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Forensic Markers of Lampricide Toxicity in Oncorhynchsus mykiss

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by

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Abstract

The lampricide 3-trifluoromethyl-4-nitrophenol (TFM) has been used for decades to control invasive sea lamprey (*Petromyzon marinus*) populations in the Great Lakes, normally with little harm to other fish populations. However, due to the nature of lampricide treatments and the chemical properties of TFM, adverse effects to non-target fishes and non-target mortality occasionally occur. However, investigations of non-target mortality can be complex, particularly if fish deaths are not noticed immediately, due to decomposition of the carcasses. The objectives of this thesis were to expose rainbow trout (*Oncorhynchsus mykiss*) to TFM in order to: (i) identify forensic markers of lampricide toxicity and (ii) determine the stability of these markers when the fish carcass was decomposing in air or water, at different temperatures (4°C, 15°C or 20°C). To complete these objectives, LC-MS/MS was used to determine the concentration of TFM and the relative amounts of TFM metabolites in the liver and white muscle of rainbow trout exposed to their 9-h LC_{50} of TFM (the concentration of TFM that results in 50% mortality during a 9 h exposure) for 6 h. These experiments showed that the greatest accumulation of TFM occurred in the liver, in which concentrations were 15-30 fold greater than in the white muscle tissue. These observations were likely the result of the much higher blood flow to the liver compared to the muscle, and the likely presence of organic anion transporters in the hepatocytes of the liver, which would facilitate TFM uptake and other xenobiotics. Although TFM accumulation was greatest in the liver, concentrations were found to be most stable in white muscle during decomposition in both water and air, which was likely due to the muscle tissues relative isolation from the GI tract of the fish and as such the anaerobic bacteria responsible for initiating putrefaction. TFM and TFM-metabolite levels (TFM-glucuronide, TFM-sulphate) were stable in both liver and muscle at 4°C over 72 h in water. However, liver TFM concentration

declined by approximately 50% when decomposition took place at 15°C and 20°C. Similar 50 % reductions in liver TFM took place in air, but proceeded more rapidly at warmer temperatures (15°C, 20°C). In white muscle, however, TFM concentrations were stable over the 24 h decomposition period in air, despite significant signs of tissue putrefaction occurring. The metabolites of TFM, TFM-glucuronide and TFM-sulphate, were also detected but much less stable in both liver and muscle, particularly at warm temperatures. It is concluded that white muscle and liver tissue should be collected from the carcasses as quickly as possible following suspected incidents of TFM-induced non-target mortality, and that TFM concentrations in both tissues are reliable forensic markers of TFM toxicity.

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Chapter 1

General Introduction

Sea Lamprey Invasion and Control

Sea lampreys (*Petromyzon marinus*) are a jawless vertebrate, which are native to the Atlantic Ocean, but have become an invasive species in the Great Lakes (Smith, & Tibbles, 1980). Sea lamprey spend majority of their life, approximately 3-7 years, burrowed into the sediment of streams filter feeding on organic matter in the water column (Potter, 1980; Beamish, 1980). After accumulating enough body mass, the sea lamprey undergo a complex metamorphosis into juvenile parasitic lamprey, emerge from the sediment and then migrate downstream to larger bodies of water. Metamorphosis results in the development of a "suction cup" like oral disc, filled with rows of teeth, and a rasping tongue which is used by the juvenile lamprey to cut through the skin of larger fishes in order to drain blood from their host/prey for nutrients (Mallatt, 1996). This can kill the fish either directly from loss of blood or from infections resulting from the attack (Swink, & Hanson, 1989). The parasitic juvenile phase last for approximately 12-20 months, during which a single sea lamprey can kill up to 18kg (~40lbs) of fish (Potter, 1980; Swink, 2003). Following this period, the sexually maturing sea lamprey then travel back to nursery streams were they spawn and die (Potter & Beamish, 1977).

Sea Lamprey were once confined to Lake Ontario due to the natural barrier provided by Niagara Falls, however following modifications made to the Welland Canal, Sea Lamprey were able to bypass Niagara Falls in the early 1900s and invade the remaining upper Great Lakes (Applegate, 1950). Following their invasion of the upper Great Lakes, sea lamprey decimated native fish species such as lake trout (*Salvelinus namaycush*), whitefish (*Coregonus clupeiformis*), and herring/cisco (*Coregonus artedi*) (Smith, & Tibbles, 1980). This increase in sea lamprey population combined with overfishing soon resulted in the collapse of many fisheries in the Great Lakes. This also

impacted the recreational fisheries, culturally significant fish populations, as well as the overall health of the Great Lakes ecosystem (Applegate, 1950; Smith, & Tibbles, 1980; Siefkes, 2017**)**.

In an attempt to eradicate and control the invasive sea lamprey population, multiple methods of control have been developed. An early attempt of control was the use of physical and electrical barriers that would prevent the mature sea lamprey from reaching their spawning grounds (Applegate, 1950). However, these barriers also affected native fish populations and sea lamprey populations continued to proliferate, as such new control methods where sought (Applegate, 1950**)**. In 1958 Vernon Applegate and colleagues investigated over 6000 different chemical compounds as possible lampricides (a pesticide designed to kill lamprey). They discovered that 3-trifluoromethyl-4-nitrophenol (TFM) was extremely effective at killing sea lamprey larvae, but relatively non-toxic to non-target fishes (Applegate et.al. 1961).

Currently TFM is frequently mixed with the granular molluscicide, 5-chloro-N-(2-chloro-4-nitrophenyl)-2-hydroxybenzamide (niclosamide) at a ratio of 0.5-2% niclosamide to TFM for treatments, as well straight niclosamide is also used to survey lentic areas. This is done to increase the effectiveness of treatments in slow moving waters which reduces the amount of TFM needed to achieve the same end results; however, niclosamide is highly toxic to all fish (Dawson et.al. 1999; McDonald and Kolar, 2007). These compounds are applied to nursery streams and rivers in the Great Lakes basin approximately every three to five years, which can eradicate multiple generations of larval sea lamprey with a single treatment (McDonald and Kolar, 2007; GLFC 2011). The dose of TFM is determined using the minimum lethal concentration

(MLC) of TFM required to kill 99.9% of larval lamprey over a specific time period, with the dosage applied in the field being 1.2-1.5 times greater than the MLC (McDonald and Kolar 2007). Despite the relatively low toxicity to non-target fishes, exposure to these chemicals may sometimes result in adverse effects including non-target mortality (Boogaard et.al. 2003).

The mode of toxicity of TFM is the same in sea lampreys and non-target fishes (Birceanu et al. 2011). TFM is believed to act as a protonophore that transfers protons across the phospholipid bilayer of the inner mitochondrial membrane destroying the proton motive force required for ATP-synthase to produce ATP (Figure 1-1). This effectively uncouples mitochondrial oxidative phosphorylation from the electron transport chain, resulting in reduced ATP production, reduced fuel stores and eventually death (Niblett, & Ballantyne, 1976; Birceanu et.al. 2011) (Figure 1-1). However, non-target fish have a greater tolerance to TFM exposure than sea lamprey because of their greater capacity to detoxify the lampricide compared to lampreys (Lech & Statham, 1975; Kane et al 1993).

Currently, the application of lampricides is one of three major methods employed by the Great Lakes Fisheries Commission's (GLFC) sea lamprey control program. In addition to lampricides, barriers are widely used to prevent the upstream migration of adults to spawn (Applegate, 1950; Smith and Tibbles, 1980), and trapping also reduces the number of spawning adults (Smith and Tibbles, 1980; McLaughlin et al. 2007). Together these methods of control have been widely successful, reducing sea lamprey populations by 90% from historic levels in the Great Lakes (Siefkes, 2017). However, the application of lampricides is the foundation of the sea lamprey control program, with

roughly 200 tributaries being treated regularly. Although TFM has been greatly successful, the GLFC is concerned about the possible adverse effects it may have on non-target species. With this in mind the main focus of this thesis is to be able to determine if a fish kill was caused by TFM application using forensic toxicological methods. Forensic science involves the use of scientific techniques and principles in order to aid investigators during a criminal or civil case. This is done by collecting, examining and analyzing physical evidence recovered from the scene of the incident (Saferstein 2001).

TFM (chemical structure and properties) & TFM (mechanism of action)

TFM is a phenolic compound composed of an aromatic ring with a hydroxyl group attached along with a nitro and trifluoromethyl side chain. The bioavailability of TFM is heavily influenced by water chemistry, particularly pH and alkalinity. Since TFM is a weak acid with a pK_a of 6.07, its more un-ionized species increases in waters of lower pH relative to the ionized form (Figure 1-2, Bills et.al 2003; McDonald and Kolar, 2007). In its un-ionized form, TFM is more lipophilic, allowing it to be taken-up across the gills (Hunn & Allen, 1974; McDonald and Kolar, 2007; Hlina et al. 2017). If pH increases, moving away from the pKa of TFM, a majority of TFM will exists in the ionized form, making it necessary to use a greater amount of TFM to achieve the same levels of toxicity during an exposure (Cummins, 1974; Meyer & Barclay, 1990; Bills et.al 2003; McDonald and Kolar, 2007).

Detoxification and Metabolites

The greater tolerance of rainbow trout and other species to TFM is their ability to biotransform this compound into less harmful or easier to eliminate conjugates (Lech & Statham, 1975). TFM's major conjugates are TFM-glucuronide (TFMOGlu) and TFM-

sulfate (TFMOS) (Lech and Statham 1975; Kane et al. 1993; Bussy et al. 2018a,b).TFMOGlu is produced using the enzyme UDP-glucuronosyltransferase (UDPGT), in which glucuronic acid is added to lipophilic compounds such as TFM, making the compound more polar and hydrophilic, making it easier to excrete via urinary and/or gastrointestinal routes. On other hand, TFMOS is produced utilizing cytosolic sulfotransferase enzymes (SULT) to conjugate an O-sulfate with the TFM resulting in a more polar molecule (Lech and Statham 1975; Kane et al. 1993; Bussy et al. 2018a, b). Although non-target organisms have a greater ability to detoxify TFM, drops in pH can result in greater amounts of unionized TFM being bioavailable to the fish allowing TFM to cross the gill at higher rates due to its lipophilic nature (Hunn & Allen, 1974, Bills et.al 2003; McDonald and Kolar 2007) (Figure 1-2), overwhelming the animal's ability to detoxify the lampricide. The major conjugates of niclosamide are niclosamide-glucuronide and niclosamide sulfate ester, but less is known about how it is taken-up by sea lampreys and non-target fishes (Hubert et.al. 2005). The focus of the present thesis, however, will be on TFM.

Forensic markers of non-target mortality

Although non-target mortality during treatments is infrequent, it can occur following sudden decreases in water pH related to respiratory processes by plants and phytoplankton or sudden, heavy rainfall, changes in water temperature or other factors. For this reason sea lamprey control agents frequently monitor water pH and TFM application rates, allowing them to adjust to changing conditions. At lower pHs, the greater proportion of un-ionized, more lipophilic TFM results in greater rates of uptake across the gills, leading to more rapid TFM accumulation which may overwhelm the non-

target fish's ability to detoxify the compound (Hunn & Allen, 1974, Bills et.al 2003; McDonald and Kolar 2007). This will lead to a reduction in aerobic ATP production forcing the organism to generate ATP through anaerobic pathways such as glycolysis and the dephosphorylation of other high energy molecules such as creatine phosphate, or argininophosphate in the case of molluscs (Viant et al. 2002; Wilkie et al. 2007; Birceanu et al. 2009; Clifford et al. 2012).

Once anaerobic energy supplies are insufficient to meet ATP demands, it can lead to the death of the animal. Although TFM can lead to fish kills mortality may result from a number of other reasons besides lampricide application. Exposure to municipal wastes, agricultural or industrial runoff, oxygen depletion leading to hypoxia, disease, as well as the application of other pesticides, can all result in large scale fish kills (Meyer & Barclay, 1990). When a fish kill does occur, during or around the time of a lampricide treatment, it is therefore important to ascertain if the treatment could have contributed to the event. Knowledge of the internal concentrations of lampricides that are associated with death is also important for effectively investigating incidents of non-target mortality. To achieve these goals, it is therefore important to be able to reliably quantify how much TFM can lead to incidents of non-target mortality in fishes, to determine where the TFM is distributed, and how stable TFM and its metabolites are in different tissues under different environmental conditions. Moreover, decomposition following death can further complicate fish kill investigations. Since fish kills may result in legal action, as well as poor public perception of the program, it is imperative to be certain of the cause of death if it is suspected that lampricides contributed to the event. Indeed TFM application has in the past been contested in court due to the perceived dangers it would have on the

wildlife present (see Elliott v. U.S. Fish and Wildlife Service, Oct 2 1990). Thus, knowing how stable lampricides and their metabolites are in various tissues over time will help biologists and regulators to develop or modify standard operating procedures (SOPs) so that fish kill investigations related to lampricide applications are more thorough, accurate and legally admissible.

Due to the nature of lampricide application, fish kills may go un-noticed for some time; as well fish may drift away from the site of application or into it from upstream locations not treated with lampricide. The presence or absence of lampricides and their metabolites could provide investigators with forensic markers of TFM toxicity following a fish kill, and would help identify the cause of death in such investigations. However, the effectiveness of such investigations will also depend upon how stable lampricides and their metabolites are in decomposing tissues. The stability of lampricides and their metabolites in fish tissues could depend upon such factors as whether the fish remain floating in the water, or whether they wash up on shore where there are left exposed to the air. Other variables include variations in the temperature of the air or water, which tissues are analyzed and the extent of carcass decomposition (Butzbach, 2010).

The post-mortem interval (time elapsed since death) of the carcass will therefore have a large impact on the stability of parent TFM and its metabolites by directly influencing the extent of carcass decomposition. As well, proteolytic enzyme activity could alter the concentrations of these compounds or even degrade them (Butzbach, 2010). Due to the breakdown of tissue boundaries, these compounds may be able to move down concentration gradients changing the concentration of compounds found in specific tissues, as well as expose these chemicals to exogenous sources of degradation

(Butzbach, 2010). Thus, better knowledge of which tissues are best suited for measuring post-mortem concentrations of TFM, niclosamide, and their metabolites, and how their concentrations and distribution are influenced by decomposition would greatly improve our understanding of the reliability of forensic analysis in non-target fish kill investigations.

Stages and effects of Decomposition

When an organism dies, decomposition begins rapidly, starting at the cellular level. Upon death cells begin to digest themselves through a process known as autolysis. During this process cell membranes breakdown releasing hydrolytic enzymes which then break-down structural protein and lipids (Butzbach, 2010; Hau et al. 2014). After death cells of the body begin to utilize anaerobic metabolism, due to a lack of oxygen this results in the pH of the tissues to drop which activates additional proteolytic enzymes. Once cell membranes have broken down, anaerobic bacteria from the digestive and respiratory tracts begin to invade the body. The metabolic activity of these bacteria breaks down and liquefies tissues in a process known as putrefaction **(**Butzbach, 2010; Jones & Karch, 2011; Schmitt et.al. 2006**)**. These processes can be influenced by external factors such as temperature, humidity, access to air, as well as the activity of insects, vertebrates, microbes, and fungi (Payne, 1965; Schmitt et.al. 2006; Jones & Karch, 2011; Iscan & Steyn, 2013). Because the process is affected by multiple variables, there is no well-defined time line for decomposition, but according to Payne (1964) there are five or six general stages of decomposition. If a carcass is exposed to arthropods it will undergo six stages of decomposition; fresh, bloated, active decay, advanced decay, dry, and remains. Whereas a carcass protected from arthropods will undergo five stages of decomposition

including fresh, bloating and decomposition, flaccidity and dehydration, mummification, and desiccation and disintegration (Payne, 1965). During the fresh stage of decomposition, tissues no longer receive oxygen and as such cells turn to anaerobic forms of metabolism, this eventually causes cell membrane gradients to breakdown releasing hydrolytic enzymes into the cytosol leading to autolysis (Butzbach, 2010; Hau et al. 2014). Once cell membranes have degraded opportunistic bacteria invade the body, also referred to as post-mortem migration. These bacteria further degrade the tissues of the body through metabolic processes. The "bloated stage" occurs due to microbial activity, as bacteria breakdown constituent carbohydrates, lipids and protein into their base components gases are released leading to a swelling of the carcass, this may lead to the rupture of the carcass creating an aerobic environment with in the body as well as allowing liquefied tissues to leak from the carcass signaling the "active decay stage" (Payne, 1965; Butzbach, 2010). During this third stage arthropods, insects and other scavengers will begin to consume the carcass. Advanced decay is reached when majority of the soft tissue is removed, and finally the final "dry stage" is marked by only skeletal remains (Payne, 1965; Carter et al. 2007).

Decomposition is twice as quick when the carcass is exposed to air when compared to submerged in water, and eight times faster than when buried undergo, which is known as Casper's rule (Payne, 1965; Iscan & Steyn, 2013; Schmitt et.al. 2006). This may have to do with the fact that the carcass will be more or less protected from arthropods when in water, whereas in air arthropods could play an important role in the decomposition process. However it is important to note that other abiotic factors such as

variations in temperature and water influx/efflux could dramatically affect rates of decomposition and the stability of xenobiotics in tissues.

Research Objectives

Although the application of TFM is closely monitored, large scale fish kills may still occur. In the case of an unexplained fish kill it is imperative to be able to distinguish if the cause of death was due to lampricide applications, and/or whether death was related to other natural or anthropogenic causes. This would not only be important from the stand-point of legal liability, it could also rule out lampricides in some fish kill cases. In instances, where lampricides were involved in incidents of non-target mortality, alone or in association with other factors (e.g. temperature, swings in pH), it may be possible to develop measures to prevent future occurrences under similar conditions. However, to achieve such a goal, it will first be necessary to develop methods that can be used to investigate incidents of non-target mortality including the identification of the most appropriate tissue(s) to collect, how to handle and store samples, and to ascertain how potential confounding factors such decomposition affect the accuracy of subsequent analyses. With this background, the overarching goal of my thesis was to quantify lampricide and lampricide metabolite concentrations in the tissues of rainbow trout immediately following death and how decomposition would affect such measurements. Focusing on TFM, the objectives of my M.Sc. thesis were to:

i) Identify the most appropriate markers and tissues upon which to perform forensic chemical analysis following TFM-induced mortality in rainbow trout.

ii) Determine how tissue decomposition in water affected TFM and metabolite stability in rainbow trout subjected to TFM exposure.

iii) Compare the effects of air exposure on TFM and metabolite stability, to observations made on immersed fishes.

iv) Develop a practical protocol for tissue sampling collection, handling, storage and analysis that can be used to investigate suspected instances of non-target mortality following TFM application.

The model non-target species that I used in my research was the rainbow trout (*Oncorhynchus mykiss*), which is commonly found in freshwater streams and rivers in and around the Great Lakes basin. Like most other non-target fishes, rainbow trout are able to detoxify TFM by converting it to TFMOGlu through UDP-glucuronyl transferase, greatly increasing its tolerance to the compound (Lech & Statham, 1975; Kane et.al. 1993). I exposed rainbow trout to their 9 h LC_{50} of TFM for 6 h prior to euthanization. Once deceased, the carcass transferred to an exposure system that either mimicked the shore of a stream or the stream itself. For the in air decomposition experiments fish where left at room temperature in humidified air for 8 h or 24 h prior to collection of white muscle, and liver tissue. Whereas for the in water decomposition experiments fish where left in water at either 4°C, 15°C, or 20°C for 8 h, 24 h, or 72 h prior to collection of the same tissue. Both experiments had control tissue samples that did not decompose. Tissue samples were analyzed using LC-QTOF (LC-MS/MS) at the Upper Midwest Environmental Sciences Center in La Crosse, WI for both parent TFM and metabolites.

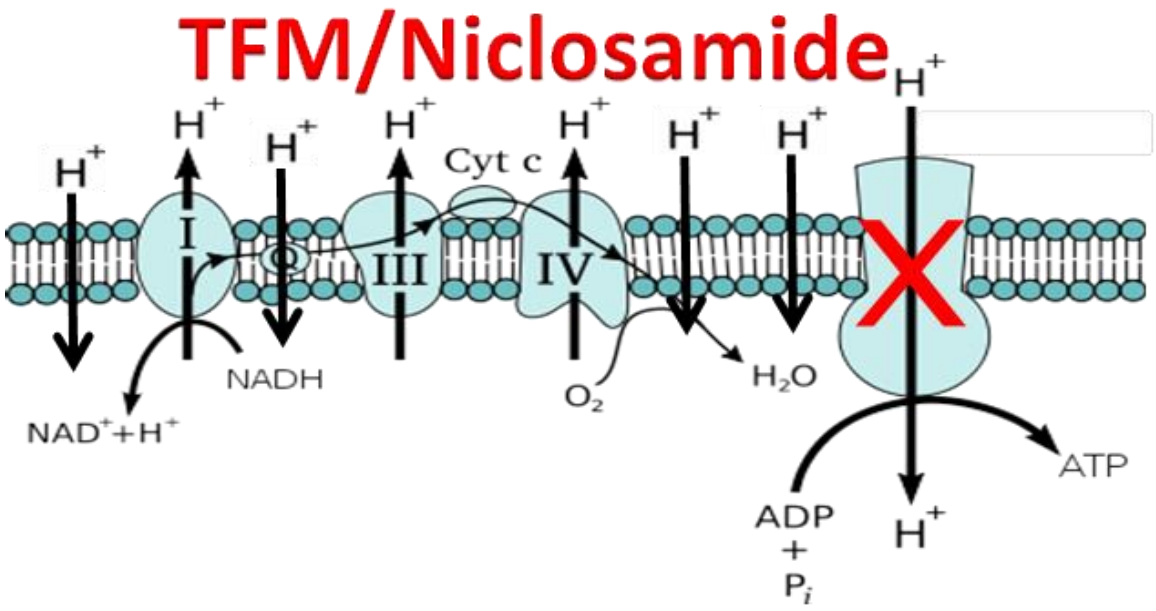

Figure 1-1: Uncoupling of Mitochondrial Oxidative Phosphorylation
Proposed mechanism of action of TFM/niclosamide. Lampricides are thought to target the inner mitochondrial membrane, where they act as protonophore that "carry" H^+ ions across the phospholipid bilayer destroying the proton gradient and the proton motive force required for ATP synthase to function. (Image modified from: https://upload.wikimedia.org/wikipedia/commons/thumb/f/f1/Mitochondrial_electron_transport_chain_short_PL.svg/467px-Mitochondrial_electron_transport_chain_short_PL.svg.png)

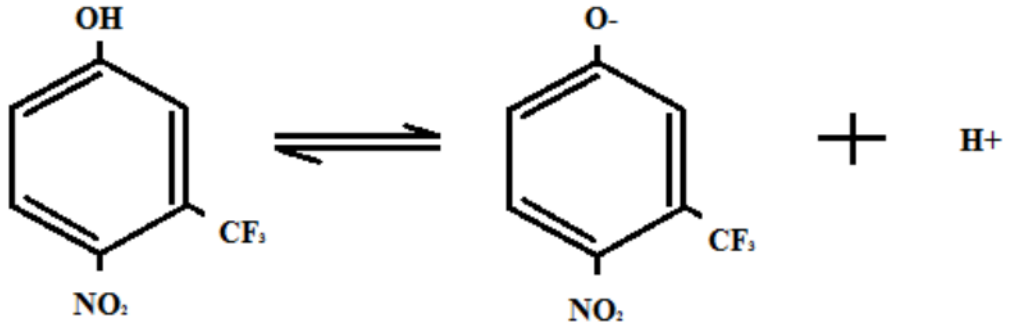

Figure 1-2: Speciation of TFM at low pH (left) and high pH (right)

The un-ionized (left) and the ionized (right) forms of TFM. The unionized form is much more lipophilic than the hydrophilic ionized form of the compound. TFM becomes ionized at more alkaline pHs due to TFM's weak acidic properties (pKa = 6.07) (Hubert, 2003).

Chapter 2

Effects of Water Exposure and Temperature on Lampricide Stability in Decomposing Rainbow Trout (*Oncorhynchsus mykiss*) Tissue.

Introduction:

An integrative pest management program to reduce the number of invasive sea lamprey (*Petromyzon marinus*) in the Great Lakes has been in place since the 1950s (Applegate et al. 1961; GLFC 2011; Siefkes 2017). This program uses barriers and traps to prevent the upstream spawning migration by adult sea lamprey, the release of sterilized males onto spawning beds, and applying piscidides (aka. lampricides) that selectively target larval sea lamprey in nursery streams (Siefkes 2017). Two lampricides are used to control sea lamprey, 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide, which is usually used in combination with TFM, in a 0.5-2% niclosamide mixture. Lampricides are the mainstay of the sea lamprey control program in the Great Lakes, but TFM is predominately used, because unlike niclosamide, it specifically targets larval sea lamprey with minimal effects on non-target organisms (Applegate et al. 1961; McDonald and Kolar 2007; Siefkes 2017).

Although TFM is more toxic to larval sea lampreys, its mechanism of toxicity is the same in different organisms. It is known to uncouple mitochondrial oxidative phosphorylation in rat, rainbow trout (*Oncorhynchus mykiss*) and sea lamprey, which results in a decrease in cellular ATP production (Niblett and Ballantyne 1976; Birceanu et al. 2011). In fishes and other vertebrates, decreased aerobic (oxygen requiring) ATP production forces them to rely on anaerobic ATP production processes such as glycolysis and the dephosphosphorylation of high energy phosphagens (e.g. creatine phosphate) to make up for the shortfall in aerobic ATP production (Viant et al. 2002; Wilkie et al. 2007; Birceanu et al. 2009; Clifford et al. 2012). Once creatine phosphate stores and glycogen are insufficient to meet ATP demands, the animal will die.

The selectivity of TFM to sea lamprey is due to their relative inability to detoxify TFM using phase II metabolic pathways, which involves the conjugation of TFM with the UDP-glucuronyl acid to generate TFM-glucuronide (TFMOGlu) or with O-sulfate to produce sulphated TFM (TFMOS), resulting in a more water soluble product that can be excreted by the animal (Lech and Statham 1975; Kane et al. 1993; Bussy et al. 2018a, b). Nevertheless, non-target mortality does occur during TFM applications, which is often the result of changes in the water chemistry during TFM application (Meyer & Barclay, 1990; McDonald and Kolar 2007; Scholefeild et al. 2008). Decreased water pH is often the cause of non-target mortality which, due to TFM's weak acidic properties (pK_a of TFM = 6.07) makes the lampricide more bioavailable to the organism. At lower, more acidic pHs TFM is more likely to be found in its un-ionized form which tends to be more lipophilic (Hunn and Allen 1974; Bills et al. 2003, McDonald and Kolar 2008). Due to this, TFM is able to cross the gill boundary layer easier than at higher pHs. As the pH becomes higher than the pKa of TFM, the TFM is more commonly found in its ionized form resulting in more TFM being needed to achieve the same levels of toxicity (Cummins 1974; Meyer & Barclay, 1990; Bills et.al 2003; McDonald and Kolar 2007). Therefore with TFM present at lower pHs non-target organisms may be unable to detoxify TFM quickly enough, leading to death (Bills et al. 2003; McDonald and Kolar 2007). However mortality could occur for any number of reasons besides the application of lampricides, such as xenobiotics originating from industrial, agricultural or municipal sources, drastic declines in oxygen levels in the water leading to hypoxia, increases in water temperature leading to increased metabolic states, as well as various diseases or viruses all of which can make investigations of fish kills difficult (Meyer & Barclay,

1990). It is therefore imperative to know if incidents of unexpected mortality that take place during or after TFM treatments are the result of TFM toxicity, other toxicants, or a combination of both.

After death, fish carcasses will either remain floating or submerged in the water as they decay, or are washed onto shore downstream. It is therefore important to know how different environments will affect the rate of decomposition as well as the stability of toxicants and their metabolites within various tissues. According to Casper's Rule, which states that "At a tolerable similar average temperature, the degree of putrefaction present in a body lying in the open air for one week (month) corresponds to that found in a body after lying in the water for two weeks (months), or lying in the earth in the usual manner for eight weeks (or months)" (Giersten 1977, as quoted in Keh 1985), it can be assumed that the decomposition of a fish carcass would be slower in water than in air accordingly (Iscan & Steyn, 2013; Schmitt et al. 2006). On the one hand, slower decomposition could be expected to lead to better preservation of toxicants and their metabolites for post-mortem toxicological analysis. On the other, a number of additional factors could complicate analysis and interpretation of toxicological data in fish carcasses including factors such as variation in temperature and water uptake by the carcass.

Lampricide treatments take place from the early spring to autumn, when water temperatures experience pronounced temporal variation. However, temperature can vary spatially within a river or stream based on water depth, groundwater inflow (upwelling) or run-off from the shoreline, which can be many degrees warmer, leading to wide differences in the rate of decomposition of a dead fish. For instance, in warmer waters microbial activity would be higher, which accelerates decomposition (Payne, 1965;

Yarema & Becker, 2005; Schmitt et al. 2006; Iscan & Jones & Karch, 2011; Iscan & Steyn, 2013). Another factor that needs to be considered when dealing with decomposition in water is the influx of external water into the tissues which could dilute toxicant concentrations or, in the case of more polar molecules, "flush" chemical agents of out of the tissues (Butzbach, 2010).

Time also plays a critical role in post-mortem toxicological analysis. As decomposition progresses it becomes increasingly likely that toxicants migrate from certain tissues and deposit in others, due to post-mortem redistribution (Yarema & Becker, 2005; Butzbach, 2010). Post-mortem redistribution (PMR) tends to be more prominent in tissues/organs closely associated with large blood vessels or the heart, such as the left lobe of the liver in humans (Yarema & Becker, 2005). Luckily other tissues seem to be relatively immune to PMR, at least until decomposition progresses, such as skeletal muscle, vitreous humor, and the right lobe of the human liver. However as decomposition proceeds, liquefaction of the internal organs could lead to greater PMR to other tissues, including tissues that are normally less vulnerable to this process such as the muscle (Yarema & Becker, 2005; Butzbach, 2010).

The goal of this study, was to ascertain how the decomposition of a fish carcass (rainbow trout, *Oncorhynchus mykiss*) in water over 3 days, and at different temperatures, affected the measurement of TFM and its metabolites in the liver and white muscle. With this aim, rainbow trout where exposed to TFM (9- h LC_{50}) for 6 h, at which time they were euthanized and either sampled immediately, or left to decompose for 8 h, 24 h, or 72 h at 4, 15, and 20°C, followed by analysis for TFM and TFM-metabolites using LC-MS/MS.

Material and Methods:

Experimental animals and holding

Juvenile rainbow trout (*Oncorhynchus mykiss*) (N = 120; 100-210g) were purchased from Rainbow Springs Trout Farm in Thamesford, Ontario in 2016 and 2017, and housed in a large, circular, holding-tank (>1000L) receiving re-circulated, City of Waterloo, de-chlorinated tap water in Laurier's Centre of Cold Regions and Water Science (CCRWS). The re-circulating water was 15 ± 1°C, drained into a large sump (volume = 1000L), from which it was filtered through a mechanical filter, followed by UV filtration before its return to the tank. Dissolved oxygen (DO %), pH, and conductivity were measured daily (DO% = 81-87%, pH = 8.2-8.6, alkalinity = 150 mg/L $CaCO_3$, temperature = 14.9-15.2). The holding room was set to a 12 hour light/dark cycle, and the trout were feed 5PT Martin complete floating trout grower feed at 2% body weight (BW) 2-3 times weekly. Food was withheld in the 48 h preceding TFM exposure, to prevent fouling of the water and interference with TFM measurements. All experiments were approved by the Wilfrid Laurier Animal Care Committee, and followed guidelines and principles of the Canadian Council of Animal Care (CCAC).

Experimental Set-up

For each experiment, rainbow trout (N = 8) where placed into individual test chambers (volume ~ 4 L; dimensions: length = 29.3 cm, width = 19.4 cm, depth = 9.5 cm) approximately 12 h prior to TFM exposure. Each separately aerated chamber was positioned on a large tray that received well-aerated water from a head tank (65 L) positioned immediately above the tray. Water drained into each chamber via flow splitters running from the head tank, before draining into a lower reservoir from which it

was returned to the head tank via a submersible pump. The entire system was cooled using a chiller set to 15°C (measured temperature range = 15.1-15.8°C) positioned in series between the lower reservoir and the head tank.

Experimental Protocol

Immediately prior to experiments pH, DO%, and alkalinity were measured, followed by the removal of the water inflow lines from each chamber (pH = 7.8-8.15, DO% = 80-91%, alkalinity = 300-350 mg/L $CaCO_3$). The system was then dosed with a known amount of TFM to establish target exposure concentrations, left for 15 min to allow sufficient mixing, and the water inflow lines re-connected to the exposure chambers. Water samples (volume = 1 mL) were collected at various time intervals during the TFM exposure period (0 h, 2 h and 5 h) to confirm that TFM concentrations were at target values. Concentrations of water TFM were determined using a NovaSpec II (Bauch and Lombe, Cambridge, England) spectrophotometer using 1.5 mL polystyrene cuvets, at a wavelength of 390 nm and standards were provided courtesy of the Sea Lamprey Control Centre, Fisheries and Oceans Canada, Sault Ste. Marie, Ontario. After the trout where exposed to the 9-h LC_{50} of TFM for 6 h, they were euthanized with a lethal dose of anesthetic (0.5g L^{-1} tricane methansulfonate buffered with 1 g L^{-1} sodium bicarbonate).

Following death, either by euthanasia or TFM toxicity, each fish was transferred to a separate chamber (identical in design to the exposure chambers) contained in an identical re-circulating system filled with TFM-free de-chlorinated water set to 4, 15, or 20°C (averaging 5.3 ± 0.5, 15.2 ± 0.3, and 21°C ± 1 respectively). Each chamber was separately aerated, and was continuously supplied with temperature-controlled water. The carcasses where left to decompose for 8, 24, or 72 h prior to the collection of whole liver,

white muscle filet, gallbladder bile, intact heart, and head kidney. Blood was collected from control fish (0 h of decomposition), but due to the decomposition process, collection of further samples was untenable. Samples of gallbladder bile, intact heart, and head kidney where not analyzed and remain in storage at -80°C. Throughout all experiments only 9 fish died due to TFM toxicity (7.5% of total fish) as well during the 4°C decomposition experiment four fish were excluded from sampling due to complications during euthanasia (4°C 8h N = 7, 4°C 24h N = 7, 4°C 72h N = 6).

Analytical Procedures

All tissue preparation and analysis was conducted at the Upper Midwest Environmental Sciences Centre (UMESC), United States Geological Survey (USGS), in La Crosse Wisconsin, USA. Tissue was delivered to UMESC on dry ice and then kept frozen at -80°C until ready for processing. Processing entailed breaking off chunks from each tissue (approximately 50-100 mg) and transferring the sample into pre-labeled and weighed, 2mL polypropylene micro-centrifuge tubes; the remaining tissues were then returned to the -80°C freezer. The samples where then diluted with a mixture of 1:1 of acetonitrile with 1% formic acid. Two 2mm stainless steel ball bearings where then added to the centrifuge tube, followed by homogenization using a GenoGrinder (1225U01,Thomas Scientific, Swedesboro, NJ) at 1200 strokes/min for 90 seconds. Exactly, 400 µL of acetonitrile with 1% formic acid was then added to the homogenate and vortexed for 15 s, after which the samples where refrigerated for 20 min, followed by centrifugation at 12000 relative centrifugal force (RCF) at room temperature for 10 min. The samples were then transferred to Phree phospholipid removal 96 well plate (8B-S133-TAK., Phenomenex, Torrance, CA) and left at room temperature. Meanwhile the centrifuge

tubes where rinsed with 500 µL of a 1% formic acid:acetonitrile mixture and then vortexed for 15 seconds. The tubes where then centrifuged again at 12000 RCF at room temperature for 10 min. Prior to adding the rinse to the sample plate, the original aliquots were filtered through the Phree cartridge into a 2 mL reservoir on a 96 well plate by centrifuging the Phree cartridges at 500 RCF for 5 min. Once the Phree cartridge was empty of the first aliquot the second aliquot was added to the cartridge followed by another round of centrifugation at 500 RCF at room temperature for 10 min. The plate was then sealed and either run directly on the LC-QTOF or kept frozen at -20°C until ready for injection.

Sample analysis was conducted using Liquid Chromatography-Quadrupole Time of Flight (LC-QTOF) LC-MS/MS instrument set-up comprising of a high-performance liquid chromatography (290 Infinity LC, Agilent, Santa Clara, CA) with a mass spectrometer (6530 Accurate-Mass Q-TOF LC/MS, Agilent, Santa Clara, CA). Reverse phased high-performance liquid chromatography was used in this study with a lipophilic column (Kinetex 1.7um EVO C18 100Angstrom 100 x 2.1mm, Phenomenex, Torrance, CA) in order to separate the parent molecule from the metabolites. Samples where then ionized at the source of the mass spectrometer before proceeding past the qudrapoles. Parent TFM was identified by retention time using HPLC and a TFM standard, as well as a mass/charge library. Metabolites where identified also using retention time with metabolites eluting faster than parent TFM, the metabolites where confirmed using a mass/charge library. TFM concentrations are expressed in nmol per gram of wet tissue (nmol g-1), while TFM metabolite data is expressed as relative amount compared to control tissue.

Dry weight and water tissue percent was determined by dehydrating tissue samples of liver and muscle taken from the same fish. Tissues where placed into pre-labeled, pre-weighed centrifuge tubes and then placed in an oven at 70°C for 24 h, after which they were weighed again. Tissue water was determined by taking the wet tissue weight and subtracting the dried tissue then dividing by the wet tissue weight and multiplying by 100. This was done for every tissue collected from the fish (Table 2-1).

Calculations and Statistics

One way ANOVAs where used to determine differences in TFM concentration between temperature at the same decomposition time point of either 8, 24 or 72 h in both white muscle and liver tissue, as well as to determine differences within the same decomposition temperature over the three decomposition time points compared to a control (8, 24 or 72 h). When a significant difference was found ($p \leq 0.05$) a Tukey post-hoc test was conducted on parametric data sets whereas if the data sets where non parametric a Kruskal-Wallis test was conducted to determine significant difference followed a Dunn's post-hoc test being conducted if applicable.

Results:

Effects of Decomposition on Tissue water in Liver and White Muscle Tissue

The percent tissue water found in fresh liver tissue taken from rainbow trout was 75.9 ± 1.13% which was comparable to the percent tissue water found in white muscle tissue which was 79 ± 0.63%. Over all temperatures and decomposition time points percent tissue did not significantly change in either liver or white muscle tissues. Due to this TFM concentrations where not corrected according to tissue water.

Qualitative Aspects of Decomposition

The carcasses where monitored over the course of the decomposition period at all temperatures. The first sign of decomposition while viewing the external surface of the carcass was a clouding of the eyes, a loss of scales/skin from the carcass, as well as rigor mortis (the stiffening of muscles throughout the body), which was observable between 12-24h post mortem in carcasses exposed to 15°C and 20°C (Figure 2-1). Following 24h of decomposition at 15°C and 20°C there was a notable bloating effect around the mid-section of the carcass as well as a leaking of fluid from the gills. Upon dissection of the carcasses following 8h of decomposition at 15°C the carcass was fairly well preserved however the muscle tissue of the carcass was soft and was easy to tear compared to fresh tissue. Following 24h of decomposition at 15°C the organs of the carcass began to liquefy and muscle began to pull away from bone after 72h and the previous effects were much more pronounced. At 20°C decomposition progressed much quicker, with the effects seen after 24h of decomposition at 15°C becoming apparent after only 8h of decomposition. Following 24 h and 72h of decomposition the carcass was heavily liquefied with multiple bones protruding (Figure 2-2). However at 4°C decomposition was reduced greatly, and at all-time points the carcass was comparable to the control carcasses (Figure 2-3).

Effects of Decomposition on TFM Concentration in Liver and White Muscle Tissue

The concentration of TFM found in fresh liver tissue taken from rainbow trout exposed to a measured concentration of TFM of 19.0 mg L^{-1} (CI = 15.0-19.2, nominal TFM 16.8 mg L^{-1}) for 6 h was 455.2 ±155.7 nmol g^{-1} wet tissue, which was ~30-fold greater than measured in the white muscle tissue in which the TFM concentration was 14.1 ± 3.45 nmol g^{-1} of wet tissue. At all stages of decomposition, the concentration of TFM in the

liver was significantly higher than the corresponding amount found in muscle (Compare Figure 2-4to Figure 2-5).

Decomposition in cooler (4°C) water did not significantly affect liver TFM concentration , which averaged 571.3 ± 110.8 nmol g^{-1} of wet tissue after 8 h, and 522.2 ± 152.7 nmol g^{-1} wet tissue and 617.1 ± 139.4 nmol g^{-1} wet tissue, after 24 h and 72 h, respectively (Figure 2-4A). Liver tissue that decomposed for 8 h at 15°C, had a slightly lesser amount of TFM present than the control liver, 332.7 ± 45.47 nmol g^{-1} wet tissue whereas liver tissue that decomposed for 24 h, or 72 h had much less TFM present, with a ~75% reduction at 24 h, 113.7 ± 62.06 nmol g^{-1} wet tissue, and ~53% reduction at 72 h 212.4 ± 74.11 nmol g^{-1} wet tissue respectively (Figure 2-4B). Through the use of a Kruskal-Wallis test with a Dunn's post-hoc test it was found that liver tissue TFM concentration decreased significantly after 24 h at 15°C ($P = 0.02$) of decomposition. As well liver decomposition at 15°C for 24 h was significantly different the decomposition at 4°C ($p = 0.013$). TFM concentration in liver that had decomposed at 15°C for 24 h was significantly different then decomposition at 4°C ($p = 0.013$) (Figure 2-4A,B). Liver tissue that decomposed for 8 h, 24 h, or 72 h at 20°C had much less TFM present than concentrations measured at 4°C and 15 °C. After 8 h, 24 h or 72 h of decomposition liver tissue concentration was 127.6 ± 48.37 nmol g^{-1} wet tissue, 196.1 ± 100.4 nmol g^{-1} wet tissue, and 178.6 ± 48.46 nmol g^{-1} wet tissue which represents a 71%, 56%, and 60.7% reduction in TFM concentration respectively compared to the control measurements made immediately following TFM exposure at 15°C (Figure 2-4C).

Similar to the liver, no significant differences were observed between muscle tissue samples that had decomposed for 8 h, 24 h, or 72 h at 4°C. Under these conditions,

the respective TFM concentrations averaged 14.6 ± 2.73 nmol g^{-1} of wet tissue, 23.72 ± 3.61 nmol g^{-1} of wet tissue and 19.48 ± 4.01 nmol g^{-1} of wet tissue respectively (Figure 2-5A). Muscle tissue that was allowed to decompose for 8 h, 24 h, or 72 h at 15°C had no significant differences in TFM concentration, averaging 16.0 ± 2.38 nmol g^{-1} wet tissue, 20.7 ± 1.62 nmol g^{-1} wet tissue and 16.8 ± 4.17 nmol g^{-1} wet tissue respectively (Figure 2-5B). Muscle tissue that was allowed to decompose for 8 h, 24 h, or 72 h at 20°C also had no significant differences in TFM concentration, averaging 10.0 ± 1.77 nmol g^{-1} wet tissue, 5.9 ± 0.99 nmol g^{-1} wet tissue and 7.1 ± 2.42 nmol g^{-1} wet tissue respectively (Figure 2-5C).

Through the use of a Kruskal-Wallis test it was found that at each temperature (4°C, 15°C, or 20°C) TFM concentration did not significantly change compared to control tissue over all decomposition stages in white muscle tissue. Through the use of both a one-way ANOVA and a Kruskal-Wallis test, with accompanying Tukey and Dunn's post-hoc tests respectively, it was determined that liver TFM concentration after decomposition at 20°C over all time points (8 h, 24 h or 72 h) was significantly different then decomposition at 4°C ($p = 0.001$, $p = 0.049$, and $p = 0.04$ respectively) but not 15°C. Using one-way ANOVAs with accompanying Tukey post-hoc it was found that white muscle TFM concentrations where significantly lower after 24 h of decomposition at 20°C compared to the same time point at 4°C and 15°C ($p < 0.0001$ and $p = 0.618$ respectively). At all other time points and temperatures TFM concentrations where not significantly different.

Effects of Decomposition on Relative Amounts of TFM Metabolites

TFM metabolite levels are presented relative to the amounts detected in control tissue samples, based on the peak areas measured from LC/MS-MS chromatographs, as there was no standard available to quantify the concentrations TFMOGlu and TFMOS. At 4°C, the relative amounts of TFMOGlu in liver after 8 h decomposition was 0.3% (SEM ± 0.09%) and following 24 h, or 72 h of decomposition no TFMOGlu was detectable (Table 2-2). In contrast, the relative amount TFMOGlu measured in white muscle tissue at the same temperature after 8 h, 24 h, and 72 h of decomposition where 25% (SEM ± 5%), 29% (SEM ± 12%), and 14% (SEM ± 2%) respectively (Table 2-2).

At 15°C, TFMOGlu levels were less stable. No TFMOGlu was detected in the liver after decomposition of 8 h 24 h, or 72 h. Whereas, the relative amount of TFMOGlu in muscle tissue after were 35% (SEM ± 8%) and 36% (SEM ± 20%) after 8 h and 24 h, respectively. However, by 72 h no TFMOGlu was detected in the white muscle (Table 2-2). At 20°C, TFMOGlu was even less stable, with none detected in the liver, and only trace amounts in the white muscle after 8 h, averaging 9% (SEM ± 2%), and none detected after 24 h and 72 h of decomposition(Table 2-2)

The relative amount recovered of TFMOS measured in the liver at 4°C, averaged 21% (SEM ± 12%), 4% (SEM ± 5%), at 8 h and 24 h, respectively. However, no TFMOS was detected after 72 h (Table 2-3). Like TFMOGlu, TFMOS was more stable in muscle at 4°C, averaging 56% (SEM ± 37%), 106.9% (SEM ± 51%) at 8 h and 24 h. Unlike TFMOGlu, however, no TFMOS was detected after 72 h of decomposition (Table 2-3). At 15°C, no TFMOS was detected in the liver or the muscle at 8 h, 24 h or 72 h. Notably; some TFMOS was detected in the liver at 20°C but not in the muscle. In liver, the relative

amounts of TFMOS after decomposition for 8 h, 24 h, or 72 h was 10 % (SEM ± 9%), 2% (SEM ± 1 %), and 0% respectively (Table 2-3).

Discussion:

Decomposition: Influence of Autolysis and Putrefaction on Xenobiotic Measurements

The decomposition of dead animals is a multi-staged process, which ultimately results in the breakdown of cells and tissues into their simpler organic components. Decomposition has not been studied in detail in fishes, but there are likely many similarities to the process in terrestrial animals, in which decomposition is a six-stage process (reviewed by Carter et al. 2007). Decomposition begins with "fresh stage decomposition" which is associated with cardiac arrest, followed by oxygen depletion within the animal leading to *autolysis*, in which cell membrane gradients breakdown and the hydrolytic enzymes released by cells break-down structural protein and lipids (Butzbach, 2010; Hau et al. 2014). Due to ongoing anaerobic metabolism in the animal, the pH of the tissues also drops which activates additional proteolytic enzymes. Later in fresh stage decomposition, the process of *putrefaction* begins, which is characterized by the breakdown and liquefaction of tissues and organs, that results primarily from microbial activities. During putrefaction, anaerobic bacteria, arising from the gastrointestinal tract and respiratory tract, begin to breakdown constituent carbohydrates, lipids and protein into various organic acids (lactic acid, propionic acid) and gases (methane, hydrogen sulphide, ammonia, etc.). The liberation of gases leads to swelling, which is the second stage of decomposition, the "bloating stage", this leads to the rupture of the skin which allows oxygen to enter and provide a more suitable environment for fly larvae and aerobic microbes. It is important to note that during this stage of decomposition, tissues and blood will become more alkaline due to microbial processes when compared to the

autolytic stage (Butzbach, 2010). This is followed by the third "active decay" stage in which fluids leak from the body, and other organisms including larval flies (maggots), other insects or worms, and invertebrate and vertebrate scavengers begin to devour the organism, leading to mass loss. Soft tissues disappear completely during the "advanced decay" stage, which is followed by the "dry stage" where the tissues desiccate or even mummifies, and lastly the "remains" stage in which only the skeletal remains are left (Carter et al. 2007).

From an aquatic forensic toxicology perspective, the first two stages of decomposition characterized by autolysis and putrefaction are probably the most relevant in fishes because these are most likely to impact the interpretation of analytical findings. However, decomposition is also affected by abiotic factors such as temperature and humidity, which can either accelerate decomposition or slow it down. Since fish are water dwelling organisms, immersion or emersion are also likely to profoundly impact post-mortem xenobiotic concentrations in the tissues, making it imperative to understand how the combined effects of water, temperature and decomposition affect the forensic analysis of such substances. The present study clearly demonstrates that the post-mortem concentration of the piscicide, 3-trifluoromethy-4-nitrophenol (TFM) and its metabolites, TFM-sulfate (TFMOS) and TFM-glucuronide (TFMOGlu), exhibit pronounced regional differences in concentration, which vary according to temperature and with time.

The Effects of Temperature on Tissue Decomposition and TFM Distribution

Temperature has a large impact on the rate of decomposition of a carcass, with colder temperatures preserving the carcass longer and warmer temperatures accelerating decomposition. At 4°C liver and white muscle TFM concentration where relatively stable

over 72 h but declined as temperature increased, indicating that tissue degradation was accelerated. This is likely because at higher temperature rates of autolysis are increased, promoting the breakdown of cells and tissues using their own enzymes, followed by liquefaction (Zhou and Byard, 2010). This would also cause cells to spill their cytosolic contents, including xenobiotics such as TFM, into the extracellular space and body cavities, which would tend to lower tissue concentrations of the substance in some tissues such as the liver (Yarema and Becker 2005; Skopp 2010), in which TFM was much more concentrated.

The high ratio of liver to blood TFM immediately following sampling is likely due to active uptake of TFM into liver hepatocytes, during TFM exposure. The liver is supplied with blood via the hepatic artery, which would contain TFM that was taken up across the gills. The hepatic portal system could also be a source of TFM, if appreciable amounts entered the digestive tract via ingestion, but this seems unlikely because freshwater fishes tend to drink very little (Marshall and Edwards, 2013). While the transport properties of TFM have not yet been fully characterized, with a pK_a of 6.07 (Hubert 2003), the majority of TFM would have been in its ionized state at physiological pH (e.g. at 15 °C blood pH = 7.8; hepatocyte pH ~ 7.4; Milligan and Wood 1986; Wilkie and Wood 1995), with the remainder in its un-ionized form. Under these conditions, hydrophilic anions such as TFM could have been concentrated in the hepatocytes via organic anion transporters located basolaterally on the cells, before excretion into the biliary canniculus which ultimately drains into the gall bladder and/or bile ducts (Bévalot et al. 2016). With decomposition, active transport would cease, ion and osmotic gradients would break down, and as cell membranes degraded in the liver, and

liquefaction proceeded, TFM would therefore tend to diffuse from the liver to other regions down concentration gradients that are present, due to differences in its distribution, into different body compartments. While TFM concentrations decreased in the liver as the period of decomposition proceeded at 15°C and 20°C, there was no evidence that TFM concentrations increased in the muscle, suggesting the post-mortem redistribution of TFM to the muscle was negligible at all temperatures tested.

The decomposition process, and hence redistribution/loss of TFM from the liver was much more pronounced at warmer temperatures. This was because the invasion of the tissues and bodily fluids of the carcass by gut and respiratory micro-biota is proportional to temperature, which leads to further tissue degradation as putrefaction proceeds (Yarema & Becker, 2005; Butzbach, 2010; Hau et al. 2014). Indeed, the effects of higher temperature on TFM loss was most pronounced at 20°C compared to 15°C. This relationship also at least partially explains the relatively stable TFM concentrations in the liver at 4°C. Since temperature is one of the main factors that regulate bacterial growth and metabolism along with availability of substrate (Scofield et al. 2015), gut microbe populations in fish can fluctuate depending on the water temperature, geographical location and what they are feeding on (Pond et al. 2006). Many of these bacteria are mesophilic, meaning they have an optimum temperature of 20-40°C (Yarema & Becker, 2005). Thus, at warmer temperatures microbial metabolism would be expected to be faster and tissue degradation would be accelerated resulting in a greater breakdown of tissue structure and water influx into tissues. This was true for TFM loss, and it was also time dependent, with greater loss of TFM from liver tissue at 24 h, when there was approximately a ~34% loss of TFM found in liver tissue, compared to the 8 h time-point

(Figure 2-4). It is important to note, however, that appreciable amounts of TFM were still present in the liver tissue after 72 h of decomposition, which suggests that TFM could still be detected after even a few days if non-target mortality were to occur in the field. However, it should be kept in mind that, unlike the laboratory conditions employed here, decomposition would not be limited to microbial processes as insect larvae, arthropods and other decomposers could accelerate these processes and therefore the viability of tissues (Schmitt et al. 2006; Iscan & Steyn, 2013). Despite initial predictions, there were no significant changes in liver water content during the decomposition process, suggesting that artifacts due to water influx would be minimal if liver was collected from fish carcasses in the field.

The effects of temperature on TFM loss were much less pronounced in muscle tissue, only seeing a decrease in concentration during decomposition at 20°C. This is most likely due to the fact that muscle tissue is relatively isolated from the GI tract protecting it from the putrefying bacteria that breaks down tissues **(**Butzbach, 2010). As well blood flow differences between the tissues could help explain the difference seen in decomposition. The liver when compared to white muscle tissue is much more vascularized and has a much higher perfusion rate (Johnston, 1976, Farrell, 1993), this would also give bacteria greater access to the organ and as such putrefaction would occur quicker. Another possible explanation for the more pronounced loss of TFM in liver tissues is that the capillaries often associated with the liver are highly fenestrated, meaning that the capillaries themselves are perforated allowing for easy transport of molecules from the heaptocytic extracellular space to the capillaries themselves (Bévalot et al. 2016). In contrast white muscle tissue, specifically type IIb muscle fibers, have a

less dense capillary network when compared to red muscle tissue as well as liver tissue (Korthuis, 2011). Lastly, the hepatocytes of the liver can have numerous transporters which could facilitate the movement of xenobiotics such as TFM in or out of the tissue, as noted above (Bévalot et al. 2016).

Post-Mortem Redistribution of TFM

The concentrations of compounds found in a specific tissue following death can help forensic investigators determine the cause of death. However, many xenobiotics can be redistributed into other tissues primarily from major organs such as the heart or liver into the blood, making cause of death determinations more difficult (Yarema & Becker, 2005). The relevant factors that could lead to post-mortem redistribution of TFM in non-target fishes include events related TFM's chemical characteristics, cell death and putrefaction. Lipophilic compounds that tend to concentrate in tissues such as the liver and adipose tissue are more prone to redistribute to the blood stream and to other tissues as the compounds diffuse down its concentration gradients following death. This process would be facilitated by cell autolysis, which would further breakdown membrane barriers allowing for diffusive loss of TFM from the tissues (Pélissier-Alicot et al. 2003; Yarema & Becker, 2005). If TFM is actively transported into the hepatocytes and bile caniculae, as seems likely (see above), then the absence of ATP would remove additional barriers to TFM movement. PMR in the liver is also more likely due to the multiple organs and vessels connected to it such as the GI tract, portal veins, hepatic artery, and hepatic vessels, allowing for PMR from the stomach and gall bladder or to the heart (Pélissier-Alicot et al. 2003).

As a weak acid, with a pK_a of 6.07, a greater proportion of TFM would likely exist in its un-ionized form under the more acidic conditions that arise from anaerobic conditions associated with cell death. This would explain why it would be retained by the liver and muscle in the early stages of decomposition. However, as decomposition proceeded there would be a greater tendency for TFM to undergo re-distribution. For instance, at 4°C there was no increase in TFM concentration in both muscle and liver tissue, suggesting that post-mortem redistribution was likely minimal under these conditions. This was likely because decomposition, a key factor influencing post-mortem redistribution was slowed in lower temperatures tissue, allowing these tissues to retain the accumulated TFM. At warmer temperatures, however, the possibility of post-mortem redistribution would have been greater, because decomposition would have proceeded more quickly, and this was illustrated by the marked decreases in TFM observed in both the liver and the muscle at 15°C.

As decomposition progresses, specifically during autolysis, many enzymes tend to lose their activity in the acidic environment; however β-glucuronidase is slower to denature (Butzbach, 2010). This allows for the enzyme to convert the glucuronidated compound (TFMOGlu) back to the parent molecule where it may be reabsorbed into tissues (Butzbach, 2010; Bévalot et al. 2016). This may be another factor that contributed to the loss of TFMOGlu observed in the liver and the muscle, particularly at warmer temperatures. It is key to note that intestinal glucuronidases have been shown to convert xenobiotic compounds back to their parent molecule where they are reabsorbed (Bévalot et al. 2016), which could exacerbate toxicity in the organism. The present study, however, provides little evidence to support this possibility in rainbow trout.

In humans, post-mortem redistribution could also depend on the orientation of the carcass after death, because blood will tend to pool in the lowest point of the carcass (Yarema and Becker 2005). This could cause isolation of certain organs from the blood depending on concentration gradients this could result in a lower or higher amount of toxicant detected in the tissue (Pélissier-Alicot et al. 2003; Yarema & Becker, 2005). However, the importance of body position in non-target fishes, fully or partially immersed in water, is likely minimal because of waters high density and non-compressible properties, which would tend to support the organs.

The Effects of Water Exposure and Temperature on TFM Metabolite stability

The present study is not only in agreement with previous observations that the TFM detoxification in rainbow trout proceeds via phase II metabolic pathways involving glucuronidation (Lech 1974; Lech and Statham 1975; Kane et al. 1994; Birceanu et al. 2014), but it also involves sulfation. Using liver slices, Bussy et al. (2018) demonstrated that TFM could potentially undergo sulfation, but this is the first study to demonstrate that this phase II process takes place *in vivo* in the trout. It is known, that niclosamide, a salicylanilide molluscicide, also used to control sea lamprey, undergoes sulfation (Dawson et al. 2003). Glucuronidation of TFM primarily takes place in the liver through the use of the enzyme UDP-glucuronosyltransferase which facilitates the conjugation of glucuronic acid with TFM, forming the metabolite TFM-glucuronide (TFMOGlu) which is much more hydrophilic and as such easier to excrete via biliary excretion into the intestine, and the ultimately out of the animal via defecation (King et.al. 2000; Hunn & Allen, 1974; Clarke et.al. 1991; Bévalot et al. 2016).

In was not surprising, that TFMOGlu accumulated predominantly in the liver, in which relative concentrations were 1000-fold greater compared to the muscle tissue immediately following TFM exposure. However, in both muscle and liver the relative amounts of TFMOGlu rapidly disappeared as the fish decomposed, even at 4°C. However, the rate of TFMOGlu loss was much greater in the warmer water, resulting in virtually no TFMOGlu detected in the liver after 8 h at 15 and 20°C, respectively. The rate of TFMOGlu loss from the white muscle was noticeably slower, however. This was likely because muscle was more isolated from putrefying bacteria than liver (Yarema & Becker, 2005; Butzbach, 2010). Bacteria can decrease the amount of TFMOGlu in the tissue in two manners, either by degrading the tissues themselves enough to release the metabolite allowing it to flow down natural concentration gradients, or by converting the metabolite back to the parent molecule through the use of β- glucuronidases (Bévalot et al. 2016). This decrease is more pronounced at higher temperatures as increased temperature will accelerate the rate at which the reaction proceeds. Water influx may also contribute to the loss of TFM metabolites in tissues due to their hydrophilic nature.

Time is not the only factor that affects the relative amount of metabolites in tissue. As shown in this study, low temperature (4°C) tends to preserve the concentration of TFMOGlu, and as temperature increases losses increase. In cold temperatures putrefaction is slowed due to reduced microbial activity resulting in tissues remaining stable longer which allows them to retain xenobiotic compounds and metabolites longer, as well the process of autolysis would be delayed as well further preserving the tissues. This protective effect is lost at warmer temperatures as both aspects of decomposition, autolysis and putrefaction, are accelerated consequential the liver and muscle tissue

become less stable resulting in no TFMOGlu being recovered from muscle tissue after 24 h of decomposition and virtually none being recovered in liver tissue after 24 h.

TFMOGlu is not the only metabolite formed in rainbow trout, in this study TFM sulfate (TFMOS) was also discovered in both liver and muscle after exposure to the 9 h LC_{50} of TFM for rainbow trout. TFMOS is formed through the use of cytosolic sulfotransferase enzymes (SULT) and a phase II reaction which facilitates the addition of sulfonate group to the parent compound, typically from 3-phosphoadenosine-5-phosphosulfate (PAPS), which is a universal donor, this makes the compound easier to excrete out of the body (Kauffman, 2004; Bussy et al. 2017). As with TFMOGlu there is approximately 1000x greater concentration of TFMOS found in the liver tissue as opposed to muscle tissue at 0 h of decomposition. As the liver is the main site of detoxification in teleost fishes it stands to reason that there would be a higher concentration of the SULTs and as such a higher concentration of TFMOS present (Chambers & Yarbrough, 1976).

Colder temperature also had a protective effect on TFMOS in both liver and muscle tissue with recoveries of ~25% being accomplished after 72 h of decomposition, this again points to a slower rate of putrefaction at colder temperatures due to lower microbial metabolic activity (Yarema & Becker, 2005; Scofield et al. 2015). However, as temperature increased (15, 20°C) the recovery rate of TFMOS was 0% in muscle tissue over all time points. Due to the hydrophilic nature of TFMOS it is possible that as decomposition progressed the metabolite was flushed from the tissues, as well since the muscle tissue is not a site of detoxification low levels of TFMOS would have been present initially (Chambers & Yarbrough, 1976).

Conclusion:

The rapid collection and preservation of tissue samples should be the first priority following a fish kill, along with details of the environmental conditions before, during and after the incident including the timing, duration, and concentrations of TFM used during the lampricide application. Water chemistry data, especially water pH and temperature, are essential, as are physical measurements including water flow, discharge and clarity (appearance). The condition of the carcass(es) of the fish(es) should also be noted. Decomposition could profoundly influence post-mortem toxicology investigations because it could compromise the ability to detect and measure the concentrations of lampricides and/or their metabolites in the tissues. Decomposition of the tissues could also potentially interfere with the accurate detection and quantification of TFM and/or its metabolites by contributing to more rapid degradation of the compounds and/or producing interfering compounds that compromise analysis. Whether or not the fish is immersed in water, or found on-shore in air, could markedly influence decomposition and the integrity of post-mortem forensic toxicological analysis.

It is evident from this study that temperature has a powerful impact on the rate of decomposition in tissues as well as the stability of TFM and its metabolites in those tissues. As temperature increases microbial degradation (putrefaction) accelerates causing a breakdown of tissues and a loss of TFM. This is important because TFM is applied to streams from spring to fall, over a wide range of water temperatures. Thus, water temperature is a critical variable to record following any incidents of non-target mortality that may or may not be related to TFM toxicity. From a forensic toxicology perspective, the present study clearly demonstrates that TFM is detectable for up to 72 h after death in

liver tissue. Although, levels continue to decline with decomposition, the presence of TFM in the liver would be strong evidence of recent TFM exposure. Despite accumulating much lower concentrations of TFM than in the liver, the white muscle is ideal for post-mortem TFM analysis because TFM concentrations are much more stable over time, at least up to 15°C over 3 days. At warmer, 20°C temperatures, the concentrations of parent TFM also decline in the muscle, but again TFM's continued presence or absence in the tissue could be important for resolving the cause of death if unexpected fish kills are preceded by lampricide applications. Due to their relative ease of collection, storage, and utility in measuring TFM, liver and white muscle tissue should be collected from fish(es) if TFM is suspected in any incidences of unexplained fish mortality.

Table 2-1: Relationship between percent tissue water in liver and white muscle at different stages of decomposition and different temperatures.

Total percent tissue water of each tissue (white muscle and liver) throughout all experiments. Tissue water was determined by taking the wet tissue weight and subtracting the dried tissue then dividing by the wet tissue weight and multiplying by 100. Tissue water is presented as an average percent (%) for each experiment (N=8) ± SEM.

Tissue and Temperature	**0 h**	**8 h**	**24 h**	**72 h**
Control Liver	75.9% ± 1.13	N/A	N/A	N/A
Control White Muscle	79% ± 0.63	N/A	N/A	N/A
4°C Liver	N/A	80.3% ± 0.67	79.7% ± 0.47	80.2% ± 0.67
4°C White Muscle	N/A	81.2% ± 1.01	79.1% ± 0.54	80.7% ± 0.42
15°C Liver	N/A	79.5% ± 0.25	80% ± 0.6	80.5% ± 0.4
15°C White Muscle	N/A	77.7% ± 0.82	78% ± 0.74	80% ± 1.05
20°C Liver	N/A	80% ± 0.68	80.1% ± 0.6	82.2% ± 0.61
20°C White Muscle	N/A	79.8% ± 0.8	81.3% ± 0.73	80.6% ± 1.67

Table 2-2: Effects of water exposure and decomposition at different temperatures on TFMOGlu concentration in liver and white muscle tissue.

Differences in TFMOGlu recovery between white muscle tissue and liver tissue decomposed while exposed to low (4°C), medium (15°C), or high (20°C) temperature water. Data is presented as the mean compared to TFMOGlu at time 0 h ± SEM for each tissue (N = 8 fish at each time and temperature point). If no tissue sample was taken at a specific time point N/A (not applicable) was used.

Tissue and Temperature	0 h	8 h	24 h	72 h
Control Liver	1 ± 0.41	N/A	N/A	N/A
Control Muscle	1 ± 0.28	N/A	N/A	N/A
4°C Liver	N/A	0.003 ± 0.0009	0	0
4°C White Muscle	N/A	0.25 ± 0.05	0.29 ± 0.12	0.14 ± 0.02
15°C Liver	N/A	0	0	0
15°C White Muscle	N/A	0.35 ± 0.08	0.36 ± 0.2	0
20°C Liver	N/A	0	0	0
20°C White Muscle	N/A	0.09 ± 0.02	0	0

Table 2-3: Effects of water exposure and decomposition at different temperatures on TFMOS concentration in liver and white muscle tissue.

Differences in TFMOS recovery between white muscle tissue and liver tissue decomposed while exposed to low (4°C), medium (15°C), or high (20°C) temperature water. Data is presented as the mean compared to TFMOS at time 0 h ± SEM for each tissue (N = 8 fish at each time and temperature point).

Tissue and Temperature	0h	8 h	24 h	72 h
Control Liver	1 ± 0.52	N/A	N/A	N/A
Control Muscle	1± 0.49	N/A	N/A	N/A
4°C Liver	N/A	0.21 ± 0.12	0.04 ± 0.05	0
4°C White Muscle	N/A	0.56 ± 0.37	1.06 ± 0.51	0
15°C Liver	N/A	0	0	0
15°C White Muscle	N/A	0	0	0
20°C Liver		0.1 ± 0.09	0.02 ± 0.01	0
20°C White Muscle		0	0	0

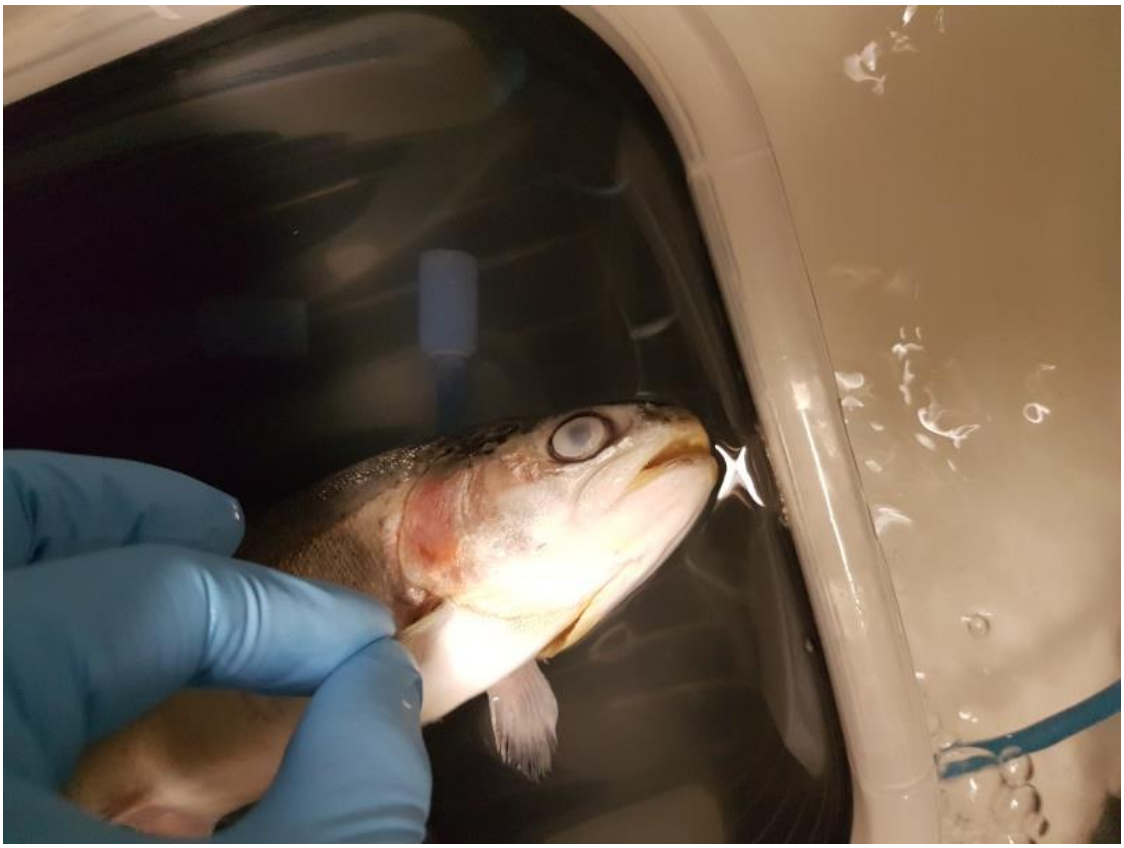

Figure 2-1: Early effects of decomposition.

The carcass of a fish decomposed in 15°C water for approximately 12h. There was a notable clouding of the eye, a loss of scales/skin from the carcass, as well as rigor mortis (the stiffening of muscles throughout the body.

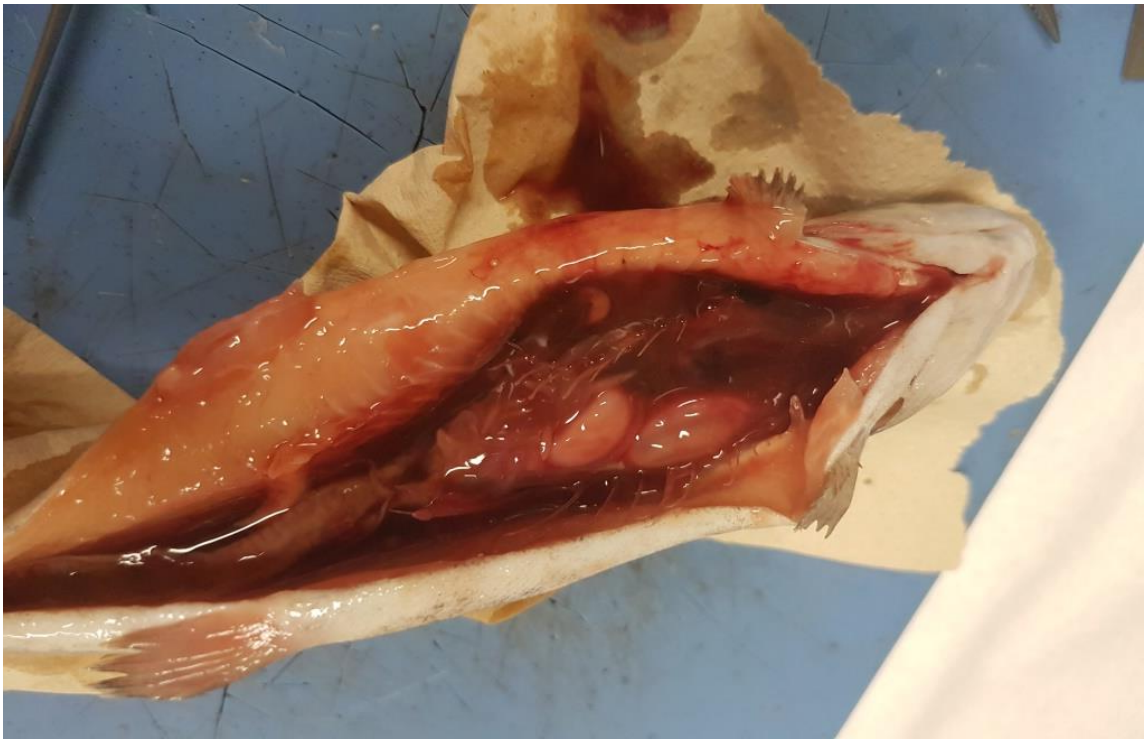

Figure 2-2: Prolonged decomposition in warm (15-20°C) water.

The carcass of a fish decomposed in 20°C water for 72h. Majority of the internal organs have liquefied and the muscle has become soft and pulled away from the skeletal structure.

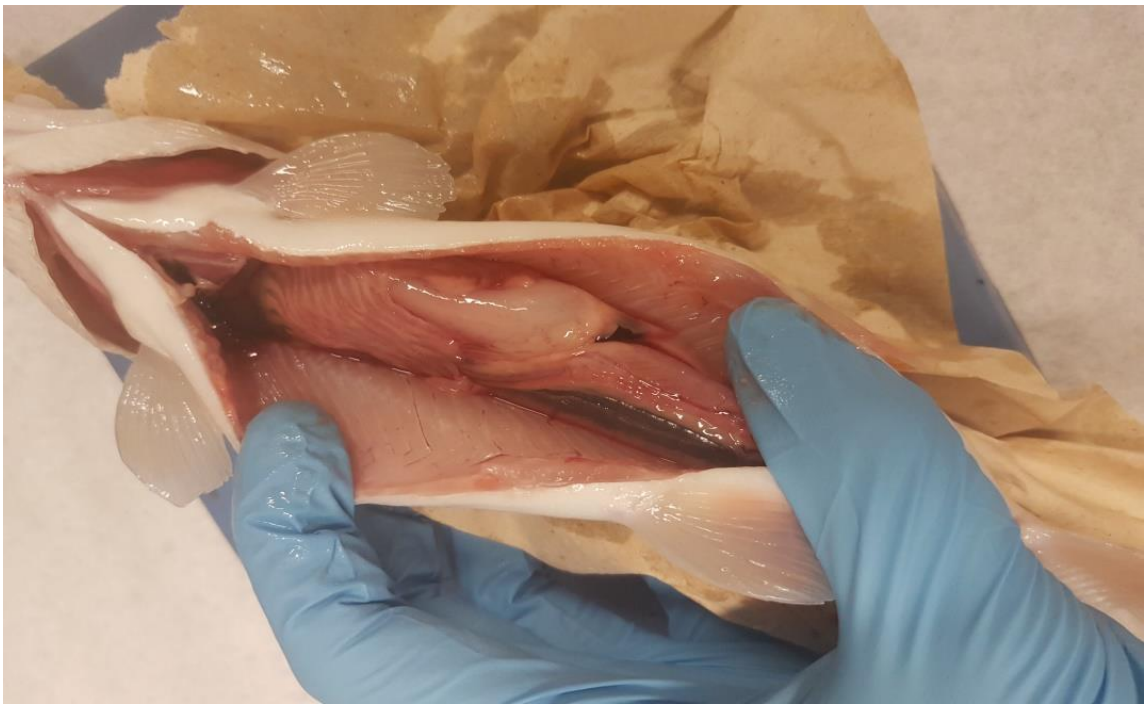

Figure 2-3: Prolonged decomposition in cold (4°C) water.

The carcass of a fish decomposed in 4°C water for 72h. Almost know decomposition has taken place and is comparable to control (non-decomposed) carcasses.

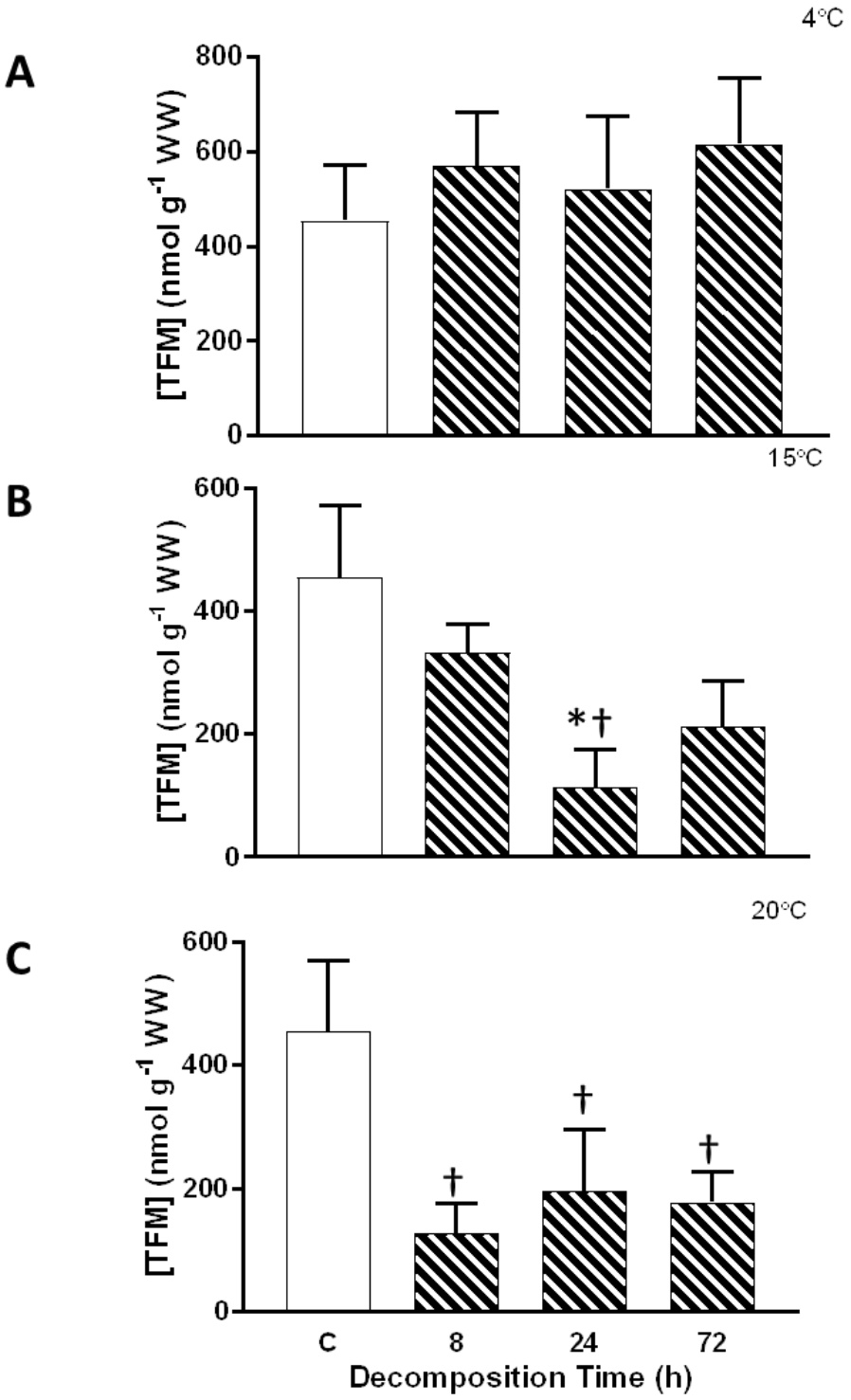
A
4°C
800
600
400
200
0
[TFM] (nmol g-1 WW)
B
15°C
600
400
200
0
*†
[TFM] (nmol g-1 WW)
C
20°C
600
400
200
0
†
†
†
[TFM] (nmol g-1 WW)
C
8
24
72
Decomposition Time (h)

Figure 2-4: Effects of water exposure and decomposition at different temperatures on TFM concentration in liver tissue.

Changes in the TFM concentration of liver tissue decomposed over 3 days while exposed to 4°C (Panel A), 15°C (Panel B), or 20°C (Panel C) water. Rainbow trout exposed to the LC_{50} (16.8 mg L^{-1}) of TFM for 6 h were euthanized, and liver tissue was collected immediately (time zero, open bars) or after 8 h (hashed bars), 24 h (grey bars) and 72 h (black bars) of decomposition. Data is presented as the mean ± SEM (N = 8 fish at each time point and each temperature). An asterisk denotes a significant difference ($p \leq 0.05$) between that experiment and the control (C) while a single dagger denotes a significant difference ($p \leq 0.05$) from 4°C at corresponding time points at 15°C and 20°C.

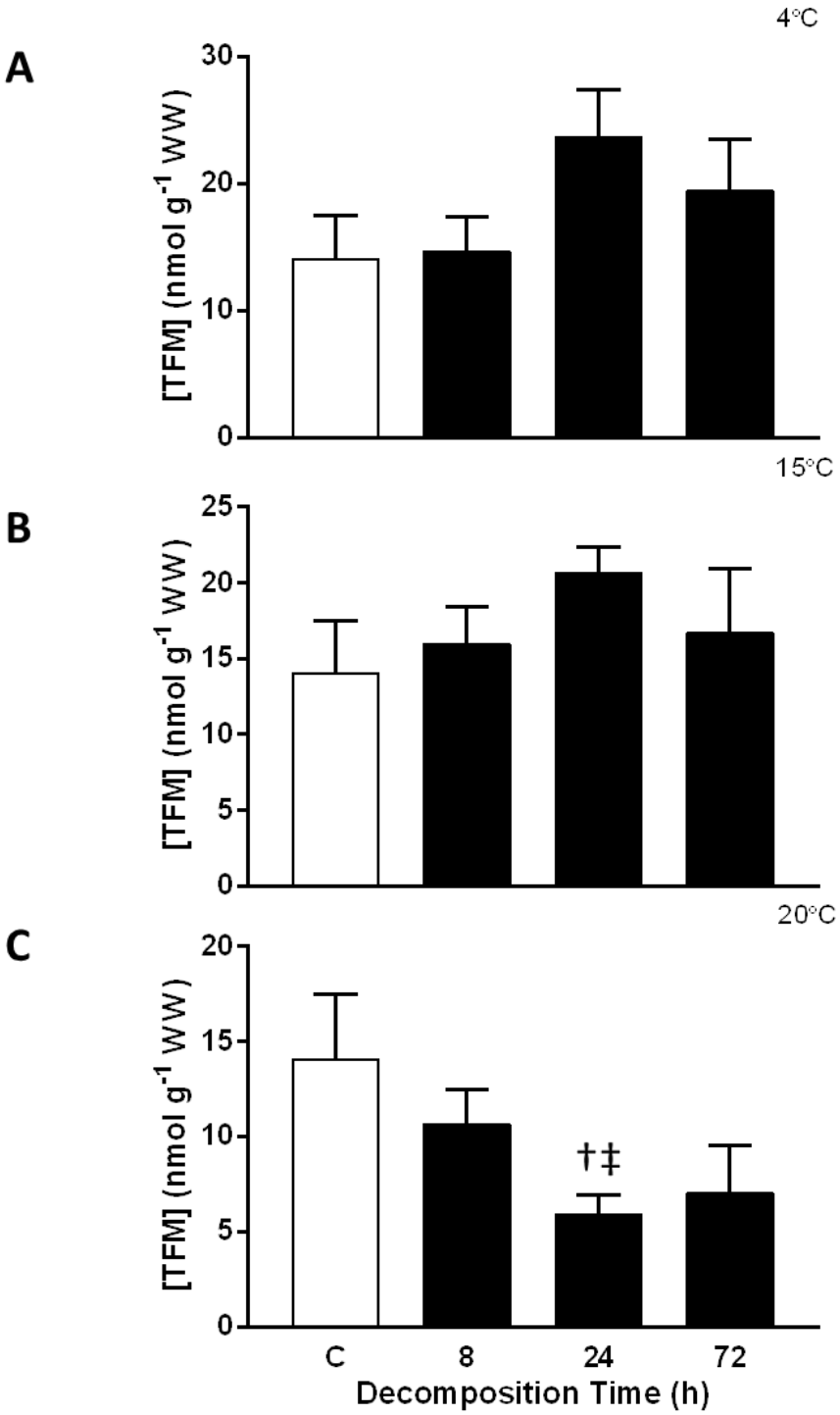
A
4°C
[TFM] (nmol g-1 WW)
30
20
10
0
B
15°C
[TFM] (nmol g-1 WW)
25
20
15
10
5
0
C
20°C
[TFM] (nmol g-1 WW)
20
15
10
5
0
†‡
C
8
24
72
Decomposition Time (h)

Figure 2-5: Effects of water exposure and decomposition at different temperatures on TFM concentration in white muscle tissue.

Changes in the TFM concentration of muscle tissue decomposed over 3 days while exposed to 4°C (A), 15°C (B), or 20°C (C) water. Rainbow trout exposed to the LC_{50} (16.8 mg L^{-1}) of TFM for 6 h, were euthanized, and white muscle tissue was collected immediately (time zero, open bars) or after 8 h (hashed bars), 24 h (grey bars) and 72 h (black bars) of decomposition. Data is presented as the mean ± SEM (N = 8 fish at each time point and each temperature). An asterisk denotes a significant difference ($p \leq 0.05$) between that experiment and the control (C) while a single dagger denotes a significant difference ($p \leq 0.05$) from 4°C at corresponding time points at 15°C and 20°C. A double dagger denotes a significant difference ($p \leq 0.05$) from 15°C at corresponding time points at 20°C.

Chapter: 3

Effects of Air Exposure on Lampricide Stability in Decomposing Rainbow Trout (*Oncorhynchsus mykiss*) Tissue.

Introduction:

The piscidide (lampricide), 3-triflouromethyl-4-nitrophenol (TFM) is administered to streams and tributaries around the Great Lakes basin every three to five years to control invasive sea lamprey larva populations (McDonald and Kolar, 2007; GLFC 2011). It is specific to sea lamprey, normally causing minimal harm to non-target species including invertebrates and fishes which have a greater capacity to detoxify TFM (Lech and Statham 1975; Kane et al. 1994; Boogaard et. al. 2003). The specificity of TFM can be explained by its mode of detoxification, which appears to mainly take place via phase II biotransformation in the liver of non-target fishes such as rainbow trout. Here, the enzyme UDP-glucuronosyltransferase (UDPGT) promotes glucuronidation, in which a glucuronic acid molecule is attached to each molecule of TFM, making it more polar (hydrophilic) and easier to excrete via urinary and/or gastrointestinal routes (Hunn & Allen, 1974; Clarke et al. 1991; King et al. 2000). Unlike non-target fishes including rainbow trout, channel catfish (*Ictalurus punctatus*), and bluegill (*Lepomis macrochirus*), sea lampreys have a much lower capacity to use this pathway to detoxify TFM. Like many substances, however, if the amount of drug or toxin taken-up by an organism exceeds its existing capacity to detoxify the compound, it can lead to toxicity or death. This is also true for TFM, which can have non-target effects or cause mortality if the fish take-up too much TFM, due to sudden changes in water flow and/or related changes in water pH (McDonald and Kolar 2008). At lower pH the bioavailability of TFM is greater because a greater proportion of TFM exists in un-ionized, more lipophilic form (Hunn and Allen 1974; Bills et al. 2003, McDonald and Kolar 2008). However, fluctuations in pH occur naturally in streams and rivers of the Great Lakes due to photosynthesis by

algae, cyanobacteria and macrophytes, not to mention precipitation or agricultural, industrial or municipal runoff (Meyer & Barclay, 1990; McDonald and Kolar 2007).

In some instances, it may be difficult to establish whether or not a fish kill was due to TFM, particularly if the fish are not discovered until long after the event. When a fish kill occurs the carcass will either remain in the water or it may wash up on shore exposing the decomposing carcass to air. In either case, the collection of tissues for quantification of TFM and/or its metabolites could be a valuable investigative tool. However, the utility of this approach would depend on the stability of the lampricides and/or their metabolites in the tissues of the fish. The stability of lampricides and their metabolites in fish tissues could depend on many different factors such as the temperature of the air or water, stage of decomposition and/or which tissues are analyzed (Yarema & Becker, 2005; Butzbach, 2010; Skopp, 2010; Zhou and Byard, 2010; Hau et al. 2014). Analysis on air-exposed carcasses could pose greater challenges, because the animal (fish or mammal) will decompose twice as fast as a body submerged in water (Iscan & Steyn, 2013). This is because the atmospheric temperature is generally warmer than that of nearby water, which accelerates bacterial decomposition (Schmitt et al. 2006). As well, immersion in water offers protection from invertebrate decomposers such as insects, and fungi as well as mammalian predators which can accelerate decomposition (Iscan & Steyn, 2013; Schmitt et.al. 2006). The presence or absence of lampricides and their metabolites following a fish kill could be the key to identifying the cause of death in fish following a lampricide treatment, particularly if the cause of death is in dispute or not readily apparent, or due to other factors from the surrounding environment (Meyer & Barclay, 1990). Indeed death can arise from numerous factors including anthropogenic

toxicants arising from industrial or agricultural activities, disease, or even natural events such as the depletion of oxygen in the water (Meyer & Barclay, 1990). Thus, it is imperative that investigators know what happens to lampricides and their metabolites following death in organisms.

One of the goals of this study was to measure TFM and its metabolites in various different tissues allowed decomposing in humidified air, similar to that found near a river bank, and determine how this process affects the stability of TFM and its metabolites in the tissues of rainbow trout. I hypothesized that decomposition will be accelerated when a carcass is exposed to air, and that this makes reliable measurements of TFM and its metabolites more difficult. I also predicted that as the period of decomposition proceeds, that there would be accompanying degradation of TFM and TFM-metabolites. To test these hypotheses, and how decomposition influences the measurement of TFM, rainbow trout where exposed to their LC_{50} of TFM for 6 h, sacrificed, followed by the collection of tissues (liver, muscle, blood) at different sample periods (0 h, 8 h, 24 h), which were subsequently analyzed using LC-MS/MS.

Material and Methods:

Experimental Animal and Holding conditions

Juvenile rainbow trout (*Oncorhynchus mykiss*) weighing 100-200 g (N = 120), were purchased from Rainbow Springs Fish Hatchery, Thamesford, Ontario, in 2016 and 2017. The trout were housed in a large circular tank (>1000L) continuously fed with aerated, temperature controlled (15°C) fresh water from a ~2500 L recirculating system. The water drained from the tank to a sump, from which it was pumped through a mechanical filter, and then subject to UV filtration prior to returning to the holding tank. The trout

were fed 5PT Martin complete floating trout grower feed at a ratio of 2% body weight (BW) 2-3 times weekly. Dissolved oxygen (DO %), pH, and conductivity were measured daily (DO% = 81-87%, pH = 8.2-8.6, alkalinity = 150 mg/L $CaCO_3$, temperature = 14.9-15.2), and the fish were held under a 12 hour light/dark cycle. All experiments were approved by the Wilfrid Laurier Animal Care Committee, and followed guidelines and principles of the Canadian Council of Animal Care (CCAC).

Experimental Set-up

Fish were held for a minimum of 2-3 weeks prior to starting experiments. Prior to experiments, the trout were fasted for 2 days to prevent them from fouling their experimental holding chambers during TFM exposure. Prior to each experiment, N = 8 rainbow trout where transferred into their individual test chambers (volume = ~4 L; dimensions = length 29.3 cm, width 19.4 cm, depth 9.5 cm), and left to acclimate to the TFM exposure system overnight (~ 12 h). Each chamber was separately aerated, and was fed with aerated water draining from a head tank positioned above the tray holding the containers at a rate of ~1 L min-1. The head tank was partially replenished with de-chlorinated city of Waterloo water, and cooled (15.1-15.8°C) using a Coralife 1/4 HP Aquarium Chiller (Coralife, Franklin, WI). During TFM exposure, water flow was cut-off to the containers by removing the water inflow lines, and temperature was maintained by immersing the containers in the 15 °C water which flowed through the tray.

Experimental Protocol

After the water lines were removed from all of the chambers, the system was dosed with a known amount of TFM. After a 15 minute mixing period, the water inflows lines where re-inserted into each chamber, beginning the exposure period. Water samples (~10 mL)

were collected from each container throughout the exposure, and the concentrations of TFM where monitored using a spectrophotometer (NovaSpec II, Bauch and Lombe, Cambridge, England), against precision standards provided by the Sea Lamprey Control Centre, Fisheries and Oceans Canada (DFO), Sault Ste. Marie, ON.

The trout where exposed to the 9 h LC_{50} of TFM which was previously determined by acute toxicity tests in an accompanying study by Foubister et al. (2018), and those that died were immediately removed from their holding containers, and were either immediately sampled, or left in humidified air for 8 h or 24 h, before tissue collection. Surviving fish were exposed to TFM for a maximum of 6 h after which they were euthanized with a lethal dose of anesthetic (0.5g L^{-1} tricane methansulfonate buffered with 1 g L^{-1} sodium bicarbonate), and either sampled immediately or left to decompose in air as described above. To simulate the humid environment of a river or stream bank, the dead fish were placed into individual decomposition chambers, in which the fish were placed on a plastic mesh grate suspended over ~1 L of water (depth = 2.5 cm). The chambers were otherwise identical to the TFM exposure chambers, and the water beneath the mesh grate was gently aerated using air stones placed under each end of the grate to generate a fine mist inside the chamber, which was covered with a plastic lid. Immediately following death (time 0 h controls), or after 8 h or 24 h of decomposition, samples of liver, muscle, bile, heart, and kidney where collected from each animals, and snap-frozen in liquid nitrogen. Blood samples, which were impossible to collect from the decomposing fish, were also collected from the control fish, and frozen in liquid nitrogen. Only muscle and liver tissue where analyzed after decomposition had progressed and only blood, muscle, and liver where analyzed from

control fish. The remaining tissues (bile, heart, and kidney) remained frozen at -80°C for future analysis.

Analytical Procedures

The snap frozen tissues were stored at -80°C, until they were transported on dry ice to the Upper Midwest Environmental Sciences Centre (UMESC), US Geological Survey, La Crosse Wisconsin, USA. Upon arrival, the samples were stored at -80°C, until they were processed for LC-MS/MS analysis. Liver and muscle were prepared for LC-MS/MS by breaking apart the tissue under nitrogen using a mortar pestle yielding coarse chunks of tissue (50-100mg) which, were transferred into pre-labeled, pre-weighed 2mL, polypropylene micro-centrifuge tubes that could be fitted with a screw able cap (SC Micro-tube – 2 mL, with conical base, Sardstedt, Inc., Newton, North Carolina). The samples where then diluted 1:1 using a mixture of acetonitrile containing 1% formic acid. Two small, stainless steel ball bearings (2mm diameter) where then added to the tube, which was capped, and the samples homogenized using a GenoGrinder bead homogenizer at 1200 strokes/min for 90 sec. Exactly 400 µL of the acetonitrile 1% formic acid mixture was added to the homogenate, which was vortexed for 15 seconds, and then refrigerated at 4°C for 20 min. The tubes were then centrifuged at 12000 *g* at room temperature for 10 min, transferred to Phree phospholipid removal 96 well plates (8B-S133-TAK., Phenomenex, Torrance, CA) and left at room temperature. The original centrifuge tubes were rinsed with 500 µL of the acetonitrile 1% formic acid solution, vortexed for 15 sec, capped and centrifuged at12000 *g* at room temperature for 10 min. The solution from the rinsed tube was then transferred to the Phree cartridge making sure to place the second aliquot in the same well as the first aliquot. The aliquots where

filtered through the Phree cartridge into a 2 mL reservoir well plate by centrifuging the Phree cartridges at 500 *g* for 5 min. The plate was then sealed and either run directly on the LC-QTOF or kept frozen at -20°C until ready for injection. Sample analysis was conducted using Liquid Chromatography-Quadrupole Time of Flight (LC-QTOF) LC-MS/MS in the same as described in Chapter 2.

Calculations and Statistics

TFM concentrations are expressed in nmol per gram of dry tissue (nmol g^{-1}). Dry weight and water tissue percent was determined by dehydrating tissue samples of liver and muscle taken from the same fish. Tissues where placed into pre-labeled, pre-weighed centrifuge tubes and then placed in an oven at 70°C for 24 h, after which they were weighed again. Tissue water was determined by taking the original tissue and tube weight and subtracting the dried tissue and tube weight. This was done for every tissue collected from the fish. Tissue water percent was then averaged for each tissue and each experiment.

One-way ANOVAs where used to determine if parent TFM and/or its metabolites varied significantly over time within each tissue. In instances in which significant variation was observed, a Tukey post-hoc test was used to determine significance between time points at the $P < 0.05$ level. A paired student's t-test was used to make comparisons between tissue types at the same decomposition time point. Tissues where collected from the same fish at the same time. Significance was evaluated at the $P < 0.05$ level. In the case of non-parametric data a Kruskal-Wallis test and a Dunn's multiple comparison post-hoc test it was used.

Results:

Effects of Air Decomposition on Tissue water in Liver and White Muscle Tissue

All solid tissue samples where dried and tissue water percent was calculated (Table 3-2). The percent tissue water found in liver tissue taken from rainbow trout immediately following death was 75.5 ± 0.45% which was similar to the percent tissue water found in white muscle tissue taken immediately after death which was 79 ± 0.63%. Following 8 h of decomposition liver and white muscle tissue water was 77.2 ± 1.17% and 76.8 ± 1.08% respectively. After 24 h of decomposition liver tissue water was 78.8 ± 0.6% and white muscle tissue water was 79.2± 1.53%. No significant differences were observed between in tissue water over the decomposition period in either liver or white muscle tissue and as such TFM concentrations where not corrected according to tissue water and where expressed in wet weight.

Qualitative Aspects of Decomposition

The carcasses where monitored over the course of the decomposition periods. As opposed to Chapter 2, fish where allowed to decompose while exposed to humidified air for 8h or 24h while suspended on a mesh grate above fresh water. It was noted that a clouding of the eyes was visible following approximately 12h of decay (see Figure 2-1 in previous chapter) as well as a drying out of the skin of the fish leaving a rough texture. Foam was also present in the water and on the mesh grate surrounding the fish after 8h of decomposition (Figure 3-1). During dissections it was noted that there was little to no liquefaction of the tissues, however, the carcasses tended to be more bloated than the carcasses recovered from water.

TFM Tissue Distribution and Effects of Air Decomposition on TFM concentration

The 9 h LC50 of TFM was determined to be 16.8 mg L^{-1} (CI = 15.0-19.2) in preceding toxicity tests conducted by Foubister et al., which was the target exposure concentration in these experiments. The measured concentration of TFM to which the fish were exposed averaged 16.80 ± 0.36 mg L^{-1} during the 6 h exposure period (Table 3-1). The concentration of TFM in liver collected immediately (0 h) from the fish following the 6 h TFM exposure period was 211.8 ± 3.86 nmol g^{-1} wet tissue, whereas white muscle tissue collected at the same time had a much lower TFM concentration of 13.0 ± 1.82 nmol g^{-1} wet tissue (Figure 3-2). Through the use of a paired student's t-test it was found that liver tissue TFM concentration was significantly greater than that of the white muscle at 0 h ($p < 0.0001$).

Whole blood and blood fractions were also collected immediately (0 h) from the fish following TFM exposure. The concentration of TFM in whole blood was 20.1 ± 3.26 nmol ml^{-1} wet tissue, in blood plasma it was 3.5 ± 0.7nmol ml^{-1} wet tissue and in red blood cell pellet it was 41.5 ± 4 nmol ml^{-1} wet tissue (Figure 3-3). Through the use of a Kruskal-Wallis test and a Dunn's multiple comparison post-hoc test it was found that plasma TFM concentration was significantly lower than that of the red blood cell pellet ($p < 0.0001$).

After decomposition in air for 8 h and 24 h, the respective concentrations of TFM in liver decreased by just over 50 % to 100.1 ± 17.79 nmol g^{-1} wet tissue and 108.9 ± 29.17 nmol g^{-1} wet tissue (Figure 3-2). There were no significant changes in muscle tissue TFM concentration following the 8 or 24 h decomposition period, averaging 15.9 ± 1.91 nmol g^{-1} wet tissue and 15.25 ± 1.16 nmol g^{-1} wet tissue, respectively (Figure 3-2). Through the

use of a paired student's t-tests it was found that liver tissue TFM concentration was significantly greater than that of the white muscle at both 8 h and 24 h ($p = 0.002$ and $p = 0.0137$ respectively). Through the use of a one-way ANOVA followed by a Tukey post-hoc test it was determined that at both 8 h and 24 h of decomposition TFM concentration in liver decreased significantly ($p = 0.0062$ and $p = 0.0115$ respectively) (Figure 3-2).

Since a standard for TFM-glucuronide (TFMOGlu) or TFM-sulfate (TFMOS) was not available, amounts of each were expressed relative to the peak area of the metabolite measured immediately following death, which was set at 100 %. Compared to fresh liver, collected immediately following TFM exposure, the relative amount of TFM-glucuronide had declined by approximately 70 % after 8 h. By 24 h, TFM-glucuronide was below levels of detection (Figure 3-4). In contrast, there was no significant decrease in the relative amount of TFM-glucuronide in the white muscle collected after 8 h of decomposition, compared to freshly collected tissue. After 24 h of decomposition, however, the relative amount of TFM-glucuronide had decreased by almost 80 % compared to the original amount (Figure 3-5). As for TFM-sulfate compared to fresh liver, the relative amount of TFM-sulfate declined by approximately 60% after 8 h of decomposition, but by 24 h TFM-sulfate was below levels of detection (Figure 3-5). There was no TFM-sulfate detected in white muscle tissue at any time point (data not shown).

Discussion:

Differences in TFM distribution between liver, white muscle tissue and blood

As in immersed carcasses (Chapter 2), highest initial concentrations of TFM found in rainbow trout, exposed to lampricide for 6 h, were in the liver tissue, in which TFM was approximately six times the amount of that present in white muscle tissue. This

observation was expected given the role of the liver's essential role in xenobiotic detoxification and elimination (Chambers & Yarbrough, 1976; Wolf and Wolfe 2005; Bévalot et al. 2015). Under resting conditions, the liver of rainbow trout receives three times the arterial blood flow of the white muscle tissue, despite the fact that the white muscle comprises 60 % of the body mass (Farrell, 1993). However, blood flow to the liver is also augmented by the hepatic portal system, further elevating the relative amount of blood perfusing the liver compared to the white muscle. The liver's role in xenobiotic detoxification is further augmented by a different variety of transport proteins found on the basolateral membrane of the hepatocytes, including organic anion transporters (OATs) and organic anion transporting polypeptides (OATP) that actively transport hydrophilic and hydrophobic anions, respectively, into and out of the cytosol of the hepatocyte (Bévalot et al. 2016). The hepatocyte also contains cytosolic and microsomal enzymes involved in phase I and phase II biotransformation resulting in more hydrophilic compounds that are easier to excrete via biliary routes (Wolf and Wolfe 2005). As a result, far greater amounts of xenobiotic substances such as TFM are deposited in the liver than other organs, making it ideal for post-mortem analysis.

The high lipid content of the liver also makes it an ideal reservoir for lipophilic substances to accumulate. With a log K_{ow} of 2.87 (TFM, MSDS, 2015), TFM is moderately lipophilic, which would also promote TFM sequestration in this tissue compared to the white muscle, which is leaner. In addition to lower rates of blood flow (Johnston, 1976, Farrell, 1993), the white muscle has significantly greater water content and lower lipid content, making it a less likely storage reservoir for TFM than liver.

There are two types of skeletal muscle tissue present in trout, white muscle tissue and red muscle tissue. White muscle fibers, or fast twitch muscle, tend to have fewer mitochondria present and are better suited for anaerobic metabolism, whereas red muscle fibers, or slow twitch muscle, are better suited for oxidative metabolism (Cassens and Cooper, 1971; Johnston, 1976). This results in the red muscle being more vascularized then white muscle tissue (Johnston, 1976). It is important to note, that following exhaustive exercise, blood flow to the white muscle increases dramatically, to promote recovery by facilitating the re-charging of high energy phosphagens (e.g. phosphocreatine), correction of metabolic acidosis in the tissue, and the restoration of glycogen reserves and elimination of lactate (Wood 1991; Farrell 1993). Therefore if the animal is actively attempting to escape the TFM block in the water, greater amounts of TFM could accumulate in the white muscle than reported here.

The majority of the TFM that concentrated in the blood of the rainbow trout was accumulated in the red blood cells (RBC) as opposed to the plasma. RBC TFM concentration was ~90% greater than that of the plasma, which may indicate that TFM is being trapped in the erythrocytes. TFM is taken up into the organism in its un-ionized lipophilic form where it can easily pass through cell membranes (Hunn and Allen 1974; Bills et al. 2003, McDonald and Kolar 2008). TFM enters the organism mainly by diffusion across the gills into the blood stream, where it is most likely converted to its ionized form due to physiological pH (e.g. at 15 °C blood pH = 7.8; Milligan and Wood 1986; Wilkie and Wood 1995). In a study by Thomas and Egée (1998) it was shown that anion exchangers are present on RBC and as such could be a route of entrance for TFM-O^-. Another possible explanation for the increased amount of TFM in the RBC is through

mass action; however, more research is needed in this area. The RBC of teleost fish are mitochondria rich and as such TFM may end up trapped inside the inner mitochondria membrane once inside the cell, leading to further ion trapping (Thomas and Egée, 1998).

The Effects of Air Exposure on Tissue Decomposition and TFM stability

Despite accumulating less TFM than the liver, the muscle was a more robust reservoir for TFM. During decomposition of the whole animal exposed to humidified air, TFM concentrations in the liver tissue rapidly decreased over the 24 h time period, whereas TFM concentrations remained relatively stable in the white muscle (Figure 3-2). This was likely because (i) the initial post-mortem concentrations of TFM in the liver were markedly higher than in white muscle, and (ii) the liver decomposes substantially faster than the muscle tissue due to its close proximity to the gastrointestinal (GI) tract and opportunistic populations of anaerobic bacteria that initiate the process of putrefaction (Butzbach, 2010).

Putrefaction is the breakdown and liquefaction of tissues and organs primarily due to microbial metabolism. Putrefaction begins when bacteria from the GI tract and respiratory tract enter the blood stream and tissues closely associated with them following the death of the organism, which is also known as post mortem migration (Yarema & Becker, 2005; Butzbach, 2010). During putrefaction the tissues and blood of the deceased organism will become more alkaline due to microbial process when compared to the preceding "autolytic" stage of decomposition (Butzbach, 2010). As noted in Chapter 2, the corresponding microbial activity breaks down protein and fatty tissue, generating gaseous compounds such as hydrogen, hydrogen sulfide, carbon dioxide, methane, and ammonia causing the carcass to bloat. The subsequent buildup of pressure may lead to

the purging of fluids (liquefied tissues, blood, bacteria, etc.) through open wounds or orifices', such as the gills of fish (Butzbach, 2010; Hau et al. 2014). Compared to an animal immersed in relatively cool waters, decomposition of the liver would accelerate if the animal were air-exposed. In contrast to air, the high thermal conductivity and heat capacitance of water would tend to keep the carcass cooler if immersed. In air, however, it would tend to warm, even under the relatively humid conditions characteristic of a stream bank in the spring or summer accelerating microbial breakdown of cell boundary layers, leading to the loss of compounds from tissues (Yarema & Becker, 2005; Butzbach, 2010).

It is known that certain species of the bacteria, such as *Moraxella* sp. as well as some *Arthrobacter* sp. are found in the rainbow trout intestine (Kim et al. 2006) and are capable of aerobically degrade nitro phenolic ring compounds (Spain and Gibson, 1991; Jain et.al., 1994). Seeing as TFM is a para-nitrophenolic compound it is reasonable to assume that degradation in the same manner may occur following the bloating phase of decomposition as the carcass ruptures creating an aerobic environment, and may have contributed to the loss of TFM (Hubert, 2003). Studies have also shown microbial degradation of TFM under anaerobic conditions in bottom sediments. In a GLFC technical report from 1973, Kempe showed a reduction in TFM concentration in water with sediment collected from Lake Erie and showed microbial action by inoculating bacteriological culture media that had TFM present with the same sediment (Kempe, 1973). This was also shown in a study by Bothwell et al. (1973), where under anaerobic conditions TFM was reduced to 3-trifluoromethyl-4-aminophenol (RTFM). The study used formalin to stop microbial growth, with resulted in reduced TFM degradation,

implicating microbial activity in the process (Bothwell et al. 1973, Hubert, 2003). Although these studies focused on sediment bound bacteria it is possible that, due to the fishes close proximity to the sediments and diet, these bacteria could be found in the GI tract of non-target organisms and as such could lead to the degradation of TFM within the animal.

However, bacterial degradation is not the only factor in degradation of tissues. Cell death will begin in tissues exposed to ischemia (an inadequate or absent supply of blood to an organ) the time frame for irreversible cell damage is different for various tissues, in liver tissue a lack of blood for 1-2 hours will result in mass cell death (Yarema & Becker, 2005). During cell death cell lyses will result in the spillage of cytosolic contents into the extracellular space, these contents would include any TFM captured by the cell resulting in a loss of concentration (Yarema & Becker, 2005). Once in the extracellular space TFM has a greater likelihood of traveling down concentration gradients out of the tissue as well TFM metabolites being more hydrophilic would likely be flushed out of the space due to fluid shifts and tissue water. Muscle tissue is more resistant to ischemia, being able to last 4-6 hours before irreversible cell damage is done which likely contributed to its greater resistance to decomposition. It is important to note, however, that the effects of ischemia can be dependent on multiple factors such as the ambient temperature, and tissue mass (Blaisdell, 2002). Necrosis due to ischemia is accelerated at warmer temperatures as well muscle fiber type seems to impact the speed of necrosis with fast-twitch muscle fibers (white muscle tissue) being more greatly effected after extended periods of ischemia (Petrasek et al. 1994).

Muscle tissue, being relatively isolated form the gut micro-biome, is not affected (initially) by the bacteria responsible for decomposition which allows the tissue to retain its structure and as such traps the toxicant and metabolites inside the tissue (Butzbach, 2010). It is to be expected, however, that as decomposition time increases muscle tissue will eventually become affected by decomposition and as such will not be suitable for analysis for forensic purposes. This was noted in the qualitative aspects of this study. The muscle tissue itself following prolonged decomposition was seen to pull away from the bone structure of the fish and become extremely soft and delicate indicating a breakdown in cellular structure. This break down of cellular structure is likely caused by the activity of proteolytic enzymes that are released during autolysis. As membranes, such as those of the lysosomes, breakdown these enzymes are released into the cytosol and are activated due to the buildup of lactic acid from anaerobic metabolism (Butzbach, 2010). This lower pH activates the proteolytic enzymes causing them to degrade larger molecules such as the membranes of cells and other organelles leading to the destruction of cells and tissues.

TFM Metabolite Differences in Air Decomposed Tissues

Detoxification occurs through the use of UDP-glucuronosyltransferase, an enzyme found predominantly in the microsomes of the liver, which facilitates phase-II metabolism by adding a glucuronic acid to a xenobiotic in order to make it more hydrophilic and easier to excrete mainly via the gastrointestinal tract, and to a lesser extent the urine (Lech & Costrini, 1971; Kawatski & Bittner 1975; Kane et.al., 1994). TFM glucuronide (TFMOGlu), the main metabolite formed from TFM detoxification, is formed using the enzyme UDP-glucuronosyltransferase and a phase II reaction. UDP-

glucuronosyltransferase (UDPGT) facilitates the addition of a glucuronic acid to TFM which makes the molecule more hydrophilic and thus easier to excrete through renal and dietary pathways (King et.al. 2000; Hunn & Allen, 1974; Clarke et.al. 1991). This compound was found in the liver tissue, which was expected as the liver is the main site of detoxification in the organism as well it has the highest concentration of UDPGT necessary for the biotransformation to occur (Chambers & Yarbrough, 1976; Lech & Costrini, 1971; Kawatski & Bittner 1975; Kane et.al., 1994). This likely explains why the relative amounts of TFMOGlu found in the liver were approximately 1000-fold greater than in the white muscle. The TFMOGlu found in the muscle suggests it may have some capacity to detoxify xenobiotic compounds using this phase II biotransformation pathway, however at the present time there is no evidence of UDPGT being present in the muscle. More likely the TFMOGlu detected in the muscle was trapped in the extracellular fluid, which comprises the plasma plus the interstitial fluid. Because TFMOGlu is more hydrophilic, greater amounts would be present in the extracellular fluid space. In an accompanying study (Foubister, 2018), it was noted that the plasma to red blood cell ratio of TFMOGlu was more than 20:1, compared to about 1:20 for the parent TFM, suggesting that significant TFMOGlu is released to the ECF.

As decomposition progressed, liver TFMOGlu concentration decreased rapidly, following 8 hours of decomposition only approximately 27% of the TFMOGlu was recovered from the tissue, and after 24 hours of decomposition virtually no TFMOGlu remained (Figure 3-4). However in the muscle tissue 87.9% of the TFMOGlu remained after 8 hours of decomposition and roughly 21% remained after 24 hours of decomposition (Figure 3-5). A possible explanation for the difference is that the speed of

decomposition is much greater in liver tissue, which putrefies more quickly than muscle tissue, as well once the animal has died and blood flow has stopped liver tissue cells will rapidly undergo necrosis hours before the muscle tissue begins to die (Yarema & Becker, 2005). This increased time may allow for more TFMOGlu to escape the tissues. Liver tissue is also much more susceptible to autolysis due to its high abundance of catabolic enzymes. This would accelerate its decomposition and as such may explain the loss of metabolites (Powers, 2005; George et al. 2016). TFMOGlu is also hydrophilic and as such would be eliminated by the animal via normal excretion pathways, and when not trapped inside a cell would be quickly eliminated from the body flowing down concentration gradients. TFM-sulfate (TFMOS) is another metabolite formed from TFM detoxification. TFMOS is also formed through the use a phase II biotransformation pathway and the use of cytosolic sulfotransferase enzymes (SULT). This facilitates the addition of sulfonate group, normally from the universal donor 3-phosphoadenosine-5-phosphosulfate (PAPS), to the parent molecule making the compound easier to excrete out of the body (Kauffman, 2004; Bussy et al. 2017). There was no TFMOS found in the muscle tissue throughout the experiments indicating that the tissue is unable to form this metabolite. However it was found in liver tissue after 8 h of decomposition. A similar recovery rate to TFMOGlu was found for TFMOS (~40%) which likely occurs due to the compounds hydrophilic nature.

Decomposition in air vs in water at varying temperature

It was to be expected that TFM concentrations would remain more constant in tissues recovered from animals left to decompose in water, especially at colder temperatures due to Casper's rule (Iscan & Steyn, 2013; Schmitt et al. 2006). Comparing the tissue

concentrations from Chapter 2 to the tissue concentrations presented in this chapter it was found that indeed decomposition at colder water temperatures protects against tissue degradation and as such TFM loss. It was found that at 4°C liver TFM concentration did not decline over 72 h of decomposition whereas an approximately 50% reduction was seen in tissues decomposed in air up to 24 h. This is most likely explained by the temperature difference between the two studies, as during air decomposition the fish where left at room temperature (~17.5°C). As water temperature increased the protective effect of water began to decline, at 15°C TFM concentrations where stable up to 8 h, however after 24 h TFM concentration reduced by 75%. This could indicate that at moderate water temperatures decomposition is delayed for a short time period, however, as cell membranes breakdown and tissue water increases redistribution or loss of xenobiotics becomes more likely. Finally at 20°C large reductions in TFM concentration where observed after 8 h (71%), this was most likely caused by the increased metabolic activity of bacteria during the putrefaction stage of decomposition at this warmer temperature combined with an accelerated autolytic stage resulting in a faster breakdown of tissues (Butzbach, 2010; Zhou and Byard, 2010). White muscle tissue showed greater resilience to decomposition in both studies with no reduction in TFM being observed over all time points when exposed to air or water at 4°C and 15°C. However at warm temperatures (20°C) TFM concentrations reduced by 25% after 8 h and 50-60% after 24 h and 72 h. These findings suggest that the two most important factors to consider when recovering tissues from a fish kill are time and temperature.

Conclusion:

As this study has shown, TFM is found in greater concentrations in the liver when compared to the white muscle of rainbow trout. This is due to the physiology of the animal, as the liver has a greater perfusion rate, as well as organic anion transporters (OATs) and organic anion transporting polypeptides (OATP). This allows for more TFM to pass through the tissue and therefore it is more likely to accumulate there. The liver tissue also tends to have a higher lipid content then white muscle, and as such has a greater ability to trap TFM due to its lipophilic nature. TFM was detectable in both liver and muscle tissue after 24 h of decomposition, with muscle tissue TFM concentration being more stable over the decomposition period. This is due to the greater effect of autolysis on the liver as a result of a greater amount of proteolytic and hydrolytic enzymes located in the tissue.

The effects of putrefaction are also more pronounced in liver tissue due to its close association with the GI tract and major blood vessels which expose it to the putrefying bacteria of the digestive system. Therefore after prolonged periods of decomposition muscle tissue may be the ideal tissue to sample for forensic purposes, however this study has shown liver tissue is viable up to 24 h. Other tissues that may be forensically relevant but where not analyzed in this study would be the bile, kidney, and heart. Bile would be an extremely valuable sample to obtain, since TFM is excreted through digestive as well as urinary pathways, it would likely have a high concentration of TFM.

TFM metabolites give insight into the cause of and time of death of the organism. If TFM metabolites are present in liver and muscle tissues it is likely that the animal was

exposed to the toxicant for a prolonged period of time, which ultimately lead to its death. Combining the presence of TFM metabolites with the water quality parameters collected at the fish kill site, information on any recent TFM application sites, and taking into account any other phenomenon that could alter the water chemistry of a stream or tributary (such as rain, agricultural or industrial discharges, etc.), could help to explain how the fish kill occurred and how a similar incident could be prevented in the future.

Table 3-1: TFM exposure concentration.

Differences in average TFM concentration between experiments with standard error. A target of 16 .8 mg L^{-1} of TFM was used in each experiment.

Experiment	Average TFM Concentration	Standard Error ±
Control	16.21	0.27
8 h Decomposition	17.29	0.35
24 h Decomposition	16.9	0.47
Average	**16.8**	**0.36**

Table 3-2: Relationship between percent tissue water in liver and white muscle at different stages of decomposition.

Total percent tissue water of each tissue (muscle and liver) throughout all experiments.

Tissue	0 h	8 h	24 h
White Muscle	79 ± 0.63	76.8 ± 1.08	79.2 ± 1.53
Liver	75.5 ± 0.46	77.2 ± 1.17	78.8 ± 0.6

A

B

Figure 3-1: Effects of short and prolonged decomposition in air.

Carcass A was decomposed for 8h in air, while carcass B was decomposed for 24h. There is a notable foam forming around carcass A. Carcass B's eye had become cloudy and the mid-section of the carcass had become bloated.

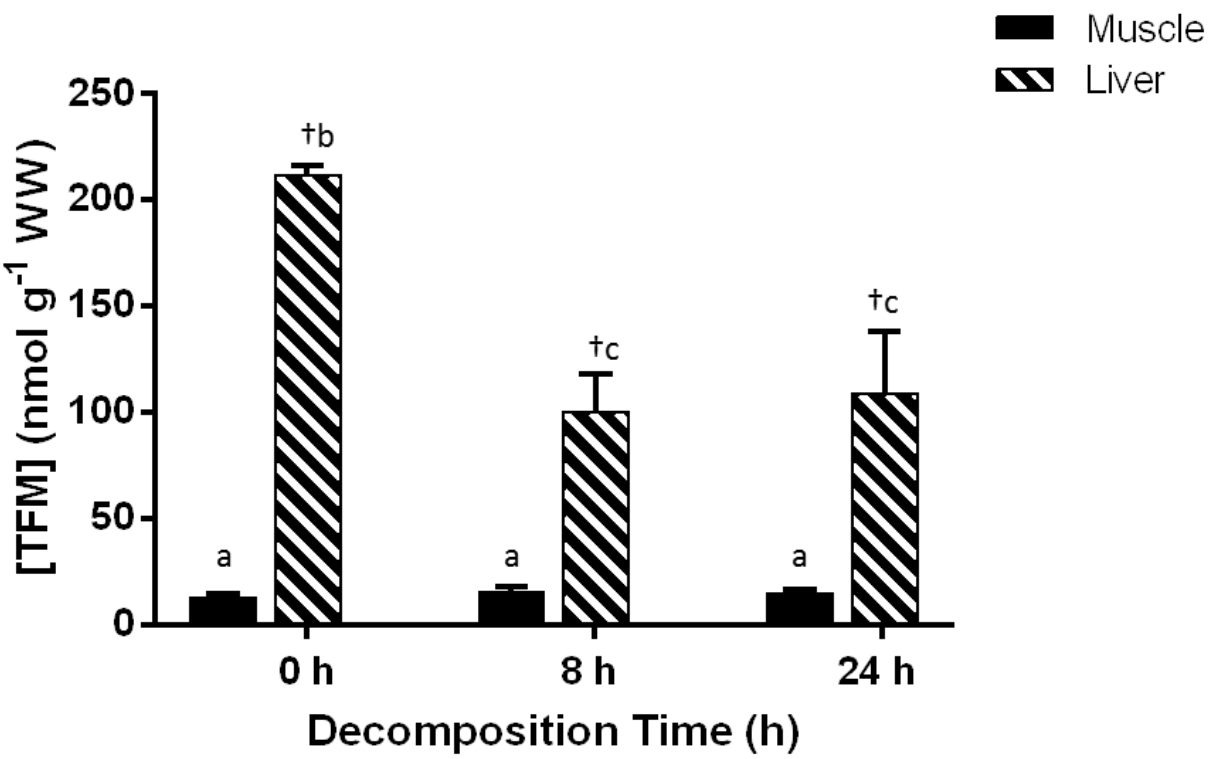

Figure 3-2: Effects of decomposition in air on the distribution and amounts of TFM in the liver and white muscle tissue of rainbow trout.

Changes in the TFM concentration of decomposing liver (hashed bars) and white muscle (solid bars) tissue versus time, while exposed to humidified air. Rainbow trout exposed to the LC_{50} (16.80 mg L^{-1}) of TFM for 6 h were euthanized, and liver and white muscle tissue collected at immediately (time zero control) or after 8 h and 24 h of decomposition. Data is presented as the mean ± SEM (N = 8 fish at each time point). Bars sharing the same letters are not significantly different from each other for each respective tissues. Significance between the respective concentrations of TFM between the liver and muscle are denoted by a single dagger.

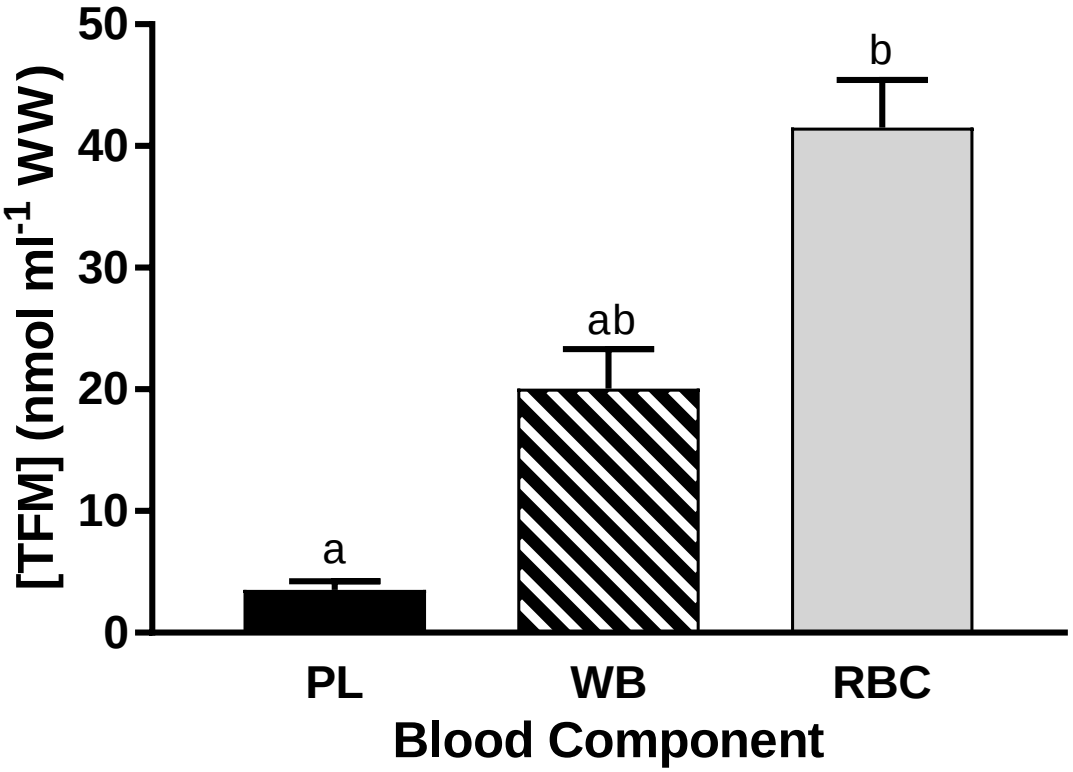

Figure 3-3: Differences in the distribution of TFM between whole blood, red blood cells and the plasma.

Rainbow trout were exposed to the LC_{50} (16.80 mg L^{-1}) of TFM for 6 h prior to blood sample collection via caudal puncture (mixed venous-arterial blood), and centrifuged at 10,000 *g*. TFM was quantified in the whole blood (WB), red blood cells (RBC) and the plasma (PL) fractions. Due to deterioration of the fish carcass due to decomposition, blood was only collected immediately following TFM exposure as described in Figure 3-3. Data is presented as the mean ± SEM (N = 8 fish). Bars sharing the same letters denote data that are not significantly different from each other. TFM concentrations where significantly different between plasma and red blood cell pellet ($P \leq 0.05$).

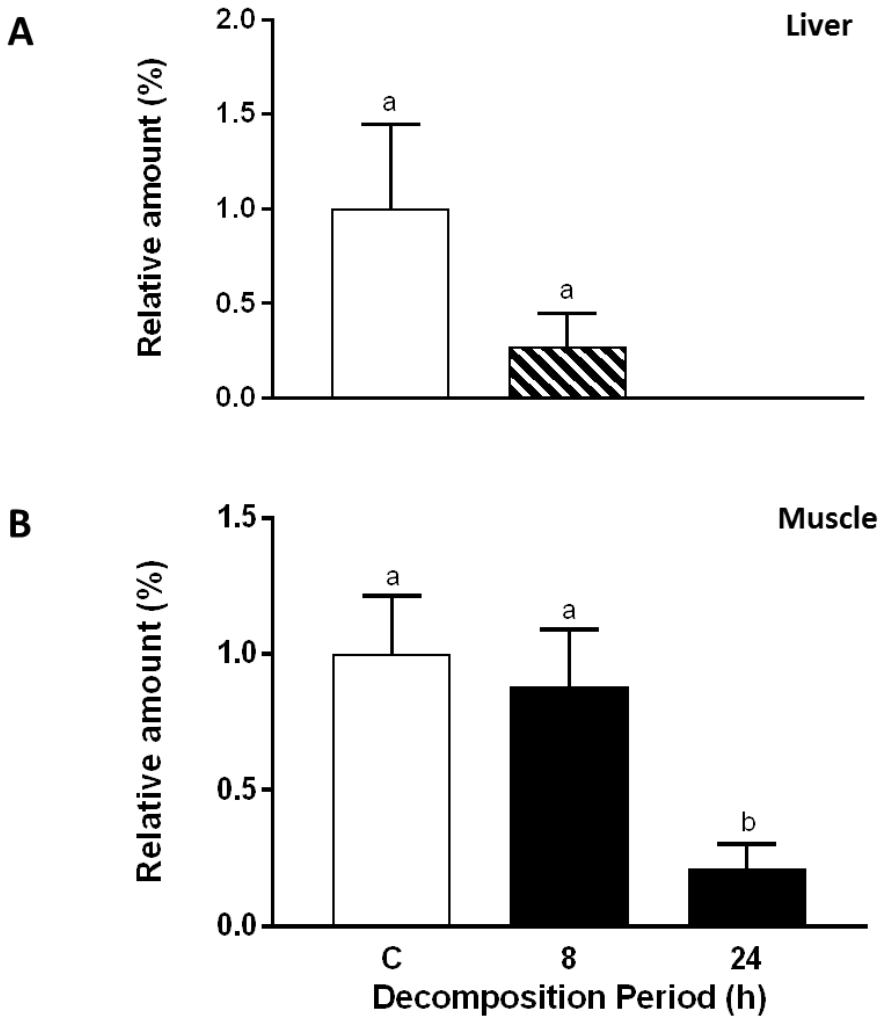

Figure 3-4: Effects of air exposure and decomposition TFM-glucuronide concentrations rainbow trout.

Changes in the TFM-glucuronide concentration of (A) decomposing liver and (B) white muscle tissue versus time, while exposed to humidified air. Rainbow trout exposed to the LC_{50} (16.80 mg L^{-1}) of TFM for 6 h were euthanized, and liver and white muscle tissue collected at immediately (time zero control) or after 8 h and 24 h of decomposition. Data is presented as the mean ± SEM (N = 8 fish at each time point). Bars sharing the same letters are not significantly different from each other.

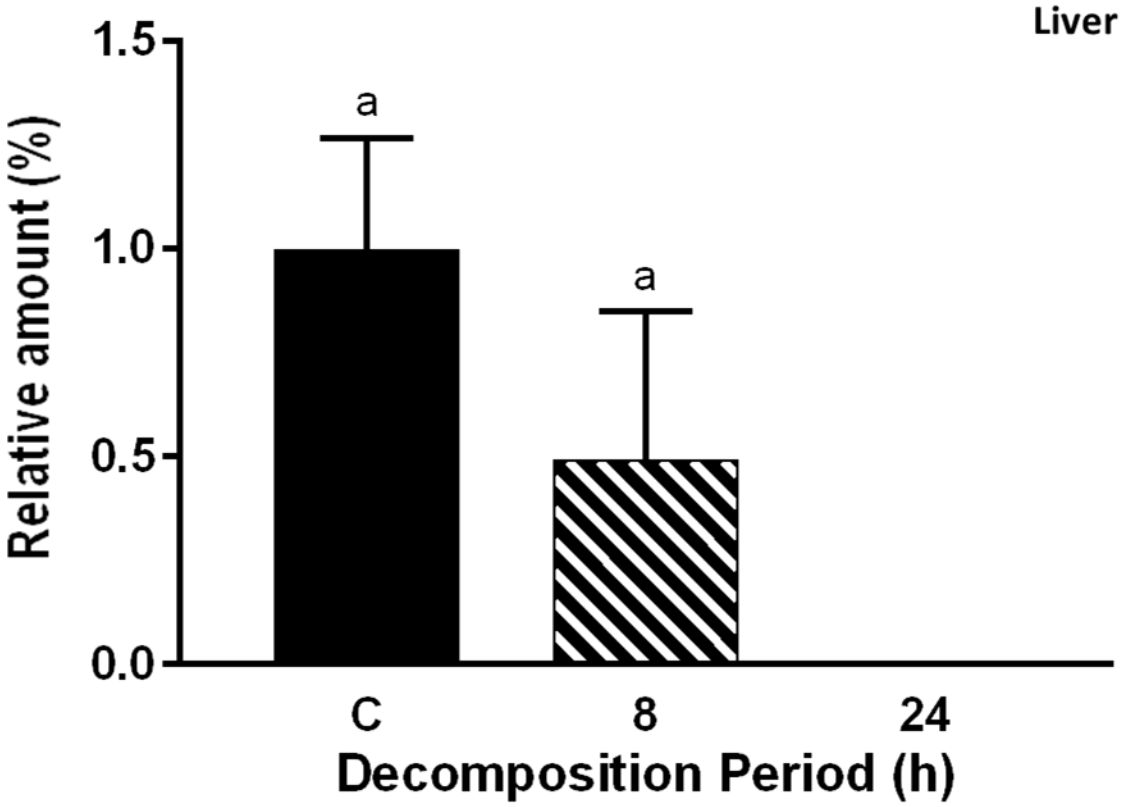

Figure 3-5: Effects of air exposure and decomposition on TFM-sulphate concentrations in rainbow trout.

Changes in the TFM-sulphate concentration of decomposing liver tissue versus time, while exposed to humidified air. Rainbow trout exposed to the LC_{50} (16.80 mg L^{-1}) of TFM for 6 h were euthanized, and liver and white muscle tissue collected at immediately (time zero control) or after 8 h and 24 h of decomposition. Data is presented as the mean ± SEM (N = 8 fish at each time point). Bars sharing the same letters are not significantly different from each other. Data are not shown for TFM-sulphate in the white muscle, which was not detected at any sample time.

Chapter 4:

A Practical Protocol for Tissue Sample Collection, Handling, Storage and Analysis in Instances of Non-target Mortality Following TFM Application

Introduction:

The piscide (lampricide) 3-trifluoromethly-4-nitrophenol has been used since its discovery in the 1960's to control the proliferation of the invasive sea lamprey (Applegate et.al. 1961; Dawson et.al. 1999; McDonald and Kolar, 2007; Siefkes, 2017). TFM is applied to nursery streams and rivers in the Great Lakes basin approximately every three to five years in order to kill multiple generations of sea lamprey larva and has been widely successful in the controlling sea lamprey populations, reducing their presence in the Great Lakes by 90% from historic levels, when combined with other methods of control such as the use of barriers and traps (Applegate, 1950; Smith and Tibbles, 1980; McLaughlin et al. 2007; Siefkes, 2017).

However, non-target fish can also affected by the use of TFM if their uptake exceeds their ability to detoxify the compound which may lead to non-target mortality (Boogaard et.al. 2003). As such it is imperative that in the event of an incident involving lampricides that investigators be well equipped to determine the cause of death. Indeed fish mortality could occur due to any number of reasons such as disease, oxygen depletion or from anthropogenic causes such as pollution (Meyer & Barclay, 1990). The present thesis identifies the effects that decomposition can have on TFM concentrations in fish tissue exposed to various environments. Below I outline a practical protocol for tissue sample collection, handling, storage and analysis which will aid in the investigation of suspected instances of non-target mortality following TFM application.

Ideal tissues to sample for analysis

Both liver and white muscle tissue where analyzed for TFM in this study with liver tissue having the greater abundance of TFM at all decomposition time points. However white muscle tissue TFM concentration was much more stable over time indicating that the

effects of decomposition where lesser on it. Both tissues are ideal to collect for analysis if available as liver tissue would provide a large reservoir of detectable TFM and white muscle tissue is stable over long periods of time retaining its TFM concentration. Other tissues that may be of analytical interest include the gallbladder bile, and head kidney. The bile of the gallbladder would likely be high in TFM concentration due to organic anion transporters concentrating TFM in the liver which ultimately drains into the gallbladder. Kidney tissue may be of interest due to its involvement in renal excretion.

When assessing a fish kill, the fish present may be in various stages of decay as they may not all be exposed to the same environment (various water temperatures and depth, amount immersed in water, on the shore, etc.). Collecting samples from the freshest looking carcasses would help to ensure that detectable levels of xenobiotics would still be present in the tissues, however make sure to note the various stages of decay in order to estimate when the incident may have occurred. Along with tissue samples it is imperative to collect water quality measurements such as temperature, pH, and alkalinity as well as water clarity and any possible influxes of anthropogenic contamination (i.e. sewage, agricultural run-off, etc.) as these factors could accelerate decomposition.

Longevity of tissues exposed to different environments

Fish kills may go over looked for long periods of time as the fish wash up on shore downstream or remain immersed in the water. The present study has determined that over the course of 72 h in water it is possible to detect TFM in both white muscle and liver tissue however as temperature increases decomposition accelerates. Therefore in warmer seasons such as the late spring and summer it is possible that complete loss of TFM could

occur prior to 72 h. In the case of advanced decomposition collecting samples of various tissues becomes increasingly important as the detectable levels of concentrations may not be present in white muscle tissue.

Ideal storage method

Storage of tissues after collection in the field should be done as soon as possible. Ideally tissues would be immediately frozen to prevent any further decomposition from taking place and as such the loss of TFM, and then stored at -80°C until analysis. However in the field it is unlikely that this would be possible. To slow down the processes of autolysis and putrefaction tissue samples should be kept as cold as possible, however keeping tissues at or below 4°C should slow bacterial metabolism and as such the decomposition process enough until long term storage options are available. If analyzing white muscle tissue from carcasses stored at 4°C or 15°C TFM concentrations should be stable up to 72 h, whereas if liver tissue is to be analyzed fish carcasses should be stored at 4°C to prevent loss of TFM.

Future directions and Conclusion

While we now know how TFM is distributed in the tissues of non-target fish and how decomposition affects these concentrations it is unknown how niclosamide as well as other xenobiotics may react under the same conditions. Expanding this bank of knowledge could help build a forensic library that could be used when attempting to investigate the source of a fish kill associated with any number of events from lampricide treatments, to industrial activities. Studies should be conducted on xenobiotic free carcasses left to decompose in TFM burdened water in order to determine if there is a difference in forensic markers between a fish killed by TFM and a fish that expired due to

other causes. Decomposition experiments of longer length and with different tissues such as bile, kidney, and heart should be conducted in order to increase the number of relevant collectable samples should some be unattainable in the field due to degradation, contamination or any other reason. Lastly decomposition experiments should be conducted in the field in order to more accurately observe the effects of decomposition. This study since conducted under laboratory conditions lacked a key component of decomposition; vertebrate scavengers, and arthropods which could significantly increase the rate of decomposition as well as the over degradation of tissues.

This thesis showed that TFM is detectable in tissues decomposed in air up to 24 h and in water up to 72 h. As well it indicated the importance of temperature on the rate of decomposition and the loss of TFM from tissues. At warmer temperatures tissue degradation occurs faster resulting in a greater loss of TFM making it more difficult to determine the cause of death. The TFM metabolites TFM-sulphate and TFM-glucorinide were also detected in this study, indicating that the organism was actively detoxifying TFM prior to death. The presence of metabolites in tissues is a good indicator that the organism's ability to detoxify the compound was overwhelmed and the cause of death was indeed TFM. When comparing decomposition in water to decomposition in air this study showed that at lower temperature (4°C) TFM concentrations are more stable in tissues recovered from carcasses found in the water. At moderate temperatures (15°C) TFM concentrations were also more stable in water recovered carcasses up to 8 h however after 24 h TFM reduction was much approximately 25% greater than air recovered tissues.

The sea lamprey control program, specifically the use of lampricides, is integral in regulating the populations of invasive sea lamprey, without the use of these compounds sea lamprey populations would proliferate resulting in large scale death of larger fish species, such as the lake trout (*Salvelinus namaycush*) and whitefish (*Coregonus clupeiformis*), and other commercial and game fish species (Smith, & Tibbles, 1980). If left unchecked the overall health of the Great Lakes could decline due to population explosions of smaller fishes at lower trophic levels creating a similar situation to that seen in the 1950's, when massive die-offs of these populations occurred and contributed to the degradation of water quality and beaches. This would adversely affect the lives of the many people that rely on the Great Lakes for employment, food/water, as well as recreation. For this reason lampricides remain important for maintaining ecosystem health in the Great Lakes. However, with the possibility of fish mortality arising from the use of these compounds, it is necessary to fully understand how TFM could adversely affect non-target fishes, making it possible to develop certain checks and balances to minimize harm. It is therefore important to determine how and if lampricides contribute to non-target mortality events. By using the forensic toxicological methods of this study, it will be possible to identify and quantify TFM levels in fish tissues and help determine if this lampricide contributed to the death of non-target organisms in unexplained fish kills. Not only will it make it possible to attribute such events to lampricide treatment, but to also exclude them in cases where TFM toxicity is suspected. Using such knowledge could also lead to measures that result in safer and more effective use of TFM for the sea lamprey control program, and the health of the Great Lakes ecosystem.

Appendix A

List of Abbreviations

ATP	Adenosine triphosphate
GLFC	Great Lakes Fisheries Commission
HPLC	High performance liquid chromatography
LC	Lethal Concentration
MS	Mass Spectrometry
OAT	Organic anion transporters
OATP	Organic anion transporting polypeptides
PMR	Post Mortem Redistribution
QTOF	Quadrupole time-of-flight
SULT	Sulfotransferase enzymes
TFM	3-trifluoromethyl-4-nitrophenol
TFMOGlu	TFM-glucuronide
TFMOS	TFM-sulfate
UDPGT	UDP-glucuronosyltransferase

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