exposure concentrations compared to the levels of parent TFM, which were greatest at the high exposure concentrations. Differences in metabolites in the blood and tissues of fish that survived and those that did not were transient and generally no different from each other (Figures 2-6, 2-7).

Discussion

Comparing the Toxicity of TFM to Two Non-Target Fish Species

The present study shows the 12h-h LC_{50} of rainbow trout in water with a pH of 8.24 and an alkalinity of 231 mg l^{-1} $CaCO_3$ to be 13.27 mg l^{-1}, which more-or-less matches predictions of the expected LC_{50}s for brown trout under similar water chemistries. The sensitivity of white suckers to TFM is still unclear, as some studies have shown them to be slightly more tolerant to TFM (Marking and Olson, 1975), but other suggest that they are slightly more sensitive to TFM, compared to rainbow trout (Applegate and King, 1962; Boogaard et al., 2003). The white suckers were exposed to a similar range of nominal TFM concentrations, and although this experiment shows that white suckers are slightly more tolerant to TFM, as seen by the higher 12-h LC_{50} 14.99 mg l^{-1}, compared to 13.27 mg l^{-1} for the trout, the difference in sensitivity between rainbow trout and white sucker to TFM could be even greater due to the slightly lower pH measured during the white sucker experiment. Lower water pH is known to increase the toxicity of TFM due to the increased uptake of the more lipophilic, un-ionized form of TFM (Applegate et al., 1961; Hunn and Allen, 1974; Howell et al., 1980; McDonald and Kolar, 2007; Hlina et al., 2017). Based on these observations, it seems likely that if the experiments were run at the same pH, as for rainbow trout, the TFM tolerance of white sucker could be even higher.

It should also be noted that a difference in temperature was measured between the two experiments. The rainbow trout were exposed to TFM at an

average temperature of 17.3˚C in the summer, and the white sucker experiment averaged a temperature of 11.7˚C in the winter. TFM is mainly taken up by passive diffusion across the gills, but warmer temperatures can lead to an increase in metabolic rate, leading to increased ventilation rates (Hunn and Allen, 1974; Moffit and Crawshaw, 1983), leading to increased rates of TFM uptake via the gills, and greater toxicity (Scholenfield et al., 2008; Patra et al., 2009; Hooper et al., 2013). It has been shown that warmer temperatures cause an increase in the rates of TFM uptake by sea lamprey (Hlina. 2015). However, Howe et al., (1994) determined that the toxicity of nitrophenols decrease as temperatures increase from 7 to 17˚C. In larval lampreys, Muhametsafina (2018) also noted much greater survival during TFM exposure in warmer (21°C) versus cooler water (5°C). Such observations could be due to temperature dependent increases in metabolism and excretion of TFM, which could ultimately lower toxicity as suggested for other toxicants (Noyes et al., 2009). A study by Patra et al. (2015) supports this hypothesis, based on reductions in phenol toxicity as temperature increased from 5 to 15˚C, but they also reported a subsequent increase in phenol toxicity as temperatures continued to increase to 25˚C.

Distribution of TFM in the Tissues of Non-target Fishes Exposed to Their Respective 9-h LC_{25}

To determine how TFM and its metabolites are distributed in the tissues of non-target fishes, rainbow trout and white sucker were exposed to their respective 9-h LC_{25}. For both species, by far the greatest TFM concentration was found in the liver, in which TFM was more than an order of magnitude grater than observed in

the muscle and blood of rainbow trout, as shown previously by Lech and Statham, (1975). In white sucker, the difference in TFM concentration was less pronounced, but still 4-5-fold greater in liver compared to muscle and whole blood. This was expected because the liver is the main site of detoxification of endogenous and xenobiotic compounds, with past studies demonstrating that TFM preferentially accumulates in the liver of non-target fishes (Lech, 1974; Lech and Statham, 1975; Clark et al., 1991; Vue et al., 2002; Hubert et al., 2005). As TFM diffuses across the gill epithelium into the blood of the fish, it enters circulation, from which it will reach the liver via hepatic arteries. The hepatic arteries, along with the hepatic portal vein, which delivers blood to the liver from the gastrointestinal tract, deliver massive amounts of blood to the liver in most vertebrates, including fishes (McLean and Ash, 1989; Satchell, 1971). This is one reason why TFM rapidly accumulates in this organ, for subsequent biotransformation via Phase II metabolism (Lech and Statham, 1975; Kane et al., 1993). Due to its additional roles in digestion and lipid metabolism, the liver also has a relatively high lipid content which also aids in the accumulation of lipophilic TFM.

The accumulation of TFM could be further augmented by the presence of organic anion transporters (OATs) on the basolateral membrane of hepatocytes. These OATs transport negatively charged organic compounds, such as TFM, into the hepatocyte, where the compounds undergo biotransformation and subsequent secretion into the biliary caniculus of the bile ducts, which lead to either the gall bladder or the gastrointestinal tract, from which the more water soluble metabolite is excreted to the environment (Bévalot et al., 2016). At physiological pH (7.2-7.8

for a trout; Wilkie and Wood, 1995), the majority of TFM would be in its ionized, phenolate form, making it an ideal candidate for anion transport into the hepatocyte, where it would be trapped until cleared from the cell in glucuronide and/or sulfate conjugate form.

While the concentration of TFM is the highest in the liver on a per gram wet tissue basis, it should be noted that the muscle comprises about 55% of the total weight of the rainbow trout compared to the liver at 1.3% and to the blood at 3.1% of the total fish weight (Giblin and Massaro, 1972). In the the present study, the total TFM stored in the whole body of rainbow trout was actually greatest in the white muscle at a value of 2.07 nmol g^{-1} total fish weight compared to 0.84 nmol g^{-1} total fish weight in the liver. Although TFM concentrates in the liver due to its high blood flow and role in detoxification, the skeletal muscle serves as a large overall sink in which TFM accumulates.

The much higher concentration of TFM found in the red blood cell fraction of the blood of rainbow trout compared to the plasma could be explained partially by the movement of TFM across cell membranes in its un-ionized (phenolic) form, as well as the binding of TFM to cellular proteins in its ionized (phenolate) form. TFM is a weak acid with a dissociation constant (pK_a) of 6.07 (Applegate, 1961), meaning that at physiological pH (7.2-7.8; Wilkie and Wood, 1995), more than 90% of the TFM will exist in its ionized form, and the rest in its un-ionized form (Howell et al., 1980; Bills et al., 2003; McDonald and Kolar, 2007). Due to the lipophilic nature of un-ionized TFM, it is able to enter red blood cells by passive diffusion down its concentration gradient. Once inside the red blood cell, 93% of the un-ionized TFM

will naturally dissociate to its ionized (phenolate) form, which could possibly bind to possibly bind to positively charged amino acid residues on hemoglobin proteins inside the erythrocyte (Steck, 1974). This binding of TFM could, in turn, sustain the inward directed concentration gradient of TFM between the red blood cells and extracellular fluid (plasma), essentially creating a sink within the cells to promote TFM uptake (Figure 2-8). If TFM binding to hemoglobin caused a conformational change in the protein, it could affect its affinity for oxygen. Therefore, the effect of TFM on the binding of oxygen to hemoglobin deserves future investigation. In the muscle, TFM could be taken-up and sequestered in a similar manner, but it is less clear why concentrations would be lower in the muscle than in the RBCs.

Although it is well established that TFM-glucuronide was a major metabolite of TFM in non-target fishes (Lech and Costrini, 1972; Lech et al., 1973; Kane et al., 1994), TFM-sulfate was recently been discovered to be another TFM metabolite (Bussy et al., 2017b). With this new information, it now appears that when TFM reaches the liver, it undergoes phase II biotransformation, in which it is conjugated to TFM-glucuronide via uridine diphosphate glucuronsyltransferase (UDPGT; Lech and Costrini, 1972; Lech et al., 1973; Kane et al., 1994) and to TFM-sulfate, most likely via phenol sulfotransferase (PST; Bussy et al., 2017a). The present study demonstrates for the first time, that both rainbow trout and white sucker accumulate significant amounts of each metabolite, primarily in the liver. However, the relative amounts of TFM-sulfate levels in whole blood were much higher than for the glucuronide conjugate. It is not clear why TFM-sulfate was relatively higher than TFM-glucuronide, but it may be related to differences in how the two

metabolites are handled by the liver. Both TFM-glucuronide and TFM-sulfate are highly hydrophilic, and are bi-directionally transported between the sinusoidal blood of the liver and the basolateral membrane of hepatocytes and the biliary ducts using organic transporting polypeptides and/or organic anion transporters (Zamek-Gliszczynski et al., 2006; Bévalot et al., 2016). These transporters are responsible for delivering conjugated compounds to the bile ducts for excretion (Bévalot et al., 2016), but also protect the liver from damage due to the accumulation of these and other substances (Deeley et al., 2006). Differences in the affinities and capacities of these transporters for sulfated vs glucuronidated compounds can influence the concentrations of biotransformed substances excreted from the liver into the blood (Deeley et al., 2006). Similar differences in the handling of TFM-glucuronide compared to TFM-sulfate may explain the relative differences in the blood.

While TFM-sulfate seems to be evenly distributed between the different blood fractions of rainbow trout, TFM-glucuronide exhibited an opposite pattern. The relatively high amount of TFM-glucuronide found in the plasma compared to the red blood cell pellet is likely related to its greater hydrophilicity due to its more polar structure. As a result, the TFM-glucuronide tended to remain in the plasma because it was unable to cross the plasma membrane of the red blood cell. This also lends further support to the hypothesis that TFM accumulation by the red blood cells and white muscle by passive diffusion, when its un-ionized, more lipophilic form, before getting trapped in its ionized, less lipid permeable form.

Dose-dependent Changes in the Distribution of TFM and its Metabolites in Rainbow Trout Tissues

The present study suggests that at sub-lethal exposure concentrations, relatively stable steady state levels of TFM are maintained in the liver of rainbow trout. Beyond these concentrations, the liver TFM spikes, suggesting that the livers capacity to detoxify TFM is exceeded, leading to a spill over of TFM characterized by increased TFM concentrations in the blood and white muscle and likely other tissues such as the brain, ultimately causing death. Survival to TFM exposure is directly related to detoxification capacity, as determined by a fish's ability to metabolize TFM via glucuronidation and sulfation (Lech and Statham, 1975; Clark et al., 1991; Kane et al., 1993; Bussy et al., 2017b). Many studies have shown that the selective toxicity of TFM to sea lamprey is due to their lower capacity to detoxify the lampricide via glucuronidation, compared to other non-target fishes (Lech, 1974; Lech and Statham, 1975; Kane et al., 1993). Lech and Statham (1975) reported the higher ratio of free to conjugated TFM in sea lamprey compared to rainbow trout, which indicates that biotransformation of TFM to hydrophilic metabolites is critical for its elimination and subsequent survival during exposure to TFM. The present study demonstrates that at sub-lethal exposure concentrations, biotransformation of TFM by rainbow trout liver occurs at a rate high enough to prevent spillover into blood and other tissues, resulting in subsequent toxicity. Further support for this hypothesis is illustrated by the increases in relative levels of TFM-glucuronide and TFM-sulfate in the liver at increasing, yet still sub-lethal exposure concentrations, even as the parent TFM concentrations remain more or less stable. However, when

fish were experiencing mortality at higher exposure concentrations, the levels of conjugated metabolites plateaued and then decreased at the highest exposure concentration. In other words, the capacity of the fish to detoxify TFM was overwhelmed.

In the present study, the levels of TFM quantified in rainbow trout liver, blood and white muscle were similar to earlier studies on the non-target effects of TFM. Birceanu et al. (2014) measured a peak TFM concentration of about 15 nmol g^{-1} wet tissue in the muscle of rainbow trout exposed to a concentration of 11 mg l^{-1} and Lech and Statham, (1975) observed a TFM concentration of about 25 nmol ml^{-1} in the blood of rainbow trout exposed to 8 mg l^{-1} TFM. The similar concentrations of TFM in livers of fish that survived the treatments and fish that did not, combined with the significant difference in TFM levels in the blood and muscle of the surviving fish and those experiencing mortality, strongly suggests that when liver TFM concentrations exceed a threshold of about 20-25 nmol g^{-1} wet weight, it could cause death. Concentrations below these values make it less likely that TFM directly caused death. Of course other exacerbating factors such as water temperature, dissolved oxygen or the presence of other natural or anthropogenic stressors would need to be considered when investigating a fish kill following lampricide applications, and should be addressed in future studies. In cases where blood samples are available, concentrations in excess of 60 nmol ml^{-1} would also be indicative of death arising from TFM exposures. In many cases, however, decomposition will make the collection of blood, and possibly liver, impractical, if not impossible. In such instances, muscle should be collected because it decomposes

at a much slower rate than liver or blood and provides a reliable measure of postmortem TFM concentrations.

Relevance for non-target mortality investigations

It is important to note that non-target fishes typically will not be exposed to concentrations of TFM as those encountered by the fish in this experiment because TFM is applied in the field at 1.2-1.5 times the minimum lethal concentration (MLC) of TFM to lamprey. The MLC is defined as the concentration of TFM required to produce 99.9% mortality to larval sea lamprey during a 9-h exposure (Brege et al. 2003), and this value is typically much lower than 9-h LC_{25} of most non-target fishes, including rainbow trout (Howell et al., 1980; Boogaard et al., 2003, McDonald and Kolar, 2007). Nevertheless, these findings provide investigators of non-target fish kills with important tools to investigate such incidents, particularly if TFM toxicity is suspected.

The present data shows that, in the case of non-target fish kills, muscle can provide very useful quantitative information that can be used to ascertain if TFM might have contributed or caused death. If mortalities are relatively recent, the present study suggests that blood can also be used to quantify TFM concentrations, which in some cases would corroborate conclusions based on muscle TFM measurements. Recent studies from our lab have also demonstrated that TFM concentrations in liver rapidly decrease in the 24-72 h following death due to the rapid decomposition of the fish (White, 2018). Muscle, however, is relatively robust to decomposition over the same time frame and TFM concentrations do not significantly change. Taken together, these findings suggest that the collection of

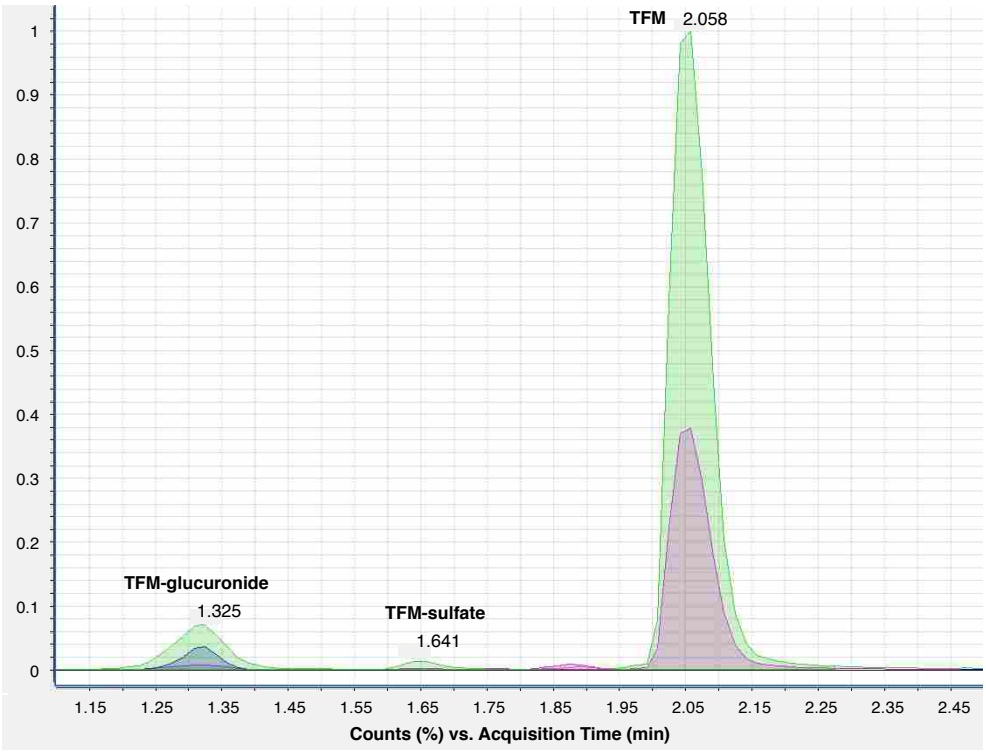
white muscle should always be collected when TFM is suspected of causing non-target mortality, because it provides stable, quantifiable and interpretable postmortem data.

Table 2-1. Species and tissue specific extraction efficiency and corresponding extraction coefficients. All tissue TFM and metabolite measurements were multiplied by their respective extraction coefficient to correct for efficiency of the solid phase extraction method, which was measured by dividing TFM measurements from spiked tissues that went through the extraction process by TFM measurements from spiked tissues that did not go through the extraction process.

Species	Tissue	Extraction Efficiency (%)	Extraction Coefficient
Rainbow Trout	Muscle	86.3	1.137
Rainbow Trout	Liver	77.5	1.225
Rainbow Trout	Whole Blood	24.6	1.754
Rainbow Trout	Plasma	2.0	1.98
Rainbow Trout	RBC Pellet	46.2	1.538
White Sucker	Muscle	37.1	1.629
White Sucker	Liver	67.8	1.322
White Sucker	Whole Blood	3.2	1.968

Figure 2-1. QTOF-MS ion chromatograms of TFM and its metabolites in rainbow trout. A) Muscle and B) Liver samples with total ion count peaks representing measurements of TFM-glucuronide (382 m/z; blue) with its breakdown products at acquisition time 1.326 min, TFM-sulfate (285 m/z; black, not seen) with its breakdown products at acquisition time 1.641 min, and TFM (206 m/z; green) with its breakdown breakdown product (176 m/z; pink) at acquisition time 2.058 min.

(A)


TFM 2.058
TFM-glucuronide
1.325
TFM-sulfate
1.641
Counts (%) vs. Acquisition Time (min)

(B)

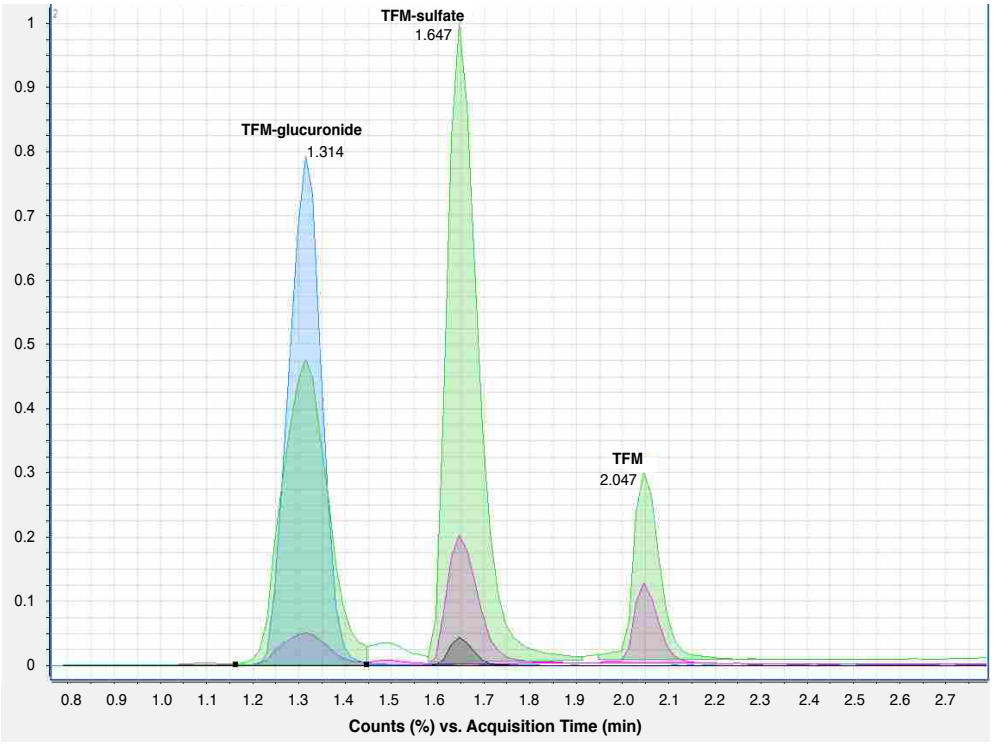
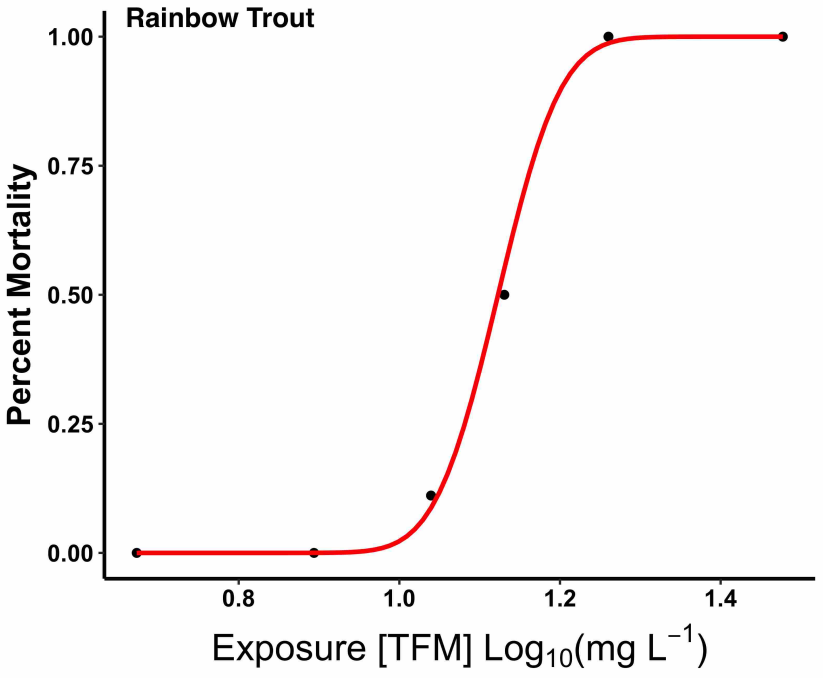

TFM-sulfate
1.647
TFM-glucuronide
1.314
TFM
2.047
Counts (%) vs. Acquisition Time (min)

Figure 2-2. Dose response curves in rainbow trout and white Sucker exposed to TFM. Percent mortality of (A) rainbow trout (*Oncorhynchus mykiss;* N = 18) and (B) white sucker (*Catostomus commersonii*; N = 5) exposed to various concentrations of 3-trifluoromethyl-4-nitrophenol (TFM) or held under control conditions (no TFM exposure) for 12 h. Lethal concentrations (LC) and acute toxicity was determined by probit analysis. Exposure concentrations were measured as 4.71, 7.83, 10.95, 13.51, 18.21 and 30.02 mg l^{-1} TFM for rainbow trout and 2.17, 4.27, 7.34, 12.25, 18.34, 19.59, 22.38, 23.91 and 32.67 mg l^{-1} TFM for white sucker. TFM concentration is expressed on a Log_{10} scale against a percent mortality expressed as a percentage of 1.

(A)


Rainbow Trout
Percent Mortality
1.00
0.75
0.50
0.25
0.00
0.8
1.0
1.2
1.4
Exposure [TFM] Log10(mg L−1)

(B)

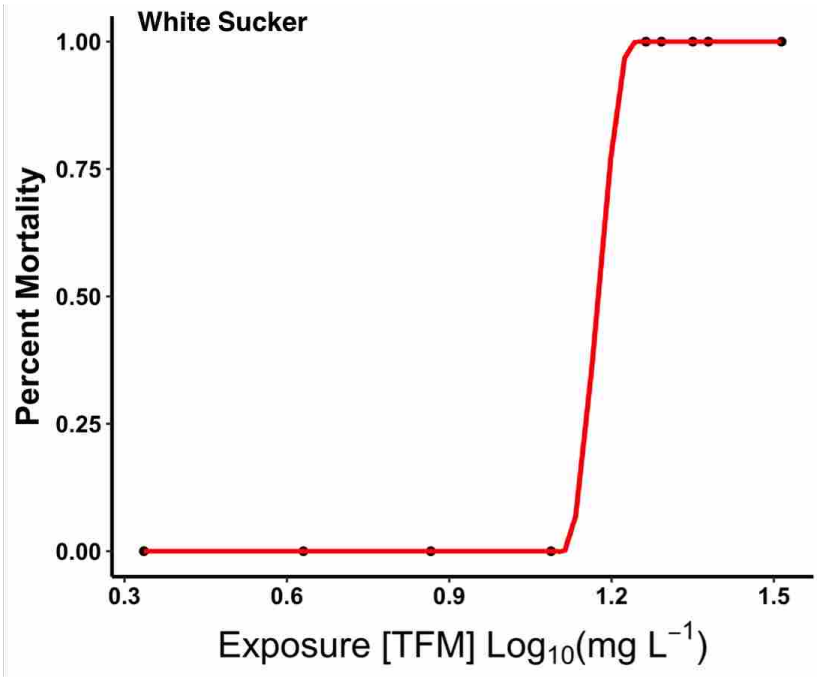
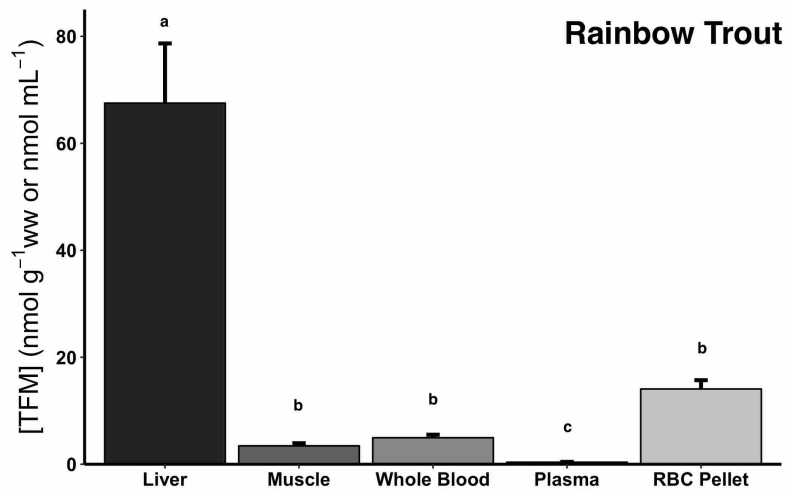
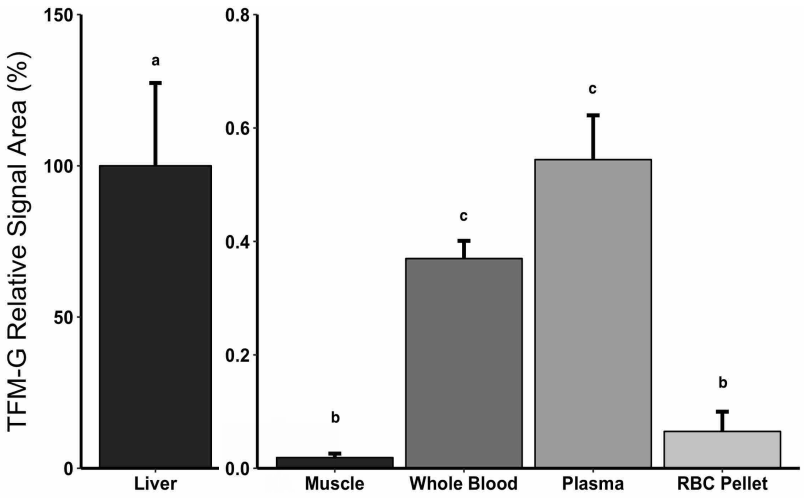

White Sucker
Percent Mortality
1.00
0.75
0.50
0.25
0.00
0.3
0.6
0.9
1.2
1.5
Exposure [TFM] Log10(mg L−1)

Figure 2-3. Distribution of TFM and its metabolites in rainbow trout blood and tissues. (A) Quantification of 3-trifluoromethyl-4-nitrophenol (TFM) in different tissues and blood fractions of rainbow trout (*Oncorhynchus mykiss*) exposed to their pre-determined 9 h LC_{25} (12.9 mg l^{-1} TFM). Relative signal area of (B) TFM-glucuronide conjugate and (C) TFM-sulfate conjugate in the same tissues of rainbow trout. The metabolite signal area of tissue samples were normalized by expressing their values as a percentage of the value obtained from the liver samples. All data are expressed as the mean + 1 SEM (N = 10). Bars sharing the same letter are not significantly different from one another ($P < 0.05$).

(A)


Rainbow Trout
[TFM] (nmol g^{-1}ww or nmol mL^{-1})
0
20
40
60
80
a
b
b
c
b
Liver
Muscle
Whole Blood
Plasma
RBC Pellet

(B)


TFM-G Relative Signal Area (%)
0
50
100
150
0.0
0.2
0.4
0.6
0.8
a
b
c
c
b
Liver
Muscle
Whole Blood
Plasma
RBC Pellet

(C)

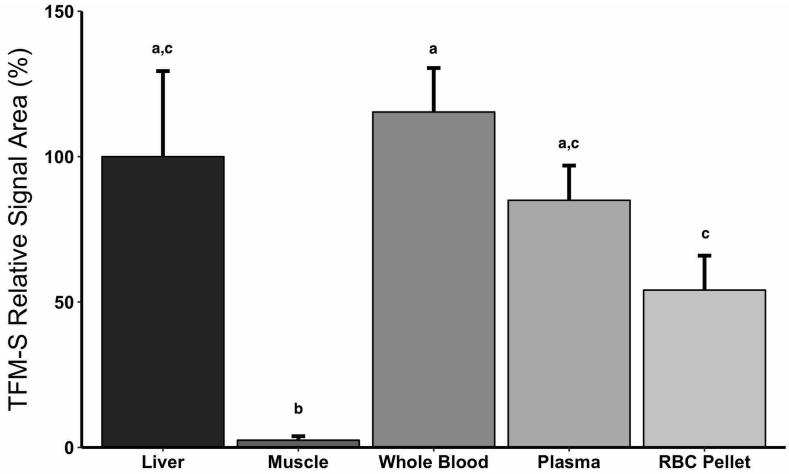
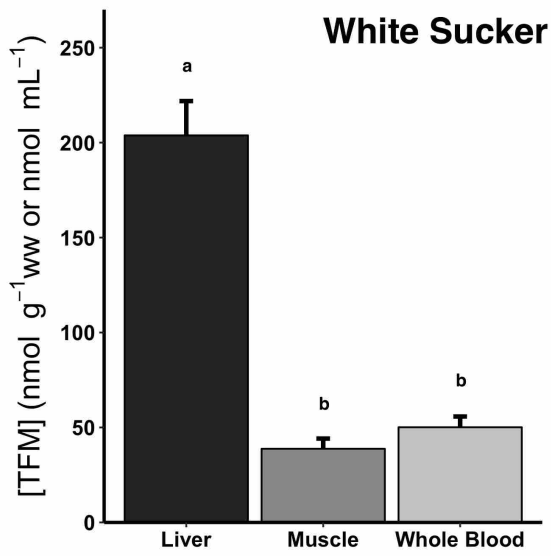
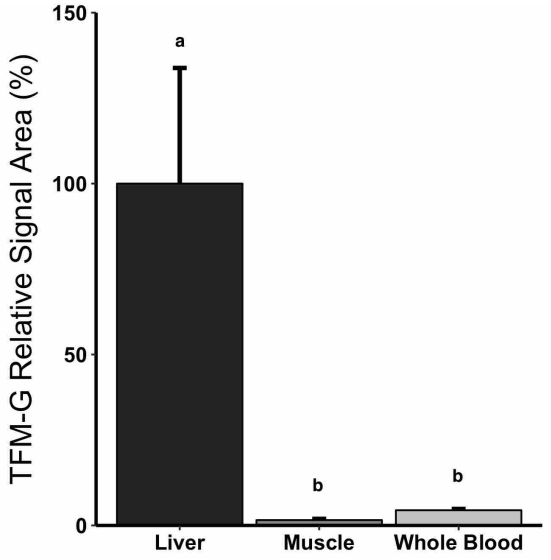

TFM-S Relative Signal Area (%)
0
50
100
150
a,c
b
a
a,c
c
Liver
Muscle
Whole Blood
Plasma
RBC Pellet

Figure 2-4. Distribution of TFM and its metabolites in white sucker blood and tissues. (A) Quantification of 3-trifluoromethyl-4-nitrophenol (TFM) in blood and tissues of white sucker (*Catostomus commersonii*) exposed to their pre-determined 9 h LC_{25} (18.3 mg l^{-1} TFM). Relative signal area of (B) TFM-glucuronide conjugate and (C) TFM-sulfate conjugate in the same tissues of white sucker. The metabolite signal area of tissue samples were normalized by expressing their values as a percentage of the value obtained from the liver samples. All data are expressed as the mean + 1 SEM (N = 10). Bars sharing the same letter are not significantly different from one another ($P < 0.05$).

(A)


White Sucker
[TFM] (nmol g^{-1}ww or nmol mL^{-1})
250
200
150
100
50
0
a
b
b
Liver
Muscle
Whole Blood

(B)


TFM-G Relative Signal Area (%)
150
100
50
0
a
b
b
Liver
Muscle
Whole Blood

(C)

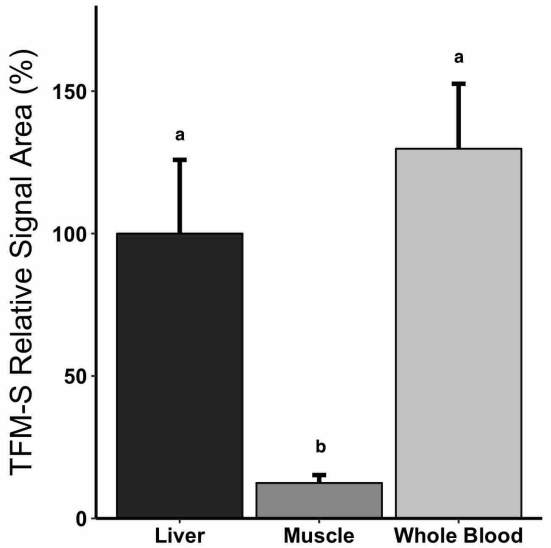
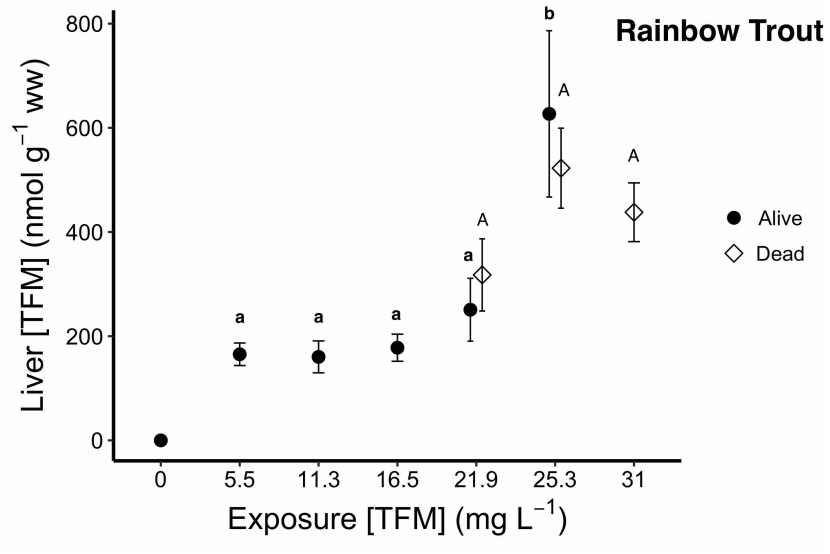
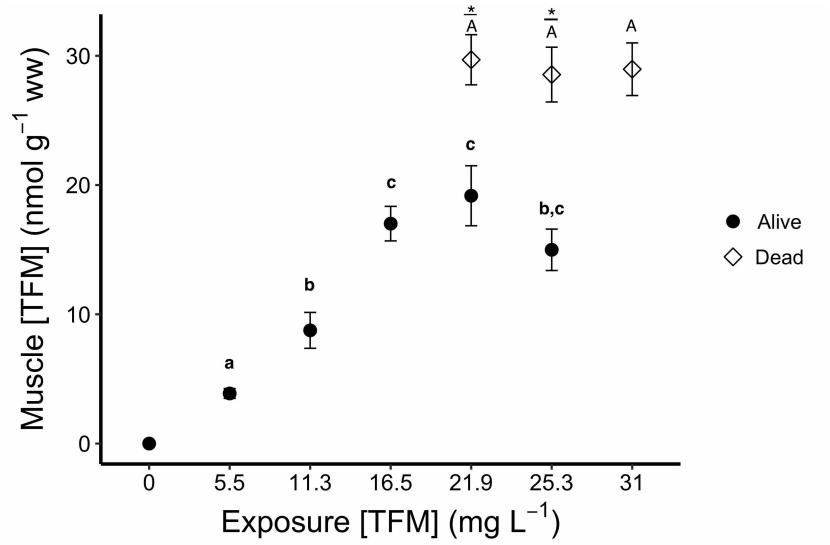

TFM-S Relative Signal Area (%)
150
100
50
0
a
b
a
Liver
Muscle
Whole Blood

Figure 2-5. Dose dependent changes in the distribution of TFM in rainbow trout tissues. Quantification of 3-trifluoromethyl-4-nitrophenol (TFM) in (A) liver (B) muscle and (C) blood of rainbow trout (*Oncorhynchus mykiss*) exposed to various TFM concentrations (measured 5.45, 11.34, 16.47, 21.89, 25.33 and 30.98 mg l^{-1} TFM). All data are expressed as the mean + 1 SEM (See appendix C for sample sizes). Different lower case letters denotes significant differences between "surviving" groups of fish at the different concentrations. Different upper case letters denotes significant differences between fish experiencing mortalities at each concentration. Asterisks denote significant difference between fish surviving TFM exposure and those experiencing mortality at the same concentration ($P < 0.05$).

(A)


Rainbow Trout
Liver [TFM] (nmol g^{-1} ww)
800
600
400
200
0
a
a
a
a
A
b
A
A
0
5.5
11.3
16.5
21.9
25.3
31
Exposure [TFM] (mg L^{-1})
Alive
Dead

(B)


Muscle [TFM] (nmol g^{-1} ww)
30
20
10
0
a
b
c
c
b,c
*
A
*
A
A
0
5.5
11.3
16.5
21.9
25.3
31
Exposure [TFM] (mg L^{-1})
Alive
Dead

(C)

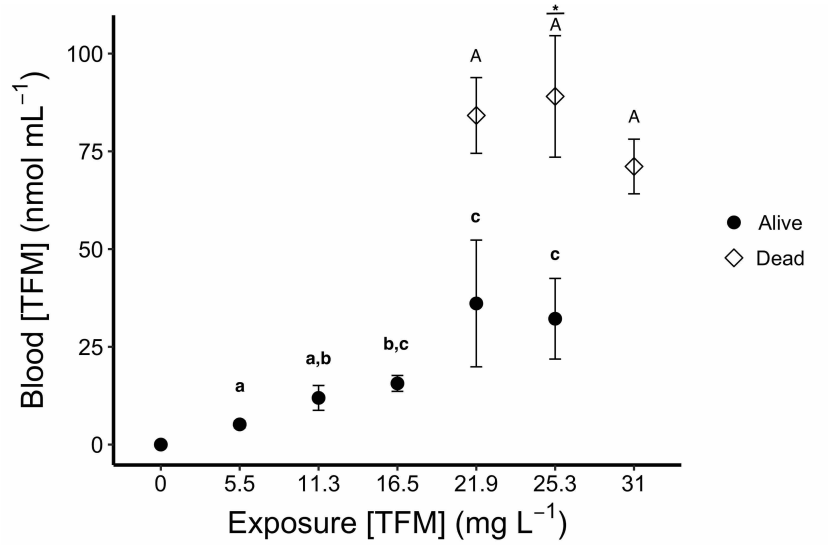
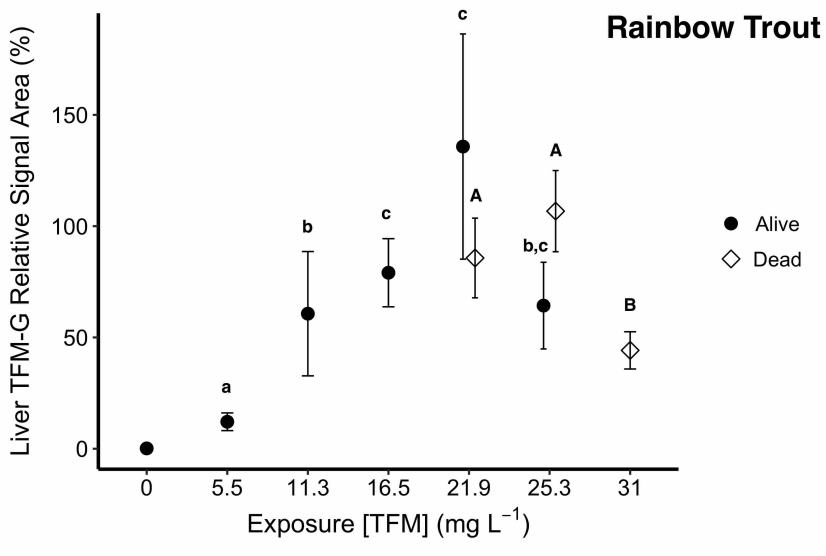
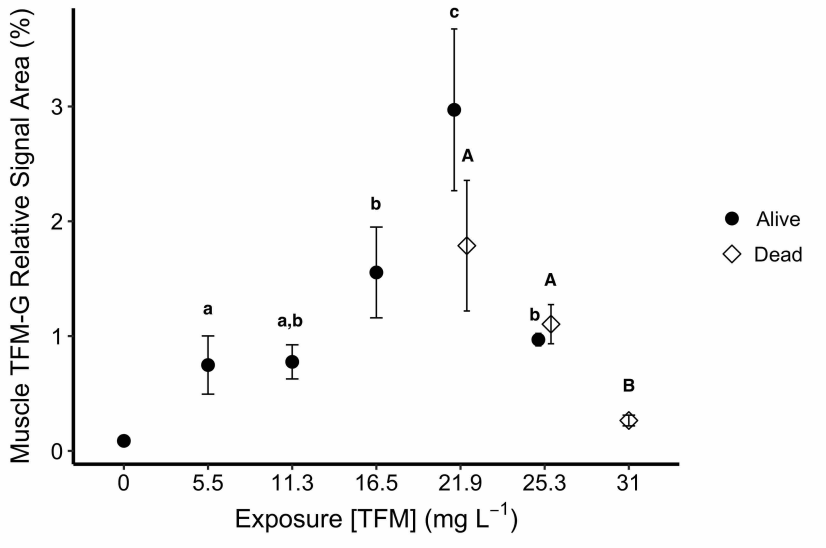

Blood [TFM] (nmol mL^{-1})
100
75
50
25
0
a
a,b
b,c
c
c
A
*
A
A
0
5.5
11.3
16.5
21.9
25.3
31
Exposure [TFM] (mg L^{-1})
Alive
Dead

Figure 2-6. Dose dependent changes in the distribution of TFM-glucuronide in rainbow trout tissues. Relative amounts of TFM-glucuronide in (A) liver (B) muscle and (C) blood of rainbow trout (*Oncorhynchus mykiss*) exposed to various TFM concentrations (measured 5.45, 11.34, 16.47, 21.89, 25.33 and 30.98 mg l^{-1} TFM). The metabolite signal areas of tissue samples at all concentrations were normalized by expressing their values as a percentage of the greatest average value obtained from the liver samples. All data are expressed as the mean + 1 SEM (See Appendix C for sample sizes). Different lower case letters denotes significant differences between "surviving" groups of fish at the different concentrations. Different upper case letters denotes significant differences between fish experiencing mortalities at each concentration. Asterisks denote significant difference between fish surviving TFM exposure and those experiencing mortality at the same concentration ($P < 0.05$).

(A)


Rainbow Trout
Liver TFM-G Relative Signal Area (%)
0
50
100
150
a
b
c
c
b,c
A
A
B
0
5.5
11.3
16.5
21.9
25.3
31
Exposure [TFM] (mg L^{-1})
Alive
Dead

(B)


Muscle TFM-G Relative Signal Area (%)
0
1
2
3
a
a,b
b
c
b
A
A
B
0
5.5
11.3
16.5
21.9
25.3
31
Exposure [TFM] (mg L^{-1})
Alive
Dead

(C)

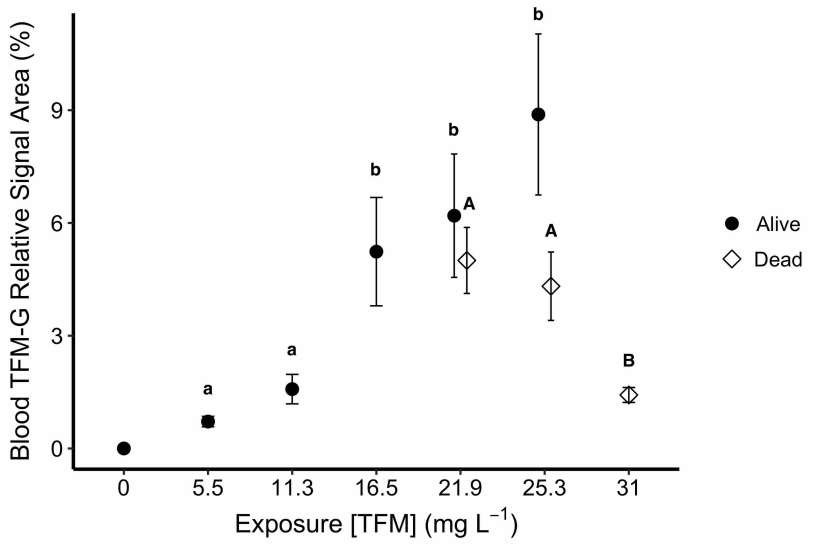
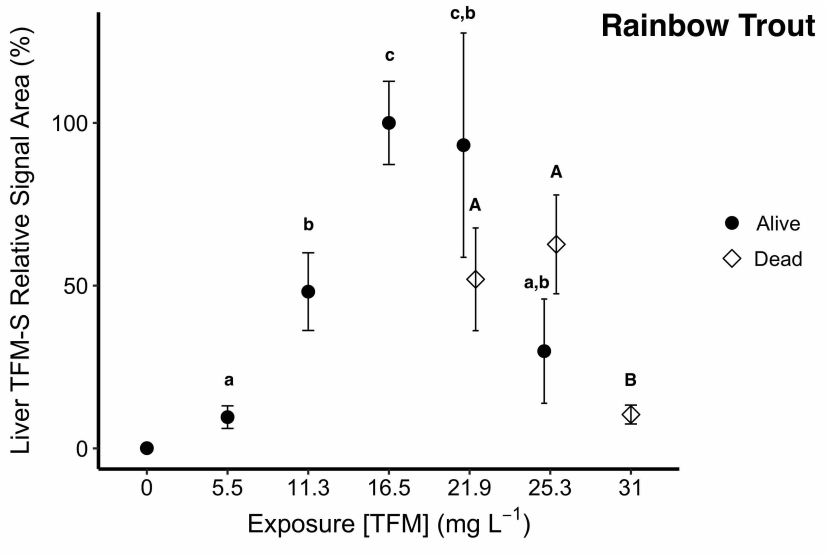
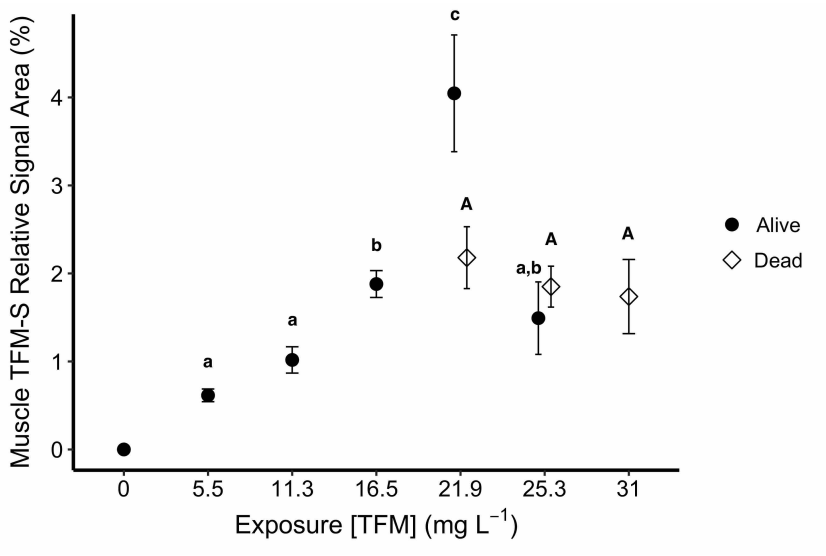

Blood TFM-G Relative Signal Area (%)
0
3
6
9
a
a
b
b
b
A
A
B
0
5.5
11.3
16.5
21.9
25.3
31
Exposure [TFM] (mg L^{-1})
Alive
Dead

Figure 2-7. Dose Dependent changes in the distribution of TFM-sulfate in rainbow trout tissues. Quantification of TFM-sulfate in (A) liver (B) muscle and (C) blood of rainbow trout (*Oncorhynchus mykiss*) exposed to various TFM concentrations (measured 5.45, 11.34, 16.47, 21.89, 25.33 and 30.98 mg l^{-1} TFM). The metabolite signal areas of tissue samples at all concentrations were normalized by expressing their values as a percentage of the greatest average value obtained from the liver samples. All data are expressed as the mean + 1 SEM (See Appendix C for sample sizes). Different lower case letters denotes significant differences between "surviving" groups of fish at the different concentrations. Different upper case letters denotes significant differences between fish experiencing mortalities at each concentration. Asterisks denote significant difference between fish surviving TFM exposure and those experiencing mortality at the same concentration ($P < 0.05$).

(A)


Rainbow Trout
Liver TFM-S Relative Signal Area (%)
0
50
100
a
b
c
c,b
A
a,b
A
B
Alive
Dead
0
5.5
11.3
16.5
21.9
25.3
31
Exposure [TFM] (mg L^{-1})

(B)


Muscle TFM-S Relative Signal Area (%)
0
1
2
3
4
a
a
b
c
A
a,b
A
A
Alive
Dead
0
5.5
11.3
16.5
21.9
25.3
31
Exposure [TFM] (mg L^{-1})

(C)

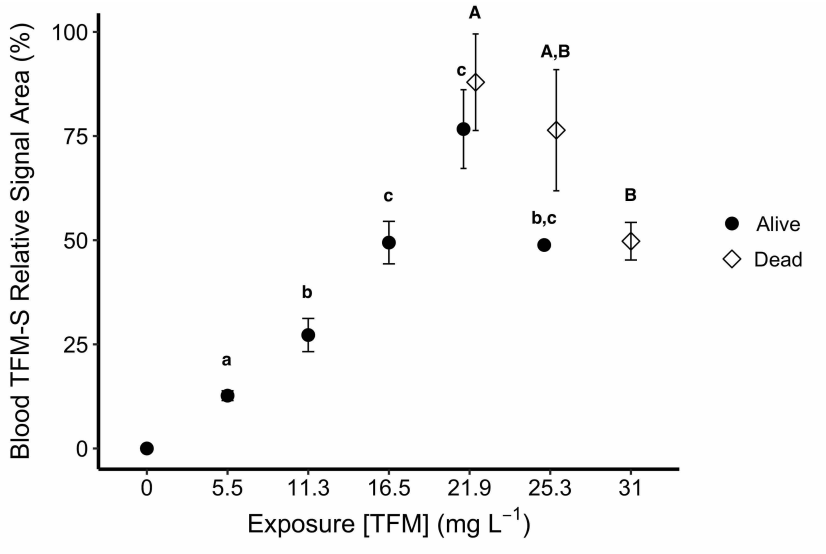
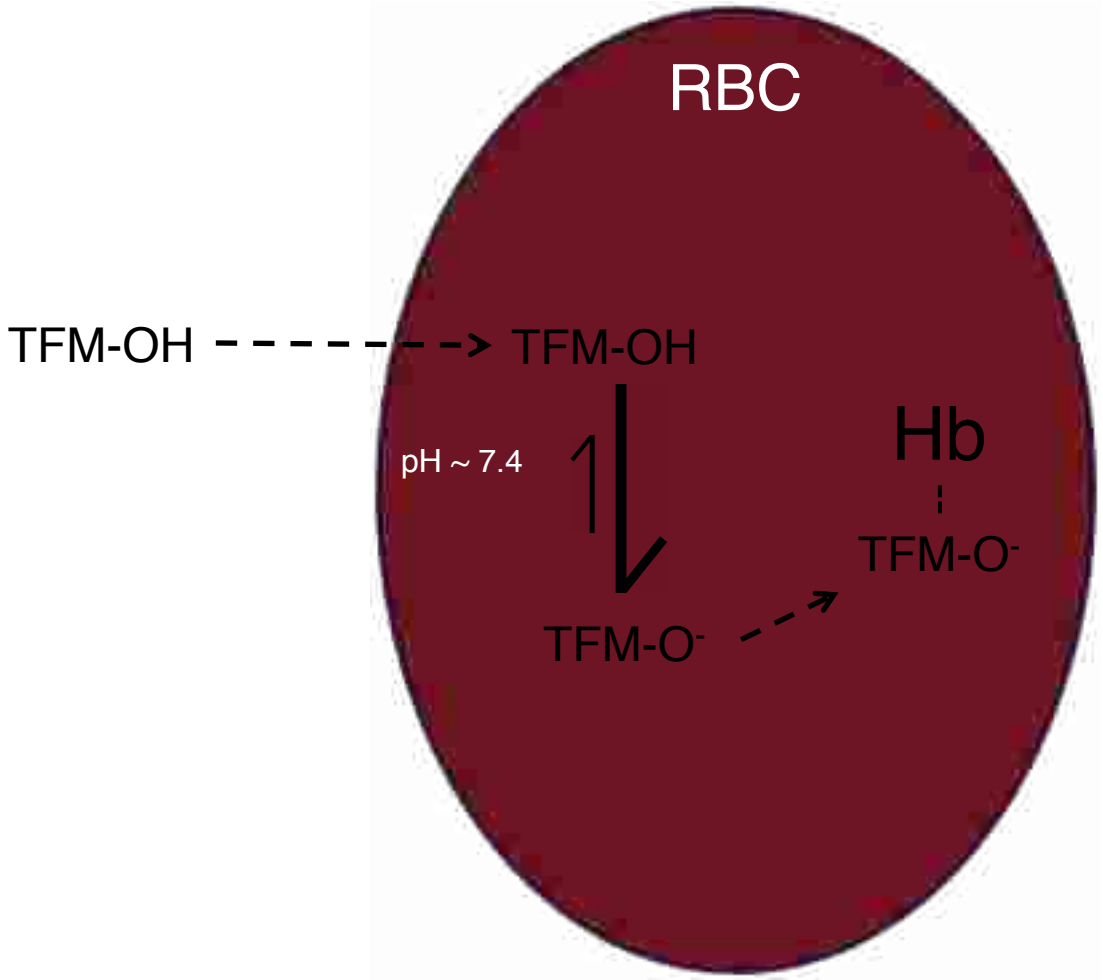

Blood TFM-S Relative Signal Area (%)
0
25
50
75
100
a
b
c
c
A
b,c
A,B
B
Alive
Dead
0
5.5
11.3
16.5
21.9
25.3
31
Exposure [TFM] (mg L^{-1})

Figure 2-8. Proposed model of TFM-protein binding in erythrocytes. The un-ionized form of TFM enters red blood cells by passive diffusion down its concentration gradient. Once inside the red blood cell, 95% of the un-ionized TFM will naturally dissociate to its un-ionized form, which then binds to positively charged proteins inside the erythrocyte. The binding of TFM to a protein, in turn, increases the concentration gradient of TFM between the red blood cells and extracellular fluid (plasma), creating a sink within the cells causing more TFM to be taken up.


RBC
TFM-OH
TFM-OH
pH ~ 7.4
Hb
TFM-O-
TFM-O-

Chapter 3:

Postmortem Handling and Storage of Non-Target Fish Tissues After Exposure to TFM

Introduction

The piscicide 3-trifluoromethyl-4-nitrophenol (TFM) has been key to the successful control of invasive sea lamprey *(Petromyzon marinus)* populations in the Great Lakes for over 60 years (Great Lakes Fishery Commission, 2011). Its application to streams and tributaries around the Great Lakes has lead to the resurgence of commercial, recreational and culturally significant fisheries (Lawrie, 1970; Christie, 1974; Christie and Goddard, 2003; Zimmerman and Krueger, 2009; Great Lakes Fishery Commission, 2017). TFM is selectively toxic to larval sea lampreys because non-target fishes are able to detoxify it via glucuronidation and sulfation to a greater extent than sea lamprey (Lech, 1974; Lech and Statham, 1975; Kane et al., 1994; Bussy et al., 2017b).

Although concentrations of TFM applied to streams are typically sub-lethal for non-target fishes, mortality can result from accidental over-application and/or decreases in stream pH (McDonald and Kolar, 2007). As a weak acid, with a pK_a of 6.07, the proportion of TFM in its more diffusible un-ionized form increases as pH decreases, leading to greater TFM uptake at low compared to higher water pH (Hunn and Allen, 1974; Hlina et al., 2017. Drops in water pH can result from precipitation, agricultural runoff, or photosynthesis and aerobic respiration by aquatic vegetation and algae (Wetzel, 1983; Poudel et al., 2013). Non-target fish kills can also result from natural causes such as extreme temperatures, oxygen depletion, disease, or xenobiotics (Meyer and Barclay, 1990).

In instances where there is uncertainty about whether or not lampricides contributed to fish kills, it is important to reliably ascertain the amount of lampricide that has accumulated in the fishes, and whether or not the measurements are indicative of toxic exposure concentrations. In the previous chapter, threshold concentrations of TFM in the liver, muscle and blood were established that could cause death in non-target fishes. However, uncertainty about the reliability of such measurements could undermine interpretations of postmortem liver, muscle or blood TFM concentrations. There is currently a lack of information regarding how to best store and preserve fish tissues for the quantification of TFM, not to mention, the identification of its metabolites TFM-glucuronide and TFM-sulfate during investigations of non-target fish kills.

In forensic toxicology, the pre-analytic sampling and storage phase is a source of great variation and can drastically affect the reliability of analyte measurements in tissue samples (Skopp, 2004). Poor sample quality due to inadequate sampling, handling and/or storage can potentially compromise the accuracy and confidence in measurements of postmortem TFM and/or metabolites, undermining the effectiveness of non-target mortality investigations.

The purpose of the current study was to determine the effects that variables such as tissue type, storage temperature, storage length and the use of preservatives have on stability of TFM and its metabolites in the blood and tissues of two non-target fishes, rainbow trout (*Oncorhynchus mykiss)* and white sucker (*Catostomus commersonii*). Accordingly, after exposure to TFM, blood,

liver and muscle were collected and either immediately snap-frozen in liquid N_2 and stored at -80˚C, or left on ice or at room temperature for a period of one hour before freezing and storage at -80˚C until measurement of TFM and the characterization of TFM metabolites. The effects of longer-term storage conditions on TFM stability were then investigated by measuring TFM and its metabolites in the blood and tissues after storing the samples for a one-week period at -80˚C, freezing at -20˚C, refrigeration at 4˚C or storage at room temperature. The effectiveness of storing muscle and liver in sodium fluoride preservative was also determined as well as the utility of collecting and storing whole blood in vacutainers containing different additives including the anticoagulants lithium heparin, sodium citrate and Na_2 EDTA, and the preservative sodium fluoride (NaF).

Material and Methods

Experimental Animals and Holding

Rainbow trout were purchased from Rainbow Springs Trout Hatchery (Thamesford, Ontario) and white suckers were captured by seine netting from Canagagigue Creek, Elmira, Ontario. Prior to importing the white suckers into Laurier's fish holding facilities, they were treated for ectoparasites in a formalin bath (0.75% formalin; SOP I23 - Wilfrid Laurier Animal Care Committee). Both the trout and white sucker were held in separate 800 L holding tanks in Wilfrid Laurier's Centre for Cold Regions and Water Science, each tank receiving a mixture of reverse osmosis water and de-chlorinated, City of Waterloo tap water (pH ~ 8.0, alkalinity ~150 mg l^{-1} as $CaCO_3$, temperature ~15˚C) at a flow rate of 5-10 L min^{-1}. The tanks holding rainbow trout were supplied with water from a ~2000 L recirculating system, equipped with mechanical and UV filtration. The water supplied to the white suckers drained directly to waste to prevent mixing of water with that supplying other fish being held in the facility. Rainbow trout were fed with EWOS 3mm floating pellets and white suckers were fed a mixture of bloodworms and EWOS #1 micro crumble feed 3 times per week to satiation. Fish were held for at least 2 weeks but food was withheld for approximately 72 h before commencing experiments to prevent fouling of TFM exposure containers and build-ups of ammonia in the water. Both groups of fish were held under a 12 h light and 12 h dark photoperiod. All fish husbandry and experiments were

approved by the Wilfrid Laurier University Animal Care Committee and followed guidelines of the Canadian Council of Animal Care (CCAC).

Experimental Protocol

Series 1- Effects of One-Hour Storage Methods on the Stability of TFM

The goal of these experiments was to ascertain how the stability of TFM and its metabolites in the blood, muscle and liver of rainbow trout (N = 9, 293.1 ± 13.6 g, 28.2 ± 0.4 cm) and white sucker (N = 9, 85.9 ± 7.6 g, 18.8 ± 0.5 cm) was affected by the method of sample preservation following TFM exposure. Accordingly, fish were exposed for 6 h to their respective 9-h LC_{25} of TFM, which was determined in Chapter 2. The goal was to expose the fish to TFM, with minimal mortality over the 6 h exposure period and to achieve more-or-less uniform TFM accumulation within each species of fish

The experimental setup was identical for both species, with each acclimated overnight to darkened, individual 4 L exposure chambers contained within a 200 L flow-through system continuously receiving dechlorinated, city of Waterloo tap water at a flow rate of 0.5 liters per minute. Water chemistry was more or less similar between the two experiments, but temperature varied between the experiments (rainbow trout: water pH = 8.0 ± 0.01, T = 16.0 ± 0.07˚C, alkalinity = 255 mg l^{-1} as $CaCO_3$, DO > 90%; white sucker: water pH = 8.1 ± 0.03, T = 10.4 ± 0.02˚C, alkalinity = 238 mg l^{-1} as $CaCO_3$, DO > 90%). Prior to the addition of TFM, incoming water flow to the system was shut off to yield a closed re-circulating system in which a submersible pump was used to pump water

from a lower reservoir (~70 L) to a head tank (~ 70 L), from which water was drained into the individual chambers of the fish. Based on the total volume of the system, sufficient field formulation TFM (35% active ingredient dissolved in isopropanol; Clariant SFC GMBH WERK, Griesheim, Germany; provided courtesy of Fisheries and Oceans Canada) was added to achieve the desired TFM exposure concentration. After a 20 minute mixing period, TFM concentrations were verified spectrophotometrically using a NovaSpec II spectrophotometer (Pharmacia Biotech, Cambridge, England, UK) at a wavelength of 395 nm following Standard Operating Procedures of the Department of Fisheries and Oceans, Sea Lamprey Control Centre, Sault Ste. Marie, Ontario (IOP: 012.4). After 6 h of TFM exposure, all fish were euthanized with an overdose of tricaine methanesulfonate (MS222; 0.5 g l^{-1}, buffered with 1.0 g l^{-1} $NaHCO_3$) before collection of blood, liver and muscle. To determine the effects of different storage temperatures on the stability of TFM and its metabolites, whole blood, muscle and liver from each fish were split into three sub-samples. The first sub-sample was snap-frozen in liquid N_2, the second was placed into a small tin-foil pouch and placed on ice, and the third was placed into a tin-foil pouch and kept at room temperature (measured 18.1˚C). After one hour all samples were stored at -80˚C until processed for quantification of TFM, and characterization of TFM-glucuronide and TFM-sulfate.

Series 2 – Effects of One-Week Storage Methods on TFM Stability

The goal of these experiments was to ascertain how storage under different conditions for 1 week affected the stability of TFM and its metabolites

in the blood, muscle and liver of rainbow trout (N = 8, 373.7 ± 20.1 g, 28.2 ± 1.57 cm) and white sucker (N = 9, 123.1 ± 9.5 g, 21.2 ± 0.55 cm) following 6 h of exposure to TFM.

The TFM exposure protocol was identical to that described above (Series 1), with more or less the same water chemistry and temperature (rainbow trout: water pH = 8.0 ± 0.03, T = 14.1 ± 0.01˚C, alkalinity = 238 mg l^{-1} as $CaCO_3$, DO > 90%; white sucker: water pH = 7.8 ± 0.01, T = 12.9 ± 0.01˚C, alkalinity = 272 mg l^{-1} as $CaCO_3$, DO > 90%). As described in Series 1, after the incoming water flow to the system was shut off to yield a closed re-circulating system, sufficient TFM was added to achieve the desired TFM exposure concentration. After the 6 h exposure period, the fish were euthanized as described earlier (Series 1), followed by the collection of blood, liver and muscle. The samples of muscle and liver that were collected from each rainbow trout were separated into 6 sub-samples, while whole blood was sub-divided into 7 sub-samples each. The sub-samples of muscle and liver (1-3g) were either snap-frozen in liquid N_2, or placed into a 15 mL polypropylene centrifuge tube and stored under the following conditions for one week: -20˚C, 4˚C while submerged in 5 mL sodium fluoride solution (+NaF), 4˚C without NaF (-NaF), room temperature (measured 20.2˚C) plus NaF, or room temperature (20.2˚C) minus NaF. Sodium fluoride solution (1%), which is often used to help preserve forensic blood samples (Skopp, 2004), was prepared using 1g of 99% pure NaF (Bioshop, Burlington, Canada) added to 100mL of deionized water. Rainbow trout blood was either snap-frozen in liquid N_2, kept at -20˚C, 4˚C, or room temperature for a period of

one week. The three remaining aliquots of rainbow trout blood were separated into one of three different, colour-coded vacutainer blood collection tubes (green, blue, grey; BD Vacutainer, Franklin Lakes, NJ, U.S.A.), and stored at 4˚C for one week. The three types of containers contained (i) lithium heparin (green caps), (ii) 0.109 M sodium citrate (blue caps) and (iii) NaF and Na_2 ethylenediaminetetraacetic acid (EDTA; grey caps). White sucker muscle and liver samples were sub-divided into 4 sub-samples each and either snap-frozen in liquid N_2, or stored at -20˚C, 4˚C or room temperature (20.2˚C) for a period of one week. White sucker blood was sub-divided into four sub-samples each and either snap-frozen in liquid N_2 or separated into one of the three different vacutainers and stored at 4˚C. After one week all trout and white sucker samples were stored at -80˚C until processed for the quantification of TFM, and characterization of TFM-glucuronide and TFM-sulfate at the Upper Midwest Environmental Sciences Center (UMESC), U.S. Geological Survey, La Crosse, Wisconsin, U.S.A.

Analytical Methods

Water TFM Measurements

Water TFM concentrations were measured spectrophotometrically using either a 96-well microplate spectrophotometer (Epoch 2, Biotek Instruments, USA) or a standard spectrophotometer (NovaSpec II), using 1.5 mL polystyrene cuvettes. Unknown absorbances were measured at wavelength of 395 nm against precision standards as described above.

Quantitation of TFM and Characterization of TFM-metabolites in Liver, Muscle and Blood

All blood and tissue samples were analyzed at UMESC, following the protocol described in Chapter 2 (refer to Chapter 2 for details). Briefly, solid phase extraction was performed on aliquots liver, muscle or blood using acetonitrile containing 1% formic acid to extract TFM and TFM-metabolites. The samples were homogenized and filtered using Phree phospholipid removal 96-well plates (Phenomenex, Torrance, California, USA). The extracted sample was then immediately injected into the reversed phase liquid chromatography column (Phenomenex, Kinetex 1.7 µm Evo C18) interfaced with a quadrupole time of flight mass spectrometry system (Agilent Technologies, 1290 Infinity II LC and 6530 Accutate-Mass Q-TOF LC/MS system) for quantification of TFM and its metabolites. Unused samples were frozen at -80˚C for later analysis.

In the present study, an LC mobile phase was used to separate TFM and its metabolites through the liquid chromatography column. The eluent was then pumped into the mass spectrometer source and accelerated through a vacuum by an electrical field for analysis based on the mass to charge ratio (*m/*z) of the ion fragment (Pitt, 2009). The ion counting detector measures the signal from the ions and a chromatogram is produced with peak areas relating to the intensity of signal from the ions, and thus the amount of ions in the sample (Guilhaus, 1995). By comparing the relative signal size, the m/z ratio vs time of flight against known TFM standards, the concentrations of parent compounds were quantified. Due to the lack of standards for TFM metabolites TFM-

glucuronide and TFM-sulfate, a standard curves could not be generated for these compounds, making it impossible to report metabolite concentrations; therefore all measurements of metabolites were reported in relative peak areas as described by Bussy et al. (2017b).

Calculations and Statistical Analysis

Concentrations of TFM in tissues were determined by applying peak area counts of the sample to the standard curve equation produced by measuring the peak areas from standards of known concentration. A dilution factor taking into account the volume of the extraction solution and homogenized tissue was applied to the calculated concentration of the sample in ng ml^{-1}. The resulting concentration in ng g^{-1} was then converted into nmol g^{-1} TFM by dividing by the molecular weight of TFM (207.11 g mol^{-1}). Dilution factors were also applied to the peak areas of the metabolites TFM-glucuronide and TFM-sulfate. The extraction efficiency of TFM was calculated in different tissue matrices by spiking the different TFM-free tissues with a known amount of TFM (spike tissue) and performing the extraction as described above. Another sample was measured that had the same spike solution of know concentration but without the tissue (spike null). Extraction efficiency was then calculated using the equation below:

$$\text{Extraction Efficiency (\%)} = \frac{\text{Spike tissue (ng/g)}}{\text{Spike null (ng/g)}} \times 100\ \% \qquad (1)$$

A tissue specific extraction coefficient (EC; Table 2-1) was then applied to the raw TFM concentration measured in each sample in ng ml^{-1}, followed by the

dilution factor (DF) then divided by the molecular weight of TFM to yield the true concentration of TFM in the tissue in nmol g^{-1} for muscle and liver or nmol ml^{-1} for blood via the following formula:

$$\text{Tissue [TFM]} = \frac{\text{Sample [TFM] X EC X DF}}{207.11} \quad (2)$$

To determine if submersion in sodium fluoride had any effect on tissue water, rainbow trout muscle and liver was dried to a constant mass at 60°C using a laboratory oven (Precision Scientific, Chicago, IL, U.S.A.). The corresponding percent water content was then calculated using the formula below:

$$\% \text{ Tissue Water} = \frac{\text{(Wet mass – Dry Mass)}}{\text{Wet Mass}} \text{ X } 100\ \% \quad (3)$$

Submersion of muscle and liver into sodium fluoride preservative increased the water content of each by about 9-10% in muscle and about 10-12% in liver (Table 3-1). Storage at -20˚C, 4˚C or at room temperature, in the absence of NaF, had no significant effect on tissue water content

Signal area measurements of TFM metabolites in the tissues of fish exposed to their 9-h LC_{25} were normalized to the signal area in snap-frozen samples and represented as a percentage.

Concentrations of TFM in tissues were analyzed using a one-way analyses of variance (ANOVA) followed by a Tukey Honest Significant Difference post-hoc test when data were homoscedastic and normally distributed. If these assumptions were not met (even after transformations; inverse, square root, Log_{10}), a Kruskal-Wallis rank sum test was performed followed by a Dunn's test

of multiple comparisons was used. Interactive effects of sodium fluoride preservative on the concentrations of TFM in liver and muscle kept 4˚C and room temperature for one week were analyzed using a two-way ANOVA. The level of significance was set at a P value ≤ 0.05. Statistical analysis and figures were produced using R version 3.4.2, RStudio version 1.1.383 (RStudio, 2016), and 'ggplot2' (Wickham, 2016). All data are presented as the mean ± 1 standard error of the mean (SEM).

Results

Series 1- Effects of One-Hour Storage Methods on the Stability of TFM

Following exposure to respective TFM concentrations of 12.9 ± 0.03 and 17.9 ± 0.18 mg l^{-1} TFM (~ the respective 9h LC_{25}) for 6 h, the greatest TFM concentration was found in the liver for both rainbow trout and white sucker, averaging 560 ± 53 and 182 ± 8 nmol g^{-1} wet weight, respectively (Figures 3-1A, 3-2A). After an hour on ice, TFM concentrations in rainbow trout liver dropped significantly to 357 ± 91 nmol g^{-1} wet weight ($P < 0.04$), while 1 h at room temperature resulted in an increase in TFM to 720 ± 134 nmol g^{-1} wet weight, although this difference was not significantly different from snap-frozen levels due to the high inter-sample variation ($P = 0.76$; Figure 3-1A). TFM in white sucker liver was largely unaffected by storage temperature for a period of one hour, remaining just below 200 nmol g^{-1} wet weight (Figure 3-2A).

The concentrations of TFM in snap-frozen rainbow trout muscle was approximately 8.2 ± 0.8 nmol g^{-1} wet weight and remained stable after an hour on ice and at room temperature (Figure 3-1A). The TFM in snap-frozen white sucker muscle was 37.2 ± 2.0 nmol g^{-1} wet weight, and not significantly different from the concentrations measured in muscle kept on ice and at room temperature were which averaged 41.5 ± 3.3 and 26.7 ± 4.3 nmol g^{-1} wet weight, respectively (Figure 3-2A).

Blood from rainbow trout kept on ice or at room temperature for an hour showed no changes in TFM concentration from levels found in snap-frozen blood

at 16.1 ± 1.6 nmol ml^{-1} (Figure 3-1A). White sucker blood also showed minimal differences in TFM concentrations as samples held on ice or at room temperature for one hour were not significantly different from snap-frozen samples at 43.7 ± 2.3 nmol ml^{-1}, although there was a slight decrease to 34.6 ± 3.7 nmol ml^{-1} TFM in samples kept on ice (P = 0.15), as well as a slight increase to 50.7 ± 3.6 nmol ml^{-1} TFM in samples kept at room temperature (P = 0.31; Figure 3-2A).

The relative amounts of TFM-glucuronide in rainbow trout liver kept on ice for one hour were approximately 69 % greater than that found in snap-frozen livers, while samples kept at room temperature contained almost no TFM-glucuronide (7 %) compared to that measured in snap-frozen samples (Figure 3-1B). The relative amounts of TFM sulfate in trout liver were unchanged following one-hour storage on ice or at room temperature (Figure 3-1C). The relative amount of TFM-glucuronide in trout muscle was reduced by about 25 % after one-hour on ice or at room temperature (Figure 3-1B). The levels of TFM-sulfate in muscle kept on ice and at room temperature declined by about a third compared to snap-frozen muscle (Figures 3-1C). This same trend was observed for TFM-glucuronide in trout blood samples, while TFM-sulfate in the same blood samples was not affected by storage temperature for a period of one hour (Figures 3-1B, C).

The relative amounts of TFM-glucuronide in white sucker livers kept on ice for one hour dropped about 50 % compared to levels in snap-frozen livers, declining further after being kept at room temperature for an hour, to levels that

were only 12 % of those observed in snap-frozen livers (Figure 3-2B). A similar, yet slightly less pronounced trend occurred for TFM-sulfate in white sucker livers as levels in samples kept on ice dropped by about 40 % and samples kept at room temperature dropped by about 60 % of those found in snap-frozen livers (Figure 3-2C). Although TFM-glucuronide in sucker muscle kept on ice was similar to samples that were snap-frozen, there was a loss of about 50 % after keeping the muscle at room temperature for an hour (Figure 3-2B). Levels of both TFM-glucuronide and TFM-sulfate in white sucker blood were unaffected by storage on ice or at room temperature for one a period of one hour (Figures 3-2B, C).

Series 2 – Effects of One-Week Storage Methods on the Stability of TFM

Parent TFM

Concentrations of TFM found in rainbow trout liver after a week of storage at -20˚C did not appear to deviate significantly from levels found in livers snap-frozen in liquid N_2 ($P = 0.36$; Figure 3-3A). Conversely, the concentrations of TFM in all other liver samples held at 4˚C and room temperature, plus or minus NaF, underwent greater than 90 % reductions in TFM ($P < 0.012$; Figure 3-3A).

Concentrations of TFM in trout muscle were again more stable than in the liver, ranging from approximately 6-9 nmol g^{-1} wet weight in samples stored at -20˚C and at 4˚C in both the presence and absence of NaF, compared to the levels measured in snap-frozen muscle (Figure 3-3A). Storage at room temperature

however, saw TFM significantly decrease to lower levels of 3.9 ± 2.4 and 0.83 ± 0.68 nmol g^{-1} wet weight for with and without sodium fluoride, respectively ($P < 0.003$; Figure 3-3A).

There was a slight interaction between storage temperature and NaF preservative on liver samples kept at 4˚C and room temperature ($P = 0.03$). However, NaF did not appear to affect concentrations of TFM in the muscle ($P = 0.85$)

In contrast to liver and white muscle, TFM concentrations in trout blood significantly increased in an incremental fashion from 8.5 ± 1.5 nmol ml^{-1} in snap-frozen samples to 11 ± 2.8, 23 ± 6.5, and finally 39.5 ± 5.7 nmol ml^{-1} after storage at -20˚C, 4˚C and room temperature for a period of one week (Figure 3-3A).

Keeping samples of white sucker liver at -20˚C for one week did not affect the concentration of TFM (173 ± 13.6 nmol g^{-1} wet weight), compared to snap-frozen samples (169 ± 11.7 nmol g^{-1} wet weight; Figure 3-4A). In contrast, storage of the livers at 4˚C and at room temperature resulted in significant decreases in TFM concentrations of 40 and 2% to 68.8 ± 18.7 nmol g^{-1} wet weight ($P = 0.01$) and 3.70 ± 2.0 nmol g^{-1} wet weight ($P < 0.01$), respectively (Figure 3-4A). The concentration of TFM in white sucker muscle was unchanged from 25.6 ± 3.9 nmol g^{-1} wet weight after one week at -20˚C and 4˚C. After a week at room temperature, however, TFM was below levels of detection (Figure 3-4A).

TFM-Metabolites

After one week at -20˚C, the relative levels of TFM-glucuronide in rainbow trout liver decreased by over 90% and TFM-sulfate by close to 60% compared to levels found in snap-frozen samples (Figures 3-3B, C). All other treatments resulted in TFM metabolite levels that were undetectable (Figures 3-3B, C). A similar trend was seen in rainbow trout muscle, other than at -20˚C, in which the relative amount of TFM-glucuronide was unchanged (Figure 3-3B). Keeping trout muscle at 4˚C with or without NaF preservative resulted in a greater than 80% decrease in TFM-glucuronide after one week. At room temperature however, TFM-glucuronide was undetectable after a week in both the presence and absence of NaF (Figure 3-3B).

The relative amounts of TFM-sulfate in trout muscle samples kept at -20˚C and 4˚C without NaF at approximately 57 and 20% of that found in snap-frozen muscle samples, respectively, while all other treatments resulted in levels that were undetectable (Figure 3-3C). The relative amount of TFM-glucuronide in trout blood was about 38% greater after one week at -20˚C, whereas after a week at 4˚C the levels decreased to about 39% of that found in snap-frozen blood samples (Figure 3-3B). These results, however, were highly variable and not significantly different from those found in snap-frozen samples, other than at room temperature, in which relative levels of TFM-glucuronide were undetectable. TFM-sulfate did not appear to change in trout blood in all treatments, but values were highly variable. At room temperature, however,

relative levels decreased by approximately 35% after one week, compared to snap-frozen blood samples (Figure 3-3C).

Compared to snap-frozen samples, the relative amounts of both TFM-glucuronide and TFM-sulfate in white sucker liver after a week at -20˚C remained relatively constant, but were highly variable. However, after a week at 4˚C and at room temperature, both metabolites were undetectable (Figures 3-4B, C). After a week at -20˚C and 4˚C, relative levels of TFM-glucuronide in white sucker muscle were unchanged from levels found in snap-frozen samples, while levels of TFM-sulfate decreased by 25 and 61%, respectively (Figures 3-4B, C). Neither metabolite was measured in white sucker muscle after a week at room temperature (Figures 3-4B, C).

Effects of Temperature, Anticoagulants, and Preservatives on Blood TFM Stability

After one week of storage at 4˚C in the green or grey vacutainers, containing lithium heparin or NaF and Na_2 EDTA, respectively, parent TFM in rainbow trout blood significantly increased by more than 4-fold compared to the snap-frozen blood samples (< 0.004; Figure 3-5A). Whereas storage of the blood in the blue vacutainer containing sodium citrate at 4˚C, resulted in only a slight, non-significant increase in TFM concentration (P = 0.36 Figure 3-5A). TFM in white sucker blood also increased by more than 30 and 100%, respectively, after storage in the green and grey vacutainers, relative to the snap-frozen samples preserved in liquid N_2 (Figure 3-6A). Conversely, after a week in the blue vacutainer at 4˚C, TFM significantly decreased by more than 80% (P < 0.0004; Figure 3-6A).

The relative levels of TFM-glucuronide in rainbow trout blood significantly decreased by greater than 50% after a week at 4˚C in the green vacutainer, 80% after a week in the blue vacutainer, and over 95% after a week in the grey vacutainer (Figure 3-5B), while levels of TFM-sulfate remained relatively stable in all vacutainers. Although the relative levels of TFM-glucuronide in the blood of white sucker-fish stored in the green vacutainer at 4˚C were similar to levels found in snap-frozen samples, there was almost no TFM-glucuronide found in the blood stored in the blue or the grey vacutainers for one week (Figure 3-6B), TFM-sulfate was not significantly different in blood kept in the grey vacutainer for a week compared to snap-frozen blood samples, while barely any metabolite was measured after a week in the blue vacutainer (Figure 3-6C) . Levels of TFM-sulfate found after a week of storage in the green vacutainer were about 40% of that found in snap-frozen blood samples (Figure 3-6C).

Discussion

Distribution and Metabolism of TFM and its Metabolites

Quantification of TFM, along with the characterization of its metabolites, can indicate if fish were exposed to TFM. In the present study, quantifiable amounts of TFM were detected in the liver, muscle and blood of rainbow trout and white sucker after just 6 h of exposure to their 9-h LC_{25} of TFM. Although, I was unable to generate quantitative data on the two main TFM metabolites, TFM-glucuronide and TFM-sulfate were detected by LC-MS analysis. The presence of these substances, as confirmed by LC-MS analysis, would also provide strong evidence of prior TFM-exposure in non-target fishes in the field. The presence of TFM metabolites was confirmed by the detection of ions with calculated mass to charge ratios specific to those of TFM-glucuronide (382.0391) and TFM-sulfate (285.9633), which are based on molecular weight. It is known that the metabolites break down in the source of the mass spectrometer to produce an TFM molecule, therefore the metabolite ions were further confirmed to be metabolites of TFM because of the detection of ions with calculated a mass to charge ratio specific to that of TFM (206.0071) at the same elution times as each of the metabolites (Figure 2-1).

The vast majority of TFM was measured in the liver of both rainbow trout and white sucker. This was not unexpected because the vertebrate liver is well supplied with blood, arising from both the hepatic veins, and the hepatic portal system, which receives blood from the gastrointestinal tract (Olson, 2011; Bévalot et al., 2015). The hepatocytes of liver are also equipped with organic anion

transporters (OATs) and organic anion polypeptides (OATPs), which can be used to transport xenobiotics, which could include ionized TFM into the cells, as well as its metabolites (Deeley et al., 2006; Bevalot et al., 2016). Due to its weak acid properties and pKa of ~ 6.07 (25°C; Hubert, 2003), the majority of TFM exists in its ionized form at physiological pH (pH 7.2-7.8 in trout; Wilkie and Wood, 1995). This likely explains the preferential accumulation of TFM and its metabolites in the liver relative to the muscle and the blood. However, further work is required to determine if and how OATs and OATPs function in these tissues in fishes. The presence, let alone physiological relevance, of such transporters in the blood and muscle are also unresolved in mammals, not to mention fishes.

Increases in TFM-glucuronide were observed in both rainbow trout and white sucker liver, white muscle and blood following TFM exposure. As the main site of detoxification in the body, the liver has high amounts of the enzyme uridine diphosphate glucuronsyltransferase (UDPGT), which catalyzes the addition of glucuronic acid to the TFM, resulting in a more polar molecule that can be excreted by the animals (Lech et al., 1972; Lech, 1974; Clark et al., 1991; Kane et al., 1994). Until recently, however, there was no evidence to suggest that fish accumulated physiologically or toxicologically relevant levels of TFM-sulfate during TFM exposure. The present study demonstrates, for the first time, that non-target rainbow trout and white sucker produce physiological relevant amounts of TFM-glucuronide, as well as TFM-sulfate, suggesting that the metabolism of TFM is more complicated than previously thought. Bussy et al., (2018a) detected TFM-glucuronide and TFM-sulfate in sea lampreys and teleosts including trout, bluegill

(*Lepomis macrochirus*) and lake sturgeon using extracts (S9 fraction) of liver tissue. *In vivo* experiments were limited to sea lamprey, however, in which it was shown that TFM-glucuronide and TFM-sulfate were present at very low levels in lamprey compared to the parent TFM. Notably, high amounts of an amino metabolite of the parent TFM were detected *in vivo* in the lamprey, likely because insufficient TFM was metabolized using glucuronidation and sulfation pathways (Bussy et al., 2018b). However, they did not complete similar *in vivo* experiments on non-target fishes. The present study builds on these findings by further demonstrating that the greater tolerance of non-target fishes, in this case rainbow trout and white sucker, to TFM results from their greater capacity to produce significantly relevant amounts of not only TFM-glucuronide but also TFM-sulfate. The goal of future studies should be to better define the quantitative importance of these metabolites in TFM detoxification by non-target fishes and sea lampreys.

Tissue Collection and Handling During the Pre-analytic Phase

The investigation of fish kills can be complicated during the pre-analytic phase by the decomposition of tissues for prolonged periods of time, improper sample collection and preservation, and inadequate conditions during shipping and storage. It is therefore critical that procedures be used to eliminate or minimize confounding factors that could compromise sample integrity. This could be difficult in practice if fish mortality takes place in remote locations where a lack of access to resources or facilities could complicate sample collection, preservation and storage. Indeed, this could be the situation in instances of non-target mortality that may arise during or following lampricide applications. Here, I report that parent TFM is

relatively stable if it is preserved within an hour and either snap frozen in liquid N_2, kept on ice, or ensuring that the tissue is kept at room temperature for 1 h or less, before freezing or refrigerating the sample for longer-term storage.

It is well established that the best way to preserve blood and tissue samples for the postmortem analysis of endogenous substances and metabolites, as well as exogenous compounds including xenobiotics, drugs and pesticides is to freeze the samples as fast as possible and keep them frozen until analysis (Wang et al., 1994; Butzbach, 2010). Snap freezing in liquid N_2 combined with storage at -80˚C will protect the sample from enzymatic or microbial decomposition by slowing down or stopping these processes while also keeping them viable upon thawing (Wang et al., 1994; Lin et al., 2007). Failure to adequately preserve tissues, such as having to store tissues at ambient temperatures due to a lack of refrigeration, ice or freezing capacity can cause the breakdown of tissue structures and organelles. Autolysis and edema begins minutes after the removal of the tissue, as blood flow and oxygen supply is cut off and endogenous enzymes (e.g. lipases, proteases) continue to function (Zhou and Byard, 2011). Without oxygen, ATP is temporarily produced via anaerobic pathways producing lactate and acid, resulting in decreased intracellular and extracellular pH, that can activate proteolytic enzymes released from intracellular compartments such as lysosomes (Skoop, 2004; Yarema and Becker, 2005; Butzbach, 2010; Zhou and Byard, 2011). Further complications can arise if ambient temperatures favour microbial growth and enzymatic activity, which can further accelerate postmortem cellular and tissue degradation (Skoop, 2004; Yarema and Becker, 2005, Zhou and Byard, 2011). It is therefore not only critical to

select tissues that can be easily collected, preserved and stored, but to understand how concentrations of the analytes of interest, in this case TFM and its metabolites, respond to variations in sampling, preservation, handling and storage methods. As the present study demonstrated, the liver, muscle and blood meet these criteria, with limitations related to the method of preserving and handling of each tissue.

The present study demonstrates that TFM can be reliably measured in liver, muscle and blood, and that the most effective means of preserving TFM concentration is by snap-freezing the sample in liquid N_2. However, preservation of tissues on ice or even leaving them at room temperature (1 h) did not markedly compromise sample integrity. The preservation of the liver on ice or at room temperature for 1 hour only resulted in slight decreases in TFM concentration in the rainbow trout, but not in white sucker in which tissues were more or less stable. In both fishes, TFM was also relatively stable in both muscle and blood, suggesting that these tissues are somewhat robust to different sampling procedures. Taken together these findings suggest that reliable measurements of TFM can be collected from liver, muscle or blood if liquid nitrogen (or other rapid deep-freeze method such as dry ice) is not available. Putting the samples on ice helps to preserve TFM in instances where liquid N_2 is not available. Even if ice is unavailable, temporarily leaving the samples in air (Capped) will only result in slight increases in variation of TFM, so getting the samples collected and stored (e.g. -20°C or 4°C) as quickly as possible, would likely yield samples of analytical value.

Although the relative amounts of TFM-glucuronide were more or less stable in tissues that were kept on ice, the metabolite was susceptible to degradation in the

liver if the samples were kept at room temperature for 1 hour, after which only traces of the metabolite remained. The loss of TFM-glucuronide may have been due to hydrolysis of the metabolite by the enzyme β-glucuronidase, which is found in the intestinal microbial community of mammals (Gadelle et al., 1985; Paigen, 1989). β-glucuronidase is a hydrolytic enzyme commonly found in animal tissues including liver, kidney, spleen, intestinal epithelium as well as gut bacteria that can hydrolyze glucuronide conjugates to liberate unconjugated, parent compounds (Paigen, 1989; Lampe et al., 2002; Butzbach, 2010). While *in vivo* studies on β-glucuronidase activity in fish are limited, especially in relation to TFM, Lech (1973) has used β-glucuronidase *in vitro* to study the metabolism of TFM by rainbow trout. It is not known if the microbial community of fishes contains similar microbial biota, but this information should become available in the next few years as more is learned about the gut microbiome of fishes (Tarnecki et al., 2017).

The relative amounts of TFM-sulfate on the other hand were relatively constant in rainbow trout liver suggesting that little to no sulfatase-mediated degradation of the metabolite took place within one hour on ice or at room temperature in the liver or blood. Curiously, TFM-sulfate was less stable in muscle. This is somewhat counterintuitive because the muscle would be less prone to bacterial contamination than the liver or blood, due to its relative isolation from the gut. Perhaps decreases in intramuscular pH associated with the lack of blood flow promoted the hydrolysis of TFM-sulfate?

In contrast to the rainbow trout, there was no significant difference in parent TFM concentrations after keeping the liver on ice or at room temperature for one

hour. As in the trout, however, the concentrations of TFM were relatively stable in the white muscle and blood.

A notable observation was that compared to rainbow trout, white sucker accumulated 3-4 fold as much TFM in the muscle and blood. Although the relative TFM exposure was the same for both species (9-h LC_{25}), the white suckers were exposed to a higher absolute concentration of TFM (17.9 mg l^{-1} vs. 12.9 mg l^{-1}) These findings suggest that the detoxification capacity of the trout liver is likely greater than that of the white sucker. At first glance, this observation would appear counterintuitive because TFM concentrations were 1.5-3.0 fold greater in the trout. As pointed-out in Chapter 2, however, the accumulation of TFM in the blood and white muscle was relatively stable as TFM exposure concentrations were increased, before exceeding an "upper concentration" in the liver, at which point muscle and blood concentrations began to increase or "spike". This likely was the result of a "spill-over" of TFM that occurred when the liver's capacity to detoxify TFM was exceeded, resulting in the accumulation of parent TFM by blood and muscle. Thus, greater accumulation of TFM in the blood and white muscle of white sucker may because the liver of this animal has a lower overall capacity to store and detoxify TFM than in rainbow trout.

The higher liver TFM in the rainbow trout could also reflect higher rates of TFM uptake by the trout compared to the white sucker. Indeed, the routine metabolic rates of comparably sized trout are much higher than those of white sucker (Beamish, 1964), which would be associated with greater oxygen demands and rates of TFM uptake due to greater ventilation at the gill. Indeed, Tessier et al.,

(2018) demonstrated that TFM uptake was closely correlated with routine rates of oxygen consumption in larval sea lamprey. It would be very useful to conduct similar experiments on non-target species to establish inter-species relationships between TFM uptake and species-dependent metabolic rate.

Compared to rainbow trout exposed to their 9-h LC_{25} in Chapter 2, the fish in this experiment were, on average, about 100 g larger. This may explain why there was greater accumulation of TFM in the in the blood, muscle and liver of the larger fish. This was somewhat un-expected because larger fish have a relatively lower metabolic rate compared to smaller fish (Gillooly et al., 2001). Because smaller fish have higher metabolic demands and therefore greater rates of O_2 uptake, gill ventilation and delivery of TFM to the fish would be expected to be higher, resulting in a greater accumulation of the parent TFM in the blood and tissues of the smaller fishes (Gillooly et al., 2001). In fact, Tessier et al. (2018) determined that relatively smaller sea lamprey took up TFM at much higher rates compared to larger ones. However, it should be kept in mind that sea lamprey have a low capacity to detoxify TFM compared to trout. Perhaps, the smaller trout in the present study had a relatively higher capacity to detoxify and/or eliminate TFM than their larger counterparts, leading to less total accumulation of TFM compared to the larger fish

Effects of One-week Storage Methods on Postmortem Stability of TFM and its Metabolites

Compared to snap freezing and storage at -80˚C, storage at -20˚C appears to be equally as effective for preserving TFM in the tissues of non-target fishes for up to a period of at least one week. While parent TFM levels in both species were not

affected by storage at -20˚C, compared to snap-frozen tissues, the metabolites were much less stable. After one week at -20˚C, levels of TFM-glucuronide and TFM-sulfate in rainbow trout liver dropped about 90 and 60% respectively, suggesting that hydrolytic enzymes in the liver such as the β-glucuronidase and sulfatases might still be active at this temperature. These hydrolytic enzymes are normally found inside lysosomes (Zhou and Byard, 2011; Mindell, 2012), which prevent them from hydrolyzing newly generated TFM-glucuronide or TFM-sulfate arising from conjugation reactions. However, at -20˚C it is likely that the formation of ice crystals during freezing damages cellular membranes including those of lysosomes resulting in the leakage of these and other enzymes into inter and extracellular spaces (Rehbein and Çakli, 2000; Leygonie et al., 2012). As a result, any TFM metabolites that had accumulated would be more susceptible to hydrolysis, and subsequent degradation at this temperature. This would also be complicated by the longer time needed for the sample to completely become completely frozen at -20°C compared to snap freezing in liquid N_2, which occurs almost instantaneously. With more time for the enzymes to hydrolyze the TFM conjugates back into TFM, the relative amounts of TFM-glucuronide and TFM-sulfate would be expected to be lower following one week at -20˚C. This degradation could be further exacerbated if the tissues were thawed during the homogenization of the tissues for extraction and measurement of TFM and its metabolites.

After one week at storage temperatures of 4˚C (with or without preservative) and at room temperature (with or without preservative), parent TFM and its metabolites were almost completely degraded in rainbow trout and white sucker

liver. The loss of TFM from the liver was likely due to autolysis and putrefaction associated with more advanced decomposition that took place in the unfrozen tissue over one week. Autolysis is the self-digestion of cells by intracellular enzymes and putrefaction is the decomposition of cellular structures by microorganisms (Skopp, 2004; Butzbach 2010; Zhou and Byard, 2011). The liver is an enzyme-rich tissue due to its role in detoxification (Bévalot et al., 2016), which can also become contaminated with bacteria due to its close proximity to, as well as connection to the intestines via the hepatic portal vein (Skopp, 2010, Bévalot et al., 2016).

In contrast to the liver, the relative stability of TFM in muscle of trout and white sucker at 4°C, and to some extent at room temperature, was likely because of its relative isolation from sources of bacteria, and a relative lack of endogenous hydrolytic enzymes. However, at room temperature, muscle TFM was almost completely degraded, although the presence of sodium fluoride preservative slowed down the process somewhat.

Sodium fluoride (1 % in deionized water) is frequently used as a preservative in forensic blood samples to prevent the breakdown different drugs including paracetamol, cocaine, and benzodiazepines by inhibiting residual enzymatic and microbial activity (Baselet, 1983; Mahjoub and Staub, 2000; Battal et al., 2013; Butzbach, 2010). While NaF helped to preserve TFM in the white muscle of trout, it had little effect on the stability of TFM or its metabolites in rainbow trout liver. This may be because the solution was unable to fully penetrate the tissue in time to prevent the rapid onset of decomposition that takes place in the liver compared to the muscle..

The increase in parent TFM concentrations that was observed in rainbow trout blood with storage temperature suggests that endogenous hydrolytic enzymes were active during one-week storage at 4˚C and room temperature. The decomposition of blood occurs in a similar manner as described above as the onset of hemolysis can occur rapidly after sampling (Forrest, 1993). White blood cells that contain hydrolytic enzymes within lysosomes will inevitably rupture during decomposition at elevated temperatures along with other blood cell membranes, and could cause a release of cellular contents into the blood plasma resulting in the hydrolysis of any TFM-glucuronide that was present in the blood as direct result of TFM exposure (Fishman et al., 1947; Lorbacher et al., 1967; Avila and Convit,, 1973), the net effect being increased concentrations of parent TFM.

The difference in stability of the TFM metabolites in the blood suggests that the liberation of parent TFM comes primarily from hydrolysis of TFM-glucuronide as the levels of TFM-sulfate remains relatively stable at elevated temperatures. In contrast to liver and muscle, relative levels of TFM-sulfate in rainbow trout blood were not affected by one week storage other than a slight decrease at room temperature, perhaps due to the lower metabolic activity of blood compared to liver and muscle, resulting in an overall lower decrease in pH as decomposition occurred over one week, ultimately keeping levels of TFM-sulfate relatively stable.

While blood may not contain significant levels of conjugative enzymes due to their role in detoxification being mainly limited to transportation of xenobiotics to the liver it appears that decomposition of TFM-glucuronide occurs at an increasingly

higher rate as temperatures increase, indicating that this metabolite is best preserved at very low temperatures due to the inhibition of glucuronidase activity.

Method of Blood Sampling and Storage

To determine the effectiveness of different methods for storing whole blood at 4˚C, vacutainers containing different preservatives and anticoagulants were tested. These sterile test tubes are vacuum-sealed, allowing for the quick and easy drawing of blood. The vacutainers are colour-coded universally, with each color corresponding to the additives that are present in each tube. For instance, green-capped tubes contain lithium heparin, an anticoagulant that activates antithrombins to inhibit the coagulation cascade (Bjork and Lindahl, 1982). Blue tubes contain, sodium citrate, an anticoagulant that works by chelating calcium, which is needed for clotting activity (Mollison, 2000). Finally, grey tubes contain sodium fluoride, which as noted above reduces rates of metabolism of drugs in the blood by enzymatic (Baselt, 1983) and microbial degradation (Butzbach, 2010). Grey tube also contains sodium EDTA, another Ca^{2+}-chelating agent that prevents clotting (Bowen and Remaley, 2014).

Somewhat surprisingly, the concentration of TFM in the blood collected from both trout and white sucker increased after one week at 4°C when lithium heparin (green) or the combination of NaF plus EDTA (grey) were used. In contrast, the use of sodium citrate (blue) effectively preserved parent TFM in whole blood at 4°C in rainbow trout, but parent TFM was just above detection in white sucker suggesting that there may be interspecies differences in the effectiveness of this blood preservation technique. Given the life style and habitat differences between trout

and white sucker, it is possible that the reduction of TFM in the latter was because of different microbes present in the blood.

The upward trend in TFM concentration in the lithium heparin and NaF/EDTA treated blood samples stored at 4˚C was coupled with decreases in TFM-glucuronide and/or TFM-sulfate, which again suggest that hydrolytic enzymes remained active under these conditions. If the goal is to simply obtain a semi-quantitative measure of TFM in the blood, then collecting and preserving the samples in lithium heparin would likely be the most-effect method. However, due to the tendency of parent TFM concentrations to increase with this method, and with Na citrate/EDTA treated vacutainers, interpretation would likely be limited to presence or absence of TFM exposure. This could nevertheless be very important when investigating fish kill events that take place following TFM applications.

Relevance for non-target mortality investigations

Where possible, liver, muscle and whole blood should be collected if non-target fish mortalities take place during or after TFM application. The best way to preserve blood and tissue samples for postmortem measurements of analytes is to freeze the samples as fast as possible in liquid N_2, and keep them frozen at -80°C until analysis (Wang et al., 1994; Butzbach, 2010). However, sea lamprey control agents or investigators in the field may not have access to liquid N_2 or dry ice, which would likely be a suitable alternative. Thus, keeping the samples on ice until they can be stored at -20°C or shipped to investigative laboratories on dry ice, would be an acceptable alternative. Samples can be kept at room temperature for periods of up to 1 hour, but this would be considered the least desirable option. Storage of

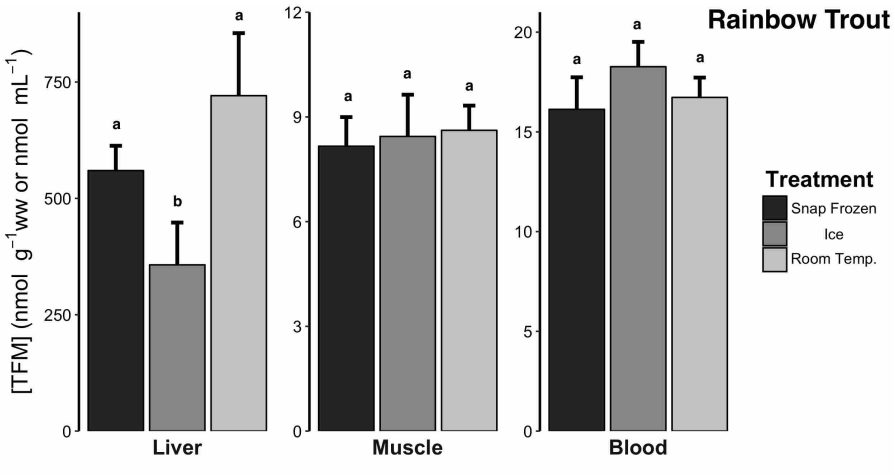
samples at room temperature, even with NaF preservative, is ineffective. Under such sub-optimal storage conditions, the concentrations of TFM can be drastically altered due to the degradation of TFM in the liver, and the degradation of TFM metabolites can lead to marked increases in blood TFM concentration. While liver would be the tissue of choice under optimal conditions, because it is prone to decomposition, it should be rapidly frozen and stored at -20°C until shipped to the lab. In less than optimal conditions, muscle appears to be the most reliable tissue for forensic analysis when TFM is the suspected cause of a fish kill. If blood can be samples from living or recently killed fish, vacutainers containing either lithium heparin or NaF/EDTA would be the best option because TFM and its metabolites appear to be stable in blood under even sub-optimal storage conditions for at least one week.

Table 3-1. Percent water measured in rainbow trout muscle and liver from Series 2: Effects of Storage Methods on Postmortem Stability of TFM and its metabolites. Tissues were dried to a constant mass at 60°C and corresponding percent water content was then calculated. All data are expressed as the mean ± 1 SEM (N = 8). Asterisks denote significant difference from snap-frozen values.

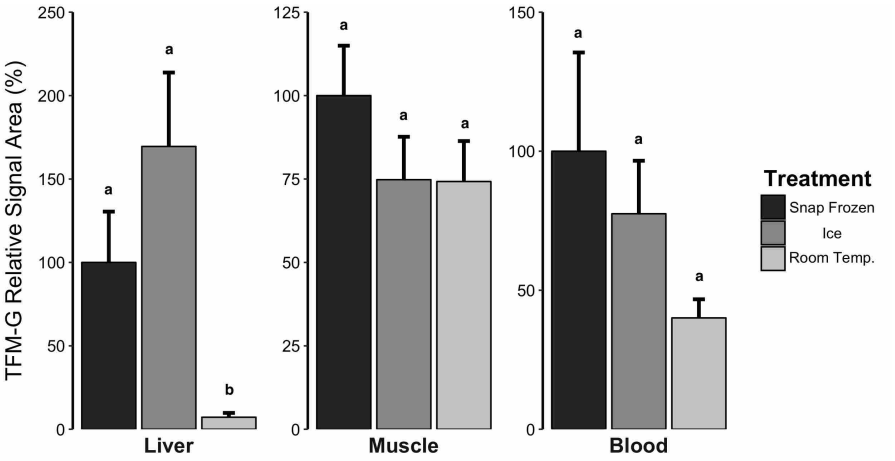
Treatment	Muscle % Water	Liver % Water
Snap-Frozen	76.4 ± 1.3 (8)	76.9 ± 0.6 (8)
-20˚C	78.3 ± 0.5 (8)	76.6 ± 0.6 (8)
4˚C (+ NaF)	85.6 ± 0.7 (8) *	87.1 ± 0.4 (8) *
4˚C (- NaF)	79.7 ± 0.6 (8)	75.1 ± 0.4 (8)
Room Temperature (+NaF)	86.2 ± 0.9 (8) *	89.0 ± 1.6 (8) *
Room Temperature (-NaF)	79.1 ± 0.7 (8)	75.4 ± 1.7 (8)

Figure 3-1. Effects of different collection and handling methods on postmortem stability of TFM and its metabolites in rainbow trout blood and tissues. A) Quantification of 3-trifluoromethyl-4-nitrophenol (TFM) in liver, muscle and blood of rainbow trout (*Oncorhynchus mykiss*) exposed to their pre-determined 9-h LC_{25} (12.9 mg l^{-1} TFM). Blood and tissues from each fish were separated into 3 different aliquots and either snap-frozen in liquid N_2, kept on ice for one hour or kept at room temperature for one hour. Relative signal area of (B) TFM-glucuronide conjugate and (C) TFM-sulfate conjugate in the same tissues of rainbow trout that were either snap-frozen in liquid N_2, kept on ice for one hour or kept at room temperature for one hour. The metabolite signal areas of samples kept on ice or at room temperature were normalized by expressing values as a percentage of values obtained from the snap-frozen (liquid N_2) samples. All data are expressed as the mean + 1 SEM (N = 10). Bars sharing the same letter are not significantly different from one another ($P < 0.05$).

(A)


Rainbow Trout
[TFM] (nmol g^{-1}ww or nmol mL^{-1})
Liver
Muscle
Blood
Treatment
Snap Frozen
Ice
Room Temp.

(B)


TFM-G Relative Signal Area (%)
Liver
Muscle
Blood
Treatment
Snap Frozen
Ice
Room Temp.

(C)

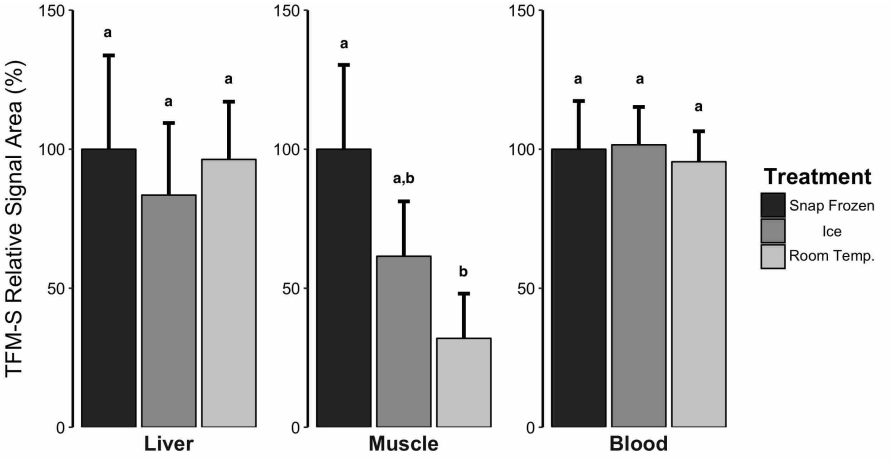
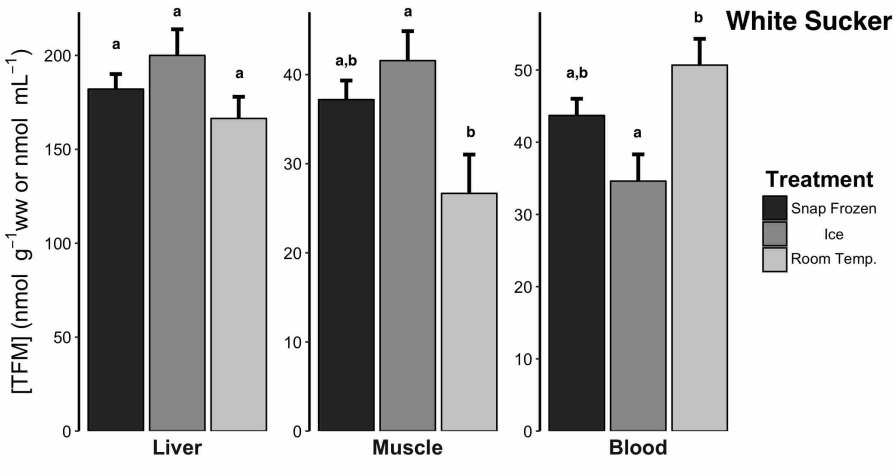
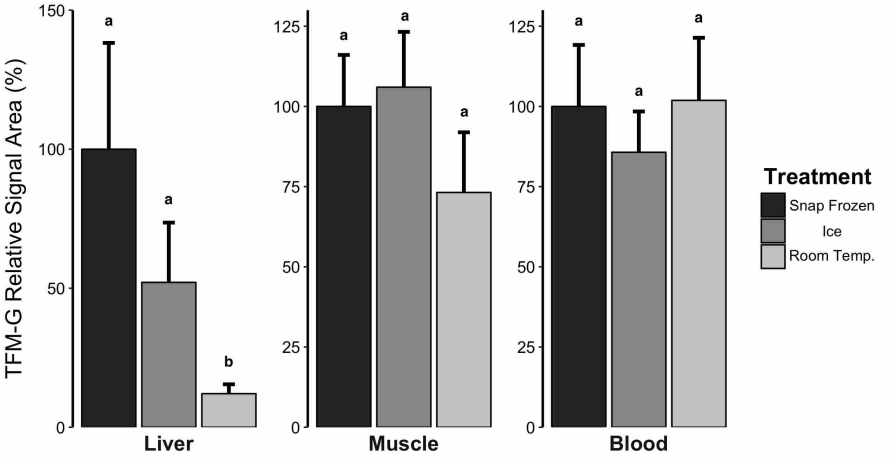

TFM-S Relative Signal Area (%)
Liver
Muscle
Blood
Treatment
Snap Frozen
Ice
Room Temp.

Figure 3-2. Effects of different collection and handling methods on postmortem stability of TFM and its metabolites in white sucker blood and tissues. A) Quantification of 3-trifluoromethyl-4-nitrophenol (TFM) in liver, muscle and blood of white sucker (*Catostomus commersonii*) exposed to their pre-determined 9-h LC_{25} (18.88 mg l^{-1} TFM). Blood and tissues from each fish separated into 3 different aliquots and either snap-frozen in liquid N_2, kept on ice for one hour or kept at room temperature for one hour. Relative signal area of (B) TFM-glucuronide conjugate and (C) TFM-sulfate conjugate in the same tissues of white sucker that were either snap-frozen in liquid N_2 kept on ice for one hour or kept at room temperature for one hour. The metabolite signal areas of samples kept on ice or at room temperature were normalized by expressing values as a percentage of values obtained from the snap-frozen (liquid N_2) samples. All data are expressed as the mean + 1 SEM (N = 10). Bars sharing the same letter are not significantly different from one another ($P < 0.05$).

(A)


White Sucker
[TFM] (nmol g^{-1}ww or nmol mL^{-1})
Liver
Muscle
Blood
Treatment
Snap Frozen
Ice
Room Temp.

(B)


TFM-G Relative Signal Area (%)
Liver
Muscle
Blood
Treatment
Snap Frozen
Ice
Room Temp.

(C)

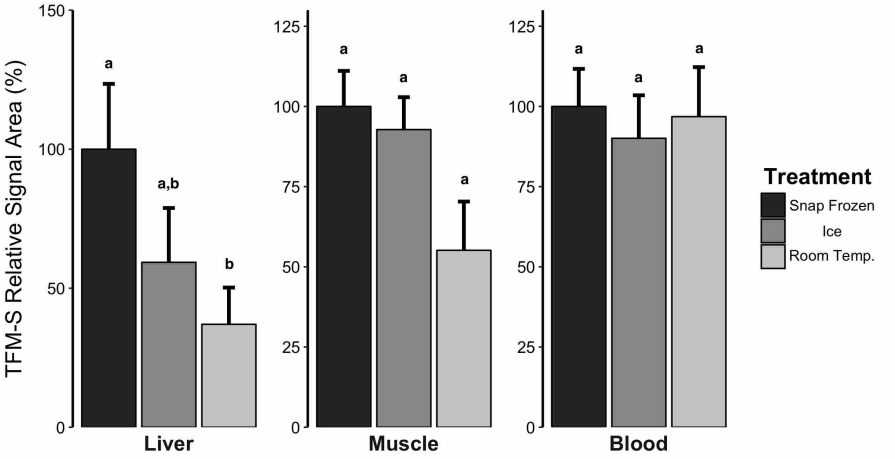
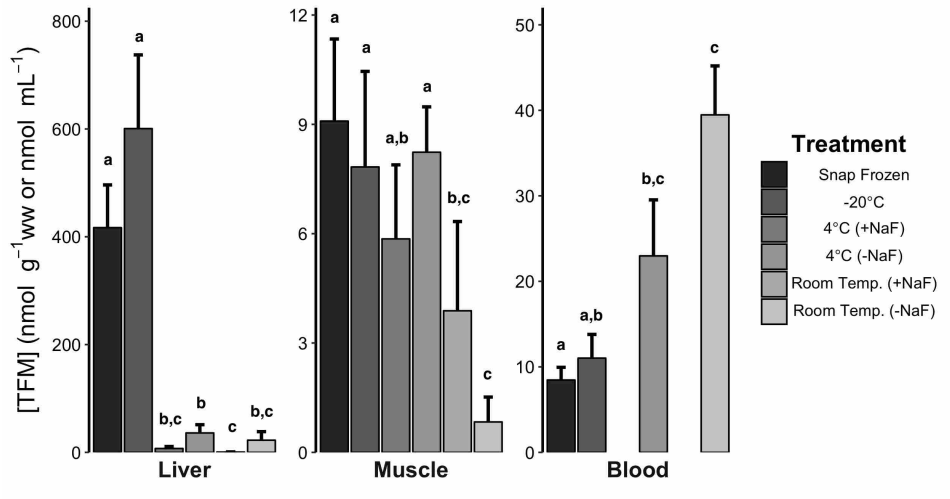
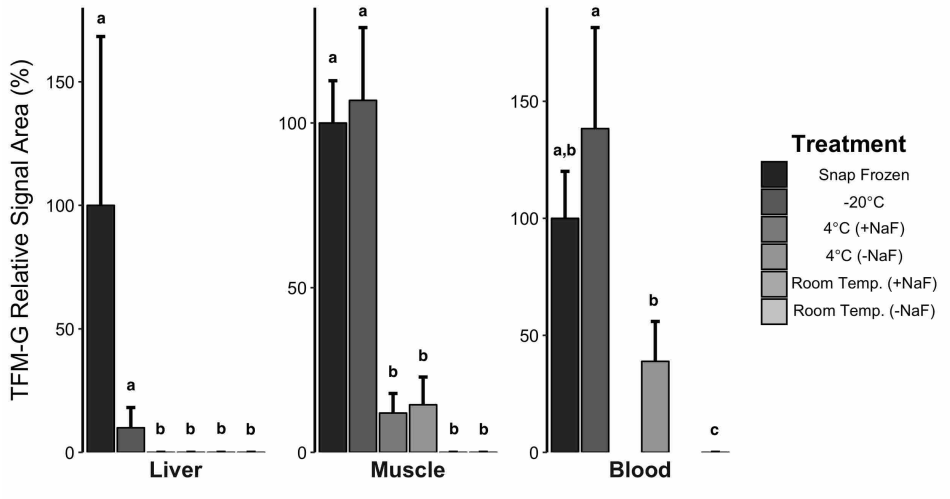

TFM-S Relative Signal Area (%)
Liver
Muscle
Blood
Treatment
Snap Frozen
Ice
Room Temp.

Figure 3-3. Effects of different storage methods on postmortem stability of TFM and its metabolites in the blood and tissues of rainbow trout. A) Quantification of 3-trifluoromethyl-4-nitrophenol (TFM) in liver, muscle and blood of rainbow trout (*Oncorhynchus mykiss*) exposed to their pre-determined 9-h LC_{25} (12.9 mg l^{-1} TFM). Blood and tissues from each fish separated into 6 different aliquots and either snap-frozen in liquid N_2 or kept at -20˚C, 4˚C with NaF preservative, 4˚C without NaF preservative, room temperature with NaF preservative, or room temperature without NaF preservative for one week. Relative signal area of (B) TFM-glucuronide conjugate and (C) TFM-sulfate conjugate in the same tissues and treatments as above. The metabolite signal areas of all samples kept on at -20˚C, 4˚C and room temperature were normalized by expressing their values as a percentage of values obtained from the snap-frozen (liquid N_2) samples. All data are expressed as the mean + 1 SEM (N = 10). Bars sharing the same letter are not significantly different from one another ($P < 0.05$).

(A)


[TFM] (nmol g^{-1}ww or nmol mL^{-1})
Liver
Muscle
Blood
Treatment
Snap Frozen
-20°C
4°C (+NaF)
4°C (-NaF)
Room Temp. (+NaF)
Room Temp. (-NaF)

(B)


TFM-G Relative Signal Area (%)
Liver
Muscle
Blood
Treatment
Snap Frozen
-20°C
4°C (+NaF)
4°C (-NaF)
Room Temp. (+NaF)
Room Temp. (-NaF)

(C)

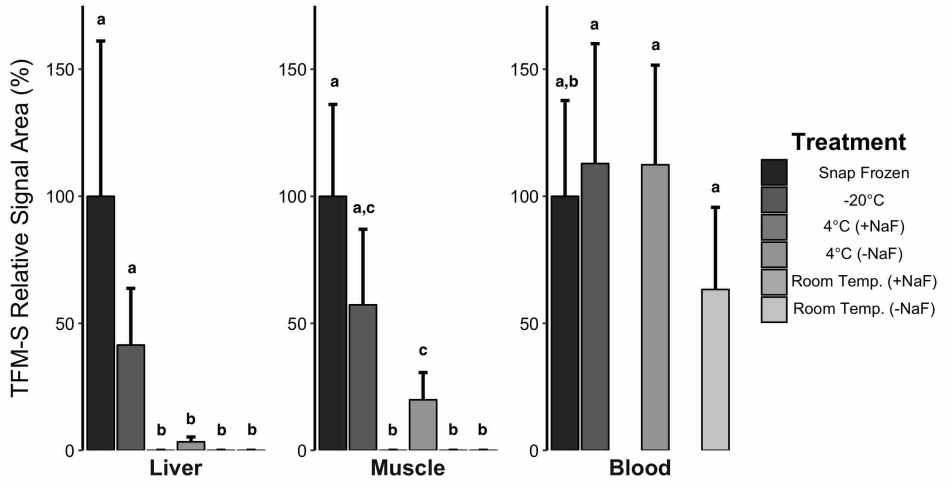
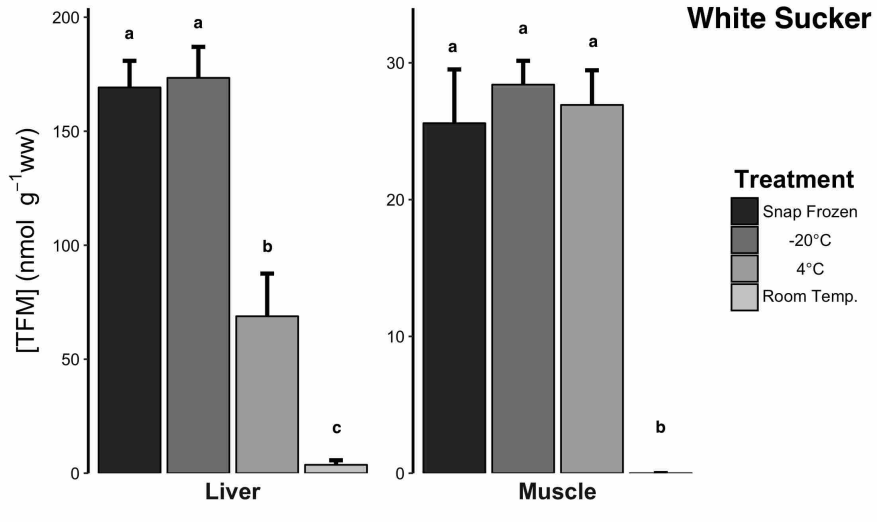
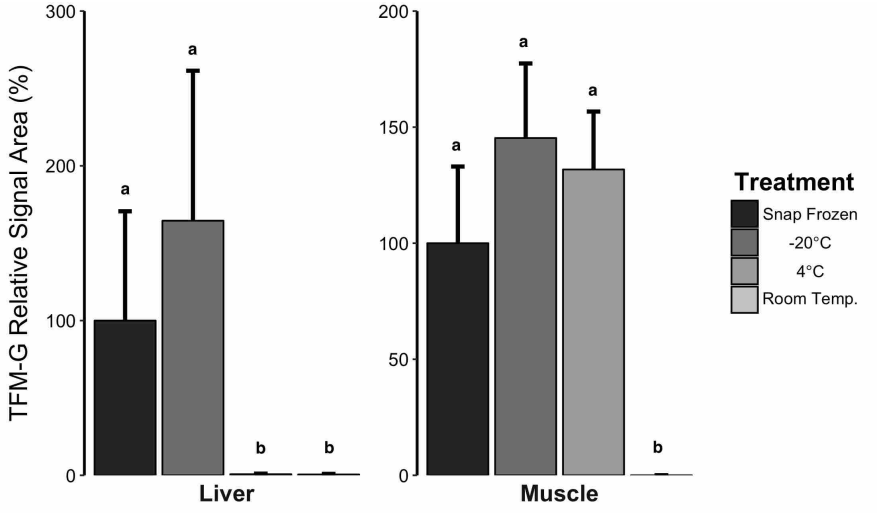

TFM-S Relative Signal Area (%)
Liver
Muscle
Blood
Treatment
Snap Frozen
-20°C
4°C (+NaF)
4°C (-NaF)
Room Temp. (+NaF)
Room Temp. (-NaF)

Figure 3-4. Effects of different storage methods on postmortem stability of TFM and its metabolites in the blood and tissues of white sucker. A) Quantification of 3-trifluoromethyl-4-nitrophenol (TFM) in liver, muscle and blood of white sucker (*Catostomus commersonii*) exposed to their pre-determined 9-h LC_{25} (18.88 mg l^{-1} TFM). Blood and tissues from each fish separated into 6 different aliquots and either snap-frozen in liquid N_2 or kept at -20˚C, 4˚C without NaF preservative, or room temperature without NaF preservative for one week. Relative signal area of (B) TFM-glucuronide conjugate and (C) TFM-sulfate conjugate in the same tissues and treatments as above. The metabolites signal areas of samples kept at-20˚C, 4˚C and at room temperature were normalized by expressing their values as a percentage of the values obtained from the snap-frozen (liquid N_2) samples. All data are expressed as the mean + 1 SEM (N = 10). Bars sharing the same letter are not significantly different from one another ($P < 0.05$)

(A)


White Sucker
[TFM] (nmol g^{-1}ww)
Liver
Muscle
Treatment
Snap Frozen
-20°C
4°C
Room Temp.

(B)


TFM-G Relative Signal Area (%)
Liver
Muscle
Treatment
Snap Frozen
-20°C
4°C
Room Temp.

(C)

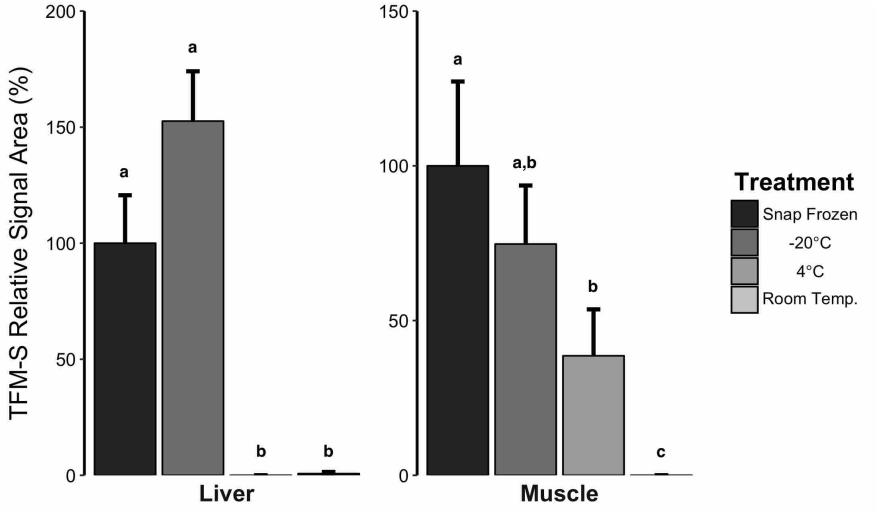
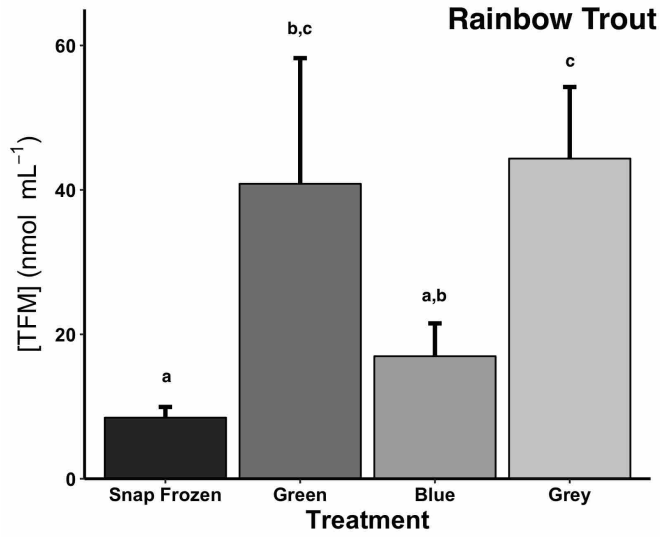
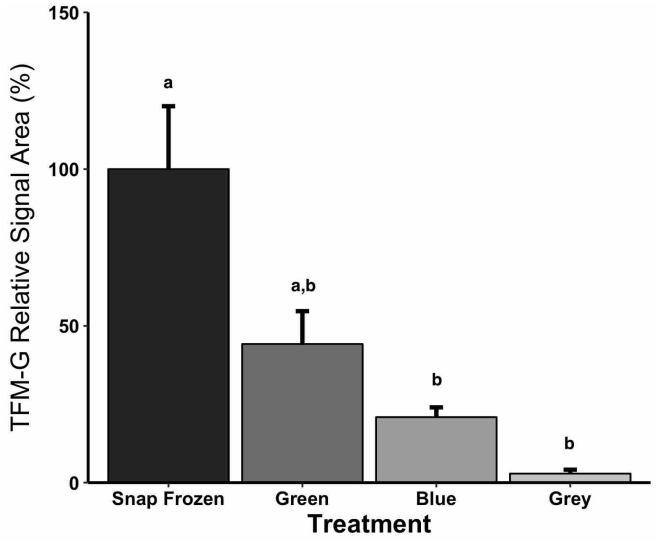

TFM-S Relative Signal Area (%)
Liver
Muscle
Treatment
Snap Frozen
-20°C
4°C
Room Temp.

Figure 3-5. Effects of anticoagulants and preservatives on the stability of TFM and its metabolites in rainbow trout blood. A) Quantification of 3-trifluoromethyl-4-nitrophenol (TFM) in the blood of rainbow trout (*Oncorhynchus mykiss*) exposed to their pre-determined 9-h LC_{25} (12.9 mg l^{-1} TFM). Blood from each fish was separated into 4 different aliquots and either snap-frozen in liquid N_2 or kept in one of 3 different vacutainers and kept at 4˚C for one week. The three types of containers contained (i) lithium heparin (green), (ii) 0.109 M sodium citrate (blue) and (iii) NaF and Na_2 Ethylenediaminetetraacetic acid (EDTA; grey). Relative signal area of (B) TFM-glucuronide conjugate and (C) TFM-sulfate conjugate in the same tissues and treatments. The metabolite signal areas of samples kept in vacutainers were normalized by expressing their values as a percentage of values obtained from the snap-frozen (liquid N_2) samples. All data are expressed as the mean + 1 SEM (N = 10). Bars sharing the same letter are not significantly different from one another ($P < 0.05$).

(A)


Rainbow Trout
[TFM] (nmol mL^{-1})
60
40
20
0
a
b,c
a,b
c
Snap Frozen
Green
Blue
Grey
Treatment

(B)


TFM-G Relative Signal Area (%)
150
100
50
0
a
a,b
b
b
Snap Frozen
Green
Blue
Grey
Treatment

(C)

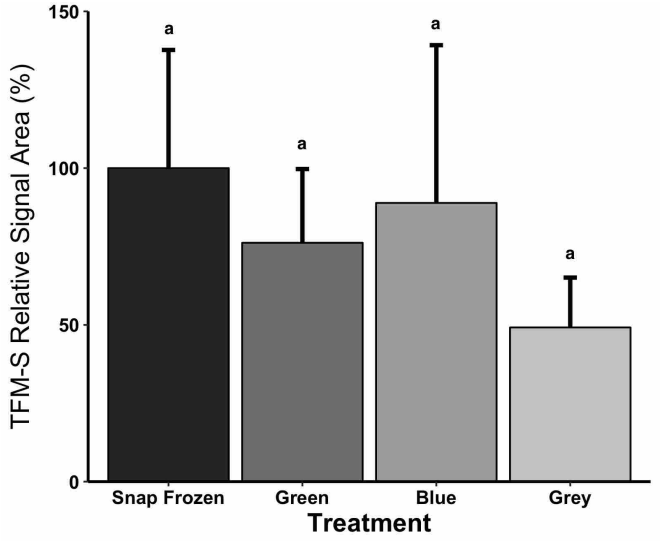
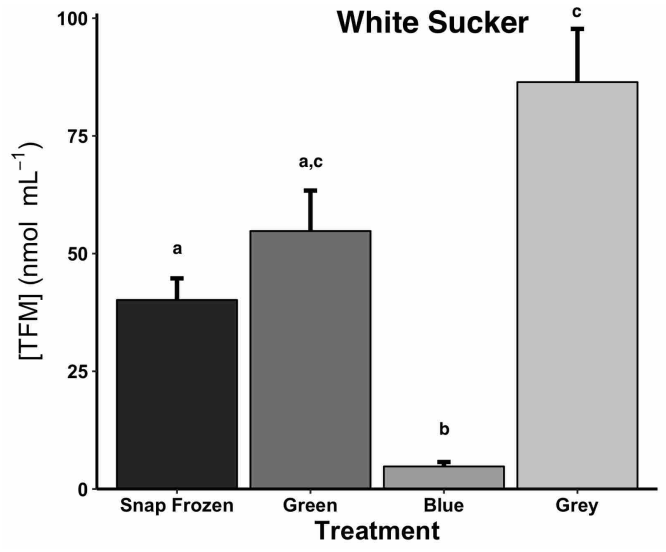
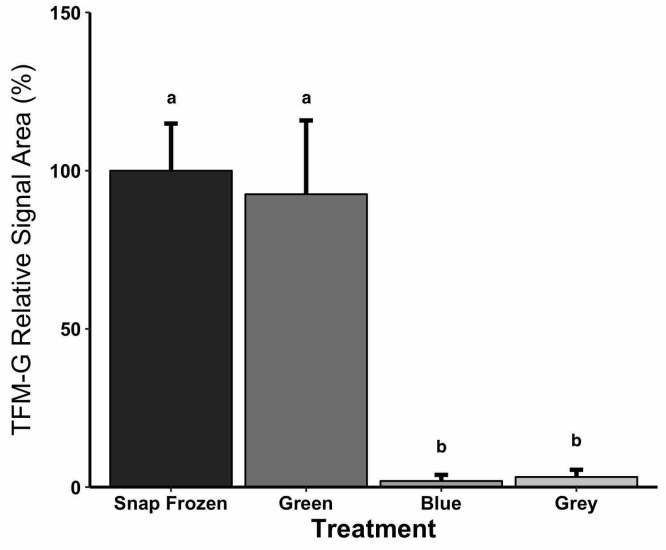

TFM-S Relative Signal Area (%)
150
100
50
0
a
a
a
a
Snap Frozen
Green
Blue
Grey
Treatment

Figure 3-6. Effects of anticoagulants and preservatives on the stability of TFM and its metabolites in white sucker blood. A) Quantification of 3-trifluoromethyl-4-nitrophenol (TFM) in the blood of white sucker (*Catostomus commersonii*) exposed to their pre-determined 9-h LC_{25} (18.88 mg l^{-1} TFM). Blood from each fish was separated into 4 different aliquots and either snap-frozen in liquid N_2 or kept in one of 3 different vacutainers and kept at 4°C for one week. The three types of containers contained (i) lithium heparin (green), (ii) 0.109 M sodium citrate (blue) and (iii) NaF and Na_2 Ethylenediaminetetraacetic acid (EDTA; grey). Relative signal area of (B) TFM-glucuronide conjugate and (C) TFM-sulfate conjugate in the same tissues and treatments. The metabolite signal areas of samples kept in vacutainers were normalized by expressing their values as a percentage of values obtained from the snap-frozen (liquid N_2) samples. All data are expressed as the mean + 1 SEM (N = 10). Bars sharing the same letter are not significantly different from one another ($P < 0.05$).

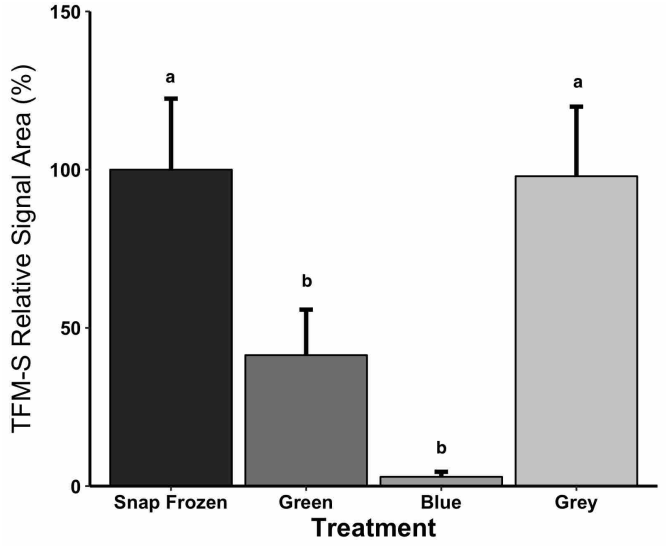
(A)


White Sucker
[TFM] (nmol mL^{-1})
100
75
50
25
0
a
a,c
b
c
Snap Frozen
Green
Blue
Grey
Treatment

(B)


TFM-G Relative Signal Area (%)
150
100
50
0
a
a
b
b
Snap Frozen
Green
Blue
Grey
Treatment

(C)


TFM-S Relative Signal Area (%)
150
100
50
0
a
b
b
a
Snap Frozen
Green
Blue
Grey
Treatment

Chapter 4:

Integrated Tissue Sampling Protocol for the Postmortem Measurement of TFM and its Metabolites

Introduction

The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) is selectively toxic to larval sea lampreys (*Petromyzon marinus*) due to their lower capacity to detoxify the compound via glucuronidation and sulfation compared to other non-target fishes (Lech, 1974; Lech and Statham, 1975; Kane et al., 1994; Bussy et al., 2017b). During typical stream treatments, TFM is applied at concentrations that are sub-lethal to most non-target fishes, however, unexpected events such as sudden drops in water pH or changes in water flow could increase the bioavailability and toxicity of TFM resulting in non-target mortality (Bills et al. 2003; Boogaard et al 2003; McDonald and Kolar, 2007). Other, unrelated factors including disease, contamination, oxygen depletion and drastic temperature changes could also cause death to fishes (Meyer and Barclay, 1990). Therefore in situations when the cause of death is uncertain, measuring tissue levels of TFM and its metabolites could provide insight into whether or not TFM played a role in the death of the fishes. The present thesis provides qualitative and quantitative data on the forensic markers of lampricide toxicity for the facilitation of non-target morality investigations.

Current Sampling and Storage Protocols and Areas of Improvement

Cold temperatures are known to slow down the process of decomposition that inevitably occurs in tissues following death. However, little is known about the effects that various storage techniques, as well as how different tissues can affect the stability of TFM and its metabolites. The Great Lakes Fishery

Commission currently has standard operating procedures (SOPs) in place for the collection, handling and storage of fish samples when it is not obvious whether or not non-target mortality is due to lampricide exposure. The procedures include the collection of blood, muscle and liver, and storage of all samples on ice (or dry ice if it available) for delivery to UMESC and subsequent analysis of lampricide concentrations (Great Lakes Fishery Commission, 2016). In some cases, dry ice may not be available on site, or within a reasonable distance of the fish kill. It is therefore imperative to ascertain whether or not TFM and its metabolites are stable under sub-optimal storage conditions, and which tissues are most reliable in the case when optimal storage is not an option.

While it would be difficult to be able to conclude for certain that an internal concentration of TFM or any of its metabolites are indicative of TFM toxicity, especially if the fish kill was not observed for a few days, data comparing various external exposure concentrations with corresponding internal tissue concentrations could provide a valuable reference for these investigations. This thesis set out to characterize how the internal distribution of TFM and its metabolites are affected by various exposure concentrations, and whether or not these measurements can provide useful information that can help distinguish between death caused by lampricide toxicity and death caused by other possible factors for the investigations unexplained fish kills that coincide with lampricide applications. A second major goal of this thesis was to provide further recommendations for the collection, handling and storage of

blood and tissue samples to be incorporated into current standard operating procedures for investigations of unexplained non-target mortality.

Legal Implications

Tissue samples are collected in the case of a moderate to major fish kill, which is defined as 100 or more non-target fish deaths per 1.6 km (Great Lakes Fishery Commission, 2016). Besides taking biological samples from up to 20 of each species of fish found dead, water samples are collected along with quantifying the number of dead fish, recording properties of water chemistry and completing a number of different fish kill investigation forms found in TOP:026.8: Protocol for investigations of and responses to unexplained mortality of non-target fish. The collection of samples and the recording of all data is very important and must be performed following outlined procedures because it could possibly be used in legal proceedings, including cases of civil litigation, or in a worse case scenario, criminal proceedings in the event that charges are laid by government authorities in Canada or the United States. It is therefore critical that all samples must follow chain of custody to ensure the integrity of the evidence that is provided by the tissue samples during the investigation. Data from this thesis will help augment these sampling procedures to ensure that the tissues are stored and shipped in a manner so that the chain of custody is maintained and the samples are reliable and admissible for legal proceedings.

What Concentrations of TFM Could Cause Death in Non-Target Fishes

The observation of a "spill over" effect that occurs in blood and muscle when the detoxification capacity of the liver becomes overwhelmed due to exposure to lethal concentrations of TFM could have major implications in improving the quality of information that is gathered from this analysis. By measuring TFM concentrations in the blood, muscle and liver it may be possible to determine a lethal threshold for each of these tissues, namely a threshold in which the uptake of TFM will be too high for the liver to actively detoxify at a rate high enough to prevent spill over of the TFM into general circulation, ultimately causing death due to the inhibition of mitochondrial ATP production (Birceanu et al., 2011). In agreement with the current protocol for collection of tissues from non-target fishes (Appendix D; TOP:026.8; Great Lakes Fishery Commission, 2016), liver, muscle and blood (whole), should be collected, along with water samples to determine approximate exposure concentrations, which could be compared to charts similar to Figure 2-5 to determine if the tissue thresholds were exceeded, which would indicate whether or not TFM toxicity could have caused death.

Quickly collecting blood and tissue samples directly after death and freezing the samples as soon as possible would be the ideal scenario to generate samples for the reliable determination of TFM that could cause death. However, in the case of non-target mortality, this is typically not the case because mortalities could go unobserved for some time, and ideal sampling and storage conditions might not always be available or practical. A a recent study by White

(2018) found that whole body decomposition of rainbow trout in warm water (20˚C) causes a significant decrease in TFM levels in muscle and liver by 40-50%. At 4˚C, however, TFM concentrations in trout muscle and liver were no different from control levels of fish sampled immediately after death. It was also note-worthy that TFM was more stable in muscle than in the liver, suggesting that muscle may be the most reliable and important sample to collect, followed by liver and blood, when conditions are less than optimal (Figure 4-1; e.g. no refrigeration available; lack of ice).

The current GLFC SOP for sample collection also directs investigators to freeze samples as quickly as possible. The present study supports this practice, but it also demonstrates that even under sub-optimal conditions, measurements of value can be collected from the muscle, liver and blood. However, in both liver and blood, the quantification of TFM becomes highly complicated and less reliable because liver concentrations of TFM decrease, and TFM increases in blood if the samples remain at room temperature or 4°C for more than 24 h. Thus, it is crucial to note with care the sampling conditions and storage conditions of tissues so that the most accurate interpretation of TFM values possible are obtained.

Implications for the Great Lakes

One of the major goals of the Great Lakes Fishery Commissions Integrated Sea Lamprey Control program is to increase the effectiveness and efficiency of sea lamprey control to further reduce populations of sea lampreys in the Great

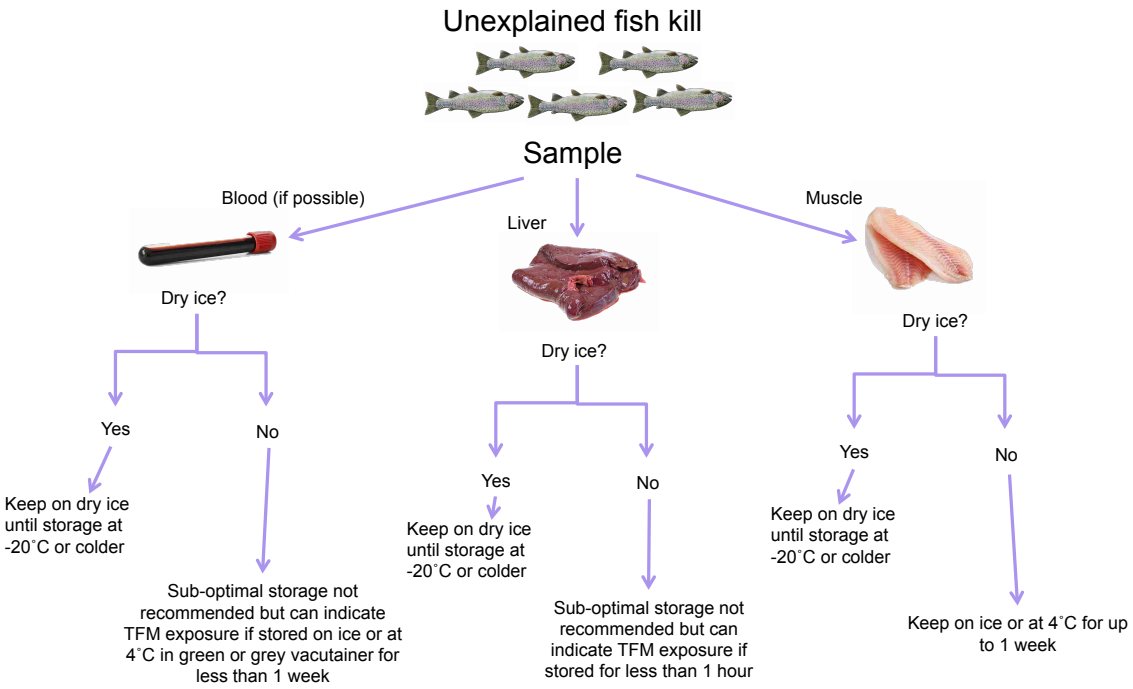
Lakes (Great Lakes Fishery Commission, 2017). Application of TFM in the tributaries of the Great Lakes is the backbone of sea lamprey control, and its effectiveness is highly dependent on applying a concentration high enough to kill as close to 100% of the lampreys present as possible. While harm to non-target fish can occur at higher concentrations, allowing the survival of a significant number sea lampreys can have far greater implications not only to those non-target fishes in the streams where the applications take place, but in the greater ecosystem of the Great Lakes. The ability to rule out TFM as the cause of a major non-target fish kill is imperative to the continuing success of the sea lamprey control program because a fish kill at a large enough scale, not to mention one that harms endangered or culturally significant species of fish could have major consequences like charges laid by Canadian or American Governments and/or retraction of funding for future programs. By improving sampling, handling and storage protocols for the analysis of non-target fish tissues in the case of unexplained mortality, greater reliability and confidence in the measurements of lampricides could provide stronger cases for the role that TFM has played in the death of the fishes.

Further Directions

Further research should be aimed at providing measurement of TFM and its metabolites in the blood and tissues of other non-target fishes that may be exposed to lethal concentrations of TFM. If a database on the internal thresholds of TFM in the different tissues can be built for a range of species, it could provide

a reliable resource to help ascertain if TFM could have caused death in instances of non-target mortality. Indeed the difference in stability of TFM in white sucker and rainbow trout liver after 1 h suggests that there could be substantial variation in the stability of TFM in the tissues of other species. For this reason it might be appropriate to develop specific storage protocols for different species. Further study could also be useful for determining the effectiveness of sodium fluoride preservative at different strengths for storage of blood and plasma.

Figure 4-1. Recommended biological sample collection and preservation for the investigation of unexplained non-target mortality. After an unexplained fish kill that coincides with a lampricide treatment, investigations are conducted and include the collection of biological samples for the measurement of lampricide concentrations to determine the cause of death of the fishes. While blood, liver and muscle can all be used to determine if fish were exposed to lampricides, not all measurements of TFM in these tissues will be reliable if they are stored at sub-optimal conditions before analysis. To accurately determine if lampricides contributed to death of the fishes, sub-optimal storage of blood and liver is not recommended, while muscle can be stored on ice or at 4˚C for up to one week.


Unexplained fish kill
Sample
Blood (if possible)
Liver
Muscle
Dry ice?
Dry ice?
Dry ice?
Yes
No
Yes
No
Yes
No
Keep on dry ice until storage at -20˚C or colder
Sub-optimal storage not recommended but can indicate TFM exposure if stored on ice or at 4˚C in green or grey vacutainer for less than 1 week
Keep on dry ice until storage at -20˚C or colder
Sub-optimal storage not recommended but can indicate TFM exposure if stored for less than 1 hour
Keep on dry ice until storage at -20˚C or colder
Keep on ice or at 4˚C for up to 1 week

Appendix A

List of Abbreviations

TFM	3-trifluoromethyl-4-nitrophenol
GLFC	Great Lakes Fishery Commission
MLC	Minimum lethal concentration
ATP	Adenosine triphosphate
UDPGT	Uridine diphosphate glucuronyltransferase
PST	Phenol sulfotransferase
PAPS	3'-phosphate 5'-phosphosulphate
APHA	American Public Health Association
FWS	United States Fish and Wildlife Service
DFO	Department of Fisheries and Oceans Canada
UMESC	Upper Midwest Environmental Sciences Center
LC	Liquid Chromatography
MS	Mass Spectrometry
Q-TOF	Quadrupole Time-of-Flight
CCAC	Canadian Council on Animal Care
DO	Dissolved Oxygen
MS222	Tricaine methanesulfonate
EC	Extraction coefficient
DF	Dilution factor
ANOVA	Analysis of Variance

SEM	Standard error of the mean
CI	95% Confidence interval
MCHC	Mean cell hemoglobin concentration
EDTA	Ethylenediaminetetraacetic acid
NAD+ -	Nicotinamide adenine dinucleotide

Appendix B

P-values

Rainbow trout tissue distribution
Parent TFM

Tissue comparison	p-value
Liver - Muscle	<.01
Liver - Whole blood	<.01
Liver - Plasma	<.01
Liver - RBC pellet	<.01
Muscle - Whole blood	0.38
Muscle - plasma	<.01
Muscle - RBC pellet	<.01
Whole blood - Plasma	<.01
Whole blood - RBC pellet	<.01
Plasma - RBC pellet	<.01

TFM-OG

Tissue comparison	p-value
Liver - Muscle	0.0000
Liver - Whole blood	0.0024
Liver - Plasma	0.0271
Liver - RBC pellet	0.0000
Muscle - Whole blood	0.0034
Muscle - plasma	0.0002
Muscle - RBC pellet	0.3501
Whole blood - Plasma	0.1859
Whole blood - RBC pellet	0.0102
Plasma - RBC pellet	0.0007

TFM-OS

Tissue comparison	p-value
Liver - Muscle	0.0001
Liver - Whole blood	0.1262
Liver - Plasma	0.3881
Liver - RBC pellet	0.1724
Muscle - Whole blood	0.0000
Muscle - plasma	0.0000
Muscle - RBC pellet	0.0032
Whole blood - Plasma	0.1948
Whole blood - RBC pellet	0.0184
Plasma - RBC pellet	0.1096

White sucker tissue distribution
Parent TFM

Tissue Comparison	p-value
Muscle – Liver	<.01
Blood - Liver	<.01
Blood - Muscle	.34

TFM-OG

Tissue Comparison	p-value
Muscle – Liver	<.01
Blood - Liver	<.01
Blood - Muscle	<.01

TFM-OS

Tissue Comparison	p-value
Muscle – Liver	.02
Blood - Liver	0.67
Blood - Muscle	<.01

Dose dependent changes in the distribution of TFM in rainbow trout tissues
Parent TFM
Alive fish - Liver

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
11.3 - 5.5	0.9583094
16.5 - 5.5	0.9999979
21.9 - 5.5	0.6801090
25.3 - 5.5	0.0052211**
16.5- 11.3	0.9617530
21.9 - 11.3	0.3712477
25.3 - 11.3	0.0011885**
21.9 -16. 5	0.6355750
25.3 -16.5	0.0037964**
25.3 -21. 9	0.2283349

Parent TFM
Alive fish - Muscle

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
11.3 - 5.5	0.0000286
16.5 - 5.5	0.0000000
21.9 - 5.5	0.0000000
25.3 - 5.5	0.0000062

16.5 -11.3	0.0000239
21.9 -11.3	0.0014261
25.3 -11.3	0.0707421
21.9 -16.5	0.9691163
25.3 -16.5	0.9942229
25.3 - 21.9	0.9293646

Parent TFM
Alive fish - Blood

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
11.3 - 5.5	0.2679474
16.5 - 5.5	0.0084949
21.9 - 5.5	0.0002308
25.3 - 5.5	0.0016867
16.5 -11.3	0.5813251
21.9 -11.3	0.0128039
25.3 -11.3	0.0447665
21.9 -16.5	0.1325901
25.3 -16.5	0.2687082
25.3 - 21.9	0.9999843

Parent TFM
Dead fish - Liver

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
25.3 - 21.9	0.1202222
31 - 21.9	0.3908462
31 - 25.3	0.6409272

Parent TFM
Dead fish - Muscle

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
25.3 - 21.9	0.9255447
31 - 21.9	0.9656749
31 - 25.3	0.9882983

Parent TFM
Dead fish - Blood

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
25.3 - 21.9	0.82
31 - 21.9	0.92
31 - 25.3	0.93

Parent TFM
Dead vs. alive - Liver

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
21.9	0.8782
25.3	0.576

Parent TFM
Dead vs. alive - Muscle

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
21.9	0.00914
25.3	0.0005057

Parent TFM
Dead vs. alive - Blood

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
21.9	0.06955
25.3	0.01103

TFM-OG
Alive fish - Liver

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
11.3 - 5.5	0.0057
16.5 - 5.5	0.0000
21.9 - 5.5	0.0002
25.3 - 5.5	0.0042
16.5- 11.3	0.0125
21.9 - 11.3	0.0289
25.3 - 11.3	0.1199*
21.9 -16. 5	0.3286*
25.3 -16.5	0.4529*
25.3 -21. 9	0.3355*

TFM-OG
Alive fish - muscle

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
11.3 - 5.5	.67
16.5 - 5.5	<.01
21.9 - 5.5	<.01
25.3 - 5.5	<.01

16.5- 11.3	.06
21.9 - 11.3	<.01
25.3 - 11.3	.07
21.9 - 16. 5	.02
25.3 - 16.5	.99
25.3 -21. 9	.01

TFM-OG
Alive fish - blood

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
11.3 - 5.5	0.1262564
16.5 - 5.5	0.0000004
21.9 - 5.5	0.0000360
25.3 - 5.5	0.0000138
16.5- 11.3	0.0014251
21.9 - 11.3	0.0054605
25.3 - 11.3	0.0013832
21.9 -16. 5	0.8557336
25.3 -16.5	0.4166185
25.3 -21. 9	0.9527528

TFM-OG
Dead fish - Liver

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
25.3 - 21.9	0.6691551
31 - 21.9	0.1127327
31 - 25.3	0.0121414

TFM-OG
Dead fish - Muscle

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
25.3 - 21.9	0.3018
31 - 21.9	0.0001
31 - 25.3	0.0004

TFM-OG
Dead fish - Blood

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
25.3 - 21.9	.85
31 - 21.9	.01
31 - 25.3	.02

TFM-OG
Dead vs. alive - Liver

Exposure Concentration ($mg\ l^{-1}$)	Comparison	p-value
21.9		0.4065
25.3		0.1617

TFM-OG
Dead vs. alive - Muscle

Exposure Concentration ($mg\ l^{-1}$)	Comparison	p-value
21.9		0.05731
25.3		0.4655

TFM-OG
Dead vs. alive - Blood

Exposure Concentration ($mg\ l^{-1}$)	Comparison	p-value
21.9		0.5515
25.3		0.1518

TFM-OS
Alive fish - Liver

Exposure Concentration ($mg\ l^{-1}$)	Comparison	p-value
11.3 - 5.5		0.0052
16.5 - 5.5		0.0000
21.9 - 5.5		0.0053
25.3 - 5.5		0.1466
16.5- 11.3		0.0148
21.9 - 11.3		0.1860
25.3 - 11.3		0.3343
21.9 -16. 5		0.3018
25.3 -16.5		0.0460
25.3 -21. 9		0.1557

TFM-OS
Alive fish - muscle

Exposure Concentration ($mg\ l^{-1}$)	Comparison	p-value
11.3 - 5.5		0.0512394
16.5 - 5.5		0.0000001
21.9 - 5.5		0.0000000
25.3 - 5.5		0.0200320

16.5- 11.3	0.0010363
21.9 - 11.3	0.0000058
25.3 - 11.3	0.5269505
21.9 - 16. 5	0.0304156
25.3 - 16.5	0.9076576
25.3 -21. 9	0.0373940

TFM-OS
Alive fish - blood

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
11.3 - 5.5	0.0363
16.5 - 5.5	0.0000
21.9 - 5.5	0.0000
25.3 - 5.5	0.0150
16.5- 11.3	0.0028
21.9 - 11.3	0.0004
25.3 - 11.3	0.1284
21.9 -16. 5	0.0632
25.3 -16.5	0.3206
25.3 -21. 9	0.0653

TFM-OS
Dead fish - Liver

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
25.3 - 21.9	0.2942
31 - 21.9	0.0077
31 - 25.3	0.0010

TFM-OS
Dead fish - Muscle

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
25.3 - 21.9	0.8401628
31 - 21.9	0.0986484
31 - 25.3	0.2701480

TFM-OS
Dead fish - Blood

Exposure Concentration Comparison ($mg\ l^{-1}$)	p-value
25.3 - 21.9	0.4590045
31 - 21.9	0.0177448
31 - 25.3	0.2340224

TFM-OS
Dead vs. alive - Liver

Exposure Concentration (mg l^{-1})	Comparison	p-value
21.9		0.3336
25.3		0.1859

TFM-OS
Dead vs. alive - Muscle

Exposure Concentration (mg l^{-1})	Comparison	p-value
21.9		0.05728
25.3		0.5014

TFM-OS
Dead vs. alive - Blood

Exposure Concentration (mg l^{-1})	Comparison	p-value
21.9		0.4675
25.3		0.08471

Chapter 3
One-hour storage techniques

Parent TFM
Rainbow trout – Liver

Treatment comparison	p-value
Snap-Frozen – Ice	0.0399455
Snap-Frozen – Room Temp.	0.7646412
Ice – Room Temp.	0.0080712

Parent TFM
Rainbow trout – Muscle

Treatment comparison	p-value
Snap-Frozen – Ice	0.9967155
Snap-Frozen – Room Temp.	0.8202734
Ice – Room Temp.	0.8599578

Parent TFM
Rainbow trout – Blood

Treatment comparison	p-value
Snap-Frozen – Ice	0.4887906
Snap-Frozen – Room Temp.	0.9438065
Ice – Room Temp.	0.6859794

TFM-OG
Rainbow trout – Liver

Treatment comparison	p-value
Snap-Frozen – Ice	.62
Snap-Frozen – Room Temp.	.02
Ice – Room Temp.	.02

TFM-OG
Rainbow trout – Muscle

Treatment comparison	p-value
Snap-Frozen – Ice	0.3910163
Snap-Frozen – Room Temp.	0.3758350
Ice – Room Temp.	0.9995344

TFM-OG
Rainbow trout – Blood

Treatment comparison	p-value
Snap-Frozen – Ice	0.4527
Snap-Frozen – Room Temp.	0.0578
Ice – Room Temp.	0.0728

TFM-OS
Rainbow trout – Liver

Treatment comparison	p-value
Snap-Frozen – Ice	0.9997087
Snap-Frozen – Room Temp.	0.9272097
Ice – Room Temp.	0.9356394

TFM-OS
Rainbow trout – Muscle

Treatment comparison	p-value
Snap-Frozen – Ice	0.2442
Snap-Frozen – Room Temp.	0.0389
Ice – Room Temp.	0.1421

TFM-OS
Rainbow trout – Blood

Treatment comparison	p-value
Snap-Frozen – Ice	0.8503111
Snap-Frozen – Room Temp.	0.9791039
Ice – Room Temp.	0.9354114

Parent TFM
White sucker – Liver

Treatment comparison	p-value
Snap-Frozen – Ice	0.5162217
Snap-Frozen – Room Temp.	0.6023828
Ice – Room Temp.	0.1149167

Parent TFM
White sucker – Muscle

Treatment comparison	p-value
Snap-Frozen – Ice	0.6382117
Snap-Frozen – Room Temp.	0.0926962
Ice – Room Temp.	0.0129017

Parent TFM
White sucker – Blood

Treatment comparison	p-value
Snap-Frozen – Ice	0.1473842
Snap-Frozen – Room Temp.	0.3094014
Ice – Room Temp.	0.0063128

TFM-OG
White sucker – Liver

Treatment comparison	p-value
Snap-Frozen – Ice	0.1175
Snap-Frozen – Room Temp.	0.0007
Ice – Room Temp.	0.0233

TFM-OG
White sucker – Muscle

Treatment comparison	p-value
Snap-Frozen – Ice	0.9744073
Snap-Frozen – Room Temp.	0.2873049
Ice – Room Temp.	0.2027739

TFM-OG
White sucker – Blood

Treatment comparison	p-value
Snap-Frozen – Ice	0.8358935
Snap-Frozen – Room Temp.	0.9821298
Ice – Room Temp.	0.9191391

TFM-OS
White sucker – Liver

Treatment comparison	p-value
Snap-Frozen – Ice	0.0955
Snap-Frozen – Room Temp.	0.0188
Ice – Room Temp.	0.2199

TFM-OS
White sucker – Muscle

Treatment comparison	p-value
Snap-Frozen – Ice	0.9101754
Snap-Frozen – Room Temp.	0.0424586
Ice – Room Temp.	0.0989332

TFM-OS
White sucker – Blood

Treatment comparison	p-value
Snap-Frozen – Ice	0.2183
Snap-Frozen – Room Temp.	0.4298
Ice – Room Temp.	0.2739

One-week storage Techniques
Parent TFM
Rainbow Trout - Liver

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.3605
Snap-Frozen – 4˚ C (+NaF)	0.0005
Snap-Frozen – 4˚ C (- NaF)	0.0117
Snap-Frozen – Room temp. (+NaF)	0.0000
Snap-Frozen – Room temp. (- NaF)	0.0008
-20˚C – 4˚ C (+NaF)	0.0001
-20˚C – 4˚ C (-NaF)	0.0043
-20˚C – Room temp. (+NaF)	0.0000
-20˚C – Room temp. (- NaF)	0.0002
4˚ C (+NaF) – 4˚ C (-NaF)	0.1544
4˚ C (+NaF) – Room temp. (+NaF)	0.1587
4˚ C (+NaF) – Room temp. (- NaF)	0.4503
4˚ C (- NaF) – Room temp. (+NaF)	0.0218
4˚ C (- NaF) – Room temp. (- NaF)	0.1860
Room temp. (+NaF) – Room temp. (- NaF)	0.1303

Parent TFM
Rainbow trout - Muscle

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.2106
Snap-Frozen – 4˚ C (+NaF)	0.0579
Snap-Frozen – 4˚ C (- NaF)	0.4857
Snap-Frozen – Room temp. (+NaF)	0.0030
Snap-Frozen – Room temp. (- NaF)	0.0001
-20˚C – 4˚ C (+NaF)	0.2211
-20˚C – 4˚ C (-NaF)	0.2004
-20˚C – Room temp. (+NaF)	0.0257
-20˚C – Room temp. (- NaF)	0.0019
4˚ C (+NaF) – 4˚ C (-NaF)	0.0538
4˚ C (+NaF) – Room temp. (+NaF)	0.1191
4˚ C (+NaF) – Room temp. (- NaF)	0.0167
4˚ C (- NaF) – Room temp. (+NaF)	0.0026
4˚ C (- NaF) – Room temp. (- NaF)	0.0001
Room temp. (+NaF) – Room temp. (- NaF)	0.1717

Parent TFM
Rainbow trout - Blood - temperatures

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.9371841
Snap-Frozen – 4˚ C	0.0203747
Snap-Frozen – Room temp.	0.0000633
-20˚C – 4˚ C	0.0679020
-20˚C – Room temp.	0.0002593
4˚ C – Room temp.	0.1591646

Parent TFM
Rainbow trout - Blood - vacutainers

Treatment comparison	p-value
Snap-Frozen – Green	0.0042802
Snap-Frozen – Blue	0.3648472
Snap-Frozen – Grey	0.0003679
Green – Blue	0.1535785
Green – Grey	0.7646271
Blue – Grey	0.0191225

TFM-OG
Rainbow Trout - Liver

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.1481
Snap-Frozen – 4˚ C (+NaF)	0.0000
Snap-Frozen – 4˚ C (- NaF)	0.0000
Snap-Frozen – Room temp. (+NaF)	0.0000
Snap-Frozen – Room temp. (- NaF)	0.0000
-20˚C – 4˚ C (+NaF)	0.0002
-20˚C – 4˚ C (-NaF)	0.0006
-20˚C – Room temp. (+NaF)	0.0002
-20˚C – Room temp. (- NaF)	0.0002
4˚ C (+NaF) – 4˚ C (-NaF)	0.3691
4˚ C (+NaF) – Room temp. (+NaF)	0.5000
4˚ C (+NaF) – Room temp. (- NaF)	0.5000
4˚ C (- NaF) – Room temp. (+NaF)	0.3691
4˚ C (- NaF) – Room temp. (- NaF)	0.3691
Room temp. (+NaF) – Room temp. (- NaF)	0.5000

TFM-OG
Rainbow trout - Muscle

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.4767
Snap-Frozen – 4˚ C (+NaF)	0.0005
Snap-Frozen – 4˚ C (- NaF)	0.0007
Snap-Frozen – Room temp. (+NaF)	0.0000
Snap-Frozen – Room temp. (- NaF)	0.0000
-20˚C – 4˚ C (+NaF)	0.0006
-20˚C – 4˚ C (-NaF)	0.0009
-20˚C – Room temp. (+NaF)	0.0000
-20˚C – Room temp. (- NaF)	0.0000
4˚ C (+NaF) – 4˚ C (-NaF)	0.4535
4˚ C (+NaF) – Room temp. (+NaF)	0.1826
4˚ C (+NaF) – Room temp. (- NaF)	0.1826
4˚ C (- NaF) – Room temp. (+NaF)	0.1534
4˚ C (- NaF) – Room temp. (- NaF)	0.1534
Room temp. (+NaF) – Room temp. (- NaF)	0.5000

TFM-OG
Rainbow trout - Blood - temperatures

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.4460
Snap-Frozen – 4˚ C	0.0525
Snap-Frozen – Room temp.	0.0001
-20˚C – 4˚ C	0.0400
-20˚C – Room temp.	0.0001
4˚ C – Room temp.	0.0285

TFM-OG
Rainbow trout - Blood - vacutainers

Treatment comparison	p-value
Snap-Frozen – Green	0.0653
Snap-Frozen – Blue	0.0034
Snap-Frozen – Grey	0.0000
Green – Blue	0.1077
Green – Grey	0.0004
Blue – Grey	0.0182

TFM-OS
Rainbow trout - Liver

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.3524
Snap-Frozen – 4˚ C (+NaF)	0.0000
Snap-Frozen – 4˚ C (- NaF)	0.0031
Snap-Frozen – Room temp. (+NaF)	0.0000
Snap-Frozen – Room temp. (- NaF)	0.0000
-20˚C – 4˚ C (+NaF)	0.0001
-20˚C – 4˚ C (-NaF)	0.0093
-20˚C – Room temp. (+NaF)	0.0001
-20˚C – Room temp. (- NaF)	0.0001
4˚ C (+NaF) – 4˚ C (-NaF)	0.0674
4˚ C (+NaF) – Room temp. (+NaF)	0.5000
4˚ C (+NaF) – Room temp. (- NaF)	0.5000
4˚ C (- NaF) – Room temp. (+NaF)	0.0674
4˚ C (- NaF) – Room temp. (- NaF)	0.0674
Room temp. (+NaF) – Room temp. (- NaF)	0.5000

TFM-OS
Rainbow Trout - Muscle

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.1885
Snap-Frozen – 4˚ C (+NaF)	0.0001
Snap-Frozen – 4˚ C (- NaF)	0.0114
Snap-Frozen – Room temp. (+NaF)	0.0001
Snap-Frozen – Room temp. (- NaF)	0.0001
-20˚C – 4˚ C (+NaF)	0.0019
-20˚C – 4˚ C (-NaF)	0.0816
-20˚C – Room temp. (+NaF)	0.0019
-20˚C – Room temp. (- NaF)	0.0019
4˚ C (+NaF) – 4˚ C (-NaF)	0.0667
4˚ C (+NaF) – Room temp. (+NaF)	0.5000
4˚ C (+NaF) – Room temp. (- NaF)	0.5000
4˚ C (- NaF) – Room temp. (+NaF)	0.0667
4˚ C (- NaF) – Room temp. (- NaF)	0.0667
Room temp. (+NaF) – Room temp. (- NaF)	0.5000

TFM-OS
Rainbow trout - Blood - temperatures

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.4464
Snap-Frozen – 4˚ C	0.2730
Snap-Frozen – Room temp.	0.0568
-20˚C – 4˚ C	0.3176
-20˚C – Room temp.	0.0430
4˚ C – Room temp.	0.0168

TFM-OS
Rainbow trout - Blood - vacutainers

Treatment comparison	p-value
Snap-Frozen – Green	0.9686782
Snap-Frozen – Blue	0.2067774
Snap-Frozen – Grey	0.3690494
Green – Blue	0.4209110
Green – Grey	0.6321339
Blue – Grey	0.9888159

Parent TFM
White Sucker - Liver

Treatment comparison	p-value
Snap-Frozen – -20˚C	1
Snap-Frozen – 4˚ C	.01

Snap-Frozen – Room temp.	<.01
-20˚C – 4˚ C	.01
-20˚C – Room temp.	<.01
4˚ C – Room temp.	<.01

Parent TFM
White sucker - Muscle

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.7699943
Snap-Frozen – 4˚ C	0.9423835
Snap-Frozen – Room temp.	0
-20˚C – 4˚ C	0.9297287
-20˚C – Room temp.	0
4˚ C – Room temp.	0

Parent TFM
White sucker - Blood – vacutainer

Treatment comparison	p-value
Snap-Frozen – Green	.46
Snap-Frozen – Blue	<.01
Snap-Frozen – Grey	.01
Green – Blue	<.01
Green – Grey	.16
Blue – Grey	<.01

TFM-OG
White Sucker - Liver

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.2991
Snap-Frozen – 4˚ C	0.0004
Snap-Frozen – Room temp.	0.0002
-20˚C – 4˚ C	0.0000
-20˚C – Room temp.	0.0000
4˚ C – Room temp.	0.4363

TFM-OG
White sucker - Muscle

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.1782
Snap-Frozen – 4˚ C	0.1667
Snap-Frozen – Room temp.	0.0013
-20˚C – 4˚ C	0.4821
-20˚C – Room temp.	0.0000
4˚ C – Room temp.	0.0000

TFM-OG
White sucker - Blood – vacutainer

Treatment comparison	p-value
Snap-Frozen – Green	0.2933
Snap-Frozen – Blue	0.0001
Snap-Frozen – Grey	0.0003
Green – Blue	0.0008
Green – Grey	0.0018
Blue – Grey	0.3974

TFM-OS
White Sucker - Liver

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.1782
Snap-Frozen – 4˚ C	0.0003
Snap-Frozen – Room temp.	0.0006
-20˚C – 4˚ C	0.0000
-20˚C – Room temp.	0.0000
4˚ C – Room temp.	0.4157

TFM-OS
White sucker - Muscle

Treatment comparison	p-value
Snap-Frozen – -20˚C	0.4907
Snap-Frozen – 4˚ C	0.0496
Snap-Frozen – Room temp.	0.0002
-20˚C – 4˚ C	0.0520
-20˚C – Room temp.	0.0003
4˚ C – Room temp.	0.0333

TFM-OS
White sucker - Blood – vacutainer

Treatment comparison	p-value
Snap-Frozen – Green	0.0330
Snap-Frozen – Blue	0.0004
Snap-Frozen – Grey	0.4458
Green – Blue	0.0671
Green – Grey	0.0242
Blue – Grey	0.0003

Appendix C

Sample sizes: Dose Dependent Changes in the distribution of TFM in Rainbow Trout Tissues

Liver

TFM Concentration (mg l^{-1})	Dead or alive	Sample size
0	Alive	10
5.5	Alive	12
11.3	Alive	15
16.5	Alive	15
21.9	Alive	4
21.9	Dead	10
25.3	Alive	3
25.3	Dead	11
31	Dead	14

Muscle

TFM Concentration (mg l^{-1})	Dead or alive	Sample size
0	Alive	10
5.5	Alive	15
11.3	Alive	15
16.5	Alive	15
21.9	Alive	4
21.9	Dead	11
25.3	Alive	3
25.3	Dead	12
31	Dead	15

Blood

TFM Concentration (mg l^{-1})	Dead or alive	Sample size
0	Alive	10
5.5	Alive	15
11.3	Alive	15
16.5	Alive	15
21.9	Alive	4
21.9	Dead	11
25.3	Alive	3
25.3	Dead	12
31	Dead	15

Appendix D

TOP:026.8

December 10, 2016

U.S. Fish and Wildlife Service Marquette Biological Station 3090 Wright Street Marquette, Michigan 49855 U.S.A.

and

U.S. Fish and Wildlife Service Ludington Biological Station 229 South Jebavy Drive Ludington, Michigan 49431 U.S.A.

and

Fisheries and Oceans Canada Sea Lamprey Control Centre 1219 Queen Street East Sault Ste. Marie, Ontario P6A 2E5 Canada

TECHNICAL OPERATING PROCEDURE

PROCEDURE TITLE:

Protocol for investigations of, and responses to unexplained mortality of nontarget fish

APPLICABILITY:

Protocol applies to all unexplained nontarget fish mortality that coincides with lampricide applications and cannot be initially attributed to the treatment. The extent that the procedures are followed is at the discretion of the Treatment Supervisor.

PRINCIPLE:

To provide a framework for investigations of, and responses to unexplained nontarget fish mortality that coincides with the use of lampricides.

EQUIPMENT REQUIRED:

See Investigation Equipment Checklist (Attachment 1)

POTENTIAL INTERFERENCES:

Not applicable

SAFETY:

Wear minimum safety equipment (gloves, boots, apron, and safety goggles). Insulated gloves are required when handling tanks of CO2 and dry ice.

DISPOSAL:

Minor fish kill Disposal of dead fish (<100) can be done by burying fish in a pit away from human or animal activity after permission is granted from property owner. All fish can be disposed in a landfill after they have been double bagged.

Moderate or major fish kill All fish should be disposed in a landfill after they have been double bagged. Contact the local landfill near the body of water where the fish kill occurred.

REAGENTS:

Not applicable

DEFINITIONS:

I. Typical Survey Effort Biological surveys are routinely conducted during a treatment and after the lampricide block has passed in order to assess treatment effectiveness, verify sea lamprey distribution and age class structure where assessments are questionable, or document nontarget mortality. Typically, staff walk a stream and collect organisms using scap nets (Fyke nets are not recommended). Nontarget organisms are identified to species and sea lampreys are counted and measured. Survey types and detailed procedures are contained in TOP:029.x.

II. Fish Kill The American Public Health Association (APHA) et al. (1985) definitions for levels of impact on fish are used for all fish except lampreys.

A. Minor kill is defined as <100 fish/1.6 km (1.0 mi.)

B. Moderate kill is defined as 100 - 1000 fish/1.6 km (1.0 mi.)

C. Major kill is defined as >1000 fish/1.6 km (1.0 mi.)

III. Environmental Protection Agency (EPA) Threshold for Nontarget Organisms Killed Involves any incident caused by a pesticide in Formal

Review for ecological concerns.

A. Fish: Affected 1,000 or more individuals of a schooling species or 50 or more individuals of a non-schooling species (AOP:007.x).

PROCEDURES:

The Flowchart for Response to Fish Mortality Concurrent with Lampricide Treatment (Appendix W) outlines the following procedures in graphical form.

Nontarget fish mortality may be discovered by Sea Lamprey Control Program (SLCP) field personnel or by personnel external to the program, including the general public. When the SLCP is notified of fish mortality by a public source the incident must be confirmed. If notification occurs after the field crew has left the area, the treatment supervisor may choose to contact another natural resources agency located in the vicinity of the treatment and ask that they confirm the report. If there is positive confirmation, the treatment supervisor will determine the appropriate amount of resources required to respond.

I. Evaluation

A. Field crew estimates severity and range of the fish kill and determines whether kill is minor, moderate, or major based on typical chemical treatment survey effort (See DEFINITIONS).

B. Field crew identifies species involved

C. Field crew notifies Treatment Supervisor that a fish kill has occurred

D. Evaluate potential impacts

1. Risk to human health

a. Exposure to disease carrying animals

b. Exposure to large amounts of decomposing organisms

c. Nontarget fish kill large enough to affect public activities

2. Fish population

a. Spawning stock versus pre-recruited stock

b. Species type 1) Valued or non-game species 2) Schooling or non-schooling (AOP:007.x)

c. Species sensitive to lampricides

E. Evaluate immediate response/consider options

1. Treatment Supervisor determines whether to deploy additional personnel and resources to assist in nontarget investigation

2. Treatment Supervisor notifies Unit Supervisor of nontarget fish kill

II. Notification

A. Unit Supervisor decides which agencies or individuals need to be notified based on consideration of the following: 1. Severity/scope of the fish kill 2. Species impacted 3. Potential for public concern

B. Agencies, organizations, and individuals that may require notification

1. Canada

a. Federal agencies 1) DFO 2) Health Canada

b. Provincial agencies 1) Ministry of Natural Resources 2) Ministry of Environment

c. Local agencies 1) Conservation authorities 2) Municipalities 3) First Nations

d. Great Lakes Fishery Commission 1) Sea Lamprey Program Director 2) Communications Director

2. United States

a. Federal agencies 1) EPA

2) USFWS (Region 3) (a) Sea Lamprey Control Field Supervisor

b. State agencies c. Local agencies

1) Municipalities 2) County Health Department 3) Native American Tribes

d. Great Lakes Fishery Commission 1) Sea Lamprey Program Director 2)

Communications Director

III. Investigation

A. Minor fish kill; follow routine procedures (see TOP:029.x)

1. Fill out collection forms (Appendices K and M)

a. U.S. records mortality data on the Larval Assessment Form

b. DFO records mortality data on the Secondary Application Data Form

2. Treatment Supervisor will determine if a 6(a)(2) report is required (AOP:007.x/US or AOP:007.x/CAN)

B. Moderate and major fish kill; investigation warranted In addition to actions taken under minor fish kill, conduct the following:

1. Quantify numbers of dead nontarget organisms. This task is accomplished immediately by field personnel using at least one of the following methods:

a. Absolute counting method 1) Sample entire area and count all nontarget fish killed 2) Measure area affected 3) Estimate collection efficiency

b. Empirical method of providing estimate based on severity and range of fish kill (See Attachment 3– Fish Counting Procedures) 1) Subsample area and count nontarget fish killed 2) Estimated area affected 3) Estimate collection efficiency

2. Collect water samples for toxicant analysis (Attachment 4 - Sample Collection and Preservation)

a. Inside the treatment area

b. In a control area outside of the treatment area

3. Collect biological samples of fish (Attachment 4 - Sample Collection and Preservation)

REFERENCES:

4. Define the stream reach or lentic area in which the fish kill occurred

a. GPS coordinates

b. Zone/Station codes

c. Branch/Lentic

5. Complete the Fish Kill Investigation Packet (Attachment 1) which would include but is not limited to:

a. List the species involved

b. List of contacts 1) Employees involved in the treatment 2) Observers (General public witnesses)

c. Possible outside contributing factors 1) Commercial/recreational fishing 2) Fire 3) Toxic algal blooms 4) Lightning 5) Electrofishing (State, Tribal, Federal, Universities) 6) Ammonia and pesticides from farming activities

d. Take photographs of anything suspected of contributing to the fish kill 1) Area treated 2) Fish killed 3) Shoreline 4) Agriculture 5) Industry 6) Other boats in the area

6. Compile water chemistry data collected prior to and during treatment which may include

a. Temperature

b. pH

c. Alkalinity

d. Dissolved oxygen

e. Ammonia

7. Compile lampricide application and concentration data collected during treatment

a. Lampricide application forms

b. Lampricide analysis forms

c. Secondary application forms

American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 1985. Standard methods for the examination of water and wastewater. 16th edition. American Public Health Association, Washington, D.C. 1268 pp.

Field Manual for the Investigation of Fish Kills. 1990. United States Department of the Interior, Fish and Wildlife Service/ Resource Publication 177.119 pp.

Queensland Department of Environment and Heritage. 1998. Fish Kill Reporting and Investigation Manual. QDEH, Brisbane.

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This procedure has been reviewed and approved by the undersigned representatives of the U.S. Fish and Wildlife Service and Fisheries and Oceans Canada.

REVIEWED/APPROVED_____
DATE_____ Field Supervisor (U.S.)

REVIEWED/APPROVED_____
DATE_____ Division Manager (Canada)

SAMPLE COLLECTION AND PRESERVATION

Collections are conducted at the discretion of the Treatment Supervisor. The intent of this section is to provide procedures to collect samples of fish and water in the event that a fish kill occurs under circumstances where the cause is not obvious. Data from the analysis of these samples is intended to assist in determining the cause(s) of the fish kill. Two types of samples will be collected. One set of samples will be collected for chemical toxicant screening. One set will be collected for determining if pathogens contributed to the fish kill. Because these data could potentially be used in litigation, it is critical that the samples be properly collected and that chain of custody is followed to ensure sample integrity. These procedures are not required when it is obvious fish mortality is due to lampricide exposure (toxicity).

I. Collection of samples for chemical toxicant screening

A. Water

1. Collect 200 mL of water from mid-depth at multiple points outside of (control) and in the treatment area(s) using a suitable grab device (Van Dorn or Kemmerer).

a. If the treatment area is a granular Bayluscide application, collect sample from each corner and in the center of the plot.

b. If the treatment area is a stream lampricide application, collect samples from upstream of the main AP (control), in the section of stream where the fish mortality occurred (upper limit, mid-way, lower limit).

2. Transfer the samples to a suitable container for storage and transport. The containers should be made of an inert substance (Teflon is preferred). The use of glass is discouraged because of the potential for breakage on freezing of the sample.

3. Place the samples in a freezer for storage. If it is not possible to immediately place the samples in a freezer, place them on ice (dry ice preferred) in a cooler until they can be transferred to a freezer or shipped.

B. Fish blood

1. Samples of approximately 2 mL of blood should be collected with a heparinized syringe from 20 fish of each species.

a. Caudal Sample

1) Position fish so that the dorsal side is down.
2) Using a heparin syringe (one per fish) position the needle between the anus and caudal fin (Photo 1).
3) Insert needle until the vertebra is hit.
4) Draw back on the syringe while pulling back the needle until vein is hit and 2 ml

of blood is drawn. May need to rotate needle to start blood flow.
5) Remove needle and place blood in Teflon tube that is completely wrapped in foil (Photo 2). Foil protects sample from light degradation.
6) Label sample as a caudal blood sample and record on Incident Synopsis and Samples Catalogue and Chain of Custody Record forms.
7) Place on ice (dry ice preferred) and deliver to UMESC (FWS) and *** (DFO).

b. Heart Sample
1) Position fish so that the dorsal side is down.
2) Using a heparin syringe (one per fish), position the needle equally between where the pectoral fins attach (Photo 3).
3) Insert needle and draw back until 2ml of blood is drawn.
4) Remove needle and place blood in Teflon tube that is completely wrapped in foil (Photo 2). Foil protects sample from light degradation.
5) Label as a heart blood sample and record on Incident Synopsis and Samples Catalogue and Chain of Custody Record forms.
6) Place on ice (dry ice preferred) and deliver to UMESC (FWS) and *** (DFO).

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